Immunohistochemistry: a Novel Tool for the Diagnosis of Animal Disease

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Abstract

Immunohistochemistry (IHC) is a novel technique for diagnostic and research purposes. In the last decade, the ability to detect antigens (Ag) in tissue sections has improved dramatically, mainly by countering the deleterious effects of formaldehyde with antigen retrieval (AR) and increasing sensitivity of the detection systems. IHC provides the most direct method for identifying both the cellular and sub-cellular distribution of your protein.Two types of antibodies are used in IHC—polyclonal antibodies, and monoclonal antibodies.IHC is used for disease diagnosis, drug development and biological research. Using specific tumor markers, physicians use IHC to diagnose a cancer as benign or malignant, determine the stage and grade of a tumor, and identify the cell type and origin of a metastasis to find the site of the primary tumor. IHC is also used in drug development to test drug efficacy by detecting either the activity or the up or down-regulation of disease targets. Samples are prepared on individual slides, or multiple samples can be arranged on a single slide for comparative analysis, such as with tissue micro-arrays. IHC slides can be processed and stained manually, while technological advances now provide automation for high-throughput sample preparation and staining. Samples can be viewed by either light or fluorescence microscopy, and advances in the last 15 years have improved the ability to capture images, quantitative multi-parametric IHC data and increase the collection of that data through high content screening.

1. Introduction

Immunohistochemistry (IHC) is a novel technique for diagnostic and research purposes. In the last decade, the ability to detect antigens (Ag) in tissue sections has improved dramatically, mainly by countering the deleterious effects of formaldehyde with antigen retrieval (AR) and increasing sensitivity of the detection systems. IHC provides the most direct method for identifying both the cellular and sub-cellular distribution of protein.

Two types of antibodies are used in IHC—polyclonal antibodies and monoclonal antibodies. Polyclonal antibodies are produced by immunizing rabbits or another species with the antigen to be detected. Polyclonal antibodies are multivalent, meaning that they contain antibodies for several regions of the antigen molecule, providing a strong detection capacity. However, polyclonal antibodies can cross-react with antigens from different organisms, e.g. polyclonal antibodies raised against bacterial lipopolysaccharide might also recognize different species of Gram-negative bacteria (Shi et al., 1997; Taylor et al., 1996).

Monoclonal antibodies are produced in mice and are the result of fusing immunoglobulin-producing B cells with myeloma cells. This fusion results in hybrid cells, which continue to grow and divide in culture and which also produce antibodies. Monoclonal antibodies have the advantage of being highly specific for a single sequence or 'epitope' of the antigen molecule. However, because monoclonal antibodies will bind only to one site (epitope) of the antigen molecule, fewer antibody molecules will bind to the antigen and be subsequently detected by the labeling method. Fixation may modify that particular binding site, making the antibody unable to recognize (bind to) the antigen; and thus fail to detect it (Shi et al., 1991).

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analysis, such as with tissue micro-arrays. IHC slides can be processed and stained manually. Technological advances now provide automation for high-throughput sample preparation and staining. Samples can be viewed by either light or fluorescence microscopy, and advances in the last 15 years have improved the ability to capture images, quantitative multi-parametric IHC data and increase the collection of that data through high content screening (Polak and van Noorden, 1997).

2. Antigens (Ag) and Antibodies (Ab)

IHC is based on the binding of Ab to a specific Ag in tissue sections. The most common immunoglobulin (Ig) used in IHC is IgG. IgM is less commonly used.Igis Y-shaped and consists of two identical light chains and two identical heavy chains. The heavy chains determine the Ab class. The tail of the Y is called Fc. The light chains of most vertebrates have two distinct forms, called kappa and lambda. In any Ig molecule, both light chains and both heavy chains are of the same type. The light chains consist of two distinct regions—the C-terminal half of the chain is constant and called CL (constant: light chain), whereas the N-terminal half of the chain has abundant sequence variability and is called the VL (variable: light chain) region. The Fab region is the Ag-binding portion of the Ig and contains variable and constant segments of the heavy and light chains. The Fc portion of the Ab determines the biological functions and permits Ab binding to other Ab, complement, and inflammatory cells with Fc receptors. This portion of Ig is needed for multi-step immunohistochemical techniques. This part is also responsible for background staining resulting from non-immune adherence of Ab to the tissue section; to avoid that, there has been a trend to use only Fab or F(ab)2 portions of the Ig molecule for IHC. The major problem with this approach is that the Fc portion of Igs tends to stabilize Ab binding to solid substrates such as tissue. The specific binding of an Ab to an Ag occurs via hypervariable regions of both heavy and light chains of the amino terminus. The Ag binding site of an Ab is called the paratope. Epitopes are the regions of an Ag that bind to Abs. Epitopes are usually 5-21 amino acids long. One of the most important criteria for binding of an Ab is the tertiary structure of the epitope, or the way in which the peptide chains of a protein are folded together or interact with adjacent peptides. The paratope interacts with the tertiary structure of the epitope through a series of non-covalent bonding interactions. The more bonding interactions, the greater the affinity and avidity (defined as the overall binding strength between the Ag and Ab) of the Ab. IgG Abs are bivalent (have two identical arms used in Ag recognition). This is a key feature necessary to perform multiple-layer immunohistochemical methods (Shi et al., 1991; Werner et al., 1996).

Ag can have different structures (isoforms). A single gene can generate several different protein isoforms via the principal mechanisms—alternative splicing of the primary gene transcript can produce multiple different matured transcripts, each of which codes for a slightly different protein. Many proteins undergo posttranslational modifications, such as glycosylation, phosphorylation, and proteolytic processing, which add more complexity to proteins derived from a single gene. As a result of these mechanisms, a single gene can generate numerous protein isoforms and even this repertoire can change with time, e.g. tenascin and hemoglobin isoforms that change from fetal development to adulthood (Morgan et al., 1994; Werner et al., 1996).

3. Ag-Ab Interaction

From a chemical and biochemical point of view, Ag-Ab interactions are somewhat unusual. The bonds involved are weak (mostly hydrophobic and electrostatic) and not covalent. Hydrophobic bonds happen between macromolecules with surface tensions lower than that of water. They can be interatomic or intermolecular. These hydrophobic interactions are imparted primarily through the side chain amino acids phenylalanine, tyrosine, and tryptophan. By their lower attraction to water molecules, these amino acids tend to link to one another. Electrostatic (coulombic) interactions are caused by attractive forces between one or more ionized sides of the Ag determinant and oppositely charged ions on the Ab-active site. These typically are the carboxyl and the amino groups of polar amino acids of the Ag and Ab molecules. Van der Waals forces are weak electrostatic interactions between dipolar molecules or atoms. Van der Waals forces and electrostatic attractions are maximal at the shortest distances. Therefore, precise juxtaposition of oppositely charged ions on epitopes and paratopes favors strong electrostatic bonding. Hydrogen bonds are the result of dipole interactions between OH and CO, NH and CO, and NH and OH groups. The binding energy is of the same order of magnitude as that of van der Waals and electrostatic interactions. Its significance in Ag-Ab interactions is probably not great because of the necessity of a very precise fit between both molecules for it to happen. Although there are cases in which only one of these types of interactions is significant in Ag-Ab binding, for most polysaccharide, glycoprotein, and polypeptide Ag the Ag-Ab bond is a combination of van der Waals forces and electrostatic interactions. Ab, except for IgM (which is dekavalent) is divalent. Most protein Ag is multivalent. Each valency site of protein Ag generally is an antigenic determinant (epitope) with a completely different configuration from all the other valency sites, e.g. a monoclonal Ab can react with only one valency site of such a protein Ag. Affinity is a thermodynamic expression of the binding energy of an Ab

(paratope) to an antigenic determinant (epitope). Affinity can be defined in mathematical terms as an affinity constant (KA), which represents the amount of Ag-Ab complex that will be formed at equilibrium. The range of Ag-Ab affinity constants is wide and varies from below 105 to 1012 $lmol^{-1}$. What do these numbers mean? An Ag-Ab complex with a KA of 1012 $lmol^{-1}$ has a thousand-fold greater affinity than an Ag-Ab complex with a KA of 109 $lmol^{-1}$. The affinity of the Ag-Ab reaction is important for practical reasons—high-affinity Ab will bind more Ag in a shorter incubation time than low-affinity Ab; and in general, the higher affinity detects the more dilution of the Ab solution (Werner et al., 1996; Chu et al., 1982).

4. Sample Preparation

While using the right Abto target the correct Ag and amplify the signal is important for visualization, complete preparation of the sample is critical to maintain cell morphology, tissue architecture and the antigenicity of target epitopes (Shi et al., 1997; Taylor et al., 1996).

5. Tissue Collection and Perfusion

Patient or animal biopsies, or whole animal organs, are collected for preservation and IHC analysis, depending on the requirements of the assay. Tissue must be rapidly preserved to prevent the breakdown of cellular protein and tissue architecture. Often, the tissue is perfused, or rinsed of blood, prior to preservation to prevent the detection of hematologic antigens that may interfere with the detection of target antigens. Tissue perfusion is performed on anesthetized animals by using a peristaltic pump to exsanguinate the animal and rinse the vasculature with sterile saline to remove all blood components from the entire animal. The target organ or tissue can then be collected for IHC (Morgan et al., 1994; Holgate et al., 1983).

6. Tissue Fixation

Fixation chemically cross-links proteins or reduces protein solubility, which can mask target Ag during prolonged or improper fixation. Therefore, the right fixation method must be optimized based on the application and the target antigen to be stained. The most common fixative is formaldehyde, a semi-reversible, covalent cross-linking reagent that can be used for perfusion or immersion fixation for any length of time, depending on the level of fixation required. Other fixatives are available, and their use depends on the Ag being sought (Morgan et al., 1994).

7. Tissue Embedding

Fixed tissue samples are embedded in paraffin to maintain the natural shape and architecture of the sample during long-term storage and sectioning for IHC. Samples too sensitive for either chemical fixation or the solvents used to remove the paraffin are encased in cryogenic embedding medium and then snap-frozen in liquid nitrogen.

8. Sectioning and Mounting

The decision to section tissue is dependent upon the application used. For example, whole mount IHC, with sample thickness up to 5 mm, does not require sectioning, while small samples for which multiple staining procedures are needed may require sectioning. Formalin-fixed, paraffin-embedded tissues are sectioned into slices as thin as 4-5 μm with a microtome. These sections are then mounted onto glass slides that are coated with an adhesive. This adhesive is commonly added by surface-treating glass slides with 3-aminopropyltriethoxysilane (APTS) or poly-L-lysine, which both leave amino groups on the surface of the glass to which the tissue directly couples. Alternatively, slides may be coated with physical adhesives, including gelatin, egg albumin or Elmer's glue. After mounting, the sections are dried in an oven or microwave for deparaffinization. Frozen sections are cut using a pre-cooled cryostat and mounted to adhesive glass slides. These sections are often dried overnight at room temperature and fixed by immersion in pre-cooled (-20°C) acetone, although the drying step is sometimes skipped depending on the target Ag and tissue used(Shi et al., 1997; Taylor et al., 1996).

9. Epitope (Ag) Recovery

The paraffin from formalin-fixed, paraffin-embedded sections must be completely removed for the Ab to reach the target Ag. Xylene, a flammable, toxic and volatile organic solvent is commonly used to remove the paraffin from IHC slides, although commercial alternatives are available. Formaldehyde fixation generates methylene bridges that cross-link proteins in tissue samples. These bridges can mask Ag presentation and prevent Abbinding. Formalin-fixed, paraffin-embedded sections commonly require a treatment to unmask the antibody epitopes, either by heat, i.e. heat-induced epitope retrieval (HIER) or enzymatic degradation, i.e. proteolytic-induced epitope retrieval (PIER)[Cattoretti and Suurjmeijer, 1995].

10. Quenching/Blocking Endogenous Target Activity

For staining approaches that depend on biotin, peroxidases or phosphatases for the amplification or enzymatic detection of target Ag, quenching or masking endogenous forms of these proteins prevents false positive and high background detection. The general strategies include physically blocking or chemically inhibiting all endogenous biotin or enzyme activity,

respectively (Cattoretti and Suurjmeijer, 1995).

11. Blocking Non-specific Sites

Although shows preferential avidity for specific epitopes, Abmay partially or weakly bind to sites on non-specific proteins (also called reactive sites) that are similar to the cognate binding sites on the target Ag. In the context of Ab-mediated Ag detection, non-specific binding causes high background staining that can mask the detection of the target antigen. To reduce background staining in IHC, ICC and any other immunostaining application, the samples are incubated with a buffer that blocks the reactive sites to which the primary or secondary antibodies may otherwise bind. Common blocking buffers include normal serum, non-fat dry milk, BSA or gelatin, and commercial blocking buffers with proprietary formulations are available for greater efficiency (Cattoretti and Suurjmeijer, 1995).

12. Immunodetection

Detecting the target Ag with Abis a multi-step process that requires optimization at every level to maximize the signal detection. Both primary and secondaryAbis diluted into a buffer to help stabilize the Ab, promote the uniform dissemination throughout the sample and discourage non-specific binding. While one diluent may work with one Ab, the same diluent may not work with another Ab, demonstrating the need for optimization for each Ab. Rinsing the sample in between Abapplication is critical to remove unbound Ab and also to remove Abthat is weakly bound to non-specific sites. Rinse buffers are usually simple solutions of only a few components, but the right components must be considered to maximize sample washing and minimize interference with the signal detection. Ab-mediated Ag detection approaches are separated into direct and indirect methods. Both these methods use Abto detect the target Ag, but the selection of the best method to use depends on the level of target Ag expression and availability and also the readout desired. Most indirect methods employ the inherent binding affinity of avidin to biotin to localize a reporter to the target Ag and amplify the signal that is detected. IHC target Ag is detected through either chromogenic or fluorescent means and the type of readout depends on the experimental design. For fluorescent detection, the reporter that the primary or secondary Abis conjugated to is a fluorophore that is detected by fluorescent microscopy. Chromogenic detection is based on the activities of enzymes, most often horseradish peroxidase (HRP) or alkaline phosphatase (AP), which forms colored, insoluble precipitates upon the addition of substrate, such as DAB and NBT/BCIP, respectively (Holgate et al., 1983).

13. β-Galactosidase Staining

Instead of using antibodies to detect the target antigen, β-galactosidase can be identified in tissue samples by employing the inherent enzymatic activity of the protein. β-galactosidase catalyzes 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (BCIG or X-Gal) to form 5,5'-dibromo-4,4'-dichloro-indigo, an insoluble blue enzymatic product. For IHC, frozen sections are incubated with X-Gal along with an inducer, IPTG, to generate the chromogenic response (Lackie, 1996).

14. Counter-staining

Counter-stains give contrast to the primary stain and can be cell structure-specific. These single-step stains are usually added after Ab staining (Figure 1).

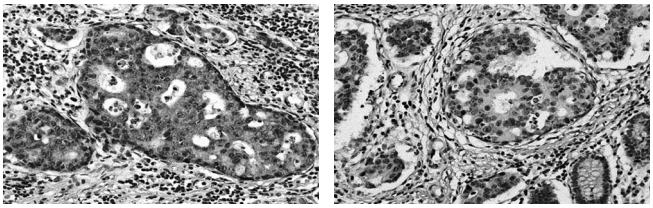

Figure 1: Detection of biotinylated GAPDH in colon carcinoma by chromogenic IHC-fixed and paraffin-embedded human colon carcinoma sections were incubated with biotinylated cytokeratin18 Ab (left panel) or blocking buffer alone as a negative control (right panel). The samples were then incubated with Thermo Scientific Pierce Streptavidin Poly-HRP Conjugate, and the signal was developed with Thermo Scientific Pierce Metal Enhanced DAB

15. Sealing the Stained Sample

After all staining is complete; the sample should be preserved for long-term usage and storage and to prevent enzymatic product solubilization or fluorophore photobleaching. Sealing the sample by mounting a coverslip with an appropriate mountant stabilizes the tissue sample and stain. An antifade reagent should also be included if fluorescent detection will be performed to prolong fluorescence excitation. The coverslip can then be sealed with clear nail polish or a commercial sealant after the mountant has cured to prevent sample damage(Lackie, 1996).

16. Sample Visualization

Once the sections are prepared, the samples are viewed by light or fluorescent microscopy. Depending on the Abdetection method, one can perform confocal microscopy for greater detail and enhanced imaging capabilities. Additionally, samples can be analyzed by high content screening for rapid quantification and comparison of data from multiple samples(Holgate et al., 1983).

17. IHC Methods

17.1. Blocking

Background staining may be specific or non-specific. Inadequate or delayed fixation may give rise to false positive results due to the passive uptake of serum protein and diffusion of the Ag. Such false positives are common in the center of large tissue blocks or throughout tissues in which fixation was delayed. Ab, speciallypolycolonalAb, is sometimes contaminated with other Abdue to impure Ag used to immunize the host animal. The main cause of non-specific background staining is non-immunological binding of the specific immune sera by hydrophobic and electrostatic forces to certain sites within tissue sections. This form of background staining is usually uniform and can be reduced by blockingthose sites with normal serum. Endogenous peroxidase activity is found in many tissues and can be detected by reacting fixed tissue sections with DAB substrate. The solution for eliminating endogenous peroxidase activity is by the pretreatment of the tissue section with hydrogen peroxide prior to incubation of primary antibody. Many tissues also contain endogenous alkaline phosphatase (AP) activity and should be blocked by the pre-treatment of the tissue section with levamisole if using AP as a label. Some tissues such as liver and kidney have endogenous biotin. To avoid unwanted avidin binding to endogenous biotin if using biotin-avidin detection system, a step is necessary for these tissues by the pre-treatment of un-conjugated avidin which is then saturated with biotin. Auto-fluorescence or natural fluorescence exists in some tissues and can cause background problems when fluorescent dyes are used in the experiments. The simplest test is to view the tissue sections with a fluorescence microscope before any antibody incubation. If auto-fluorescence is detected in the tissue sections, the best solution is to avoid use of fluorescent method but choose enzyme or other labeling methods(Haines and Clark, 1991).

17.2. Controls

Special controls must be run in order to test the protocol and for the specificity of the Abbeing used. Positive control is to test a protocol or procedure and make sure it works. It will be ideal to use the tissue of known positive as a control. If the positive control tissue shows negative staining, the protocol or procedure needs to be checked until a good positive staining is obtained. Negative control is to test for the specificity of an Abinvolved. First, no staining must be shown when omitting primary Abor replacing a specific primary Abwith normal serum (must be the same species as primary Ab). This control is easy to achieve and can be used routinely in immunohistochemical staining. Second, the staining must be inhibited by adsorption of a primary Abwith the purified Ag prior to its use, but not by adsorption with other related or unrelated Ag. This type of negative control is ideal and necessary in the characterization and evaluation of new Abbut it is sometimes difficult to obtain the purified Ag, therefore it is rarely used routinely in immunohistochemical staining(Lackie, 1996).

17.2.1. Directmethod

Direct method is one step staining method, and involves a labeled Ab, i.e. FITC conjugated antiserum, reacting directly with the antigen in tissue sections. This technique utilizes only one Aband the procedure is short and quick. However, it is insensitive due to little signal amplification and rarely used since the introduction of indirect method.

17.2.2. Indirectmethod

Indirect method involves an unlabeled primary Ab(first layer) which react with tissue Ag, and a labeled secondary Ab (second layer) react with primary Ab. Secondary Abmust be against the IgG of the animal species in which the primary Abhas been raised. This method is more sensitive due to signal amplification through several secondary Abreactions with different antigenic sites on the primary Ab. In addition, it is also economic since one labeled second layer Abcan be used with many first layer Ab(raised from the same animal species) to different Ag.

The second layer Abcan be labeled with a fluorescent dye such as FITC, rhodamine or Texas red, and this is called indirect immunofluorescence method. The second layer Abmay be labeled with an enzyme such as peroxidase, AP or glucose oxidase, and this is called indirect immunoenzyme method.

17.3. PAP method (peroxidase anti-peroxidase method)

PAP method is a further development of the indirect technique and it involves a third layer which is a rabbit Abto peroxidase, coupled with peroxidase to make a very stable peroxidase anti-peroxidase complex. The complex, composed of rabbit γ-globulin and peroxidase, acts as a third layer Ag and becomes bound to the unconjugated goat anti-rabbit gaba-globulin of the second layer. The sensitivity is about 100-1000 times higher since the peroxidase molecule is not chemically conjugated to the anti IgG but immunologically bound, and loses none of its enzyme activity. It also allows for much higher dilutionof the primary Ab, thus eliminating many of the unwanted Ab and reducing non-specific background staining(Haines and Chelack, 1991).

17.4. Avidin-biotin complex (ABC) method

ABC method is standard IHC method and one of the widely used techniques for immunhistochemical staining. Avidin, a large glycoprotein, can be labeled with peroxidase or fluorescein and has a very high affinity for biotin. Biotin, a low molecular weight vitamin, can be conjugated to a variety of

biological molecules such as Ab. The technique involves three layers. The first layer is unlabeled primary Ab. The second layer is biotinylated secondary Ab. The third layer is a complex of avidin-biotin peroxidase. The peroxidase is then developed by the DAB or other substrate to produce different colorimetric end products(Haines and Chelack, 1991).

17.5. Labeled streptavidin biotin (LSAB) method

Streptavidin, derived from streptococcus avidin, is a recent innovation for substitution of avidin. The streptavidin molecule is uncharged relative to animal tissue, unlike avidin which has an isoelectric point of 10, and therefore electrostatic binding to tissue is eliminated. In addition, streptavidin does not contain carbohydrate groups which might bind to tissue lectins, resulting in some background staining. LSAB is technically similar to standard ABC method. The first layer is unlabeled primary Ab. The second layer is biotinylated secondary Ab. The third layer is enzyme-streptavidin conjugates (HRP-streptavidin or AP-streptavidin) to replace the complex of avidin-biotin peroxidase. The enzyme is then visualized by application of the substrate chromogen solutions to produce different colorimetric end products. The third layer can also be fluorescent dye-streptavidin such as FITC-streptavidin if fluorescence labeling is preferred (Haines and Chelack, 1991).

17.6. Polymeric methods

EnVision systems are based on dextran polymer technology. This unique chemistry permits binding of a large number of enzyme molecules (horseradish peroxidase or AP) to a secondary Abvia the dextran backbone. The benefits are many, including increased sensitivity, minimized non-specific background staining and a reduction in the total number of assay steps as compared to conventional techniques. The simple protocol is: i) Application of primary Ab; ii) Application of enzyme labeled polymer; iii) Application of the substrate chromogen. EnVision+was developed after EnVision to provide increased sensitivity (Table 1).

ImmPRESS polymerized reporter enzyme staining system is based on a new method of polymerizing enzymes and attaching these polymers to Ab. The novel approach employed to form enzyme 'micro-polymers' avoids the intrinsic shortcomings of using large dextrans or other macromolecules as backbones. Attaching a unique micro-polymer with a high density of very active enzyme to a secondary Abgenerates a reagent that overcomes steric interference and provides enhanced accessibility to its target. The result is outstanding sensitivity, signal intensity, low background staining, and reduced non-specific binding. The simple protocol is: i) Application of primary Ab; ii) Application of enzyme labeled polymer; iii) Application of the substrate chromogen.

Table 1: IHC sensitivity chart

Standard sensitivity	Moderate sensitivity	Most sensitivity
ABC Method	EnVision	CSA Method
PAP Method	EnVision+	CSA II Method
LSAB Method	ImmPRESS	

17.7. CSA methods

CSA systems use tyramide signal amplification. It is ideal for the following applications: i) Detecting small quantities of Ag; ii) Enhancing performance of low affinity mouse and rabbit Ab; iii) Enabling compatibility of certain 'tough' mouse and rabbit Ab with paraffin embedded tissue sections. The simple protocol is as follows:

- Application of primary Ab
- Application of biotinylated linking Ab
- Application of the tyramide amplification reagent
- Application of streptavidin-HRP
- Application of the substrate chromogen

CSA II-Biotin-free tyramide signal amplification system is a highly sensitive immunohistochemical staining procedure incorporating a signal amplification method based on the peroxidase-catalyzed deposition of a fluorescein-labelled phenolic compound, followed by a secondary reaction with a peroxidase-conjugated anti-fluorescein. In the procedure, a mouse' primary Abis first detected with a peroxidase-conjugated secondary Ab. The next step utilizes the bound peroxidase to catalyze oxidation of a fluorescein-conjugated phenol (fluorescyl-tyramide) which then precipitates onto the specimen. The procedure is continued with detection of the bound fluorescein by a peroxidase-conjugated anti-fluorescein. Staining is completed using diaminobenzidine/hydrogen peroxide as chromogen/substrate, and can be observed with a light microscope. In comparison to standard immunohistochemical methods, such as labeled streptavidin biotin (LSAB) or avidin-biotin complexes (ABC), tyramide amplification methods have been reported to be many folds more sensitive. The CSA II system is a simplified version of the extremely sensitive Catalyzed Signal Amplification System (code K1500) that utilizes biotinyl-tyramide. The highly sensitive CSA II system allows for the detection of very small quantities of target protein, as well as for the use of low affinity Ab. This reagent system utilizes fluorescyl-tyramide, rather than biotinyl-tyramide, and does not contain avidin/biotin reagents, thus eliminating potential background staining due to reactivity with endogenous biotin (Speel, 1995).

The principle of IHC has been known since the 1930s, but it was not until 1942 that the first IHC study was reported. Coons and Kaplan (1950) used FITC-labeled Abto identify *Pneumococcal*Ag in infected tissue. Since then, improvements have been made in protein conjugation, tissue fixation methods, detection labels and microscopy, making IHC a routine and

essential tool in diagnostic and research laboratories.

18. Conclusion

IHC is used for disease diagnosis, drug development and biological research. Using specific tumor markers, physicians use IHC to diagnose a cancer as benign or malignant, determine the stage and grade of a tumor, and identify the cell type and origin of a metastasis to find the site of the primary tumor. IHC is also used in drug development to test drug efficacy by detecting either the activity or the up or down-regulation of disease targets.

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