

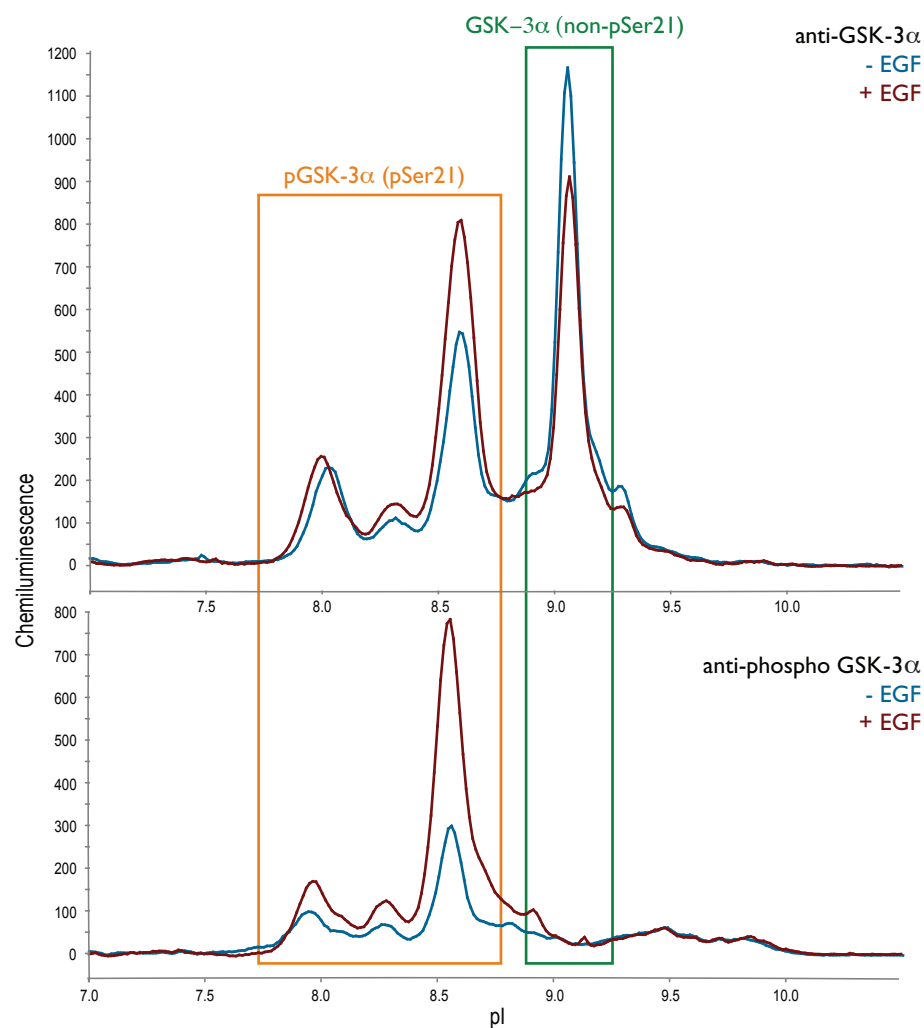
# NanoPro™ Assay: Glycogen Synthase Kinase 3α (GSK-3α)

## SUMMARY

Primary Antibody: Anti-GSK-3α (Cell Signaling Technology, cat# 9338) and  
Anti-phospho GSK-3α (Ser21) (Cell Signaling Technology, cat# 9316)  
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-3α antibody as well as an increase of the same peaks detected by the total anti-GSK-3α antibody in response to EGF treatment in MCF10A cells. At the same time, the peaks not recognized by the anti-phospho Ser21 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. In other cell systems (A549 for example), both the anti-total GSK-3α antibody as well as the anti-phospho GSK-3α antibody also recognize peaks around pI 6. Alignment of the GSKα and GSKβ 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-3α at Ser21

MCF10A cells were stimulated with epidermal growth factor (EGF) for 15 minutes. The peaks labeled pSer21 in the figure reproducibly showed increased signal after EGF stimulation, as detected by anti-GSK-3α (upper traces) and antiphospho GSK-3α (Ser21) (lower traces). The peak labeled non-pSer21 in the upper trace showed slightly decreased signal after EGF treatment and was not recognized by anti-phospho GSK-3α (Ser21), which suggests that this peak represents a lower phosphorylation state of GSK-3α with insignificant phosphorylation at Ser21.

*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, 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<sub>2</sub> 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<sub>2</sub>.
- 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 pl 3–10, 1% TEMED (Sigma, cat# T7024)
- pl standards:** pl Standards 5.5 and 7.0 (Cell Biosciences, p/n 040-028 and p/n 040-031), 1:100
- Wash:** Wash Buffer (Cell Biosciences, p/n 040-654)
- Primary antibody:** Anti-GSK-3α (Cell Signaling Technology, cat# 4818) and Anti-phospho GSK-3α (Ser21) (Cell Signaling Technology, cat# 9316) 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](http://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:** 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-3α (Cell Signaling Technology, cat# 4818)  
Anti-phospho GSK-3α (Ser21)  
(Cell Signaling Technology, cat# 9316)

### Other antibody suggestions

Anti-GSK-3α (Cell Signaling Technology, cat# 9338)  
Anti-GSK-3α/β (Millipore, cat# 05-412)

# 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 G1 and pl standards.	In a separate tube, mix Premix G2 and pl standards.
Step 3	Mix equal parts of diluted lysate prepared in Step 1 with the Premix G1 + pl 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 + pl 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.