NanoPro™ Assay: Glycogen Synthase Kinase 3β (GSK-3β)

Cell Biosciences™

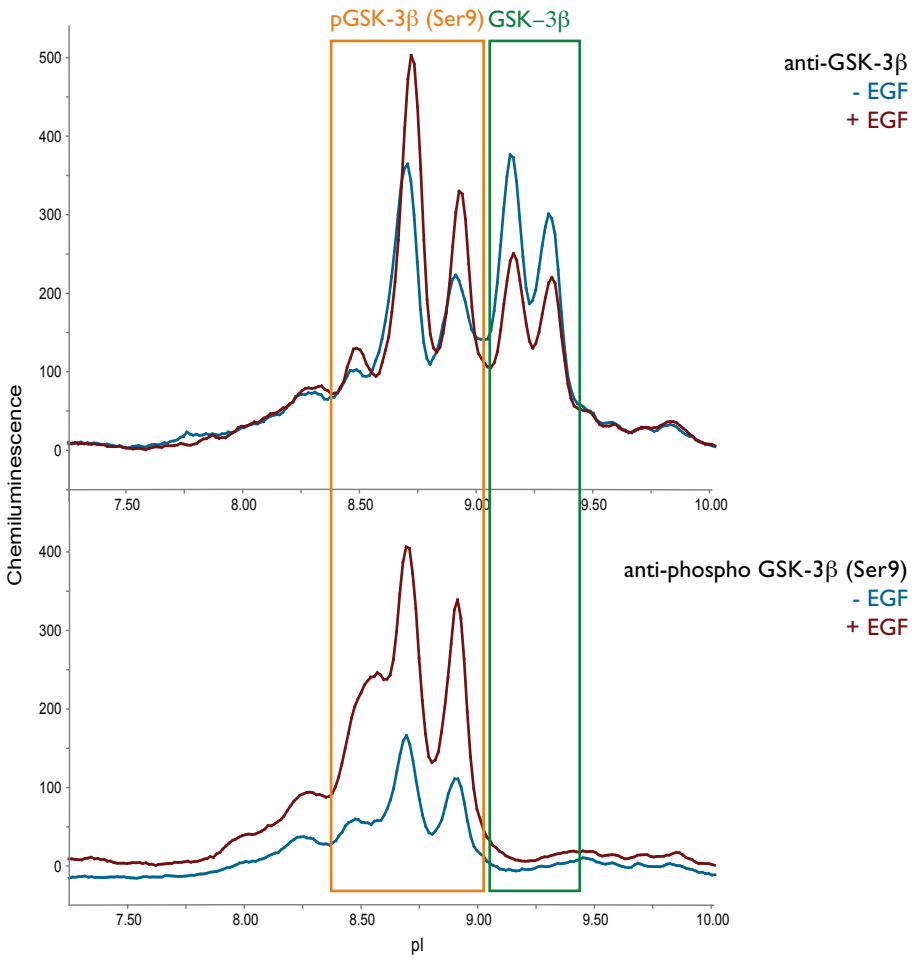
APPLICATION BRIEF No. 1006

SUMMARY

Primary Antibody: Anti-GSK-3β (Abcam, cat# ab69739) and Anti-phospho GSK-3β (Ser9) (Cell Signaling Technology, cat# 9336)
Detection Antibody: Anti-Rabbit HRP (Cell Biosciences, p/n 040-656)

GSK3 is a critical downstream element of the PI3 kinase/AKT cell survival pathway whose activity can be inhibited by AKT-mediated phosphorylation at Ser21 of GSK-3α and Ser9 of GSK-3β. While GSK-3α and GSK-3β have high sequence homology, their biological function differs. The data presented shows an increase of peaks detected by anti-phospho GSK-3β antibody as well as an increase of the same peaks detected by the total anti-GSK-3β antibody in response to EGF treatment in MCF10A cells. At the same time, the peaks not recognized by the anti-pS21 antibody decrease in size, implying that these peaks represent non-phospho or non-pS21 phospho GSK-3α forms. The position of these peaks around pI 9 is in accordance with the theoretical pI for this sequence. Alignment of the GSK-3α and GSK-3β profiles, in addition to use of antibodies recognizing both isoforms, confirmed the specificity of the anti-GSK-3α versus the anti-GSK-3β antibodies used (data not shown).

RESULTS



EGF stimulation results in increased phosphorylation of GSK-3β at Ser9

MCF10A cells were stimultated with epidermal growth factor (EGF) for 15 minutes. The peaks labeled pGSK-3β (Ser9) in the figure showed increased signal after EGF stimulation, as detected by anti-GSK-3β (upper traces) and anti-phospho GSK-3β (Ser9) (lower traces). The two peaks labeled "GSK-3β" in the upper trace showed decreased signal after EGF treatment and were not recognized by anti-phospho GSK-3β (Ser9), which suggests that these peaks are in lower phosphorylation states of GSK-3β with insignificant phosphorylation at Ser9.

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: MCF10A cells (ATCC, cat# CRL-10317) were cultured in MEGM (Lonza, cat# CC-3151) containing 5% FBS (Hyclone, Scientific, cat# 1677-006), 1x Penicillin/Streptomycin/Glutamine (JRS Scientific, cat# 20020), 100 ng/mL Cholera Toxin (Calbiochem, cat# 227035), and MEGM SingleQuot (Lonza, cat# CC-4136) with final concentration of 13 mg/mL BPE, 0.5 mg/mL hydrocortisone, 10 µg/mL hEGF, 5 mg/mL insulin. 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 5.

Pre-treatment: Before EGF stimulation, cells were placed at 37 °C, 5% CO_2 overnight in starvation medium containing MEGM, 1% FBS, 1x Penicillin/Streptomycin/Glutamine, 100 ng/mL cholera toxin, 13 mg/mL BPE, and 0.5 mg/mL hydrocortisone.

Treatment: 20 ng/mL EGF (Millipore, cat# 01-107) in full media without MEGM SingleQuot for 15 minutes at 37 °C, 5% CO_2.

Lysis buffer: RIPA Lysis Buffer (Cell Biosciences, p/n 040-483) 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: Ampholyte-free premix (Cell Biosciences Premix G1, p/n 040-611 or Premix G2, p/n 040-967) with 12% Pharmalyte pI 3-10, 1% TEMED (Sigma, cat# T7024)

pI standards: pI Standards 5.5 and 7.0 (Cell Biosciences, p/n 040-028 and p/n 040-031), 1:100 and pI Standard 9.7 at 2 µM (Anaspec, cat# 61750)

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

Primary antibody: Anti-GSK-3β (Abcam, cat# ab69739) and Anti-phospho GSK-3β (Ser9) (Cell Signaling Technology, cat# 9336), both 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, 50 minutes (Premix G1) or 21000 µW, 50 minutes (Premix G2)
Immobilization:	80 seconds
Wash 1:	2 x 150 seconds (default)
Primary antibody incubation:	60 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-GSK-3β(Abcam, cat# 69739)
Anti-pGSK-3β (Cell Signaling Technology, cat# 9336)

Other antibody suggestions

Anti-GSK-3α/β (Millipore, cat# 05-412)

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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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