



PREPARATION OF VECTASTAIN® WORKING SOLUTIONS

For convenience, VECTASTAIN® ABC-AP Kits include mixing bottles to prepare working solutions of reagents. As supplied, the drop dispenser tip is in an inverted position and is not inserted into the bottle. After the buffer and appropriate reagents are added to the bottle, insert the drop dispenser top into the white opaque cap in correct orientation. Place the entire unit onto the bottle and twist on the cap. As the cap is tightened, the drop dispenser will snap into place. To remove the drop dispenser top for refilling, merely press laterally with thumb until the top snaps off. Care should be taken to replace the dispenser top on the correct bottle to avoid cross-contaminating reagents. All bottles have color-coded labels to minimize inadvertent use of the wrong mixing bottle. When dispensing drops, hold the bottle in an inverted **vertical** position and squeeze gently. To prevent evaporation, secure the white opaque caps on the bottles when they are not in use.

When using dropper bottles to dispense reagents, apply a sufficient number of drops on the slide to cover the entire section. Slides should then be placed in a humidified chamber during the incubation period. Staining dishes or Coplin jars may also be used in the staining procedure. To make up these working solutions, use the same drop/volume ratio as recommended in the instructions for preparation of dropper bottle reagents but increase the amounts as desired.

A number of different buffers can be used in the VECTASTAIN® ABC-AP system. One of the most common is 10 mM sodium phosphate, pH 7.5, 0.9% saline (PBS). The VECTASTAIN® working solutions are prepared as follows:

- **Blocking Serum (Normal Serum):** add three (3) drops* of stock (yellow label) to 10 ml of buffer in mixing bottle (yellow label).
- **Biotinylated Antibody:** add one (1) drop of stock (blue label) to 10 ml of buffer in mixing bottle (blue label).
- **VECTASTAIN® ABC-AP Reagent:** add exactly two (2) drops of REAGENT A to 10 ml of buffer in the ABC Reagent mixing bottle. Then add exactly two (2) drops of REAGENT B to the same mixing bottle, mix immediately, and allow VECTASTAIN® ABC-AP Reagent to stand for about 30 minutes before use.

* one drop is approximately 50µl.

SUBSTRATES

Alkaline phosphatase catalyzes the hydrolysis of a variety of phosphate-containing substances in the alkaline pH range. The enzymatic activity of alkaline phosphatase can be localized by coupling a soluble product generated during the hydrolytic reaction with a "capture reagent", producing a colored insoluble precipitate. Four different substrate kits are available for use with the VECTASTAIN® ABC-AP Kits. Vector® Red, Vector® Black, Vector® Blue, and BCIP/NBT produce reaction products which are red, black, blue and purple/blue, respectively.

The Vector® Red, Vector® Black, and BCIP/NBT reaction products can be permanently mounted in non-aqueous media. Vector® Blue can also be permanently mounted in non-xylene mounting media if xylene substitutes are used to clear tissue sections. The reagents are supplied in dropper bottles at optimal concentrations to provide at least 200 ml of working substrate solutions. Development times may differ depending upon the level of antigen, the staining intensity desired or the substrate kit used. Generally, the Vector alkaline phosphatase substrate kits should be developed for 20-30 minutes. BCIP/NBT (unlike the other alkaline phosphatase substrates) will provide increased levels of sensitivity if the substrate incubation time is lengthened (up to 24 hours).

Note: The Vector® Red reaction product is a highly fluorescent, bright red precipitate when viewed with rhodamine or Texas Red® filter systems. Vector® Red fluorescence may also be visible with fluorescein or AMCA filter systems using broad band emission filters.

ENDOGENOUS ALKALINE PHOSPHATASE ACTIVITY

Endogenous alkaline phosphatase activity is less common in paraffin sections than in frozen sections and is generally completely absent in sections treated with high temperature to unmask antigens. 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. Intestinal alkaline phosphatase can be inhibited by treating the sections, prior to staining, with 20% acetic acid at 4 °C for 15 seconds or with 2.3% periodic acid for 5 minutes and 0.02% potassium borohydride for 2 minutes (Bulman AS and Heyderman E; J. Clin. Pathol. 34, 1349-1351, 1981).

STAINING PROCEDURE FOR PARAFFIN SECTIONS

1. Deparaffinize and hydrate tissue sections through xylenes or other clearing agents and graded alcohol series.
2. Rinse for 5 minutes in tap water.
3. Wash in buffer for 5 minutes.
4. Incubate sections for 20 minutes with diluted normal blocking serum from the species in which the secondary antibody is made. (In cases where nonspecific staining is not a problem, Steps 4 and 5 may be deleted).
5. Blot excess serum from sections.
6. Incubate sections for 30 minutes with primary antiserum diluted in buffer.
7. Wash slides for 5 minutes in buffer.
8. Incubate sections for 30 minutes with diluted biotinylated secondary antibody solution.
9. Wash slides for 5 minutes in buffer.
10. Incubate sections for 30 minutes with VECTASTAIN® ABC-AP Reagent.
11. Wash slides for 5 minutes in buffer.
12. Incubate sections for 20-30 minutes in alkaline phosphatase substrate solution.
13. Rinse sections in tap water.
14. Counterstain, clear and mount.

STAINING PROCEDURE FOR FROZEN SECTIONS

This procedure is generally appropriate for frozen sections, cell smears or cytocentrifuge preparations.

1. Sections are air dried.
2. Immediately before staining, fix sections with acetone or the appropriate fixative for the antigen under study.
3. Transfer slides directly into buffer.
4. Follow steps 4-14 of the procedure recommended for paraffin sections.

After completion of the staining procedure, dilute working solutions should be discarded, and the containers washed with distilled water and stored together with the stock reagents in the kit box.

MULTIPLE ANTIGEN LABELING ON SAME TISSUE SECTIONS

For photomicrographic examples of substrates, counterstains, and general protocols, please refer to our website or request a free brochure on multiple labeling - "Discovery through color".

VECTASTAIN® ABC-AP KIT

INSTRUCTIONS FOR IMMUNOHISTOCHEMICAL STAINING

INTRODUCTION

Avidin is a 68,000 molecular weight glycoprotein with an extraordinarily high affinity ($10^{15}M^{-1}$) for the small molecular weight vitamin, biotin. Because this affinity is over one million times higher than that of antibody for most antigens, the binding of avidin to biotin (unlike antibody-antigen interactions) is essentially irreversible. In addition to this high affinity, the Biotin/Avidin System can be effectively exploited because avidin has four binding sites for biotin and most proteins (including antibodies and enzymes) can be conjugated with several molecules of biotin. These aspects provide the potential for macromolecular complexes to be formed between avidin and biotinylated enzymes.

An immunoperoxidase procedure based on these properties was devised for localizing a variety of histologically significant antigens and other markers. (Hsu SM, Raine L, Fanger H: *Am. J. Clin. Pathol.* 75, 734-738, 1981; Hsu SM, Raine L, Fanger H: *J. Histochem. Cytochem.* 29, 577-580, 1981.) This technique employs unlabeled primary antibody, followed by biotinylated secondary antibody and then a preformed Avidin and Biotinylated horseradish peroxidase macromolecular Complex. This has been termed the ABC technique. The reagents necessary for the ABC technique have been specially formulated and made available as VECTASTAIN® ABC kits.

VECTASTAIN® ABC Kits are also available with alkaline phosphatase (AP) as the enzyme marker. VECTASTAIN® ABC-AP Kits contain a special form of Avidin DH and biotinylated alkaline phosphatase H. Although the structure of the ABC-AP has not been determined, it is likely that the complex is similar to that of the avidin - biotinylated peroxidase ABC. It probably consists of many biotinylated alkaline phosphatase molecules crosslinked by avidin into a three dimensional array. The complex apparently has few exposed biotin residues but retains at least one biotin binding site. Formation of the complex is achieved by mixing Avidin DH and biotinylated alkaline phosphatase H in dilute solution and in defined amounts prior to use. The complex remains stable for approximately 24 hours after formation.

The sensitivity of the VECTASTAIN® ABC-AP system is equivalent to the peroxidase-based *Elite* ABC System. This feature allows lower amounts of antigen to be detected or permits higher dilutions of primary antibody to be used. The ABC-AP system is recommended in staining situations where high sensitivity is a prerequisite. The VECTASTAIN® ABC-AP system is also recommended when a high level of endogenous peroxidase activity precludes the use of the peroxidase-based ABC system. Alkaline phosphatase substrates produce a more translucent reaction product than peroxidase substrates and provide additional color choices for single or double labeling.

RAPID STAINING PROCEDURE

The sensitivity of the VECTASTAIN® ABC-AP Kit permits development of shortened alkaline phosphatase staining protocols. In this section some guidelines are provided for a rapid staining method having a sensitivity and staining quality equivalent to the full-length VECTASTAIN® ABC-AP protocol.

1. Prepare paraffin-embedded or frozen sections for staining as described previously.
2. Prepare VECTASTAIN® ABC-AP Kit reagents as follows:
For the Biotinylated Antibody, add one drop concentrated stock to 5 ml of PBS containing 1.5% normal serum. If background staining is a problem, increase the concentration of normal serum up to 10%. For the ABC-AP Reagents, add two drops of Reagent A to 5.0 ml buffer, mix, then add two drops of Reagent B, mix. Allow to stand for 5-30 minutes before use.
3. If background staining is a problem, incubate sections for 5-10 minutes in 2% -10% normal serum in buffer.
4. Incubate sections with primary antibody.*
5. Wash gently with a stream of buffer from a wash bottle.
6. Incubate sections for 10 minutes with diluted biotinylated secondary antibody.
7. Wash as in step 5.
8. Incubate sections for 5 minutes with VECTASTAIN® ABC-AP Reagent.
9. Wash as in step 5.
10. Incubate sections in alkaline phosphatase substrate solution until desired stain intensity develops.
11. Wash as in step 5.
12. Counterstain, clear and mount.

*The concentration, staining time, and incubation temperature is dependent upon the primary antibody used.

NOTE: A very rapid procedure that provides excellent staining results can also be performed. Prepare diluted biotinylated secondary antibody 1 drop/2.5 ml. Prepare VECTASTAIN® ABC-AP Reagent as in the above protocol. Apply diluted VECTASTAIN® ABC-AP Kit reagents preheated to 37 °C. Incubate sections in each reagent for 2 minutes.

NOTES:

1. The section should be well prepared. Fixation (generally, in buffered formalin not exceeding 4 percent formaldehyde) should be sufficient to maintain the integrity of the section throughout the staining procedure but not so harsh as to destroy the antigen under study. During the staining procedure, do not allow the section to dry out. Use a humidified chamber for incubations.

2. Primary antibodies can be used at higher dilutions because of the sensitivity of the VECTASTAIN® ABC-AP system. To avoid adsorption of the antibody to the plastic or glass container in which the final dilution is made, the primary antibody may be diluted in buffers containing 0.1% immunohistochemical grade bovine serum albumin (Cat. No. SP-5050) or dilute Blocking Serum (2 drops of concentrated stock in 10 ml of buffer) included in the kit.

3. Use only freshly prepared buffers. Bacterial contamination which can occur in buffers stored at room temperature may affect the quality of the staining. It is recommended that the VECTASTAIN® ABC-AP Reagent and substrate solution be prepared in buffers made with glass distilled water.

4. Stock VECTASTAIN® ABC-AP Kit reagents should be stored under refrigeration. For best results, the VECTASTAIN® ABC Kit reagents should be used before the date shown on the bottom of the box.

5. Use of alkaline phosphatase detection systems in neural tissues may not produce optimal staining results of fibers, processes or terminals. The VECTASTAIN® *Elite* ABC peroxidase system is recommended for neural tissue.



6. Although the affinity-purified biotinylated secondary antibody and the normal serum provided in VECTASTAIN® ABC-AP Kits can be purchased individually, the Avidin DH and biotinylated alkaline phosphatase H are prepared especially for the VECTASTAIN® ABC-AP Kits and are matched reagents. Do not use an A reagent from one kit with a B reagent from another kit. We recommend that they be kept in the box in which they were supplied. If reagents are removed from the box please note on them the date shown on the bottom of the box so that specific lots of reagents can be traced. Do not confuse these reagents with Cat. Nos. A-2000 and B-2005. We recommend using only ABC-AP reagents provided in the VECTASTAIN® ABC-AP Kits. Biotinylated secondary antibody and normal serum can be purchased separately, and the Avidin DH and biotinylated alkaline phosphatase H are available as the VECTASTAIN® ABC-AP Standard Kit.

7. Sections which are thicker than normal may require longer incubation times for optimal staining.

8. Specimens should not be embedded in paraffin heated higher than 60 °C. Too much heat can destroy antigens. After mounting, paraffin sections should be dried in a hot air oven at 50-56 °C. Some slide warmers contain "hot spots" that can overheat tissues.

9. To prevent sections from detaching from the glass, slides can be treated with VECTABOND™ Reagent (Cat. No. SP-1800), a non-protein tissue section adhesive. Do not use egg albumin coated slides. Traces of egg white avidin may affect staining quality.

10. Complete deparaffinization is important. Clearing agents and alcohol solutions should be changed regularly. All steps of the deparaffinization should be sufficiently long to completely remove the paraffin from the sections.

11. Hand lotions prevent adequate penetration of reagents. Avoid touching rinse baths with oily hands.

NOTE: If more dilute reagents are used, first prepare the biotinylated antibody and VECTASTAIN® ABC-AP reagent as described in the instructions. Subsequent dilutions should be made in a buffer containing 0.1% immunohistochemical grade bovine serum albumin (Cat. No. SP-5050), as other BSA preparations can contain undesired impurities. Dilution of these reagents may require longer incubation times and/or higher incubation temperatures to achieve maximum sensitivities.

VECTASTAIN® ABC-AP Kits Available:

Each kit contains sufficient reagents to prepare approximately 220 ml of each working solution. Generally 1000-2000 sections can be stained per kit.

VECTASTAIN® ABC-AP Kit (Standard)	AK-5000
This Standard Kit consists of only the ABC-AP reagent	
VECTASTAIN® ABC-AP Kit (Goat IgG)	AK-5005
VECTASTAIN® ABC-AP Kit (Guinea Pig IgG)	AK-5007
VECTASTAIN® ABC-AP Kit (Human IgG)	AK-5003
VECTASTAIN® ABC-AP Kit (Human IgM)	AK-5009
VECTASTAIN® ABC-AP Kit (Mouse IgG)	AK-5002
VECTASTAIN® ABC-AP Kit (Mouse IgM)	AK-5010
VECTASTAIN® ABC-AP Kit (Rabbit IgG)	AK-5001
VECTASTAIN® ABC-AP Kit (Rat IgG)	AK-5004
VECTASTAIN® ABC-AP Kit (Sheep IgG)	AK-5006
VECTASTAIN® ABC-AP Kit (Universal)	AK-5200

Alkaline Phosphatase Substrate Kits Available:

Each kit provides sufficient stock reagents to prepare approximately 200 ml of substrate solution.

VECTOR® Red	- Alkaline Phosphatase Substrate Kit I	SK-5100
VECTOR® Black	- Alkaline Phosphatase Substrate Kit II	SK-5200
VECTOR® Blue	- Alkaline Phosphatase Substrate Kit III	SK-5300
BCIP/NBT	- Alkaline Phosphatase Substrate Kit IV	SK-5400

The following biotinylated antibodies can be used in conjunction with the VECTASTAIN® ABC-AP Kit:

Biotinylated "Universal" Anti-Mouse/Rabbit IgG (H + L) † made in horse	2.1 mg	BA-1400
Biotinylated "Universal" Pan-Specific Anti-Mouse/Rabbit/Goat IgG (H + L) made in horse	2.2 ml	BA-1300
Biotinylated Anti-Cat IgG (H + L) made in goat	1.5 mg	BA-9000
Biotinylated Anti-Chicken IgG (H + L) made in goat	1.5 mg	BA-9010
Biotinylated Anti-Goat IgG (H + L) * † made in rabbit	1.5 mg	BA-5000
Biotinylated Anti-Goat IgG (H + L) * made in horse	1.5 mg	BA-9500
Biotinylated Anti-Guinea Pig IgG (H + L) † made in goat	1.5 mg	BA-7000
Biotinylated Anti-Hamster IgG (H + L) made in goat	1.5 mg	BA-9100
Biotinylated Anti-Horse IgG (H + L) made in goat	1.5 mg	BA-8000
Biotinylated Anti-Human IgA (α chain specific) made in goat	0.5 mg	BA-3030
Biotinylated Anti-Human IgE (ε chain specific) made in goat	0.5 mg	BA-3040
Biotinylated Anti-Human IgG (H + L) † made in goat	1.5 mg	BA-3000
Biotinylated Anti-Human IgG (γ chain specific) made in goat	0.5 mg	BA-3080
Biotinylated Anti-Human IgM † (μ chain specific) made in goat	0.5 mg	BA-3020
Biotinylated Anti-Human Kappa Chain (κ chain specific) made in goat	0.5 mg	BA-3060
Biotinylated Anti-Human Lambda Chain (λ chain specific) made in goat	0.5 mg	BA-3070
Biotinylated Anti-Mouse IgG (H + L) † made in horse	1.5 mg	BA-2000
Biotinylated Anti-Mouse IgG (H + L) made in goat	1.5 mg	BA-9200
Biotinylated Anti-Mouse IgG (H + L) (Rat Adsorbed) made in horse	0.5 mg	BA-2001
Biotinylated Anti-Mouse IgM † (μ chain specific) made in goat	0.5 mg	BA-2020
Biotinylated Anti-Rabbit IgG (H + L) † made in goat	1.5 mg	BA-1000
Biotinylated Anti-Rat IgG (H + L) † made in rabbit	1.5 mg	BA-4000
Biotinylated Anti-Rat IgG (H + L) made in goat	1.5 mg	BA-9400
Biotinylated Anti-Rat IgG (H + L) (Mouse Adsorbed) made in rabbit	0.5 mg	BA-4001
Biotinylated Anti-Sheep IgG (H + L) † made in rabbit	1.5 mg	BA-6000
Biotinylated Anti-Swine IgG (H + L) made in goat	1.5 mg	BA-9020

* Use with Bovine IgG primary antibodies.

† Antibodies included in VECTASTAIN® ABC-AP Kits.

Other related reagents also available are:

Antigen Unmasking Solution (dilutes to 25 liters)				
Citrate-based	250 ml	H-3300		
High pH	250 ml	H-3301		
Avidin/Biotin Blocking Kit	1 Kit	SP-2001		
ImmEdge™ Hydrophobic Barrier Pen	2-pen set	H-4000		
ImmPrint™ Permanent Marking Histology Pen	5-pen set	H-6100		
Levamisole Solution	18 ml	SP-5000		
Vectabond™ Reagent (dilutes to 350 ml)	7 ml	SP-1800		
VectaMount™ Permanent Mounting Medium	60 ml	H-5000		
VectaMount™ AQ Aqueous Mounting Medium	60 ml	H-5501		
Vector® Hematoxylin	500 ml	H-3401		
Vector® Hematoxylin QS	100 ml	H-3404		
Vector® Methyl Green	500 ml	H-3402		
Vector® Nuclear Fast Red	500 ml	H-3403		

Heat-treated, ultrafiltered normal serum from				
Goat	20 ml	S-1000	Chicken	20 ml
Horse	20 ml	S-2000	Swine	20 ml
Rabbit	20 ml	S-5000		

Control Antibodies		
Goat IgG	5 mg	I-5000
Mouse IgG	1 mg	I-2000
Rabbit IgG	5 mg	I-1000
Rat IgG	1 mg	I-4000

Detailed product listings, specifications and protocols are available on our website: www.vectorlabs.com

VECTASTAIN® ABC-AP Reagents and Kits are designed for laboratory use only.