

PathScan® Phospho-IκB-α (Ser32) 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.

Species Cross-Reactivity: H, M

Introduction: CST's PathScan® Phospho-IκB-α (Ser32) Sandwich ELISA Kit is a solid phase sandwich enzyme-linked immunosorbent assay (ELISA) that detects endogenous levels of Phospho-IκB-α (Ser32) protein. An IκB-α Mouse mAb #7361* has been coated onto the microwells. After incubation with cell lysates, both nonphospho- and phospho-IκB-α proteins are captured by the coated antibody. Following extensive washing, Phospho-IκB-α (Ser32) Antibody #2859* is added to detect the captured phospho-IκB-α (Ser32) protein. HRP-linked anti-rabbit 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-IκB-α (Ser32) protein.

* Antibodies in kit are custom formulations specific to kit.

Companion Products:

PathScan® Total IκB-α Sandwich ELISA Kit #7360

Anti-rabbit IgG, HRP-linked Antibody #7074

Cell Lysis Buffer (10X) #9803

Specificity/Sensitivity: CST's PathScan® Phospho-IκB-α (Ser32) Sandwich ELISA Kit detects endogenous levels of Phospho-IκB-α (Ser32) protein. As shown in Figure 1, using this Sandwich ELISA Kit #7355, a significant induction of Phospho-IκB-α (Ser32) in HeLa cells treated with TNF-α can be detected. However, the level of total IκB-α (phospho- and nonphospho-), detected by the Total IκB-α Sandwich ELISA Kit #7360, remains unchanged.

Products Included	Volume	Solution Color
IκB-α (L27H11) Mouse mAb Coated Microwells*	96 tests	
Phospho-IκB-α Detection Ab	11 ml	green
Anti-rabbit IgG HRP-Linked Ab	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).

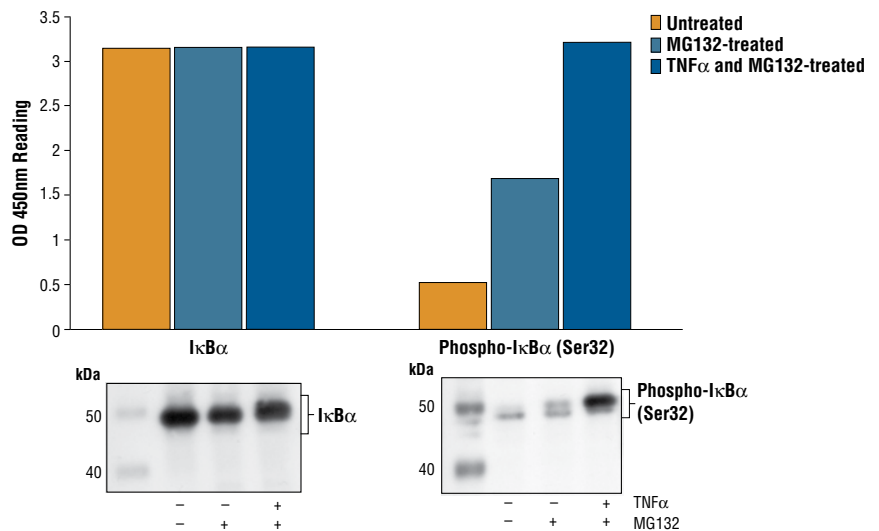


Figure 1: Treatment of HeLa cells with TNF α stimulates phosphorylation of IκB-α at Ser32, detected by PathScan® Phospho-IκB-α (Ser32) Sandwich ELISA kit, #7355, but does not affect the level of total IκB-α protein detected by PathScan® Total IκB-α Sandwich ELISA kit, #7360. Treatment with MG132, a proteasome inhibitor (37°C for 180 min before TNF α induction), causes accumulation of phospho-IκB-α in control and TNF- α -treated cells, shown in both Sandwich ELISA and Western blot analysis. OD450 readings are shown in the top figure, while the corresponding Western blot using Phospho-IκB-α (Ser32) Ab #9241 (right panel) or IκB-α (L27H11) Mouse mAb #7361 (left panel), is shown in the bottom figure.



Background: The NF- κ B/Rel transcription factors are present in the cytosol in an inactive state complexed with the inhibitory I κ B proteins (1-3). Activation occurs via phosphorylation of I κ B- α at Ser32 and Ser36 followed by proteasome-mediated degradation, resulting in the release and nuclear translocation of active NF- κ B (3-7). I κ B- α phosphorylation and resulting Rel-dependent transcription are activated by a highly diverse group of extracellular signals including inflammatory cytokines, growth factors and chemokines.

Kinases that phosphorylate I κ B at these activating sites have been identified (8). Because phosphorylation of I κ B-alpha at Ser32 is essential for release of active NF- κ B, phosphorylation at this site is an excellent marker of NF- κ B activation (1-3).

Background References:

- (1) Baeuerle, P.A. and Baltimore, D. (1988) *Science* 242, 540-546.
- (2) Beg, A.A. et al. (1993) *Genes Dev.* 7, 2064-2070.
- (3) Finco, T.S. et al. (1994) *Proc. Natl. Acad. Sci. USA* 91, 11884-11888.
- (4) Brown, K. et al. (1995) *Science* 267, 1485-1488.
- (5) Brockman, J.A. et al. (1995) *Mol. Cell. Biol.* 15, 2809-2818.
- (6) Traenckner, E.B. et al. (1995) *EMBO J.* 14, 2876-2883.
- (7) Chen, Z.J. et al. (1996) *Cell* 84, 853-862.
- (8) Karin, M. and Ben-Neriah, Y. (2000) *Annu. Rev. Immunol.* 18, 621-663.

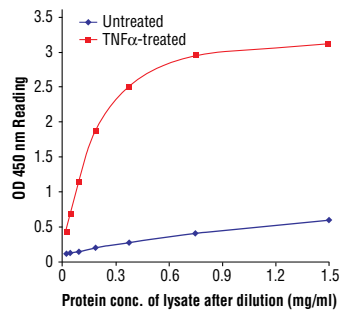


Figure 2: Linear relationship between protein concentration of lysates from untreated and TNF α -treated HeLa cells and kit assay optical density readings. HeLa cells (70-85% confluence) were treated with TNF α (10 ng/ml) and lysed after incubation at 37°C for 5 minutes.

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 Na₃VO₄, 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 cm in diameter) 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. Generally, 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. However, dilution factors need to be titrated when specific cell lysates are used.

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.
 - d. 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 4.
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 4.
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.