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A Guide to Multiple Antigen Labeling

VECTOR
LABORATORIES

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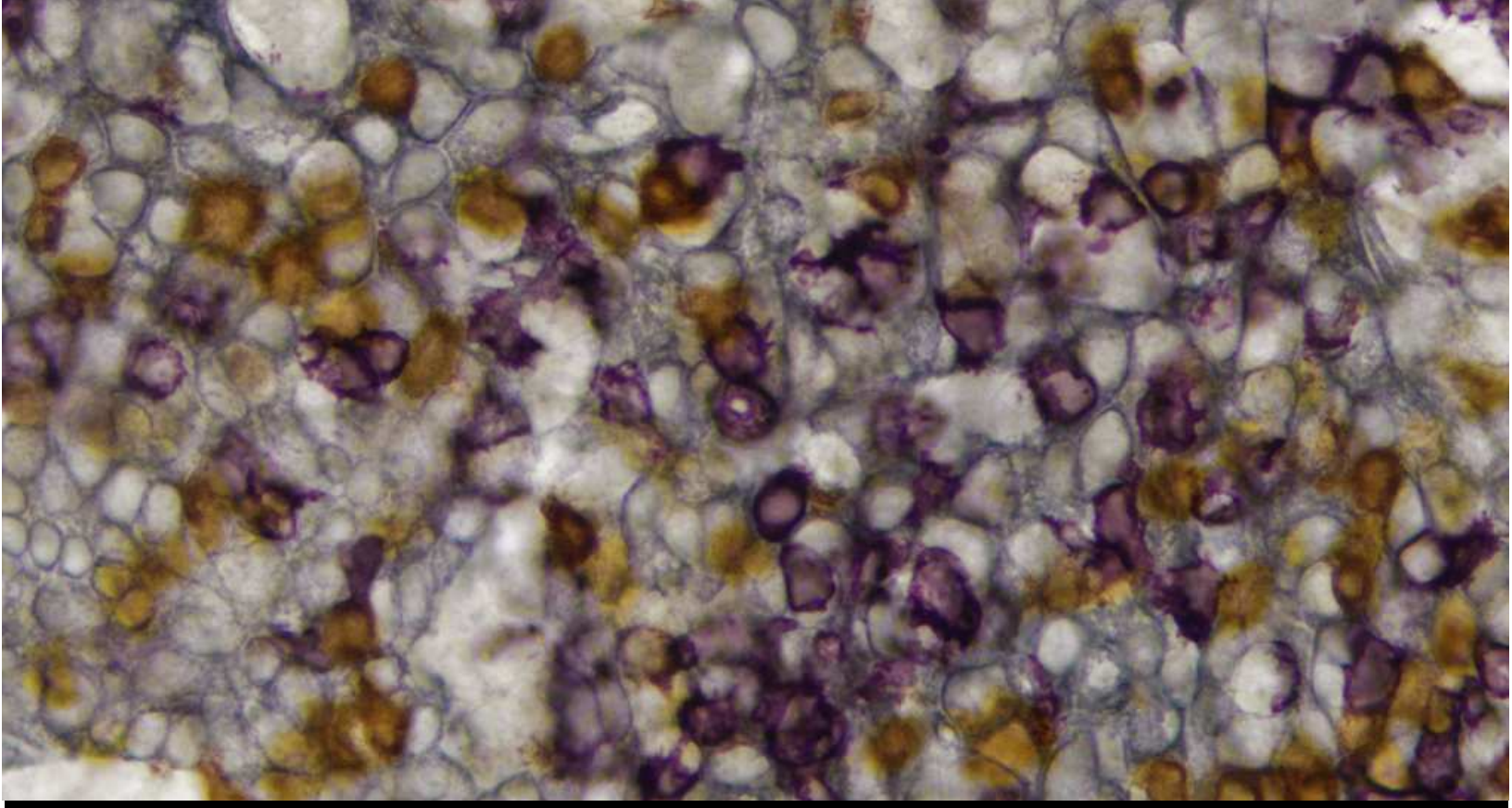


Vector Laboratories has been at the forefront of developing novel, innovative labeling and detection reagents for over a quarter of a century. For immunohistochemistry, that accomplishment was first achieved with our VECTASTAIN® ABC kits and continues with the ImmPRESS™ polymerized reporter enzyme staining system. The wide popularity of these products is a consequence of their high sensitivity, low background, reliability, reproducibility and value pricing. Ease of use and versatility also enable the VECTASTAIN® ABC kits and ImmPRESS™ reagents to be incorporated into applications such as multiple antigen labeling, without requiring significant procedural alterations or additional reagents.

Accurate, reliable localization of two or more antigens on the same tissue section is a powerful research tool that can be easily applied in standard laboratory settings. The majority of multiple labeling applications use either enzyme or fluorescent-based techniques for visualization. This booklet, with an emphasis on the most sensitive detection methods, is intended as a guide to introduce double or triple labeling into the laboratory.

A number of the reagents featured here are unique to Vector Laboratories. While other methods for multiple labeling not described here have been used in the past, the methods included herein provide optimal sensitivity coupled with exquisite color combinations, possible only with detection reagents from Vector Laboratories.

Immunohistochemistry has certainly evolved over the last 25 years to become an invaluable technique in many facets of biological science. Through our expertise and diligence Vector Laboratories will continue to generate new products and methods to assist investigators in new discoveries.

Note: All of the photomicrograph examples depicted here are human, paraffin embedded, formalin-fixed tissue (unless otherwise noted) and were generated using the simple protocols outlined in this booklet.

Abbreviations used in figure legends: AP - alkaline phosphatase, HRP - horseradish peroxidase, m - mouse monoclonal antibody, rm - rabbit monoclonal antibody, rp - rabbit polyclonal antibody, s - sheep polyclonal antibody.

Shown above: Tonsil – Triple label
- CD3 (m), VECTASTAIN® Universal Elite® ABC Kit, DAB HRP substrate (brown).
- CD20 (m), VECTASTAIN® Universal Elite® ABC Kit, Vector® VIP HRP substrate (purple).
- Multi-Cytokeratin (m), VECTASTAIN® Universal Elite® ABC Kit, Vector® SG HRP substrate (blue/gray).

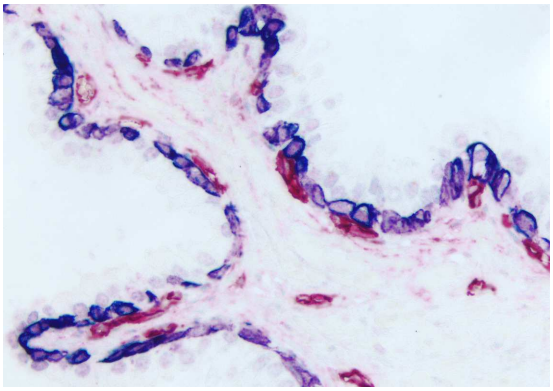
Multiple Antigen Labeling in the Same Tissue Section

Table of Contents

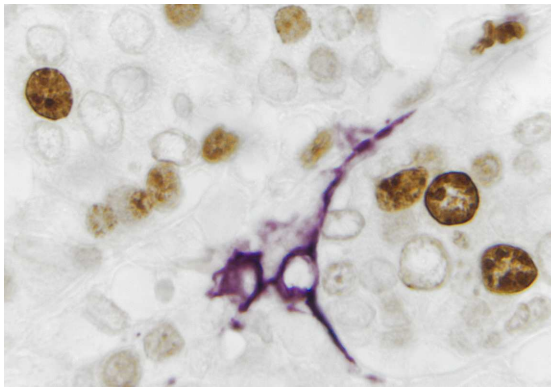
Vector Laboratories offers many solutions for the localization of two or more antigens in the same tissue section. Choosing the optimal method will depend on several factors:

- the type of tissue being stained
- whether the antigens are co-localized in the same cellular compartment
- the limitations in sensitivity of a staining method

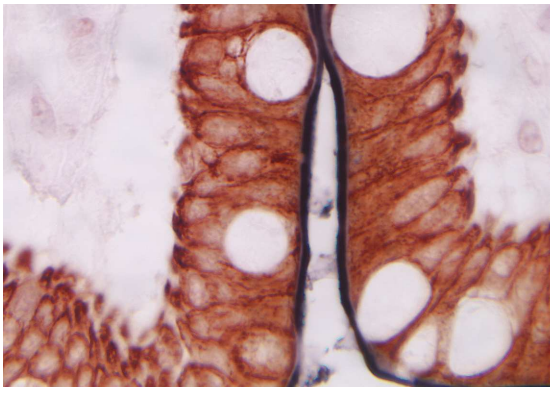
In general, immunoenzymatic methods can be used to stain two or more antigens in the same tissue section when the antigens are located in different cell types or different compartments of the same cell. In cases where the two antigens are co-localized in the same compartment of the same cell, e.g., two nuclear antigens, immunofluorescence techniques may be preferred.



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



Breast Carcinoma – Double label
- Ki67 (rm), ImmPRESS™ Universal Reagent, DAB HRP substrate (brown).
- CD34 (m), ImmPRESS™ Universal Reagent, Vector® VIP HRP substrate (purple).



Small Bowel – Double label
- CD10 (m), ImmPRESS™ Anti-Mouse Ig Reagent, DAB+Ni HRP substrate (gray/black).
- Cytokeratin 20 (m), ImmPRESS™ Anti-Mouse Ig Reagent, Vector® NovaRED™ HRP substrate (red).

Immunoenzymatic Staining Methods

Enzyme/Substrate Choices

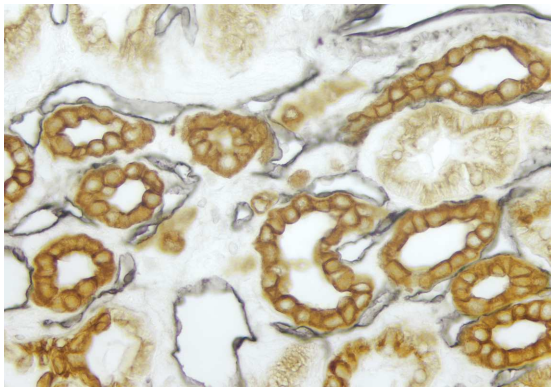
Successfully labeling multiple antigens in the same tissue section requires the use of detection systems with high sensitivity and low background as well as substrate choices that yield good color contrast. Because Vector Laboratories' peroxidase and alkaline phosphatase detection systems and their substrates are widely acknowledged for having these qualities, they are particularly suitable for this application.

The wide range of substrates from Vector Laboratories, both classic and unique, provide numerous color choices for optimizing a multiple labeling procedure. The choice and combinations of substrates depends on various factors. Substrates should provide high color contrast so that each antigen is clearly delineated.

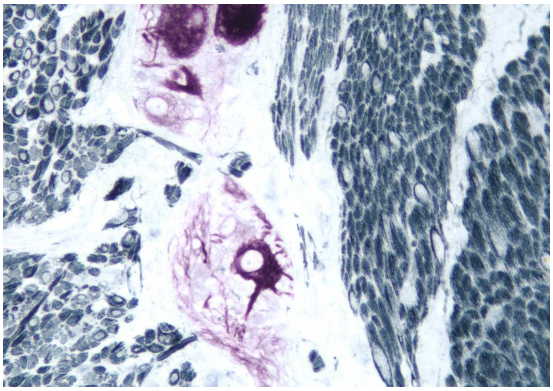
Substrates should fulfill sensitivity requirements, precipitation characteristics for a given tissue, and stability requirements in the mounting medium. Finally, the ability to inhibit endogenous enzyme activity must also be addressed when choosing a combination of substrates.

The VECTASTAIN® kits and ImmPRESS™ reagents and their respective substrate kits can be used for immunoenzymatic staining methods that fall into two main categories:

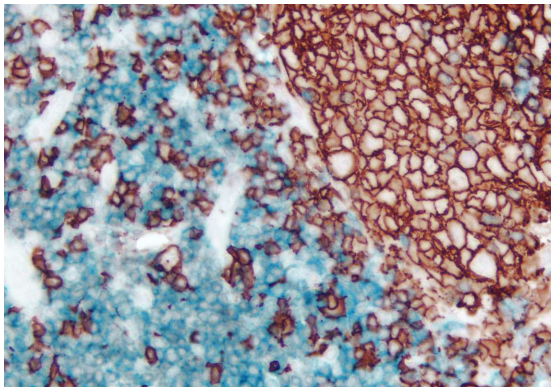
- The same enzyme system used with different substrates to detect each antigen, or
- Different enzyme systems and their substrates used to label each antigen.



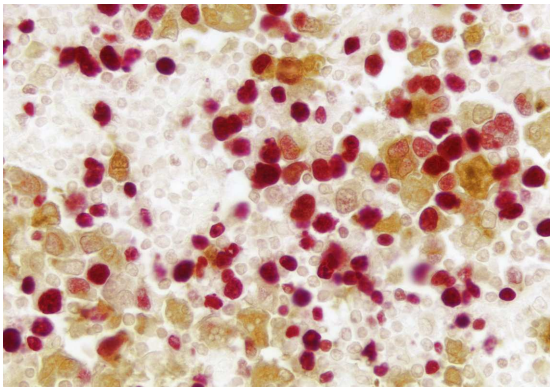
Kidney – Double label
• CD34 (m), VECTASTAIN® Universal Elite® ABC Kit, DAB+Ni HRP substrate (gray/black).
• Cytokeratin 8/18 (m), VECTASTAIN® Universal Elite® ABC Kit, DAB HRP substrate (brown).



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



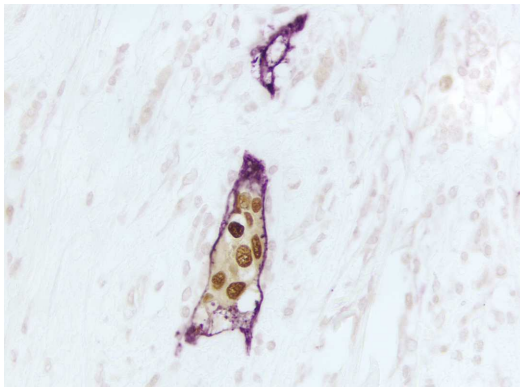
Tonsil – Double label
• CD3 (m), VECTASTAIN® Universal ABC-AP Kit, Vector® Blue AP substrate (blue).
• CD20 (m), ImmPRESS™ Universal Reagent, Vector® NovaRED™ HRP substrate (red).



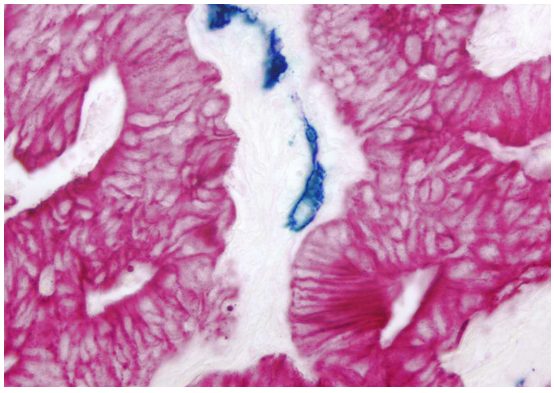
Lymph Node – Double Label
• Ki67 (m), VECTASTAIN® Universal ABC-AP Kit, Vector® Red AP substrate (red).
• Multi-Cytokeratin (m), VECTASTAIN® Universal Elite® ABC Kit, DAB HRP substrate (brown).

Peroxidase Substrates

In general, peroxidase substrates produce sharper, denser precipitates with crisp localization. Vector® NovaRED™ (red), Vector® VIP (purple) and DAB and DAB/Ni (brown or black) are essentially equivalent in sensitivity and can be permanently mounted. Vector® SG (blue/gray) and AEC (red) are less sensitive than DAB. Vector® SG can be permanently mounted, but AEC **must** be aqueously mounted as it is soluble in alcohols and other organic solvents. Although TMB is the most sensitive peroxidase substrate, it produces a more diffuse, less localized reaction product. It is not recommended for multiple labeling unless it is necessary for the detection of antigens that are present in very low concentrations. When staining neural tissue, peroxidase substrates are usually preferred because they give more consistent labeling of both cell body and processes.



Breast Carcinoma – Double Label
- Estrogen Receptor (rm), ImmPRESS™ Universal Reagent, DAB HRP substrate (brown).
- M2A Antigen (m), ImmPRESS™ Universal Reagent, Vector® VIP HRP substrate (purple).



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

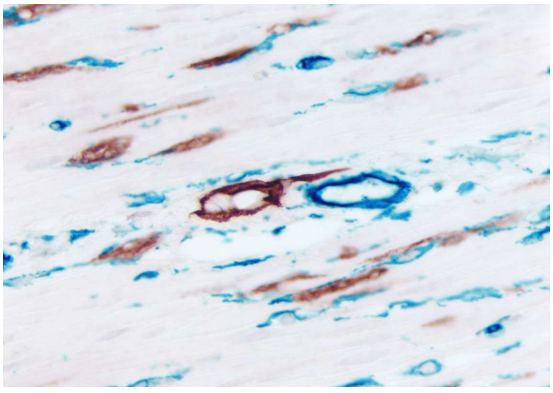
Alkaline Phosphatase Substrates

Alkaline phosphatase substrates are more diffuse and translucent than peroxidase substrates. Vector® Red (red) and Vector® Blue (blue) are equivalent in sensitivity to the peroxidase substrate DAB. Both are dehydratable. BCIP/NBT substrate (blue/violet) is the most sensitive of all the chromogenic substrates because it continues to develop over many hours increasing in sensitivity with time. It can be used for multiple labeling in situations of low antigen concentrations, and it is dehydratable for permanent mounting.

Vector® Red is unique. It is fluorescent and dehydratable. It can be viewed with a fluorescence microscope using a rhodamine or Texas Red® filter. Vector® Red can be used in fluorescent multiple label protocols in combination with fluorochromes like fluorescein, and is compatible with fluorescent counterstains like DAPI.

Vector® Red, as well as DAB, is also recommended for sequential, multiple label in situ hybridization (ISH) or IHC/ISH, because of its stability throughout ISH procedures.

Relative intensities of substrates should be taken into account - and primary antibody concentrations adjusted if possible - to ensure optimal results in any multiple labeling procedure.

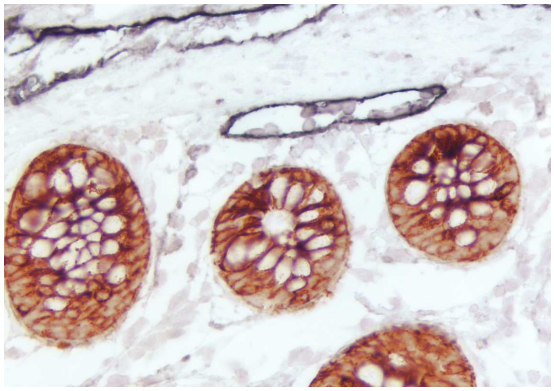


Colon – Double label
- M2A Antigen (m), ImmPRESS™ Universal Reagent, Vector® NovaRED™ HRP substrate (red).
- CD34 (m), VECTASTAIN® Universal ABC-AP Kit, Vector® Blue AP substrate (blue).

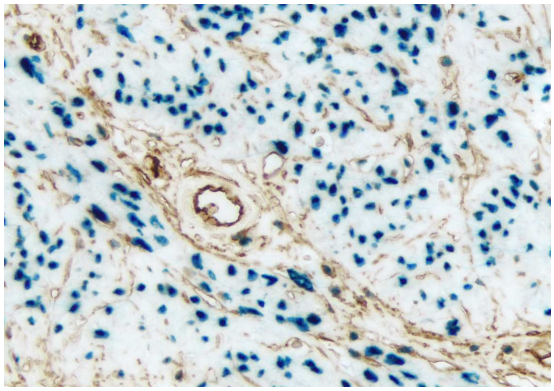
Multiple Antigen Labeling

The preferred method for double or triple labeling involves sequential staining of each primary antibody. With proper color development of substrate, the reaction product from the first substrate usually prevents most of the subsequent antibodies and detection reagent(s) from interacting with components used to stain the first antigen. This feature allows multiple labeling with primary antibodies raised in the same species using a single VECTASTAIN® ABC kit or ImmPRESS™ reagent. The order of the primary antibodies and the substrates is very important. Several controls and additional blocking steps may be necessary to obtain optimal results. Numerous combinations of colors are possible using Vector Laboratories' products and our multiple labeling protocols.

The following protocols are for formalin-fixed, paraffin-embedded tissue. These protocols can be adapted for other tissue preparations.



Colon – Double label
- M2A Antigen (m), ImmPRESS™ Universal Reagent, DAB+Ni HRP substrate (gray/black).
- Cytokeratin 8/18 (m), ImmPRESS™ Universal Reagent, Vector® NovaRED™ HRP substrate (red).



Endometrium – Double label
- Progesterone Receptor (rm), VECTASTAIN® Universal ABC-AP Kit, Vector® Blue AP substrate (blue).
- CD34 (m), VECTASTAIN® Universal Elite® ABC Kit, DAB HRP substrate (brown).

Order of Labeling for Primary Antibodies

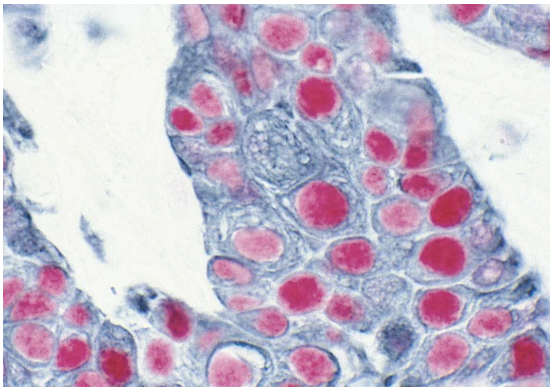
The order of labeling may significantly affect the quality and labeling pattern of each antigen in the stained section.

– Once the detection systems and substrates have been selected, it is best to try single labeling protocols for each of the primary antibodies with each labeling system.

– Optimize the staining conditions for each primary antibody. Include any necessary pretreatments such as high temperature antigen unmasking or proteolytic digestion and titer the antibodies to find the optimal dilutions and incubation times. All pretreatments must be appropriate for the staining of all subsequent antigens in the procedure. Once optimized, the same conditions should then be used in the double labeling protocol.

– Use sections stained with the optimized single label conditions as controls to compare the quality of staining and the labeling pattern for each antigen in the double label protocol.

– To verify that steric hindrance is not adversely affecting the labeling of the second antigen, first perform the double label protocol using one sequence of the two primary antibodies. Then, keeping the order of the detection systems the same, reverse the sequence of the two primary antibodies. Select the protocol giving the best labeling patterns. For example, nuclear antigens are generally best labeled before cytoplasmic antigens, and cell membrane antigens are often best labeled before cytoplasmic antigens.



Tumor – Double Label
- p53 protein (m), VECTASTAIN® ABC-AP Kit, Vector® Red AP substrate (red).
- Pan-Cytokeratin (s), VECTASTAIN® Elite® ABC Kit, Vector® SG HRP substrate (blue/gray).

Order of Substrates in Labeling Protocol

This Enzyme Substrate Combinations Table 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.

Enzyme Substrate Combinations

First Substrate \ Second Substrate	Vector® Red (red)	Vector® Blue (blue)	Vector® Black (brown/black)	BCIP/NBT (blue/violet)	Vector® VIP (purple)	DAB (brown)	DAB-Ni (gray/black)	Vector® NovaRED™ (red)	Vector® SG (blue/gray)	AEC (red)	TMB* (blue)
Vector® Red (red) Cat. No. SK-5100		–	–	–	–	+	+	–	+	–	–
Vector® Blue (blue) Cat. No. SK-5300	+		–	–	+	+	+	+	+	+	–
Vector® Black (brown/black) Cat. No. Sk-5200	+	+		+	+	–	–	–	–	+	–
BCIP/NBT (blue/violet) Cat. No. SK-5400	+	–	–		+	+	+	+	+	+	–
Vector® VIP (purple) Cat. No. SK-4600	–	+	–	–		+	+	–	+	–	–
DAB (brown) Cat. No. SK-4100	+	+	–	+	+		–	–	+	+	+
DAB-Ni (gray/black) Cat. No. SK-4100	+	–	–	–	+	+		+	–	+	+
Vector® NovaRED™ (red) Cat. No. SK-4800	–	+	–	+	–	+	+		+	–	+
Vector® SG (blue/gray) Cat. No. Sk-4700	+	–	–	–	+	+	–	–		+	–
AEC (red) Cat. No. SK-4200	–	–	–	–	–	+	–	–	+		–
TMB* (blue) Cat. No. SK-4400	–	–	–	–	–	–	–	–	–	–	

KEY:
Alkaline Phosphatase Peroxidase

\+ indicates good contrast, – indicates incompatibility of substrates for various reasons

* TMB does not provide optimal reactivity in double label applications.

Protocol: Multiple Antigen Labeling Using the VECTASTAIN® Systems

Staining for First Antigen

1. Preparation of tissue. Deparaffinize and rehydrate tissue sections following standard protocols.

2. Rinse in distilled water for 5 minutes.

3. If endogenous enzyme activities are present inactivate using appropriate methods (See Appendix 2).

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. Avidin/biotin blocking step. Perform Avidin/Biotin blocking if required (Avidin/Biotin Blocking Kit, Cat. No. SP-2001). Incubate sections with Avidin Solution for 15 minutes. Rinse briefly with buffer, then incubate in the Biotin Solution for 15 minutes. Wash sections 2 x 2 minutes in buffer. This blocking step may be eliminated if suitable controls have determined such background not to be a concern.

6. Protein blocking step. Incubate sections for 20 minutes with buffer containing 5% normal serum (NS) prepared from the first VECTASTAIN® kit, or incubate for 5-10 minutes in 10% NS.

7. Primary antibody. Blot excess blocking solution from sections and incubate with the first primary antibody diluted in 5% NS from the first VECTASTAIN® kit using appropriate concentration and length of incubation.

8. Wash 2 x 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 x 3 minutes in buffer.

11. 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 x 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 x 3 minutes in buffer.

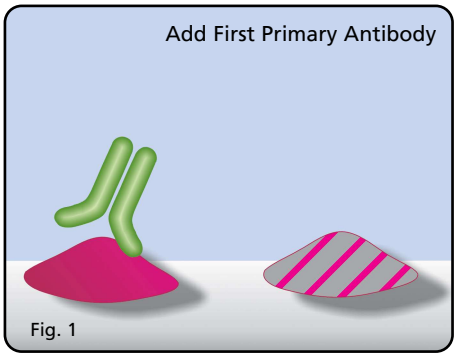


Fig. 1

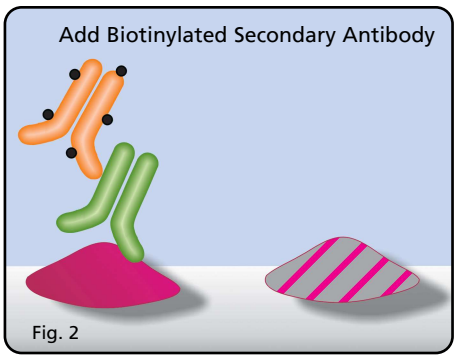


Fig. 2

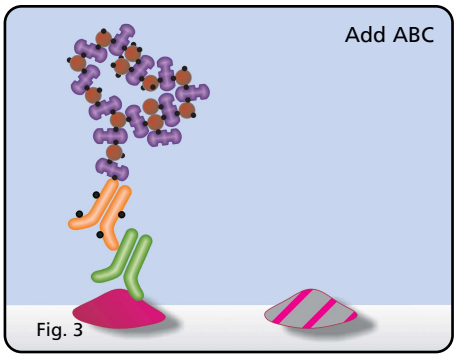


Fig. 3

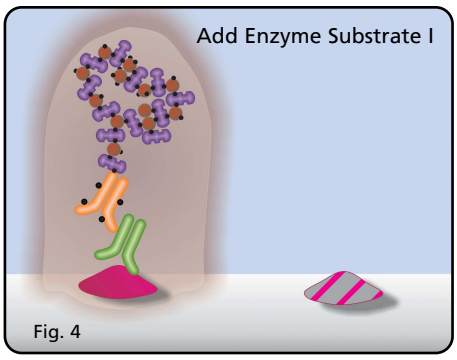


Fig. 4

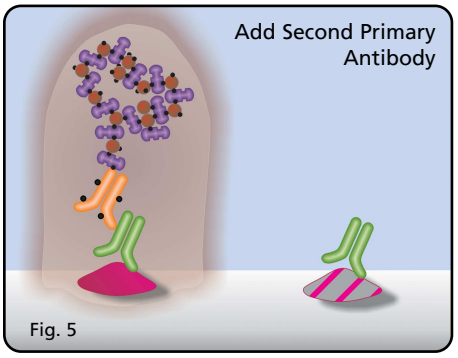


Fig. 5

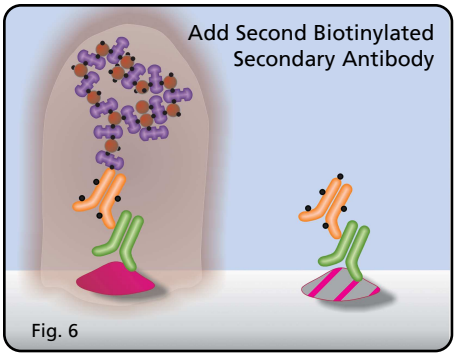


Fig. 6

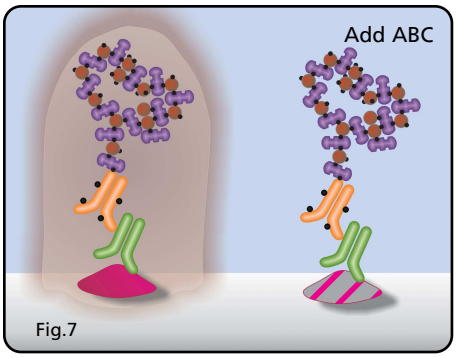


Fig.7

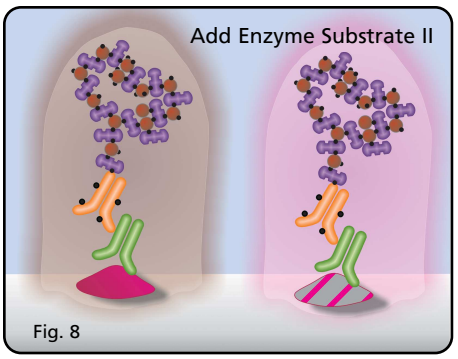


Fig. 8

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. Blot excess blocking solution from sections 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 x 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. 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.

21. Wash sections 2 x 3 minutes in buffer.

22. 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.

23. Wash sections in tap water for 5 minutes.

24. Counterstain, clear, and mount in appropriate mounting medium.

Protocol: Multiple Antigen Labeling Using the ImmPRESS™ Reagents

Staining for First 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 an appropriate method (See Appendix 2).

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 horse serum (NHS).

6. Primary antibody. Incubate sections with mouse or rabbit primary antibody diluted in appropriate antibody diluent (buffer containing diluted (2.5%) NHS or 0.1% immunohistochemical grade bovine serum albumin (BSA)).

7. Wash slides for 5 minutes in buffer.

8. ImmPRESS™ Reagent. Incubate sections for 30 minutes with appropriate ImmPRESS™ Reagent (anti-mouse Ig, anti-rabbit Ig, or universal).

9. Wash slides for 5 minutes in buffer.

10. Substrate. Incubate sections in peroxidase substrate solution until optimal color develops.

11. Rinse for 5 minutes in buffer.

Staining for Second Antigen

12. Protein blocking step. Incubate sections for 20 minutes with ready-to-use NHS.

13. Primary antibody. Incubate sections with the second mouse or rabbit primary antibody diluted in appropriate antibody diluent (See step 6).

14. Wash for 5 minutes in buffer.

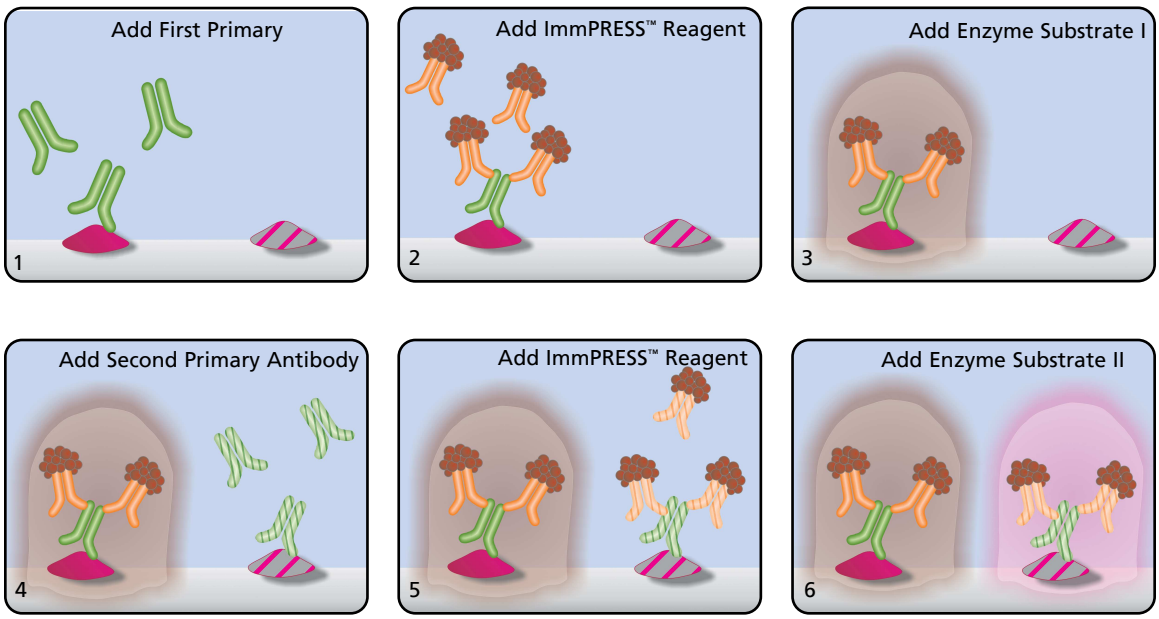
15. ImmPRESS™ Reagent. Incubate sections for 30 minutes with appropriate ImmPRESS™ Reagent (anti-mouse Ig, anti-rabbit Ig, or universal).

16. Wash for 5 minutes in buffer.

17. Substrate. Incubate sections in second, contrasting peroxidase substrate solution until optimal color develops.

18. Wash for 5 minutes in buffer.

19. Counterstain, clear, and mount in appropriate mounting medium.



General Notes

Control Sections

Control sections are important for the correct interpretation of results from multiple labeling procedures. A Troubleshooting Guide that outlines necessary controls for single labeling is available online or upon request.

– As in the case of single labeling protocols, "deletion controls" should be used in multiple labeling procedures. For deletion controls in multiple labeling, the first antigen should be labeled to completion with the first substrate in each section. In each control section, successive steps in the second staining protocol, i.e., second primary antibody and second detection system components, should be deleted. This series of controls will help determine whether any staining is due to the reagents of the second staining procedure binding to elements of the first labeling protocol; or, in the case of using the same enzyme detection system, the first enzyme reacting with the second substrate.

– Besides the usual deletion controls mentioned above, an additional control is necessary when primary antibodies from the same species are used in a multiple antigen labeling application. In this control, the second primary antibody should be substituted with an irrelevant antibody of the same species and in the same concentration range. This will help determine if any staining is due to binding of the second primary antibody by the first secondary antibody.

Background Staining

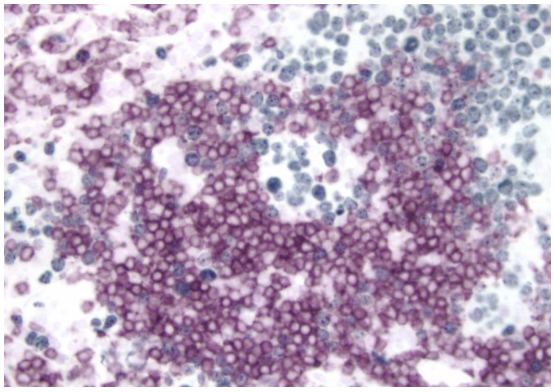
The chances of developing background staining are greater when immunostaining with multiple labels. The control sections for single and multiple labels mentioned previously will help determine which steps in a labeling protocol are contributing to the background. Described below are solutions to some common background issues.

– If endogenous enzyme is present in the section, an appropriate inactivation step should be introduced to eliminate staining (See Appendix 2).

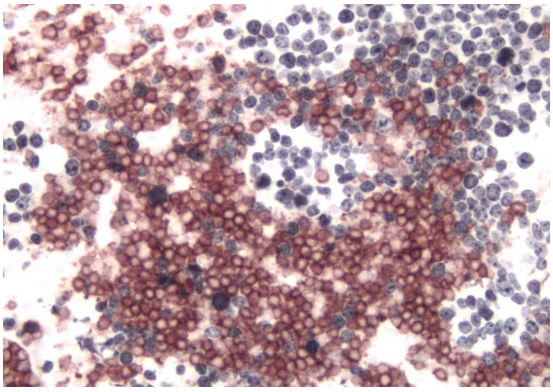
– An avidin/biotin blocking step may be required before (for endogenous activity) and/or between labels (in case of interaction between the second labeling system and the first).

– To reduce background staining due to nonspecific interactions of detection reagents with the tissue, increase the serum concentration or use immunohistochemical grade bovine serum albumin (BSA; Cat. No. SP-5050) in the blocking/antibody solution. A detergent, such as Tween 20 (0.05%-0.2%) can also be added to these solutions.

– The biotinylated secondary antibody used in the second labeling system can bind to the immunoglobulins in the normal serum used in the first staining procedure. To avoid the background staining due to this crossreactivity of reagents, use a blocking serum from another species that is not recognized by either of the secondary antibodies, or use another blocking agent such as immunohistochemical grade BSA instead.



Tonsil – Double label
- CD3 (m), ImmPRESS™ Anti-Mouse Ig Reagent, Vector® VIP HRP substrate (purple).
- Ki67 (m), ImmPRESS™ Anti-Mouse Ig Reagent, Vector® SG HRP substrate (blue/gray).



Tonsil – Double label
- CD3 (m), ImmPRESS™ Anti-Mouse Ig Reagent, Vector® NovaRED™ HRP substrate (red).
- Ki67 (m), ImmPRESS™ Anti-Mouse Ig Reagent, DAB+Ni HRP substrate (gray/black).

General Notes (continued)

Intensity and Clarity of Staining

The relative intensities of the two colored precipitates is an important factor in obtaining the best visualization of the antigens.

– Adjust the concentrations of the two primary antibodies to balance the staining intensities.

– Observe substrate development under the microscope to monitor color intensity, especially when developing the substrate for the second antigen.

– In multiple label applications, a counterstain may be unnecessary or may even detract from the staining. If a counterstain is desired, it must contrast with the labeling colors and be compatible with all detection systems used. Please see Appendix 1: Counterstain/Substrate Compatibility Table (page 19).

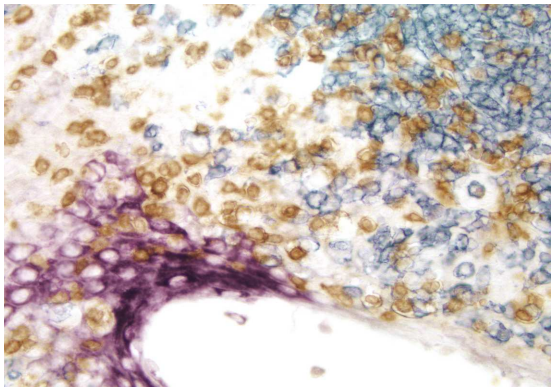
– Visualizing two antigens within the same cell is possible using precipitating enzyme substrates if the antigens are located in separate cellular compartments (e.g. detecting one nuclear and one cytoplasmic antigen). If blocking of antigenic sites occurs from the first substrate, fluorescence techniques may be preferable, especially in situations when antigens are located in the same part of the cell (e.g. two nuclear antigens). See the section on "Immunofluorescence Staining Methods".

Shortening the Staining Protocol

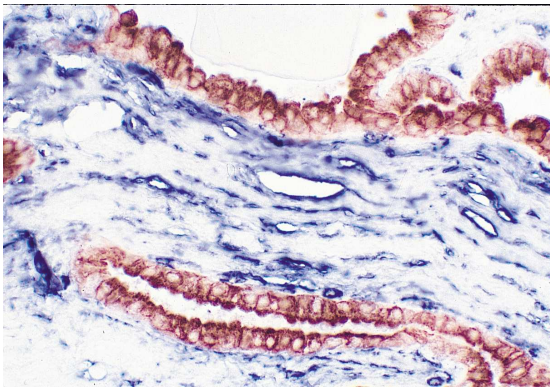
Several strategies are available for shortening a multiple antigen labeling procedure. A rapid staining protocol is available for the VECTASTAIN® ABC systems which involves using more concentrated detection reagent solutions for shorter incubation times. Alternatively, the ImmPRESS™ polymer peroxidase reagents offer a convenient, one-step detection protocol that yields sensitivities equivalent to the VECTASTAIN® Elite® ABC systems. Since the ImmPRESS™ system is a non-biotin system, it also eliminates the Avidin/Biotin blocking step(s).

Interrupting the Staining Protocol

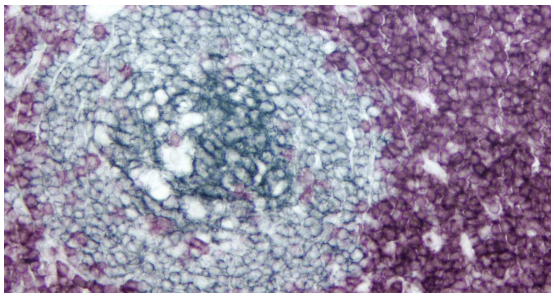
It is recommended that the multiple labeling protocol be completed the same day. However, if the staining protocol must be interrupted, complete the staining procedure through the development of the first substrate. Then store sections in PBS at 4 °C overnight. After an overnight PBS incubation some substrates may appear less crisp than if the procedure had been completed without interruption.



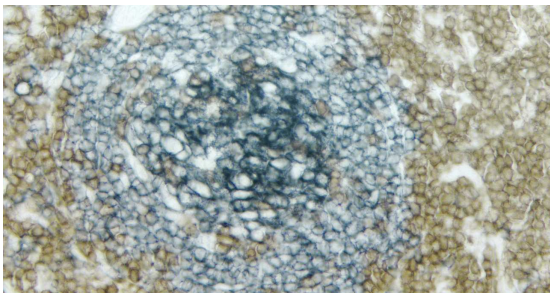
Tonsil – Triple label
- CD3 (m), VECTASTAIN® Universal Elite® ABC Kit, DAB HRP substrate (brown).
- CD20 (m), VECTASTAIN® Universal Elite® ABC Kit, Vector® SG HRP substrate (blue/gray).
- Multi-Cytokeratin (m), VECTASTAIN® Universal Elite® ABC Kit, Vector® VIP HRP substrate (purple).



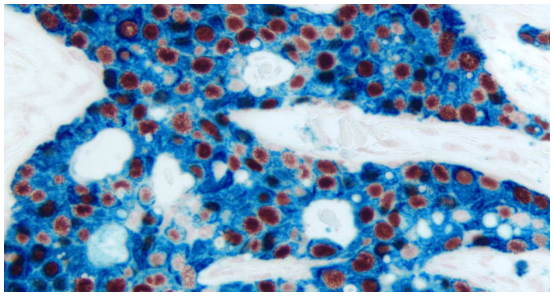
Tumor – Double label
- Multi-Cytokeratin (m), VECTASTAIN® Elite® ABC Kit, Vector NovaRED™ HRP substrate (red).
- CD34 (m), VECTASTAIN® ABC-AP Kit, BCIP/NBT AP substrate (blue).



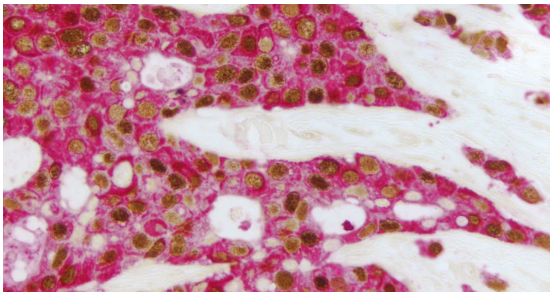
Tonsil – Double label
• CD3 (m), VECTASTAIN® Universal Elite® ABC Kit, Vector® VIP HRP substrate (purple).
• CD20 (m), VECTASTAIN® Universal Elite® ABC Kit, Vector® SG HRP substrate (blue/gray).



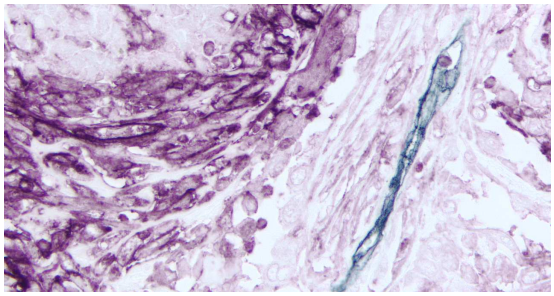
Tonsil – Double label
• CD3 (m), VECTASTAIN® Universal Elite® ABC Kit, DAB HRP substrate (brown).
• CD20 (m), VECTASTAIN® Universal Elite® ABC Kit, Vector® SG HRP substrate (blue/gray).



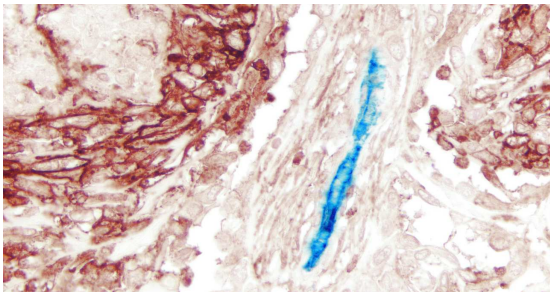
Breast Carcinoma – Double label
• Estrogen Receptor (m), ImmPRESS™ Universal Reagent, Vector® NovaRED™ HRP substrate (red).
• Cytokeratin 8/18 (m), VECTASTAIN® Universal ABC-AP Kit, Vector® Blue AP substrate (blue).



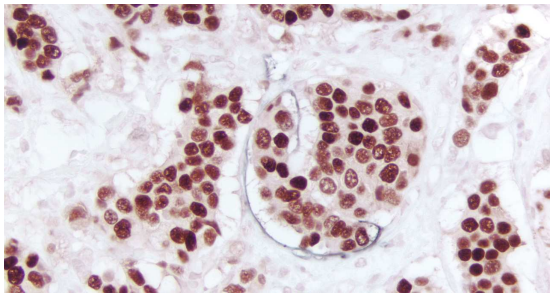
Breast Carcinoma – Double Label
• Estrogen Receptor (m), ImmPRESS™ Universal Reagent, DAB HRP substrate (brown).
• Multi-Cytokeratin (m), VECTASTAIN® Universal ABC-AP Kit, Vector® Red AP substrate (red).



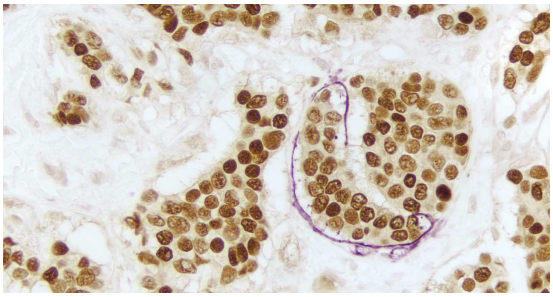
Mesothelioma – Double label
• M2A Antigen (m), VECTASTAIN® Universal Elite® ABC Kit, Vector® VIP HRP substrate (purple).
• CD34 (m), VECTASTAIN® Universal Elite® ABC Kit, Vector® SG HRP substrate (blue/gray).



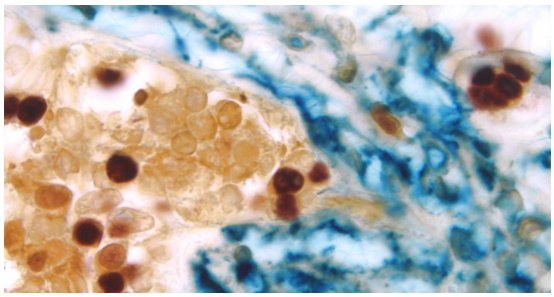
Mesothelioma – Double label
• M2A Antigen (m), VECTASTAIN® Universal Elite® ABC Kit, Vector® NovaRED™ HRP substrate (red).
• CD34 (m), VECTASTAIN® Universal ABC-AP Kit, Vector® Blue AP substrate (blue).



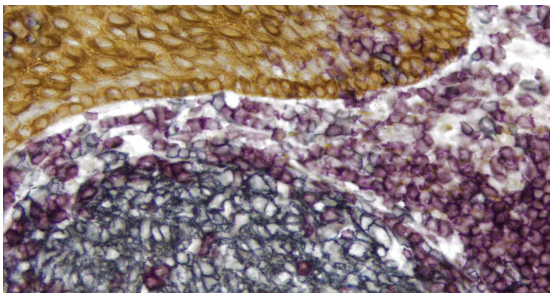
Breast Carcinoma – Double label
• Estrogen Receptor (rm), ImmPRESS™ Universal Reagent, Vector® NovaRED™ HRP substrate (red).
• M2A Antigen (m), ImmPRESS™ Universal Reagent, DAB+Ni HRP substrate (gray/black).



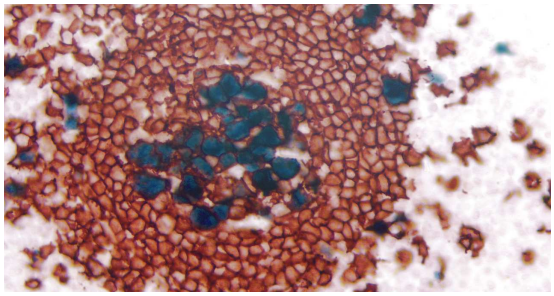
Breast Carcinoma – Double label
• Estrogen Receptor (rm), ImmPRESS™ Universal Reagent, DAB HRP substrate (brown).
• M2A Antigen (m), ImmPRESS™ Universal Reagent, Vector® VIP HRP substrate (purple).



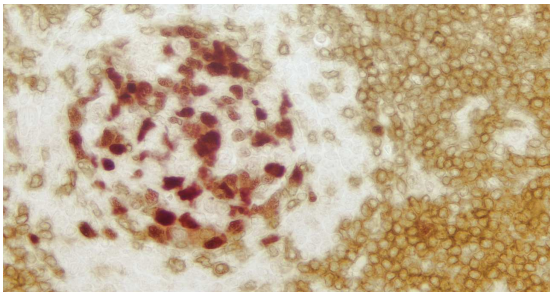
Breast Carcinoma – Triple label
- Estrogen Receptor (m), VECTASTAIN® Elite® ABC Kit, Vector® NovaRED™ HRP substrate (red).
- CD34 (m), VECTASTAIN® ABC-AP Kit, Vector® Blue AP substrate (blue).
- Cytokeratin 8/18 (m), VECTASTAIN® Elite® ABC Kit, DAB HRP substrate (brown).



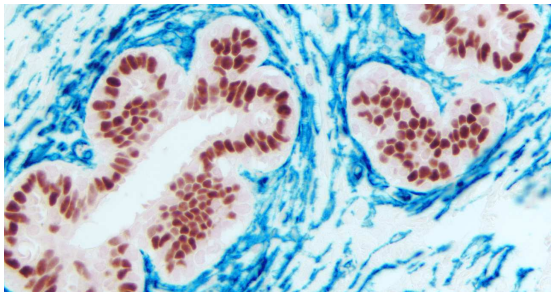
Tonsil- Triple label
- Multi-Cytokeratin (m), VECTASTAIN® Universal Elite® ABC Kit, DAB HRP substrate (brown).
- CD3 (m), VECTASTAIN® Universal Elite® ABC Kit, Vector® VIP HRP substrate (purple).
- CD20 (m), VECTASTAIN® Universal Elite® ABC Kit, Vector® SG HRP substrate (blue/gray).



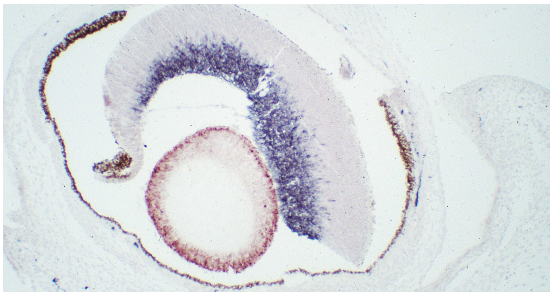
Tonsil – Double label
- Cyclin A (m), VECTASTAIN® Universal ABC-AP Kit, Vector® Blue AP substrate (blue).
- CD20 (m), VECTASTAIN® Universal Elite® ABC Kit, Vector® NovaRED™ HRP substrate (red).



Tonsil – Double label
- Cyclin A (m), VECTASTAIN® Universal ABC-AP Kit, Vector® Red AP substate (red).
- CD3 (m), VECTASTAIN® Universal Elite® ABC Kit, DAB HRP substrate (brown).

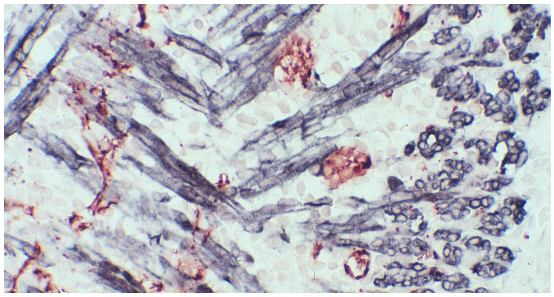


Breast Carcinoma – Double label
- Estrogen Receptor (m), ImmPRESS™ Universal Reagent, Vector® NovaRED™ HRP substrate (red).
- CD34 (m), VECTASTAIN® Universal ABC-AP Kit, Vector® Blue AP substrate (blue).

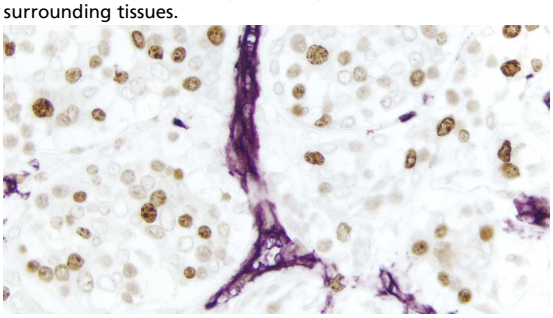


Mouse, Newborn (eye) – Double label
- GFAP (m), M.O.M.™ Peroxidase Kit, Vector® NovaRED™ HRP substrate (red).
- Synapsin (m), M.O.M.™ Peroxidase Kit, DAB+Ni HRP substrate (gray/black).

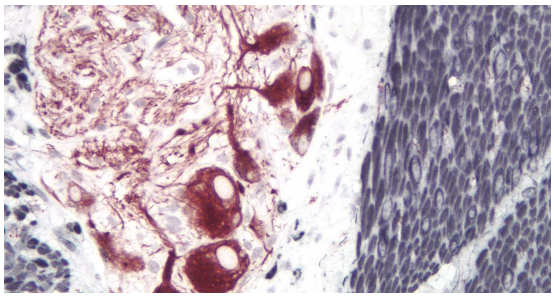
Note contrast with endogenous pigment (brown) seen in surrounding tissues.



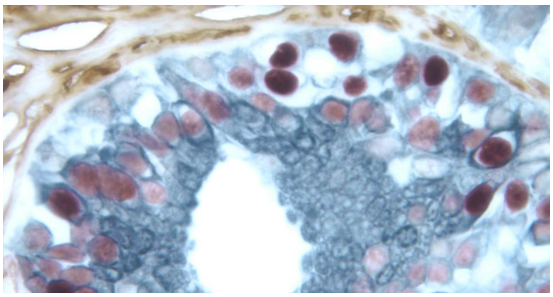
Mouse, Newborn (tongue) – Double label
- Synapsin (m), M.O.M.™ Peroxidase Kit, Vector® NovaRED™ HRP substrate (red).
- Desmin (m), M.O.M.™ Peroxidase Kit, DAB+Ni HRP substrate (gray/black).



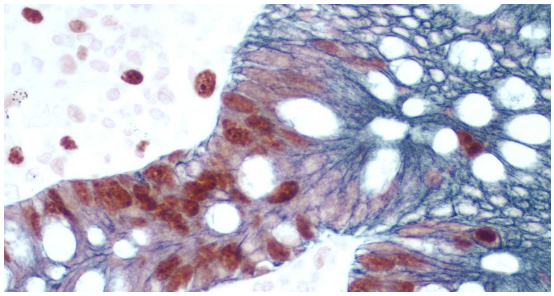
Breast Carcinoma – Double label
- Ki67 (rm), ImmPRESS™ Universal Reagent, DAB HRP substrate (brown).
- CD34 (m), ImmPRESS™ Universal Reagent, Vector® VIP HRP substrate (purple).



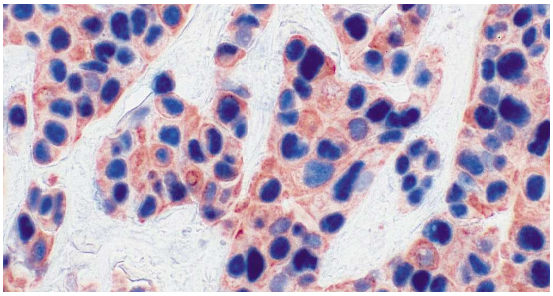
Small Bowel – Double label
• Peripherin (m), ImmPRESS™ Anti-Mouse Ig Reagent, Vector® NovaRED™ HRP substrate (red).
• Desmin (m), ImmPRESS™ Anti-Mouse Ig Reagent, DAB+Ni HRP substrate (gray/black).



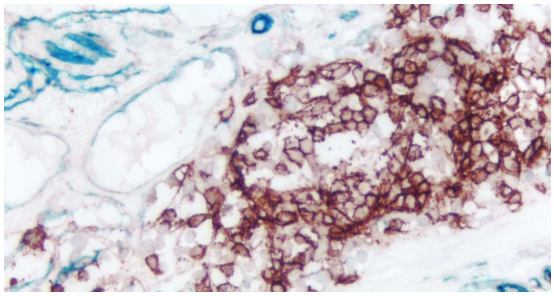
Breast Carcinoma – Triple label
• Estrogen Receptor (m), VECTASTAIN® Elite® ABC Kit, Vector® NovaRED™ HRP substrate (red).
• CD34 (m), VECTASTAIN® Elite® ABC Kit, DAB HRP substrate (brown).
• Cytokeratin 8/18 (m), VECTASTAIN® Elite® ABC Kit, Vector® SG HRP substrate (blue/gray).



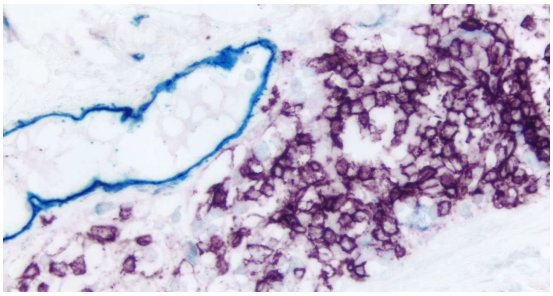
Small Bowel - Double label
• Ki67 (rp), ImmPRESS™ Universal Reagent, Vector® NovaRED™ HRP substrate (red).
• Cytokeratin 8/18 (m), ImmPRESS™ Universal Reagent, Vector® SG HRP substrate (blue/gray).



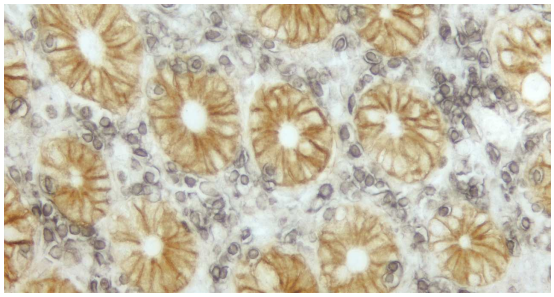
Tumor – Double label
• p53 (m), VECTASTAIN® ABC-AP Kit, Vector® Blue AP substrate (blue).
• Multi-Cytokeratin (m), VECTASTAIN® Elite® ABC Kit, AEC HRP substrate (red).



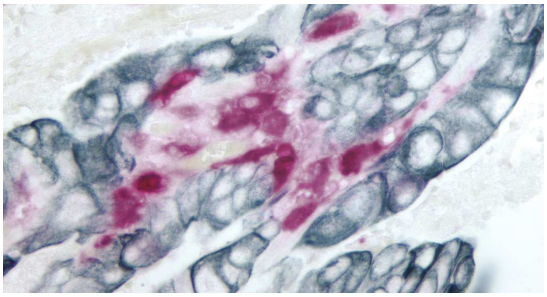
Colon – Double label
• CD34 (m), VECTASTAIN® Universal ABC-AP Kit, Vector® Blue AP substrate (blue).
• CD20 (m), ImmPRESS™ Universal Reagent, Vector® NovaRED™ HRP substrate (red).



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



Small Bowel – Double label
• CD45 (m), VECTASTAIN® Universal Elite® ABC Kit, DAB+Ni HRP substrate (gray/black).
• Epithelial Specific Antigen (m), VECTASTAIN® Universal Elite® ABC Kit, DAB HRP substrate (brown).



Colon Carcinoma – Double label
• S100 (rp), VECTASTAIN® Universal ABC-AP Kit, Vector® Red AP substrate (red).
• Cytokeratin 8/18 (m), VECTASTAIN® Universal Elite® ABC Kit, Vector® SG HRP substrate (blue/gray).

Immunofluorescence Staining Methods

Immunofluorescence staining methods can also be used successfully for labeling multiple antigens in the same preparation. These methods are especially useful for co-localization of antigens in the same compartment of a cell, and in this regard offer a distinct advantage over enzyme-based detection systems.

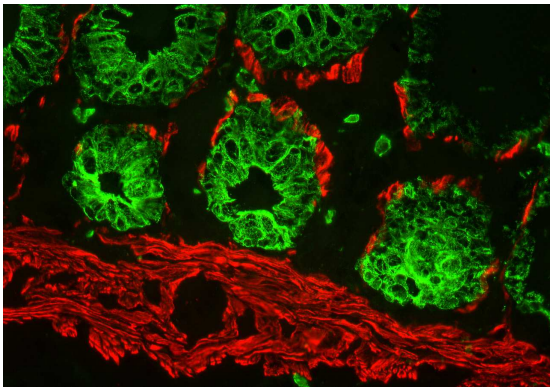
Traditionally, double fluorescent labeling has been achieved by using fluorescently conjugated secondaries against primary antibodies from different species. Although this technique in general tends to be less sensitive than enzymatic staining, the sensitivity of the fluorescent stain can be increased by using a (strept)avidin-biotin system for amplification of the label. Many of the principles regarding multiple antigen labeling will apply to both immunofluorescent and enzymatic methods (see section on "Immunoenzymatic Staining Methods"). For best results, sequential staining of each primary antibody is recommended. As in enzymatic applications, the order of labeling, appropriate controls, and additional blocking steps may be important to obtain optimal results. In contrast to enzymatic staining, special consideration must be given to the species of the primary antibodies to be used. For fluorescent applications, the two primary antibodies usually should be from different species, otherwise, artifactual co-staining of antigens may result. However, the use of two mouse primary antibodies is the exception. See section on "Multiple Immunofluorescent Labeling Using Two or More Mouse Monoclonal Primary Antibodies".

In addition, if the (strept)avidin biotin system is used for the visualization of both antigens, a (strept)avidin-biotin block MUST be used before the second primary antibody step.

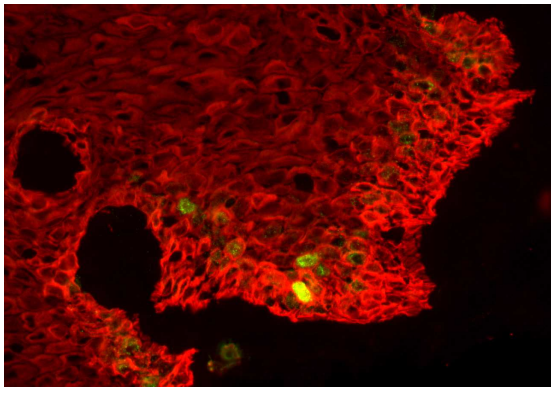
Following labeling it is important to preserve the intensity of the fluorescent signal, as some fluorescent products are prone to rapid fading when viewed under the microscope. In most instances this is easily accomplished by coverslipping the preparation with an anti-fade, anti-photobleaching agent such as VECTASHIELD® mounting media. The use of VECTASHIELD® also allows the preparation to be stored for extended periods without significant loss of intensity.

Autofluorescence of the tissue may obscure staining depending on the type of tissue, the fixation method, and the microscope filter used for visualization. This should be evaluated before staining, and autofluoresence quenched, if necessary, using appropriate methods.

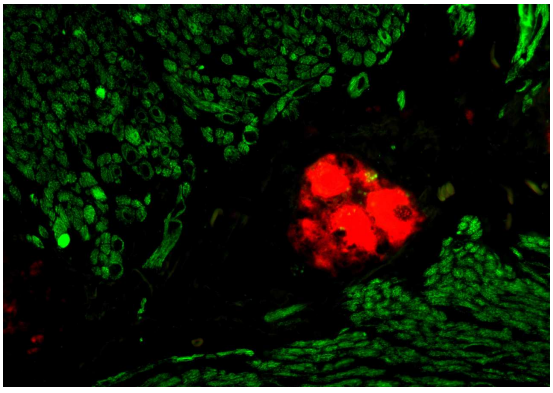
The following protocols are for frozen tissue sections. These protocols can be adapted for other tissue preparations.



Colon (frozen) – Double label
- Multi-Cytokeratin (m), M.O.M.™ Fluorescein Kit (green).
- Desmin (m), M.O.M.™ Basic Kit, Texas Red® Avidin DCS (red).



Tonsil (frozen) – Double label
- Ki67 (m), M.O.M.™ Fluorescein Kit (green).
- Pan-Cytokeratin (m), M.O.M.™ Basic Kit, Texas Red® Avidin DCS (red).



Small Bowel (frozen) – Double label
- PGP9.5 (m), M.O.M.™ Basic Kit, VECTASTAIN® ABC-AP Standard Kit, Vector® Red AP substrate (red).
- Desmin (m), M.O.M.™ Fluorescein Kit (green).

Protocol: Double Immunofluorescent Labeling Using Two Primary Antibodies From Different Species

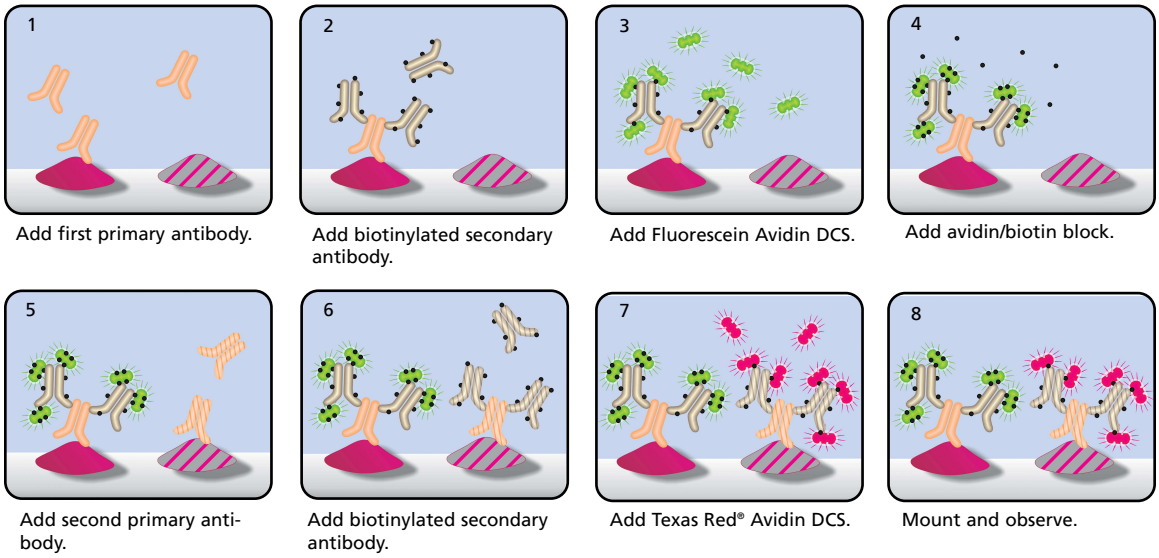
Staining for First Antigen

1. Preparation of tissue. Fix sections with the appropriate fixative for the antigen under study (Please see Note 1).
2. Air dry sections.
3. Wash sections 2 x 2 minutes in buffer (PBS).
4. Avidin/biotin blocking step. Perform Avidin/Biotin blocking if required (Avidin/Biotin Blocking Kit, Cat. No. SP-2001). Incubate sections with Avidin Solution for 15 minutes. Rinse briefly with buffer, then incubate in the Biotin Solution for 15 minutes. Wash sections 2 x 2 minutes in buffer. This blocking step may be eliminated if suitable controls have determined this step to be unnecessary.
5. Protein blocking step. Incubate sections for 20 minutes with buffer containing 5% normal blocking serum (NS) which was prepared from the species in which the secondary antibody is made.
6. Blot excess serum from sections.
7. Primary antibody. Incubate sections with the first primary antibody diluted in appropriate antibody diluent (buffer containing 5% NS) using an appropriate concentration and length of incubation.
8. Wash for 5 minutes in buffer.
9. Secondary antibody. Incubate sections for 30 minutes with biotinylated secondary antibody (5-10 µg/ml diluted in buffer containing 5% NS).
10. Wash slides for 5 minutes in buffer.
11. Avidin conjugate. Incubate sections with Fluorescein Avidin DCS (15-20 µg/ml diluted in buffer) for 5-10 minutes.
12. Wash slides for 5 minutes in buffer.

Staining for Second Antigen

13. Avidin/biotin blocking step. Apply an Avidin/Biotin block according to Step 4. (This step must be done to prevent the interaction of the second set of labeling reagents with the first set of labeling regents).
14. Protein blocking step. Incubate sections for 20 minutes with 5% NS.
15. Blot excess serum from sections.
16. Primary antibody. Incubate sections with second primary antibody diluted in appropriate antibody diluent (buffer containing 5% NS) using an appropriate concentration and length of incubation.
17. Wash slides for 5 minutes in buffer.
18. Secondary antibody. Incubate sections for 30 minutes with biotinylated secondary antibody (5-10 µg/ml diluted in buffer containing 5% NS).
19. Wash slides for 5 minutes in buffer.
20. Avidin conjugate. Incubate sections with Texas Red® Avidin DCS (15-20 µg/ml diluted in buffer) for 5-10 minutes.
21. Wash slides for 5 minutes in buffer.
22. Mount with the appropriate VECTASHIELD® mounting media.
23. Observe under a fluorescence microscope.

NOTES:
1. Aldehyde-fixed tissues (e.g. formalin) tend to be autofluorescent and may make interpretation of specific fluorescein signal difficult.

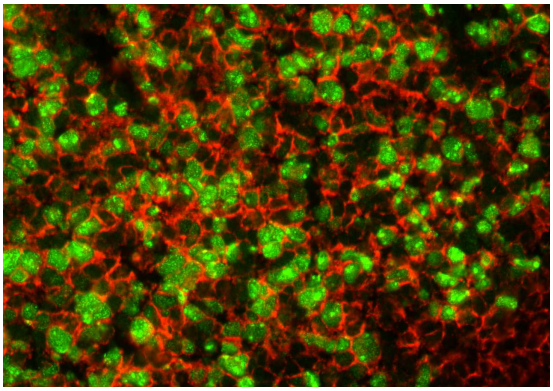


Multiple Immunofluorescent Labeling using Two or More Mouse Monoclonal Primary Antibodies

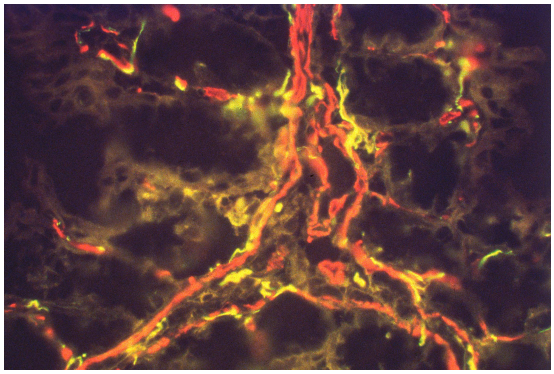
Specific localization of a mouse primary antibody on mouse tissue was problematic until the introduction of our Vector® Mouse on Mouse (M.O.M.™) Immunodetection Kits.

These M.O.M.™ kits block endogenous mouse immunoglobulins in mouse tissue, allowing for accurate recognition of the mouse primary antibody by the biotinylated anti-mouse IgG secondary antibody, eliminating confusing background.

Using this same mouse Ig blocking technology, it is possible to fluorescently detect several mouse primary antibodies on the same tissue section whether or not the tissue is of mouse origin.

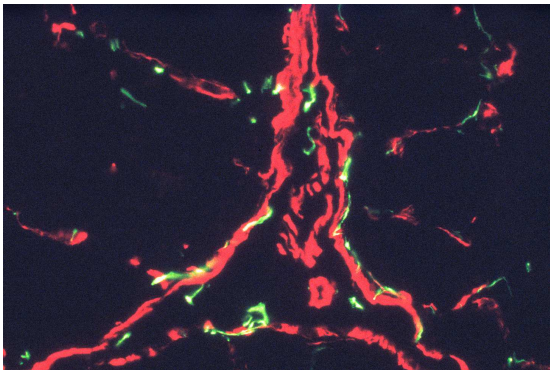


Tonsil (frozen) – Double label
- Ki67 (m), M.O.M.™ Fluorescein Kit (green).
- CD20 (m), M.O.M.™ Basic Kit, Texas Red® Avidin DCS (red).



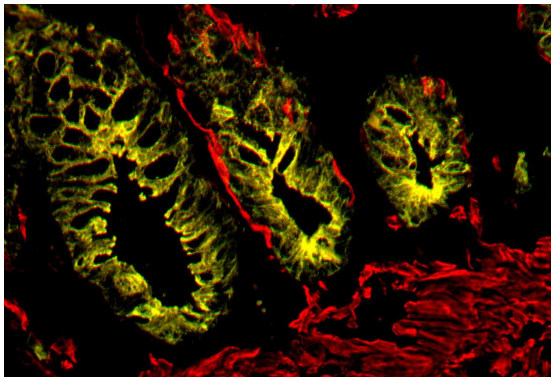
No M.O.M™. Kit: Mouse Intestine (frozen) – Double label
- Peripherin (m), Biotinylated horse anti-mouse IgG, Fluorescein Avidin DCS (green).
- Desmin (m), Biotinylated horse anti-mouse IgG, Texas Red® Avidin DCS (red).

NOTE BACKGROUND AND SIGNAL MIXING.



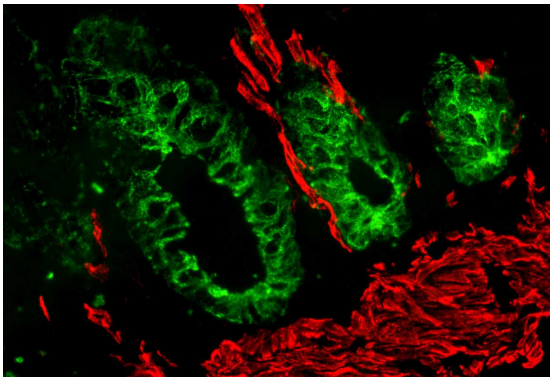
With M.O.M.™ Kit: Mouse Intestine (frozen) – Double label
- Peripherin (m), M.O.M.™ Fluorescein Kit (green).
- Desmin (m), M.O.M.™ Basic Kit, Texas Red® Avidin DCS (red).

COMPARE WITH ADJACENT NO M.O.M.™ KIT PHOTO.



No M.O.M.™ Kit: Colon (frozen) – Double label
- Multi-Cytokeratin (m), Biotinylated horse anti-mouse IgG, Fluorescein Avidin DCS (green).
- Desmin (m), Biotinylated horse anti-mouse IgG, Texas Red® Avidin DCS (red).

NOTE BACKGROUND AND SIGNAL MIXING.



With M.O.M.™ Kit: Colon (frozen) – Double label
- Multi-Cytokeratin (m), M.O.M.™ Fluorescein Kit (green).
- Desmin (m), M.O.M.™ Basic Kit, Texas Red® Avidin DCS (red).

COMPARE WITH ADJACENT NO M.O.M.™ KIT PHOTO

Protocol: Multiple Immunofluorescent Labeling using Two or More Mouse Monoclonal Primary Antibodies

Staining for First Antigen

1. Preparation of tissue. Fix sections with the appropriate fixative for the antigen under study (Please see Note 1).

2. Air dry sections.

3. Wash sections 2 x 2 minutes in buffer (PBS).

4. Avidin/biotin blocking step. Perform Avidin/Biotin blocking if required (Avidin/Biotin Blocking Kit, Cat. No. SP-2001). Incubate sections with Avidin Solution for 15 minutes. Rinse briefly with buffer, then incubate in the Biotin Solution for 15 minutes. Wash sections 2 x 2 minutes in buffer. This blocking step may be eliminated if suitable controls have determined this step to be unnecessary.

5. Mouse Ig blocking step. Incubate sections for 1 hour in working solution of M.O.M.™ Mouse Ig Blocking Reagent (Please see Note 2).

6. Wash sections 2 x 2 minutes in buffer (Please see Note 2).

7. Protein blocking step. Incubate tissue sections for 5 minutes in working solution of M.O.M.™ diluent.

8. 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 (Please see Note 3).

9. Wash sections 2 x 2 minutes in buffer.

10. Secondary antibody. Apply working solution of M.O.M.™ Biotinylated Anti-Mouse IgG Reagent. Incubate sections for 10 minutes.

11. Wash sections 2 x 2 minutes in buffer.

12. Avidin conjugate. Apply Fluorescein Avidin DCS prepared as described in M.O.M.™ kit instructions. Incubate sections for 5 minutes (Please see Note 4).

13. Wash sections 2 x 5 minutes in buffer.

Staining for Second Antigen

14. Avidin/biotin blocking step. Perform Avidin/Biotin blocking according to step 4. (This step must be done to prevent the interaction of the second set of labeling reagents with the first set of labeling reagents).

15. Mouse Ig blocking step. Incubate sections for 1 hour in working solution of M.O.M.™ Mouse Ig Blocking Reagent.

16. Wash sections 2 x 2 minutes in buffer.

17. Protein blocking step. Incubate sections for 5 minutes in working solution of M.O.M.™ diluent.

18. Primary antibody. Tip off excess M.O.M.™ diluent from sections. Dilute second primary antibody in M.O.M.™ diluent to the appropriate concentration. Incubate section for 30 minutes (Please see Note 3).

19. Wash sections 2 x 2 minutes in buffer.

20. Secondary antibody. Apply working solution of M.O.M.™ Biotinylated Anti-Mouse IgG Reagent. Incubate sections for 10 minutes.

21. Wash sections 2 x 2 minutes in buffer.

22. Avidin conjugate. Apply Texas Red® Avidin DCS at a concentration of 15-20 µg/ml in buffer. Incubate sections for 5-10 minutes (Please see Note 4).

23. Wash sections for 2 x 5 minutes in buffer.

24. Mount with appropriate VECTASHIELD® mounting media.

NOTES:

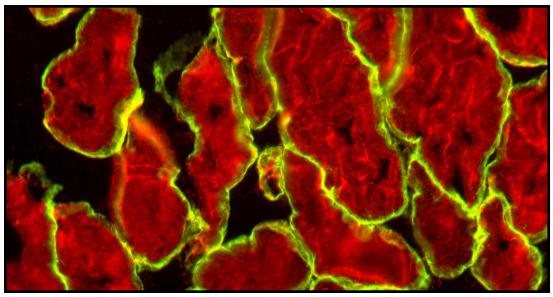
1. Aldehyde-fixed tissues (e.g. formalin) tend to be autofluorescent and may make interpretation of specific fluorescein signal difficult.

2. For non-murine tissue, omit step 5 and step 6.

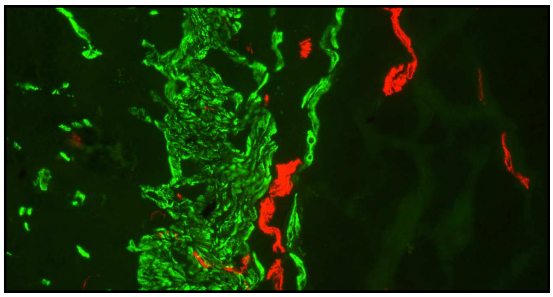
3. Optimal results with the M.O.M.™ kit are usually obtained with a primary antibody incubation of 30 minutes. Primary antibody concentrations should be optimized for multiple labeling applications.

4. Optimal order of the fluorescent label should be determined. Other fluorochrome conjugated streptavidin or avidin reagents can be substituted once optimal signal/noise has been established.

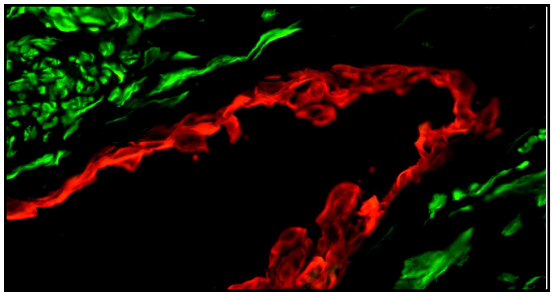
5. A M.O.M.™ Troubleshooting Guide is available online or upon request.



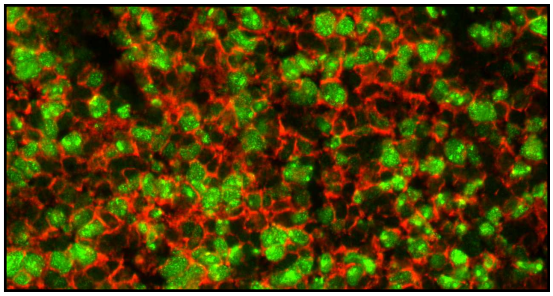
Skeletal Muscle (frozen, unfixed) – Double label
- Alpha-Sarcoglycan (m), M.O.M.™ Fluorescein Kit (green).
- Muscle Specific Actin (m), M.O.M.™ Basic Kit, Texas Red® Avidin DCS (red).



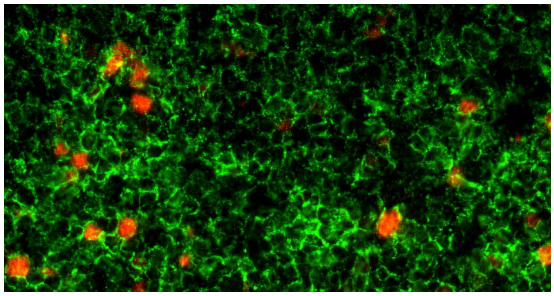
Colon (frozen) – Double label
- Desmin (m), M.O.M.™ Fluorescein Kit (green).
- Peripherin (m), M.O.M.™ Basic Kit, Texas Red® Avidin DCS (red).



Prostate (frozen) – Double label
- Muscle Specific Actin (m), M.O.M.™ Fluorescein Kit (green).
- Multi-Cytokeratin (m), M.O.M.™ Basic Kit, Texas Red® Avidin DCS (red).

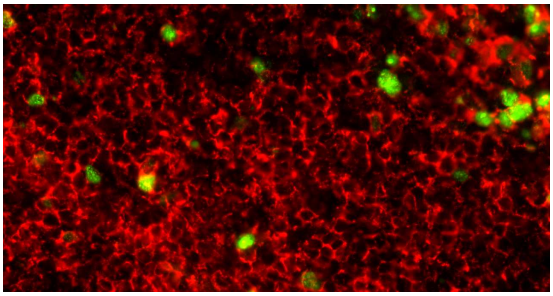


Breast Carcinoma (frozen) – Double label
- Estrogen Receptor (m), M.O.M.™ Fluorescein Kit (green).
- Multi-Cytokeratin (m), M.O.M.™ Basic Kit, VECTASTAIN® ABC-AP Standard Kit, Vector® Red AP substrate (red).



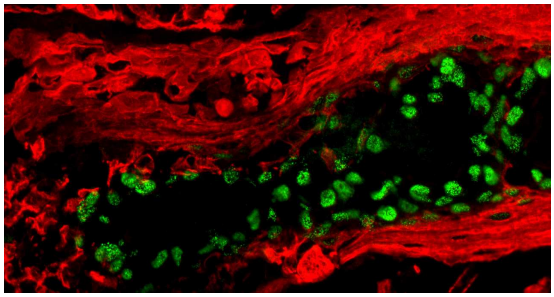
Tonsil (frozen) – Double label
- Ki67 (m), M.O.M.™ Basic Kit, Texas Red® Avidin DCS (red).
- CD20 (m), M.O.M.™ Fluorescein Kit (green).

Compare color contrast with adjacent photo.



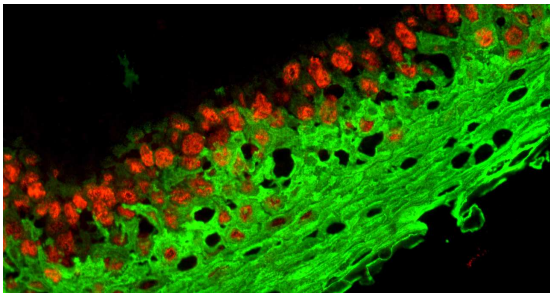
Tonsil (frozen) – Double label
- Ki67 (m), M.O.M.™ Fluorescein Kit (green).
- CD20 (m), M.O.M.™ Basic Kit, Texas Red® Avidin DCS.

Compare color contrast with adjacent photo.



Tonsil (frozen) – Double label
- p63 (m), M.O.M.™ Fluorescein Kit (green).
- Multi-Cytokeratin (m), M.O.M.™ Basic Kit, Texas Red® Avidin DCS (red).

Compare color contrast with adjacent photo.



Tonsil (frozen) – Double label
- p63 (m), M.O.M.™ Basic Kit, Texas Red® Avidin DCS (red).
- Multi-Cytokeratin (m), M.O.M.™ Fluorescein Kit (green).

Compare color contrast with adjacent photo.

Appendix 1: Counterstain/Substrate Compatibility Table

This table is designed as a reference to determine the optimal counterstain/substrate combination for your application.

		Vector® Hematoxylin & Hematoxylin QS H-3401, H-3404	Vector® Methyl Green H-3402	Vector® Nuclear Fast Red H-3403
Substrate	Catalog No.			
DAB (brown)	SK-4100	Excellent Contrast	Excellent Contrast	Fair Contrast
DAB-Ni (gray/black)	SK-4100	Excellent Contrast	Fair Contrast*	Good Contrast
AEC (red)	SK-4200	Excellent Contrast	Counterstain Incompatibility**	Color Incompatibility
TMB (blue)	SK-4400	Color Incompatibility	Counterstain Incompatibility	Excellent Contrast
VECTOR® VIP (purple)	SK-4600	Fair Contrast	Excellent Contrast	Poor Contrast
VECTOR® SG (blue/gray)	SK-4700	Poor Contrast	Good Contrast	Excellent Contrast
VECTOR® NovaRED™ (red)	SK-4800	Excellent Contrast	Excellent Contrast***	Color Incompatibility
VECTOR® RED (red)	SK-5100	Excellent Contrast	Excellent Contrast	Color Incompatibility
VECTOR® BLACK (brown/black)	SK-5200	Excellent Contrast	Excellent Contrast*	Excellent Contrast
VECTOR® BLUE (blue)	SK-5300	Color Incompatibility	Good Contrast	Excellent Contrast
BCIP/NBT (blue/violet)	SK-5400	Color Incompatibility	Excellent Contrast*	Excellent Contrast
GLUCOSE OXIDASE NBT (purple/blue)	SK-3100	Color Incompatibility	Excellent Contrast	Excellent Contrast
GLUCOSE OXIDASE TNBT (black)	SK-3200	Excellent Contrast	Excellent Contrast	Excellent Contrast
GLUCOSE OXIDASE INT (red/purple)	SK-3300	Good Contrast	Counterstain Incompatibility**	Fair Contrast

* This substrate shows a slight decrease in sensitivity following the Methyl Green protocol. This decrease can be minimized by reducing the heat incubation and acetone rinse times in the Methyl Green protocol.
** Substrate dissolves in acetone wash.
***A slight color change in Vector® NovaRED™ reaction product may be seen using Methyl Green.
Counterstains should be optimized for each tissue type, antigen unmasking protocol, and immunocytochemical staining intensity desired.

Appendix 2: Quenching Endogenous Enzyme Activity

Quenching Endogenous Peroxidase Activity

Method 1. 3% H_2O_2 in water. Incubate for 5 minutes. Rinse with water for 2-3 minutes. This is the most rapid and simplest technique for quenching, however the bubbling that might occur may damage the morphology of frozen sections and specimens with large amounts of endogenous enzyme activity (e.g., blood smears, etc.). This is a good general block.

Method 2. 0.3% H_2O_2 in methanol. Incubate for 20-30 minutes. Rinse with water for 2-3 minutes. This is the method of choice for frozen sections and specimens with large amounts of endogenous enzyme activity (blood smears, cytospins, etc.). The concentration of H_2O_2 can be doubled and/or incubation time shortened as appropriate for the specimen. Methanol accelerates the destruction of the heme groups so a lower concentration of H_2O_2 can be used for a longer period of time. This is also a good general block except for cell surface markers.

Method 3. 0.180 g β-D(+) glucose, 5 mg glucose oxidase, 6.5 mg sodium azide in 50 ml PBS. Incubate sections for 1 hour at 37 °C. Rinse in PBS 3 x 5 minutes. This reaction slowly and steadily produces very low concentrations of H_2O_2 by enzymatic reaction. This method consistently and completely inhibits peroxidase activity.

(Andrew S.M., Jasani, B.; Histochem J. 19, 426-430,1987.)

Quenching Endogenous Alkaline Phosphatase Activity

Method 1. If the endogenous activity is an isoenzyme other than the intestinal form, it can be inhibited by the addition of levamisole (Cat. No. SP-5000) to the buffer used to prepare the substrate solution. Levamisole is a competitive inhibitor of most alkaline phosphatase (AP) activity in tissues but is not bound by the isoform of AP used in detection systems.

Intestinal alkaline phosphatase can be inhibited by either of the following two methods:

Method 2. Prior to staining, treat the sections with 20% acetic acid at 4 °C for 15 minutes.

Method 3. Treat the sections with 2.3% periodic acid for 5 minutes and 0.02% potassium borohydride for 2 minutes.

(Bulman A.S. and Heyderman E.; J. Clin. Pathol. 34, 1349-1351, 1981).

Appendix 3: Buffer Recipes for Substrate Solutions

PBS (10 mM phosphate, 150 mM NaCl, pH 7.5)

1.42 g Na_2HPO_4

8.75 g NaCl

to 950 ml distilled water

pH to 7.5 with phosphoric acid, bring up to final volume of 1 liter

100 mM Tris-HCl, pH 8.2 or 9.5

12.11 g Tris Base

to 950 ml distilled water

pH to 8.2 or 9.5 with HCl, bring up to final volume of 1 liter

NOTE: For 200 mM Tris-HCl, double the quantity of Tris Base.