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Original Article

The Use of the Immunohistochemical Biotin-Free Visualization Systems for Estrogen Receptor Evaluation of Breast Cancer

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Abstract

Background: A novel generation of immunohistochemical visualization systems based on a biotin-free polymeric (BFP) technology has recently been released. We have compared the new BFP and the classical streptavidin-biotin (SAB) systems to evaluate estrogen receptor in breast carcinomas. **Methods:** Serial sections from a tissue microarray containing 320 invasive breast carcinomas were stained by immunohistochemistry for estrogen receptor using the rabbit monoclonal antibody SP1. Eleven different visualization systems were used, including seven BFP systems (six second-generation: DAKO Advance ™ , Leica Novolink ™, Zymed SuperPicTure™ , Zymed PicTure Max ™ , Biogenex Super Sensitive Non-Biotin HRP ™ , CellMarque Mouse/Rabbit Polydetector HRP/DAB™ ; one first-generation: DAKO EnVision+™) and four SAB systems (DAKO LSAB+™ ; Signet EasyPath™ ; Biogenex Super Sensitive™ and CellMarque Mouse/Rabbit Immunodetector HRP/DAB™). All visualization systems were used following the instructions provided by the manufacturers. All slides were scanned using Zeiss Mirax Scan™, and the intensity of immunohistochemistry staining was automatically quantified using HistoQuant™ software. The cytoplasm staining was visually evaluated as absent (0), weak (1), moderate (2), or strong (3). **Results:** The BFP Advance and Novolink , and the SAB LSAB + showed the highest staining intensity among all the systems (P<0.01). However, LSAB+ showed the highest cytoplasm staining among those used (p<0.01). The other second-generation BFPs showed similar staining intensities among those used and also similar to the SABs. The first-generation EnVision+ showed the weakest staining intensity. The seven BFPs showed sharper signal without cytoplasm staining compared to all SAB systems (p<0.05). BFP PicTureMAX showed the least cytoplasm staining. **Conclusion:** The BFP systems provide sharper and better localized immunohistochemical signal without cytoplasm staining compared to the SAB systems. The BFP Advance and Novolink showed the strongest staining intensity and, followed by all the other second-generation BFPs, represent a powerful tool for immunohistochemistry standardization of estrogen receptor evaluation of breast carcinomas.

Keywords: Immunohistochemistry; Breast Neoplasms; Estrogen Receptor.

Introduction

The success of immunohistochemistry is largely a result of a development of reliable markers and of highly sensitive visualization procedures. The demonstration of antigens in tissues and cells by immunostaining is a two-step process involving first, the binding of an antibody to the antigen of interest, and second, the detection and visualization of bound antibody by one of a variety of enzyme chromogenic systems. The choice of visualization systems will impact the sensitivity, utility, and ease-of-use of the method, including also reduction in incubation times.[1]

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Developments in immunohistochemistry techniques, especially antigen retrieval methods and sensitive visualization systems have allowed the detection of very small quantities of protein. The staining intensity depends on the staining procedures used in addition to the protein content itself. [2] The mostly used streptavidin-biotin visualization systems are based on the sequential application of biotinylated link antibody and streptavidin labeled with one or two molecules of alkaline phosphatase or peroxidase. These conventional biotin-rich procedures advertise the advantage that streptavidin presents high affinity for biotin (DakoCytomation Product Catalog 2005/2006).

Recently, a novel generation of polymeric biotin-free visualization systems has been released. The polymer-based, biotin-free detection reagent is based on polymeric technology, which uses compact enzyme-antibody conjugates. They are based in a polymeric HRP, which is a unique enzyme-conjugated polymer backbone that also carries secondary antibody molecules.[3] The polymeric visualization systems are claimed to achieve signal amplification and thereby an enhanced sensitivity by increasing the number of enzyme molecules which are conjugated to the secondary antibody. According to the suppliers, endogenous biotin will not affect polymeric staining results due to their biotin-free characteristic.

In this study, we aimed to compare the performance of the biotin-free polymeric visualization system kits to the streptavidin-biotin-system kits for evaluating estrogen receptor using an automated image analysis system.

Materials and Methods

Case Selection

Three hundred and twenty cases of invasive breast carcinomas, diagnosed between 1990 and 2005, were randomly selected from the files of the Breast Pathology Laboratory of Federal University of Minas Gerais, State University of Campinas Medical School and Cancer Hospital A.C. Camargo of Sao Paulo, Brazil. All original slides were reviewed to confirm the diagnosis and to select representative areas of tumors. One cylinder (1mm diameter) of each tumor with representative area of neoplasm was selected from paraffin blocks to build a tissue microarray (TMA).[4] Two cylinders of tumors from previously tested and whose results were positive and negative were also included as internal controls for the TMA. Sequential 4μm sections were obtained and stained for hematoxylin and eosin (first and last sections) to confirm diagnosis, and the interval sections were used for the immunohistochemical study. Slides containing full sections of previously tested positive breast tumor were included in all batches as external control.

Immunohistochemical Procedures

The sections were mounted on glass slides coated with silane (3-aminopropyltriethoxysilane) and dried for 30 minutes at 37°C. The sections were deparaffinized in xylene and rehydrated via a series of graded alcohols. Endogenous peroxidase activity was blocked by following the procedures and the reagents supplied by each visualization system manufacturer. All sections were initially submitted to heat-induced epitope retrieval in steamer for 25 minutes under the same environment conditions. Each manufacturer supplied its antigen retrieval reagents, which were all citrate pH=6.0.[5-6] The rabbit monoclonal antibody SP1, Roche™, against estrogen receptor, was used as primary antibody for all the visualization systems,[7-8] preliminary testing was performed in our laboratory to identify the best concentration for the primary antibody and to choose the negative and positive controls using the dilution data supplied by the manufacturer as the starting point. The best primary antibody dilution achieved was 1:300 and this dilution was used for all the visualization systems for a 30 minute incubation. After washing the primary antibody with phosphate buffered saline (PBS), the slides were incubated with the reagents supplied by the manufacturers of each visualization system following all the procedures and incubation times suggested in the specification sheets. Eleven different visualization systems were used, including seven biotin-free polymer systems(six second-generation: DAKO Advance™, Leica Novolink ™, Zymed SuperPicTure™, Zymed PicTure Max ™, Biogenex Super Sensitive Non-Biotin HRP ™, CellMarque Mouse/Rabbit Polydetector HRP/DAB™; one first-generation: DAKO EnVision+™) and four streptavidin-biotin systems (DAKO LSAB+™; Signet EasyPath™; Biogenex Super Sensitive™ and CellMarque Mouse/Rabbit Immunodetector HRP/DAB™ as shown in Table1. Freshly prepared DAB solution was applied following the procedures and incubation times suggested. DAB was removed by rinsing with distilled water. The slides were counterstained with hematoxylin, dehydrated in ethanol, cleared in xylene and mounted using Entelan™.

Table 1: Specification, type, and supplier of each visualization system

Visualization System	Type	Supplier
EnVision+	Biotin-free polymer - 1st generation	Dako, Carpinteria, CA, USA
Advance	Biotin-free polymer - 2st generation	Dako, Carpinteria, CA, USA
NovoLink	Biotin-free polymer - 2st generation	Leica, New Castle, UK
SuperPicTure	Biotin-free polymer - 2st generation	Zymed, San Francisco, CA, USA
PicTure Max	Biotin-free polymer - 2st generation	Zymed, San Francisco, CA, USA
Super Sensitive non-biotin HRP	Biotin-free polymer - 2st generation	Biogenex, San Ramon, CA, USA
Mouse/Rabbit Polydetector HRP/DAB	Biotin-free polymer - 2st generation	CellMarque, Rocklin, CA, USA
LSAB +	Streptavidin-biotin based system	Dako, Carpinteria, CA, USA
EasyPath	Streptavidin-biotin based system	Signet, Dedham, MA, USA
Super Sensitive	Streptavidin-biotin based system	Biogenex, San Ramon, CA, USA
Mouse/Rabbit Immunodetector HRP/DAB	Streptavidin-biotin based system	CellMarque, Rocklin, CA, USA

Immunostaining Analysis

All slides submitted to immunohistochemistry were labeled and the examiner was blinded to the visualization system used. The automated analysis was made using digital microscopy to quantify the intensity of immunohistochemical staining. All the slides were digitalized using Mirax Scan (developed and produced by 3DHISTECH Ltd., Budapest, Hungary; distributed worldwide by Carl Zeiss, Jena, Germany) and the images provided by the software were exhibited in an LCD monitor under contrast, focus, saturation, and white balance standardization.

To evaluate the intensity of staining, the image analysis system HistoQuant™, 3DHISTECH™, Budapest, Hungary, was used (Figure 1). The software identified the

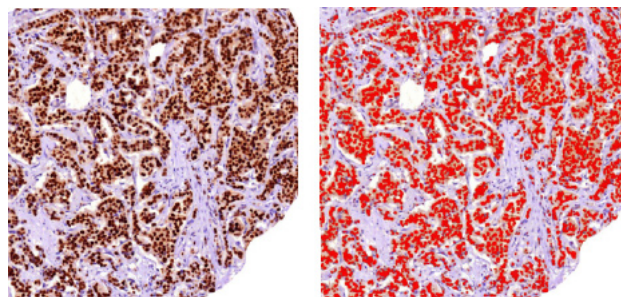

Figure 1 - The same TMA spot in 200x magnification. A) Original immunohistochemical staining. B) Selected positive nuclei to have their intensity of immunohistochemical staining numerically quantified.

immunohistochemical staining to be quantified by minimizing background-staining artifacts using image filters. Since the software recognizes the positive nuclei staining of all different intensities, the quantification was processed in each TMA spot automatically by the software and all the numerical data was exported to a Microsoft Excel file.

The cytoplasm staining was semi-quantitatively evaluated as absent (0), weak (1), moderate (2), and strong (3) by creating a TMA project, which exhibits each TMA spot at a time on the computer screen (Figure 2).

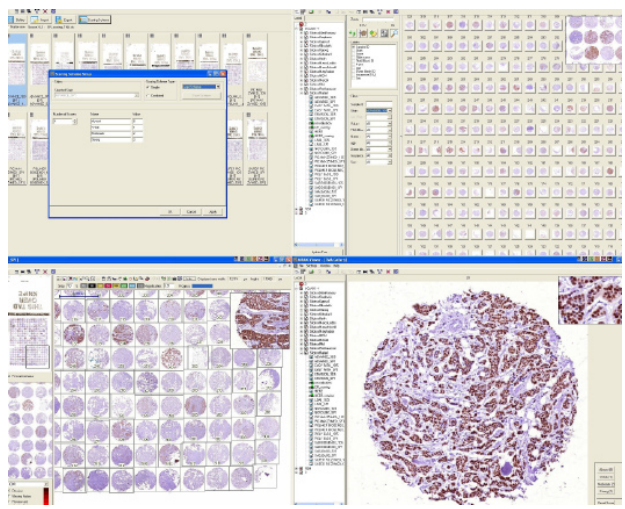

Figure 2 -TMA project built in MiraxViewer software. A) All slides in the background and a window containing the scoring scheme proposed for the background staining evaluation. B) 320 ordered and numerated TMA spots from one single slide shown individually in the screen. C) The same TMA spots shown in higher magnifications. D) One single TMA spot ready to be analyzed. The scoring scheme is shown in the picture footnote and a 400x magnification of this spot is shown in the window on the top.

Statistical Analysis

The Software WINKS – Statistical Data Analysis, Version 6.0, was used for the statistical analysis. Wilcoxon signed rank test was used to compare the different groups of paired variables. Chi-square test was used to evaluate the difference in frequencies of score among the groups of categorical variables of the cytoplasm staining evaluation.

Ethics

The procedures described in this section are in compliance with the Helsinki Declaration and are also in accord with the ethical standards established by the institutions where this study took place.

Results

The biotin-free polymeric visualization systems Advance and Novolink showed the strongest staining intensity together with the SAB LSAB+, which showed stronger staining intensity among all streptavidin-biotin systems and also among most of the polymeric systems. The polymeric systems PicTure Max and Super Sensitive Non-Biotin HRP showed similar intermediate staining intensity to the streptavidin-biotin system Super Sensitive. The polymeric systems Super PicTure and Mouse/Rabbit Polydetector HRP/DAB showed low intermediate staining intensity similar to the streptavidin-biotin systems Mouse/Rabbit Immunodetector HRP/DAB and EasyPath. The polymer EnVision + showed the weakest staining intensity among all the visualization systems.

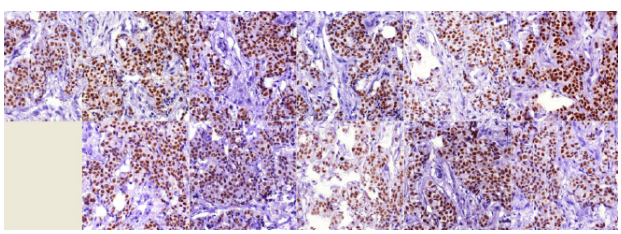

Figure 3 - Intensity of immunohistochemical staining in the same tumor using 11 different visualization systems A) Advance, B) EasyPath, C) EnVision, D) LSAB+, E) Novolink, F) PicTure Max, G) Super Sensitive non-biotin HRP, H) Mouse/Rabbit Polydetector HRP/DAB, I) Super Sensitive, J) Mouse/Rabbit Immunodetector HRP/DAB, K) Super PicTure.

All visualization systems and p values of statistical analysis are shown in Table 2 according to their intensity of immunohistochemical staining.

The polymeric systems showed a sharper nuclear signal with no cytoplasm staining when compared to streptavidin-biotin systems ($p<0.01$). The biotin-free polymer Zymed PicTure MAX™, showed the least cytoplasm staining and the streptavidin-biotin LSAB+ showed the most ($p<0.01$).

Table 2 - Eleven different visualization systems grouped according their staining intensity level expressed by the p value

Staining intensity level	Biotin-free polymer system (p value*)	Streptavidin-biotin polymer (p value*)
Stronger	Advance (0.0034) NovoLink (0.0061)	LSAB+ (0.03)
Up intermediate	Super Sensitive non-biotin HRP (0.01) PicTure Max (0.01)	Super Sensitive (0.02)
Low intermediate	SuperPicTure (0.01) Mouse/Rabbit Polydetector (0.01)	Mouse/Rabbit imunodetector EasyPath
Weaker	EnVision+	

* p value of the statistical analysis between the staining intensity of each visualization system and the group of systems from the level below. There was no significant difference between the staining intensity of the polymeric systems and the streptavidin-biotin systems of the same level, neither between two systems of the same level group.

Discussion

The present study showed that highly sensitive visualization systems enhanced immunohistochemistry staining intensity. We have compared the kits of biotin-free polymeric visualization systems to the conventional streptavidin-biotin-system kits to evaluate estrogen receptor in breast carcinomas. Due to the great number of different reagents and protocols from all the visualization system kits, every immunohistochemical reaction was carried out manually in the same environmental conditions. Since we wanted to compare the efficacy of the combination between the particular retrieval method and the visualization system provided by each kit, all the procedure steps, times and reagents supplied by each manufacturer were followed accordingly.

Interpretation of immunohistochemistry is usually done manually and is, therefore, dependent

on the experience and ability of the interpreter.[9-10] Computerized mage analysis systems have been used since the late 1980s and were shown to provide a more accurate means of quantification of ER.[11-12-13] Quantification of immunohistochemistry for ER using different types of image analysis has also shown strong agreement with manual scoring from experienced observers. According to our automated quantification, the biotin-free Advance and Novolink showed the strongest staining intensity among the polymers. Advance, a ready-to-use, peroxidase-based visualization system is compatible with suitably diluted rabbit and mouse primary antibodies. According to its manufacturer, it is a super-sensitive, non-biotin based, immunohistochemical visualization system that is useful for the detection of antigens in low concentrations, for short incubation time or for higher dilution of primary antibodies. It consists of two main reagents: an HRP Link, which contains anti-mouse and anti-rabbit secondary antibodies in a Tris-HCl buffer and stabilizing protein and an anti-microbial agent, and an HRP Enzyme, which contains antibodies polymerized with horseradish peroxidase in a Tris-HCl buffer and stabilizing protein and anti-microbial agent (DakoCytomation Product Catalog 2005/2006). None of the biotin-free polymer systems employed in this study supply a link antibody between the primary and the polymer, except by Advance™ and Zymed PicTure Max polymer™. This might be one reason for the increased staining intensity observed. The Advance™ kit also supplies an enhancer reagent, which acts in DAB, and may provide improvements in sharpness and staining intensity. According to the Leica datasheet, the polymer Novolink contains increased number of peroxidase molecules bound to an activated dextran backbone with lack of major gaps among them to improve chromogen reaction, and that might contribute for increasing staining intensity in this visualization system´s reaction. The Novolink kit also supplies the Post Primary Block, which is used to enhance penetration of the subsequent polymer reagent and might be one reason for its high performance. On the other hand, the first generation polymer EnVision+, which neither supplies a link antibody nor an enhancer, and yet shows larger gaps among its peroxidase molecules bound to the dextran backbone, showed the weakest staining intensity among the polymers tested in our study.

Our results show that some polymers present equivalent staining intensity to streptavidin-biotin systems according to the automated image analysis (Table 2). It is relevant to mention that the polymeric systems present macromolecules containing secondary antibodies and nearly 100 enzyme molecules polymerized in a dextram backbone, while the streptavidin biotin systems present up to four enzyme molecules bound to one molecule of streptavidin. This great difference in molecule size might result in different membrane penetration capacities between these two groups of visualization systems. Although the presence of biotin is the cause of important background and cytoplasm staining in some tissues,[14] the high affinity between streptavidin and biotin may be the most important reason for the strong staining intensity observed in some streptavidin biotin visualization system reactions compared to some biotin-free polymers.

All the polymeric visualization systems used in this study showed sharper staining without cytoplasm staining when compared to the streptavidin-biotin systems. This may be explained by the fact that the polymers utilize a novel controlled polymerization technology to prepare polymeric HRP-linker antibody conjugates, which are free of biotin. Therefore, the problem of non-specific staining that can appear with streptavidin-biotin visualization systems due to endogenous biotin does not occur. However, streptavidin from the kit and endogenous biotin may bind to each other leading to background and cytoplasm staining in most tissues when streptavidin-biotin visualization systems are used.[15] As an example, the streptavidin-biotin system LSAB+, which showed one of the highest staining intensities among the systems, also shows the highest cytoplasm staining among all of them. That applies unreliability to the reaction. Certainly, adjustments could be made in order to reduce or eliminate cytoplasm staining seen in LSAB+, such as reducing primary antibody dilution associated with increased incubation time.

According to some authors, besides allowing cheaper immunohistochemical assays, the high dilutions of the primary antibodies achieved by the systems shown to present stronger staining intensity also enable more reliability to the results. High dilutions prevent background and cytoplasm staining, formation of electrostatic or other non-immunological non-specific bonds, or unexpected cross-reactivities (especially with polyclonal primary antibodies).[16]

Finally, but equally important, an additional advantage of the polymeric systems would be the reduction of the staff workload and assay time due to the fewer number of steps present in their protocol.

In the light of these results, the kits of biotin-free polymeric visualization systems provide sharper immunohistochemical signal without cytoplasm staining

when compared to the streptavidin-biotin-system kits. They present variable staining intensity among them due to their differences in molecule structures, reagent types and concentrations, and buffers supplied in the kit. Advance and Novolink were seen to present the highest staining intensity among the biotin-free systems and, followed by the other polymers of second-generation, represent a powerful tool for clinical testing and research work. They can contribute to immunohistochemistry standardization of estrogen receptor evaluation of breast cancer.

Competing Interests

The authors declare that they have no competing interests. The authors have no relationship with the manufacturers of the visualization systems cited in the manuscript.

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