Cell Biosciences™

NanoPro™ Assay: p-STAT3

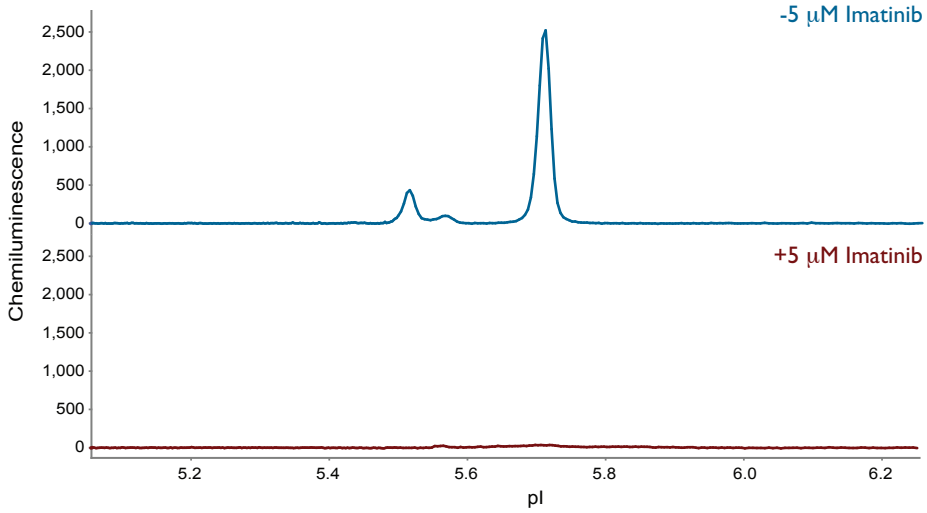
APPLICATION BRIEF No. 1012

SUMMARY

Primary Antibody: Anti-phospho STAT3 (Cell Signaling Technology, cat# 9131) and anti-phospho STAT3 (Abcam, cat# ab30646)
Detection Antibody: Anti-Rabbit HRP (Cell Biosciences, p/n 040-656)

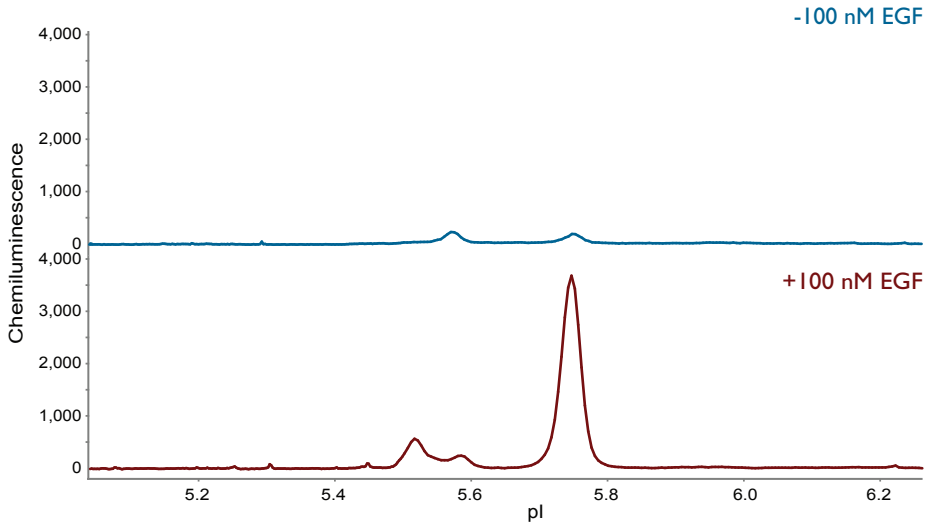
The Signal Transducer and Activator of Transcription (STAT) proteins regulate many aspects of cell growth, survival and differentiation. The transcription factors in this family are activated by Janus Kinase (JAK). Dysregulation of this pathway is frequently observed in primary tumors and leads to increased angiogenesis, enhanced survival of tumors and immunosuppression. STAT3 is constitutively active in overexpressing BCR-ABL K562 myelogenous leukaemia cells. Imatinib (also known as Gleevec®), a BCR-ABL inhibitor, reduces STAT3 phosphorylation in these cells. STAT3 is also part of the Epidermal Growth Factor (EGF) signaling cascade as shown in MCF10A cells.

RESULTS



Imatinib treatment reduces STAT3 phosphorylation in K562 cells

K562 cells were treated -/+ 5 μM imatinib for 24 hours. The CST 9131 anti-phospho STAT3 antibody recognized three peaks in the control cells (blue trace). These peaks were strongly inhibited by imatinib treatment (red trace). A similar phospho-STAT3 signature was detected in EGF-treated MCF10A cells using Abcam 30646 (see figure below).



EGF treatment increases STAT3 phosphorylation in MCF10A cells

MCF10A cells were treated -/+100 nM EGF for 5 minutes. The Abcam 30646 anti-phospho STAT3 antibody detected little phospho-STAT3 signal in the untreated MCF10A cells (blue trace). Exposure to EGF significant increase phospho-STAT3 signal in these cells (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: K562 cells (ATCC, cat# CCL-243) were cultured in RPMI 1640 media (Cellgro, cat#10-041-CV) containing 10% FBS (Irvine Scientific, cat# 3000-A) and 1x Penicillin/Streptomycin/Glutamine (JRS Scientific, cat# 20020). Cells were split 1:5 every 3 days by removing an aliquot of cells and transferring them 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 (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: 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

Cell culture: MCF10A cells (ATCC, cat# CRL-10317) were cultured in MEGM (Lonza, cat# CC-3151) containing 5% FBS, 1x Penicillin/Streptomycin/Glutamine and MEGM SingleQuot (Lonza, cat# CC-4136). Cells were split 1:5 every 3 days using 0.25% Trypsin at 37 °C for 3–5 minutes. Data shown from cells at passage 4.

Pre-treatment: Before EGF stimulation, cells were placed at 37 °C, 5% CO_2 for 20 hours in starvation medium.

Treatment: 600 ng/mL EGF in starvation medium for 5 minutes at 37 °C, 5% CO_2.

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

Assay Reagents

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

Protein concentration:	0.1 mg/mL final in capillary by BCA assay
Sample diluent:	Sample Diluent (Cell Biosciences, p/n 040-649) plus 1x DMSO Inhibitor Mix
Ampholyte premix:	Premix 5-8 (Cell Biosciences Premix G1, p/n 040-643 or Premix G2, p/n 040-972)
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 STAT3 (Cell Signaling Technology, cat # 9131 and Abcam, cat# ab30646), 1:100 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:	80 seconds
Wash 1:	2 x 150 seconds (default)
Primary antibody incubation:	120 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 antibodies

Anti-phospho STAT3 (Abcam, cat# ab30646)
Anti-phospho STAT3 (Cell Signaling Technology, cat# 9131)

Other antibody suggestions

Anti-STAT3 (Santa Cruz Biotechnology, cat# sc-483)
Anti-STAT3 (Genscript, cat# A00276)
Anti-STAT3 (Cell Signaling Technology, cat# 4904 and cat# 9132)
Anti-phospho STAT3 (Genscript, cat# A00251)

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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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