NanoPro™ Assay: Heat Shock Protein 70 (Hsp70) Loading Control

Cell Biosciences™

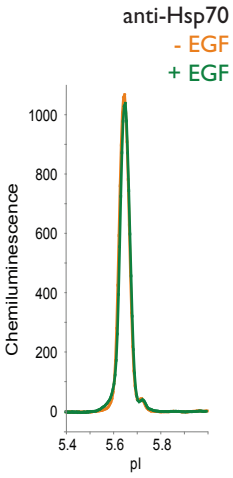
APPLICATION BRIEF No. 1009

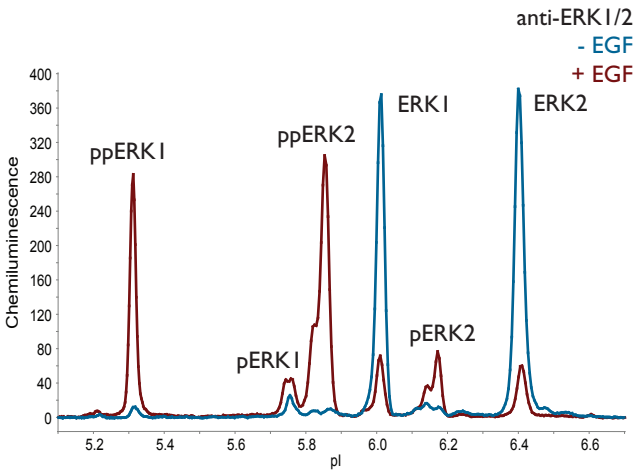
SUMMARY

Primary Antibody: Anti-Hsp70 [W27] (Novus Biologicals, cat# NB600-571)
Detection Antibody: Anti-Mouse HRP (Cell Biosciences, p/n 040-655)

The 70 kilodalton heat shock proteins (Hsp70) are a family of ubiquitously expressed heat shock proteins. Proteins with similar structure exist in virtually all living organisms. The Hsp70s are an important part of the cell's machinery, and help to protect proteins from misfolding under stress. We describe its use as loading control for EGF stimulation in HeLa and MCF10A cells. In the NanoPro assay, Hsp70 is present as a single peak around pI 5.8 under the conditions described.

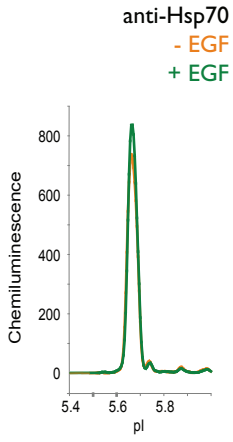
RESULTS

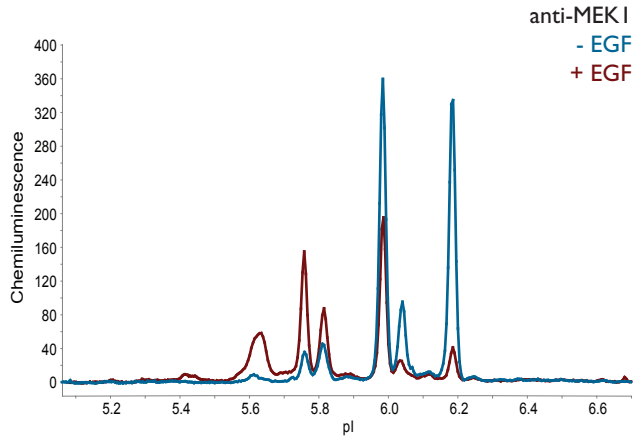




Hsp70 serves as a consistent loading control; levels are equivalent in EGF stimulated and non-stimulated HeLa cells

HeLa cells were stimulated with 50 ng/mL epidermal growth factor (EGF) for 15 minutes. Changes in phosphorylation of ERK1 and ERK2 were observed in stimulated cells as compared to non-stimulated while Hsp70 levels remained unchanged.





Hsp70 serves as a consistent loading control; levels are equivalent in EGF stimulated and non-stimulated MCF10A cells

MCF10A cells were stimulated with 20 ng/mL EGF for 15 minutes. Changes in amounts of MEK1 were observed in stimulated cells as compared to starved cells while Hsp70 levels remained unchanged.

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 (ATCC, cat# CCL-2) were cultured in DMEM (ATCC, cat# 30-2002) containing 5% FBS (VWR, cat# 1677-006) and 1x Penicillin/Streptomycin/Glutamine (JRS Scientific, cat# 20020). Cells were split 1:5 every 3 days using 0.25% Trypsin (Mediatech, cat# 25-053-CI) 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 for 14 hours in starvation medium containing DMEM and 1% FBS.

Treatment: 50 ng/mL EGF (Millipore, cat# 01-107) in starvation medium for 15 minutes at 37 °C, 5% CO_2.

Lysis buffer: RIPA Lysis Buffer (Cell Biosciences, p/n CBS401) 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

Cell culture: MCF10A cells (ATCC, cat# CRL-10317) were cultured in MEGM (Lonza, cat# CC-3151) containing 5% FBS, 1x Penicillin/Streptomycin/Glutamine, 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, and 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, 13 mg/mL BPE, 0.5 mg/mL hydrocortisone, 100 ng/mL cholera toxin, and 1x Penicillin/Streptomycin/Glutamine.

Treatment: 20 ng/mL EGF in full media for 15 minutes at 37 °C, 5% CO_2.

Lysis buffer: RIPA Lysis Buffer plus 1x DMSO Inhibitor Mix and 1x Aqueous Inhibitor Mix.

Lysis details: Wash cells with 10 mL of ice-cold PBS, 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: 0.03 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 Standards: 4.92, 5.5, 7.0 (Cell Biosciences, p/n 040-027, p/n 040-028, p/n 040-031), 1:100
Wash: Wash Buffer (Cell Biosciences, p/n 040-654)
Primary antibody: Anti-Hsp70 [W27] (Novus Biologicals, cat# NB600-571)1:500 in Antibody Diluent (Cell Biosciences, p/n 040-309)
Detection antibody: Anti-Mouse HRP (Cell Biosciences, p/n 040-655), 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 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: 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, and 240 seconds

Our favorite antibody

Anti-HSP-70 (Novus Biologicals, cat# NB600-571)

Cell Biosciences, Inc.
3040 Oakmead Village Drive
Santa Clara, CA 95051
tel: 408.510.5500
fax: 408.510.5599
www.cellbiosciences.com

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.

Cell Biosciences, Inc.
3040 Oakmead Village Drive
Santa Clara, CA 95051
tel: 408.510.5500
fax: 408.510.5599
www.cellbiosciences.com