



VECTASTAIN® ABC 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.*

VECTASTAIN® ABC KITS contain Avidin DH and biotinylated horseradish peroxidase H reagents, which have been specially prepared to form ideal complexes for immunoperoxidase staining. Although the structure of the Avidin DH : biotinylated horseradish peroxidase H complex is still undefined, evidence suggests that it consists of many biotinylated horseradish peroxidase 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 horseradish peroxidase H in dilute solution and in defined amounts prior to use. After the initial incubation there appears to be little change in the complex as judged by only a marginal increase in immunoperoxidase staining sensitivity and the complex remains stable for several hours after formation.

The high sensitivity and shorter incubation times reported for the VECTASTAIN® ABC system are likely due to the number of active horseradish peroxidase molecules associated with the complex and the rapid, irreversible interaction of the complex with biotinylated antibody. The low background staining obtainable with the VECTASTAIN® ABC Kits is probably due to the high dilutions of primary antisera and other reagents employed in the method, the quality of our affinity-purified biotinylated secondary antibodies, and the specially prepared Avidin DH and biotinylated horseradish peroxidase H.

* A further improvement in this original principle is the basis for a more sensitive version: the VECTASTAIN® *Elite* ABC Kit. Refer to our website for more information on the VECTASTAIN® *Elite* ABC Kits.

VECTASTAIN® ABC KIT INSTRUCTIONS

Reagents supplied:

- Blocking Serum (Normal Serum) in yellow-labeled small bottle – 3 ml
- Biotinylated, Affinity-purified Anti-Immunoglobulin in blue-labeled small bottle – 1 ml
- Reagent A (Avidin DH) in orange-labeled small bottle – 2 ml
- Reagent B (Biotinylated Horseradish Peroxidase H) in brown-labeled small bottle – 2 ml

NOTE: The VECTASTAIN® ABC Kit (Standard), Cat. No. PK-4000, contains only Reagent A and Reagent B.

Reagents not supplied:

- Primary Antibody
- Buffer
- Hydrogen Peroxide
- Oxidizable Peroxidase Substrate

PREPARATION OF VECTASTAIN® WORKING SOLUTIONS

For convenience, VECTASTAIN® ABC 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 tip 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 tip for refilling, merely press laterally with thumb until the tip snaps off. Care should be taken to replace the dispenser tip 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.

The VECTASTAIN® ABC Kit has been designed to be used with any of our extensive range of biotin-labeled reagents. As specific examples, the instructions below detail the use of normal serum and biotinylated secondary antibody from VECTASTAIN® ABC Kits, which are supplied in dropper bottles. If purchased separately from the VECTASTAIN® ABC Kit, the biotinylated antibody is supplied lyophilized and should be reconstituted in 1 ml of distilled water or according to instructions.

A number of different buffers can be used in the VECTASTAIN® ABC 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). The preferred serum for blocking is prepared from the same species in which the biotinylated secondary antibody is made.
- **Biotinylated Antibody:** add one (1) drop of stock (blue label) to 10 ml of buffer in mixing bottle (blue label).
- **VECTASTAIN® ABC Reagent:** add exactly two (2) drops of REAGENT A (orange label) to 10 ml of buffer in the ABC Reagent mixing bottle. Then add exactly two (2) drops of REAGENT B (brown label) to the same mixing bottle, mix immediately, and allow VECTASTAIN® ABC Reagent to stand for about 30 minutes before use.

* one drop is approximately 50µl.

ENZYME SUBSTRATES

A variety of chromogens can be used to localize peroxidase in tissue sections. Vector Laboratories offers the traditional substrates DAB and AEC as well as several proprietary substrates, producing colors as listed below:

ImmPACT™ DAB, SK-4105, brown
ImmPACT™ AEC*, SK-4205, red
ImmPACT™ VIP, SK-4605, purple
ImmPACT™ SG, SK-4705, blue/gray
ImmPACT™ NovaRED™, SK-4805, red
Diaminobenzidine (DAB), SK-4100, brown
DAB + Ni²⁺, SK-4100, gray/black
3-amino-9-ethyl carbazole (AEC)*, SK-4200, red
TMB, SK-4400, blue
Vector® VIP, SK-4600, purple
Vector® SG, SK-4700, blue-gray
Vector® NovaRED™, SK-4800, red

* AEC is soluble in alcohol and clearing agents and must be mounted in aqueous mounting media. All other substrates are not soluble in alcohol or clearing agents. They may be dehydrated, cleared, and permanently mounted.

These substrates can be used as single labels or to introduce multiple colors in a tissue section.

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. If quenching of endogenous peroxidase activity is required, incubate the sections for 30 minutes in 0.3% H₂O₂ in methanol or water. Incubation times may be shortened by using higher concentrations of H₂O₂. If endogenous peroxidase activity does not present a problem, step 3 may be deleted.
4. Wash in buffer for 5 minutes.
5. Incubate sections for 20 minutes with diluted normal serum (large yellow-labeled bottle) from the species in which the secondary antibody is made. (In cases where non-specific staining is not a problem, Steps 5 and 6 may be deleted).
6. Blot excess serum from sections.
7. Incubate sections for 30 minutes with primary antiserum diluted in buffer. (If background staining occurs, dilutions of the primary and secondary antibodies may be made in buffer containing 1-2% of the appropriate blocking serum.)
8. Wash slides for 5 minutes in buffer.
9. Incubate sections for 30 minutes with diluted biotinylated secondary antibody solution (large blue-labeled bottle).
10. Wash slides for 5 minutes in buffer.
11. Incubate sections for 30 minutes with VECTASTAIN® ABC Reagent.
12. Wash slides for 5 minutes in buffer.
13. Incubate sections in peroxidase substrate solution until desired stain intensity develops. (See Note 1)
14. Rinse sections in tap water.
15. Counterstain, clear and mount.

* If antigen unmasking is required, perform this procedure after step 2, using Cat. No. H-3300 (citrate-based) or H-3301 (high pH).

If unwanted staining occurs in the absence of biotinylated secondary antibody, endogenous protein-associated biotin may be present in the tissue. To eliminate this unwanted staining, use an Avidin/Biotin blocking step (Cat. No. SP-2001) between steps 4 and 5.

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 into buffer.
4. If quenching of endogenous peroxidase is required, follow the procedure in step 3 of the paraffin section protocol. In some cases, especially when using monoclonal antibodies, the antigen may be destroyed by treatment with H₂O₂. In these cases, this treatment should be used only after incubation with biotinylated antibody.
5. Follow steps 4-15 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 SECTION

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".

NOTES:

1. Solutions containing sodium azide or other inhibitors of peroxidase activity should not be used in diluting the peroxidase substrate or the VECTASTAIN® ABC Reagent. Do not add normal serum, non-fat dried milk, culture media, or other potential sources of biotin to the ABC reagent. This may result in reduced sensitivity.
2. Development times may differ depending upon the level of antigen, the intensity of the stain that is required, or the substrate used. ImmPACT™ DAB and DAB generally should be developed for 2-10 minutes; ImmPACT™ VIP and Vector® VIP for 2-15 minutes; ImmPACT™ SG and Vector® SG for 2-10 minutes; ImmPACT™ NovaRED™ and Vector® NovaRED™ for 2-15 minutes; ImmPACT™ AEC and AEC for 10-30 minutes; TMB for 5-20 minutes. Some counterstains may not be compatible with certain peroxidase substrates because of solubility of the reaction products or lack of color contrast. A counter-stain compatibility chart is available upon request. Refer to the instructions in the respective substrate kits for further details.
3. In the presence of nickel ions, the precipitate formed by DAB is gray/black rather than brown. This may enhance the sensitivity of the staining procedure and, because of the difference in color from DAB alone, has been used in double-labeling techniques. The DAB Substrate Kit (Cat. No. SK-4100) contains nickel chloride and allows two colors to be introduced into the section.
4. If the reagents are to be diluted beyond their recommended concentrations, first prepare the diluted biotinylated antibody and VECTASTAIN® ABC 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). Only immunohistochemical grade BSA should be used, as other preparations can contain undesired impurities. Dilution of these reagents may require longer incubation times and/or higher incubation temperatures to achieve maximum sensitivities.
5. 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. In some cases, use of Antigen Unmasking Solution (Cat. No. H-3300 or H-3301) and exposure to high temperatures can overcome loss of antigens due to fixation.

6. 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 or dilute Blocking Serum.
7. Incubation times may be shortened. In cases where the antigen concentration in the section is high, suggested incubation times with primary antibody, biotinylated secondary antibody, and VECTASTAIN® ABC Reagent may be reduced. Incubation times as short as five minutes have been reported to be sufficient in some cases when incubation temperatures are raised to 37 °C. If the antigen concentration is low, steps 7 and 9 may be lengthened to achieve maximal staining.
8. 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 Reagent and substrate solution be prepared with glass distilled water. Deionized water (even with low conductivities) may contain inhibitors of peroxidase and can reduce sensitivity.
9. Stock VECTASTAIN® ABC 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. The A and B reagents in the kits are matched. 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.
10. Although the affinity-purified biotinylated secondary antibody and the normal serum provided in VECTASTAIN® ABC Kits can be purchased individually, the Avidin DH and biotinylated horseradish peroxidase H are prepared especially for the VECTASTAIN® ABC Kits and are matched reagents. Do not confuse these with Cat. Nos. A-2000 and B-2004. We recommend using only ABC reagents provided in the VECTASTAIN® ABC kits.
11. Sections of neuronal tissue or sections which are thicker than normal may require longer incubation times for optimal staining.
12. Specimens should not be embedded in paraffin heated higher than 60 °C. Too much heat can destroy antigens.
13. 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.
14. 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.
15. Hand lotions can cause sections to detach from slides or may prevent adequate penetration of reagents. Avoid touching rinse baths with oily hands.
16. Paraffin tissue blocks should be stored in sealed containers in a cool location.
17. 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.
18. If smaller volumes of working solutions are desired, it is recommended that a drop from the stock solution be dispensed into a small, conical plastic tube. A suitable aliquot can then be withdrawn. To avoid the risk of introducing contaminants, do not remove the drop dispensers from the stock solution bottles.
19. If staining of mast cells or other tissue elements occurs in the absence of primary and secondary antibodies, prepare the ABC reagent in buffer containing 0.3M-0.5M NaCl. This will eliminate undesirable ionic interactions. If this fails to eliminate unwanted staining, use an Avidin/Biotin blocking step (Cat. No. SP-2001) after the serum block step.

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 Kit (Standard)	1 Kit	PK-4000
This Standard Kit consists of only the ABC reagents.		
VECTASTAIN® ABC Kit (Goat IgG)	1 Kit	PK-4005
VECTASTAIN® ABC Kit (Guinea Pig IgG)	1 Kit	PK-4007
VECTASTAIN® ABC Kit (Human IgG)	1 Kit	PK-4003
VECTASTAIN® ABC Kit (Human IgM)	1 Kit	PK-4009
VECTASTAIN® ABC Kit (Mouse IgG)*	1 Kit	PK-4002
VECTASTAIN® ABC Kit (Mouse IgM)*	1 Kit	PK-4010
VECTASTAIN® ABC Kit (Rabbit IgG)	1 Kit	PK-4001
VECTASTAIN® ABC Kit (Rat IgG)	1 Kit	PK-4004
VECTASTAIN® ABC Kit (Sheep IgG)	1 Kit	PK-4006

* For staining mouse primary antibodies on mouse tissue use the **Vector® M.O.M.™ (Mouse on Mouse) Peroxidase Kit** (Cat. No. PK-2200), providing sufficient reagents to stain about 250 sections.

The following biotinylated antibodies can be used in conjunction with any VECTASTAIN® ABC 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-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 IgG (H + L)◇ made in goat †	1.5 mg	BA-3000
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-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) (Mouse Adsorbed) made in rabbit	1.5 mg	BA-9400
Biotinylated Anti-Sheep IgG (H + L) made in rabbit †	0.5 mg	BA-4001
Biotinylated Anti-Swine IgG (H + L) made in goat	1.5 mg	BA-6000
Biotinylated Anti-Swine IgG (H + L) made in goat	1.5 mg	BA-9020

* Use with Dog IgG primary antibodies.

** Use with Bovine IgG primary antibodies.

† Antibodies included in VECTASTAIN® ABC Kits.

‡ Anti-Mouse/Rabbit IgG

◇ Chain-specific antibodies are also available.

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™ Histology Pen	5-pen set		H-6100	
Vectabond™ Reagent (dilutes to 350 ml)	7 ml		SP-1800	
VectaMount™ Mounting Medium	60 ml		H-5000	
VectaMount™ AQ 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	S-3000
Horse	20 ml	S-2000	Swine	20 ml	S-4000
Rabbit	20 ml	S-5000			

Peroxidase Substrate Kits (Approximately 300 ml of working solution.)

ImmPACT™ DAB (brown)	120 ml	SK-4105
ImmPACT™ AEC (red)	120 ml	SK-4205
ImmPACT™ VIP (purple)	120 ml	SK-4605
ImmPACT™ SG (blue/gray)	120 ml	SK-4705
ImmPACT™ NovaRED™ (red)	120 ml	SK-4805
DAB/Ni Substrate (brown or gray/black)	1 Kit	SK-4100
AEC Substrate (red)	1 Kit	SK-4200
TMB Substrate (blue)	1 Kit	SK-4400
Vector® VIP Substrate (purple)	1 Kit	SK-4600
Vector® SG Substrate (blue/gray)	1 Kit	SK-4700
Vector® NovaRED™ Substrate (red)	1 Kit	SK-4800

VECTASTAIN® ABC Reagents and Kits are designed to be used for laboratory use only.

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