Immunohistochemistry is the marriage of immunologic and histochemical techniques, allowing phenotypic markers to be detected and interpreted within a morphologic context. The birth of immunohistochemistry is attributable to Coons and colleagues, who, in 1941, introduced fluorescent-labeled antibodies. The development of enzyme-labeled antibodies that were reactive in fixed, paraffin-embedded tissue, provided the basis for modern immunohistochemistry. Subsequent modifications of techniques, including the peroxidase-antiperoxidase method and avidin-biotin-peroxidase method, as well as the development and commercial availability of a wide range of polyclonal and monoclonal antibodies, have combined to make immunohistochemistry an essential element of contemporary pathology practice.

The most widespread use of immunohistochemistry in pathology is to supplement morphologic criteria in determining the appropriate classification of an undifferentiated neoplasm. For this type of application the immunohistochemistry results are used to aid in the differential diagnosis suggested by the clinical setting and histology of the lesion. In the detection of infectious agents or identification of physiologic substances in aberrant locations, immunohistochemical assays more directly determine the diagnosis. In other applications, such as the detection of prognostic markers including hormone receptors, oncogene products, or proliferation markers, immunohistochemistry is used as an analytical assay. The addition of analytical assays has served to alert the pathology community to the need for more stringent controls in all aspects of immunohistochemistry.

Antigen-Antibody Reaction

Central to the immunohistochemical assay is the specific binding of an antibody to its corresponding antigen. The binding is dependent on noncovalent interactions between the molecules. The strength (avidity) and specificity of that binding are attributable to the multiplicative effect of numerous weak bonds, including electrostatic bonds, hydrophobic bonds, van der Waals forces, and hydrogen bonds. A detailed consideration of all aspects of the biology and biochemistry of these molecules is beyond the scope of this chapter, but a brief description emphasizing aspects important for development and use of immunohistochemical assays follows.

Antigens

Briefly, an antigen is any substance that evokes an antibody response. Antigens are composed of multiple molecular binding sites (epitopes), each of which may be a target for antibody. Epitopic determinants on an antigen are contiguous amino acids, nucleotides, or sugars that are in a linear sequence in the molecule or that join as a result of conformational arrangements. One antigenic determinant stimulates production of multiple different clonal antibodies of varying degrees of affinity. The normal immune response in vivo involves the selection of high-affinity antibodies over low-affinity antibodies (affinity maturation) and is probably driven by competitive binding. Affinity of an antibody is a reflection of the quality of the fit between a single antigen-binding site with the antigen and is independent of the number of antigenic sites. Avidity of an antibody is the total binding strength of all its binding sites together. The nature of many common antigens is discussed in detail in Chapter 2.

Antibodies

An antibody is an immunoglobulin with a specific binding domain for its corresponding antigen. The majority of antibodies pertinent to immunohistochemistry are of the immunoglobulin G class, with a small percentage belonging to the immunoglobulin M class. The immunoglobulin molecule is composed of two heavy chains and two variable light chains. The latter form
the antigen-binding sites (Fab) and have hypervariable domains, which account for the diversity and specificity of the immunoglobulin-antigen interaction. The other major functional component of the immunoglobulin molecule from the immunohistochemical perspective is the Fc binding domain. The Fc domain is itself antigenic. Depending on its class, it can also bind complement, protein A of Staphylococcus species, and cells including neutrophils, macrophages, and trophoblast cells. In some applications, the Fc portion of the immunoglobulin molecule is removed, leaving only Fab fragments to participate in the reaction.

**Polyclonal Antibodies.** Polyclonal antibodies are the product of a particular animal that has been challenged (immunized) with an antigen. Adjuvants, complex mixtures of lipids, emulsifying agents, and other compounds are often used to stimulate the antigenic response of the animal. In response to the antigenic challenge, the animal produces an idiosyncratic mixture of antibodies of varying specificity and affinity. One of the major limitations of polyclonal antibodies is the reliance on the individual animal’s response to an antigen that contributes to significant lot-to-lot variability. Adjuvants may result in the production of an antibody mixture of low avidity and specificity.7 The heterogeneous nature of the antibody response contributes to the potential for antibodies to be present that are directed against a contaminant in the immunogen or that cross-react with other antigens. Affinity-purification techniques may be used to fractionate polyclonal antibodies on the basis of antigenic recognition to eliminate such undesired reactivities. However, affinity-purification can result in an antiserum that contains predominantly low-avidity antibodies.8 Polyclonal antibodies are relatively easy to prepare and have a broader tolerance to antigen fixation than monoclonal antibodies9(54) (see later). Because of the presence of a mixture of antibodies against different epitopes of the same antigen, polyclonal antibodies may result in a more intense signal than a corresponding monoclonal antibody.9(54)

**Monoclonal Antibodies.** The polyclonal antibody response is the product of multiple clones of plasma cells, each secreting a unique immunoglobulin molecule. Isolation of a single clone of plasma cells would result in the production of a single immunoglobulin species, a monoclonal antibody (Fig. 3–1). This has been achieved through the use of cell fusion technology, referred to as hybridoma techniques. An animal is immunized, and immunoglobulin-producing lymphocytes are isolated. A lymphocyte producing the antibody of interest is fused with a nonsecreting myeloma cell to produce an immortalized hybrid cell line that can produce the desired antibody in cell culture or as a tumor in the body cavity of an animal. Monoclonal antibody from ascitic fluid of an animal may be contaminated by constitutive polyclonal antibodies. The use of cell culture supernatant avoids this difficulty. Whichever production method is used, the result is the ability to indefinitely produce a unique well-characterized immunoglobulin.

Despite the cost of the technology to produce monoclonal antibodies, these purified reagents are increasingly used in immunohistochemistry because of greatly reduced background staining, absence of contaminating antibodies, consistency between production lots, and applicability as detection reagents in the form of secondary antibodies (see “Immunohistochemical Assay Formats,” later). However, these reagents do have limitations. Because the initial cells for fusion are isolated by affinity techniques, the selected clones may be of lowered affinity compared with the starting polyclonal response.9(53) A mixture of monoclonal antibodies with specificities directed against different epitopes can result in sensitivity comparable to corresponding polyclonal antibodies.10,11 An additional problem unique to monoclonal antibodies is that intrinsic cross-reactivity may occur, owing to shared epitopes between unrelated antigens. Because epitopic determinants are often short sequences of amino acids or carbohydrates, the chance that the same sequence may be shared by other cells or structures is significant.
This undesired cross-reaction cannot always be anticipated and may not be discovered until the antibody is in use.12

**Conditions Affecting Antigen-Antibody Reaction**

Time, temperature, and pH during the incubation of antigen and antibody will influence the binding of antibody to antigen. Increased temperature generally increases the rate of antigen-antibody complex formation. Within limits, the time of incubation may be shortened as the temperature of the incubation is raised. For polyclonal antibodies, which are composed of a mixture of antibodies with potentially different antigen-binding characteristics, long or repeated incubations should favor binding by the highest avidity antibodies, owing to displacement of low-avidity antibodies. However, these steps have little enhancing effect on the ultimate signal achieved with monoclonal antibodies. Although numerous permutations of incubation time and temperature have been utilized, the trend is away from 24- to 48-hour incubations at room temperature, or 37°C, to facilitate the rapid turnaround time in demand in the current laboratory environment.

The buffer used as the antibody diluent controls the pH during the incubation. An alteration in the reaction pH can alter both the strength and specificity of the antigen-antibody interaction.13(p160) The majority of reactions between antigen and polyclonal antibody are tolerant of a pH range between 6.5 and 8.5. Monoclonal antibodies, however, are less tolerant of pH changes<sup>9(p42)</sup> Most incubations utilize a pH between 7.2 and 7.6 maintained by phosphate-saline or TRIS-saline.

Antibody dilution is a critical factor in the antigen-antibody reaction. Antibody should be used at as high a dilution as possible without loss of detection of specific staining. This is economical, will diminish any prozone effect (false negative owing to high antigen content), and favors lower background staining. The highest dilution is selected during optimization of an antibody before implementing its use in the laboratory.

The cellular antigen must be accessible for the antigen-antibody reaction to occur optimally. For antigens located on the cell surface, this is usually not problematic. Intracellular antigens are made more accessible to antibody, which normally does not cross the cell membrane, by disrupting the cell membrane. This is partially accomplished by tissue fixation and sectioning. Antibody penetration is also enhanced by use of digestion techniques or by addition of detergent to the various reagents used in the immunohistochemical sequence. Addition of detergent, however, can occasionally result in antigen damage.13(p155) Lower-molecular-weight reagents will penetrate tissue sections more uniformly, hence enhancing reactivity with antigen. Because of this effect, in some settings Fab fragments of immunoglobulins may enhance the immunohistochemical result. Even penetration will occur only when antiserum uniformly covers the tissue section on the glass slide. Humidity is important to minimize dehydration of the tissue sections and reagents that can lead to a higher antibody concentration in solution than is desirable.

**Immunohistochemical Assay Formats**

**DIRECT IMMUNOHISTOCHEMISTRY**

The direct immunohistochemical method was originally developed by Coons and colleagues,<sup>1</sup> who conjugated a fluorescent molecule to antibody for use as a label (1941). For direct immunohistochemistry, the detection system (i.e., the enzyme or fluorochrome) is covalently linked (conjugated) directly to the primary antibody (Fig. 3–2). The primary antibody is applied to the specimen, followed by washing and direct detection by viewing or development with substrate. Because so few steps are involved, the direct technique is quite rapid. The principal utility of the direct method is in the immunofluorescent detection of immune complexes in skin and kidney biopsy specimens. The final sensitivity of the method is dependent on the chemistry of labeling the primary antibody. The chemical conjugation must be carefully controlled to ensure that the antigen-binding site is not damaged and that the reaction is nearly 100% efficient. Unlabeled primary antibody will efficiently compete with the labeled molecules for antigen binding and greatly reduce the signal. In practice, it is usually more efficient to use unlabeled primary antibody and devise alternative methods for detection.

**INDIRECT IMMUNOHISTOCHEMISTRY**

Indirect immunohistochemical methods exploit the natural capacity of immunoglobulin to act as antigen. In this approach, the primary antibody is raised in one species, for example, the rabbit, and is not conjugated.

![FIGURE 3–2](image) Direct immunohistochemistry. The label is attached directly to the antibody having specificity for the antigen under study. F, fluorescein; Px, peroxidase. (From Taylor CR: Principles of immunomicroscopy. In Taylor CR, Shi SR, Cote RJ [eds]: Immunomicroscopy: A Diagnostic Tool for the Surgical Pathologist ed 2. Philadelphia, WB Saunders, 1994, p 10.)
Immunoglobulins from this species are then used as an immunogen in a second species, for example, the goat, resulting in antibodies recognizing immunoglobulin in the primary serum (in this example, goat anti-rabbit). Immunoglobulin from the secondary serum is then conjugated to a detection system as described earlier for the direct system (Fig. 3–3). The complete assay involves incubation with unlabeled primary antibody, washing, incubation with secondary antibody, followed by washing and detection.

The labeled secondary antibody can allow for signal amplification compared with the direct method, and this permits the primary antibody to be used at a higher dilution. Sensitivity is generally increased compared with direct detection. The labeled secondary antibody can be used to detect any primary antibody that has been raised in the species it recognizes. This versatility allows for commercial development and standardization of these detection reagents. Background staining associated with polyvalent secondary antibodies can be a problem with this technique.7

A recently developed variation of this technique uses a large dextran backbone to covalently link secondary antibody and enzyme molecules. The complex includes up to 100 enzyme molecules and 20 secondary antibody molecules. Improved sensitivity, attributed to increased localization of enzyme activity, has been reported.14

Antibody Bridge Techniques

The antibody bridge technique (“three-step method”) is a further modification of the indirect method. In addition to the primary and secondary antibodies used in the indirect method, a tertiary labeled antibody is used in the antibody bridge technique. The primary and tertiary antibodies must be from the same or closely related species, and the secondary or bridge antibody is directed against that species, hence binding both primary and tertiary antibodies as a “bridge.” (In this application the secondary antibody is unlabeled.)

In a variation on the same theme, the tertiary antibody can be an unlabeled antienzyme antibody. Addition of the enzyme (usually horseradish peroxidase) as the fourth step results in an immunologic reaction between enzyme (e.g., horseradish peroxidase) and antienzyme (anti-horseradish peroxidase) for detection. Preformed enzyme antienzyme complexes such as peroxidase antiperoxidase (PAP),5 alkaline phosphatase anti-alkaline phosphatase (APAAP), and glucose oxidase anti-glucose oxidase (GAG), are available. This combination of tertiary antibody and enzyme into one complex that is linked to the primary antibody by an unlabeled bridge antibody reduces the four-step technique to a three-step technique (Fig. 3–4). Reproducibility and specificity are enhanced with use of PAP, which is a small, stable complex from the same or closely related species that shares antigenic determinants against which the bridge antibody is directed. The efficacy of these techniques depends on the development of high-affinity bridging antibodies, tertiary antibodies, and antienzyme antibodies.

Affinity Labeling Methods

Avidin-Biotin Methods. The extremely high affinity of avidin for biotin has been used as an alternative to relying on the antigenic nature of immunoglobulins to provide links between antigen localization and detection reagents. Avidin is a high-molecular-weight protein (mol. wt. 67,000) found in egg white that has very high affinity for biotin, a low-molecular-weight water-soluble vitamin. The avidin-biotin disassociation constant approaches the strength of a covalent bond ($K_d = 10^{-15}$), and the chemistry of utilizing these molecules as reagents for detection is identical.
Affinity purification has been well characterized. One molecule covalently labeled with avidin will bind to another molecule covalently labeled with biotin, assuming labels are introduced in a fashion that does not sterically limit the interaction of the affinity labels. Because avidin molecules have four binding sites for biotin, the geometry and proportions may be arranged to allow for multi–molecular complex formation between avidin- and biotin-labeled reagents.

Although biotin can be attached to any component of the immunohistochemical reaction—primary, secondary, or histochemical enzyme—the use of a biotinylated secondary antibody is employed most frequently because of the great flexibility in the approach for detection. A simple layered approach may be used. After localization of the biotinylated secondary antibody, detection can be achieved with unlabelled avidin followed by biotinylated label as the fourth step, known as the bridged avidin–biotin method. The biotinylated label is most commonly a histochemical enzyme. The third and fourth steps of the bridged avidin–biotin method could be combined though complex formation between avidin and biotinylated enzyme, in the same way that the PAP complex eliminated one step. This was first described for biotinylated peroxidase and is known as the avidin–biotin complex (ABC) method (Fig. 3–5). Because of the polyvalent avidin–biotin–binding capacity of avidin, mixing of the biotinylated enzyme and avidin can be done in a ratio that allows for multiple enzyme molecules to be complexed to avidin while the overall complex still retains the excess biotin-binding sites required for interaction with the biotinylated secondary antibody. The significant advantage of the three-step ABC is its great sensitivity, which has been attributed to the localization of several molecules of peroxidase at the antigenic site. The avidin–biotin peroxidase-antiperoxidase (ABPAP) complex involves sequential use of the PAP and ABC techniques in an attempt to further increase the number of enzyme molecules at an antigen site. Signal amplification by these techniques reaches a limit because of steric effects. Binding of large complexes is less efficient, and enzymes buried within layers of other molecules may not be as accessible to substrates required to generate the colored products. Although first described for peroxidase, alkaline phosphatase can be biotinylated and complexed with avidin in a similar fashion.

Avidin reagents have been made by covalent reaction to a histochemical enzyme. For horseradish peroxidase, the resulting conjugate can include two or three enzymes bound to an avidin molecule. The use of conjugates is referred to as labeled avidin–biotin (LAB). The conjugates require no mixing before use, and the sensitivity of complex versus that of the conjugate is similar. Well-standardized commercial reagents are available for either approach.

Whereas the previous discussion has centered on avidin, a very similar protein, streptavidin, is available. It is produced by Streptomyces avidinii and has nearly identical biotin-binding characteristics. Unlike avidin, streptavidin has a neutral isoelectric charge because it lacks the carbohydrate side chains of avidin. In some applications, substituting streptavidin for avidin may contribute to lower background.

**Protein A Methods.** Protein A is a major cell wall component of most strains of *Staphylococcus aureus*. It has a very high affinity for the Fc component of immunoglobulin, particularly IgG. Its affinity for different subclasses of IgG and for other immunoglobulins varies from species to species. It can be conjugated to various labels (e.g., fluorescein, gold, alkaline phosphatase, peroxidase) without impairment of its immunoglobulin-binding capacity, allowing for shorter incubation times. Lack of sensitivity limits widespread application in light microscopy; however, because of its low molecular weight, protein A is quite useful in immunoelectron microscopy.

**Lectin Histochemistry.** Lectins are proteins and glycoproteins of plant origin that bind in a noncovalent fashion to specific polysaccharides. In most applications, the lectin is substituted for a primary antibody to specifically localize a complex carbohydrate group. The bound lectin is then detected in a fashion similar to immunohistochemistry, for example, peroxidase with an appropriate label or a fluorescent or metallic label. Lectin histochemistry and diagnostic applications have been reviewed.

**Immunoelectron Microscopy**

Immunoelectron microscopy serves to accurately localize antigen at the ultrastructural level. The technique is complex because of the need to balance antigen...
preservation and accessibility with adequate fixation for ultrastructural morphology. In pre-embedding techniques the antibody is applied to relatively thick sections of unfixed tissue. Subsequently, these sections are fixed and processed for electron microscopy. Although useful for very labile antigens susceptible to fixation, significant disadvantages include the need for fresh tissue and relatively poor ultrastructural preservation. Postembedding techniques, whereby the tissue is fixed, processed, and resin embedded before thin sectioning for immunochemistry, are much more practical. Morphology is improved, less tissue is needed, double staining is possible, and retrospective studies can be performed. Immunochemistry has been reviewed, and a detailed discussion is beyond the scope of this chapter.

Detection Systems

As implied in the previous description of assay formats, antibody alone cannot be visualized. Each assay format uses a different technique for localizing a reporter molecule or detection system to specifically bound primary antibody. These detection systems include nonenzymatic systems as well as systems using enzyme to generate a signal.

Nonenzymatic Labels

Fluorescent labels, such as fluorescein isothiocyanate, were among the first labels used in immunohistochemistry and are most commonly used for detection of the abnormal deposition of physiologic substances in renal and skin biopsy specimens. Disadvantages associated with fluorescent labels include the need for a special light source, limited morphology because of poor counterstaining, fading of the signal on storage, and intrinsic tissue autofluorescence that is increased by fixation.

Heavy metals, such as silver, gold, ferritin, and mercuryl compounds may be used as labels, particularly at the ultrastructural level. Depending on particle size, metal labels may be visible with the light microscope. Immunogold techniques, involving conjugation of colloidal gold particles to antibody, protein A, or avidin, are increasingly being used at both the light and electron microscopic levels. The size of gold particles used ranges from 1 to 20 nm. Smaller particles improve signal resolution but may require silver enhancement for detection. Particles of 20 nm are readily visible at the light microscopic level but may be associated with steric hindrance and produce a granular signal.

Histochemical Enzymes and Substrates

Histochemical enzymes are enzymes that have been used as visualization tools for the localization of targets in tissues or cells. To be useful in such a capacity, the enzyme must have a substrate system that generates a product that can be visualized (i.e., a chromogen) and has minimal diffusion from the site of production. This latter characteristic is referred to as the substantivity of the chromogen. The final sensitivity of detection is determined by the combined efficiency of the enzyme-substrate system in generating signal. The enzyme-specific activity, substrate concentration and purity, time, and temperature of reaction will all impact results. In practice, these variables have been minimized because of the commercial availability of these key reagents. Table 3–1 summarizes the commonly used enzyme-substrate chromogens, color of product, and solubility of product in alcohol.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Chromogens</th>
<th>Color</th>
<th>Solubility in Alcohol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Horseradish peroxidase</td>
<td>Diaminobenzidine (DAB)</td>
<td>Brown</td>
<td>Insoluble</td>
</tr>
<tr>
<td></td>
<td>Aminoethylcarbazole (AEC)</td>
<td>Red</td>
<td>Soluble</td>
</tr>
<tr>
<td></td>
<td>Alpha-naphthol pyronin</td>
<td>Red</td>
<td>Soluble</td>
</tr>
<tr>
<td></td>
<td>4-chloro-1-naphthol</td>
<td>Blue</td>
<td>Soluble</td>
</tr>
<tr>
<td></td>
<td>Paraphenylenediamine pyrocatechol</td>
<td>Blue</td>
<td>Insoluble</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>Fast red TR</td>
<td>Red</td>
<td>Soluble</td>
</tr>
<tr>
<td></td>
<td>Fast blue BB</td>
<td>Blue</td>
<td>Soluble</td>
</tr>
<tr>
<td>Glucose oxidase β-Galactosidase</td>
<td>BCIP/NBT</td>
<td>Blue/black</td>
<td>Insoluble*</td>
</tr>
<tr>
<td></td>
<td>Tetrazolium blue</td>
<td>Blue</td>
<td>Insoluble</td>
</tr>
<tr>
<td></td>
<td>BCI</td>
<td>Indigo</td>
<td>Insoluble</td>
</tr>
</tbody>
</table>

* Slight loss of intensity in alcohol.

BCI, 5-bromo-4-chloro-3-indoly1-β-D-galactopyranoside; BCIP/NBT, 5-bromo-4-chloro-3-indoly1 phosphate/nitroblue tetrazolium.
Horseradish Peroxidase. Horseradish peroxidase is the most commonly used histochemical enzyme. It is a relatively small protein (mol. wt. 40,000) that uses peroxide as a substrate and is inactivated by substrate excess. The enzyme-substrate reaction is rapid and enzyme activity is quenched within 15 to 30 minutes. Color development occurs using an electron donor as histochemically demonstrable material. There are several different histochemical systems for this enzyme. The two most commonly used are diaminobenzidine (DAB) and 3-amino-9-ethyl carbazole (AEC), either of which yields comparable results, although less diffusion from site of liberation is noted for DAB. Peroxidase is most active at neutral pH and is reversibly inhibited by azide, cyanide, and sulfide.

DAB results in a mahogany brown reaction product that does not fade and is insoluble in organic solvents. The contrast with a light hematoxylin counterstain is good, and standard mounting media can be used. Intensification of the DAB reaction product, changing the color to black, can be accomplished by addition of heavy metals (e.g., nickel chloride, cobalt chloride, copper chloride, imidazole or osmium tetroxide). DAB results in a mahogany brown reaction product that does not fade and is insoluble in organic solvents. A sequential procedure is used when both primary antibodies are raised in different species, a simultaneous procedure using different enzyme-chromogen combinations can be performed. The optimal end result in a case containing the antigens of interest will be two highly contrasting visually different signals. Combining of immunogold-silver staining with horseradish peroxidase and alkaline phosphatase results in successful triple staining.

Glucose Oxidase. Glucose oxidase is most often employed with tetrazolium blue as the chromogen. Although this enzyme-substrate system is less sensitive than methods using peroxidase- and alkaline phosphatase–based colorimetric systems, it has the advantage that human tissues lack endogenous glucose oxidase activity. It is also useful in double immunohistochemical labeling techniques.

β-Galactosidase. β-Galactosidase is isolated from Escherichia coli. At the optimal pH for use (7.0 to 7.5), interference from mammalian β-galactosidase is not a problem. The substrate, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, results in an insoluble indigo-colored reaction product.

The substrate, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, results in an insoluble indigo-colored reaction product.
subsequently detected with a second round of histochemical enzyme localization and conventional signal generating substrates. The systems have been called varying names, including catalyzed reported deposition (CARD), tyramide signal amplification (TSA), and catalyzed signal amplification (CSA). The most common approach uses any of the conventional histochemistry methods to localize horseradish peroxidase. The horseradish peroxidase activity is used against a tyramide substrate covalently linked to an affinity label. Horseradish peroxidase acts on the phenolic part of the affinity-labeled tyramide to produce a highly reactive short-lived intermediate that forms a covalent link with adjacent protein molecules. The newly deposited affinity labels are subsequently detected with another round of histochemical detection (either horseradish peroxidase or alkaline phosphatase, with standard colorimetric substrates) or fluorescence. Alternatively, additional rounds of signal amplification with the tyramide reagent may be used to generate still more targets. Tyramide conjugates have been made with biotin or fluorescein affinity tags. This approach has been reported to result in greatly improved sensitivity. Because the reactive affinity-labeled intermediates have an extremely short half-life, signal localization remains good, although some loss of precision occurs with additional rounds of amplification. Care must be taken to optimize the system because any background in the initial histochemical localization is amplified in the subsequent reactions. Signal amplification applies equally to detection of in situ hybridization reactions and is an attractive alternative to in situ PCR reactions for detection of low copies of target.

**Optimization of Test Material**

A crucial requirement for a valid immunohistochemical assay is to have adequate test material that will yield valid results. Although this component of the assay is often taken for granted, it is clear that no refinement of immunohistochemical technique can compensate for inadequate starting material. Adequate test material means that the sample must be representative of the lesion and be prepared to allow for preservation of morphologic detail and antigenicity. Some method of fixation is undertaken to arrest autolysis and to preserve morphologic detail. Unfortunately, from the perspective of the immunohistochemist, fixation may result in variable target antigen loss or may render the antigen target inaccessible.

Under ideal situations, the tissue specimen would be rapidly submitted to the pathologist in the fresh state and equally rapidly examined and processed. The pathologist would then control the nature and duration of fixation. A representative sample of all tumors could be snap frozen and maintained in a tumor bank, in case a particular target antigen is susceptible to fixation and to allow for validation of results or extension of results by current or future molecular assays. Although this idealized strategy is unlikely to be implemented, some improvement on the widely used practice of immersing a whole specimen in fixative for an unspecified period of time before sending it to the pathologist needs to be considered.

The biochemical and ultrastructural alterations known as autolysis occur immediately after tissue is removed from the body, or after the body dies. Just as tissues do not autolyze at a standard rate, so tissue antigens and nucleic acids autolyze at variable rates. Minor changes are inevitable, and even extensively autolyzed tissue may still be useful for immunohistochemistry. Knowledge about the effect of autolysis on any particular antigen is necessary for reliable interpretation. The goal of each method of tissue fixation and processing is to obtain reproducible preservation of morphology and biochemical macromolecules.

**Methods of Tissue Fixation**

**Freezing.** Rapidly frozen tissue is of use for immunohistochemistry because of superior preservation of antigens, in particular cell membrane surface antigens. The pattern of immunoreactivity in frozen sections remains the gold standard against which all other assays are judged. If antigen is detected in frozen sections but not in paraffin sections of formalin-fixed tissue, then the fixation and processing sequence has resulted in antigen loss, change, or masking, rendering it unavailable. Frozen tissue is used to demonstrate the optimal pattern of reactivity of an antibody. A wider range of antibodies is applicable to frozen tissues, and generally frozen tissues have been promptly handled and are well preserved.

Snap freezing tissue for immunohistochemical analysis is not routine, so frozen material will not always be available. The frozen sample must be protected from freeze-thaw cycles and from dehydration during storage. The morphology of the cryostat section is not as good as can be obtained with paraffin sections, and adherence of frozen tissue to the glass slide can be difficult. In addition, unfixed antigens may diffuse or be dislocated to other areas during sectioning. Rapid fixation of cryostat sections in acetic acid or periodate lysine paraformaldehyde or by microwave irradiation is used to minimize the problem of antigen dislocation and to improve morphology. Variations in duration of fixation and nature of the fixative will influence antigen preservation and the eventual staining intensity and patterns. In current diagnostic practice, the use of frozen section immunohistochemistry is largely confined to hematopathology and the diagnosis of renal and dermatologic diseases. The availability of antibodies reactive in fixed tissues and the use of plastic embedding media and enhanced antigen
retrieval systems have reduced the need for frozen tissue studies. Freeze-drying or freeze-substitution of tissue as a means of fixation can be used for either electron or light microscopic study. In combination with both liquid- and vapor-phase fixatives and resin-embedding, excellent preservation of small antigens, such as intracellular enzymes, is achieved. Intracellular location of antigen within specific organelles can also be demonstrated morphologically by such means.

**Cross-linking Fixatives.** Formaldehyde at 37% (wt/wt) or 40% (wt/vol) in aqueous solution is diluted 1 in 10 and buffered to pH 7.0 to make a working solution that is known as 10% buffered formalin or 4% formaldehyde. Formalin is inexpensive, readily available, widely used, and probably the closest approach to a universal fixative. Morphologic preservation and antigen immobilization is superior when compared with coagulant fixatives. Although formaldehyde penetrates tissues rapidly, fixation occurs slowly. It acts to form covalent cross-links between adjacent reactive groups on macromolecules. The highest reactivity is with amino groups such as lysine, and thiols such as cysteine, but amides will also react with the aldehyde. This cross-linking process can result in direct loss of epitopes that include these reactive groups or indirect loss of antigenicity through cross-linking of adjacent molecules resulting in steric hindrance.

Tissue fixation is dependent on reagent penetration and on the rate of reaction. For the chemical reaction to be even, the tissues must be thinly cut, 0.2 cm ideally, and no larger than 1.5 cm in diameter. The rate of chemical reaction is temperature dependent, and times range from 16 hours at 37°C to 24 hours at room temperature for the chemical reaction to reach equilibrium. Prolonged fixation time, 72 or 96 hours, has no deleterious effect on morphology; however, increased cross-linkage limits antigen accessibility. For this reason the fixation time should be minimized. The response of each antigen to fixation is somewhat different, making broad generalizations about optimal fixation times difficult. A minimum of 12 hours and a maximum of 24 hours formalin fixation have been advocated. The use of freshly made 10% buffered formalin, rather than commercial preparations that may contain methanol or other contaminants, may also enhance target preservation. With shorter fixation times in formalin, the periphery of the specimen is formalin fixed, but fixation of the central portion is accomplished by the dehydrating alcohols of the tissue processor, resulting in uneven immunohistochemical signal limited to either the central portion or the edge of the tissue. At the other extreme, formalin fixation for prolonged periods of time can result in potentially irretrievable antigen loss. Antigens such as vimentin filaments are particularly susceptible to formalin fixation. Tissues fixed in formalin for more than 72 hours show marked decrease or complete loss of vimentin immunoreactivity. The use of such ubiquitous targets as internal controls of fixation is useful in the interpretation of immunohistochemical panels wherein results seem erratic or aberrant. Addition of other substances to formalin may enhance morphologic detail and antigenicity. However, the improved antigen preservation with any of these modified formalin fixatives may be more related to control of the duration of fixation than to the chemical reaction of the fixative with the tissue. Mercuric chloride, picric acid, acetic acid, and periodate lysine paraformaldehyde (PLP) are the most common additives. Mercuric chloride–containing fixatives such as B5 (formalin/mercuric chloride/sodium acetate mixture) and Zenker’s fluid result in improved nuclear morphology. Enhancement of intracellular immunoglobulin staining is reported. The mercury results in a precipitate that must be removed from the tissue and adds to cost for appropriate disposal of an environmental toxin. Fixatives containing picric acid, such as Bouin’s fluid or Zamboni’s fluid, have been shown to enhance cytoplasmic immunoglobulin signal, obviating the need for predigestion of tissue sections. Picric acid reacts with histones and basic proteins, with the formation of crystalline picrates. PLP fixation permits the use of monoclonal antibodies directed against some lymphocyte surface antigens in paraffin-fixed tissue and is useful for immunofluorescent techniques because it does not induce tissue autofluorescence. PLP requires fresh preparation for each specimen.

Glutaraldehyde is another cross-linking aldehyde fixative that differs from formaldehyde in that it penetrates tissues slowly but cross-links tissue rapidly. It is the preferred fixative for small pieces of tissue intended for electron microscopic study or for light microscopic immunohistochemistry of low-molecular-weight substances. However, its potent cross-linking ability can result in antigen masking. Strong background fluorescence after fixation in glutaraldehyde renders it an unsuitable fixative for immunofluorescence studies.

**Coagulant Fixatives.** Coagulant fixatives fix tissues by precipitating proteins without introducing covalent bonds. The primary protein structure may be relatively unaffected by these methods. Absolute ethanol is probably the most commonly used coagulant fixative; and in comparison with formalin, it appears to afford greater preservation of immunoreactivity of filament proteins. Another advantage of non–cross-linking fixatives is that a predigestion step can often be avoided. Coagulant fixatives are not without disadvantages. Low-molecular-weight antigens are susceptible to loss (extraction) during fixation and processing. Dislocation artifacts occur; for example, instead of the usual cytoplasmic distribution of myeloperoxidase, ethanol-fixed neutrophils demonstrate perinuclear
myeloperoxidase immunohistochemical signal.\textsuperscript{43} Tissue shrinkage and distortion can also be a problem. Enzymes responsible for autolysis may not be completely inactivated by ethanol fixation; and if a lengthy incubation step is necessary subsequent to fixation, focal tissue autolysis may occur. Proprietary alcohol-based fixatives are available and reportedly result in less tissue shrinkage than ethanol. Other coagulant fixatives include Carnoy’s fluid, methacarn, acetone, and methanol.\textsuperscript{53} Carnoy’s fluid and methacarn reportedly penetrate tissue more rapidly than ethanol.

**Microwave Irradiation Fixation.** The physics of microwave technology in histochemistry has been reviewed in detail.\textsuperscript{54} The predominant physical effect of microwaving is to increase the temperature, resulting in acceleration of reagent permeation and chemical reactions. Microwave-assisted fixation in dilute aldehydes is reported to give superior morphology and eliminate the need for a predigestion step.\textsuperscript{55} This approach may result in rapid fixation of the outer zone of tissue with subsequent decrease in diffusion of fixative into the tissue. The central portion of the tissue would be stabilized secondary to heat coagulation of proteins rather than by cross-linking.\textsuperscript{56}

In another variation, microwave stabilization of tissue in buffered salt solution such as normal saline can result in good preservation of morphology and enhanced immunoreactivity against common antigens compared with conventional formalin fixation.\textsuperscript{57} Additional advantages include speed of fixation and use of normal saline rather than chemical fixatives; however, preservation of fine ultrastructural detail may be suboptimal.

All microwave methods are highly temperature dependent. The size, shape, and volume of the material to be microwaved, dilution and ionic strength of the medium, position in the microwave oven, and presence of other loads in the oven may all significantly affect temperature. In addition, the size and power of the microwave oven and age of the magnetron may affect the temperature achieved. Some control of these variables can be achieved by using the same oven for defined periods of time in a standardized fashion.\textsuperscript{54,58} Horobin and Flemming\textsuperscript{56} have summarized artifacts and hazards associated with microwave use and provided examples of useful flow charts for pinpointing specific problems.\textsuperscript{46}

**Tissue Processing and Embedding Media**

In the majority of histology laboratories, tissue is dehydrated and infiltrated with paraffin on automated tissue processors with or without vacuum. It is then subsequently embedded in paraffin wax at its melting point. In most instances all tissues cut throughout the day are processed together in group fashion to allow for the processing to be complete for embedding the following morning. Because the process is automated and “routine,” the impact of tissue processing on immunohistochemical and molecular preservation tends to be overlooked. As part of tissue processing, fixation is completed. For optimal results, reagents on the tissue processors must be changed at appropriate intervals, depending on the number of cases processed. Times and temperatures at each step of the processor should be controlled. As commonly used, the processor includes one or two additional changes of formalin before initiation of the alcohol-based dehydration steps. These are added to ensure adequate fixation, but significant variation in fixation occurs depending on the size of the tissues and time of fixation before the tissue is placed on the processor. Weekend processing introduces further variation because of the common practice of holding tissue blocks in the formalin step from Friday through Sunday. Separate processing of smaller tissues (biopsy specimens) with appropriate adjustment of times has been used by some laboratories to minimize variation. Weekend holding stations may be modified to avoid prolonged exposure to formalin.

At the completion of the dehydration steps, tissue is infiltrated with paraffin at its melting temperature in preparation for manual embedding. The melting temperature of the paraffin embedding medium varies depending on the commercial preparation and additives but varies between 50°C and 60°C. Prolonged exposure of tissue to this elevated temperature may result in antigen loss or denaturation.\textsuperscript{59}

Alternative tissue processing procedures can be developed to attempt to introduce more standardization. One, known as the AMeX procedure, involves initial fixation in acetone and clearing in methylbenzoate and xylene before paraffin embedding, has been reported to result in improved antigen detection using antibodies usually only reactive in frozen sections.\textsuperscript{60}

Numerous alternative embedding media in combination with various fixatives have been used on various tissues for “routine” light microscopy immunohistochemistry and for immunoelectron microscopy. Briefly, the main alternatives are acrylic resins, of which the best known are methyl methacrylate or glycol methacrylate, epoxy resins, and, more recently, LR resins and lowicryl. Advantages of plastic embedding media include ability to embed tissue at low temperature, minimal tissue shrinkage, ability to cut 1- to 2-\(\mu\)m sections (of use for serial sections of the same cell), improved preservation of hematolymphoid antigens, and obviation of the need for decalcification.\textsuperscript{49} Disadvantages include expense, need for additional equipment and expertise, difficulty with antigen preservation, and need for removal of embedding medium before immunohistochemistry. There are reports of simplified methods and documented success with immunohistochemistry on plastic embedded tissues.\textsuperscript{39,41,42,61–64} Plastic embedded blocks
may require special storage conditions either at low temperatures or in conditions of low humidity. Epoxy resins are most commonly used for electron microscopy because of their sturdiness. Removal of epoxy resins requires a harsh chemical procedure that may result in antigen loss. Combinations of freeze-drying or freeze-substitution fixation and resin embedding are used for immunoelectron microscopy. Most antigens and intracellular enzymes are preserved well with this technique.

**Digestion and Antigen Retrieval**

Recognizing that tissue fixation is often a poorly controlled part of the immunohistochemistry sequence and that tissue antigens may undergo deleterious yet reversible alteration as a result of excessive or harsh fixation, both enzymatic and nonenzymatic methods have been used in attempts to retrieve or “unmask” target antigen. Such techniques may lead to unanticipated or aberrant cross-reactivity and should be introduced with appropriate controls to exclude that possibility.

**Enzyme Digestion.** Enzymes such as trypsin, pepsin, pronase, or DNase, either singly or in combination or sequence, have been used to digest tissue sections before incubation with antibody. The digestion may function to reduce steric hindrance of antibody and detection reagents. Variables to be considered during optimization and standardization of the digestion include enzyme concentration, time and temperature of digestion, nature of the tissue, and duration of fixation as well as thickness of tissue section. Time of digestion needed for maximum target antigen unmasking varies inversely with the duration of fixation. For formalin-fixed tissues, no significant advantage of any one protease over another has been identified. Optimal conditions for enzyme digestion vary considerably between antibodies, and a decrease or abolition of immunoreactivity with certain antibodies is reported after enzyme digestion. The digestion conditions must be optimized whenever a new antibody is introduced and must be closely monitored to ensure that maximum antigen unmasking is not achieved at the cost of suboptimal tissue morphology. Because lot-to-lot variability can occur, the activity of a new lot of enzyme used for digestion must be compared with the previous lot before it is put into use. Similarly, the enzyme must be stored under appropriate conditions to minimize loss of activity.

**Antigen Retrieval.** Nondenzyometric methods of antigen retrieval are becoming more popular. Refixation of the tissue block in zinc formalin or immersion of tissue sections in strong alkaline solution is reported to improve immunohistochemical staining. “Antigen retrieval solutions” of proprietary composition are available commercially, and enhanced immunoreactivity has been reported. Microwave-assisted antigen retrieval has been reviewed. Reversal of formalin-induced protein cross-linkage at high temperature was noted originally in the 1940s and this is thought to be a partial basis for microwave irradiation enhancement of immunohistochemical signal. Various solutions (ranging from heavy metal solutions or salt solutions to distilled water) have been used. Similar enhancement of signal is observed after simple boiling of tissue sections in a domestic pressure cooker, whereas heating of tissue sections in a conventional oven has proved less successful. Various factors intrinsic to microwave ovens as discussed in the previous section on microwave fixation need to be at least partially standardized for the procedure to be reproducible. Different conditions result in optimal antigen retrieval depending on the target and its cellular location. Enhancement of detection is reported for most antigens tested, although some examples of reduced or abolished signal are demonstrated. It is not yet clear which antigenic characteristics can predict whether enhancement or abolition of signal will occur after antigen retrieval.

There are several advantages associated with microwave antigen retrieval. There is potential for reduction in incubation time with primary antibody resulting in cost savings. Positive immunohistochemical signal can be identified after microwave antigen retrieval in tissues that have been formalin fixed for extended periods of time or in tissues that are usually unreactive for that particular antibody after formalin fixation. Improved signal-to-noise ratio is usually demonstrated. A disadvantage is that in tissues prone to high background, antigen retrieval can lead to exacerbation of background staining. Use of microwave antigen retrieval in determining the differentiation of neoplasms does not appear to result in aberrant staining patterns. The enhanced sensitivity of microwave antigen retrieval in the immunohistochemical detection of potentially prognostically significant antigens such as cellular proliferation markers or overexpression or mutation of oncogenes is more problematic. Correlation with results of molecular assays will be required to establish the appropriate cutoff for a true positive result.

**Testing Samples of Minimal Size**

**Previously Stained Slides.** In some instances, such as in the examination of consultation material, immunohistochemistry is required but additional material may not be available. In such a situation, hematoxylin-and-eosin–stained tissue sections can successfully be submitted for immunostaining after de-staining, or without de-staining because multiple washes during immunostaining will lighten the stain. Gentle treatment of slides is necessary to ensure that tissue remains adherent to the slides. If only one slide with multiple tissue levels is
available, two different approaches can be used so that more than one immunostain and control can be performed. Tissue can be removed and placed on other slides using the “peel and stick” method, or the slide can be divided into “wells” for different antibodies using a diamond pen. When removing the coverslip from previously stained slides, immersion time in xylene should be minimized to avoid false-negative results or increased background staining. Higher antibody concentration may be needed to achieve a positive result in previously stained tissues. 

**Cytology Specimens.** Immunohistochemistry can also be a valuable adjunct to cytopathology. Cytology cell blocks that are processed as “tissue” are the preferred material for immunohistochemistry. Some special considerations apply to other cytology specimens. Filter preparations are not suitable for immunohistochemistry because the filter adsorbs immunologic reagents, resulting in very high background staining. Smears, cytospins, or imprints that are briefly fixed (1–20 minutes) in formalin or alcohol are all suitable substrates for immunohistochemistry. Cellular loss can be minimized by the use of coated slides during preparation of smears, cytospins, or imprints. If detection of surface antigen is sought, the cytospins, smears, or imprints are best air-dried and subsequently briefly fixed. Cytologic material is particularly useful for detection of surface antigens because these are more easily demonstrated in cytologic material and because loss of intracellular antigen in cytologic material predisposes to false-negative results for those targets. Cytology preparations for estrogen and progesterone receptor study must be kept frozen unfixed, because these antigens are labile at room temperature and sensitive to fixation. Interpretive pitfalls in immunohistochemistry of cytologic preparations include nonspecific positive signal in three-dimensional cell clusters or in cells that contain phagocytic materials such as neutrophils within their cytoplasm.

**Decalcified Specimens.** Immunohistochemistry can be successfully performed on sections of fixed decalcified bone. Individual antigens vary in their resistance to decalcification procedures, however; immunoglobulins are particularly sensitive. Successful immunohistochemistry is reported on glycol methacrylate–embedded bone marrow biopsy specimens in which the decalcification step is avoided altogether. 

**Previously Frozen Tissue.** Immunohistochemistry performed on a tissue block that has been frozen for intraoperative diagnosis, with subsequent slow thawing, fixation, and processing, can give aberrant or suboptimal results, possibly associated with antigen diffusion or destruction. This is an important consideration in intraoperative evaluation of tumors by frozen section. If at all feasible, some tissue should be routinely fixed or rapidly frozen in anticipation of the need for immunohistochemistry or molecular studies.

### QUALITY CONTROL FOR IMMUNOHISTOCHEMISTRY

#### Titration and Optimization of Reagents

As discussed briefly in the description of the antibody antigen reaction, optimal results are obtained with the primary antibody diluted to the point at which background is minimized and signal is maximized. This optimal dilution is determined empirically for a given set of working conditions, that is, time and temperature of incubation as well as detection conditions. The tissue used for titration should be processed in a manner as close as possible to that used for test tissues.

In practice, most primary antibodies are obtained commercially and laboratories rely on the manufacturer to extensively validate the reactivity of the primary antiserum. Commercial preparations include recommendations for optimal working dilutions as well as for storage of the reagent. Some antibodies are sold as working solutions. Such “pre-diluted” solutions are intended to be used without alteration by the consumer. Although the quality of commercial reagents can in general be trusted, good laboratory practice requires verification of results with several dilutions on either side of the manufacturer’s recommendation. As an additional component of this titration process, the reactivity of the primary antibody may be evaluated using one or more tissue or reagent controls described in the next section. Detection reagents also require empirical optimization. Commercial reagents are generally sold in sets with each component adjusted to give optimal results. In general, components from one detection system should not be mixed with those from another if reproducible results are to be expected. Even different lots from one manufacturer may yield slightly different results.

#### Reagent Quality Control

Once the reactivity of the primary antibody has been characterized, appropriate storage conditions are required to ensure that there is no deterioration. The manufacturer’s instructions on commercial kits should be followed strictly. Repeated freezing and thawing can lead to antibody degradation or precipitation. Overgrowth of microorganisms in antiserum can be prevented by storage of antiserum aliquots at −20°C. Storage at 4°C of working dilutions prevents repeated freezing and thawing of the antiserum. Addition of sodium azide 0.1% suppresses microorganism growth. Addition of proteins such as bovine serum albumin stabilizes the working dilutions stored at 4°C. A possible explanation for loss of antibody activity in working dilutions in the absence of albumin additives is that immunoglobulin may adsorb to the walls of the glass or plastic containers used.
for storage. Deterioration in the working dilutions or in the stock antiserum will lead to inconsistencies in the final results.

Although the primary antibody is central to the immunohistochemistry assay, it is clear that accurate reproducible results will be obtained only when all reagents used throughout the assay are prepared and stored under controlled conditions. Dewaxing agents, buffers, protease, detection reagents, and substrates all will impact on the quality of the final product. Changes in buffers and dewaxing agents should be noted in case variable results are seen, but testing before introducing new lots is not required. Change in all other reagents requires comparison of new and old lot results to ensure reproducibility.

**Assay Controls**

Because so many variables exist in the immunohistochemistry staining sequence, controls are of paramount importance to monitor each assay for validity. The purpose of controls is to provide assurance that tissues without signal are devoid of the antigen and that tissues with signal contain the antigen. Controls also allow problems in any assay to be detected so that appropriate corrective measures can be instituted. Routine controls for immunohistochemistry are briefly summarized in Table 3–2. Controls can be divided into tissue controls and reagent controls.

**Tissue Controls.** Positive tissue controls contain a known quantity of the antigen of interest and have been fixed and processed identically to the patient material to be tested. In practice, antigen quantitation is seldom achieved in tissues. It is recognized that neoplasms may not express antigen in the same density as normal tissues, so a positive result in the control tissue and negative result in the tumor may reflect differences in antigen density rather than true absence of antigen. In some instances, the patient material may include an internal positive control, such as normal nerve that is S-100 protein positive. This can be helpful in monitoring loss of antigenicity because of problems with fixation and processing. The most commonly used positive control is tissue known to contain the antigen of interest in either normal or abnormal cells. When feasible, use of a “low-positive” control, that is, a control in which the target antigen is present in small quantities, will establish the limits of sensitivity of the procedure. This concept is of particular importance with respect to hormone receptor, oncogene, and proliferation assays, in which the clinical importance of a strong versus weak positive result may be as yet unknown.

Some authors advocate the use of multi-tissue blocks as control tissues. This allows confirmation of reactivity with appropriate antigens and evaluation of any unexpected spurious reaction. The tissue control, whether multi-tissue or single tissue, can be sectioned and placed on the same slide as the patient’s section, allowing fewer slides to be manipulated during the assay and providing exact monitoring of reagents used for the patient material. This facilitates interpretation and retrospective review of immunostains, in addition to removing variables in the staining steps, because the tissues are stained simultaneously; however, valuable control blocks can be rapidly depleted with this approach.

The main purpose of the negative control is to confirm that any positive signal in patient tissue represents specific rather than nonspecific reaction. The simplest approach to the negative tissue control is to study the patient tissue section, to ensure that unexpected staining patterns are not detected (e.g., antibodies directed against keratin demonstrating positive signal in segments of peripheral nerve). Alternatively, a tissue section known to lack the antigen of interest can be used in parallel with the patient’s

<table>
<thead>
<tr>
<th>Control</th>
<th>Requirement</th>
<th>Purpose</th>
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<tbody>
<tr>
<td>Positive control tissue (handle as additional tissue sample)</td>
<td>Processed in manner identical to test tissue; Known to contain antigen that binds to the primary antibody</td>
<td>Positive results verify reaction of antibody and detection reagents</td>
</tr>
<tr>
<td>Negative control tissue (handle as additional tissue sample; may be a component of test tissue)</td>
<td>Processed in manner identical to test tissue; Known to lack the antigen that binds to the primary antibody</td>
<td>Negative results monitor specificity of primary antibody and detection reagents</td>
</tr>
<tr>
<td>Negative antibody control (reagent control, use on each tissue)</td>
<td>Omission of primary antibody; nonimmune serum or buffer diluent for antibody is substituted for primary antibody</td>
<td>Negative results monitor specificity of detection reagents</td>
</tr>
</tbody>
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tissue to confirm absence of staining. The remainder of the issues that pertain to negative control tissues are best discussed under reagent control.

**Reagent Controls.** Reagent controls serve to determine that any positive signal detected is not caused by spurious reaction associated with each individual step in the immunohistochemical staining procedure. Therefore, under ideal conditions, the immunohistochemical staining procedure would be repeated multiple times, each time omitting one particular step. This is particularly important when multistep procedures involving more than one antibody are performed. The more commonly used negative reagent control represents substitution of preimmune or hyperimmune serum from the same species as the primary antibody for the primary antibody in the incubation step. Preimmune serum is generally not available for commercial marketed antibodies. In practice, substitution of diluent or of an antibody of irrelevant specificity from the same species as the primary antibody, in place of the primary antibody at the incubation step, is the recommended negative control for nonspecific staining and should be performed on the patient’s tissue.

Another approach to the negative control is to use adsorbed antiserum. Incubation of antibody with excess purified antigen should result in abolition of signal. The specificity of this method relies on the purity of the antigen used for adsorption. Any extraneous contaminating material in the adsorption antigen would allow removal of cross-reacting antibodies as well as those directed against the truly pure antigen. This problem can be minimized by rigorous purification and the use of different preparations for immunization and adsorption. The lack of signal adsorption should raise questions about the validity of the reaction, but there are situations in which the reacting epitopes, usually part of the secondary structure, are modified in the adsorbing antigen. Similarly, lack of adsorption may reflect inability of the antigen-antibody complex to precipitate. Although this approach has these difficulties, it is a useful control for those laboratories preparing their own serum and when a poorly characterized antibody is being introduced into the laboratory.

**Independent Validation of Results.** Independent validation of the results of immunohistochemistry assays with assay using different technology is an ideal approach. For example, when introducing hormone receptor immunohistochemical assays, samples could be also tested by the biochemical method for comparison. Similarly, for an infectious agent, aliquots could be tested by the biochemical method for comparison. For example, when introducing hormone receptor immunohistochemical assays, samples could be also tested by the biochemical method for comparison. Similarly, for a poorly characterized antibody, aliquots could be tested by the biochemical method for comparison. The following example illustrates this approach. For example, when introducing hormone receptor immunohistochemical assays, samples could be also tested by the biochemical method for comparison.

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**Troubleshooting**

Appropriate controls will allow the laboratory to recognize that a problem exists. Recognition that there is a problem is a key first step. Troubleshooting can be thought of as the subsequent steps to specifically identifying and correcting the problem.

**SIGNAL IN NEGATIVE CONTROLS**

**OR ABERRANT LOCATIONS**

**Endogenous Enzyme Activity.** When the signal is generated by histochemical enzymes, any enzyme activity remaining in the tissues will contribute to the signal. Endogenous peroxidase or pseudo-peroxidase (catalase and cytochrome oxidase) is present in granulocytes, hepatocytes, and erythrocytes and can survive freezing or fixation and paraffin-embedding. Difficulty in interpretation of immunostains performed on tissues naturally rich in these cells may occur. An extra step can be undertaken to quench endogenous peroxidase, and numerous methods have been reported. Taylor has used 80% methanol containing 0.6% hydrogen peroxide for 15 minutes without detectable loss of antigenicity. A need for quenching endogenous peroxidase may not be present in all situations. When a quenching step is used, the appearance of signal should suggest preparation of fresh quenching solution, because peroxide is labile.

Endogenous alkaline phosphatase activity is present in intestinal mucosa, placenta, renal tubular cells, osteoblasts, lymphoid cells, and some mesenchymal cells. Although quite problematic in frozen sections, formalin fixation and embedding inactivates the majority of such activity. Endogenous alkaline phosphatase activity is most efficiently removed by quenching with acid. Alkaline phosphatase as an enzyme marker may be particularly useful in tissues that possess much intrinsic peroxidase activity such as bone marrow. It is also useful in dual staining when combined with the peroxidase-antiperoxidase technique. Glucose oxidase, which is not present in human tissues, can also be used as an enzyme marker.

**Cellular Pigment.** Cells containing abundant pigment such as melanin or hemosiderin may cause difficulty in interpretation of signal when diaminobenzidine is used as chromogen. The pigment may be the reason why
the negative control section appears positive. Switching
to a substrate producing a different colored product, such
as AEC, or changing the histochemical enzyme system
may allow the problem to be verified as caused by
endogenous pigment. By careful comparison with the
negative control tissues, signals in the test tissues can be
“read around” the endogenous pigment.

Avidin-Biotin Interactions. Nonspecific binding
of detection reagents, resulting in spurious signal,
can occur as the result of interaction between cellular
components and the highly charged avidin molecules in
solution. Similarly, human tissue may contain endoge-
rous biotin that will bind to avidin. Both undesired
reactions can be partially ameliorated by incubating in
nonfat dry milk or by preincubation with free avidin or
biotin, but this approach will also block specific avidin
binding. A useful alternative is simply to “read around”
the unwanted background or to substitute an alterna-
tive detection system that does not rely on avidin-biotin
interactions.

Endogenous Fc Receptors. Fc receptors on mono-
nuclear cells do not survive tissue fixation and processing.
In frozen tissue subject only to brief fixation, Fc receptors
on lymphoid and monocytic cells can result in unwanted
binding of primary antiseraum. The Fc portion of
antibody can also bind to basic groups present in
collagen fibers. Substitution of Fab fragments for primary
antibody, bridge antibody, and antiperoxidase complex
eliminates the problem.

Antigen Dislocation. Diffusion of antigen from its
primary location and subsequent attachment to or
permeation of other cells can result in “aberrant” signal.
Antigen dislocation reflects events that occurred during
fixation and processing and cannot be corrected.
Thyroglobulin and immunoglobulin can be particularly
problematic and may result in thyroglobulin signal in
thyroid endothelial cells or immunoglobulin signal in
Reed-Sternberg cells of Hodgkin’s disease. Recognition
of such a signal as aberrant with respect to the cellular
morphology will aid the pathologist in avoiding a pitfall
of interpretation.

Edge Artifact. Other spurious reactions such as the
positive tissue “edge artifact” in which the only signal is
identified at the edge of the tissue section, plague the
unsuspecting pathologist who must interpret the stain. A
possible explanation for edge artifact is that tissue may
curl slightly at the edge, allowing nonspecific trapping of
antibody and subsequent spurious positive signal.
Alternatively, the edge of the tissue may be the only
part that is adequately fixed and may represent the only
true detection of signal in the tissue.

LACK OF SIGNAL IN POSITIVE CONTROL

Error in Assay. Probably the simplest and most
frustrating possibility is omission of one step or use of
incorrect reagents in one step. This type of error is reduced
with experience and may be almost eliminated with the
introduction of automated methods. In most instances the
problem will affect more than one assay in the run.

Change in Reagents. When an assay that has been
working at an acceptable level suddenly no longer works,
or works with reduced signal, the first areas to
investigate is the key reagents of antibody and detection.
Out-of-date reagents cannot be used reliably. Changes
of lots can result in different results. Careful intro-
duction of new lots of any reagent by comparison with old
lots will allow the laboratory to pinpoint the problem
before introduction of the faulty reagent. This not only
eliminates errors on patient material but also provides
cogent evidence to the manufacturer when the problem
occurs with commercial reagents.

If loss of signal is only observed in a test that requires
protease digestion, a change in that reagent’s activity
could be suspected. Similarly, microwave unmasking
reagents and conditions should be investigated. Other
reagents, such as buffers and dewaxing agents, are less
likely sources of error but should not be overlooked.

Fixation Problems. These problems are difficult to
detect with routine controls and may be restricted to a
specific tissue. Once they occur, there is no way to
correct for them, although microwave antigen retrieval
techniques can be tried to overcome overfixation.
Problems with fixation can be suspected when there is
difficulty identifying good normal controls processed in
the laboratory. Similarly, difficulty with antigens known
to be susceptible to fixation, such as vimentin, also
suggest that fixation and processing should be investi-
gated. Finally, a laboratory that gets better results with
the interlaboratory comparison programs than it does
with its own tissues should seriously review protocols to
improve standardization of fixation and processing.

OTHER PROBLEMS

Loss of Tissue from Slides. Some method of
enhancement of tissue adherence to glass slides is usually
necessary, owing to extensive manipulations of the tissue
on the glass slides during the immunohistochemical
staining procedure. Clean glass slides must be used. A
number of different products such as poly-L-lysine,
albumin, and Elmer’s glue can be used to coat the slides.
Description of six different methods is available.11,38 Use
of electrostatically charged glass slides can also enhance
tissue adherence. Gentle handling during the various
steps of the staining procedure is necessary. Excessive
use of digestion techniques or microwave antigen
retrieval methods can also result in loss of tissue from
the slides, and particular care is necessary when using
these methods.

Excess Chromogen on Glass Surrounding Tissue.
The presence of chromogen on the glass slides on
completion of the immunohistochemical procedure
indicates an imbalance between primary and secondary
antibodies or between primary antibody and detection reagents.

**IN SITU HYBRIDIZATION**

### Principles of Hybridization Assay

The basis for any hybridization assay is the specificity of the interaction of a probe to target nucleic acid. This interaction shares some similarities to the antibody-antigen reaction that forms the basis for immunohistochemistry. In both instances the remarkable specificity of the interaction is caused by many weak noncovalent interactions that will be influenced by assay conditions. However, it is nucleic acid chemistry rather than protein chemistry that dictates the nature of the noncovalent interactions and the optimal conditions for these interactions. Nucleic acid chemistry and principles of hybridization assays have been reviewed elsewhere, and readers not familiar with nucleic acids may benefit from additional reading.

Briefly, in an aqueous environment, the thermodynamically stable conformation of nucleic acid is a double-stranded helix. The negatively charged sugar-phosphate backbone is exposed, and the hydrophobic bases are shielded from the aqueous phase. The bases are flat and stack centrally, interacting between strands through hydrogen bonds. Chemistry and geometry dictate that only certain base pairs fit into this stable configuration; adenine pairs with thymine (or uracil) and cytosine with guanine. Bases that “fit together” are termed complementary, as are two strands of nucleic acid that will combine to form a stable double-stranded helix. Clearly, the sequence of bases in one strand will determine the sequence of bases in its complementary strand.

A double-stranded molecule may be separated or denatured into two single strands by conditions that disrupt the stabilizing hydrogen bonds between bases. This process is referred to as **melting** because the change is from “order” to “disorder” often through the application of heat. Formamide, which breaks hydrogen bonds, is another commonly used denaturant. The melting process is reversible, and complementary strands will re-form a helix when denaturants are removed (Fig. 3–6). When complementary strands from two different sources are mixed, some of the duplex structures will be composed of one strand from each source, molecules known as “hybrids.” In a hybridization assay, the two sources are the target (sample) and the probe nucleic acids. A probe is simply a known fragment of nucleic acid with a label that can be detected in some fashion. A probe will form a hybrid molecule with a sample that contains nucleic acids complementary to its sequence. The process of searching a sample for specific nucleic acid sequences is termed a **hybridization reaction**. Both DNA and RNA may participate in the hybridization process, and duplex structures may be composed of DNA:DNA, DNA:RNA, or RNA:RNA, in order of increasing stability. Depending on the conditions, some degree of base-pair mismatching will be tolerated in a duplex structure. Conditions that require exact or nearly exact base-pair matching are called “stringent.”

All hybridization assays share some similarities. They require the probe and sample nucleic acid to be mixed under conditions allowing complementary base-pairing as well as a method to detect that hybridization has occurred. Although there is a bewildering array of techniques published for in situ hybridization, all share the same basic steps. These steps are shown in Figure 3–7 and are in turn compared with the basic steps for an immunohistochemical assay. In situ hybridization is a specialized form of a solid support assay in which sample nucleic acid is affixed to a solid matrix and interacts with a probe in solution. The microscopic slide is the solid support, and the affixed tissue with preserved morphology is the sample. It is the nature of the sample that makes the in situ hybridization unique. The goal of in situ hybridization is to allow the assay to occur and to be interpreted within a morphologic context. The requirement for morphologic preservation means that the sample nucleic acid must remain at least partially...
associated with membranes, proteins, and other cellular components forming the framework recognized as cell and tissue structure by light microscopy. This places a restraint on the availability of the target nucleic acids for interaction with probe. The final conditions therefore represent a compromise between morphologic integrity and sample availability.

**Probes**

**PROBE TYPES**

Genomic probes are composed of purified genomic material from a particular organism. Before the advent of recombinant nucleic acid technology, these were the only probes available. For example, to get a probe for a virus, the virus was grown in tissue culture and then the virions were isolated and nucleic acid was extracted. The chemical nature of the probe (i.e., DNA or RNA, double- or single-stranded) depended on the isolated organism’s genomic structure. Genomic probes are still used in some circumstances, but the vast majority of probes are now produced either by recombinant nucleic acid technology or through chemical synthesis.

Cloned probes consist of a known segment of DNA inserted into a plasmid vector that is propagated by growth in bacterium (Fig. 3–8). The small circular plasmids are easily separated from the bacterial chromosomal DNA on the basis of size. Either DNA or RNA probes may be produced, depending on the nature of the plasmid. Purified recombinant plasmid may be used as the probe. This produces a double-stranded DNA probe with both insert and vector sequences; alternatively insert sequences may be purified from the vector sequences and used as a probe. If the plasmid contains an RNA promoter region, then RNA probes may be produced using RNA polymerase to transcribe the insert sequences. Because only one strand is transcribed, the resulting RNA probes are single stranded. (From Unger ER: In situ hybridization: Principles and practice. Clin Immunol Newslett 1990;10[8]:121.)

New vectors contain RNA promoter regions adjacent to the inserted DNA sequence that permit generation of
RNA transcripts from the DNA insert. Because only one strand is copied during the RNA synthesis, single-strand RNA probes are generated. Controlling the orientation of the insert in relation to the promoter region allows the production of transcripts in the “sense” (i.e., same as messenger RNA [mRNA]) or “antisense” (i.e., complementary to mRNA) direction. These probes have several advantages. No self-hybridization is possible, favoring maximal interaction with target sequences. RNA probes oriented in the “sense” direction provide excellent negative controls for “antisense” probes. Nonspecifically bound RNA probe can be removed through RNase digestion that will degrade single strands but spare hybrids. The disadvantages of these probes are related to the requirement for placing the insert in a vector with one or two RNA promoters and the relative instability of RNA. RNA probes require careful handling and storage to prevent degradation. RNA is much more labile than DNA, and enzymes that digest RNA (known as RNase) are virtually everywhere. The use of RNA probes therefore dictates use of sterile technique and preparation of reagents and glassware to remove RNase.

Oligonucleotide probes are short segments (15 to 45 bases) of DNA that are chemically synthesized to a specified base sequence. Automated accurate methods of synthesis continue to lower the price of production. Sequence information is increasingly available in data banks. The probes can be generated to be “sense” or “antisense.” These features combine to make chemical synthesis very competitive. However, selection of sequences and hybridization conditions to optimize specificity is somewhat empirical. The limited amount of genetic information in these short probes results in lower sensitivity than that achieved with recombinant DNA or RNA probes, which contain several kilobases of information. The result of increasing the genetic complexity of probe is that more of the total target sequence will hybridize with the probe. Thus, for equal numbers of targets, probes with highest representation of target sequence will result in highest signal. This problem has been addressed through the use of multiple oligonucleotide probes to increase the representation of the target sequence in the probe. This approach is somewhat reminiscent of combining monoclonal antibodies to increase the number of recognized epitopes.

A variation of the synthetic approach to probe production is the use of the PCR polymerase chain reaction to produce amplified segments of DNA. In this application the PCR is used not to identify the presence of a target sequence but rather to produce copies of the sequence that can be used as probes. The resultant probe is double-stranded DNA, although asymmetric synthesis can be used to produce single-stranded probes. If the primers are designed to incorporate an RNA promoter, RNA probes can be produced with a subsequent reaction using RNA polymerase.

### PROBE SIZE

The physical size of the probe (as opposed to complexity or amount of genetic information) is a crucial consideration for in situ hybridization. Most investigators have found that relatively short probes (fewer than 500 bases) favor increased signal and decreased background. This observation is attributable to penetrance of probe into morphologically constrained target. Oligonucleotide probes are short by nature of their construction; however, genetically complex recombinant probes have the potential for being too large for optimal hybridization. DNA probes can be reduced in size by treatment with DNase I, an endonuclease, whereas RNA probes are subjected to controlled alkaline hydrolysis. Often this reduction in size is achieved through the methods of label incorporation.

### PROBE LABELING: ISOTOPIC LABELS

A wide variety of radioactive and nonradioactive labels have been described. Nucleic acid technology was founded with the use of isotopic labeling and detection. Radioactive labels are still favored in many research settings because of the unsurpassed sensitivity achieved with probes of high specific activity. Thus, in situ hybridization with radioactive probes and autoradiographic detection is generally accepted as the standard to which all other methods are compared. Tritium and sulfur 35 ($^{35}$S) are the two radioisotopes most commonly employed. Higher-energy isotopes, such as phosphorus 32 ($^{32}$P) and iodine 125, do not permit adequate localization of signal. Tritium has a very long half-life, so the probes are stable for years. This is in contrast to $^{35}$S probes, which have a short half-life (80 to 90 days). Exposure times with $^{35}$S are shorter than those with tritium because of its higher emission, with some loss of signal localization. Isotopic labeling is achieved through enzymatic incorporation of radioactive nucleotides.

The principles of enzymatic incorporation of label are exemplified by the nick translation reaction, which was the first widely used method of labeling double-stranded DNA probes. As shown in Figure 3–9, single-stranded nicks are randomly introduced into the probe DNA by an enzyme, DNase I. The 3’-hydroxy groups generated form priming sites for the initiation of DNA synthesis by another enzyme, DNA polymerase, which incorporates labeled triphosphate nucleotides in the 5’ to 3’ direction using the opposite strand as template and removing unlabeled strand with its 5’ exonuclease activity. The single-stranded nicks are introduced randomly throughout the probe, the number of nicks being dependent on the amount of DNase I activity. The net result of the action of these two enzymes is to reduce the size of the probe and to incorporate label into both strands of the probe. Fragments will be of random size, but the size range can be controlled by the ratio of the two enzymes in the reaction mixture. The activity of both
enzymes is stopped with heat or by treating the sample with ethylenediaminetetraacetic acid (EDTA). The probe must be separated from unincorporated nucleotides by precipitation or gel filtration.

A newer method of labeling double-stranded DNA probes is referred to as random-priming. The probe is denatured and allowed to anneal with short hexanucleotides (oligos) of random sequence. The 3'-hydroxy end of an annealed oligo forms the initiation site for the DNA polymerase enzyme, which will incorporate labeled nucleotides using single-stranded regions of the DNA as the template. This reaction also generates short random-sized fragments with both strands of plasmid template labeled. Depending on the reaction conditions, net synthesis of probe may occur and higher specific activity may result.

The generation of RNA probes with use of a transcription vector containing a promoter for RNA polymerase enzyme has been illustrated schematically in Figure 3–8. The transcription vector is linearized by digestion with a restriction endonuclease and then serves as a template for the RNA polymerase as directed by the promoter. The first such vector contained a promoter for SP6 polymerase. When labeled ribonucleotides are present in the reaction mixture, the RNA polymerase will incorporate them using the DNA strand as template. Some vectors contain promoters for two different polymerases permitting generation of sense or anti-sense probes with the same plasmid depending on the choice of RNA polymerase. If conditions are optimized, the RNA polymerase will generate full-length single-stranded RNA transcripts, all the same length as the insert.

Different enzymatic reactions are useful for oligonucleotide probes. T4 polynucleotide kinase may be used to label the 5' end with ³²P, but because that isotope is not generally useful for in situ work, an alternative labeling technique is more common. Terminal deoxynucleotidyl transferase (TdT) will add nucleotides onto the 3' end in a ‘‘tailing’’ reaction. No template is required, and the number and type of nucleotide can be controlled by the reaction conditions. This will result in a slightly longer probe than the original oligonucleotide with additional ‘‘noncoding’’ labeled bases at the 3'-hydroxy end.

PROBE LABELING: NONRADIOACTIVE

The first practical example of nonisotopic labeling of a probe was achieved through production of a biotin-labeled analogue of deoxyuridine triphosphate, shown in Figure 3–10. The biotin side chain is quite bulky, but despite the altered steric configuration, this nucleotide will be incorporated by DNA polymerase with only slightly lower efficiency than isotopic analogues. Biotin itself cannot generate signal. Polynucleotides with biotin incorporated into their structure are detected indirectly through high-affinity interaction with avidin or streptavidin chemically linked or complexed to a colorimetric enzyme or fluorescent tag. (Antibodies against biotin may be used in place of avidin.) Other functional groups such as bromodeoxyuridine or digoxigenin may be used as affinity labels for DNA and RNA probes. In each case the functional group is chemically linked to a nucleotide (e.g., digoxigenin-DUTP) with subsequent incorporation of the nucleotide analogue by polymerase enzymes. High-affinity antibodies specifically directed against the functional group hapten are produced. The incorporated label is then detected with this antibody linked with a colorimetric
enzyme or fluorescent tag. Although either the nick translation or random priming methods may be used to incorporate affinity labels, nick translation is more frequently used. The higher incorporation achieved with random priming is not advantageous for these indirectly detected labels. Final size of labeled product is more easily controlled with nick translation.

Incorporation of affinity-labeled ribonucleotides by RNA polymerases to generate nonradioactive RNA probes can be problematic. RNA polymerases have difficulty with efficient incorporation of these analogues, and short, poorly labeled transcripts may result. As an alternative, analogues with an activated amino group have been synthesized that are more efficiently utilized by the RNA polymerase. Once incorporated, the active amino group can be affinity-labeled in a second direct chemical reaction.

Oligonucleotide probes can be affinity-labeled by several different methods. One of the most efficient is through incorporation of affinity-labeled nucleotide probes at the 3' end with terminal transferase ("tailing reaction"). Affinity-labeled nucleotides may be incorporated during the synthetic reaction, resulting in label in the hybridizing portion of the probe. Affinity labels may also be added to the 5' end of the molecule by beginning the synthesis with noncoding nucleotides with active amino groups that are subsequently derivatized. Labels at either the 5' or the 3' end have been found to be most efficient.

Nonenzymatic methods of affinity-labeling probes have been described. These include activated analogues of biotin that chemically link to DNA, RNA, or protein when exposed to the light (photo-biotin). Probes can be modified through sulfonation or addition of other large groups (e.g., AAIF) that are subsequently detected with antibodies. Enzymes and fluorescent groups have also been directly chemically linked to probes. These methods have potential because of their simplicity; however, none has been optimized to compete with enzymatic methods of affinity labeling in terms of sensitivity and reproducibility.

COMMERCIAL PROBES

Although numerous well-characterized primary antibodies are readily available through commercial sources, nucleic acid probes are only beginning to be developed. For most clinical laboratories, the lack of commercial reagents is one of the most significant factors limiting diagnostic application.

ASSAY CONDITIONS

The sensitivity and specificity of the hybridization reaction is greatly influenced by the physical-chemical
The hybridization reaction is controlled by the formation of specific base-pairing. Isotopically labeled probes are especially prone to this problem and are generally used at much lower concentrations than affinity-labeled probes. Similar to the situation with immunohistochemistry, the time of the hybridization reaction can be modulated by the concentration of a saline sodium citrate (SSC) buffer composed of 0.15 mol/L sodium chloride and 0.015 mol/L trisodium citrate with a pH of 7.0. The shorthand notation for these conditions refers to the ionic strength of both the hybridization cocktail and the subsequent washes is most often modulated by the concentration of a saline sodium citrate (SSC) buffer composed of 0.15 mol/L sodium chloride and 0.015 mol/L trisodium citrate with a pH of 7.0. The final stringency of the hybridization reaction is controlled by the formamide and salt concentration of the hybridization cocktail, the temperature of the hybridization reaction, and the temperature and salt concentration of the washing steps.

The concentration of the probe in the hybridization cocktail is another important variable. Unlike antibody, serial dilutions or titrations are not used to determine optimal concentrations. Within limits, kinetics of the solid liquid hybridization rate are influenced by the probe concentration. If probe is double-stranded, solution phase self-hybridization is favored at higher concentration and with increased time, limiting the final sensitivity of probe target interaction. High probe concentration also contributes to high background, that is, interaction with tissues through charge or other mechanisms not involving specific base-pairing. Isotopically labeled probes are especially prone to this problem and are generally used at much lower concentrations than affinity-labeled probes. Similar to the situation with immunohistochemical assays, the time of the hybridization reaction can be reduced with increased probe concentration.

After hybridization, excess probe is removed with buffer washes. The salt concentration and temperature during the washes control the final stringency of the hybridization reaction. Even exactly matched hybrids may be destabilized and removed with improper washing. Detection methods are, of course, dictated by the type of label on the probe. Detection of radiolabeled probes is achieved with autoradiography. Slides are dipped in a silver emulsion while protected from light and, after time of exposure, developed to demonstrate deposition of silver grains over the areas of localized isotope. The time of exposure and degree of signal localization depend on the isotope. After an optimal hybridization assay, the specific activity of the radiolabeled probe is the major determinant of sensitivity. Within limits, increasing the time of exposure can increase the sensitivity of detection. Longer times will increase background, and background eventually limits resolution of signal. Low-energy isotopes such as tritium require long exposure (month) and provide highest resolution of signal.

Detection of affinity-labeled probes is achieved with methods identical to those previously discussed for immunohistochemistry. Colorimetric detection is guided by the same considerations as those in immunohistochemistry, except that final sensitivity is an even more important consideration for in situ hybridization. In most applications the amount of antigen greatly exceeds the amount of nucleic acid target in tissues. Low background is essential for optimal results and is greatly influenced by the colorimetric detection reagents. The histochemical enzyme/substrate combination also needs to be selected with final sensitivity in mind. Alkaline phosphatase systems with NBT-BCIP substrate are reported to yield the best results, although there are many options.

**Optimization of Test Material**

**FIXATION AND PROCESSING**

These topics have been considered in detail in the section on immunohistochemistry, and the same considerations apply for in situ hybridization. Optimal results will be achieved only with carefully fixed and processed tissue samples. Although snap freezing and coagulant fixatives have been used with success in research settings, formalin-fixed paraffin-embedded material remains the best choice in a diagnostic setting. Cross-linking fixatives result in improved morphology and increased retention of small nucleic acids. At the same time, cross-linking limits permeation of probe into the tissue, so final probe size, denaturation conditions, and protein digestion must be adjusted to maximize target accessibility.

The concerns with the adequacy of “routine” histopathology practice for adequate preservation of antigens are magnified for preservation of nucleic acids, especially RNA. The extreme lability of RNA requires
tissues to be rapidly cut thin enough to allow for even penetration of fixative. DNA is much more stable than RNA and will tolerate more variation in fixation and processing conditions. Because the nucleic acids are bound to tissue, more degradation of target is tolerated for an in situ hybridization assay than for extraction-based hybridization assays such as Northern or Southern hybridization. Nonetheless, there is a point at which the molecular information becomes inadequate for analysis. Clearly variations in fixation and processing that may go unnoticed by hematoxylin-and-eosin staining will result in significant alterations in nucleic acid preservation and cross-linking and will influence the results of molecular analysis.

**Protease Pretreatment**

Protease treatment is required for all cross-linked samples. Digestion removes proteins and makes the target more accessible to the probe. Acid conditions also contribute to tissue permeabilization and protein removal. Conditions depend on the type of sample and degree of cross-linking. Because of variations in tissue fixation and processing, optimal digestion conditions must be established for each tissue using hybridization to an endogenous target as a guide (see "Controls"). Proteinase K, pronase, and pepsin, as well as other proteases have all been used successfully.

**Controls**

The same basic considerations discussed for controls and quality assurance in immunohistochemistry apply to in situ hybridization so need not be repeated in detail. With so many facets of the in situ hybridization assay being empirically determined, it is clear that laboratories must carefully control and monitor the results of each test. Interpretation of a precipitated product as evidence of an identified segment of nucleic acid requires that all other explanations be eliminated. Ideally, the specificity of the probe should be established through the use of other hybridization assay formats, such as Southern or Northern blot. Although there are many different kinds of controls that can be included, the three essential controls are listed in Table 3–3. The College of American Pathologists (CAP) Laboratory Checklist for molecular pathology requires these three as a minimum.

With each assay, a sample known to contain the target must be run to demonstrate that all components of the assay are working. This positive control tissue should be fixed and processed identically to test tissues and should be run as an additional sample in each assay. It is often helpful to use a sample with a low level of target to verify the lower limit of assay sensitivity. This allows for small variations between runs to be noticed and corrected. Because of unavoidable variations in tissue fixation and processing, the use of an endogenous positive control probe on each sample is absolutely essential. This probe is selected to be positive on all tissues if the target nucleic acid has been adequately preserved and made available. This probe should be labeled and used at the same concentration as the test probe. If the positive control probe does not give positive results on a tissue, a negative result for the test probe cannot be interpreted. Adjustment of digestion conditions will often allow positive results to be obtained, but, at times, preservation is poor or tissues are so overfixed that the assay must be termed unsatisfactory.

<table>
<thead>
<tr>
<th>Control</th>
<th>Requirement</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive control tissue (handle as additional tissue sample)</td>
<td>Processed in manner identical to test tissue Known to contain target that hybridizes to test probe</td>
<td>Positive results verify reaction of probe and detection reagents</td>
</tr>
<tr>
<td>Positive control probe (use on each tissue)</td>
<td>Hybrids with a target present in all tissues Labeled in similar manner to test probe and used at similar concentration</td>
<td>Positive result verifies preservation of nucleic acid and availability to probe</td>
</tr>
<tr>
<td>Negative control probe (use on each tissue)</td>
<td>Probe of similar base-pair composition to test probe, that should not hybridize to test and control tissues Labeled in similar manner to test probe and used at similar concentration</td>
<td>Negative results monitor specificity of hybridization and detection</td>
</tr>
</tbody>
</table>
The negative control probe is selected to evaluate the specificity of probe target interaction. It should be of similar size and base-pair composition as the test probe but should not hybridize in the absence of the specific target. For recombinant DNA probes, unmodified plasmid sequences are commonly used as the negative control. The negative control probe is labeled and used at the same concentration as the test probe. A negative result with the negative control probe does not prove specificity of the test probe interaction but is at least a guide to monitoring that specificity. The negative control also monitors nonspecific interaction of detection reagents and tissue. If the negative control probe yields positive results, use of a cocktail-only reaction (no probe) will demonstrate if the problem is attributable to detection reagents.

Troubleshooting

Again, because of the similarities between the techniques of in situ hybridization and immunohistochemistry, the approach to troubleshooting problems in the in situ hybridization assay will be similar to that for immunohistochemistry and will not be repeated.

Tissue Loss

Because the conditions for hybridization assays are more harsh than those used for immunohistochemistry, tissue loss is a bigger problem. The use of silanized glass is almost required. The tissue will form a covalent bond to the glass. This will occur immediately as the tissue is picked up from a protein-free tap water bath. If the glass is properly treated, the tissue will not be able to be “refloated” or moved around on the slide once it has been lifted from the surface of the water. Difficulties with the procedure can be attributed to poorly treated glass, dirty glass, or protein in the water bath.

Overdigestion of tissues will cause poor morphology and result in increased tissue loss. Digestion conditions should be adjusted to the minimum required for probe penetrance.

No Signal with Endogenous Positive Control Probe

Assuming the positive control probe has been properly prepared and stored, this problem reflects either inadequate preservation of target or inadequate pretreatment to make target available. Obviously, if target is degraded, nothing can be done. Increasing concentration of protease, time, and temperature of digestion may all be attempted to improve detection. Conditions of denaturation should also be verified because no hybridization will occur if the target is not denatured. Increasing the time and temperature of denaturation can be attempted.

Signal with Negative Control Probe

This problem could result from endogenous histochemical enzyme, nonspecific binding of detection reagent, or nonspecific binding of probe. The first two possibilities are discussed earlier in the immunohistochemistry section. Nonspecific binding of probe in most instances reflects low-stringency conditions in hybridization and/or wash. The probe may be used at a concentration that is too high or when the time of hybridization is too long. Drying of tissue during the hybridization reaction will result in very high nonspecific sticking of a probe.

REFERENCES


42. Murray GI: Enzyme histochemistry and immunohistochemistry with freeze-dried or freeze-substituted resin-embedded tissue. Histochem J 1992;24:399–408.


APPENDIX

ANTIGEN DETECTION IN TISSUE BY ABC IMMUNOHISTOCHEMISTRY

PRINCIPLE
The presence of a certain antigen is demonstrated using the avidin-biotin-peroxidase complex (ABC) method of immunohistochemistry. Tissue samples are submitted on appropriate slides.

SPECIMEN
Specimens are 4- to 6-μm sections of formalin-fixed, paraffin-embedded tissue that has been placed on coated glass microscope slides. Two slides are required at a minimum.

MATERIALS AND SOLUTIONS
Oven set at 60°C
Water bath set at 35°C to 40°C
Magnetic stir plate, stir bars
Gloves
Pipettes
Xylene
100% ethanol
0.05% protease VIII (Sigma, St. Louis) in 0.1 mol/L sodium phosphate buffer, pH 7.8
100% methanol with 3% hydrogen peroxide (H₂O₂)
0.01 mol/L sodium phosphate, pH 7.40, 0.15 mol/L NaCl (PBS)
Normal serum (specific for secondary antibody species)
Primary antibody
Biotinylated secondary antibody
Avidin-biotin complex (ABC) reagent
0.016% diaminobenzidine tetrahydrochloride (DAB), 0.24% H₂O₂ in PBS
Gill’s hematoxylin
2% concentrated ammonia in deionized water
Permount (Fisher Scientific)

CONTROL

POSITIVE CONTROL SLIDE. This is a slide known to demonstrate the antigen of interest; it should be weakly or moderately (not strongly) positive. A positive control is run with every patient specimen. This control ensures that the reagent system is functioning properly.

NEGATIVE CONTROL SLIDE. This is a patient slide with PBS substituting for the primary antibody, to control for nonspecific binding of the detection system. Section should not give a signal.

PROCEDURE
1. Paraffin sections mounted on pretreated slides are heated in an oven for 30 minutes.
2. Deparaffinize sections using four changes of xylene for 5 minutes each, followed by four changes of 100% for 3 minutes each. For sections that do not require digestion, go directly to step 6.
3. For sections requiring digestion, rinse in deionized water for 3 minutes.
4. Incubate sections in buffered protease VIII at 37°C for 1 to 30 minutes. The standard time for digestion is 3 minutes.
5. Rinse sections in two changes of deionized water for 2 minutes each, then three changes of 100% ethanol for 3 minutes each.
6. Block endogenous peroxidase activity with 3% H₂O₂ methanol for 30 minutes.
7. Rinse sections three times with deionized water.
8. Reduce nonimmunologic binding of antiserum by placing sections in 10% normal serum-PBS derived from the same species as the biotinylated (secondary) antibody (e.g., if biotinylated antibody is goat anti-rabbit antiserum, use normal goat serum) at 4°C overnight.
9. Shake off excess normal serum and place slides in leveled staining trays. Distribute the slides according to the primary antiserum that is to be applied. Care should be taken so that the sections do not touch during incubation with primary antisera. The need to separate the slides into groups to preclude any chance of contaminating a section with the wrong antibody cannot be overemphasized. Cover section with primary antiserum (e.g., rabbit antiserum) specific for desired antigen for 30 minutes at room temperature. Make sure the chamber lid is on to prevent air-drying during the incubation.
10. Wash sections with PBS three times.
11. Flood sections twice with 10% normal serum for 10 minutes each, and then rinse in PBS.
12. Shake off excess buffer and cover sections with biotinylated secondary antibody. Incubate for 30 minutes at room temperature, making sure the chamber lid is on to prevent air-drying of sections.
13. Wash sections three times with PBS.
14. Shake off excess buffer and cover sections with ABC reagent, prepared according to the recommendations of the manufacturer, for 30 minutes at room temperature, making sure the chamber lid is on to prevent air-drying of the sections.
15. Wash sections three times with PBS. Place sections in staining racks for a final 10-minute soak in PBS.
16. Develop in 0.016% DAB and 0.24% H$_2$O$_2$ in PBS solution for 10 to 25 minutes at room temperature. The usual time for optimal development of DAB is 15 minutes, but this may vary with the lot of DAB being used.
17. Wash sections 1 or 2 minutes three times with deionized water.
18. Counterstain with Gill’s hematoxylin.
19. “Blue” sections with ammonia water.
20. Wash in deionized water for 5 minutes.
21. Dehydrate and mount sections with Permount.

REPORTING RESULTS

Positive antibody-antigen reaction is brown, with nuclei blue. The assay is indeterminate if the assay is negative and the positive control section shows no reactivity. The negative control should be carefully examined to control for nonspecific staining.

PROCEDURE NOTES

Throughout the procedure, keep drying of the sections to a minimum to prevent destroying epitopes on the cell membrane. Drying also causes nonspecific background staining.

In step 2, it is important to complete deparaffinization because paraffin can mask epitopes from the primary antibody.

In step 16, special precautions should be taken when handling DAB because of its possible carcinogenic properties.

REFERENCES