



VECTASTAIN® *Elite*® ABC KIT

INSTRUCTIONS FOR IMMUNOHISTOCHEMICAL STAINING

INTRODUCTION

The VECTASTAIN® ABC system is widely accepted as one of the most sensitive, economical and reliable immunoperoxidase systems available. For several years our efforts were devoted to improving the original ABC system. This research led to the development of the VECTASTAIN® *Elite* ABC Kit, which is considerably more sensitive than the original VECTASTAIN® ABC peroxidase system without increased background staining. This enhanced sensitivity is particularly important in the localization of antigens present in low amounts or in cases where the cost of primary antibodies is significant. The increased sensitivity also provides an option to substantially reduce staining times. The VECTASTAIN® *Elite* ABC Kit is based on the same patented principles as the other VECTASTAIN® ABC Kits outlined below.

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® *Elite* 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. The complex remains stable for several hours after formation. For long term stability, we recommend using the R.T.U. VECTASTAIN® *Elite* ABC products. The *Elite* is 5 times more sensitive than the original ABC kit.

PREPARATION OF VECTASTAIN® WORKING SOLUTIONS

For convenience, VECTASTAIN® *Elite* 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 or gray 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. When dispensing drops, hold the bottle in an inverted vertical position and squeeze gently. To prevent evaporation, secure the opaque white or gray caps on the bottles when they are not in use. 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.

When using mixing 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 mixing bottle reagents but increase the amounts as desired.

The VECTASTAIN® *Elite* 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.

A number of different buffers can be used in the VECTASTAIN® *Elite* 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 (150 µl) 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 three (3) drops (150 µl) of normal blocking serum stock (yellow label) to 10 ml buffer in mixing bottle and then add one (1) drop (50 µl) of biotinylated antibody stock (blue label).
- VECTASTAIN® *Elite* ABC Reagent: add exactly two (2) drops of REAGENT A (gray label) to 5 ml of buffer in the ABC Reagent large mixing bottle. Then add exactly two (2) drops of REAGENT B (gray label) to the same mixing bottle, mix immediately, and allow VECTASTAIN® *Elite* ABC Reagent to stand for about 30 minutes before use.

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 either 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 omitted.
 4. Wash in buffer for 5 minutes.
 5. Incubate sections for 20 minutes with diluted normal blocking serum which was prepared 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 omitted).
 6. Blot excess serum from sections.
 7. Incubate sections for 30 minutes with primary antibody diluted in buffer. (see Note 6).
 8. Wash slides for 5 minutes in buffer.
 9. Incubate sections for 30 minutes with diluted biotinylated secondary antibody solution.
 10. Wash slides for 5 minutes in buffer.
 11. Incubate sections for 30 minutes with VECTASTAIN® *Elite* ABC Reagent.
 12. Wash slides for 5 minutes in buffer.
 13. Incubate sections in peroxidase substrate solution until desired stain intensity develops (see Note 2).
 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, use gentle H₂O₂ blocking to reduce the risk of antigen destruction or tissue loss: 0.3% H₂O₂ in 0.3% Normal Sera in PBS for 5 minutes; or 0.3% H₂O₂ in methanol for 30 minutes, or use other published methods (e.g. Andrew, S. M., Jasani, B., *Histochem J.* 1987, **19**, 426-430). If necessary, H₂O₂ treatment may also be performed after the biotinylated secondary antibody step.
5. Follow steps 4-15 of the procedure recommended for paraffin sections.

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".

RAPID STAINING PROCEDURE

The sensitivity of the VECTASTAIN® *Elite* ABC Kit permits development of shortened immunoperoxidase 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® *Elite* ABC protocol.

1. Prepare paraffin-embedded or frozen sections for staining as described elsewhere. Prepare VECTASTAIN® *Elite* ABC 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 VECTASTAIN® *Elite* ABC Reagent, add two drops of Reagent A to 2.5 ml buffer, mix, then add two drops of Reagent B. Mix and allow to stand for 5-30 minutes before use.
2. If quenching of endogenous peroxidase is required, an accelerated quenching procedure can be employed. Treat sections with 3% hydrogen peroxide in water for 3-5 minutes.
3. Wash gently with a stream of buffer from a wash bottle.
4. If background staining is a problem, incubate sections for 5-10 minutes in 2% -10% normal serum in buffer.
5. Incubate sections with primary antibody.*
6. Wash as in step 3.
7. Incubate sections for 10 minutes with diluted biotinylated secondary antibody.
8. Wash as in step 3.
9. Incubate sections for 5 minutes with VECTASTAIN® *Elite* ABC Reagent.
10. Wash as in step 3.
11. Incubate sections in peroxidase substrate solution until desired stain intensity develops (see Note 2).
12. Wash as in step 3.
13. Counterstain, clear and mount.

*The concentration, staining time and temperature of the primary antibody should be tailored to an investigator's particular requirements. The increased sensitivity of the VECTASTAIN® *Elite* ABC Kit allows shorter primary antibody incubation times. For example, at primary antibody concentrations optimal for the regular VECTASTAIN® ABC Kit, incubation times can be reduced at least in half when using the VECTASTAIN® *Elite* ABC Kit. Higher concentrations of primary antibody allow even shorter incubation times.

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® *Elite* ABC Reagent as in the above protocol. Apply diluted VECTASTAIN® *Elite* ABC Kit reagents preheated to 37 °C. Incubate sections in each reagent for 2 minutes.

A VECTASTAIN® Universal **Quick** Kit (Cat. No. PK-8800), based on a pre-formed streptavidin/peroxidase complex, is also available to perform rapid immunohistochemical staining.

NOTES:

1. Solutions containing sodium azide or other inhibitors of peroxidase activity should not be used in diluting the peroxidase substrate or the VECTASTAIN® *Elite* 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 counterstain 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® *Elite* 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. 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. During the staining procedure, do not allow the section to dry out. Use a humidified chamber for incubations.
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. 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® *Elite* 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.
8. Stock VECTASTAIN® *Elite* ABC Kit reagents should be stored under refrigeration. For best results, the VECTASTAIN® *Elite* 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.
9. Although the affinity-purified biotinylated secondary antibody and the normal serum provided in VECTASTAIN® *Elite* ABC Kits can be purchased individually, the Avidin DH and biotinylated horseradish peroxidase H are prepared especially for the VECTASTAIN® *Elite* 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® *Elite* ABC kits.
10. Sections of neuronal tissue or sections which are thicker than normal may require longer incubation times for optimal staining.
11. Specimens should not be embedded in paraffin heated higher than 60 °C. Too much heat can destroy antigens.
12. 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.
13. 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.
14. Hand lotions can cause sections to detach from slides or may prevent adequate penetration of reagents. Avoid touching rinse baths with oily hands.
15. Paraffin tissue blocks should be stored in sealed containers in a cool location.
16. 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.
17. 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.
18. 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.

The VECTASTAIN® *Elite* ABC Kit contains sufficient reagents to prepare approximately 110 ml of each working solution (500-1000 sections).

VECTASTAIN® <i>Elite</i> ABC Kit (Standard)	1 Kit	PK-6100
This Standard Kit consists of only the ABC <i>Elite</i> reagents.		
VECTASTAIN® <i>Elite</i> ABC Kit (Goat IgG)	1 Kit	PK-6105
VECTASTAIN® <i>Elite</i> ABC Kit (Human IgG)	1 Kit	PK-6103
VECTASTAIN® <i>Elite</i> ABC Kit (Mouse IgG)	1 Kit	PK-6102
VECTASTAIN® <i>Elite</i> ABC Kit (Rabbit IgG)	1 Kit	PK-6101
VECTASTAIN® <i>Elite</i> ABC Kit (Rat IgG)	1 Kit	PK-6104
VECTASTAIN® <i>Elite</i> ABC Kit (Sheep IgG)	1 Kit	PK-6106
VECTASTAIN® <i>Elite</i> ABC Kit (Universal) ‡	1 Kit	PK-6200

The VECTASTAIN® *Elite* ABC Reagent and VECTASTAIN® *Elite* ABC Universal Kit are available in ready-to-use (R.T.U.), prediluted formats.

R.T.U. VECTASTAIN® <i>Elite</i> ABC Reagent	50 ml	PK-7100
R.T.U. VECTASTAIN® <i>Elite</i> ABC Kit (Universal) ‡	50 ml	PK-7200

The following biotinylated antibodies can be used in conjunction with the VECTASTAIN® *Elite* 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-9500
Biotinylated Anti-Hamster IgG (H + L) made in goat	1.5 mg	BA-7000
Biotinylated Anti-Horse IgG (H + L) made in goat	1.5 mg	BA-9100
Biotinylated Anti-Human IgG (H + L) †	1.5 mg	BA-8000
Biotinylated Anti-Mouse IgG (H + L) †	1.5 mg	BA-3000
Biotinylated Anti-Mouse IgG (H + L) †	1.5 mg	BA-2000
Biotinylated Anti-Mouse IgG (H + L) (Rat Adsorbed) made in horse	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) 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 Dog IgG primary antibodies.

** Use with Bovine IgG primary antibodies.

† Antibodies included in VECTASTAIN® *Elite* 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 Substrates

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

The kits provide sufficient stock reagents to prepare about 300 ml of substrate solution.

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

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VECTASTAIN® *Elite* ABC Reagents and Kits are designed for laboratory use only.

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