NanoPro™ Assay: GAPDH Loading Control

SUMMARY

Primary Antibody: Mouse Anti–GAPDH (Millipore, cat# MAB374)

Detection Antibody: Goat Anti–Mouse HRP (ProteinSimple, p/n 040-655)

GAPDH (Glyceraldehyde-3-phosphate dehydrogenase) is a housekeeping gene found in most tissues and cells. Because GAPDH expression is fairly constant in a variety of tissue and cell types, this protein is often used as a control in comparisons of protein expression levels. Here we describe its use as a loading control for cIAP1 inhibition in PBMCs. In the NanoPro assay, GAPDH is present with a major peak around pI 8.9 under the conditions described. We also demonstrate the multiplexing capabilities of the NanoPro assay and the detection of cIAP1 and GAPDH in the same capillary.

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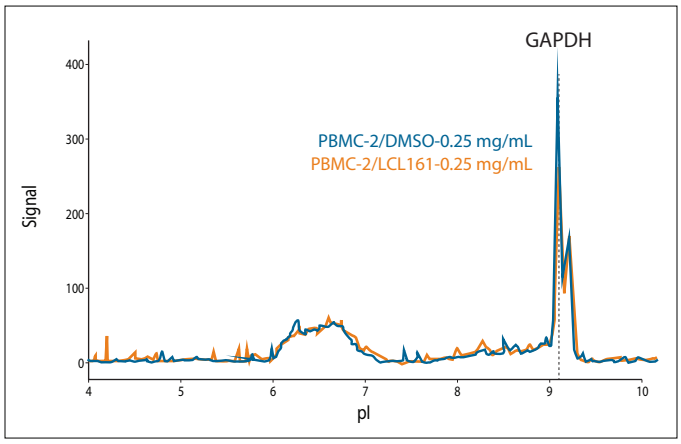
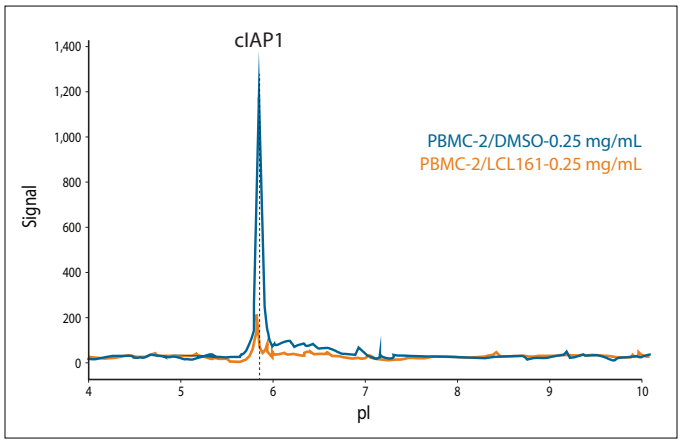
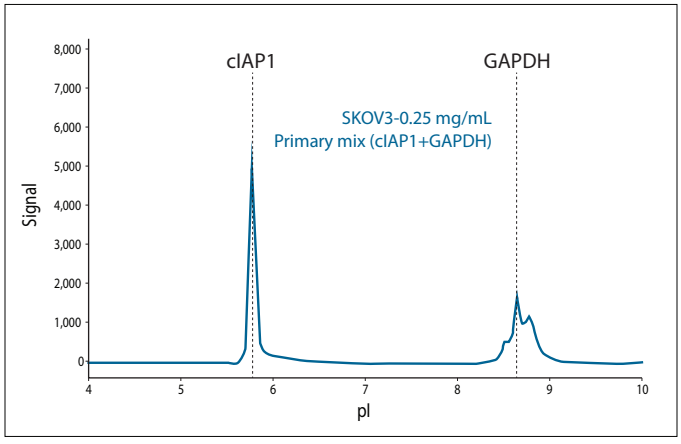
RESULTS





GAPDH serves as a consistent loading control; levels are equivalent in SMAC-mimetic inhibitor treated and untreated PBMCs. Blood from healthy donors (8 mL) was treated with 2 µM LCL161 or DMSO for 2 hours. Changes in cIAP1 levels were observed in treated cells as compared to untreated cells while GAPDH levels remained unchanged.



GAPDH and cIAP1 can be multiplexed in the same capillary

Because the pIs of cIAP1 and GAPDH are different, they can be detected simultaneously. The highly specific primary antibodies were mixed together for labeling and their species-specific conjugated secondary antibodies were introduced separately for detection.

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PROTOCOL

CELL PREPARATION

Cell culture:	SKOV3 cells are maintained in a humidified 5% CO_2 incubator at 37 °C. Cell lines are grown in RPMI1640 medium, penicillin/streptomycin and 10% fetal bovine serum.
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:	Cells are washed with ice cold 1x PBS and lysed with 150 µL of Bicine CHAPs Lysis Buffer with Aqueous Inhibitor Mix and DMSO Inhibitor Mix for 30 min on ice. Lysates are cleared by centrifugation at 13,000 x g for 15 minutes at 4 °C and protein concentrations are determined by the Lowry method (Bio-Rad).
Storage:	-80 °C

PBMC PREPARATION

Sample collection:	Collect 8 mL of blood from healthy donors in CPT tubes (BD Vaccutainer, ref# 362753) and invert 8 – 10 times. Centrifuge the blood sample at room temperature in a horizontal rotor (swing-out head) at 1500 to 1800 Relative Centrifugal Force (RCF) for 20 minutes. After centrifugation, mononuclear cells and platelets will be in a whitish layer just under the plasma layer. Immediately after centrifugation, use a serological pipette to remove and discard as much of the plasma layer as possible without disturbing the white layer. Next, collect the white layer and transfer to a 15 mL conical tube with cap (BD, cat# 352096). Add 10 mL of ice cold PBS and centrifuge at 1000 RCF for 10 minutes at 4 °C. Aspirate and discard the supernatant without disturbing the cell pellet. Add 0.5 mL of ice cold PBS to the pellet to resuspend and then transfer to a 1.5 mL eppendorf tube. Centrifuge the tube at 13000 x g for 5 minutes in a microfuge at 4 °C. Discard the supernatant without disturbing the pellet.
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:	Add 100-150 µL of Bicine CHAPs Lysis Buffer with Aqueous Inhibitor Mix and DMSO Inhibitor Mix to the PBMC pellet. Pipette up and down until the pellet is resuspended. Incubate for 30 minutes on ice. Lysates are cleared by centrifugation at 13,000 x g for 15 minutes at 4 °C and protein concentrations are determined by the Lowry method (Bio-Rad).
Storage:	–80 °C

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ASSAY REAGENTS

Protein concentration:	0.25 mg/mL for cell lysates and 0.5 mg/mL for PBMCs final concentration in capillary by Lowry method (Bio-Rad).
Sample diluent:	Bicine/CHAPS Lysis Buffer plus 1x DMSO Inhibitor Mix
Ampholyte premix:	Premix 3-10 (Premix G1, ProteinSimple, p/n 040-806)
pI standards:	pI Standard Ladder 1 (ProteinSimple, p/n 040-644) and pI 9.7 Standard (ProteinSimple, p/n 040-790)
Wash:	Wash Buffer (ProteinSimple, p/n 040-654)
Primary antibody:	Mouse Anti-GAPDH (Millipore, cat# MAB374), 1:500 in Antibody Diluent (ProteinSimple, p/n 040-309)
Detection antibody:	Goat Anti-Mouse HRP (ProteinSimple p/n 040-655), 1:100 in Antibody Diluent
Anolyte:	Phosphoric Acid, 10 mM (ProteinSimple, p/n 040-650)
Catholyte:	Sodium Hydroxide, 100 mM (ProteinSimple, p/n 040-651)
Luminol/Peroxide:	Mixed 1:1 (ProteinSimple, 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 proteinsimple.com, contact your ProteinSimple Field Applications Specialist or call Technical Support at (888) 607-9692.

ASSAY CONDITIONS

System:	NanoPro 1000
Sample loading time:	10 seconds (Premix G1)
Focus conditions:	15000 µW, 40 minutes (Premix G1)
Immobilization:	40 seconds
Wash 1:	2 x 150 seconds (default)
Primary antibody incubation:	120 minutes
Wash 2:	2 x 150 seconds (default)
Detection antibody incubation:	30 minutes
Wash 3:	2 x 150 seconds (default)
Chemiluminescence exposure:	60, 120, 240, 480 and 960 seconds

OUR FAVORITE ANTIBODIES

Mouse Anti – GAPDH (Millipore, cat# MAB374)
Goat Anti – cIAP1 (R&D systems, cat# AF8181)

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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 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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