

## Immunohistochemistry: pSmad1/5/9 (by Frank Ko)

Updated: 9/30/14

### 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 H<sub>2</sub>O
  - 900 mL H<sub>2</sub>O
- Prepare humid chambers (w/ Whatman paper)
- Make 4L 1X TNT
  - 400 mL 10x TBS
  - 3600 mL dH<sub>2</sub>O
  - 2 mL Tween-20 (Sigma P2287): add while stirring

### 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. 3% H<sub>2</sub>O<sub>2</sub>/MeOH 10 min RT
  - a. 25 ml 30% H<sub>2</sub>O<sub>2</sub> + 225 mL MeOH
5. dH<sub>2</sub>O, 5 min, RT
6. Circle sections with Pap-pen
7. Equilibrate sections by rinsing with 1X TBS
8. Incubate with TNB at RT in moist chamber for 30 min
9. 1° antibody diluted in TNB, RT in moist chamber for O/N at 4°C
  - a. pSMAD (Cell Signaling 9511) 1:100 dilution in TNB
10. Wash: 1X TNT, 3x 5 mins at RT
11. Biotinylated 2° Ab diluted in TNB, in moist chamber for 30 min
  - a. antiRabbit IgG, 1/300 (Vector BA1000)
12. Wash: 1X TNT, 3x 5 min at RT
13. SA-HRP (from kit) 1:100 dilution in TNB, 30 mins at RT

14. Wash: 1X TNT, 3x 5 min at RT
15. Biotynil Tyramid (kit) 1:50 dilution in amplification diluent, 5 min RT
16. Wash: 1X TNT, 3x 5 min at RT
17. SA-HRP (from kit) 1:100 dilution in TNB, 30 min RT
18. Wash: 1X TNT, 3x 5 min at RT
19. For HRP detection, use DAB (VECTOR SK-4100)  
In 5ml dH<sub>2</sub>O:
  - Add 2 drops buffer; mix well
  - Add 4 drops DAB; mix well
  - Add 2 drops H<sub>2</sub>O<sub>2</sub>; mix well
  - Add 2 drops NiCl (optional)
20. Incubate until color develops (2-10 mins)
21. Wash 5 mins in dH<sub>2</sub>O
22. Counterstain (optional)
23. 95% EtOH 2 min
24. 100% EtOH 2x 3 min
25. Xylene 2x 5 min