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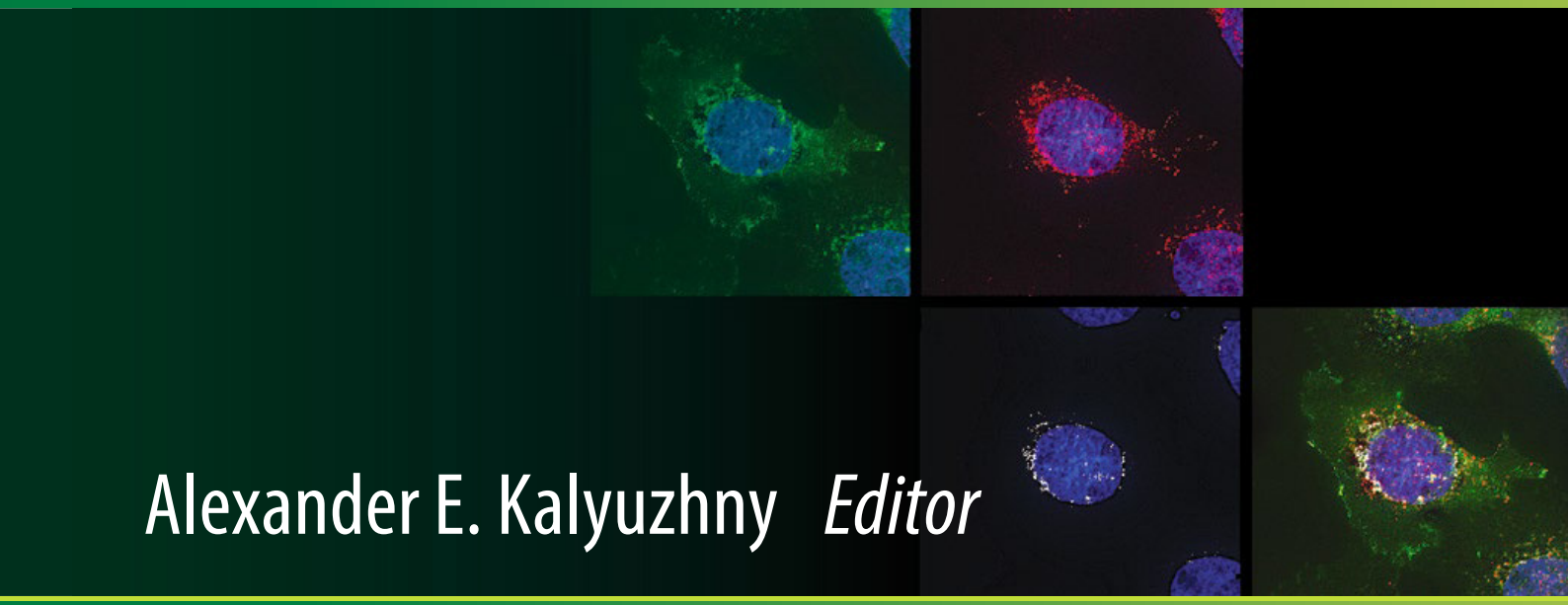

Alexander E. Kalyuzhny *Editor*

Signal Transduction Immunohistochemistry

Methods and Protocols

Third Edition

Humana Press

Recent Advances in Chromogens for Immunohistochemistry

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Abstract

Various staining strategies and color combinations have been developed to perform single- and double-immunohistochemical staining on biological samples. However, until recently the lack of appropriate chromogen color combinations has severely limited many of these methods. Fortunately, this situation has dramatically improved with the introduction of new chromogens and methods of analysis. This article reviews recent trends in multi-color immunohistochemical staining methods that are finding broad applications in both research and clinical laboratories.

Introduction

Immunohistochemistry (IHC) has played a vital role in both research and clinical applications for the evaluation of molecular targets in histological samples. These methods have provided the necessary tools to localize proteins and other molecules of interest in biological samples. Similarly, the closely related method of In Situ Hybridization (ISH) is able to localize nucleic acids within these samples. The ability to localize molecular targets within the broader context of the morphological architecture of the tissue has revolutionized the study of tissues by brightfield microscopy.

From its inception IHC has relied upon a variety of colored chromogens to identify a single target molecule within a tissue. Graham and Karnovsky (1) first introduced the use of 3,3'-diaminobenzidine tetrahydrochloride (DAB) as a chromogen in 1966. The widespread use of DAB in brightfield microscopy continues to this day, nearly 6 decades later. This method relies upon DAB forming an insoluble brown precipitate at sites of peroxidase activity(Figure 1).

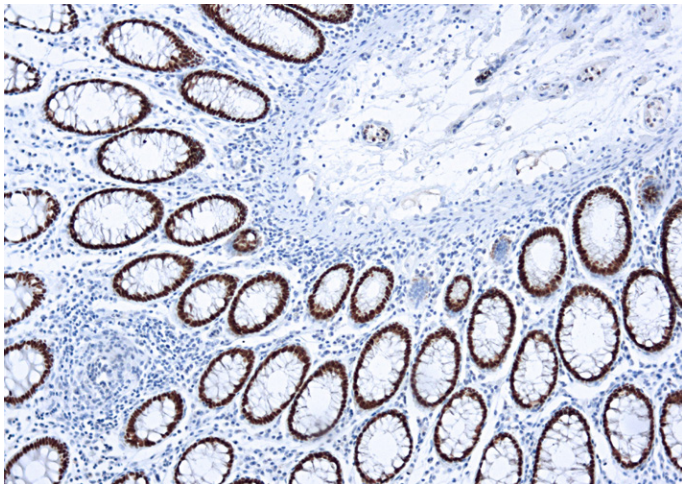


Figure 1. DBS HRP/DAB Detection System: FFPE human colon stained with Cdx2 antibody (DBS Cat Mob432).

DAB continues to remain one of the most popular chromogens for IHC, and for good reason. It exhibits many desirable features including the fact that DAB precipitates are virtually insoluble in aqueous and organic solvents. This allows the stained slides to be mounted and cover-slipped by virtually any method without concern for dissolving or damaging the final DAB stain. Such mounted slides are considered permanent and have shown remarkable longevity with the DAB stains remaining stable for many years.

At Diagnostic BioSystems we have developed an improved formulation: the Stable DBA/Plus. It offers improvements and benefits, when compared to traditional working DAB solution. Upon preparation Stable DAB/Plus is stable for five days as opposed to 6 hours for traditional DAB working solution. This feature provides users with the convenience of making one working solution for the entire work week. Stable DAB/Plus is highly sensitive, providing the cost-effective option of diluting the primary antibody, and is the preferred option for high volume and automated stains.

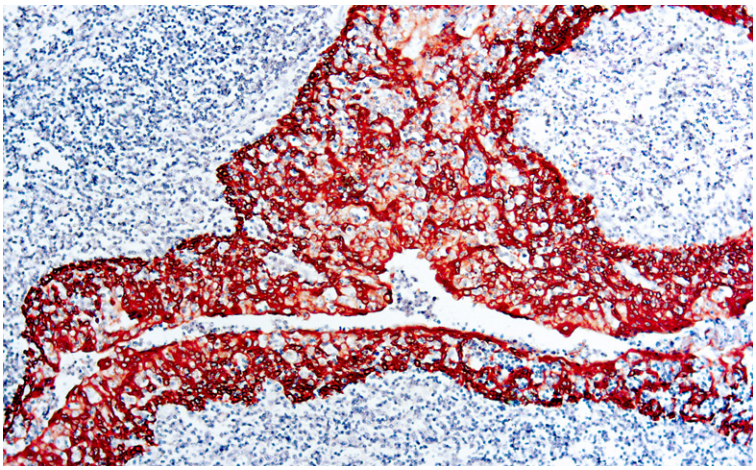
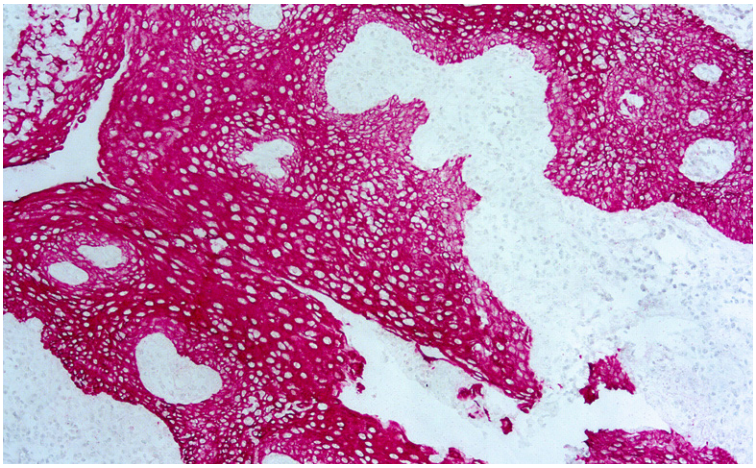
However, there are circumstances in which a chromogen other than brown DAB is desirable, particularly if endogenous brown pigments are present in the tissue sample. Such indications would include the presence of lipofuscin, hemosiderin, and melanin pigments.
Other indications might include circumstances where the localization of more than one molecular target is to be visualized within the confines of a single slide. Such multi-staining methods necessitate the use of multiple chromogens of different colors. Over the intervening years since the introduction of DAB, a number of different colored chromogens have been developed that have the potential to greatly expand the usefulness of IHC in both research and clinical fields. (Table 1).

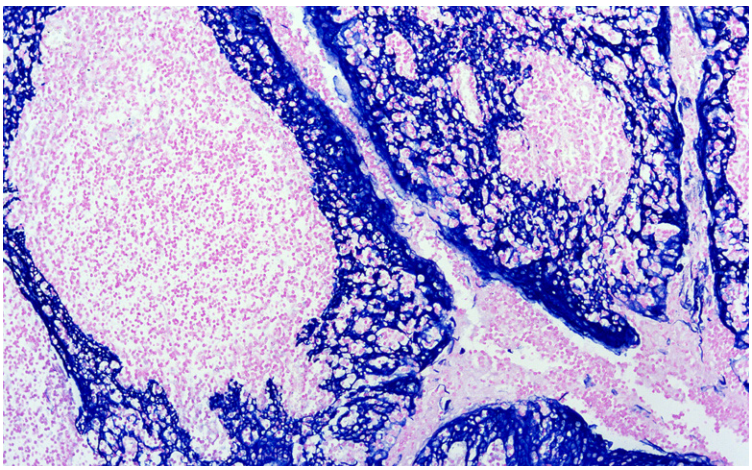
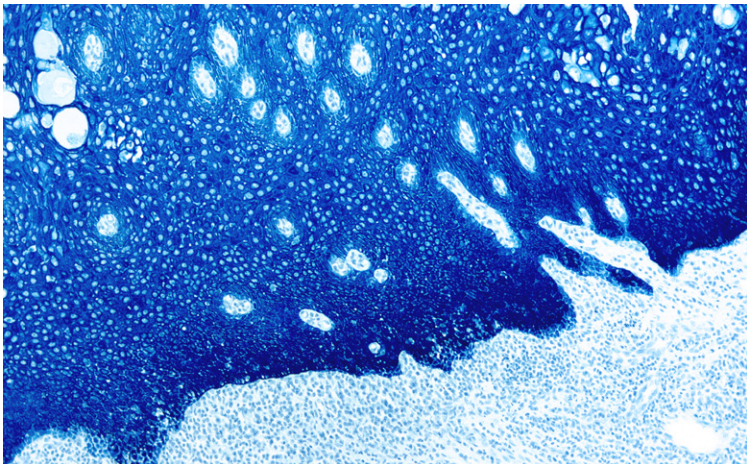
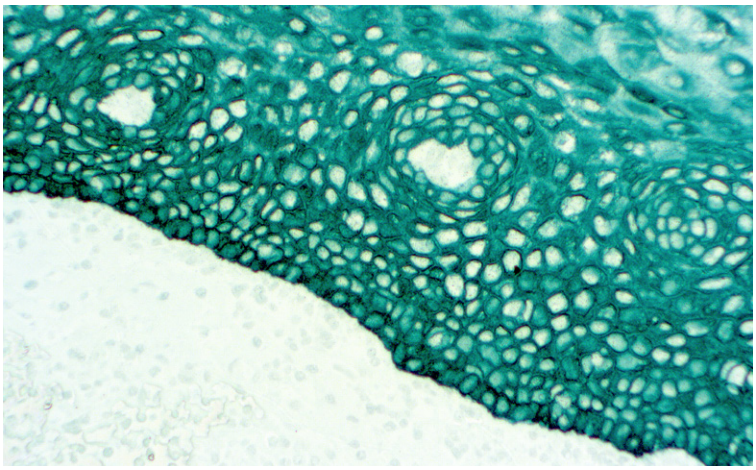
Table 1. Chromogens for Immunohistochemistry

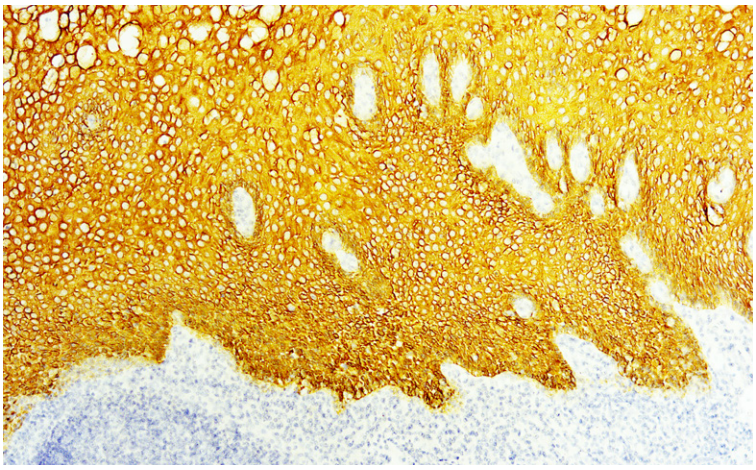
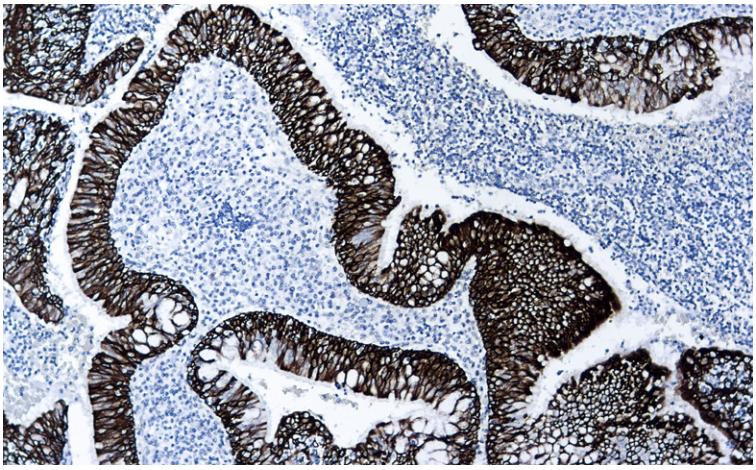
Alkaline Phosphatase		
Color	**Name**	**Source**
Red	Vector Red	Vector Laboratories
Black	Vector Black	Vector Laboratories
Blue	Vector Blue	Vector Laboratories
Red	Fast Red	BioCare Medical
Blue	Ferangi Blue	BioCare Medical
Blue	PermaBlue AP	Diagnostic BioSystems
Green	PermaGreen AP	Diagnostic BioSystems
Red	PermaRed AP	Diagnostic BioSystems

Peroxidase		
Color	**Name**	**Source**
Purple	Vector VIP Purple	Vector Laboratories
Blue-Gray	SG	Vector Laboratories
Red	NovaRed	Vector Laboratories
Blue-Gray	TMB	Vector Laboratories
Purple	Bajoran Purple	BioCare Medical
Green	Vina Green	BioCare Medical
Black	Deep Space Black	BioCare Medical
Brown	Stable DAB	Diagnostic BioSystems
Dark Brown	High Contrast DAB	Diagnostic BioSystems
Black	PermaBlack HRP	Diagnostic BioSystems
Blue	PermaBlue HRP	Diagnostic BioSystems
Green	PermaGreen HRP	Diagnostic BioSystems
Yellow	PermaYellow HRP	Diagnostic BioSystems
Red	PermaRed HRP	Diagnostic BioSystems
Red	AEC	Diagnostic BioSystems

Table 2. Chromogen Description and Characteristics

#	Chromogen	Description	IHC Staining Image
1	3-Amino-9-Ethyl-carbazole (AEC)	In the presence of peroxidase enzyme, ACE produces a vivid red end- product that is soluble in alcohol and must be used with an aqueous counterstain and mounting media.	 Fig.1a FFPE human tonsil stained with HMW Cytokeratin and labeled with Mono AEC/Plus (DBS cat. K050).
2	PermaRed/AP and PermaRed/ HRP	Substrate-chromogen systems designed to be used for either IHC or ISH by using alkaline phosphatase detection and/or horseradish peroxidase detection. The chromogens produce a brilliant dark red color that is insoluble in organic solvents. Therefore, sections can be dehydrated in alcohol, cleared in xylene or a xylene substitute, and permanently mounted. The chromogens can be applied for both automation and manual.	 Fig.1b FFPE squamous cell carcinoma human tissue stained with CK 5/6 antibody (DBS Cat. Mob362) labeled with PermaRed AutoPlus (DBS Cat K057) produces a brilliant red color.

#	Chromogen	Description	IHC Staining Image
3	PermaBlue/AP and PermaBlue/ HRP	These substrate-chromogen systems designed for the use for either IHC or ISH when utilizing alkaline phosphatase and horseradish peroxidase. They produce a vibrant blue color. PermaBlue/AP is insoluble in alcohol and xylene substitute and therefore, sections can be dehydrated in alcohol, cleared in xylene substitute, and permanently mounted. PermaBlue Plus/AP has been reformulated to increase stability of the working solution while producing a distinct bright blue color. PermaBlue Plus/AP is insoluble in alcohol and xylene substitute and therefore, sections can be dehydrated in alcohol, cleared in xylene substitute, and permanently mounted. The PermaBlue Plus/AP working solution is light sensitive and should be kept away from the light. Working solution is stable for 6 hours in the dark; and any solution not used during this 6-hour period should be discarded. For optimal staining, freshly made solution should be used	 Fig.1a FFPE human tonsil stained with HMW Cytokeratin and labeled with Mono AEC/Plus (DBS cat. K050).  Fig.1d FFPE human tonsil stained with Cytokeratin H.M.W and labeled with PermaBlue/HRP (DBS cat. K063)
4	PermaGreen Plus/AP and PermaGreen/ HRP	These substrate-chromogen systems designed to use either in IHC or ISH when utilizing alkaline phosphatase and/or horseradish peroxidase. PermaGreen Plus/AP has been modified to increase stability and staining intensity producing a strong green color that is insoluble in alcohol and xylene substitute, and permanently mounted. With a 6-hour working stability, this PermaGreen Plus/AP can be used for both automation and manual. PermaGreen/ HRP can be permanently mounted to generate a bright green color that can be easily distinguished from other stains.	 Fig.1e PermaGreen Plus/AP FFPE human tonsil stained with HMW CK Ab (DBS cat, PDM074) labeled with PermaGreen Plus/ AP Chromogen (DBS cat. K059)

#	Chromogen	Description	IHC Staining Image
5	PermaYellow/ HRP	A substrate-chromogen system designed for use in IHC or ISH when using horseradish peroxidase. It produces a distinct yellow color that can be easily distinguished from other stains. With approximately one-day working stability, this PermaYellow/HRP system can be used in automation as well as manual staining.	 Fig.1f PermaYellow/HRP FFPE human tonsil stained with Cytokeratin H.M.W labeled with PermaYellow/HRP (DBS Cat. K060)
6	PermaBlack/HRP	A horseradish peroxidase-based substrate-chromogen system, produces a sharply contrasting ebony black stain, and serves as a useful tool in IHC as well as ISH staining procedures. PermaBlack/HRP is a permanent stain that can go through alcohol and xylene,and has been optimized for both automated and manual staining	 Fig.1g PermaBlack/HRP FFPE human adenocarcinoma stained with Cytokeratin H.M.W and labeled with PermaBlack/HRP (DBS Cat. K062).

Single-color IHC

Current IHC methods are enzyme driven with peroxidase and alkaline phosphatase being the most commonly used enzymes. These enzymes react with a chromogenic reagent composed of a chromogen and a substrate. In the peroxidase reaction the substrate is generally a peroxide such as hydrogen peroxide. The chromogen is oxidized in the presence of peroxidase and hydrogen peroxide resulting in the deposition of a colored insoluble precipitate at the site of enzymatic activity. In the case of DAB, the chromogen is transformed from a colorless soluble state into a highly colored brown precipitate that is easily visualized by brightfield microscopy. Similarly, a useful substrate for alkaline phosphatase in naphthol-phosphate. Upon cleavage of the phosphate group, the free naphthol is now capable of reacting with a chromogen, such as Fast Red to produce an insoluble red precipitate at the site of the alkaline phosphatase enzyme.

In situations where a brown chromogen is not desirable, other colored chromogens can be used. In particular melanin presents an additional challenge in IHC because this brown pigment provides no visual contrast with the brown DAB reaction product (2). The interpretation of specific staining upon a background of endogenous pigments may be compromised under these circumstances. In such situations alternative chromogens can be selected such as 3-Amino-9-ethylcarbazole (AEC) or Fast Red. Both produce a red reaction product with AEC being a chromogen for peroxidase and Fast Red being a chromogen for alkaline phosphatase. In addition, there are now many other useful chromogens that can be employed to produce a variety of colors (see Table 1).

Double-Stain IHC

Double-Stain IHC provides many advantages over single-color IHC. Double-Stain IHC provides the tools necessary to identify two molecular targets within a single tissue on a single slide. In comparison traditional single color IHC would require the use of two separate slides to generate the same information. Double-stain IHC allows for the simultaneous visualization of two different molecular targets allowing the topographical relationship between the two targets to be evaluated within the context of the tissue morphology. Furthermore, when combined with the advanced methods of image analysis and spectral analysis, it is possible to accurately quantify the targets in terms of numbers, ratios, and distances. Even with the co-localization of targets where the two chromogens overlap, it may be possible to separate these two overlapping signals by spectral analysis (3,4).

For double-stain IHC to be technically successful the methods and chromogens must be carefully considered. In general, the double-stain methods fall into one of two different categories, either simultaneous double-stain or sequential double-stain. In simultaneous double-stain both primary antibodies, usually derived from different species, are applied together as a cocktail. Next, two species-specific secondary antibodies are applied also as a cocktail. In this step one of the secondary antibodies carries the peroxidase enzyme, and the other secondary antibody carries the alkaline phosphatase enzyme. In the final step the two chromogens, one for peroxidase and one for alkaline phosphatase are sequentially applied. It is common that the chromogen for peroxidase is DAB and the chromogen for alkaline phosphatase is Fast Red, although this is by no means the only color combination that can be used, as shown in Table 1.

The sequential method of double-stain, although technically more complicated offers greater flexibility and is less constrained by the selection of antibodies, enzymes, and chromogens. In the sequential method the first IHC stain is performed to completion, from primary antibody through chromogen. When the first stain has been completed, the second stain is performed to completion from primary antibody to chromogen. In this method both primary antibodies can be generated from the same species and the secondary antibodies can carry the same enzyme. Likewise, two chromogens can be selected from the same enzyme family. As shown in Table 1 there are a variety of colored chromogens that can be selected, many belonging to the peroxidase enzyme family.

Whether by simultaneous or sequential staining, there are a number of factors that must be considered for successful double-staining. First the two colors chosen must contrast sufficiently from each other, as well as any counterstains, such that the observer can clearly separate the individual staining patters as well as any co-localized staining. Second the chromogens should not interact with each other to the extent that the color of the first chromogen is altered making it difficult to distinguish from the second chromogen. Such color interactions could be acceptable provided the individual stains are still clearly distinguishable from each other.

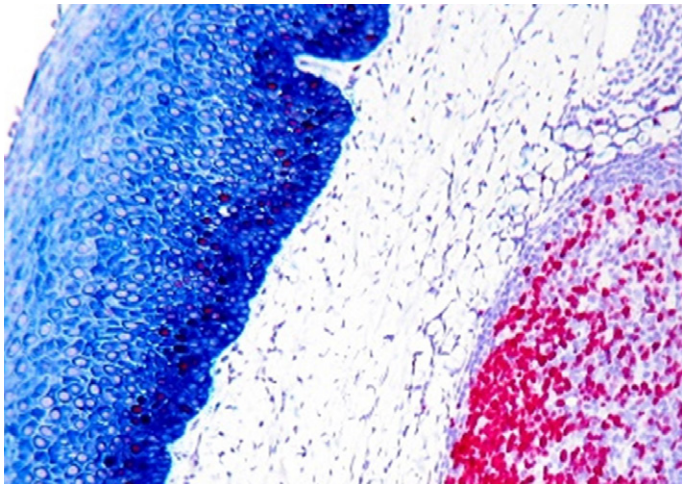


Figure 2. An example of double-staining: Human FFPE tonsil stained with cytokeratin H.M.W. and labeled with PermaBlue/HRP (DBS Cat. K063) and with Ki67 (DBS Cat. RP026) using PermaRed/HRP (DBS Cat. K075)

Third, the order of the chromogens must be considered. In this context certain chromogens can act as blocking chromogens such that the underlying antibody-enzyme complexes are completely blocked from interacting in any subsequent IHC steps. In fact, this blocking effect can be exploited beneficially in double-stain IHC by blocking the antibody-enzyme complex of the first IHC reaction such that the reactants do not need to be stripped or inactivated prior to initiating the second IHC reaction. Fortunately, DAB is an excellent blocking chromogen, and for this reason it is frequently selected as a first chromogen in double-stain IHC. Another example of a blocking chromogen is PermaBlue HRP. Figure 2 depicts a tonsil tissue that has been double stained using the sequential staining method. The first stain is for cytokeratin and has been developed with PermaBlue HRP chromogen, while the second stain is for Ki67 and has been developed with the PermaRed HRP chromogen.

Multiplex IHC

With the development of numerous colored chromogens, it is now possible to produce multiplex IHC stains. The use of three or more colored chromogens on a single slide is now possible, although the analysis of such stained specimens becomes significantly more difficult as the number of different chromogens increases. Nevertheless, the use of image analyzers can be employed to separate the images into layers or to perform other complex analyses. Even without such complex tools, it is possible manually to evaluate multiplex images using a standard brightfield microscope by careful selection of the molecular targets and chromogens, thereby minimizing the co-localization of chromogens.

Clinical Indications

Having considered some of the technical aspect of the various colored chromogens in IHC, we can next turn our attention to some of the clinical applications.

Clinical Applications

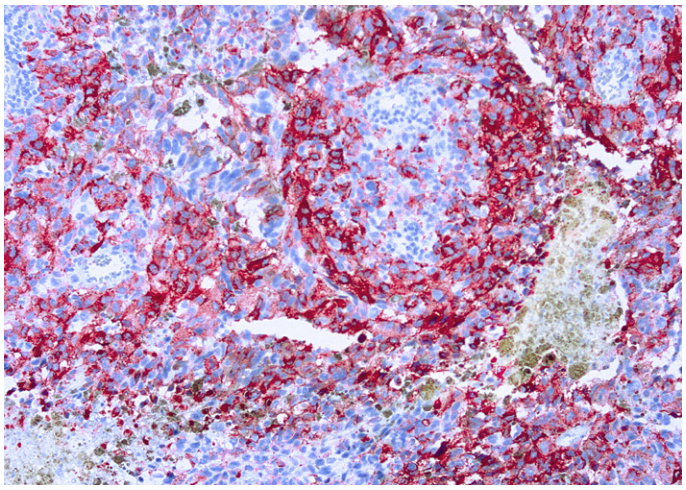


Figure 3. FFPE human melanoma stained with Melanoma antibody (DBS cat. Mob079).

1. Melanoma and morphologic mimics in the clinical laboratories need to be separated from each other. IHC staining using different antibodies and methods with DAB as a colored substrate can serve the purpose. Because the heavily pigmented melanocytic lesions usually contain melanin and other pigment that may require bleaching of the sample to remove the confounding melanin pigment. Bleaching can affect cellular antigenicity and may be incomplete. In this case, PermaRed/AP or PermaRed/HRP can be used for IHC and present vivid red color without visible negative impact on interpretation under bright microscopic. (Figure 3).

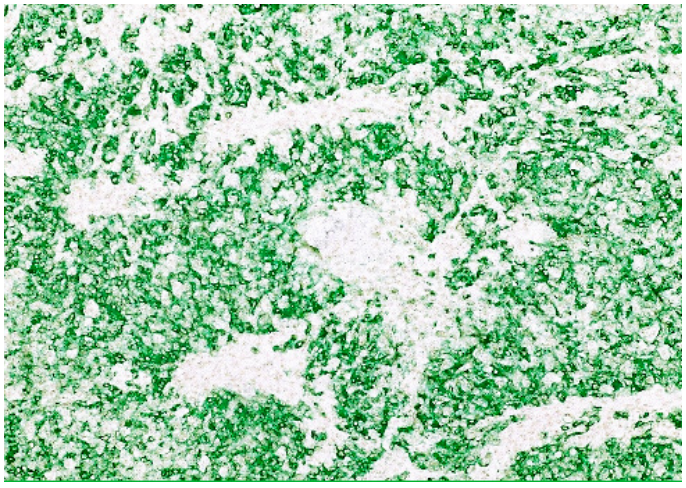


Figure 4. Mohs HRP-Green Kit (DBS Cat. K092) MART-1 Melanoma (DBS Cat. PDM153)

2. MOHs: The Diagnostics BioSystems provides Mohs HRP-Green Kit that consists of a ready-to-use HRP-polymer, a concentrated solution of PermaGreen HRP chromogen, and a PermaGreen HRP Substrate Buffer for dilution of the HRP Green Chromogen. This Kit is optimized for detection of mouse primary antibodies. It has the following unique features: 1) improved accessibility of antigens in frozen tissue sections; 2) better contrast to endogenous melanin by green chromogen; 3), rapid two-step immunohistochemistry protocol and time to results in < 20 minutes; 3) No rinse step required after primary antibody; 4) optimized for use with Diagnostic BioSystems Ready-to-use IHC antibodies. Mohs HRP-Green Kit is a very useful detection system for melanin-rich melanoma in quick service time on frozen sections. (Figure 4).

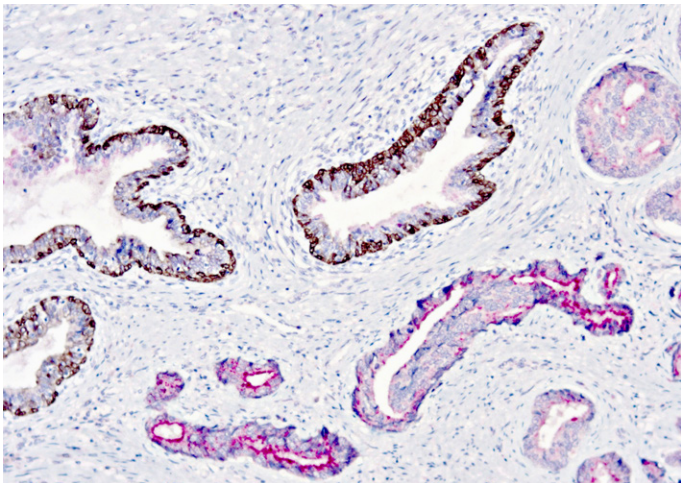


Figure 5. PIN5 Cocktail Rabbit anti-p40 + Mouse anti-Cytokeratin (HMW) and Rabbit anti-p504S (AMACR) (DBS Cat.: PDR057 Prediluted)

3. In distinguishing Prostatic Adenocarcinoma for other benign or malignant mimics, PIN5 cocktail was developed. High molecular weight cytokeratin and p40 (DAB, brown), the basal cell markers, were combined with p504 (AMACR) (AEC, PermaRed) for detection prostatic adenocarcinoma versus mimics. The PIN5 antibody combination may be particularly useful for confirming the diagnosis of prostatic adenocarcinoma in small needle biopsy specimens. The positive prostate carcinoma marker, p504S (AMACR), in conjunction with the negative basal cell marker (p40 and high molecular weight cytokeratin) offers confirmation in identifying prostatic intraepithelial neoplasia in difficult cases with limited tissue. (Figure 5) .

Discussion

In both the research and clinical setting, IHC has become a standard tool for tissue evaluation and study. It has become one of the most powerful diagnostic approaches in clinical studies. Prior to the advent of multiple-colored chromogens IHC was primarily limited to a small group of chromogens of which DAB was the most prominent. Although DAB still remains one of the most widely used chromogens in IHC, a number of alternative and supplemental chromogens are now available that have the potential to greatly expand the use of IHC going forward. Theoretically multiplex IHC can identify nearly every protein target, thereby greatly facilitating the evaluation of targets including their identification, interactions, localization, and function.

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