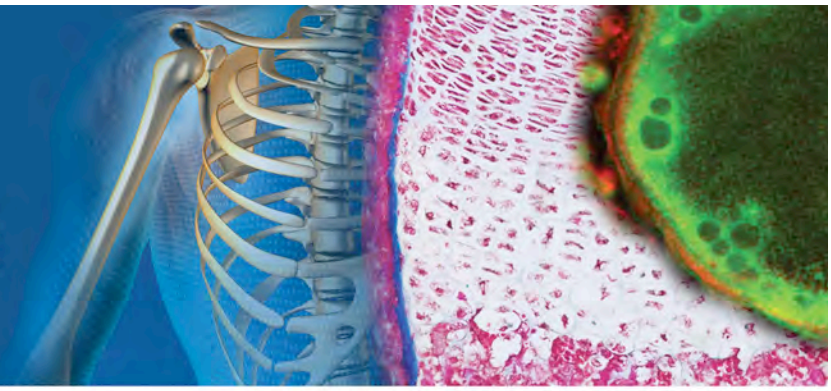

Phosphorylated-Smad 1/5/8

Millipore Catalog # AB3848
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, 5 minutes each), and rehydrate through graded ethanols (2 changes 100% and 95%, 1 change 70%, 5 minutes each).
3. Wash twice in deionized water for 5 minutes each.
4. Perform antigen retrieval using a **10mM Sodium Citrate Buffer pH 6.0** – This is done in a water bath at a constant temp. **Set the temp to 65°C** – leave slides in the hot water bath for **1 hour**. Then remove cover and leave outside to cool for about 10- 20 min.
5. Rinse in 3 changes of deionized water.
6. Outline each section with a **PAP pen.**
7. Quench endogenous peroxidase **DAKO Dual Endogenous Enzyme Blocking Reagent for 30 min (*Dako S2003*)**
8. Rinse with 3 changes of deionized water and once in PBST.
9. Block non-specific binding sites with diluted **Vectastain Normal Goat Serum (***Vectastain Elite Rabbit IgG Kit PK-6101***) for 30 minutes.**
10. **Do not Rinse Slides.**
11. Drain off serum and incubate **overnight at 4°C** with a **1:200 dilution of PSmad 1/5/8 primary antibody** (*Millipore Cat# AB3848)* **for 2-3 month old adult sections**.
12. Use a **1:400 dilution for embryo (E18.5) sections**.
13. Prepare the primary antibody in **2% Normal Goat Serum**. This decreases non-specific staining.
14. Negative Control slides need to be incubated with 2% Normal Goat Serum only**.**

DAY 2

1. Let slides warm up to room temperature for 30 minutes, then wash 5 times with 1X PBST for 5 min each.
2. Incubate with **1:200 Biotinylated Goat anti-rabbit (***Vectastain Elite Rabbit IgG Kit PK-6101***)** secondary antibody for **30 minutes.**
3. Reconstitute the **Vectastain ABC reagent** and incubate at **room temperature for 30 min (***Vectastain Elite Rabbit IgG Kit PK-6101***)**
4. Wash 5 times with 1X PBST for 5 minutes each.
5. Incubate with the **Vectastain ABC reagent** for **30 minutes.**
6. Wash 4 times with 1X PBST, then twice in deionized water.
7. Detect color reaction with **Vector Immpact DAB** *(Vector SK-4105)* for a few minutes (check under microscope)
8. Stop the reaction with deionized water.
9. Counterstain the sections with **Hematoxylin (***Zymed Cat # 93-3943***)** for 5 minutes.
10. Rinse in tap water.
11. Place slides in 1X PBS for 1-3 minutes.
12. Rinse with deionized water.
13. Dehydrate through 95% ethanol (3 changes) and 2 changes of 100% ethanol.
14. Clear in 3 changes of xylene and mount with cytoseal.

¶ Buffers:-

10mM Na Citrate, 0.05% Tween-20 Buffer; pH 6.0

1. Tri-Sodium Citrate Dihydrate 2.94g
2. dH2O 1000 ml

Mix well to dissolve**.** Adjust pH to 6.0 with 1N HCl
Add 0.5ml of Tween 20 and mix. Store the buffer at 4ºC for longer storage.

¶ Vectastain Elite Rabbit IgG Kit (PK-6101):

1:20 BSA-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, sporadically present in the trabecular region. Columnar cells of the Growth plate and Articular Cartilage chondrocytes are negative for pSmad 1/5/8 in wild type sections.