

IHC Multiplexing Resource Guide

A guide to enzyme-based multiple antigen labeling

Discovery through color

Visualizing two or more target antigens expressed in the same tissue section using immunohistochemistry (IHC) can be accomplished reliably and reproducibly utilizing established secondary detection systems and combinations of enzyme substrates.

This guide was written as a resource to aid investigators new to IHC, for labs expanding their IHC applications to include multiple antigen labeling and to those investigators looking for methods to streamline double or triple antigen staining on the same tissue section.

What is presented here is a culmination of many years of diligent research and development by lab personnel at Vector Laboratories. While there are other approaches to double and triple antigen staining, the procedures and products featured in this guide are known to generate clear, unambiguous localization of the target antigens. It should be noted, unless otherwise stated, all of the images featured in this brochure were generated at Vector Laboratories using these same procedures.

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Cover image: Breast carcinoma—double label: CD31 (m), ImmPRESS HRP Reagent, ImmPACT® DAB EqV HRP substrate (brown); MRC1 (r), ImmPRESS AP Reagent, ImmPACT Vector® Red AP substrate (magenta). Image courtesy of Richard Allen, University of Sheffield.

Quick Start to Easy Double Staining

For investigators new to IHC and double labeling, or investigators experiencing issues with the current IHC double labeling, the ImmPRESS Duet Double Staining Polymer kits are designed to simplify the visualization of two target antigens on the same tissue section using IHC methods.

If your double label application meets the following criteria, then the ImmPRESS Duet Double Staining Polymer kits will enable quick, accurate double staining using prediluted, ready-to-use matched peroxidase (HRP) and alkaline phosphatase (AP) detection reagents:

1. One primary antibody is raised in mouse and the other primary antibody is raised in rabbit.
2. The tissue specimen is not from a mouse, rabbit or rat source.
3. The target antigens are not co-localized (overlapping in their expression).

If your application meets the above criteria, then select an ImmPRESS Double Staining Polymer kit based on your choice of which enzyme (HRP or AP) will be used to detect your two target antigens.

Using the ImmPRESS Duet Double Staining System

1. **Preparation of tissue.** For paraffin sections, deparaffinize and hydrate tissue sections using standard procedures. For frozen sections or cell preparations, fix with acetone. Wash for 5 minutes in tap water.
2. **Antigen Unmasking.** If antigen unmasking is required, use Antigen Unmasking Solution, Citrate-based, pH 6.0 or Tris-based, pH 9.0.
3. **Enzyme Quench.** If quenching of endogenous enzyme (HRP or AP) is required, incubate sections in BLOXALL® Blocking Solution for 10 minutes.
4. Wash in buffer for 5 minutes.
5. Incubate sections for 20 minutes with ready-to-use (2.5%) Normal Horse serum (NHS).
6. **Add Primary antibody.** Tip off serum and incubate with rabbit and mouse primary antibodies diluted in appropriate antibody diluent.
7. Wash in buffer for 5 minutes.
8. **Apply ImmPRESS Duet Reagent.** Incubate for 10 minutes.
9. Wash in buffer 2 × 5 minutes.
10. **Apply ImmPACT DAB EqV Substrate.** 2-10 minutes.
11. Wash in buffer 2 × 5 minutes.
12. **Apply ImmPACT Vector Red Substrate.** 20-30 minutes.
13. Wash slides in water 5 minutes.
14. Counterstain (optional), clear and mount.

Fig. 1 Add Primary Antibodies*

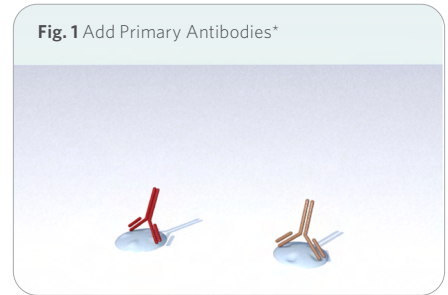


Fig. 2 Add ImmPRESS Duet Reagent

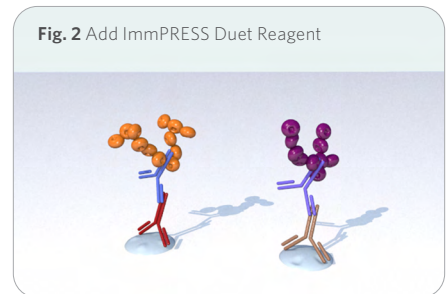
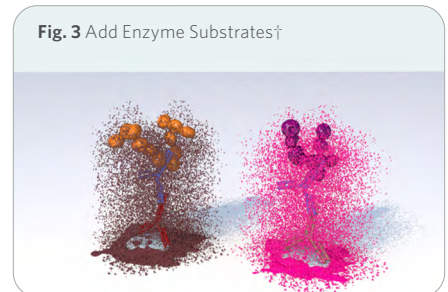


Fig. 3 Add Enzyme Substrates†



* Primary antibodies may be applied separately or mixed together.

† Enzyme substrates are applied sequentially.

Reagent Selection to Achieve Multiple Antigen Labeling

A) Detection System

If your specific application does not meet the criteria for using the ImmPRESS Duet Polymer kits (see previous page), then we recommend a more customized approach using individual detection reagents and enzyme substrate kits.

Within this brochure we present three different double labeling protocols that can be considered:

- 1) Using only ImmPRESS HRP and/or AP Polymer detection reagents
- 2) Using only VECTASTAIN ABC HRP and/or AP detection reagents
- 3) Using a combination of ImmPRESS Polymer Reagents and VECTASTAIN ABC Reagents

The choice of whether to use an ImmPRESS Polymer System and/or a VECTASTAIN ABC System to generate multiple antigen staining is largely at the end-user's discretion. As showcased in this brochure, either system or combination of systems can be used. However, it should be noted that using the ImmPRESS Polymer Systems will reduce the number of steps required to perform a double label, and as such, would be a faster, more convenient methodology over using the VECTASTAIN ABC System.

Selection of a specific detection system, or systems, for double labeling would primarily be based upon the species in which the primary antibodies are raised, and then a choice of HRP and/or AP options.

B) Enzyme/Substrate Options

The choice of using HRP and/or AP detection systems should be based on several criteria:

- 1) Presence of pigment or extensive endogenous enzyme in the tissue specimen
- 2) Extent of antigen expression
- 3) Level of sensitivity required to visualize the targets
- 4) Compatibility of enzyme substrates when used in combination

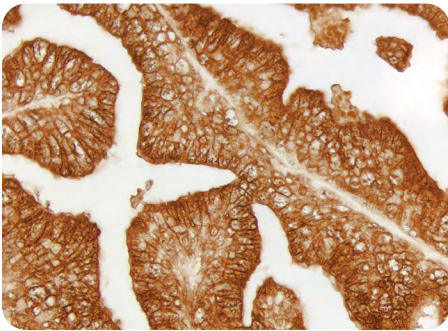
Generally, HRP substrates generate sharper, more dense reaction product (precipitate) compared with AP substrates. This characteristic may be helpful if the target is located intracellularly, expressed in a punctate manner, or in situations that require highly delineated localization. AP substrate precipitates are more diffuse and translucent. These characteristics may be preferred when target antigens are expressed broadly and to allow visualization of underlying tissue morphology. Please refer to representative images of the color and appearance of our HRP and AP substrates on page 4.

The charts presented on pages 5 and 6 are intended as helpful reference guides to select enzyme substrates based on their relative sensitivities and compatibility when used together. Note that the color combinations indicated are intended to be used where two or three antigens are expressed in different cell types or different compartments of the same cell. If two antigens are co-localized (overlap) in the same compartment of the same cell, please refer to the "Frequently Asked Questions" on page 19.

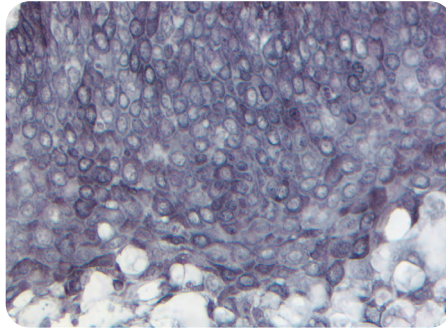
Enzyme Substrate Choices

Representative color and appearance of reacted (precipitated) HRP and AP enzyme substrates on tissue sections. See the chart on page 6 for recommended combinations of substrates for double labeling IHC.

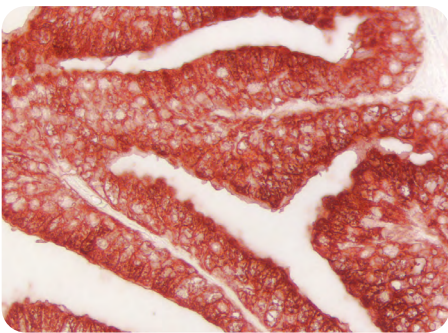
HRP Substrates



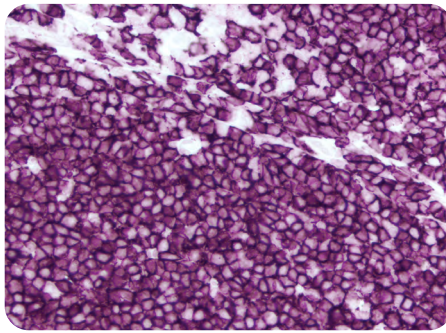
Brown: ImmPACT DAB, ImmPACT DAB EqV and DAB.



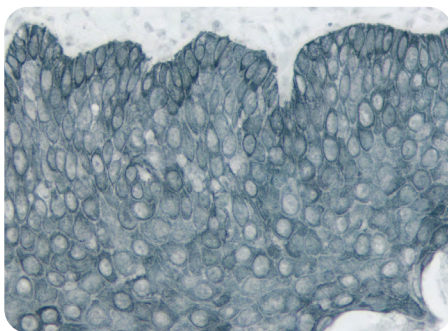
Gray-Black: DAB-Ni.



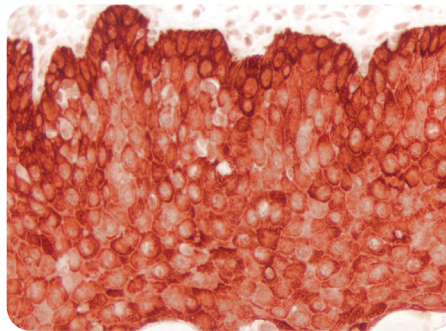
Red: ImmPACT AEC, ImmPACT AMEC Red and AEC.



Purple: ImmPACT VIP and Vector VIP.

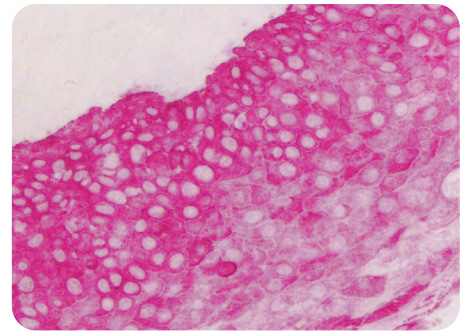


Blue-gray: ImmPACT SG and Vector SG.

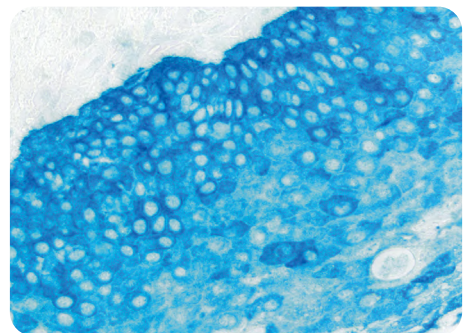


Red: ImmPACT NovaRED and Vector NovaRED.

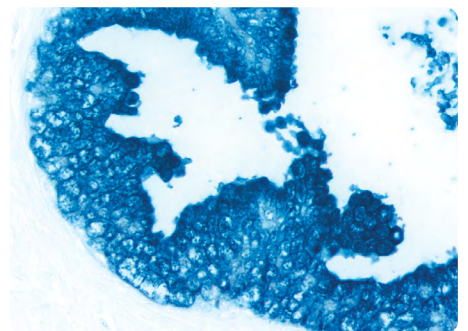
AP Substrates



Magenta: ImmPACT Vector Red and Vector Red.



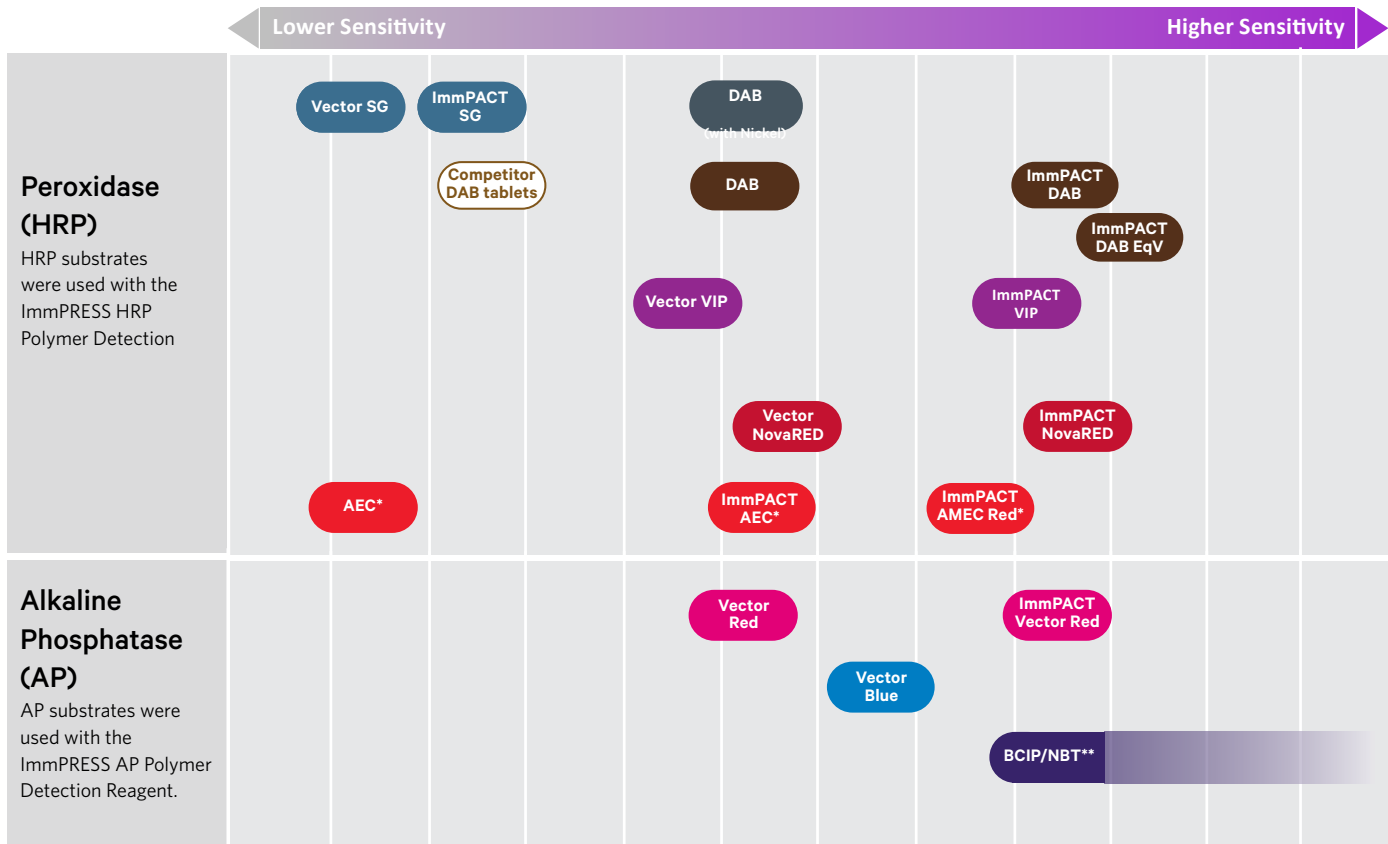
Blue: Vector Blue.



Indigo: BCIP/NBT.

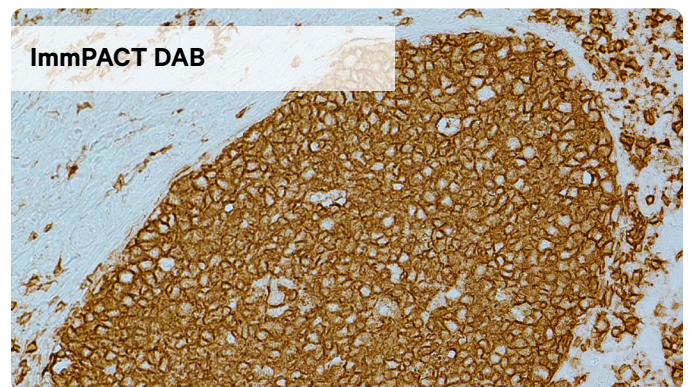
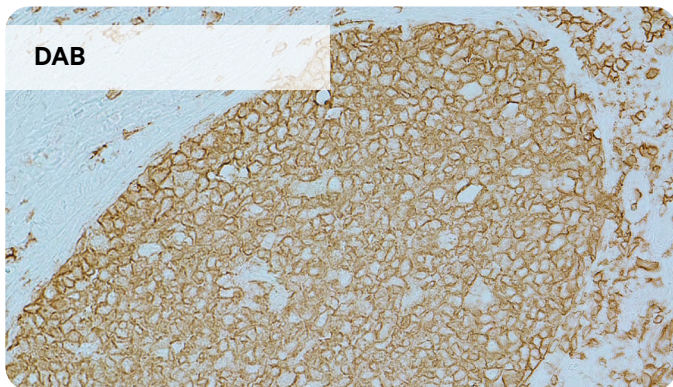
Enzyme Substrate Sensitivity Chart

Not all substrates have equivalent sensitivity and indeed some formulations of the same substrate are more sensitive than others. In staining applications that require maximum sensitivity, it would be recommended to use a more sensitive substrate. Examples of these instances would be where target antigens are weakly expressed, have unknown expression in a disease state or treated specimen,



* AEC & AMEC substrates must be aqueously mounted ** Longer incubation times increase sensitivity

Different sensitivity between two different formulations of DAB



Serial sections of human tonsil stained for CD20 using mouse primary antibody and ImmPRESS anti-mouse HRP polymer kit. The only IHC workflow variable was the different DAB formulation as indicated. The more intense staining of the ImmPACT DAB substrate may allow for detection of weaker expressed antigens, further dilution of the primary antibody, better contrast with surrounding tissue elements and greater clarity of the extent of staining for the target antigen.

Enzyme Substrate Combinations

Recommended Order of Substrates in Double Labeling Protocol

This enzyme substrate combination chart is designed as a reference for optimal multiple labeling, because the order of the two colored precipitates can significantly affect the quality, color, and labeling pattern of each antigen in the stained section. This chart ensures that distinct colors are visible after the labeling reactions are completed using an optimized multiple labeling protocol. See tissue section staining examples using the combinations indicated throughout this brochure.

Second Substrate \ First Substrate		Alkaline Phosphatase			Peroxidase					
		ImmPACT Vector Red & Vector Red (magenta) SK-5105 & SK-5100	Vector Blue (blue) SK-5300	BCIP/NBT (indigo) SK-5400	ImmPACT VIP & Vector VIP (purple) SK-4605 & SK-4600	ImmPACT DAB, ImmPACT DAB EqV & DAB (brown) SK-4105, SK-4103, SK-4100	DAB-Ni (gray-black) SK-4100	ImmPACT NovaRED & Vector NovaRED (red) SK-4805 & SK-4800	ImmPACT SG & SG (blue-gray) SK-4705 & SK-4700	ImmPACT AEC, ImmPACT AMEC Red & AEC (red) SK-4205, SK-4285, SK-4200
Alkaline Phosphatase	ImmPACT Vector Red & Vector Red (magenta) SK-5105 & SK-5100		-	-	-	+	+	-	+	-
	Vector Blue (blue) SK-5300	+		-	+	+	+	+	+	+
	BCIP/NBT (indigo) SK-5400	+	-		+	+	+	+	+	+
Peroxidase	ImmPACT VIP & Vector VIP (purple) SK-4605, SK-4600	-	+	-		+	+	-	+	-
	ImmPACT DAB, ImmPACT DAB EqV & DAB (brown) SK-4105, SK-4103, SK-4100	+	+	+	+		-	-	+	+
	DAB-Ni (gray-black) SK-4100	+	-	-	+	+		+	-	-
	ImmPACT NovaRED & Vector NovaRED (red) SK-4805, SK-4800	-	+	+	-	+	+		+	-
	ImmPACT SG & SG (blue-gray) SK-4705, SK-4700	+	-	-	+	+	-	-		+
	ImmPACT AEC, ImmPACT AMEC Red & AEC (red) SK-4205, SK-4285, SK-4200	-	-	-	-	+	-	-	+	

+ Indicates good contrast

- Indicates incompatibility of substrates for various reasons

Double Label Protocol

Using only ImmPRESS HRP and/or AP Polymer Systems

Staining for First Antigen

1. **Preparation of tissue.** Deparaffinize and rehydrate tissue sections following standard protocols. For frozen sections fix in acetone.
2. Rinse in distilled water for 5 minutes.
3. If endogenous enzyme (HRP or AP) activity is present in the section, inactivate using BLOXALL Blocking Solution.
4. Wash sections 2 X 3 minutes in 10 mM sodium phosphate buffer, pH 7.5, 150 mM NaCl (PBS). (Other buffers may be used).
5. **Protein blocking step.** Incubate sections for 20 minutes with ready-to-use (2.5%) normal serum.
6. **Primary antibody.** Tip off serum. Incubate sections with primary antibody diluted in appropriate antibody diluent [buffer containing diluted (2.5%) NHS].
7. Wash slides for 5 minutes in buffer.
8. **ImmPRESS Reagent.** Incubate sections for 30 minutes with appropriate ImmPRESS Reagent (e.g., anti-mouse IgG, anti-rabbit IgG, etc..).
9. Wash slides for 5 minutes in buffer.
10. **Substrate.** Incubate sections in enzyme substrate solution until optimal color develops.
11. Rinse for 5 minutes in buffer.

Staining for Second Antigen

1. **Protein blocking step.** Incubate sections for 20 minutes with ready-to-use (2.5%) normal serum (NS).
2. **Primary antibody.** Incubate sections with the second primary antibody diluted in appropriate antibody diluent (See step 6 above).
3. Wash for 5 minutes in buffer.
4. **ImmPRESS Reagent.** Incubate sections for 30 minutes with appropriate ImmPRESS Reagent (e.g., anti-mouse IgG, anti-rabbit IgG, etc..).
5. Wash for 5 minutes in buffer.
6. **Substrate.** Incubate sections in second, contrasting enzyme substrate solution until optimal color develops.
7. Wash for 5 minutes in buffer.
8. Counterstain (optional), clear, and mount in appropriate mounting medium.

NOTE: For triple labeling repeat Steps 12-18, after second substrate development.

Fig. 1 Add First Primary Antibody

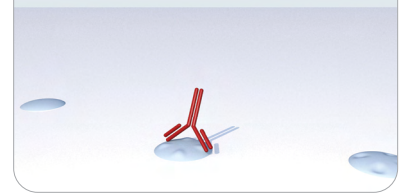


Fig. 2 Add ImmPRESS Reagent

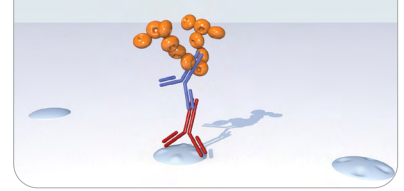


Fig. 3 Add First Enzyme Substrate

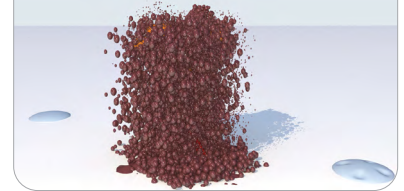


Fig. 4 Add Second Primary Antibody

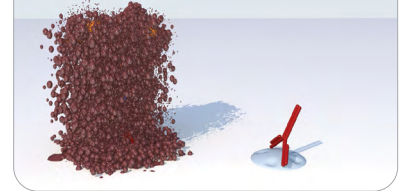


Fig. 5 Add ImmPRESS Reagent

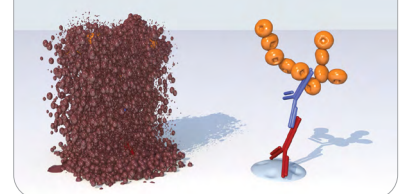
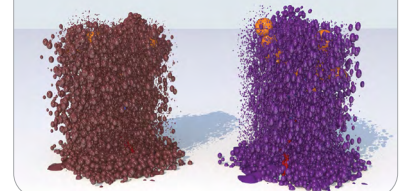
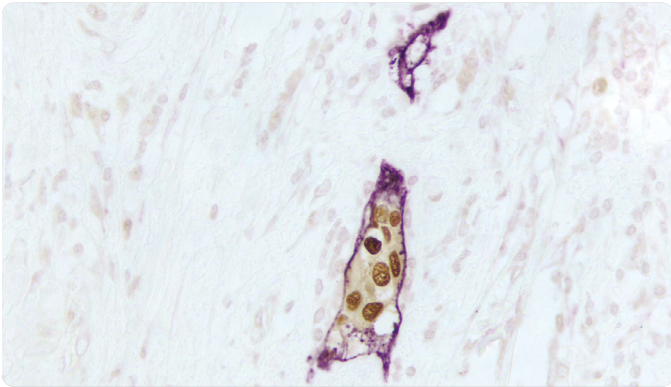


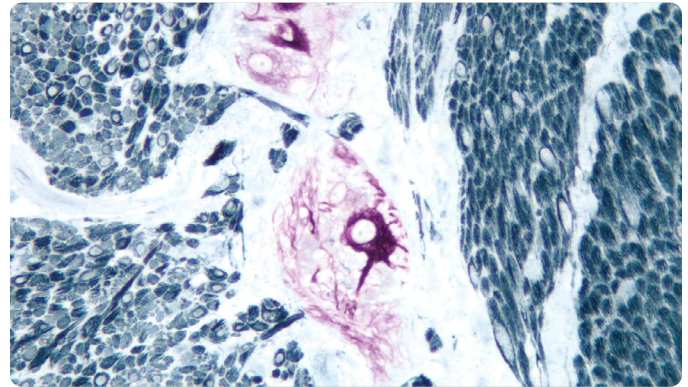
Fig. 6 Add Second Enzyme Substrate



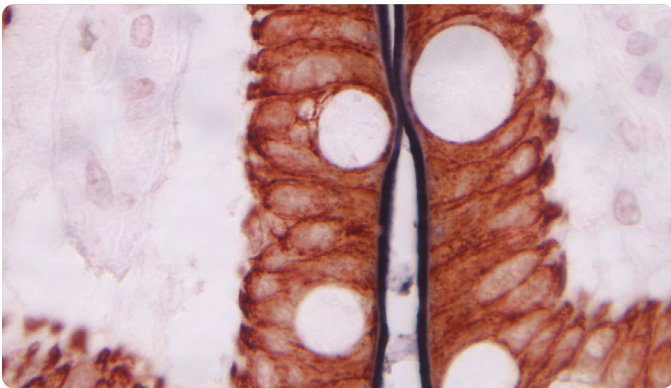
Double label tissue section staining using ImmPRESS HRP Polymer Reagents and combinations of HRP substrates



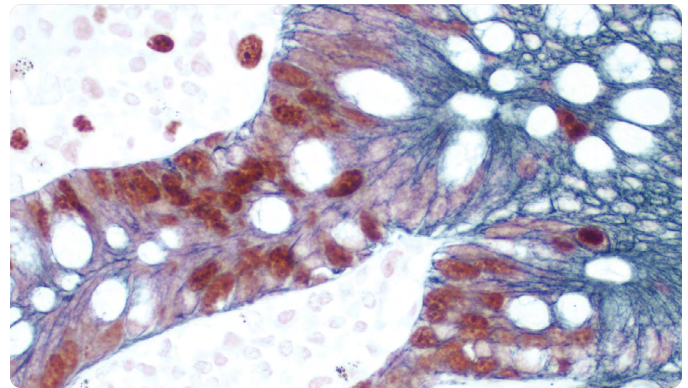
Breast Carcinoma–Double Label • Estrogen Receptor (m), ImmPRESS Universal Reagent, DAB substrate (brown) • M2A Antigen (m), ImmPRESS Universal Reagent, Vector VIP substrate (purple).



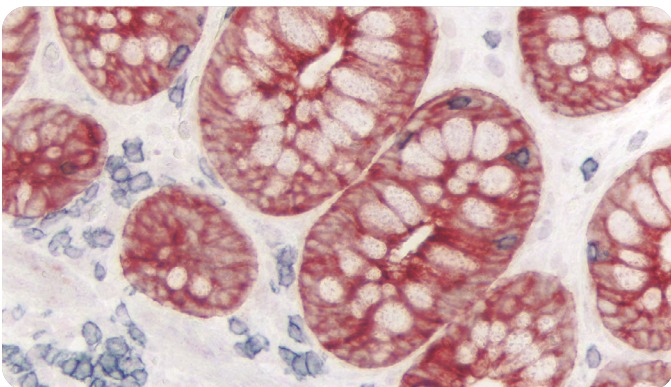
Small Bowel–Double label • Neurofilament 200kD (m), ImmPRESS Anti-Mouse IgG Reagent, Vector VIP substrate (purple) • Desmin (m), ImmPRESS Anti-Mouse IgG Reagent, Vector SG substrate (blue/gray).



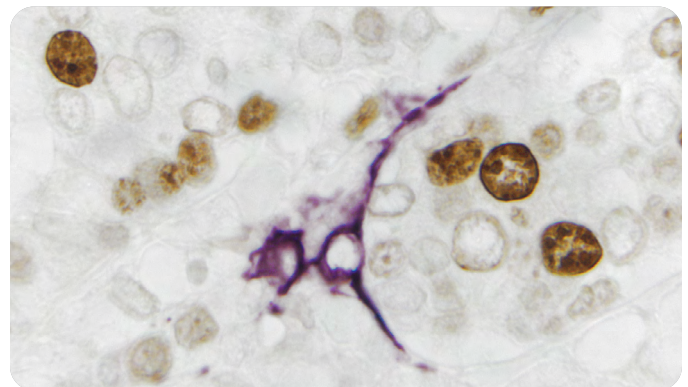
Small Bowel–Double label • CD10 (m), ImmPRESS Anti-Mouse IgG Reagent, DAB+Ni substrate (gray-black) • Cytokeratin 20 (m), ImmPRESS Anti-Mouse IgG Reagent, Vector NovaRED substrate (red).



Small Bowel–Double label • Ki67 (rp), ImmPRESS Universal Reagent, Vector NovaRED substrate (red) • Cytokeratin 8/18 (m), ImmPRESS Universal Reagent, Vector SG substrate (blue-gray).

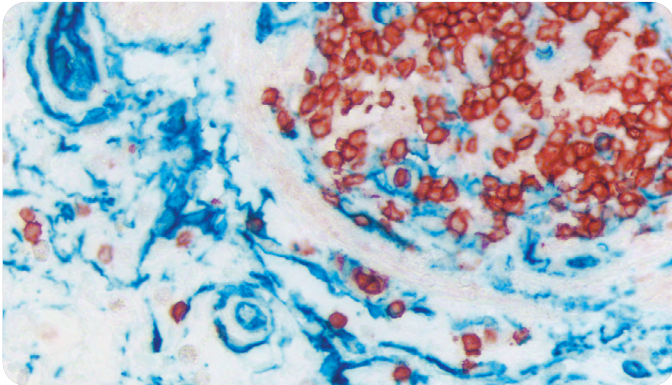


Colon–Double label • CD3 (m), ImmPRESS Anti-Rabbit IgG Reagent, ImmPACT SG (blue-gray) • Cytokeratin AE1/AE3 (m), ImmPRESS Anti-Mouse IgG Reagent, ImmPACT AMEC Red (red).

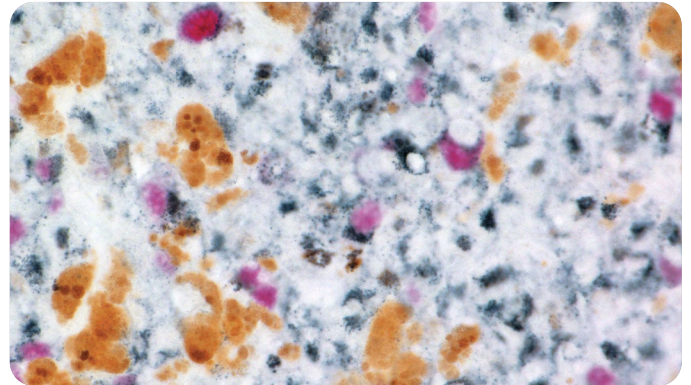


Breast Carcinoma–Double Label • Ki67 (m), ImmPRESS Universal Reagent, DAB substrate (brown) • CD34 (m), ImmPRESS Universal Reagent, Vector VIP substrate (purple).

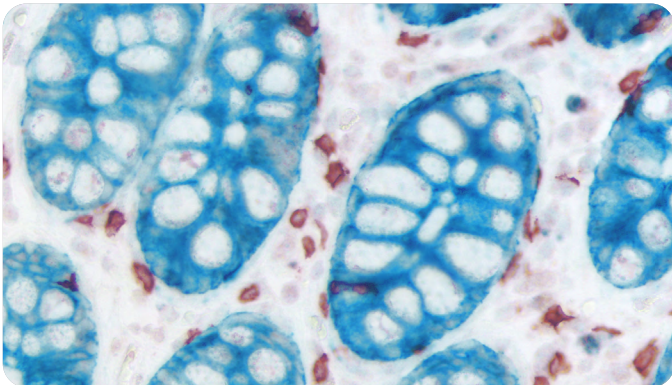
Double label tissue section staining using ImmPRESS HRP with ImmPRESS AP Polymer Reagents and combinations of HRP and AP substrates



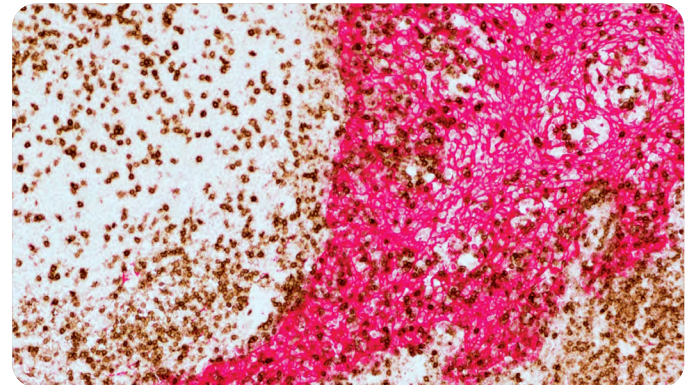
Colon-Double label • CD3 (rm), ImmPRESS Anti-Rabbit IgG Reagent (HRP), ImmPACT AMEC Red • CD34 (m), ImmPRESS AP Anti-Mouse IgG Reagent (AP), Vector Blue (blue).



Melanoma-Double label • Cyclin A (m), ImmPRESS AP Anti-Mouse IgG Reagent (AP), ImmPACT Vector Red AP Substrate (magenta) • Melanoma Marker (m) ImmPRESS Anti-Mouse IgG Reagent (HRP), Vector SG HRP Substrate (gray). Note contrast of double stain with the brown pigments in the tissue.

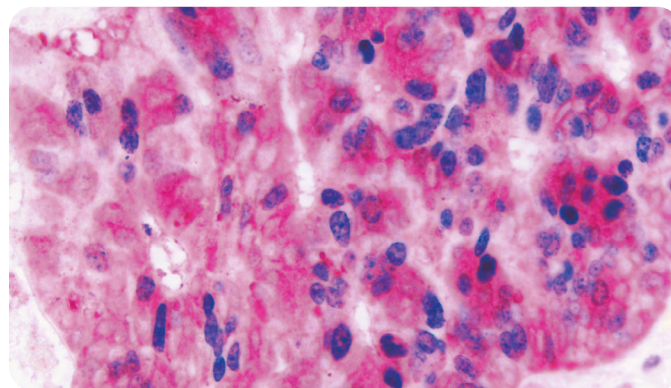


Colon-Double label • Cytokeratin (AE1/AE3, m), ImmPRESS AP Anti-Mouse IgG Reagent, Vector Blue AP Substrate (blue) • CD3 (rb), ImmPRESS Anti-Rabbit IgG HRP Reagent, ImmPACT AMEC Red HRP Substrate (red).



Small Bowel-Double label • Ki67 (rp), ImmPRESS Universal Reagent, Vector NovaRED substrate (red) • Cytokeratin 8/18 (m), ImmPRESS Universal Reagent, Vector SG substrate (blue-gray).

Double label tissue section staining using an ImmPRESS AP Reagent and two different AP substrates



Colon Cancer-Double label: • Ki67 (rm), ImmPRESS AP Anti-Rabbit IgG Reagent, Vector Blue AP substrate (blue) • Cox2 (m), ImmPRESS AP Anti-Rabbit IgG Reagent, ImmPACT Vector Red AP substrate (magenta).

Double Label Protocol

Using only VECTASTAIN HRP and/or AP ABC Systems

Staining for First Antigen

1. **Preparation of tissue.** Deparaffinize and rehydrate tissue sections following standard protocols. For frozen sections fix in acetone.
2. Rinse in distilled water for 5 minutes.
3. If endogenous enzyme (HRP or AP) activity is present in the section, inactivate using BLOXAL Blocking Solution.
4. Wash sections 2 × 3 minutes in 10 mM sodium phosphate buffer, pH 7.5, 150 mM NaCl (PBS). (Other buffers may be used).
5. **Avidin/biotin blocking step.** Perform Avidin/Biotin blocking if required (Avidin/Biotin Blocking Kit).
6. **Protein blocking step.** Incubate sections for 20 minutes with buffer containing 5% NS prepared from the first VECTASTAIN kit, or incubate for 5-10 minutes in 10% NS.
7. **Primary antibody.** Tip off serum and incubate with first primary antibody diluted in 5% NS from the first VECTASTAIN kit using appropriate concentration and length of incubation.
8. Wash 2 × 3 minutes in buffer.
9. **Secondary antibody.** Incubate sections for 30 minutes with biotinylated secondary antibody from the first VECTASTAIN kit diluted in 5% NS. For a 5-10 minute incubation, double the concentration of the biotinylated antibody and normal serum.
10. Wash sections 2 × 3 minutes in buffer.
11. **VECTASTAIN ABC.** Incubate sections for 30 minutes with the first VECTASTAIN ABC Reagent prepared in advance as described in the kit instructions. For a 5-10 minute incubation, use the VECTASTAIN ABC Reagent at twice the recommended concentration.
12. Wash sections 2 × 3 minutes in buffer.
13. **Substrate.** Incubate sections with the appropriate enzyme substrate until optimal color develops. Use the recommended times given in the substrate kit instructions as a guideline.
14. Wash sections 2 × 3 minutes in buffer.

Fig. 1 Add First Primary Antibody

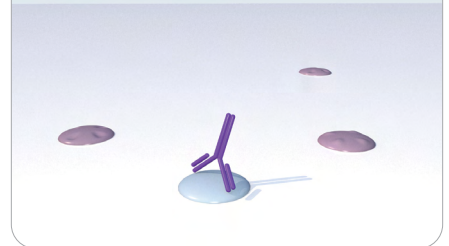


Fig. 2 Add Biotinylated Secondary Antibody

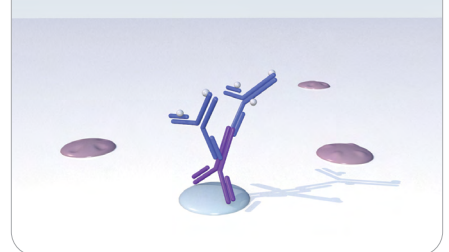


Fig. 3 Add VECTASTAIN ABC

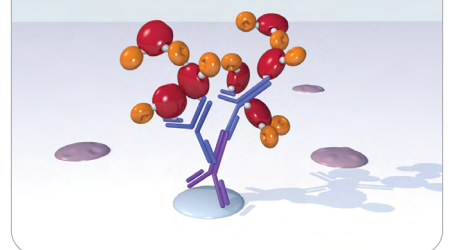
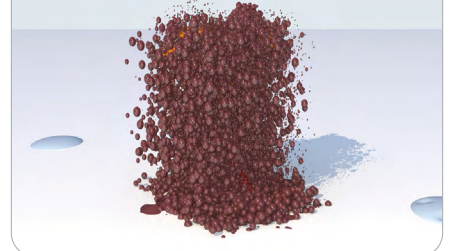


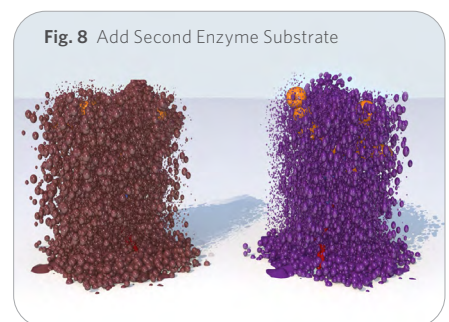
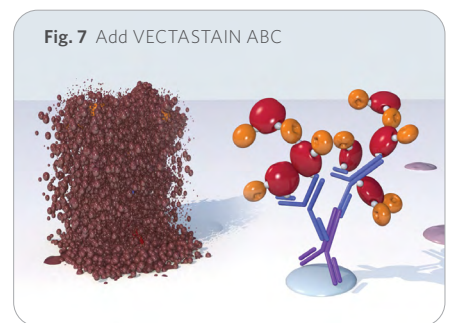
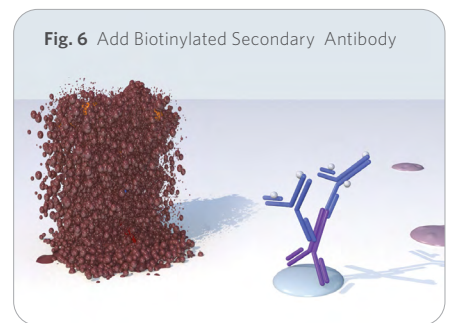
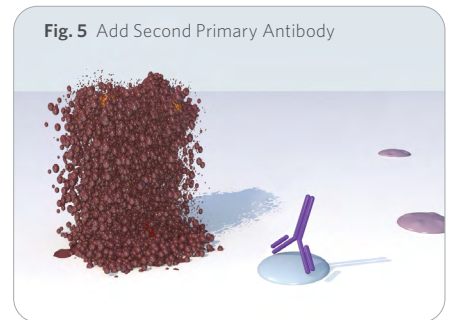
Fig. 4 Add First Enzyme Substrate



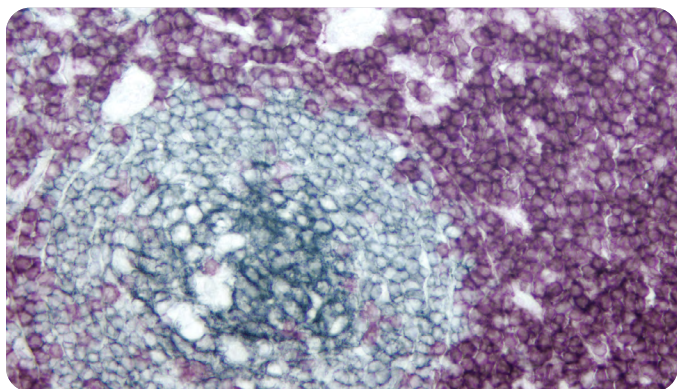
Staining for Second Antigen

15. **Avidin/Biotin blocking step.** Perform Avidin/Biotin blocking step if required. (This step may be necessary to prevent the interaction of the second set of labeling reagents with the first set of labeling reagents).
16. **Protein blocking step.** Incubate sections for 20 minutes with buffer containing 5% NS prepared from the second VECTASTAIN kit, or incubate for 5-10 minutes in 10% NS.
17. **Primary antibody.** Tip off serum and incubate with the second primary antibody diluted in 5% NS from the second VECTASTAIN kit using appropriate concentration and length of incubation.
18. Wash 2 × 3 minutes in buffer.
19. **Secondary antibody.** Incubate sections for 30 minutes with biotinylated secondary antibody appropriate for labeling the second primary antibody diluted in 5% NS. For a 5-10 minute incubation, double the concentration of the biotinylated antibody and normal serum.
20. Wash 2 × 3 minutes in buffer.
21. **VECTASTAIN ABC.** Incubate sections for 30 minutes with the second VECTASTAIN ABC Reagent prepared in advance as described in the kit instructions. For a 5-10 minute incubation, use the second VECTASTAIN ABC Reagent at twice the recommended concentration.
22. Wash sections 2 × 3 minutes in buffer.
23. **Substrate.** Incubate sections with the appropriate second, contrasting enzyme substrate until optimal color develops. Use the recommended times given in the substrate kit instructions as a guideline.
24. Wash sections in tap water for 5 minutes.
25. Counterstain (optional), clear, and mount in appropriate mounting medium.

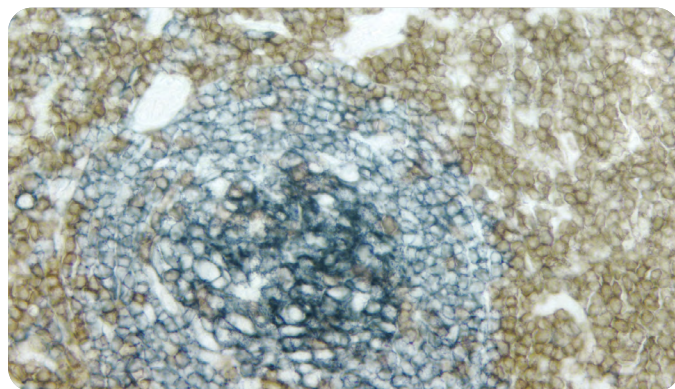
NOTE: For triple labeling repeat Steps 15-23, after second substrate development. See triple staining images on page 12.



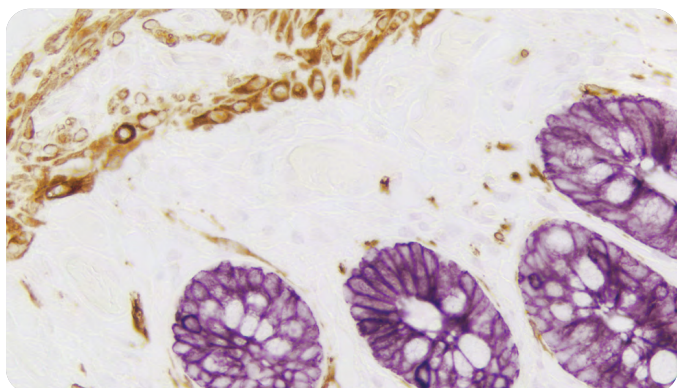
Double label tissue section staining using VECTASTAIN Elite ABC HRP Kits and combinations of HRP substrates



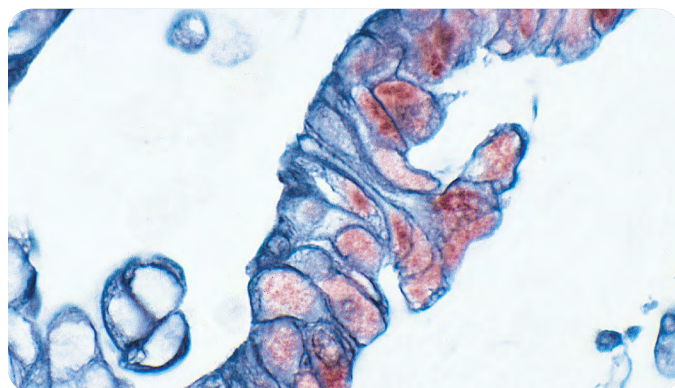
Tonsil-Double label • CD3 (m), VECTASTAIN Universal Elite ABC Kit, Vector VIP substrate (purple) • CD20 (m), VECTASTAIN Universal Elite ABC Kit, Vector SG substrate (blue-gray).



Tonsil-Double label • CD3 (m), VECTASTAIN Universal Elite ABC Kit, DAB substrate (brown) • CD20 (m), VECTASTAIN Universal Elite ABC Kit, Vector SG substrate (blue-gray).

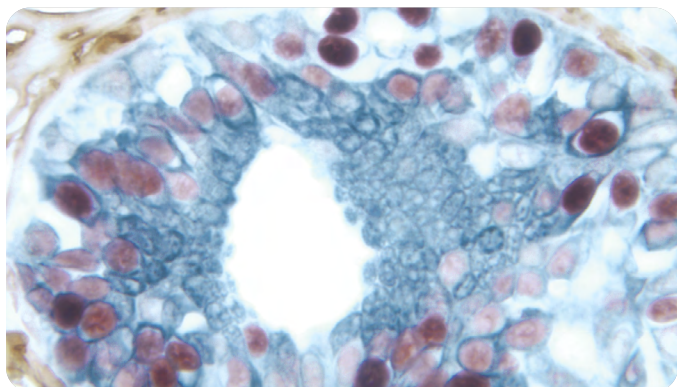


Intestine-Double label: • Desmin (m), VECTASTAIN Elite ABC Kit, ImmPACT DAB substrate (brown) • Cytokeratin (m), VECTASTAIN Elite ABC Kit, Vector VIP substrate (purple).

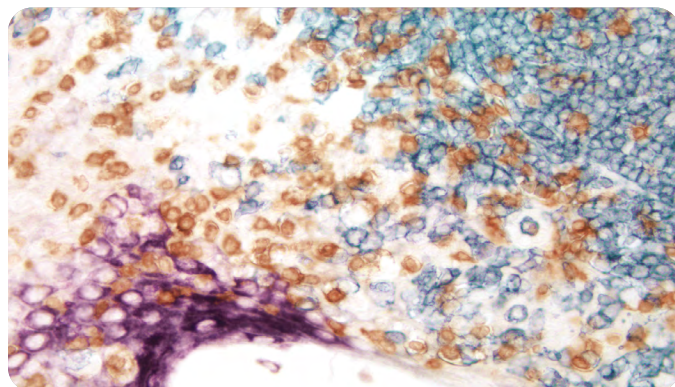


Tumor-Double label • p53 (m), VECTASTAIN Elite ABC Kit, Vector NovaRED substrate (red) • Cytokeratin (s), VECTASTAIN Elite ABC Kit, Vector SG substrate (blue-gray).

Triple label tissue section staining using VECTASTAIN Elite ABC HRP Kits and three different HRP substrates

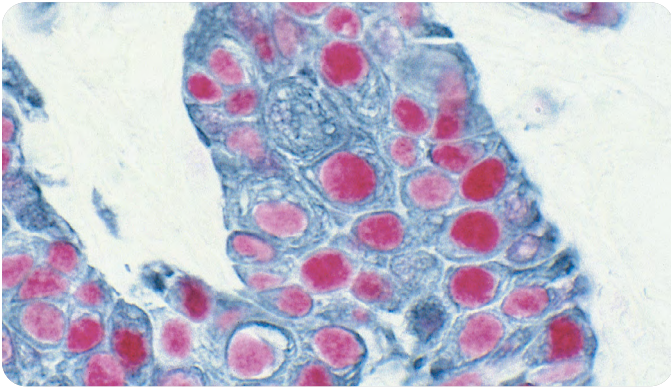


Breast Carcinoma-Triple label • Estrogen Receptor (m); VECTASTAIN Elite ABC Kit, Vector NovaRED substrate (red) • CD34 (m), VECTASTAIN Elite ABC Kit, DAB substrate (brown) • Cytokeratin 8/18 (m), VECTASTAIN Elite ABC Kit, Vector SG substrate (blue-gray).

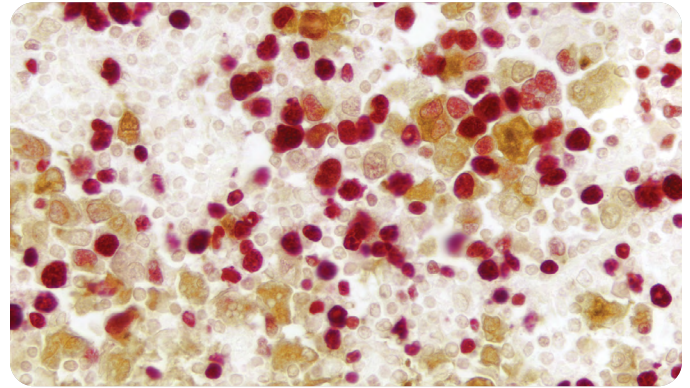


Tonsil-Triple label • CD3 (m), VECTASTAIN Universal Elite ABC Kit, DAB substrate (brown) • CD20 (m), VECTASTAIN Universal Elite ABC Kit, Vector SG substrate (blue-gray) • Multi-Cytokeratin (m), VECTASTAIN Universal Elite ABC Kit, Vector VIP substrate (purple).

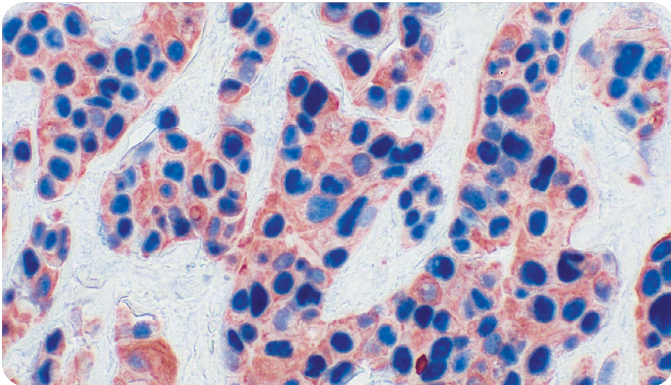
Double label tissue section staining using VECTASTAIN ABC HRP Kits with VECTASTAIN ABC AP Kits and combinations of HRP and AP substrates



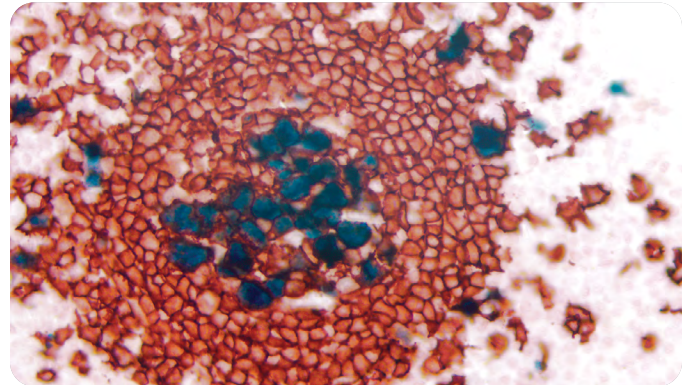
Tumor-Double label • p53 protein (m), VECTASTAIN ABC-AP Kit, Vector Red substrate (red) • Pan-Cytokeratin (sheep), VECTASTAIN Elite ABC Kit, Vector SG substrate (blue/gray).



Lymph Node-Double label • Ki67 (m), VECTASTAIN Universal ABC-AP Kit, Vector Red substrate (red) • Multi-cytokeratin (m), VECTASTAIN Universal Elite ABC Kit, DAB substrate (brown).

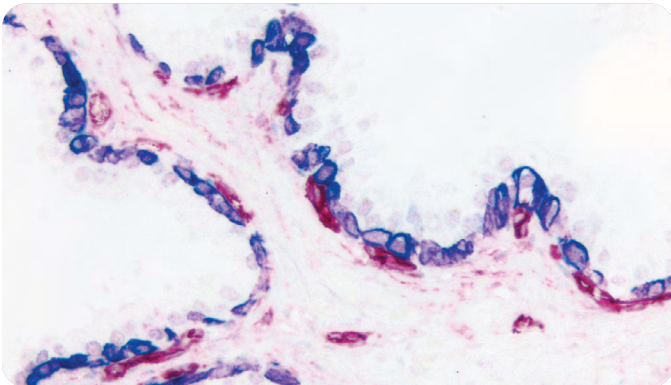


Tumor-Double label: • p53 (m), VECTASTAIN ABC-AP Kit, Vector Blue substrate (blue) • Multi-Cytokeratin (m), VECTASTAIN Elite ABC Kit, AEC substrate (red).

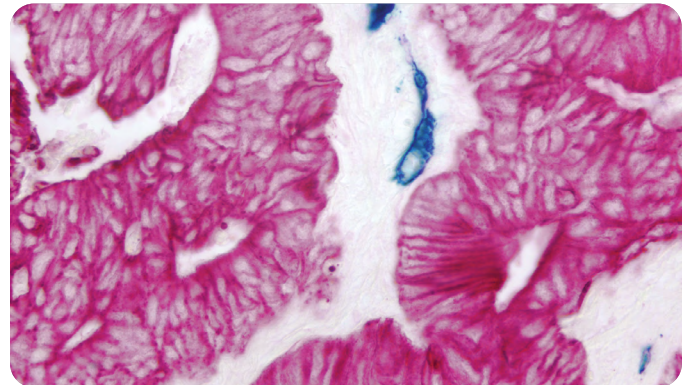


Tonsil-Double label • Cyclin A (m), VECTASTAIN Universal ABC-AP Kit, Vector Blue substrate (blue) • CD20 (m), VECTASTAIN Universal Elite ABC Kit, Vector NovaRED substrate (red).

Double label tissue section staining using VECTASTAIN Universal ABC AP Kits and two different AP substrates.



Prostate-Double label • Cytokeratin 5 (m), VECTASTAIN Universal ABC-AP Kit, Vector Blue substrate (blue) • CD34 (m), VECTASTAIN Universal ABC-AP Kit, Vector Red substrate (red).



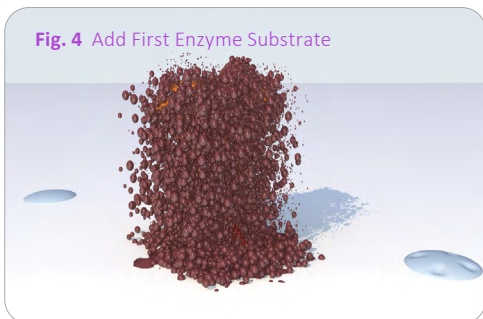
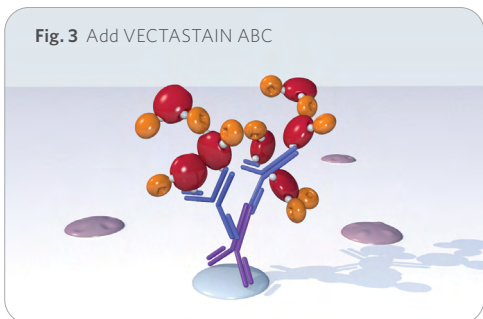
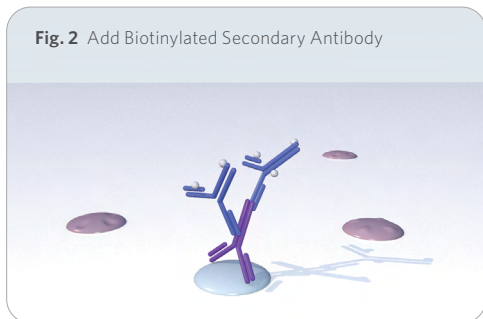
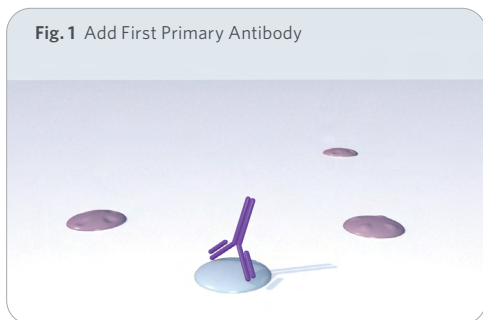
Tumor-Double label • CD34 (m), VECTASTAIN Universal ABC-AP Kit, Vector Blue substrate (blue) • Cytokeratin 8/18 (m), VECTASTAIN Universal ABC-AP Kit, Vector Red substrate (red).

Double Label Protocol

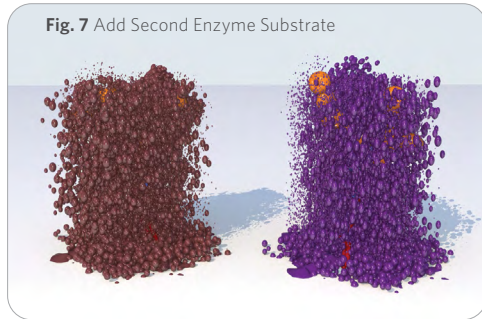
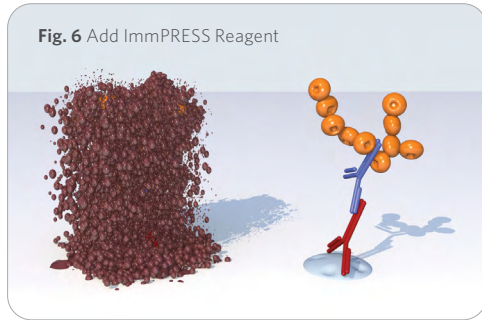
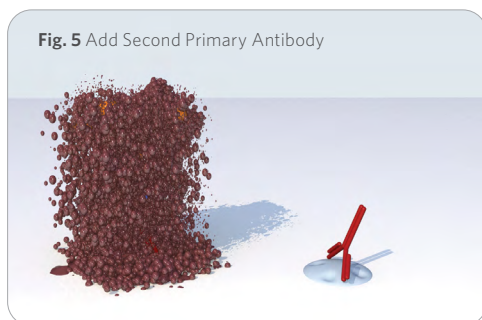
Using VECTASTAIN ABC System combined with the ImmPRESS Polymer System

For labs that are looking to combine both a VECTASTAIN ABC System with an ImmPRESS Polymer System to achieve double labeling, we have provided a recommended approach in the graphic below. Essentially the two labels are run sequentially, one after the other, for maximum reproducibility and avoid potential cross-reactivity between the reagents of the two detection systems.

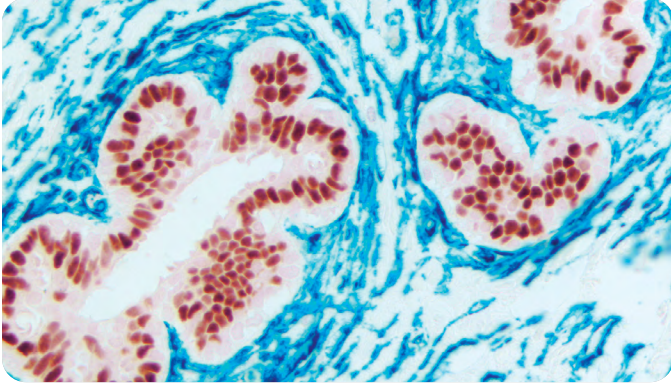
Staining for first antigen using VECTASTAIN ABC System (see page 10).



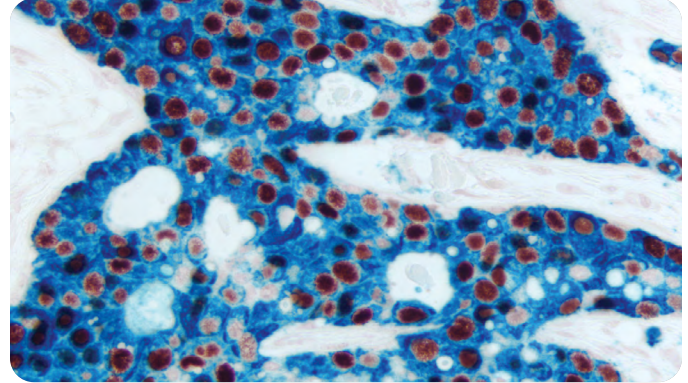
Staining for second antigen using ImmPRESS Polymer System (see page 7).



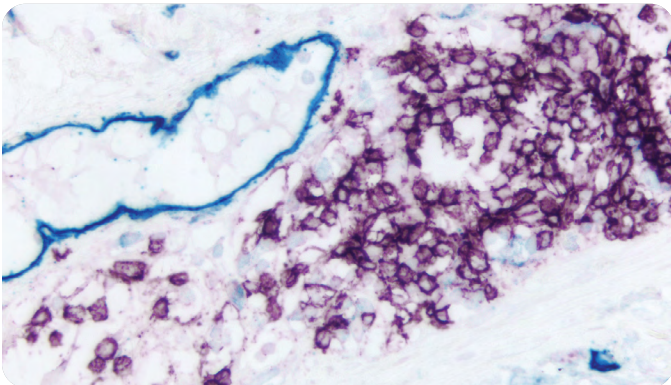
Double label tissue section staining using VECTASTAIN ABC AP Kits with ImmPRESS HRP Polymer Reagents and combinations of HRP and AP substrates.



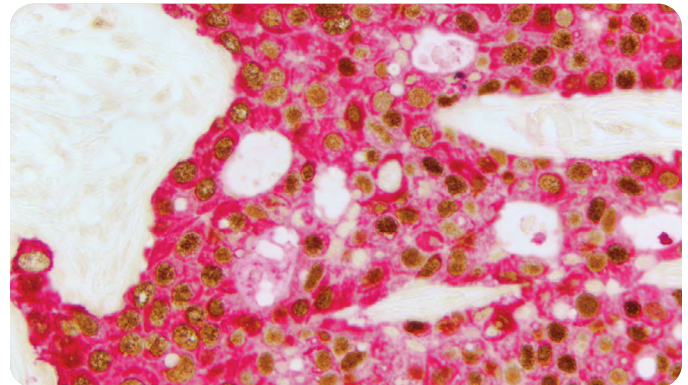
Breast Carcinoma–Double label • Estrogen Receptor (m), ImmPRESS Universal HRP Reagent, Vector NovaRED HRP substrate (red) • CD34 (m), VECTASTAIN Universal ABC-AP Kit, Vector Blue AP substrate (blue).



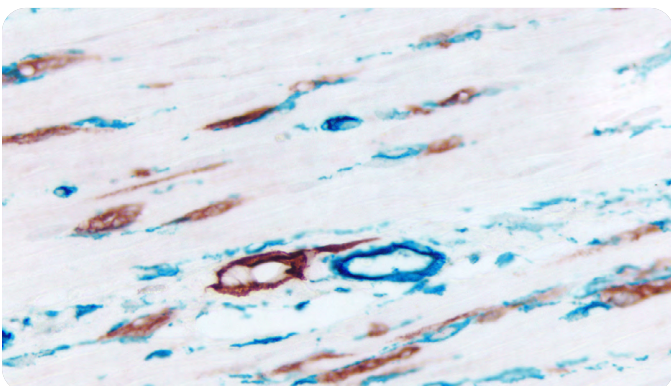
Breast Carcinoma–Double label • Estrogen Receptor (m), ImmPRESS Universal HRP Reagent, Vector NovaRED HRP substrate (red). • Cytokeratin 8/18 (m), VECTASTAIN Universal ABC-AP Kit, Vector Blue AP substrate (blue).



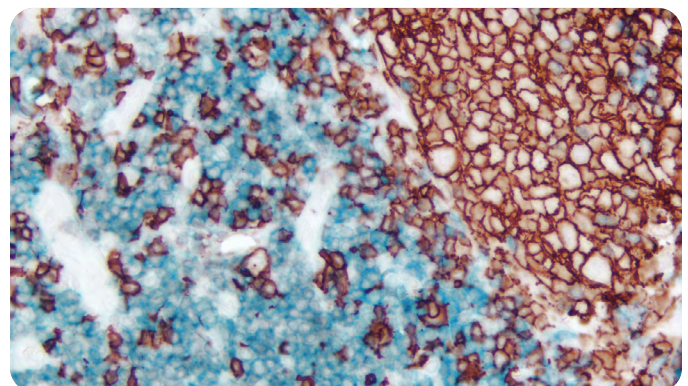
Colon–Double label • M2A antigen (m), VECTASTAIN Universal ABC-AP Kit, Vector Blue AP substrate (blue) • CD20 (m), ImmPRESS Universal HRP Reagent, Vector VIP HRP substrate (purple).



Breast Carcinoma –Double label • Estrogen Receptor (m), ImmPRESS Universal HRP Reagent, DAB HRP substrate (brown) • Cytokeratin AE1/AE3 (m), VECTASTAIN Universal ABC-AP Kit, Vector Red AP substrate (magenta).



Colon–Double label • M2A Antigen (m), ImmPRESS Universal HRP Reagent, Vector NovaRED HRP substrate (red) • CD34 (m), VECTASTAIN Universal ABC-AP Kit, Vector Blue AP substrate (blue).



Tonsil–Double label • CD3 (m), VECTASTAIN Universal ABC-AP Kit, Vector Blue AP substrate (blue) • CD20 (m), ImmPRESS Universal Reagent, Vector NovaRED HRP substrate (red).

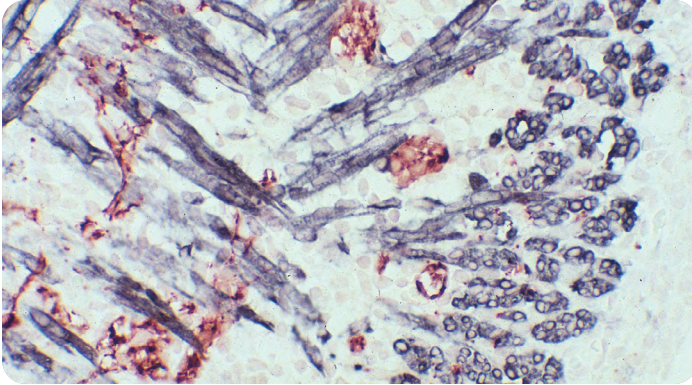
Double labeling on mouse tissue using one or more mouse primary antibodies

Background free visualization of target antigens in mouse tissue using a mouse primary antibody in a multiple label staining application requires a specialized methodology. The problem arises when an anti-mouse IgG secondary detection reagent is applied to detect the mouse IgG primary antibody and fails to distinguish between the primary antibody and endogenous mouse IgG in the specimen.

Several approaches have been used to circumvent this problem. One method is to conjugate the mouse primary antibody directly with an enzyme such as HRP, or biotin or another hapten. This avoids the need for using an anti-mouse IgG secondary detection system. The shortcomings of this approach however include potential changes in binding characteristics of the primary antibody to the antigen, leading to off-target binding, and a low level of sensitivity which is problematic when detecting weakly expressed antigens.

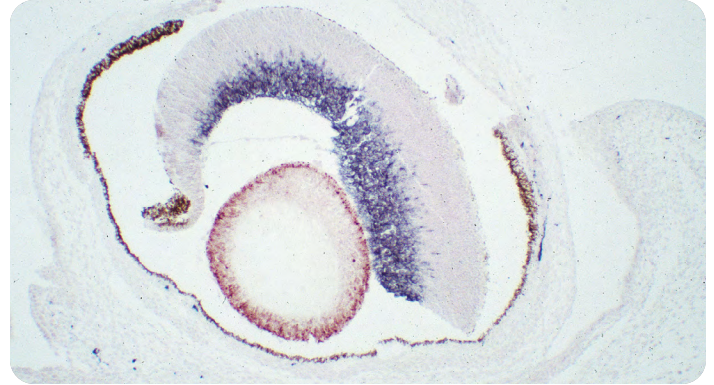
Use of a M.O.M. (mouse on mouse) detection kit does not involve conjugation of the primary antibody and allows for higher levels of sensitivity. These kits include a defined mouse Ig blocking reagent that binds to endogenous mouse Ig, preventing subsequent binding by the anti-mouse IgG secondary detection system. Presented below are two images showing examples using the M.O.M. Peroxidase Kit for double labeling.

The double label procedure used to generate the images shown below is provided on the next page. In the situation where only one of the primary antibodies being applied to the mouse specimen is raised in mouse, then use of the M.O.M. reagents would be recommended for that one label. The end-user should determine if better staining results are achieved applying the M.O.M. reagents first, or as the second detection system in a double label methodology.



Mouse, Newborn (tongue)–Double label

- Synapsin (m), M.O.M. Peroxidase Kit, Vector NovaRED HRP substrate (red).
- Desmin (m), M.O.M. Peroxidase Kit, DAB+Ni HRP substrate (gray/black).



Mouse, Newborn (eye)–Double label

- GFAP (m), M.O.M. Peroxidase Kit, Vector NovaRED HRP substrate (red).
 - Synapsin (m), M.O.M. Peroxidase Kit, DAB+Ni HRP substrate (gray/black).
- Note contrast with endogenous pigment (brown) seen in surrounding tissues.*

Staining for First Mouse Antigen

1. **Preparation of tissue.** Deparaffinize and rehydrate tissue sections following standard protocols.
2. Rinse in distilled water for 5 minutes.
3. If endogenous peroxidase activity is present in the section, inactivate using BLOXALL Blocking Solution.
4. Wash sections 2 × 3 minutes in PBS. (Other buffers may be used).
5. **Avidin/biotin blocking step.** Perform avidin/biotin blocking if required (Avidin/Biotin Blocking Kit).
6. **Mouse Ig blocking step.** Incubate tissue sections for 1 hour in working solution of M.O.M. Mouse Ig Blocking Reagent.
7. Wash sections 2 × 2 minutes in buffer.
8. **Protein blocking step.** Incubate tissue sections for 5 minutes in working solution of M.O.M. diluent.
9. **Primary antibody.** Tip off excess M.O.M. diluent from sections. Dilute primary antibody in M.O.M. diluent to the appropriate concentration. Incubate section in diluted primary antibody for 30 minutes (See Note 1 below).
10. Wash sections 2 × 2 minutes in buffer.
11. **Secondary antibody.** Apply working solution of M.O.M. Biotinylated Anti-Mouse IgG Reagent. Incubate sections for 10 minutes.
12. Wash sections 2 × 2 minutes in buffer
13. **VECTASTAIN ABC.** Incubate sections for 30 minutes with VECTASTAIN ABC Reagent prepared in advance as described in the kit instructions.
14. Wash sections 2 × 3 minutes in buffer.
15. **Substrate.** Incubate sections with the appropriate first enzyme substrate until optimal color develops.
16. Wash sections 2 × 3 minutes in buffer.

Staining for Second Mouse Antigen

17. **Avidin/biotin blocking step.** Perform avidin/biotin blocking step, if required. (This step may be necessary to prevent the interaction of the second set of labeling reagents with the first set of labeling reagents.)
18. **Mouse Ig blocking step.** Incubate tissue sections for 1 hour in working solution of M.O.M. Mouse Ig Blocking Reagent.
19. Wash sections 2 × 2 minutes in buffer.
20. **Protein blocking step.** Incubate tissue sections for 5 minutes in working solution of M.O.M. diluent.
21. **Primary antibody.** Tip off excess M.O.M. diluent from sections. Dilute primary antibody in M.O.M. diluent to the appropriate concentration. Incubate section in diluted primary antibody for 30 minutes
22. Wash 2 × 3 minutes in buffer.
23. **Secondary antibody.** Apply working solution of M.O.M. Biotinylated Anti-Mouse IgG Reagent. Incubate sections for 10 minutes.
24. Wash sections 2 × 2 minutes in buffer.
25. **VECTASTAIN ABC.** Incubate sections for 30 minutes with VECTASTAIN ABC Reagent prepared in advance as described in the kit instructions.
26. Wash sections 2 × 3 minutes in buffer.
27. **Substrate.** Incubate sections with the appropriate second, contrasting enzyme substrate until optimal color develops.
28. Wash sections in tap water for 5 minutes.
29. Counterstain, clear (optional), and mount in appropriate mounting medium.

Notes:

1. Optimal results with the M.O.M. Kit are usually obtained with a primary antibody incubation of 30 minutes. Primary antibody concentrations should be optimized for multiple labeling applications.
2. M.O.M. ImmPRESS HRP Polymer Kit is also available. Use of this kit would reduce the number of steps required, retain high sensitivity, and omit the need for using an avidin/biotin blocking step.

General Notes and Considerations

The procedures and images presented in this brochure were developed on formalin-fixed, paraffin-embedded specimens that were cut at <10 um with sections subsequently adhered to glass slides. Unless otherwise noted, staining was performed at room temperature (20–25 degrees Celsius) under standard laboratory lighting conditions. The same procedural parameters would be pertinent to fresh frozen, acetone-fixed, thin-cut specimens. However, thicker preparations such as wholemounts (e.g., embryos) or free-floating sections (e.g., CNS specimens) would require modifications to the incubation times, frequency and duration of buffer washes, use of permeabilization reagents (i.e. detergents) and possible variations with incubation temperature.

Order of Antigen Labeling

- Once the detection systems and substrates have been selected, it is recommended to optimize each label on separate tissue sections, as a single staining procedure. Optimization would include any pretreatments such as high temperature antigen unmasking, primary antibody dilution and incubation times. Once established, the same conditions should be used in the multiple label procedure. Note that any pretreatments need to be compatible for staining all subsequent target antigens in the procedure.
- In general, weaker expressed antigens should be detected first using the most sensitive detection method. Ensure compatibility of the substrates in the order of their application (see chart on page 6). In some cases, reversing the order of antigen staining may generate better results.

Controls

- Use sections stained with the optimized single label conditions initially established as positive controls to compare with staining results for the same antigens in the double label procedure.
- Defined deletion controls should be run to ensure absence of cross-reactivity between the labels. The first antigen should be labeled to completion with the first substrate. In each control section, the second primary antibody and second detection system should be omitted. This series of controls determines whether any staining is due to reagents of the second stain binding to elements of the first stain. This control series would also be crucial if the two labels use the same enzyme detection system.
- A further negative control would be necessary when primary antibodies from the same species are used in a multiple label application. The second primary antibody should be substituted with an irrelevant antibody or non-immune IgG of the same species and at the same concentration. This will determine if any staining is due to binding of the second primary antibody by the first secondary antibody.

Background Interference

- Ensure appropriate blocking steps have been incorporated into the workflow. Use BLOXALL Blocking Solution to quench endogenous enzyme activity and include an avidin/biotin blocking step before and/or between labels if using a VECTASTAIN ABC System.
- Nonspecific binding to tissue elements can be reduced or eliminated with increased serum concentrations as well as the use of a detergent such as Tween 20 (0.1% to 0.5%) in wash buffers and antibody diluents.
- If the second or third labeling reagents are cross-reacting with blocking serum used in the preceding steps (i.e. first label) then substitute the serum with R.T.U. Animal Free Blocker and Diluent. The R.T.U. Animal Free Blocker and Diluent is a universal reagent that can be used with both HRP and AP based ImmPRESS polymer and VECTASTAIN ABC Systems.

Frequently Asked Questions Regarding Double/Triple Labeling

1. Can I mix the primary antibodies together and detect them separately instead of running the complete IHC assays sequentially?

Yes, but only if the primary antibodies are raised in different species such as mouse and rabbit. This will allow for specific detection of each primary antibody by the corresponding secondary detection system. Initially though, ensure that each label has been developed with the same detection system and enzyme substrate and optimized as a single label first. Also, when applying the primary antibodies together, make sure the working concentrations of each match what was established in the single label validated procedure.

2. Can I use the same secondary detection reagent (ImmPRESS polymer or VECTASTAIN ABC) to detect two primary antibodies raised in the same species?

Yes. Indeed, there are several examples of this exact application in some of the images in this brochure. The two labels must be run sequentially (one after the other) from incubation of primary antibody right through to enzyme substrate development.

3. If my specimen is mouse tissue and one of my primary antibodies is raised in rat and the other in rabbit, what would you recommend I use for my secondary detection reagents?

The detection of a rat IgG primary antibody on mouse tissue does require more than a standard anti-rat IgG detection reagent. We would suggest using a specific anti-rat IgG, mouse adsorbed secondary reagent such as ImmPRESS anti-rat (mouse adsorbed) HRP or AP Polymer kits. Alternatively, you could use a biotinylated anti-rat IgG, mouse adsorbed secondary antibody combined with a VECTASTAIN ABC kit. Detection of the rabbit primary antibody would be more straightforward using either an ImmPRESS anti-rabbit IgG Polymer Reagent or VECTASTAIN ABC anti-rabbit IgG Kit. The choice of using either HRP or AP detection systems and enzyme substrates would be something you would decide using the tools provided in this brochure.

4. If I am performing double labeling with two HRP detection systems, do I need to quench the HRP enzyme from the first label before applying the HRP substrate for the second label?

Before undertaking a double or triple staining application, each label should be optimized as single label on separate tissue sections. Once these conditions have been established (primary antibody concentration, incubations times etc.), run the double or triple labeling sequentially. If conditions for the single label have been optimized appropriately, there is no need to perform an enzyme quench step after the development of each substrate in a double or triple label application.

5. Both of the target antigens in my specimen are co-expressed in the same cell compartment of the same cell type. Can I still use enzyme based IHC to visualize them?

There are studies in scientific literature that describe co-localization of antigens using standard IHC staining methods. These studies involved careful choice of enzyme system, enzyme substrate, order of staining. The reproducibility was dependent on the extent of co-localization. The methods presented in this brochure are largely limited to visualization of two or more antigens expressed in different parts of the same cell type or different cells in the same specimen. This is evident in all of the images presented in this brochure. If the antigens are co-localized in the structure or cell compartment of interest, then your options would be to explore more complicated IHC methods involving rounds of staining and de-staining or move to an immunofluorescence methodology.

6. At what point during the double label procedure is a good place to stop or interrupt the staining?

If the double label staining protocol must be interrupted, we recommend stopping after substrate development of the first antigen. Sections usually can be kept in buffer in the refrigerator until staining is resumed. Ensure that sections be kept moist and under conditions that will preserve antigens during any pause in the staining protocol.

Detection Systems—Product Selection Tables

VECTASTAIN® ABC Systems

Product	Elite®-Peroxidase (HRP)	Elite®-Peroxidase PLUS* (HRP)	Original- Peroxidase (HRP)	Quick-Peroxidase (HRP)	Alkaline Phosphatase (AP)
VECTASTAIN® ABC Kit, Standard	PK-6100		PK-4000		AK-5000
VECTASTAIN® ABC Kit, Rabbit IgG	PK-6101		PK-4001		AK-5001
VECTASTAIN® ABC Kit, Mouse IgG	PK-6102		PK-4002		AK-5002
VECTASTAIN® ABC Kit, Mouse IgM			PK-4010		
VECTASTAIN® ABC Kit, Human IgG	PK-6103				
VECTASTAIN® ABC Kit, Rat IgG	PK-6104		PK-4004		AK-5004
VECTASTAIN® ABC Kit, Goat IgG	PK-6105		PK-4005		
VECTASTAIN® ABC Kit, Sheep IgG	PK-6106				
VECTASTAIN® ABC Kit, Guinea Pig IgG			PK-4007		
VECTASTAIN® ABC Kit, Universal	PK-6200	PK-8200			AK-5200
VECTASTAIN® ABC Reagent, R.T.U.	PK-7100				
VECTASTAIN® ABC Kit, R.T.U., Universal	PK-7200				
VECTASTAIN® Universal Quick Kit, R.T.U.				PK-7800	
VECTASTAIN® Universal Quick Kit (Concentrate)				PK-8800	

* BLOXALL Blocking Solution and enzyme substrate included.

ImmPRESS® Polymer Systems

Product	Peroxidase (HRP)	Peroxidase PLUS* (HRP)	Veterinary Reagents (VR)-Peroxidase (HRP)	Excel Amplified* Peroxidase (HRP)	Alkaline Phosphatase (AP)	Duet Double Staining* (HRP & AP)
ImmPRESS® Anti-Rabbit IgG Kit (made in horse)	MP-7401	MP-7801	MP-6401	MP-7601	MP-5401	
ImmPRESS® Anti-Rabbit IgG Kit (made in goat)	MP-7451					
ImmPRESS® Anti-Mouse IgG Kit (made in horse)	MP-7402	MP-7802	MP-6402	MP-7602	MP-5402	
ImmPRESS® Anti-Mouse IgG Kit (made in goat)	MP-7452					
ImmPRESS® Anti-Mouse IgG, Rat Adsorbed, Kit (made in horse)	MP-7422					
ImmPRESS® Anti-Rat IgG Kit (made in goat)	MP-7404				MP-5404	
ImmPRESS® Anti-Rat IgG, Mouse Adsorbed, Kit (made in goat)	MP-7444				MP-5444	
ImmPRESS® Anti-Goat IgG Kit (made in horse)	MP-7405				MP-5405	
ImmPRESS® Universal Antibody Kit Anti-Rabbit/Mouse Kit (made in horse)	MP-7500	MP-7800				
ImmPRESS® Duet Anti-Rabbit (HRP, Brown) Anti-Mouse (AP, magenta)						MP-7714
ImmPRESS® Duet Anti-Mouse (HRP, Brown) Anti-Rabbit (AP, magenta)						MP-7724

* BLOXALL Blocking Solution and enzyme substrate included.

M.O.M.® (Mouse on Mouse) Kits

Product	Catalog Number
M.O.M.® Elite® Immunodetection Kit, Peroxidase	PK-2200
M.O.M.® Immunodetection Kit, Fluorescein†	FMK-2201
M.O.M.® Immunodetection Kit, Basic	BMK-2202
M.O.M.® ImmPRESS® HRP Polymer Kit	MP-2400
M.O.M.® Blocking Reagent	MKB-2213
M.O.M.® Biotinylated Anti-Mouse IgG Reagent*	MKB-2225
M.O.M.® ImmPRESS® Polymer Reagent, Anti-Mouse IgG, HRP	MPX-2402

† This is a fluorescent-based kit not intended for enzyme-based multiple antigen labeling.

* This reagent must be used with the M.O.M.® Mouse Ig Blocking Reagent (MKB-2213) and is not intended to be a stand-alone reagent for mouse on mouse applications.

Related Products

Product	Catalog Number
Antigen Unmasking Solution, Citrate-based, pH 6.0	H-3300
Antigen Unmasking Solution, Citrate-based, pH 9.0	H-3301
Avidin/Biotin Blocking Kit	SP-2001
BLOXALL® Blocking Solution	SP-6000
ImmEdge® Hydrophobic Barrier Pap Pen	H-4000
Normal Goat Serum Blocking Solution, 2.5%	S-1012
Normal Horse Serum Blocking Solution, 2.5%	S-2012
Normal Sera (Goat, Horse, Chicken, Swine, Rabbit)	S-1000, S-2000, S-3000, S-4000 S-5000
Animal-Free® Blocker and Diluent, R.T.U.	SP-5035
VectaMount® Express Mounting Medium	H-5700
VectaMount® Permanent Mounting Medium	H-5000
VectaMount® AQ Aqueous Mounting Medium	H-5501

Contact Details

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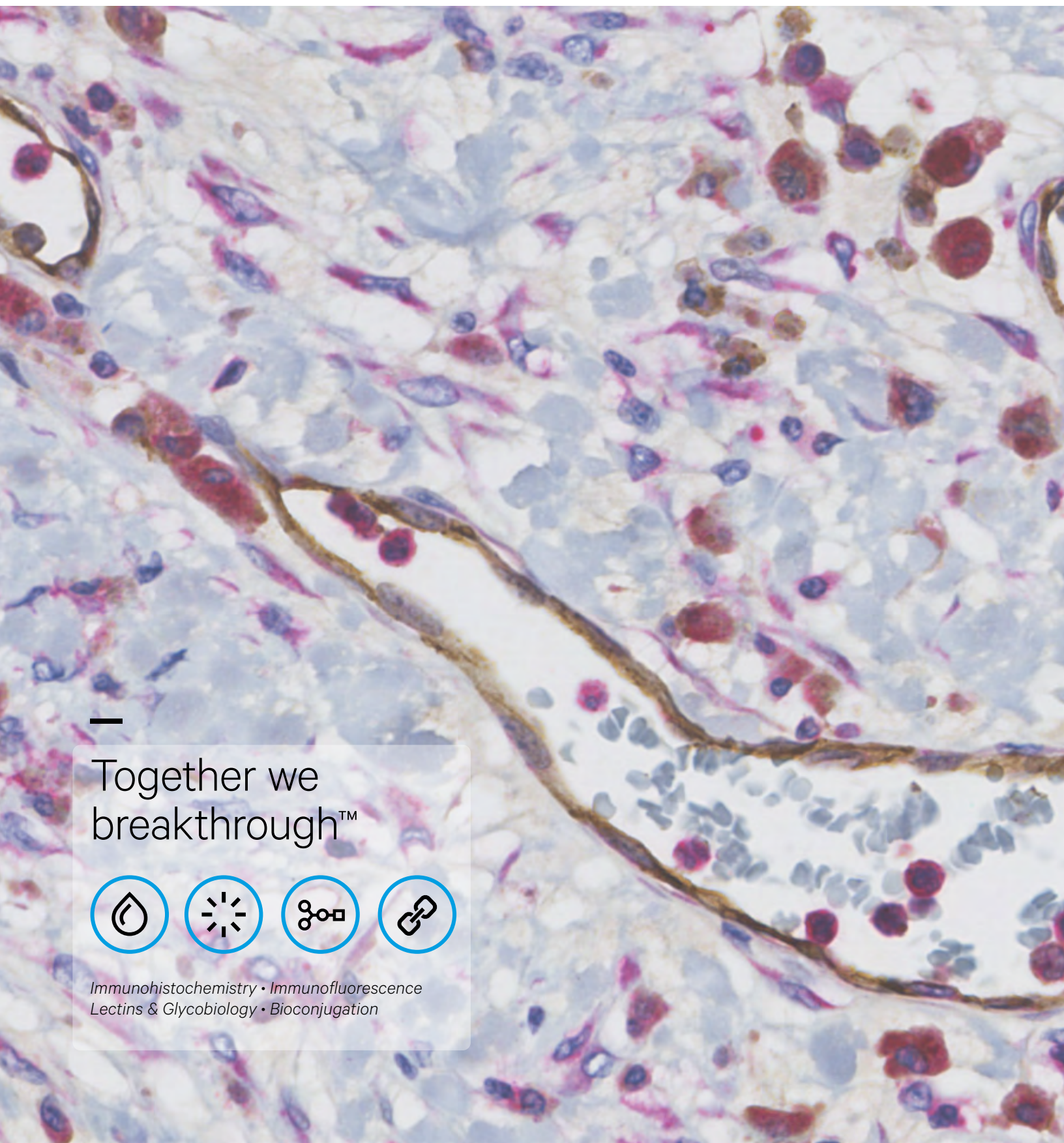
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