

# PathScan® Inflammation Multi-Target Sandwich ELISA Kit



✓ 1 Kit  
(96 assays)

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

## Species Cross-Reactivity: H, M

**Introduction:** CST's PathScan® Inflammation Multi-Target Sandwich ELISA Kit is a solid phase sandwich enzyme-linked immunosorbent assay (ELISA) that combines the reagents necessary to detect endogenous levels of NF-κB p65, phospho-NF-κB p65 (Ser536), phospho-SAPK/JNK (Thr183/Tyr185), phospho-p38 MAPK (Thr180/Tyr182), phospho-Stat3 (Tyr705) and phospho-IκB-α (Ser32). These molecules represent convergence points and key regulatory proteins in signaling pathways controlling the stress and inflammation response. Sixteen tests are provided for each target protein. Specific assay formulations for the indicated target proteins can be found in the datasheets associated with the individual PathScan® Sandwich ELISA Kits‡. Briefly, a capture antibody† has been coated onto the microwells. After incubation with cell lysates, the target protein is captured by the coated antibody†. Following extensive washing, a detection antibody is added to detect the captured target protein. An HRP-linked secondary antibody is then used to recognize the bound detection antibody. HRP substrate, TMB, is added to develop color. The magnitude of absorbance for this developed color is proportional to the quantity of bound target protein.

† Antibodies in kit are custom formulations specific to kit.

‡ See companion products.

**Specificity/Sensitivity:** CST's PathScan® Inflammation Multi-Target Sandwich ELISA Kit #7276 detects endogenous levels of six proteins: NF-κB p65, phospho-NF-κB p65 (Ser536), phospho-SAPK/JNK (Thr183/Tyr185), phospho-p38 MAPK (Thr180/Tyr182), phospho-Stat3 (Tyr705) and phospho-IκB-α (Ser32). Differential activation of these proteins can be observed over time in response to various cytokine treatments. As shown in Figure 1, the relationship between the protein concentration of the lysate and the absorbance at 450 nm can be found in the datasheets associated with the individual PathScan® Sandwich ELISA kits‡. ‡See companion products.

## Companion Products:

PathScan® Total NF-κB p65 Sandwich ELISA Kit #7174

PathScan® Phospho-NF-κB p65 (Ser536) Sandwich ELISA Kit #7173

PathScan® Phospho-SAPK/JNK (Thr183/Tyr185) Sandwich ELISA Kit #7325

PathScan® Phospho-p38 MAPK (Thr180/Tyr182) Sandwich ELISA Kit #7946

PathScan® Phospho-Stat3 (Tyr705) Sandwich ELISA Kit #7300

PathScan® Phospho-IκB-α (Ser32) Sandwich ELISA Kit #7355

Products Included	Quantity	Solution Color	Cap Color
NF-κB p65 Mouse Antibody Coated Microwells	16 tests		
NF-κB p65 Rabbit Detection Antibody	1.8 ml	Green	<b>Gray</b>
Anti-Rabbit IgG HRP-Linked Antibody	1.8 ml	Red	<b>Gray</b>
Phospho-NF-κB p65 (S536) Mouse Antibody Coated Microwells	16 tests		
NF-κB p65 Rabbit Detection Antibody	1.8 ml	Green	<b>Orange</b>
Anti-rabbit IgG HRP-Linked Antibody	1.8 ml	Red	<b>Orange</b>
Phospho-SAPK/JNK (Thr183/Tyr185) Rabbit Antibody Coated Microwells	16 tests		
SAPK/JNK (L7E7) Mouse Detection Antibody	1.8 ml	Green	<b>Purple</b>
Anti-mouse IgG, HRP-Linked Antibody	1.8 ml	Red	<b>Purple</b>
Phospho-p38 MAP Kinase (Thr180/Tyr182) Mouse Antibody Coated Microwells	16 tests		
p38 MAPK Rabbit Detection Antibody	1.8 ml	Green	<b>Yellow</b>
Anti-Rabbit IgG HRP-Linked Antibody	1.8 ml	Red	<b>Yellow</b>
Stat3 Rabbit Antibody Coated Microwells	16 tests		
Phospho-Stat3 (Tyr705) Mouse Detection Antibody	1.8 ml	Green	<b>Dark Pink</b>
Anti-Mouse IgG HRP-Linked Antibody	1.8 ml	Red	<b>Dark Pink</b>
IκB-α Mouse Antibody Coated Microwells	16 tests		
Phospho-IκB-α (Ser32) Rabbit Detection Ab	1.8 ml	Green	<b>Blue</b>
Anti-rabbit IgG HRP-Linked Antibody	1.8 ml	Red	<b>Blue</b>
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	
Cell Lysis Buffer (10X)	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).

Anti-rabbit IgG, HRP-linked Antibody #7074

Anti-mouse IgG, HRP-linked Antibody #7076

Cell Lysis Buffer (10X) #9803

TMB Substrate #7004

STOP Solution #7002

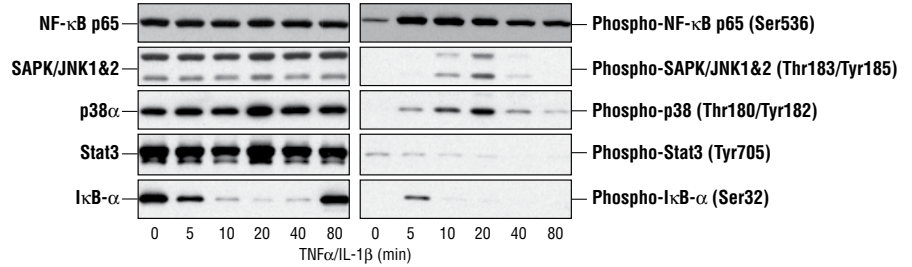
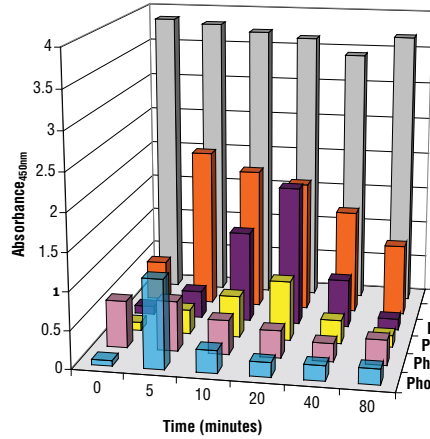
Phosphate Buffered Saline (PBS-20X) #9808

Phosphate Buffered Saline with Tween 20 (PBST-20X) #9809

U.S. Patent No. 5,675,063

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

**A. TNF $\alpha$ /IL-1 $\beta$**



**B. IL-6**

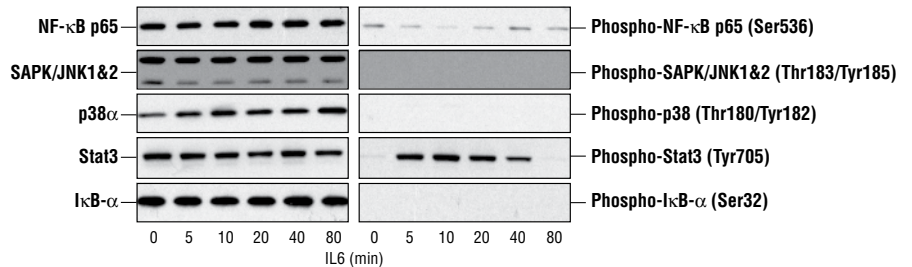
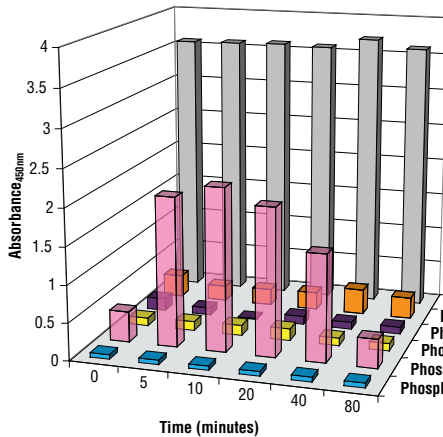


Figure 1. Treatment of HeLa cells with TNF $\alpha$ /IL-1 $\beta$  (A) or IL-6 (B) induces differential phosphorylation of NF- $\kappa$ B p65 at Ser536, SAPK/JNK at Thr183/Tyr185, p38 MAPK at Thr180/Tyr182, Stat3 at Tyr705 and I $\kappa$ B- $\alpha$  at Ser32 as detected by the PathScan® Inflammation Multi-Target Sandwich ELISA Kit #7276. While dynamic phosphorylation is observed throughout the time course, the level of total NF- $\kappa$ B p65, SAPK/JNK, p38 MAPK, Stat3 and I $\kappa$ B- $\alpha$  remains unchanged as demonstrated by sandwich ELISA and Western analysis. HeLa cells (80-90% confluent) were serum starved and stimulated with IL-6 (100 ng/mL) for 5, 10, 20, 40 and 80 minutes at 37°C. Alternatively, exponentially growing cultures of HeLa (80-90% confluent) were treated simultaneously for the indicated times at 37°C with 20 ng/mL TNF- $\alpha$  and 10 ng/mL IL-1 $\beta$ . Lysates were assayed at a protein concentration of 0.5 mg/mL. The absorbance readings at 450 nm are shown as a 3-dimensional representation in the left figure, while the corresponding Western blots are shown in the right figure. The antibodies used for the Western analyses include NF- $\kappa$ B p65 Antibody #3034, Phospho-NF- $\kappa$ B p65 (Ser536) (93H1) Rabbit mAb #3033, SAPK/JNK (56G8) Rabbit mAb #9258, Phospho-SAPK/JNK (Thr183/Tyr185) (G9) Mouse mAb #9255, p38 $\alpha$  MAP Kinase Antibody #9218, Phospho-p38 MAPK (Thr180/Tyr182) (28B10) Mouse mAb #9216, Stat3 Antibody #9132, Phospho-Stat3 (Tyr705) (3E2) Mouse mAb #9138, I $\kappa$ B- $\alpha$  Antibody #9242 and Phospho-I $\kappa$ B $\alpha$  (Ser32) (14D4) Rabbit mAb #2859.

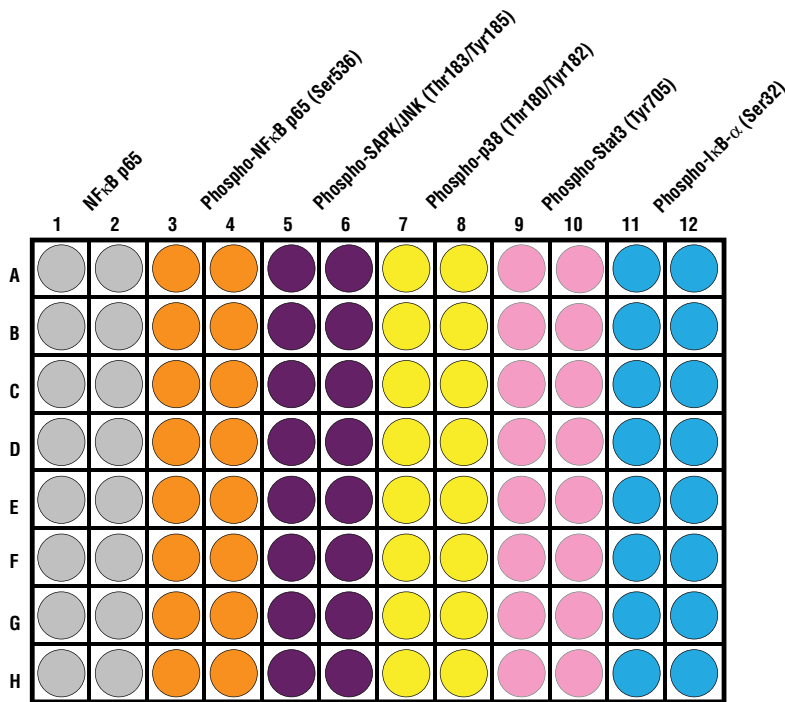


Figure 2. Schematic representation of a 96-well plate depicting the color-code of the reagents used to detect endogenous levels of NF-κB p65 (gray; 1 & 2), Phospho-NF-κB p65 (Ser536) (orange; 3 & 4), Phospho-SAPK/JNK (Thr183/Tyr185) (purple; 5 & 6), Phospho-p38 MAPK (Thr180/Tyr182) (yellow; 7 & 8), Phospho-Stat3 (Tyr705) (dark pink; 9 & 10) and Phospho-IκB-α (Ser32) (blue; 11 & 12).

**Background References:**

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**Background:** Transcription factors of the nuclear factor κB (NF-κB)/Rel family play a pivotal role in inflammation, stress and immune responses. There are five family members in mammals: RelA/p65, c-Rel, RelB, NF-κB1 (p105/p50) and NF-κB2 (p100/p52). These proteins function as dimeric transcription factors. In unstimulated cells, NF-κB/Rel proteins are sequestered in the cytoplasm and inhibited by the IκB proteins. NF-κB-activating agents induce phosphorylation of IκB’s, targeting them for degradation and thereby releasing the NF-κB/Rel complexes. Active NF-κB/Rel complexes are further activated by phosphorylation (1-4).

The stress-activated protein kinase/Jun-amino-terminal kinase SAPK/JNK is activated by a variety of environmental stresses, including UV and gamma radiation, ceramides, inflammatory cytokines and in some instances, by growth factors and GPCR agonists (5-10). As with the other MAPKs, the core-signaling unit is composed of a MAPKKK, typically MEKK1-4, or by a mixed lineage kinase (MLK), which phosphorylates and activates MKK4-7, which then phosphorylates Thr183 and Tyr185 to activate the SAPK/JNK kinase (6). Stress signals are delivered to this cascade by small GTPases of the Rho family (Rac, Rho, cdc42) (7). Both Rac1 and cdc42 mediate the stimulation of MEKs and MLKs (7). Alternatively, MKK4-7 can be activated by a pathway independent of small GTPases via stimulation of a member of the germinal center kinase (GCK) family (8).

p38 MAP kinase (MAPK) participates in a signaling cascade controlling the cellular response to pro-inflammatory cytokines and a variety of cellular stresses. MKK3, MKK6 and SEK (MKK4) activate p38 MAP kinase by phosphorylation at Thr180 and Tyr182 (11-14).

The Stat3 transcription factor is an important signaling molecule for many cytokines and growth factor receptors. Stat3 is activated by phosphorylation at Tyr705, which induces dimerization, nuclear translocation and DNA binding (15,16).

## 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 Na<sub>3</sub>VO<sub>4</sub>, 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-colored solution) to each well. **Be sure to match the cap color of the Detection Antibody with the corresponding color code of the 8-well strip.** 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-colored solution) to each well. **Be sure to match the cap color of the Detection Antibody with the corresponding color code of the 8-well strip.** 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.