



Multiple Antigen Labeling Simplified using ImmPRESS™ Reagents

Introduction

The simplest system for use in a double labeling application is the ImmPRESS™ Polymer Detection System. ImmPRESS™ Polymer Reagents are available with either micropolymers of peroxidase (HRP) or alkaline phosphatase (AP) detection enzymes attached to a variety of our affinity-purified secondary antibodies. These one-step, non-biotin reagents are very sensitive with low non-specific background. In addition, pairing ImmPRESS™ Polymer Reagents with Vector's ImmPACT™ line of substrates produces sensitivities at least 2-4 times greater than with conventional substrates. Because the ImmPRESS™ Polymer System is a single step, the time for any multiple labeling experiment is shortened and the protocol simplified.

General Protocol for Double Antigen Labeling using Primary Antibodies from different species and ImmPRESS™ Polymer Reagents with different enzymes*

This is the simplest method of double antigen labeling using chromogenic staining, since using primary antibodies from different species (e.g. mouse and rabbit) eliminates concern over cross-reaction of antibody reagents. In addition, using two different enzymes eliminates substrate cross interaction and allows for a broader choice of substrate colors and counterstains. For example, a combination of ImmPACT™ DAB (HRP detection) and ImmPACT™ Vector® Red (AP detection) allows a hematoxylin counterstain.

All staining protocols need to be optimized for a particular application, and the appropriate deletion controls should always be run to allow for the accurate interpretation of results.

Step 1. Antigen Retrieval: Perform antigen retrieval, if required by the primary antibody, using the appropriate Vector Antigen Unmasking Solution (citrate based or high pH based) with high temperature.

Please Note: A heat denaturing step does not inactivate endogenous peroxidases. If endogenous peroxidase activity is present in the tissue, an endogenous enzyme blocking reagent such as BLOXALL™ Blocking Solution should be used.

Step 2. Endogenous Enzyme Blocking: Block endogenous enzyme activity, if required, using BLOXALL™ Endogenous HRP/AP Blocking Solution.

Step 3. Protein Blocking: Block potential non-specific protein interactions by incubating the section with the ready-to-use serum block included in ImmPRESS™ Polymer Detection Kit.

Step 4. Primary antibody: Incubate with the primary antibody solution according to manufacturer's suggestions. Primary antibody titer should be re-optimized for multiple labeling applications.

Step 5. ImmPRESS™ Polymer Detection Reagent: Incubate the section with the Ready-to-use ImmPRESS™ Reagent according to the instructions. The wash time listed in the protocol, especially after the ImmPRESS™ Reagent, should be followed and can be lengthened if necessary.

Step 6. Enzyme Substrate: Incubate in the Vector® substrate of choice. When choosing the substrate consider properties such as sensitivity, color contrast, type of microscopy, and heat resistance.



Charts showing the relative substrate sensitivities, substrate properties and compatibilities, staining sequence, heat resistance and counterstain compatibility as well as images of each substrate are available on the Vector Labs website (www.vectorlabs.com).

Repeat Steps 3-6 for detecting the second antigen.

Additional Considerations for General Double Labeling Protocol Variations*

1) When using primary antibodies from the same species:

The above general protocol can be easily modified for use with primary antibodies of the same species where there is potential for “cross-reactivity” between the detection reagents of the sequential labeling steps. This cross-reaction can be prevented by one of two methods:

- a) The reaction product of the first chromogen can provide an effective “covering” or “sheltering” of all the reagents used in the preceding staining protocol, eliminating cross-reactivity with subsequent steps. Titering the primary antibody to optimize substrate sheltering is required.
- b) A heat denaturing step (HIER, heat induced antigen retrieval) used before each subsequent labeling protocol removes antibody reagents in the first labeling system, thus preventing most antibody/antibody cross-reactions. Repeat step 1 (Antigen Retrieval) before the second labeling protocol. If a heat denaturing step is used, choose a substrate for the first label that is heat resistant.

2) When using primary antibodies from the same species and ImmPRESS™ Reagents with the same detection enzymes:

When using the same detection enzyme for both labeling protocols, the enzyme from the first labeling protocol must be completely inactivated in order to prevent artifactual co-staining.

Fortunately, the methods used above - (1) “sheltering” by the first substrate deposition or (2) removing the antibody reagents in the first system using HIER - will also address this concern.

3) When using primary antibodies from different species and ImmPRESS™ Reagents with the same detection enzymes:

In the case where the species of primary antibody is different and only the detection enzyme is the same, possible HRP or AP enzyme activity from first label can be easily quenched by a 5-10 minute incubation in BLOXALL™ Blocking Solution between the sequential labeling steps.

4) When the availability of one antigen is low:

If one of the antigens has lower availability and extra sensitivity is required, the amplified ImmPRESS™ Excel Kit can be used to stain that antigen, and the single-step ImmPRESS™ Polymer Reagent used to stain the second antigen.

* Detailed multiple labeling protocols, controls, trouble-shooting information, and images are available on the Vector Labs website or in our booklet: “Discovery through Color - A Guide to Multiple Antigen Labeling”, or contact Vector Labs Technical Services.