

Detection of Vascular Endothelial Growth Factor (VEGE) - Immunoperoxidase Method (DAB-NiCl₂ with Methyl Green Counterstain)

Don C. Wilson

University of Washington Medical Center

I. SLIDE PREPARATION

- 1) Using microtome, cut 5 µm thick sections from formalin fixed paraffin embedded tissue.
- 2) Place tissue sections on SuperFrost/Plus Fisher brand slides.
- 3) Place slides in a 45°C drying oven overnight.
- 4) Deparaffinize slides in 3 changes of xylene for 5 minutes each.
- 5) Rehydrate the slides in the following order:
 - a) 100% ethyl alcohol - 3 changes, 5 minutes total
 - b) 95% ethyl alcohol - 2 changes, 4 minutes total
 - c) 70% ethyl alcohol - 1 change, 1 minute
- 6) Rinse slides in distilled water.
- 7) Place slides in 3% hydrogen peroxide for 5 minutes (save 5 mL for DAB solution).
- 8) Rinse slides in distilled water.
- 9) Rinse slides in PBS twice.

Antigen retrieval - pronase method:

- 10) Place slides in room temperature pronase solution for 5 minutes.
- 11) Rinse slides in PBS and one change of PBS/Triton for total of 5 minutes.
- 12) Proceed with antibody application.

Antigen retrieval - microwave method:

- 10) Place slides in a plastic rack (*no metal*). Rack must always be full. Load with blank slides if necessary.
- 11) Place slides in 200 mL of 10 mM citrate buffer, cover with a lid loosely and

place at edge of microwave tray.

- 12) Microwave at high power setting for 10 minutes.

Note: Do not let fluid level evaporate below level of tissue. Stop microwave every 3 minutes and add distilled water.

- 13) Remove slides from microwave and let sit in citrate at room temperature for a full 20 minutes.

- 14) Rinse slides in PBS and one change of PBS/Triton for a total of 5 minutes.

If tissue is lost from slides during microwave procedure, use alternate method of antigen retrieval (enzyme incubation = pronase method).

II. ANTIBODY APPLICATION

We use a Vectastain ABC Kit supplied by Vector Laboratories (Burlingame, CA). The Kit contains:

- i) Normal goat serum (blocking agent)
- ii) biotinylated anti-rabbit IgG (secondary antibody)
- iii) avidin DH (reagent A)
- iv) biotinylated horseradish peroxidase (reagent B)

One doesn't know the concentrations of the reagents. You are told to mix 2 drops of stock solution in 10 mL of buffer, for example.

- 15) Incubate specimens (humidified chamber, room temperature) with 10% goat serum in PBS for 20 minutes. Use sufficient reagent to cover the specimen (usually 20-50 µL).

- 16) Wash slides in 2 changes of PBS and 1 change of PBS with BSA/Triton for a total of 5 minutes.

- 17) Prepare primary antibodies by diluting:

- a) Control antibody (non-immune Rabbit IgG)
 - 5 µL stock solution (5 mg/mL) diluted in 12.5 mL PBS + 1% BSA
- b) 1:50 (Santa Cruz A-20) polyclonal anti-VEGF antibody
 - 25 µL stock solution (100 µg/mL) plus 1225 µL PBS with 1% BSA (sufficient volume to stain 20 slides).

SAMx

Are you searching for an Automation system?

Would you like EDS/WDS Imaging?

Would you like to have EDS/WDS Analysis?

Then we are the company you're looking for.

We are the leading developer of EPMA/SEM automation, imaging, and combined EDS/WDS analysis under Windows95/NT. We are the pioneers in Thin Film analysis with STRATA released in 1991. We are fully Internet compatible, all software upgrades can be downloaded directly from our web site.

We listen to our customers needs and we deliver. Here are just a few:

- **XMAS Automation:** We can upgrade your EPMA / SEM equipped with WDS.
- **PCIMAX with IDFIX:** Get your SEM equipped with imaging and combined EDS.
- **TN2WIN and KV2WIN:** Convert your old TRACOR/KEVEX data files to PC.
- **STRATAGem:** Our 2nd generation of Thin Film STRATA is ready. Get your copy today.

For more detailed information on our products, we invite you to visit our web site at: www.samx.com

Please be sure to stop by our Booth #236 at Microscopy & Microanalysis '98 in Atlanta for your personalized demonstration. We look forward to seeing you there.

SAMx Support for Applications in Microanalysis and x-ray

4, rue Galilée / 78280 Guyancourt - France / Tel: 011 33 1 30 57 90 25 / Fax: 011 33 1 30 48 95 65 / email: jfthiot@samx.com
Bldg. M2048, Swarr Run Road, Lancaster PA 17601 / Tel.: (717)299-0599 / Fax: (717)299-2022 / email: jbarney@samx.com

- 18) Apply an adequate amount (usually about 75 μ L) of the primary antibody to cover the entire tissue.
- 19) Incubate the slides for 60 minutes in a moist chamber at room temperature.
 - At this time, prepare the secondary antibodies, avidin, and biotinylated peroxidase. These solutions should be made up in PBS.
 - Thaw the appropriate number of DAB aliquots.
 - Warm 175 mL of 0.05 M Tris buffer in a beaker (foil covered) in a water bath at 37°C.
 - Warm 200 mL of 0.05 M Tris buffer in a slide boat in a water bath at 37°C.
- 20) Wash slides in 1 change of PBS and 1 change of PBS with BSA/Triton for a total of 5 minutes.
- 21) Apply the biotinylated secondary antibody (goat anti-rabbit IgG) and incubate for 30 minutes in a moist chamber at room temperature.
- 22) Rinse with 3 changes of PBS.
- 23) Cover sections with peroxidase labeled streptavidin (ABC solution) and incubate for 30 minutes in a moist chamber at room temperature.
- 24) Wash slides in one change of PBS and one change of 0.05 M Tris buffer (at 37°C in a water bath) for a total of 5 minutes.

III. COLOR DEVELOPMENT

Note: DAB may be carcinogenic. Use rubber gloves and prepare under a fume hood. DAB is light sensitive and *should be prepared in the dark and just prior to use.*

25) Preparation of working DAB solution:

Note: Always add reagents in the following order:

- a) Add 4 mL of thawed DAB to the warmed Tris buffer.
 - b) While stirring, add 1 mL of 8% NiCl₂.
 - c) Add 12 drops of 3% hydrogen peroxide with a Pasteur pipette.
 - d) Mix solution thoroughly.
- 26) Incubate slides in the DAB solution for 10 minutes.

- 27) Wash slides in distilled water for 1 change.
- 28) Counterstain slides in methyl green for 5 minutes.
- 29) Dehydrate slides in the following order:
 - a) 95% ethyl alcohol - 1 quick dip (methyl green is extremely soluble in water)
 - b) 100% ethyl alcohol - 2 changes for a few quick dips
 - c) Leave the slides in the last change of 100% ethyl alcohol for 5 minutes.
- 30) Rinse slides in 3 changes of xylene for a total of 5 minutes.
- 31) Apply coverslips with Permount.

IV. REAGENT PREPARATION FOR IMMUNOPEROXIDASE STAINING.

1) 10 mM Citrate Buffer:

Reagents:

- a) Citric acid, monohydrate, granular HOC(COOH)CH₂COOH₂H₂O
- b) Filtered distilled water
- c) 10 M sodium hydroxide (NaOH)

Procedure:

- a) Dissolve 8.41 g citric acid monohydrate in 4 L distilled H₂O.
- b) pH to 6.0 \pm 0.3 with 10 M NaOH.

Storage: Room temperature - Shelf life: 3 months

2) 3,3' - Diaminobenzidine (DAB):

Reagents:

- a) 3,3' - Diaminobenzidine (3,3',4,4' - tetraaminobiphenyl) tetrahydrochloride 97%

Caution: Possible carcinogen. Use safety goggles, rubber gloves, and work under a fume hood when handling this reagent.

- b) 0.05 M Tris buffer (see reagent preparation for 0.05 M Tris buffer)

Procedure:

- 1) Stock DAB solution

Continued on page 26

Only from

MICRO STAR

**Flawless quality
proven with one year guarantee.**

MICRO STAR DIAMOND KNIVES 800 533 2509 FAX 409 294 9861 E-MAIL MISTAR@MSN.COM

Detection of Vascular Endothelial Growth Factor (VEGE) - Immunoperoxidase Method (DAB-NiCl₂ with Methyl Green Counterstain)

Continued from page 25

- Add 132 mL of 0.05 Tris buffer to 5.0 g of DAB.
 - Mix solution well with magnetic stir bar. (It takes a long time for the DAB to go into the solution.)
 - When the DAB is completely dissolved, aliquot the solution into 4 mL portions.
 - Store the aliquots in the refrigerator for a few hours, then place the tubes in the -70°C freezer for storage.
- Storage: -70°C freezer - Shelf Life: Indefinite

2) Working DAB solution

Reagents:

- Stock DAB solution
- 0.05 M Tris buffer (see reagent preparation for 0.05 M Tris buffer)
- 3% hydrogen peroxide
- 1% FeCl₃ (see reagent preparation for 1% FeCl₃)

Procedure:

DAB is light sensitive preparation of the working solution which should be done in the dark. Prepare the working DAB solution immediately before use.

Note: Always add the reagents in the following order:

- Prewarm 175 mL of 0.05 M Tris buffer in a slide boat to 37°C in a waterbath.
- Add 4 mL of thawed DAB solution to the 0.05 M Tris buffer.
- While stirring, add 1 mL of 8% NiCl₂.
- Add 12 drops of 3% hydrogen peroxide with a Pasteur pipette.
- Mix this solution thoroughly.

Storage: N/A - Shelf Life: Good for 2-3 racks of slides

3) 1% Ferric Chloride (FeCl₃):

Reagents:

- Ferric chloride (FeCl₃·6H₂O)
- Filtered distilled water

Procedure:

- Add 1 g of FeCl₃·6H₂O to 100 mL of filtered distilled water.
- Mix solution well with a magnetic stir bar.

Storage: Refrigerator - Shelf Life: 1 week

4) Harris Hematoxylin:

Reagents:

- Harris hematoxylin
- Filtered distilled water

Procedure:

- Add 125 mL of Harris hematoxylin to 125 mL of distilled water.
- Filter hematoxylin solution with Whatman #2 filter paper.

Storage: Room temperature - Shelf Life: 2 weeks or until a precipitate builds up

5) Methyl Green:

Reagents:

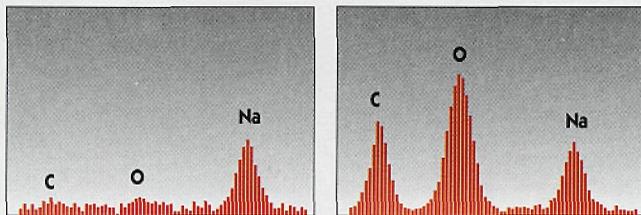
- Glacial acetic acid (aldehyde free) CH₃COOH
- Sodium acetate, anhydrous CH₃COONa
- 10 N NaOH
- Methyl green

Procedure:

- 0.1 N acetic acid
Add 1.5 mL of glacial acetic acid to 250 mL of filtered distilled water and mix.
- 0.1 N sodium acetate
Add 1.026 g of sodium acetate to 125 mL of filtered distilled water and

Light Element Peaks Revealed!

Oil Film on EDX Windows Removed:



Oily Window

Clean Window

Oil build-up on EDX detector windows can ruin sensitivity for light element X-rays in SEMs. To stop oil condensation and keep the system clean, smart SEM users rely on the XEI Scientific SEM-CLEAN™ system.

Result: Consistent light element X-ray results and contamination-free pictures. The Nitrogen purge of the inexpensive SEM-CLEAN system actively cleans your electron microscope while you're away.

SEM-CLEAN™ Stops the Oil

XEI
SCIENTIFIC

3124 Wessex Way, Redwood City, CA 94061-1348
650-369-0133 • Fax 650-363-1659

<http://www.msa.microscopy.com/SM/XEI/XEIHomePage.html>

Optimizing Microscopy

Get the BEST
from your
light microscope!
Attend this energetic lecture/demo
with author Barbara Foster

Space is limited so reserve now!



Barbara Foster or Ken Piel
Microscopy/Microscopy Education
(413) 746-6931
e-mail: mme@map.com



mix.

c) Buffer

- 1) Add 189 mL of 0.1 N acetic acid to 66.3 mL of 0.1 N sodium acetate.
- 2) pH to 4.2 with NaOH.

d) Methyl green

- 1) Add 5.0 g of methyl green to the buffer solution.
- 2) Filter the methyl green solution through Whatman #2 filter paper.

Storage: Room temperature - Shelf Life: Indefinite. When in use, the shelf life is 2 weeks or until staining becomes weak.

6) 8% NiCl₂:

Reagents:

- a) Nickel chloride hexahydrate (NiCl₂ · 6H₂O, abbreviated as NiCl₂)
Caution: Possible carcinogen. Use rubber gloves, safety goggles, and work under a fume hood when handling.
- b) Filtered distilled water

Procedure:

- a) Add 2.0 g of NiCl₂ and make up to 25 mL with filtered distilled water.
- b) Mix thoroughly with magnetic stir bar.

Storage: Refrigerator, in dark - Shelf Life: 1 week

7) Phosphate Buffered Saline (PBS):

Reagents:

- a) Sodium chloride, crystals (NaCl)
- b) Sodium phosphate, dibasic, anhydrous, granular (Na₂HPO₄)
- c) Potassium chloride, crystals (KCl)
- d) Potassium phosphate, monobasic, crystals (KH₂PO₄)
- e) Filtered distilled water

Procedure:

- a) Add 32 g NaCl, 4.6 g Na₂HPO₄, 0.8 g KCl, 0.8 g KH₂PO₄ to filtered distilled water and make up to 4 litres.
- b) The pH should be between 7.28 and 7.40. If the pH falls outside this range, remake the PBS solution.

Storage: Refrigerator - Shelf Life: 2 months

8) Phosphate Buffered Saline with 1% Bovine Serum Albumin (PBS with 1% BSA):

Reagents:

- a) PBS (See reagent preparation for PBS)
- b) Albumin, bovine fraction V

Procedure:

- a) Add 1.0 g of bovine serum albumin to 100 mL of PBS.
- b) Mix thoroughly with magnetic stir bar.

Storage: Refrigerator - Shelf Life: 1 week

9) PBS with BSA and Triton X-100:

Reagents:

- a) PBS (see reagent preparation for PBS)
- b) Albumin, bovine fraction V
- c) Triton X-100 (Octyl Phenoxy Polyethoxyethanol)

Procedure:

- a) Add 2.0 g BSA and 200 µL of Triton X-100 to 2.0 L of PBS. (Draw the Triton X-100 up slowly because it is very viscous and also add slowly for the same reason).
- b) Mix thoroughly by shaking.

Storage: Refrigerator - Shelf Life: 2 months

10) Pronase

- a) Add 20 mg (0.02 g) of pronase to 200 mL of PBS.
- b) Mix thoroughly by shaking.

Storage: Refrigerator - Shelf life: 1 week

11) 0.05 M Tris Buffer:

Reagents:

- a) Trizma base (Tris[hydroxymethyl]aminomethane) (C₄H₁₁NO₃)

b) Filtered distilled water

- c) Hydrochloric acid, concentrated (HCl approximately 37%)

Procedure:

- a) Add 24.22 g of Trizma base to 3800 mL filtered distilled water.
- b) pH with concentrated hydrochloric acid to 7.6 to 7.7.
- c) Make up to 4.0 L with filtered distilled water.

Storage: Refrigerator - Shelf Life: 2 months ■



RESEARCH POSITION

UNIVERSITY OF FLORIDA

MAJOR ANALYTICAL INSTRUMENTATION CENTER

DEPARTMENT OF MATERIALS SCIENCE AND ENGINEERING

A non-tenure track research faculty position is available in the Major Analytical Instrumentation Center (MAIC) for persons with expertise and a strong academic record in the area of transmission electron microscopy and other analytical techniques. The research faculty member will be expected to develop an independent research program, provide analytical expertise and training, organize and participate in short courses, and provide technical support to industrial and university research and development activities. Persons with a doctoral degree in materials science and engineering or closely related fields are encouraged to apply.

The MAIC provides a user-oriented facility to support the University of Florida, the State University System of Florida, and the industrial community with access to modern analytical instrumentation. MAIC is housed within the Department of Materials Science and Engineering (MSE) at UF. MSE is one of the nation's leading departments in the field and has 32 faculty, 160 graduate students, 100 upper division undergraduates, and over \$10 million of annual research expenditures. The Department provides integrated materials science and engineering education, as well as multidisciplinary research programs devoted to biomaterials, ceramics, composites, electronic and optical materials, metals and polymers.

The position is open at the level of Assistant or Associate Research Scientist. The deadline for application is July 30, 1998. Applicants should submit a curriculum vitae, statement of research plans and the names of at least three references to:

Chair of the Search Committee
Major Analytical Instrumentation Center
107 MEL
PO Box 116400
University of Florida
Gainesville, FL 32611-6400

The University of Florida is an Affirmative Action/Equal Opportunity Employer