PATHOLOGY

Education Guide

Immunohistochemical Staining Methods
Fourth Edition

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Immunohistochemical Staining Methods, Fourth Edition

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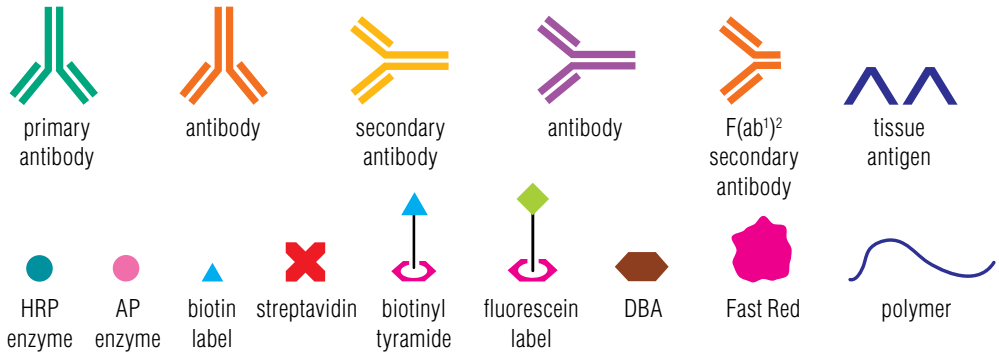

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Preface to the Fourth Edition of the Dako Educational Guide to Immunohistochemical Staining Methods

It is my pleasure to introduce this fourth edition of Dako's Guidebook to Immunohistochemical Staining Methods. This unique reference is provided by Dako to academic research investigators, pathologists, histopathologists and students from medical and scientific disciplines around the world as part of their continuing commitment to foster excellence in the fields of immunology and histopathology.

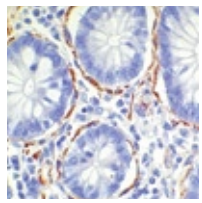
In order to provide the most up-to-date information in these fields, Dako periodically revises this book's content to reflect advancements in these disciplines. For example, this edition, like previous ones, contains relevant information on the well-established theoretical basis and methodology that is employed for these techniques. Of equal importance, contemporary approaches in automation, image analysis, molecular diagnostics and multi-staining have been updated and expanded in this edition. Finally, of great practical importance, knowledgeable practitioners have provided very helpful information related to interpretation of staining results and troubleshooting to resolve unexpected problems that can arise.

On behalf of Dako, I would like to thank all the contributors to this fourth edition. Most of the authors are Dako employees directly active in the development of these methods providing unique insights in this highly specialized field. The other authors, Thomas Boenisch, Marc Key, Mehrdad Nadji, Kenneth Bloom, W. Roy Overton and Ron Zeheb are recognized experts in their fields and greatly respected by all of us here at Dako. We value their individual contributions highly and appreciate the fact that they would participate so fully in this endeavor.

All available copies of previous editions of this handbook have been distributed. It is our goal to continue this practice in order to expand knowledge in this area and serve interested scientists around the world. We hope you find this edition to be as useful as the prior ones and we ask that you share your learning with your colleagues.

Dennis E. Chenoweth, Ph.D., M.D.

Corporate Vice President, Business Development



Chapter 1 ■ Antibodies

Thomas Boenisch

Introduction

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

Immunoglobulins

Antibodies belong to a group of proteins called immunoglobulins (Ig) that are present in the blood of immunized animals. The removal of cells and fibrin from blood is used to collect the serum fraction frequently referred to as antiserum. Listed in order of decreasing quantity found in plasma or serum, immunoglobulins comprise five major classes: Immunoglobulin G (IgG), IgA, IgM, IgD and IgE. Each 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 of type kappa (κ) or lambda (λ). Distribution of κ and λ chains differs in all Ig classes 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.

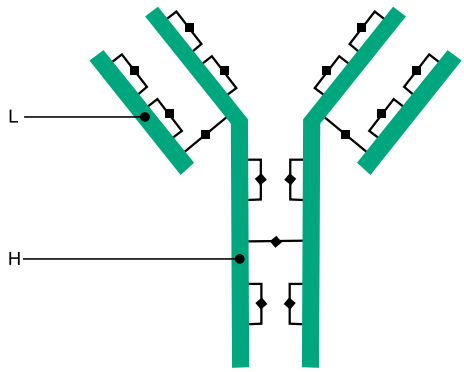


Figure 1.1. Diagram showing the structure of an immunoglobulin molecule. It comprises two identical heavy (H) chains and two identical light (L) chains. Inter- and intrachain disulfide bonds (|—•—|) contribute to the structure and stability of the molecule.

Of the five classes of immunoglobulins, IgG and IgM will be considered in more detail here, as these antibodies are utilized by far the most frequently 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 IgG1.

IgG

The heavy chains of IgG are denoted as gamma (γ) chains. 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 γ heavy chains, and two light chains of either type κ or type λ (Figure 1.1). The structure of the IgG molecule has been determined in part by proteolytic digestions and reductive dissociation of the molecule (Figure 1.2). 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 γ 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).

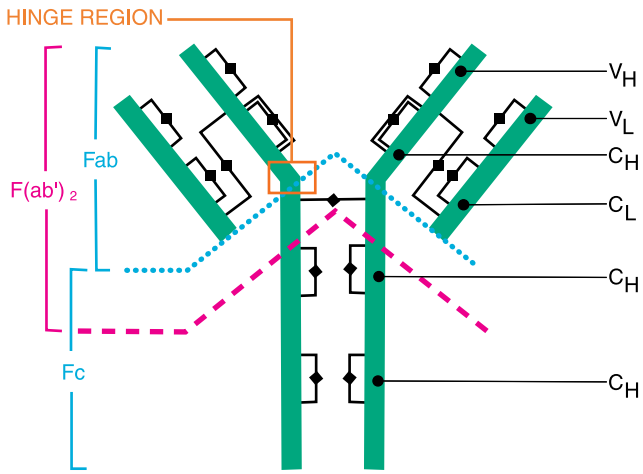
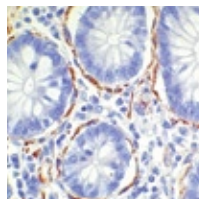


Figure 1.2. Diagram showing the structure of rabbit IgG (which exists as a single major subclass). The heavy (H) and light (L) chains are composed of variable (V) and constant (C) domains and are linked by inter- and intrachain disulfide bonds (|—•—|). Proteolytic digestion with papain (– – – –) yields two antigen-binding fragments $(Fab)_2$ and one crystalline fragment (Fc), whereas digestion with pepsin (• • • • •) yields one $F(ab')_2$ fragment.

The IgG molecule can be divided further into so-called domains, namely the variable domains (V) and the constant domains (C). Each domain contains 110 to 120 amino acids and one intrachain disulfide bond. The amino terminals of the immunoglobulin



molecule are located on the variable domain of the light chain (V_L), and on the variable domain of the heavy chain (V_H). Together, V_L and V_H form the antigen-combining site. Several hypervariable (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 HV regions of the antibody is approximately 0.2 to 0.3 nm.

Unique structural specificities called idiotypic determinants are located in this region. 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_H^1, C_H^2 and C_H^3) and carries the carboxyl terminal portion of the immunoglobulin. Located on the C_H^2 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_H^1 and C_H^2 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 κ to λ 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 percent κ chains, and therefore most monoclonal IgG antibodies from this species have κ chains. The number of disulfide bridges linking the heavy chains also varies among 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 a range of distances between identical antigenic determinants.

IgM

IgM is a pentamer (MW approximately 900 kD) consisting of five subunits of approximately 180 kD each (Figure 1.3). The general formula can be expressed as ($\mu_2 \kappa_2$) 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 μ and two light chains of type κ or λ. 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 percent mercaptoethanol cleaves the disulfide bridges between the subunits to yield five monomers. Subclasses of IgM_1 and IgM_2 have been reported.

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.

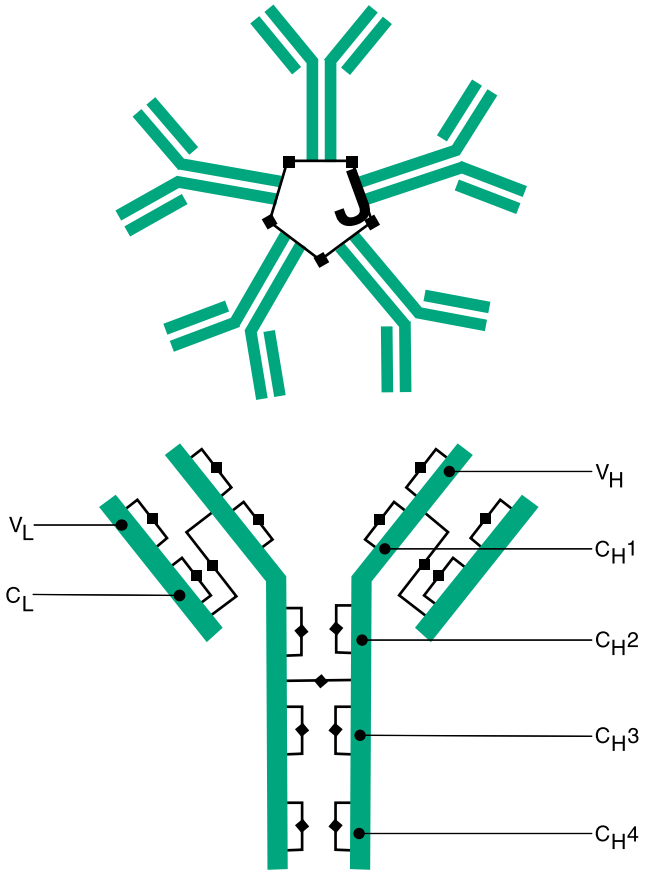
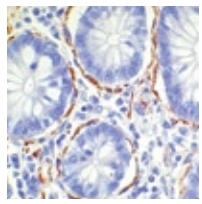


Figure 1.3. Diagram showing (A) the five subunits of mouse IgM linked by disulfide bridges (|—•—|) and the J chain to form a pentameric ring structure. Each subunit (B) comprises two mu heavy (H) chains and two light (L) chains each composed of constant (C) and variable (V) domains.

Antibody formation on the molecular level is a complex process, and a detailed account of it is beyond the scope of this guidebook. 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 1.4). 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. An additional advantage is that human antibodies to rabbit proteins are much more rare than to proteins from ruminants, such as goat. In addition, rabbit antibodies precipitate human proteins over a wider range of either 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).

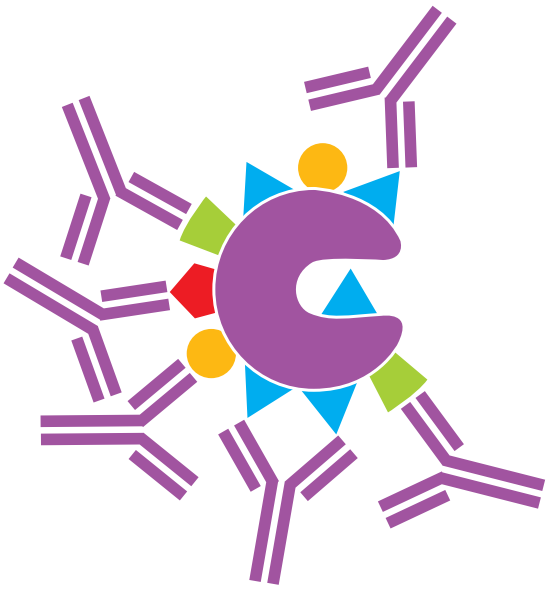


Figure 1.4. Schematic diagram of polyclonal antibodies binding to various epitopes on an antigen.

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 injected most often intradermally or subcutaneously, but injections into the footpad muscle or peritoneal cavity are used also. In rabbits, volumes of 0.1–0.5 mL are given usually 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 collected most often 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 purified immunoglobulin fractions. For the latter, 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 nonspecific antibodies.

Monoclonal Antibodies

Monoclonal antibodies are the product of an individual clone of plasma cells. Antibodies from the same clone are immunochemically identical, and react with a specific epitope on the antigen against which they are raised (Figure 1.5). Probably for reasons of economy, mice are used most frequently 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. 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 and identical characteristics can be produced.

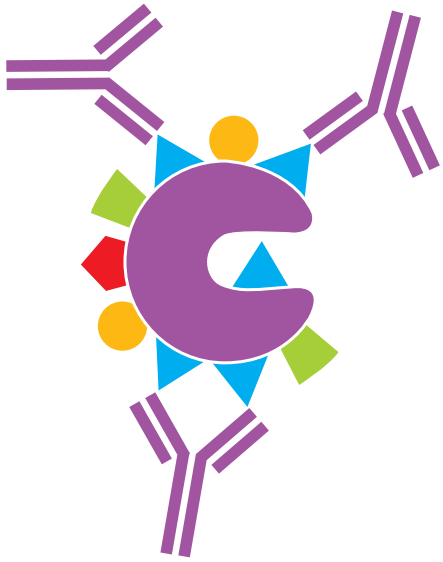
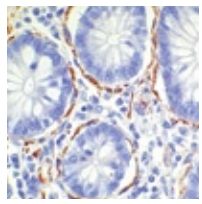


Figure 1.5. A given clone of monoclonal antibodies reacts with a specific epitope on an antigen.



In immunohistochemistry, monoclonal antibodies have certain advantages over their polyclonal counterparts. These include high homogeneity, absence of nonspecific antibodies, ease of characterization and minimal batch-to-batch or lot-to-lot variability.

Some pitfalls in the use of monoclonal antibodies should be noted. Test methods for selection of useful clones and for quality control must be identical to the methods for which they ultimately will be used. For example, monoclonal antibodies must be characterized on formalin-fixed tissues and not on frozen tissue, if they ultimately are intended for use on formalin-fixed specimens.

Similarly, results from testing reactivity of a new antibody on optimally fixed tissue must not be relied upon to predict its reactivity on sub-optimally fixed tissue, such as tissue fixed for a prolonged or inconsistent length of time. Also, as improved antigen retrieval procedures are being published continuously, it is imperative that the screening of new antibodies consider these additional variables (see Antigen Retrieval, Chapter 6).

Targeted epitopes also must 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 cross-reactivity of a polyclonal antibody can be removed usually by absorption, that is not possible with a monoclonal antibody.

Screening methods also should consider that monoclonal antibodies, unlike 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. Primarily ionic (electrostatic) interactions, but also 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 forms last and has a stabilizing effect on the cultivated immune complex, and, with soluble reactants, usually leads 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 and is the reciprocal of concentration in moles per liter. The higher the intrinsic affinity of the antibody, the lower the concentration of the 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 may result in lower titers of antibody, and vice versa.

In immunohistochemistry, the functional affinity of an antibody or an antiserum can be defined very loosely 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 a higher functional affinity. The term “avidity” has been used synonymously to describe functional affinity (5), but also has been used to denote the strength of the binding reached between antibody and its antigen (6). The term avidity also has been used to describe the sum total of all intrinsic affinities found in a polyclonal antibody population.

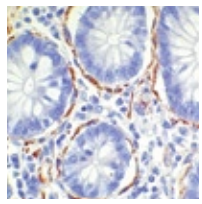
Because antigen-antibody reactions are reversible, the simple immune complexes formed on tissue occasionally may dissociate during the washing cycles used in immunohistochemistry. The ease and extent of this dissociation vary from antibody to antibody, and low salt concentrations as well as low temperatures 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 dissociation is less likely to occur than with low-affinity antibodies during washing. 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 a primary antibody 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 same is low, loss of staining may be due to the dissociation of the antibody from its epitope. Therefore, if possible, monoclonal antibodies of high affinity should be selected. As indicated above, factors that weaken the antigen-antibody bond such as high salt concentrations, high temperature and very low pH during the washing of the specimens should be avoided. Experience in the handling of antibodies in immunohistochemistry has shown that the washing and incubation in buffer baths can be reduced safely and that gentle agitation helps to reduce background staining (7).

Affinity of antibodies also is related to their capacity 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, do not 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 that was first noted in antibody-induced agglutinations. It is the observation that some antibodies, when insufficiently diluted, fail to agglutinate cells



even though higher dilutions will do so. While prozone also can be observed in precipitin reactions, in immunohistochemistry, it is a rare event (7).

As most antibodies carry a net positive electrostatic charge, the strength of the antibody's affinity for the targeted tissue antigen also depends on the availability and abundance of the net negative electrostatic charges present on the latter. Excessive formalin-fixation times of many tissues were held largely responsible for alteration of these charges, and as a consequence, for the unpredictably erratic immune reactivity with the primary antibody. Lost affinities, however, were restored largely by the routine use of heat-induced retrieval for all antigens (8).

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-λ (or -κ) 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 (9), 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 usually is subject to change because of affinity maturation during immunization.

Cross-reactivity of antibodies to human antigens with identical or similar antigens of other species, or "cross-species cross-reactivity," can be of interest to the researcher and veterinarian because of the scarcity of animal-specific antibodies. In an effort to overcome this, two groups published reports on the results of cross-species reactivity studies using commercially available antihuman polyclonal and monoclonal antibodies (10, 11). 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 antibody on animal tissues, the reader is referred to 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 of whether or not they contain common antigens, as this would distort the strict immunochemical definition of the term.

Antibody Reaction Rates

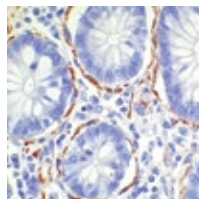
Although under ideal conditions antibodies react with their antigens very rapidly, in immunohistochemistry the conditions are rarely ideal. Depending on length of tissue fixation, antibody concentration, ambient temperature and other variables, primary antibody incubation times of up to 48 hours may be required for maximum reactivity (12). It is not surprising therefore, that as immunohistochemical procedures have become increasingly useful in surgical pathology, the need for shortened processing times also has been voiced. Very short incubation periods are made feasible by the relatively rapid reaction rates that occur when higher concentrations of high-affinity primary and link antibodies are used.

In these situations equilibrium between antigen-bound and free antibody rarely is achieved. To achieve equilibrium, very long incubation periods with more dilute antibody preparations are required. 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 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 actually is 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.

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 (see Immunohistochemistry Staining Methods, Chapter 7). However, it is reasonable to assume that gross overfixation of tissue may make penetration more difficult for antibodies and their complexes.

Antibody Stability

Polyclonal antibodies, when stored unfrozen and used subsequently in immunohistochemistry, are 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 frequent changes noted after prolonged storage. 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 in immunohistochemistry (see Chapter 16, Background) (7). Removal of these aggregates and polymers from IgG fractions is therefore prudent prior to their application for immunohistochemistry.

Just as storage of purified antibodies may augment their hydrophobicity due to aggregation and polymerization, so may their conjugation to other molecules (13). Conjugation with glutaraldehyde involves the epsilon-amino 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 percent of monoclonal antibodies investigated by Underwood and Bean showed changes in specificity, affinity and cross-reactivity (14). Antibodies of class IgM and subclass IgG_{2b} were especially sensitive.

It must be noted that actual-time testing of proteinaceous reagents is not feasible. While commonly practiced in the pharmaceutical field (15, 16), high-temperature accelerated degradation testing when applied to immunochemicals such as antisera and antibodies, can be irrelevant or even misleading (17, 18).

Antibody stability in commercially produced reagents is determined best by real-time and real-temperature testing by each manufacturer. Most manufacturers demonstrate stability by testing during a pre-determined period of time, ie, the “shelf life.” While many antibodies may retain activity longer, 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 testing until the antibody loses activity.

In addition, 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 manufacturer’s shelf-life studies. Because of the possibility of adverse storage conditions after the purchase of the product, the manufacturer can offer only a limited liability instead of predicting the actual demise of a reagent.

The only possible corollary to these requirements is to allow laboratories to document the activity of the product until the loss of the same. Alternatively, laboratories may aliquot and freeze undiluted antibody at –20 °C for later use. At this time, laboratories must confirm activity prior to the use of the antibody in any test.

Finally, expiration dating as practiced today also serves the purpose of conforming to regulatory requirements. Regulatory guidelines in place in the United States for clinical laboratories have been mandated by the Clinical Laboratory Improvement Act of 1988 and by the College of American Pathologists. These regulations mandate that expired reagents cannot be used in the clinical diagnostic laboratory on human tissue.

Handling of Antibodies

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

Receiving

Although many commercially produced immunochemicals are guaranteed to be stable for up to several years, ready-to-use (RTU) antibodies have a shorter shelf life (see Antibody Stability). Upon receipt, immunochemicals should be stored promptly according to the manufacturer's recommendations. Log 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 should become necessary.

Storage

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

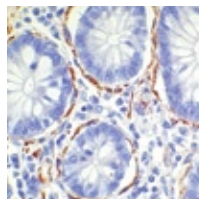
Storage Containers

Ideally, 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 (ie, less than 10–100 µg/ml), should receive an addition of immunochemically inert protein. Generally, 0.1 percent to 1.0 percent 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 RTU antibodies and their conjugates 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 ready-to-use reagents, including monoclonal antibodies. Store concentrated protein solutions such as antisera and immunoglobulin fractions in aliquots and frozen at –20 °C or below, in order 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 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 (19), 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 four hours at 37 °C has proven useful.

Mild to moderate hemolysis in antiserum resulting from suboptimal bleeding techniques probably does not interfere with most immunohistochemical staining procedures, but excessive hemolysis should be avoided. If excessive hemolysis or lipemia is encountered, isolation of the immunoglobulin fraction from the antiserum may be necessary. Such isolates usually will appear colorless and clear. Discard all immunochemicals, including antisera and normal non-immune 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 and also provide detailed information about the ancillary reagents and procedures used in immunohistochemistry.

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Footnote

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

Chapter 2 ■ Basic Immunochemistry

Thomas Boenisch

Introduction

In immunohistochemistry (IHC), antibody titer and dilutions as well as incubation time and temperature are tightly interwoven in their effect on staining quality. These factors can be changed independently, or as is more often the case, in complementary fashion to bring about positive differences. Generally, when making changes the overriding goal should be to achieve optimal specific staining accompanied by minimal interference from background staining. This chapter will highlight these variables.

Antibody Titer

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 titers have been expressed traditionally as micrograms of antigen precipitated per milliliter of antiserum. While this is of interest, it is not necessary information to the immunohistochemist. Augmenting polyclonal antisera titers by isolating and enriching immunoglobulin fractions produces little benefit for immunohistochemical applications, because nonspecific antibodies and soluble aggregates - frequent sources of nonspecific background - also become enriched (see Background, Chapter 16). For monoclonal antibody preparations, the absolute concentration of specific antibodies can be determined readily, and frequently forms the basis for making required dilutions.

An optimal antibody dilution also is 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.

In more practical terms, titers may vary from 1:100 to 1:2000 for polyclonal antisera, from 1:10 to 1:1,000 for monoclonal antibodies in cell culture supernatants, and up to 1:1,000,000 for monoclonal antibodies in ascites fluid. These dilutions may be exceeded in the future due to ever-increasing sensitivities of newer detection methods, including the use of an appropriate antigen retrieval procedure.

Antibody Dilution

Correct dilutions will contribute to the quality of staining if they are prepared accurately and consistently. Often a manufacturer offers ready-to-use (RTU) reagents, or

recommends dilution ranges compatible with other variables such as method, incubation time and temperature. If this information is not provided, optimal working dilutions of immunochemical reagents must be determined by titration. Correct dilutions are determined best 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. It should be noted that at least 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 has been found, 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 restricted molecular conformation and well-defined pI, monoclonal antibodies are more sensitive to the pH and ions of the diluent buffer (1). Indeed, it has been demonstrated that with the exception of the relatively rare IgG_3 isotype, all monoclonal antibodies could be diluted higher and stained more intensely at pH 6.0, especially after the use of heat-induced epitope retrieval (HIER) (2). IgG_3 isotype antibodies retained a preference for a more alkaline pH both before and after HIER. Almost all monoclonal antibodies stained more intensely in the absence of NaCl. Of several diluents used in this investigation, phosphate buffered saline (PBS), although still widely used as a diluent for primary antibodies, was found to suppress the reactivity of all monoclonal antibodies tested (2). Differences in the net negative electrostatic charges of the target antigen are likely the explanation for these pH- and ion-related observations (3).

Dilutions usually are 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 stock solution with nine parts diluent. Two-fold serial dilutions are made by successive 1:2 dilutions of the previous 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 intermediate dilution (10 µl + 990 µl).

The use of adjustable pipets for preparing dilutions allows for greater flexibility and more accurate delivery. To measure volumes in excess of 1.0 mL, serological or volumetric pipets can be used. Table 2.1 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 the following example of a checkerboard titration, the optimal dilutions of the primary antibody and the streptavidin-HRP reagent are found, while the dilution of the biotinylated link antibody is held constant. Nine tissue sections are required for testing three dilutions.

Table 2.1. Volumes of stock reagents and diluents.

Streptavidin-Peroxidase	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

If results achieved by use of several different dilutions are identical or similar, reagent costs may become an additional factor in selecting optimal dilutions.

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. For example, it has been found to be more restricted with the use of unlabeled enzyme-antienzyme complexes (PAP, APAAP), than with methods utilizing the streptavidin-biotin technology (4). This is probably consistent with the observation that as opposed to the PAP method, the avidin-biotin method cannot distinguish between high and low concentrations of tissue antigens (5). For additional information on immunohistochemistry staining methods the reader is referred to Immunohistochemistry Staining Methods, Chapter 7.

Antibody Incubation

As mentioned above, 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.

Incubation times for the primary antibody may vary within up to 24 hours, with 10-30 minutes probably being the most widely used incubation time. For an antibody to react sufficiently strongly with the bound antigen in a short period of time, it must be of high affinity and concentration, as well as have the optimal reaction milieu (pH and diluent ions). Variables believed to contribute to increased nonspecific background staining should be kept to a minimum (see Background, Chapter 16). Primary antibody incubations with a 24-hour duration allow for greater economy, because higher dilutions of the same may be used. Low affinity and/or low titer antibodies must be incubated for long periods in order to reach equilibrium*. But 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 this step is therefore important. Inconsistent incubation

times can cause variations in overall stain quality and intensity, and may lead to incorrect interpretation of results. These criteria are particularly essential in efforts that attempt to assess the degree of tumor differentiation.

Incubation Temperature

Because antigen-antibody reactions reach equilibrium more quickly at 37 °C compared to room temperature, some workers prefer to incubate at the higher temperature. However, while increases in incubation temperature allow for greater dilution of the antibody and/or a shortened incubation time, consistency in incubation time becomes even more critical. It is not known whether an increased temperature promotes the antigen-antibody reaction selectively, rather than the various reactions that give rise to background.

A temperature of 4 °C is used frequently 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 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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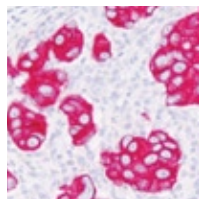
Footnote

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



Chapter 3 ■ Basic Enzymology

Thomas Boenisch

Introduction

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 used only rarely today.

This chapter also will discuss the various chromogens and substrates that can be used in conjunction with peroxidase and phosphatase, together 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 → 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 an enzyme (or any protein). Two basic types of inhibitions

are recognized: Competitive inhibition and noncompetitive inhibition. Competitive inhibition is the result of a reversible formation of an enzyme-inhibitor complex (EI):

E + Inhibitor (1) + 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 → E I S

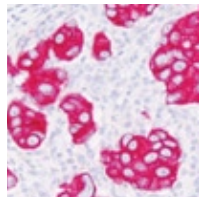
Selecting the enzyme most suitable for a particular immunohistochemical application depends on a number of criteria:

1. The enzyme should be available in highly purified form and be relatively inexpensive.
2. Conjugation (covalent binding to antibody or avidin, for example) or noncovalent binding should not abolish enzyme activity, although it may diminish it.
3. The bound enzyme should be stable in solution.
4. Endogenous enzyme activity should interfere only minimally with specific antigen-related staining.
5. Products of the enzyme 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 (*Cochlearia armoracia*). 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 (H_20_2), and then causes it to decompose, resulting in water and atomic oxygen. HRP oxidizes several substances, two of which are polyphenols and nitrates. It should be noted that similar to 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 (eg, chromogenic substance), is reversibly inhibited. It is the excess hydrogen peroxide and the absence of an electron donor that brings about quenching of endogenous peroxidase activities. Cyanide and azide are two other strong (reversible) inhibitors of peroxidase.



HRP can be attached to other proteins either covalently or noncovalently. Covalent binding of HRP to other proteins can be performed using either one-step or two-step procedures and glutaraldehyde. The chemical 4,4'-difluoro-3,3'-dinitrophenyl sulfone (FNPS) is used less commonly for this purpose. In all cases, the epsilon-amino 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 a paucity of 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 labeling and no polymerization. The subsequent conjugates are predominantly of 200,000 - 240,000 kD.

HRP also is conjugated to (strept)avidin using the two-step glutaraldehyde procedure and is used in this form in the Labeled Streptavidin Biotin (LSAB) procedure 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 unlabeled 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, "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 formed briefly. The chief metal activators for AP are Mg^{++}, Mn^{++} and Ca^{++}.

AP had not been used extensively in immunohistochemistry until publication of the unlabeled 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 earlier peroxidase techniques was the lack of interference posed by endogenous peroxidase activity. Because of the potential distraction of endogenous peroxidase activity, the alkaline phosphatase techniques were recommended particularly 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 one mM levamisole to the substrate solution (4), although five 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, which upon being oxidized, become colored products and therefore are called chromogens. This along with the property of becoming insoluble upon oxidation, make such electron donors useful in immunohistochemistry.

3,3'-diaminobenzidinetrahydrochloride (DAB)

This produces a brown end product that 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). DAB has been classified as a potential carcinogen and therefore should be handled and disposed of with appropriate care.

3-amino-9-ethylcarbazole (AEC)

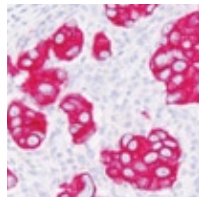
Upon oxidation, AEC forms a rose-red end product, which is alcohol soluble. Therefore, specimens processed with AEC must not be immersed in alcohol or alcoholic solutions (for example, Harris' hematoxylin). Instead, an aqueous counterstain and mounting medium should be used. AEC is unfortunately susceptible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

4-chloro-11 -naphthol (CN)

CN precipitates as a blue end product. Because it 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)

This 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

This 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

This 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.

Additional substrates include naphthol AS-BI phosphate, naphthol AS-TR phsophate and 5-bromo-4-chloro-3-indoxyl phosphate (BCIP). Other possible chromogens include Fast Red LB, Fast Garnet GBC, Nitro Blue Tetrazolium (NBT) and iodonitrotertrazolium 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 five to 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. (Solution is stable for one hour at room temperature.)
3. Add solution to tissue and incubate for three to 10 minutes at room temperature.
4. Rinse with distilled water.
5. Counterstain and coverslip with either organic- or aqueous-based medium.

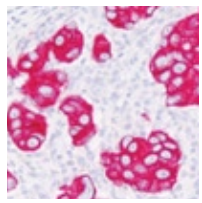
Alkaline Phosphatase

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 HCI) with 0.35 mL of freshly prepared 4% sodium nitrite (Sigma S 2252, 40 mg in 1 mL distilled water). Stir for 60 seconds.
4. Mix Solutions A and B, then add Solution C; adjust to pH 8.7 with HCI. 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 seconds. Add 100 mL of 0.05 M Tris buffer, pH 8.7, and 100 ~Ll 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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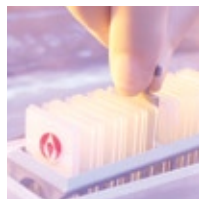


Chapter 4 ■ Fixation and Processing

A. J. Farmilo and Ronald H. Stead, Revised by A. J. Farmilo

Introduction

Immunohistochemistry (IHC) has advanced considerably since the first edition of this handbook was published in 1983 (1), and the driving force behind that change has been the need for standardization. If tissue staining is to provide consistent, reproducible diagnostic information, it must continue to evolve from an "art form" to a science. That evolution demands quantitation and reproducibility of methodology and, extending from that, consistency of results.

One of the last of these IHC "art forms" is tissue fixation and processing. Laboratory professionals are little closer to uniformity in this part of the process, and achieving that uniformity, or "standardization," remains one of the true unknowns in diagnostic interpretation.

Fixation

Part of the challenge is the finite amount of antigen in each tissue sample, and the fact that most steps in the IHC process destroy some of this antigen. This is especially problematic at the critical step of tissue fixation, because it is at this step that we intentionally try to change protein structure in order to preserve them from elution, degradation, or other modifications that occur in normal, unfixed tissue samples.

In addition to preventing antigen elution or degradation, fixation also should preserve the position of the antigen, whether nuclear, cytoplasmic or membrane-bound, and preserve as much antigenic secondary and tertiary structure as possible, to provide a target for antibodies that will be used to detect the antigen.

As a result of poor or inadequate fixation, many examples exist of situations that have led to incorrect interpretation of staining patterns. One example is elution of estrogen receptor protein from nucleus to cytoplasm. In this situation, the antigen is detected in the cytoplasm and therefore the cell stains "positive." But in fact the antigen should be primarily localized in the nucleus, and therefore diagnostically the stain is useless.

The same antigen can be used to demonstrate the importance of fixation and antibody-antigen reactions. Fixation in neutral buffered formalin will result in the destruction of an epitope against which some monoclonal antibodies react. Use of those antibodies would indicate a "negative" reaction for estrogen receptor, while the use of antibodies for a different epitope, one that is not destroyed by the fixation, would indicate a "positive" reaction.

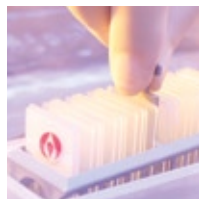
What is the solution to this complex issue? Standardization of fixative and fixation protocols would be an ideal start. Many fixatives have been developed over the years and at least two fairly recent ones have been promoted as possible "standards." But

so far no single fixative has proven ideal for all markers, antibodies and applications. Therefore standardization and validation will have to focus on particular antibodies and their corresponding staining protocols.

The acceptance of a common procedure for fixation is also extremely important and essential to achieving reproducible results. This means that reagent preparation must be done exactly the same way each time a particular staining protocol is performed. Reagents and protocols need validation, which would include determining the limits of the reagent's shelf life, optimal fixation time and conditions such as temperature and humidity. Many fixation reagents are concoctions of reactive and moderately toxic chemicals, and often little is known about the exact reactions that occur within them. For example, formalin preparations vary greatly, and concentrations of aldehydes, acids, and other by-products in each preparation may change with time and storage, and those changes will vary from product to product.

Validation is an initial step for two reasons: First, to ensure that a certain standardized procedure will give consistent and diagnostically useful results. Second, to test the limits of changes in the procedure that will continue to provide those results. For example, users can validate fixation time by running a series of tests using fixation times of zero, four, eight, 12, 24 and 36 hours; plus times of five, 15 and 30 days. For a given antigen and antibody combination, users might find that the zero-, four- and eight-hour fixations gave sub-optimal results, perhaps because the antigen was not fixed completely, and diffused through the cell or tissue. They then might determine that a range of 12 hours to five days is optimal, and that the 15- and 30-day results are sub-optimal due to over-fixation. They therefore have validated their procedure with respect to fixation time, and now know that the tissue requires a minimum fixation of 12 hours and a maximum fixation of five days. Practically speaking, that would mean that overnight fixation would be required, that weekend fixation would be OK, but a longer fixation time would not be useful. With this information, users would be able to evaluate the results obtained from tissues received from outside sources by comparing fixation procedures.

For smaller laboratories, the work involved in validation is often difficult, but there are two alternatives. Users can choose a system with an existing standardized and validated protocol and validated interpretation system. Commercially available kits generally provide these, and when utilized exactly as described in the kit insert, are guaranteed to provide diagnostically useful results. A second option would be to use one of the more common "standard" systems of fixatives with known antibodies, in which publication data has provided some evidence of functionality. As an example, a laboratory could use a 10 percent neutral buffered formalin fixation with a standard protocol, followed by a biotin-streptavidin HRP system, using a monoclonal antibody combination called AE1/AE3. This has been proven to be a reliable measure of cytokeratin in tissue sections.



Tissue Handling

The computer-related adage, “garbage-in, garbage-out” can apply to IHC as well, because the first steps of tissue handling arguably dictate the quality of results, more than do any steps that follow. Therefore a good foundation is to remember that the “first steps” start the very moment that tissue becomes a sample. Necrotic degradation begins immediately once the tissue is separated from its source of nutrients, so the time to processing is quite often critical.

For most IHC procedures, it is imperative that tissue not dry out. Collection from the surgical arena should be onto moist absorbent paper, in a covered container, followed by rapid delivery to the pathology lab for processing.

Tissue then should be trimmed and cut for fixation. The area of interest should be cut into blocks no more than two cm square by four mm thick. Thickness is important. The fixative must penetrate tissue in order to be effective. Fast penetration is desirable – the thinner the tissue, the faster fixation can begin. The most common formalin fixatives penetrate quickly, then fix tissue slowly.

The most frequently used fixative is a solution of 10 percent neutral buffered formalin. Due to its cross-linking characteristic, it is an especially good fixative for small molecules such as hormones (2). Optimum fixation time is critical and will vary from one antigen-antibody combination to another. Generally, six to 12 hours is acceptable, but longer fixation is needed occasionally. Over-fixation can pose problems, in that the cross-linking can mask epitopes needed to react with the antibody. A frequently used method of repairing this damage involves heating the fixed tissue in distilled water to a temperature of 95 degrees for 15 to 20 minutes. This will be discussed later in this chapter as part of the overall staining procedure.

Many other fixatives are available and a considerable body of literature exists that describes situations in which one of these performs better than others. Some of these fixatives will be discussed later in this chapter. Other specific applications exist in which tissue is frozen and cut, rather than fixed.

Formalin always should be fresh (see above reference to formaldehyde and formic acid formation with time), and buffered to a pH of 7.0-7.6. As this is a slow reacting fixative, acidic mixtures may induce structural or antigenic changes resulting in poor morphology and low detection.

Table 4.1. Ten percent neutral buffered formalin, pH 7 (10 percent NBF).

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

There are other aldehyde-based fixatives, such as those using glutaraldehyde as a base, but they all act similarly to 10 percent NBF and are used much less frequently.

Another class of fixatives used significantly in the past is mercuric-chloride fixatives. These do not initiate aldehyde linkages, but react with a number of amino acid residues such as thiols, amino groups, imidazole, phosphate and hydroxyl groups. On the positive side, fixation times are short, in the order of five to eight hours. On the negative side, it should be noted that mercuric chloride is highly toxic, and special disposal procedures are required. For these reasons and because of the prevalence of viable alternatives, these fixatives are used less and less in laboratories today.

Table 4.2. B5 Fixative

Reagent A:	Mercuric chloride	60 g
	Sodium acetate	12.5 g
	Distilled water	1000 mL
Reagent B:	10 percent neutral buffered formalin	

Working solution is 90 mL of Reagent A with 10 mL of Reagent B

Table 4.3. Zenker's Fixative

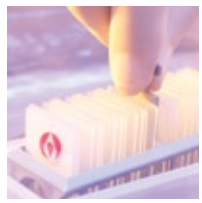
Distilled water	900 mL
Potassium dichromate	25 g
Mercuric chloride	50 g
Glacial acetic acid	50 g

NOTE: Fixation times are four to 24 hours, with an overnight wash or removal of mercuric chloride crystals required. This can be accomplished by one wash in 0.5 percent iodine in 70 percent ethanol, and a second wash in five percent sodium thiosuphate in water.

Alcoholic Fixatives

This class includes Carnoy's, Methacarn and others. They have been used for IHC purposes primarily to avoid the loss of antigenicity caused by excessive formalin fixation, or for monoclonal antibodies that reacted against an epitope destroyed by formalin. These fixatives typically found most of the application in looking at lymphocytes using CD-specific markers, and in looking for immunoglobulins such as IgG, A, and M.

Specialty fixatives, such as Osmium tetroxide, which is used primarily in electron micrography and acetone, is used in fixation of frozen sections. Others used for research



purposes on specific tissue, organs or even whole organisms are not discussed in this publication.

The final fixative class that is becoming more significant is the "combination fixative." These often combine alcohol with formalin, calcium or other heavy metals, and also with some kind of buffering mixture. Many are commercial, and as such their exact formulations are typically not disclosed by their manufacturers. Most are designed to address the search for a universal fixative that can standardize this element of IHC. To be truly universal, most of these fixatives also address RNA and DNA fixation, for genetic studies in fixed tissue (3, 4, 5).

Many of these fixatives are used widely, including Omnifix (AnCon Genetics, Melville, New York, USA), Tissufix #2 (Chaptec, Montreal, Quebec, Canada), UMFIX (Sakura Finetek USA Inc., Torrance, California, USA) and HistoChoice (AMRESCO, Solon, Ohio, USA). So far none have become accepted universally as the standard fixative.

Specialized Tissue Preparations

No discussion of fixation would be complete without mentioning a few specialized tissue preparations that have been popular in the past. Frozen sections used to be required for some studies, particularly for lymph node. Their use was due primarily to antigen destruction caused by formalin fixation, or for example, to the need to examine a thicker (10 micron) section to study axons in nerve tissue. The introduction of antigen unmasking methods using heated water has reduced the need for frozen sections.

The primary remaining motive for using frozen sections in routine practice is the need for a quick examination that eliminates the time required for fixation, processing and de-waxing. Frozen tissue sections also are used when direct or indirect immunofluorescence is the detection method, in which case formalin fixation can produce weaker results. Frozen sections should be fixed with acetone (room temperature, five seconds) before storing. They are then re-processed in acetone (4 °C, 10 minutes) and then re-hydrated in buffer for five minutes before immunostaining.

Blood smears, tissue imprints, cell cultures and purified cells may be examined as fresh tissue or as fixed tissue. These cells can be centrifuged to make a pellet that is then fixed just as in tissue fixation. Alternatively, a fresh smear may be made on the slide, and the cells fixed either with acetone or 10 percent NBF for 10 minutes. It is important to incubate the slide with an endogenous peroxidase blocking solution prior to staining if there are a large number of erythrocytes present, as these will stain due to endogenous peroxidase.

Finally, many of the newer fixation methods incorporate microwave treatments, either for the fixation itself or to speed fixation of other reagents (6). Direct microwave fixation is probably fixation due to heat, and is primarily a coagulation of the proteins. In conjunction with fixatives, microwaving probably speeds the reaction by heating the solution. It also perhaps speeds the penetration of the solution due to the relaxing of the cell structure.

Tissue and Slide Processing

Once the tissue is well-fixed, subsequent steps seem to have little effect on antigen detection. Variations in xylol processing, alcohol re-hydration, wax temperature, time or formulation, instrumentation used etc., provide satisfactory results in most cases. Some basic processing principles are:

- No processes should raise tissue temperature to higher than 60 °C, as this will cause severe loss of antigenicity that may not be recoverable.
- Tissue fixation medium must be replaced by wax, generally done through a series of incubations in increasing alcohol concentrations to 100 percent, followed by xylene and then hot wax. This is to provide stability of the tissue (wax) in order to make cutting the sections easier.
- The tissue sections should be cut at three or four microns or so in thickness, and certainly no thicker than five microns. Thick sections have multiple layers of cells, and make interpretation extremely difficult.
- When cut, sections are floated on water and picked up on slides that are coated with some adherent material. Some commercially available slides come with a positive charge that attracts the negative charges of tissue proteins. Slides can also be bought or prepared with a coating of albumin or lysine, either of which will provide a sticky surface for creating flat, adherent sections. Sections that are not flat and that have non-adherent ridges likely will be digested or torn off of the slide during immunostaining.
- Once on the slide, wax must be removed completely, in order that the aqueous antibody solution can adhere properly to and penetrate the tissue. This usually is done by heating the slides to about 60 °C to soften the wax, and then reversing the procedure described in Detailed De-Waxing Protocol, A, below. The slide is immersed in xylene, 100 percent alcohol and then diminishing concentrations of alcohol until the final buffer is fully aqueous. Note that 50 slides per 250 mL of xylene is the limit before the xylene is no longer effective, and residual wax begins causing artifacts in the final stained tissue.

Detailed De-Waxing Protocol

A. Circle and label the specimen with a diamond pencil.

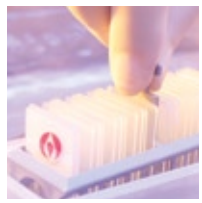
B. Place in 60 °C oven for 30 minutes.

C. Transfer immediately to a fresh xylene bath for three minutes.

D. Repeat step C above with a second xylene bath.

E. Place in a fresh bath of absolute alcohol for three minutes.

F. Repeat step E above with a second bath of absolute alcohol.



G. Place in a bath with 95 percent ethanol for three minutes.

H. Repeat step G with a second 95 percent ethanol bath.

I. Rinse under gently running water.

J. Do not let dry; store in buffer; begin required antigen treatment (see previous section) or immunostaining.

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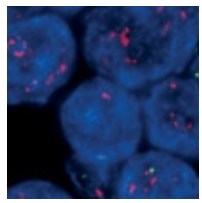


Chapter 5 ■ Molecular-Friendly Tissue Processing

Mehrdad Nadji, MD

Why Molecular Histopathology?

Most pathology laboratories use fixation and processing technologies that are more than 100 years old. These traditional methods continue to be used widely because of their excellent qualities for routine morphological examination and their cost-effectiveness (1). However, at the molecular level their impact on biomolecule preservation is variable and unpredictable. Because of the rapidly advancing era of molecular medicine, it is not unreasonable to predict that molecular methods eventually will either replace existing morphologic approaches, or more likely will be used in conjunction with them. Most of today's diagnostic and therapeutic decisions are based on the evaluation of small tissue biopsies, and laboratories are expected to use the same precious small volume of tissues for histology and molecular tests. Pathology laboratories therefore should devise molecular-friendly tissue handling systems that allow for morphological diagnosis while rendering the same archival tissue suitable for advanced molecular testing (2).

What Is a Complete Molecular-Friendly Histology Platform?

A complete molecular-friendly histopathology platform requires that specimens are properly handled from the moment they are removed from a patient to the time they are archived. With the introduction of fixatives and processing systems that protect molecular properties of tissue, two major steps toward this goal have been taken. It should be remembered, however, that a molecular-friendly fixative alone or a simple formalin-free processing system by itself does not guarantee that the final product is suitable for advanced molecular studies. Molecularly compatible fixatives and tissue processing are merely two components of a complete molecular histopathology platform. No matter how efficient the fixation and processing systems, if the time interval between surgical excision and fixation is long, there will be considerable degradation of biomolecules. Therefore, an overall "molecularly conscious" laboratory philosophy with provisions for proper pre-fixation and post-processing handling of tissue is sometimes more important than the fixative or the processor alone.

Fixation

It has been suggested that the best fixative for preservation of tissue macromolecules is "no" fixative. This is because past experiences have shown that all chemical fixatives, in one way or another, modify, degrade or destroy nucleic acids and proteins. For this reason fresh or fresh-frozen tissues have been used for most molecular studies. But these have limited value for assessment of histomorphology or for the performance of routine ancillary tests such as histochemistry (HC) and immunohistochemistry

(IHC). In addition, transportation and long-term storage of frozen tissue creates logistical problems that renders it impractical for routine use in most diagnostic histopathology laboratories.

Formalin-fixed, paraffin-embedded histologic sections, however, are quite suitable for most HC and IHC tests, although they yield degraded nucleic acids suitable only for slide-based in situ hybridization as well as PCR amplification of short amplicons. An example is less than 500 bp for DNA and less than 100 for RNA. The material therefore is of limited value for performing molecular assays that require intact molecules, such as high-molecular-weight RNA and biologically viable proteins. For this reason a search for alternative non-formaldehyde molecular fixatives has been ongoing the past several years (3, 4).

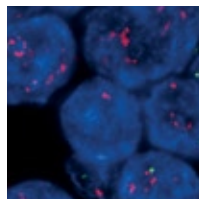
Chemical solutions that potentially can protect macromolecules in tissue can be grouped into two general classes. One class protects nucleic acids well, but the same tissue is unsuitable for histomorphologic evaluation. These “molecular preservatives” are therefore excellent alternatives to freezing the tissue, but of limited value as practical histology fixatives. In other words they must be used in addition to formalin, and that limits their utilization for small biopsies.

The second class could be regarded as true “molecular fixatives,” because they not only protect macromolecules but preserve acceptable histomorphology. They therefore can be used as a single universal reagent to fix tissue and to preserve its molecular properties at the same time. Such complete molecular fixatives have not been available until recently.

In addition to protecting intact biomolecules and preserving microanatomy, an ideal molecular fixative preferably should be non-toxic, non-volatile, active at ambient temperatures, economically priced and cost-effective. A molecular fixative recently introduced by Vincek et al for the most part meets these requirements (5). That fixative - a mixture of methanol and polyethylene glycol - is non-volatile and active at room temperature. It protects tissue DNA, RNA and proteins along with histomorphology. In addition, long exposure of tissue to the fixative, up to six months at room temperature, does not significantly alter its molecular properties or histomorphology.

Processing

The value of harvesting microwave energy for histopathology in general and for tissue processing in particular, has been well established (6, 7). In most published works conventional microwave ovens are used, including some that are adapted for histology purposes. In 2002, Morales and coworkers reported the development of a microwave-assisted, continuous-specimen-flow, one-hour tissue-processing method (8). This manual rapid-tissue-processing (RTP) system utilized microwave energy along with vacuum and a combination of common histologic reagents – minus formalin and xylene. It permitted preparation of paraffin blocks from either fresh or prefixed tissue in about one hour. The system utilized a specially designed cylindrical, low-energy microwave



that distributed energy uniformly throughout the chamber, thus avoiding the creation of hot-cold spots commonly observed in conventional microwave processing. The manual procedure is automated now into a rapid-tissue-processing system, complete with robotics, internal reagent containers, and user-friendly operating software. As a corollary, it has been observed that tissue samples processed by this methodology show improved RNA preservation, particularly when they are not prefixed in formalin. This unexpected but highly desirable "molecular-friendliness" led to a search for a fixative that similarly could protect tissue biomolecules. The result was development of the molecular fixative by Vincek et al, referred to above.

Validation

Molecular fixative and the RTP system must be used together to preserve macromolecules. Use of molecular fixative with conventional processing, or the use of RTP without a molecular fixative, will result in degradation of tissue nucleic acids and proteins. Since the complete system was intended for use as a molecular-friendly alternative to formalin fixation and conventional processing, a detail morphologic, immunohistochemical and molecular biologic evaluation of processed tissue was carried out as summarized in the following:

The Processing System

The histologic quality of the RTP system for formalin-fixed tissues was validated through comprehensive parallel studies, including a blinded review of slides by an expert external panel (9). In addition to integrity of histomorphology, quality of histochemical and immunohistochemical properties of processed tissues was established through extensive testing and reviewing. Altogether results confirmed that when formalin-fixed tissues were processed in the RTP system, no modification of protocols was necessary for Hematoxylin and Eosin (H&E) staining, histochemistry, immunohistochemistry, and in situ hybridization.

Combined Molecular Fixation and Processing Platform (MP)

When molecular fixative was used as an alternative to formalin, the RTP-processed tissues were subjected to new sets of validation studies. These experiments used parallel slices from surgically removed specimens. One slice was fixed in formalin and the other in molecular fixative; they both then were processed by the RTP system.

Summary of Results

Histomorphology

As safe alternatives to formalin, alcohol-containing fixatives have been in use for many years and most pathologists are familiar with their histologic properties. As with other alcohol-based fixatives, the molecular fixative produces a histomorphology that is

similar, but not identical to formalin. For example, the molecular fixative-exposed tissues show an overall brighter, shiny appearance in hematoxylin and eosin-stained slides. The minor morphologic differences, however, in no way interfere with establishing the correct diagnosis.

Immunohistochemistry

IHC of MP specimens is different from that of formalin-exposed tissue, and therefore modification of staining protocols may be necessary. A comparative study showed that for most routinely-used antibodies the sensitivity of immunohistochemistry performed on molecular-fixed tissue is either comparable or superior to formalin-fixed specimens. This is not surprising, because the superiority of alcohol-containing fixative for preservation of certain tissue antigens in general and intermediate filaments in particular has been observed before. A word of caution, however: Since a number of stand-alone immunohistochemical tests are designed solely for use on formalin-fixed tissue, one must adjust the antibody concentration or remove the antigen retrieval step to achieve comparable sensitivity in MP specimens.

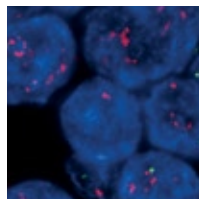
Molecular Properties

As a general rule, any molecular test that can be performed on formalin-fixed tissue could be done on specimens processed in the molecular system. This includes PCR amplification of small segments of DNA and RNA, as well as in situ hybridization. The latter requires shorter predigestion time, usually about one-third of the time required to digest formalin-fixed tissues. This is because the formalin-induced cross-linking of tissue proteins is not a problem in a formalin-free system. Tests that require intact biomolecules cannot be performed on formalin-fixed paraffin-embedded specimens. The same tests, however, are feasible on archival tissue prepared by the molecular platform.

The following summarizes validation of some of these tests as they apply to the preservation of intact RNA and proteins.

RNA Preservation

Tissues processed by the molecular platform yield an intact RNA comparable to that of fresh tissue, whereas in formalin-fixed specimens, RNA is degraded significantly as evidenced by the absence of 28S and 18S ribosomal bands. Similarly, in quantitative real-time PCR, the copy number of templates of molecular system is similar to fresh tissue, and significantly higher than that of formalin-fixed samples. In addition, extracted RNA from molecular and fresh tissue yields similar cDNA microarray profiles. Finally, it has been demonstrated that high–molecular-weight RNA can be extracted successfully by laser capture microdissection from H&E sections of paraffin blocks processed by the molecular platform (10). It should be remembered, however, that validation of tissue RNA integrity has to be carried out under well-controlled RNase-free conditions. This includes maintenance of an RNase-free laboratory environment, meaning RNase-free



instruments, glassware and reagents. Even more important is to ensure that endogenous RNase activity of tissue is blocked immediately and efficiently. To that end one has to establish strict operating room protocols that require immediate fixation of small biopsies, and timely delivery of larger specimens to the laboratory for proper handling.

Protein Preservation

Protein extracts from MP blocks show distinct spot patterns on 2D-gel electrophoresis similar to that of fresh tissue. This is in contrast to formalin-fixed specimens that produce a small number of distinguishable spots. On Western blots, MP samples also reveal distinct bands with most antibodies tested, including some antibodies against phosphorylated proteins. Only a few antibodies react with protein extracts of formalin-fixed tissues, and the intensity of bands is usually weaker and less distinct. Recent studies also have shown that proteins isolated from MP blocks are suitable for surface enhanced laser desorption and ionization spectrometry (SELDI-TOF), yielding high-resolution protein-profile patterns. No such pattern is observed with formalin-fixed specimens.

Conclusion

At this writing, several molecular preservatives/fixatives and formalin-free, tissue-processing systems are available. While an all-inclusive, combined system similar to MP has yet to be marketed and validated, such systems hopefully one day will be commonplace. The most formidable obstacle to this might be existing mind-sets, but the benefits of establishing a complete molecular histopathology laboratory as a key component of “personalized” diagnosis and treatment outweigh all challenges.

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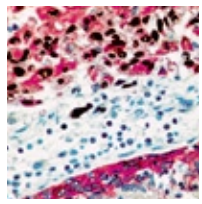


Chapter 6 ■ Antigen Retrieval

Marc Key and Tom Boenisch

Introduction

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

Inconsistent use of formalin fixation between laboratories, especially as it pertains to the variables of concentration, pH and exposure time, has contributed to this complexity, as these factors influence immunohistochemistry (IHC) stain results. Physiological and pathological changes in tissue composition, including the juxtaposition of tissue proteins and their antigenic sites (epitopes), make fixation outcomes unpredictable. Each antigen may contain from one to many epitopes, and each may be composed of five or more amino acids. These in turn may be linked continuously in sequence, or be spatially arranged in three-dimensional proximity as a result of intermolecular folding. Formalin fixation will allow some epitopes to emerge unchanged. Those are considered "formalin-resistant" epitopes. Others that undergo substantial changes are considered "formalin-sensitive" epitopes. In this process, cross-linking unrelated proteins to target antigens is also possible, resulting in the antigen's partial or complete loss of immunoreactivity. Such loss of immunoreactivity may be irreversible or reversible. If it is reversible, the epitope frequently is referred to as "masked."

A Short History of Antigen Retrieval*

The first attempt to improve formalin-fixed tissue antigens' immunoreactivity used tryptic digestion prior to immunofluorescent staining (3). Proteolytic digestion compensates for the impermeable nature of non-coagulant fixatives by "etching" tissue, and exposing hidden determinants. Today other proteolytic enzymes, including bromelain, chymotrypsin, ficin, pepsin, pronase and other proteases have been reported to restore immunoreactivity to tissue antigens with varying success. Enzyme use may, however, also entail the risk of destroying some epitopes. Formalin fixation in conjunction with digestion procedures needs to be optimized and then adhered to firmly (4).

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

The concept of recovering lost immunoreactivity through exposure to heat near water's boiling point was met at first with skepticism, because it went against the tenet of

protecting proteins from the denaturing effect of heat. However, Cattoretti et al developed another major step forward in the use of heat by employing 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 (6). Shortly thereafter, Gown (7) and Leong (8) were able to apply their modifications of AR methods to a wide variety of additional markers. Their modifications improved staining of many tissue markers, but more importantly they showed that a whole new class of antigens, previously found to be non-reactive in FFPE tissue, could be demonstrated successfully for the first time. These included additional proliferation markers, hormone receptors (ER and PR), growth factor receptors (HER2/neu), CD markers and others. Antigen retrieval is now a widely accepted method for heat-assisted retrieval of antigens in FFPE tissues prior to IHC staining (9, 10).

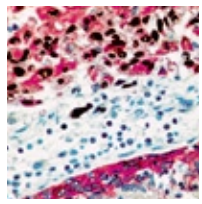
More recently, combinations of enzymatic digestion and heat-induced antigen retrieval have been reported. Iczkowski et al (11) combined steam heat with protease digestion, an EDTA buffer of pH 8.0, and obtained staining with monoclonal anti-keratin antibody 34ßE12. This staining was found to be superior to that obtained when only one of these measures was applied. Detailed information on optimal methods of antigen retrieval are usually available in the manufacturer's product specification sheet.

Principle and Technique

Antigen retrieval relies on application of heat to FFPE tissue sections in an aqueous medium. After deparaffinizing and rehydrating tissue sections, 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 two, seven or 10. Recent systematic comparisons of several retrieval solutions showed that 0.01 M TRIS-HCl, pH 1 or 10, was slightly superior to citrate buffer of pH 6.0 and gave the best overall results (12).

Following immersion in the preheated retrieval solution, containers holding the slides are exposed to heat. This step is critical and the degree to which immunoreactivity can be restored is related directly to the duration of incubation and the attained temperature. The most commonly used heating methods include microwave ovens, autoclaves, steamers, pressure cookers and water baths (7, 8, 13-16). Their advantages and disadvantages, however, are subject to ongoing experimentations whose preliminary results have been summarized by Battifora et al (17). Although an optimal temperature has not been established, most AR methods apply temperatures near the boiling point of water. Optimal length of exposure to heat may vary from 10 minutes 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 usually is allowed to take place slowly, requiring another 20 minutes to 30 minutes.

At higher elevations (above 4,500 feet or 1,200 meters), boiling of the AR 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 AR solution until



the desired staining intensity is achieved (18). An additional possible solution is to use a closed pressure system, such as a pressure cooker or autoclave to achieve temperatures of at least 95 °C. However, each laboratory must determine the best method and antigen retrieval time for their particular circumstances.

Several studies have now confirmed that pressurized systems supporting higher temperatures (up to about 120 °C) have yielded superior results in terms of intensity and number of sites stained, compared to non-pressurized systems operating at lower temperatures (19-22).

Mechanism of Action

The precise mechanism of action of AR has long fascinated researchers and fueled numerous investigations into this mystery (23-27). However, in spite of these efforts the exact mechanism still remains largely unknown today. In view of the complexity of antigens, this observation is not surprising. Heat is obviously of great importance in reversing the damage caused by fixation with formalin and embedding in paraffin. Whatever the mechanism, some of the cross-linking induced by formalin must remain intact, as without this stabilizing structure, proteins would be denatured irreversibly by the heat used during AR. This seemingly contradictory observation can be explained only 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 almost certainly will provide new insights and help us to understand what we can presently only accept.

Cytology

Methods of AR also have been used successfully for some cytology specimens. It has been shown that by certain modifications, AR procedures can be used successfully 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 immunoreactivity was facilitated by an increase in cell membranes' permeability, 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. For alcohol-fixed specimens it was also necessary to reduce the temperature to 37 °C in order to maintain morphology. However by including formalin in the fixative, no modifications were necessary, and standard high-temperature AR yielded optimal results without compromising morphology (see Methods of Immunocytology for Slide-Based Cellular Analysis, Chapter 12, for further details).

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 high-temperature target retrieval. This combined protocol provides better overall results than either method alone. For greater detail, see In Situ Hybridization, Chapter 11.

Antigen Retrieval and Its Use in Doublestaining

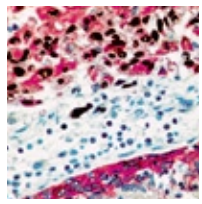
One of the prerequisites for the successful sequential staining of several antigens in the same tissue section is the removal of all reactants prior to applying the subsequent primary antibody. Dako's EnVision™ Doublestain System accomplishes this using of an acid-elution step, leaving behind only the converted chromogen of the first cycle. However, depending on the affinity of the first antibody, this method occasionally left some primary antibody still bound to the tissue. An alternative method that we have found to be superior is the intervening use of antigen retrieval prior to the application of the subsequent primary antibody. Apparently high temperature results in nearly complete "removal" of bound antibodies, because these proteins have not been fixed, while the fixed proteins of the tissue remain intact.

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 employed. The following chromogens 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 useful. Antigen retrieval has made a significant contribution in this endeavor, as many markers previously believed to be lost to the process of FFPE now can be demonstrated routinely. 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 reevaluating staining results and clinical interpretation (12).

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, 12), and very likely optimized for each separate antigen (28).



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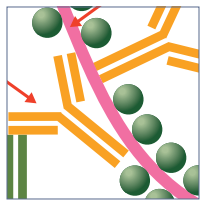
Footnote

*Alternate terminology 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 also have been applied to the retrieval of nucleic acid targets for in situ hybridization.



Chapter 7 ■ Immunohistochemistry Staining Methods

Marc Key

Introduction

Immunohistochemistry has emerged as a powerful investigative tool that can provide supplemental information to the routine morphological assessment of tissues. The use of immunohistochemistry to study cellular markers that define specific phenotypes has provided important diagnostic, prognostic, and predictive information relative to disease status and biology. The application of antibodies to the molecular study of tissue pathology has required adaptation and refinement of immunohistochemical techniques, particularly for use in fixed tissues. In contrast to solution-based immunoassays that detect relatively abundant native proteins, in fixed tissues the preservation of antigen is variable and unpredictable. Thus, the history of immunohistochemistry has been a constant effort to improve sensitivity for detection of rare surviving antigenic targets with the ultimate goal of integrating tissue-based analysis with proteomic information.

Immunohistochemistry: In the Beginning

Because of the superior morphology provided by formalin-fixed paraffin-embedded tissues, this has become the medium of choice for most clinical and research studies. The peroxidase-labeled antibody method, introduced in 1968, was the first practical application of antibodies to paraffin-embedded tissues and overcame some of the limitations of earlier fluorescence antibody methods (1). These pioneering studies using enzyme labels instead of fluorescent dyes opened the door to the development of modern methods of immuohistochemistry.

The successful application of immunohistochemical methods to formalin-fixed surgical pathology specimens stimulated rapid progress in this newly emerging field, and in quick succession came the introduction of the immunoperoxidase bridge method (2) and the peroxidase anti-peroxidase (PAP) complex method (3).

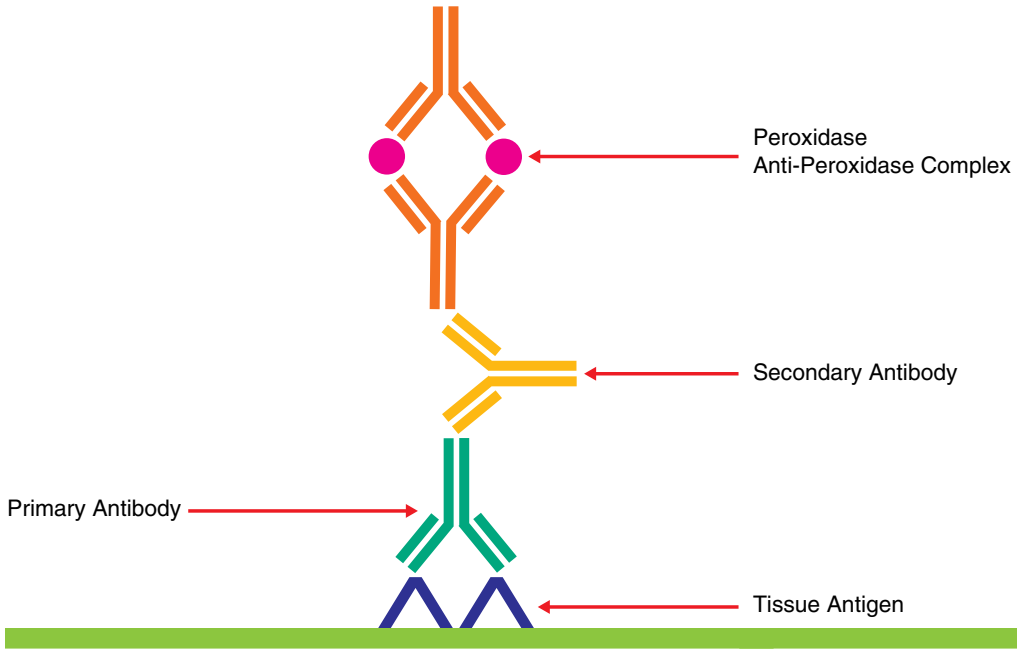
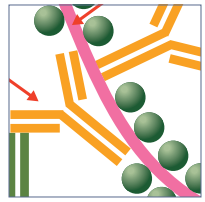


Figure 7.1. Peroxidase Anti-Peroxidase (PAP) Complex Method

Avidin-Biotin Immunohistochemistry

In 1981 a new generation of immunohistochemical methods emerged with the advent of the avidin-biotin methods, which remains widely used today (4). All avidin-biotin methods rely on the strong affinity of avidin or streptavidin for the vitamin biotin.

Streptavidin (from Streptomyces avidinii) and avidin (from chicken egg) both possess four binding sites for biotin. The biotin molecule is conjugated easily to antibodies and enzymes. In the avidin-biotin complex (ABC) method secondary antibodies are conjugated to biotin and function as links between tissue-bound primary antibodies and an avidin-biotin-peroxidase complex (5).



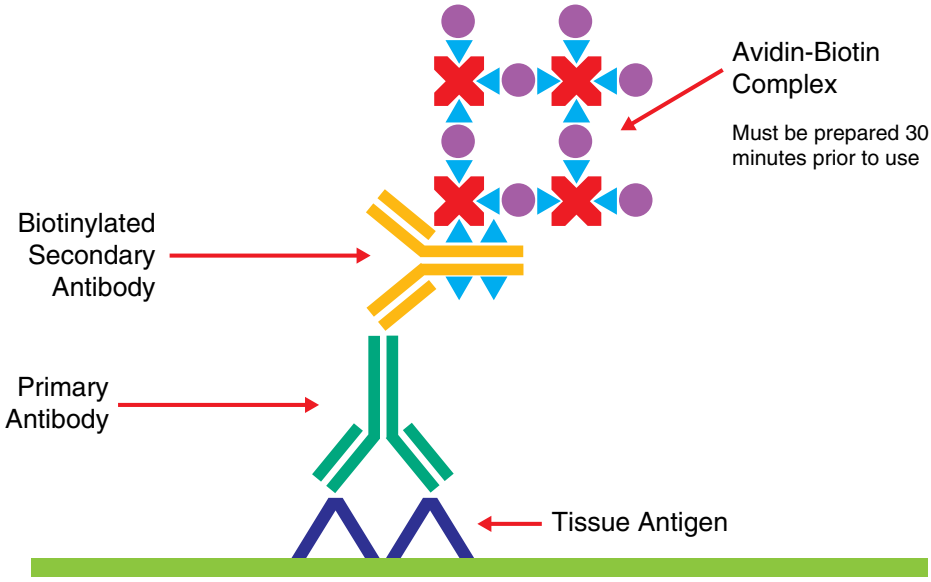


Figure 7.2. Avidin-Biotin Complex (ABC) Method

In a similar method, the labeled streptavidin-biotin (LSAB) method also utilizes a biotinylated secondary antibody that links primary antibodies to a streptavidin-peroxidase conjugate (6). In both methods a single primary antibody subsequently is associated with multiple peroxidase molecules, and because of the large enzyme-to-antibody ratio, a considerable increase in sensitivity is achieved compared to direct peroxidase-conjugate methods.

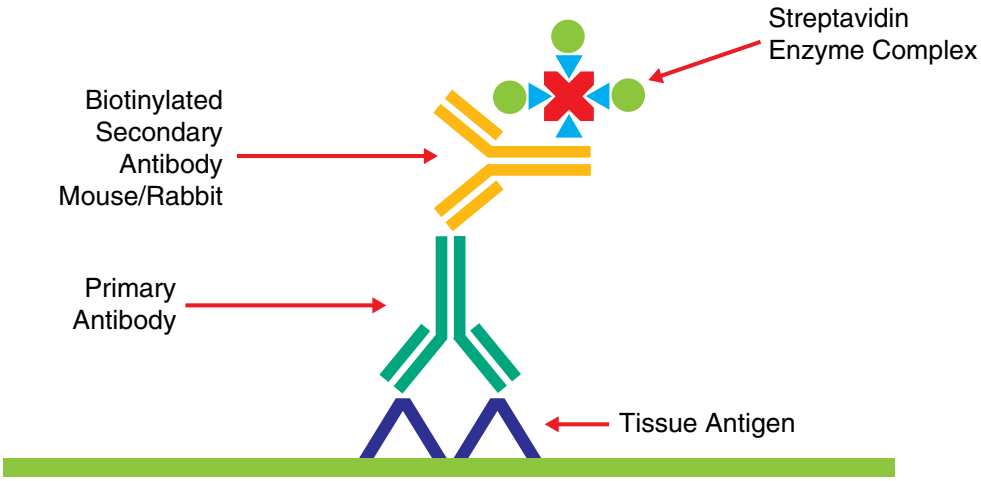


Figure 7.3. Labeled Streptavidin-Biotin (LSAB) Method

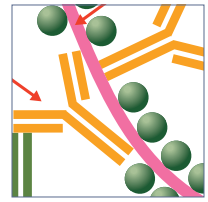
Because avidin is a glycoprotein and has an isoelectric point (pI) of 10, it has a propensity to bind non-specifically to lectin-like and negatively charged tissue components at physiological pH. In contrast to avidin, streptavidin has a more neutral isoelectric point and lacks the carbohydrate moieties. These differences result in less nonspecific tissue binding.

Polymer-Based Immunohistochemistry

Although many of these (strept) avidin-biotin methods are still in widespread use, there are certain limitations characteristic of these methods. The presence of endogenous biotin in tissues can lead to significant background staining in certain circumstances. Formalin fixation and paraffin embedding has been shown to significantly reduce the expression of endogenous biotin, but residual activity can still be observed in tissues such as liver and kidney. Furthermore, with the advent of heat-induced antigen retrieval, the recovery of endogenous biotin can appear as an unwanted side effect. Methods to block endogenous biotin are partially effective, but add another layer of complexity to an already complex procedure. These limitations are further exacerbated by the use of frozen tissue sections, in which levels of endogenous biotin are usually even higher than those encountered in paraffin-embedded specimens.

Because of these limitations, polymer-based immunohistochemical methods that do not rely on biotin have been introduced and are gaining popularity (5). These methods utilize a unique technology based on a polymer backbone to which multiple antibodies and enzyme molecules are conjugated. In the EPOS (Enhanced Polymer One Step)* system, as many as 70 enzyme molecules and about 10 primary antibodies are conjugated to a dextran backbone. This allows the entire immunohistochemical staining procedure, from primary antibody to enzyme, to be accomplished in a single step (6). On the other hand, one limitation of this method is that it is restricted to a select group of primary antibodies provided by the manufacturer, and not suitable for user-supplied primary antibodies.

To overcome this limitation a new type of dextran polymer, EnVision™ +*, was introduced. This polymer system contained a dextran backbone to which multiple enzyme molecules were attached. However, unlike EPOS, which contained primary antibodies, the EnVision™ system contained secondary antibodies with anti-mouse Ig and anti-rabbit Ig specificity. This universal reagent could be used to detect any tissue-bound primary antibody of mouse or rabbit origin. The utility of this method opened the door to a new family of polymer-based immunohistochemical methods. The sensitivity of these methods compared to LSAB and ABC methods was comparable or even slightly greater in most cases (7). However, because of the large molecular size of the polymer conjugates, accessibility to certain epitopes was restricted, presumably due to steric hindrance, in a minority of cases.



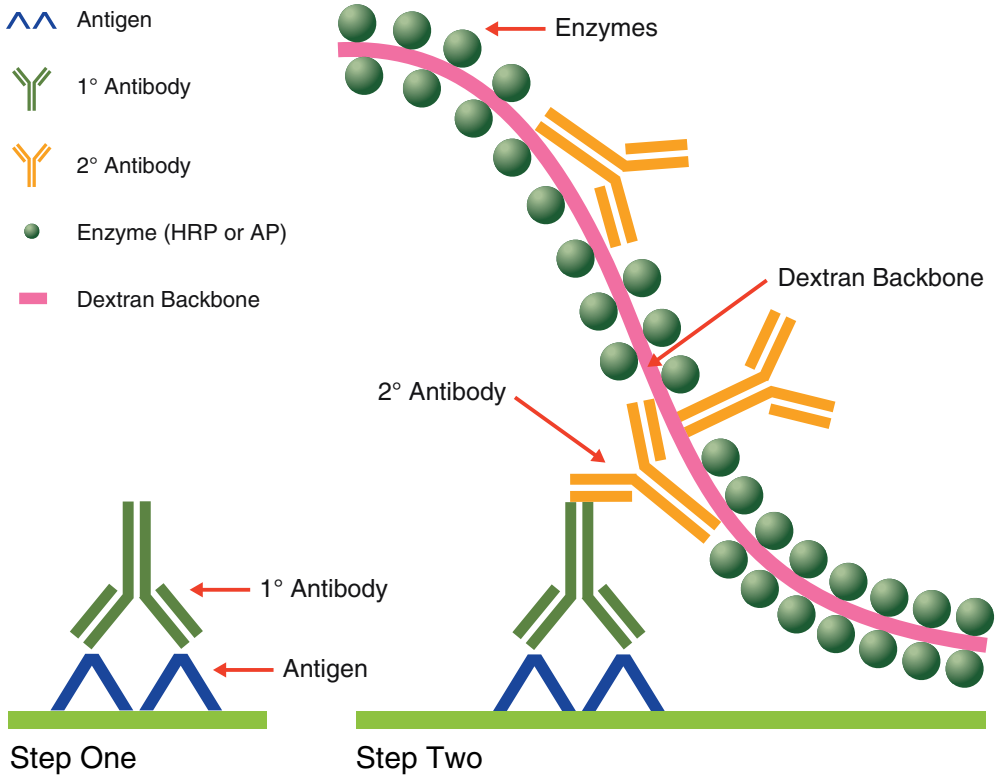


Figure 7.4. Two-Step Polymer Method (Envision™)

Tyramide Amplification

The tyramide amplification technique is based on the ability of phenolic compounds to become oxidized to highly reactive and unstable intermediates (8). When biotinyl tyramide is oxidized, dimerization with electron-rich aromatic compounds, such as those found in protein molecules, occurs (9). This reaction can be harnessed in immunohistochemistry to generate highly reactive biotinyl-tyramide intermediates that bind rapidly to protein molecules in the immediate vicinity of peroxidase enzymes. This reaction results in the deposition of numerous biotin signals. In a typical immunohistochemistry procedure, peroxidase enzymes are associated first with primary antibodies by any of the standard immunohistochemical methods, for example by the ABC or LSAB methods. Biotinyl tyramide and hydrogen peroxide are applied as a substrate to generate numerous biotin (biotinyl tyramide) signals. These biotin molecules then can be used to capture subsequent streptavidin-peroxidase enzymes that are converted to a chromogenic endpoint via diaminobenzidine or similar chromogenic substrates (10).

Cycled Tyramide Amplification

The sequence of streptavidin-peroxidase and biotinyl-tyraminde can be applied alternately to perform a cycled tyramide amplification procedure. In practicality, however, cycling usually cannot exceed two or three cycles before background staining limits the utility of this approach. Commercial tyramide amplification products are available and include Tyramide Signal Amplification (TSA, DuPont NEN Life Sciences, Boston, MA) and Catalyzed Signal Amplification (CSA)*.

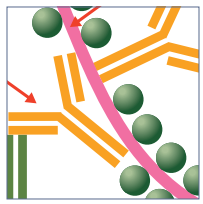
Fluorescyl-Tyramide Amplification

In keeping with current trends in immunohistochemistry to develop alternatives to biotin-streptavidin detection methods, a fluorescyl-tyramide amplification system has been introduced recently (FT-CSA)*. In this procedure peroxidase is associated with a tissue-bound primary antibody by application of a secondary anti-mouse Ig antibody to which peroxidase has been conjugated. The peroxidase catalyzes the conversion and deposition of fluorescyl-tyramide onto the tissue section. At this point the reaction can be terminated and viewed by fluorescence microscopy, or the signal can be converted to a colorimetric reaction by the sequential application of an anti-fluorsecein antibody conjugated to peroxidase followed by a diaminobenzidine-hydrogen peroxide substrate.

In comparison to standard IHC methods, tyramide amplification methods typically have increased sensitivity by at least 50 fold or greater (11). As with any amplification method, background tends to increase along with signal. Therefore it is essential to run appropriate positive and negative controls and interpret any positive staining within the context of the negative control.

Rolling Circle Amplification

Rolling Circle Amplification (RCA) is a novel signal amplification system that generates a local signal via extension and amplification of an oligonucleotide tail. Although initially developed for nucleic acid detection, this method also can be applied to immunohistochemistry. RCA-mediated immunohistochemistry has been applied successfully to the detection of a variety of cell surface and intracellular molecules (12). The method utilizes a short oligonucleotide sequence coupled to a primary or secondary antibody molecule. After binding to the tissue, a circularized nucleic acid probe with a complementary sequence is hybridized to the oligonucleotide. The oligonucleotide then acts as a primer and is extended linearly using a DNA polymerase and the rolling circle. Then the extended DNA is hybridized with labeled oligonucleotide probes. These labels may include, for example, biotin, which then can be visualized by any one of the many avidin-biotin detection methods. RCA derives its specificity from an antigen-antibody reaction and its sensitivity from nucleic acid synthesis. RCA has been reported to generate a 10^5-fold increase in signal (13).



Conclusion

As immunohistochemical techniques continue to evolve, their application to surgical and research pathology is becoming increasingly valuable. Various amplification methods have made significant improvements to this technology such that many antigens, previously believed to have been lost to the process of fixation and embedding, now can be demonstrated routinely. However, as the sensitivity of immunohistochemistry continues to increase, accepted staining criteria and clinical interpretation may require re-evaluation.

New signal amplification methods continue to be developed, each with their own unique strengths and weaknesses, and this can present a bewildering assortment of choices that profoundly influence the practice of immunohistochemistry to the investigator or clinician. As technology marches forward, new arrays of tissue markers are emerging that are providing the tools to generate important new discoveries. As new markers are added to this list, our knowledge of the underlying biology and pathogenesis of disease is increased. The full impact is still many years away.

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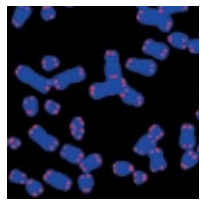
Footnote

* A proprietary methodology developed by Dako.



Chapter 8 ■ Immunofluorescence

W. Roy Overton, Revised by Jim Hudson and Karen Atwood

Introduction

Fluorescent dyes were developed first in the late 19th century. But it wasn't until 1950 that antibodies conjugated with fluorescein isothiocyanate were utilized to produce specific biological staining to generate fluorescence in a specimen. These stained tissues and cells could be examined readily by fluorescence microscopy. Today, attaching fluorescent compounds, or "fluorochromes," to antibodies enables scientists and clinicians to gain tremendous information about the biology and pathology of cells from humans, animals, plants and microbes. The following is a discussion of these compounds.

Among the properties of many compounds is the ability to absorb one color of light and then emit a different color of light. This causes the compound to appear to be glowing, or fluorescing, and the compound is referred to as a fluorochrome. A "fluorophore" is a component of a molecule that causes a molecule to fluoresce. It is a functional group in a molecule that absorbs energy of a specific wavelength and re-emits energy at a different wavelength. Fluorescein isothiocyanate is an example of a fluorophore that can be attached chemically to a different, non-fluorescent molecule to create a new and fluorescent molecule.

To better understand how a fluorochrome works, it is necessary to examine this process at the subatomic level. Electrons normally spin around the nucleus of an atom at a distance that is referred to as the electron's "ground state" or "ground level." If the atom is hit by photons of light that can excite the electron, such as ultraviolet radiation, then the electron will move up to a higher energy state that is farther from the nucleus. The electron is unable to maintain that distance from the nucleus due to the electron's magnetic attraction to the protons in the nucleus, so quickly drops to a slightly closer distance, called the "lowest singlet excited state." Moving to the lowest singlet excited state causes the electron to release a little of the energy that it got from the light, but it releases energy as heat. The electron then returns to its original ground state, releasing the rest of the energy that it absorbed from the light. This energy is released as light, but since some energy was released as heat into the lattice of the molecule, there is less energy in that light. Thus, the emitted light appears to be a different color than the light that excited the atom.

Light travels in waves that determine the color of the light. If the light has a lot of energy, its waves are shorter than a wave of light with less energy. The wavelength of the light determines the color of the light. Very high-energy light such as ultraviolet (UV) light has very short wavelengths. The wavelength of UV light is less than 400 nm. Because the wavelength is so short, the human eye is unable to see UV light, but electronic

photodetectors are able to detect and measure this invisible light. Visible light has wavelengths from 400 nm to 700 nm, that produce the colors violet, blue, green, yellow, orange and red. Above 700 nm is the infrared range, which is also invisible to the human eye, but can be felt as heat or detected by electronic photodetectors.

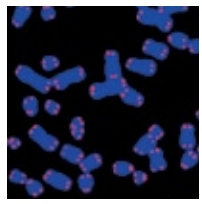
In immunofluorescent staining, mercury-vapor, xenon or halogen lamps in the fluorescent microscope usually are used to excite the fluorochromes. The chemical properties of the fluorochrome determine whether its electrons can be excited to the higher energy state by a specific wavelength. If the electrons can be excited to the higher energy state, the chemical properties of the fluorochrome also will determine the amount of energy lost as heat when the electrons drop back down to the lowest singlet excited state and the wavelength of light produced when the electrons return to their ground state. The difference in the wavelength of the light that excites the electrons and the light that is emitted is called the "Stokes shift," and is determined by the amount of energy lost as heat. The emitted wavelength is always longer (if single photons are absorbed) or equal to the incident wavelength, due to energy conservation.

Some fluorochromes have a small Stokes shift, and the excitation and emission wavelengths have almost the same wavelengths. But other fluorescent compounds have large Stokes shifts. For example, the fluorochrome, fluorescein, can be excited by blue-green light, and its Stokes shift is only about 25 nm, which means that the light emitted is green. This contrasts with another fluorochrome, phycoerythrin, which also can be excited by blue-green light, but has a large Stokes shift. Thus, the light emitted is yellow-orange. In immunofluorescence, a single wavelength can be used to excite several fluorochromes with different Stokes shifts and, thereby, produce a variety of fluorescent colors.

The electrons of a fluorochrome can be excited by a range of wavelengths of light. For example, the fluorochrome, fluorescein, will fluoresce when hit by light with a wavelength between 430 nm and 520 nm. However, the closer the excitation wavelength is to 495 nm, the more fluorescence will be produced. This optimal wavelength is called the excitation peak. Similarly, the light produced by fluorochromes has a range of wavelengths. The emission of light from fluorescein ranges from 490 nm to 630 nm, and the emission peak is approximately 520 nm.

Knowing the excitation and emission properties of fluorescent compounds makes it possible to select combinations of fluorochromes that will work together. However, for a fluorochrome to be useful in a biological application, it must attach to or be contained within a particle of biological significance. Many fluorochromes can be attached to antibodies, which then will bind to specific chemical structures on or inside of cells.

There are many other chemical and physical properties of fluorochromes that determine where and when these dyes are useful in various biological assays. For example, some of the fluorochromes that bind to DNA, such as Hoechst 3342, can get into living cells, but most DNA-binding fluorochromes cannot get past the cell membrane. The fluorescent dyes that cannot get past a viable cell membrane, such as propidium iodide, often are used to distinguish live from dead or dying cells.



Fading, Quenching and Photobleaching

Fluorescence detection can be affected adversely by “fading.” This is a reduction of fluorescent emission intensity caused by photobleaching or quenching.

Photobleaching is an irreversible degradation of the activated or excited fluorochrome as a result of its interaction with molecular oxygen (1). Illumination of the fluorochromes by light of the appropriate wavelength can result in an excited state that renders it more chemically reactive. It then may react irreversibly with any available oxygen molecules. This interaction may result in decomposition, polymerization, or oxidation and subsequent reaction with another molecule.

Photobleaching can be minimized by using the lowest illumination possible. To minimize the effects of photobleaching, fluorescence microscopy can be combined with other techniques that are non-destructive to fluorochromes, such as differential interference contrast (DIC), Hoffman modulation contrast (HMC), and phase contrast. The operator locates the area of interest on the slide using a non-destructive low level light before switching on the fluorescence excitation light.

Quenching of an excited fluorochrome may occur due to a non-radiative or radiative energy loss and is mediated through compounds in the micro-environment. Non-radiative energy loss and reduction in fluorescence intensity can be caused by the presence of oxidizing agents, salts, heavy metals or halogen compounds. In some cases, quenching results from radiative energy loss or the transfer of energy from the fluorochrome (donor molecule) to another molecule (acceptor) that resides physically close to the excited fluorochrome. This is called fluorescence resonance energy transfer (FRET).

There are several commercially available anti-fading mounting media available that significantly reduce this phenomenon (2).

Fluorescein

Purified antibodies are conjugated with fluorescein isothiocyanate isomer (FITC). After conjugation, unreacted FITC is removed completely by gel filtration on Sephadex G-25. Further purification is carried out by ion exchange chromatography. This process removes unconjugated antibody molecules and antibody molecules to which more than four molecules of FITC are attached. These conjugates consist of optimally labeled antibody molecules for immunofluorescent use. Then fluorescein/protein ratio measured as the absorbance ratio measured as the absorbance ration A495nm/A278nm is 0.65, corresponding to a molar FITC/protein ratio of 2.3-2.5. FITC conjugates with a higher F/P value, such as those prepared for flow cytometric analysis, are not suitable for immunofluorescence, as they will produce high background, false positive staining and possible self-quenching of the FITC molecule.

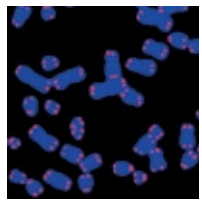
The FITC-labeled immunofluorescent stain does not require strong ultra-violet radiation for excitation. With a peak absorption of 495, light generated by a quartz-halogen or xenon lamp is sufficient.

Procedure for Formalin-Fixed Paraffin-Embedded Tissue

- Cut four micron sections onto charged or silanized slides.
- Allow sections to dry at 37 °C for four hours.
- De-wax and hydrate tissue sections.
- Rinse in deionized (DI) water.
- Wash in PBS for 10 minutes at 4 °C.
- Enzyme digest tissue sections if proteolytic treatment is required.
- Stop enzyme digestion by incubation in TBS for 60 minutes at 4 °C.
- Wash in PBS for 10 minutes at 4 °C.
- An optional serum block may be used at this point to block Fc receptors. Tap off excess.
- Incubate with FITC conjugated antibodies, diluted in PBS and one percent bovine serum albumin, in a moist chamber for 20 minutes at room temperature.
- Rinse slides in PBS to remove excess antiserum.
- Wash in PBS for 10 minutes at 4 °C (2x).
- Mount with fluorescent mounting medium .
- Seal coverslip edges with clear nail polish and store at 2-8 °C in the dark.

Procedure for Frozen Sections

- Cut four micron cryostat sections.
- Fixation:
  - Fix in acetone for 10 minutes at 4 °C, air dry for 30 minutes, post fix in acetone for 90 seconds at 4 °C;
  - Air dry for 1-12 hours, fix in acetone for 10 minutes at 4 °C.
- Wash in PBS for one to five minutes at 4 °C.
- An optional serum block may be used at this point to block Fc receptors
- Tap off excess.
- Incubate with FITC conjugated antibodies, diluted in PBS and one percent bovine serum albumin, in a moist chamber for 20 minutes at room temperature.
- Rinse slides in PBS to remove excess antiserum.
- Wash in PBS for 10 minutes at 4 °C (2x).
- Mount with fluorescent mounting medium.
- Seal coverslip edges with clear nail polish and store at 2-8 °C in the dark.



Buffers:

- Phosphate buffered saline: pH 7.2:
  - 33.99 g NaCl;
  - 9.258 g Na2HP42H2O;
  - 2.15 g KH2OPO4.
  - Bring to five liters with DI water.
- Phosphate buffered saline: pH 7.0;
  - Dako Code S3024.
- Tris-Buffered Saline:
  - Dako Code S1968 or S3001.
- Pronase:
  - Dako Code S2013.

Table 8.1. Antibody dilution chart for direct immunofluorescence on frozen sections.

Code	Dako FITC Conjugated Antibody	Dilution in PBS
F0117	Albumin	1:20-1:40
F0254	C1q Complement	1:10-1:20
F0201	C3c Complement	1:75-1:100
F0111	Fibrinogen	1:50-1:70
F0204	IgA	1:20-1:40
F0202	IgG	1:20-1:40
F0203	IgM	1:20-1:40
F0198	Kappa Light Chains	1:20-1:40
F0199	Lambda Light Chains	1:20-1:40
X0929	Negative Reagent Control	See NOTE

NOTE: The negative reagent control is used on an additional tissue section. To determine the dilution factor, calculate the total protein (TP) of each antibody to be used in the test run (TP of primary antibody/optimized dilution factor). Select the highest calculated TP and divide it into the TP of the negative reagent control (TP negative reagent control/ highest calculated TP). This is the dilution factor of the negative reagent control for the run.

Positive and negative controls are required now by the Clinical Laboratory Improvement Amendments of 1988 (CLIA) for each staining run. Positive and negative tissues are stained with the antibody.

An unstained control slide may be used to determine if autofluorescence is present. Primary or autofluorescence is seen when a specimen has not been labeled with a fluorescing dye, but still manifests fluorescence during exposure to short wavelength energy. Tissue autofluorescing substances include: Elastic fibers, lipofuscins, collagen, porphyrins, vitamin A, cartilage, keratin, cardiac muscle, fat, waxes, hormones, calcium and powdered crude drugs. Autofluorescence also may be caused by free aldehydes from fixation.

A number of “home-brew” procedures have been shown to quench autofluorescence. Those include pronase digestion; 100 mM glycine in PBS (pH 7.4) for 10-20 minutes; 50 mM ammonium chloride in PBS for 10 minutes; and one percent sodium borohydride in PBS for 10-20 minutes.

Other factors that can contribute to unexpected or undesired staining include heat-induced epitope retrieval (HIER) lipofusion artifacts; reagent contamination; sub-optimal temperature and duration at which the reagents are incubated; dead or necrotic cells and Tween 20 added to the wash buffer.

In addition, due to the size of some fluorochromes such as RPE, some conjugated antibodies may become trapped in an intact cell during analysis for intracellular antigens.

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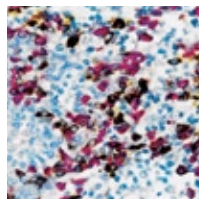
2. Ono M, Murakami T, Kudo A, Isshiki M, Sawada H, and Segawa A. Quantitative Comparison of Anti-Fading Mounting Media for Confocal Laser Scanning Microscopy. J Histochem Cytochem 2001;49:305-12.



Chapter 9 ■ Multi-Staining Immunohistochemistry

Nanna K. Christensen and Lars Winther

Introduction

Immunohistochemistry (IHC) has become established as an important tool for both research and diagnostic purposes. However, in some cases there is a need for knowledge about the relative localizations of targets, which can be obtained only by visualizing all relevant targets on one slide. This chapter describes the advantages of multiple staining, as well as the considerations that have to be made to ensure successful staining. This article will discuss the choice of appropriate protocols as well as the choice of visualization systems.

Advantages of Multiple Staining

Multiple staining can be defined as the detection of two or more targets on one slide, thus increasing the information obtained from each slide. Hands-on time per slide depends on the method used. Sequential staining does not reduce hands-on time compared to combining single staining; whereas simultaneous staining does reduce turn-around time (see below). With increasing demand for reduced turn-around-time, multiple staining may offer at least part of the solution. Furthermore, there is a demand for less invasive sampling techniques giving smaller and fewer specimens and available slides. In such cases multiple staining also may be a great advantage.

Equally important, multiple staining makes it possible to assess the topographic relationship of the targets, for example, to determine whether targets are present in different cells, in the same cell or even in the same cellular compartment. Information also can be obtained on possible cell-to-cell spatial contacts of different cell types. Some of this information also can be obtained using single staining on serial sections. However, this is laborious and time consuming and the sections must be very thin to ensure all structures or cells are present in the entire series of sections. Multiple staining allows the combination of in situ hybridization (ISH) and IHC, giving information about a particular target both at protein level and DNA/mRNA level.

The diagnosis of prostatic epithelial neoplasia (PIN) is just one example of the clinical importance of multiple staining. Prostatic needle biopsy is the preferred method for diagnosing early prostate cancer. But in some cases an ambiguous diagnosis is made due to the fact that the biopsy has identified only a few malignant glands or several histological benign mimics of cancer (1). Since basal cells are present in the benign cancer mimics but absent in the malignant glands, these cells can be used to distinguish between the two cases. Basal cells are labeled using high-molecular-weight cytokeratin, cytokeratin 5/6 or p63 immunostaining. In addition, the gene product of p504s, alpha-methylacyl-CoA-racemase is expressed in a high percentage of prostate carcinomas, but is negative or only weakly expressed in benign prostate tissue. Thus it is used as

a positive cancer marker. In cases of small foci, ambiguous lesions may disappear when using serial sections, causing suspected malignancies to remain undiagnosed. A multiple staining protocol reduces the percentage of residual ambiguous lesions and the need for additional biopsies.

Multiple staining is well known from flow cytometry, where staining of three to four different targets on the same cell is routine. A single sample elicits considerable information, allowing unambiguous separation of different cell-types and identification of abnormalities. ISH routinely uses multiple staining on slides to determine gene amplification from the ratio of the signals from the gene probe of interest to a reference probe.

ISH also can be used in multiple staining to detect chromosome translocations using split-signal FISH. Probes directed towards stretches immediately upstream and downstream of the breakpoint are labeled green and red, respectively. Thus when probes are co-localized, the mixture of green and red results in a yellow signal, but when the chromosome breaks, the signals separate and individual green and red signals can be seen. In this case, single-target staining would not give the desired information. Multiple staining is well established in ISH, and there are obvious advantages to extending this to an IHC format to gain the benefit of the additional information.

Technical Challenges

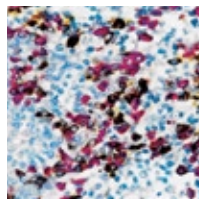
Users experienced with the challenges of single-target staining will find the demands for multiple staining are similar but more complex.

Before embarking on a multi-staining project, some important issues should be considered:

- To avoid target or species cross-reactivity, complex protocols may be necessary.
- Spectral differentiation of stain colors may be difficult, especially if the targets are co-localized. Rare targets that are co-localized with more abundant targets may not show, and if colors are mixed, results may be difficult to separate from single colors.
- Even if targets are not co-localized it is difficult to balance signals enabling rare targets to be visible in the same slide as highly abundant targets. This is due to the narrow dynamic range of IHC. An adjustment in concentration of the primary antibodies may solve this problem.
- If different targets are viewed under different magnifications, it may not be possible to get the topographic information desired.

Pretreatment

Multiple staining, like single staining, can be performed on both formalin-fixed, paraffin-embedded tissue, cryosections, cell smears and cytospin preparations. Multiple staining is constrained by the fact that it may not be possible to find one tissue pre-treatment



protocol that is optimal for all targets. Often protocols optimized for individual staining differ from one target to the other; for example, different target retrieval methods are used. If that is the case, it is necessary to determine a method that allows all targets to be stained, although the method may be sub-optimal for some targets.

In cases where targets of different abundances are to be stained, a method must be selected that gives the best balancing of the signals. Combining ISH and IHC on one slide is challenging, particularly because targets require very different pre-treatment protocols. Since ISH processes such as DNA denaturing are not compatible with the presence of the antibodies for IHC, the ISH protocol normally is performed first.

Staining Method Selection

To ensure success, IHC staining must be planned carefully. This is even more important with multi staining. If primary antibodies are commercially available both directly labeled and unlabeled and from different host-species, there are several different staining methods to choose from; however, very often the choice is limited by the available reagents (2). Care must be taken to avoid cross-reactivity between reagents. A flow chart or similar aid might prove useful in selecting the best method.

In general, staining methods can be divided in the following classes:

Sequential Staining

An indirect technique using unlabeled primary antibodies where the staining of one target is completed including the application of the chromogenic dye, before the application of the next.

The primary and secondary antibodies from the first staining are eluted before staining the next target (for an example, see Figure 9.1). This avoids cross-reactivity-related problems; however, elution may be difficult with some high-affinity primary antibodies, leading to spurious double stained structures. This technique therefore is not recommended for evaluation of mixed colors at sites of co-localization. Elution also risks denaturing epitopes of antigens that are to be subsequently visualized. Furthermore, for some chromogens there is a risk that the first chromogen (DAB in particular) shields other targets. Not all reaction products are capable of surviving the rigorous washing required to remove the antibodies. Thus in order to avoid blurry staining results the most robust dyes should be applied first.

Simultaneous Staining

A direct method with directly labeled primary antibodies, or an indirect method based on unlabeled primary antibodies raised in different host species, or of different Ig isotype or IgG subclass (3).

A simple example of this method is when the primary antibodies are fluorescently or enzyme labeled to allow direct visualization. This avoids cross-reactivity but is rarely

practical since some form of amplification is necessary to get sufficient signal. In that case, primary antibodies are conjugated directly with enzymes, biotin, haptens or fluorochromes, subsequently employing the corresponding antibody or streptavidin reagent as the second layer. This is less time-consuming than the sequential method, since primary and secondary antibodies can be mixed together in two incubation steps. However, it requires avoiding all cross-reactivity.

With the indirect method it is also possible to apply timesaving antibody cocktails. Generally it is advantageous to use secondary antibodies raised in the same host in order to prevent any unexpected inter-species cross-reactivity.

Multi-Step Technique

An indirect/direct method combining unlabeled primary antibodies with antibodies that are directly conjugated. The method starts with the staining of the unlabeled antibody/antibodies.

Sequential staining avoids the problem of cross-reactivity but cannot be used for co-localized targets. The technique often leads to a long staining protocol and carries an inherent risk of incorrect double staining due to insufficient elution of one set of reagents before application of the next. Simultaneous staining is less time-consuming since the reagents of each layer can be mixed together. However, the technique can be used only if suitable primary antibodies are available. Multi-step staining can be used when the selection of primary antibodies is limited. However, when using this method, it is not possible to mix reagents.

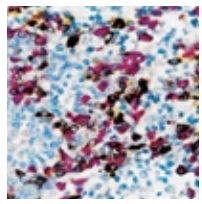
Users often will find that the choice of staining method is limited by the availability of the primary antibodies with respect to species origin or label.

When targets are known or suspected to be co-localized and the only available primary antibodies are unlabeled monoclonal mouse antibodies of the same IgG subclass, none of the techniques described above are applicable.

One solution is the Dako Animal Research Kit (ARK™) Peroxidase, which contains reagents for labeling mouse primary antibodies with a biotinylated anti-mouse Fab fragment, followed by blocking of the remaining reagent with normal mouse serum. This can be applied to the tissue as part of the multi-step technique (4). The kit gives a non-covalently labeled antibody, thus avoiding the risk of reducing the affinity. In addition, only small amounts of primary antibody are needed and the kit does not require time-consuming purification steps.

Another solution is Zenon Technology (Invitrogen) developed for flow cytometry. It uses essentially the same technique, and offers labeling kits for mouse primary antibodies available as enzyme conjugates or conjugated to one of a wide variety of fluorescent dyes.

Visualization systems with dual recognition such as the EnVision™+ Dual Link system do not discriminate between species, and are thus only suitable for multiple staining when



using the sequential method. Visualization kits with amplification layers that are not well specified should be avoided, since possible cross-reactivity cannot be predicted.

Selection of Dyes

The primary choice to make when deciding how to make the targets visible is whether to use immunoenzyme staining or fluorescence. Both have advantages and disadvantages and in the end, decisions should be made based on conditions of the individual experiment.

Chromogenic Dyes

When selecting color combinations for multiple staining with chromogenic dyes, it is advisable to choose opposing colors in the color spectrum, to facilitate spectral differentiation. If using a counterstain, this also must be included in the considerations. When working with co-localized targets, dyes must be chosen so that it is possible to distinguish the mixed color from the individual colors. Double staining using chromogenic dyes is well established, but it is demanding if the targets are co-localized. For triple staining, it is more difficult to get colors that can be unambiguously differentiated and it is very difficult if targets are co-localized.

A narrow dynamic range is a handicap for immunoenzymatic staining. The precipitation process, which is crucial for this method, is only triggered at a certain threshold concentration of substrate/product. On the other hand, at high concentrations the precipitated product may inhibit further reaction. Therefore it is difficult to visualize rare targets and highly abundant targets in the same slide. To reduce this problem using the strongest dye to stain the most rarely expressed target is advantageous. Or if possible use extra amplification like the Catalyzed Signal Amplification (CSA) System to bring rare targets within the same dynamic range as highly expressed targets.

There is a limited range of chromogenic dyes to choose from. These are examples of enzyme/chromogen pairs suitable for triple staining:

- GAL/XGAL/Turquoise, AP/Fast blue, HRP/AEC/Red;
- HRP/DAP/Brown, GAL/XGAL/Turquoise, AP/Fast red;
- HRP/DAP/Brown, AP/New Fucsin/Red, HRP/TMB/Green.

In conclusion, chromogenic dyes can be used successfully for double staining; however, identifying co-localized targets may be a problem. Triple staining is also possible, but great care must be used in selecting dyes.

Fluorescent Dyes

Double immunofluorescence labeling is also quite well established (5). Some of the same considerations as with chromogenic dyes apply when working with immunofluorescence. It is equally necessary to select dyes with distinguishable spectral properties. However, there are more colors available and the emissions spectra of the fluorescent molecules are narrower than the spectra of the chromogenic dyes. DAB in particular has a very broad spectrum. The use of multiple fluorescent colors is also already well established in FISH and flow cytometry, where dichroic filters and bandpass filters are employed to separate different fluorescent signals. The spectral separation can be aided by digital compensation for overlapping emission spectra.

When staining targets that are co-localized fluorescent dyes, allow separate identification of targets. This makes it possible to discern targets even in widely different concentrations, whereas subtly mixed colors may pass unnoticed easily with immunoenzyme staining.

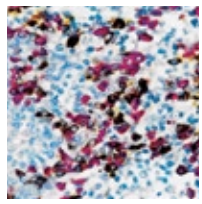
Immunofluorescence potentially has a wider dynamic range than immunoenzyme staining. Using this method, there is no enzymatic amplification involved and thus the dynamic range is determined solely by the sensitivity of the detectors.

On the other hand, there are some inherent problems with the use of immunofluorescence:

- Fluorescence signal is quenched when the fluorochromes are in close proximity.
- Dyes undergo photo bleaching when subjected to light and will thus only fluoresce for a limited time unless stored in the dark.
- Even when stored protected from light, some fluorochromes will deteriorate slowly at room temperature.
- The morphology viewed in slides is different from what is observed in immunoenzyme staining with counterstains.
- Increased background due to autofluorescence can pose a problem when working with some formalin-fixed tissues.
- Leaching of stored antibody conjugates may pose a problem.

In spite of these drawbacks, immunofluorescence gives clear, sharp localization of targets and has advantages over chromogenic dyes when working with co-localized targets.

Some chromogenic dyes fluoresce as well, such as Fast Red, an AP-substrate that is brighter in fluorescence microscopy than in bright field microscopy.



Other Labels

Colloidal gold-labeled antibodies were developed originally for electron microscopy, but with silver-enhancement they are visible with normal light microscopy.

Another example of inorganic stains is Quantum dots (Q-dots). These are fluorescent nanoparticles with varying emission wavelengths, depending on the size of the particle. They can be linked to antibodies or streptavidin as an alternative to fluorochromes (6). However, the size of their conjugates may pose diffusion problems.

Automated Image Acquisition and Analysis

Digital image analysis will increase the number of usable dyes since it does not rely on the human eye for detection and differentiation. A digital image is acquired at excitation wavelengths relevant for the dyes applied, and separate detectors record individual colors. So, for example, digital image analysis will allow the combination fluorescent and immunoenzyme dyes.

Detectors, however, have biased color vision. They amplify colors differently than does the human eye. Therefore dyes used on image analysis should be optimized for the best possible fit with the detector's filter properties.

Image analysis systems contain algorithms that allow compensation for overlapping emission spectra comparable to flow cytometry. They also allow signal gating within an interesting range of wavelengths, enabling users to see only signals within the desired range. Visualizing a combination of several gates with color selected independently of the dyes used for staining may clarify pictures and make conclusions easier to reach. This also makes it possible to set a threshold on signal intensity to exclude unspecific staining or background from final images.

Another advantage of digital image analysis is that it allows signal quantitation. Through software manipulation users, can count how many signal clusters exceed a certain level of intensity, and, potentially, calculate the ratio of different cell-types. For example, an image analysis algorithm can calculate the percentage of cells that stain positive for a certain target, combine that percentage with information of other stained targets and, based on this, highlight diagnosis.

Conclusion

Multiple-target staining will one day be as routine as single-target staining is today.

Use of the technique will expand, since it offers reduced turn-around time and information not obtainable from single-target staining. Availability of labeled primary antibodies, antibodies raised in different host species and multiple staining kits also is likely to increase.

Software for automated image acquisition and analysis will play a key role in this evolution since the limit to how many colors the human eye can distinguish will be

reached soon. Analysis algorithms will never entirely replace a skilled pathologist, but algorithms will improve gradually as the amount of information loaded into underlying databases increases. Eventually algorithms will become sufficiently “experienced” to be able in many cases to suggest a diagnosis, and only the final decision will be left for the pathologist.

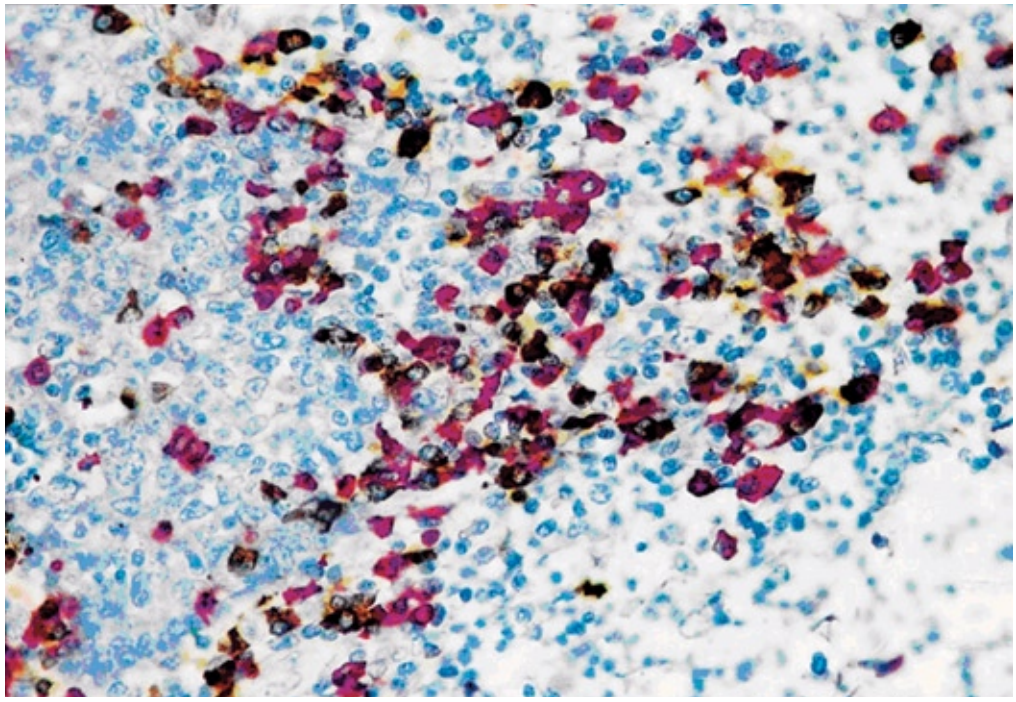


Figure 9.1. Sequential doublestaining method performed with the EnVision™ G|2 Doublestain Kit * using polyclonal anti-kappa (red) and polyclonal anti-lambda (brown) as primary antibodies. Formalin-fixed paraffin-embedded section from tonsil.

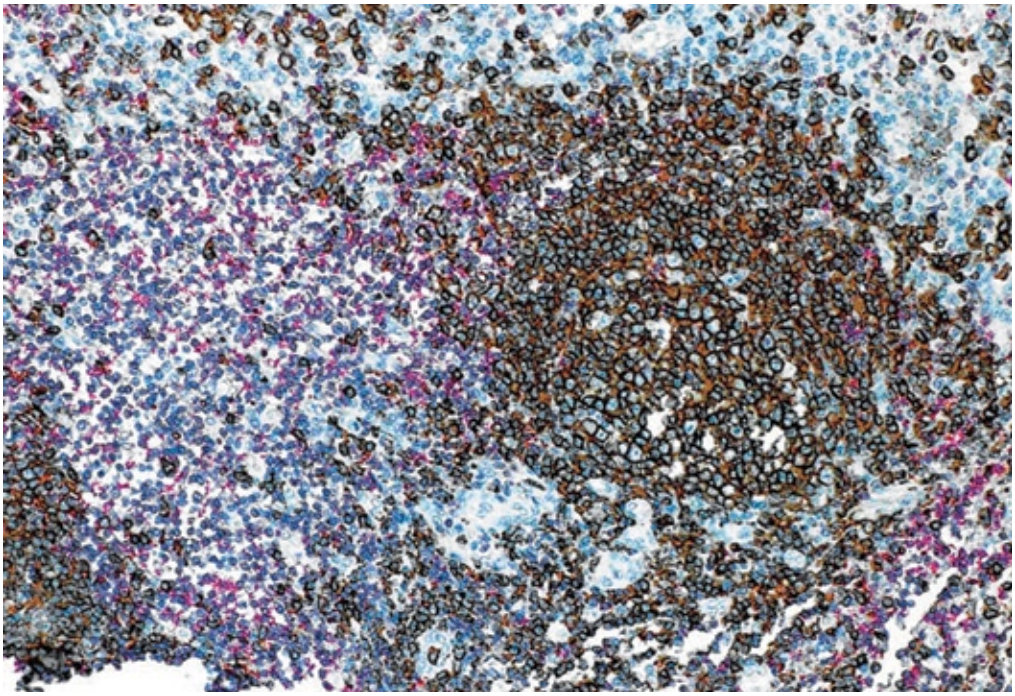


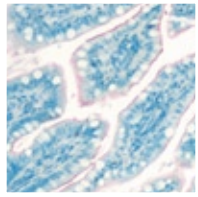
Figure 2. Sequential doublestaining method performed with the EnVision™ G|2 Doublestain Kit * using monoclonal anti-CD3 (red) and monoclonal anti-CD20 (brown) as primary antibodies. Formalin-fixed paraffin-embedded sections from tonsils.

References

1. Mol Molinié V et al. Modern Pathology 2004;17:180-90.
2. Van der Loos CM Immunoenzyme Multiple Staining Methods, BIOS Scientific Publishers Ltd 1999.
3. Chaubert P et al. Modern Pathology 1997;10(6):585-91.
4. Van der Loos CM and Göbel H J Histochem Cytochem 2000;48(10):1431-7.
5. Mason DY et al. J Pathol 2000;191:452-61.
6. Wu X et al. Nat Biotechnol 2003;21(1):41-6.

Footnote

* A proprietary methodology developed by Dako.



Chapter 10 ■ Ancillary Methods in Immunohistochemistry

Gale E. Pace

Introduction

Ancillary reagents in immunohistochemistry are supplemental components – peripheral elements that maximize quality and reliability. This chapter covers these components, overlapping topics from other chapters, but addressing them from a different perspective.

These reagents are subordinate to the main reaction of an antibody with its target epitope. They include some of the following components: Protein blocks, endogenous enzyme blocks, avidin/biotin reducing agents, chromogen enhancers, antibody diluents, enzymatic epitope retrieval solutions, wash buffers and water. Understanding the effects of each type of reagent when used independently or in conjunction with other ancillary reagents is crucial to obtaining the desired results.

Enzymatic epitope retrieval is defined as a method used to relax the rigidity of the protein structure that results from the cross linkages of formalin fixation. Proteolytic enzymes are used in an attempt to restore the immunodominant structure in the epitope of interest. This method makes an epitope available to associate with its antibody. As noted in the antigen retrieval chapter, the retrievable epitopes may be associated with Schiff bases, while the methylene bridges are considered irreversible to maintain structural integrity. Proteolytic enzymes are thought to cleave proteins at specific locations depending on the specificity of the enzyme. If cleavage points are in proximity to a cross-link, then the resulting effect is a relaxation of the rigid protein structure facilitating contact between the primary antibody and the corresponding antigenic determinant.

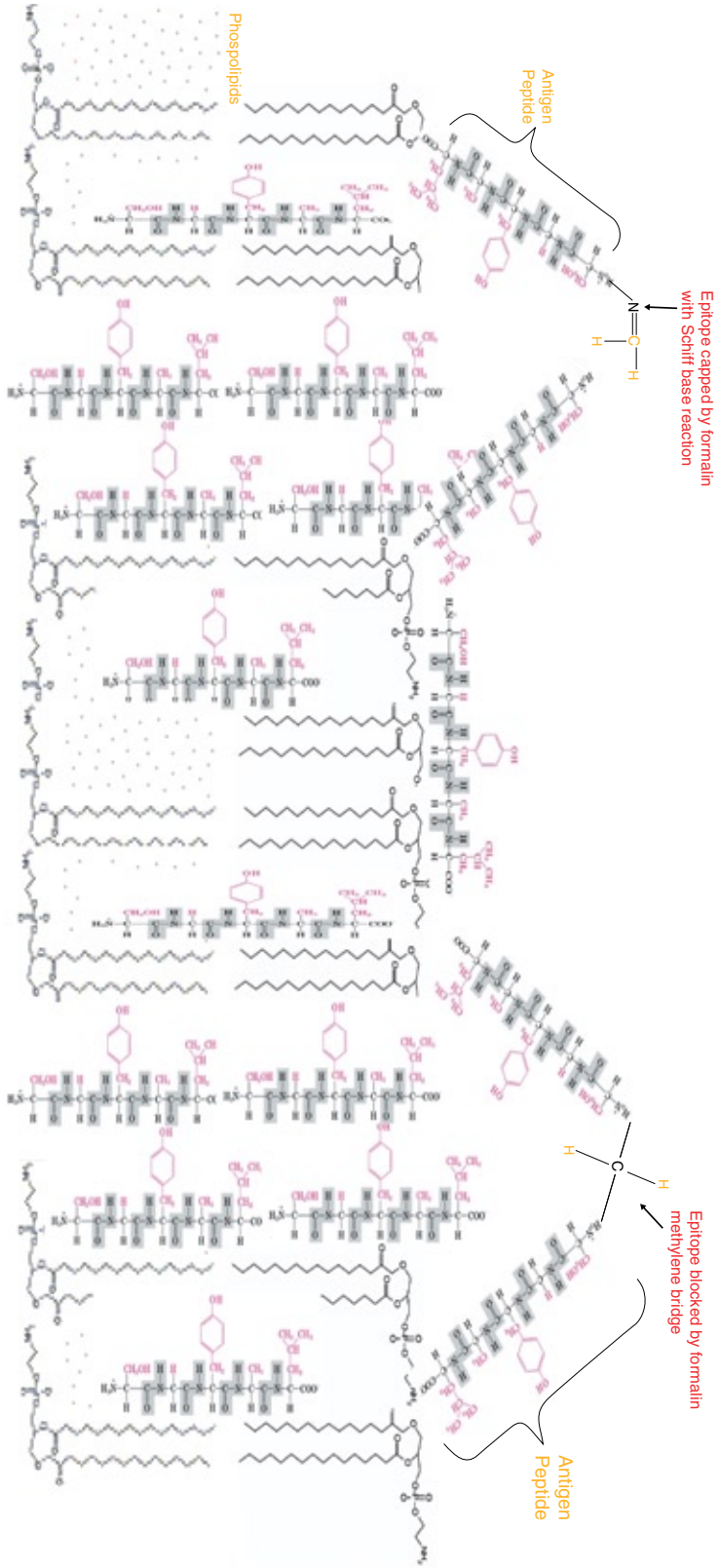
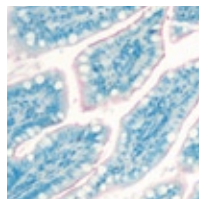


Figure 10.1. Chemical illustration of a cell membrane, demonstrating the differences between a methelyne bridge and an epitope that has been fixed with a Schiff base.



Several enzymatic solutions contribute to IHC, as illustrated in the chart below. Each enzyme responds to a specific amino acid sequence. Since the specific cleavage sites are usually unpredictable, the procedure is not always successful and sometimes results in the loss of certain epitopes. Typically enzymatic digestion doesn't affect epitopes with high carbohydrate content. However, it can be appropriate for glycoprotein-rich targets, such as the epitope for glucagon immunoreactivity in certain tumors (1).

Theoretically, conditions and enzymes used for unmasking could be different for each antigen. For example, proteinase K may be required to unmask an epitope for primary antibody cytokeratin AE1/AE3 but it may alter a CD20 epitope. Proteinase K is an effective proteolytic enzyme, however, it has proven to be less selective than protease XXIV for retrieving certain epitopes. Analysis through preliminary experiments should be done to determine incubation times, temperatures, morphological effect and concentrations of enzymes for proper optimization.

The optimal temperature for most proteolytic enzymes used for IHC is about 37 °C. However, lower temperatures are possible and in some cases are preferable because they allow a greater degree of control over the digestive process.

Table 10.1 shows several enzymatic reagents and their incubation conditions that have been used successfully in IHC.

Table 10.1. Enzymatic reagents and their incubation conditions.

Enzyme	Approximate activation temperature or range in ° Celsius	Incubation time in minutes
Proteinase K	25-37	5
Trypsin	37	10
Pepsin	37	5–20
Protease XXIV	37	5–10
Pronase	25-37	30

NOTE: Formalin does not preserve tissue proteins by coagulation but it is thought to form cross links with basic amino acids. Ethanol and mercuric chloride-based fixatives are based on coagulation. With few exceptions retrieval should not be performed on ethanol fixed tissues. It should only be conducted with limited controlled protocols in mercuric–chloride-based fixatives.

Endogenous Enzyme Blocks

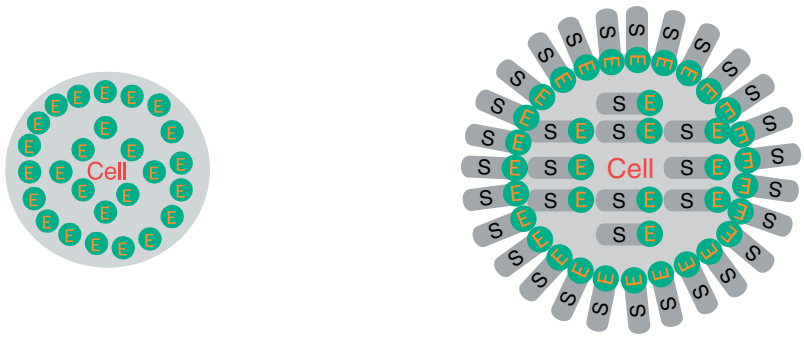
This block is an inhibitor that prevents an enzyme that originates within a cell or tissue from causing a reaction with another substance (substrate).

The development of immunohistochemistry detection systems was founded on the principle of enzyme-substrate reactions. Specifically, this is done to convert colorless chromogens into colored end products for visualization. However, certain types of cells have endogenous enzymes that can convert colorless chromogens to colored end products, independent from detecting the antigen antibody complexes producing false-positive results.

There are two common enzymes that effect most IHC clinical applications: Horseradish peroxidase and alkaline phosphatase.

Table 10.2. Endogenous enzymes found in a variety of cells and tissue types.

Enzyme: Peroxidase	Enzyme: Alkaline Phosphatase*
Red Blood Cells	Placenta Intestine - situated between cellular components of mucosa
Granulocytes	Proximal tubules of kidney
Eosinophils	Osteoblast in bone
Hepatocytes	Arterial & capillary endothelial cell surfaces
Muscle	Stromal reticulum cells
Kidney	Neutrophils
Monocytes	Follicle and mantle zones in most lymphoid tissue
*Alkaline Phosphatase is destroyed by routine fixation and paraffin-embedding procedures.	



Cell with Endogenous Enzyme

Enzyme Substrate Complex

Figure 10.2. Illustration of a cell with endogenous enzyme and enzyme substrate complex. Enzymes inhibited by excess substrate are rendered inactive and are unable to respond to chromogens. These enzymes are considered "blocked," meaning that the substrate has become an inhibitor.

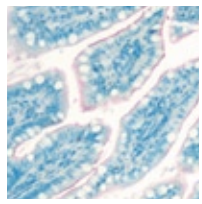


Table 10.3. Common endogenous enzyme blocking reagents for horseradish perioxidase and alkaline phosphatase systems.

Dual endogenous enzyme block, Dako code S2003	HrP and AP Labels
Hydrogen peroxide	Horseradish peroxidase label
Levamisole + chromogen except intestinal alkaline phosphatase	Alkaline phosphatase label
Weak acid (0.3 N HCl), including intestinal alkaline phosphatase	Alkaline phosphatase label

Figure 10.3. Figures 10.3a and 10.3b: Endogenous peroxidase reaction.

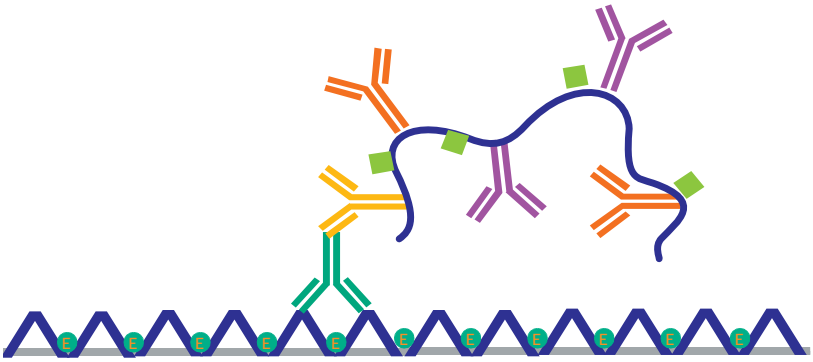


Figure 10.3a. A typical IHC HrP detection reaction, showing a primary antibody (green) binding to its target antigen (blue) without an endogenous enzyme block or DAB substrate.

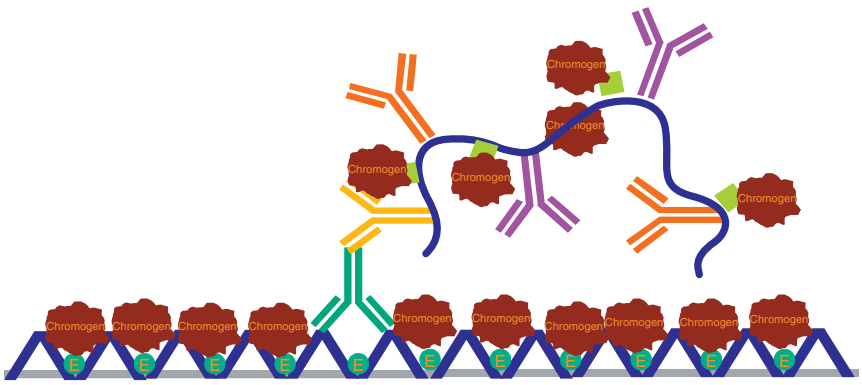


Figure 10.3b. A typical IHC HrP detection reaction with added DAB (brown) in the presence of peroxidase.

Figure 10.4. Figures 10.4a, 10.4b, and 10.4c: An example of a teaction using an endogenous enzyme block.

Figure 10.4a. Substrate (gray) responding to enzyme in an endogenous peroxidase reaction.

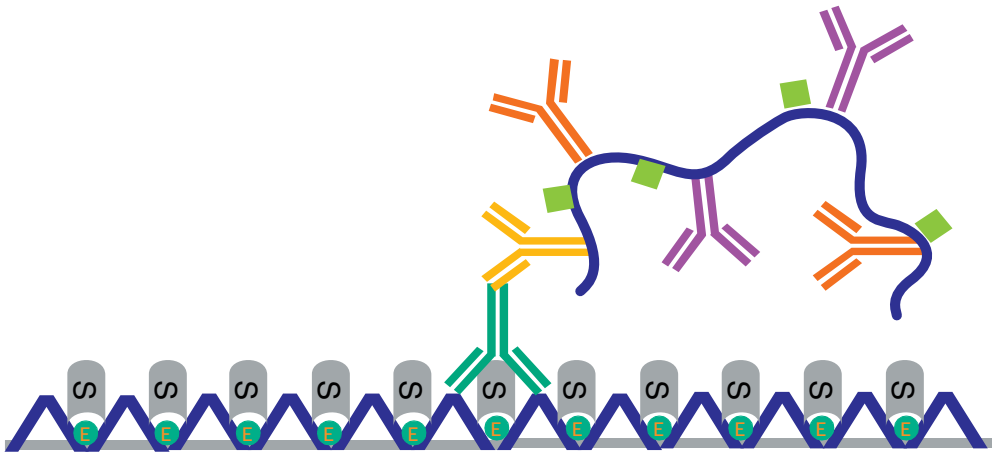


Figure 10.4b. Substrate (gray) responding to enzyme in an endogenous peroxidase reaction, with the addition of a primary antibody and detection.

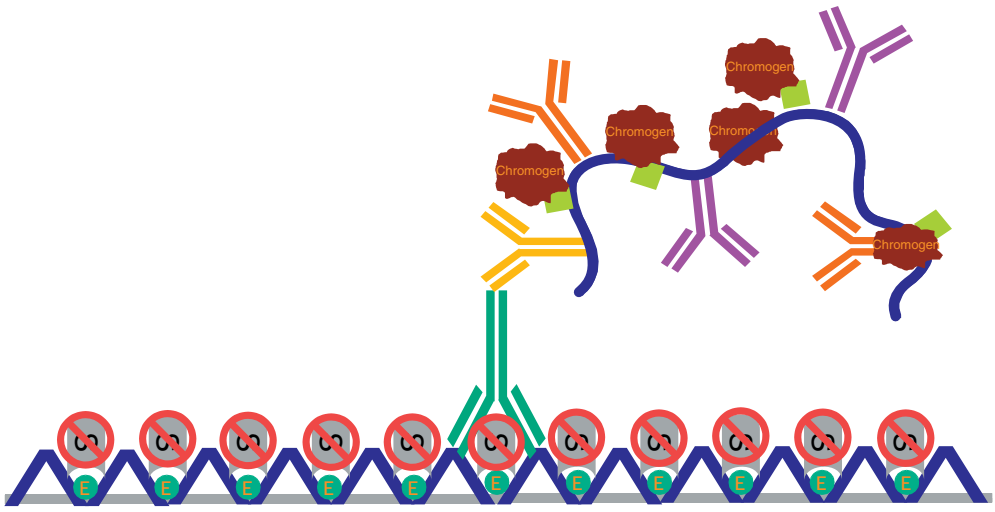


Figure 10.4c. Substrate (gray with red blocked circles) responding to enzyme in an endogenous peroxidase reaction, with the addition of a primary antibody, detection and chromogen (brown). Red blocked circles indicate that chromogen only reacts to uninhibited enzymes on the detection system.

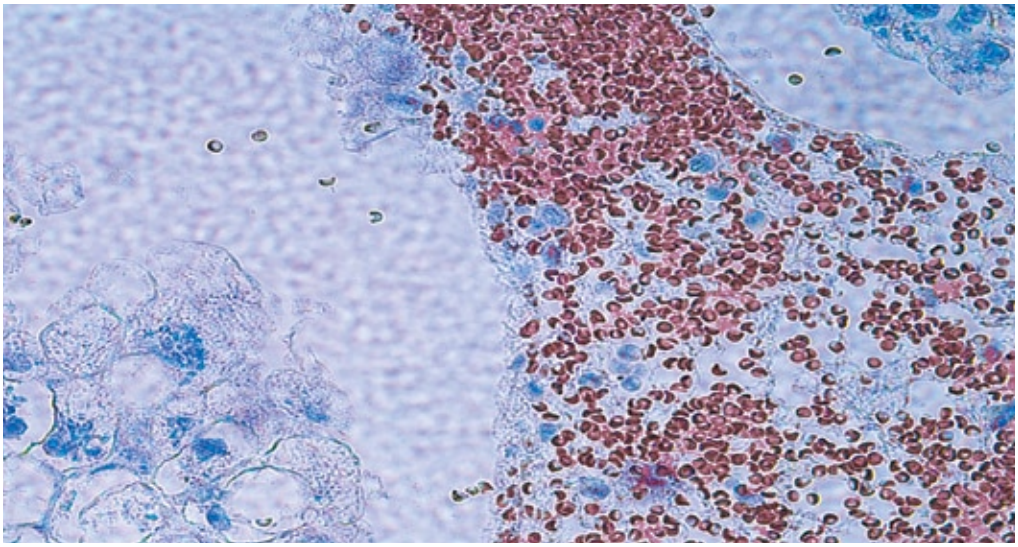


Figure 10.5. Example of endogenous peroxidase in RBC of Kidney with DAB.

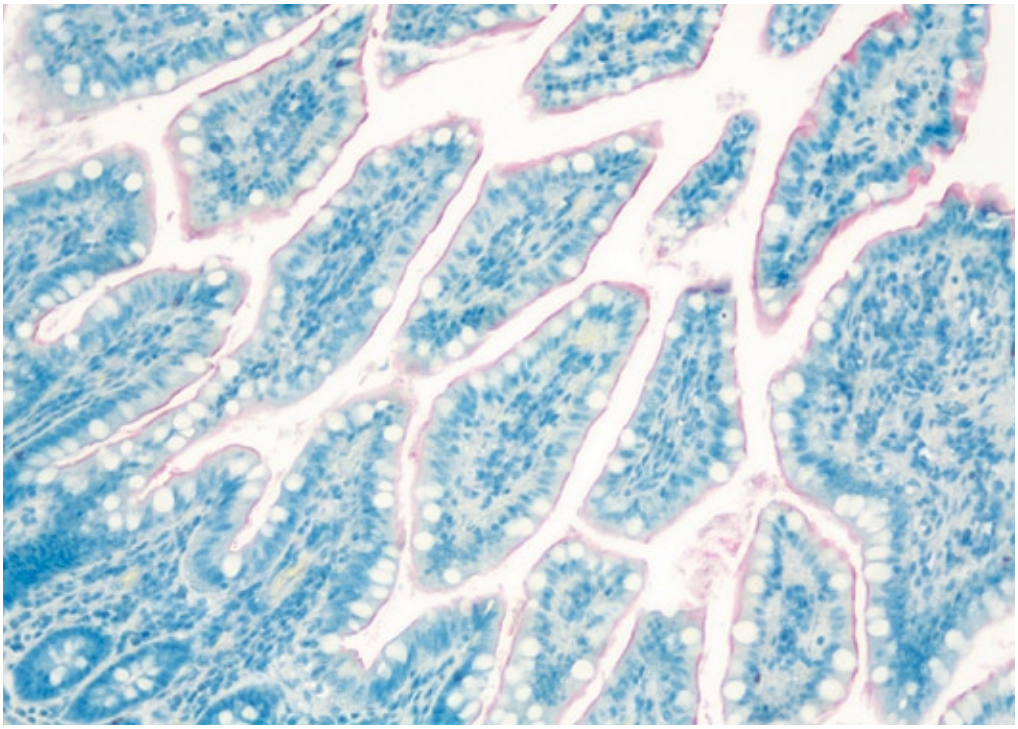


Figure 10.6. Example of endogenous alkaline phosphatase in ileum with Permanent Red.

In rare instances, the enzyme-blocking reagent may alter a specific epitope. It then may be appropriate to apply the primary antibody prior to the enzymatic block to insure its reaction. In such cases the blocking reagent can be applied at any point after the primary and before the enzyme-labeled components. Endogenous peroxidase, pseudo-peroxidase and alkaline phosphatase activity should be quenched when enzyme conjugated detection systems are utilized for visualization methods with chromogens. Omitting this vital protocol step may result in unwanted chromogenic reactions.

Protein Blocks

These are reagents used to reduce the chances of nonspecific reactions of an antibody with components other than its target antigen.

Figure 10.7. Figures 10.7a, 10.7b, and 10.7c: Example of a protein block

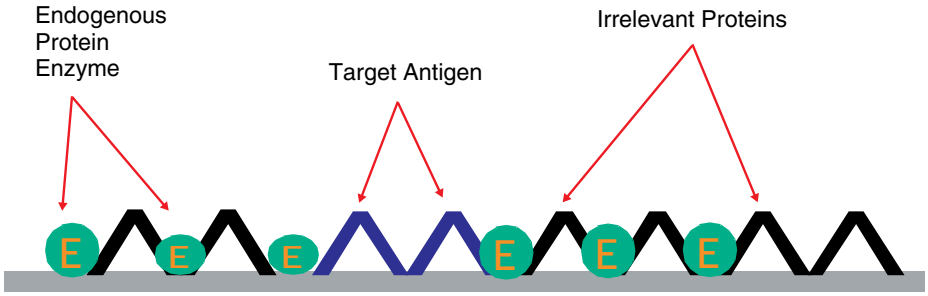


Figure 10.7a. Tissue prior to protein block application, showing endogenous enzyme and irrelevant proteins (black) and target antigen (blue).

Figure 10.7b. Endogenous enzyme block has been added, followed by protein block. Protein block should not be rinsed off.

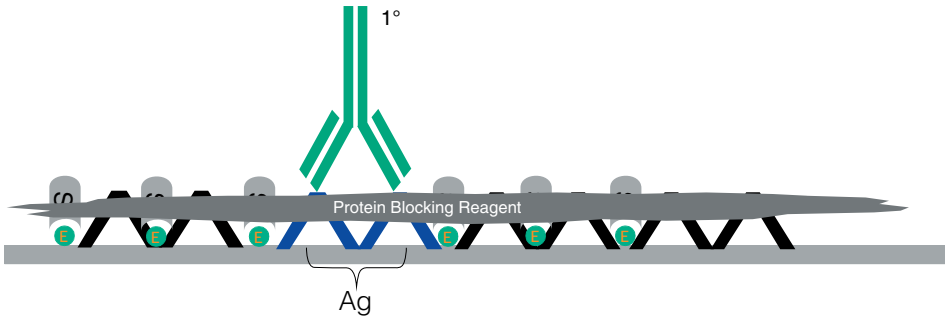
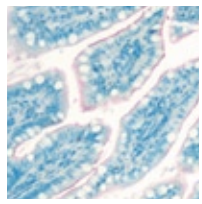


Figure 10.7c. Primary antibody has been added. Its high affinity for the antigen overpowers the attraction of the protein block. The protein block masks irrelevant sites with less affinity.



A primary antibody's high affinity for the antigen will displace the low affinity of the protein block. Within any given tissue, highly charged molecules exist as normal components. These molecules may not be the target antigen of a given immunohistochemical protocol. When applying a primary antibody, if the target antigen is present, the primary antibody will bind to it, resulting in an immunospecific reaction. However, in circumstances where the tissue has not been adequately blocked the primary antibody also may combine with non-target sites, resulting in a non-immunospecific reaction. If this happens, the secondary antibody also will bind, leading to background staining.

Antibody Diluents

These are inert fluids or reagents used in immunohistochemistry to dilute a particular antibody stock solution for the purpose of preparing a working antibody reagent.

Ionic interactions are one of the primary forces controlling immunochemical interactions between antigens and antibodies. Buffers near physiological pH (pH = 7.0-7.2) are normally utilized for dilution of primary antibodies. Diluents can have different isoelectric points. The isoelectric point (pI) is the pH value at which the net electric charge of a molecule in a solution is zero. The pI for immunoglobulins can range from 5.8 to 8.5 so diluents can cause a net negative or positive charge on antibodies resulting in specific and nonspecific reaction (2).

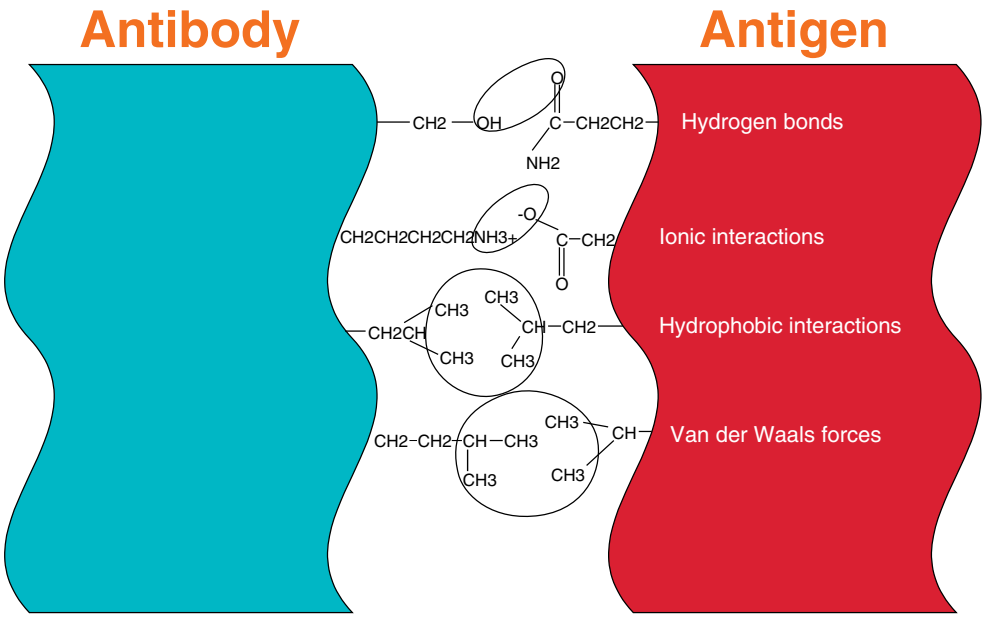


Figure 10.8. Antibodies are attracted to antigens initially through electrostatic, and subsequently Van Der Waals and hydrophobic interactions. The illustration below shows the importance of pi and how it influences spatial complementarities. If the pH of the environment is close to pi, the immunoreactivity can be impaired.

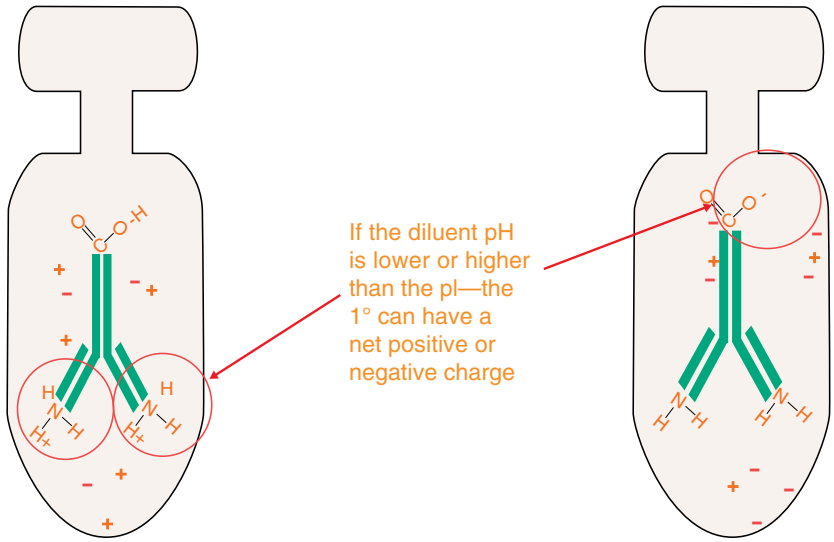


Figure 10.9. If the overall ionic strength of the environment is varied, it can alter the 1° conformation and/or the ionic interactions, allowing nonspecific binding of the primary to charged components, as well as no reaction with the intended epitope with negative results.

Antibody diluents purchased from manufacturers can provide stability to the working primary antibody solution.

Some of the choices available include:

- Antibody diluent with standard Tris HCl, detergent and stabilizers.
- Antibody diluent with background reducing components (bovine serum albumen,or normal serum, or other proteins).

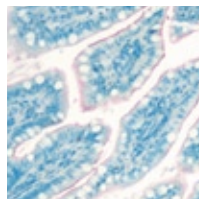
Beware of diluents with normal serum components:

- Can nonspecifically bind with secondary antibodies resulting in false positive results.
- Can cause a reduction in sensitivity of the primary antibody.

Because of numerous unknown factors influencing the overall stability of diluted antibodies there is little room for a general and safe recommendation for how long a diluted antibody is stable.

Technicians are advised to follow proper quality control procedures for validation if diluted primaries are utilized for extended periods of time.

An advantage to using commercially diluted primary antibodies is the built-in customer protection provided by regulatory mandates. Manufacturers must demonstrate stability in commercially produced reagents for a set period of time, with the antibodies subjected to real-time shelf-life quality control and accelerated shelf-life studies through controlled heating processes. However, manufacturers are required only to certify the time period, and antibodies actually may retain immunoreactivity for a longer period of time. There



is no requirement for manufacturers to continue testing until the antibody looses activity. For some commercial reagents, manufacturers add sufficient preservatives to maintain viability of concentrated or ready-to-use antibodies for 12 to 18 months. CLIA 88 and CAP regulations in the United States allow laboratories to document antibody activity until an antibody has entirely lost its immunoreactivity based on good quality control practices.

Other Important Points about Diluents

- Antibody diluents' effectiveness can be a direct function of its pH.
- High concentrations of sodium chloride or azides are used frequently as preservatives, but can reduce antibody reactivity.
- Phosphate diluents can add to ionic strength, causing decrease in specific and nonspecific staining.
- Stability of a diluted antibody can vary over time depending upon the dilution.
- Generally speaking, over time the more dilute the antibody, the less stable the working solution.
- Refrigeration can cause changes in pH.
- Reagents must come to room temperature before use.
- Phosphate buffered saline (PBS) should not be used as a diluent unless recommended by the manufacturer.

Table 10.4. Diluent factors that may affect antibody/antigen reaction.

Reagent	Added component	Subtracted component
Bovine Serum Albumin (BSA)	Decreases nonspecific staining	Increases nonspecific staining
NaCl PO_4 ions	May cause negative results	Increases nonspecific staining
Phosphate Buffered Saline	Increases nonspecific staining	If Tris HCl is used then specific staining
Tween 20 or Berol	Decreases nonspecific staining	Increases nonspecific staining
1% Normal Serum	Decreases nonspecific staining	Increases nonspecific staining

Wash Buffers

These are useful in immunohistochemistry for removing excess or unwanted reagents or complexes formed during each step. Common wash buffers commercially available include Tris Buffered Saline (TBS) and Phosphate Buffered Saline (PBS). Both have their benefits and pitfalls depending on the conditions encountered.

Tris Buffered Saline

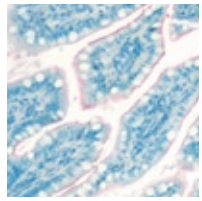
Tris(hydroxymethyl)aminomethane is utilized in IHC to help reduce the effects of nonspecific staining, because of its stability and chemical properties when combined with sodium chloride (NaCl), Tween 20, and 0.01% sodium azide.

Pure Tris, and the crystalline hydrochloride salt of Tris, have little if any buffering capacity when in solution independently. But mixing the two compounds together will produce a range of pH's with buffering capacity ranging from a pH of 7.0-9.0. The pH values of all buffers are temperature and concentration interdependent. As the solution decreases in temperature, pH increases at a rate of approximately 0.03 units per degree centigrade.

When conditions require a higher specificity due to a selection of more sensitive detection methods, the saline and detergent content can be increased to minimize the potential of non-specific binding of reagents. A dilution of 0.05% Tween 20 can be used to reduce the effects of nonspecific labeled polymer attachments. As an example, Table 10.5 describes the differences between two Dako wash buffers, S3006 (recommended for general IHC procedures) and S3306 (recommended for high-sensitivity IHC procedures).

Table 10.5. Wash buffer comparison.

Standard Tris Buffered Saline, Dako Code S3006	High Salt Tris Buffered Saline, Dako Code S3306
150mM NaCl	300mM NaCl
0.05% Tween 20	0.1% Tween 20
pH 7.6 at 25 °C	pH 7.6 at 25 °C



Chapter 10, Figures 10.10a and 10.10b. Human prostate tissue with Cytokeratin 34BE12, Dako Code M0630, demonstrating differences in nonspecific staining results when standard or high-salt rinse buffers are used.

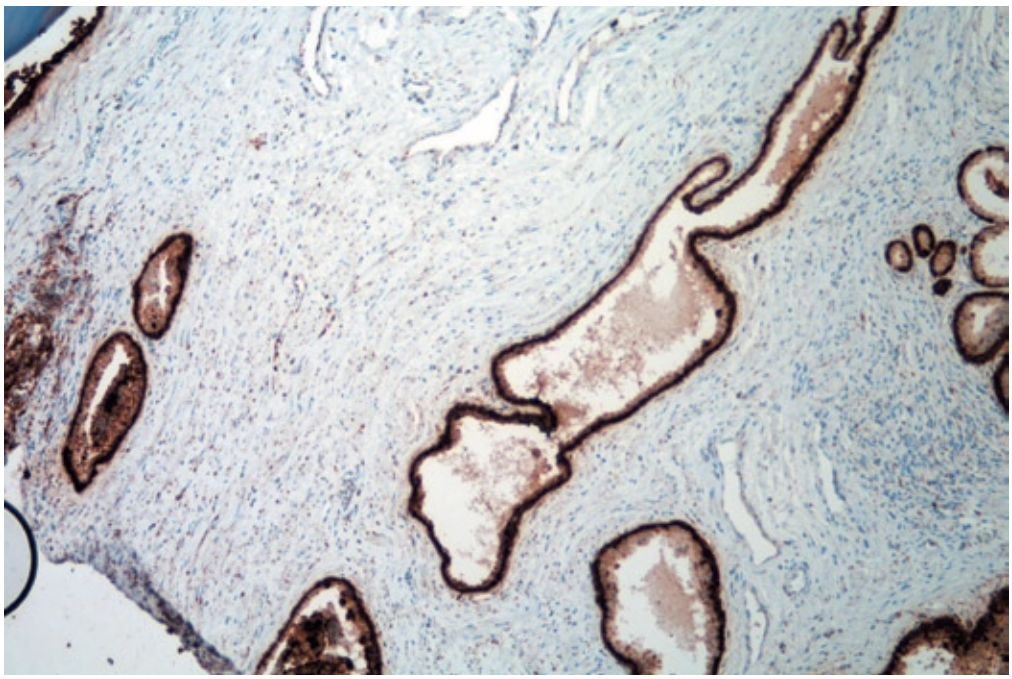


Figure 10.10a. Cytokeratin 34BE12, Dako Code M0630, on human prostate tissue, rinsed with 150mM NaCl 0.05% Tween 20 Tris Buffered Saline, Dako Code S3006, pH 7.6 at 25 °C.

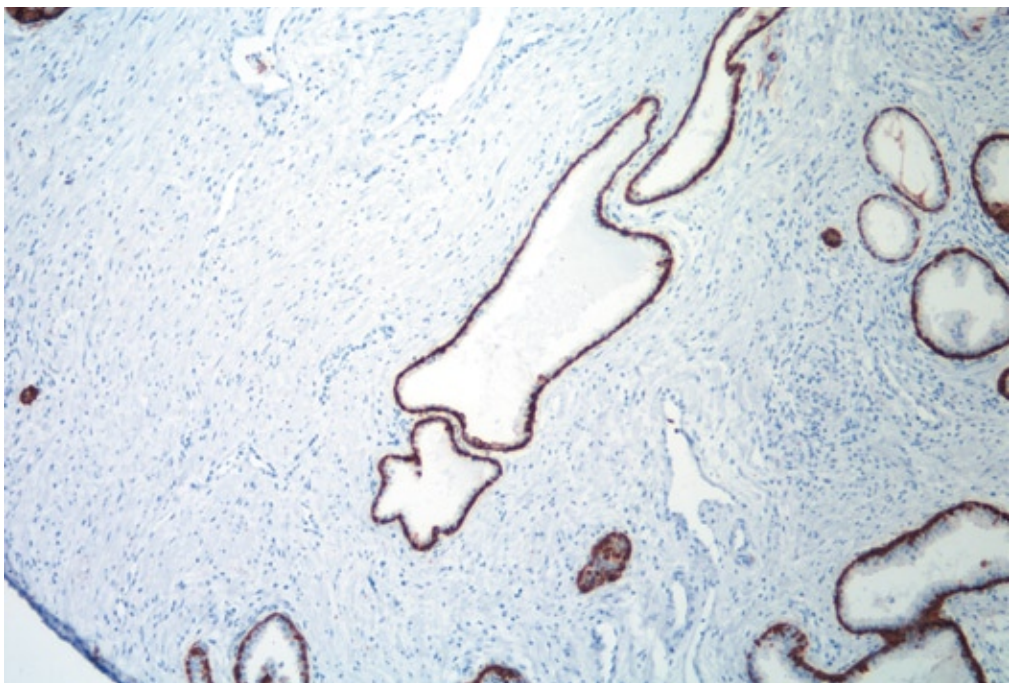


Figure 10.10b. Cytokeratin 34BE12, Dako Code M0630, on human prostate tissue, rinsed with 300mM NaCl 0.05% Tween 20 Tris Buffered Saline, Dako Code S3306, pH 7.6 at 25 °C.

Table 10.6. Effects of temperature on pH of Tris buffered saline.

Temperature in °C	pH
5 °C	8.18
25 °C	7.6
37 °C	7.30

Phosphate Buffer Saline (PBS)

This has a long history in IHC and still is used in certain applications where specified by protocol (kidney biopsies, direct skins, Aspergillus). PBS is better at reducing auto fluorescence in immunofluorescent assays than Tris buffer, and is inexpensive to make. However, it can cause a higher incidence of nonspecific staining, producing primary antibody shielding, and subsequently reducing specific binding capacity to the targeted epitopes with certain monoclonal antibodies (CD30, for example).

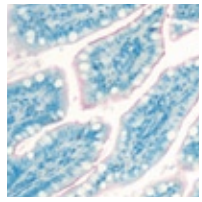
Buffer Storage

Working solutions of Tris buffers generally are stable when stored at 25 °C for four days. Commercially available wash solutions that come in concentrated configurations generally can be stored at 2 °C to reduce bacterial growth and should be discarded per expiration date to maintain reagent viability. However, highly concentrated buffers may form crystals due to lower solubility at cold temperatures. Therefore the stock buffer solutions stored in the cold should be inspected carefully before use. Crystals forming in stock solutions generally will re-dissolve upon warming to room temperature.

Measuring pH of Buffers

When measuring pH of Tris buffer, the electrode used should be of appropriate type such as Glass-Calomel (mercurous chloride). Silver/silver chloride reference electrodes with Tris buffers containing protein may cause spurious results. Strict guidelines should be enforced to insure proper utilization of laboratory reagents, thereby giving more consistent and reliable results.

NOTE: Technologist should be mindful to keep accurate records of pH values prior to using a wash buffer. These records should be observed for trends of increasing or decreasing pH values. Use appropriate pH probe for the respective buffers. pH should be recorded at room temperature 25 °C.



Twenty-Day Validation Protocol

During a 20-day validation period of a primary antibody and staining system, measure and record the pH of buffers used in the protocol. Retain those values where acceptable staining is observed.

NOTE: If using a pre-set Tris buffer, such as commercial Tris buffered saline (TBS), these values should remain constant (at 25 °C). At the end of 20 days, calculate the standard deviation or dispersion of a set of values from the mean (3).

The following is a suggested method for measuring pH:

- Calculate the mean of the 20 values.
- Subtract each sample value from the sample mean and square the product.
- Total the squares.
- The square root of the summed squares gives a standardized value or standard deviation (SD).
- Multiply the standard deviation by two.
- Subtract 2SD and add the initial mean. This will produce a range of acceptable values.

Table 10.7. The Levy Jennings Chart. May be useful in recording pH values.

The Levy Jennings Chart may be useful in recording the pH values:
2SD_____ 1 SD_ Mean_____ 1 SD_ 2SD_____
NOTE: Two standard deviations from the mean is sufficient for acceptable ranges in pH.

Suggestions for Making Wash Buffer Solutions

- Do not add old wash buffer to new wash buffer.
- Make fresh buffer according to manufacturer's specifications.
- Do not dilute reagents beyond the recommended dilution.
- Place a label on all solution containers when they are opened.
- Write your initials and the date that the solution was opened on the label.
- Do not mix different types of buffers.
- Make buffer with either distilled, organically filtered deionized, or reagent grade water. Do not use tap water.

Antimicrobials such as chloramines, in tap water as well as unfiltered deionized water can remove buffering effects of Tris buffer in particular, contributing to nonspecific staining and creating harmful byproducts such as methane, hydrazine, and formaldehyde.

Chromogen Enhancers

These are considered a color modification process, and should be considered as a separate chromogen in the grading system.

Technically, DAB enhancers contain compounds that continue the DAB reaction, allowing for further enhancement. Enhancers should be applied immediately after rinsing slides with reagent grade or filtered, deionized water.

Incubation times are determined by the individual laboratory, based on the desired hue of the chromogen. The end product will be deeper chocolate-brown color. Enhancers require the presence of the original reaction and should not turn a non-reactive stain result into a positive result – it only works with what already is deposited.

Enhancers are usually heavy metals, which continue the reduction process with elements such as copper, silver, nickel, gold, or cobalt. Some studies show that if the diaminobenzadine reaction is stopped with tap water instead of reagent-grade water, a gold color develops.

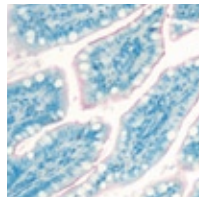


Figure 10.11. Examples for various DAB enhancements.

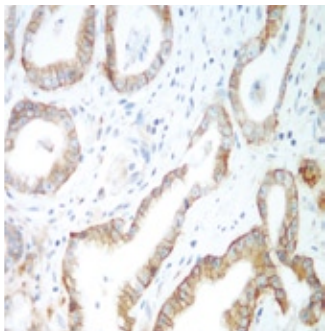


Figure 10.11a.
No enhancement (Di water)

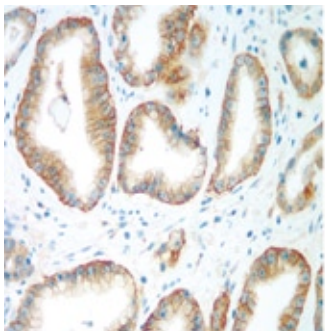


Figure 10.11b.
Tap water enhancement

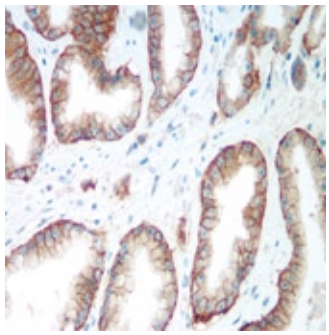


Figure 10.11c.
Enhanced with Dako DAB Enhancer

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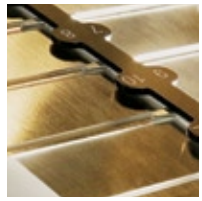


Chapter 11 ■ In Situ Hybridization

Richard Harvey, Updated by Andreas Schønau

Introduction

In situ hybridization is a powerful technique for detection of nucleic acid sequences. It allows the user to detect the presence of specific targets inside individual cells while preserving cell and tissue morphology. This allows for simultaneous assessment of the morphological alterations associated with the lesion and highly detailed information on the genetic composition of the cells.

The technique has developed significantly over the last decades. Different kinds of probes and labeling have been developed to accommodate the very different needs of the relevant targets and patient samples. The following section addresses some of the most relevant probes and labels, and provides a few clinically relevant examples of how in situ hybridization is being used to answer important clinical questions.

Types of Probes

DNA

DNA probes are still the most frequently used type of probes both in 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 Peptide Nucleic Acid (PNA) probes (see below). Thirdly, the advent of nucleic acid amplification techniques such as Polymerase Chain Reaction (PCR) has increased their availability greatly. Finally, DNA probes can come in all sizes (from short oligonucleotides to megabase constructs).

RNA

RNA probes are not used quite as frequently as DNA probes, although they do have their own niche applications. Often referred to as “riboprobes,” these are single-stranded materials that typically are synthesized from a vector via an RNA polymerase. Some of their advantages are that they are already single stranded (for example, 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 due to the ubiquitous presence of RNAses. RNAses are quite abundant in the environment and are extremely difficult to inactivate.

PNA

PNA probes are the newest members of this category (1). 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 do 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

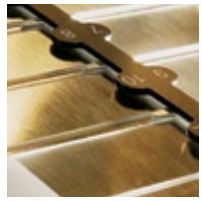
The length of a probe is highly dependent upon the application for which it is intended. Consider both the high and low extremes of probe size. An oligonucleotide of just 16 bases is statistically large enough to be unique in the human genome if the 3.2 x 109 bases of the human genome can be assumed 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

The purpose of introducing a label on the probe is to allow for subsequent detection of the probe, thereby retrieving relevant information on target localization and target abundance. Direct fluorescence in situ hybridization (FISH) requires the use of probes labeled directly with fluorescent molecules, while other in situ hybridization techniques normally make use of secondary molecules to translate the primary label into a visual signal.

Directly labeled FISH probes can be detected immediately after hybridization and stringent wash. The fluorescent labels are instantly detectable and are normally compatible with standard fluorescence microscopes. Labels typically include red



and green fluorochromes, but others are also available. The number of different fluorochromes that can be used in the same assay depends on the configuration of the fluorescence microscope including the filters. As the method does not include any amplification steps, directly labeled FISH probes are normally fairly large (> 100,000 bp) or target repetitive regions.

Probes for ISH applications usually are detected by use of secondary molecules. The primary label, which could be any hapten (including fluorescent molecules), is attached to the probe and recognized by a secondary molecule – normally an antibody or streptavidin. The secondary molecule can be either directly detectable, for example, a fluorescent label; be conjugated to an enzyme for chromogenic detection; or form the basis for further amplification steps, depending on the required level of sensitivity.

Types of Samples

The term *in situ* means "in the normal place." This means that in situ hybridization takes place in the cell where the target DNA or RNA is normally present. Cells normally are handled by placing them on a glass slide, and basically any collected cell sample that can be applied as a mono-layer on a glass slide also can be hybridized.

Cytology samples are intact cells from a liquid sample that are spread on a slide. Fixation is usually fairly mild, and the hybridization procedure includes steps like fixing cells to the slide, applying the probe mix, denaturing probe and specimen, hybridizing and conducting a stringent wash to remove excess probe. Cytology samples include hematological samples, lavage samples, cervix smears, etc. DNA and RNA are usually well-preserved in these kinds of samples, provided that fixation has occurred shortly after extraction of the sample

Tissue samples are typically either frozen or embedded in paraffin, and both can be used for hybridization assays. Formalin-fixed and paraffin-embedded tissue is very suitable for both DNA and RNA analyses, although the stability of RNA is very dependent on the time span between extraction of the sample and fixation. Fixed and embedded tissue requires pre-treatment similar to that for IHC methods, to allow for efficient hybridization. As a consequence, the procedure typically includes steps such as heat-induced target retrieval and/or enzymatic digestion of the tissue, prior to the step mentioned for cytology samples above. Evaluation of a tissue sample is also different from that of a cytology sample. This is mainly due to the high density of the material and the fact that preparation of tissue samples includes cutting of cells, resulting in fragments of cells and nuclei on the slide. This affects the scoring algorithms that can be applied, especially when enumerating nuclear targets.

Examples

Split-Signal FISH Probes for Detection of BCR-ABL Translocation in Acute Lymphoblastic Leukemia (ALL)

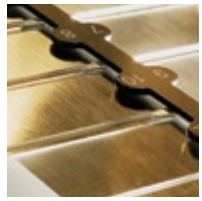
A hematological sample is spread on a glass slide and fixed. A probe mix containing a red probe and a green probe located on either side of the BCR breakpoint region on chromosome 22 is applied. Sample and probe are denatured and the probe is allowed to hybridize overnight before a stringent wash is conducted to remove excess probe. The slide is dried and mounted using fluorescence mounting media containing an anti-fade agent such as diamidino phenylindole dihydrochloride (DAPI). Results are evaluated using a fluorescence microscope. A normal cell will have two sets of co-localized signals corresponding to the normal situation in which no translocation has occurred, and the red and the green probe will bind in close proximity on the intact chromosome. When viewed microscopically the two probes appear to co-localize, which produces a yellow color. Translocated cells will have one set of co-localized signals corresponding to the normal allele, and one set of split signals. A split signal indicates that the two probes have been separated physically as part of the translocation process. For this reason the probes are no longer co-localized, and the resulting split can be identified easily as individual green and red signals by means of a fluorescence microscope.

Assessment of *HER2* Gene Amplification Status in Breast Cancer

A formalin-fixed, paraffin-embedded tissue is cut into sections of four to six µm and placed on a glass slide. After deparaffinization and rehydration, specimens are heated in pre-treatment solution for 10 minutes. The next step involves a proteolytic digestion using ready-to-use pepsin at room temperature for five to 15 minutes. Following the heating and proteolytic pre-treatment steps, a probe mix including a Texas Red-labeled DNA probe covering the *HER2* gene on chromosome 17, and a mixture of fluorescein-labeled PNA probes targeted at the centromeric region of chromosome 17 (CEN-17) is applied. Sample and probe are denatured and the probe is allowed to hybridize overnight. After a stringent wash the specimens are mounted with fluorescence mounting medium containing DAPI, and coverslipped.

Results are evaluated using a fluorescence microscope and the number of *HER2* (red) and *CEN-17* (green) signals in 20 nuclei are counted. Finally the ratio of *HER2* to *CEN-17* signals is calculated and compared with the cut-off for amplification. A value of greater than 2.0 is indicative of amplification.

Amplification of the *HER2* gene and/or overexpression of its protein have been demonstrated in 25 to 30 percent of breast cancers. This up-regulation is associated with poor prognosis, increased risk of recurrence, and shortened survival. Several studies have shown that HER2 status correlates with sensitivity or resistance to certain chemotherapy regimens (2).



Demonstration of high HER2 protein overexpression or *HER2* gene amplification is essential for initiating therapy with Herceptin™, a monoclonal antibody to HER2 protein. Clinical studies have shown that patients whose tumors have high HER2 protein overexpression and/or amplification of the *HER2* gene benefit most from Herceptin™ (1).

Detection of High-Risk HPV Infections in a Cervical Smear

A cervical smear is spread on a glass slide and fixed. A cocktail of biotin-labeled DNA probes targeting 13 different HPV genotypes associated with poor prognosis is applied. Sample and probe are denatured and the probe is allowed to hybridize. Following a stringent wash, the probe is detected by use of the GenPoint™ Tyramide Signal Amplification System, resulting in clear and distinct precipitation of DAB chromogen at the localization of the probe. Results are visualized using a light microscope.

Presence of a signal indicates the presence of either episomal or integrated viral DNA. The appearance of the signal (allows the microscopist to distinguish between the episomal (confluent) and integrated (punctuate) forms of the viral target DNA. The intensity of the signal may reflect the copy number of the viral target.

HPV DNA testing may reduce health care costs by triaging patients into appropriate management strategies such as replacing some unnecessary colposcopies with regular screening in low-risk patients. The clearest role for HPV DNA testing is to improve diagnostic accuracy and to limit unnecessary colposcopies in patients with borderline or mildly abnormal cytologic test results (4).

Conclusion

There are numerous probes, along with methods for their labeling. This leaves opportunities for optimal design to a specific desired application. Regardless of steps taken toward optimizing probe performance, specimen processing and sample pretreatment remain two of the largest sources of variability in assay performance and have to be standardized to ensure consistent results.

Compared to other nucleic acid detection techniques, in situ hybridization is a powerful tool, allowing for low-level detection of specific nucleic acid targets inside individual cells while preserving morphology. This technology undoubtedly will lead to the development of new tests for genetic markers, provide an expanded role for ISH in clinical testing, and improve patient management.

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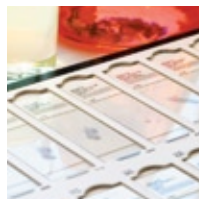


Chapter 12 ■ Methods of Immunocytology for Slide-Based Cellular Analysis

Marc Key

Introduction

Cytological examination of single cells and small groups of cells provides a wealth of diagnostic and prognostic information to laboratory professionals with specialized training in deciphering complex morphological information (1, 2). These interpretations usually are based on the characteristics of cells stained with a variety of organic and inorganic dyes that can highlight differentially various cellular and subcellular components. Immunocytology adds an additional dimension to cytology by further providing the means for molecular analysis. By employing specific antibodies that target well-characterized molecular targets, it is possible to combine molecular analysis with subcellular analysis (3-6).

Many of the methods of immunocytology derive from the general methods of immunohistology, where whole tissue samples are analyzed by specific antibody probes. Despite these similarities there are critical distinctions between the analysis of isolated cellular samples and whole tissue samples. An understanding of these differences is crucial in achieving optimal immunocytochemical staining.

Procedures

Sample Collection

There are three primary methods of sample collection, 1) collecting the sample into a transport or collection medium containing a fixative, 2) collecting the sample into a container without fixative, or 3) collecting the sample directly onto the microscope slide in the unfixed state. The latter two methods are similar in that the cells are not initially exposed to a fixative before being placed on the microscope slide.

Sample Collection with Fixative

Cytology samples may be collected directly into a medium containing fixative. This is particularly true when using an automated monolayer preparation instrument where this method of collection is required by the manufacturer. Although these methods have been optimized for ease of collection, transport, and morphological analysis, only a few studies have tested their compatibility with immunocytology (7, 8). Because the manufacturer's transport medium is proprietary, little information is available on its effects in preserving epitopes for subsequent antibody staining. Many of these transport media contain mixtures of ethanol and polyethylene glycol. Such fixatives are

generally compatible with immunocytology procedures, whereas fixatives containing high amounts of methanol, isopropanol, or formalin may cause denaturation of certain antigens thus producing weak immunostaining.

In order to ensure optimal immunostaining, samples collected into fixatives should be processed and stained as soon as possible. Samples held up to 48 hours are suitable for immunocytochemical analysis.

Sample Collection without Fixative

Samples collected without fixative should be processed and stained as soon as possible. The following table provides a preferred schedule for sample collection, processing, and staining.

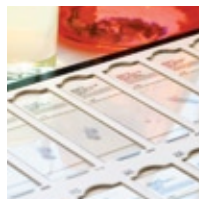
Table 12.1. Schedule for processing cytology specimens.

Procedure	Time from initial sampling
Cell smears	Prepare slide immediately after sample collection
Cell imprints	Prepare slide immediately after sample collection
Red blood cell removal	Perform within 24 hours
Cell wash and resuspension	Perform within 24 hours
Cell enrichment methods	Perform within 24 hours
Preparation of monolayer	Perform within 24 hours
Slide storage	Up to 48 hours (room temperature)
Stain slides	Perform within 48 hours

The optimal schedule requires that all steps up through preparation of the microscope slide should be completed within the first 24 hours. All subsequent staining steps should be completed within 48 hours.

Comparison of Pre-Fixed to Unfixed Specimens

The process of fixation renders the cell membranes rigid. If cells are fixed in suspension, as is the case when cells are collected into transport medium, the cells retain their three-dimensional shapes as free-floating cells. For squamous epithelial cells this shape generally is elongated and flattened, whereas most other cell types, particularly white blood cells and many types of tumor cells, retain a spherical conformation. When these fixed preparations are deposited onto the slide, they tend to retain a rounded appearance with densely staining nuclei and scant cytoplasm. In contrast, when cells are applied to a microscope slide in the unfixed state, they tend to flatten and spread, providing more nuclear and cytoplasmic detail. Thus the morphology of the same cell type can be vastly different depending on how the sample was processed (9).



The choice of whether to fix before or after application of the cells to the slide depends on the sample type, and also on the manufacturer's requirements when using an automated monolayer device. Both methods are compatible with antibody staining. However, fixation after application of cells to the slide frequently provides better morphological detail.

Sample Preparation

Microscope Slides

In order to ensure adequate cellular adhesion, the slides must be treated chemically to promote cell adhesion. Positively charged slides or silanized slides are available from several commercial sources and are preferred for immunocytology applications.

Application of Specimens to Microscope Slides

Cells may be applied to slides manually, using cell smear methods, or with the aid of an automated monolayer device or cytocentrifuge. For automated methods, follow the manufacturer's instructions. After applying samples to the slides, the slides should be either dry or nearly dry without excess liquid. Complete the process by rapidly air drying any slides containing residual liquid. If slides storage is required before staining, store slides in the unfixed state.

Slide Storage

Stain slides as soon as possible after preparation. If it is necessary to delay staining:

- Store unfixed slides at room temperature in a sealed container for no more than 24 hours.
- If a longer storage period is necessary, slides may be stored for up to seven days at –20 °C or up to 30 days at –70 °C.
  - Individually wrap slides with two layers of aluminum foil, securely sealing all seams. Special care is required to avoid scratching or otherwise damaging the area of cellular deposition.
  - Place wrapped slides in a plastic bag, expel excess air, and seal bag.
  - Store at –20 °C to –70 °C.
  - When slides are removed for staining, first equilibrate slides to room temperature for 30 minutes prior to removal from the plastic bag. In order to prevent condensation on the unfixed cells, it is important that the slides reach room temperature before unwrapping the aluminum foil.
  - Unwrap slides and proceed immediately to fixation and staining.

Fixation

The method of fixation is perhaps the most critical step in achieving optimal results. For optimal morphology, strong fixation is preferred in order to preserve cellular detail. In contrast for antibody staining, weak fixation is preferred in order to retain protein molecules in their native conformation. The precise balance between these two opposing requirements is critical for optimal staining. Fixatives containing ethanol and propylene glycol commonly are used for cytology and are generally compatible with antibody staining. A further consideration is that immunocytology procedures are generally more harsh than standard cytology methods, making the balance between over- and under-fixation particularly challenging. While the goal for immunocytology is to achieve both acceptable morphology and high-sensitivity immunostaining, in general practice morphology frequently is compromised in order to achieve the high sensitivity of the latter.

Fixatives are divided generally into two categories depending on their mode of action. Agents that combine with proteins are called additive fixatives, and agents that precipitate proteins are called coagulating fixatives. Because of the harsh nature of immunocytology, strong fixation is required in order to achieve optimal morphology. A fixative combining both the additive properties of formalin and the coagulating properties of ethanol provides an ideal solution. A general fixative for immunocytology is given below:

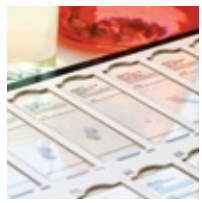
General Fixative for Immunocytology

- 50 mL of absolute (100 percent) ethanol;
- 5 mL of 40 percent (w/v) solution of polyethylene glycol in deionized water;
- 5 mL of formalin from 37 percent formaldehyde stock;
- 40 mL of deionized water.

Fixation Procedure

The following procedure is applicable for all samples, whether or not they have been pre-fixed.

- Place slides in fixative for 10 minutes at room temperature.
- Rinse briefly in buffered saline (phosphate-buffered saline (PBS), or Tris-buffered saline (TBS).
- Proceed to staining, without allowing slides to dry.



Immunostaining Methods

Permeabilization and Antigen Retrieval

Cells must be permeabilized to allow antibodies and visualization reagents to penetrate the cell membranes. A suitable permeabilization reagent can be prepared from a buffer containing detergent.

Formalin is a cross-linking fixative that can denature epitopes by forming methylene bridges. These cross-links may be broken, restoring the epitopes to their native configuration, by applying heat. A suitable antigen retrieval reagent can be prepared from a buffer containing detergent. A combined method of performing permeabilization and antigen retrieval is outlined below.

Permeabilization/Retrieval Reagent

- 1.92 g Citric acid, anhydrous.
- Dissolve in 900 mL deionized H_20.
- 0.1% Nonidet P40 (NP40).
- pH to 6.0 with concentration NaOH.
- Bring up to 1000 mL with deionized H_20.

Procedure

- Place permeabilization/retrieval reagent into a Coplin jar and heat to 95 °C;
- Add slides to Coplin jar and incubate for five minutes at 95 °C;
- Rinse slides with buffered saline.

Blocking endogenous enzymes

Peroxidase and alkaline phosphatase are the two enzymes most frequently employed in immunocytology. However, both of these enzymes occur naturally in a variety of cells and tissues. In order to avoid false-positive staining, these endogenous enzymes must be blocked prior to immunocytochemical analysis. Depending on the visualization method either endogenous peroxidase or endogenous alkaline phosphatase must be blocked.

Blocking methods for peroxidase generally employ solutions of hydrogen peroxide up to three percent. However, for cytology specimens three percent hydrogen peroxide can severely damage cellular morphology. Therefore weaker concentrations of hydrogen peroxide containing sodium azide are recommended. Commercial blocking reagents are available. However, the blocking reagent should specify that it is intended for use with cytology samples.

Endogenous peroxidase blocking reagent

- 0.03% hydrogen peroxide in deionized water.
- 0.2% (w/v) sodium azide.

Endogenous alkaline phosphatase blocking reagent

- 0.1 N HCl in deionized water.

Procedure for blocking endogenous enzymes

- Incubate slides with endogenous enzyme blocking reagent for five minutes at room temperature.

Antibody Preparation and Staining Methods

The antibody preparation and staining methods for immunocytochemistry are similar to those previously discussed for immunohistochemistry. For a review of immunohistochemical staining methods the reader is referred to Immunohistochemistry Staining Methods, Chapter 7.

Controls

Positive Control

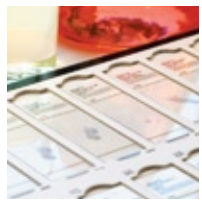
In every staining procedure a positive control must be run in order to establish the proper performance of the staining reagents and methods. The most appropriate positive controls are cytology samples containing known positive cells of interest. Once a positive sample is identified it is possible to make a repository of positive slides that can serve as future positive controls for up to two months. Positive control cell slides can be stored frozen at -20 °C, as previously described, and used for up to two months. After prolonged storage, a decrease in staining intensity frequently is observed. If staining becomes noticeably weaker the slides should be discarded even if they are less than two months old.

In the absence of appropriate cytology material, a tissue section containing known positive elements may be used to verify the performance of the reagents. However, the procedural elements of the protocol cannot be verified with tissue sections.

Negative Control

An appropriate negative control is performed on a second identical cytology sample collected and prepared at the same time as the patient test sample.

An isotype-matched negative control reagent, diluted to the same concentration as the primary antibody, is used in place of the primary antibody.



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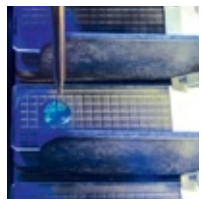


Chapter 13 ■ Automating Immunohistochemistry

Ron Zeheb

Introduction

In today's busy histology lab, an instrument to automate immunohistochemical (IHC) stains has become almost as essential as a Hematoxylin and Eosin (H&E) stainer or an automated coverslipper. Skilled histotechnologist time is too valuable to be spent on hand pipetting, egg timers and ensuring moist incubation chambers, when effective alternatives are readily available. IHC automation leverages technologist time, by having an instrument perform the many reagent additions, incubations and washes. While the instrument is running, the histotech who would otherwise tend the IHC, could help keep up with the day's routine workload by embedding, sectioning, and labeling slides.

Choosing the Right IHC Stainer

So what exactly is automated IHC, and how do you know if it's right for your lab? In essence, an automated IHC stainer is simply a robot that follows pre-programmed instructions that mirror the steps that a skilled histotech would use when staining a slide. Like any other critical piece of equipment that's brought into the lab, the automated IHC stainer should: 1) produce consistent, high-quality slides, 2) be robust and reliable, 3) be cost effective, 4) be easy to use and maintain and 5) fit in well with the lab's workflow and reporting requirements.

A properly functioning and maintained instrument will perform its pre-programmed instructions exactly the same way again and again every time it is run. This is one of the great advantages of an automated system, and the principal reason why an instrument's slide staining consistency is typically superior to manually stained slides. The operator of the instrument and the reagent manufacturers share responsibility for ensuring that the primary antibodies, detection (visualization) reagents, and any washes or buffers are made correctly and positioned on the instrument; are contamination-free; and are used within appropriate expiration dates.

Because tissue handling and processing may vary widely from institution to institution, prior to the routine implementation of any new IHC stain for clinical diagnostic use, the user of an automated IHC stainer should verify that the staining results are appropriate. Antibody titer and reaction conditions should be adjusted to achieve the desired staining results, and the instrument should lend itself to making such modifications when needed. With these simple prerequisites, the user of an automated IHC stainer can expect consistent, high-quality slides every time. Some instruments are designed to permit the user considerable flexibility in choice of reagents and staining protocol design. That is, the user can choose the reagents they want, and program the reagent additions and wash steps to produce the stain of their choosing. This approach is well

suited for a lab that is knowledgeable and comfortable with the IHC staining procedure and desires a high degree of flexibility. Other instruments permit a more limited degree of user-modification to the staining protocol, and typically little or no flexibility (other than choice of primary antibody) to use other than instrument-matched detection reagents. This may suit labs that prefer a more hands-off approach.

Maintaining Your IHC Stainer

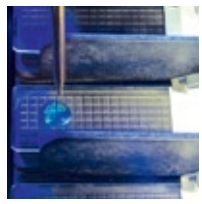
With proper maintenance, most automated instruments can be expected to provide many years of reliable service. You probably will be running your IHC stainer at least once every day and in many cases two or three times per day depending on case-load and work shifts. Once installed and operating, it's easy to develop a dependence on the instrument. Any unexpected interruption in the ability to use the instrument can throw the entire lab's schedule into turmoil. The best way to keep your stainer humming along is to read, understand and follow the directions in the manufacturer's user's manual. Make sure you pay attention to and follow recommended cleaning and maintenance schedules. If something doesn't look right or sound right, call the manufacturer's customer service department. An active approach might make the difference between a simple, rapid fix and a complex, lengthy one. When shopping around for a new IHC stainer, one of the criteria you may wish to consider when making your selection is the manufacturer's proven history of instrument reliability as well as its commitment to customer support and service.

Economics of IHC Stainers

The cost to acquire and operate an automated IHC stainer varies from manufacturer to manufacturer. In addition to the outright purchase of a new instrument, most companies make available numerous purchase options, including the purchase of a refurbished instrument at a reduced price and various leases, rentals and reagent acquisition plans. The options should be reviewed to determine which works best for your particular needs, time of year, funding cycle, etc. Of course, once the instrument is acquired, you still have the daily operating expenses incurred through the purchase of antibodies, detection reagents, buffer solutions and other ancillaries. Estimating these operating expenses can be done with the help of the instrument company's representatives and will help you plan your budget. In many cases the instrument's operating expenses may be offset partially by freeing histotechnologists' time. The additional labor freed by use of the instrument may help reduce the need for expensive overtime or increased personnel. The consistent, high-quality staining that instruments provide can also help save money by reducing the number of re-stains and by making it easier and faster for a pathologist to render a diagnosis.

Flexibility and Ease of Use

In some respects, a more flexible platform is by necessity more complex. However, well-designed software and a clean, intuitive user interface goes a long way toward



making that platform easy to use as well as flexible. Not everyone feels comfortable and competent operating computer-driven devices. An instrument that's easy to use will be accepted and embraced by more of the histology staff, thus easing entry of the (possibly) new technology into the lab and making the scheduling of work rotations easier. The instrument's ease of use also will come in handy when the lab decides to branch out into more complex but very useful home-brewed assays, such as multiplex IHCs that utilize cocktails of several antibodies and potentially multi-colored detection.

Workflow and Data Management

Finally, any decision to make an investment in automation should take into account how well the device fits into the natural workflow of the lab, as well as the data management system of the institute.

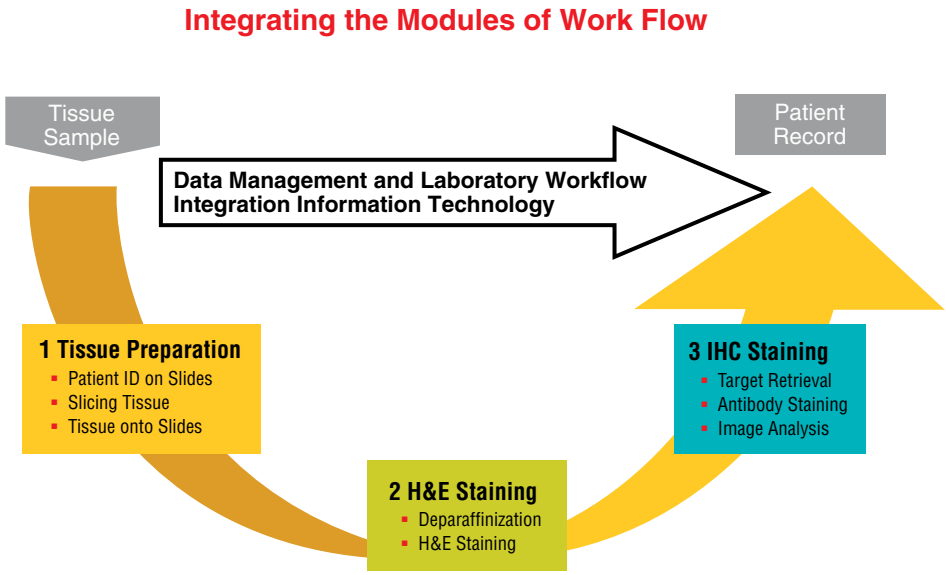


Figure 13.1. Data management and workflow.

What is the lab's cutoff time for new IHC orders? When does the pathologist expect to receive the stained slides for review? What is the expected turn-around time for reporting the results, and will use of the instrument improve that? Just as an instrument can provide flexibility in staining, it also can provide flexibility to the lab with respect to scheduling runs and reporting results. For example, most histology labs operate on a single shift that may begin at 5:30 a.m. and end by 2:30 p.m. The day begins with the removal of the previous night's run of tissue from the processor and continues with embedding, sectioning and staining with H&E. A pathologist examines these slides and may order additional testing, including IHC and/or ISH (in situ hybridization testing).

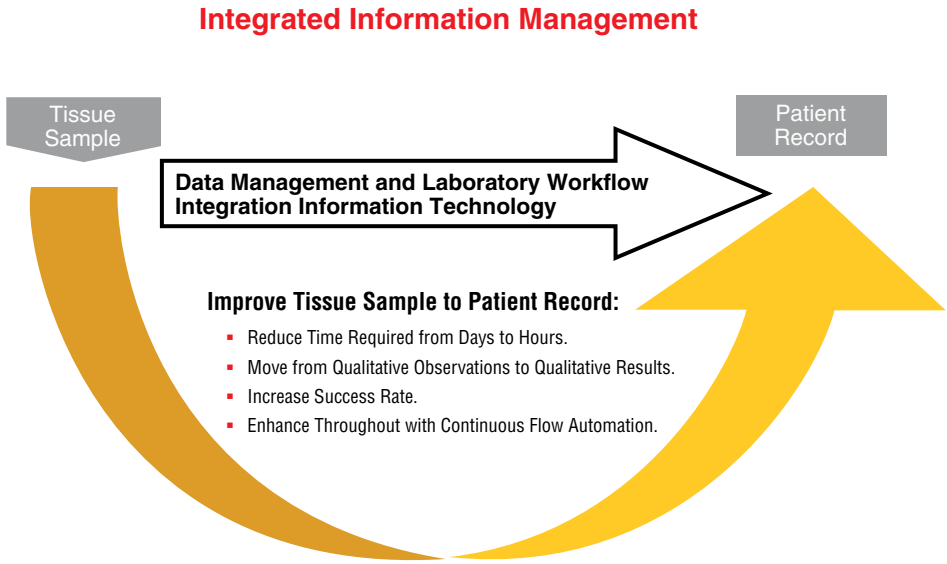


Figure 13.2. Objectives of Integrated Information Management.

Additional slides are sectioned for the requested new procedures. There may be a cutoff time by which the IHC tests must be ordered, in order for the slides to be sectioned that day. By that time it's getting late, and could almost be time for the histotechnologists to wrap up for the day. The next day these slides are processed and IHC stained, and the whole process repeated. In comparison, an automated IHC stainer capable of running unattended overnight could allow those slides to be stained on the same day they are sectioned, and ready for review by a pathologist first thing in the morning, instead of mid-afternoon. In other words, automation provides options that could streamline lab operations in addition to providing consistent, high-quality results. Exactly how an instrument would benefit any given lab depends on the lab itself and the willingness of the staff to consider and implement beneficial changes to their usual routine.

Chapter 14 ■ Virtual Microscopy and Image Analysis

Kenneth J. Bloom

The History

Automating manual microscopy has been evolving since the first demonstration of telepathology in 1968. The concept has evolved along two distinct pathways, one based on technology, the other on need. The technology folks focused on adapting technology developed for other disciplines in an effort to prove that automated microscopy was possible. Improvements in digital camera resolution, speed and fidelity, the invention of the robotic microscope and stage, the development of the internet and the exponential advancements in computer technology including processor speed, memory and storage have contributed to eliminating the hurdles that prevented a viable automated microscopy system. The second group focused on resolving problems in anatomic pathology, such as rapid second opinion, pathology staffing of remote frozen sections, cost reduction for reference laboratories, medical student and resident teaching, continuing medical education, and improved storage and retrieval of slides - to name just a few (1-7).

The concept of developing a functional robotic telepathology network, with the aim of providing real-time expert opinions for frozen sections and difficult cases was put forth by Ronald Weinstein in the mid 1980s (8). I spent a large part of my residency developing the software and system integration necessary to build a prototype so that human performance data could be generated (9). All of the performance studies supported the feasibility of telepathology, and a demonstration project for the U.S. Department of Defense showed how slides prepared in El Paso, Texas could be read remotely via satellite, at the Armed Forces Institute of Pathology in Washington, D.C. Although the prototype was successful, there were many limitations to commercializing a telepathology system, most involving a lack of telecommunication infrastructure and standards. But just as importantly, there was little perceived need for the technology.

Since it was not cost effective to purchase a satellite, cheaper alternatives were sought, and the concept of “static telepathology” was introduced as an alternative. In static telepathology, a pathologist captures and saves a digital image or series of images from a camera mounted on a microscope, and then forwards the images to a remote computer, where they may be reviewed by a second pathologist. E-mail and File Transfer Protocol (FTP) servers were readily available to facilitate the transfer of images and standards in image formats were evolving rapidly; however, the static nature of the images severely limited clinical use. To fully represent a standard pathology slide, it would be necessary to acquire thousands of static images, making routine use impractical. Although few pathologists used these systems diagnostically, many pathologists use them for tumor boards, teaching and other educations purposes.

The next step in evolution was the creation of "stitching software." This technology allowed the digital representation of an entire microscopic slide by digitizing individual microscopic fields and then stitching them together to create a virtual slide. This process was laborious and time consuming, and the computer processing and storage requirements pushed the limits of available technology. Because of these limitations, the next systems that were created were hybrids, containing elements of both static and dynamic systems. These systems would digitize an entire slide at low power magnification creating a tissue map for the pathologist to select areas of interest, which would then be re-digitized at higher magnification and forwarded as a series of static images. Most of the previous limitations now have been overcome, such that we can effectively digitize an entire microscopic slide at resolutions comparable to that obtained with a standard light microscope.

Scanning the Slide

The first step in virtual microscopy is to obtain a digital representation of a pathology slide. Although the resulting image typically is stored as a two-dimensional file, pathology slides are three-dimensional structures, with the z-axis ranging from three to seven microns in thickness. With low magnification lenses, a single focal plane suffices, but with higher magnification lenses, the depth of focus is less than the thickness of the slide, necessitating that the scanning system have the ability to capture and average several focal planes. Before scanning the slide, the system must first establish a focal plane. All systems have the ability to autofocus, but because of patent issues, they all do it in a slightly different manner. Some start above the tissue plane and focus down, some start below the tissue plane and focus up, while others start within the tissue plane. Advanced systems use a second camera to monitor and adjust the focus continuously as the slide is scanned, but most methods simply create a map of the tissue on the slide to be scanned, and then using principles such as "triangulation," create "focus points." Then as the slide is scanned the system performs an autofocus at each calculated focus point. In this way systems hope to overcome problems such as variations in thickness and tissue folds.

The process of scanning a slide also differs between various systems. All processes involve acquiring images in some fashion and then stitching them together to create a representation of the slide. Older systems acquire images as tiles, while newer systems use other methods such as "linear scanning," or using an array of lens (10). Many of the newer methods were developed merely to workaround patent issues while others offered significant improvements in image acquisition.

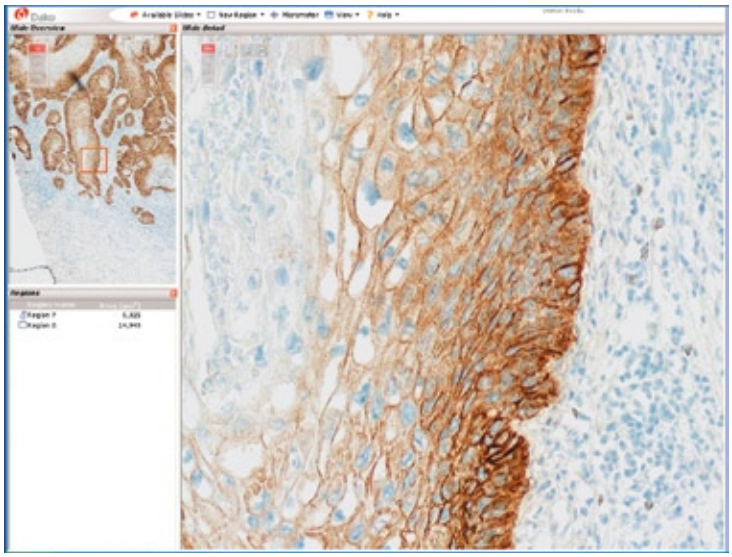
Scanning systems generally are judged by two criteria: speed and resolution. The total speed of acquisition involves not only acquiring the image, but also stitching the images together and storing the resulting image on a computer. Since the purpose of acquiring a virtual slide is to view it in the future, some additional processing often is implemented to facilitate the viewing process. For example, to implement the full functionality of a standard light microscope, the scanned image must be viewable at the same objective

magnifications found on a standard microscope, namely, 1x, 2x, 4x, 10x, 20x and 40x. Assuming the slide was scanned, stitched and stored at the equivalent of a 40x objective, viewing the 40x image would not require additional processing. However, to view the image at the equivalent of a 4x objective, the 4x image would have to be derived from the 40x image before it was displayed. This would result in a significant delay between the time an objective was selected and the time it was displayed on a monitor. To overcome this delay, some vendors process the acquired image in a format that already has calculated the image at all intermediate magnification levels. While this format, known as a pyramidal format, decreases the time to load intermediate magnification views, it increases the image processing necessary following image acquisition and produces a larger file to be stored (11).

The ability of the image acquisition system to resolve features present in the microscopic slide is known as the absolute or, “point-to-point resolution” and is dependent on the microscope objectives, the camera lens and the analog to digital conversion process. What is more important is the actual resolution, which is also dependent on hardware and software compression techniques, the video card and monitor used to display the image. Many commercial digital cameras, for example, have chips that automatically “clean” the image by performing tasks such as edge sharpening.

Viewing the Virtual Slide

There is currently no accepted standard for viewing virtual slides. Each hardware vendor has created software for viewing the image files that they create. All software programs perform similar tasks such as changing the apparent objective, moving the slide in any direction, saving screenshots as image files, and annotating specific areas of the virtual slide. Many programs have advanced features, including viewing multiple slides simultaneously, slide synchronization and video conferencing.



The quality of the image viewed on a monitor is not only a function of the resolution with which the slide was acquired, but also the resolution and color depth of the video card and monitor itself. The apparent resolution viewed by the pathologist may be deemed unsuitable, even if the image is acquired in high resolution. For example, if the image is viewed on a poor resolution monitor, or a monitor being fed by a low resolution video card, the image will be only as good as the hardware allows. Virtual microscopy systems that use your existing desktop computer as the viewing station will be only as good as the video card and monitor in that system. On the other hand, virtual microscope systems are similar to a standard microscope, in that the pathologist can and should manipulate the image. In the case of a standard light microscope, the pathologist alters the light intensity, the condenser and the diaphragm to create the desired level of brightness and contrast, while in video microscopy, the pathologist is provided with controls to adjust the brightness and contrast to produce the desired image. It should be noted that the video card/monitor systems can reduce the quality of the scanned image, but cannot improve the resolution beyond the initial quality from when the slide was scanned.

Capturing Images for Image Analysis

Collecting an image for subsequent analysis has a few more requirements than systems that obtain images for virtual microscopy. Since the image is going to be analyzed, procedures must be put in place to ensure that the image is captured reproducibly, and that the system is operating in the detectable range of whatever is to be analyzed. For example, when acquiring an image for the purpose of quantifying the amount of antigen such as HER2/*neu*, the same image file must be produced whether the slide is scanned today, tomorrow or six months from now. While this sounds trivial, it is not. It is well known that all analog imaging systems are subject to drift over time, including CCD cameras, which is just a camera with a built-in analog-to-digital converter. The problem of drift is especially acute in color systems, because color is, in effect, the ratio of the different color channels. Small changes in the signal of any channel can give rise to large changes in apparent color. Other sources of variation that must be considered include variation in the light source, variation in the transmission of the light, variation in the camera, and variation in the analog-to-digital conversion process. It is well known that as a bulb ages it becomes hotter, until the filament eventually burns out. Unfortunately, as the bulb becomes hotter it alters the color spectrum that the bulb emits. Adding further complexity, some of these factors vary at different level of magnification. Luckily, calibration can be performed to adjust these variables.

All light sources can drift, and the rate of drift can be fast enough to require daily calibration. An image collection system must either stabilize the bulb, adjust the camera gain, or collect a reference image of a known-to-be-stable calibration object to correct for this. Since the color temperature of the light source also might drift, this correction needs to be done in each channel. Care must be taken to perform this calibration at an intensity that will not saturate any channel, since saturation of the channels can give rise to a false appearance of equality.

The systems for illuminating a glass slide do not provide truly uniform illumination. Unless corrected for, this will cause algorithms to give different results in different parts of the field of view. A system must collect calibration images of known-to-be-stable, dust-free, and blank fields to have enough data to correct the captured image to that of a flat field. Since the calibration will be different at each level of magnification, this data must be collected for each objective. Since dust profoundly affects calibration, algorithms must be able to detect and correct for dust on the calibration target.

When a pathologist reviews a slide on a standard light microscope, the pathologist manually adjusts for many of these variables as described previously. This is possible because the human eye is very adept at correcting for these variations, and pathologists use features for diagnosis that have proven to be reproducible over time. Don't be lulled into thinking that an image analysis system works like the human eye, it doesn't. Of course anyone can apply image analysis software to a digitized slide, but if the digitized image is even subtly different each time the slide is acquired, over time the results from the analysis will lack precision.

Systems used for image analysis therefore must undergo calibration prior to use. At a minimum, the calibration must adjust for all of the aforementioned variables, and also include a set of calibrators that help determine the minimal amount of chromogen that can be detected; the maximal amount of chromogen that can be detected before saturation of the signal occurs; and help establish that the system can detect the specific chromogen in a linear fashion over the reportable range. This is identical to the sort of calibration routinely performed on equipment in a clinical pathology laboratory.

Image analysis represents a significant step in standardizing the interpretation of histologic slides. The creation of an image analysis is not an easy task. Just as it takes considerable time and effort to create and validate a pharmacodiagnostic assay, so too does it takes significant effort to create and validate a clinically useful image analysis system. These systems are meant to complement pathologists, not replace them. Accordingly they should not attempt to emulate what pathologists can do well, but rather they should aid pathologists in tasks that the human eye does not do well. Additionally, the principle of garbage-in, garbage-out cannot be overstated. There is no point in attempting to quantify an immunostain unless one is sure that the immunohistochemical procedure has been validated and is known to be linear, or at least follow a known curve, across the range of analysis.

Virtual microscopy is an import new technology that already has penetrated pathology education significantly. With recent improvements in the technology, other uses are sure to follow quickly. If performing image analysis is an important consideration, care must be taken to select acquisition systems that have calibrators appropriate for the type of analysis to be performed (12).

Applications for Virtual Microscopy

- Telepathology
- Image analysis
- Education
- Quality assurance
- Cost reduction
- Higher throughput
- Research

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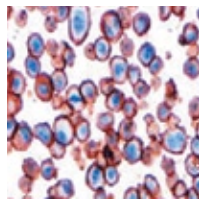


Chapter 15 ■ Controls

Ole Feldballe Rasmussen

Introduction

Many factors may introduce variations in immunohistochemistry: Differences in tissue fixative and fixation time, day-to-day variations due to temperature, variations due to different workers' interpretations of protocol steps or in the conditions of reagents applied on a particular day.

Most diagnostic reagents suppliers have implemented measures to safeguard the quality of their reagents. However, many factors may influence an immunohistochemical staining, so it is not always sufficient to assume that any given staining is correct. It is important, therefore, to include reagent and tissue controls for verification of immunohistochemical staining results for in vitro diagnostic use. It is also important to understand what information a given control can provide and what information a control cannot provide. This chapter will describe the range of controls that should be adapted in a diagnostic laboratory.

Reagent Controls

The most important reagent in immunohistochemistry is the primary antibody. Without good specificity of the primary antibody the IHC stain will be jeopardized. In addition to the manufacturer's quality guarantee, it is important for the user to ensure the quality of the primary antibody prior to its use.

During development, most manufacturers ensure specificity using a range of immunochemical techniques. These may include immunoelectrophoresis, Western Blot, double diffusion, rocket immunoelectrophoresis and ELISA. Testing on transgenic cells expressing the specific as well as closely related antigens also may be performed. It is, however, imperative to test the primary antibody in immunohistochemistry. In general, manufacturers first test antibodies on a range of positive tissues to identify optimal antibody dilution in combination with chosen staining protocols. Next, immunohistochemistry testing is extended to an expanded panel of additional tissues known to either contain or not contain targeted antigens. For new antibody lots, manufacturers typically perform quality control to ensure specificity and sensitivity documented during development.

Users must control reagents within routine quality programs, documenting reagents, dilutions, diluents, incubation times and dates to which any procedural changes are introduced by proper record keeping. In laboratories with changing environmental conditions, it is also advisable to keep track of the relative humidity and temperature.

Negative Controls

For monoclonal primary antibodies, nonspecific negative reagent controls may be developed by different methods. The optimal method is an antibody of the same isotype, present in the same immunoglobulin concentration, using the same diluent and exhibiting no specific reactivity with the given human tissues tested. A less optimal alternative is to use mixtures of antibodies representing all or most relevant IgG subtypes. Finally, the diluent itself also may be used as an alternative, which, however, is neither efficient nor desirable.

For polyclonal antibodies, negative reagent controls should be a dilution of immunoglobulin fractions or whole serum of normal/non-immune serum of the same animal source. Again, the negative reagent control should be applied in the same concentration as the test antibody, and the same diluent should be used.

Using the same protocol as the primary antibody, the negative reagent control should be applied to a sequential section of each patient specimen, to evaluate nonspecific staining in that particular tissue.

NOTE: A special situation to be aware of is when two or more antibodies are applied to serial sections. In this case, negative stain areas of one slide may serve as the negative/nonspecific binding background control for other antibodies.

In cases where it is necessary to evaluate non-specific binding potentially caused by sources other than the primary antibody, additional patient tissue sections may be stained with selected reagents. For example, tissues may be stained with just the secondary antibody and/or the enzyme followed by application of the substrate/chromogen. In cases where the suspected non-specific staining may be the result of endogenous enzyme present within the tissue, this can be confirmed by application of the substrate/enzyme only.

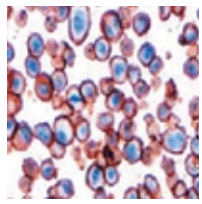
Tissue controls

Tissue controls can be negative, positive, or internal. Each serves a different purpose.

Positive Tissue Controls

These are indicative of proper staining techniques and provide a measure of whether the target retrieval procedure has been carried out correctly. They should assess correct temperature and incubation period of water baths or other retrieval methods. Likewise, positive tissue controls verify that all reagents were applied, that they performed correctly, and the proper incubation time and temperature were used.

These controls are also indicative of properly prepared tissue. To be as accurate as possible, positive tissue controls should be prepared in the same manner as patient samples. Optimally autopsy/biopsy/surgical specimens should be fixed, processed and embedded as soon as possible for best preservation of antigens. Please see the section, Processing Control Indicator, next page.



One positive tissue control should be included for each set of tests. Ideally, this control should contain a spectrum of weak to strongly positive reactivity. If such tissue is not available, another option is to select a weakly positive tissue, as this provides the best basis to evaluate whether a particular staining reaction is too weak or too strong.

During a staining run, positive tissue controls may be run on a separate slide, or included on the same slide as the test specimen. If this second option is chosen, one method is to use small arrays with selected tissue or cell lines to serve as a positive control for a range of stains. In this method, one tissue may serve as a positive control, a different tissue may serve as a negative control (see below).

If positive tissue controls do not perform as expected, results from test specimens should be considered invalid.

Positive controls cut and stored in bulk with cut surfaces exposed for extended periods should be tested to determine if the antigens are stable under these storage conditions.

Internal Tissue Controls

Internal controls, also known as “built-in” or intrinsic controls, contain the target antigen within normal tissue elements, in addition to the tissue elements to be evaluated. Thus, they can replace external positive controls. This is ideal, as the tissue elements to be evaluated have been treated exactly as the positive control. One example of a “built-in” control is the presence of S-100 protein in both melanoma and normal tissue, such as peripheral nerves and dendritic reticulum cells. Other examples include vimentin, which is distributed ubiquitously, and desmin, which is present in blood vessel musculature.

Negative Tissue Controls

Positive staining of negative controls could indicate a lack of specificity of the antibody or nonspecific background staining. Just as in positive controls, tissue used for negative controls should be prepared in the same manner as the patient sample. Additionally, the selected tissue should not contain the specific antigen to be tested. One example would be use of normal liver tissue as a control for hepatitis B surface antigen, or a HER2/neu-negative tissue for testing with Dako HercepTest™ kit.

If positive staining occurs in the negative tissue control, consider test specimen results invalid.

Processing Control Indicator

There is currently no optimal way to evaluate whether tissue processing has occurred satisfactorily. Battifora (1) has suggested using vimentin, a substance present in virtually every tissue specimen. Furthermore, the vimentin V9 clone recognizes an epitope that is partially susceptible to fixation with formaldehyde and can function as a “reporter” for the quality of fixation. Proper processing should give uniform distribution of vimentin reactivity in tissue vessels and stromal cells. Good, uniform vimentin staining

demonstrates adequate fixation, while heterogeneous staining indicates variable and suboptimal fixation. In these cases only fields demonstrating the best staining intensity and homogeneity should be used in the patient sample analysis.

Cell Line Controls

Several FDA-approved predictive IHC assays contain cell line controls as part of the diagnostic kit or are sold separately. These are developed specifically to monitor staining of the antigen of interest, and should be included in all stain runs as an additional protocol control.

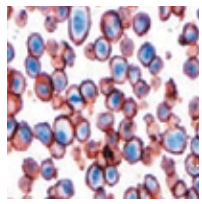
Just as with tissue controls, cell line controls may be positive or negative. Positive cell line controls monitor staining performance by assessing target retrieval, blocking, antibody incubation and visualization. Negative cell line controls assess specificity and, depending on the characteristics of the chosen cell line, also may provide information on performance.

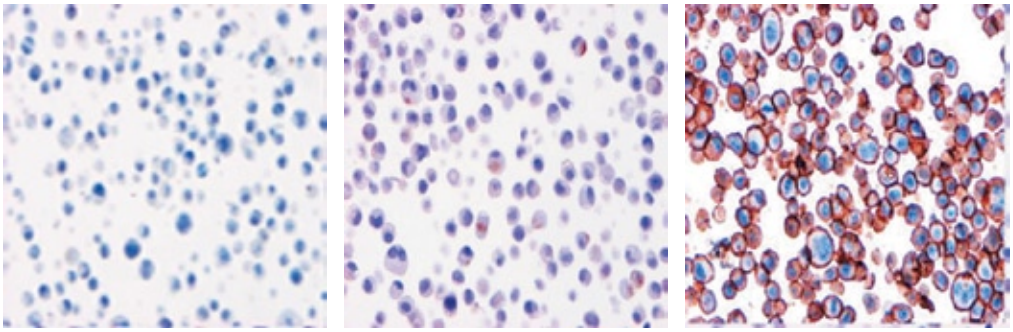
An ideal negative cell line control will contain an amount of target antigen, sufficiently low to produce no staining if the procedure has been performed correctly. At the same time, the amount should be sufficiently high to produce a weakly positive stain if the run has been performed under conditions that produce an excessively strong stain.

An ideal positive cell line control would contain a number of target antigens producing a medium intensity stain. This would allow the control to assess both stains that are too weak and stains that are too strong.

An example of the way in which cell line controls can be used is illustrated by Dako's HercepTest™ kit, which contains three cell line controls: A negative, a 1+ (weak staining) and a 3+ (strong staining). All are designed to be placed on the same microscope slide.

Figure 15.1, next page, shows examples of staining HercepTest™ control cells. Fluorescent in situ hybridization kits such as *HER2*FISH pharmDx™ do not contain cell line controls because they include probes against both the target gene and the respective centromere, in order to evaluate the amplification ratio (or deletion) of the target gene. In this way, the centromere probe also serves as internal control.





Negative cell line | 1+ cell line | 3+ cell line

Figure 15.1. Cell line controls for Dako HercepTest™

Control Programs

Immunohistochemical stain test results have no common quantitative measures. Instead, results typically are based on subjective interpretation by microscopists of varying experience (2-4). Quality control and assurance, therefore, remain crucial and need high attention by both manufacturers and laboratory users.

A number of scientific bodies have quality programs or quality assessment services. These programs should be seen as an aid or external assistance and should never replace national requirements for internal quality control.

One highly regarded program is the non-profit United Kingdom National External Quality Assessment Service (UK NEQAS), established in 1995 to "advance education and promote the presentation of good health by providing external quality assessment services for clinical laboratories." UK NEQAS includes a range of specific external quality assessment (EQA) services, each focusing on areas such as breast screening pathology.

Likewise, in the United States the College of American Pathologists (CAP) provides a similar proficiency testing service for its member laboratories.

Also the external quality assessment program run by Nordic Immunohistochemical Quality Control (NordiQC), established in 2003, should be mentioned. It now has more than 100 laboratories participating.

Each organization provides proficiency testing in IHC for participating laboratories by sending out tissue samples to be included as part of a laboratory's routine IHC staining procedures. Laboratories then return their results, which are compared with all other participating laboratories and summarized in a final report.

Contact information for CAP can be found at the Web site, www.cap.org, for UK NEQAS at the Web site, www.ukneqas.org.uk, and for NordiQC at the Web site, www.nordiqc.org.

Future Aspects

To ensure quality diagnosis, immunohistochemistry quality control will become even more important. It is expected that the next five years will see increased participation in proficiency testing, as an increase in the number of laboratories that become accredited. New technologies on the horizon also will facilitate more efficient means of quality control.

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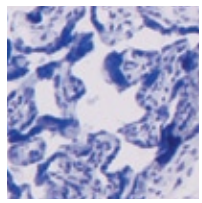


Chapter 16 ■ Background

Helle Grann Wendelboe and Kirsten Bisgaard

Introduction

As immunostaining of histological tissue specimens becomes more diversified in methodology and more sensitive in detection, background staining has developed into one of the most common problems in immunohistochemistry. Background staining in tissue sections may be due to several factors, some of which are specific to the antigen and antibody reaction or detection method, and others, which are of a more general character. The terminology used in this chapter uses the term "unwanted specific staining" if the staining is mediated by interactions between any antibodies and their respective epitopes and "nonspecific staining" for all other interactions. The following description will cover the major causes of background staining related to antibodies, detection methods, and other general factors and will offer possible solutions to these problems.

Background Associated with Detection Methods

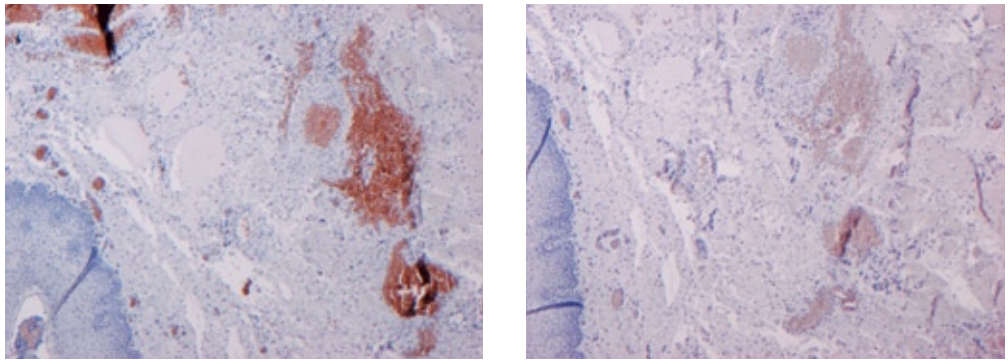
Horseradish Peroxidase-Based Detection Methods

Endogenous Peroxidase Activity

For practical purposes in immunohistochemistry, endogenous peroxidase activity can be defined as any activity that results in the decomposition of H_20_2. Such activity is a common property of all hemoproteins, such as hemoglobin (red cells), myoglobin (muscle cells), cytochrome (granulocytes, monocytes) and catalases (liver and kidney). Peroxidase activity also may be encountered in tissue areas adjacent to vascularized areas 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 three percent H_2O_2 for five to 10 minutes (Figure 16.1). Methanolic H_2O_2 treatment (11 parts, three percent H_2O_2 plus four parts absolute methanol) for 20 minutes also is used, but is not recommended for specimens where cell surface markers are to be stained. Methanolic treatment also may detach frozen sections from their carrier glass. Endogenous peroxidase activity also can be suppressed by a mixture of sodium azide and H_2O_2 (1). However, in most work with formalin-fixed tissue sections, the interpretation of specific staining is not impaired by any endogenous peroxidase activity. If the formalin-fixed tissue is rich in blood-containing elements then it will be a good idea to quench endogenous peroxidase activity. In cell preparations and frozen sections, routine quenching of endogenous peroxidase is also advisable.

Specimens rich in endogenous peroxidase activity may be processed using an alkaline phosphatase detection method instead of a peroxidase method, eliminating the background.



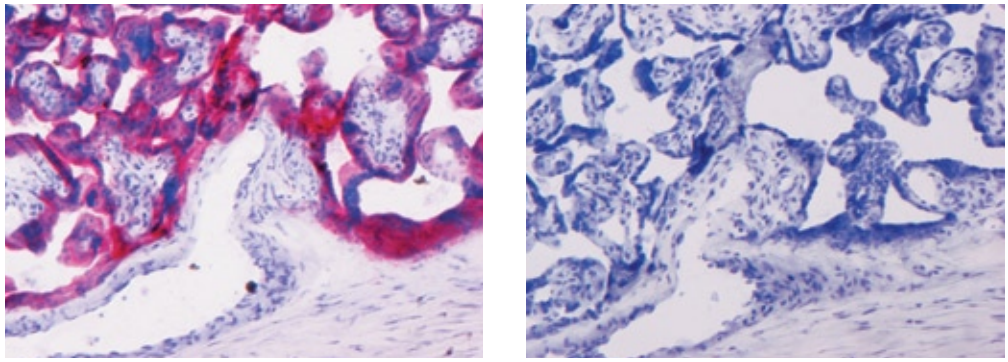
(a) Before (b) After

Figure 16.1. Red blood cells showing endogenous peroxidase activity (a) before, and (b) after blocking with three percent hydrogen peroxide.

Alkaline Phosphatase-Based Detection Methods

Endogenous Alkaline Phosphatase

Endogenous alkaline phosphatase activity is encountered frequently in intestine, kidney, osteoblasts, endothelial cell surfaces, neutrophis, stromal reticulum cells, lymphoid tissues, and placenta. In frozen tissue, where endogenous alkaline phosphatase activity is pronounced most, routine quenching of endogenous alkaline phosphatase is recommended. In most formalin-fixed tissue sections, interpretation is not usually impaired by endogenous alkaline phosphatase, which makes quenching an optional choice. Most forms of endogenous alkaline phosphatase can be quenched by including five mM levamisole in the chromogen substrate solution (Figure 16.2). The intestinal form of alkaline phosphatase is the exception and resists this treatment, but it can be quenched by treating the tissue sections with a weak acid wash prior to the application of the primary antibody.



(a) Before (b) After

Figure 16.2. Placenta showing endogenous alkaline phosphatase activity (a) before, and (b) after blocking with levamisole.

Double Staining

Combined Endogenous Peroxidase and Alkaline Phosphatase

Double staining using these enzymes requires quenching of both endogenous activities. To achieve this, use the H_2O_2 method for endogenous peroxidase and the weak acid method for endogenous alkaline phosphatase. The sequence of blocking endogenous activities is optional: The procedure will work effectively conducting either step first. Also, reagents that block both endogenous peroxidase and alkaline phosphatase in one step are available.

Biotin/Streptavidin-Based Detection Methods

Endogenous avidin-binding activity (EABA) has been observed with all biotin-based techniques, due to its presence in a wide variety of tissues. Biotin is bound to enzymes and other protein, especially in the liver (hepatic nodules), kidney (tubular epithelia) and lymphoid tissue (paracortical histiocytes) (Figure 16.3). EABA usually is observed within cytoplasm and is pronounced most when using frozen tissue sections. Paraffin-embedded tissues also hold substantial endogenous biotin. Other examples of EABA include the nonimmunochemical staining of myelin (2) and mast cells (Figure 16.4) in both frozen and paraffin-embedded tissue (3). Guesdon et al (4) found EABA in granulocytes from mouse spleen.

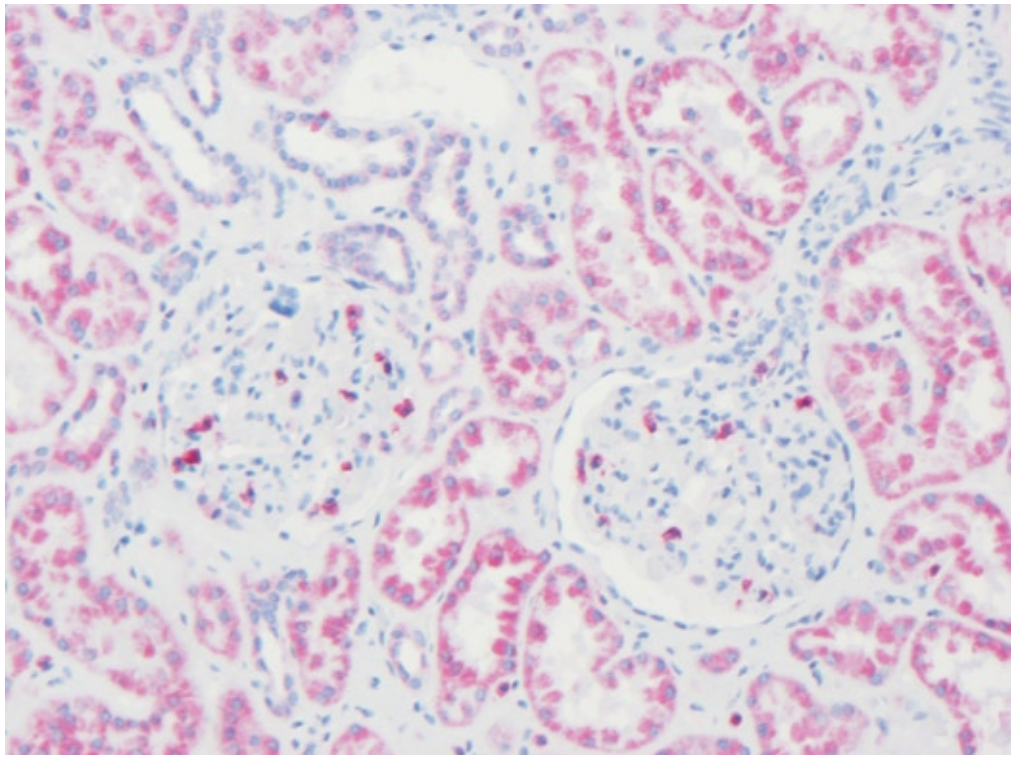
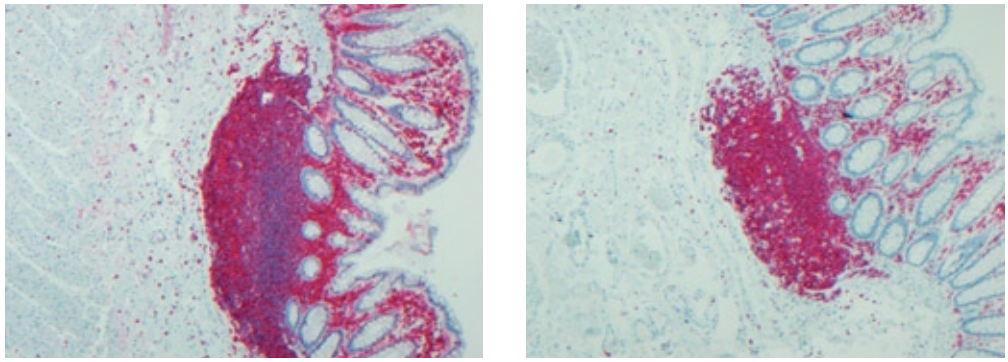


Figure 16.3. Endogenous biotin expression in the kidney.

EABA is suppressed best by sequential incubations of 10 to 20 minutes of tissue sections, first with 0.01 percent to 0.1 percent, avidin followed by 0.001 percent to 0.01 percent biotin prior to the staining protocol (5). Avidin has four binding sites for biotin while each biotin molecule can bind to only one avidin molecule. The first incubation with avidin effectively blocks endogenous biotin but simultaneously adds three more potential biotin-binding sites to the specimen. This means there are extra biotin binding sites open to link antibodies or detection systems that can give background staining. Therefore, it is important to block these extra biotin binding sites with a subsequent biotin incubation.

Because avidin is a glycoprotein containing 10 percent carbohydrates and has a pI of 10, it tends to bind nonspecifically to lectin-like and negatively charged tissue components at physiological pH. Streptavidin contains no carbohydrates and has a pI of five. Its introduction to IHC largely has eliminated these problems. A sugar solution can block the lectin-like elements. Many commercially available Avidin detection systems contain modified avidin to minimize nonspecific avidin background.



(a) Before (b) After

Figure 16.4. Avidin-biotin-complex (ABC) binding to mast cells in submucosa (a) before, and (b) after blocking for endogenous avidin binding activity (EABA).

Polymer-Based Detection Methods

Use of polymer detection systems avoids endogenous avidin/biotin background completely. General overall background staining may occur if insufficient washing is performed after polymer application. Due to the large size of polymer conjugates, the diffusion rate of these molecules is lower than for low-molecular weight conjugates. In addition, polymer conjugates based on a hydrophobic backbone have a tendency to be sticky. This can be resolved by applying multiple wash steps, adding detergent to the wash buffer and by prolonging washing time.

Antigen Retrieval

This has been reported both to eliminate and introduce cytoplasmic and nuclear background in immunohistochemical procedures (6). A possible explanation is that antigen retrieval influences antigen-antibody binding activity, and thereby affects binding of the antibody to tissue proteins. Different types of antigen retrieval solutions with different buffer compositions, pH and chelating abilities exist. Citrate pH 6.0, TRIS/EDTA pH 9.0, and TRS pH 6.0 retrieval solutions vary in the way they influence antigen-antibody binding. Retrieval time also can influence antigen-antibody binding, so for new antibodies it is advisable to investigate which antigen retrieval solution and time are best to optimize signal and minimize background.

General Factors

Antigen Diffusion

Unwanted 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. Because many fixatives penetrate tissues slowly, it is important to keep tissue specimens as

small as possible and to fix immediately. Otherwise the antigens may not be adequately fixed and may be extracted or displaced by the subsequent tissue processing steps. Extracellular antigens or those of low molecular weight are more likely to diffuse than high-molecular-weight antigens.

A typical example is the diffusion of thyroglobulin from thyroid follicular epithelium and colloid-containing lumen into surrounding stromal tissue. Similarly, specific background may result when the tissue marker also is present in high concentrations in blood plasma and has diffused in the tissue prior to fixation. This can be seen when tonsil tissue is stained for immunoglobulin heavy and light chains (Figure 16.5), particularly when fixation was not performed promptly and when antisera were not diluted sufficiently. Ingestion of target antigens by phagocytes also may produce specific background staining, resulting in stain patterns not normally seen in such cells.

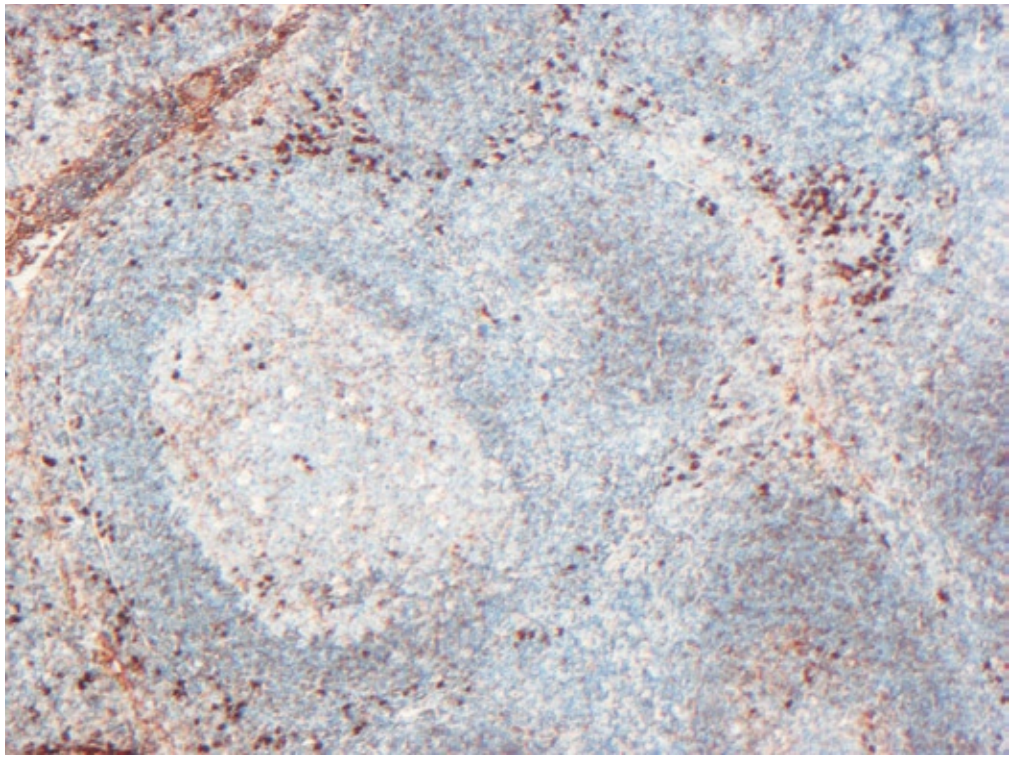
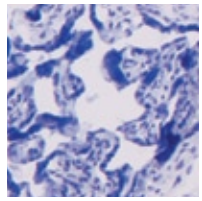


Figure 16.5. Undesirable staining of plasma proteins with antibody to kappa light chain. Plasma cells stain specifically.



Natural and Contaminating Antibodies

Natural Antibodies

Low-level natural antibodies present in the antiserum as a result of prior environmental antigenic stimulation may increase in titer during immunization with use of adjuvants. As a consequence, they can give rise to nonspecific staining. In 1979, Osborn et al (7) reported that sera from non-immunized 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 (8).

Most natural antibodies are of the nonprecipitating type and occur only in relatively low concentrations. These antibodies usually are 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 a host's immune system reacts to impurities, contaminated 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.

Contaminating antibodies may be related to infectious agents, other animal species kept in the same facilities, or carrier proteins used for immunization. These antibodies may be of special concern when dealing with antisera against synthetic peptide. Small peptides are not antigenic, and therefore must be coupled to carrier proteins prior immunization. The antisera produced therefore will contain antibodies against the carrier protein and the peptide.

However, if contaminating antibodies do interfere with specificity, affinity absorption of the antiserum usually is 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 (8).

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

Problems stemming from natural and contaminating antibodies, of course, do not occur with monoclonal antibodies produced in tissue culture, but may be present in monoclonal antibodies prepared from ascites fluid.

Cross-Reactivity

Background staining due to antibody cross-reactivity may result when target tissue antigen epitopes are shared with other proteins. A typical example is the use of 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 antibody cross-reactivity with similar or dissimilar epitopes on different antigens may also be the cause of confusing background staining. This is rare however, and can be avoided by using antibodies from hyper-immunized animals or carefully selected clones.

Cross-reactivity of antigens from related species is a common problem in multi-staining. This often can be avoided by using affinity purified antibodies, sub-type specific antibodies or site/ region specific antibodies. For more detail on cross-reactivity, see, Antibodies, Chapter 1.

Fc Receptors

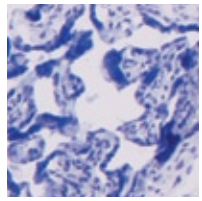
Fc receptors (FcR) are a family of detergent-soluble membrane glycoproteins with approximate molecular weights of 50–70 kD. They comprise less than one percent of the total membrane proteins and are present most frequently on macrophages and granulocytes. They also have been reported on B cells and some T cells. The intrinsic affinity of the FcR for monomeric IgG is approximately 1x106 to 1x108 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 IgG2a and IgG3 but not other IgG subclasses (9). Goat sera do not react with FcR's of human leucocytes (10).

Background staining due to FcR is more common in frozen sections, smears and in lightly fixed 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.

Hydrophobic Interaction

In aqueous media, hydrophobic interactions between macromolecules occur when surface tensions are lower than that of water (called van der Waals forces). These interactions 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 primarily through the side chains of 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 between different protein molecules.

Tissue Proteins

In tissue, proteins are rendered more hydrophobic by fixation with aldehyde-containing reagents such as formalin and glutaraldehyde. Increased hydrophobicity often results from cross-linking reactive epsilon- and alpha-amino acids within and between adjacent tissue proteins. The extent of this hydrophobic cross-linking during fixation is primarily a function of time, temperature and pH. Changes in these factors likely will 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 and squamous epithelium (keratins) and adipocytes (lipoids) if incompletely removed during processing with xylene. Excessive background staining due to overfixation with formalin may be remedied by postfixation with Bouin's, Zenker's or B5 fixative (11).

Antibodies

Of the major serum proteins, immunoglobulins unfortunately are particularly hydrophobic. In general, mouse 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 also may increase their hydrophobicity and lead to aggregation and polymerization. This frequently leads to a diminution in, or loss of, immune reactivity. 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 (12).

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

Anions: PO_4^{-3}, SO_4^{-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 adding detergent, for example 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 to use a protein blocking solution either in a separate step, or by adding it to the antibody diluent. However this will be successful only if the blocking protein is a type that can compete effectively with IgG or its aggregates or conjugates, for hydrophobic binding sites. Separate incubation with a solution containing blocking protein is best when carried out immediately prior to application of the primary antibody. The solution should contain proteins identical to those present in the secondary link or labeled antibody, but not to those in the primary antibody, in order to prevent nonspecific binding of the secondary antibody.

The addition of one percent bovine serum albumin (BSA) to the primary antibody diluent is probably the most widely practiced step for reducing non-specific binding due to hydrophobic interaction. Use of non-fat dry milk (13) or of casein (14) for reducing background staining also is recommended. Casein, when used as a blocking agent, an antibody diluent and in the wash buffer, was found to result in significantly less background staining compared to normal swine and sheep sera (14).

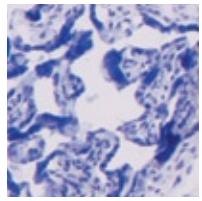
Because of the different uses of biotinylated antibodies today, it should be of interest to note that biotinylation can change the pI of the antibody in excess of three units, for example from a pI of eight for the antibody to less than five for the conjugate (15). This may have a marked effect on the 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. However, they also may contribute 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 have been reported to interact with cationic conjugates composed of rabbit Fab fragments and horseradish peroxidase type VI (pI 10.0) (16). In general, interactions of the ionic type can be reduced by use of diluent buffers with higher ionic strength. Addition of NaCl to the diluent buffer can reduce background staining stemming from ionic interactions but its routine use in diluents for monoclonal antibodies is not recommended (17).

Unfortunately, most diffuse background staining results from a combination of ionic and hydrophobic interactions. Remedies for one type of interaction may aggravate the other.



Complement-mediated Binding

Complement-mediated binding occasionally may 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 usually are inactivated.

Miscellaneous Sources

Physical injury to tissue, drying out prior to fixation or incomplete penetration of fixative may cause diffuse staining of all or most tissue elements within an affected area. 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 also may show this type of diffuse background, especially in procedures of high staining sensitivity. Water baths should be free of bacterial or yeast contamination.

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

Nonimmunologic binding of horseradish peroxidase (either in free form or as a conjugate) to HbsAg in hepatocytes was reported by Omata et al (18). The precise nature of this binding was not known.

Necrotic areas of tissue may stain with all reagents. Nadji and Morales (19) provide an excellent collection of color plates illustrating background staining and accompanying explanations.

Excessive counterstaining may compromise the specific staining signal.

General Aspects

While it is clear that background staining can be caused by the factors outlined above, it is also important to work with well-characterized reagents and established protocols in order to avoid background or to troubleshoot background staining. Many IHC reagent providers offer "system solutions," which are IHC product lines with carefully optimized buffers, target retrieval reagents, primary antibodies, detection reagents and substrates to be run on an automated platform. These are designed to provide users with consistent, optimal staining. Several countries have established national quality programs, such as United Kingdom National External Quality Assessment Service (UK NEQAS) (20), and Nordic Immunohistochemical Quality Control (NoridQC) (21), formed to raise awareness of the need for quality and best practices in IHC laboratories, including improving the reduction of background staining. See Chapter 15, Controls, for further discussion. Information on individual national programs can be found on each program's Web site.

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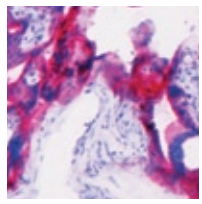


Chapter 17 ■ Troubleshooting

Karen N. Atwood and Dako Technical Support Group

Introduction

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. The information contained in this chapter should enable you to rapidly pinpoint and resolve problems encountered during the staining procedure.

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 inadequate 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, streptavidin-biotin 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. You are encouraged to copy this chart and use it to help troubleshoot any problems you may encounter with your staining systems.

Section Four is a guide to reading manufacturers' specification sheets for IVD antibodies. This includes general information for use in immunohistochemistry including fixation, recommended visualization systems, recommended titer and diluent, pretreatment, and selection of required controls.

Section One:

Troubleshooting problems commonly encountered during immunohistochemical staining.

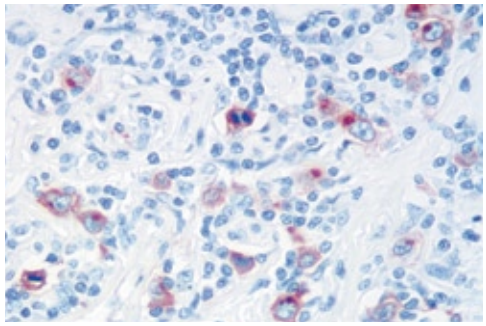


Figure 17.1a. anti-CD30 diluted 1:50 with Tris-HCl, pH 7.6.

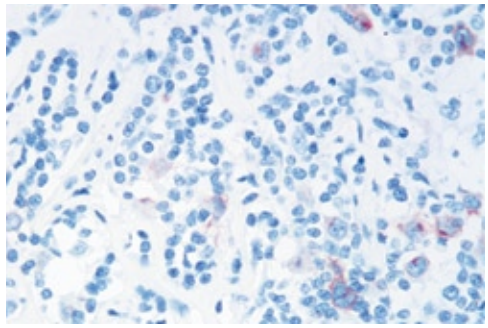


Figure 17.1b. anti-CD30 diluted 1:50 with PBS, pH 7.0.

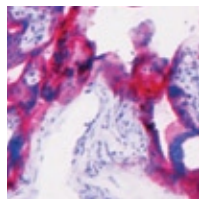
Figure 17.1a and 1b. Ongoing studies performed in the DakoCytomation Research & Development laboratory confirm that the pH and ion content of the antibody diluent may have a significant effect on the sensitivity of monoclonal antibodies. (Hodgkin's lymphoma stained with CD30 (clone Ber-H2) antibody, using a three-stage immunoperoxidase staining system.)

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 labeled reagent omitted. Reagent used in wrong order.	Repeat the procedure using the manufacturer's staining system specification sheet or standard operating procedure reagent checklist as established by the individual laboratory.	47-53
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 two- to five-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.	10, 15-16

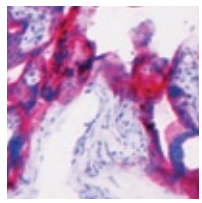


Inadequate Staining (continued)

Possible Cause	Solution	See Page
Primary antibody diluted with inappropriate buffer. ■ Use of PBS or TBS as an antibody diluent. ■ Lack of stabilizing or carrier protein. ■ Detergent in diluent.	Check formula and compatibility of antibody diluent. A change of ion content 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 seen primarily with monoclonal antibodies.	47-53
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 specification sheet or package insert. ■ If using a neat or concentrated antibody, and directed by the manufacturer to store frozen, it may be aliquoted to 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.	10-11
Dissociation of primary antibody during washing or incubation with link antibodies.	A feature of low-affinity antibodies: ■ Polyclonal primary antiserum: Attempt staining at low dilutions. ■ Monoclonal primary antibody: Replace with higher affinity antibody of identical specificity. ■ Re-optimize incubation times for washing buffer and link antibody.	7-8
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/DAB+, that is not affected by organic solvents.	21-22
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.	119-129
Incorrect preparation of substrate-chromogen mixture.	■ Repeat substrate-chromogen treatment with correctly prepared reagent. ■ Staining intensity is decreased when excess DAB/DAB+ is present in the working reagent.	Spec Sheet

Inadequate Staining (continued)

Possible Cause	Solution	See Page
Incompatible buffer used for preparation of enzyme and substrate-chromogen reagents: ■ Use of PBS wash buffer with an alkaline phosphatase 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 phosphates activity. ■ Avoid sodium azide in diluents and buffers. A concentration of 15 mM/L sodium azide, which is added routinely to IHC reagents to inhibit bacterial growth, will not impair HRP conjugated labels.	47-53
Antigen levels are too low for detection by the employed visualization system. 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, using a range of pH buffers.	47-53
Steric hindrance due to high antigen level and possible prozone effect.	Re-optimize concentration of the primary antibody and ancillary reagents. Antibody concentration of the primary antibody may be too high.	47-53
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.	27-33



Inadequate Staining (continued)

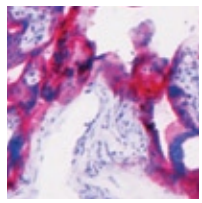
Possible Cause	Solution	See Page
Immunoreactivity diminished or destroyed during embedding process.	Use a paraffin wax with a melting temperature ~ 55-58 °C. Wax used for embedding should not exceed 60 °C.	27-33
Immunoreactivity diminished or destroyed during dewaxing at high oven temperature.	Oven temperature 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.	27-33
Immunoreactivity diminished or destroyed on pre-cut tissue sections.	The intensity of immunostaining may be diminished when pre-cut tissue sections are exposed to air. Use freshly cut sections and reseal paraffin-embedded blocks.	27-33
Immunoreactivity diminished or destroyed by the enzyme blocking reagent altering a specific epitope.	More common on frozen sections: apply the primary antibody prior to the enzymatic block to insure its reaction. In such cases the blocking reagent can be applied at any point after the primary and before the enzyme labeled components.	13, 30, 31, 58, 107, 119, 126
Excessive wash buffer or blocking serum remaining on tissue section prior to application of IHC reagents.	Excess reagent will dilute the next consecutive reagent. Repeat staining, making sure to wipe away excess washing buffer and blocking serum.	15-16
Demasking protocol is inappropriate or has been omitted.	Some tissue antigens require proteolytic enzyme digestion or heat-induced antigen retrieval performed prior to staining. The 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.	27-33
Repeated reuse of antigen retrieval buffer.	Do not reuse buffer.	Spec Sheet
Sections incorrectly dewaxed.	Prepare new sections and deparaffinize according to standard laboratory protocol, using fresh xylene or xylene substitute.	119-129

Inadequate Staining (continued)

Possible Cause	Solution	See Page
Failure to achieve the optimal temperature required for heat induced antigen retrieval.	■ When using a waterbath or steamer, allow sufficient time for the retrieval buffer to equilibrate to a temperature range of 95-99 °C. ■ At high altitude (greater than ~ 4,500 feet), the buffer will boil at less than 95 °C. ■ Utilize a closed heating system such as a pressure cooker, autoclave or Pascal, or utilize a low temperature protocol if standardization of the validated procedure is not affected.	41-44
Excessive or incomplete counterstaining.	Re-optimize concentration of counterstain and incubation time.	41-44
Instrument malfunction.	Ensure automated stainer is programmed correctly and is running to manufacturer's specifications.	95

Positive control tissue shows adequate specific staining with little or no background staining. Specimen tissue shows little or no specific staining with variable 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 aldehydes 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.	41-44
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.	119-129
Sectioned portion of specimen contains necrotic or otherwise damaged elements.	Ignore physically damaged portions of stained tissue sections.	41-44



Inadequate Staining (continued)

Possible Cause	Solution	See Page
Section 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.	27-33, 119-129

General Background

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

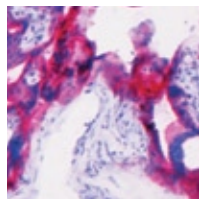
Possible Cause	Solution	See Page
Excessive incubation with substrate-chromogen reagent.	Reduce incubation time.	Spec Sheet
Substrate-chromogen reagent prepared incorrectly.	Repeat incubation with correctly prepared chromogen reagent.	Spec Sheet
Secondary or link antibody cross-reacts with antigens from tissue specimen.	Absorb link antibody with tissue protein extract or species-specific normal serum from tissue donor.	47-53
Secondary or 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.	16-18
Slides inadequately rinsed.	Gently rinse slide with wash buffer bottle and place in wash bath for five minutes. Gentle agitation of the wash bath may increase effectiveness when used with cytoplasmic or nuclear staining protocols.	7-8
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.	119-129

General Background (continued)

Possible Cause	Solution	See Page
Blocking serum or wrong blocking serum used.	Block with serum from the host of the secondary or link antibody. Avoid serum that contains auto-immune immunoglobulins. Alternatively, a serum-free protein block, lacking immunoglobulins, may be substituted for the serum block.	119-129
Sections incorrectly dewaxed.	Prepare new sections and deparaffinize according to standard laboratory protocol using fresh xylene or xylene substitute.	119-129
Non-specific binding of the secondary antibody with an animal tissue specimen.	Use a secondary antibody that has been absorbed against a species specimen, or use a secondary antibody produced in a host that exhibits little or no cross-reactivity with the tissue source.	47-53
Instrument malfunction.	Ensure automated stainer is programmed correctly and is running to manufacturer's specification.	95

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 aldehydes 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.	27-33
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.	27-33



General Background (continued)

Possible Cause	Solution	See Page
Sectioned portion contains crush artifact caused by grossing tissue with dull scalpel or razor. Serum proteins diffuse through tissue and are fixed in place.	Serum proteins diffuse through tissue and are fixed in place. Re-cut tissue using sharp blade.	119-129
Sectioned portion of specimen contains necrotic or otherwise damaged elements.	Ignore physically damaged portions of stained tissue sections.	119-129
Excessive or unevenly applied subbing agent on poly-L-lysine, charged, or silanized slides.	Some IHC reagents may 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.	119-129
Antigen diffusion prior to fixation causing specific background outside the expected antigen site.	Avoid delays in fixation of the tissue.	119-129
Tissue sections too thick.	Cut tissue sections thinner. Formalin-fixed paraffin-embedded tissue sections should be approximately 4-6 µm; cryostat section 10 <µm.	27-39
Incomplete permeabilization of tissue sections.	Seen in frozen sections, cell smears and non-paraffin embedded tissue: Incomplete permeabilization of cells allows unattached reagents to become trapped within the cells and resistant to removal by wash buffer.	99

General Background (continued)

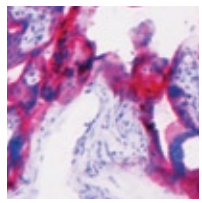
Negative reagent control slide shows background. Positive control tissue, negative control tissue and specimen tissue show expected specific staining.

Possible Cause	Solution	See Page
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 the primary antibody.	113-118
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.	113-118
Negative reagent control serum contaminated with bacterial or fungal growth.	Replace product with non-contaminated serum.	12-13

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. Avoid allowing water from an initial section mounting to flow over an area where additional sections will be mounted. This is particularly important when using charged or silanized slides.	35-39, 119-129
Undissolved granules of chromogen.	Insure that chromogen in tablet or powder form is completely dissolved, or switch to a liquid chromogen.	119-129



Limited Background (continued)

Possible Cause	Solution	See Page
Incomplete removal of embedding medium.	Remove embedding medium thoroughly, using fresh reagents	119-129
Incomplete dezenkerization of tissue fixed with B5 or mercury containing reagents.	Perform dezenkerization with fresh reagents.	27-33
Bacterial or yeast contamination from mounting waterbath.	Clean and refill waterbath.	119-129
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. ■ When using an automated staining instrument, addition of wet towels to the sink may prevent drying of slides.	119-129
Instrument malfunction.	Ensure automated stainer is programmed correctly and is running to manufacturer's specification.	95, 119-129

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 usually can be disregarded.	119-129
Primary antibody and negative reagent control serum are diluted insufficiently.	Reoptimize the dilution of the primary antibody and negative control serum.	15-16

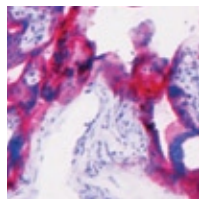
Limited Background (continued)

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.

Possible Cause	Solution	See Page
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.	5-7, 119-129
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.	27-33, 119-129

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.	119-129



Limited Background (continued)

Background seen in all control and specimen tissue when using an immunoperoxidase staining system.

Possible Cause	Solution	See Page
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 and mast cells are particularly resistant to peroxidase quenching. Use a peroxidase blocker. ■ Use special stains: Eosin will stain eosinophils a bright red-orange.	119-129

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. Pretreat the tissue with 0.03 N HCl.	119-129

Limited Background (continued)

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

Possible Cause	Solution	See Page
Endogenous protein-bound biotin (water-soluble B vitamin). High amounts of biotin are found in adrenal, liver, and kidney. Lesser amounts are found in the GI tract, lung, spleen, pancreas, brain, mammary gland, adipose tissue, lymphoid tissue, and cells grown in culture media containing biotin as a nutrient.	Use a biotin block or chose another non-biotin based staining system.	119-129

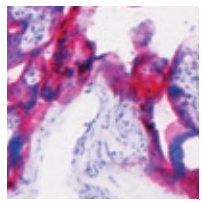
Background of skeletal or smooth muscle tissue in positive control tissue, negative control tissue, specimen tissue and negative reagent control.

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.	119-129

Undesired "Specific" Staining

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

Possible Cause	Solution	See Page
Binding of the 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 intact antibodies. Add detergent to the wash buffer.	119-129



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

Possible Cause	Solution	See Page
Phagocytosis of antigens may render phagocytes positive for the same.	Rare. Should not interfere with interpretation of specific staining.	119-129

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.	119-129

Miscellaneous

Loss of viability of cell cultures.

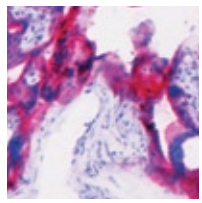
Possible Cause	Solution	See Page
Some manufacturers produce antibodies and reagents for in vitro use only. These products may contain preservatives, usually sodium azide, which is a known poison.	Utilize an in vivo product for application on viable cells. For use on cell cultures only: Sodium azide may be dialyzed out of some reagents. Contact Dako Technical Support for additional information.	119-129

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 endogenous pigment (such as 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 three percent hydrogen peroxide or other peroxidase blocking reagent. Using a new bottle of hydrogen peroxide, perform a three percent H_2O_2 peroxidase block, followed by DAB and an appropriate counterstain.
NO STAINING SEEN. GO TO NEXT STEP. ▼	
SLIDE #3 — Positive Control Tissue: Peroxidase Block + Secondary Antibody + 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 a biotin block or switch to a staining system that is not dependent on the streptavidin/biotin reaction.
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 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 a biotin block or switch to a staining system that is not dependent on the streptavidin/biotin reaction.

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	Brown/Red color observed: ■ May indicate non-specific binding of the primary antibody carrier-protein. Perform a protein block with normal serum from the host of the link antibody 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

NO STAINING SEEN. GO TO NEXT STEP.

▼

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 levamisole (Intestinal alkaline phosphatase may be quenched by the addition of 0.03 N HCl prior to the addition of the alkaline phosphatase).

SLIDE #2

Positive Control Tissue:
Streptavidin-AP + Fast Red, Fuchsin or BCIP/NBT + Counterstain

NO STAINING SEEN. GO TO NEXT STEP.

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 a biotin block or switch to a staining system that is not dependent on the streptavidin/biotin reaction.

SLIDE #3

Positive Control Tissue:
Biotin Block (if required) + Secondary Antibody + Streptavidin-AP + Fast Red, Fuchsin or BCIP/NBT + Counterstain

NO STAINING SEEN. GO TO NEXT STEP.

Red/Blue color observed:

- Indicates non-specific or undesired binding 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 10 µL of normal serum (from the species of tissue to be stained) per 100 µL of the secondary antibody.

SLIDE #4

Positive Control Tissue:
Biotin Block (if required) + Negative Reagent Control + Secondary Antibody + Streptavidin-AP + Fast Red, Fuchsin or BCIP/NBT + Counterstain.

NO STAINING SEEN. GO TO NEXT STEP.

Red/Blue color observed:

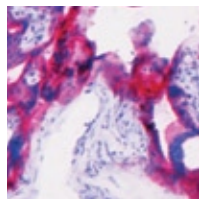
- May indicate non-specific binding of the primary antibody carrier-protein. Perform a protein block with normal serum from the host of the link antibody or a protein block; add 0.05-0.1% TWEEN 20 to wash buffer to decrease protein attachment.
- Antigen retrieval lipofusion-artifact may appear as granule staining in liver and cardiac tissue or as specific staining in pancreatic sections.

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.



Negative Control Reagent

Reagents	Result/Action
Negative Control Reagent: Perform complete staining protocol.	■ (Human tissue) Perform the peroxidase blocking protocol from Slide #2 under "Background Staining Encountered with HRP-Peroxidase Reagents." ■ Perform a biotin block if required, protein block if required, apply the appropriate negative reagent control (see below), apply biotinylated secondary antibody, apply streptavidin/HRP reagent and DAB.

Prepare a negative reagent control

- Polyclonal: Non-immunized sera from the same species, diluted to the same protein concentration as the primary antibody.
- Monoclonal: Negative reagent control that matches the isotype as the primary antibody. Additionally, the diluent used to manufacture a monoclonal primary antibody and isotypic negative control should contain the same ions. Diluents containing sodium or phosphate ions may change the sensitivity of some monoclonal antibodies.
- Calculation:
  - Ig or total protein concentration of primary antibody divided by dilution factor of primary antibody = x.
  - Ig or total protein concentration of negative reagent control divided by x = dilution factor of negative reagent control.

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.

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:

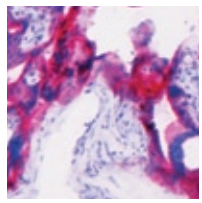
Type of fixative _____

Length of time _____

Size of specimen _____

Tissue mounting:

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



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 0.03-0.5 N 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.

Peroxidase block:

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

Alkaline Phosphatase block:

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

Biotin block:

- ☐ Biotin Block (Dako Code X0590)
- ☐ Other _____

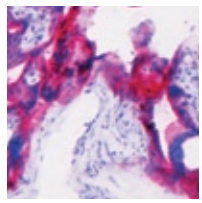
Protein block:

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

Section Four:

Using a Typical Specification Sheet for an IVD Antibody

Information You Need to Know	Information Located on the Specification Sheet	Comments
Regulatory Status of the Primary Antibody	Intended use ■ For in vitro diagnostic use.	Indicates that a product meets the FDA requirements as a clinical diagnostic product. Likewise, a CE icon indicates the reagent meets European Union requirements. Patient test results do not require an FDA disclaimer.
Tissue Preparation	Specimen preparation ■ Paraffin sections: The antibody can be used for labeling paraffin- embedded tissue sections fixed in formalin. Pre-treatment of tissues with heat-induced epitope retrieval is required. Optimal results are obtained with 10 mmol/L citrate buffer, pH 6.0. Less optimal results are obtained with 10 mmol/L Tris buffer, 1 mmol/L EDTA, pH 9.0. The tissue sections should not dry out during the treatment or during the following immunocytochemical staining procedure. ■ Frozen sections and cell preparations: The antibody can be used for labeling frozen sections or fixed cell smears .	Indicates the type of specimen that was used during validation studies. In many cases this would include formalin-fixed tissue and frozen sections. Use of other fixatives requires validation by each individual laboratory. This section also indicates the optimal epitope retrieval procedure and warns against procedures that may destroy the epitope. Specimen preparation and staining procedure sections can and will change periodically, to reflect changes in technology. So remember to retain copies of each version of the reagent specification sheet. Version numbers are usually found on each page.

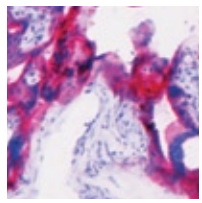


Using a Typical Specification Sheet for an IVD Antibody (continued)

Information You Need to Know	Information Located on the Specification Sheet	Comments
Choosing the Visualization System	Staining procedure ■ Visualization: This antibody can be used with an immunoperoxidase staining method. Follow the procedure enclosed with the selected visualization kit. ■ Automation: The antibody is well-suited for immunocytochemical staining using automated platforms.	Indicates the recommended visualization system to be used with the antibody. It also indicates that the antibody can be used for automated staining. NOTE: If your state regulatory agency requires written documentation that a reagent can be used for automated staining and this indication is not listed on the specification sheet, you may wish to contact the manufacturer's technical support group for further information.
Diluting the Primary Antibody	Staining procedure ■ Dilution: Monoclonal Mouse Anti-Vimentin, may be used at a dilution range of 1:50-1:100 when applied on formalin-fixed, paraffin-embedded sections of human tonsil.	Includes a suggested dilution range for the antibody and the recommended diluent. The dilution range is merely a suggested starting point for an individual laboratory. Optimal conditions may vary depending on specimen, preparation method, temperature of the laboratory or automated instrumentation.
Negative Reagent Control	Reagent provided ■ Isotype: IgG1, kappa. Staining procedure ■ The recommended negative control is mouse monoclonal IgG1, diluted to the same mouse IgG concentration as the primary antibody. Positive and negative controls should be run simultaneously with patient specimen.	Use of a negative reagent control is required by the College of American Pathologists (CAP), based on Clinical Laboratory Improvement Amendments (CLIA 2003), for each patient or patient block in a staining run.

Using a Typical Specification Sheet for an IVD Antibody (continued)

Information You Need to Know	Information Located on the Specification Sheet	Comments
Positive Control Tissue	Performance characteristics ■ Normal tissues: In general, most human mesenchymal cells are labeled by the antibody, including fibrocytes, lipocytes, smooth muscle cells, vascular endothelial cells, astrocytes, peripheral nerve (Schwann) cells, macrophages (including Kupffer cells), as well as myoepithelial cells of sweat and salivary glands and of breast, which are all labeled strongly. Also positive, with variable intensity and distribution, are the follicular cells of the thyroid, adrenal cortex, renal distal tubules, and mesangial and endothelial cells of the renal glomerulus, as well as pancreatic acinar cells (1,2). In the human eye, the antibody labels the pigmented posterior and the anterior epithelia of the human iris, including the muscle portion (dilator pupillae) of the anterior epithelium, as well as the nonpigmented and pigmented ciliary epithelia (4). In the ciliary epithelia, vimentin was coexpressed with cytokeratin (4). ■ Abnormal tissues: The antibody labeled 17/20 sarcomas, 16/18 melanomas, 4/4 meningeomas, and 3/3 schwannomas, and was the sole intermediate filament present in these tumours. In addition, variable percentages (10 to 57 percent) of carcinomas, neuroendocrine carcinomas, neuroblastomas, thymomas and mesotheliomas were positive with the antibody. With the exception of the neuroblastomas, cytokeratin was coexpressed with vimentin in these tumours. Among adenocarcinomas, more than 50 percent of papillary carcinomas of the thyroid as well as renal, endometrial, ovarian and lung carcinomas were labeled by the antibody and coexpressed keratins and vimentin.	CLIA 2003 Sec. 493.1273 (3) Mandates that fluorescent and immunohistochemical stains must be checked for appropriate positive and negative reactivity each time they are used. Most IVD antibody specification sheet will list tissue that will exhibit positive and negative staining patterns in the Performance Characteristics section. NOTE: abnormal tissue will not necessarily be labeled. Both negative and positive tissue controls should be processed using the same fixation, embedding, mounting, drying, epitope retrieval and immunostaining protocols as the patient tissue.



Using a Typical Specification Sheet for an IVD Antibody (continued)

Information You Need to Know	Information Located on the Specification Sheet	Comments
Negative Control Tissue	Performance characteristics ■ Normal tissues: Skeletal and cardiac muscle cells, epidermal, squamous, urothelial, colonic and gastric mucosal, and glial cells, as well as neurons are consistently negative with the antibody .	

References

1. Wood G, et al. Suppression of Endogenous Avidin-Binding Activity in Tissues and Its Relevance to Biotin-Avidin Detection Systems. Journal of Histochemistry and Cytochemistry 2981;29(10):1196-204.
2. Sayaki H, et al. Azure B as a Counterstain in the Immunohistological Evaluation of Heavily Pigmented Nevomelanocytic Lesions. Applied Immunohistochemistry 1995;3(4):268-71.
3. Federal Register: January 24, 2003;68(12) 42CFR Part 493.
4. College of American Pathology; Anatomic Pathology Checklist, October 2005.

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 Guide.

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 commonly is 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, eg, 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 (ie, 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 most commonly is 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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