Rabbit (polyclonal) Anti-NFκB [pS^{529}] Phosphospecific Antibody, Unconjugated

PRODUCT ANALYSIS SHEET

Catalog Number: 44-711G (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 NFκB. The final product is generated by affinity chromatography using a NFκB-derived peptide that is phosphorylated at serine 529.

Immunogen: The antiserum was produced against a chemically synthesized phosphopeptide derived from a region of human NFκB that contains serine 529.

Target Summary: Nuclear factor-κB (NFκB) is a family of transcription factors that consists of homo- and heterodimers of NFκB1/p50 and RelA/p65 subunits, and controls a variety of cellular events including development and immune responses. All members share a conserved amino-terminus domain that includes dimerization, nuclear localization, and DNA binding regions, and a carboxy-terminal transactivation domain. Serines 529 and 536 in the transactivation domain of RelA/p65 are phosphorylated in response to several stimuli including phorbol ester, IL-1β and TNF-α as mediated by IκB kinase and p38 MAPK. Serine 529 is located in a negatively charged region (amino acids 422-540) that is phosphorylated in response to phorbol myristate acetate plus calcium ionophore activation. Phosphorylation of serines 529 and 536 is critical for RelA/p65 transcriptional activity.

Reactivity: Human NFκB. Mouse NFκB (91% homologous) has not been tested, but is expected to react.

Applications: The antibody has been used in Western blotting.

Suggested Working Dilutions: For Western blotting applications, we recommend using the antibody at a 1:1000 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: Jurkat cells treated with PMA and a Ca^{2+} ionophore.

Related Products:

Antibodies:

IKKα [pSpS$^{176/180}$], Cat. # 44-714

IκBα [pSpS$^{32/36}$], Cat. # 44-726G

c-Ret [pS^{696}], Cat. # 44-274

NFAT1 [pS^{54}] (mouse), Cat. # 44-944

PKCθ [pT^{538}], Cat. # 44-960

PKCι [pT^{555}] / λ [pT^{563}], Cat. # 44-968G

This product is for research use only. Not for use in diagnostic procedures.

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References:

Jeong, S.J., et al. (2005) A novel NF-κB pathway involving IKKβ and p65/RelA ser-536 phosphorylation results in p53 inhibition in the absence of NF-κB transcriptional activity. J. Biol. Chem. 280(11):10326-10332.

Jijon, H., et al. (2004) NF-κB inducing kinase activates NF-κB transcriptional activity independently of IκB kinase γ through a p38 MAPK-dependent RelA phosphorylation pathway. Cell Signal. 16(9):1023-1032.

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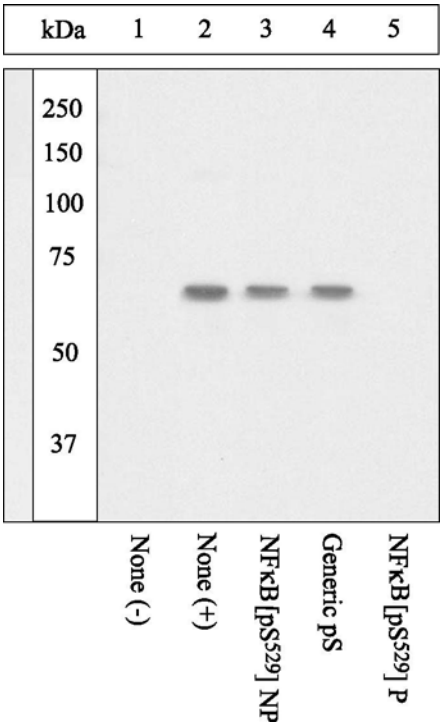
Hunter, R.B., et al. (2002) Activation of an alternative NF-κB pathway in skeletal muscle during disuse atrophy. FASEB J. 16(6):529-538.

Madrid, L.V., et al. (2001) Akt stimulates the transactivation potential of the RelA/p65 subunit of NF-κB through utilization of the IκB kinase and activation of the mitogen-activated protein kinase p38. J. Biol. Chem. 276(22):18934-18940.

Wang, D., et al. (2000) Tumor necrosis factor α-induced phosphorylation of RelA/p65 on Ser529 is controlled by casein kinase II. J. Biol. Chem. 275(42):32592-32597.

Wang, D. and A.S. Baldwin, Jr. (1998) Activation of nuclear factor-κB-dependent transcription by tumor necrosis factor-α is mediated through phosphorylation of RelA/p65 on serine 529. J. Biol. Chem. 273(45):29411-29416.



Peptide Competition

Extracts of Jurkat cells unstimulated (lane 1) or stimulated with 100 ng/mL PMA for 20 minutes then 0.5 µM Ca^{2+} ionophore for 10 additional minutes (lanes 2-5) were resolved by SDS-PAGE on a 10% Tris-glycine gel and transferred to PVDF. The membrane was blocked with a 5% non-fat dried milk-TBST buffer for one hour at room temperature, then incubated with the NFκB [pS^{529}] antibody for two hours at room temperature in a 3% non-fat dried milk-TBST buffer, following prior incubation with: no peptide (lanes 1, 2), the non-phosphopeptide corresponding to the phosphopeptide immunogen (lane 3), a generic phosphoserine-containing peptide (lane 4), or the phosphopeptide immunogen (lane 5). After washing, the membrane was 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 only the phosphopeptide corresponding to NFκB [pS^{529}] blocks the antibody signal, demonstrating the specificity of the antibody. The data also show the induction of the phosphorylated signal after addition of PMA and a Ca^{2+} ionophore in this cell system.

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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 1:1000 starting dilution in Tris buffered saline supplemented with 3% non-fat dried milk 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.
14. 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
5 gm non-fat dried milk
0.1 mL Tween 20

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