

# NF- $\kappa$ B Pathway Sampler Kit

1 Kit  
 (7 x 20  $\mu$ l)



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**For Research Use Only. Not For Use In Diagnostic Procedures.**

Products Included	Product #	Quantity	Mol. Wt.	Source
IKK $\alpha$ (3G12) Mouse mAb	11930	20 $\mu$ l	85 kDa	Mouse IgG1
IKK $\beta$ (D30C6) Rabbit mAb	8943	20 $\mu$ l	87 kDa	Rabbit IgG
Phospho-IKK $\alpha$ / $\beta$ (Ser176/180) (16A6) Rabbit mAb	2697	20 $\mu$ l	85 kDa IKK $\alpha$ 87 kDa IKK $\beta$	Rabbit IgG
NF- $\kappa$ B p65 (D14E12) XP <sup>®</sup> Rabbit mAb	8242	20 $\mu$ l	65 kDa	Rabbit IgG
Phospho-NF- $\kappa$ B p65 (Ser536) (93H1) Rabbit mAb	3033	20 $\mu$ l	65 kDa	Rabbit IgG
I $\kappa$ B $\alpha$ (L35A5) Mouse mAb (Amino-terminal Antigen)	4814	20 $\mu$ l	39 kDa	Mouse IgG1
Phospho-I $\kappa$ B- $\alpha$ (Ser32) (14D4) Rabbit mAb	2859	20 $\mu$ l	40 kDa	Rabbit IgG
Anti-rabbit IgG, HRP-linked Antibody	7074	100 $\mu$ l		Goat
Anti-mouse IgG, HRP-linked Antibody	7076	100 $\mu$ l		Horse

See [www.cellsignal.com](http://www.cellsignal.com) for individual component applications, species cross-reactivity, dilutions and additional application protocols.

**Description:** The NF- $\kappa$ B Pathway Sampler Kit contains reagents to examine the activation state and total protein levels of key proteins in the NF- $\kappa$ B pathway: IKK $\alpha$ , IKK $\beta$ , NF- $\kappa$ B p65/RelA and I $\kappa$ B $\alpha$ . The kit includes enough antibody to perform two western blot experiments with each primary antibody.

**Background:** The transcriptional nuclear factor  $\kappa$ B (NF- $\kappa$ B)/Rel transcription factors are present in the cytosol in an inactive state, complexed with the inhibitory I $\kappa$ B proteins. Activation occurs via phosphorylation of I $\kappa$ B $\alpha$  at Ser32 and Ser36, resulting in the ubiquitin-mediated proteasome-dependent degradation of I $\kappa$ B $\alpha$  and the release and nuclear translocation of active NF- $\kappa$ B dimers. The regulation of I $\kappa$ B $\beta$  and I $\kappa$ B $\epsilon$  is similar to that of I $\kappa$ B $\alpha$ , however, the phosphorylation and degradation of these proteins occurs with much slower kinetics. Phosphorylation of I $\kappa$ B $\beta$  occurs at Ser/Thr19 and Ser23, while I $\kappa$ B $\epsilon$  can be phosphorylated at Ser18 and Ser22. The key regulatory step in this pathway involves activation of a high molecular weight I $\kappa$ B kinase (IKK) complex, consisting of three tightly associated IKK subunits. IKK $\alpha$  and IKK $\beta$  serve as the catalytic subunits of the kinase. Activation of IKK

depends on phosphorylation at Ser177 and Ser181 in the activation loop of IKK $\beta$  (176 and 180 in IKK $\alpha$ ). NF- $\kappa$ B-inducing kinase (NIK), TANK-binding kinase 1 (TBK1), and its homolog IKK $\epsilon$  (IKKi), phosphorylate and activate IKK $\alpha$  and IKK $\beta$ .

The NF- $\kappa$ B family of transcription factors is comprised of five proteins in mammals, p65/RelA, c-Rel, RelB, NF- $\kappa$ B1 (p105/p50) and NF- $\kappa$ B2 (p100/p52). p105 and p100 are proteolytically processed to produce p50 and p52, respectively. The 50 kDa active form is produced through proteolytic processing following IKK-mediated phosphorylation of p105 at multiple sites (Ser922, 924, 928 and 933), while p100's processing to p52 is induced by phosphorylation of Ser864 and Ser868. The p50 and p52 products form dimeric complexes with Rel proteins, which are then able to bind DNA and regulate transcription. Phosphorylation of p65/RelA at Ser276 by PKA C and MSK1 enhances transcriptional activity. p65 phosphorylation at Ser536 regulates activation, nuclear localization, protein-protein interactions, and transcriptional activity. PMA-induced NF- $\kappa$ B transcriptional activity is dependent on the region of p65 containing the potential phosphorylation sites Ser457, Thr458, Thr464 and Ser468. Phosphorylation of

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

**Recommended Antibody Dilutions:**

Western blotting 1:1000

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Ser468 by GSK-3 $\beta$  inhibits basal p65 activity.

**Specificity/Sensitivity:** Phospho-IKK $\alpha$ / $\beta$ , phospho-NF- $\kappa$ B p65, and phospho-I $\kappa$ B $\alpha$  antibodies recognize endogenous levels IKK $\alpha$ / $\beta$ , p65, and I $\kappa$ B- $\alpha$ , respectively, only when phosphorylated at the indicated residues. They do not cross-react with other family members at physiological levels. Total IKK $\alpha$ , IKK $\beta$ , p65 and I $\kappa$ B $\alpha$  antibodies recognize endogenous levels of their respective targets regardless of phosphorylation state and do not cross-react with other family members at physiological levels.

**Source/Purification:** Monoclonal antibodies are produced by immunizing animals with recombinant human proteins or synthetic peptides.

**Background References:**

- (1) Yamamoto, Y. and Gaynor, R.B. (2004) *Trends Biochem. Sci.* 29, 72–79.
- (2) Ghosh, S. and Karin, M. (2002) *Cell* 109, S81–S96.
- (3) Viatour, P. et al. (2005) *Trends Biochem. Sci.* 30, 43–52.

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**Applications Key:** W—Western IP—Immunoprecipitation IHC—Immunohistochemistry ChIP—Chromatin Immunoprecipitation IF—Immunofluorescence F—Flow cytometry E-P—ELISA-Peptide

**Species Cross-Reactivity Key:** H—human M—mouse R—rat Hm—hamster Mk—monkey Mi—mink C—chicken Dm—D. melanogaster X—Xenopus Z—zebrafish B—bovine

Dg—dog Pg—pig Sc—S. cerevisiae All—all species expected Species enclosed in parentheses are predicted to react based on 100% homology.

# Western Immunoblotting Protocol

For western blots, incubate membrane with diluted primary antibody in either 5% w/v BSA or nonfat dry milk, 1X TBS, 0.1% Tween® 20 at 4°C with gentle shaking, overnight.

**NOTE:** Please refer to primary antibody datasheet or product webpage for recommended primary antibody dilution buffer and recommended antibody dilution.

## A. Solutions and Reagents

**NOTE:** Prepare solutions with reverse osmosis deionized (RODI) or equivalent grade water.

- 20X Phosphate Buffered Saline (PBS):** (#9808) To prepare 1 L 1X PBS: add 50 ml 20X PBS to 950 ml dH<sub>2</sub>O, mix.
- 10X Tris Buffered Saline (TBS):** (#12498) To prepare 1 L 1X TBS: add 100 ml 10X to 900 ml dH<sub>2</sub>O, mix.
- 1X SDS Sample Buffer:** Blue Loading Pack (#7722) or Red Loading Pack (#7723)  
Prepare fresh 3X reducing loading buffer by adding 1/10 volume 30X DTT to 1 volume of 3X SDS loading buffer. Dilute to 1X with dH<sub>2</sub>O.
- 10X Tris-Glycine SDS Running Buffer:** (#4050) To prepare 1 L 1X running buffer: add 100 ml 10X running buffer to 900 ml dH<sub>2</sub>O, mix.
- 10X Tris-Glycine Transfer Buffer:** (#12539) To prepare 1 L 1X transfer buffer: add 100 ml 10X transfer buffer to 200 ml methanol + 700 ml dH<sub>2</sub>O, mix.
- 10X Tris Buffered Saline with Tween® 20 (TBST):** (#9997) To prepare 1 L 1X TBST: add 100 ml 10X TBST to 900 ml dH<sub>2</sub>O, mix.
- Nonfat Dry Milk:** (#9999)
- Blocking Buffer:** 1X TBST with 5% w/v nonfat dry milk; for 150 ml, add 7.5 g nonfat dry milk to 150 ml 1X TBST and mix well.
- Wash Buffer:** (#9997) 1X TBST
- Bovine Serum Albumin (BSA):** (#9998)
- Primary Antibody Dilution Buffer:** 1X TBST with 5% BSA or 5% nonfat dry milk as indicated on primary antibody datasheet; for 20 ml, add 1.0 g BSA or nonfat dry milk to 20 ml 1X TBST and mix well.
- Biotinylated Protein Ladder Detection Pack:** (#7727)
- Prestained Protein Marker, Broad Range (Premixed Format):** (#7720)
- Blotting Membrane and Paper:** (#12369) This protocol has been optimized for nitrocellulose membranes. Pore size 0.2 µm is generally recommended.
- Secondary Antibody Conjugated to HRP:** anti-rabbit (#7074); anti-mouse (#7076)
- Detection Reagent:** LumiGLO® chemiluminescent reagent and peroxide (#7003) or SignalFire™ ECL Reagent (#6883)

## B. Protein Blotting

**A general protocol for sample preparation.**

- 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 for a 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 sec to complete cell lysis and shear DNA (to reduce sample viscosity).
- Heat a 20 µl sample to 95–100°C for 5 min; cool on ice.
- Microcentrifuge for 5 min.
- Load 20 µl onto SDS-PAGE gel (10 cm x 10 cm). **NOTE:** Loading of prestained molecular weight markers (#7720, 10 µl/lane) to verify electrotransfer and biotinylated protein ladder (#7727, 10 µl/lane) to determine molecular weights are recommended.
- Electrotransfer to nitrocellulose membrane (#12369).

## C. Membrane Blocking and Antibody Incubations

**NOTE:** Volumes are for 10 cm x 10 cm (100 cm<sup>2</sup>) of membrane; for different sized membranes, adjust volumes accordingly.

### I. Membrane Blocking

- (Optional) After transfer, wash nitrocellulose membrane with 25 ml TBS for 5 min at room temperature.
- Incubate membrane in 25 ml of blocking buffer for 1 hr at room temperature.
- Wash three times for 5 min each with 15 ml of TBST.

### II. Primary Antibody Incubation

- Incubate membrane and primary antibody (at the appropriate dilution and diluent as recommended in the product datasheet) in 10 ml primary antibody dilution buffer with gentle agitation overnight at 4°C.
- Wash three times for 5 min each with 15 ml of TBST.
- Incubate membrane with the species appropriate HRP-conjugated secondary antibody (#7074 or #7076 at 1:2000) and anti-biotin, HRP-linked Antibody (#7075 at 1:1000–1:3000) to detect biotinylated protein markers in 10 ml of blocking buffer with gentle agitation for 1 hr at room temperature.
- Wash three times for 5 min each with 15 ml of TBST.
- Proceed with detection (Section D).

## D. Detection of Proteins

- Incubate membrane with 10 ml LumiGLO® (0.5 ml 20X LumiGLO® #7003, 0.5 ml 20X peroxide, and 9.0 ml purified water) or 10 ml SignalFire™ #6883 (5 ml Reagent A, 5 ml Reagent B) with gentle agitation for 1 min at room temperature.
- Drain membrane of excess developing solution (do not let dry), wrap in plastic wrap and expose to x-ray film. An initial 10 sec exposure should indicate the proper exposure time.  
**NOTE:** Due to the kinetics of the detection reaction, signal is most intense immediately following incubation and declines over the following 2 hr.

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