Rabbit (polyclonal) Anti-IKKα [pSpS$^{176/180}$] Phosphospecific Antibody, Unconjugated

PRODUCT ANALYSIS SHEET

Catalog Number/Size: 44714 (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), 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 IKKα protein. The final product is generated by affinity chromatography using a IKKα-derived peptide that is phosphorylated at serines 176 and 180.

Immunogen: The antiserum was produced against a chemically synthesized phosphopeptide derived from the region of human IKKα that contains serine 176 and serine 180. The sequence is conserved in mouse.

Target Summary: IκB Kinase-α (IKKα) is an 85 kDa cytoplasmic serine kinase that forms a complex (IKK complex) with a second serine kinase, IKKβ, and a structural/regulatory component, IKKγ (also known as NEMO or IKKAP). The IKK complex plays a central role in the regulation of transcription mediated by the NFκB transcription factor. In the absence of a stimulus, NFκB is sequestered in the cytoplasm in an inactive form through its binding to inhibitory proteins, primarily Inhibitor of NFκB (IκB). In response to a variety of stimuli (e.g., proinflammatory cytokines, tumor necrosis factor, and interleukin-1β) the IKK complex is activated through phosphorylation of IKKα and IKKβ by members of the MAPK kinase kinase family, including NFκB-Induced Kinase (NIK), TGF-β-Activated Kinase 1 (TAK1), and MEKK1. Activation of the IKK complex may also occur stepwise through stimulus-induced phosphorylation of IKKα which then phosphorylates and activates IKKβ. The now activated IKK complex phosphorylates IκB leading to its subsequent ubiquitination and degradation by the proteasome. The free NFκB translocates to the nucleus and stimulates transcription of NFκB-responsive genes. IKKα activation is dependent on phosphorylation of serine 176 and serine 180 residues within the kinase activation loop.

Reactivity: Human IKKα. Mouse (100% homologous) IKKα has not been tested, but is expected to react. IKKβ (93% homologous) has not been tested, but may cross-react in a system expressing high levels of this protein.

Applications: The antibody has been used in Western blotting. When examining phosphorylation of endogenous IKKα protein, we recommend that IKKα first be immunoprecipitated. Other applications may work but have not been tested.

Suggested Working Dilutions: For Western blotting applications, we recommend using the antibody at 0.5-1.5 µg/mL. At 0.75 µg/mL, the dilution provides 100 mL working solution, which at 10 mL/blot allows 10 blots to be performed. The optimal antibody concentration should be determined empirically for each specific application.

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

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Storage: Store at –80°C. Upon initial thawing, we recommend that this vial be briefly centrifuged to ensure recovery of the entire volume. The antibody should then be apportioned into working aliquots and stored at –80°C. Avoid repeated freeze/thaw cycles.

Expiration Date: Expires one year from date of receipt when stored as instructed.

Positive Control Used: Immunoprecipitates of Jurkat cells pre-treated with ALLN and treated with TNF-α.

Related Products:

Antibodies:

PKCα [pT^{638}], Cat. # 44962

JNK1&2 [pTpY$^{183/185}$], Cat. # 44682G

ERK1&2 [pTpY$^{185/187}$], Cat. # 44680G

p38 [pTpY$^{180/182}$], Cat. # 44684G

ELISAs:

IκBα [pS^{32}], Cat. # KHO0221

IκBα total, Cat. # KHO0211

References:

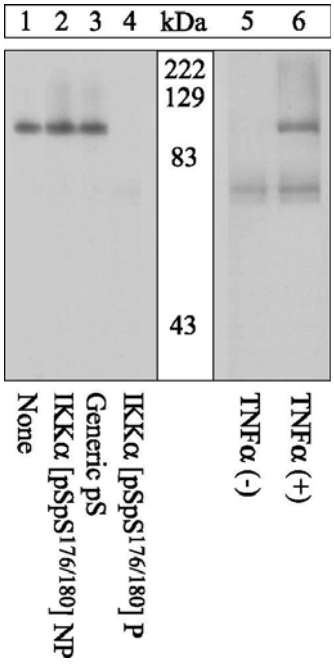
Senftleben, U. and M. Karin (2002) The IKK/NF-κB pathway. Crit. Care Med. 30(l Suppl.):S18-S26.

Ghosh, S. and M. Karin (2002) Missing pieces of the NF-κB puzzle. Cell 109(Suppl.):S81-S96.

O'Mahony, A., et al. (2000) Activation of the heterodimeric IκB kinase α (IKKα)-IKKβ complex is directional: IKKα regulates IKKβ under both basal and stimulated conditions. Mol. Cell. Biol. 20(4):1170-1178.

Ling, L., et al. (1998) NF-κB-inducing kinase activates IKK-α by phosphorylation of Ser-176. Proc. Nat'l. Acad. Sci. USA 95(7):3792-3797.

Lee, F.S., et al. (1998) MEKK1 activates both IκB kinase α and IκB kinase β. Proc. Nat'l. Acad. Sci. USA 95(16):9319-9324.



Antibody-Peptide Competition and Up-regulation

Immunoprecipitates using an IKKα pan antibody of Jurkat cells pre-treated with 100 µM ALLN (protease inhibitor) for 1 hour with (1-4, 6) or without stimulation using 1 nM TNF-α for 30 minutes (5) were resolved by SDS-PAGE on a 10% Tris-glycine gel and transferred to PVDF. The membrane was blocked with a 5% BSA-TBST buffer overnight at 4°C and incubated with the IKKα [pSpS$^{176/180}$] antibody for two hours at room temperature in a 3% BSA-TBST buffer, following prior incubation with: no peptide (1, 5, 6), the non-phosphopeptide corresponding to the phosphopeptide immunogen (2), a generic phosphoserine-containing peptide (3), or the phosphopeptide immunogen (4). 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 IKKα [pSpS$^{176/180}$] blocks the antibody signal, demonstrating the specificity of the antibody. The data also show up-regulation of the phospho signal upon ALLN and TNFα treatment 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 (Invitrogen catalog number 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.
7. Soak the 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) overnight at 4°C or for one hour at room temperature.
12. Incubate the blocked blot with primary antibody at a concentration of 0.5-1.5 µg/mL in Tris buffered saline supplemented with 3% Ig-free BSA and 0.1% Tween 20 overnight at 4°C or for two hours at room temperature.
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 (catalog number ALI4405) or goat $F(ab')_2$ anti-rabbit IgG horseradish peroxidase conjugate (catalog number 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 catalog number 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 Ig-free BSA
0.1 mL Tween 20

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Peptide Competition Experiment

To demonstrate the specificity of a Phosphorylation Site Specific Antibody, we recommend the following peptide competition experiment which uses our control peptides. These control peptides have the sequences of the phosphopeptide immunogen used to raise the antibody and the corresponding non-phosphorylated peptide. In the competition experiment, 200-500 fold molar excess of the phosphorylated and non-phosphorylated peptides are pre-incubated with aliquots of the antibody prior to use in immunoassay procedures.
A sample calculation for the determination of the 200 fold molar excess of peptide to antibody is presented below. The following assumptions have been made:

- The molecular mass of an IgG molecule is 150,000 daltons.
- Each mole of antibody binds two moles of peptide.
- The Phosphorylation Site Specific Antibody is used at a concentration of 0.5 µg/mL.

The optimal antibody concentration for use in peptide competition experiments is below saturating as determined by previous experiments in your system. If an optimal concentration has not been determined, it is suggested that the concentration provided on the antibody Product Analysis Sheet be used. A final antibody concentration of 0.5 µg/mL is satisfactory for most applications.
The molarity of the 0.5 µg/mL antibody solution is:
(0.5 µg/mL)(1000 mL/L)/(150,000 µg/µmole) = 0.00333 µM.
Because each mole of antibody binds two moles of peptide, 0.5 µg/mL antibody can bind 0.00667 µM of peptide.

A 200 fold molar excess of peptide is (200)(0.00667 µM) = 1.334 µM.

The following procedure describes peptide competition experiments using antibody at a concentration of 0.5 µg/mL and a 200 fold molar excess of peptides based on the calculation above, in a total volume of 2 mL.

Procedure:

1. Prepare three identical test samples, such as identical nitrocellulose or PVDF strips with transferred protein. The test samples should be blocked with BSA or non-fat dried milk in a buffer compatible with an antibody based detection method, such as Tris buffered saline or phosphate buffered saline.
2. Slowly thaw the Phosphorylation Site Specific Antibody on ice.
3. Prepare 3 mL of a 2x (1 µg/mL) antibody stock solution in a buffer appropriate for the application. Suggested buffer formulations are TBS or PBS supplemented with blocking protein such as BSA or non-fat dried milk.
4. Apportion the unused Phosphorylation Site Specific Antibody into working aliquots and store at -80°C for future use.
5. The lyophilized control peptides should be warmed to room temperature, ideally under desiccation.
6. Reconstitute each of the control peptides to a concentration of 100 µM using nanopure water at room temperature. As indicated on the peptide labels, each vial contains 0.1 mg. For a peptide with a molecular mass of 1500, reconstitution with 0.67 mL water yields a solution with a concentration of 100 µM.
7. Allow the peptides to dissolve at room temperature, then gently triturate several times using a pipette. Avoid introducing air bubbles.
8. Label 3 test tubes as follows:
   - tube 1: water only no peptide control
   - tube 2: phosphopeptide
   - tube 3: non-phosphopeptide
9. Prepare 2x peptide stock solutions (2.66 µM) or water control by pipetting the following:
   - tube 1: water control stock: 0.973 mL buffer (TBS or PBS plus blocking protein) plus 27 µL water.
   - tube 2: phosphopeptide 2x stock: 0.973 mL buffer (TBS or PBS plus blocking protein) plus 27 µL reconstituted (100 µM) phosphopeptide.
   - tube 3: non-phosphopeptide 2x stock: 0.973 mL buffer (TBS or PBS plus blocking protein) plus 27 µL reconstituted (100 µM) non-phosphopeptide.
10. Apportion unused reconstituted peptide solutions into working aliquots and store at –20°C for future use.
11. Pipette 1 mL of the 2x antibody stock into each of the tubes marked 1, 2, and 3. The tubes should be incubated for 30 minutes at room temperature with gentle rocking.
12. The pre-incubated antibody in each of the three tubes is then ready for use. Pipette the contents of each tube onto the three identical test samples.
    For Western blotting strips:
    - ♦ Incubate these strips for 2 hours at room temperature, followed by several washes to remove unbound antibody.
    - ♦ Transfer each strip to a new solution containing a labeled secondary antibody (example goat anti-rabbit IgG-alkaline phosphatase conjugate).
    - ♦ Remove unbound secondary antibody by thorough washing and develop bands.

The signals obtained with antibody incubated with "(1) water only no peptide control", which represents the maximum signal, and the signals obtained with "(2) phosphopeptide and "(3) non-phosphopeptide" are readily compared under these conditions.

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