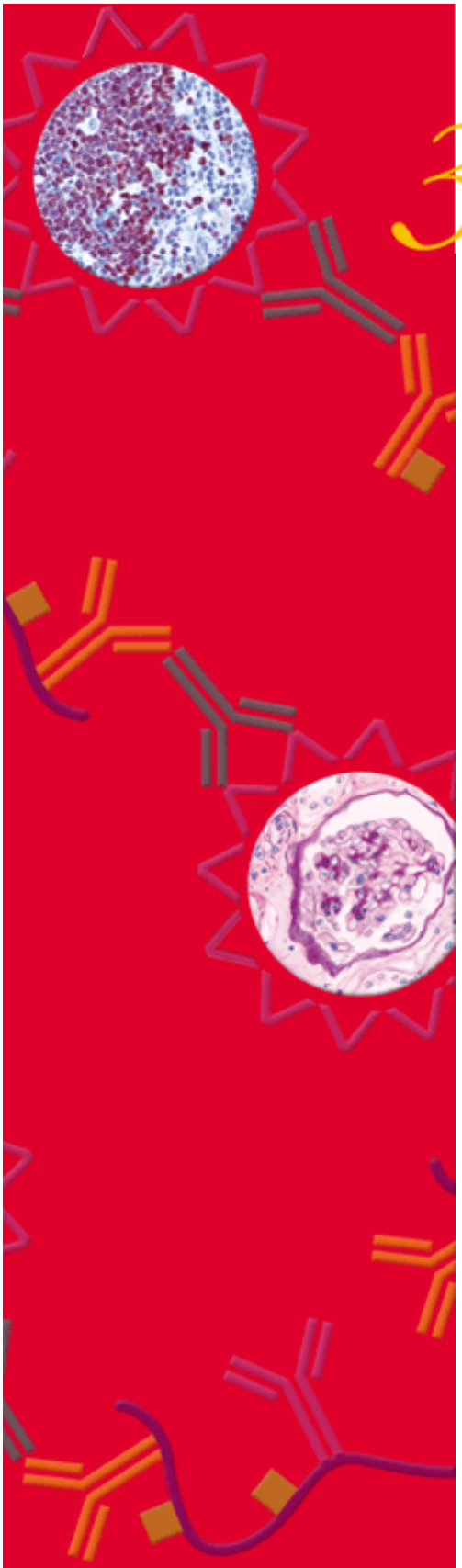

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HANDBOOK

Immunochemical Staining Methods

3rd EDITION

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ANTIBODIES

THOMAS BOENISCH

■ The pivotal reagent common to all immunohistochemical* techniques is the antibody. The availability of antisera, immunoglobulin fractions and monoclonal antibodies to an ever-increasing number of clinically useful tissue antigens has enormously expanded the quantity and quality of the immunohistologic repertoire. To better comprehend the potential of immunohistochemical staining methods as well as any latent problems that may be associated with the same, it is necessary to have a basic knowledge of antibodies and their potentials, as well as their limitations.

IMMUNOGLOBULINS

■ Antibodies belong to a group of proteins called immunoglobulins (Ig). Listed in order of decreasing quantity found in plasma or serum, the immunoglobulins comprise five major classes: immunoglobulin G (IgG), IgA, IgM, IgD and IgE. Each immunoglobulin is composed of two identical heavy chains (H) and two identical light chains (L). The H chains differ in antigenic and structural properties and determine the class and subclass of the molecule. The two L chains are either type kappa or lambda. The distribution of kappa and lambda chains differs in all Ig clases and subclasses, as well as between different species. Covalent interchain disulfide bridges join L to H and H to H chains. By participating in the tertiary structure, they confer greater stability to the immunoglobulin molecule.

Of the five classes of immunoglobulins, IgG and IgM will be considered in more detail here, as they are by far the most frequently utilized antibodies in immunohistochemistry. Unless otherwise noted, most of what is described of the IgG structure in this text was learned from studies with human IgG of subclass IgG_1.

■ **IgG** IgG has the general formula of $gamma_2$ $kappa_2$ or $gamma_2$ $lambda_2$, which denotes that one molecule of IgG (MW = 150 kD) is composed of two gamma heavy chains, and two light chains of either type kappa or type lambda (Figure 1). The structure of the IgG molecule has been determined in part by proteolytic digestions and reductive dissociation of the molecule (Figure 2). Digestion by papain results in the cleavage of a susceptible bond on the N-terminal side of the inter-heavy chain disulfide bridges. This yields two monovalent antigen-binding fragments (Fab) and one crystalline fragment (Fc). Pepsin cleaves the gamma chains on the C-terminal side of the inter-heavy chain disulfide bridges, resulting in one bivalent antigen-binding fragment, $F(ab')_2$. In this case, the Fc fragments are destroyed. Reductive dissociation of an IgG molecule splits the interchain disulfide bridges and, if the free sulfhydryl groups are blocked, results in the formation of two H chains (molecular weight 50 kD each) and two L chains (25 kD each).

The IgG molecule can be further divided into so-called domains, namely the variable domains (V) and the constant

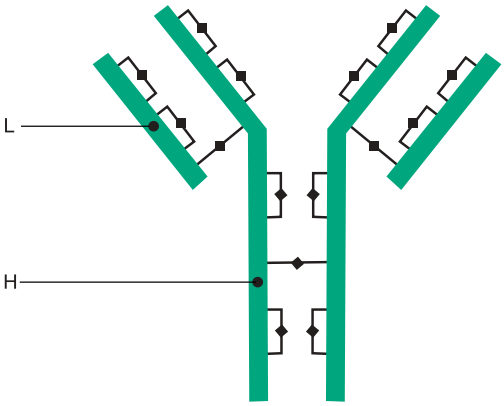

Figure 1: Diagram showing the structure of an immunoglobulin molecule. It comprises two identical heavy (H) chains and two identical light (L) chains. Inter- and intrachain disulfide bonds ( ) contribute to the structure and stability of the molecule.

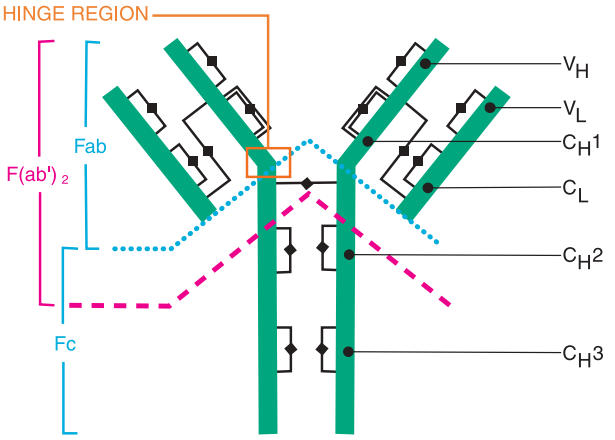

Figure 2: Diagram showing the structure of rabbit IgG (which exists as a single major subclass). The heavy (H) and light (L) chains are composed of variable (V) and constant (C) domains and are linked by inter- and intrachain disulfide bonds ( ). Proteolytic digestion with papain ( ) yields two antigen-binding fragments (Fab) and one crystalline fragment (Fc), whereas digestion with pepsin ( ) yields one $F(ab')_2$ fragment.

*It should be understood that the term “immunohistochemistry” as used in this chapter, denotes and includes the term “immunocytochemistry” also.

domains (C). Each domain contains 110 to 120 amino acids and one intrachain disulfide bond. On the variable domain of the light chain (V_L), and on the variable domain of the heavy chain (V_H) are located the amino terminals of the immunoglobulin molecule. V_L and V_H together form the antigen-combining site. Several hyperactive (HV) regions are located within the V_L and V_H domains of the antibody. During their reaction with antigens, HV regions are brought into close proximity to the antigenic determinant (epitope). The distance between the antigen and the HV regions of the antibody is approximately 0.2 to 0.3 nm. In this region, unique structural specificities called idiotypic determinants are located. Each antibody clone expresses its own idiotype. Each L chain also has one constant domain (C_L) in addition to the V_L domain. The H chain also has three constant domains (C_H1, C_H2 and C_H3) and carries the carboxyl terminal portion of the immunoglobulin. Located on the C_H2 domain is the carbohydrate moiety of the IgG molecule and several strongly hydrophobic neutral aromatic amino acids. The hinge regions are located between the C_H1 and C_H2 domains of the H chains. Minor differences within these hinge regions contribute to the subclass specificity of immunoglobulin G. The same are designated by subscripts as in IgG_1, IgG_{2a}, IgG_{2b}, IgG_3 and IgG_4. Whereas in human IgG the overall ratio of kappa to lambda is 2:1, in the subclasses IgG_2 and IgG_4 for example the ratios are 1:1 and 8:1, respectively. Mice have approximately 95% kappa chains and therefore most monoclonal IgG antibodies from this species have kappa chains. The number of disulfide bridges linking the heavy chains also varies amongst the IgG subclasses. IgG_1 and IgG_4 each have two, while IgG_2 and IgG_3 have four and five, respectively. Because of the flexibility of the hinge region, the angle that both Fab fragments form can vary to accommodate varying distances between identical antigenic determinants.

■ **IgM** IgM is a pentamer (MW approximately 900 kD) consisting of five subunits of approximately 180 kD each (Figure 3). The general formula can be expressed as $(mu_2\ kappa_2)^5$ or $(mu_2\ lambda_2)^5$. Each subunit is linked by a sulfhydryl-rich peptide, the J chain (15 kD), and consists of two heavy chains (mu) and two light chains of type kappa or lambda. The J-chains contribute to the integrity and stability of the pentamer. As with IgG, IgM subunits can be fragmented by enzymatic and reductive cleavage into $F(ab')_2$, Fab and Fc portions, as well as heavy and light chains, respectively. The Fc fragment of IgM is a cyclic pentamer (molecular weight approximately 340 kD). Treatment of pentameric IgM with 0.1% mercaptoethanol cleaves the disulfide bridges between the subunits to yield five monomers. Subclasses of IgM_1 and IgM_2 have been reported.

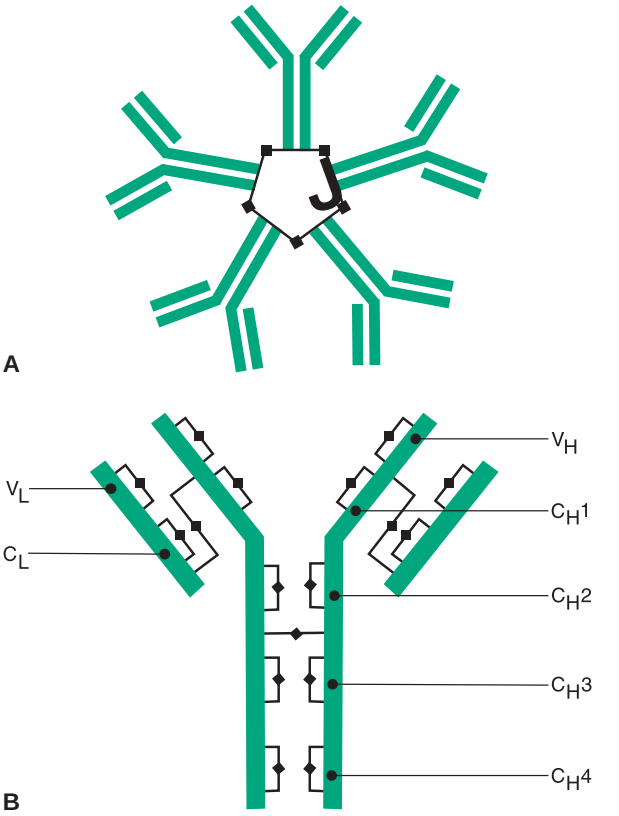

Figure 3: Diagram showing (A) the five subunits of mouse IgM linked by disulfide bridges (┌◆┐) and the J chain to form a pentameric ring structure. Each subunit (B) comprises two mu heavy (H) chains and two light (L) chains each composed of constant (C) and variable (V) domains.

Whereas IgG is the most abundant antibody in the hyperimmunized host, in the newly immunized animal, IgM is the first humoral antibody detectable. The primary antibody formation proceeds in several major stages. Injected immunogen first reaches equilibrium between extra- and intravascular spaces, then undergoes catabolism resulting in smaller fragments, and finally is eliminated from the intravascular spaces by the newly formed antibodies. The period from the introduction of an immunogen until the first appearance of humoral IgM antibodies is called the latent period and may last approximately one week. Within two weeks, or in response to a second injection, IgG class antibodies usually predominate. Like all proteins, antibodies are subject to catabolism. Whereas antibodies of class IgM have a relatively short half-life of only four to six days, IgG antibodies have a mean survival of approximately three weeks. Unless repeated booster injections with the immunogen are given, the serum antibody level will decrease after this period.

Antibody formation on the molecular level is a complex process and a detailed account of it is beyond the scope of this handbook. The interested reader is referred to the textbook *Molecular Immunology* by Atassi et al.[1]

POLYCLONAL ANTIBODIES

■ Polyclonal antibodies are produced by different cells, and in consequence, are immunochemically dissimilar; they react with various epitopes on the antigen against which they are raised (Figure 4). By far, the most frequently used animal for the production of polyclonal antibodies is the rabbit, followed by goat, pig, sheep, horse, guinea pig and others. The popularity of rabbits for the production of polyclonal antibodies is attributed primarily to their easy maintenance. Furthermore, an additional advantage in their use is that human antibodies to rabbit proteins are much rarer than to proteins from ruminants, such as goat. In addition, rabbit antibodies precipitate human proteins over a wider range of antigen or antibody excess and pools of antibodies made from many rabbits are less likely to result in major batch-to-batch variations than pools made from only a few, larger animals. Many years of selective breeding for favorable immunization response has made the New Zealand White rabbit the most frequently used animal for the production of polyclonal antibodies.[2]

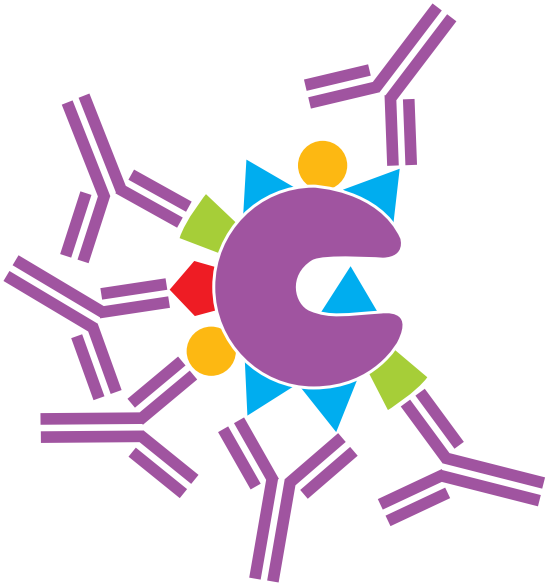

Figure 4: Schematic diagram of polyclonal antibodies binding to various epitopes on an antigen.

Depending on the immunogenicity of the antigen, doses of from 10 µg to 200 µg are traditionally administered to generate an immune response in animals. The antigen is most often injected intradermally or subcutaneously, but injections into the footpad muscle or peritoneal cavity are also used. In rabbits, volumes of 0.1-0.5 mL are usually given intradermally and distributed over several sites; the antigen is suspended in an equal volume of adjuvant, such as Complete or Incomplete Freund's Adjuvant. Booster shots, repeated once a month or when decreasing titers are noted, are intended to maintain or increase antibody levels. Blood is most often harvested from the ear (rabbits), the jugular vein (larger animals) or from the heart, sometimes by sacrificing the animal. After the removal of cells from the blood, polyclonal antibodies can be obtained either in the form of stabilized antisera or as immunoglobulin fractions purified to varying degrees. Precipitation by salts, followed by ion exchange chromatography, serves to remove the bulk of other serum proteins. Affinity chromatography can be used to isolate the antigen-specific antibodies and thereby free them of cross-reacting antibodies to other species.

MONOCLONAL ANTIBODIES

■ Monoclonal antibodies are the product of an individual clone of plasma cells. Antibodies from a given clone are immunochemically identical and react with a specific epitope on the antigen against which they are raised (Figure 5). Probably for reasons of economy, mice are currently used almost exclusively for the production of monoclonal antibodies. After an immune response has been achieved, B lymphocytes from spleen or lymph nodes are harvested and fused with non-secreting mouse myeloma cells under specific conditions. While the B lymphocytes convey the specific antibody, myeloma cells bestow upon the hybrid cells (hybridoma) longevity in culture medium. Non-reactive B cells and myeloma cells are discarded and the antibody-producing hybridoma is cultured and tested for desired reactivity. Propagation can be carried out in culture medium or by transplantation of the hybridoma into the peritoneal cavity of syngeneic mice from where the antibodies are harvested in ascites fluid. Thus, large and, at least theoretically, unlimited quantities of monoclonal antibodies of specific characteristics can be produced.

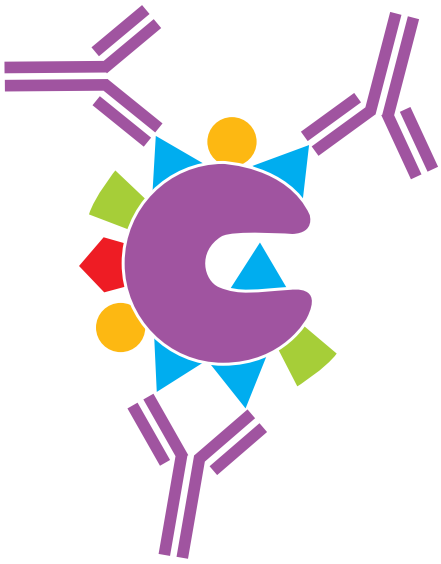

Figure 5: A given clone of monoclonal antibodies reacts with a specific epitope on an antigen.

In immunohistochemistry, there are certain advantages monoclonal antibodies have over their polyclonal counterparts; these include high homogeneity, absence of nonspecific antibodies, ease of characterization and no batch-to-batch or lot-to-lot variability. However, some pitfalls in the use of monoclonal antibodies should be noted.

The test methods for the selection of useful clones and for quality control must be identical to the methods of use. All too often, monoclonal antibodies are characterized using frozen tissue when they are intended for use on formalin-fixed specimens. For this, the targeted epitope must survive fixation. In some cases, antigens have been shown to survive formalin fixation by use of polyclonal antibodies, but the particular epitope with which the monoclonal antibody interacts does not.

Similarly, reactivity of an epitope after optimal fixation does not necessarily assure its survival under suboptimal fixing conditions. As continuously new and improved antigen retrieval procedures are being published, it is imperative that any screening for new monoclonal antibodies also consider this additional variable (see Antigen Retrieval chapter).

The targeted epitope must also be unique to a given antigen. Specificity, one of the greatest benefits of monoclonal antibodies, is lost if the antibody is directed against an epitope shared by two or more different antigens (see Antibody Cross-Reactivity). While the cross-reactivity of a polyclonal antibody can usually be removed by absorption, that of a monoclonal antibody cannot be.

The screening method should also consider that monoclonal antibodies, compared to polyclonal antibodies, depend more on environmental factors such as pH and solute for optimum performance.[3]

ANTIBODY AFFINITY

■ Antibodies from hyperimmunized animals not only differ with regard to the determinants they recognize on multivalent antigens, but also differ in their affinities for the same. The term affinity has been used to describe both “intrinsic” and “functional” affinities.[4]

The *intrinsic* affinity of an antibody resides in the HV region and is determined by the same sequence of amino acids that determines specificity. However, to say that the greater the specificity, the stronger the affinity, is probably an oversimplification. Ionic interactions, hydrogen bonding and van der Waals forces are the major contributors to the intrinsic affinity between the paratope on the antibody and the epitope on the antigen. Hydrophobicity appears to have a stabilizing effect on the formed immune complex and may lead to its precipitation. Covalent binding between antibody and antigen does not occur. The association constant (Ka) of the binding between an antibody and its antigenic determinant is a measure of the antibody's affinity. It can range from 10^3 to 10^{10} liters per mole, which is the reciprocal of concentration in moles per liter. The higher the affinity of the antibody, the lower the concentration of free antigen needed for the available binding sites of the antibody to become saturated (reach equilibrium). Just as the quantity (titer) of an antibody increases with time during immunization, so does its quality (affinity). This has been called "affinity maturation”.[5] Lower doses of immunogen increase the rate of affinity maturation, but result in lower titers of antibody, and vice versa.

In immunohistochemistry, the *functional* affinity of an antibody or an antiserum can be very loosely defined by the time required to reach equilibrium with the tissue antigen. If equal aliquots of two antibodies or antisera of identical titer are incubated for increasing periods of time with the antigen on the tissue, the antibody that reaches a plateau of maximum staining intensity first is of higher functional affinity. The term “avidity” has been used synonymously to describe functional affinity,[5] but has also been used to denote the strength of the binding between antibody and its antigen.[6] Frequently, the term avidity has also been used to describe the sum total of all intrinsic affinities found in a polyclonal antibody population.

Because antigen-antibody reactions are reversible, the simple immune complexes formed on the tissue may dissociate during the washing cycles used in immunohistochemistry. The ease and degree of dissociation vary from antibody to antibody, and low salt concentrations as well as low temperatures will reduce the likelihood of weak staining due to dissociation of an already formed immune complex. Thus, high affinity antibodies are desirable and have the advantage that during washing, dissociation is less likely to occur than with low affinity antibodies. As mentioned before, a polyclonal population of antibodies contains a more or less continuous spectrum of low to high affinities against several epitopes on a given antigen. Therefore, after incubation with primary antibodies of this type, excessive washing is unlikely to result in any appreciable loss of staining.

On the other hand, monoclonal antibodies are of uniform affinity and, if the affinity is low, loss of staining is likely due to dissociation of the antibody from its epitope. Thus, monoclonal antibodies of high affinity should be selected, if possible. As indicated above, avoid factors that weaken the antigen-antibody bond such as high salt concentrations, high temperature and very low pH during the washing of the specimens. Experience in the handling of antibodies in immunohistochemistry has shown that the washing and incubation in buffer baths can be safely reduced and that gentle agitation helps to reduce background staining.[7]

The affinity of antibodies is also related to their capability to form insoluble immune complexes. Generally, the higher the affinity of an antibody, the greater its tendency to form a precipitate. Precipitation proceeds through a rapid stage in which soluble antigen-antibody complexes form, followed by slower aggregation and, eventually, precipitation. Non-precipitating antibodies are mostly of lower affinity and are incapable of forming the lattice required for precipitation to occur.

Monoclonal antibodies, regardless of whether they are of high or low affinity, cannot form a lattice with antigen, and, hence only rarely form insoluble precipitates. However, in immunohistochemistry, the capability of a primary antibody to form precipitating immune complexes is of little importance because reaction with immobilized tissue antigen entails antibody capture onto tissue rather than precipitation.

Prozone is a property which was first noted in antibody-induced agglutinations. It is the observation that some antisera, when insufficiently diluted, fail to agglutinate cells even though higher dilutions will do so. While prozone can also be observed in precipitin reactions, in immunohistochemistry, it is a rare event.[7]

ANTIBODY CROSS-REACTIVITY

■ The term “cross-reactivity” denotes an immunochemical activity that can occur either between an antibody and two or more antigens or vice versa, when an antigen reacts with several different antibodies. Typical examples are when anti-L (or -K) chain antibodies interact with all five Ig classes or when carcinoembryonic antigen (CEA) reacts with antibodies against CEA, blood group antigens and normal tissue proteins, respectively. The common denominator in each case is the sharing of at least one common epitope between several antigens.

Another valid use of the term cross-reactivity denotes the experimentally or accidentally induced changes within one or several epitopes, through antigen retrieval,[8] leading to a possible loss of specificity by a given monoclonal antibody for this antigen. The term cross-reactivity also describes the interaction of an antibody with similar or dissimilar epitopes on unrelated antigens. This latter phenomenon however is frequently a property of low affinity antibodies, and is usually subject to change because of affinity maturation during immunization.

Cross-reactivity of antibodies to human antigens with identical or similar antigens of other species (“Cross-species cross-reactivity”) can be of interest to the researcher and veterinarian because of the scarcity of animal-specific antibodies. To overcome this, two publications reported the results of cross-species reactivity studies using commercially available antihuman polyclonal and monoclonal antibodies.[9,10] It was demonstrated that the majority of animal antigens selected showed strong reactivity with antihuman antibodies. However, for more technical detail on the use of a given mouse primary antibodies on animal tissues, the reader is referred to the DAKO ARK (animal research kit) products.

The terminology of cross-reactivity however is misplaced when describing any observed staining by the same antibody of different cells or tissue components, regardless whether they contain common antigens, as this would distort the strict immunochemical definition of the term.

ANTIBODY REACTION RATES

■ Generally, the size and shape of the antibody molecule and its conjugates or complexes appear to be of little consequence in immunohistochemistry. Insufficient tissue penetration, even when staining intranuclear or cytoplasmic antigens, has never been observed, regardless of whether primary antibodies of class IgM (900 kD), large complexes like PAP (400–430 kD) or APAAP (approximately 560 kD) or dextran-linked reagents* were used. However, it is reasonable to assume that gross overfixation of tissue may make penetration more difficult for antibodies and their complexes.

Although under ideal conditions, antibodies react with their ligands (antigens) very rapidly, in immunohistochemistry the conditions are rarely ideal. Depending on tissue fixation, antibody concentration, ambient temperature and other variables, primary antibody incubation times of up to 48 hours may be required for maximum reactivity. It is not surprising therefore that, as immunohistochemical procedures have become increasingly useful in surgical pathology, the need for shortened processing times has also been voiced. Very short incubation periods are made feasible by the relatively rapid reaction rates which occur when higher concentrations of high affinity primary and link antibodies are used.

Equilibrium between antigen-bound and free antibody is rarely achieved. For this purpose, very long incubation periods with more dilute antibody preparations are being used. It is not known whether shorter incubations with more concentrated antibody preparations would establish equilibrium sooner because, as a rule, nonspecific background staining may result under these conditions, preventing unambiguous interpretation. Incubates of primary antibody have been salvaged experimentally after their first use by aspiration from one section and transferred to additional sections.[7] With some antibodies, up to seven identical tissue specimens could be stained with equal quality

*See Staining Methods chapter

when the primary antibody was used in concentrations required for routine 10 minute incubations. This suggests that only a very small fraction of the available antibody is actually utilized during these relatively short incubation times. Needless to say, once an incubation time has been selected, it must be maintained uniformly or staining will not be consistently reproducible.

ANTIBODY STABILITY

■ Polyclonal antibodies, when stored unfrozen and used subsequently in immunohistochemistry, are to be somewhat less stable as immunoglobulin fraction compared to whole antiserum.[7] However, this reduced stability was found to depend largely on the method of purification and storage as well as on the method of application. Exposure of antibodies to extreme pH, as well as high or very low concentrations of salts during purification tends to decrease their stability more than does exposure to mild conditions such as ion exchange chromatography. Formation of soluble aggregates and subsequently, precipitated polymers are the most frequently resulting changes noted. These changes are probably the result of hydrophobic interaction between the IgG molecules in solution. While the presence of soluble aggregates may enhance their performance as precipitating antibodies, their increased hydrophobicity has been shown to cause increased nonspecific binding (see Background chapter) in immunohistochemistry.[7] Removal of these aggregates and polymers from IgG fractions is therefore important prior to applications for immunohistochemistry.

Just as storage of purified antibodies may augment their hydrophobicity due to aggregation and polymerization, so may their conjugation to other molecules.[11] Conjugation with glutaraldehyde involves the epsilonamino groups of lysine and alpha-amino groups of the N-terminal amino acids resulting in their cross-linking. Because there are many glutaraldehyde-reactive sites in IgG molecules, the hydrophobicity of the conjugated antibodies may increase significantly, resulting in augmented attraction to hydrophobic sites in the fixed tissue and increased background.

■ Monoclonal antibodies also have been shown to be influenced in their performance by methods of purification and storage; 42% of monoclonal antibodies investigated by Underwood and Bean showed changes in specificity, affinity and cross-reactivity.[12] Antibodies of class IgM and subclass IgG_{2b} were especially sensitive.

■ Of special interest therefore, is a study by Balaton et al.[13] No significant differences were observed in the immunohistochemical specificity, sensitivity or staining patterns between 65 polyclonal and monoclonal antibodies that either had or had not exceeded the manufacturer's recommended expiration date. The authors recommended a revision of the guidelines for establishing expiration dates as shelf lives of up to eleven years were observed.

It must be noted however that actual-time testing of proteinaceous reagents is not feasible. While commonly practiced in the pharmaceutical field,[14,15] high-temperature accelerated degradation testing when applied to immunochemicals such as antisera and antibodies can be irrelevant or even misleading.[16,17]

■ There are however, regulatory guidelines in place in the United States. In the clinical laboratory, this has been mandated by the Clinical Laboratory Improvement Act of 1988 and by the College of American Pathologists. The only possible corollary to these requirements is to allow the laboratory to document the activity of the product until the loss of the same. Alternatively, the laboratory may aliquot and freeze the undiluted antibody at -20°C for later use. At this time, the laboratory must confirm the activity prior to use of the antibody in any test.

Antibody stability in commercially produced reagents is determined by real-time and real-temperature testing by each manufacturer. Most manufacturers demonstrate stability for a set period of time. While many antibodies may retain activity for a longer period of time, the only regulatory requirement for the manufacturer is to certify the period of time that the antibody has been tested. There is no requirement to continue further testing until the antibody loses activity.

Furthermore, it is this writer's experience that the conditions for the storage of reagents in the user's laboratory are frequently not identical to those that prevailed during the shelf life studies. Because of the possibility of adverse storage conditions after the purchase of the product, the manufacturer can only offer a limited liability instead of predicting the actual demise of a reagent.

HANDLING OF ANTIBODIES

■ In order to achieve optimal performance from the reagents used in immunohistochemistry, it is imperative to observe certain basic rules for their handling and storage. If properly maintained, most reagents will remain stable for months and even years. The recommendations given by the manufacturer on specification sheets and on vial labels should always be followed.

■ **RECEIVING** Although many commercially produced immunochemicals are guaranteed to be stable for up to several years, prediluted antibodies have a shorter shelf life (see Antibody Stability). Upon receipt, immunochemicals should be promptly stored according to the manufacturer's recommendations. Log in reagents by entering the manufacturer's lot numbers, expiration date, date of receipt and invoice number. These entries provide valuable information for the user, especially if later reclamations become necessary.

■ **STORAGE** Perhaps the two most important considerations when storing antibodies are the storage container and the temperature.

STORAGE CONTAINERS Ideally, the preferred materials for storage containers of protein solutions should have negligible protein adsorptivity. Polypropylene, polycarbonate or borosilicate glass are recommended and are used widely. Solutions containing very low concentrations of protein (i.e., less than 10–100 µg/ml), should receive an addition of bulk protein. Generally, 0.1% to 1.0% bovine albumin is used to reduce loss through polymerization and adsorption onto the container. Containers made of clear and colorless materials are preferred as these will allow ready inspection of contents. Container labels also should allow access for inspection.

STORAGE TEMPERATURE Probably more than any other factor, observe proper storage temperature as recommended by the manufacturer. Monitor refrigerators and freezers used for storage of immunochemicals for accurate and consistent temperatures. Store valuable or large quantities of immunochemical reagents in equipment with temperature alarm and emergency back-up power systems.

Store most prediluted (“ready to use”) antibodies, their conjugates and monoclonal antibody solutions at 2–8°C because freezing and thawing is known to have a deleterious effect on their performance. This also applies to entire kits that contain prediluted reagents including monoclonal antibodies. Store concentrated protein solutions, such as antisera and immunoglobulin fractions, in aliquots and frozen at -20°C or below to prevent cycles of repeated freezing and thawing. Bring frozen protein solutions to room temperature slowly, and avoid temperatures above 25°C.

■ **USE AND CARE** Proper reagent care can reduce problems stemming from contamination, heat or excessive light exposure. Reagent contamination can be avoided by the use of clean pipet tips. Prompt return of reagents to proper storage conditions will prolong their shelf life.

The appearance of immunochemical reagents, particularly undiluted antisera, is not always indicative of their performance. Although beta-lipoproteins have a very strong hydrophobic property, neither lipemia nor lipolysis in antisera has been studied systematically for interference with immunohistochemical staining. Where obvious lipemia is encountered in an antiserum and thought to be the cause of interference with successful staining, removal of the lipids by use of dextran sulfate and calcium,[18] or by extraction with organic solvents is recommended. Alternatively, the addition of 2 g Aerosil (Degussa, NY) to 100 mL antiserum followed by incubation for 4 hour at 37°C has proven useful.

Mild to moderate hemolysis in serum (plasma) resulting from suboptimal bleeding techniques, probably does not interfere with most immunohistochemical staining procedures, but avoid excessive hemolysis. If excessive hemolysis or lipemia is encountered, isolation of the immunoglobulin fraction from the antiserum or normal serum may be necessary. Such isolates will usually appear colorless and clear. Discard all immunochemicals, including antisera and normal nonimmune sera contaminated with bacterial growth. Their use in immunohistochemical procedures most likely will introduce artifacts and nonspecific staining.

Familiarity with the nature of antibodies, their capabilities and limitations, will allow the user to better utilize these reagents and to more efficiently solve problems, if they occur. The following chapters will further contribute to the understanding of antibodies; they will also provide detailed information about the ancillary reagents and procedures used in immunohistochemistry.

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BASIC IMMUNOCHEMISTRY

THOMAS BOENISCH

■ Antibody titer and dilutions, as well as incubation time and temperature, are tightly interwoven in their effects on the quality of immunohistochemical staining. These factors can be changed independently or, as is more often the case, in complementary fashion, to bring about marked differences in the quality of staining. Generally, when making any changes, the overriding goal should be the achievement of optimal specific staining accompanied by minimal interference from background staining. This chapter will highlight these variables.

ANTIBODY TITER

■ In immunohistochemistry, the optimum antibody titer may be defined as the highest dilution of an antiserum (or monoclonal antibody) that results in maximum specific staining with the least amount of background under specific test conditions. This highest dilution is determined primarily by the absolute amount of specific antibodies present.

With polyclonal antisera, antibody levels have traditionally been expressed as micrograms of antigen precipitated per milliliter of antiserum. Although of interest, to the immunohistochemist this is not necessary information.

For monoclonal antibody preparations, the absolute concentration of specific antibodies can be readily measured and frequently forms the basis for making the required dilutions. The highest dilution is also governed by the intrinsic affinity of an antibody; if the titer is held constant, a high affinity antibody is likely to react faster with the tissue antigen and give more intense staining within the same incubation period than an antibody of low affinity.

Augmenting titers by isolating and enriching the immunoglobulin fractions from polyclonal antisera produces little benefit for immunohistochemical applications because nonspecific antibodies and soluble aggregates—frequently an additional source for nonspecific background—become enriched also (see Background chapter).

Titers as defined above may vary from 1:100 to 1:2000 for polyclonal antisera; from 1:10 to 1:1000 for monoclonal antibodies in cell culture supernatants; and up to 1:1,000,000 for monoclonal antibodies in ascites fluid. These dilutions may well be exceeded in the future by the ever increasing sensitivities of newer detection methods and, in some cases, by the use of an appropriate antigen retrieval procedure.

ANTIBODY DILUTION

■ Often the manufacturer offers prediluted reagents ready for use, or recommends dilution ranges compatible with other variables such as method, incubation time and temperature. If this information is not provided, determine optimal working dilutions of immunochemical reagents by titration. Correct dilutions will contribute to the quality of staining if they are prepared accurately and consistently. They are best determined by first selecting a fixed incubation time and then by making small volumes of a series of experimental dilutions. Depending on specimen size, applications of 0.1–0.4 mL of solution per section is generally adequate. On paraffin sections, optimal dilutions of primary antibodies are not only signaled by a peak in staining intensity, but also by the presence of minimal background (maximal signal-to-noise ratios). Once the optimal working dilution is determined, larger volumes can be prepared according to need and stability.

The extent to which monoclonal antibodies can be diluted is subject to additional criteria. Because of their more restricted pI and molecular conformation, monoclonal antibodies are more sensitive to the pH and ions of the diluent buffer.[1] It was proposed therefore that any evaluation of monoclonal antibodies also include their titration at pH 6.0 and 8.6 in the *absence* of NaCl. That highest dilution and pH retaining the strongest immunoreactivity was called the optimal dilution and recommended for future use. Of the diluents tested, phosphate buffered saline, although widely used as a diluent for primary antibodies, was found to suppress the reactivity of most monoclonal antibodies tested.

Dilutions are usually expressed as the ratio of the more concentrated stock solution to the total volume of the desired dilution. For example, a 1:10 dilution is made by mixing one part of the stock solution with nine parts of diluent. Two-fold serial dilutions are made by successive 1:2 dilutions of the previous stock dilution. In order to make a very small volume of a highly diluted solution, it may be necessary to make it in two steps. For example, to prepare 1.0 mL of a 1:1000 dilution, first make 100 µl of a 1:10 dilution (10 µl + 90 µl), and then 1000 µl of a 1:100 dilution using 10 µl of the first dilution (10 µl + 990 µl).

When making dilutions, the use of adjustable pipettes allows for greater flexibility and more accurate delivery. To measure volumes in excess of 1.0 mL, use serological or volumetric pipettes. Table 1 indicates the volumes of stock reagents and diluents necessary to obtain dilutions

ranging from 1:50 to 1:200. Checkerboard titrations are used to determine the optimal dilution of more than one reagent simultaneously. In this example of a checkerboard titration, the optimal dilutions of a primary antibody and the streptavidin-HRP reagent are to be found, while the dilution of the biotinylated link antibody is held constant (not shown). Nine tissue sections are required if three dilutions are to be tested.

TABLE 1

Streptavidin-HRP	Primary Antibody Dilutions		
1:50	1:50	1:100	1:200
1:100	1:50	1:100	1:200
1:200	1:50	1:100	1:200

As noted earlier, staining results achieved by use of several different dilutions will often be identical or similar, in which case the cost of the reagent may become an additional factor in selecting the optimal dilution.

Precise definition of the optimal signal-to-noise ratio as a function of the primary antibody dilution is likely to be more critical with some methods than with others. For example, it was found to be more restricted with the use of unlabelled enzyme-antienzyme complexes (PAP, APAAP), than with methods utilizing the (strept)avidin-biotin technology.[2] This is probably consistent with the observation that, as opposed to the PAP method, the ABC method cannot distinguish between high and low concentrations of tissue antigens.[3]

ANTIBODY INCUBATION

As mentioned previously, incubation time, temperature and antibody titers are interdependent; a change in one factor will affect the others.

INCUBATION TIME There is an inverse relationship between incubation time and antibody titer—the higher the antibody titer, the shorter the incubation time required for optimal results. In practice, however, it is expedient to first set a suitable incubation time before determining the optimal antibody dilution. Higher concentrations of specific antibodies (and higher affinities) allow for the shortening of the incubation time.

Incubation times for the primary antibody may vary up to 48 hours, with 10–30 minutes probably being the most widely used. For an antibody to react sufficiently strong with the bound antigen in a very short period of time, it must be of high affinity and of relatively high concentration. Variables believed to contribute to increased nonspecific background staining should be kept to a minimum (see Background chapter). Primary antibody incubations of 48-hour duration allow, more than anything else, for greater economy because very high dilutions of antiserum may be used. While antibodies of low affinity and/or low titer must be incubated for long periods in order to reach equilibrium*, nothing can be gained by prolonging primary antibody incubation beyond the time at which the tissue antigen is saturated with antibody.

Equilibrium is usually not reached during primary antibody incubations of less than 20 minutes. Consistent timing of the primary antibody incubation step is of great importance. Inconsistent incubation times can cause variations in the overall quality and intensity of staining. Consistency in the intensity of staining is particularly essential in efforts that attempt to assess the degree of tumor differentiation.

INCUBATION TEMPERATURE Because equilibrium in antigen-antibody reactions is reached more quickly at 37°C compared to room temperature, some workers prefer to incubate at the higher temperature. An increase in incubation temperature allows for a greater dilution of the antibody or a shortened incubation time. It is not known whether temperature promotes the antigen-antibody reaction selectively rather than the various reactions that give rise to background.

A temperature of 4°C is frequently used in combination with overnight or longer incubations. Slides incubated for extended periods or at 37°C should be placed in a humidity chamber to prevent evaporation and drying of the tissue sections. Similarly, tissue incubated at room temperature in a very dry or drafty environment will require the use of a humidity chamber.

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*The term equilibrium here denotes saturation of antigen with antibody.

BASIC ENZYMOLOGY

THOMAS BOENISCH

■ Immunoenzymatic staining methods utilize enzyme-substrate reactions to convert colorless chromogens into colored end products. Of the enzymes used in these applications, only horseradish peroxidase and calf intestine alkaline phosphatase will be considered in some detail. Because of its low sensitivity, glucose oxidase *(Aspergillus niger)* is only rarely used today.

This chapter will also discuss the various chromogens and substrates that can be used in conjunction with peroxidase and phosphatase, along with suggested procedures for the preparation of some substrate solutions.

ENZYMES

■ Enzymes are proteinaceous catalysts peculiar to living matter. Hundreds have been obtained in purified and crystalline form. Their catalytic efficiency is extremely high–one mole of a pure enzyme may catalyze the transformation of as many as 10,000 to 1,000,000 moles of substrate per minute. While some enzymes are highly specific for only one substrate, others can attack many related substrates. A very broad classification of enzymes would include hydrolytic enzymes (esterases, proteases), phosphorylases, oxidoreductive enzymes (dehydrogenases, oxidases, peroxidases), transferring enzymes, decarboxylases and others.

Enzymatic activity is dependent upon several variables, such as enzyme and substrate concentrations, pH, salt concentration of the buffer milieu, temperature and light. Many enzymes also possess non-proteinaceous chemical portions termed prosthetic groups. Typical prosthetic groups are the iron-protoporphyrin of peroxidase, and biotin of $C0_2$ transferases. In addition, many enzymes require the presence of metal ions such as Mg^{++}, Mn^{++} and Zn^{++}, which function as electrophilic (electron-attracting) agents.

The general formula, which describes the reactions of an enzyme with its substrate, may be written as follows:

1. Enzyme (E) + Substrate (S) = ES complex
2. ES $\rightarrow$ E + Products (P)

Thus, before formation of the product, a transient enzyme-substrate complex is formed at the “active site” (prosthetic group) of the enzyme.

Substances that interfere with the specific binding of the substrate to the prosthetic group are specific inhibitors and differ significantly from agents which cause nonspecific denaturation of enzyme (or any protein). Two basic types of inhibition are recognized, competitive inhibition and non-competitive inhibition. Competitive inhibition is the result of a reversible formation of an enzyme-inhibitor complex (EI):

E + Inhibitor (I) + S = EI + S

The formation of the complex EI can be reversed by a change in the concentration of either the substrate or the inhibitor, unless the affinity of I for E is greater than of S for E. The action of carbon monoxide or azides on the heavy metals of respiratory enzymes is a typical example of competitive inhibition.

In noncompetitive inhibition, the inhibition depends solely on the concentration of the inhibitor and generally, is not reversible. Noncompetitive inhibition may or may not involve the prosthetic group of the enzyme and manifests itself by slowing down or halting the velocity of the enzyme’s reaction upon the substrate.

E + I + S $\rightarrow$ E I S

Selecting the enzyme most suitable for a particular immunohistochemical application depends on a number of criteria:

- The enzyme should be available in highly purified form and be relatively inexpensive.
- Conjugation (covalent binding to antibody or avidin, for example) or noncovalent binding should not abolish enzyme activity, although it may diminish it.
- The bound enzyme should be stable in solution.
- Endogenous enzyme activity should interfere only minimally with specific antigen-related staining.
- The products of the enzymic reactions should be readily detectable and stable.

Horseradish peroxidase and calf intestine alkaline phosphatase meet most of these criteria and the following will list their properties in more detail.

■ **HORSERADISH PEROXIDASE (HRP):** This enzyme (molecular weight 40 kD) is isolated from the root of the horseradish plant. HRP has an iron-containing heme group (hematin) as its active site and in solution is colored brown. The hematin of HRP first forms a complex with hydrogen peroxide and then causes it to decompose resulting in water and atomic oxygen. HRP oxidizes several substances, two of which are polyphenols and nitrates. Like many other enzymes, HRP and some HRP-like activities can be inhibited by excess substrate. The complex formed between

HRP and excess hydrogen peroxide is catalytically inactive and in the absence of an electron donor (e.g. chromogenic substance), is reversibly inhibited. It is the excess hydrogen peroxide and the absence of an electron donor that brings about quenching of endogenous HRP activities. Cyanide and azide are two other strong (reversible) inhibitors of HRP.

HRP can be attached to other proteins either covalently or noncovalently. The covalent binding of HRP to other proteins can be performed using either one-step or two-step procedures involving glutaraldehyde. The chemical 4,4'-difluoro-3,3'-dinitrophenyl sulfone (FNPS) is less commonly used for this purpose. In all cases, the epsilonamino groups of lysine and N-terminal amino groups of both proteins are involved in this reaction. The two-step conjugation procedure is preferred because, relative to the antibody molecule, the HRP molecule has few reactive groups. As a consequence, adding glutaraldehyde to a solution containing an admixture of HRP and antibody will result in more antibody molecules being conjugated to each other than to the enzyme. In the two-step procedure, HRP reacts with the bifunctional reagents first. In the second stage, only activated HRP is admixed with the antibody resulting in much more efficient labelling and no polymerization.

HRP is also conjugated to (strept)avidin using the two-step glutaraldehyde procedure. This form is used in the LAB and LSAB procedures for example. Conjugation with biotin also involves two steps, as biotin must first be derivatized to the biotinyl-N-hydroxysuccinimide ester or to biotin hydrazide before it can be reacted with the epsilonamino groups of the enzyme.

Noncovalent binding of HRP to antibody (also known as unlabelled antibody binding) is described in great detail by Sternberger.[1] Instead of the use of bifunctional reagents, IgG-class antibodies to HRP are used to form a soluble semicyclic immune complex consisting of two antibody and three enzyme molecules. The molecular weight of the peroxidase-antiperoxidase or PAP complex is 400–430 kD.

■ **CALF INTESTINE ALKALINE PHOSPHATASE (AP):** Calf intestine alkaline phosphatase (molecular weight 100 kD) removes (by hydrolysis) and transfers phosphate groups from organic esters by breaking the P-0 bond; an intermediate enzyme-substrate bond is briefly formed. The chief metal activators for AP are Mg^{++}, Mn^{++} and Ca^{++}.

AP had not been used extensively in immunohistochemistry until publication of the unlabelled alkaline phosphatase-antialkaline phosphatase (APAAP) procedure.[2,3] The soluble immune complexes utilized in this procedure have molecular weights of approximately 560 kD. The major advantage of the APAAP procedure compared to the PAP technique is the lack of interference posed by endogenous peroxidase activity. Because of the potential distraction of endogenous peroxidase activity on PAP staining, the APAAP technique is recommended for use on blood and bone marrow smears. Endogenous alkaline phosphatase activity from bone, kidney, liver and some white cells can be inhibited by the addition of 1 mM levamisole to the substrate solution,[4] although 5 mM has been found to be more effective.[5] Intestinal alkaline phosphatases are not adequately inhibited by levamisole.

SUBSTRATES AND CHROMOGENS

■ **PEROXIDASE** As described above, HRP activity in the presence of an electron donor first results in the formation of an enzyme-substrate complex, and then in the oxidation of the electron donor. The electron donor provides the "driving" force in the continuing catalysis of H_20_2, while its absence effectively stops the reaction.

There are several electron donors that when oxidized, become colored products and are therefore called chromogens. This, and the property of becoming insoluble upon oxidation, make such electron donors useful in immunohistochemistry.

3,3'-DIAMINOBENZIDINE (DAB) produces a brown end product which is highly insoluble in alcohol and other organic solvents. Oxidation of DAB also causes polymerization, resulting in the ability to react with osmium tetroxide, and thus increasing its staining intensity and electron density. Of the several metals and methods used to intensify the optical density of polymerized DAB, gold chloride in combination with silver sulfide appears to be the most successful.[6]

3-AMINO-9-ETHYLCARBAZOLE (AEC), upon oxidation, forms a rose-red end product which is alcohol soluble. Therefore, specimens processed with AEC must not be immersed in alcohol or alcoholic solutions (e.g., Harris' hematoxylin). Instead, an aqueous counterstain and mounting medium should be used. AEC is unfortunately susceptible to further oxidation and, when exposed to excessive light, will fade in intensity. Storage in the dark is therefore recommended.

4-CHLORO-1-NAPHTHOL (CN) precipitates as a blue end product. Because CN is soluble in alcohol and other organic solvents, the specimen must not be dehydrated, exposed to alcoholic counterstains, or coverslipped with mounting media containing organic solvents. Unlike DAB, CN tends to diffuse from the site of precipitation.

p-PHENYLENEDIAMINE DIHYDROCHLORIDE/pyrocatechol (Hanker-Yates reagent) gives a blue-black reaction product which is insoluble in alcohol and other organic solvents. Like polymerized DAB, this reaction product can be osmicated. Varying results have been achieved with Hanker-Yates reagent in immunoperoxidase techniques.

ALKALINE PHOSPHATASE In the immunoalkaline phosphatase staining method, the enzyme hydrolyzes naphthol phosphate esters (substrate) to phenolic compounds and phosphates. The phenols couple to colorless diazonium salts (chromogen) to produce insoluble, colored azo dyes. Several different combinations of substrates and chromogens have been used successfully.

NAPHTHOL AS-MX PHOSPHATE can be used in its acid form or as the sodium salt. The chromogens Fast Red TR and Fast Blue BB produce a bright red or blue end product, respectively. Both are soluble in alcoholic and other organic solvents, so aqueous mounting media must be used. Fast Red TR is preferred when staining cell smears.

NEW FUCHSIN also gives a red end product. Unlike Fast Red TR and Fast Blue BB, the color produced by New Fuchsin is insoluble in alcohol and other organic solvents, allowing for the specimens to be dehydrated before coverslipping. The staining intensity obtained by use of New Fuchsin is greater than that obtained with Fast Red TR or Fast Blue BB.

OTHER SUBSTRATES AND CHROMOGENS Additional substrates include naphthol AS-BI phosphate, naphthol AS-TR phosphate and 5-bromo-4-chloro-3-indoxyl phosphate (BCIP). Other possible chromogens include Fast Red LB, Fast Garnet GBC, Nitro Blue Tetrazolium (NBT) and iodonitrotetrazolium Violet (INT).

Detailed descriptions and information for the preparation of the most commonly used substrate-chromogen mixtures for HRP[7] and AP[8] as well as their appropriate use and advantages or disadvantages are available.[9-12]

SUGGESTED PROCEDURES FOR SUBSTRATE-CHROMOGEN REAGENTS

PEROXIDASE

AEC SUBSTRATE SOLUTION (recommended for cell smears)

1. Dissolve 4 mg AEC in 1 mL N,N-dimethylformamide.
2. Add 14 mL 0.1 M acetate buffer, pH 5.2 and 0.15 mL 3% hydrogen peroxide.
3. Mix, and filter if precipitate forms.
4. Add solution to tissue and incubate for 5–15 minutes at room temperature.
5. Rinse with distilled water.
6. Counterstain and coverslip with aqueous-based medium.

DAB SUBSTRATE SOLUTION

1. Dissolve 6 mg DAB in 10 mL 0.05 M Tris buffer, pH 7.6.
2. Add 0.1 mL 3% hydrogen peroxide. Mix, and filter if precipitate forms.
3. Add solution to tissue and incubate for 3–10 minutes at room temperature.
4. Rinse with distilled water.
5. Counterstain and coverslip with either organic- or aqueous-based medium.

ALKALINE PHOSPHATASE

FAST RED SUBSTRATE SOLUTION (recommended for cell smears)

1. Dissolve 2 mg naphthol AS-MX phosphate, free acid (Sigma N 4875) in 0.2 mL N,N-dimethylformamide in a glass tube.
2. Add 9.8 mL 0.1 M Tris buffer, pH 8.2.
3. Add 0.01 mL of 1 M levamisole (Sigma L 9756) to block endogenous alkaline phosphatase. (Solution can be stored at 4°C for several weeks, or longer at -20°C.)
4. Immediately before staining, dissolve 10 mg Fast Red TR salt (Sigma F 1500) in above solution and filter onto slides.
5. Incubate for 10–20 minutes at room temperature.
6. Rinse with distilled water.
7. Counterstain and coverslip with aqueous-based medium.

NEW FUCHSIN SUBSTRATE SOLUTION (recommended for tissue sections)

1. Solution A: Mix 18 mL of 0.2 M 2-amino-2-methyl-1, 3-propanediol (Merck 801464) with 50 mL 0.05 M Tris buffer, pH 9.7 and 600 mg sodium chloride. Add 28 mg levamisole (Sigma L 9756).
2. Solution B: Dissolve 35 mg naphthol AS-BI phosphate (Sigma N 2250) in 0.42 mL N,N-dimethylformamide.
3. Solution C: Under fume hood, mix 0.14 mL 5% New Fuchsin (Sigma N 0638, 5 g in 100 mL 2 N HCl) with 0.35 mL of freshly prepared 4% sodium nitrite (Sigma S 2252, 40 mg in 1 mL distilled water). Stir for 60 sec.
4. Mix Solutions A and B, then add Solution C; adjust to pH 8.7 with HCl. Mix well and filter onto slides.
5. Incubate for 10–20 minutes at room temperature.
6. Rinse with distilled water.
7. Counterstain and coverslip with either organic- or aqueous-based medium.

NEW FUCHSIN SUBSTRATE SOLUTION (alternative procedure)

1. Solution A: In fume hood add 0.2 mL of 5% New Fuchsin (Merck 4041, in 2 N HCl) to 0.5 mL of fresh 4% sodium nitrite. Agitate for 30-60 sec. Add 100 mL of 0.05 M Tris buffer, pH 8.7, and 100 µL of 1 M levamisole to block endogenous alkaline phosphatase.
2. Solution B: Dissolve 50 mg naphthol AS-BI phosphate (Sigma N 2250) in 0.6 mL N,N- dimethylformamide.
3. Add Solution B to Solution A and mix well. Filter directly onto slides.
4. Incubate for 10–20 minutes at room temperature.
5. Rinse with distilled water.
6. Counterstain and coverslip with either organic- or aqueous-based medium.

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FIXATION

A. J. FARMILO AND RONALD H. STEAD, REVISED BY KAREN N. ATWOOD

An essential part of all histological and cytological techniques is the preservation of cells and tissues in as reproducible and lifelike manner as possible. To accomplish this, tissue blocks, sections or smears are usually immersed in a fixative fluid, although in the case of smears, merely drying the preparation acts as a form of preservation. The fixatives employed prevent autolysis by inactivating lysosomal enzymes and inhibit the growth of bacteria and molds, which would give rise to putrefactive changes. Furthermore, fixatives stabilize the cells and tissues to protect them from the rigors of subsequent processing and staining techniques.

In performing their protective role, fixatives denature proteins by coagulation, by forming additive compounds or by a combination of the two. Thus, conformational changes in the structure of proteins occur causing inactivation of enzymes. The resulting complexes differ from the undenatured proteins in both chemical and antigenic profiles. The dilemma of fixation has always been that it is necessary to introduce some artifact in order to have a protective effect; by definition, fixatives alter the original chemical composition of the tissues involved.

In addition to altering the chemical nature of the cells and tissues to which they are applied, fixatives also cause physical changes to cellular and extracellular constituents. Viable cells are encased in an impermeable membrane. Fixation breaks down this barrier and allows relatively large molecules to penetrate and escape. Furthermore, the cytoplasm undergoes what is essentially a sol-gel transformation, with the formation of a proteinaceous network sufficiently porous to allow further penetration of large molecules. It must be recognized, however, that different fixatives result in different degrees of porosity; coagulant fixatives, such as B5 and formal sublimate, result in a larger pore size than do non-coagulant fixatives, like formalin. Such changes are obviously of great importance in immunochemistry when it is necessary to demonstrate all but the most superficial antigens in a section or smear.

Most fixative solutions contain chemicals, which stabilize proteins, since this is how protection of the cellular structure is best accomplished. Some fluids have been designed to preserve carbohydrates or lipids. These have not been routinely employed, since histologists and anatomists have mainly required the preservation of microanatomy.

Fixation is always a compromise and the requirements of a fixative vary according to the different techniques employed in visualizing the structure of the cells or tissues. Thus, the techniques for cytology differ completely from those for histology or electron microscopy. Furthermore, the application of different staining methods necessitates other alterations in the fixation protocol, such as air-drying prior to Giemsa staining, and wet-fixation for Papanicolaou's method.

BLOOD SMEARS AND CYTOCENTRIFUGE PREPARATIONS

Blood smears are preserved by air-drying. The subsequent fixation method used depends upon the staining technique. For routine Romanowsky-type stains, the fixative of choice is a high-grade methanol for 1–3 minutes.

In order to produce the desired morphology with routine blood dyes, it is important that the smear be dry, as is evidenced by the morphology of leucocytes in the thick end of a routine blood film. Fortunately, routine air-drying for 1–2 hours does not have a deleterious effect on most antigens studied immunocytochemically. In fact, it has been shown that many leucocyte surface markers can be preserved for over a week following routine air-drying by storage at room temperature.[1] Thus, it is possible to obtain preparations of these markers of a quality similar to those used for routine morphological evaluation. Using an appropriate chromogen such as diaminobenzidine, it is even possible to counterstain with routine Romanowsky-type methods.

One advantage of air-drying smears is that the cells are more firmly attached to the slide than they are following wet-fixation. This is very important if the slide is to survive the rigors of an immunocytochemical technique. With regard to the choice of fixative, however, methanol is not optimal for all antigens; it is often necessary to fix tissue with anhydrous acetone for 1–3 minutes, especially in the case of the leucocyte surface antigens. Notwithstanding this comment, alternative fixatives are used successfully for these and other antigens. For example, formalin-based fixatives are suitable for use on cytoplasmic antigens and membrane- bound immunoglobulins, while formal-acetone mixtures are employed with certain lymphocytic markers.[2]

Finally, it is noteworthy that air-dried preparations often exhibit relatively weak immunostaining. This is probably because the dried cells exhibit an overall lower antigen density. This can be compensated for by extending antibody and/or chromogen incubation times or by using more sensitive, multiple-step immunocytochemical techniques.

CYTOLOGY SMEARS

■ Unlike hematologists, most cytologists prefer smears to be wet-fixed immediately after preparation. This preserves the fine structure of the chromatin and helps in the evaluation of nuclear changes. Thus, most cytology smears are immediately fixed in 95% ethanol or are spray-fixed with a carbowax containing alcoholic fluid. Ethanol is satisfactory for preserving many antigens, particularly those used to differentiate melanoma from carcinoma. However, ethanol fixation precludes staining for most leucocyte markers, such as T and B cell antigens. We suggest, therefore, that two preparations be made, one wet-fixed and one air-dried. With wet-fixed smears, one of the main problems is the loss of cells, particularly clumps, during the immunostaining incubations. It is essential to cut incubation times as short as possible by using higher antibody concentrations or by using elevated temperatures.

CRYOSTAT SECTIONS

■ For immunocytochemistry, cryostat sections give much better antigen preservation than paraffin sections. Additionally, fixative can be used with cryostat sections, allowing the immunochemist to select a different and optimal fixative for each antigen, all from the same block. However, the morphological detail and resolution of the frozen sections is usually considerably inferior to tissue that has been embedded during specimen processing.

Many antigens, such as leucocyte surface markers, survive neither paraffin processing nor fixation with additive fixatives. Accordingly, use either alcohol or acetone. For leucocyte surface antigens, acetone is preferred by most laboratories. Unfortunately, the preservation engendered by acetone is not complete; frozen sections subjected to extended immunochemical procedures often show deleterious morphological changes, including chromatolysis and apparent loss of membranes. Numerous attempts to improve acetone fixation have included the addition of chloroform or dessication, neither of which has proven to be entirely satisfactory.[3] However, thin sections and extended drying prevent the artifacts often seen in frozen-section immunostains of lymphoid tissues fixed in acetone. Extending the drying period to 48 hours will usually result in improved morphology. If it is necessary to stain sections the same day they are cut, sections may be fixed in cold acetone or freshly cut sections may be placed under an infra-red lamp so that the section is heated to 70°C after a 5 second exposure. Slides are then removed, allowed to cool, and the sections are immunostained. This procedure must be carefully controlled to avoid overheating the sections.

Since there are almost as many different procedures as there are laboratories, it is up to the individual technologist and pathologist to determine what sequence of fixation and drying steps will produce the best results for them with the antigens that they are attempting to detect. For further information, see the Tissue Processing chapter for a working procedure.

PARAFFIN-EMBEDDED SECTIONS

■ By far the largest proportion of samples used for immunostaining is embedded in paraffin, and a number of fixatives have been formulated with this in mind. The most commonly used fixatives are discussed here. There is a plethora of specialty fixatives which will not be covered, but which may appear in literature references given in the bibliography.

■ **FORMALDEHYDE-BASED FIXATIVES** The most popular fixatives contain formalin (40% w/v formaldehyde in water), usually a neutral salt to maintain tonicity and often a buffering system to maintain pH. These fixatives are well tolerated by tissues and have good penetration. This is important since specimens are often large, and fixation may be extended beyond optimal times in routine situations. There may be shrinkage or distortion during fixation or subsequent paraffin-embedding, but generallyformalin-based fixatives are excellent for most immunostains.

Formaldehyde fixes not by coagulation, but by reacting primarily with basic amino acids to form cross-linking “methylene bridges.” This means that there is relatively low permeability to macromolecules and that the structures of intracytoplasmic proteins are not significantly altered. Although many people dislike formalin fixatives, their opinion is often based on studies using suboptimally formalin-fixed tissue. Small (10x10x3 mm) tissue pieces fixed promptly in neutral buffered formalin for 6–24 hours will generally show good cytological preservation and immunolocalization, with a minimum of antigen masking. It is the great variation in time and conditions for fixation that cause the majority of problems in immunochemistry.

Although some antigens are not well demonstrated after fixation in formaldehyde-based fixatives, many can be demonstrated after the use of appropriate pretreatment methods, such as proteolytic enzyme digestion and/or antigen retrieval, particularly if polyclonal antisera are used. If monoclonal antibodies are to be utilized on formalin-fixed, paraffin-embedded tissue sections, there are three considerations, which should be kept in mind:

- Does formaldehyde react with the epitope under investigation?
- Does it react with adjacent amino acids causing conformational changes?
- Does paraffin processing destroy the epitope under investigation?

Monoclonal antibodies are selected for their ability to bind specifically to an epitope on the immunogen. This selection is usually based on immunoenzyme techniques or radioimmunoassays employing the native antigen. If formaldehyde reacts with amino acids within the epitope, the antibody will be unable to bind and therefore will be of no use in formaldehyde-fixed tissue. The same problem may arise with other fixatives and may affect different antibodies.

If there are conformational changes resulting from the reaction of formaldehyde with amino acids adjacent to the epitope, these can often be reversed using proteolytic enzyme digestion or antigen retrieval. If there are conformational changes in the epitope due to tissue processing, these are irreversible. Thus, it is clear that fixatives forming additive compounds may block immunoreactivity, but with the appropriate selection of monoclonal antibodies and the appropriate postfixation treatment, formaldehyde is suitable for monoclonal antibodies against many antigens.

Conformational changes, which destroy epitopes, or alter them to reduce reactivity with the antibody, can occur in a number of ways. The most common alterations occur chemically by fixation, or physically by heat during paraffin-embedding. Many epitopes are sensitive to heat, and during the paraffin-embedding step, tissues are heated to the melting point of wax, usually between 50–60°C. Studies have shown that epitopes of vimentin reacting with some monoclonal antibodies have a half-life of 10–15 minutes at 60°C. Thus, overheating of tissues during embedding or overheating of sections during drying can induce detrimental effects on immunostaining. It is essential not to overheat at any stage of processing if immunostaining is to be optimally sensitive.

When discussing formaldehyde or formalin-based fixatives and comparing the immunocytochemical results obtained in two laboratories, the results often differ. This is hardly surprising since there are many formulas for these fixatives, and even particular batches of formaldehyde may contain different amounts of formic acid and methanol. All of these factors can affect the results of staining fixed tissues. Therefore, although results obtained in different laboratories may be similar, they cannot be expected to be identical.

Given all of these caveats, the following is one standard method of fixation using a neutral buffered formalin solution:

NEUTRAL BUFFERED FORMALIN SOLUTION (NBF)

Formalin (40% w/v formaldehyde)	100 mL
Sodium phosphate, monobasic, monohydrate	4 g
Sodium phosphate, dibasic, anhydrous	6.5 g
Distilled water	to 1 liter

This solution is stable for many months at room temperature. Fix small blocks of tissue (10x10x3 mm) for up to 24 hours. Large pieces may be immersed in NBF for a few hours, but blocks should be processed as soon as possible.

Another common fixative is Bouin's solution, which is a mixture of formalin and picric acid. This fixative penetrates rapidly and fixes all tissues very well, except kidney. Fixation time is 1–12 hours depending on tissue thickness. Tissues fixed for longer than 12–24 hours become very brittle. Lipids are reduced in quantity and altered, so lipid-containing antigens may be affected. Tissues fixed in Bouin's solution must be washed in 70% ethanol to precipitate soluble picrates prior to aqueous washes. After cutting sections, the yellow color in the tissue can be removed by treatment with 5% (w/v) sodium thiosulfate, followed by a water wash.

BOUIN'S SOLUTION

Saturated (1.2% w/v) aqueous picric acid	75 mL
Formalin (40% w/v formaldehyde)	25 mL
Glacial acetic acid	5 mL

Saturated picric acid is available commercially so handling of highly explosive picric acid crystals can be avoided. A saturated solution (w/v) contains 1.17 g/100 mL distilled water.

MERCURIC CHLORIDE-BASED FIXATIVES In an effort to improve cytological preservation and minimize distortion associated with formaldehyde-based fixatives, as well as to improve on the tinctorial qualities of the stained paraffin sections, mercuric chloride-based fixatives such as formal sublimate and B5 have gained some popularity in histopathology. Like the formalin-based fluids discussed above, these often include a neutral salt to maintain tonicity and may be mixed with other primary fixatives in an attempt to provide a balanced solution. These fluids are generally poor penetrators and are not well tolerated by the tissues. Consequently, small blocks should be employed and the fixation period should be short. Frequently, mercuric chloride-based fixatives are used secondarily to formalin. Tissues are initially fixed in formal saline or neutral buffered formalin, blocks are taken and these are immersed in the mercuric chloride-containing fluid for a further period of fixation.

Mercuric chloride-containing fixatives are additive and coagulative. It is their coagulative properties that cause considerable hardening of the tissue, hence their use originally as "hardening agents." Before the advent of embedding media, it was necessary to immerse tissues in a fluid, which in addition to preventing autolysis and putrefaction, hardened the tissue sufficiently to allow thin slices to be made. These types of fixatives are particularly suitable for the demonstration of intracytoplasmic antigens. Because the permeability of the tissue is greater following coagulant fixatives, antibody penetration is better, resulting in a more intense immunostain. It must be remembered, however, that these fluids are also additive. Therefore, as with formaldehyde-based fixatives, loss of immunoreactivity will occur through blockage of specific epitopes and this

will be particularly evident with monoclonal antibodies. The other major advantage of these coagulant, mercuric chloride-based fixatives, is that the cytological detail is well preserved, improving the localization of the end product of the immunochemical technique, as well as allowing easier morphological interpretation.

B5 is widely advocated for the fixation of lymph node biopsies, both to improve the cytological detail and to enhance the immunoreactivity with anti-immunoglobulin antisera used in phenotyping B cell neoplasms. The immunohistochemical results from such B5-fixed, paraffin-embedded tissue sections are excellent, if only cytoplasmic immunoglobulin staining is required. However, surface membrane immunoglobulin is not stained as readily. Since B5 contains a low percentage of formalin, it may be the formalin that reacts with the surface immunoglobulin. Limited proteolytic enzyme digestion or antigen retrieval will compensate for this, allowing surface immunoglobulin to be demonstrated clearly. However, as a general rule, enzyme pretreatments do not improve, and may actually hinder, immunostaining of tissues fixed in coagulative fixatives.

B5 STOCK SOLUTION

Mercuric chloride	2 g
Sodium acetate	2.5 g
Distilled water	200 mL

B5 WORKING SOLUTION

B5 Stock solution	20 mL
Formalin (40% w/v formaldehyde)	2 mL

Another of the more popular mercuric chloride fixatives is Zenker's solution. Zenker's is subject to the same advantages and disadvantages as B5. Morphological detail is generally well preserved, but penetration is very poor if blocks are more than 3–4 mm thick. For Zenker's, blocks and sections must be cleared of mercury deposits before immunostaining. Fixation time is 2–15 hours at room temperature, depending on tissue thickness. Sections are washed in running water for at least 1 hour to remove potassium dichromate deposits. The tissue is then dehydrated using clearing agent A as the first bath, which helps to remove mercuric deposits prior to sectioning. After cutting sections and collecting on clean glass slides, the tissue is again incubated in clearing solution A for 1–2 minutes. The sections are rinsed in water, and placed in clearing solution for 1–2 minutes.

ZENKER'S STOCK SOLUTION

Mercuric chloride	50 g
Potassium dichromate	25 g
Sodium sulfate	10 g
Distilled water	to 1 liter

ZENKER'S WORKING SOLUTION

Zenker's stock solution	100 mL
Glacial acetic acid	5 mL

CLEARING SOLUTION A

Iodine	0.5 g
70% ethanol	100 mL

CLEARING SOLUTION B

Sodium thiosulfate 5% (w/v) in distilled water

■ **ACETIC ACID-ZINC CHLORIDE** This fixative is becoming increasingly popular, partly because it preserves membrane proteins.

Zinc chloride	500 g
Formalin (40% w/v formaldehyde)	3 L
Glacial acetic acid	19 mL
Distilled water	20 L

■ **PERIODATE-LYSINE-PARAFORMALDEHYDE (PLP)** Numerous compound fixatives have been prescribed for immunochemistry. As with routine histological techniques, these fluids must preserve the microanatomical relationship of cells and cytological details. Periodate-lysine-paraformaldehyde (PLP), first described by McLean and Nakane[4] is thought to be particularly useful since the periodate oxidizes sugars to produce aldehydes which are cross-linked with lysine. The paraformaldehyde stabilizes proteins. A recent modification to this solution involves the addition of potassium dichromate (PLDP).[5] This chemical is added to preserve lipids and the fixative should therefore preserve all protein, carbohydrate and lipid antigenic determinants. It must be noted, however, that since additive compounds are formed, immunoreactivity may be blocked. Furthermore, cytological detail is not as good as with other fluids.

PLP WORKING SOLUTION

3% (w/v) Paraformaldehyde	50 mL
M Disodium hydrogen orthophosphate	100 mL
Lysine	0.9 g
Sodium periodate	0.15 g

Adjust to pH 7.4.

■ **ETHANOL** Ethanol is not widely employed as a fixative for routine histological techniques due to its poor penetrating ability. However, small pieces of tissue are fixed rapidly and show good cytological preservation. Since alcohols fix through coagulation, without the formation of additive compounds, they are in some ways ideal fixatives for immunocytochemistry. Sainte-Marie first described the use of alcohol fixation and paraffin processing for immunocytochemistry.[6] Alcohol fixation has been widely employed since that time, particularly in research laboratories where the size of specimens and handling requirements are different from the routine histopathology situation.

Since alcohols are coagulant fluids and do not form additive compounds, they permit good antibody penetration and do not block immunoreactive determinants. However, conformational changes can occur. Alcohols precipitate carbohydrates and are therefore particularly useful for surface membrane antigens, which often display carbohydrate-containing epitopes. In this context, they are generally applied to frozen sections or smears. Proteolytic digestion or antigen retrieval is of no use following alcohol fixation and results in destruction of the tissue section or smear.

■ **ACETONE** Acetone is an excellent preservative of immunoreactive sites, leaving most sites intact, but it is a very poor penetrator. For this reason, it is used only for smears and cryostat sections. Fixation is not complete, and extended incubation in buffers may result in chromatolysis and loss of membranes. Refer to sections on blood smears and cryostat sections for procedures.

FIXATION FOR IMMUNOELECTRONMICROSCOPY

■ The dilemma of trying to obtain good morphological preservation while maintaining immunoreactivity is even more apparent in ultrastructural immunochemical studies. For routine electronmicroscopy, it is usual to employ glutaraldehyde primary fixation followed by postfixation in osmium tetroxide. This combination produces excellent ultrastructural detail with good preservation of membranes. With immunochemistry, however, the combination of glutaraldehyde and osmium tetroxide is not generally useful. In some instances, it is possible to pretreat ultrathin sections with hydrogen peroxide or sodium metaperiodate to counteract the deleterious effects of osmium. Unfortunately, this is not possible in all cases. Furthermore, the glutaraldehyde primary fixation is not suitable for many antigens, at least when employing the concentrations of glutaraldehyde used for routine electronmicroscopy. Consequently, for immunoelectron microscopy studies, paraformaldehyde is often employed either alone or in mixtures containing very low (0.1–0.2%) concentrations of glutaraldehyde. Either perfuse the animal with fixative after flushing with saline, or immerse small (1x1x1 mm) pieces for 2 hours at room temperature. Do not postfix in osmium. Process routinely to Epon, Lowicryl, LR white or LR gold.

PARAFORMALDEHYDE-GLUTARALDEHYDE WORKING SOLUTION

0.2 M Phosphate buffer, pH 7.4	50 mL
8% (w/v) Paraformaldehyde	25 mL
25% (w/v) Glutaraldehyde	0.8 mL
Distilled water	24.2 mL

For a more detailed discussion on fixation in electron immunocytochemistry, refer to the texts listed in the Bibliography.

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ANTIGEN RETRIEVAL

MARC KEY

Because of the superior preservation of morphology, formalin-fixed paraffin-embedded (FFPE) tissue remains today the medium of choice for most clinical and research studies. However, the loss of immunoreactivity by many antigens caused as a result of fixation in formalin has introduced many challenges. To more fully appreciate the chemical complexity of the fixation of tissue in formalin, the reader is encouraged to consult with two reviews on this topic.[1,2]

The lack of consistency in the use of formalin for fixation between laboratories, especially as it pertains to the variables of concentration, pH and exposure time, has contributed to this complexity as these factors markedly influence the outcome of staining in immunohistochemistry (IHC). Furthermore, the physiological and pathological changes in tissue composition, including the juxtaposition of tissue proteins and their antigenic sites (epitopes), do not allow predicting the outcome of fixation with any degree of certainty. Each antigen may contain from one to many epitopes and each of these may be composed of five or more amino acids. The same may be linked continuously and/or are arranged in three-dimensional proximity as a result of intermolecular folding. Although formalin fixation will allow some epitopes to emerge unchanged (formalin-resistant), others will undergo substantial changes (formalin-sensitive). In this process, cross-linking of unrelated proteins to the target antigen is also possible. The ultimate results are the partial or complete loss of immunoreactivity by the antigen and/or the "masking" of the same.

The first attempt to "improve" the immunoreactivity of formalin-fixed tissue antigens was by use of tryptic digestion prior to immunofluorescent staining.[3] Proteolytic digestion compensates for the impermeable nature of the non-coagulant fixatives by "etching" the tissue and allowing hidden determinants to be exposed. Since then, other proteolytic enzymes including bromelain, chymotrypsin, ficin, pepsin, pronase and various other proteases have been reported for restoring immunoreactivity to tissue antigens with different degrees of success. Use of enzymes may however also entail the risk of destroying some epitopes. Formalin-fixation in conjunction with the digestion procedures need to be optimized and then firmly adhered to.[4]

An entirely new approach for the restoration of immunoreactivity in FFPE tissue sections was reported by Shi et al 1991.[5] This technology used solutions containing various metals and microwave heating for this restoration and the term "antigen retrieval" (AR) was applied for the first time.*

The concept of recovering lost immunoreactivity through exposure to heat near the boiling point of water was at first met with skepticism as it went against the tenet of protecting proteins from the denaturing effect of heat. However, another major step forward in the use of heat was reported by Cattoretti et al[6] who employed a citrate buffer of pH 6.0 instead of the original metal solution for the first successful demonstration in FFPE tissue of the proliferation marker Ki-67. Shortly thereafter, Gown[7] and Leong[8] were able to apply their modifications of AR methods to a wide variety of additional markers. Not only was the staining of many tissue markers improved, but more importantly, a whole new class of antigens, previously found to be non-reactive in FFPE tissue, could be demonstrated successfully for the first time. This included additional proliferation markers, hormone receptors (ER and PR), growth factor receptors (HER2/*neu*), CD markers and others.

More recently, combinations of enzymatic digestion and heat-induced antigen retrieval have been reported. Iczkowski et al[9] combined steam heat with protease digestion, an EDTA buffer of pH 8.0 and obtained staining with monoclonal anti-keratin antibody 34ßE12. This staining was found to be superior to that obtained when only one of these measures was applied. Detailed information can be found in the Product Specification Sheets for IVD approved antibodies.

PRINCIPLE AND TECHNIQUE

The principle of antigen retrieval relies on the application of heat for varying lengths of time to FFPE tissue sections in an aqueous medium. After deparaffinizing and rehydrating the tissue sections, the slides are immersed in an aqueous solution commonly referred to as a "retrieval solution". Although many different chemicals have been proposed, most retrieval solutions share a pH near 2, 6, 8 or 10. Recent systematic comparisons of several retrieval solutions showed that 0.01M TRIS-HCl, pH 1 or 10, was slightly superior to citrate buffer of pH 6.0 and gave the best overall results.[10]

*Alternate terminology used today for "antigen retrieval" includes epitope retrieval, heat-induced epitope retrieval (HIER), target retrieval and target unmasking. The latter two versions have a more generic appeal and have also been applied to the retrieval of nucleic acid targets for in situ hybridization.

Following their immersion in the preheated retrieval solution, the containers holding the slides are exposed to heat. This step is the most critical and the degree to which immunoreactivity can be restored is directly related to the duration of incubation and the attained temperature. The most commonly used heating methods include the use of microwave ovens, autoclaves, steamers, pressure cookers and water baths.[7,8,11-14] Their advantages and disadvantages however are subject to ongoing experimentations whose preliminary results have been summarized by Battifora et al.[15] Although the optimal temperature has not been established, most AR methods apply temperatures near the boiling point of water. The optimal length of exposure to heat may vary from 10 to 60 minutes and depends to some extent on the length of formalin fixation. Twenty minutes appears to be the most satisfactory for most antigens and fixation protocols. Cooling is usually allowed to take place slowly, requiring another 20–30 minutes.

A *Protocol for Antigen Retrieval* (38031) is available upon request. The same has been used with consistently good results at DAKO Corporation for considerable time.

At higher elevations (above 4500 feet or 1200 meters), boiling of the target retrieval solution may occur prior to achieving the desired optimal temperature. In such situations, a recommended alternative procedure is to heat the slides at the maximum achievable temperature and to extend the incubation time of the slides in the target retrieval solution until the desired staining intensity is achieved.[16] An additional possible solution is to use a closed pressure system such as a pressure cooker or autoclave to achieve 95°C. However, each laboratory must determine the best method and target retrieval time for its particular circumstances.

MECHANISM OF ACTION

■ Despite numerous speculative publications regarding the exact mechanism of action by AR procedures, the same remains largely unknown today. In view of the complexity of many different antigens and the largely unknown changes formalin fixation entails, this is not surprising. Heat is obviously of great importance in reversing the damages caused by the fixation with formalin and embedding in paraffin. Whatever the mechanism, some of the cross-linking induced by formalin must remain intact as without the same, proteins would be denatured by the heat used during AR. This seemingly contradictory observation can only be explained by the fact that some cross-links are reversible (Schiff bases), thus restoring the immunochemical integrity of the protein, while others are not (methylene bridges).

Although much remains to be learned, our primary concern is that AR works. Future studies will almost certainly provide insight and help us to understand what we can presently only accept.

CYTOLOGY

■ Methods of AR have also been used successfully for some cytology specimens. It has been shown that by certain modifications, AR procedures can be successfully used for the recovery of estrogen receptor, Ki-67, LCA, HER2/*neu* and cytokeratin. In contrast to FFPE material, the success of this method is not so much related to the mode of fixation as it is readily applicable to aldehyde- and alcohol-based fixatives as well. It was proposed that the immunoreactivity was facilitated by an increase in permeability of cell membranes thus providing access to previously masked cell and nuclear antigens. The modification includes the incorporation into the retrieval solution of a small amount of detergent. It was also necessary to reduce the temperature to 37°C in order to maintain morphology.

TARGET RETRIEVAL FOR IN SITU HYBRIDIZATION

■ Soon after the discovery of AR for immunohistochemistry, investigators applied similar approaches for the recovery of nucleic acid targets in FFPE. Today, many retrieval methods optimized for nucleic acids combine proteolytic digestion with target retrieval. This combined protocol provided better overall results than either method alone.

For greater detail, read the chapter on DNA Probe Technology.

ANTIGEN RETRIEVAL AND ITS USE IN DOUBLESTAINING

■ One of the prerequisites for the successful staining of several antigens in the same tissue section is the removal of all reactants prior to the application of the subsequent primary antibody. This was accomplished by use of an acid elution step, leaving behind only the converted chromogen of the first cycle.

Use of the DAKO EnVision Doublestain System (see Staining Methods chapter) allowed for the staining of two or more tissue antigens separated by the intermittent use of an antigen retrieval reagent instead of the acid elution step. A method for doublestaining based on this procedure is available.

The action of the antigen retrieval reagent is to either physically remove the reactants and/or to alter them sufficiently so they are no longer immunoreactive. This basic method can be extended to accommodate multiple staining within the same tissue specimen provided different chromogens are being employed. The following chromogens (and their colors) were used for simultaneous staining: DAB (brown), Fuchsin (red), Fast Red (red), BCIP/NBT (purple) and nickel-DAB (gray).

CONCLUSION

As immunohistochemical techniques continue to be refined, their application in routine and research pathology is becoming increasingly more useful. Antigen retrieval has made a significant contribution in this endeavor as many markers, previously believed to be lost to the process of FFPE, can now be routinely demonstrated. The benefits are especially obvious with such important diagnostic markers as estrogen and progesterone receptors, Ki-67 and HER2/*neu*. The greater sensitivity in their demonstration gained through AR may however require the reevaluation of the staining results and its clinical interpretation.[10]

As many recent publications have born out, heat-induced AR has been decidedly more successful than the use of proteolytic enzymes and therefore has profoundly affected the practice of immunohistochemistry. However, because of the ongoing proliferation of alternative AR methods, including new and better retrieval solutions for different antigens, some bewilderment exists today among pathologists and histologists. In the future therefore, greater attention will have to be directed to the standardization of fixation in conjunction with, antigen retrieval,[4,10] and very likely optimized for each separate antigen.[17]

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STAINING METHODS

THOMAS BOENISCH

This chapter will briefly review some of the older methods and draw attention to aspects that were not given their deserved dues in the two previous editions of the Handbook. This will be followed by a more detailed description of several newer working procedures, including their advantages and disadvantages. Because of the ever-increasing repertoire of immunohisto(cyto)chemical methodology to choose from, the individual investigator will have to make a careful selection based primarily on the type of specimen to be investigated, the available primary antibody, the degree of sensitivity and the processing time required as well as the cost of the reagents.

Although published more than ten years ago, today the *Special Report* by Nadji and Ganjei[1] on the use of immunochemical staining methods in diagnostic cytology is considered a landmark review on this topic. Among the subjects covered were important technical aspects, the evaluation of the staining results and their specific diagnostic applications. The preparation of cell block specimens including their immunocytochemical staining has been covered also by others.[2]

Another subject not dealt with in our last edition was the influence that decalcification of bone biopsies may have on the immunoreactivity of several antigens. For important contributions on this topic, read the publications by Mullink et al[3] and Mukai et al.[4]

Regarding primary antibodies of the monoclonal type, some attention should be directed to the type of diluent buffer used. Rather than extrapolate from many years of experience in the use of homogeneous polyclonal antibodies,[5] diluents to be used for monoclonal antibodies of widely different characteristics should be selected more carefully.[5-9] For example, 0.02M phosphate buffered saline (PBS) was highly recommended[8] and is still widely used as a diluent for primary antibodies, including those of the monoclonal type. However, two recent systematic studies showed that this diluent buffer decreased the sensitivity of the immune reaction between most of the tissue antigens investigated and their monoclonal antibodies.[7,9] This study also confirmed that addition of 0.15M Na^+ ions to Tris diluent buffers could negatively affect the performance of monoclonal antibodies when compared to Tris buffers without this ion.[7,10]

When alkaline phosphatase serves as an enzyme label in a procedure, avoid using phosphate buffers as they inhibit the activity of the enzyme. Sodium azide, an antibacterial agent present in many commercially prepared buffers, can prevent binding of the peroxidase enzyme to its substrate and inhibit color development. Its use in wash and diluent buffers should be avoided.

DIRECT METHOD

In this oldest technique, an enzyme-labelled primary antibody reacts with the antigen in the tissue (Figure 6). Subsequent use of substrate-chromogen concludes the reaction sequence. Because this method utilized only one antibody, it could be completed quickly, and nonspecific reactions were limited. However, since staining involves only one labelled antibody, little signal amplification was achieved and the method is no longer sufficiently sensitive for today's demands.

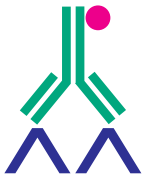

Figure 6: Direct method: Enzyme-labelled *primary* antibody reacts with tissue antigen.

TWO-STEP INDIRECT METHOD

In this method, an unconjugated primary antibody first binds to the antigen. An enzyme-labelled secondary antibody directed against the primary antibody (now the antigen) is then applied (Figure 7), followed by the substrate-chromogen solution. If the primary antibody is made in rabbit or mouse, the secondary antibody must be directed against rabbit or mouse immunoglobulins, respectively.

This method is more versatile than the direct method because a variety of primary antibodies from the same species can be used with the same conjugated secondary antibody. The procedure is also more sensitive than the direct method as several secondary antibodies are likely to react with a number of different epitopes of the primary antibody thus amplifying the signal as more enzyme molecules are attached per each target site.

Figure 7: Two-step indirect method: Enzyme-labelled *secondary* antibody reacts with *primary* antibody bound to tissue antigen.

Undesired reactions may occur if the secondary antibody cross-reacts with immunoglobulins of the tissue specimen. This cross-reactivity however is now routinely eliminated by using preabsorbed secondary antiserum, i.e., antiserum that has been absorbed with immunoglobulins from the species under investigation.

Probably one of the oldest uses of this technique was for the detection of autoimmune antibodies (e.g., antinuclear antibodies) in human serum. In this case, the patient's serum served as the source of the primary antibody and was applied onto tissue specimens containing the nuclear antigen, to be followed by an enzyme-linked secondary antibody to human immunoglobulin. If the patient's serum contained antibodies to this antigen, the enzyme-linked secondary antibody would react with the patient's antibody and thereby indicate a positive result.

THREE-STEP INDIRECT METHOD

■ In the three-step indirect method, a second enzyme-conjugated antibody is added to the previously described indirect technique. The two enzyme-conjugated secondary antibodies are applied sequentially (Figure 8). For example, if the secondary antibody was made in goat, the third antibody must be specific for goat immunoglobulin. Both, the secondary and tertiary antibodies must be conjugated to the same enzyme.

The addition of a third layer of antibody serves to further amplify the signal, since more antibodies are capable of binding to the previously bound secondary reagent. This procedure is particularly helpful when staining antigens with a limited number of epitopes.

The three-step indirect method provides a simple way to further increase the staining intensities compared to the two previous techniques.

Figure 8: Three-step indirect method: Enzyme-labelled *tertiary* antibody reacts with enzyme-labelled *secondary* antibody.

SOLUBLE ENZYME IMMUNE COMPLEX TECHNIQUES

■ These techniques, sometimes also called unlabelled antibody methods, utilize a preformed soluble enzyme-anti-enzyme immune complex. To obtain this soluble complex, excess enzyme is added to its antibody and any precipitate is removed.

The staining sequence of this technique consists of the use of an unconjugated primary antibody, a secondary antibody, the soluble enzyme-anti-enzyme complex and substrate solution. The primary antibody and the antibody of the enzyme immune complex must be made in the same species. The secondary antibody must be directed against immunoglobulins of the species producing both the primary antibody and the enzyme immune complex. The secondary antibody is added in excess so that one of its two Fab sites binds to the primary antibody leaving the other site available for binding the antibody of the enzyme immune complex.

Soluble enzyme-anti-enzyme immune complex techniques were named after the particular enzyme immune complex they used. For example, the PAP method utilized a peroxidase-antiperoxidase complex, APAAP used an alkaline phosphatase-antialkaline phosphatase complex. The PAP complex consists of three molecules of peroxidase and two antibodies and the APAAP complex has two molecules of alkaline phosphatase and one antibody (Figure 9).

Figure 9: The APAAP immune complex reacts with secondary antibody. Primary antibody and the antibody of the immune complex must be made in the same species.

These methods were more sensitive than those described previously. The technique made use of the affinity of antibody for antigen (enzyme) to form a stable immune complex as opposed to the harsher chemical conjugation process. The greater degree of sensitivity compared to the previously described methods was mainly attributed to the greater number of enzyme molecules localized per antigenic site.

For many years, the PAP and APAAP procedures represented the most sensitive and reliable and hence most popular techniques in many pathology laboratories. However, today these techniques are only rarely used.

(STREPT)AVIDIN-BIOTIN TECHNOLOGIES

■ Most of the immunochemical staining methods in use today are based on the high affinity ($K = 10^{-15}M$) that (strept)avidin (*Streptomyces avidinii*) and avidin (chicken egg) have for biotin. Both possess four binding sites for biotin, but due to the molecular orientation of the binding sites, fewer than four molecules of biotin will actually bind. Because avidin is a glycoprotein and has an isoelectric point (pI) of 10, it has a propensity to non-specifically bind to lectin-like and negatively charged tissue components at physiological pH. It has been largely replaced today by streptavidin (also see Background chapter).

The inherent amplification of sensitivity made the avidin- and streptavidin-biotin methods more desirable than the previously described PAP and APAAP methods.The basic sequence of reagent application consists of primary antibody, biotinylated secondary antibody, followed either by the preformed (strept)avidin-biotin-enzyme complex of the avidin-biotin complex (ABC) technique (Figure 10) or by the enzyme-labelled streptavidin (Figure 11). Both conclude with the substrate solution. Horseradish peroxidase and alkaline phosphatase are the most commonly used enzyme labels. While the authors of the ABC method[11] reported this procedure to have a greater sensitivity than the PAP method, Giorno[12] subsequently found the sensitivity of a labelled avidin-biotin (LAB) method to be approximately four- to eight-fold greater than the ABC method. In both methods, the avidin has now been largely replaced by the use of streptavidin leading to the labelled streptavidin-biotin (LSAB) method and a modified ABC procedure, respectively.

Several modifications of these technologies have either increased the sensitivity or contributed to facilitating the staining process. These will be discussed later in this chapter.

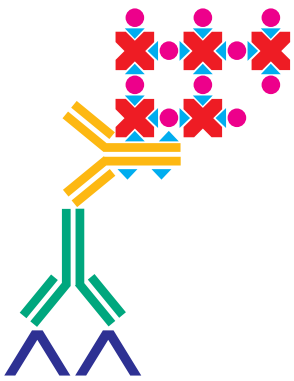

Figure 10: In the ABC technology, the avidin- or streptavidin-biotin-enzyme complex reacts with the biotinylated secondary antibody.

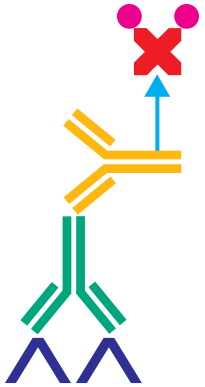

Figure 11: In the LAB or LSAB technology, the enzyme-labelled (strept)avidin reacts with the biotinylated secondary antibody.

■ **GENERAL ABC PROCEDURE** Formation of the ABC complex requires that the solutions of the (strept)avidin and biotinylated enzyme are mixed in an optimal ratio and be prepared at least 30 minutes before use. All incubations are carried out at room temperature.

1. Incubate tissue 30 minutes with normal swine (rabbit) serum.
2. Tap off serum and wipe away excess. DO NOT RINSE.

   Incubate 30 minutes with each of the following 3 reagents; rinse with and place 3–5 minutes in wash buffer after each step.
3. Primary antibody.
4. Biotinylated secondary antibody.
5. (Strept)avidin-biotin complex (prepared at least 30 minutes before use).
6. Incubate with substrate until desired staining intensity has developed.
7. Rinse with distilled water, counterstain and coverslip.

ABC PROCEDURE UTILIZING CATALYZED SIGNAL AMPLIFICATION (CSA)

■ Catalyzed signal amplification (CSA) uses an amplification reagent subsequent to the use of the streptavidin-biotin-peroxidase complex of the ABC protocol. The amplification reagent contains a phenolic substrate (biotinyl-tyramide) that is catalyzed by the bound peroxidase to form insoluble biotinylated phenols.[13-15] The deposited biotins are then reacted with streptavidin-labelled peroxidase, thereby resulting in the deposition of additional enzyme molecules (Figure 12). Sanno et al[16] reported staining for pituitary hormones by CSA with a sensitivity estimated to be 100 fold greater than that of the standard ABC method. In a comparison with the LSAB method, over 40 primary antibodies were found to react positively by the CSA system at dilutions from 20 to

200 times greater.[17] Furthermore, the CSA system was shown to be sensitive enough to detect many antigens of formalin-fixed and paraffin-embedded tissue previously considered unreactive in this medium. In combination with thermally induced antigen retrieval (see Antigen Retrieval chapter), CSA has vastly expanded the horizons of immunohistochemistry.

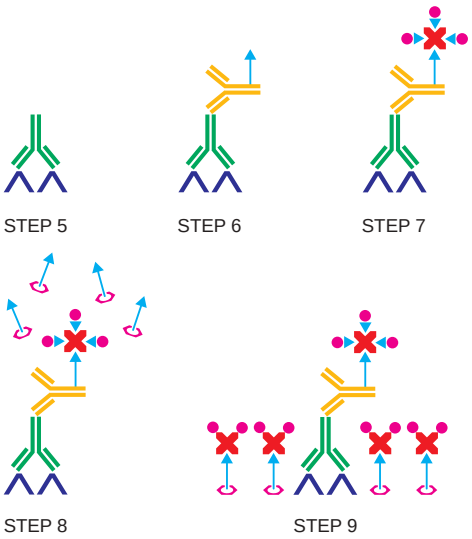

Figure 12: In the CSA technology, the primary antibody (step 5) is followed by a biotinylated secondary antibody (step 6), the streptavidin-biotin complex (step 7), the amplification reagent (step 8), and concludes with the streptavidin-enzyme complex (step 9).

■ GENERAL CSA PROCEDURE FOR USE WITH MONOCLONAL MOUSE PRIMARY ANTIBODIES.

1. Incubate tissue 5 minutes with peroxidase blocking reagent (optional).
2. Rinse with and place 3–5 minutes in buffer.
3. Incubate 5 minutes with a protein-blocking reagent to reduce background.
4. Tap off excess protein block. DO NOT RINSE.

   Incubate 15 minutes with each of the following 5 reagents, repeat Step 2 after each:
5. Primary mouse antibody (or negative control reagent).
6. Biotinylated rabbit anti-mouse link antibody.
7. Streptavidin-biotin complex.
8. Amplification reagent.
9. Streptavidin-peroxidase complex.
10. Incubate 5 minutes with substrate-chromogen solution.
11. Rinse with distilled water.
12. Counterstain with hematoxylin (optional) and coverslip.

LSAB TECHNOLOGIES

■ The LSAB reagents are applied in the sequence of primary rabbit (mouse) antibody, biotinylated anti-rabbit (anti-mouse) immunoglobulins and streptavidin-enzyme conjugate (Figure 13). The color reaction is then developed with the appropriate substrate/chromogen. Although relatively short incubations of 10 minutes are generally recommended for routine applications, a substantial increase in sensitivity can be obtained by incubations with these reagents (especially primary antibody) for 30 minutes.

Except for the use of appropriately diluted enzyme-labelled streptavidin, the same protocol should be followed as for the ABC procedure. It should be noted that the biotinylated antibody and the enzyme-labelled streptavidin can be pre-mixed and applied as a complex, thereby shortening this procedure by one step.

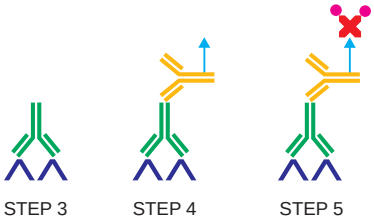

Figure 13: The three steps of the LSAB technology consist of the primary antibody (step 3), biotinylated link antibody (step 4) and enzyme-labelled streptavidin (step 5).

■ GENERAL LSAB PROCEDURE (HRP) FOR USE WITH MONOCLONAL MOUSE PRIMARY ANTIBODY

1. Incubate 5 minutes with peroxidase blocking reagent (optional).
2. Rinse with and place 3–5 minutes in wash buffer.

   Incubate 10 minutes with each of the following 4 reagents; repeat Step 2 after each:
3. Primary antibody (or negative control reagent).
4. Biotinylated link antibody.
5. Streptavidin-HRP.
6. Substrate-chromogen solution.
7. Counterstain with hematoxylin (optional) and coverslip.

CHAIN POLYMER-CONJUGATED TECHNOLOGY

■ This technology (DAKO EPOS™ and DAKO EnVision Systems) is protected by patents both in the USA as well as in Europe and utilizes an enzyme-labelled inert “spine” molecule of dextran (Figure14). In addition to an average of 70 molecules of enzyme, 10 molecules of antibody can be attached to the spine molecule. In the EPOS System (Enhanced Polymer One-step Method) it is the primary

antibody that is conjugated to the enzyme-labelled dextran, thus staining is reduced to one immunochemical step. Conjugation of the secondary antibody instead results in the EnVision System in which staining is performed first by incubation with the primary antibody followed by the polymer. Conjugation of both anti-rabbit and -mouse secondary antibodies renders the system useful for both poly- and monoclonal primary antibodies, respectively. Because these systems avoid the use of (strept)avidin and biotin, nonspecific staining as a result of endogenous biotin is eliminated.

As mentioned, the chief advantage gained by the EPOS and EnVision technologies was the reduced number of incubation steps of the staining protocol normally required. Using the EPOS system, Chilosi et al[18] reported rapid staining completed in a single step and within 10 minutes. On the other hand, prolonging the incubation times with the primary antibody and the labelled polymer of the EnVision system can also result in antibody dilutions several-fold higher than those used in the standard ABC or LSAB protocols.

To what extent, if any, the molecular size of the polymer conjugate may constitute an impediment to unobstructed penetration of tissue sections has not been established.[19]

GENERAL EPOS PROCEDURE (PEROXIDASE)

1. Quench for endogenous peroxidase activity (optional).
2. Rinse with and place 3–5 minutes in wash buffer.
3. Incubate 10–60 minutes with EPOS conjugate.
4. Repeat Step 2.
5. Incubate 5–15 minutes with substrate-chromogen
6. Counterstain (optional) and coverslip.

GENERAL ENVISION PROCEDURE (PEROXIDASE)

1. Incubate 5 minutes with peroxidase blocking reagent (optional).
2. Rinse with and incubate 3–5 minutes in wash buffer.
3. Incubate with primary antibody for 10* minutes.
4. Repeat Step 2.
5. Incubate for 5–10 minutes in polymer solution.
6. Repeat Step 2 twice.
7. Incubate 5–10 minutes in substrate substrate-chromogen.
8. Repeat Step 2.
9. Counterstain and coverslip.

Incubations of 30 minutes are used in the EnVision+ system and help to increase sensitivity.

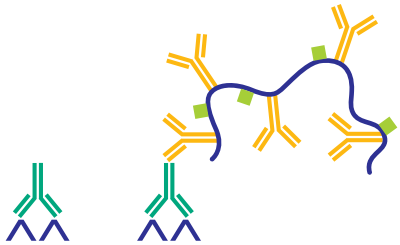

Figure 14: In the two-step EnVision System, the primary antibody (step 3) is followed by a spine molecule (step 5) which contains an average of 10 molecules of secondary antibody and 70 molecules of enzyme. (Peroxidase block and wash steps omitted.)

ENVISION PROCEDURES FOR THE SIMULTANEOUS STAINING OF SEVERAL TISSUE MARKERS

Procedures for the simultaneous staining of two or more tissue antigens have frequently suffered from limitations which rendered their use impractical. The chain-polymer based technology of the EnVision system however has been utilized successfully for this application.[20] Figure15 illustrates the system in which peroxidase- and alkaline phosphatase-labelled polymers are conjugated with secondary antibodies against rabbit or mouse. An elution step prior to the use of additional primary antibodies serves to remove previously bound primary and link antibodies leaving only the deposit of chromogen from the previous steps,[21] thus eliminating any potential for cross-reactivity.

GENERAL DOUBLESTAIN PROCEDURE

1. Quench for endogenous peroxidase activity (optional).
2. Rinse and incubate for 3–5 minutes in wash buffer.

   Incubate for 10* minutes with each reagent in Steps 3, 4, 8 and 9; repeat Step 2 after each:
3. First primary antibody.
4. First link antibody/peroxidase-conjugated polymer.
5. Incubate 5–10 minutes with first substrate-chromogen.
6. Rinse with distilled water.
7. Incubate in doublestain block reagent for 3 minutes.
8. Second primary antibody.
9. Second link antibody/alkaline phosphatase-conjugated polymer.
10. Incubate 5–10 minutes with second substrate-chromogen.
11. Rinse with distilled water.
12. Counterstain (optional) and coverslip.

Incubations of 30 minutes are used in the EnVision+ system and help to increase sensitivity.

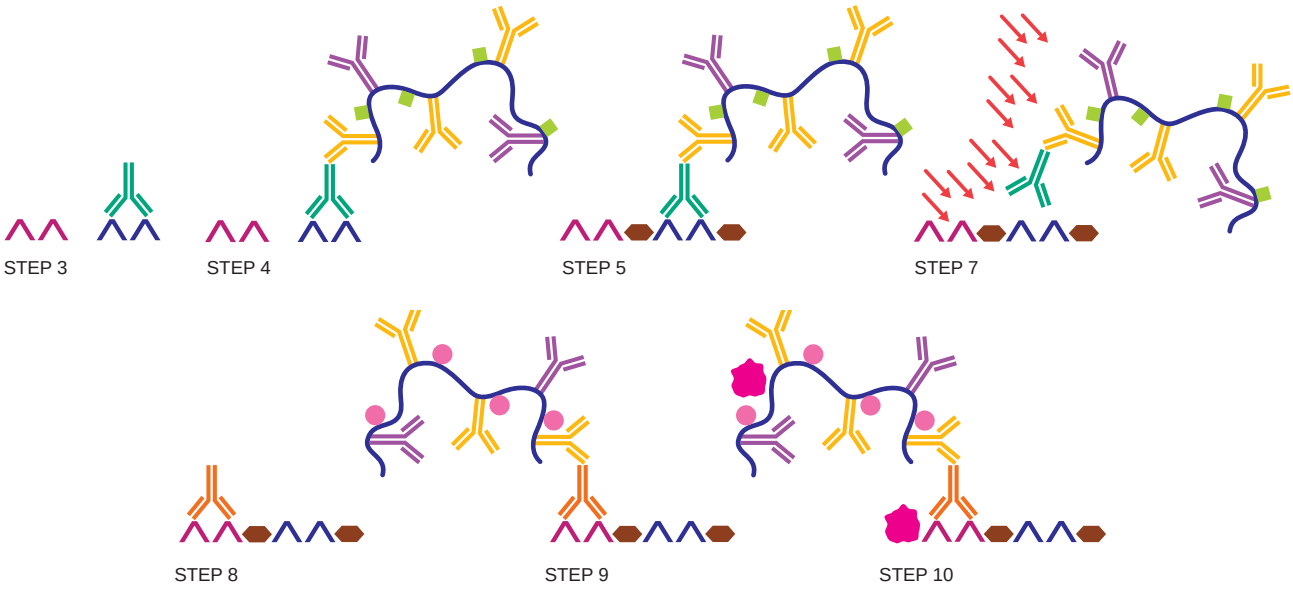

Figure 15: For the simultaneous staining of two or more tissue markers by the EnVision System, the first primary antibody (step 3) is followed by the first spine molecule (step 4), use of a chromogen for the staining of the first antigen (step 5) is followed by a blocking reagent (step 7), application of the second primary antibody (step 8), the second spine molecule (step 9) and is concluded with the use of the second chromogen (step 10). The System allows for the use of a combination of rabbit polyclonal and/or mouse monoclonal primary antibodies, peroxidase and/or alkaline phosphatase, as well as chromogens such as DAB, Enhanced DAB, Fast Red, Fuchsin and BCIP/NBT. (Peroxidase block and wash steps omitted.)

QUICK STAINING METHODS

■ Situations may arise when rapid histopathological evaluation becomes either necessary or desirable, as for example, during surgery. Traditionally, such evaluation was based almost entirely on morphological parameters following routine hematoxylin and eosin staining. The development of high-quality primary antibodies and more sensitive staining techniques, has made it possible to also obtain rapid immunochemical parameters to complement morphology.

Careful selection of methodology (e.g., EPOS), including primary antibody and antibody diluent (pH, ions), have significantly contributed to successful rapid staining with minimal background interference.[7,18,22] However, rapid staining protocols do not lend themselves to the processing of many tissue sections at one time.

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CONTROLS

THOMAS BOENISCH

■ Reagent and tissue controls are necessary for the validation of immunohistochemical staining results. Without their use, interpretation of staining would be haphazard and the results of doubtful value. More specifically, controls determine if the staining protocols were followed correctly, whether day-to-day and worker-to-worker variations have occurred, and that reagents remain in good working order. Furthermore, all procedures designed for in vitro diagnostic use must be monitored by reagent and tissue controls.

REAGENT CONTROLS

■ Because of the subjective nature of the test, the relevant reagents must be controlled within routinely conducted quality assurance programs both by the manufacturer as well as by the user. The prime objective is to ascertain whether the primary and secondary antibodies are specific for their target antigens. Various immunochemical techniques, such as double diffusion, immunoelectrophoresis and rocket electrophoresis may be useful for obtaining this information. It is however imperative to test the primary antibody in immunohistochemistry, first for optimal dilution on positive tissue and then against an expanded panel of additional tissues known to either contain or not contain the antigen. The secondary (link) antibody should be affinity-absorbed in order to render it non-reactive to the tissue proteins. Quality control programs should be documented by proper record keeping on dilutions, diluents, incubation times, and dates on which any procedural changes are introduced.

Of all the components used in an immunochemical staining system, the primary antibody is, without doubt, the most critical, although occasionally other reagents may need to be replaced.

To ascertain the specificity of a primary antibody of the polyclonal type, it is best to replace the same with an affinity-absorbed antiserum or immunoglobulin fraction. Affinity absorption of the primary antibody with highly purified antigen is the ideal means to obtain a valid negative control for differentiating specific from nonspecific staining. The problem is that purified antigen is rarely available in clinical histology laboratories, and that such preparations are very expensive. Practically, therefore, most laboratories use as a control either non-immune serum or its immunoglobulin fraction from the same species as the primary antibody or select a primary antibody of irrelevant specificity.

Because soluble aggregates present in immunoglobulin fractions may contribute to nonspecific staining, these fractions, if used as controls, should be the product of identical isolation methods, be of comparable age and contain nearly identical protein concentrations. They are therefore likely to contain similar amounts of the aggregates. Omission of the primary antibody or use of diluent in its place are ineffective controls. An example for the preparation of a negative reagent control for an IgG fraction of a polyclonal antiserum is illustrated as follows:

PROTEIN CONCENTRATION OF IGG FRACTION: 4.8 g/L, recommended dilution is 1:200, resulting in a final protein concentration of 4.8/200=0.024 g/L.

NON-IMMUNE RABBIT IMMUNOGLOBULIN FRACTION: 20 g/L; required dilution to be determined as follows: 20 g/L ÷ 0.024 g/L = 833

DILUTION OF NEGATIVE REAGENT CONTROL:1:833, or one part non-immune rabbit immunoglobulin fraction in 832 parts buffer.

For primary antibodies of the monoclonal type, use of another irrelevant antibody is probably the best negative reagent control, although non-immune mouse antibodies of the same subclass are now available also for this application. Tissue culture media used for the propagation of monoclonal antibodies have been employed occasionally but are not recommended.

The paramount objective in the selection of a good control in all cases is to imitate all facets of the primary antibody step except for the antigen specificity. Buffers used for the dilution of antibodies and controls must be identical. If these points are not observed, confusion may result from background staining in the positively stained section, but not in the negatively processed control, or vice versa.

■ **TISSUE CONTROLS** can be of either the negative, positive or the internal type.

NEGATIVE TISSUE CONTROLS Specimens serving as negative controls must be processed (fixed, embedded) identically to the unknown, but not contain the relevant tissue marker. An example would be normal liver serving as control for hepatitis B surface antigen-positive liver.

POSITIVE TISSUE CONTROLS Again, these controls must be processed identically to the specimen but contain the target protein. In some cases, it will be advantageous to have this control tissue stain only marginally positive, so

as to monitor not only for the presence of the antigen, but also for any possible *loss of sensitivity.* This loss might not be apparent if only intensely staining controls are used. Controls for loss of sensitivity would be particularly important, for example, when staining tumors. In this case, staining intensity frequently varies with the degree of tumor differentiation.

INTERNAL TISSUE CONTROLS Also known by the name "built-in" control, this control is ideal because the variables of tissue fixation between specimens and controls are eliminated. Built-in controls contain the target antigen not only in the tissue elements under scrutiny, e.g., tumors, but also in adjacent normal tissue elements. One example is the presence of S-100 protein in both melanoma and normal tissue elements, such as peripheral nerves and melanocytes. Built-in controls have the additional advantage that no separate positive control sections are required.

Battifora[1] recommended staining for vimentin as a means for an internal control. Because of its presence in blood vessels and stromal cells, vimentin is distributed ubiquitously and therefore encountered in every tissue sample. Monoclonal antibody V9 recognizes an epitope on vimentin that is partially susceptible to fixation with formaldehyde and thus functions as a "reporter" for the quality of tissue fixation. Other diagnostically useful tissue markers often show fixation-induced alterations that parallel those of vimentin. Through extrapolation to other antigens, it is possible to assess the quality of fixation and to facilitate the selection of fields for diagnostic interpretation.

STATUS QUO

■ As in the clinical laboratory, quality control in immunohistochemistry is of great importance. However, contrary to quantitative immunoassays, e.g., enzymeimmunoassays (EIA or ELISA) where numbers afford definitive quantitative information over standardization and control, the results of immunohistochemical staining, itself an art, must also undergo subjective interpretation by variably experienced pathologists.[2-4]

Quality control and quality assurance in immunohistochemistry therefore will continue to remain one of the most important issues in need of attention, both by the manufacturers as well as by the user in the histopathology laboratory. In 1993, Taylor[2] proposed the development and distribution of tissue reference standards for all pathology laboratories for quality control and quality assurance. This has yet to be realized. The continuing introduction of a multitude of different antigen retrieval procedures promoting the advantages of one retrieval buffer or heating source over another, has made efforts in the standardization and control over a qualitative procedure even more challenging.[5,6] However, as in other branches of the clinical laboratory, automation did contribute significantly to the increase in consistency in, and the control over immunohistochemical staining.

Many publications have appeared during the last ten years on this subject in general and on specific issues and applications of quality control. For more information, read the editorial by Taylor,[4] and the approved guideline for internal quality control.

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BACKGROUND

THOMAS BOENISCH

■ Background staining is probably the most common problem in immunohistochemistry. The following discussion will cover the major causes of background staining and offer possible solutions to this problem.

HYDROPHOBIC INTERACTION

■ In aqueous media, hydrophobic interactions between macromolecules occur when their surface tensions are lower than that of water. The mutual attractions resulting from this are called van der Waals forces. They can be interatomic as well as intermolecular and originate through the fluctuating dipolar structure within these macromolecules.

Hydrophobicity is a property shared, to varying degrees, by most proteins and is imparted to the same primarily through the side chains of the neutral aromatic amino acids phenylalanine, tyrosine and tryptophan. By their lower attraction for water molecules, these amino acids tend to link to one another, thus expelling water from the molecule. While hydrophobicity is one of the natural forces that confer stability on the tertiary structure of peptides, it also imparts stability to formed immune complexes and, depending on environmental factors, can exist also between different protein molecules.

TISSUE PROTEINS In tissue, proteins are rendered more hydrophobic by fixation with aldehyde-containing reagents such as formalin and glutaraldehyde. The increased hydrophobicity is often the result of cross-linking of reactive epsilon- and alpha-amino acids, both within and between adjacent tissue proteins. The extent of this hydrophobic cross-linking of tissue proteins during fixation is primarily a function of time, temperature and pH. Changes in these factors will likely result in variable hydrophobicity due to variable cross-linking of tissue proteins. Therefore, once optimized, fixation procedures must be maintained and controlled. Tissues that commonly have the most background staining as a result of hydrophobic (as well as ionic) interactions are connective tissue (collagen laminin, elastin, proteoglycans and others) (Figure16), squamous epithelium (keratins) (Figure17) and adipocytes (lipoids) if incompletely removed during processing with xylene. Excessive background staining due to overfixation with formalin may be remedied by post-fixation with Bouin's, Zenker's or B5 fixative.[1]

ANTIBODIES Of the major serum proteins, immunoglobulins unfortunately are particularly hydrophobic. In general, antibodies of subclass IgG_3 and IgG_1 are more hydrophobic than those belonging to subclasses IgG_2 and IgG_4. Furthermore, some isolation procedures for IgG class antibodies promote the formation of aggregates, thereby further increasing their hydrophobicity. Storage of immunoglobulins may also increase their hydrophobicity and lead to aggregation and polymerization thereby frequently leading to a diminution in or loss of immune reactivity. The attendant increase in non-specific background staining by use of a polyclonal IgG fraction when compared to that obtained by use of the original whole antiserum has been demonstrated.[2] However, this hydrophobic interaction between the cross-linked proteins of fixed tissue and the antibody fraction (or their aggregates and conjugates) can be minimized by careful characterization of the reagents and by stringent observation of and adherence to optimal fixing conditions. Furthermore, it is imperative to remember that optimal fixation may vary from tissue to tissue.

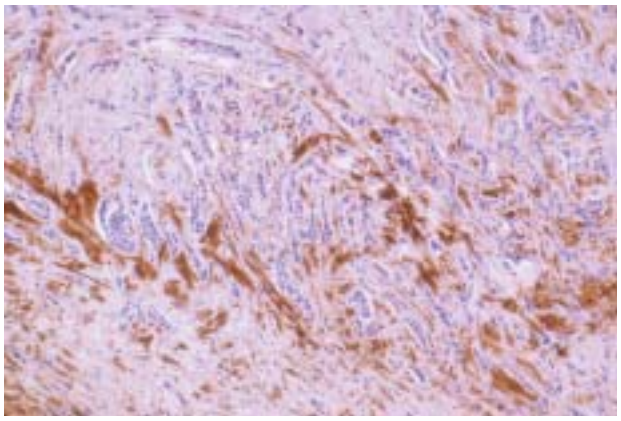

Figure 16: Nonspecific staining of collagen.

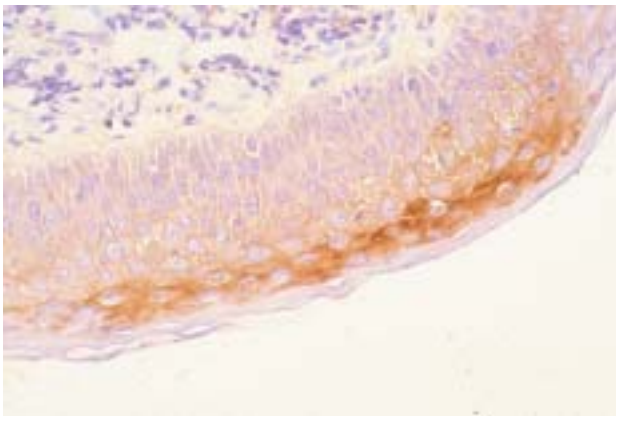

Figure 17: Nonspecific staining of squamous epithelium.

Hydrophobic binding between monoclonal IgG and tissue proteins can also be influenced by the formulation of the diluent buffer. The greater the proximity of diluent pH and the isoelectric point (pI) of the antibody, the stronger the hydrophobic interaction will be. The lower the ionic strength of the diluent, the weaker the strength of hydrophobic attraction. The following anions and cations are arranged in order of their diminishing effect on hydrophobicity:

ANIONS: $PO4^{-3}$, $SO4^{-2}$, Cl^{-}, NO^{3-}, SCN^{-}
CATIONS: NH_4^{+}, K^{+}, Na^{+}, Ca^{2+}

Other possible methods to reduce hydrophobic interactions between tissue and reagent proteins include addition of detergent (e.g., Tween 20) or ethylene glycol to the diluent, or by raising the pH of the diluent used for polyclonal antibodies only.

The most widely practiced measure to reduce background due to hydrophobic interaction is the use of blocking protein either in a separate step, or through its addition to the antibody diluent. However, this will be only successful if the blocking protein is of the type that can compete effectively with IgG (or its aggregates or conjugates) for hydrophobic binding sites in the tissue. Separate incubation with a solution containing blocking protein is best carried out immediately prior to the application of the primary antibody. The solution should contain proteins identical to those present in the secondary link or labelled antibody (but not those in the primary antibody), in order to prevent nonspecific binding of the secondary antibody.

The addition to the primary antibody diluent of 1% bovine serum albumin (BSA) is probably the most widely practiced step for reducing non-specific binding due to hydrophobic interaction. Use of non-fat dry milk[3] or, more recently, of casein[4] for reducing background staining was also recommended. When compared to normal swine and sheep sera, casein, when used as a blocking agent, as an antibody diluent and in the wash buffer, was found to result in significantly less background staining.[4]

Because of the different uses of biotinylated antibodies today, it should be of interest to note that biotinylation can change the pI of the antibody in excess of three units, e.g. from a pI of 8 for the antibody to less than 5 for the conjugate.[5] It was pointed out that this may have a marked effect on the solubility of these conjugates, possibly due to increased hydrophobicity.

IONIC AND ELECTROSTATIC INTERACTIONS

■ Ionic interactions are one of the prime forces that control immunochemical interaction between antigens and their corresponding antibodies. They may however also be one of the factors contributing to non-specific background. The pI of the majority of polyclonal IgG ranges from approximately 5.8 to 8.5. At physiological pH and at the pH commonly used for diluents, antibodies can have either net negative or positive surface charges. Ionic interaction of some antibodies with tissue proteins can be expected if the latter possess opposite net surface charges. Negatively charged sites on endothelia and collagen fibers were reported to interact with cationic conjugates composed of rabbit Fab fragments and horseradish peroxidase type VI (pl 10.0).[6] In general, interactions of the ionic type can be reduced by use of diluent buffers with higher ionic strength. Although addition of NaCl to the diluent buffer can reduce background staining stemming from ionic interactions, its routine use in diluents for monoclonal antibodies is not recommended.[7]

Despite the use of antibodies to cytoplasmic antigens, non-specific nuclear staining was recently reported as a consequence of antigen retrieval by either 1% zinc sulfate, 0.01 M citrate (pH 6.0) or 0.01 M Tris (pH 9.0).[8] In 2.7% of tissues examined, repeated and intense nuclear immunoreactivity could only be eliminated by the use of higher dilutions of the primary antibody. A combination of electrostatic and polar (electron-acceptor/electron-donor) forces were postulated to be the cause for this background. The authors also reviewed five additional reports of both non-specific cytoplasmic as well as nuclear staining as a consequence of antigen retrieval.

Unfortunately, most diffuse background staining is the result of a combination of ionic and hydrophobic interactions, and remedies for one type of interaction may aggravate the other. One example of probable concurrent hydrophobic and ionic interactions is the nonspecific binding of IgG molecules to collagen and elastin. Another example is the adherence of biotinylated antibody complexes to both plastic[5] and glass surfaces.[9] The causes of this binding may be unfavorable conditions during biotinylation resulting in antibody aggregation and reduced solubility,[5] possibly due to increased hydrophobicity (see above). Similar circumstances may contribute to background staining on tissue as well.

ENDOGENOUS ENZYME ACTIVITIES

■ For practical purposes in immunohistochemistry, “endogenous peroxidase activity” and “pseudoperoxidase activity” can be considered the same. Peroxidase activity results in the decomposition of H_20_2 and is a common property of all hemoproteins such as hemoglobin (red cells), myoglobin (muscle cells), cytochrome (granulocytes, monocytes) and catalases (liver and kidney). Interstitial peroxidase activity may be encountered due to the diffusion of blood prior to fixation.

The most commonly used procedure for suppressing endogenous peroxidase activity in formalin-fixed tissue is the incubation of sections in 3% H_20_2 for 5–10 minutes. Methanolic H_20_2 treatment (11 parts 3% H_20_2 plus 4 parts absolute methanol) for 20 minutes is also used, but is not recommended for specimens where cell surface markers are to be stained. Methanolic treatment may also detach frozen sections from their carrier glass. Endogenous peroxidase activity can also be suppressed by a mixture of sodium azide and H_20_2.[10] However, in most work with formalin-fixed tissue, the successful interpretation of specific staining is not impaired by any endogenous peroxidase activity. In cell preparations and frozen sections, routine quenching of endogenous peroxidase is advisable.

Also, specimens rich in endogenous peroxidase activity may be processed by use of an enzyme label of calf intestine alkaline phosphatase instead of peroxidase. Immunoalkaline phosphatase technology does not require suppression of endogenous peroxidase activity. Quenching of endogenous tissue alkaline phosphatase activity other than that of intestine is achieved simply by including 5 mM levamisole in the substrate solution. Gastrointestinal alkaline phosphatase activity can be abolished by treatment of these tissue sections with a weak acid wash.

NATURAL AND CONTAMINATING ANTIBODIES

■ **NATURAL ANTIBODIES** Low-level natural antibodies, present in the antiserum as a result of prior environmental antigenic stimulation, may likely increase in titer during immunization with use of adjuvants and, as a consequence, can give rise to nonspecific staining. In 1979, Osborn et al[11] reported that sera from nonimmunized rabbits and goats, but not from guinea pigs, contained environmental antibodies to keratins. This may be an example of specific epithelial background staining caused by natural antibodies. Although also observed by others, attempts to isolate or remove these antibodies from the antiserum were not successful.[2]

Most natural antibodies are of the nonprecipitating type and occur only in relatively low concentrations. These antibodies are usually rendered non-reactive on tissue if the antiserum is used at a sufficiently high dilution or by shortening the incubation periods.

■ **CONTAMINATING ANTIBODIES** Isolated antigens used for immunization are rarely pure. If the host's immune system reacts to the impurities, contaminating antibodies will result. Usually these contaminating antibodies are present in low concentration and will not detract from the immunohistochemical specificity of high titered antisera provided they are diluted sufficiently. However, if contaminating antibodies do interfere with specificity, affinity absorption of the antiserum is usually performed. "Batch-absorbed" antisera almost always contain residual levels of contaminating antibodies (mostly of the non-precipitating type) and will cause nonspecific staining of tissue if used at excessively high concentration.[2]

Monitoring and evaluating the results of absorption by use of such techniques as immunodiffusion, immunoelectrophoresis and rocket immunoelectrophoresis can only be used to determine non-specificity, but cannot establish the specificity of an antiserum. Ultimate monospecificity must be demonstrated by use of the designated technique and by extensive use of tissues.

The problems stemming from natural and contaminating antibodies, of course, do not occur with monoclonal antibodies.

ENDOGENOUS (STREPT)AVIDIN-BINDING ACTIVITY (EABA)

■ Endogenous avidin-binding activity (EABA) has been observed with all biotin-based techniques and is primarily due to endogenous biotin. Biotin, a vitamin (B7) and coenzyme, is distributed in a wide variety of tissues, especially in the liver (hepatic nodules), kidney (tubular epithelia) and lymphoid tissue (paracortical histiocytes), where it is bound to enzymes and other proteins. Lesser amounts are found in the tissues of the central nervous system and in adipose tissue (breast). EABA usually observed within the cytoplasm and most pronounced when using cryostat sections but has been reported in paraffin-embedded tissues as well.[12]

The biochemical basis for EABA is better understood by remembering the fact that while avidin has four binding sites for biotin, each biotin molecule can bind one avidin molecule thereby adding three more potential biotin-binding sites to the specimen. Practically, EABA therefore is best suppressed by sequential 10–20 minutes incubations of the sections first with 0.01%–0.1% avidin followed by 0.001%–0.01% biotin prior to the staining protocol.[13]

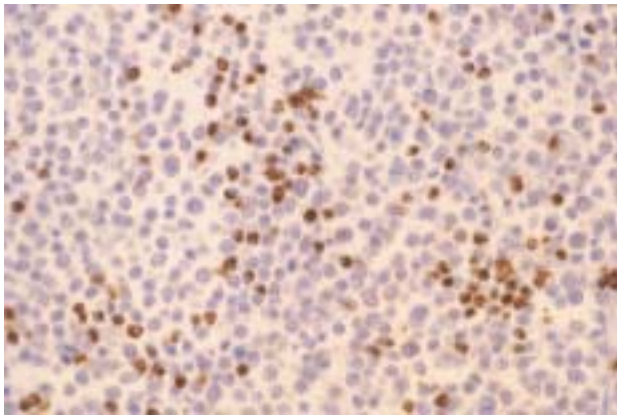

Figure 18: Avidin-binding-complex (ABC) binding to mast cell.

Other reports of EABA include the nonimmunochemical staining of myelin[14] and mast cells (Figure 18) in both frozen as well as paraffin-embedded tissue.[12] Also, Guesdon et al[15] found EABA in granulocytes from mouse spleen.

Because avidin is a glycoprotein containing 10% carbohydrates and has a pI of 10, it has the propensity to non-specifically bind to lectin-like and negatively charged tissue components at physiological pH, respectively. Streptavidin contains no carbohydrates and has a pI of 5, its introduction to IHC therefore has largely eliminated these problems.

ANTIGEN DIFFUSION

■ Specific background staining may occur when the tissue marker to be stained has diffused from its sites of synthesis or storage into the surrounding tissue. A typical example is the diffusion of thyroglobulin from the thyroid follicular epithelium and colloid-containing lumen into the surrounding stromal tissue (Figure 19). Similarly, specific background may result when the tissue marker is also present in high concentrations in blood plasma and has perfused the tissue prior to fixation. This can be seen when tonsil tissue is stained for immunoglobulin heavy and light chains (Figure 20), particularly when fixation was not performed promptly and when the antisera used were not diluted sufficiently. Another form of specific background staining may result from the ingestion of target antigens by phagocytes, resulting in staining normally not seen in such cells.

CROSS-REACTIVITY

■ Background staining due to cross-reactivity of the antibody (monoclonal and polyclonal) may result when one or several epitopes of the target tissue antigen are shared with other proteins. A typical example is the use of an unabsorbed antiserum to carcinoembryonic antigen (CEA). Because CEA shares epitopes with some normal tissue proteins and blood group antigens, non-specific staining may result. Careful absorption of such antisera or, in the case of monoclonal antibodies, careful screening of clones will eliminate this type of background staining.

Nonspecific cross-reactivity of an antibody with similar or dissimilar epitopes on different antigens may also be the cause for confusing background. This, however, is rare and can be avoided by use of antibodies from hyper-immunized animals or carefully selected clones.

For more detail on cross-reactivity see the Antibodies chapter.

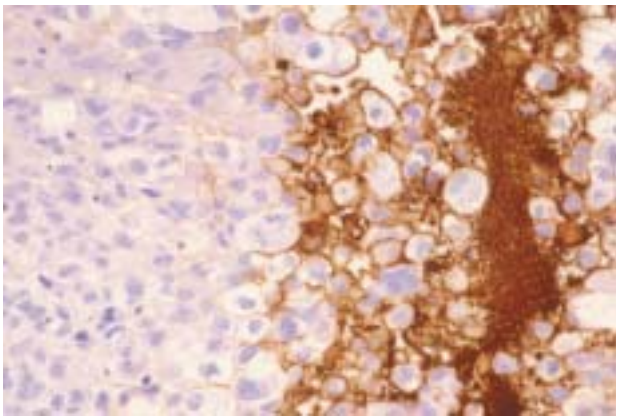

Figure 19: Undesirable staining due to diffusion of antigen (thyroglobulin).

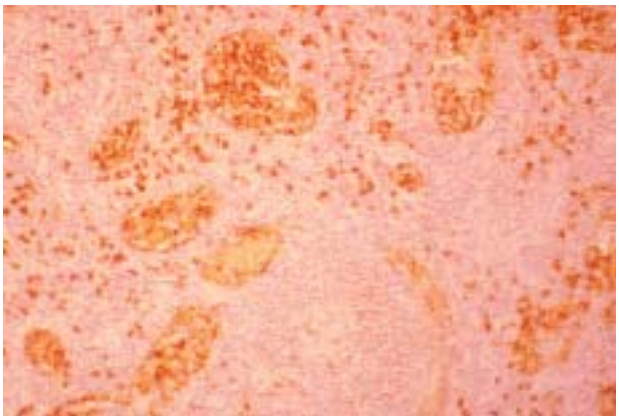

Figure 20: Undesirable staining of plasma protein with antibody to kappa chain. Plasma cells stain specifically.

FC RECEPTORS

■ Fc receptors (FcR) are a family of detergent-soluble membrane glycoproteins with approximate molecular weights of 50–70 kD. They comprise less than 1% of the total membrane proteins and are most frequently present on macrophages and granulocytes, but have also been reported on B cells and some T cells. The intrinsic affinity of the FcR for monomeric IgG is approximately $1x10^6$ to $1x10^8$ M^{-1}, but is higher for polymers and immune complexes of IgG. There is considerable class/subclass and species specificity among different FcR's. For example, the FcR on some human cells was found to bind mouse monoclonal IgG_{2a} and IgG_3 but not other IgG subclasses.[16] Goat sera do not react with FcR's of human leucocytes.[17]

Nonspecific background staining due to FcR is more common in frozen sections and smears than in tissues fixed by harsher procedures. It can be avoided by use of $F(ab')_2$ fragments instead of whole IgG molecules, and by careful screening of monoclonal antibodies.

COMPLEMENT-MEDIATED BINDING

■ Complement-mediated binding may occasionally be a cause of background in frozen tissue when whole antisera are used; however, by the time large pools of antisera have been prepared for use, several of the complement factors are usually inactivated.

MISCELLANEOUS SOURCES

■ Diffuse staining of all or most tissue elements within an affected area may be caused by physical injury to tissue (Figure 21), by tissue drying out prior to fixation or by incomplete penetration of the fixative into the tissue (Figure 22). Similar diffuse background staining of both the section and the glass slide, usually limited to the area of antibody incubate, has been observed and may be due to residual embedding medium. Sections mounted routinely in water baths containing protein additives such as Knox gelatin or Elmer's glue may also show this type of diffuse background when used in immunohistochemistry, especially in procedures of high staining sensitivity. Water baths should be free of bacterial or yeast contamination.

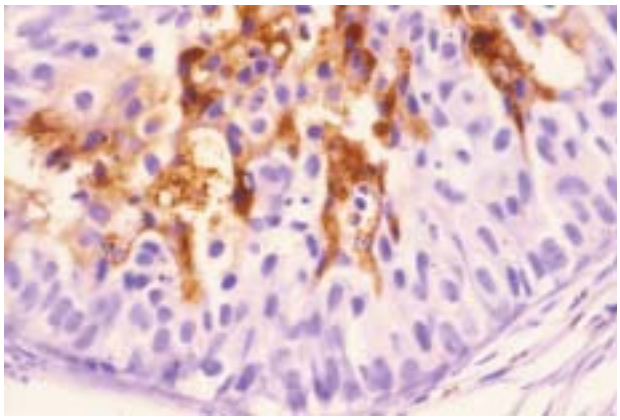

Figure 21: Nonspecific staining of crushed cells.

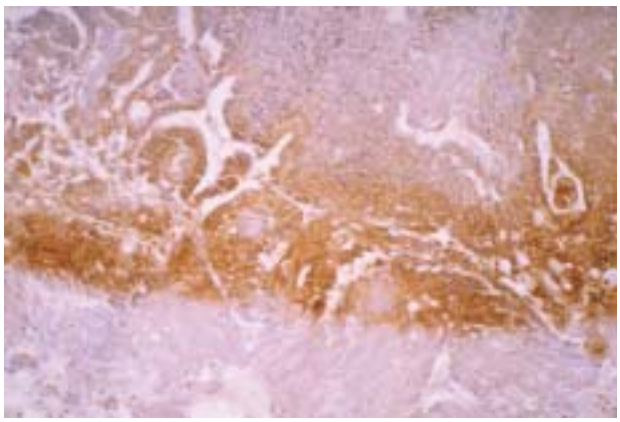

Figure 22: Nonspecific staining due to poor penetration of fixative (lower part).

Nonspecific staining due to undissolved chromogen granules may, on occasion, be encountered.

The nonimmunologic binding of horseradish peroxidase, either in free form or as conjugate, to HbsAg in hepatocytes was reported by Omata et al.[18] The precise nature of this binding was not known.

Necrotic areas due to autolysis of tissue may stain with all reagents. Nadji and Morales[19] provide an excellent collection of color plates illustrating background staining, together with explanations of the artifacts that caused the same.

Excessive counterstaining may compromise the specific staining signal.

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AUTOMATION IN IMMUNOHISTOCHEMISTRY

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■ Automation is often an intrinsic development when manual efforts no longer suffice. Due to high output demands and stringent requirements for reproducibility this tenet held for many branches of the medical sciences including clinical chemistry, hematology and bacteriology. These services are to a large extent automated today. Although immunohistochemistry (IHC) was initially believed by many to be too intricate and therefore exempt from this trend, in the last few years there has been a dramatic reversal of this attitude.

There were several requirements that contributed to the automation of IHC, none of which were significantly different from those that had prevailed prior to the automation of other clinical services, namely:

- Increase output
- Improve reproducibility and quality
- Reduce the cost of labor and material
- Allow better standardization and inter-laboratory comparisons

The types of automated IHC stainers on the market today vary in several aspects, including the technology for the processing of slides, the slide capacity per run (20 to 500) and in the flexibility of reagents (“open” versus “closed” systems).

CAPILLARY GAP METHOD

One of the first technologies employed for the automated staining of slides was the use of the *capillary gap principle.* In this technology, tissue specimens on two microscope slides, or one microscope slide and a cover plate, are placed together to form a gap of defined width between them. Typically, this gap is 50 microns wide and will draw and hold a consistent volume, e.g. 150 microliters, thereby contributing significantly to the reproducibility in staining. During the run, the slides are partially immersed in reagent wells resulting in the filling of the gaps by capillary attraction. After each incubation period, the gaps between the slides are emptied of fluid by touching onto blotting pads. Refilling of the capillary gaps and blotting is repeated for each reagent and wash step. In systems employing a cover plate in place of one slide, reagents are pipetted through a small funnel located on the top of the plate. The capillary gap retains the defined amount of fluid by surface tension while the excess overflows and is automatically discarded. Washing is performed by the flow of buffer through the gaps. Sufficient fluid is retained at all times to prevent any drying of the sections.

One minor deficiency of this approach is that the ease of filling or emptying the capillary gap depends somewhat on the individual surface tension of the fluid. In systems utilizing the cover plate, the formation and trapping of air bubbles can be minimized by the gentle one-way flow of reagents through the gap.

LIQUID COVERSLIP METHOD

Another technology that was utilized in automated staining is known as the *liquid cover slip* method. Here, the reactions are allowed to proceed inside a heat-equilibrated incubation chamber. Oil is used to cover the reagents to prevent evaporation during their incubation. This system requires reagents that are specifically optimized to perform best at the raised temperature.

OPEN SYSTEM

Other instruments used for automation mimic manual staining, i.e., the slides are secured in a horizontal position and reagents are dispensed onto the tissue by use of teflon-coated pipettes or disposable pipette tips. Incubations proceed at room temperature. This technology permits use of the same reagents as used for manual procedures. It is frequently called an *open system* because reagents and protocols from any source can be employed.

The need for automation in anatomic pathology is expanding into other areas such as in situ hybridization and Special Stains. However, these applications require features not commonly found in today's instruments.

Because the acquisition of instrumentation for automation is usually a major capital investment, the individual laboratory should proceed while considering the following:

■ **SLIDE CAPACITY** As different instruments vary in the number of slides they can process, present as well as future needs should be carefully considered.

■ **PERSONNEL** Evaluate the impact of automation on hands-on time.

■ **FLEXIBILITY** Some laboratories require more flexibility in protocols and reagents than others. This must be taken into account in the selection of an open versus closed reagent system.

■ **AVAILABILITY OF BENCH SPACE** Some instruments require more laboratory space than others. Consider if the laboratory may be better served by one large or several smaller instruments.

■ **SELECTION OF VENDOR** The specific needs of the laboratory may determine the selection of vendors. Initial costs of instrument, reagents and software, as well as the quality of maintenance are important factors.

In the future, clinical laboratory services in general, and anatomic pathology in particular, will increasingly rely on automation. Effective initiation of automation in laboratory services has in the past provided better patient care and future advances will certainly continue this trend.

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IN SITU HYBRIDIZATION

RICHARD HARVEY

INTRODUCTION

■ Nucleic acids have been used as probes to assay biological materials for approximately three decades. Today, there are two major categories of nucleic acid probe assays: solid support and solution-based. The solid support assays are further divided into two distinct areas: in situ hybridization (ISH) and synthetic support. For the purposes of this overview, only these solid support assays will be discussed in detail.

The oldest and most widely used nucleic acid assays are in the synthetic support category. Southern and Northern blots, first described in the 1970's, are perhaps the best known.[1,2] The basic principle of all solid support assays is that the nucleic acids are fixed to an artificial surface yet still remain accessible for hybridization. These nucleic acids may behave either as the targets or the probes. Interestingly, some of the most recently developed assays of this type, microarrays, are essentially just variations on the standard blots. Whereas in Southern and Northern blots the nucleic acids are separated by electrophoresis and then transferred to a membrane, microarrays are constructed by synthesizing or spotting the nucleic acids directly to specific areas of the surface.[3] Other common types of solid supports used in nucleic acid assays are microparticles or microspheres.

ISH assays are relatively closely related to the solid support category. The principal distinction is that the nucleic acids in ISH assays are fixed in situ within the cell rather than being extracted first. The significant advantage of an ISH assay is that it permits the localization of the hybridizing material within a cell or tissue. The major disadvantages are that by maintaining the morphology and structure of the cells, one also introduces more potentially inhibitory elements. Historically, most of the tissues used for ISH assays have been fixed in formalin and embedded in paraffin. In order to render the nucleic acids in these tissue sections accessible for hybridization, protease pretreatment is typically performed. Either as an adjunct or alternative to the protease treatment, reports have described the use of a microwave or autoclave step.[4,5]

SAMPLE COMPLEXITY

■ One of the more important factors when selecting a probe and detection system is the complexity of the sample. The greater the complexity of the specimen, the more likely that a non-specific hybridization event will occur. Even when the extent of cross-hybridization is low, if a non-specific target is present in sufficient abundance, a false positive signal is likely to occur.

There are two major steps in an assay at which the issue of complexity can be addressed: specimen processing and probe design. When one performs a Northern or Southern blot, removal of contaminating DNA or RNA, respectively, during the preparation of the sample dramatically reduces the complexity of the specimen. In the case of a Northern blot to identify an mRNA target, further removal of ribosomal RNA will add another 20 to 50 fold enrichment. Similarly, when performing an ISH experiment, the tissue may be pretreated either chemically or enzymatically to remove some of the undesired nucleic acids. Additionally, if one is performing an ISH assay for an RNA target, the potential for cross-hybridization to genomic DNA might be minimized by avoiding strongly denaturing conditions. Usually the secondary structure of RNA can be removed under much milder conditions than those required for denaturing DNA.

Probably the best opportunity to minimize potential cross-hybridization is at the stage of probe selection. In part, good probe selection requires some knowledge of the specimen. With information about the complexity of the specimen, it is easier to design a probe that limits homology to unwanted sequences that are likely to be present. Thanks, in part, to the significant progress made by the Human Genome Project, it is becoming increasingly easier to design better probes for human DNA sequences. The rapidly expanding database and sequence analysis tools that have come from this project have made it much easier to evaluate potential probes.

For RNA ISH assays, however, this is still a formidable problem. Because of the highly variable expression of genes across different tissues, it is unlikely that one can predict either a comprehensive list of potential cross-hybridizing RNAs or their abundance. Although there are now some databases with information on human gene expression in different tissues, this information is still quite limited and sometimes biased. As a first step in developing an ISH assay, it is probably still worthwhile to first perform Northern blots on some specimens. This should aid in determining whether the signal seen in the tissues is coming from the true target or a homolog.

REQUIRED SENSITIVITY

■ Possibly the most frequently overlooked, yet most important aspect of any probe assay is the requisite sensitivity for the task. Abundant targets require fewer hybridizing probe signals to generate a result. While this seems obvious, it is frequently overlooked. As a first step in selecting a probe, it is important to estimate the number of targets that are to be detected. In Southern blotting, for example, a single-copy human gene or sequence is present approximately three million times in a lane with 10 µg of DNA.[6] Although the expression of RNA varies widely across different tissues, many cells have about equal amounts of RNA and DNA. As a rule of thumb, a lane with 10 µg total RNA would contain roughly 1.4 million cells worth of material. Because the number of target molecules is relatively high in some of these assays, smaller probes or those with a lower specific activity may still be useful.[7]

Obviously, for ISH the situation is quite different. For DNA targets in a normal diploid cell, there are only two copies of each sequence per intact cell. Considering that the fixation and specimen processing also render some of this material unsuitable for analysis, this rapidly begins to approach single-copy detection. For this reason, the majority of ISH assays for the detection of low copy number DNA use very large probes (typically >100 kb). In this way, a detectable signal can be generated from even a small number of target molecules.

The prospects for RNA analysis are slightly better. A relatively highly expressed gene might be present in hundreds or thousands of copies per cell. Both the inherent instability of mRNA and specimen processing procedures, however, will negatively impact this figure. Nevertheless, if the message is expressed at more than two copies per cell, this should be no worse than the single-copy DNA analysis described above. Depending upon the spliced nature and abundance of the RNA, the potential for false positive signals resulting from the genomic DNA may also be of concern.

DETECTION METHOD

■ There are a variety of different detection methods available for nucleic acid probes. Essentially, they fall into two categories: direct and indirect. The oldest, and one of the most sensitive direct methods, is radioactivity. The labelled probe is detected either by exposure to film or a comparable imaging approach. Depending upon the isotope used, typical exposure times might range from minutes to months. Another relatively old method of direct detection is fluorescence. The fluorescent labels of the probe are visualized directly using either a fluorometer or fluorescent microscope. Other direct methods include chemiluminescence and gold. The sensitivity of these is usually much lower than either radioactivity or fluorescence.

There is also a wide variety of indirect methods for probe detection. Similar to the immunohistochemistry field, a probe may be labelled with a hapten or a biotin that is used to bring an enzyme to the site of the hybridization. The enzyme then creates the detectable event (e.g., chemiluminescent, colorimetric or fluorescent). In addition, there are also some newer, more extensive signal amplification methods that are useful for low abundance targets.[8,9]

PROBE COMPOSITION

■ **DNA** DNA probes are still the most frequently used type of probes both in the clinical and research laboratories. This is true for a variety of reasons. First, they are relatively easy to make in large quantities, either by synthesis or growth in a vector. Secondly, they are the best characterized. The kinetics and properties of DNA probes are better understood than RNA or PNA probes (see below). Thirdly, the advent of nucleic acid amplification techniques such as PCR has greatly increased their availability. Finally, DNA probes can come in all sizes (from short oligonucleotides to megabase constructs).

■ **RNA** RNA probes are also used quite frequently. Often referred to as “riboprobes”, these are single-stranded materials that are typically synthesized from a vector via an RNA polymerase. Some of their advantages are that they are already single stranded (i.e., need no initial denaturation) and they hybridize slightly better to DNA targets than their DNA counterparts. Their sizes range from short oligonucleotides to several kilobases. Riboprobes larger than a couple of kilobases are uncommon. One of the principal disadvantages to RNA probes is their inherent instability. RNAses are quite abundant in the environment and they are extremely difficult to inactivate.

■ **PNA** Peptide nucleic acid (PNA) probes are the newest members of this category.[10] PNAs can have the same bases as DNA and RNA probes, however they are joined by a backbone of amide linkages (like proteins) instead of the sugars and phosphates of DNA and RNA. The end result of this modified structure is that, while the bases still conform to Watson-Crick base bonding rules, the kinetic properties are much different. PNAs tend to hybridize much more rapidly than their DNA counterparts and they are also quite effective at discriminating single-base mismatches. PNAs are also very useful in hybridizing to regions that are involved in extensive secondary structure. The primary disadvantages of PNA probes are that their properties are not yet as well understood as DNA oligonucleotides and their solubility is much lower than a corresponding DNA. They are typically quite short (usually less than 30 bases) and presently must be made synthetically.

PROBE LENGTH

■ The length of a probe is highly dependent upon the application for which it is intended. Consider both the high and low extremes of probe size. An oligonucleotide of just 16 bases is statistically large enough to be unique in the human genome if the 3.2×10^9 bases of the human genome can be assumed to be comprised of random sequences. While the genome is most certainly not made up of completely random sequences, this is still a useful number for starting probe design. Sequences of less than 16 bases are quite likely to occur multiple times, whereas those larger than 16 bases have a better chance of being unique.

On the opposite end of the spectrum, large probes also have limits. Because of the repetitive elements found throughout the genome, the larger a probe becomes, the more likely that it contains some form of repeat. Additionally, for ISH experiments it is important that the probe be sufficiently small to make it through the cellular scaffolding and reach its target. Although this size limit is debatable, generally an upper limit of approximately 500 bases is considered acceptable for ISH. Probes larger than this are typically fragmented (sonication or enzymatically) down to this size.

TYPES OF LABELS

■ As was mentioned previously, the choice of label depends upon the method for detection. The most commonly used labels are radioactive, fluorescent, chemiluminescent and bioreactive (i.e., biotin, hapten or enzyme). Although there are a few other labels being used for probes, these are the most common. In the case of the first three, if the target is sufficiently abundant, these labels may be detected directly. Otherwise, an indirect or signal amplification technique can be used. Over the past decade there has been a trend towards reduced use of radioactivity. Partly this is due to regulatory and safety restrictions. It is also a result of other detection methods yielding comparable sensitivity.

LABELLING METHODS

■ There are a number of ways in which a label can be attached to a probe. In the case of a radioactive probe, the isotope is usually a modified atom of one of the bases. Typically, the label is introduced during the polymerization of the probe (either synthetically or enzymatically). In some cases, the radioactivity is added subsequently to the synthesis (i.e., kinase). For some of the other types of label, a linker is used to attach the label to the probe (either to, or between, the bases). In these situations, the modified base can be incorporated during the synthesis, or the linker may be introduced during the synthesis and then the label attached afterwards. Another method is to synthetically add a label either to the 5' or 3' end of the nucleic acid. Enzymes might also be attached directly to probes using one of these techniques.

There are also less direct methods for adding labels to probes. Psoralen and platinum are two other ways in which labelled material may be added to a probe following synthesis. Both methods permit the addition of a wide variety of labels. Another advantage to these methods is that the probe size can be carefully adjusted prior to the labelling. Overall, most of these methods permit probes to be selectively labelled at varying degrees of specific activity.

CONCLUSION

■ There are numerous different types of probes and methods for their labelling. The choice of any particular one of these is highly dependent upon the desired application. It is very important to combine the design of the probe with its intended use and method of detection. Regardless of the steps taken towards optimizing probe performance, specimen processing and sample pretreatment remain two of the largest sources of variability in assay performance.

Recent advances in probe design and signal amplification have now made it possible to look at mRNA expression by ISH. Once these methods become more quantitative, it is likely that ISH mRNA assays will start to become routine tests in the clinical laboratory. Until that time, they remain primarily research tools.

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TISSUE PROCESSING

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As shown in the Troubleshooting chapter, proper tissue processing and fixation is mandatory when preparing specimens for immunohistochemical staining. Although proper techniques represent the very basis for accountable IHC staining, they are often overlooked. The following specimen-processing methods presented in this chapter should not be considered all inclusive. Rather, they are offered as possible protocols to be adapted in the IHC laboratory.

SPECIMEN PROCESSING

PREPARATION OF CELL SMEARS

PERIPHERAL BLOOD SMEARS:

PUSH FILMS:

1. Collect fresh blood and prepare slides immediately or collect in an anti-coagualant treated tube and prepare slides within 2 hours.
2. Place a drop of fresh or anti-coagulated whole blood on a clean, grease-free slide, a short distance from one end.
3. With the thumb and forefinger of the right hand, hold the end of a second spreader slide against the center of the first at an angle of approximately 30–45 degrees.
4. Draw the spreader slide back to contact the drop of blood, allowing the sample to spread along its edge. Push the spreader slide forward until all the blood has been spread into a thin film.
5. Air-dry for 2–18 hours.

PULL FILM:

1. Collect fresh blood and prepare slides immediately or collect in an anti-coagualant treated tube and prepare slides within 2 hours.
2. Place a drop of fresh or anti-coagulated whole blood on a clean, grease-free slide, a short distance from one end.
3. Place a second slide over the specimen and allow the blood to spread between the two pieces of glass. Avoid exerting pressure on the two slides.
4. Separate the slides by pulling them apart in a direction parallel to their surfaces.
5. Air-dry for 2–18 hours.[1]

BONE MARROW SMEARS

DIRECT FILMS:

1. Place a drop of fresh, uncoagulated marrow on a glass microscopy slide a short distance from one end. Grey particles of marrow should be visible with the naked eye.
2. With the thumb and forefinger of the right hand, hold the end of a second spreader slide against the center of the first at an angle of approximately 30–45 degrees. The spreader slide should not be wider than 2 centimeters.
3. Draw the spreader slide back to contact the drop of marrow, allowing the sample to spread along its edge. Push the spreader slide forward until the cellular elements have been spread into a 3–5 centimeters long film. Marrow particles are dragged behind the spreader, but care should be taken not to crush the cells. Optimally, a trail of cells is left behind each particle.
4. Air-dry for 2–18 hours.[1]

CRUSH PREPARATIONS:

1. Place a drop of fresh, uncoagulated marrow on a glass microscopy slide a short distance from one end.
2. Place a second slide over the specimen and exert slight pressure until cellular elements are flattened.
3. Separate the slides by pulling them apart in a direction parallel to their surfaces. The appearance of irregular holes in the film is due to fat and gives the assurance that marrow and not just blood has been obtained.
4. Air-dry for 2–18 hours.[1]

CYTOCENTRIFUGED CELLS

1. Collect peripheral blood or bone marrow in an heparin treated tube. Cytocentrifuge preparations of normal human mononuclear cells must be prepared from freshly collected specimens.
2. Separate leukocytes by first diluting with equal parts of Hank's balanced salt solution (GIBCO) and then layering over FicollPaque lymphocyte separation medium (Pharmacia) and centrifuging at 750xg for 15 minutes. Alternatively, Lymphoprep (Nycomed) may be use for cell separation.

3. The mononuclear cell layer is then gently aspirated and resuspended in sufficient medium to give a final cell density of 1x106 cells/mL in TRIS-buffered saline (TBS), containing 1–5% bovine serum albumin.
4. Cytocentrifuge preparations are made from four drops of the cell suspension by centrifuging at 500 rpm (approx. 20xg) for 3–4 minutes.
5. Slides should be removed quickly from the rotor and rapidly air-dried for 2–18 hours.

LEUKOCYTE SMEARS (BUFFY COAT)

1. Mix fresh whole blood with an equal volume of dextran solution, such as 6% Lomodex, and allow the erythrocytes to sediment.
2. Centrifuge and discard the supernatant plasma.
3. Using resuspended cells from the white cell pellet, place a drop of on a glass microscopy slide a short distance from one end.
4. Place a second slide over the specimen and allow the cells to spread between the two pieces of glass. Avoid exerting pressure on the two slides.
5. Separate the slides by pulling them apart in a direction parallel to their surfaces.
6. Air-dry for 2–18 hours.[1]

AFTER DRYING: All slides which are not to be stained immediately should be placed back-to-back, wrapped in aluminum foil and stored at -20 to -70°C for a maximum of 6 months.

BEFORE FIXATION: Allow frozen and refrigerated slides to reach room temperature before unwrapping.

FIXATION:

1. Fix in acetone:methanol* or acetone:methanol*:formalin for 90 seconds at room temperature.
2. Transfer the slide directly to TBS.
3. Leave in TBS for 1–5 minutes.

Do not allow slides to dry out at any stage after fixation.

Acetone: methanol*

Acetone	1 part
Methanol*	1 part

Acetone: methanol*:formalin

Acetone	19 parts
Methanol	19 parts
Formaldehyde (40%)	2 parts

*If the antigen to be stained is sensitive to methanol, ethanol may be used as a substitute reagent.

PREPARATION OF TISSUE SECTIONS

CRYOSTAT FROZEN SECTIONS

1. Trim tissue to approximately 4mm^3.
2. Snap-freeze in liquid nitrogen and store at -70°C. If desired, the tissue may be embedded in a mixture of polyvinyl alcohol/polyethylene glycol or gelatin.
3. Using forceps, slowly immerse the base mold in approximately 2 inches of liquid nitrogen, until it is completely frozen.
4. Frozen blocks are wrapped in aluminum foil and stored at -70°C.
5. Blocks to be sectioned are placed in the -20°C cryostat for at least one hour prior to sectioning.
6. Cut sections 4–10µ thick and thaw mount onto clean glass slides. Use of an adhesive coating or subbing agent, such as poly-L-lysine, charging, or silanization, may improve tissue adherence.
7. Immediately fix in acetone at room temperature for 10 seconds.
8. Air dry 12–24 hours.

Alternate method: To accelerate the drying process, air dry for 30 minutes, fix for 10 minutes in fresh acetone, then air dry again for 30 minutes.

PARAFFIN SECTIONS

1. Fix tissue specimens according to established laboratory protocols. The most commonly used fixatives contain formalin. To determine if these fixatives are compatible with an intended marker, check the specification sheet of the primary antibody for further information. In addition to formalin, several other fixatives are commonly used for tissue preservation. Please refer to the Chapter on Fixation in this Handbook for further information.
2. Trim tissue to approximately 4mm^3 and dehydrate in graded alcohols. Typically, the grading is as follows:
    a. 60%
    b. 65%
    c. 95%
    d. 95%
    e. absolute
3. Clearing of alcohol from the tissue may be performed using xylene or a xylene substitute.
4. Infiltrate and embed tissue in liquid paraffin. The temperature should not exceed ~58°C. Note: Depending on the molecular weight of paraffin, the melting point (MP) will range from 48–66°C. Typically, paraffin used in the histology laboratory has a MP ~55–58°C.

5. Cut sections 4–6µ thick and mount onto clean glass slides. Use of an adhesive coating or subbing agent, such as poly-L-lysine, charging or silanization, may improve tissue adherence.
6. Air-dry 12–24 hours at room temperature, overnight at 37°C or at 60°C for one hour.

NOTE: Use of protein-containing products, such as commercially available compounds that may contain gelatin, Elmer's Glue or Knox gelatin, in the mounting water bath is contraindicated if tissue is being mounted for IHC staining. Proteins in the waterbath will bind with the adhesive coating before the tissue section is mounted on the glass slide. During pretreatment and/or IHC procedures, the tissue may partially lift off the glass slide, allowing IHC reagents to become trapped beneath, or total detachment of the specimen may occur. Additionally, IHC reagents may non-specifically bind to this protein layer, contributing to background staining.

AFTER DRYING: All cryostat section slides which are not to be stained immediately, should be placed back-to-back, wrapped in aluminum foil and stored at -20 to -70°C. Paraffin section slides should be stored at room temperature or 2–8° C.

BEFORE FIXATION: Allow frozen and refrigerated slides to reach room temperature before unwrapping.

FIXATION AND DEWAXING

CRYOSTAT FROZEN SECTIONS

1. Fix slide for 10 minutes in cold 100% acetone.
2. Place in TBS for 1–5 minutes.
3. Do not perform a peroxidase block using H_2O_2 or H_2O_2/methanol until after the primary antibody step.

PARAFFIN SECTIONS

1. Place slides in a xylene bath and incubate for 5 minutes. Change baths and repeat once.
2. Tap off excess liquid and place slides in 95–96% ethanol for 3 minutes. Change baths and repeat once.
3. Tap off excess liquid and place slides in 70% ethanol for 3 minutes. Change baths and repeat once.
4. Tap off excess liquid and place slides in DI water for a minimum of 30 seconds.

REFERENCES

1. Henry, John Bernard MD; Clinical Diagnosis & Management by Laboratory Methods 18th edition; Philadelphia, W. B. Saunders, 1991 p. 621-622.
2. Sheehan, Dezna; Hrapchak, Barbara; Theory and Practice of Histotechnology, 2nd edition; Columbus, Battelle Press, 1987.
3. National Committee for Clinical Laboratory Standards; Approved Guideline; Internal Quality Control Testing: Principles and Definitions. NCCLS Document C24-A; vol 11, number 6.

TROUBLESHOOTING

KAREN N. ATWOOD AND DAKO CORPORATION TECHNICAL SERVICE GROUP

■ Immunohistochemistry is a multi-step process that requires specialized training in the processing of tissue, the selection of appropriate reagents and interpretation of the stained tissue sections. In general, IHC staining techniques allow for the visualization of antigens by sequential application of a specific antibody to the antigen, a secondary antibody to the primary antibody, an enzyme complex and a chromogenic substrate. The enzymatic activation of the chromogen results in a visible reaction product at the antigen site. Because of its highly complex nature, the causes of unexpected negative reactions, undesired specific staining or undesired background could be difficult to isolate. It is our hope in DAKO Corporation Technical Service that the information contained in this chapter will enable you to rapidly pinpoint and resolve problems encountered during the staining procedure.

To this end, we have developed troubleshooting tools for use in the histology laboratory.

Section One is a compilation of common problems encountered when using immunohistochemical-staining reagents, the underlying causes of staining failure and recommended corrective actions. The chart is divided into sections describing little or no staining, general background staining and limited background staining.

Section Two presents a method of systematically adding one IHC reagent at a time to determine at which stage non-specific or undesired staining may be occurring in a peroxidase or alkaline phosphatase staining system.

Section Three is a simple chart used to define the type of tissue specimen, the IHC staining and ancillary reagents already in place in the laboratory, and the staining protocol used by the laboratory personnel. We encourage you to copy this chart and use it to help troubleshoot any problems you may encounter with your staining systems.

SECTION ONE

■ Troubleshooting problems commonly encountered during immunohistochemical staining.

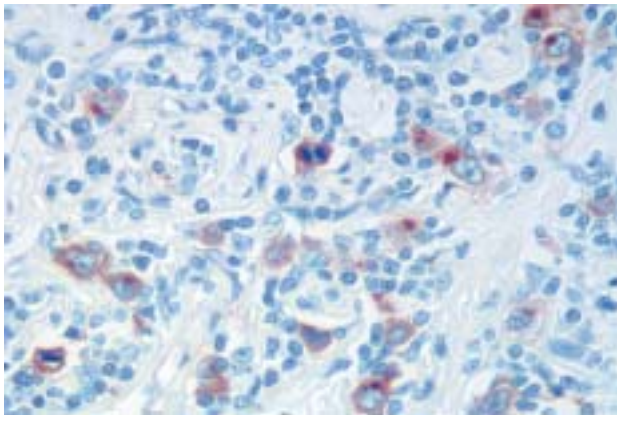

Figure 23

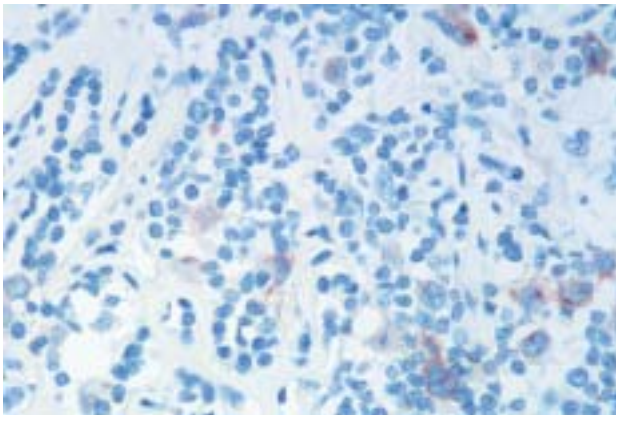

Figure 24

Ongoing studies performed in the DAKO Corporation Research & Development laboratory confirm that the pH and ion content of the antibody diluent may have a significant effect on the sensitivity of monoclonal antibodies.

Hodgkin's lymphoma stained with CD30 (clone Ber-H2) antibody, using a three-stage immunoperoxidase staining system. Figure 23: anti-CD30 diluted 1:50 with Tris-HCl, pH 7.6. Figure 24: anti-CD30 diluted 1:50 with PBS, pH 7.0.

INADEQUATE STAINING

■ Little or no staining of controls or specimen tissue, except for counterstain. May show little or no background staining.

POSSIBLE CAUSE	SOLUTION	SEE PAGE
Primary antibody or labelled reagent omitted. Reagents used in the wrong order.	Repeat the procedure using the manufacturer's staining system specification sheet or standard operating procedure reagent check-list as established by the individual laboratory.	26
Excessively diluted or excessively concentrated reagents; inappropriate incubation time and temperature.	Determine correct concentration for each reagent. Depending on the degree of staining obtained, if any, a 2- to 5- fold change in concentration may be needed. Incubation temperature and incubation time are inversely proportional and will affect results. To determine optimal incubation protocol, vary either the time or temperature for each reagent in the IHC staining system. Generally, incubation times can be extended if little or no background was detected.	12
Primary antibody diluted with inappropriate buffer. ■ Use of PBS or TBS as an antibody diluent. ■ Lack of stabilizing or carrier protein. ■ Detergent in the diluent.	Check formula and compatibility of antibody diluent. A change of ion and/or pH of the antibody diluent can cause a diminution in the sensitivity of the antibody. Addition of NaCl should be avoided. This problem is primarily seen with monoclonal antibodies.	26
Primary antibody defective; one or several secondary or ancillary reagents defective. Do NOT use product after expiration date stamped on vial.	Replace defective or expired antibody; repeat staining protocol, replacing one reagent at a time with fresh, in-date reagents. ■ Store products according to each product specific package insert. ■ If using a neat or concentrated antibody, it may be aliquoted and frozen. Avoid repeated freezing and thawing. ■ Do not freeze ready-to-use or customer diluted products. Follow manufacturer recommendations on product specification sheets, package inserts and reagent labels.	10
Dissociation of primary antibody during washing or incubation with link antibodies.	A feature of low affinity antibodies: ■ Polyclonal primary antiserum: Attempt staining at lower dilutions. ■ Monoclonal primary antibody: Replace with higher affinity antibody of identical specificity. Re-optimize incubation times for washing buffer and link antibody.	8
Use of alcohol-based counterstain and/or alcohol-based mounting media with aqueous- based chromogens.	■ Repeat staining, using water-based counterstain and mounting media. ■ Use a permanent chromogen, such as DAB, that is not affected by organic solvents.	15
Excessive counterstaining may compromise proper interpretation of results.	Use a counterstain that: ■ Will not excessively stain tissue sections. ■ Can be diluted so as not to obliterate the specific signal. ■ Reduce incubation time of the counterstain.	38

INADEQUATE STAINING (continued)

POSSIBLE CAUSE	SOLUTION	SEE PAGE
Incorrect preparation of substrate-chromogen mixture. (See Specification Sheet)	■ Repeat substrate-chromogen treatment with correctly prepared reagent. ■ Staining intensity is decreased when excess DAB is present in the working reagent.	
Incompatible buffer used for preparation of enzyme and substrate-chromogen reagents: ■ Use of PBS wash buffer with an alkaline phosphatase labelled staining system. ■ Sodium azide in reagent diluent or buffer baths for immunoperoxidase methodologies.	Check compatibility of buffer ingredients with enzyme and substrate-chromogen reagents. Repeat staining. ■ Commercial phosphate buffers may contain additives that will inhibit alkaline phosphatase activity. ■ Avoid sodium azide in diluents and buffers. A concentration of 15mM/L sodium azide, which is routinely added to IHC reagents to inhibit bacterial growth, will not impair HRP conjugated labels.	26
Antigen levels are too low for detection by the employed staining method. May be due to loss of antigenic differentiation in some tumors or loss of antigenicity due to sub-optimal tissue fixation.	■ Utilize a higher sensitivity staining system. ■ Prolong incubation time of primary antibody. ■ Re-optimize incubation times and concentrations of ancillary reagents. ■ Perform antigen retrieval if applicable.	29
Steric hindrance due to high antigen level and possible prozone effect	Re-optimize concentration of the primary antibody and ancillary reagents. Antibody concentration may be too high.	29
Use of inappropriate fixative. ■ Use of certain fixatives may damage or destroy antigens or epitopes in the tissue specimen. ■ Use of non-cross linking fixatives may allow the elution of antigens soluble in IHC reagents. ■ Different fixatives may affect standardization of cells.	Check manufacturer's specifications regarding recommended fixative.	20
Immunoreactivity diminished or destroyed during embedding process.	Use a paraffin wax with a melting temperature less than or equal to 58°C. Wax used for embedding should not exceed 60°C.	20
Immunoreactivity diminished or destroyed during dewaxing at high oven temperature.	Oven temperature not to exceed 60°C. Note: The intensity of immunostaining may be diminished when tissue is exposed to prolonged heat. Refer to the primary antibody specification sheet for additional information.	20
Excessive wash buffer or blocking serum remaining on tissue section prior to application of IHC reagents.	Excess reagent left on the tissue section will dilute the next consecutive reagent. Repeat staining, wiping away excess washing buffer and blocking serum.	12
Demasking protocol is inappropriate or has been omitted.	Some tissue antigens require proteolytic enzyme digestion or heat induced antigen retrieval performed prior to staining. Need for pretreatment depends on the type and extent of fixation, specific characteristics of the antigen and the type of antibody used. Use the pretreatment method recommended by the manufacturer. No single pretreatment is suitable for all applications.	23
Repeated reuse of antigen retrieval buffer. (See Specification Sheet)	Do not reuse buffer.	

INADEQUATE STAINING (continued)

POSSIBLE CAUSE	SOLUTION	SEE PAGE
Sections incorrectly dewaxed.	Prepare new sections and deparaffinize according to standard laboratory protocol, using fresh xylene or xylene substitute.	38
Failure to achieve the optimal temperature required for heat induced antigen retrieval. (See Specification Sheet)	■ Allow sufficient time for the retrieval buffer to equilibrate to a temperature range of 95-99°C. ■ At high altitude, the buffer will boil at less than 95°C. Utilize a closed heating system such as a pressure cooker or autoclave or a low temperature protocol if standardization of procedure is not affected.	24
Excessive or incomplete counterstaining. (See Specification Sheet)	Re-optimize concentration of counterstain and incubation time.	38
Instrument malfunction (See Manufacturer's Instrument Manual)	Ensure automated-stainer is programmed correctly and is running to manufacturer's specifications.	
■ **Positive control tissue shows adequate specific staining with little or no background staining. Specimen tissue shows little or no specific staining with variably background staining of several tissue elements.**		
Specimen held for too long in a cross-linking fixative, usually in formalin, causing "masking" of antigenic determinants due to aldehyde cross-linking and increased hydrophobicity of tissue.	Standardize routine fixation. Proteolytic digestion or antigen retrieval will break down cross-linking and render some tissue antigens reactive. Refer to the primary antibody specification sheet for additional information.	23
Sectioned portion contains crush artifact caused by grossing tissue with dull scalpel or razor. Serum proteins diffuse through tissue and are fixed in place.	Re-cut tissue using sharp blade.	35
Sectioned portion of specimen contains necrotic or otherwise damaged elements.	Ignore physically damaged portions of stained tissue sections.	38
Sectioned portion of specimen not penetrated by fixative. Loss of antigenicity in unfixed tissue.	Fix tissue biopsy for longer period of time or fix smaller pieces to ensure complete penetration. Unfixed tissue tends to bind all reagents nonspecifically.	19, 38

GENERAL BACKGROUND

■ **Background seen in all control tissues and specimen tissue. May see marked background staining in several tissue elements such as connective tissue, adipose tissue and epithelium.**

POSSIBLE CAUSE	SOLUTION	SEE PAGE
Excessive incubation with substrate-chromogen reagent. (See Specification Sheet)	Reduce incubation time.	
Substrate-chromogen reagent prepared incorrectly. (See Specification Sheet)	Repeat incubation with correctly prepared chromogen reagent.	
Link antibody cross-reacts with antigens from tissue donor.	Absorb link antibody with tissue protein extract or species-specific normal serum from tissue donor.	27

GENERAL BACKGROUND (continued)

POSSIBLE CAUSE	SOLUTION	SEE PAGE
Secondary (link antibody) and/or tertiary reagents too concentrated.	Repeat staining. Determine correct concentration for each reagent. Incubation temperature and incubation time will affect results. To determine optimal incubation protocol, vary both the time and temperature for each reagent in the IHC staining protocol.	13
Slides inadequately rinsed.	Gently rinse slide with wash buffer bottle and place in wash bath for 5 minutes. Gentle agitation of the wash bath may increase effectiveness when used with cytoplasmic or nuclear staining protocols.	8
Insufficient saline or detergent in wash buffer.	High sensitivity staining systems may require higher concentrations of saline or detergent in the wash buffer. Refer to the staining system specification sheet for optimal formulation.	35
Wrong blocking serum used.	Blocking serum and link antibody should come from the same species. Alternatively, a universal serum-free protein block, lacking immunoglobulins, may be substituted for the serum block.	35
Sections incorrectly dewaxed.	Prepare new sections and deparaffinize according to standard laboratory protocol using fresh xylene or xylene substitute.	38
Non-specific binding of the secondary antibody with an animal tissue specimen.	Use a secondary antibody that has been absorbed against species specimen.	27
Instrument malfunction (See Manufacturer's Instrument Manual)	Ensure automated stainer is programmed correctly and is running to manufacturer's specifications.	
■ **Specimen tissue and negative reagent control slides show background staining. Positive and negative control tissue show appropriate specific staining. May involve several tissue elements such as connective tissue, adipose tissue and epithelium.**		
Specimen held for too long in a cross-linking fixative, usually in formalin, causing "masking" of antigenic determinants due to aldehyde cross-linking and increased hydrophobicity of tissue.	Standardize routine fixation. Proteolytic digestion or antigen retrieval will break down cross-linking and render some tissue antigens reactive. Refer to the primary antibody specification sheet for additional information.	19
Sectioned portion of specimen not penetrated by fixative. Loss of antigenicity in unfixed tissue. Unfixed tissue tends to bind all reagents nonspecifically.	Fix tissue biopsy for longer period of time or fix smaller pieces to ensure complete penetration.	20
Sectioned portion contains crush artifact caused by grossing in tissue with dull scalpel or razor. Serum proteins diffuse through tissue and are fixed in place.	Re-cut tissue using sharp blade.	35
Sectioned portion of specimen contains crushed, necrotic or otherwise damaged elements.	Ignore physically damaged portions of stained tissue sections.	35
Excessive or unevenly applied adhesive (subbing agent) on poly-L-lysine, or silanized slides.	IHC reagents bind to these products, resulting in a light stain over the entire slide surface. Some slides may be unevenly coated, and will exhibit the above problems on only a portion of the tissue or glass.	35

GENERAL BACKGROUND (continued)

POSSIBLE CAUSE	SOLUTION	SEE PAGE
Antigen diffusion prior to fixation causing specific background outside the expected antigen site.	Avoid delays in fixation of the tissue.	37
Tissue sections too thick.	Cut tissue sections thinner. Formalin fixed paraffin embedded tissue should be approximately 4-6µ; cryostat sections <10µ.	45
■ Negative reagent control slide shows background. Positive control tissue, negative control tissue and specimen tissue shows expected specific staining.		
Negative control serum insufficiently diluted.	Use properly diluted negative reagent control serum. ■ For polyclonal antibodies, dilute the negative reagent control serum until the protein concentration is equal to that of the primary antibody. ■ For monoclonal antibodies, dilute the negative reagent control serum until the Ig concentration is equal to that of primary antibody.	32
Contaminating antibodies in the negative control serum are cross-reacting with proteins from the specimen tissue.	Replace the negative reagent control serum; repeat staining protocol.	32
Negative reagent control serum contaminated with bacterial or fungal growth.	Replace product with non-contaminated serum.	11

LIMITED BACKGROUND

■ Areas of inconsistent staining on controls, specimens and glass slides

POSSIBLE CAUSE	SOLUTION	SEE PAGE
Protein trapped beneath the tissue during the mounting process will allow partial lifting of the section. Pooling of IHC reagents beneath the section, or partial detachment of the tissue from the slide may occur.	Avoid the use of commercial adhesives, glue, starch or gelatin in water baths when mounting tissue sections. This is particularly important when using charged slides.	38, 46
Undissolved granules of chromogen.	Insure that chromogen in tablet or powder form is completely dissolved, or switch to a liquid chromogen.	38
Incomplete removal of embedding medium.	Remove embedding medium thoroughly, using fresh reagents.	38
Incomplete dezenkerization of tissue fixed with B5 or mercury containing reagents.	Perform dezenkerization with fresh reagents.	21
Bacterial or yeast contamination from mounting waterbath.	Clean and refill waterbath.	38

LIMITED BACKGROUND (continued)

POSSIBLE CAUSE	SOLUTION	SEE PAGE
Partial drying of tissue prior to fixation. Unaffected areas show normal staining.	■ Immerse tissue promptly in fixative or holding reagent. ■ Keep moist during the entire staining process. ■ Use a humidity or moist chamber during incubation steps.	38
Instrument malfunction	Ensure automated-stainer is programmed correctly and is running to manufacturer's specifications.	38
■ **Adipose or connective tissue in specimen, negative control tissue, positive control tissue and negative reagent control slides. Background in connective and epithelial tissue.**		
Hydrophobic and ionic interactions between immunoglobulins and lipoid substances in fatty tissue.	Nonspecific staining of fatty tissue rarely interferes with interpretation of specific staining and can usually be disregarded. Special stain with Sudan IV; fat will stain an orange to red color (normal myelin and fatty acids do not stain).	34
Primary antibody and negative reagent control serum are insufficiently diluted.	Re-optimize the dilution of the primary antibody and negative control serum.	12
■ **Epithelial tissue in specimen, negative control tissue, positive control tissue and negative reagent control slides. Staining is moderate to marked, especially in epidermal epithelium. Background in epithelia accompanies background in connective tissue.**		
Both the primary antibody and negative control serum contain contaminating antibodies to epithelial elements, possibly cytokeratins.	■ Use a higher dilution of the primary antibody and negative control serum. ■ Increase the incubation time. ■ Replace the antibody.	7, 36
Excessive formalin fixation of tissues may increase protein cross-linking, resulting in tissue hydrophobicity.	Proteolytic digestion or antigen retrieval will break down cross-linking and render some tissue antigens reactive. Refer to the primary antibody and/or the negative reagent control specification sheet for appropriate pretreatment.	23, 34
■ **Focal cytoplasmic staining observed in epithelium in the specimen tissue.**		
Focal cytoplasmic staining is seen, particularly in intermediate and superficial layers of the epidermis. May be caused by passive absorption of plasma proteins into degenerating epidermal cells.	This observation is rare and should not interfere with interpretation of specific staining.	37
■ **Background seen in all control and specimen tissue when using an immunoperoxidase staining system.**		
Unquenched endogenous peroxidase activity may be seen in all hemoprotein-containing specimens, including hemoglobin in erythrocytes, myoglobin in muscle cells, cytochrome in granulocytes and monocytes and catalases in liver and kidney.	■ Use alternate or prolonged peroxidase blocks or use another enzyme label such as alkaline phosphatase. ■ Eosinophils are particularly resistant to peroxidase quenching. Use special stain: eosin will stain these cells a bright red-orange.	35
■ **Background seen in all control and specimen tissue when using an alkaline phosphatase staining system.**		
Unquenched endogenous alkaline phosphatase activity may be seen in leucocytes, kidney, liver, bone, ovary, bladder, salivary glands, placenta and gastro-intestinal tissue.	Add levamisole to the alkaline phosphatase chromogen reagent or use another enzyme label such as horseradish peroxidase. Intestinal alkaline phosphatase is not quenched by the addition of levamisole. If use of an alkaline phosphatase system is necessary, the tissue may be pre-treated with 0.3N HCl.	36

LIMITED BACKGROUND (continued)

■ Background seen in all control and specimen tissue when using a biotin-streptavidin (avidin) staining system.

POSSIBLE CAUSE	SOLUTION	SEE PAGE
Endogenous protein-bound biotin (water-soluble B vitamin). High to moderate amounts may be found in adrenal , kidney, liver, spleen, gastrointestinal, lung, central nervous system, adipose and lymphoid tissue. Staining may also be seen on cells grown in culture media containing biotin as a nutrient.	Use a biotin block or another non-biotin (strept) avidin based staining system.	36

■ Background of skeletal or smooth muscle tissue in positive control tissue, negative control tissue, specimen tissue and negative reagent control.

Cause is not understood. It is possibly due to antibodies to muscle antigens in primary and negative reagent control serum.	Should not interfere with interpretation of specific staining.	35, 36

UNDESIRED "SPECIFIC" STAINING

■ Positive staining of leukocyte membranes in specimen tissue, positive control, negative tissue control and negative reagent control.

POSSIBLE CAUSE	SOLUTION	SEE PAGE
Binding of Fc portion of Ig by Fc receptors on the cell membrane of macrophages, monocytes, granulocytes and some lymphocytes.	■ Use $F(ab')_2$ or F(ab) fragments for the primary and secondary antibodies rather than the intact antibodies. ■ Add detergent to the wash buffer.	37

■ Positive staining of histiocytes and granulocytes in the specimen tissue only, with a marker not normally reactive with these cells.

Phagocytosis of antigens may render phagocytes positive for the same.	Rare: should not interfere with interpretation of specific staining.	38

■ Positive membrane staining of specimen tissue and negative reagent control tissue when using a horseradish peroxidase staining system.

Tissue from persons infected with hepatitis B virus and expressing hepatitis B surface antigen may exhibit undesired staining.	Utilize a non-peroxidase staining system.	38

MISCELLANEOUS

■ Loss of viability of cell cultures.

POSSIBLE CAUSE	SOLUTION
DAKO manufactures all antibodies for in vitro use only. These products contain preservatives, usually sodium azide. Azide is a known poison. (See Product MSDS)	Utilize an in vivo product only for application or injection into viable cells. For use on cell cultures only: Sodium azide may be dialyzed out of DAKO reagents. Contact Technical Service for additional information.

SECTION TWO

Troubleshooting flow chart: Use this flow chart to determine source(s) of non-specific staining, when using an immunohistochemical protocol.

BACKGROUND STAINING ENCOUNTERED WITH HRP-PEROXIDASE REAGENTS

REAGENTS	RESULT/ACTION
SLIDE #1 Positive Control Tissue: Counterstain with hematoxylin	Brown pigment (melanin) observed: To distinguish melanin pigment from DAB chromogen, Azure B can be used as a counterstain. The melanin stains blue-green, while the DAB remains brown. An alternate method is to use AEC as the chromogen. However, if high levels of pigment exist in the tissue, the red chromogen may be partially obscured. Since bleaching protocols to remove melanin may compromise tissue antigenicity, it should be avoided if at all possible.
NO STAINING SEEN. GO TO NEXT STEP. ▼	
SLIDE #2 Positive Control Tissue: DAB/AEC + Counterstain	Brown/Red color observed: Indicates endogenous peroxidase activity in the tissue sections. It is present in all hemoprotein containing tissue including erythrocytes, muscle, liver, kidney, granulocytes and monocytes. Block with 3% hydrogen peroxide, sodium azide or DAKO Peroxidase Blocking Reagent (S2001).
NO STAINING SEEN. GO TO NEXT STEP. ▼	
SLIDE #3 Positive Control Tissue: Peroxidase Block + Streptavidin-HRP + DAB/AEC + Counterstain	Brown/Red color observed: Indicates endogenous biotin activity in the tissue sections. Protein-bound biotin may be found in adrenal, liver, kidney, GI tract, lung, spleen, brain, mammary gland, adipose tissue, lymphoid tissue and cell grown in culture media containing biotin (RPMI, NCTC, MEME). Block with DAKO Biotin Block (X0590) or switch to a staining system that is not dependent on the streptavidin/biotin reaction, such as the DAKO EnVision system.
NO STAINING SEEN. GO TO NEXT STEP. ▼	
SLIDE #4 Positive Control Tissue: Peroxidase Block + Biotin Block (if required) + Secondary Antibody + Streptavidin-HRP + DAB/AEC + Counterstain	Brown/Red color observed: Indicates non-specific or undesired attachment of the secondary antibody to the tissue sections. This primarily occurs when the secondary antiserum has not been prepared for use on a specific species tissue. To determine if this is the problem, absorb out non-specific proteins by adding 2, 5 or 10µL of normal serum (from the species of tissue to be stained) per 100µL of the secondary antibody.
NO STAINING SEEN. GO TO NEXT STEP. ▼	
SLIDE #5 Positive Control Tissue: Peroxidase Block + Biotin Block (if required) + Negative Reagent Control + Secondary Antibody + Streptavidin-HRP + DAB/AEC + Counterstain	Brown/Red color observed: ■ May indicate non-specific attachment of the primary antibody carrier-protein. Perform a protein block with normal serum from the host of the link antibody or DAKO Protein Block (X0909); add 0.05-0.1% TWEEN 20 to wash buffer to decrease protein attachment. ■ Antigen retrieval lipofusion-artifact may appear as granule staining in liver and cardiac tissue, or as specific staining in pancreatic sections.
NO STAINING SEEN. GO TO NEXT STEP. ▼	
SLIDE #6 Negative Control Tissue: Perform complete staining protocol.	Brown/Red color observed on Negative Control Tissue: ■ Monoclonal antibody: Possible contamination. ■ Polyclonal antibody: Possible contamination or undesired antibody in the host Ig fraction ■ Antigen retrieval lipofusion-artifact may appear as granule staining in liver and cardiac tissue, or as specific staining in pancreatic sections.

BACKGROUND STAINING ENCOUNTERED WITH ALKALINE PHOSPHATASE

REAGENTS	RESULT/ACTION
SLIDE #1: Positive Control Tissue: Fast Red, Fuchsin or BCIP/NBT + Counterstain	Red/Blue color observed: Indicates endogenous alkaline phosphatase activity in the tissue sections. It is present in liver, kidney, GI tract, bone, bladder, ovary, salivary gland, placenta, leukemic, necrotic or degenerated cells. Block with DAKO Levamisole X3021 (Intestinal alkaline phosphatase may be quenched by the addition of 0.3N HCl prior to the addition of the strept-avidin-AP).
NO STAINING SEEN. GO TO NEXT STEP. ▼	
SLIDE #2: Positive Control Tissue: Streptavidin-AP + Fast Red, Fuchsin or BCIP/NBT + Counterstain	Red/Blue color observed: Indicates endogenous biotin activity in the tissue sections. Protein-bound biotin may be found in adrenal, liver, kidney, GI tract, lung, spleen, brain, mammary gland, adipose tissue, lymphoid tissue and cells grown in culture media containing biotin (RPMI, NCTC, MEME). Block with DAKO Biotin Block (X0590) or switch to a staining system that is not dependent on the streptavidin/biotin reaction, such as the DAKO EnVision system.
NO STAINING SEEN. GO TO NEXT STEP. ▼	
SLIDE #3: Positive Control Tissue: Biotin Block (if required) + Secondary Antibody + Streptavidin-AP + Fast Red, Fuchsin or BCIP/NBT+ Counterstain	Red/Blue color observed: Indicates non-specific or undesired attachment of the secondary antibody to the tissue sections. This primarily occurs when the secondary antiserum has not been prepared for use on a specific species tissue. To determine if this is the problem, absorb out non-specific proteins by adding 2, 5 or 10µL of normal serum (from the species of tissue to be stained) per 100µL of the secondary antibody.
NO STAINING SEEN. GO TO NEXT STEP. ▼	
SLIDE #4: Positive Control Tissue: Biotin Block (if required) + Negative Reagent Control + Secondary Antibody + Streptavidin-AP + Fast Red, Fuchsin or BCIP/NBT + Counterstain	Red/Blue color observed: ■ May indicate non-specific attachment of the primary antibody carrier-protein. Perform a protein block with normal serum from the host of the link antibody or DAKO Protein Block (X0909); add 0.05-0.1% TWEEN 20 to wash buffer to decrease protein attachment. ■ Antigen retrieval lipofusion-artifact may appear as granule staining in liver and cardiac tissue or as specific staining in pancreatic sections.
NO STAINING SEEN. GO TO NEXT STEP. ▼	
SLIDE #5: Negative Control Tissue: Perform complete staining protocol	Red/Blue color observed on Negative Control Tissue: ■ Monoclonal antibody: Possible contamination. ■ Polyclonal antibody: Possible contamination or undesired antibody in the host Ig fraction. ■ Antigen retrieval lipofusion-artifact may appear as granule staining in liver and cardiac tissue, or as specific staining in pancreatic sections.

SECTION THREE

TISSUE SPECIMEN

■ Tissue Specimen: Successful staining of tissue with an IHC marker is dependent on the type and preparation of the specimen. Record in the chart below, the species of the animal to be tested, the tissue source or organ from which it was collected, the collection method, how the specimen was fixed and tissue preparation.

Species: _____

Organ/tissue source: _____

Collection:

- ☐ Surgical specimen/biopsy
- ☐ Post-mortem specimen
- ☐ Fine needle aspirate
- ☐ Peripheral blood (include anti-coagulant)
- ☐ Brushing
- ☐ Biologic fluid
- ☐ Cell culture
- ☐ Other _____

Tissue preparation:

- ☐ Paraffin embedded
- ☐ Plastic embedded
- ☐ Cryostat section
- ☐ Cytospin
- ☐ Cell smear
- ☐ Mono-layer cultured cells
- ☐ Other _____

Tissue fixation:

Type of fixative _____

Length of time _____

Size of specimen _____

Tissue mounting:

- ☐ Slide mount
- ☐ Tissue thickness
- ☐ Gelatin, glue commercial adhesive or starch in the water bath
- ☐ Other _____

BUFFERS

■ The type of buffer selected for dilution of the primary antibody, secondary antibody and wash buffers will have a significant impact on the quality of the IHC stain. Variations include the amount of detergent present in the buffer, the pH, ion content and temperature at which it is used.

Antibody diluent:

- ☐ Tris
- ☐ TBS
- ☐ PBS
- ☐ Other _____

Wash buffer:

- ☐ Phosphate buffered saline
- ☐ Tris buffered saline
- ☐ TWEEN 20 (percentage)
- ☐ pH at 25°C
- ☐ Other _____

DEMASKING

■ Pretreatment: Tissue specimens, when fixed with a cross-linking fixative, may require pretreatment to obtain desired results. Refer to the manufacturer's primary antibody specification sheet to determine the correct protocol.

Antigen retrieval buffer:

- ☐ DAKO Target Retrieval Solution (S1700)
- ☐ DAKO High pH Target Retrieval Solution (S3308)
- ☐ EDTA
- ☐ pH 8.0
- ☐ Other _____

Heat source:

- ☐ Steamer
- ☐ Waterbath
- ☐ Autoclave/pressure cooker
- ☐ Microwave
- ☐ Other _____

Incubation time: _____

Incubation temperature: _____

Cool down time: _____

Proteolytic enzyme digestion:

- ☐ Proteinase K
- ☐ Protease XXIV
- ☐ Pepsin
- ☐ Trypsin
- ☐ Other _____

Incubation time: _____

ENDOGENOUS BLOCKS

■ Background staining is defined as unexpected or undesirable staining seen on the test or control tissue, which does not represent the target antigen. Frequent causes of background staining are endogenous enzyme activity and endogenous biotin.

Peroxidase is an enzyme of the oxido-reductase class that reacts with a substrate containing hydrogen peroxide as the electron acceptor. To block this activity, a variety of hydrogen peroxide reagents can be applied to cells producing this enzyme.

Alkaline phosphatase is an enzyme having various isoforms, which are produced in the leukocytes, liver, bone, intestine, placenta and Regan (carcinoma). Addition of levamisole to the chromogen/substrate will inhibit endogenous alkaline phosphatase activity, with the exception of the intestinal isoform. If necessary, this can be blocked with a weak acid wash, such as 0.03-0.5N HCl.

Biotin, a B vitamin, may be protein-bound to tissue and can interfere with proper interpretation of staining patterns when using a streptavidin or avidin reagent. To block this binding, a biotin/avidin block can be applied to tissue sections containing moderate to high amounts of this vitamin.

Peroxidase block:

- ☐ 3% H_2O_2
- ☐ Methanol/H_2O_2
- ☐ Sodium azide
- ☐ DAKO Peroxidase Block (S2001)
- ☐ Other _____

Alkaline Phosphatase block:

- ☐ Levamisole
- ☐ 0.03N HCl (not for use on cryostat tissue)
- ☐ Other _____

Biotin block:

- ☐ DAKO Biotin Block (X0590)
- ☐ Other _____

Protein block:

- ☐ DAKO Protein Block (X0909)
- ☐ Normal sera from host species of the secondary antibody
- ☐ Other _____

STAINING SYSTEM

A variety of routinely used immunohistochemical systems or kits are now commercially available. These include indirect methods, labelled streptavidin-biotin (LSAB), avidin-biotin complex (ABC), biotinyl tyramide amplification (CSA) and labelled polymer technology (EnVision).

To help troubleshoot, fill in the chart below

Manufacturer: _____

- ☐ Automated
- ☐ Manual

Staining kit:

- ☐ 3-stage immunoperoxidase
- ☐ LSAB
- ☐ EnVision
- ☐ Other _____

Primary antibody: _____

Dilution: (Refer to primary antibody specification sheet) _____

Incubation time and temperature: _____

Negative Reagent Control:

- ☐ Polyclonal Ig fraction of non-immunized host of the primary serum
- ☐ Isotypic Ig (Monoclonal antibody produced as cell culture supernate or ascites fluid)
- ☐ Other _____

Secondary antibody: _____

Incubation time and temperature: _____

Label: _____

Incubation time and temperature: _____

Chromogen: _____

Incubation: _____

Counterstain compatible with the chromogen:

- ☐ Harris hematoxylin
- ☐ Mayers hematoxylin
- ☐ Gill hematoxylin
- ☐ Methyl Green
- ☐ Nuclear fast red
- ☐ Other _____

Clearing Agent:

_____

Mounting Media:

_____

GLOSSARY

This Glossary was not intended to be an all-encompassing list of terminology as used in immunochemical staining. Rather, it assumes a basic level of technical knowledge beyond which the included definitions were selected to help in clarifying the text of this Handbook.

ADJUVANT In immunology, any substance that enhances the immunogenicity of an antigen and results in a superior immune response. There are two types, those that possess the ability to enhance both cellular and humoral response to a large number of antigens (general potentiation), and those that strengthen specific response to only a few antigens (specific potentiation). Adjuvants work by several mechanisms including prolongation of antigen release, improving immunogenicity by antigen denaturation, recruitment of other immunocompetent cells and induction of inflammation.

AFFINITY ABSORPTION A method of separation by affinity chromatography. It may be used, for example, to remove unwanted antibodies from an antibody preparation. The preparation is passed through a column matrix containing antigens against which the unwanted antibodies are directed. Thus, the unwanted antibodies remain bound to the column. The antibody solution leaving the column contains only the desired antibodies, purified by affinity absorption.

AFFINITY ISOLATION A method of separation by affinity chromatography. For example, affinity isolated antibodies may be prepared by passing the antibody solution through a column matrix to which antigens are coupled. Antibodies directed against the coupled antigens remain bound on the column and may then be eluted using a solution which disrupts antigen-antibody binding.

AGGLUTINATION The clumping of cells that are distributed diffusely in a fluid. It is caused by agglutinins, antibodies developed against that specific cell type, and is seen when a bacterial culture is treated with serum from an animal immunized against that particular organism or when a suspension of cells, particularly red blood cells, is exposed to antisera. This phenomenon is commonly employed in blood banking as an indicator of antigen-antibody reaction between red cells and specific antiserum or donor plasma.

ANTIGEN A molecule that is capable of binding to an antibody.

ANTIGENIC DETERMINANT See Epitope.

ANTIGEN RETRIEVAL (AR) Also known by the terms “epitope retrieval” or “target retrieval”, pertains to the restoration of antigenicity (immunoreactivity) to an immunogen.

ANTISERUM A serum that contains antibodies.

ASCITES OR ASCITIC FLUID An accumulation of fluid in the abdominal cavity.

BACKGROUND Unless defined otherwise, background staining includes all nonspecific staining as a result of procedural artifacts. Occasionally, it may also include “undesirable” staining, e.g., due to diffused antigen.

CHROMOGEN One of a group of chemical species that can form a particular colored material or can be identified by such a reaction with an appropriate reagent.

COUNTERSTAIN A second stain that provides a contrasting effect to another stain.

CROSS-REACTIVITY The ability of an antibody to react with antigens other than the immunogen. The term should not be used when referring to reactions occurring between an antibody and different cell or tissue components.

EPITOPE The structural part of an antigen that reacts with an antibody. These are groupings of amino acids in globular proteins and sugar side-chains in polysaccharides. The most critical part is called the immunodominant point.

EPITOPE RETRIEVAL See Antigen Retrieval

EXPIRATION DATE This term signals the minimum expected shelf life of biological materials, including immunochemicals. (See Shelf Life)

HYPERIMMUNIZATION The practice of establishing a heightened state of the actively acquired immunity by the administration of repeated doses of antigen.

IDIOTYPE Traditionally, antigenic determinants that relate to the specificity of the antibody. Idiotypic arrangement of several groups of amino acids in the hypervariable regions of light and heavy chains were thought to bestow unique antigenic determinants to the antibody molecule and, as a consequence, a high degree of specificity. However, antisera directed against these antigenic determinants have since been found to cross-react with other antibody molecules. The term idiotype has yet to be redefined.

IMMUNOCHEMISTRY The branch of immunology concerned with the chemical substances and reactions of the immune system, the specific study of antigens and antibodies and their interactions with one another.

IMMUNOCYTOCHEMISTRY Immunochemistry applied to the study of intracellular activities. (Now frequently used interchangeably with immunohistochemistry.)

■ **IMMUNOGEN** Any substance capable of generating an immune reaction, in contrast to any substance that binds to an antibody (i.e., an antigen).

■ **IMMUNOGENICITY** The ability of an immunogen to elicit an immune response. Immunogenicity depends upon foreignness to the host, the size of the immunogen, the complexity of its molecular structure, the length of time it remains in the host and its ability to reach certain immunocompetent cells in order to generate immunity.

■ **IMMUNOHISTOCHEMISTRY** Immunochemistry applied to the study of cells and tissues. (Now frequently used interchangeably with immunocytochemistry.)

■ **IN SITU HYBRIDIZATION** An assay for nucleic acids "on site" in fixed tissue sections by the use of heat to first denature and then to reanneal with specific DNA, RNA or PNA probes.

■ **INTERNAL TISSUE CONTROL** A specimen from the patient donor which contains the target marker, not only in the tumor to be identified, but also in adjacent normal tissue. Thus, no separate positive control sections are needed.

■ **LIGAND** A molecule, ion or atom that is bound to the central atom (usually a metal atom) of a coordination compound or chelate.

■ **LINK ANTIBODY** See Secondary Antibody.

■ **MONOCLONAL ANTIBODIES** Immunochemically identical antibodies produced by one clone of plasma cells that react with a specific epitope on a given antigen. Produced commercially using hybridomas.

■ **MONOSPECIFIC** Having an effect only on a particular kind of cell or tissue, or reacting with a single antigen, as a monospecific antiserum.

■ **NEGATIVE TISSUE CONTROL** A tissue specimen from the same organ lacking the target antigen and processed by use of the primary antibody.

■ **NONIMMUNE SERUM** Serum obtained from animals which have not been immunized.

■ **POLYCLONAL ANTIBODIES** Immunochemically dissimilar antibodies produced by different cells and reacting with various epitopes on a given antigen.

■ **POSITIVE TISSUE CONTROL** A specimen previously shown to stain specifically for the target antigen after exposure to primary antibody. Nonspecific background staining should be at a minimum. Note that, for some target antigens (e.g., prostate specific antigen), the staining intensity ideally should be less than maximal to allow monitoring not only for positivity, but also for variation in intensity.

■ **PRIMARY ANTIBODY** The first antibody used in a staining procedure.

■ **PROZONE PHENOMENON** The phenomenon exhibited by some sera, which give effective agglutination reactions when diluted several hundred- or thousand-fold, but do not visibly react with the antigen when undiluted or only slightly diluted. The phenomenon is not simply due to antibody excess, but often involves a special class of antibodies (blocking or incomplete) which react with the corresponding antigen in an anomalous manner. The bound antibody not only fails to elicit agglutination, but actively inhibits it. The phenomenon may also occur with precipitation or other immunologic reactions.

■ **QUENCHING** Refers to the inactivation of a chemical activity by an excess of reactants or products. In enzymology, excess substrate or product may inhibit the enzymatic activity.

■ **SECONDARY ANTIBODY** The second antibody used in a staining procedure; it reacts with the primary antibody, now the antigen, and forms a bridge between the primary antibody and a subsequent reagent, if any. Also known as "link" antibody.

■ **SHELF LIFE** This term refers to the expected duration of the functional stability of biological substances, including immunochemicals, and is most commonly assessed by experimental tests, statistical work and observation. Within the user's laboratory, periodical comparisons of the working solution with aliquots kept frozen at -20°C is recommended. The shelf life is terminated by an Expiration Date.

■ **SPECIFIC STAINING** Positive staining of tissue or cells by use of primary antiserum. Occasionally this includes diffused, absorbed or phagocytosed antigen, giving rise to "undesirable" staining. The staining seen due to contaminating antibodies in the primary antiserum should be considered as nonspecific.

■ **STANDARDIZATION** Classically, to standardize means to compare with or conform an assay of unknowns to established standards. In quantitative analytical work numbers readily allow for conforming to such standards. In semi-quantitative or qualitative assays such as immunocyto- or immunohistochemistry, which frequently conclude with an opinion, only subjective comparisons to carefully selected tissue and reagent controls can be used to monitor and maintain excellence.

■ **TARGET RETRIEVAL** See Antigen Retrieval

■ **TITER** In immunohistochemistry, the highest dilution of an antiserum which results in optimal specific staining with the least amount of background.

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