Cell Biosciences™

NanoPro™ Assay: p-JNK

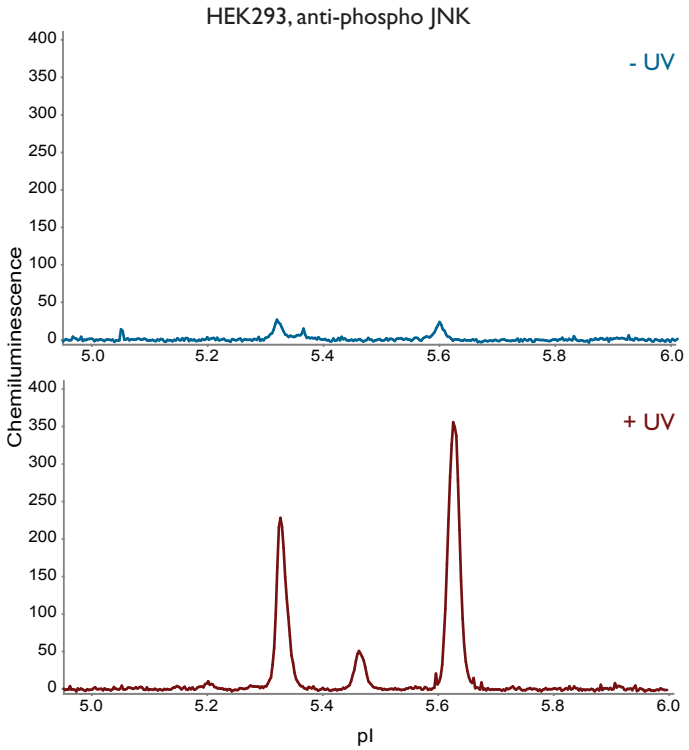
APPLICATION BRIEF No. 1019

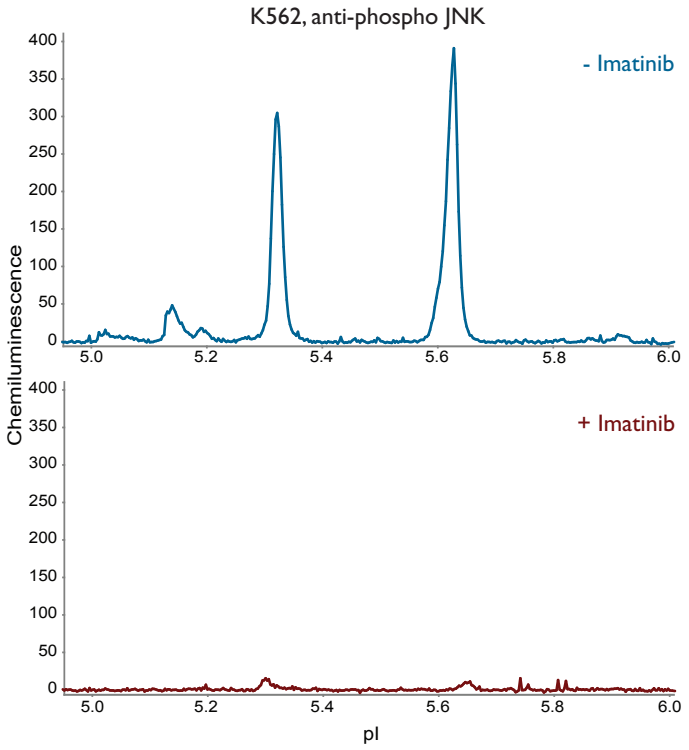
SUMMARY

Primary Antibody: Anti-phospho JNK (T183/Y185) (Cell Signaling Technology, cat# 9251)
Detection Antibody: Anti-Rabbit HRP (Cell Biosciences, p/n 040-656)

c-Jun N-terminal kinases (JNK), originally identified as kinases that bind and phosphorylate c-Jun on Ser63 and Ser73, are mitogen-activated protein kinases which are responsive to stress stimuli, such as cytokines, UV-irradiation, heat shock, and osmotic shock, and are involved in T cell differentiation and apoptosis. JNK1, 2 and 3 share a total of 10 isoforms in the pI range of 5.4–7.6. All three JNK kinases share a similar Thr/Tyr phosphorylation site (T183/Y185). We evaluate the change in JNK phosphorylation in UV-treated HEK293 cells and imatinib-treated K562 cells using a dual phospho-antibody against that site.

RESULTS





Detection of JNK phosphorylation UV-treated HEK293 cells and imatinib-treated K562 cells

HEK293 cells (left panel) were treated with -/+ UV-C exposure for 1 minute. The anti-phospho (T183/Y185) JNK antibody revealed a low level of JNK T183/Y185 phosphorylation in the untreated HEK293 cells (left panel, blue trace). After UV treatment, this antibody recognized three strongly-induced peaks (left panel, red trace). In contrast, untreated K562 cells showed significant levels of JNK T183/Y185 phosphorylation (right panel, blue trace). Treatment with imatinib drastically reduced the anti-phospho JNK signal (right panel, red trace).

NOTE: Detection of the chemiluminescent signal produced is relative. Absolute units may vary depending on cell line, treatment and assay conditions

PROTOCOL

Cell Preparation

Cell culture: HEK293 cells (ATCC, cat# CRL-1573) were cultured in EMEM (ATCC, cat# 30-2003) containing 10% FBS (Hyclone, cat# SH30070.03) and 1x Penicillin/Streptomycin/Glutamine (JRS Scientific, cat# 20020). Cells were split 1:5 every 3 days using 0.25% Trypsin (Cellgro, cat# 25-053-CI) at 37 °C for 3–5 minutes. Data shown from cells at passage 8.

Pre-treatment: Cells were starved for 20 hours before stimulation at 37 °C, 5% CO_2 in starvation medium containing RPMI 1640 without serum.

Treatment: Cells were washed with PBS (Mediatech, cat# 21-031-CV) and aspirated. Plate was exposed 6 inches from a UV-C lamp (30 mW/cm2, warmed for 5 minutes) for 0 and 60 seconds at room temperature.

Lysis buffer: Bicine/CHAPS Lysis Buffer (Cell Biosciences, p/n 040-764) plus 1x DMSO Inhibitor Mix (Cell Biosciences, p/n 040-510) and 1x Aqueous Inhibitor Mix (Cell Biosciences, p/n 040-482).

Lysis details: Wash cells with 10 mL of ice-cold PBS (Cellgro, cat# 21-031-CV), aspirate well. Add 400 µL ice-cold lysis buffer to 10-cm plate on ice, swirl around to ensure good coverage, and incubate 10 minutes on ice. Scrape plate, pipet up and down to mix. Transfer lysate to microfuge tube, lyse for an additional 30 minutes on ice. Clarify by centrifugation (14,000 x g, 15 minutes) in a cooled centrifuge. Transfer supernatant to a fresh microfuge tube. Immediately aliquot supernatant (10–30 µL) on ice and snap freeze on dry ice.

Storage: -80 °C

Cell culture: K562 cells (ATCC, cat# CCL-243) were cultured in RPMI 1640 media (Cellgro, cat#10-041-CV) containing 10% FBS and 1x Penicillin/Streptomycin/Glutamine. Cells were split 1:5 every 3 days by transferring an aliquot of cells to fresh media. Data shown from cells at passage 5.

Pre-treatment: Cells were starved for 20 hours before stimulation at 37 °C, 5% CO_2 in starvation medium containing RPMI 1640 without serum.

Treatment: 5 M Imatinib Methanesulfonate Salt (LC laboratories, cat# I-5508) in starvation medium for 24 hours at 37 °C, 5% CO_2.

Lysis buffer: Bicine/CHAPS Lysis Buffer plus 1x DMSO Inhibitor Mix and 1x Aqueous Inhibitor Mix.

Lysis details: Collect cells by centrifugation (1000 x g, 5 minutes). Transfer cells to a 15-mL centrifuge tube, spin (1000 x g, 5 minutes) to pellet the cells. Aspirate media. Wash cell pellet with 1 mL of ice-cold PBS (Cellgro, cat# 21-031-CV). Transfer cells to a 1.5-mL centrifuge tube, spin (14,000 x g, 2 minutes). Aspirate wash. Keeping tube on ice, add 400 µL ice-cold lysis buffer to pellet, pipet up and down to resuspend. Incubate for an additional 30 minutes, rotating. Clarify by centrifugation (14,000 x g, 15 minutes). Transfer supernatant to a fresh microfuge tube. Immediately aliquot supernatant (10–30 µL) on ice and snap freeze on dry ice.

Storage: -80 °C

Assay Reagent

NOTE: For specifics on sample preparation, please consult the addendum to this document.

Protein concentration: 0.05 mg/mL final in capillary by BCA assay

Sample diluent: Bicine/CHAPS Lysis Buffer with 1x DMSO Inhibitor Mix

Ampholyte premix: 80% premix 5-8 (Cell Biosciences Premix G1, p/n 040-327 or Premix G2, p/n 040-973) and 20% Ampholyte-free Premix premix (Cell Biosciences Premix G1, p/n 040-611 or Premix G2, p/n 040-967) with 12% Pharmalyte pI 3-10

pI standards: pI Standard Ladder 3 (Cell Biosciences, p/n 040-646)

Wash: Wash Buffer (Cell Biosciences, p/n 040-654)

Primary antibody: Anti-phospho JNK (T183/Y185) (Cell Signaling, cat# 9251), 1:50 in Antibody Diluent (Cell Biosciences, p/n 040-309)

Detection antibody: Anti-Rabbit HRP (Cell Biosciences, p/n 040-656), 1:100 in Antibody Diluent

Anolyte: Phosphoric Acid, 10 mM (Cell Biosciences, p/n 040-650)

Catholyte: Sodium Hydroxide, 100 mM (Cell Biosciences, p/n 040-651)

Luminol/Peroxide: Mixed 1:1 (Cell Biosciences, p/n 040-652 and p/n 040-653)

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 cellbiosciences.com, contact your Cell Biosciences' Field Applications Specialist or call Technical Support at (888) 607-9692.

Assay Conditions

System: NanoPro 1000

Sample loading time: 10 seconds (Premix G1), 25 seconds (Premix G2)

Focus conditions: 15000 µW, 40 minutes (Premix G1) or 21000 µW, 40 minutes (Premix G2)

Immobilization: 120 seconds

Wash 1: 2 x 150 seconds (default)

Primary antibody incubation: 240 minutes

Wash 2: 2 x 150 seconds (default)

Detection antibody incubation: 60 minutes

Wash 3: 2 x 150 seconds (default)

Chemiluminescence exposure: 60, 120, 240, and 480 seconds

Our favorite antibody

Anti-phospho JNK (T183/Y185) (Cell Signaling, cat# 9251)

Other antibody suggestions

Anti-phospho JNK (T183/Y185, T221/Y223) (Millipore, cat# 07-175)
Anti-JNK/SAPK1 (Millipore, cat# 06-748)
Anti-JNK1 (F-3) (Santa Cruz Biotechnology, cat# sc-1648)

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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.

Step	Premix G1 Procedure	Premix G2 Procedure
Step 1	Dilute lysate with sample diluents to 0.1 mg/mL.	Dilute lysate with sample diluents to 0.2 mg/mL.
Step 2	In a separate tube, mix Premix G1and pI standards.	In a separate tube, mix Premix G2 and pI standards.
Step 3	Mix equal parts of diluted lysate prepared in Step 1 with the Premix G1 + pI Standards prepared in Step 2 (1:1 ratio) to create final protein concentration of 0.05 mg/mL.	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.

NOTES: When working with Premix G2, thorough mixing and vortexing during sample preparation is required.
Additional sample volume may be required, 12-20 uL per sample well is recommended.
Centrifugation of the sample plate (3000 x g, 10 minutes) is required.

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

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