Cell Biosciences™

NanoPro™ Assay: Crk-L

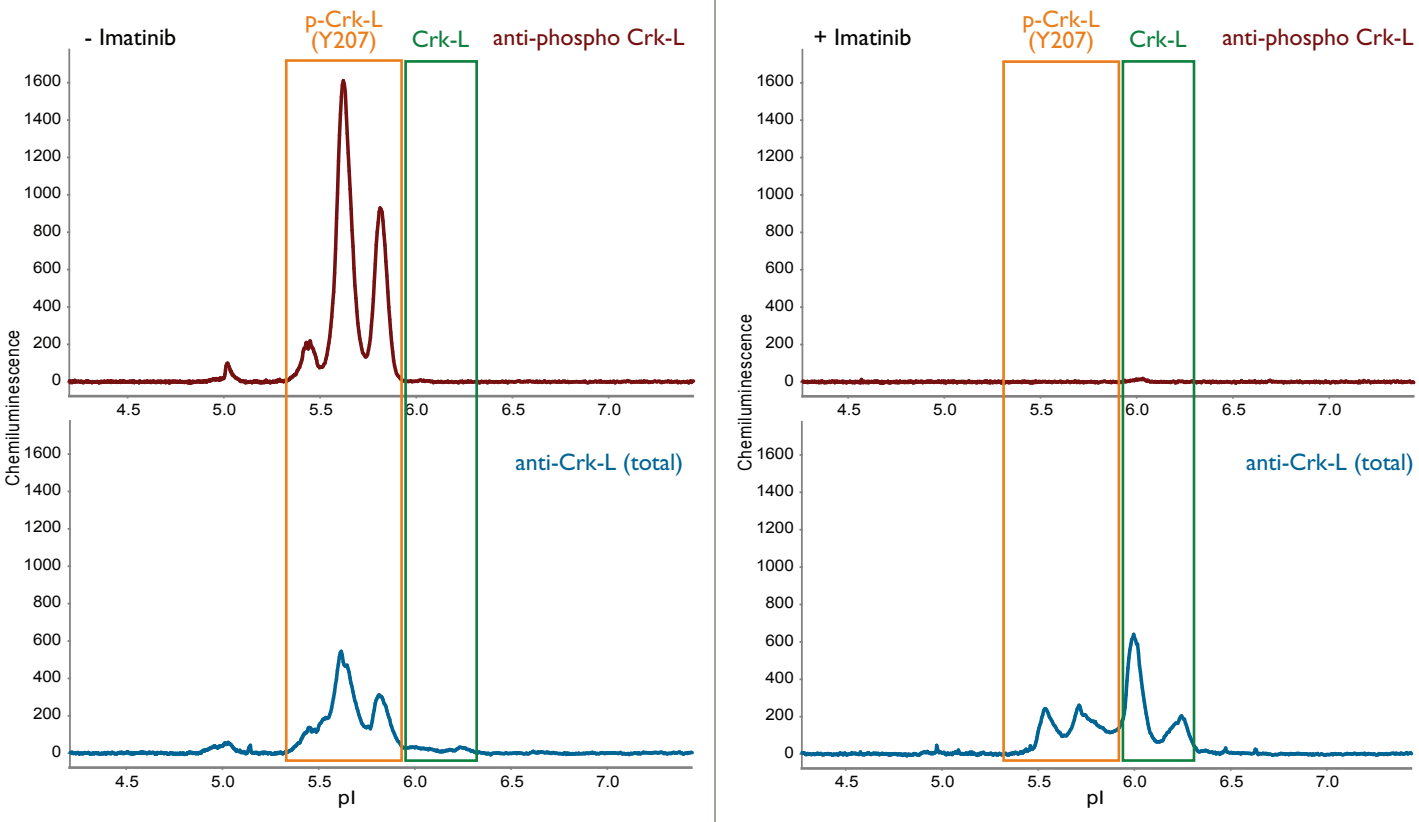
APPLICATION BRIEF No. 1023

SUMMARY

Primary Antibody: Anti-Crk-L (Cell Signaling Technology, cat# 3182) and Anti-phospho Crk-L (Abcam, cat# ab52908)
Detection Antibody: Anti-Rabbit HRP (Cell Biosciences, p/n 040-656) and Anti-Mouse HRP (Cell Biosciences, p/n 040-655)

Crk-like protein (Crk-L) is an adapter protein and phosphotyrosine-containing substrate implicated in transformation by the *bcr-abl* oncogene and in signaling by cytokines. It has been shown to activate the RAS and JUN kinase signaling pathways and transform fibroblasts in a RAS-dependent fashion. Crk-L is a substrate of the BCR-ABL tyrosine kinase and plays a role in fibroblast transformation by BCR-ABL. We show that Crk-L phosphorylation is reduced in response to Imatinib (commonly known as Gleevec®) treatment in K562 cells.

RESULTS



Detection of decreased Crk-L phosphorylation in K562 cell upon Imatinib treatment

K562 cells were treated -/+ 5 µM Imatinib for 1 hour and lysed. Traces from untreated controls are shown in the left panel and traces from Imatinib-treated cells are shown on the right. For the untreated cells, the anti-phospho (Y207) Crk-L antibody detected three discrete peaks in the pI 5.5–5.9 region (left panel, orange box, upper trace). The anti-total Crk-L antibody detected peaks at similar pIs (left panel, orange box, lower trace), putatively identified as phospho-Crk-L isoforms. Treatment with Imatinib (right panel) completely suppressed signal from phospho-Crk-L peaks as detected by the anti-phospho (Y207) Crk-L antibody (right panel, upper trace). The Imatinib treatment concurrently caused the appearance of two new peaks in the anti-total Crk-L antibody trace, putatively identified as non-phospho (Y207) isoforms.

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 the 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 1 hour 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

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:	Sample Diluent (Cell Biosciences, p/n 040-649) plus 1x DMSO Inhibitor Mix
Ampholyte premix:	Premix 5-8 (nested) (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-Crk-L (Cell Signaling Technology, cat# 3182) and Anti-phospho Crk-L (Abcam, cat# ab52908) both 1:50 in Antibody Diluent (Cell Biosciences, p/n 040-309)
Detection antibody:	Anti-Rabbit HRP (Cell Biosciences, p/n 040-656) and Anti-Mouse HRP (Cell Biosciences, p/n #040-655), both 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:	100 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, and 240 seconds

Our favorite antibodies

Anti-Crk-L (Cell Signaling Technology, cat# 3182)
Anti-Crk-L (Millipore, cat# 05-414)
Anti-phospho Crk-L (Millipore, cat# 09-466)
Anti-phospho Crk-L (Abcam, cat# ab52908)

Other antibody suggestions

Anti-Crk-L (Abcam, cat# 63491 and cat# 48578)
Anti-phospho Crk-L (Cell Signaling Technology, cat# 3181)

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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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