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Handbook of Immunoperoxidase Staining Methods

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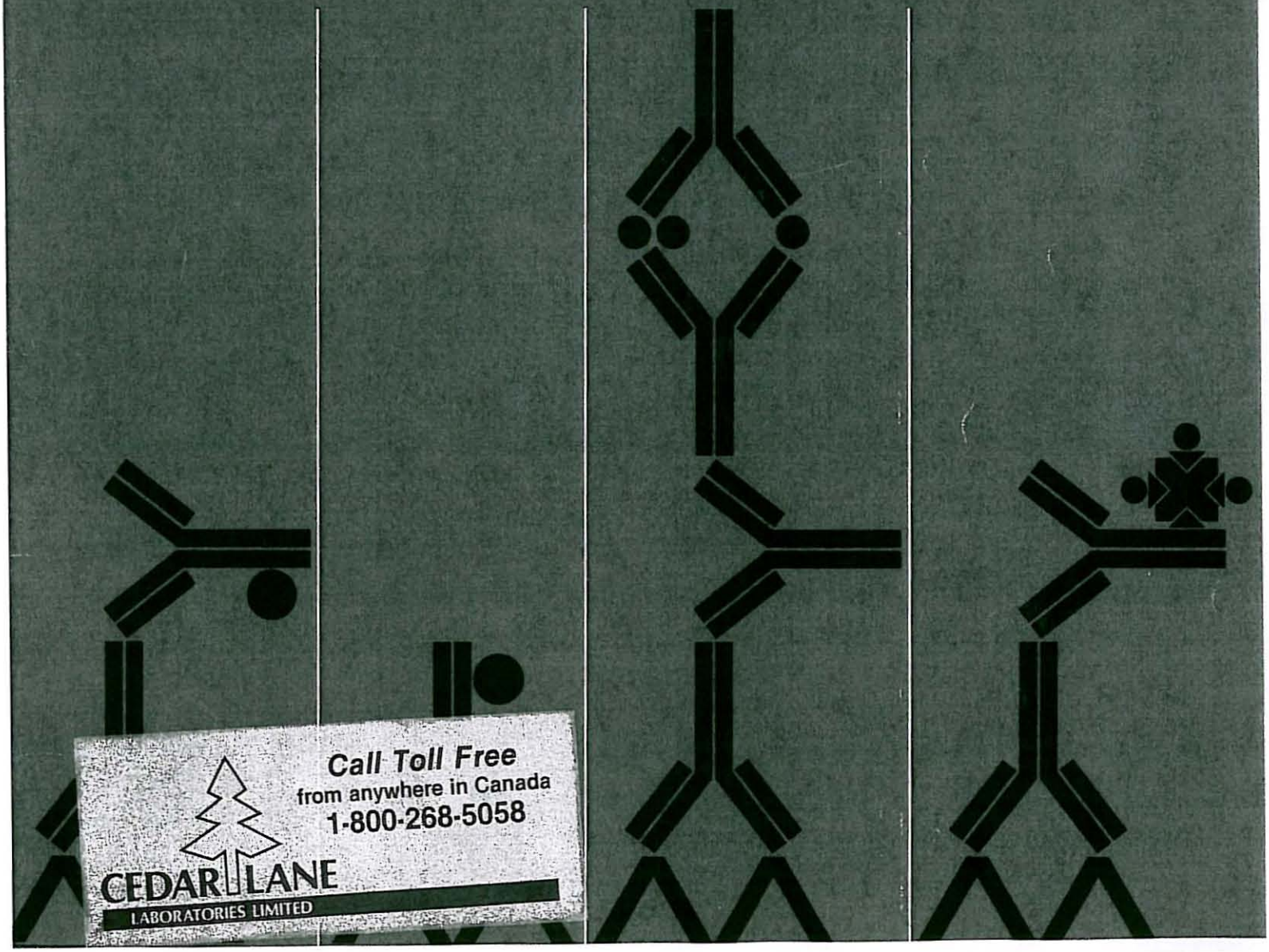

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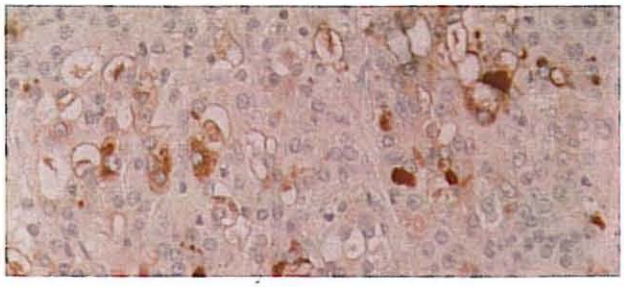
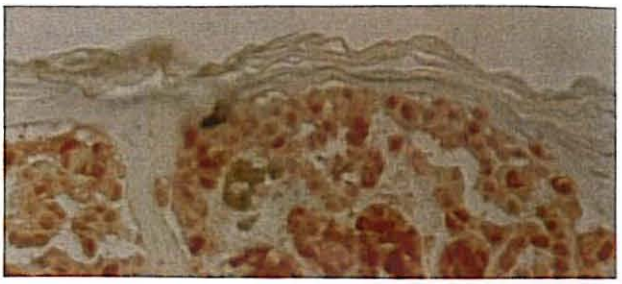
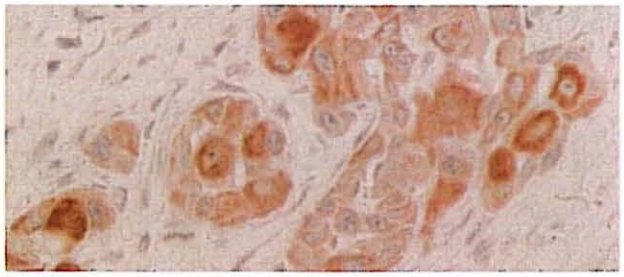


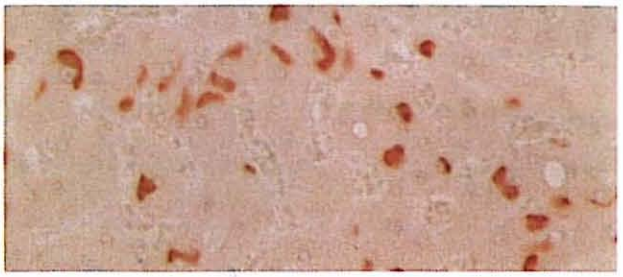
Liver, **AFP**
A liver section from a hepatoblastoma case stains positively for the oncofetal antigen alpha-1-fetoprotein (AFP). A major glycoprotein of the fetus, AFP has been localized in hepatocellular carcinoma and yolk sac tumors of the ovary and testis. AFP is a useful marker for distinguishing these types of tumors from other neoplasms.



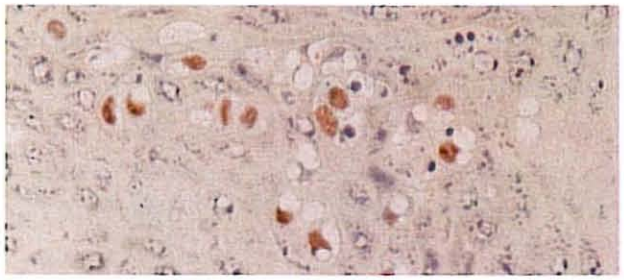
Skin, **S-100**
A skin biopsy from a case of superficial, spreading, malignant melanoma showing intense positive staining for S-100 protein. S-100 is a nervous system associated protein that is a useful marker for the identification of melanocyte derived tumors such as nevi and melanoma, regardless of melanin content. It can also aid in distinguishing poorly differentiated melanoma from tumors of obscure histological origin.



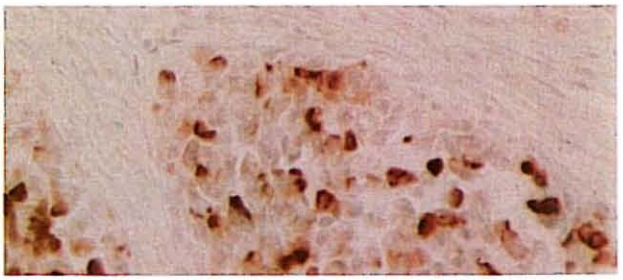
Skin, **Keratin**
A skin biopsy from a patient with well differentiated squamous cell carcinoma shows positive staining for keratin. The variation in stain intensity is due to the difference in the amount of keratin produced by the individual tumor cells. Keratin is a useful marker for establishing the epithelial nature of primary and metastatic tumors, especially for those exhibiting uncharacteristic morphology.



Liver, **HBsAg**
Hepatitis B surface antigen (HBsAg) representing the capsular material of the hepatitis B virus can be localized in the cytoplasm of infected liver cells. HBsAg can be detected in cases of type B viral hepatitis, chronic carriers, cirrhosis, and hepatocellular carcinoma. The results of immunoperoxidase staining are usually more intense and easier to interpret than those achieved by using an orcein stain.



Skin, **Papillomavirus**
Immunoperoxidase staining for papillomavirus shows localization in the nuclei of infected cells present in this skin biopsy. Papillomavirus can be identified in a variety of proliferative squamous lesions as well as in many cases of cervical dysplasia. Some types of papillomavirus have the ability to undergo malignant transformation especially in immunosuppressed patients. Indications of papillomavirus infection have been found in a high percentage of women with cervical neoplasia.



Colon, **PSA**
Positive staining for prostate specific antigen (PSA) identifies this colon tumor as being metastatic from the prostate. All primary and metastatic prostatic carcinomas show positive staining for PSA regardless of their morphological differentiation, while nonprostatic malignancies do not stain. PSA is a useful tool in the identification of tumors of prostatic origin.

The formalin fixed, paraffin embedded tissue specimens used for these photographs were all stained using the peroxidase-antiperoxidase (PAP) technique. The chromagen, 3-amino-9-ethylcarbazole, produces a red end product that precipitates at the site of the antibody reaction. Positive cells will therefore stain red, while negative cells and nuclei will appear blue due to the hematoxylin counterstain.

Note additional photographs on inside back cover.

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INTRODUCTION

With the introduction of immunochemical techniques into the routine histology laboratory, a *new era* of tissue staining evolved. These very sensitive and specific methods, utilizing antigen-antibody complexes, allow visualization of previously undetectable cell components. This handbook will guide you through the maze of scientific knowledge necessary to perform immunoperoxidase staining techniques.

We will start at the beginning by explaining exactly what immunochemicals are, and what they can do. Once we have an understanding of the various methods that are used, we can then outline step-by-step procedures; discuss controls, fixation and processing of specimens; and provide special hints to achieve successful staining. An entire chapter is devoted to troubleshooting, for those special problems. A section on stain interpretation and reference list will aid in the evaluation of the finished product.

Immunoperoxidase staining is an important and useful tool in the identification of a wide variety of cell products. *This handbook contains all the necessary information to assist you in making these techniques an integral part of your laboratory routine.*

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ANTIGENS AND ANTIBODIES

It is necessary to have a basic knowledge of the building blocks of immunology (antigens and antibodies) to more fully understand immunoperoxidase methods.

Antigens

Antigens have two main properties. The first is immunogenicity, which is the ability to induce antibody formation. The second property is specific reactivity, which means that the antigen can react with the antibody it caused to be produced. The reaction between an antigen and its antibody is one of the most specific in biology, and is the reason that immunohistochemical reactions are more precise than ordinary histochemical techniques.

An antigen then, is a substance foreign to the host which stimulates formation of a specific antibody and which will react with the antibody produced. This reaction involves the formation of immune complexes comprised of several antigen and antibody molecules. These complexes may become very large and form precipitates which can be measured by various techniques.

Antibodies

An antibody is a serum protein that is formed in response to exposure to an antigen, and reacts specifically with that antigen to form immune complexes either in the body or in the laboratory. Antibody production is a response by the body to foreign material (an antigen), and is designed to rid the body of this invader.

Antibodies are contained in the gamma globulin fraction of serum, and are often called immunoglobulins (Ig). They can be divided into five classes based on their size, weight, structure, function, and other criteria. The classes are IgA (immunoglobulin A), IgD, IgE, IgG, and IgM. Antibody solutions utilized in immunohistochemical staining contain mostly IgG type antibodies, with lesser amounts of the other classes.

Antibody Structure

Structurally, an antibody is made up of two kinds of protein chains—heavy and light chains. Immunoglobulins are named for their heavy chains, so the IgG molecule in Figure 1 will have heavy chains of the gamma (γ) type. An IgA antibody has alpha (α) heavy chains; IgD, delta (δ) heavy chains; IgE, epsilon (ε) heavy chains; and IgM has mu (μ) heavy chains. A primary antibody for immunoperoxidase staining that is "specific for gamma chains" will localize the heavy chain of an IgG molecule.

There are only two types of light chains common to all five groups: kappa (κ) and lambda (λ). An IgG molecule has two identical light chains, either two kappa chains or two lambda chains (Figure 2). A single antibody can never have both kappa and lambda chains. This is important when discussing the interpretation of light chain staining in lymphoma cases.

The orientation of an IgG antibody is shown in Figure 3. The Fc portion stands for *fragment crystalline*, and will crystallize out upon purification. This region is involved in complement fixation and transfer of antibody across the placenta. The remaining portions are called the *fragment antigen binding* or Fab regions. These are the parts of the antibody molecule capable of specifically binding to the antigen. This IgG molecule has the ability to bind two antigen molecules, one at each Fab site.

Antibody Production

In order to produce an antibody for laboratory use, it is first necessary to purify an antigen. A source for the antigen such as serum, urine or tissue is subjected to a combination of procedures including precipitation, centrifugation, dialysis, chromatography and electrophoresis to obtain a highly purified antigen. The antigen is then injected into an animal of different species than that of antigen source. The animal will identify the antigen as foreign matter, and produce an antibody directed specifically against it. Antibody production begins within twenty minutes after injection, although a measurable quantity of antibody cannot be detected for 5-10 days. Small blood samples are usually obtained and pooled at two week intervals. Booster injections of antigen are often administered every month to promote consistent antibody production.

The choice of an animal for injection depends upon the antigen used, housing facilities available, amount of antibody needed, and personal preference. Usually several animals of the species chosen will be injected with an antigen, as each animal will vary in how it responds to the antigen, and in the amount of antibody it produces. After several bleedings are pooled, contaminants present must be removed. This is usually accomplished by either liquid or solid phase antigen absorption techniques.

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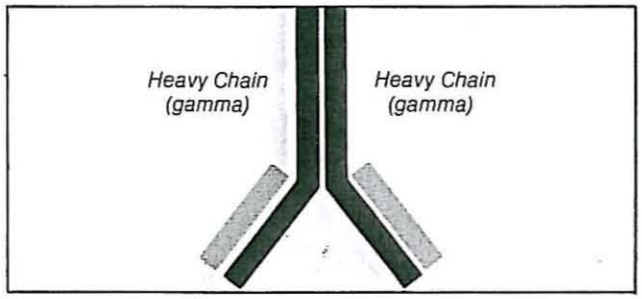

Figure 1. *IgG molecule showing paired heavy chains of gamma type.*

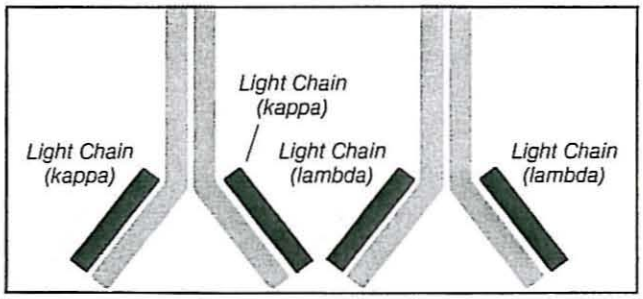

Figure 2. *IgG molecules showing only possible light chain configurations.*

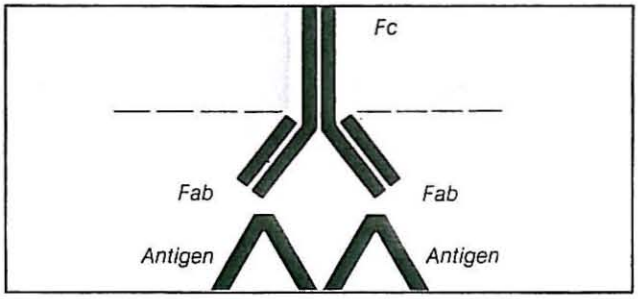

Figure 3. *Division of IgG molecule into Fc and FAb fragments. Antigen binding occurs at the two FAb sites.*

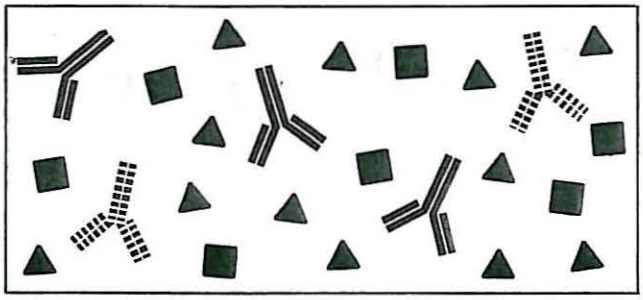

Figure 4. *Whole serum antibody solution containing all normal animal serum components in addition to specific antibody.*

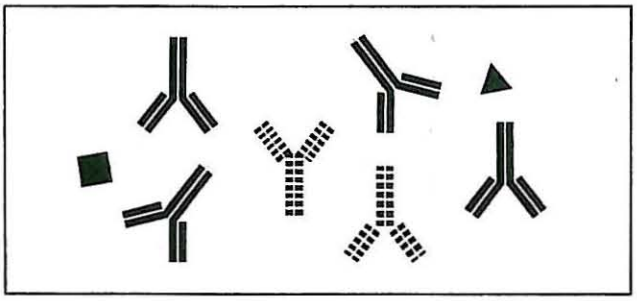

Figure 5. *Immunoglobulin fraction of serum containing only antibodies.*

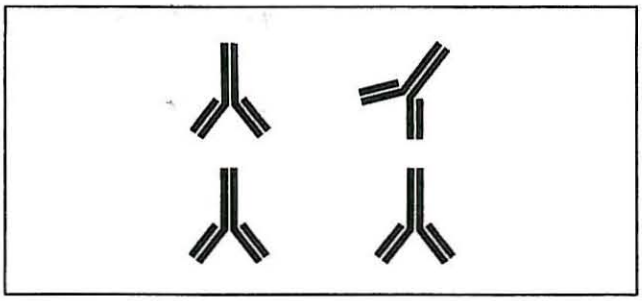

Figure 6. *Antigen specific antibody is more specific than usually necessary.*

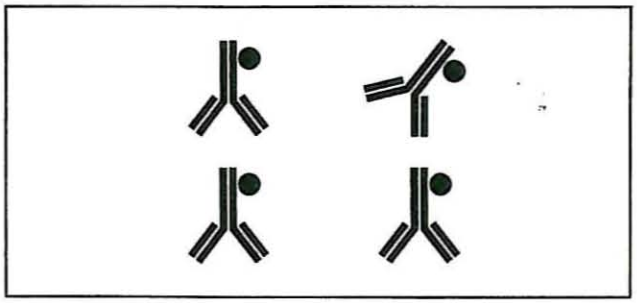

Figure 7. *A conjugate combines an antibody and some type of visual marker.*

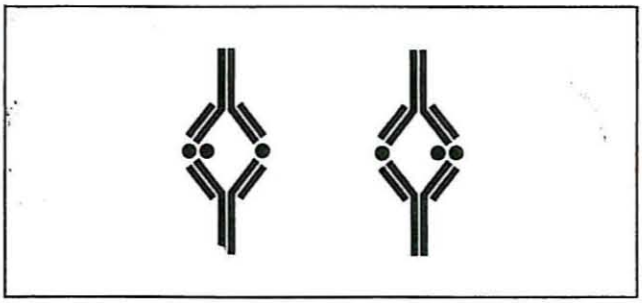

Figure 8. *A peroxidase-antiperoxidase immune complex formed by natural affinities between antigens and antibodies.*

Key *to Figures 4 through 8.*

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Types of Antibody Solutions

There are several antibody preparations available for use in immunoperoxidase procedures. The easiest to produce, and therefore the most common and least expensive, is whole serum. Animal blood containing the antibody is centrifuged to separate the cells from the serum, and any contaminating antibodies are absorbed out.

A whole serum solution is pictured schematically in Figure 4. It will contain antibodies specific for the antigen the animal was immunized with. Other antibodies which are products of the animal's normally functioning immune system will also be present. These should not interfere with staining procedures. The bulk of the whole serum fraction is made up of ordinary serum components such as enzymes, electrolytes, and serum proteins. Occasionally, these other serum elements can cause unwanted background staining in some techniques. This is due to the affinity of serum proteins, most notably albumin, alpha and beta globulins, for certain tissue components.

Since the only element necessary for immunoperoxidase methods is antibody, all other serum components can be eliminated. This type of preparation, called an immunoglobulin or Ig fraction, is depicted in Figure 5. This solution contains mostly antibodies, both specific and naturally occurring, plus a very small amount of residual serum protein. The removal of the majority of proteins will reduce the chances of nonspecific reactions in various techniques.

It is possible, as shown in Figure 6, to prepare a solution containing only antibodies directed against a specific antigen. This is called *antigen specific antibody*. It is not commonly available, and has greater specificity than is necessary for most procedures.

A fourth type of preparation, and one which is readily available, is conjugated antibody (Figure 7). Conjugation is the process of chemically linking some type of marker onto an antibody molecule. This can be a fluorescent label such as fluorescein and rhodamine, or an enzyme such as alkaline phosphatase or horseradish peroxidase. A wide variety of conjugates are available for use in various direct and indirect immunohistological stains.

Unfortunately, in the chemical process of conjugation, small amounts of antibody and label can be destroyed. This can decrease the sensitivity and specificity of these reagents. An alternative to artificially combining a marker to an antibody is an immune complex—the combination of an antigen and its specific antibody utilizing the natural affinity they have for one another. These complexes are specially prepared to remain soluble and not to form precipitates in solution. An example of this is the peroxidase antiperoxidase (PAP) complex which consists of the enzyme peroxidase (the antigen) and an antibody specific for peroxidase (Figure 8). The use of these naturally formed immune complexes instead of chemical conjugates, makes PAP staining procedures as much as 1,000 times more sensitive than immunofluorescence.

Monoclonal Antibodies

A single antigen molecule contains several characteristic antigenic determinants or epitopes. When an antigen is injected into an animal as described previously, the B-lymphocytes will make antibodies against the antigen. One B-cell can form antibodies against only one antigenic epitope. Since there are many B-cells producing antibodies against each epitope, this is called a polyclonal (many cells) antibody.

In some techniques it is desirable to have an antibody specific for a single epitope. Since this is produced by a single B-cell line (called a clone) it is termed a monoclonal (single cell) antibody.

The selection of the B-cell clone producing the desired antibody must be performed outside the animal. However, these cells cannot grow and divide once removed from their host, and will die in approximately one week. In order to preserve the antibody production capabilities of the clone, the B-cell is fused with a myeloma cell (a cancerous B-cell) that can live almost indefinitely outside the host animal. The hybrid myeloma cell (hybridoma) that is formed by this fusion can be grown in cell culture as can the myeloma cell, and will produce the antibody that was being made by the B-cell.

To obtain a consistent supply of monoclonal antibody, the hybridoma formed must be stable. First, the B-cell and the myeloma cell must come from the same animal species. The myeloma cell must be suitable for hybridizing and be easily propagated in culture. A large number of B-lymphocytes must be available for fusion. The animal that most readily fulfills these criteria is the mouse.

The first step in producing a monoclonal antibody is identical to that of making a polyclonal antibody (Figure 9). A mouse is injected with a purified antigen, and will begin making antibody against it. When large amounts of antibody are being produced, the mouse is sacrificed and the spleen, containing large quantities of B-lymphocytes, is removed. A cell suspension is made and is mixed with the myeloma cells in a medium that will cause the cells to fuse. Unfused and improperly fused cells will die, while the desired hybridomas will live and grow in culture. The hybridomas are tested to determine which clone is producing antibody against the desired epitope. This is the most difficult and time-consuming part of the procedure.

Once the appropriate cell line is identified, it can be injected back into a mouse where it will produce a tumor. The ascitic fluid from the tumor will contain high concentrations of the antibody, as well as other mouse immunoglobulins and proteins which can cause increased background staining in immunoperoxidase techniques.

Another method of producing monoclonal antibodies is to grow the hybridoma in tissue culture. The supernatant fluid will contain the antibody produced by the hybridoma. Culture supernatants contain lower concentrations of antibody than ascitic fluid, but nonspecific background staining due to undesired proteins is eliminated.

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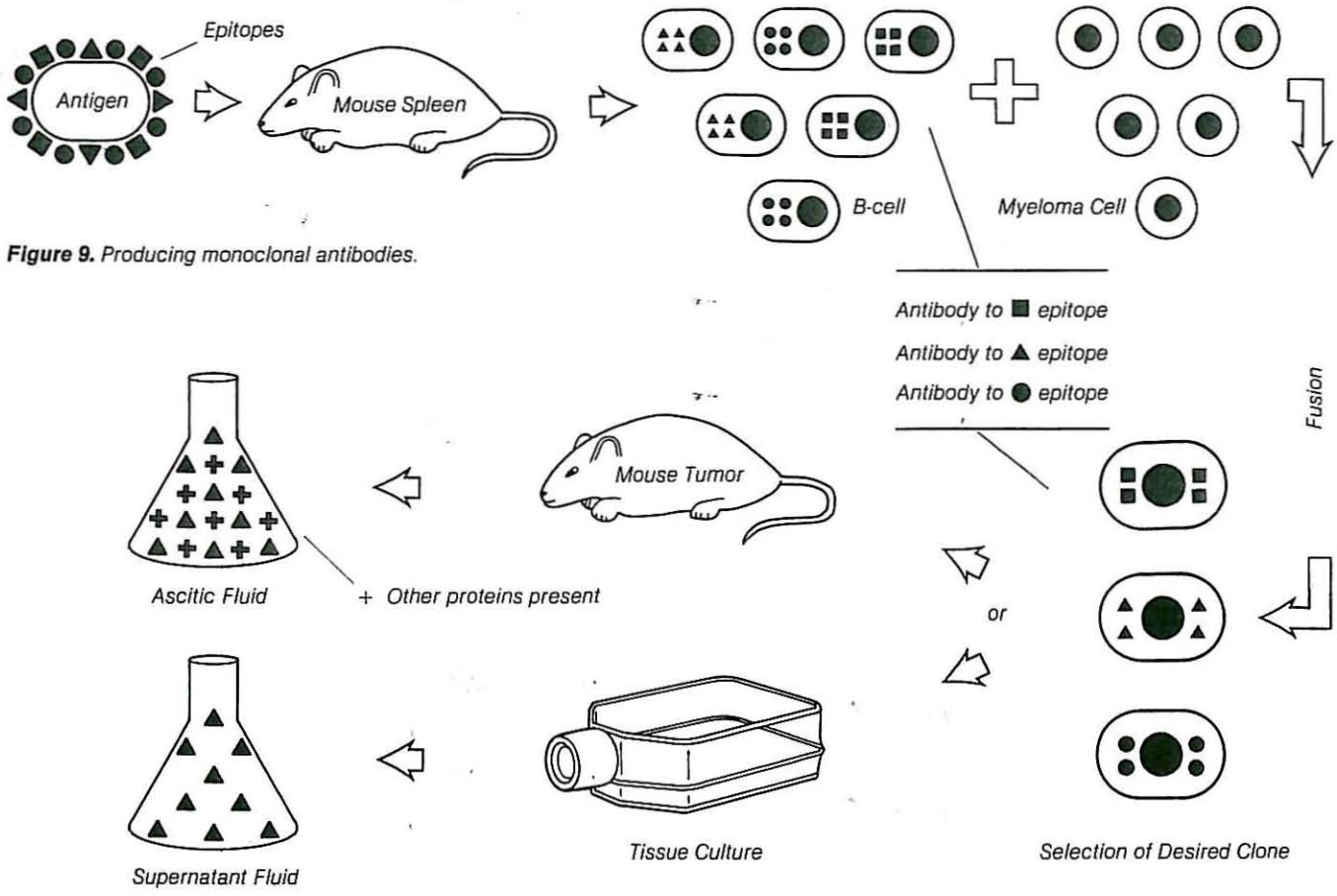

Figure 9. *Producing monoclonal antibodies.*

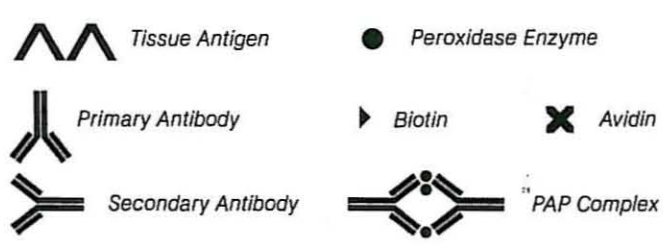

Figure 10. *Direct Method.*

Figure 11. *Indirect Method.*

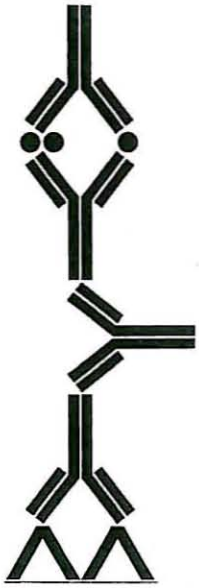

Figure 12. *PAP Method.*

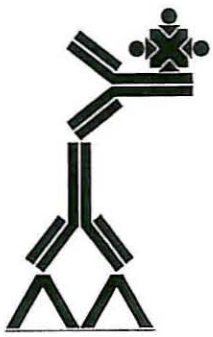

Figure 13. *Avidin-Biotin Method.*

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STAINING METHODS

There are four main methods of immunoperoxidase staining that can be used to localize cellular antigens. The direct, indirect, PAP, and avidin-biotin methods each have certain advantages and disadvantages which must be evaluated prior to selection of the most effective procedure for the work to be performed.

Immunoperoxidase procedures allow visualization of cell components in a variety of specimens including paraffin sections, cryostat sections, smears, imprints and cytospins. For a specific antigen it can be determined what type of cells produce this substance in normal and neoplastic tissue, levels of the substance produced, the identification of cells of unknown origin, and the determination of tumor cell differentiation.

Peroxidase

Immunoperoxidase staining involves the use of antibodies and the enzyme peroxidase. Peroxidase is commonly used for several reasons:

- Its small size will not hinder the binding of antibodies to adjacent sites.
- It is easily obtainable in highly purified form so that the chance of contamination is minimized.
- It is very stable, and therefore will remain unchanged during manufacture, storage and application.
- Only small amounts are present in tissue specimens, and this endogenous peroxidase activity is easily quenched.
- There is a wide availability of chromagens which can be acted upon by peroxidase to form a colored end product that will precipitate at the site of the antigen to be localized.
- It is inexpensive.

Direct Method

The simplest way to localize a certain antigen is by using an antibody directed specifically against it. In the direct immunoperoxidase method this specific antibody is chemically linked to peroxidase. The conjugated reagent is applied to the specimen, and will react with the antigen (Figure 10). A substrate is then applied which will produce a colored end product precipitating at the site, and thus mark the localized antigen.

Direct technique can be performed very quickly, with low probability of nonspecific reactions. The main drawback is that for every antigen to be localized, a different conjugated antibody is needed. If the antibody cannot be obtained in conjugated form, then the user must either perform the conjugation himself, or choose another procedure.

The most common application of the direct immunoperoxidase method is for the detection of immunoglobulin, complement and immune complex deposits in kidney biopsies from patients with various types of renal disease. These same antigens can also be localized in skin biopsies from cases of systemic lupus erythematosus (SLE) and other connective tissue disorders. The most common antigens identified in these cases are IgG, IgA, IgM, C3 and C4.

Indirect Method

In the indirect application, an unconjugated antibody will bind to the antigen in the specimen. To localize this attachment, a peroxidase conjugated antibody is needed to bind to the first antibody. For example, if the primary antibody was made in a rabbit, then the conjugated secondary antibody must be specific for rabbit antibody. A substrate is added to localize the reaction (Figure 11).

This method is more versatile than the direct method because a variety of primary antibodies made in the same animal species can be used with one conjugated secondary antibody. Therefore, this procedure can be used to advantage with any primary antibody when a peroxidase conjugated second antibody is available. However, the procedure takes approximately twice as long to complete as the direct method, and there is greater chance of nonspecific reactions occurring.

The primary use for the indirect immunoperoxidase technique is to identify antibodies in the serum of patients with various autoimmune, bacterial and parasitic diseases. In this procedure, the patient's serum is applied to the antigen containing specimen in place of the primary antibody. The peroxidase conjugated secondary antibody is specific for human immunoglobulin. If the patient has an antibody that can react wth the antigen in the specimen, the peroxidase conjugated anti-human immunoglobulin will bind to the patient antibody, and show positive staining for the antigen. If the patient's

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serum contains no antibody to that antigen, the secondary antibody cannot bind, and no staining of the antigen will be seen. Some of the more common patient antibodies identified are against nuclear, thyroidal, mitochondrial, and smooth muscle antigens; treponema pallidum; herpes simplex virus; and cytomegalovirus.

PAP Method

This method utilizes three reagents: Primary and secondary antibodies, and PAP Complex—comprised of the enzyme peroxidase and an antibody against peroxidase. The primary antibody is specific for the antigen. The secondary or "link" antibody is capable of binding to both the primary and to the PAP Complex, because both are produced in the same animal species.

Functionally, the link antibody is added in excess so that only one of its Fab sites will bind to the primary, leaving the other Fab site free to bind to the antibody in the PAP Complex. The peroxidase enzyme is visualized via a substrate-chromagen reaction (Figure 12).

Absence of conjugated antibodies in this method means greater sensitivity than that attributed to the direct and indirect techniques. This is especially evident in formalin fixed, paraffin embedded tissues where strong staining can be observed even though much of the antigen has been destroyed by fixation and processing. Due to this loss of antigen during fixation, the direct and indirect techniques must be performed on frozen sections to achieve consistent results. The greater flexibility of the PAP method in specimen processing seems to compensate for the increased time required by this method.

One of the most important applications of the PAP method is in determining the origin of tumors by identifying specific antigens the cells produce. This allows for more accurate classification—especially of poorly differentiated and metastatic tumors—than can be achieved on the basis of morphology alone. The fact that the PAP method is applicable to routinely fixed, paraffin embedded material circumvents the need for frozen tissue and also permits retrospective studies.

Some of the tumor markers commonly used are prostate specific antigen (PSA) to identify tumors of prostatic origin; immunoglobulins such as kappa and lambda light chains, IgG, IgA and IgM to distinguish B-cell lymphomas from undifferentiated carcinomas; glial fibrillary acidic protein (GFA) which stains all tumors of glial origin, both primary and metastatic; and human chorionic gonadotrophin (HCG) which can be localized in normal trophoblastic cells and the trophoblastic element of germ cell tumors of the ovary, testis and extragonadal sites.

Avidin-biotin Method

This method is based on the ability of the egg-white glycoprotein avidin to nonimmunologically bind four molecules of the vitamin biotin. As with the PAP method, three reagents are used. The first is a primary antibody specific for the antigen to be localized. The secondary antibody, capable of binding to the first, is conjugated to biotin. The third reagent is a complex of peroxidase conjugated biotin and avidin. The free sites of the avidin molecule allow binding to the biotin on the secondary antibody. The peroxidase enzyme, and therefore the original antigen, are visualized with an appropriate chromagen (Figure 13).

Even though conjugated antibodies are used in this method, the strong affinity of avidin for biotin gives this method greater sensitivity than other conjugated antibody techniques such as the direct and indirect methods. Like the PAP method, excellent results can be achieved on fixed, paraffin embedded specimens.

The avidin-biotin technique represents one of the most recent developments in immunoperoxidase staining. It lends itself to the localization of numerous antigens in a variety of specimens. Much of the early work with this method has been in the identification of pituitary hormones in normal and neoplastic tissue. Other uses include staining for cell surface markers, lectin binding sites, viral proteins and intermediate filaments.

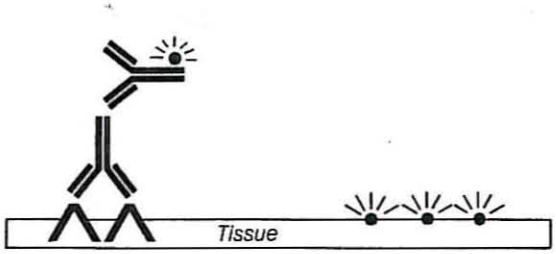

Figure 14. *Peroxidase activity already present in the tissue will show staining if not inhibited before antibody application.*

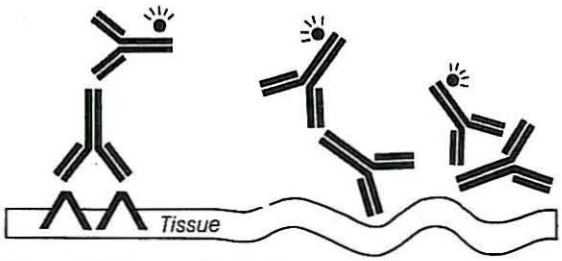

Figure 15. *Nonspecific binding of primary antibody to charged collagen and connective tissue, with subsequent immunoperoxidase staining, can be avoided by pretreatment of the specimen with animal serum.*

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Endogenous Peroxidase Activity

The substrate-chromagen reaction used to visualize peroxidase cannot distinguish between the enzyme immunologically localizing the cellular antigen, and similar enzymatic activity present in the specimen before staining. This endogenous peroxidase activity is confined mostly to red and white blood cells. If it is not removed before adding the marking enzyme, positive staining will be observed that is due not to the specific antigen alone, but also due to peroxidase activity already present in the specimen (Figure 14).

There are several ways to irreversibly inhibit endogenous peroxidase, and one of these techniques should be performed at the beginning of the staining procedure. The quickest and easiest technique is to place 4 to 6 drops of 3% hydrogen peroxide directly on the specimen, and incubate for five minutes before rinsing off.

Specimens containing large numbers of blood cells such as cytospins and blood smears will show a violent reaction with hydrogen peroxide due to the large amount of peroxidase activity present. The bubbling and fizzing that occurs can damage the cellular morphology, so 3% hydrogen peroxide should NOT be used on these specimens. A similar reaction will also occur when 3% hydrogen peroxide is placed on cryostat sections embedded in O.C.T.

Consequently, for cytospins, blood smears and frozen sections, a different procedure must be used. These specimens should be placed in a bath of hydrogen peroxide and methanol for twenty minutes. The bath of 200 ml absolute methanol plus 50 ml of 3% hydrogen peroxide must be made up fresh just before use. The formula amount can be scaled up or down depending on the number of specimens to be tested.

There are various other solutions that can be used in place of hydrogen peroxide-methanol. Slides can be placed in a bath of 100 ml of concentrated ethanol containing 0.2 ml of concentrated HCl for 15 minutes. A bath of absolute methanol with 1% sodium nitroferricyanide and 0.2% acetic acid can also be used. A slightly longer procedure is to incubate the slides in 0.1% phenylhydrazine for one hour at 37°C. A two-step procedure involves incubation with 0.01% periodic acid for ten minutes followed by a solution of 0.1 mg sodium borohydride/1 ml of water for ten minutes to reduce any aldehydes produced by the periodic acid.

Most tissues do not contain large amounts of blood cells or endogenous peroxidase, and activity is usually confined to blood vessels. In these specimens the incubation with hydrogen peroxide can be omitted, although when interpreting the staining patterns this must be taken into consideration. In specimens that do contain many blood cells—such as bone marrow, placenta and spleen—removal of endogenous peroxidase is essential to correct interpretation. These specimens also serve as excellent controls when evaluating the effectiveness of new lots of hydrogen peroxide.

Nonspecific Background Staining

Positive staining of a specimen that is not a result of antigen-antibody binding is termed nonspecific background staining. The most common cause is attachment of protein to highly charged collagen and connective tissue elements of the specimen. Antibodies are proteins. If the first protein solution applied to the tissue is the primary antibody, it can be nonspecifically adsorbed to these charged sites. The secondary antibody can still bind to the primary and the peroxidase color reaction will occur. Positive staining of these sites is due not to localization of the tissue antigen, but to nonspecific antibody attachment to collagen and connective tissue (Figure 15).

The most effective way to prevent this nonspecific staining is to add an innocuous protein solution to the specimen before applying the primary antibody. This protein will fill the charged sites, leaving no room for adsorption of the primary antibody. The most common source of the protein solution is nonimmune serum from the same animal species that produced the secondary antibody. This avoids positive staining due to binding of the secondary antibody to components in the protein solution.

The nonimmune serum is used fairly concentrated at dilutions of 1:5 to 1:20. Addition of 2-5% bovine serum albumin (BSA) will increase the protein concentration and further reduce the nonspecific staining. The nonimmune serum is applied to the specimen and allowed to remain for 10-20 minutes. The slide is not rinsed, but excess serum is tapped off leaving a thin layer coating the tissue when the primary antibody is applied. Care must be taken that only a small amount of the often viscous serum remains or the primary antibody will be diluted, resulting in pale staining of the antigen.

As with endogenous peroxidase activity, not all specimens exhibit appreciable nonspecific binding. The incubation with nonimmune serum can be eliminated in these specimens as long as it does not interfere with interpretation. Other causes of nonspecific background staining such as inappropriate antibody dilutions, incomplete removal of paraffin, improper rinsing of slides and incorrect substrate incubation will be discussed in upcoming sections.

It is important that the serum used to block nonspecific binding does not exhibit any evidence of hemolysis. When red blood cells break open (hemolyse), they release their contents into the surrounding serum. Even though the cells are subsequently separated from the serum, the products of hemolysis remain. These red cell components can react with the peroxidase substrate and give nonantigen-specific positive staining. All serum used in immunoperoxidase techniques should be carefully screened to ensure that no hemolysis has occurred.

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STAINING PROCEDURES

This section contains step-by-step instructions to perform the four immunoperoxidase staining techniques described previously. A general discussion of dilutions is followed by detailed instructions for making up buffers, substrate and counterstain solutions. The topics of incubation times and enzyme digestion are also discussed.

Introduction to Techniques

Procedure outlined herein for each staining technique represents only one possible use for the method. Suggested antibodies and dilutions are given, but optimal dilutions must be determined in each laboratory for *their* reagents and conditions. (Dilution techniques are discussed in greater detail in the following sub-section.)

Appropriate care must be taken when handling slides to avoid loss of specimens. After each antibody incubation, the slides should be thoroughly rinsed to remove unbound antibody. The most convenient and effective way to accomplish this is by using a wash bottle filled with an appropriate buffer. The buffer should not be aimed directly onto the specimen, but rather squirted above and allowed to run down over it (Figure 16). The slide is then placed in a bath of the same buffer to complete the rinsing process.

Upon removal of slides from the buffer bath, excess liquid is wiped off to avoid unwanted dilution of the subsequent reagent. This procedure should be carefully performed using a neatly folded, absorbent tissue. Touching the specimen with the wipe will damage and loosen it, and must be avoided (Figure 17). The specimen itself must never be allowed to dry out or uninterpretable staining will result.

Consequently, when processing a large number of specimens, only 3-5 slides should be wiped at one time before applying the appropriate solution.

After removal of excess buffer, the slides are laid flat with the specimen facing upward. Enough drops of diluted antibody are applied to completely cover the specimen without flooding the slide (Figure 18). To avoid evaporation of antibody in drafty laboratories, the slide should be placed in a humidity chamber. Specially designed staining boxes can be purchased from suppliers, or a simple incubation chamber can be made in the laboratory as follows:

Place a damp paper towel along the edge of a large, flat dish. Arrange the slides in the dish and apply antibody solution. Cover the dish with aluminum foil, a book or a piece of wood for the required incubation time (Figure 19). Plastic food storage containers with tight fitting lids come in a variety of sizes and make inexpensive humidity chambers.

Before beginning the staining process, the slides should be labelled with the antigen to be localized, and the specimen circled. Encircling the specimen serves several purposes.

- The specimen, most notably cell smears, is not always clearly visible once it has been hydrated. By encircling the specimen, antibody solution can be applied easily to the correct area.
- The circle serves as a guide when wiping away excess liquid to help prevent the specimen from being accidentally wiped off.
- Encircling creates surface tension and acts as a barrier to hold antibody solutions to the specimen and prevent flooding of the slide.

The most effective method of encircling a specimen is with a diamond pencil. Marking pens and wax pencils often wash off during the procedure (Figure 20).

Direct Technique

The following procedure outlines the localization of complement component C3 in a cryostat section of a kidney biopsy. All incubations are carried out at room temperature.

1. Place a 4 micron cryostat section of a kidney biopsy into a freshly made hydrogen peroxide-methanol bath for 20 minutes.
2. Place slide in a buffer bath for 5 minutes.
3. Remove slide from bath and carefully wipe away excess liquid from around the section.
4. Lay the slide flat and apply 4-6 drops of normal serum diluted 1:20. Incubate 20 minutes.
5. Tap off serum, and wipe away excess.
6. Lay the slide flat and apply 4-6 drops of peroxidase conjugated antibody to human C3 diluted approximately 1:5-1:20. Incubate 20 minutes.
7. Gently rinse slide with buffer from a wash bottle.

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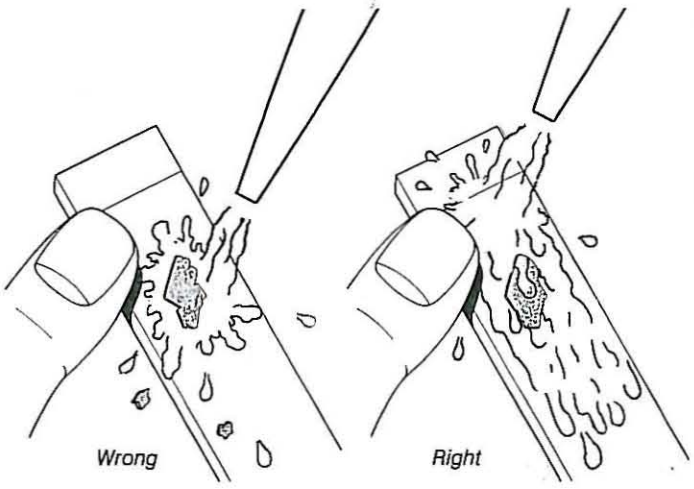

Figure 16. *Rinsing the specimen.*

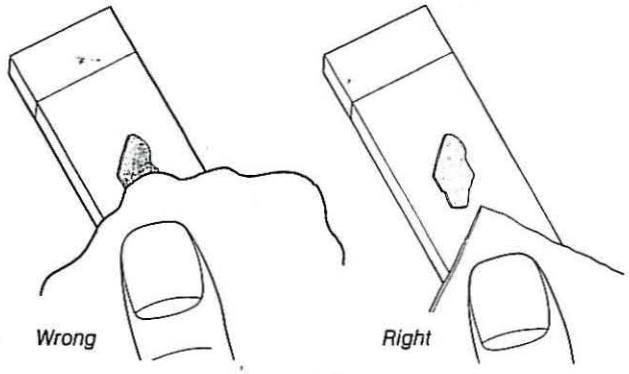

Figure 17. *Wiping away excess liquid from around the specimen.*

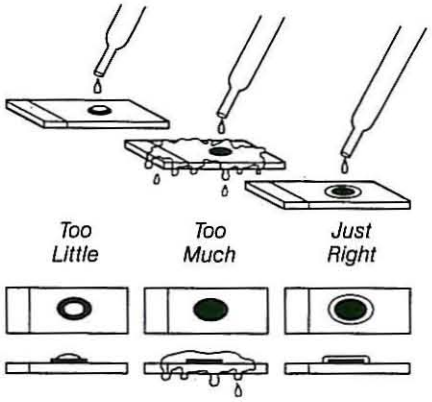

Figure 18. *Application of antibody solutions.*

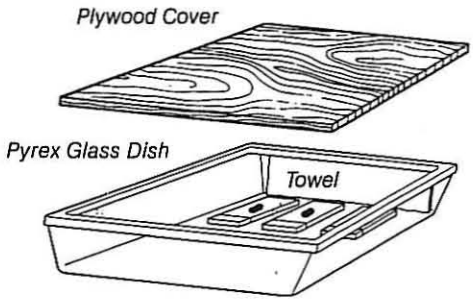

Figure 19. *An easy humidity chamber. Place damp towel as indicated.*

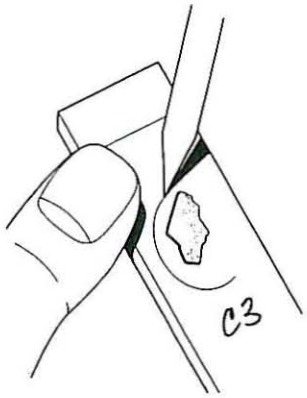

Figure 20. *Circling and labeling the specimen with a diamond pencil.*

8. Place slide in a buffer bath for 10 minutes.
9. Remove slide from bath and carefully wipe away excess liquid from around the section.
10. Apply substrate solution to give colored end product. Counterstain and coverslip.

When viewing the specimen under the microscope, positive staining for C3 appears as deposits along the glomerular basement membrane. The type and pattern of the deposits can be used to differentiate between various renal diseases.

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Indirect Technique

A smear from a herpetic lesion is stained for herpes simplex virus Type 2 by the indirect immunoperoxidase method. All incubations are carried out at room temperature.

1. Fix a freshly made lesion smear in acetone for 10 minutes.
2. Place slide in a buffer bath for 5 minutes.
3. Remove slide from bath and carefully wipe away excess liquid from around the smear.
4. Lay the slide flat and apply 4-6 drops of 3% hydrogen peroxide. Incubate 5 minutes.
5. Gently rinse slide with buffer from a wash bottle.
6. Place slide in a buffer bath for 5 minutes.
7. Remove slide from bath and carefully wipe away excess liquid from around the smear.
8. Lay the slide flat and apply 4-6 drops of normal swine serum diluted 1:5. Incubate 20 minutes.
9. Tap off serum, and wipe away excess.
10. Lay the slide flat and apply 4-6 drops of rabbit antibody to herpes simplex virus Type 2 diluted approximately 1:50-1:150. Incubate 20 minutes.
11. Gently rinse slide with buffer from a wash bottle.
12. Place slide in a buffer bath for 10 minutes.
13. Remove slide from bath and carefully wipe away excess liquid from around the smear.
14. Lay the slide flat and apply 4-6 drops of peroxidase conjugated swine antibody to rabbit immunoglobulins diluted approximately 1:20-1:80. Incubate 20 minutes.
15. Gently rinse slide with buffer from a wash bottle.
16. Place slide in a buffer bath for 10 minutes.
17. Remove slide from bath and carefully wipe away excess liquid from around the smear.
18. Apply substrate solution to give colored end product. Counterstain and coverslip.

Cells in the lesion smear infected with herpes simplex virus will show positive cytoplasmic staining, and possibly some staining in the nucleus. Some background staining is often seen in the cellular debris.

PAP Technique

Prostate specific antigen can be localized in routinely fixed, paraffin embedded tissue by the peroxidase anti-peroxidase procedure. All incubations are carried out at room temperature.

1. Deparaffinize a 4 micron, fixed section of normal prostate tissue. Rehydrate through graded alcohols to water.
2. Remove slide from water and carefully wipe away any excess liquid from around the section.
3. Lay the slide flat and apply 4-6 drops of 3% hydrogen peroxide. Incubate 5 minutes.
4. Gently rinse slide with buffer from a wash bottle.
5. Place slide in a buffer bath for 5 minutes.
6. Remove slide from bath and carefully wipe away excess liquid from around the section.
7. Lay the slide flat and apply 4-6 drops of normal swine serum diluted 1:5. Incubate 20 minutes.
8. Tap off serum, and wipe away excess.
9. Lay the slide flat and apply 4-6 drops of rabbit antibody to human prostate specific antigen diluted approximately 1:200-1:600. Incubate 20 minutes.
10. Gently rinse slide with buffer from a wash bottle.
11. Place slide in a buffer bath for 10 minutes.
12. Remove slide from bath and carefully wipe away excess liquid from around the section.
13. Lay the slide flat and apply 4-6 drops of swine antibody to rabbit immunoglobulins diluted approximately 1:40-1:100. Incubate 20 minutes.
14. Gently rinse slide with buffer from a wash bottle.
15. Place slide in a buffer bath for 10 minutes.
16. Remove slide from bath and carefully wipe away excess liquid from around the section.
17. Lay the slide flat and apply 4-6 drops of horseradish peroxidase rabbit antiperoxidase (PAP) complex diluted approximately 1:50-1:200. Incubate 20 minutes.
18. Gently rinse slide with buffer from a wash bottle.
19. Place slide in a buffer bath for 10 minutes.

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20. Remove slide from bath and carefully wipe away excess liquid from around the section.

21. Apply substrate solution to give colored end product. Counterstain and coverslip.

In normal prostate tissue, positive staining for prostate specific antigen will be seen in the glandular and ductal epithelium, and also in secretions and concretions.

Avidin-Biotin Technique

Immunoperoxidase staining by the avidin-biotin method can be used to localize various hormones such as ACTH in resin embedded pituitary sections. All incubations are carried out at room temperature.

1. Etch a 1 micron Araldite embedded pituitary section in saturated sodium methoxide for 10 minutes.

2. Rinse section in absolute ethanol.

3. Gently rinse slide with water from a wash bottle.

4. Lay the slide flat and apply 4-6 drops of 2.5% periodic acid in distilled water. Incubate 5 minutes.

5. Gently rinse slide with water from a wash bottle.

6. Lay the slide flat and apply 4-6 drops of freshly made 0.02% sodium borohydride in distilled water. Incubate 2 minutes.

7. Gently rinse slide with water from a wash bottle.

8. Place slide in buffer bath for 5 minutes.

9. Remove slide from bath and carefully wipe away excess liquid from around the section.

10. Lay the slide flat and apply 4-6 drops of normal goat serum diluted 1:20. Incubate 20 minutes.

11. Tap off serum, and wipe away excess.

12. Lay the slide flat and apply 4-6 drops of rabbit antibody to human ACTH diluted approximately 1:500-1:2000. Incubate 20 minutes.

13. Gently rinse slide with buffer from a wash bottle.

14. Place slide in a buffer bath for 10 minutes.

15. Remove slide from bath and carefully wipe away excess liquid from around the section.

16. Lay the slide flat and apply 4-6 drops of biotin conjugated goat antibody to rabbit immunoglobulins diluted approximately 1:200-1:400. Incubate 20 minutes.

17. Gently rinse slide with buffer from a wash bottle.

18. Place slide in a buffer bath for 10 minutes.

19. Remove slide from bath and carefully wipe away excess liquid from around the section.

20. Lay the slide flat and apply 4-6 drops of peroxidase conjugated biotin-avidin complex. The avidin and biotinylated peroxidase are diluted equally (approximately 1:100-1:200) 30 minutes before use. Incubate 20 minutes.

21. Gently rinse slide with buffer from a wash bottle.

22. Place slide in a buffer bath for 10 minutes.

23. Remove slide from bath and carefully wipe away excess liquid from around the section.

24. Apply substate solution to give colored end product. Counterstain and coverslip.

Positive staining for ACTH will be observed in the corticotrophic cells of the pituitary.

Dilutions

To achieve meaningful results using the previously described procedures, optimally diluted antibodies must be used. The dilution of any one antibody will depend upon several factors:

— Concentration of specific antibody in the solution is called the antibody titer. The more specific antibody molecules per milliliter, the higher the titer and the higher the working dilution.

— A number of substances other than specific antibody are often present in the solution. High levels of contaminating proteins necessitate high dilutions to prevent nonspecific background staining.

— Generally the longer the incubation time, the more dilute the antibody can be used. In the methods described, antibody incubation times were held constant at twenty minutes. By increasing the time to one hour, a higher dilution can be utilized.

— Other considerations such as the choice of diluent buffer, use of a humidity chamber, the specimen fixation and processing protocol—all will affect the dilution used. Optimal dilutions must be determined by each laboratory under its own special conditions.

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In immunoperoxidase staining a tissue antigen is localized via a system of specific antibody solutions. The concentration of the antigens and antibodies present is critical to the completion of the reaction. If too much antibody is present (if the antibody concentration is too high compared to the antigen available) reduced antibody binding may take place. A negative slide will result *not* due to lack of antigen, but due to an excess of antibody. This phenomenon is similar to the prozone effect seen in agglutination reactions. Therefore, when testing a new antibody, a wide range of dilutions should be used to avoid false negatives caused by this effect.

When interpreting dilution results, two criteria are usually evaluated: Specific antigen staining and nonspecific background staining. The goal is to obtain the greatest intensity of specific staining with the least amount of background interference. The higher the antibody dilution, the lower the background staining due to undesired protein binding. The intensity of each reaction is usually rated on a scale of 0 to 4+, or negative to very intense. The following terms and abbreviations will be used in the accompanying examples:

Specific Stain	*Background*
very good (VG)	intense
very good, slightly pale (VG, SL)	moderate (MOD)
good	slight (SL)
pale	very slight (VSL)
negative (NEG)	no background (NB)

With the direct technique there is only one antibody to be concerned about. Five identical known positive specimens are incubated with a range of antibody dilutions such as 1:5, 1:10, 1:20, and 1:40; the fifth specimen does not receive any antibody and serves as a negative control. If positive staining is observed on the fifth specimen it is not due to localization of the antigen, but represents nonspecific binding and must be taken into consideration when evaluating the other specimens. A typical result is shown below:

Dilution	*1:5*	*1:10*	*1:20*	*1:40*	*Neg Control*
Specific Stain	VG	VG	VG,SL	Pale	NEG
Background	SL	SL	NEG	NEG	NEG

Interpretation: A 1:10 dilution gives very good specific staining, but also has some background staining. A 1:20 dilution shows no background staining, but does not exhibit as strong a specific reaction. Another series of slides should be run using antibody dilutions of 1:10, 1:12, 1:14, 1:16, 1:18, and 1:20 to find the dilution that produces the strongest specific staining with the least amount of nonspecific background interference.

In the indirect technique, two antibody solutions are used. The optimal dilution is determined for each antibody by using a checkerboard titration.

Set-up of Checkerboard Titration (Indirect Technique)

		Primary Antibody		
		1:50	*1:100*	*1:150*
Secondary Antibody	1:20	1	2	3
	1:40	4	5	6
	1:60	7	8	9

Nine known positive specimens are stained in the above manner. The optimal combination of dilutions will be again represented by the slide with the most intense specific stain and the least amount of background staining.

Results of Checkerboard Titration

		Primary Antibody		
		1:50	*1:100*	*1:150*
Secondary Antibody	1:20	very good SL	good NB	pale NB
	1:40	good NB	pale NB	pale NB
	1:60	pale NB	pale NB	pale NB

After examining the results, slide number 1 clearly shows the best staining. To reduce the background, the primary antibody can be used at dilutions between 1:50 and 1:100, while keeping the secondary antibody constant at 1:20.

Three antibodies are used in the PAP technique which makes the determination of optimal dilutions a little more difficult. As the concentration of link antibody and PAP complex are interdependent, it is necessary to determine them together.

Checkerboard Titration (PAP Technique)

		PAP Complex			
		1:150	*1:100*	*1:150*	*1:200*
Link Antibody	1:40				
	1:60				
	1:80				
	1:100				

A starting dilution for the primary antibody can usually be obtained from the manufacturer. When examining the results, several different combinations will often show equal staining. The choice can then be based on the cost factor of the reagents. For example, if link 1:60, PAP 1:200 and link 1:100, PAP 1:100 give identical results, the combination utilizing the most expensive component at the highest dilution should be selected.

The next step is to determine the correct dilution for the primary antibody. This is most easily done by holding constant the link and PAP dilutions, while varying the dilution of the

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primary antibody. Only if acceptable staining cannot be achieved by this method is it necessary to repeat the titration of the link antibody and PAP complex. The objective is to dilute the primary antibody out as far as possible, and still achieve intense positive staining. As mentioned previously, a wide range of dilutions should be studied to avoid prozone-like problems. A good place to start is with dilutions of 1:100, 1:500, and 1:1,000. The following examples show interpretation of possible results, and ways to narrow the choices down to the best working dilution.

EXAMPLE 1:

Dilution	*1:100*	*1:500*	*1:1,000*
Specific stain	negative	negative	negative
Background	NB	NB	NB

Interpretation — Specimen is actually negative.
— Technical problem with stain.
— Working solution is below 1:100. Try dilutions of 1:20, 1:40, 1:60, 1:80, and 1:100.

EXAMPLE 2:

Dilution	*1:100*	*1:500*	*1:1,000*
Specific stain	very good	pale	negative
Background	MOD	NB	NB

Interpretation — Working dilution is between 1:100 and 1:500.

Dilution	*1:100*	*1:200*	*1:300*	*1:400*	*1:500*
Specific stain	VG	VG	VG	good	pale
Background	MOD	MOD	SL	NB	NB

Interpretation — Working dilution is between 1:300 and 1:400.

Dilution	*1:300*	*1:325*	*1:350*	*1:375*	*1:400*
Specific stain	VG	VG	VG	VG,SL	good
Background	SL	SL	NB	NB	NB

Interpretation — Working dilution is 1:350.

In this case, the dilution of 1:350 is very clear cut, as 1:325 showed more background, and 1:375 did not produce as intense a specific stain.

EXAMPLE 3:

Dilution	*1:100*	*1:500*	*1:1,000*
Specific stain	very good	very good	negative
Background	MOD	MOD	NB

Interpretation — Working dilution is between 1:500 and 1:1,000.

Dilution	*1:500*	*1:600*	*1:700*	*1:800*	*1:900*	*1:1,000*
Specific stain	VG	VG	VG	good	pale	negative
Background	MOD	MOD	SL	NB	NB	NB

Interpretation — Working dilution is between 1:700 and 1:800.

Dilution	*1:700*	*1:725*	*1:750*	*1:775*	*1:800*
Specific stain	VG	VG	VG,SL	good	good
Background	SL	SL	NB	NB	NB

Interpretation — Best dilution is 1:725. Take appropriate steps to reduce background.

Here, the decision is more difficult since none of the slides demonstrate optimal staining. Dilutions could be set up between 1:725 and 1:750 to see if the background can be eliminated without decreasing the specific stain. This is usually not effective as the antibody concentration differences between the dilutions are too slight to be measured accurately. A more practical course would be to use a 1:700 dilution, and take steps to reduce the background staining.

EXAMPLE 4:

Dilution	*1:100*	*1:500*	*1:1,000*
Specific stain	VG	VG	VG
Background	MOD	MOD	MOD

Interpretation: Working dilution is greater than 1:1,000. Try dilutions of 1:1,000, 1:1,200, 1:1,400, 1:1,600, 1:1,800 and 1:2,000.

Making Dilutions

This section presents a basic discussion of how to properly prepare dilutions, and is designed for those who have had little or no experience in these methods.

The results of the previously described tests are valid only if the dilutions used are accurate and can be repeated. High quality pipettes of the appropriate capacity are a necessity for these procedures. Adjustable pipettes are an advantage for they allow greater flexibility in the variety of dilutions that can be prepared. The measurable volume should cover 10ul to 1ml preferably in three ranges, for example; 1 to 20ul, 20 to 200ul, and 100 to 1,000ul. A pipette has greater accuracy near its midrange rather than at the extremes, so 100ul would be more precisely measured by the 20 to 200ul pipette than by the 100 to 1,000ul instrument. Serological pipettes can be used to measure volumes greater than 1ml.

A 1:10 dilution is made by adding one part of substance X plus nine parts substance Y. This gives one volume X in a total volume of ten parts (one X plus nine Y's). When making very dilute solutions such as 1:100, it is often just as satisfactory to add one part X plus 100 parts Y as it is to use 99 parts Y.

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The volume of solution required depends upon the size and number of specimens to be stained. The minimum amount needed is 0.2ml, which is sufficient to cover a pituitary or small lymph node section. Prostate chips, smears, and larger tissue sections require about 0.4ml of solution. When testing various dilutions of antibody, only a small amount of solution should be made to avoid wasting reagent. Once the correct dilution has been determined, larger stock volumes can be made.

When determining the correct dilution of the primary antibody, it is often helpful to start with a 1:100 stock solution, and make further dilutions from it. This eliminates making large volumes of highly dilute antibody solutions. A 1:100 dilution is easily made by adding 10ul of antibody to 1,000ul (1ml) of buffer. A 1:500 dilution can then be prepared by simply making a 1:5 dilution of the 1:100 stock. This is done by adding 50ul of the 1:100 solution to 200ul of buffer, resulting in a total volume of 250ul (enough to stain one slide).

This proportion can be scaled up to accommodate more slides or larger samples by multiplying each volume by the same factor. To stain two slides, use 100ul of 1:100 stock plus 400ul buffer. The 1:1,000 dilution used is made by diluting the stock solution 1:10, or 20ul of 1:100 stock plus 180ul buffer for a total volume of 200ul.

An easy way to figure dilutions is as follows:

A 1:800 dilution is needed to stain one prostate section. First, make a 1:100 stock solution as described previously. Now, a 1:8 dilution of the stock solution must be prepared. A 1:8 dilution means one part in a total of eight parts, 10 parts in 80 parts, or 50 parts in 400 parts. To stain one prostate section, a volume of 400ul is needed. To make the dilution add 50ul of 1:100 solution to (400ul-50ul) 350ul buffer for a total volume of 400ul.

Buffers

There are several buffers that can be used for diluents, rinsing, and wash baths in immunoperoxidase techniques. A common one is 0.05M Tris at pH 7.6. Phosphate buffered saline (PBS) can be made with a variety of molarity and pH values. Physiologic saline (0.85%) is handy to use, as it can be made quickly and easily. Combinations of these three main buffers are also used, such as a 1:10 dilution of Tris in saline or Tris in PBS. The addition of saline or PBS to the Tris gives it a higher salt concentration, effectively reducing nonspecific binding and thereby decreasing background staining.

An important consideration when choosing a buffer is that it should *not* contain sodium azide (NaN_3), an antibacterial agent often employed as a preservative in commercially available buffers. In high concentrations, sodium azide can prevent binding of the peroxidase enzyme to its substrate, and thus inhibit color development. Most undiluted antibodies contain small amounts of sodium azide to prevent bacterial contamination, but upon dilution the level is reduced to where it will not interfere.

Tris Buffer, 0.05M, pH 7.6

The classic formula for Tris buffer is given below. Tris can also be purchased ready-made or as pre-pH'd crystals that eliminate the need to handle hydrochloric acid. The pH of Tris buffer varies with the temperature of the solution, and should be measured at the temperature where the buffer will be used.

Dissolve 6.1g Tris (Trishydroxymethyl aminomethane) base in 50ml distilled water.
Add 37ml of 1N hydrochloric acid.
Dilute to a total volume of 1 liter with distilled water.
The pH should be 7.6 ± 0.2 at 25°C.

Phosphate Buffered Saline (PBS), 0.01M, pH 7.2

PBS is used at a molarity ranging from 0.01M to 0.05M with pH values of 7.0 to 7.6. A formula for the most common concentration and pH is given below:

Dissolve the following salts in 1 liter of distilled water.

Na_2HPO_4	Dibasic sodium phosphate, anhydrous	1.48g
KH_2PO_4	Monobasic potassium phosphate, anhydrous	0.43g
NaCl	Sodium chloride	7.2g

Physiologic Saline, 0.85%.

Saline can be purchased ready-made, but should not contain sodium azide as a preservative. Or it can be easily prepared as follows:

Add 8.5g sodium chloride (NaCl) to 1 liter of distilled water.

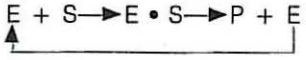
Tris Buffered Saline (TBS)

This buffer is useful for reducing background staining especially in direct smears. PBS can be substituted for the saline if desired.

Mix 100ml of 0.05 Tris buffer with 900ml of 0.85% saline.

Substrate Solutions

An enzyme (E) is a catalyst that acts upon a substrate (S) to speed up its conversion to a product (P). It does this by forming a complex with the substrate as indicated by the following reaction:



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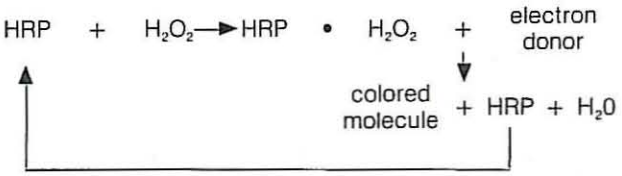
The important point to note is that the enzyme is not depleted, but can react with another molecule of substrate to make another molecule of product. A single enzyme molecule can catalyse many substrate molecules into product.

In immunoperoxidase staining the enzyme is horseradish peroxidase (HRP) which forms a complex with the substrate, hydrogen peroxide (H_2O_2). This complex reacts with an electron donor to produce the end products of the reaction—a colored molecule and water (H_2O).



Again, the peroxidase enzyme is not used up and is free to react with more hydrogen peroxide to form more colored molecules. The increased sensitivity of immunoperoxidase techiques over immunofluorescence lies in this amplification factor of enzyme techniques. One fluorescent molecule can give off only a small amount of visible light, but one peroxidase enzyme can produce many molecules of color.

There are several chromagens that can act as electron donors in the enzyme reaction. For use in immunoperoxidase techniques, the colored end product must precipitate at the site where it was produced. Chromagens that form soluble end products cannot be used in staining methods.

Of the several suitable electron donating chromagens available, the four most commonly used are as follows:

1. 3,3 diaminobenzidine tetrahydrochloride, abbreviated DAB, produces a brown color that is insoluble in alcohol. Slides can be dehydrated and coverslipped with conventional mounting media. DAB is electron dense which makes it useful for ultrastructural immunoperoxidase studies. The brown color can be enhanced by treatment with osmium tetroxide or nickel chloride. The main drawback of DAB (a benzidine derivative) is that it is considered a possible carcinogen.

2. 3-amino-9-ethylcarbazole or AEC forms a red end product that is alcohol soluble. Consequently, specimens should not be dehydrated, but rather coverslipped with a water based medium. AEC may also be a possible carcinogen, but reference sources differ on this.

3. 4-chloro-1-napthol precipitates as a blue end product that is alcohol soluble. Chloro-naphthol tends to diffuse from the site of precipitation, therefore the slides cannot be retained for a permanent record.

4. P-phenylenediamine dihydrochloride/pyrocatechol (Hanker-Yates reagent) produces a blue-black reaction product that is insoluble in alcohol and can be osmicated. Varying results have been achieved using this chromagen in immunoperoxidase techniques.

Formulas and methods for each of the above mentioned chromagens are given below. The concentrations and incubation times may be adjusted to optimize staining. To increase the intensity of the reaction product, increase the amount of chromagen used and/or increase the substrate incubation time. If too much staining occurs, decrease the time of incubation.

DAB — Substrate Solution

This solution should be prepared immediately before use. Care should be taken to avoid inhalation of the powder or contact with the skin.

Dissolve 6mg of 3,3 diaminobenzidine tetrahydrochloride in 10ml of 0.05M Tris buffer, pH 7.6.

Add 0.1 ml of 3% hydrogen peroxide.

The mixture should be filtered if precipitation occurs.

Place 4-6 drops onto each specimen and incubate 5 minutes at room temperature.

Rinse off with water and counterstain if desired.

AEC — Substrate Solution

This mixture is stable for two to three hours once prepared. The AEC-formamide mixture is stable for long periods of time and a large volume can be made up as a stock solution. Care should be taken to avoid inhalation of the powder or contact with the skin.

Dissolve 4 mg of 3-amino-9-ethylcarbazole in 1ml of N,N dimethylformamide.

While stirring, add this to 14ml of 0.1M acetate buffer pH 5.2.

Add 0.15ml of 3% hydrogen peroxide.

The mixture should be filtered if precipitation occurs.

Place 4-6 drops onto each specimen and incubate for 40 minutes at room temperature or 20 minutes at 37°C.

Rinse off with water and counterstain if desired.

Acetate Buffer, 0.1M, pH 5.2

Mix 210ml of 0.1N acetic acid (5.75ml glacial acetic acid in 1 liter distilled water) and 790ml of 0.1M sodium acetate (13.61g sodium acetate trihydrate in 1 liter distilled water).

Chloro-Naphthol — Substrate Solution

Dissolve 3mg of 4-chloro-1-naphthol in 0.1ml of absolute ethanol.

While stirring, add this to 10ml 0.05M Tris buffer, pH 7.6.

Add 0.1ml of 3% hydrogen peroxide.

A white precipitate will form which must be filtered out before use.

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Place 4-6 drops onto each specimen and incubate for 30 minutes at room temperature.

Rinse off with water and counterstain if desired.

Hanker-Yates — Substrate Solution

This solution is available commercially, or can be made as follows.

Mix 5mg of p-phenylenediamine dihydrochloride with 10mg of pyrocatechol.

Dissolve in 10ml 0.05M Tris buffer, pH 7.6.

Add 0.1ml of 3% hydrogen peroxide.

Place 4-6 drops onto each specimen and incubate for 15 minutes at room temperature.

Rinse off with water and counterstain if desired.

Hydrogen peroxide is the substrate that is acted upon by the peroxidase enzyme to form a colored end product. As seen in Chapter 2, hydrogen peroxide can also be used to inhibit endogenous peroxidase activity already present in the specimen. This occurs due to a phenomenon of enzyme kinetics called substrate inhibition (Figure 21). The principle states that an excess of substrate will reversibly or irreversibly inhibit an enzyme. In the substrate solution, a concentration of 0.1ml of 3% hydrogen peroxide in 10ml of buffer solution (0.03%) is used. To quench endogenous peroxidase activity a concentration of 3% hydrogen peroxide is applied, representing a 100-fold excess of substrate over the amount required for the reaction—an amount sufficient to irreversibly inhibit the enzyme.

Also mentioned earlier was the inhibitory effect of sodium azide on the substrate reaction. This occurs by a process known as competitive inhibition (Figure 22). Sodium azide is also a substrate for the peroxidase enzyme, and competes with hydrogen peroxide to form complexes with peroxidase. Peroxidase has a greater affinity for sodium azide, and will bind preferentially to it over hydrogen peroxide. However, the reaction will stop at this point since the sodium azide-peroxidase complex cannot react with the electron donor to form a colored end product. Hence, in the presence of sodium azide, positive staining will be weakened or absent.

Counterstaining

The procedure used for counterstaining and coverslipping the specimens depends upon the substrate solution used. With DAB and Hanker-Yates the colored end products are insoluble in alcohol and organic solvents. These specimens can be counterstained with alcoholic stains, dehydrated, and coverslipped with xylene or toluene containing mounting media. The reaction products produced by AEC and chloronaphthol are soluble in alcohol and organic solvents. These slides must not be decolorized in acid alcohol or dehydrated, and should be coverslipped using a water base mounting media. Two representative protocols are given below, one for each type of substrate solution. Other counterstains can be used besides hematoxylin, such as methyl green with Hanker-Yates, as long as the solubility of the reaction product is considered.

Counterstaining Procedure for Insoluble End Products

1. Gently rinse slide with water from a wash bottle to remove unreacted substrate.
2. Lay the slide flat on a staining rack and apply 4-6 drops of Harris' Hematoxylin. Alternatively, the slide can be placed in a staining jar containing Harris' Hematoxylin. Incubate 5 minutes.
3. Wash the slide in gently running tap water for 3 minutes.
4. Differentiate in 0.5% acid alcohol for 10 seconds.
5. Wash well in gently running tap water for 3 minutes to blue the hematoxylin.
6. Dehydrate in graded alcohols.
7. Clear in xylene.
8. Mount in DPX.

Notes:

The intensity of the counterstain can be regulated by increasing or decreasing the staining time in hematoxylin. Too intense a counterstain can obliterate the positive staining due to peroxidase.

DPX can be made by combining 10g of distrene 80, 5ml of dibutyl phthalate and 35ml of xylol. Other commercially available xylene or toluene containing mounting media may also be used.

Counterstaining Procedure for Soluble End Products

1. Gently rinse slide with water from a wash bottle to remove unreacted substrate.
2. Lay the slide flat on a staining rack and apply 4-6 drops of Mayer's Hematoxylin. Alternatively, the slide can be placed in a staining jar containing Mayer's Hematoxylin. Incubate 5 minutes.
3. Blue the hematoxylin by dipping the slide in ammonia water (2ml concentrated 17M ammonium hydroxide/1 liter tap water) 10 times.
4. Rinse under gently running tap water for 5 minutes.
5. Coverslip while still wet in glycerol gelatin.

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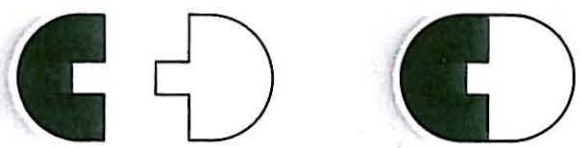
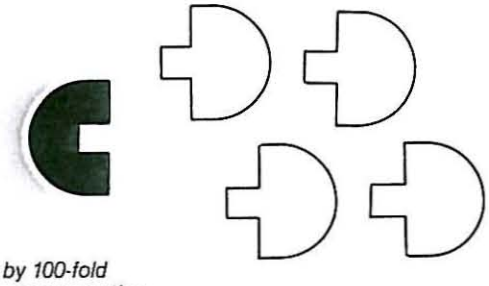
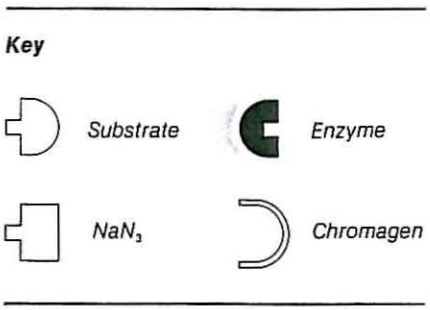
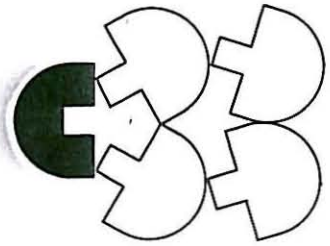


Figure 21. *Normal enzyme/substrate binding to form complex.*



Inhibition of enzyme by 100-fold increase in substrate concentration. No complex formation occurs.





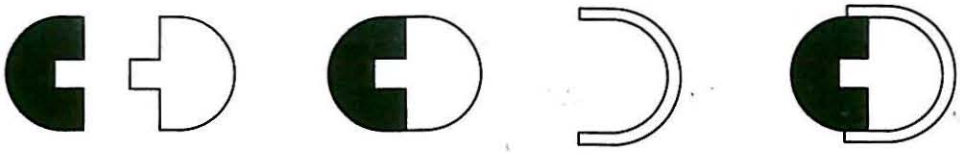
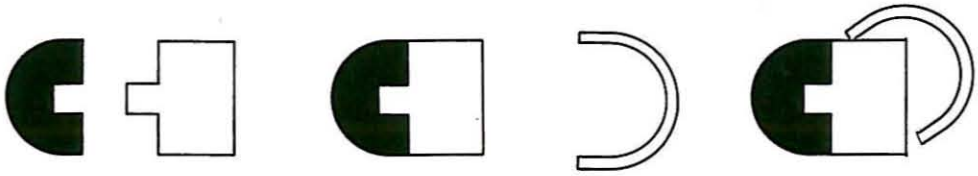


Figure 22. *Normal enzyme/substrate binding to form complex and colored end product.*

Inhibition of enzyme reaction by binding to competitive substrate—which cannot bind to chromagen to form colored end product.



Notes:

The intensity of the counterstain can be regulated by increasing or decreasing the staining time in hematoxylin. Too intense a counterstain can obliterate the positive staining due to peroxidase.

Glycerol gelatin can be purchased from several commercial suppliers. Some preparations are available that will firmly adhere the coverslip to the slide (when dry). This removes the need to rim the coverslip with nail polish, and prevents fading of the chromagen.

AEC is susceptible to oxidation which causes fading. Precautions should be taken to avoid the entrapment of air bubbles when coverslipping. A properly mounted specimen will retain its stain intensity for many years.

Mounting media containing polyvinyl alcohol (PVA) should be avoided as it can leach out the soluble colored end products over a period of time.

Incubation Timing

Incubation times, antibody dilutions, nonspecific background staining and specific staining intensity are all interrelated. The longer the incubation time, the higher the antibody dilution. The more dilute the antibody, the lower the nonspecific background stain. The lower the nonspecific stain, the greater the contrast with the specific stain, making interpretation easier. The following examples pertain to incubation of primary antibodies in the various techniques, although the principles can be applied to all antibody incubation steps (Figure 23).

Most incubations with antibody solutions are for 20-30 minutes. If a higher primary antibody dilution is required—for example to decrease background interference—then the incubation time can be increased to one hour. Often, poorly differentiated or metastastic tumors will not stain as intensely as normal tissue. To achieve greater staining intensity on these specimens without changing the antibody dilution, the 20 minute incubation time may be increased.

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Figure 23.

Time	*20 Min*		*60 Min*	*20 Min*		*18 Hr*
Antibody Dilution	*1:100*		*1:00*	*1:100*	or	*1:1000*
Temperature	*RT*		*RT*	*37°C*		*4°C*
Result	*Pale, NB*		*VG, NB*	*VG, NB*		*VG, NB*

Three methods to increase stain intensity.

Time	*20 Min*		*60 Min*	*20 Min*		*18 Hr*
Antibody Dilution	*1:100*		*1:300*	*1:300*	or	*1:1000*
Temperature	*RT*		*RT*	*37°C*		*4°C*
Result	*VG, SLB*		*VG, NB*	*VG, NB*		*VG, NB*

Three methods to reduce nonspecific background staining.

The temperature of incubation will also affect antibody dilutions. By incubating a specimen at a higher temperature (usually 37 °C), a greater antibody dilution can be used. Incubation at 37 °C for a constant time and dilution will increase the intensity of the stain over that which can be achieved at room temperature. If a poorly differentiated tumor shows weak staining with a 20 minute room temperature incubation, a 20 minute incubation at 37 °C with the same primary antibody dilution will show a more intense reaction.

The optimal temperature for maximal antibody binding is 37 °C, or body temperature. Temperatures higher than this will cause denaturation of the antibody proteins, and decreased binding. Care must be taken that incubation temperatures do not exceed 37 °C. Higher temperatures also increase the risk of evaporation which can lead to drying of the specimen. All high temperature incubations should be performed in a humidity chamber, and the slides monitored periodically for evaporation.

The lower the temperature, the longer the incubation time needed and the greater the antibody dilution. Incubating a specimen in the refrigerator (4 °C) overnight (approximately 18 hours) will afford less background staining and a more intense specific stain using a concomitant high dilution of the primary antibody.

The intense staining achieved at 37 °C incubations allows for shorter incubation times. Using the same antibody dilutions, a 20 minute room temperature incubation can be shortened to 5 minutes at 37 °C. However, certain precautions should be observed:

— When processing several slides at one time, there will be a one or two minute difference between the first slide and the last. One minute out of a 20 minute incubation is only a 5% difference. One minute out of a 5 minute incubation is a 20% difference. If the first slide is incubated for 4 minutes, and the last slide for 6 minutes, there will be a 40% difference between the two. To achieve consistency with reduced incubation times, processing of slides should be staggered so that all specimens undergo identical incubations.

— Since poorly differentiated and metastatic tumors often stain weakly with a 20 minute room temperature incubation, a five minute incubation even at 37 °C may result in weak or negative staining. In order to avoid false negative results, a known weakly positive control should be run alongside the unknown specimens. If this control shows no positive staining, then any unknown slides that were also negative should be repeated. As an additional check, all specimens that show no staining after five minute antibody incubations should be repeated using 20 minute times.

The room temperature antibody incubation times of 20 minutes are not critical, and can be lengthened or shortened by a few minutes without compromising the staining. The amount of time a specimen remains in a buffer bath is even less critical. Although the times were listed as 10 minutes, the wash baths can be shortened to 5 minutes, or lengthened for greater convenience. In fact, if the staining procedure must be interrupted for any reason, the slides can be held in buffer for several hours or several days with no decrease in staining intensity.

The number of changes of buffer is also variable. If a coplin jar is used for the bath, the buffer should be fresh for each incubation. With larger dishes, the buffer can be used twice before being changed. If desired, the baths can be changed more frequently. Instead of one ten-minute buffer bath, the slides can be placed in three separate baths for three minutes each.

Enzyme Digestion

Overfixation of tissue specimens in formalin causes formation of an excess of aldehyde linkages which will mask the tissue antigen and prevent its localization by the primary antibody. This is a particular problem when staining lymph nodes for immunoglobulin. To unmask the antigen sites, the aldehyde bonds can be digested with proteolytic enzymes. When using this technique, a few points should be considered:

1. As the enzymes digest the aldehyde linkages, the tissue section can become loosened from the glass slide. An adhesive that is resistant to digestion such as chrome alum gelatin should be used to prevent loss of the specimen.

2. Since this procedure can loosen the tissue, it is advisable to run two or three extra slides at the same time. Even if one or two sections wash off, the staining procedure can still be completed.

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3. It is essential that known positive and negative controls are also subjected to the enzyme digestion to avoid problems in interpretation.

4. The digestion time will vary depending on the condition of the specimen and the enzyme used. Excessive digestion can damage the tissue, therefore the incubation times should be kept as short as possible.

5. To ensure complete exposure of all sections to the enzyme solution, no more than five slides should be placed in a 250ml enzyme bath. The slides should be separated and the solution gently stirred during the procedure.

The most common enzyme used is trypsin. It is the least destructive to the tissue, and its reaction can be easily controlled. Trypsin, like many other enzymes, requires certain temperature and pH values for optimal activity. The incubations are carried out at 37°C using prewarmed buffer on prewarmed slides. Temperatures below 37°C will reduce the activity of the enzyme.

Note: Incubations at 37°C can be easily accomplished by immersing containers of the various solutions in a 37°C water bath.

1. Deparaffinize and rehydrate 3 or 4 identical sections.

2. Inhibit endogenous peroxidase activity using an appropriate method. Rinse slides with water.

3. Place slides in a 37°C buffer bath for 5 minutes.

4. Meanwhile, prepare a 0.1% trypsin solution in prewarmed distilled water containing 0.1% calcium chloride. Adjust the pH to 7.8 with 0.1N sodium hydroxide.

5. Immerse the slides in the trypsin bath. Incubate for 15 minutes at 37°C.

6. Stop the digestion by rinsing the slides under gently running, cold tap water for five minutes.

7. Continue with immunoperoxidase staining.

Two other proteolytic enzymes that are often used are 0.1% pronase in 0.5M Tris buffer, pH 7.5; and 0.4% pepsin in 0.01N hydrochloric acid. These solutions are prepared and used in the same manner as the trypsin.

Proteolytic enzyme digestion must be carefully controlled to avoid damage to tissue and destruction of antigen. It should not be used as a substitute for proper fixation, and used very judiciously only when absolutely necessary.

FIXATION AND PROCESSING

A specimen contains only a finite amount of antigen. Each step in the processing of that specimen destroys some antigen. Immunoperoxidase techniques can localize only that antigen in a specimen which remains recognizable by the antibody.

Fixation is one of the most critical aspects of immunoperoxidase staining. A discussion of fixation in general, and the aspects of some of the more common fixatives, will be followed by sections on processing, decalcifying, and adhesives. Special handling techniques for frozen sections, smears and plastic embedded specimens will be discussed.

Fixation

The results of immunoperoxidase staining are only as good as the tissue specimen used. If the tissue is improperly fixed and processed, a variety of artifacts will result. Since the primary purpose of staining is to localize an antigen, then the main concern in fixation is to preserve that antigen. The antigen must be fixed, available and accessible to the primary antibody.

If the antigen is not fixed, it will be washed out of the specimen and no staining will result. If the surrounding cells are not fixed, the resulting poor morphology will interfere with proper interpretation. Conversely, overfixation can cause severe problems such as masking or denaturation of the antigen. The optimal fixation then will be for the minimum time and concentration needed to produce good morphology.

The antigen must also be available to bind with the primary antibody. If the antigen diffuses from the site of synthesis, an incorrect interpretation will be made. The smaller the antigen, the more likely it is to diffuse, and the stronger the fixation needed. In this case, a cross-linking fixative such as formalin will prevent diffusion of the antigen.

If the antigen is not accessible to the primary antibody, then no binding can take place. One of the effects of overfixation by a cross-linking fixative is the formation of excess aldehyde linkages which can mask the antigen and prevent access to the antibody. Another problem develops if the fixative changes the structure of the antigen, making it unrecognizable to the antibody. The larger and more complex the antigen, the gentler the fixation needed to prevent masking or structural changes which affect its accessibility to the antibody.

Obviously, proper antigen fixation is the cornerstone of immunoperoxidase techniques; for without it, results will be poor. It should also be noted that depending on the size,

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structure and nature of the antigen to be localized, an appropriate fixation protocol must be developed. The optimal situation would involve subjecting various specimens of each antigen to several fixatives for different lengths of time to identify the best conditions for preservation of that particular antigen.

Clearly this is entirely too time consuming to be practical in most laboratories. Even if the optimal fixation scheme is identified, special processing requirements for certain specimens limit the application of immunoperoxidase methods as a routine stain. In reality, there are several fixatives that work well with the majority of antigens, and in most cases, special processing can be held to a minimum.

For optimal fixation, and to prevent staining artifacts, the following conditions should be observed:

— Tissue specimens must not be allowed to dry out, and should be placed into fixative as soon as possible. Cell smears should be fixed as promptly as possible to ensure antigen preservation.

— Small blocks of tissue, no larger than 2cm square by 4mm thick, should be placed in a minimum of 200ml of fixative. Larger blocks of tissue will prevent complete penetration of fixative, resulting in nonspecific staining.

— After fixation, tissue specimens should be thoroughly washed to remove excess fixative which can cause staining artifacts.

The most practical, and probably the best all-around fixative is 10% buffered formalin. Due to its cross-linking characteristic, it is an especially good fixative for small antigens—such as hormones. However, fixation time is critical to prevent antigen masking. Immunoglobulins are especially susceptible to overfixation with formalin. In these cases, treatment with proteolytic enzymes will digest the excess aldehyde linkages and expose the antigen.

Formalin should be fresh and buffered to a pH of 7.0-7.6. As this is a slow acting fixative, acidic solutions can cause structural disturbances and poor morphology. Due to the problem of antigen masking, fixation should be for the shortest possible time to achieve good morphology; usually 6-12 hours. Tissue fixed for longer periods of time may still exhibit intense staining depending upon the concentration of antigen present.

10% Buffered Neutral Formalin, pH 7

Formalin (40% formaldehyde)	100ml
Dibasic sodium phosphate, anhydrous	6.5g
Monobasic sodium phosphate, monohydrate	4.0g
Distilled water	900ml

Another excellent fixative is Zenker's fixative. It does not produce aldehyde linkages, and is often the fixative of choice for specimens to be stained for immunoglobulin. The fixation time is short, usually 2-4 hours. To prevent black deposits on the stained specimen, the mercuric chloride must be removed after the sections have been deparaffinized.

Zenker's Fixative

Potassium dichromate	25 mg
Mercuric chloride	50mg
Sodium sulfate	10g
Distilled water	1,000ml
Glacial acetic acid	50ml

Dissolve the salts in the distilled water by heating and stirring. Add the glacial acetic acid.

Removal of mercuric chloride crystals

1. Deparaffinize and rehydrate section to 95% alcohol.
2. Place slide in 0.5% alcoholic iodine (0.5g iodine crystals in 100ml of 70% ethanol) for 3 minutes.
3. Gently rinse in running water.
4. Place slide in 5% sodium thiosulfate (5g sodium thiosulfate in 100ml distilled water) for 2 minutes.
5. Gently rinse in running water.
6. Continue with immunoperoxidase staining.

A third fixative that is gaining in popularity is Bouin's. This fixative produces good morphology and has been recommended for localization of J-chains, immunoglobulins and alpha-fetoprotein. The fixation time is the same as Zenker's; usually 2-4 hours. To prevent artifacts, excess fixative must be removed from the tissue by thorough washing in several changes of 50% and 70% alcohol.

Bouin's Fixative

1% saturated picric acid	750ml
Formalin (40% formaldehyde)	250ml
Glacial acetic acid	50ml

For smears, cryostat sections, cytospins and imprints, fixation in acetone at 4°C for 10 minutes is recommended. As an alternative, if endogenous peroxidase activity is quenched in a hydrogen peroxide-methanol solution, the methanol will fix the specimen.

Processing

After fixation, tissue specimens can be dehydrated, cleared and embedded according to routine processing pro-

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cedures. For best results, specimens should be embedded in pure paraffin because it can be completely and easily removed from the tissue at the time of staining. The plastic contained in some embedding media is often difficult to remove, and can cause nonspecific staining and inhibition of specific staining. Since most laboratories routinely use embedding media containing plastic, certain precautions must be taken:

1. If the embedding media is heated to temperatures above 62°C, the plastics will start to form polymers. These polymers are very difficult to remove from the tissue, and can also cause nicks in the microtome knife. Consequently, paraffin baths should be kept at a maximum temperature of 60°C to prevent polymerization.

2. To aid in complete removal of the embedding media, slides should be placed in a 60°C oven for 30 minutes prior to deparaffinizing. Temperatures must not exceed 60°C to prevent antigen denaturation and damage to cellular morphology.

3. From the oven, the slides should be immediately transferred to a fresh xylene or toluene bath. The paraffin should not be allowed to solidify before deparaffinizing as this will negate the benefits of melting. Either xylene or toluene can be used to deparaffinize. Some researchers feel that toluene is more effective for complete removal of embedding media.

4. For efficient plastic removal, no more than 50 slides should be run through a 250ml xylene bath before it is changed. This may seem to be an insignificant point, but it is critical to reducing the amount of background staining. The fewer the number of slides processed through a xylene bath, the more completely the embedding media will be removed.

5. If more than 20 slides are placed in a 250ml bath at one time, xylene circulation may not be sufficient. For this reason, when processing several slides at one time, a second, fresh xylene bath should be used.

If all of these precautions are observed, there should be no problem with nonspecific background staining due to plastic residue. This type of interference is easily identified by pale staining that extends beyond the tissue specimen. All other types of background staining are confined within the borders of the specimen.

Procedure for Deparaffinizing and Rehydration

1. Circle and label specimen with a diamond pencil.

2. Place in 60°C oven for 30 minutes.

3. Transfer immediately to a fresh xylene bath for 3 minutes.

4. Place in a second, fresh xylene bath for 3 minutes if desired.

5. Place in a bath of absolute alcohol for 3 minutes.

6. Place in a second, fresh absolute alcohol bath for 3 minutes.

7. Place in a bath of 95% alcohol for 3 minutes.

8. Place in a second, fresh 95% alcohol bath for 3 minutes.

9. Rinse under gently running water.

10. Begin immunoperoxidase staining.

Bone and calcified tissue must have the insoluble calcium salts removed before they can be sectioned. This causes no problem in immunoperoxidase staining as long as the specimen has been thoroughly fixed. Adequate fixation will prevent damage to the antigen and cellular structures by the decalcifying solutions. Commercially available decalcification reagents tend to give variable results in immunoperoxidase staining. The most consistent results have been obtained using a 5% nitric acid solution for one hour. The specimens should be cut into fairly small pieces to allow complete penetration of fixative and acid. After decalcification, the specimen should be washed in running water to remove excess acid before continuing with routine processing.

Adhesives

Due to the frequent handling and rinsing of the slides, the tissue sections may become loosened and fall off. Various methods can be used to prevent this. Several slide adhesive solutions are commercially available, or they can be easily made using the formulas given below. All of the adhesives should be used sparingly as an excess can cause nonspecific background staining similar to that of unremoved embedding media.

One of the best and easiest methods to adhere the tissue to the slide is by thorough drying after sections are cut. Placing the slides in a 60°C oven for 30 minutes will melt the paraffin and promote close contact between the tissue and the glass slide. This important step should be performed even if chemical adhesives are used.

Mayer's Egg Albumin

Separate an egg placing the white into a beaker. Discard the yolk. Add an equal volume of glycerin and mix well. Filter through coarse filter paper. Add one crystal of thymol to prevent bacterial growth. Store in the refrigerator.

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To use, place a small drop on the end of a slide and distribute evenly. Drying the slides in a 60°C oven for 30 minutes after the sections are cut will coagulate the egg albumin and adhere the specimen to the slide.

This adhesive should not be used if the specimen will be trypsinized, as it will also be digested.

Elmer's Glue, 0.5%

Dissolve 0.5ml of Elmer's Glue-All (Borden, Inc. Columbus, Ohio) in 100ml of distilled water heated to approximately 60°C. Place a small drop on the end of a glass slide and distribute evenly.

Chrome Alum-Gelatin Adhesive

This is an excellent adhesive often used to assure adherence of sections during enzyme digestion.

1. Add 3.0g gelatin to one liter of distilled water heated to approximately 60°C.
2. Mix with magnetic stirrer until *completely* dissolved.
3. Add 0.5 grams chromium potassium sulfate (chrome alum). Solution will appear blue.
4. Add several thymol crystals.
5. Continue to stir until alum and thymol are completely dissolved.
6. Filter a small portion *while hot* into a 100ml beaker. Keep stock hot.
7. Place several slides, one at a time, into the beaker. Keep beaker ¾ full at all times by replenishing with hot stock solution.
8. Withdraw slides, one at a time, and blot on edge of towel. Place slides vertically in a rack, and dry in a hot oven.
9. When slides are completely dry, collect and place in a slide box.
10. Slides should be stored at room temperature. They can be used as needed.

Instead of coating the slide with an adhesive, ¼ tsp. of gelatin and/or the same amount of Elmer's Glue-All can be added to the water bath used to float the tissue ribbons. The water bath must be carefully cleaned after each use to prevent bacterial growth.

Smears

Smears should be prepared carefully to assure that they are only one cell layer thick. Thicker preparations will interfere with interpretation as reagents become trapped between layers. Smears should be fixed as soon as possible after preparation, or within 24 hours. The most common fixatives are acetone or 10% buffered formalin for 10 minutes.

After fixation the specimens are usually stable for several days at room temperature, or one to two weeks when refrigerated. When frozen at −20°C, the smears remain stable for several months. The ultimate preservation and stability of the smear is dependent upon the antigen to be localized.

Prior to staining, the specimen should be hydrated in buffer for 5 minutes before proceeding. Blood smears should be placed directly in a hydrogen peroxide/methanol solution to quench endogenous peroxidase activity. This will also serve to fix the specimen, plus pre-hydration is unnecessary. The slide is incubated for 20 minutes in the solution, rinsed in water, and stained in the usual manner.

Frozen Sections

Frozen sections are the specimens of choice for direct and indirect staining methods. Most surface antigens are destroyed by fixation and can be localized only in frozen sections. To circumvent the detrimental effects of fixation and the need for enzyme digestion, frozen specimens are often used for the identification of intracellular antigens as well.

Specimens should be small in size and quickly frozen. This allows for rapid fixation of the antigen and prevents the formation of ice crystals which disrupt cell structure. Once the tissue is frozen, it can be stored at −80°C for long periods of time.

A piece of tissue no more than 4mm thick is placed in aluminum foil and immersed in a dry ice/ethanol bath or an isopentane/liquid nitrogen slush for 1-2 minutes or until frozen. Prior to sectioning, the tissue block is attached to a metal specimen holder with a drop of water. Sections should be cut 4-6 microns thick in a cryostat at −20°C and fixed for 5 seconds in room temperature acetone. At this point the tissue can be stored up to a month at 4°C, although for best results staining should be performed shortly after sectioning.

Before beginning the immunoperoxidase staining procedure, the section is fixed in acetone at 4°C for 10 minutes. The slide is air dried and then placed in a buffer bath for 5 minutes. If endogenous peroxidase activity is quenched using a hydrogen peroxide-methanol bath, the acetone fixation step can be eliminated.

Plastic Embedded Sections

In addition to embedding tissue in paraffin, plastic compounds may also be used. Plastic embedded specimens can be cut in thinner sections than possible with paraffin, and can

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also be used in electron microscopy techniques. In this way, antigen localization at the light microscope level can be compared with that of the electron microscope. The thin sections also have the advantage of superior morphologic detail over their paraffin counterparts.

Two main types of embedding media are available. Methacrylates do not copolymerize with tissue components, thereby preventing alteration of antigenic structure. These plastics are often used when the antigen concentration is low. Methacrylates tend to polymerize unevenly which can cause shrinkage and subsequently poor morphology. The epoxy resins such as Epon and Araldite polyermize more evenly resulting in excellent preservation of structural detail. However, due to their high reactivity, they can interact with tissue structures during the polymerization process.

Immunoperoxidase staining can be performed either before the tissue is embedded (pre-embedding technique) or afterwards (post-embedding technique). Each method has its advantages and disadvantages. In the pre-embedding technique, the tissue is fixed and thick sections (5-50 microns) are cut and either placed on slides or left free floating. The specimen is stained in the usual manner except that longer incubation times (2-24 hours) must be used to allow the antibody to penetrate the thick specimen. To be certain that all unreacted antibody is removed, the buffer baths are increased to 20 minutes, with three separate baths between each antibody incubation. After staining, the specimen is postfixed for one hour in 1% osmium tetroxide, washed, dehydrated and embedded in resin. Thin sections (1 micron) are cut for use in light microscopy or ultra-thin sections for use in electron microscopy.

Long antibody incubations and buffer baths make this procedure time consuming to perform. Incomplete or uneven penetration of antibody will result in poor staining. Special care must be taken to thoroughly fix the specimen to prevent the polymerization process from altering the morphology of the stained cells. The main application for the pre-embedding staining technique is in electron microscopy.

In the post-embedding staining technique, specimens are fixed, dehydrated and embedded in plastic before being stained. For light microscopic examination, 1 micron thick sections are cut and placed on slides. For electron microscopy, ultra-thin sections are cut and picked up on nickel or gold grids. To expose the antigenic determinants, the plastic must be etched before application of the primary antibody. The most common method is by incubation in a solution of sodium hydroxide in absolute ethanol (sodium ethoxide) or methanol (sodium methoxide) for 15 minutes. A saturated solution of sodium hydroxide in absolute alcohol is prepared and allowed to mature for several days. Dilute 1:1 (1 part of a *total* of 2 parts) with absolute alcohol before use. An alternative is to incubate the section in 10% hydrogen peroxide for 15 minutes. The slides are rinsed in water and the immunoperoxidase procedure is then continued in the usual manner.

The post-embedding technique can be performed quickly and easily with little alteration in routine immunoperoxidase methods. As with antigen localization in paraffin sections, the specimen must be adequately fixed to survive plastic embedding and polymerization. If the staining is to be viewed with an electron microscope, a fixative should be chosen that will preserve ultrastructural morphology. The most commonly used fixative is 2% glutaraldehyde in 0.1M phosphate buffer pH 7.4. As this is a cross-linking fixative, it may be necessary to perform an enzyme digestion after the plastic is etched.

Immunoperoxidase staining of plastic embedded material for either light or electron microscopy is a relatively new technique. New modifications and uses are constantly being derived and evaluated. This section was designed as an introduction and overview to the adaptation of plastic sections to immunoperoxidase methods. Further information on these techniques and applications in electron microscopy can be found in the references.

CONTROLS

Positive and negative controls must be processed along with the unknown to assure the accuracy of the results in any staining technique. Therefore, various types of controls used in immunoperoxidase methods will be discussed here, along with interpretation of the results.

Certain procedural and reagent controls are essential to the validation of the results of immunoperoxidase staining. Most of these controls center around the primary antibody to ascertain that positive staining is the result of specific binding to the antigen in question. After determining the optimal working dilution of a new primary antibody, several known positive and negative specimens should be stained to certify the antibody's specificity.

Many references recommend absorption of the primary antibody with the purified antigen. If no contaminating antibodies are present, all antibodies would be absorbed by the antigen, and no staining would be observed. If positive staining occurs after absorption, it is an indication that the solution contained antibodies against other antigens present in the specimen. In theory this makes sense, but in practice it is difficult to perform.

It is not easy to find a commercial source of purified antigens to absorb all of the different primary antibodies utilized in immunoperoxidase staining. Often the manufacturer of the antibody will have the antigen available, but it is most likely the same preparation used to immunize the animals. It is inadvisable to absorb with the antigen used to produce the antibody, as any impurities in the antigen would most likely

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absorb out corresponding contaminants in the antiserum. Finally, although only small quantities of antigen are needed for absorption, the cost is still often hundreds of dollars for each antigen.

A more feasible method to test antibody specificity is to show that positive staining is not the result of any other components in the antiserum. This is accomplished by substituting an identical dilution of nonimmune serum from the same animal species as the primary antibody. This serum will contain all the components of the primary antiserum, except the specific antibody. Any other antibodies and proteins normally found in that species will be present.

Often, the serum sample is taken from the animal just before it is injected with the antigen. The serum is saved for comparison studies once the antibody has been produced. This is called pre-immune serum since it was obtained before the animal was immunized. If it is not possible to obtain serum from the same animal that made the primary antibody, serum from another nonimmunized animal of that species is used. This is termed nonimmune serum.

Once the primary antibody has been shown to be specific by staining known positive and negative specimens and by substitution with nonimmune serum, it can be put into routine use. Each time an unknown specimen is stained with a primary antibody, the following controls should be used:

— *A specimen, processed identically to the unknown, that contains the antigen in question.* This serves as a positive control for the staining. A moderately positive specimen will be sensitive to variations in the performance of the method. This is a more valuable control than a specimen that always stains intensely.

— *A specimen, processed identically to the unknown, that does not contain the antigen in question.* This serves as a negative control for the staining.

— *An additional slide of the unknown specimen that receives nonimmune serum in place of the primary antibody.* This substitution control serves as a specimen blank since any staining observed is due not to antibody localization of the antigen, but to nonspecific protein binding, endogenous peroxidase activity, or nonspecific binding of the other antibody reagents.

It is imperative that the positive and negative control undergo the same fixation and processing schedule as the unknown specimen. In this way, if no positive staining is achieved on the unknown it is not due to improper fixation.

If nonimmune serum is unavailable for the substitution control, the diluent buffer may be used. However, the control slide will no longer detect nonspecific binding of animal serum components to the tissue. When buffer is used, any staining observed will be due to either endogenous peroxidase activity or binding of the other antibody reagents to the specimen.

Another alternative to the nonimmune serum is an antibody made in the same animal species, but directed against an antigen not found in the specimen. For example, a rabbit antibody against prostate specific antigen (PSA) could be used as a substitution control for a rabbit anti-mammary epithelial antigen on breast tissue. This control will serve as an indicator for nonspecific binding of rabbit proteins, as well as for endogenous peroxidase and other protein binding. If this method is used, care must be taken that there is no antigen in the specimen for the antibody to bind to. Substituting anti-PSA for anti-IgM in a lymphoma section could be positive if the tumor was a prostatic metastasis.

The following chart lists possible interpretations of the staining results that can be achieved using the above mentioned controls.

Example No.	Positive Control	Negative Control	Substitution Control	Unknown	Interpretation
1	Negative	Negative	Negative	Negative	Procedure performed incorrectly
2	Positive	Positive	Positive	Positive	Nonspecific staining due to endogenous peroxidase or protein binding
3	Positive	Positive	Negative	Pos/Neg	Negative control contains antigen localized
4	Negative	Negative	Negative	Positive	Positive control does not contain antigen localized
5	Positive	Negative	Positive	Positive	Staining of unknown due to nonspecific reaction
6	Positive	Negative	Negative	Negative	Unknown does not contain antigen localized
7	Positive	Negative	Negative	Positive	Unknown contains antigen localized

In Examples No.'s 1-5, the results of the unknown specimen are invalid due to improper staining of the controls. Only in Examples 6 and 7 can the staining results of the unknown be accepted.

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INTERPRETATION

The most difficult aspect of immunoperoxidase staining is in the evaluation of the finished product. While the actual interpretation depends upon the particular antigen localized, several other factors also play a role. This section will discuss factors such as the condition of the tissue, extent of fixation and possible artifacts, and their relationship to accurate interpretation.

Staining Characteristics

Immunoperoxidase staining enables localization of cellular antigens via the enzyme peroxidase in combination with a specific antibody. A chromagen is added which will react with the enzyme and precipitate at the site of the antigen. The amount of chromagen precipitated, and therefore the intensity of the reaction, is proportional to the amount of antigen present. Nonantigen containing cells and cell components will be contrasted by a counterstain.

When observing a stained specimen, deposits of the colored chromagen indicate the presence of the antigen and represent specific positive staining. There are three main patterns of specific staining that can be observed: cytoplasmic, nuclear and surface. Most antigens are found in the cytoplasm of cells. Depending on antigen content, staining can involve the entire cytoplasm or only a part. Most viruses occupy the nucleus of the cell, and will be localized there by immunoperoxidase methods. Positive staining for surface markers appears on the periphery of the cell.

The staining can be further divided into focal and diffuse patterns. Focal staining will be localized in certain discrete areas of the cell. Diffuse staining involves larger areas of the cell, and often several of the adjacent cells. Certain antigens produce characteristic staining patterns which help make interpretation easier.

Not all cells will contain the same amount of antigen, and will therefore stain with varying intensity. Absence of this variable staining among cells suggests nonspecific staining.

While specific staining is localized in cells, nonspecific background staining is usually found in collagen and connective tissue. Nonspecific staining will not be confined to a single cell, but involves groups of cells with no particular pattern. The exception to this is endogenous peroxidase activity which is usually confined to red and white blood cells.

Interpretation is a comparison of the specific and nonspecific staining pattern (and comparison of staining intensity) of the unknown with that of the control. The source of the tissue, the characteristics of the antigen localized, and the procedure used must also be considered. Only when all of these factors have been taken into account can the staining pattern be properly evaluated.

Effects of Tissue Processing

The fixation protocol should be devised to produce good cellular morphology without destruction of antigen. It is hard to achieve this perfect balance for every type of specimen and antigen. Underfixation may preserve the antigen, but the morphology will be poor—causing difficulties in interpretation. Longer fixation times may produce better morphology, but larger amounts of antigen may be masked or destroyed.

If portions of the tissue were not exposed to the fixative, nonspecific staining will result. Specimens too large to allow complete penetration of fixative will exhibit this sort of staining in the center. Nonspecific staining at the edges of the tissue will be seen if the specimen dried out before being placed in the fixative.

Interpretation of staining patterns should be made based on properly fixed areas of the specimen. Close attention to immediate fixation utilizing small pieces of tissue will help ensure adequate fixation. Staining in portions of the specimen where inadequate fixation has taken place will be nonspecific and should be ignored.

Interpretation of specimens that have been treated with proteolytic enzymes may be difficult at first. Since digestion exposes the antigenic sites, a greater amount and more intense staining will be observed than on undigested specimens. It is important that positive, negative, and substitution controls be digested along with the unknown for comparison.

Artifacts

There are several artifacts that can be found in specimens which will produce staining and interfere with correct interpretation. These can be loosely divided into four categories: precipitates, tissue artifact, cell artifact, and specific background staining.

Various precipitates in the specimen are often confused with positive staining. They can be distinguished by the fact that they are not confined within cells, but are spread randomly across the specimen. Unreacted chromagen granules and counterstaining pigments are found when these solutions

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are not adequately filtered. Another type of precipitate is related to fixation. The use of unbuffered formalin will cause a black precipitate as will crystals of mercuric chloride that were not removed prior to staining.

Tissue artifact usually occurs when the sections are cut, and leads to nonspecific staining. If the sections are too thick, reagents can be trapped between the cell layers. This also happens in areas where the tissue is folded. Any staining that occurs on the very edge of the specimen or along knife marks is often nonspecific.

Positive staining of necrotic or crushed cells is always nonspecific. These cells will be distinguished from true positive cells by the fact that they all stain with the same intensity. Cells that have undergone autolysis, and hemorrhagic tissue, will also exhibit this kind of nonspecific staining. Only the staining pattern of viable cells should be considered for interpretation.

There are a few cases where specific positive staining can interfere with interpretation of results. If the specimen was very bloody or was not washed before being processed, a lot of serum will be present in the interstitial areas. If the antigen stained for is also found in high concentration in the serum, intense background staining will be seen that is actually specific localization of the antigen in the serum. Often this staining can obscure the antigen localized in the cells. Occasionally, macrophages will also demonstrate specific positive staining. These cells have the ability to phagocytize other cells. If the cell that is engulfed contains the antigen localized, then specific staining will be seen inside the macrophage.

TROUBLESHOOTING

This section will tie together all the staining hints presented previously in an effort to solve some of the most common problems encountered in immunoperoxidase techniques. In the first three situations, difficulty is encountered with all slides stained, and therefore represents a problem in the staining system itself. In the latter three situations, the control slides stain correctly, but the unknowns do not. Here the problem lies in the differences in processing the controls and unknowns.

No Staining of Any Slides

1. *Staining steps were not performed in the correct order.* For example, if the secondary antibody is applied before the primary, it will have no place to bind. The primary antibody can still bind to the tissue antigen, but there will be no way to visualize it. The antibody incubations must be carried out in the correct sequence in order to achieve binding of all the reagents. The use of a checklist will help assure that all steps are carried out properly.

2. *Omission of an antibody incubation.* As in the preceeding case, skipping an antibody reagent will prevent subsequent antibodies from binding. For example, if the link antibody is omitted in the PAP technique, there will be no place for the PAP to bind. Again, use of a checklist would be helpful to ensure that all steps are performed.

3. *Sodium azide present in buffer solutions.* This antibacterial agent will prevent development of the peroxidase color reaction. Whenever possible, the user should make up his or her own buffers without sodium azide. Commercially obtained buffers will have sodium azide listed prominently on the label if it is present. The azide content of buffers procured from colleagues or other laboratories should be verified before use.

4. *Improper concentration of hydrogen peroxide in the substrate solution.* Hydrogen peroxide is acted upon by peroxidase to ultimately form a colored end product. If too little hydrogen peroxide is present, it will prove insufficient to enable the reaction to proceed to completion. Too much hydrogen peroxide will inhibit the enzyme and prevent color formation. The substrate solution should not exceed 0.06% hydrogen peroxide, or 0.2ml 3% H_2O_2 in 10ml of substrate solution.

5. *Specimens were improperly counterstained.* This is only a problem when utilizing alcohol soluble chromagens. Counterstains containing alcohol or decolorizing solutions, dehydration, and xylene/toluene based mounting media will disolve soluble colored precipitates. Appropriate counterstaining and coverslipping techniques should be used with soluble chromagens.

6. *Improper fixation and processing of specimens.* If the antigen has not been fixed in the cell, or is not accessible to the antibody, no staining will be observed. The fixation protocol should be reviewed and adjusted for the chosen immunoperoxidase technique where necessary. If specimens were fixed in formalin, the use of proteolytic enzymes will digest excess aldehyde linkages and expose the antigen. Paraffin embedded tissue should never be exposed to temperatures above 60°C either in paraffin baths or drying ovens. High temperatures can destroy heat sensitive antigens as well as cellular morphology.

7. *Drying out of specimens during staining.* Sufficient reagent should be applied to prevent evaporation. If drafts are a problem, a humidity chamber should be

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used. When wiping off excess liquid, only a few slides should be processed at a time. Repeated drying of specimens will result in poor morphology and staining.

Weak Staining of All Slides (excluding negative control).

1. *Specimen retains too much liquid after buffer baths.* This will dilute the antibody reagent and lead to a decrease in staining intensity. As much liquid as possible should be wiped from around the specimen, taking care to get the last clinging drop. Special attention should be paid to removing viscous blocking serum to prevent overdilution of the primary antibody.

2. *Use of old substrate solution.* Once the hydrogen peroxide is added to the chromagen, the solution is stable for only a short time. For optimal results, the substrate reagent should be prepared fresh, just before use. Excess solution should be discarded. As the substrate sits, it starts to break down the chromagen. If the solution begins to change color before application, it should be discarded and a fresh solution made.

3. *Improper concentration of hydrogen peroxide.* See "No Staining of Any Slides," paragraph 4.

4. *Incubation times are too short or the antibody solutions are too dilute.* Since these two parameters are interrelated, they should be considered together. A longer incubation time, a more concentrated antibody, or both should be investigated. Refer to the chart in the Staining Procedures section for possible solutions. If 5 or 10 minute 37°C incubations were used, these should be extended to 20 minutes at this temperature.

5. *Improper storage of reagents.* Conjugated antibodies and the PAP complex are susceptible to deterioration. All reagents should be stored according to the manufacturers' instructions. Products should not be used beyond the stated expiration date.

Excess Background Staining on All Slides

1. *Endogenous peroxidase activity not removed.* If this is the case, then the staining should be observed in red and white blood cells. Repeat the staining using one of the methods described in the Staining Methods section. If endogenous peroxidase activity persists, the incubation time should be increased; or a different quenching procedure utilized. Thorough washing of bloody tissue before embedding is recommended.

2. *Nonspecific binding of protein to the specimen.* Nonimmune serum from the same animal species as the secondary antibody should be applied to reduce nonspecific binding. If background staining persists, a more concentrated solution should be used. Adding 5% bovine serum albumin to the nonimmune serum will also help. A higher concentration of salt in the buffer solutions, such as use of a 1:10 Tris:saline solution, will aid in the reduction of nonspecific binding.

3. *Nonimmune serum was hemolyzed.* This will release red cell breakdown products capable of reacting with the substrate solution. Since endogenous peroxidase activity has already been removed, background staining will result. A fresh supply of nonimmune serum should be obtained that does not show evidence of hemolysis.

4. *Improper antibody dilutions.* Use of concentrated antibody solutions can cause increased background staining. Higher dilutions of antibody should be utilized. This is especially common when changes are made in the procedure; i.e., from a 20 minute incubation to 18 hours at 4°C, paraffin sections to cryostat sections, or the indirect method to the PAP or avidin-biotin method.

5. *Use of whole serum antibodies.* These products contain proteins that can bind nonspecifically to tissue components. The use of immunoglobulin fractions will prevent this type of background. This is especially noticeable since whole antisera are often used at relatively low dilutions where the contaminating proteins are quite concentrated.

6. *Improper fixation.* If the tissue is not promptly fixed or if the specimen is too thick to permit complete fixative penetration, inadequate preservation will result. Poorly fixed specimens will exhibit intense nonspecific staining. Careful attention to prompt and adequate fixation using small specimens will eliminate this problem. Similar nonspecific staining will be observed in necrotic, crushed or damaged cells.

7. *Paraffin incompletely removed.* Residue of plastic containing paraffins causes an overall background staining that extends beyond the borders of the specimen, and often obscures the specific staining. Prior to deparaffinizing, the slides should be placed in a 60°C oven for 30 minutes. They should then be directly immersed in a fresh xylene or toluene bath. A second, fresh bath can be added if necessary. If the problem persists, a different embedding media should be chosen.

8. *Excessive application of tissue adhesive.* Too much adhesive on the slide can cause nonspecific binding of protein to the adhesive. Slides should be lightly coated with adhesive, and excess should be wiped off before picking up the specimen.

9. *Improper rinsing of slides.* To remove unreacted antibody, the specimens should be thoroughly rinsed after

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each incubation using buffer from a wash bottle. The slides should then be placed in a buffer bath to complete the rinsing process. The exception to this is the slides should NOT be rinsed after incubation with the blocking serum. Washing away the serum will expose sites that can nonspecifically bind the primary antibody and result in background staining. Excess nonimmune serum should be removed to prevent dilution of the primary antibody, but the slides should not be rinsed with buffer.

10. *Overdevelopment of substrate reaction.* There are three main conditions that can cause increased substrate staining.

    — An excess of chromagen in the solution
    — A high concentration of antigen in the specimen
    — Increased temperatures causing an accelerated reaction

In each of these areas the easiest remedy is to decrease the substrate incubation time. An alternative is to decrease the concentration of chromagen in the solution.

11. *Increased thickness of specimen.* If the specimen is greater than one cell layer thick, interpretation will be impaired due to staining in each layer. This often takes on the appearance of nonspecific staining, as antibodies become trapped between the layers. Tissue sections should be cut 4-5 microns thick, and cell smears should be spread as thinly as possible.

The next section has to do with staining problems in the unknown specimen.

Positive Control Slide Stains Well, But No Staining is Observed in a Suspected Positive Specimen.

Improper fixation and processing of unknown. This is the most common cause of staining problems, and emphasizes the need for a positive control. Ideally, controls and unknowns should be processed in an identical manner to identify false negatives due to improper fixation. If the specimen was fixed in formalin, a proteolytic enzyme digestion may be necessary. The positive control should also be carried through the digestion process.

Positive Control Slide Stains Well, But Unknown Stains Very Weakly.

1. *Antigen present in low concentration.* This is common in poorly differentiated or metastatic tumors. Staining of the control and unknown should be repeated, using a longer incubation time or a more concentrated solution of the primary antibody.
2. *Antigen partly destroyed or masked by fixation.* If the antigen was destroyed, the fixation and processing protocol should be reviewed. Utilization of a more sensitive technique, more concentrated antibody and/or an increased incubation time, will permit localization of the remaining antigen. If the antigen was masked due to overfixation in formalin, enzyme digestion should increase staining intensity.
3. *Excess buffer or nonimmune serum allowed to remain on the specimen.* This will serve to dilute the subsequently applied antibody solutions causing a weak reaction. As much liquid as possible should be removed from the specimen before applying the next reagent.

Background Staining Absent in Positive Control, But Present in Unknown.

1. *Endogenous peroxidase activity not fully quenched in unknown.* In this case the substitution control should exhibit the same type of staining. A longer incubation time in the quenching solution or a different peroxidase inhibition procedure should be utilized.
2. *Nonspecific binding not fully inhibited in unknown.* The substitution control should exhibit the same type of staining. A more concentrated solution of normal serum and/or the addition of 2-5% bovine serum albumin will decrease nonspecific binding. Use of a 1:10 Tris:saline buffer will also help reduce background staining.
3. *Unknown specimen too thick.* The substitution control may or may not show increased staining depending upon the thickness. If all cells are not in the same plane of focus, then the specimen is more than one cell layer thick. As correct interpretation of staining is impossible, the procedure should be repeated on a thinner specimen.
4. *Unknown specimen contains a high concentration of antigen.* Since this problem is due to an increase in specific staining via the primary antibody, the substitution control would show no background staining. The easiest way to reduce the degree of specific staining is to decrease the substrate incubation time. If necessary, an increased dilution of the primary antibody can also be used.

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About Janice Bourne

Janice was one of the prime movers in the development of the original peroxidase-antiperoxidase (PAP) staining kit.

In her present capacity as Product Manager for DAKO CORPORATION, she is involved in the introduction of all new products for clinical and research laboratories, and in disseminating medical information based on reference material and field experiences.

Since Janice obtained her bachelor of science degree in medical technology from Northeastern University, she has managed to move to the forefront of tissue staining technology and remain there. Today, she travels the world giving lectures, conducting hands-on workshops, and providing practical assistance where it is needed.

About DAKO CORPORATION

DAKO introduced the first PAP kit to the world on September 1, 1980. Today the company offers over 40 kit tests and nearly 250 different antisera, including monoclonals. DAKO is dedicated to the provision of reagents for state-of-the-art tissue staining technology within its broader capacity as an expanding antibody resource for medical research and clinical laboratories worldwide. The product line includes:

Antisera to human, animal, plant, viral and microbial antigens.
FITC, TRITC, HRP and alkaline phosphatase conjugates for tissue work.
Peroxidase and alkaline phosphatase immune complexes from several species.
Human proteins in bulk form.
Reagents for ELISA and EIA techniques.
In general, products for immunoelectrophoresis, immunodiffusion, immunoblotting, nephelometry, turbidimetry, immunofixation, RIA and tissue staining.

Your inquiry is welcome. Please contact the DAKO Distributor in your country or—if unknown—write directly to DAKO CORPORATION for the name and address of your nearest distributor.

In the United States, you may telephone toll free 800/235-5743 or 800/235-5763. Californians may call 805/963-9881 collect.

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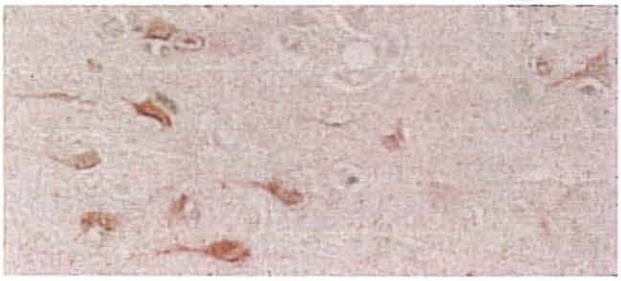
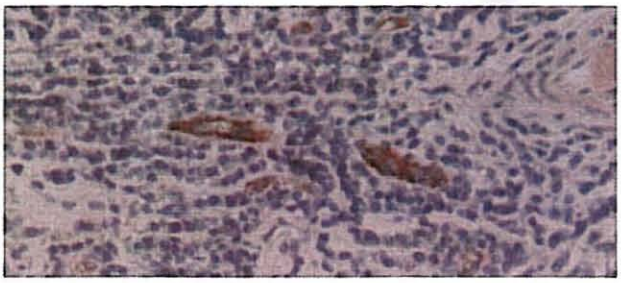
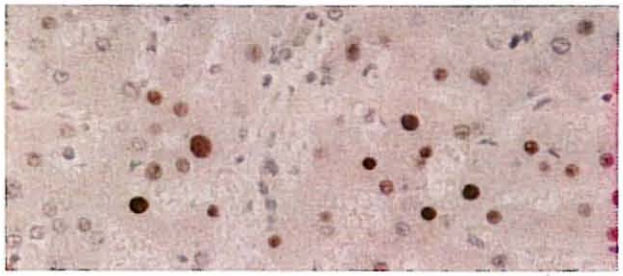


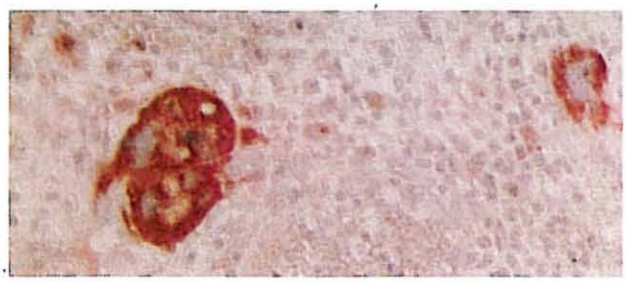
Brain, **HSV**
Formalin fixed, paraffin embedded brain tissue from a case of viral encephalitis shows positive staining of infected neurons for herpes simplex virus type 1. Immunoperoxidase staining permits rapid identification of herpes simplex virus types 1 and 2 in lesion smears as well as in paraffin sections. Comparative studies show excellent correlation with the more complex and time consuming viral isolation techniques.



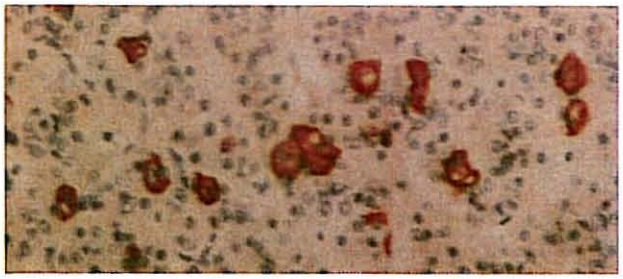
Tonsil, **Factor VIII RAg**
Factor VIII related antigen (F VIII RAg) is localized in the cytoplasm of vascular endothelial cells in this human tonsil section. F VIII RAg can be used to identify a variety of vascular tumors such as hemangiomas, hemangiosarcomas and Kaposi's sarcoma. This antigen is also useful as a marker for megakaryocytes and their precursors.



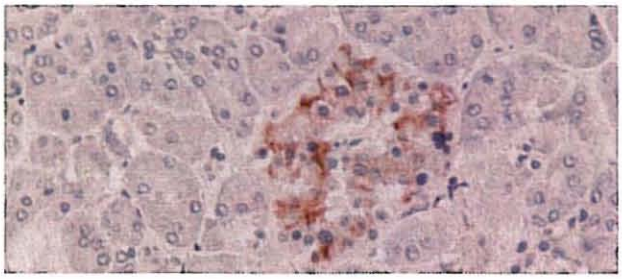
Liver, **HBcAg**
A human liver biopsy demonstrates positive staining for hepatitis B core antigen (HBcAg) in the nuclei of infected hepatocytes. HBcAg is localized primarily in immunosuppressed patients with chronic hepatitis, but will also be observed in chronic carriers and some cases of acute hepatitis. Immunohistochemical staining should always be chosen as the only reliable method to ascertain the presence of HBcAg.



Testis, **HCG**
Positive staining for human chorionic gonadotrophin (HCG) can be seen in the trophoblastic elements of this malignant germ cell tumor of the testis. HCG reflects the biochemical differentiation of cells which usually precedes the morphological differentiation. It is therefore a useful marker for identifying various types of trophoblastic tumors.



Pituitary, **ACTH**
Adrenocorticotrophin (ACTH) is localized in the corticotrophic cells of this human pituitary section. Tumors producing ACTH are usually clinically associated with either Cushing's or Nelson's Syndrome. ACTH can be identified in several endocrine and nonendocrine tumors; including oat cell carcinoma, medullary carcinoma, and ovarian tumors.



Pancreas, **Insulin**
Normal human pancreas stained for insulin identifies the B-cells of the Islets of Langerhans as the predominant cell type. A decrease in the percentage of insulin producing cells will be observed in juvenile diabetes and chronic pancreatitis. Tumors that produce insulin represent the most common type of islet cell adenoma, and are associated with increased numbers of B-cells.

The formalin fixed, paraffin embedded tissue specimens used for these photographs were all stained using the peroxidase-antiperoxidase (PAP) technique. The chromagen, 3-amino-9-ethylcarbazole, produces a red end product that precipitates at the site of the antibody reaction. Positive cells will therefore stain red, while negative cells and nuclei will appear blue due to the hematoxylin counterstain.
Note additional photographs on inside front cover.