Detection of Vascular Endothelial Growth Factor (VEGE) - Immunoperoxidase Method (DAB-$NiCl_2$ with Methyl Green Counterstain)

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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).
S) 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) biotinyated 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).

18) Apply an adequate amount (usually about 75 µL) 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% $NiCl_2$.
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 $HOC(COOH)CH_2COOH)_2H_2O$
b) Filtered distilled water
c) 10 M sodium hydroxide (NaOH)
Procedure:
a) Dissolve 8.41 g citric acid monohydrate in 4 L distilled H_20.
b) pH to 6.0 ± 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

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a) Add 132 mL of 0.05 Tris buffer to 5.0 g of DAB.
b) Mix solution well with magnetic stir bar. (It takes a long time for the DAB to go into the solution.)
c) When the DAB is completely dissolved, aliquot the solution into 4 mL portions.
d) 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:
a) Stock DAB solution
b) 0. 05 M Tris buffer (see reagent preparation for 0.05 M Tris buffer)
c) 3% hydrogen peroxide
d) 1% $FeCl_3$ (see reagent preparation for 1 % $FeCl_3$)

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:
a) Prewarm 175 mL of 0.05 M Tris buffer in a slide boat to 37°C in a waterbath.
b) Add 4 mL of thawed DAB solution to the 0.05 M Tris buffer.
c) While stirring, add 1 mL of 8% $NiCl_2$.
d) Add 12 drops of 3% hydrogen peroxide with a Pasteur pipette.
e) Mix this solution thoroughly.

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

3) 1% Ferric Chloride ($FeCl_3$):

Reagents:
a) Ferric chloride ($FeCl_3 \cdot 6H_20$)
b) Filtered distilled water

Procedure:
a) Add 1 g of $FeCl_3 \cdot 6H_20$ to 100 mL of filtered distilled water.
b) Mix solution well with a magnetic stir bar.

Storage: Refrigerator - Shelf Life: 1 week

4) Harris Hematoxylin:

Reagents:
a) Harris hematoxylin
b) Filtered distilled water

Procedure:
a) Add 125 mL of Harris hematoxylin to 125 mL of distilled water.
b) 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:
a) Glacial acetic acid (aldehyde free) CH_3COOH
b) Sodium acetate, anhydrous CH_3COONa
c) 10 N NaOH
d) Methyl green

Procedure:
a) 0.1 N acetic acid
Add 1.5 mL of glacial acetic acid to 250 mL of filtered distilled water and mix.
b) 0.1 N sodium acetate
Add 1.026 g of sodium acetate to 125 mL of filtered distilled water and

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% $NiCl_2$:

Reagents:

a) Nickel chloride hexahydrate ($NiCl_2 \cdot 6H_20$, abbreviated as $NiCl_2$)
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 $NiCl_2$ 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 ($Na_2H\ PO_4$)

c) Potassium chloride, crystals (KCl)

d) Potassium phosphate, monobasic, crystals (KH_2PO_4)

e) Filtered distilled water

Procedure:

a) Add 32 g NaCl, 4.6 g $Na_2H\ PO_4$, 0.8 g KCl, 0.8 g KH_2PO_4 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) ($C_4H_{11}NO_3$)

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