

**DAB Immuno-Staining For CD-31
(Pharmingen #01951D rat anti-mouse CD-31)**

PROCEDURE: Tissues immersion fixed in Zinc or neutral buffered formalin o/n @ 4C, processed through graded alcohols and embedded in paraffin. Cut sections @ 5 μ

A) Deparaffinize slides

Xylene	2x 5 min	_____
100%	2 min	_____
95%	2x 2 min	_____
70%	2x 2 min	_____
50%	2x 2 min	_____
ddH ₂ O	5 min	_____
PBS	2x 5 min	_____

B) Staining: DAY #1

1. 5 minutes RT Proteinase K digestion (DAKO Proteinase K in 15mM NaN₃ Cat#S3020)
2. PBS wash 3 x 3 minutes
3. Using PAP pen, "draw" around sections carefully
4. Block section in Blocking buffer (5% normal goat serum, 2.5% BSA, 1X PBS) for 30-45 min in humidified chamber @ RT (use about 150-200ul/section)
5. Aspirate block
Apply Primary antibody 1:75 (diluted in 0.5X blocking buffer) to section. Incubate in humidified chamber at 4C over night. (Lot # used _____)

Staining: Day #2

6. Aspirate antibody
7. PBS wash 3x 3min.
8. Block slide in Blocking buffer (as in step 2) for 5-10 min. @ RT
9. Apply biotinylated secondary goat anti-rat (Pierce #31831) at ~1:200 (dilute in 0.5X block buffer) to sections for 30-45 min. @ RT in humidified chamber. (Lot # used _____)

***Make up ABC-Elite** (for brown staining) according to manufacturer's specifications (2 drops ABC-A in 5 ml 0.5M NaCl, vortex, 2 drops ABC-b, vortex, **let sit 30 minutes**).

(Alternatively, for RED staining, use ABC-AP from Vector made up according to their instructions

10. PBS 2 x 3 min.
11. Block endogenous peroxidase activity: 250 ml MeOH + 5 ml 30% H₂O₂, 20 minutes @ RT. [Alternatively, block endog AP in 5% H₂O₂ in PBS without Methanol[different tissues react differentially to the methanol]]
12. PBS 3x3 min
13. Apply ABC to sections and cover in humidified chamber at RT for 30 min.
14. Wash slides in 0.1 M Tris pH 7.4 3 x 3 min.
15. Apply DAB (or AP substrate made up with Levamisol) to sections and monitor time of development
16. Wash in H₂O briefly x2
17. Counterstain in 1% Methyl green ~1min 15sec
18. **Dehydrate**
100% isobutanol 20 dips
100% isobutanol 2x 2 min _____
Xylene 2x 2 min _____
Cover section while moist with Cytoseal #60