NanoPro™ Assay: eEF2

SUMMARY

Primary Antibody: Anti-eEF2 (Cell Signaling, cat# 2332), Anti-Phospho-eEF2 (T56) (Cell Signaling, cat# 2331)
Detection Antibody: Anti-Rabbit HRP (ProteinSimple, p/n 040-656)

Eukaryotic elongation factor-2 (eEF2) catalyzes ribosome translocation during translation of mRNA. Phosphorylation of eEF2 by eEF2 kinase (also known as CaM kinase III) inactivates the protein and can block protein synthesis. Stimulation of growth is associated with a decrease in eEF2 phosphorylation. Inhibition of eEF2 phosphorylation in the hippocampus has recently been associated with an anti-depressant effect. We show that insulin treatment reduces phosphorylation of eEF2 in H4IIE cells. Inhibition of phosphorylation of eEF2 is restored in the presence of Nh125.

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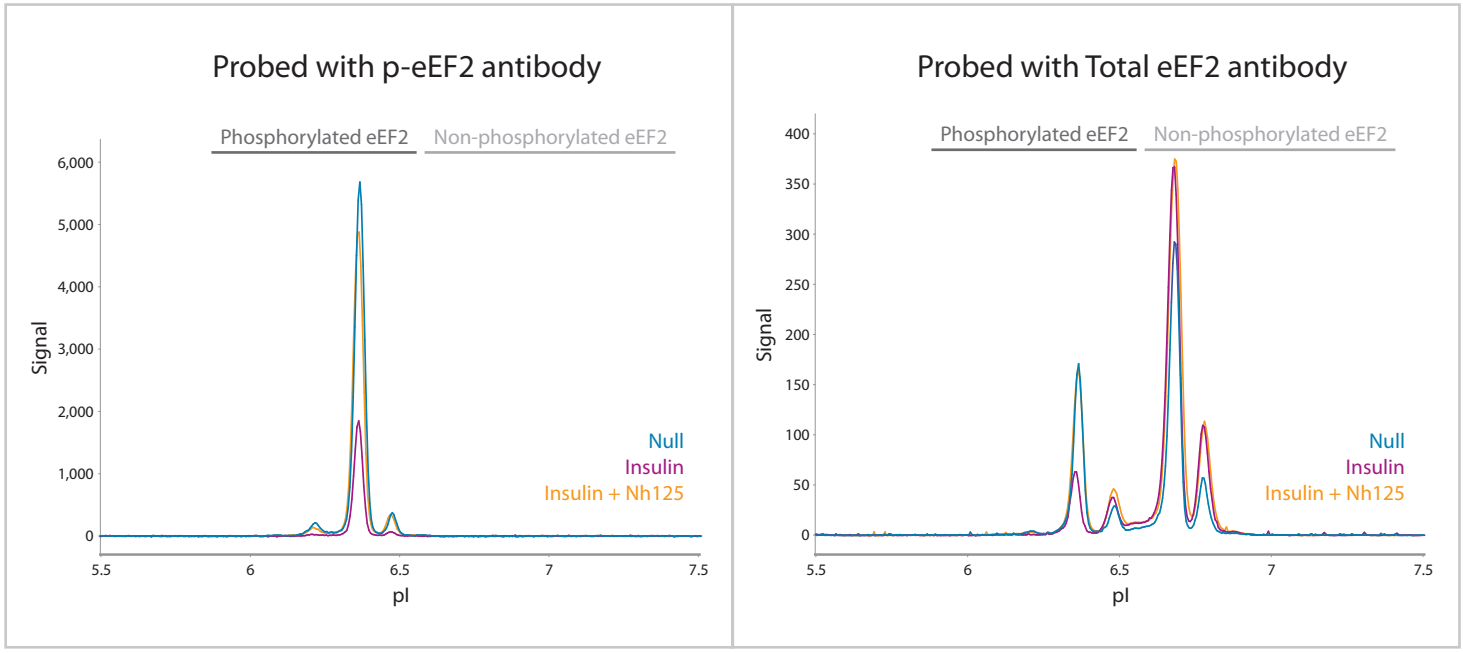
RESULTS



DETECTION OF EEF2 PHOSPHORYLATION IN H4IIE CELLS UPON TREATMENT

H4IIE cells were treated 250 nM Insulin for 10 minutes and lysed. Lysates from null (control), insulin treated and insulin + Nh125 were probed with p-eEF2 (left) and Total eEF2 (right) antibodies. Phospho peaks were detected with the phosphorylated and total eEF2 antibodies at pI 6.21, 6.35, 6.48. Non phosphorylated peaks were detected with the total antibody at pI 6.68 and 6.78. Treatment with Insulin suppressed eEF2 phosphorylation (purple traces) compared to the null samples (blue traces). In presence of 30 nM Nh125 (orange traces), insulin-mediated suppression of p-eEF2 was restored.

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PROTOCOL

CELL PREPARATION	
Cell culture:	H4IIE cells (ATCC, cat# CRL-1548) were cultured in αMEM (Invitrogen, cat#12000-063) containing 10% FBS (Invitrogen, cat#124083-020) and 1× Penicillin/Streptomycin (Invitrogen, cat#15140-122). Cells were split 1:6 every 3 days using 1.8% Trypsin (Invitrogen, cat#15090-046) at 37 °C for 3-5 minutes.
Pre-treatment:	Before treatment with Insulin cells were place at 37°C, 5% CO_2 for 72 hours in starvation medium containing αMEM with no additives.
Treatment:	250 nM Insulin for 10 minutes.
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:	Wash 2× with Cell Wash Buffer (20 mM Bicine, 250 mM Sucrose, pH 7.5) and aspirate well. Add ice-cold lysis buffer to plate (400 µL/10-cm dish), swirl and incubate 5 minutes on ice. Scrape plate, pipette up-and-down to mix and transfer to a pre-chilled 1.5-mL microfuge tube. Incubate 30 minutes on ice, vortexing briefly every 5 minutes. Clarify lysate by centrifuging for 15 minutes at 14,000 × *g* and immediately collect supernatant. Aliquot supernatant (10 µL) on ice and snap freeze in liquid nitrogen.
Storage:	–80 °C

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ASSAY REAGENTS

Protein concentration:	0.05 mg/mL final in capillary by BCA assay
Sample Diluent:	Sample Diluent (ProteinSimple, p/n 040-649) plus 1x DMSO Inhibitor Mix (ProteinSimple, p/n 040-510)
Ampholyte Premix:	Premix G2 pH 5-8 nested (ProteinSimple, p/n 040-972)
pI Standards:	pI Standard Ladder 1: 4.0, 4.9, 6.0, 6.4, 7.3 (ProteinSimple, p/n 040-644)
Procedure:	Step 1) Dilute lysate to 0.2 mg/mL with sample diluents. Step 2) In a separate tube mix ampholyte premix G2 and pI standards. Step 3) Mix step 1 and step 2 at 1:4 to create final protein concentration.
Wash:	Wash Solution (ProteinSimple, p/n 040-313)
Primary antibody:	Anti-eEF2 (Cell Signaling, cat# 2332, 1:100), Anti-Phospho-eEF2 (T56) (Cell Signaling, cat #2331, 1:100) in Antibody Diluent (ProteinSimple, p/n 040-309)
Detection antibody:	Anti-Rabbit HRP (ProteinSimple, p/n 040-656)
Anolyte:	Phosphoric Acid, 10 mM (ProteinSimple, p/n 040-337)
Catholyte:	Sodium Hydroxide, 100 mM (ProteinSimple, p/n 040-338)
Luminol/Peroxide xDR:	Mixed 1:1 (ProteinSimple, p/n 041-084 and p/n 040-652)

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
Focus Conditions:	21000 µW, 40 minutes
Immobilization:	120 seconds
Wash 1:	2 x 150 seconds
Primary antibody incubation:	120 minutes
Wash 2:	2 x 150 seconds
Detection antibody incubation:	60 minutes
Wash 3:	2 x 150 seconds
Chemiluminescence exposure:	30, 60, 120, 240, 480 and 960 seconds (240 seconds usually being sufficient)

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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.

PREMIX G1 PROCEDURE	
Step 1	Dilute lysate with sample diluent to 0.1 mg/mL.
Step 2	In a separate tube, mix Premix G1 and pI standards.
Step 3	Mix equal parts of diluted lysate prepared in Step 1 with Premix G1 + pI Standards prepared in Step 2 (1:1 ratio) to create final protein concentration of 0.05 mg/mL.

PREMIX G2 PROCEDURE	
Step 1	Dilute lysate with sample diluent to 0.2 mg/mL.
Step 2	In a separate tube, mix Premix G2 and pI standards.
Step 3	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.
NOTE: 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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