Cell Biosciences™

NanoPro™ Assay: AKT

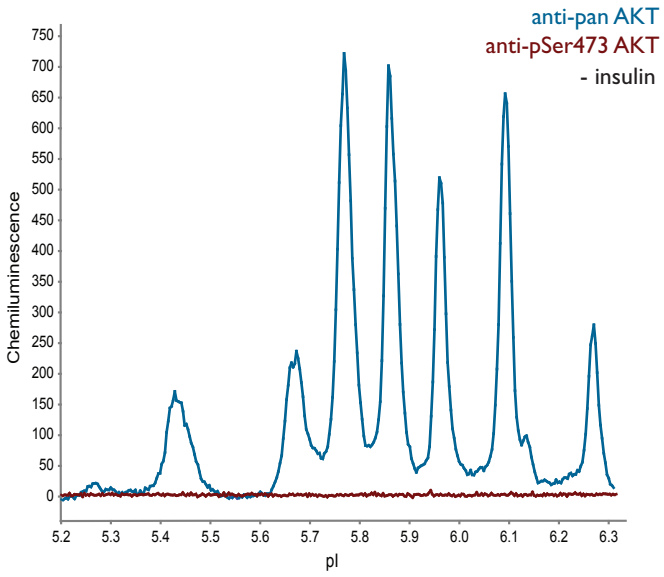
APPLICATION BRIEF No. 1002

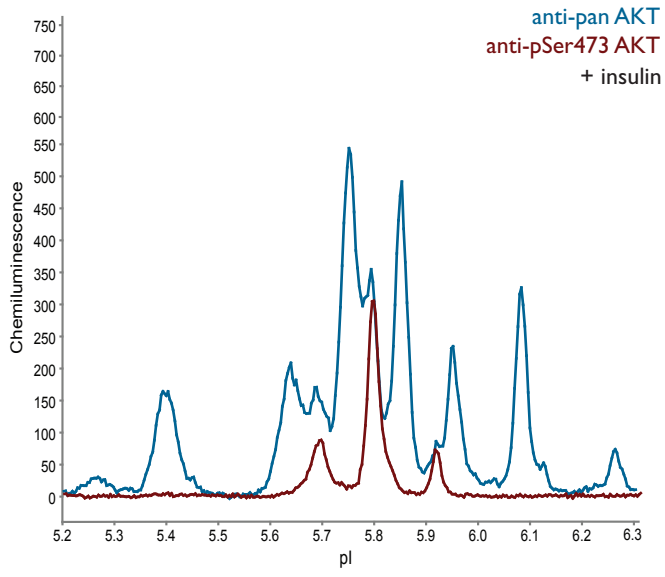
SUMMARY

Primary Antibody: Anti-AKT1/2 (Santa Cruz Biotechnology, cat# sc-8312),
Anti-phospho AKT1 (Cell Signaling Technology, cat# 9271)
Detection Antibody: Anti-Rabbit HRP (Cell Biosciences, p/n 040-656)

AKT, also referred to as PKB or Rac, plays a critical role in controlling cell survival and apoptosis. This protein kinase is activated by insulin and various growth and survival factors to function in a Wortmannin-sensitive pathway involving PI3 kinase. The main isoforms identified so far are AKT1, 2 and 3. AKT3 is mainly expressed in the brain. AKT1 and 2 play differential roles in glucose homeostasis. Activation at Thr308 and Ser473 are the main activating phosphorylation events for AKT1. Our data show increased phosphorylation of AKT using a phospho-Ser473 specific antibody. This antibody is believed to recognize sites corresponding to phospho-Ser473 on all three AKT isoforms (see Cell Signaling Technologies data sheet).

RESULTS





Insulin treatment significantly increases AKT phosphorylation at Ser473 in HeLa cells

HeLa cells were treated -/+ 200 nM insulin for 5 minutes. In the untreated cells (left panel), a number of discrete peaks were detected using the sc-8312 anti-pan AKT antibody (left panel, blue trace). However, the CST 9271 antibody detected no phospho-Ser473 signal (left panel, red trace). In contrast, three distinct phospho-Ser473 peaks were detected after insulin treatment (right panel, red trace). Note that the anti-pan AKT antibody also detected peaks with identical pIs to the three phospho-Ser473 peaks (right panel, blue trace). The peaks at pI 5.8 and 5.95 are likely to be AKT2 isoforms, as determined by a specific anti-AKT2 antibody (data not shown).

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: HeLa cells were cultured in DMEM (ATCC, cat# 30-2002) 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 using 0.25% Trypsin (Cellgro, cat# 25-053-CI) at 37 °C for 3–5 minutes to dislodge. Data shown from cells at passage 5.

Pre-treatment: Before EGF stimulation, cells were placed at 37 °C, 5% CO_2 overnight in DMEM without serum.

Treatment: 200 nM Insulin (Sigma, cat# I6634) in DMEM 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 (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-AKT1/2 (Santa Cruz Biotechnology, cat# SC8312) and Anti-phospho AKT1 (Cell Signaling Technology, cat# 9271), both 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, and 240 seconds

Our favorite antibodies

Anti-AKT1/2 (Santa Cruz Biotechnology, cat# sc-8312)
Anti-AKT1 (Millipore, cat# 05-796)
Anti-AKT2 (Cell Signaling Technology, cat# 2962)
Anti-phospho AKT1 (Cell Signaling Technology, cat# 9271)
Anti-phospho AKT1 (Epitomics, cat# 2118-1)

Other antibody suggestions

Anti-AKT1/2 (Cell Signaling Technology, cat# 9272)

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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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