Cell Biosciences™

NanoPro™ Assay: 4E-BP1

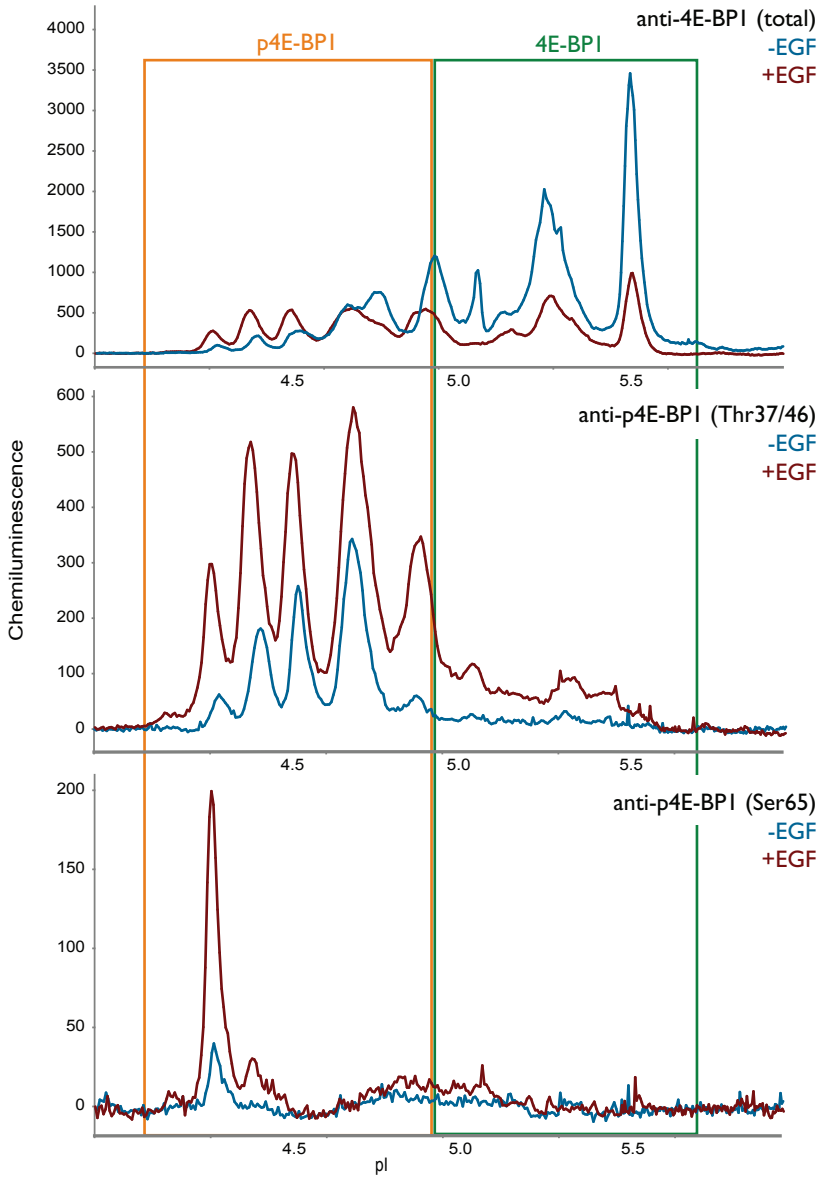
APPLICATION BRIEF No. 1021

SUMMARY

Primary Antibody: Anti-4E-BP1 (Cell Signaling Technology, cat# 9644),
Anti-phospho 4E-BP1 (Thr37/46) (Cell Signaling Technology, cat# 9459) and
Anti-phospho 4E-BP1 (Ser65) (Cell Signaling Technology, cat# 9451)
Detection Antibody: Anti-Rabbit HRP (Cell Biosciences, p/n 040-656)

Translation repressor protein 4E-BP inhibits cap-dependent translation by binding to the eIF4E translation initiation factor. Hyperphosphorylation of 4E-BP disrupts this interaction and results in activation of cap-dependent translation. Both the PI3 kinase/Akt pathway and FRAP/mTOR kinase regulate 4E-BP activity. 4E-BP1 has been implicated as a biomarker for several cancer types, while 4E-BP2 has been shown to potentially play a role in energy homeostasis. We show 4E-BP1 activation in MCF10A cells in response to EGF using total and anti-phospho 4E-BP1 antibodies that enable distinction between phospho and non-phospho peaks.

RESULTS



EGF-induced phosphorylation of 4E-BP1 in MCF10A cells

MCF10A cells were stimulated with 600 ng/mL EGF for 15 minutes. Treated and untreated lysates were probed with anti-4E-BP1 (total), (top traces), anti-p4E-BP1 (Thr37/46), (middle traces), and anti-p4E-BP1 (Ser65), (lower traces). In the top traces (total 4E-BP1 antibody), EGF treatment caused decreased signals for several basic peaks (green box, non-phospho 4E-BP1), and increased signals for several acidic peaks (orange box, phospho 4E-BP1). These acidic peaks were all detected by anti-p4E-BP1 (Thr37/46), (middle traces), identifying them as Thr37/46 phosphorylated isoforms. As expected, anti-p4E-BP1 (Thr37/46) detected no peaks in the non-phospho 4E-BP1 region (green box). Somewhat surprisingly, the anti-p4E-BP1 (Ser65) antibody (lower traces) recognized only the most acidic peak in the EGF-response profile. Based on these data, one might speculate that phosphorylation at Thr36/46 is a required prerequisite for Ser65 phosphorylation.

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-3150) containing 10% FBS (Irvine Scientific, cat# 3000-A), 1x Penicillin/Streptomycin/Glutamine (JRS Scientific, cat# 20020), and MEGM SingleQuots (Lonza, cat# CC-4136). Cells were split 1:5 every 3 days using 0.25% Trypsin (Cellgro, cat# 25-053-CI) at 37 °C for 3–5 minutes. Data shown from cells at passage 4.

Pre-treatment: Cells were starved for 20 hours before stimulation at 37 °C, 5% CO_2 in starvation medium containing MEGM.

Treatment: 600 ng/mL EGF (Sigma, cat# E1257) in starvation medium for 15 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: 0.2 mg/mL final in capillary by BCA assay

Sample diluent: Bicine/CHAPS Lysis Buffer 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-4E-BP1 (Cell Signaling Technology, cat# 9644), Anti-phospho 4E-BP1 (Thr37/46) (Cell Signaling Technology, cat# 9459) and Anti-phospho 4E-BP1 (Ser65) (Cell Signaling Technology, cat# 9451) all 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, 40 minutes (Premix G1) or 21000 µW, 40 minutes (Premix G2)
Immobilization:	100 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-4E-BP1 (Cell Signaling Technology, cat# 9644)
Anti-phospho 4E-BP1 (Thr370/46) (Cell Signaling Technology, cat# 9459)
Anti-phospho 4E-BP1 (Ser65) (Cell Signaling Technology, cat# 9451)
Anti-non-phospho 4E-BP1 (Thr46) (Cell Signaling Technology, cat# 4923)
Anti-phospho 4E-BP1/2/3 (Thr45) (Epitomics, cat# 2334-1)
Anti-4E-BP1 (cross-reacts with 4E-BP2) (Millipore, cat# 07-1416)

Other antibody suggestions

Anti-4E-BP1 (Epitomics, cat# 1557-1)
Anti-phospho 4E-BP1 (Cell Signaling Technology, cat# 2855)
Anti-phospho 4E-BP1 (Ser65) (Cell Signaling Technology, cat# 9456)
Anti-phospho 4E-BP1 (Thr46) (Abcam, cat# ab27792)

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