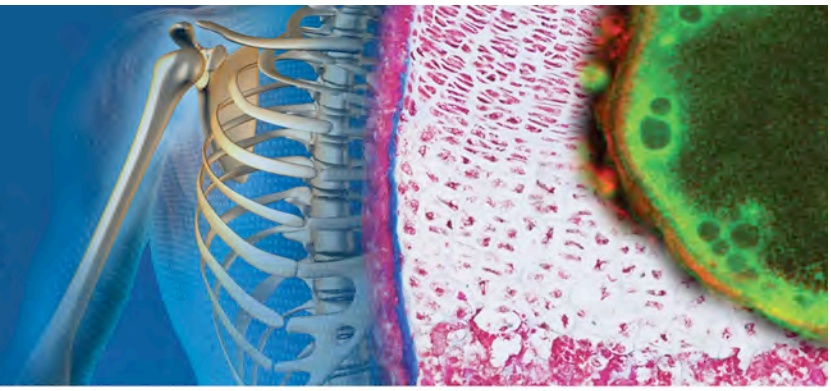

VEGF

Abcam (Cat # ab2992-500)
Immunohistochemistry Protocol For Formalin Fixed Paraffin Embedded Tissue

Cut sections at 3 microns and bake overnight at 60°C

Day 1

1. Bake slides at **60°C for minimum of 30 minutes** prior to staining.
2. Deparaffinize tissues in xylene (3 changes for 5 minutes each), and rehydrate through 2 changes in 100% Ethanol, 2 changes in 95% Ethanol and 1 change in 70% Ethanol for 5 minutes each).
3. Wash twice in deionized water for 5 minutes each.
4. Perform antigen retrieval using a **0.01 M (10mM) NaCitrate Buffer pH 6.0** – This is done in the water bath at a constant temp. **Set water bath temp to 65°C** – leave slides in a stain bucket in the water bath for **1 Hour.** After this step leave the bucket outside to cool for 20 minutes.
5. Rinse the slides in 3 changes of deionized water.
6. Outline each section with a **PAP pen.**
7. Quench endogenous peroxidase activity with **DAKO dual endogenous enzyme blocking reagent (***Dako S2003***) for 30 min.**
8. Rinse the slides in 2 changes of deionized water and once in 1X PBST.
9. Block non-specific binding sites **the diluted Vectastain Normal Goat Serum (***Vectastain Elite Rabbit IgG Kit PK-6101)* **– incubate for 30 min.**
Do not rinse slides after the incubation period.
10. Block endogenous avidin in the tissue with **Vector Avidin reagent**. (*Vector SP-2001*) for 15 minutes.
11. Rinse twice with 1X PBST.
12. Block endogenous biotin in the tissue with **Vector Biotin reagent.** (*Vector SP-2001*) for 15 minutes.
13 . Rince twice with 1X PBST.
14. Prepare the primary antibody in **2% Normal Goat Serum,** this decreases non-specific staining. Negative control slides need to be incubated with 2% normal goat serum only.
15. Incubate the slides with a **1:100 dilution of VEGF primary antibody** *(Abcam cat# ab2992-500)* **overnight at 4°C**

Day 2

1. Let slides warm up to room temperature for 15-30 minutes.
2. Wash the slides 5 times with 1X PBST for 5 minutes each.
3. Incubate with **Vectastain Biotinylated Goat anti-rabbit** secondary antibody **(***Vectastain Elite Rabbit IgG Kit PK-6101)* for **30 min.**
4. 4. Wash the slides 5 times with 1X PBST for 5 minutes each.
5. Reconstitute the **Vectastain ABC reagent** and incubate at **room temperature for 30 min.**
6. Incubate with **Vectastain ABC reagent** for **30 min.**
7. Wash the slides 4 times with 1X PBST, then twice in deionized water.
8. Detect color reaction with **Vector Immpact DAB** *(Vector SK-4105)* for a few minutes (check under microscope) until staining intensity is optimal.
9. Stop the reaction with deionized water.
10. Counterstain the sections with **Hematoxylin (***Zymed Cat # 93-3943)* for 5 minutes.
11. Rinse in tap water.
12. Place slides in 1X PBS for 1-3 minutes.
13. Rinse with deionized water.
14. Dehydrate quickly through 3 changes of 95% ethanol and 2 changes of 100% ethanol.
15. Clear in 3 changes of xylene and mount with Cytoseal.

Buffers: 10mM Na Citrate, 0.05% Tween-20 Buffer; pH 6.0 (Used for heat-induced epitope/antigen retrieval)

1. Tri-Sodium Citrate Dihydrate - 2.94g
2. Deionized water - 1000 ml
Mix well to dissolve. Adjust pH to 6.0 with 1N HCl
Add 0.5ml of Tween 20 and mix. Store at 4°C.

Vectastain Elite Rabbit IgG Kit (PK-6101)

1X PBST can be used as the diluent buffer for the blocking serum and secondary antibody. Follow the instructions written on the kit data sheet.

Evidence of positive staining:

Expression should be localized to the pre-hypertrophic zones of the growth plate, the deeper zones of the articular cartilage and bone lining cells of the trabecular bone.

(Mouse tissue protocol standardized on 3/7/2012 by Ashish Thomas, M.S.)