Rabbit (polyclonal) Anti-4E-BP1 [pT^{46}] Phosphospecific Antibody, Unconjugated

PRODUCT ANALYSIS SHEET

Catalog Number:	44-1170G (10 mini-blot size)
Lot Number:	See product label
Volume:	100 µL
Form of Antibody:	Rabbit polyclonal immunoglobulin in Dulbecco's phosphate buffered saline (without Mg^{2+} and Ca^{2+}), pH 7.3 (+/- 0.1), 50% glycerol with 1.0 mg/mL BSA (IgG, protease free) as a carrier.
Preservative:	0.05% sodium azide (Caution: sodium azide is a poisonous and hazardous substance. Handle with care and dispose of properly.)
Purification:	Purified from rabbit serum by sequential epitope-specific chromatography. The antibody has been negatively preadsorbed using a non-phosphopeptide corresponding to the site of phosphorylation to remove antibody that is reactive with non-phosphorylated 4E-BP1. The final product is generated by affinity chromatography using a 4E-BP1-derived peptide that is phosphorylated at threonine 46.
Immunogen:	The antiserum was produced against a chemically synthesized phosphopeptide derived from the region of human 4E-BP1 that contains threonine 46. The sequence is conserved in human, mouse and rat.
Target Summary:	Eukaryotic initiation factor 4E binding protein 1 (4E-BP1), also known as PHAS, is a ~20 kDa member of a family of eIF4E-binding proteins whose binding affinity to eIF4E is regulated by its phosphorylation. It inhibits cap-dependent translation by binding to eIF4E on the same site that overlaps the binding site for eIF4G, preventing its binding to the latter and eventually leading to an increase in mRNA translation. The phosphorylatin of 4E-BP1 is critical in determining cell fate by controlling translation initiation and apoptotic potency. 4E-BP1 is hyperphosphorylated in response to several external stimuli including hormones, growth factors, mitogens, cytokines and G-protein–coupled receptors and in response to stress conditions including nutrient deprivation. The phosphorylation of 4E-BP1 increases in response to activated phosphoinositol 3'-kinase (PI-3K) or its downstream effector Akt/PKB. 4E-BP1 is believed to mediate PI-3K and FRAP/mTOR signaling and is phosphorylated on at least six serine and threonine sites (Thr 37, Thr 46, Ser 65, Thr 70, Ser 83, and Ser 112). The phosphorylation of these sites is believed to occur in an orderly fashion where phosphorylation of threonine 37 and 46 by FRAP/mTOR is a priming step for subsequent phosphorylation of 4E-BP1 at the carboxy-terminal sites.
Reactivity:	Human and mouse 4E-BP1. Rat 4E-BP1 has not been tested.
Applications:	The antibody has been used in Western blotting. Other applications have not been tested at Invitrogen.
Suggested Working Dilutions:	For Western blotting applications, we recommend using the antibody at a 1:1,000 starting dilution. The optimal antibody concentration should be determined empirically for each specific application.
Storage:	Store at –20°C. We recommend a brief centrifugation before opening to settle vial contents. Then, apportion into working aliquots and store at –20°C. For shipment or short-term storage (up to one week), 2-8°C is sufficient.
Expiration Date:	Expires one year from date of receipt when stored as instructed.
Positive Control Used:	Human embryonic kidney (HEK293) cells stimulated with EGF or 15% serum.

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Related Products:

Antibodies:

mTOR [pT^{2448}], Cat. # 44-1125G	SHP2 [pY^{542}], Cat. # 44-554G
AMPK [pT^{172}], Cat. # 44-1150G	SHP2 [pS^{576}], Cat. # 44-557G
eIF4G [pS^{1108}], Cat. # 44-526G	Akt/PKB pan, Cat. # 44-609G
p70 S6 kinase [pT^{229}], Cat. # 44-918G	Akt/PKB [pT^{308}], Cat. # 44-602G
Rib. Protein S6 [pS^{236}], Cat. # 44-921G	Akt/PKB [pS^{473}] monoclonal, Cat. # 44-621G
Rib. Protein S6 [pSpS$^{235/236}$], Cat. # 44-922G	MEK1 [pSpS$^{218/222}$]/2 [pSpS$^{222/226}$], Cat. # 44-454G
c-Fos [pT^{325}], Cat. # 44-281G	ERK1&2 [pTpY$^{185/187}$], Cat. # 44-680G
c-Fos [pT^{232}], Cat. # 44-280G	NFAT-1 [pS^{54}], Cat. # 44-944G
c-Jun [pS^{73}], Cat. # 44-292G	Grb2 pan, Cat. #AHO0512

References:

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Zhou, L., et al. (2005) 4E-binding protein phosphorylation and eukaryotic initiation factor-4E release are required for airway smooth muscle hypertrophy. Am. J. Respir. Cell Mol. Biol. 33(2):195-202.

Greenberg, V.L. and S.G. Zimmer (2005) Paclitaxel induces the phosphorylation of the eukaryotic translation initiation factor 4E-binding protein 1 through a Cdk1-dependent mechanism. Oncogene 24(30):4851-4860.

Wang, X., et al. (2005) Distinct signaling events downstream of mTOR cooperate to mediate the effects of amino acids and insulin on initiation factor 4E-binding proteins. Mol. Cell Biol. 25(7):2558-2572.

Li, W. and B.E. Sumpio (2005) Strain-induced vascular endothelial cell proliferation requires PI3K-dependent mTOR-4E-BP1 signal pathway. Am. J. Physiol. Heart Circ. Physiol. 288(4):H1591-H1597.

Li, S., et al. (2002) Translational control of cell fate: availability of phosphorylation sites on translational repressor 4E-BP1 governs its proapoptotic potency. Mol. Cell Biol. 22(8):2853-2861.

Gingras, A.C., et al. (2001) Hierarchical phosphorylation of the translation inhibitor 4E-BP1. Genes Dev. 15(21):2852-2864.

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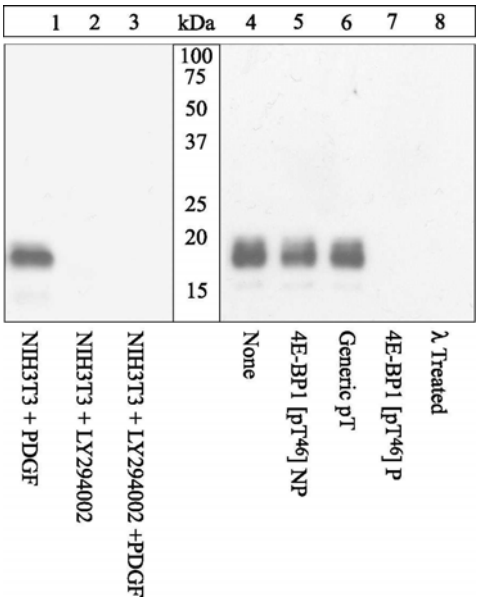

Signal Inhibition, Antibody-Peptide Competition and Phosphatase Stripping

Extracts of NIH3T3 cells untreated (1) or treated with 30 µM PI-3K inhibitor LY2904002 overnight (2, 3) prior to stimulation with 50 ng/mL PDGF for 10 minutes (1, 3), and extracts of HEK293 cells treated with 200 ng/mL EGF for 15 minutes (4-8) were resolved by SDS-PAGE on a 10% Tris-glycine gel and transferred to PVDF. The membranes were then left untreated (1-7) or treated with Lambda phosphatase (8), blocked with a 3% BSA-TBST buffer for one hour at room temperature, then incubated with the 4E-BP1 [pT^{46}] antibody for two hours at room temperature in a 3% BSA-TBST buffer, following prior incubation with: no peptide (1-4, 8), the non-phosphopeptide corresponding to the phosphopeptide immunogen (5), a generic phospho threonine-containing peptide (6), or the phosphopeptide immunogen (7). After washing, the membranes were incubated with goat F(ab')$_2$ anti-rabbit IgG HRP conjugate (Cat. # ALI4404) and signals were detected using the Pierce SuperSignal™ method.

The data show that the PI-3K inhibitor, phosphatase treatment and only the phosphopeptide corresponding to 4E-BP1 [pT^{46}] eliminates the signal, demonstrating the phospho-specificity of the antibody.

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Western Blotting Procedure

1. Lyse approximately 10^7 cells in 0.5 mL of ice cold Cell Lysis Buffer (formulation provided below). This buffer, a modified RIPA buffer, is suitable for recovery of most proteins, including membrane receptors, cytoskeletal-associated proteins, and soluble proteins. This cell lysis buffer formulation is available as a separate product which requires supplementation with protease inhibitors immediately prior to use (Cat. # FNN0011). Other cell lysis buffer formulations, such as Laemmli sample buffer and Triton-X 100 buffer, are also compatible with this procedure. Additional optimization of the cell stimulation protocol and cell lysis procedure may be required for each specific application.
2. Remove the cellular debris by centrifuging the lysates at 14,000 x g for 10 minutes. Alternatively, lysates may be ultracentrifuged at 100,000 x g for 30 minutes for greater clarification.
3. Carefully decant the clarified cell lysates into clean tubes and determine the protein concentration using a suitable method, such as the Bradford assay. Polypropylene tubes are recommended for storing cell lysates.
4. React an aliquot of the lysate with an equal volume of 2x Laemmli Sample Buffer (125 mM Tris, pH 6.8, 10% glycerol, 10% SDS, 0.006% bromophenol blue, and 130 mM dithiothreitol [DTT]) and boil the mixture for 90 seconds at 100°C.
5. Load 10-30 µg of the cell lysate into the wells of an appropriate single percentage or gradient minigel and resolve the proteins by SDS-PAGE.
6. In preparation for the Western transfer, cut a piece of PVDF membrane slightly larger than the gel. Soak the membrane in methanol for 1 minute, then rinse with ddH_2O for 5 minutes. Alternatively, nitrocellulose may be used.
7. Soak the PVDF membrane, 2 pieces of Whatman paper, and Western apparatus sponges in transfer buffer (formulation provided below) for 2 minutes.
8. Assemble the gel and membrane into the sandwich apparatus.
9. Transfer the proteins at 140 mA for 60-90 minutes at room temperature.
10. Following the transfer, rinse the membrane with Tris buffered saline for 2 minutes.
11. Block the membrane with blocking buffer (formulation provided below) for one hour at room temperature or overnight at 4°C.
12. Incubate the blocked blot with primary antibody at a 1:1,000 starting dilution in Tris buffered saline supplemented with 3% Ig-free BSA and 0.1% Tween 20 for two hours at room temperature or overnight at 4°C.
13. Wash the blot with several changes of Tris buffered saline supplemented with 0.1% Tween 20.

Detect the antibody band using an appropriate secondary antibody, such as goat $F(ab')_2$ anti-rabbit IgG alkaline phosphatase conjugate (Cat. # ALI4405) or goat $F(ab')_2$ anti-rabbit IgG horseradish peroxidase conjugate (Cat. # ALI4404) in conjunction with your chemiluminescence reagents and instrumentation..

Cell Lysis Buffer Formulation:
10 mM Tris, pH 7.4
100 mM NaCl
1 mM EDTA
1 mM EGTA
1 mM NaF
20 mM $Na_4P_2O_7$
2 mM Na_3VO_4
0.1% SDS
0.5% sodium deoxycholate
1% Triton-X 100
10% glycerol
1 mM PMSF (made from a 0.3 M stock in DMSO) or 1 mM AEBSF (water soluble version of PMSF)
60 µg/mL aprotinin
10 µg/mL leupeptin
1 µg/mL pepstatin
(alternatively, protease inhibitor cocktail such as Sigma Cat. # P2714 may be used)

Transfer Buffer Formulation:
2.4 gm Tris base
14.2 gm glycine
200 mL methanol
Q.S. to 1 liter, then add
1 mL 10% SDS.
Cool to 4°C prior to use.

Tris Buffered Saline Formulation:
20 mM Tris-HCl, pH 7.4
0.9% NaCl

Blocking Buffer Formulation:
100 mL Tris buffered saline
3 gm Ig-free BSA
0.1 mL Tween 20

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