

Immunohistochemistry: CD31 (by Masanobu Ohishi)

Tissue Preparation:

1. Fix the bone samples in 4% PFA for 24 hrs at 4°C
2. Decalcify the samples with 20% EDTA at 4°C for 7 days (change the EDTA daily)
3. Paraffin processing (histocore)

Before starting:

- Make 1L 1X TBS
 - 100 mL 10X TBS
 - 10X TBS (1L): 60.5 g Tris (Fisher BP152)
87.6g NaCl (Fisher BP358)
pH to 7.5
Bring to 1L w/ ~800 mL H₂O
 - 900 mL H₂O
- Prepare humid chambers (w/ Whatman paper)
- Make 4L 1X TNT
 - 400 mL 10x TBS
 - 3600 mL dH₂O
 - 2 mL Tween-20 (Sigma P2287): add while stirring
- Make Proteinase K
 - 10mg/ml in 10mM Tris pH 7.5/1mM CaCl₂

Staining process: (use TSA kit, Perkin Elmer #NEL700A)

1. Incubate slides at 60°C on slide warmer for at least 1 hr
2. Deparaffinize and dehydrate (use clean solutions!)
 - a. Xylene, 3x 3 min
 - b. 100% EtOH, 2x 2min
 - c. 95% EtOH 2 min
3. Rehydrate in 1X TBS, 5 min RT
4. Circle sections with Pap=pen
5. Proteinase K (Sigma P2308) 20 µg/ml, 15 min RT in moist chamber
 - a. 6 µl of 10mg/ml in 3 ml dH₂O
6. 3% H₂O₂/MeOH 10 min RT
 - a. 25 ml 30% H₂O₂ + 225 mL MeOH
7. dH₂O, 5 min, RT
8. Equilibrate sections by rinsing with 1X TBS
9. Incubate with TNB at RT in moist chamber for 30 min
10. 1° antibody diluted in TNB, RT in moist chamber for O/N at 4°C
 - a. CD31 (BD 550274) 1:200 dilution in TNB
11. Wash: 1X TNT, 3x 5 mins at RT

2 mg/ml stock sol'n
2000 µg/ml
↓ 100x
20 µg/ml

12. Biotinylated 2° Ab diluted in TNB, in moist chamber for 30 min
 - a. antiRat IgG, 1/300 (Dako E0468)
13. Wash: 1X TNT, 3x 5 min at RT
14. SA-HRP (from kit) 1:100 dilution in TNB, 30 mins at RT
15. Wash: 1X TNT, 3x 5 min at RT
16. Biotinyl Tyramid (kit) 1:50 dilution in amplification diluent, 5 min RT
17. Wash: 1X TNT, 3x 5 min at RT
18. SA-HRP (from kit) 1:100 dilution in TNB, 30 min RT
19. Wash: 1X TNT, 3x 5 min at RT
20. For HRP detection, use DAB (VECTOR SK-4100)
In 5ml dH₂O:
 - Add 2 drops buffer; mix well
 - Add 4 drops DAB; mix well
 - Add 2 drops H₂O₂; mix well
 - Add 2 drops NiCl (optional)
21. Incubate until color develops (2-10 mins)
22. Wash 5 mins in dH₂O
23. Counterstain (optional)
24. 95% EtOH 2 min
25. 100% EtOH 2x 3 min
26. Xylene 2x 5 min