

PathScan® Phospho-NF-κB p65 (Ser536) Sandwich ELISA Kit

✓ 1 Kit
(96 assays)

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

Entrez-Gene ID # 5970
Swiss-Prot Acc. # Q04206

Species Cross-Reactivity: H, M

Introduction: CST's PathScan® Phospho-NF-κB p65 (Ser536) Sandwich ELISA Kit is a solid phase sandwich enzyme-linked immunosorbent assay (ELISA) that detects endogenous levels of Phospho-NF-κB p65 protein. A NF-κB p65 Mouse mAb (8F8) #3028* has been coated onto the microwells. After incubation with cell lysates, both phospho- and nonphospho-NF-κB p65 proteins are captured by the coated antibody. Following extensive washing, Phospho-NF-κB p65 (Ser536) (93H1) Rabbit mAb #3033* is added to detect the captured phospho-NF-κB p65 protein. Anti-rabbit IgG, HRP-linked Antibody #7074* is then used to recognize the bound detection antibody. HRP substrate, TMB, is added to develop color. The magnitude of optical density for this developed color is proportional to the quantity of phospho-NF-κB p65 (Ser536).

* Antibodies in kit are custom formulations specific to kit.

Companion Products:

PathScan® Total NF-κB p65 Sandwich ELISA Kit #7174
Phospho-NF-κB p65 (Ser536) (93H1) Rabbit mAb #3033
Anti-rabbit IgG, HRP-linked Antibody #7074
Cell Lysis Buffer (10X) #9803

Specificity/Sensitivity: CST's PathScan® Phospho-NF-κB p65 (Ser536) Sandwich ELISA Kit detects endogenous levels of phospho-NF-κB p65 at Ser536. As shown in Figure 1, using the Phospho-NF-κB p65 (Ser536) ELISA Kit #7173, a significant induction of Phospho-NF-κB p65 (Ser536) in HeLa cells treated with TNF-α is detected. However, levels of NF-κB p65 (either untreated or treated), detected by the Total NF-κB p65 Sandwich ELISA Kit #7174, remain unchanged.

Products Included	Volume	Solution Color
NF-κB p65 (8F8) Mouse mAb Coated Microwells	96 tests	
Phospho-NF-κB p65 Detection Antibody	11 ml	green
Anti-rabbit IgG HRP-Linked Antibody	11 ml	red
TMB Substrate	11 ml	colorless
STOP Solution	11 ml	colorless
Sealing Tape	2 sheets	
20X Wash Buffer	25 ml	colorless
Sample Diluent	25 ml	blue
10X Cell Lysis Buffer #9803**	15 ml	yellowish

* 12 8-well modules -Each module is designed to break apart for 8 tests.

**Kit should be stored at 4°C with the exception of 10X Cell Lysis Buffer, which is stored at -20°C (packaged separately).

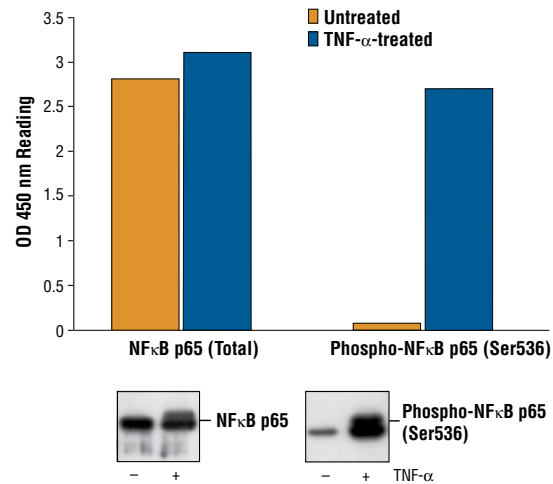


Figure 1: Treatment of HeLa cells with TNF-α stimulates phosphorylation of NF-κB p65 at Ser536, detected by PathScan® Phospho-NF-κB p65 (Ser536) Sandwich ELISA kit #7173, but does not affect the level of total NF-κB p65 detected by PathScan® Total NF-κB p65 Sandwich ELISA kit #7174. OD 450 readings are shown in the top figure, while the corresponding Western blot using Phospho-NF-κB p65 (Ser536) (93H1) Rabbit mAb #3033 (right panel) or NF-κB p65 Antibody #3034 (left panel), is shown in the bottom figure.



Background: Transcription factors of the nuclear factor κ B (NF- κ B)/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- κ B1 (p105/p50) and NF- κ B2 (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- κ B is sequestered in the cytoplasm by its inhibitory proteins, the I κ B's (3-5). NF- κ B-activating agents can induce the phosphorylation of I κ B's, targeting them for rapid degradation through an ubiquitin-proteasome pathway, releasing NF- κ B to enter the nucleus, where it regulates gene expression (6-8). NIK and IKK1 (IKK α) regulate the phosphorylation and processing of NF- κ B2 (p100) to produce p52, which is then translocated to the nucleus (9-11).

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.

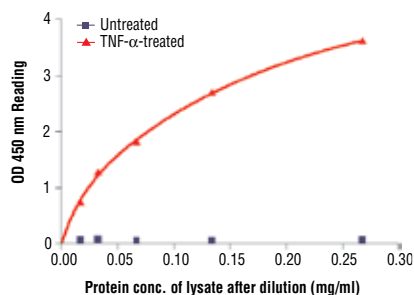


Figure 2: The relationship between protein concentration of lysates from untreated and TNF- α -treated HeLa cells and kit assay optical density readings. After starvation, HeLa cells (85% confluence) were treated with TNF- α (10 ng/ml) for 7 min at 37°C, and then lysed.

Sandwich ELISA Protocol

A Reagent Preparation

1. Bring all microwell strips to room temperature before use.
2. Prepare 1X Wash Buffer by diluting 20X wash buffer (included in each Pathscan® Sandwich ELISA Kit) in Milli-Q or equivalently purified water.
3. **1X Cell Lysis Buffer from CST #9803:** 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM ethylene diamine tetraacetate (EDTA), 1 mM ethylene glycol-bis(2-aminoethyl)-N,N,N',N'-tetraacetic acid (EGTA), 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na₂VO₄, 1 μg/ml leupeptin. This buffer can be stored at 4°C for short-term use (1–2 weeks).

B Preparing Cell Lysates

1. Aspirate media. Treat cells by adding fresh media containing regulator for desired time.
2. To harvest cells under nondenaturing conditions, remove media and rinse cells once with ice-cold PBS.
3. Remove PBS and add 0.5 ml ice-cold 1X Cell Lysis Buffer plus 1 mM phenyl-methylsulfonyl fluoride (PMSF) to each plate (10 cm²) and incubate the plate on ice for 5 minutes.
4. Scrape cells off the plate and transfer to an appropriate tube. Keep on ice.
5. Sonicate lysates on ice.
6. Microcentrifuge for 10 minutes at 4°C and transfer the supernatant to a new tube. The supernatant is the cell lysate. Store at –80°C in single-use aliquots.

C Test Procedure

1. After the microwell strips have reached room temperature, break off the required number of microwells. Place the microwells in the strip holder. Unused microwells must be resealed and stored at 4°C immediately.
2. Add 100 μl of Sample Diluent (supplied in each Pathscan® Sandwich ELISA Kit, blue color) to a microcentrifuge tube. Transfer 100 μl of cell lysate into the tube and vortex for a few seconds. (Sample applied to the well can be diluted 1:1 when the suggested cell lysis buffer is used for cell extraction.) Individual data sheets for each kit provide information regarding an appropriate dilution factor for lysates and kit assay results.

3. Add 100 μl of each diluted cell lysate to the appropriate well. Seal with tape and press firmly onto top of microwells. Incubate the plate for 2 hours at 37°C. Alternatively, the plate can be incubated overnight at 4°C, which gives the best detection of target protein.
4. Gently remove the tape and wash wells:
 - a. Discard plate contents into a receptacle.
 - b. Wash 4 times with 1X Wash Buffer, 200 μl each time for each well.
 - c. For each wash, strike plates on fresh towels hard enough to remove the residual solution in each well, but do not allow wells to completely dry at any time. Clean the underside of all wells with a lint-free tissue.
5. Add 100 μl of Detection Antibody (green color) to each well. Seal with tape and incubate the plate for 1 hour at 37°C.
6. Repeat wash procedure as in Step C4.
7. Add 100 μl of HRP-Linked secondary antibody (red color) to each well. Seal with tape and incubate the plate for 30 minutes at 37°C.
8. Repeat wash procedure as in Step C4.
9. Add 100 μl of TMB Substrate to each well. Seal with tape and incubate the plate for 10 minutes at 37°C or 30 minutes at 25°C.
10. Add 100 μl of STOP Solution to each well. Shake gently for a few seconds.

NOTE: Initial color of positive reaction is blue, which changes to yellow upon addition of STOP Solution.

11. Read results.
 - a. Visual Determination — Read within 30 minutes after adding STOP Solution.
 - b. Spectrophotometric Determination — Wipe underside of wells with a lint-free tissue. Read absorbance at 450 nm within 30 minutes after adding STOP Solution.