Improved Antigen Detection in IHC: Immunoassays utilizing HRP polymer conjugated secondary antibodies

Abstract

Traditional immunohistochemistry (IHC) methods are often inadequate when detecting low abundance antigens or under conditions of non-optimal antibody-antigen binding. Using low affinity primary antibodies or targeting epitopes that are difficult to detect may lead to poor or inconsistent antigen staining. Several signal amplification methods have been developed to improve assay sensitivity, but these methods commonly result in high background staining. Here we show an advanced method that uses a horseradish peroxidase (HRP)-polymer conjugate to dramatically increase the lower limit of detection without significantly contributing to background signal. Compared to the avidin-biotin complex (ABC) detection system, the polymer-based amplification method reduces the amount of primary antibody needed by 3-fold and shortens the incubation period from overnight to one hour. These results demonstrate the superior performance of the polymer-based signal amplification method over the ABC method in immunohistochemical analysis of target protein expression.

Background

In conventional immunohistochemistry (IHC) assays, antigens are first recognized by primary antibodies and then detected with labeled secondary antibodies. These conjugates include reporter enzymes (e.g. horseradish peroxidase, HRP) that convert chromogens such as 3,3'- Diaminobenzidine (DAB) or 3-amino-9-ethylcarbazole (AEC) into insoluble colored deposits at the reaction site. An enhanced method has been developed in which a single HRP labeled secondary antibody is replaced by a secondary antibody conjugated to a polymer backbone containing numerous HRP molecules (see Figure 1). This HRP-polymer method has shown up to a 50-fold gain in assay sensitivity compared to the standard format.

Alternatively, the sensitivity of the standard format can be improved by using additional layers to link multiple HRP enzymes to each antibody-antigen complex. In the avidin-biotin complex (ABC) method, antigen detection involves sequentially adding a biotinylated secondary antibody and the avidin-biotin peroxidase complex. Technically similar, the labeled streptavidin binding (LSAB) detection method also uses a biotinylated antibody. However, in this case, the third layer is a HRP-streptavidin (SA) conjugate, which is smaller in size and can more easily penetrate tissue sections.

One disadvantage for both of these methods is the requirement to block endogenous biotin. Even with blocking, tissues with high levels of biotin such as the liver and kidney or frozen tissue sections can still exhibit residual activity. In side-by-side experiments comparing the polymer-based method to the ABC method, we show a drastic improvement in detecting alkaline phosphatase (ALPL) in a human kidney tissue section and HAI-1 in a human liver tissue section when a HRP-polymer secondary antibody was used. Furthermore, the polymer-based amplification method is faster, requiring less steps, which helps to minimize the complexity of IHC experiments.

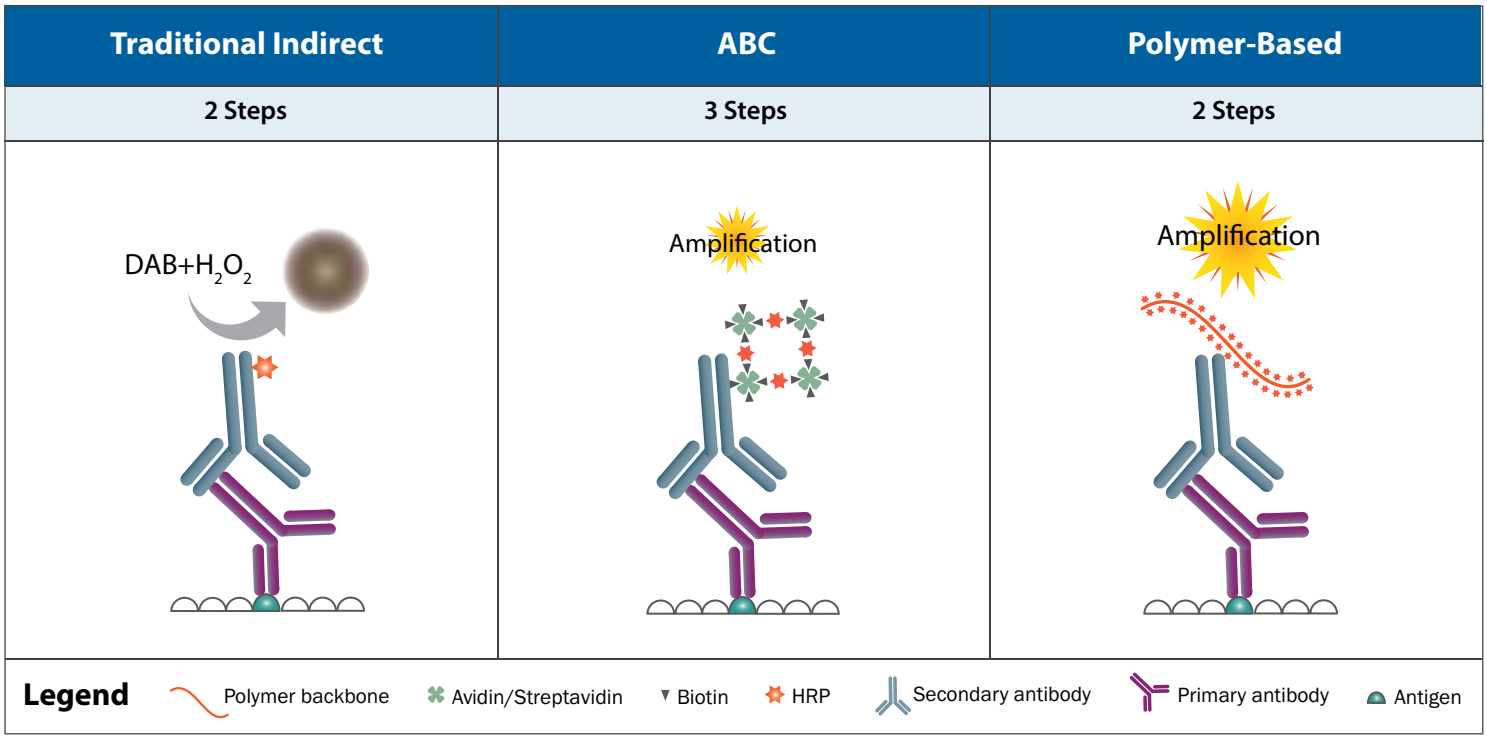

Figure 1. Comparison of indirect detection: traditional vs. signal amplification methods

Methods and Results

Paraffin-embedded tissue blocks were fixed in formalin for 24 hours. Tissue sections were cut to a thickness of 7 µM, cleared in xylene and rehydrated with decreasing concentrations of ethanol. Heat induced antigen retrieval (HIER) was applied to all slides which were immersed in a basic citrate buffer (catalog #CTS013) and heated in a vegetable steamer (95-100 °C) for 20 minutes.

In figure 2, human kidney sections were incubated with Serum Blocking Buffer (5% animal serum in PBS) for 15 minutes and endogenous peroxidase activity was quenched by incubating sections in 3% H_2O_2 in methanol for 15 minutes. The ABC kit sections were treated with Avidin Blocking Reagent for 15 minutes, followed by treatment with Biotin Blocking Reagent for an additional 15 minutes. Except for the no primary controls, all sections were incubated with 15 µg/ml of mouse anti-human ALPL antibody (catalog # MAB1448) overnight at 4 °C. Next, the ABC kit samples were incubated with anti-mouse biotinylated secondary antibody for 30 minutes followed by a 30 minute incubation with High Sensitivity Streptavidin (HSS)-HRP; while HRP polymer samples were incubated with the goat anti-mouse IgG VisUCyte HRP polymer antibody (catalog # VC001) for 30 minutes. All tissue sections were treated with DAB (brown) for 10 minutes and counterstained with hematoxylin (blue). Slides were imaged with a bright-field microscope.

Human liver tissue sections in figure 3 underwent a similar procedure as described above with the following exceptions. Sections, other than the no primary controls, were probed with 0.3 µg/ml of the HAI-1 goat anti- human HAI-1 Ectodomain Antigen polyclonal antibody (catalog #AF1048). The ABC kit sections were sequently incubated with anti-goat biotinylated secondary antibody and HSS-HRP, whereas HRP-polymer sections were incubated with the donkey anti-goat IgG VisUCyte HRP polymer antibody (catalog # VC004). As shown in figures 2 and 3, staining of ALPL and HAI-1 is substantially increased using Bio-Techne's HRP-polymer-based signal amplification reagents over the ABC method.

In an analogous IHC experiment comparing primary antibody conditions, human kidney sections for the ABC method were probed with 15 µg/ml of mouse anti-human ALPL antibody (catalog # MAB1448) overnight at 4 °C. For the polymer-based method, sections were incubated with only 5 µg/ml of primary antibody for 1 hour at room temperature to achieve equivalent antigen staining (images not shown).

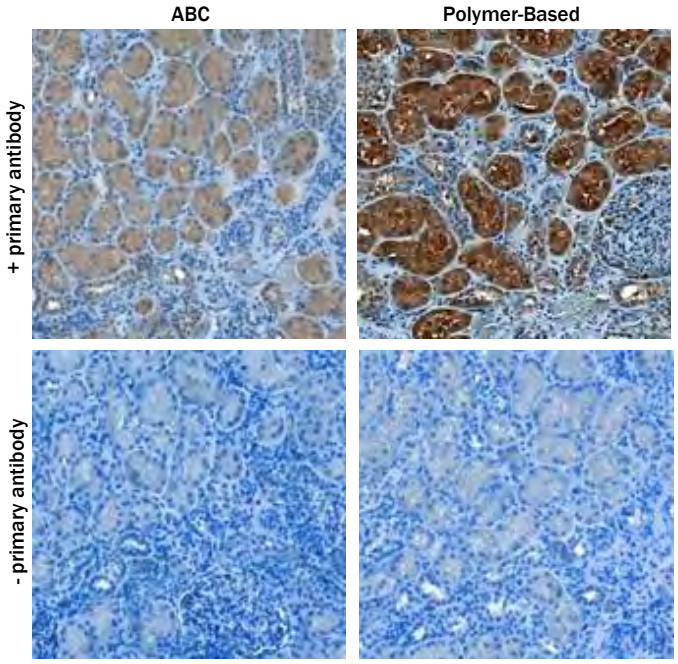

Figure 2: Improved detection of ALPL in human kidney sections with HRP-polymer conjugated secondary antibody. Formalin-fixed, paraffin-embedded (FFPE) tissue sections were stained with A) the ABC detection kit (catalog# CTS002), B) the goat anti-mouse IgG VisUCyte HRP polymer antibody (catalog # VC001), C) the ABC detection kit without the primary antibody, or D) the VisUCyte HRP polymer antibody without the primary antibody.

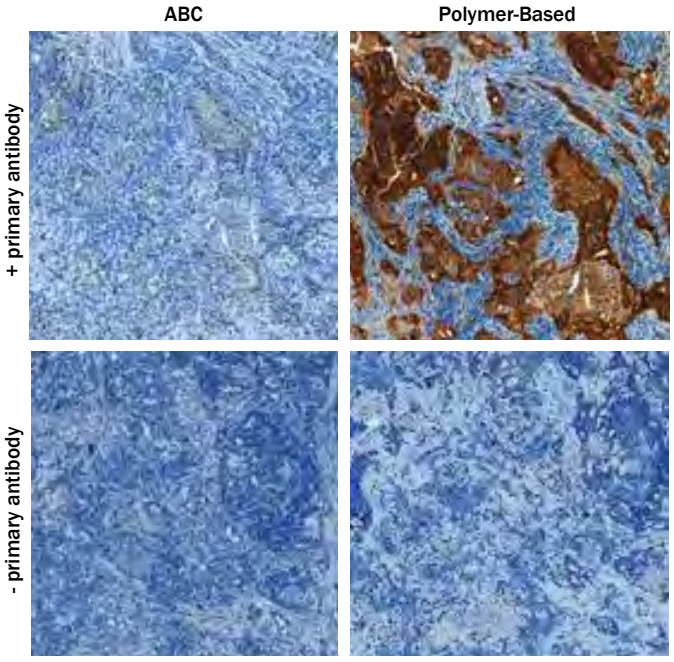

Figure 3: Improved detection of HAI-1 in human liver sections using the HRP-polymer conjugated secondary antibody. FFPE tissue sections were stained with A) the ABC detection kit (catalog# CTS008), B) the donkey anti-goat IgG VisUCyte HRP polymer antibody (catalog # VC004), C) the ABC detection kit without the primary antibody, or D) the VisUCyte HRP polymer antibody without the primary antibody.

Highlights

Improve Sensitivity	HRP-polymer antibodies increase the sensitivity of traditional IHC methods by 30-50 fold, making them ideal for detection of low abundance antigens. Under similar experimental conditions (e.g. same primary antibody concentration), the polymeric method produced intense staining of ALPL in human kidney sections and of HAI-1 in human liver sections whereas the signal was barely detectable with the ABC method.
Conserve Primary Antibody	The use of HRP polymers significantly reduces the amount of primary antibody needed. In our side-by-side comparison, the polymer-based method required 3x less primary antibody to generate an equivalent signal to samples stained using the ABC method.
Reduce Background Staining	HRP-polymer antibodies show less non-specific binding compared to biotin-avidin/ SA systems, which require a biotin blocking step.
Save Time	With the polymer-based method there's no need to block endogenous biotin or to include additional layers to enhance signal amplification. In some cases, assay protocols may permit a short incubation time (1 hour) with the primary antibody solution.
TSA Compatible	HRP-polymer secondary antibodies can be used with tyramide signal amplification (TSA) reagents to further increase assay sensitivity.

Conclusions

VisUCyte HRP Polymer is a biotin-free detection reagent that stains tissue sections in a highly specific and sensitive manner. By eliminating the endogenous biotin blocking step, reducing the number of detection steps, and shortening the primary antibody incubation time, the HRP polymer-based amplification method addresses key disadvantages of the ABC detection kits. In summary, the data presented here clearly illustrates the substantial benefits of using HRP-polymer secondary antibodies to generate IHC results with remarkable speed, sensitivity and ease.

Bio-Techne Product	Catalog Number
Goat anti-Mouse IgG VisUCyte™ HRP Polymer Antibody	VC001
Goat anti-Mouse/Rabbit IgG VisUCyte™ HRP Polymer Antibody	VC002
Goat anti-Rabbit IgG VisUCyte™ HRP Polymer Antibody	VC003
Donkey anti-Goat IgG VisUCyte™ HRP Polymer Antibody	VC004

Further Reading

Kalyuzhny, A. E., 2011. Signal Transduction Immunohistochemistry: Methods and Protocols. Totowa, NJ: Humana Press