

## **Determining whether anti-phospho-SMAD1-5 and anti-phospho-SMAD2 antibodies can recognize formaldehyde-fixed epitopes**

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### **Background:**

SMAD1, 5 and 8 are phosphorylated by active ALK2 upon stimulation by BMP4, 6, 7 and 9. The kinase activity of ALK2 in the cells can be determined using antibodies that specifically binds to phosphorylated SMAD proteins. Analysis using conventional Western blot requires large amount of samples and processing time. If the antibodies can detect phosphorylated SMAD proteins in formaldehyde-fixed cells, the quantification of ALK2 cellular kinase activity can become more high-throughput. Proteins are linearized when processed in Western blot. When fixed and stained in the cells, proteins remained folded. Epitopes that are recognized by antibodies might remain hidden or modified by formaldehyde crosslink. SMAD2 is phosphorylated by active ALK5 upon stimulation by TGFb.

### **Protocol:**

#### **Day 1**

Seeded  $1 \times 10^4$  cells per well of 96-well plate and  $1 \times 10^5$  cells per well of four 6-well plate.

#### **Day 2**

Wash once with PBS and starve cells in plain OptiMEM for 6 hours.

Stimulate cells with 2, 4 and 8ng/ml TGFb or 100, 200, 300ng/ml BMP6 or 100, 200, 300ng/ml BMP7 in OptiMEM for 1 hour.

Immunofluorescent staining

Wash once with PBS, fix 96-well and 2 of the 6-well plates at room temperature with 2% formaldehyde in PBS for 15mins.

#### **Day 3**

Immunofluorescent staining

Permeabilize in 0.1% Triton X-100 for 20mins on ice.

Block in PBAS 10% BSA for 1 hour in room temperature

Stain with primary antibodies overnight in 4 degree Celsius (p-Smad1/5, p-Smad2 and beta-Tubulin, 1:100 dilution in PBAS 1.5% BSA)

PBAS buffer recipe

0.5% BSA, 0.1% sodium azide, 0.1% saponin, in Phosphate Buffered Saline pH 7.4

#### **Day 4**

Immunofluorescent staining

Stain fixed cells with secondary antibody (anti-rabbit alexa 588, anti-mouse alexa 468, 1:200 dilution in PBAS) in room temperature for 1 hour.

Wash twice with PBAS

Wash twice with PBS

"Mount" in glycerol

### **Western Blot**

#### **Day 2**

Lyse the other 2 6-well plates for Western Blot analysis. Clarify protein lysates.

Measure protein lysates in BCA assay. Adjust protein concentration. Boil in sample loading buffer.

Run gel and transfer for Western Blotting.

(Phospho-SMAD1/5 (Ser463/465) #9516, Phospho-SMAD2 (S465/467) #3108, SMAD1 #9743, SMAD2 #5339, SMAD5 #9517, beta-Tubulin T5201 Sigma)

Composition of cell lysis buffer

150mM NaCl

20mM Tris HCl pH7.5

1% Triton X-100

25mM NaF

25mM Sodium beta-glycerolphosphate

4mM Sodium pyrophosphate

2mM Sodium orthovanadate

mini-proteinase inhibitor (Roche) 1 tablet in 10ml

Western Blot conditions

1) 1 hour 100V wet transfer onto 0.2um pore-sized PVDF membrane

2) 1 hour blocking in PBS-0.1%Tween-20 3%BSA

3) Overnight 4 degree incubation in primary antibodies 1:1000 diluted in PBS-0.1%Tween-20 with 3% BSA

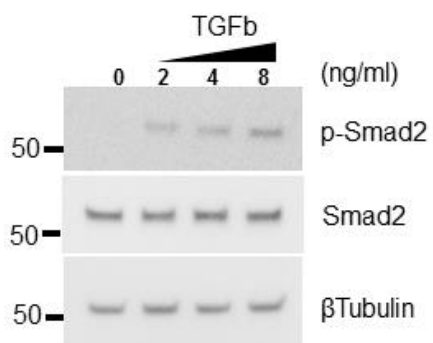
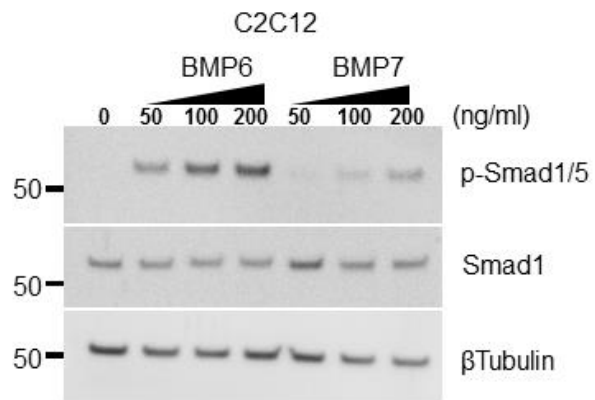
4) Washed 3X 10 minutes with PBS-0.1%Tween-20

5) 1 hour room temperature incubation in secondary antibodies conjugated to HRP (1:2000) diluted in PBS-0.1%Tween-20 with 3% skimmed milk

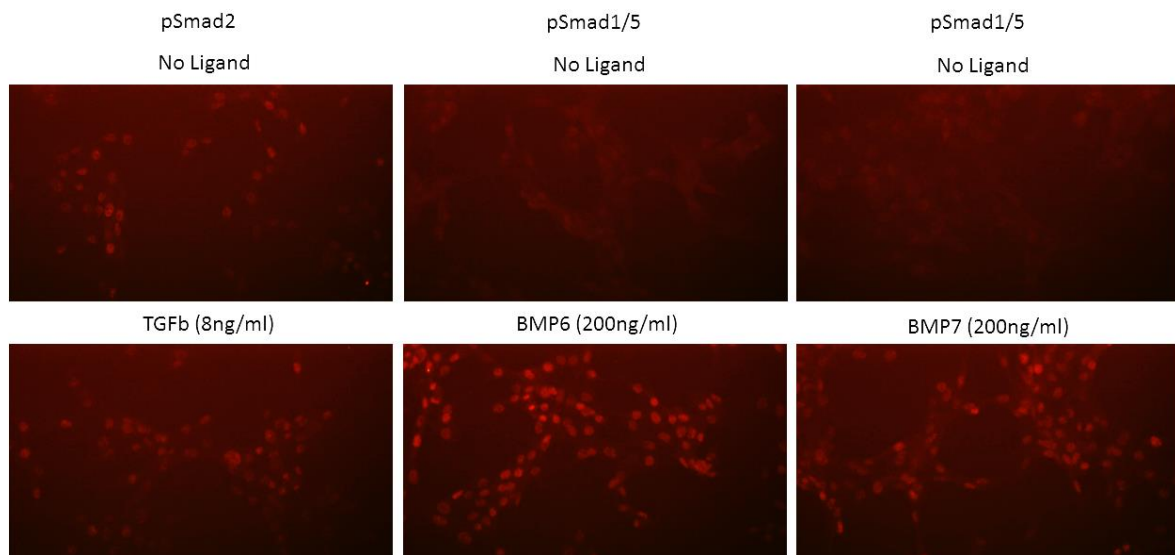
6) Washed 3X 10 minutes with PBS-0.1%Tween-20

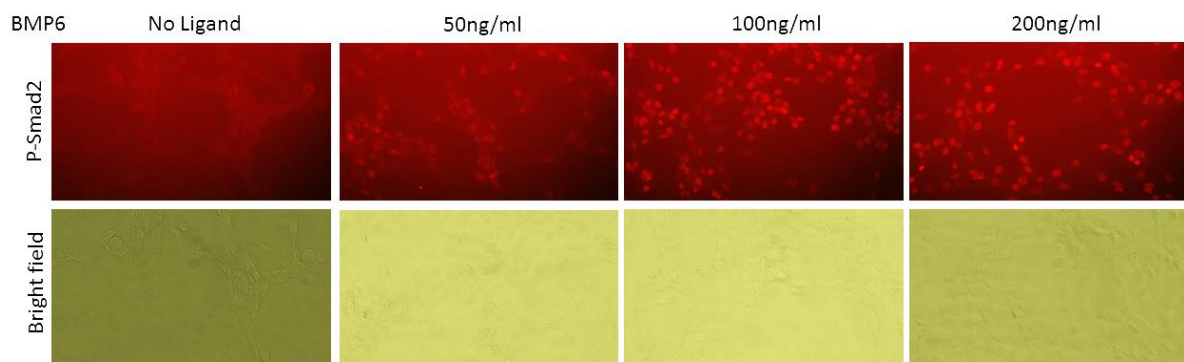
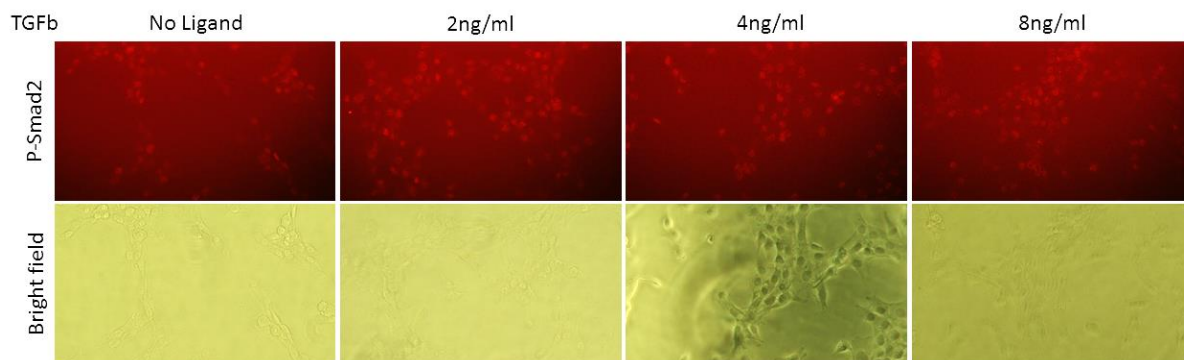
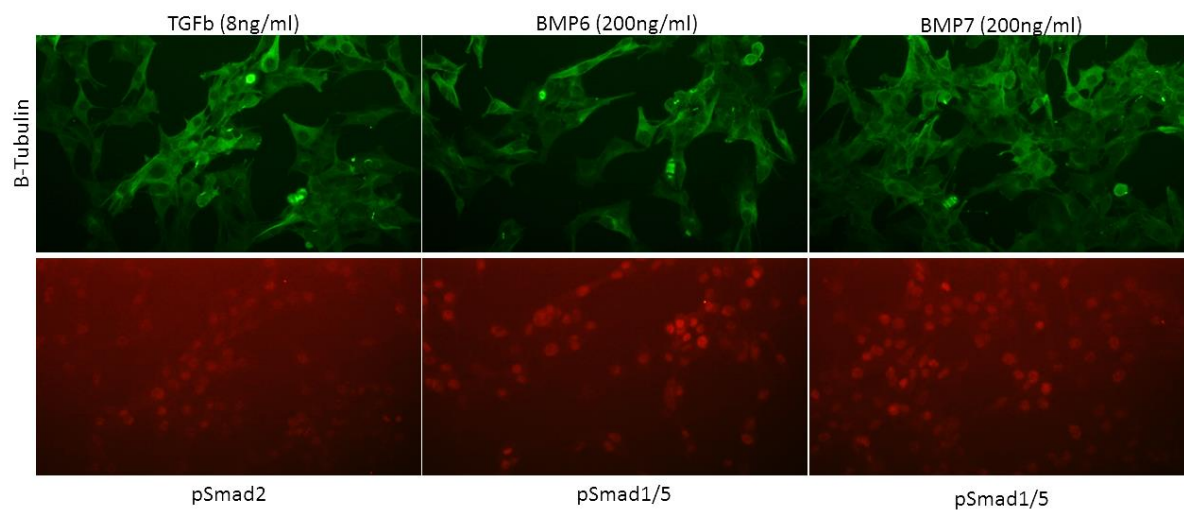
7) Blots were developed using Pierce ECL Western Blotting Substrate (Thermo Fisher Catalog no.: 32209). Images were captured using LAS-4000

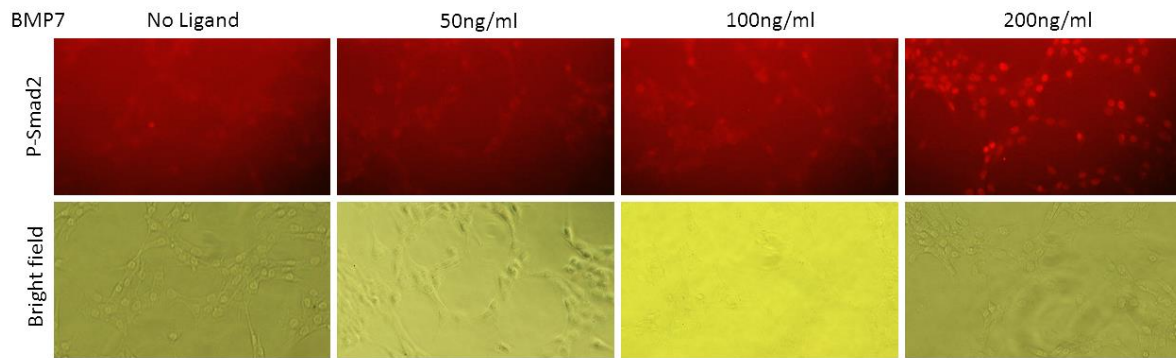
Results:



All antibodies used detected very specific bands at positions corresponding to their expected protein sizes in Western blot.







#### Conclusion:

Phospho-SMAD1/5 antibody is able to recognize epitope fixed for 15 minutes in 2% formaldehyde/PBS at room temperature.

Phospho-SMAD1/5 staining is seen only when C2C12 cells were stimulated with BMP6 or BMP7. Specific signal is seen only in cell nuclei, in accordance to the fact that phosphorylated SMAD1/5 interacts with SMAD4 and are translocated to the nucleus.

Phospho-SMAD2 antibody stained very weakly in fixed cells. Considerable amount of background staining can be seen even without ligand stimulation (TGFb).