GENERAL IMMUNOHISTOCHEMISTRY PROTOCOL
Overnight Incubation of Tissue Sections with Primary Antibody

I. STOCK SOLUTIONS

A. 0.2 M PB

184 g Na phosphate dibasic
42 g Na phosphate monobasic

Dissolve in1 L water; bring to 8 liters with water, check ph 7.2-7.4

B. 10x (1.0M) Tris Saline Buffer

242.2 g Tris 7-9 (Sigma)
90 g NaCl
2 L water

C. 10x (1.0 M acetate buffer)

164 g Na acetate
1 L water

II. WORKING SOLUTIONS

A. 0.1M Tris Saline Buffer, pH 7.6 (2 liters)

24.22 grams	Tris (Sigma 7-9)
18.00 grams	Sodium Chloride

Dissolve in 1 liter of distilled water. Adjust the pH to 7.6 with concentrated hydrochloric acid. Bring the volume to two liters with distilled water.

B. PBS Buffer, pH 7.2 (8 liters)

2.56 grams	Sodium Phosphate Monobasic (anhydrous)
10.60 grams	Sodium Phosphate Dibasic (anhydrous)
72.00 grams	Sodium Chloride

Dissolve in 1 liter of distilled water. Bring the volume to eight liters and check the pH. It should be 7.2-7.4.

C. 0.1 M Acetate Buffer, pH 6.0 (1 liter)

16.40 grams	Sodium Acetate (anhydrous)

Dissolve in 1 liter of distilled water. Adjust the pH to 6.0 and bring the volume to two liters with distilled water.

D. Tris-Cobalt, pH 7.2 (200 ml)

1.2 grams	Tris (Sigma 7-9)
1.0 gram	Cobalt Chloride
180.0 ml	Distilled Water

Dissolve in 180 ml of distilled water. Adjust the pH to 7.2 with 0.1 M HCl and bring to a volume of 200 ml. Note: You may use this solution for a period of two weeks, if used every day. It should be made fresh when it turns a dark black color.

E. 0.1 % Nuclear Fast Red in 5% Aluminum Sulfate (100 ml)

0.1 gram	Nuclear Fast Red
5.0 grams	Aluminum Sulfate
100.0 ml	Distilled Water

Dissolve the nuclear fast red and aluminum sulfate in boiling water. Stir at near boiling for at least 2 hours. Cool, filter, and add a few grains of thymol as a preservative. Filter prior to use with Whatman #4 filter paper.

F. 0.1 M PBS with Triton X-100, pH 7.4 (1 liter)

11.5 grams	Sodium Phosphate dibasic
2.62 grams	Sodium Phosphate Monobasic
9.00 grams	Sodium Chloride
2-3 ml	Triton X-100

Add salts, one at a time, to one liter of distilled water. After the salts are dissolved, add the Triton X-100 and mix well. Check the pH.

OR

PBS/TRITON

1 L 0.2 M PB
1 L water
18 g NaCl
2-3 ml Triton X-100

G. Blocking Serum (2%, 200 ml)

196 ml	0.1 M PBS with Triton X-100 pH 7.4
4 ml	Normal Goat Serum (NGS)

Add a few grains of sodium azide as a preservative. You may increase to 4%-6% NGS if background staining is still a problem. Note: You may substitute horse, donkey, or fetal bovine serum for goat. Do not use serum from same species as primary antibody.

H. Alternative Blocking Solution

20 grams	Powered Non-fat Dry Milk
200 ml	Distilled Water

III. PROCEDURE - Do not let slides dry out at any point in this procedure.

A. Deparaffinize Tissue:

1. 3 changes of xylene for 10 minutes each.
2. 2 changes of 100% EtOH for 5 minutes each.
3. 2 changes of 95% EtOH for 5 minutes each.
4. 1 change of 70% EtOH for 5 minutes each.
5. Clear in PBS pH 7.2 for 5 minutes.

Note: **Antigen retrieval is done at this point in the protocol, if required.**

B. Treatment for Removal of Endogenous Peroxidase Activity:

1. Place slides in a glass staining dish containing 180 ml of methyl alcohol and 3 ml of 30% hydrogen peroxide.
2. Incubate at room temperature for 15 minutes.
3. Clear in PBS pH 7.2 for 5 minutes.

C. Blocking of Non-Specific Binding:

1. Place slides in a glass coplin jar containing the blocking serum (Materials - G).
2. Incubate slides for 1-2 hours at room temperature on a shaker.
3. Drain slides before incubating in the appropriate primary antisera dilution. **Note: Do not rinse the slides in PBS. Do not let slides dry out.**

D. Application of the Primary Antisera:

1. Dilute the primary antisera in blocking serum (Materials - G) at the appropriate, predetermined dilution.
2. Overlay the tissue sections with the primary antibody solution, or immerse slides in a plastic slide mailer with the primary antibody solution. Incubate overnight in the cold at 4°C.
3. Wash slides in a glass coplin jar or staining dish for x5 for 5 minutes each with 0.1 M PBS with Triton X-100 on a shaker at room temperature.

E. Application of the Secondary Antibody:

1. Dilute the biotinylated, secondary antibody in the blocking serum (Materials - G), according to the instructions in the Vector Elite Kit. Note: 1:200 is the typical dilution for the secondary antibody.
2. Overlay sections or immerse slides in a plastic slide mailer and incubate for 30 minutes at room temperature on a shaker.
3. Wash slides in a glass coplin jar or staining dish x5 for 5 minutes each with 0.1 M PBS with Triton X-100 on a shaker at room temperature.

F. Application of the Avidin-Biotin Complex Reagent:

1. Dilute the avidin-biotin complex (ABC) according to the directions in the Vector Elite Kit using 0.1 M PBS with Triton. **Note: The ABC reagent must be prepared at least 30 min prior to use.**
2. Overlay sections or immerse slides in a plastic slide mailer with the ABC reagent and incubate for 30 minutes at room temperature on a shaker.
3. Wash slides in a glass coplin jar or staining dish x5 for 5 minutes each with 0.1 M PBS with Triton on a shaker at room temperature.
4. Rinse briefly in 0.1 M Acetate Buffer pH 6.0.

G. Development of Colored Reaction Product Using Diaminobenzidine (DAB).

1. Dissolve 1.6 grams of NaCl and 2 grams of Ni Sulfate in 200 ml of 0.1M Acetate buffer, pH 6.0. Add 95 mg (0.95 grams) of DAB and dissolve, using a stir bar. **Note: Always add DAB last**! (Handle DAB with care, wear safety glasses and gloves, protect your eyes, hands, face and respiratory system – refer to your MSDS for DAB.)

Note: Set up a series of 6 glass staining dishes for the following solutions/steps.

2. Filter the NiDAB solution using Whatman #1, or equivalent, filter paper.
3. Add 25 µl of 30% Hydrogen Peroxide to the NiDAB solution and pour into a glass staining dish.
4. Incubate the slides in NiDAB for 4 minutes on a shaker at room temperature.
5. Rinse briefly in Tris Saline, pH 7.6, at room temperature.
6. Incubate in Tris Cobalt for 4 minutes at room temperature.
7. Wash briefly in distilled water at room temperature.
8. Counter-stain in 0.1% Nuclear Fast Red in 5% aluminum Sulfate for 2 minutes at room temperature. **Note: Filter before use with a #4 Whatman filter paper.**
9. Wash in running water until clear.

H. Dehydration and Mounting

1. 1 change of 70% ETOH – 10 dips.
2. 2 changes of 95%% ETOH – 10 dips.
3. 3 changes of 100% ETOH – 10 dips.
4. 3 changes of Xylene – clear – 10-15 dips each.
5. Coverslip with Permount.