NanoPro™ Assay: Phospho-p38

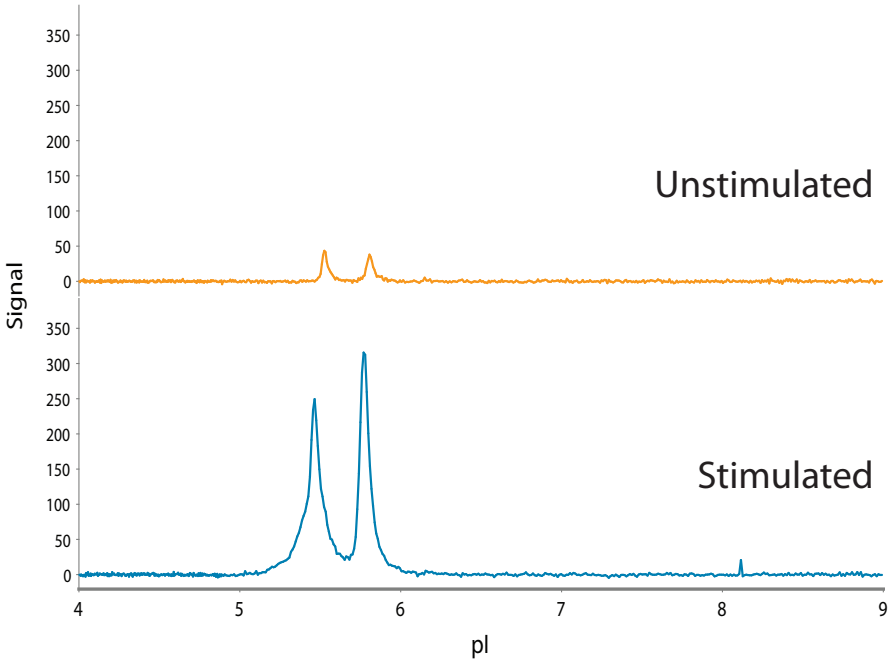
SUMMARY

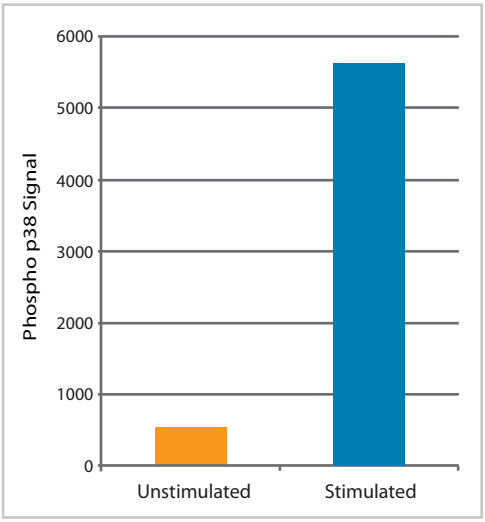
Primary Antibody: Anti-Phospho-p38 (T180/182) (Cell Signaling, cat# 9211), Anti-Phospho-p38 (T180/182) (Millipore, cat# 09-272)
Detection Antibody: Anti-Rabbit HRP (ProteinSimple, p/n 040-656)

The Mitogen Activated Protein Kinase p38 is activated by stress stimuli such as cytokines, ultraviolet irradiation, heat shock, and osmotic shock, and are involved in cell differentiation and apoptosis. We show that Interleukin-1 (IL-1) treatment induces phosphorylation of p38 in fibroblasts. In addition, two independent antibodies recognize peaks at pIs 5.4 and 5.8 for phosphorylated p38.

Authors: Joshua Hillman, Michael Rosenbach, and David Boyle, University of California, San Diego.

RESULTS





DETECTION OF P38 PHOSPHORYLATION IN FIBROBLASTS UPON TREATMENT.

Fibroblasts were treated with IL-1 for 15 minutes and lysed. Lysates from unstimulated (orange trace) and IL-1 treated cells (blue trace) were probed with Anti-Phospho-p38 antibody (Cell Signaling, cat# 9211). Phospho peaks were detected at pIs 5.4 and 5.8. Quantitation of the detected peaks shows 10-fold induction of phosphorylated p38 upon stimulation.

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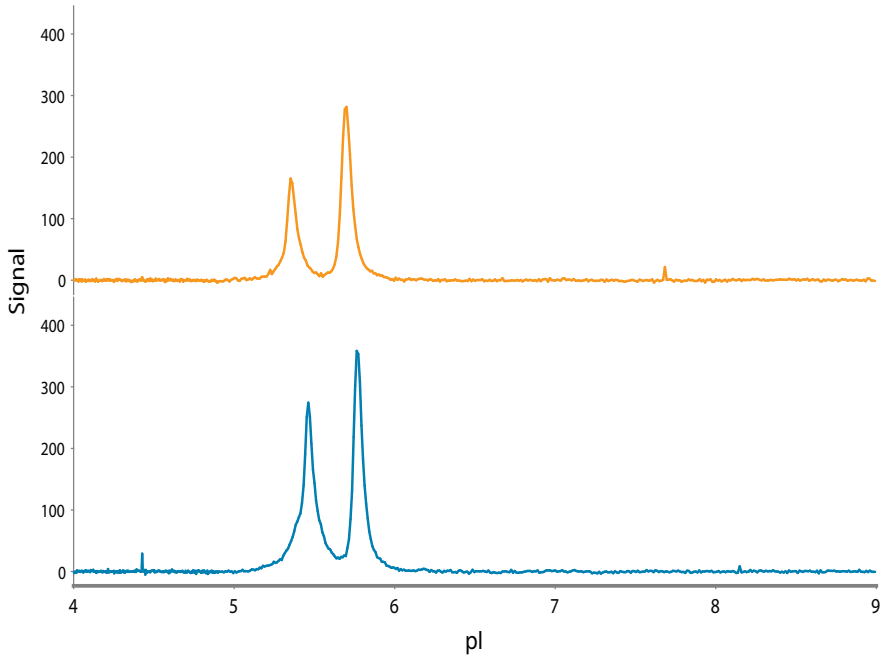

RECOGNITION OF P38 PHOSPHORYLATION IN FIBROBLASTS BY 2 INDEPENDENT P-P38 ANTIBODIES.

IL-1 stimulated fibroblasts lysates were probed with Anti-Phospho-p38 antibody from Cell Signaling (cat# 9211, orange trace) and Millipore (cat# 09-272, blue trace). Phospho peaks were detected at pIs 5.4 and 5.8 by the two antibodies.

PROTOCOL

CELL PREPARATION	
Cell culture:	Fibroblast-like synoviocytes (FLS) obtained from the joints (synovial tissue) of arthritis patients undergoing joint replacement surgery were cultured in DMEM containing 10% FBS, L-glutamine, penicillin, streptomycin and gentamicin in a humidified chamber containing an atmosphere of 5% CO_2. After 4 passages cells reached 99% homogeneity.
Pre-treatment:	Before treatment with Interleukin-1 cultured fibroblasts were placed at 37 °C, 5% CO_2 for 48 hours in starvation medium containing DMEM with no additives.
Treatment:	Interleukin-1 for 15 minutes at a final concentration of 2 ng/mL.
Lysis buffer:	Bicine/CHAPS Lysis Buffer (ProteinSimple, p/n 040-764) plus 1x DMSO Inhibitor Mix (ProteinSimple, p/n 040-510) and 1x Aqueous Inhibitor Mix (ProteinSimple, p/n 040-482).
Lysis details:	Wash 2× with Cell Wash Buffer (20 mM Bicine, 250 mM Sucrose, pH 7.5) and aspirate well. Add ice-cold lysis buffer to plate (400 µL/10 cm dish), swirl and incubate 5 minutes on ice. Scrape plate, pipette up-and-down to mix and transfer to a pre-chilled 1.5-mL microfuge tube. Incubate 30 minutes on ice, vortexing briefly every 5 minutes. Clarify lysate by centrifuging for 15 minutes at 14,000 × *g* and immediately collect supernatant. Aliquot supernatant (10 µL) on ice and snap freeze in liquid nitrogen.
Storage:	–80 °C

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ASSAY REAGENTS

Protein concentration:	0.1 mg/mL final in capillary by BCA assay
Sample Diluent:	Sample Diluent (ProteinSimple, p/n 040-649), 1x DMSO Inhibitor Mix (ProteinSimple, p/n 040-510)
Ampholyte Premix:	Premix G2 pH 3-10 (ProteinSimple, p/n 040-968)
pI Standards:	pI Standard Ladder 1: 4.0, 4.9, 6.0, 6.4, 7.3 (ProteinSimple, p/n 040-644)
Procedure:	Step 1) Dilute lysate to 0.4 mg/mL with sample diluents. Step 2) In a separate tube mix ampholyte premix G2 and pI standards. Step 3) Mix step 1 and step 2 at 1:4 to create final protein concentration.
Wash:	Wash Solution (ProteinSimple, p/n 040-313)
Primary antibody:	Anti-p38 (Millipore, cat # 09-272, 1:50), Anti-Phospho-p38 (T180/182) (Cell Signaling, cat# 9211, 1:50) in Antibody Diluent (ProteinSimple, p/n 040-309)
Detection antibody:	Anti-Rabbit HRP (ProteinSimple, p/n 040-656)
Anolyte:	Phosphoric Acid, 10 mM (ProteinSimple, p/n 040-337)
Catholyte:	Sodium Hydroxide, 100 mM (ProteinSimple, p/n 040-338)
Luminol/Peroxide xDR:	Mixed 1:1 (ProteinSimple, p/n 041-084 and p/n 040-652)

NOTE: NanoPro XDR Assays are now available with optional reagents to provide optimized assay sensitivity, extended dynamic range and both low and high end signal enrichment. Additionally, Premix G2 provides enhanced protein resolution. For more information, visit proteinsimple.com, contact your ProteinSimple Field Applications Specialist or call Technical Support at (888) 607-9692.

ASSAY CONDITIONS

System:	NanoPro 1000
Focus Conditions:	21000 µW, 40 minutes
Immobilization:	120 seconds
Wash 1:	2 x 150 seconds
Primary antibody incubation:	120 minutes
Wash 2:	2 x 150 seconds
Detection antibody incubation:	60 minutes
Wash 3:	2 x 150 seconds
Chemiluminescence exposure:	30, 60, 120, 240, 480 and 960 seconds

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SAMPLE PREPARATION ADDENDUM

Sample preparation procedures are different depending on the premix used. The following updated instructions provide guidance on sample preparation for both Premix G1 and Premix G2.

PREMIX G1 PROCEDURE	
Step 1	Dilute lysate with sample diluent to 0.1 mg/mL.
Step 2	In a separate tube, mix Premix G1 and pI standards.
Step 3	Mix equal parts of diluted lysate prepared in Step 1 with Premix G1 + pI Standards prepared in Step 2 (1:1 ratio) to create final protein concentration of 0.05 mg/mL.

PREMIX G2 PROCEDURE	
Step 1	Dilute lysate with sample diluent to 0.2 mg/mL.
Step 2	In a separate tube, mix Premix G2 and pI standards.
Step 3	Mix 1 part diluted lysate prepared in Step 1 with 3 parts Premix G2 + pI Standards prepared in Step 2 (1:3 ratio) to create final protein concentration of 0.05 mg/mL.
NOTE: When working with Premix G2, thorough mixing and vortexing during sample preparation is required. Additional sample volume may be required, 12-20 µL per sample well is recommended. Centrifugation of the sample plate (3000 x g, 10 minutes) is required.	

For further assistance, please contact your ProteinSimple Field Applications Specialist or Technical Support at (888) 607-9692.

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