

#3039 Store at -20°C

Phospho-NF-κB p65 (Ser468) Antibody

100 μl
 (10 Western mini-blot)



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This product is for *in vitro* research use only and is not intended for use in humans or animals.

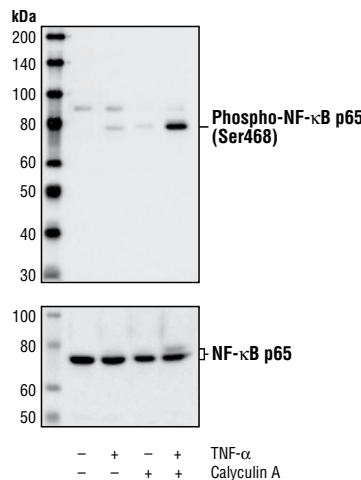
Applications	Species Cross-Reactivity*	Molecular Wt.	Source
W	H, M, R	65 kDa	Rabbit

Background: Transcription factors of the nuclear factor κB (NF-κB)/Rel family play a pivotal role in inflammatory and immune responses (1,2). There are five family members in mammals: RelA, c-Rel, RelB, NF-κB1 (p105/p50) and NF-κB2 (p100/p52). Both p105 and p100 are proteolytically processed by the proteasome to produce p50 and p52, respectively. The p50 and p52 products form dimeric complexes with Rel proteins, which are then able to bind DNA and regulate transcription. In unstimulated cells, NF-κB is sequestered in the cytoplasm by its inhibitory proteins, the IκB's (3-5). NF-κB-activating agents can induce the phosphorylation of IκB's, targeting them for rapid degradation through a ubiquitin-proteasome pathway, releasing NF-κB to enter the nucleus, where it regulates gene expression (6-8). Processing of NF-κB2 p100 is regulated by NIK and IKK1 (IKKα), which triggers the phosphorylation and processing to p52, which can then undergo nuclear translocation (9-11).

PMA-induced NF-κB transcriptional activity is dependent on the region between amino acids 442 and 470, suggesting a role for one or more of the potential phosphorylation sites (Ser457, Thr458, Thr464, or Ser468) in this region (12). T-cell costimulation and calyculin A have both been shown to increase Ser468 phosphorylation (13, 14). IKKβ (but not IKKα) and GSK-3beta both target this site (14, 15), which appears to have a negative regulatory role not involving inhibition of nuclear translocation after TNFα or IL-1β stimulation (15).

Specificity/Sensitivity: Phospho-NF-κB p65 (Ser468) Antibody detects NF-κB p65 only when phosphorylated at serine 468.

Source/Purification: Polyclonal antibodies are produced by immunizing rabbits with a synthetic phospho peptide (KLH-coupled) corresponding to residues surrounding Ser468 of human NF-κB p65. Antibodies are purified by protein A and peptide affinity chromatography.



Western blot analysis of extracts from HeLa cells treated for 5 minutes with TNF-α #2169 (20 ng/ml), Calyculin A #9902 (50 nM), or both compounds, using Phospho-NF-κB p65 (Ser468) Antibody (top) or NF-κB p65 Antibody #3034 (bottom).

Storage: Supplied in 10 mM sodium HEPES (pH 7.5), 150 mM NaCl, 100 μg/ml BSA and 50% glycerol. Store at -20°C. Do not aliquot the antibody.

*Species cross-reactivity is determined by Western blot.

Recommended Antibody Dilutions:
Western Blotting 1:1000

- Companion Products:**
- NF-κB p65 Antibody #3034
 - Phospho-NF-κB p65 (Ser536) (93H1) Rabbit mAb #3033
 - Phospho-NF-κB p65 (Ser536) (7F1) Mouse mAb #3036
 - Phospho-NF-κB p65 (Ser276) Antibody #3037
 - IκBα (44D4) Rabbit mAb #4812
 - Phospho-IκB-α (Ser32) (14D4) Rabbit mAb #2859
 - RelB Antibody #4954
 - c-Rel Antibody #4727
 - IKKα Antibody #2682
 - IKKβ (2C8) Rabbit mAb #2370
 - NF-κB p105/p50 Antibody #3035
 - Phospho-NF-κB p105 (Ser933) (18E6) Rabbit mAb #4806
 - NF-κB2 p100/p52 Antibody #4882
 - Phospho-NF-κB2 p100 (Ser864/868) Antibody #4810
 - Phototope®-HRP Western Blot Detection System, Anti-rabbit IgG, HRP-linked Antibody #7071
 - Anti-rabbit IgG, HRP-linked Antibody #7074
 - Prestained Protein Marker, Broad Range (Premixed Format) #7720
 - Biotinylated Protein Ladder Detection Pack #7727
 - LumiGLO® Reagent and Peroxide #7003

IMPORTANT: For Western blots, incubate membrane with diluted antibody in 5% w/v BSA, 1X TBS, 0.1% Tween-20 at 4°C with gentle shaking, overnight.

Applications Key: W—Western IP—Immunoprecipitation IHC—Immunohistochemistry IC—Immunocytochemistry IF—Immunofluorescence
Species Cross-Reactivity Key: H—human M—mouse R—rat Hm—hamster Mk—monkey Mi—mink C—chicken X—Xenopus
 Species enclosed in parentheses are predicted to react based on 100% sequence homology.

F—Flow cytometry E—ELISA D—DELFIATM
 Z—zebra fish B—bovine All—all species expected

Selected Application References:

Schmitz, M.L. et al. (2004) A comparative analysis of T cell costimulation and CD43 activation reveals novel signaling pathways and target genes. *Blood* 102, 3302–3304. Application: W.

Buss, H. et al. (2004) Phosphorylation of serine 468 by GSK-3 β negatively regulates basal p65 NF- κ B activity. *J. Biol. Chem.* 279, 49571–49574. Application: W.

Background References:

- (1) Baeuerle, P.A. and Henkel, T. (1994) *Annu. Rev. Immunol.* 12, 141–179.
- (2) Baeuerle, P.A. and Baltimore, D. (1996) *Cell* 87, 13–20.
- (3) Haskill, S. et al. (1991) *Cell* 65, 1281–1289.
- (4) Thompson, J.E. et al. (1995) *Cell* 80, 573–582.
- (5) Whiteside, S.T. et al. (1997) *EMBO J.* 16, 1413–1426.
- (6) Traenckner, E.B. et al. (1995) *EMBO J.* 14, 2876–2883.
- (7) Scherer, D.C. et al. (1995) *Proc. Natl. Acad. Sci. USA* 92, 11259–11263.
- (8) Chen, Z.J. et al. (1996) *Cell* 84, 853–862.
- (9) Senftleben, U. et al. (2001) *Science* 293, 1495–1499.
- (10) Coope, H.J. et al. (2002) *EMBO J.* 21, 5375–5385.
- (11) Xiao, G. et al. (2001) *Mol. Cell* 7, 401–409.
- (12) Schmitz, M.L. et al. (1995) *J. Biol. Chem.* 270, 15576–15584.
- (13) Schmitz, M.L. et al. (2004) *Blood* 102, 3302–3304.
- (14) Buss, H. et al. (2004) *J. Biol. Chem.* 279, 49571–49574.
- (15) Schwabe, R.F. and Sakurai, H. (2005) *FASEB J* Jul 26 epub, .

Western Immunoblotting Protocol (Primary Antibody Incubation in BSA)

For Western blots, incubate membrane with diluted antibody in 5% w/v BSA, 1X TBS, 0.1% Tween-20 at 4°C with gentle shaking, overnight.

A Solutions and Reagents

NOTE: Prepare solutions with Milli-Q or equivalently purified water.

- 1X Phosphate Buffered Saline (PBS)
- 1X SDS Sample Buffer:** 62.5 mM Tris-HCl (pH 6.8 at 25°C), 2% w/v SDS, 10% glycerol, 50 mM DTT, 0.01% w/v bromophenol blue or phenol red
- Transfer Buffer:** 25 mM Tris base, 0.2 M glycine, 20% methanol (pH 8.5)
- 10X Tris Buffered Saline (TBS):** To prepare 1 liter of 10X TBS: 24.2 g Tris base, 80 g NaCl; adjust pH to 7.6 with HCl (use at 1X).
- Nonfat Dry Milk (weight to volume [w/v])
- Blocking Buffer:** 1X TBS, 0.1% Tween-20 with 5% w/v nonfat dry milk; for 150 ml, add 15 ml 10X TBS to 135 ml water, mix. Add 7.5 g nonfat dry milk and mix well. While stirring, add 0.15 ml Tween-20 (100%).
- Wash Buffer:** 1X TBS, 0.1% Tween-20 (TBS/T)
- Bovine Serum Albumin (BSA)
- Primary Antibody Dilution Buffer:** 1X TBS, 0.1% Tween-20 with 5% BSA; for 20 ml, add 2 ml 10X TBS to 18 ml water, mix. Add 1.0 g BSA and mix well. While stirring, add 20 µl Tween-20 (100%).
- Phototope[®]-HRP Western Blot Detection System #7071:** Includes biotinylated protein ladder, secondary anti-rabbit (#7074) antibody conjugated to horseradish peroxidase (HRP), anti-biotin antibody conjugated to HRP, LumiGLO[®] chemiluminescent reagent and peroxide.
- Prestained Protein Marker, Broad Range (Premixed Format) #7720
- Biotinylated Protein Ladder Detection Pack #7727
- Blotting Membrane:** This protocol has been optimized for nitrocellulose membranes, which CST recommends. PVDF membranes may also be used.

B Protein Blotting

A general protocol for sample preparation is described below.

- Treat cells by adding fresh media containing regulator for desired time.
- Aspirate media from cultures; wash cells with 1X PBS; aspirate.
- Lyse cells by adding 1X SDS sample buffer (100 µl per well of 6-well plate or 500 µl per plate of 10 cm diameter plate). Immediately scrape the cells off the plate and transfer the extract to a microcentrifuge tube. Keep on ice.
- Sonicate for 10–15 seconds to shear DNA and reduce sample viscosity.
- Heat a 20 µl sample to 95–100°C for 5 minutes; cool on ice.
- Microcentrifuge for 5 minutes.
- Load 20 µl onto SDS-PAGE gel (10 cm x 10 cm).

NOTE: CST recommends loading prestained molecular weight markers (#7720, 10 µl/lane) to verify electrotransfer and biotinylated protein ladder (#7727, 10 µl/lane) to determine molecular weights.

- Electrotransfer to nitrocellulose or PVDF membrane.

C Membrane Blocking and Antibody Incubations

NOTE: Volumes are for 10 cm x 10 cm (100 cm²) of membrane; for different sized membranes, adjust volumes accordingly.

- (Optional) After transfer, wash nitrocellulose membrane with 25 ml TBS for 5 minutes at room temperature.
- Incubate membrane in 25 ml of blocking buffer for 1 hour at room temperature.
- Wash three times for 5 minutes each with 15 ml of TBS/T.
- Incubate membrane and primary antibody (at the appropriate dilution) in 10 ml primary antibody dilution buffer with gentle agitation overnight at 4°C.
- Wash three times for 5 minutes each with 15 ml of TBS/T.
- Incubate membrane with HRP-conjugated secondary antibody (1:2000) and HRP-conjugated anti-biotin antibody (1:1000) to detect biotinylated protein markers in 10 ml of blocking buffer with gentle agitation for 1 hour at room temperature.
- Wash three times for 5 minutes each with 15 ml of TBS/T.

D Detection of Proteins

- Incubate membrane with 10 ml LumiGLO[®] (0.5 ml 20X LumiGLO[®], 0.5 ml 20X Peroxide and 9.0 ml Milli-Q water) with gentle agitation for 1 minute at room temperature.

NOTE: LumiGLO[®] substrate can be further diluted if signal response is too fast.

- Drain membrane of excess developing solution (do not let dry), wrap in plastic wrap and expose to x-ray film. An initial 10-second exposure should indicate the proper exposure time.

NOTE: Due to the kinetics of the detection reaction, signal is most intense immediately following LumiGLO[®] incubation and declines over the following 2 hours.