

## Product Information

### Phospho-I $\kappa$ B $\alpha$ [pSer<sup>32</sup>] ELISA, Human

Product Number **CS0660**

Storage Temperature 2-8 °C

### Technical Bulletin

#### Product Description

Phospho-I $\kappa$ B $\alpha$  [pSer<sup>32</sup>] ELISA is a solid phase sandwich Enzyme Linked-Immuno-Sorbent Assay (ELISA) for the quantitative determination of I $\kappa$ B $\alpha$  protein phosphorylated on serine 32. A monoclonal antibody specific for I $\kappa$ B $\alpha$  (regardless of phosphorylation state) has been coated onto the wells of the multiwell plate strips provided. I $\kappa$ B $\alpha$  [pSer<sup>32</sup>] standard dilutions, control specimens, and unknown samples are pipetted into these wells. During the first incubation, the I $\kappa$ B $\alpha$  [pSer<sup>32</sup>] antigen binds to the immobilized (capture) antibody. After incubation, the excess reagents are washed away and an antibody specific for I $\kappa$ B $\alpha$  [pSer<sup>32</sup>] is added to the wells. During the second incubation, this antibody serves as a detection antibody by binding to the immobilized I $\kappa$ B $\alpha$ . After removal of excess detection antibody, horseradish peroxidase-labeled (HRP) anti-rabbit IgG is added. This binds to the detection antibody to complete the four-member sandwich. After a third incubation and washing to remove excess anti-rabbit IgG-HRP, a substrate solution is added, which is acted upon by the bound enzyme to produce color. The intensity of this colored product is directly proportional to the concentration of I $\kappa$ B $\alpha$  [pSer<sup>32</sup>] present in the original specimen. The optical density measured at 450 nm in the multiwell plate reader is used to calculate the concentration of I $\kappa$ B $\alpha$  [pSer<sup>32</sup>].

Phospho-I $\kappa$ B $\alpha$  [pSer<sup>32</sup>] ELISA is designed to detect and quantify the level of I $\kappa$ B $\alpha$  protein phosphorylated on serine 32. This ELISA can be used to detect I $\kappa$ B $\alpha$  [pSer<sup>32</sup>] in human cells. It is not recommended for detection of mouse and rat I $\kappa$ B $\alpha$ . For normalizing the I $\kappa$ B $\alpha$  content of the samples, I $\kappa$ B $\alpha$  ELISA, which is independent of phosphorylation status, is available from Sigma (Product No. CS0620).

I $\kappa$ B $\alpha$  (Inhibitor of Nuclear Factor (NF)- $\kappa$ B $\alpha$ ) is a member of the I $\kappa$ B protein family with MW of 40 kDa. I $\kappa$ B $\alpha$  is ubiquitously expressed among mammals. Located at I $\kappa$ B $\alpha$ 's amino terminus is a signal response

domain that contains serine residues capable of being phosphorylated, while the carboxyl terminus contains a PEST domain, a common feature among proteins with high turnover rates. As with all members of the I $\kappa$ B family, I $\kappa$ B $\alpha$  possesses ankyrin repeats approximately 33 amino acid residues in length, which mediate binding to the Rel homology region of NF- $\kappa$ B. The interaction of I $\kappa$ B $\alpha$  with NF- $\kappa$ B masks the nuclear localization sequence of NF- $\kappa$ B, preventing NF- $\kappa$ B translocation to the nucleus.

A variety of stimuli can activate gene expression by liberating NF- $\kappa$ B through the degradation of I $\kappa$ B $\alpha$ . These stimuli include the proinflammatory cytokines TNF- $\alpha$  and IL-1 $\beta$ , chemokines, PMA, growth factors, LPS, UV irradiation, viral infection, as well as various chemical and physical stresses. The series of events leading to this liberation is well defined. In response to stimulus, serine residues 32 and 36 of I $\kappa$ B $\alpha$  are phosphorylated, and this serine phosphorylation provides a signal for I $\kappa$ B $\alpha$  E3 ligase, a protein complex composed of SKP-1, Cul-1, Roc1, and Fbw1. I $\kappa$ B $\alpha$  E3 ligase polyubiquitinates I $\kappa$ B $\alpha$  at lysine residues 21 and 22, and the polyubiquitinated I $\kappa$ B $\alpha$  is then targeted to the 26S proteasome for degradation. Liberated NF- $\kappa$ B is transported across the nuclear membrane, where it activates transcription by binding to the consensus sequence GGRNNYYCC (where R is a purine, Y is a pyrimidine, and N is any base) which is found in the promoter regions of a large number of genes including IL-6, VEGF, VCAM-1, ICAM-1, HIV long terminal repeat, and many others.

The initial event which targets I $\kappa$ B $\alpha$  for degradation is phosphorylation of serines 32 and 36. This phosphorylation is catalyzed by a protein complex known as IKK (I $\kappa$ B kinase). IKK contains two kinase subunits designated IKK $\alpha$  (MW = 85 kDa) and IKK $\beta$  (MW = 87 kDa), and scaffold protein designated IKK $\gamma$ /NEMO (MW = 48 kDa). The kinetic properties of IKK are regulated by complex formation as well as by phosphorylation events catalyzed by upstream kinases

including members of the MAPK cascade, the SAPK/JNK cascade and NIK. Through its regulation of NF- $\kappa$ B, I $\kappa$ B $\alpha$  controls immune and inflammatory responses, cell division, and apoptosis. Numerous disease states including arthritis, asthma, and inflammatory bowel disease are associated with loss of I $\kappa$ B $\alpha$  regulation. The importance of regulation of I $\kappa$ B $\alpha$  in cancer is underscored by the observation that multiple myelomas often possess polymorphisms at I $\kappa$ B $\alpha$  regulatory sites, and certain symptoms can be ameliorated using the proteasome inhibitor PS-341 which favors the sequestration of NF- $\kappa$ B $\alpha$  in the cytoplasm.

## Reagents

- **Phospho-I $\kappa$ B $\alpha$  [pSer<sup>32</sup>] Standard, Lyophilized, 2 vials, Product No. P 3622**- Full length recombinant I $\kappa$ B $\alpha$  expressed in *E. coli* and phosphorylated by IKK $\alpha$ . Refer to vial label for quantity and reconstitution volume.
- **Standard Diluent Buffer, 25 mL, Product No. S 3943**, contains sodium azide as preservative.
- **Monoclonal-Anti- I $\kappa$ B $\alpha$  Coated 96 well plate, 1EA, Product No. I 0159** - A plate using break-apart strips coated with monoclonal antibody specific for full-length I $\kappa$ B $\alpha$  (regardless of phosphorylation state).
- **Anti-Phospho-I $\kappa$ B $\alpha$  [pSer<sup>32</sup>], 11 mL, Product No. P 3747** A detection antibody, produced in rabbit. Contains sodium azide. Ready to use.
- **Anti-Rabbit IgG-HRP, Concentrate (100X), 1 vial, Product No. I 0409** - contains 3.3 mM thymol and 50% glycerol, viscous. *See Reagent Preparation for handling, dilution and storage instructions.*
- **HRP Diluent, 25 mL, Product No. H 5788** - contains 3.3 mM thymol. Ready to use.
- **Wash Buffer Concentrate, 25X, 100 mL, Product No. W 2639** - *See Reagent Preparation for handling, dilution and storage instructions*
- **Stabilized Chromogen, Tetramethylbenzidine (TMB), 25 mL, Product No. S 3318** –Avoid prolonged exposure to light. Avoid exposure to metal. Ready to use.
- **Stop Solution, 25 mL, Product No. S 2818** – Ready to use.
- **Plate Covers, Adhesive strips, 3 each, Product No. P 4870**

## Reagents and Equipment required but not provided

- Multiwell plate reader capable of readings at 450 nm.

- Calibrated adjustable precision pipettes for volumes between 5  $\mu$ L and 1,000  $\mu$ L.
- Cell extraction buffer (see recommended extraction procedure).
- Deionized or distilled water.
- Plate washer (optional), use squirt bottle, manifold dispenser, etc.
- Glass or plastic 1.0 – 1.5 mL tubes for diluting and aliquoting standard.
- Absorbent paper towels to blot the plate.
- Calibrated beakers and graduated cylinders in various sizes.
- Vortex mixer.
- Graph paper: linear, log-log, or semi-log, as desired.

## Precautions and Disclaimer

The kit is for R&D use only, not for drug, household or other uses. Please consult the Material Safety Data Sheet for information regarding hazards and safe handling practices.

## Preparation Instructions

### Sample Preparation

- Samples of choice – extracts of cell lysates
- Samples should be frozen if not analyzed shortly after collection.
- Avoid multiple freeze-thaw cycles of frozen samples. Thaw completely and mix well prior to analysis.

### Cell Extraction Buffer

10 mM Tris, pH 7.4  
 100 mM NaCl  
 1 mM EDTA  
 1 mM EGTA  
 1 mM NaF  
 20 mM Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub>  
 2 mM Na<sub>3</sub>VO<sub>4</sub>  
 1% Triton<sup>®</sup> X-100  
 10% glycerol  
 0.1% SDS  
 0.5% deoxycholate  
 1 mM PMSF (stock is 0.3 M in DMSO) *PMSF is very unstable and must be added prior to use, even if added previously.*  
 Protease inhibitor cocktail (Sigma Product No. P 2714)

Add 250  $\mu$ l of reconstituted cocktail per 5 mL of Cell Extraction Buffer.

Stable for 2-3 weeks at 4 °C or for up to 6 months when aliquoted (without protease inhibitors and PMSF added) and stored at –20 °C.

*Thaw on ice. Add the protease inhibitors just before use.*

#### Procedure for Extraction of Proteins from Cells

This protocol has been successfully applied to several cell lines of human origin. Researchers may use the procedures that work best in their hands. They will have to assay their lysates for the satisfactory extraction and/or phosphorylation.

#### Protocol for Cell Extraction

1. Collect cells in PBS by centrifugation (non-adherent) or scraping from culture flasks (adherent).
2. Wash twice with cold PBS.
3. Remove and discard the supernatant and collect the cell pellet. (At this point the cell pellet can be frozen at –70 °C and lysed at a later date).
4. Lyse the cell pellet in Cell Extraction Buffer for 30 minutes on ice with vortexing at 10-minute intervals.
5. The volume of Cell Extraction Buffer depends on the cell number in cell pellet, expression of I $\kappa$ B $\alpha$  and phosphorylation of I $\kappa$ B $\alpha$ . For example, 1X10<sup>8</sup> Jurkat cells treated with 20 ng/mL TNF- $\alpha$  for 5 minutes can be extracted in 1 mL of Cell Extraction Buffer. Under these conditions, use of 0.4-4.0  $\mu$ L of the clarified cell extract diluted to a volume of 100  $\mu$ L/well in Standard Diluent Buffer is sufficient for the detection of I $\kappa$ B $\alpha$  [pSer<sup>32</sup>].
6. Transfer extracts to microcentrifuge tubes and centrifuge at 13,000 rpm for 10 minutes at 4 °C.
7. Aliquot the clear lysate to clean microcentrifuge tubes

Before assay: extracted cell lysate samples containing protein should be diluted with Standard Diluent Buffer at least 1:10. This dilution is necessary to reduce the matrix effect of the cell extraction buffer.

#### Reagent Preparation

##### **Standard**

*Note:* This human I $\kappa$ B $\alpha$  standard was prepared using purified, full length, human recombinant I $\kappa$ B $\alpha$  expressed in *E. coli*.

**One Unit** of standard is defined as the amount of I $\kappa$ B $\alpha$  [pSer<sup>32</sup>] derived from 40 pg of I $\kappa$ B $\alpha$ , which was phosphorylated by IKK $\alpha$ . Subsequent lots of standard will be normalized to this lot of material to allow consistency of I $\kappa$ B $\alpha$  [pSer<sup>32</sup>] quantitation.

1. Reconstitute I $\kappa$ B $\alpha$  [pSer<sup>32</sup>] Standard with *Standard Diluent Buffer*. Refer to standard vial label for instructions.
2. Swirl or mix gently and allow sitting for 10 minutes to ensure complete reconstitution. Label as 100 units/mL I $\kappa$ B $\alpha$  [pSer<sup>32</sup>]. Use standard within 1 hour of reconstitution.
3. Prepare serial standard dilutions as follows:

Tube #	Standard Buffer	Standard from tube #:	Final units/mL
1	Reconstitute according to label instructions		100 units /mL
2	0.25 mL	0.25 mL (1)	50 units/mL
3	0.25 mL	0.25 mL (2)	25 units/mL
4	0.25 mL	0.25 mL (3)	12.5 units/mL
5	0.25 mL	0.25 mL (4)	6.25 units/mL
6	0.25 mL	0.25 mL (5)	3.12 units mL
7	0.25 mL	0.25 mL (6)	1.6 units/mL
8	0.25 mL		0 units /mL

4. Remaining reconstituted standard should be discarded or frozen at –70 °C. for further use. Standard can be frozen and thawed one time only without loss of immunoreactivity.

#### **Anti-rabbit IgG Horseradish Peroxidase (HRP)**

*Note:* The *Anti-rabbit IgG-HRP* 100X concentrate is in 50% glycerol. This solution is viscous. To ensure accurate dilution:

1. Equilibrate to room temperature, mix gently, pipette slowly.
2. Remove excess concentrate solution from pipette tip with clean absorbent paper.
3. Within 1 hour of use, dilute 10  $\mu$ L of this 100X concentrated solution with 1 mL of *HRP Diluent* for each 8-well strip used in the assay. Label as Anti-rabbit IgG-HRP Working Solution.
4. Return the unused concentrate to the refrigerator
5. For more strips use the following amounts:

# of 8 well strips	IgG-HRP Concentrate $\mu$ L	Diluent mL
2	20	2
4	40	4
6	60	6
8	80	8
10	100	10
12	120	12

### Wash Buffer

1. Equilibrate to room temperature and mix to redissolve any precipitated salts.
2. Mix 1 volume Wash Buffer Concentrate 25X + 24 volumes of deionized water
3. Label as Working Wash Buffer.
4. Store both the concentrate and the Working Wash Buffer in the refrigerator. Use within 14 days.

### Storage/Stability

All components of this kit are stable at 2-8 °C. Any unused reconstituted standard should be discarded or frozen at -70 °C. Standard can be frozen and thawed one time only without loss of immunoreactivity.

Refer to the Certificate of Analysis for kit shelf life. To obtain C of A go to [www.sigma-aldrich.com](http://www.sigma-aldrich.com)

### Procedure

#### Precautions

- 20-30 minutes before use equilibrate kit and all reagents to room temperature (15-30 °C).
- Use only the coated 96 well capture plate provided with the kit
- Multiwell plate: equilibrate to room temperature in unopened foil bag. Remove desired number of strips, reseal the bag and refrigerate at 2-8 °C to maintain plate integrity.
- When not in use all kit components should be refrigerated.
- Assay all standards, controls and samples in duplicate.
- If particulate matter is present, centrifuge or filter prior to analysis.
- A standard curve must be run with each assay
- Maintain a consistent order of component and reagent addition from well to well. This ensures equal incubation times for all wells.
- Run in-house controls with every assay. If control values fall outside pre-established ranges, the accuracy of the assay may be suspect.
- All reagents are lot-specific. Do not mix reagents from different kit lots.
- Do not use reagents after the kit expiration date.
- Standards and samples can be made up in either glass or plastic tubes.
- Pre-rinse the pipette tip with the reagent and use fresh pipette tips for each sample, standard or reagent.
- Read absorbances within 2 hours of assay completion.

### Washing directions

- The purpose of washing is to remove unbound proteins and other non-specific parts of lysate.
- Incomplete washing will adversely affect the assay and render false results.
- Use only Wash Buffer provided in kit.
- Washing may be performed using automated washer, manifold pipette or squirt bottle.
- Wash cycle four times, blotting as dry as possible after the 4<sup>th</sup> wash.
- When washing manually, fill wells with Wash Buffer, aspirate thoroughly and tap dry on absorbent tissue.
- It is recommended to use laboratory tape to hold plate strips to the plate frame while performing the plate washing and drying procedure to avoid strips coming free of the frame.

### Assay Procedure

#### **Phospho-IκBα [pSer<sup>32</sup>] ELISA Assay Summary**

- 1) **100 μL of IκBα [pSer<sup>32</sup>] Standards or Samples (samples diluted 1:10 or higher in Standard Diluent Buffer)**  
**Incubate 2 hours at RT**  
**aspirate and wash 4x**
- 2) **Add 100 μL Anti-Phospho-IκBα [pSer<sup>32</sup>]**  
**Incubate 1 hour at RT.**  
**aspirate and wash 4x**
- 3) **Add 100 μL Anti-Rabbit IgG-HRP**  
**Incubate 30 min at RT.**  
**aspirate and wash 4x**
- 4) **Add 100 μL Stabilized Chromogen**  
**Incubate 30 minutes at RT**  
**(in the dark).**
- 5) **Add 100 μL of Stop Solution**  
**Read at 450nm.**

#### **Total Assay Time - 4 hours**

- Determine the number of wells for the assay run, including 2 zero wells, 2 chromogen blank wells, 14 standard dilutions wells and 2 wells for each sample to be assayed.
- Remove appropriate number of multiwell strips and return the unused strips to the pouch. Reseal pouch

1<sup>st</sup> incubation

- Add 100  $\mu$ L Standard Diluent to zero wells.
- Add 100  $\mu$ L standards, samples or controls to the appropriate wells.
- Samples prepared in cell extraction buffer must be diluted 1:10 or greater in *Standard Diluent Buffer* (for example, 10  $\mu$ L sample plus 90  $\mu$ L buffer). *The dilutions should be optimized for each assay.*
- Cell culture supernatants or buffered solutions; dilute 1:2 in *Standard Diluent Buffer* (50  $\mu$ L buffer + 50  $\mu$ L sample).
- Tap gently on the plate to mix, cover with Plate Cover and incubate 2 hours at room temperature.
- Thoroughly aspirate or decant solution from wells and discard the liquid. Wash wells for a total of 4 times following washing instructions.

2<sup>nd</sup> incubation

- Add 100  $\mu$ L Anti-phospho-I $\kappa$ B $\alpha$  [pSer<sup>32</sup>] detection antibody to all wells (except chromogen blanks).
- Tap gently on the plate to mix, cover with Plate Cover and incubate 1 hour at room temperature.
- Wash wells for a total of 4 times following washing instructions.
- After the final wash blot dry on a lint free paper towel to remove any remaining wash buffer.

3<sup>rd</sup> incubation

- Add 100  $\mu$ L Anti-Rabbit IgG-HRP Working Solution to each well except the chromogen blanks.
- Cover with Plate Cover and incubate 30 minutes at room temperature.
- Wash wells for a total of 4 times following washing instructions.
- After the final wash blot dry on a lint free paper towel to remove any remaining wash buffer.

Substrate incubation

- Add 100  $\mu$ L of Stabilized Chromogen into all wells. *The liquid in the wells will begin to turn blue.*
- Do not cover the plate
- Incubate approximately 30 minutes at room temperature in the dark (place plate in a drawer or cabinet).

Note: If your multiwell plate reader does not register optical density (OD) above 2.0, incubate only 20-25 minutes.

Stop reaction

- Add 100  $\mu$ L of Stop Solution to each well. This stops the reaction
- Tap gently to mix. *The solution will turn yellow.*

Absorbance reading

- Any commercially available multiwell plate reader capable of reading at OD 450 nm may be used.
- Blank the plate reader against the Chromogen Blank wells (containing Chromogen and Stop Solution).

Read the absorbance of the entire plate at 450 nm within 2 hours after addition of Stop Solution

**Results**

- The results may be calculated using any immunoassay software package
- The four-parameter algorithm provides the best curve fit.
- If the software program is not readily available, the concentrations of Phospho-I $\kappa$ B $\alpha$  [pSer<sup>32</sup>] may be calculated manually.
- Calculate the Average Net OD (average reading of 2 wells) for each standard dilution and samples as follows:
- Average Net OD = Average Bound OD – Average Chromogen Blank OD
- On graph paper plot the Average Net OD of standard dilutions against the standard concentration (units/mL) of phospho-I $\kappa$ B $\alpha$  [pSer<sup>32</sup>] Draw the best curve through these points to construct the standard curve.
- The I $\kappa$ B $\alpha$  [pSer<sup>32</sup>] concentrations in unknown samples and controls can be determined by interpolation from the standard curve.
- Multiply the values obtained for the samples by dilution factor of each sample.
- Samples producing signals higher than the 100 units/mL standard should be further diluted and assayed again.

**Product Profile**Typical Results

The standard curve below is for illustration only and **should not be used** to calculate results in your assay. Run standard curve in each assay.

**Standard Curve**

Standard I $\kappa$ B $\alpha$ [pSer <sup>32</sup> ] (units/mL)	Optical density (450 nm)
0	0.175
1.6	0.233
3.12	0.268
6.25	0.379
12.5	0.605
25	1.046
50	1.852
100	2.901

### Limitations

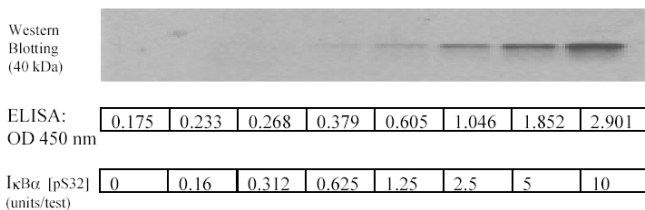
- Do not extrapolate the standard curve beyond the 100 units/mL standard point.
- The dose response is non-linear in this region and accuracy will be compromised.
- Other buffers and matrices have not been investigated.
- The influence of various drugs, aberrant sera (hemolyzed, hyperlipidemic, jaundiced, etc.) and the use of biological fluids in place of cell extract and brain homogenate samples have not been thoroughly investigated.
- The rate of degradation of native IKBA in various matrices has not been investigated.

### Performance characteristics

#### Sensitivity

The analytical sensitivity of this assay is <0.5 unit/mL of I $\kappa$ B $\alpha$  [pSer<sup>32</sup>]. This was determined by adding two standard deviations to the mean O.D. obtained when the zero standard was assayed 30 times. In Jurkat cells cultured in complete medium, this level of sensitivity was equivalent to I $\kappa$ B $\alpha$  protein extractable from 4000 Jurkat cells treated with 20 ng/mL TNF- $\alpha$  for 5 minutes.

The sensitivity of this ELISA was compared to immunoblotting using known quantities of I $\kappa$ B $\alpha$ . The data presented in Figure 1 show that this ELISA is approximately 2 times more sensitive than immunoblotting. The bands shown in the immunoblotting were developed using rabbit anti-phospho-I $\kappa$ B $\alpha$  [pSer<sup>32</sup>], a goat anti-rabbit IgG alkaline phosphatase conjugated, followed by chemiluminescent substrate.



**Figure 1** Detection of I $\kappa$ B $\alpha$  [pSer<sup>32</sup>] by ELISA vs. immunoblot

### Precision

#### 1. Intra-Assay Precision

Samples of known concentration were assayed in replicates of 16 to determine precision within an assay.

	Sample 1	Sample 2	Sample 3
Mean (units/mL)	77.10	29.55	7.77
Standard Deviation (SD)	3.28	2.01	0.52
% Coefficient of Variation	4.24	6.80	6.65

#### 2. Inter-Assay Precision

Samples were assayed 42 times in multiple assays to determine precision between assays.

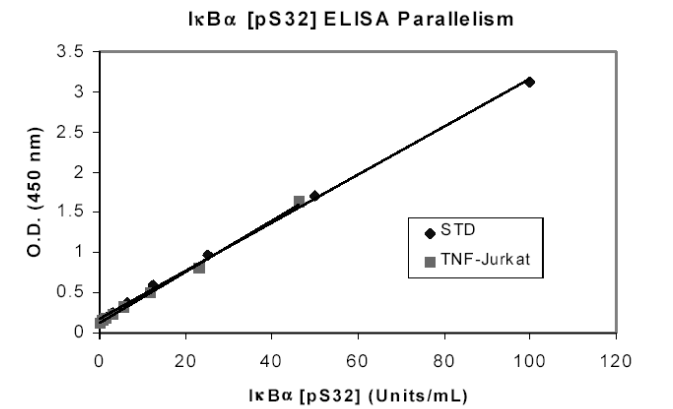
	Sample 1	Sample 2	Sample 3
Mean (units/mL)	77.39	29.55	7.77
Standard Deviation (SD)	4.21	2.22	0.91
Coefficient of Variation %	5.43	7.52	11.75

### Recovery

To evaluate recovery, I $\kappa$ B $\alpha$  was extracted from mouse 3T3L1 cells with cell lysate buffer and the extract adjusted to 100  $\mu$ g/mL. Recombinant human I $\kappa$ B $\alpha$  [pSer<sup>32</sup>] was spiked into the extract at 3 different levels and the percent recoveries over endogenous levels were calculated. The average recovery was 89%.

### Parallelism

Natural phospho-I $\kappa$ B $\alpha$  [pSer<sup>32</sup>] from human Jurkat cell lysates was serially diluted in *Standard Diluent Buffer*. The optical density of each dilution was plotted against the I $\kappa$ B $\alpha$  [pSer<sup>32</sup>] standard curve. Parallelism demonstrated in Figure 2 indicates that the standard accurately reflects I $\kappa$ B $\alpha$  [pSer<sup>32</sup>] content in samples.



**Figure 2** Parallelism: I $\kappa$ B $\alpha$  [pSer<sup>32</sup>]

### Linearity of Dilution

Jurkat cells were grown in tissue culture medium containing 10% fetal calf serum, treated with TNF- $\alpha$  for 5 minutes and lysed with Cell Extraction Buffer. This lysate was diluted in *Standard Diluent Buffer* over the range of the assay and measured for I $\kappa$ B $\alpha$  [pSer<sup>32</sup>], content. Linear regression analysis of sample values versus the expected concentration yielded a correlation coefficient of 0.99.

Dilution	Cell Lysate		
	Measured units/mL	Expected units/mL	% Expected
Neat	18.10	18.16	100
1:2	9.77	9.05	108
1:4	5.14	4.89	105
1:8	1.18	1.25	97
1:16	0.49	0.59	83

### Specificity

- The Phospho-I $\kappa$ B $\alpha$  [pSer<sup>32</sup>] ELISA is specific for measurement of human I $\kappa$ B $\alpha$ , which is phosphorylated on serine 32.
- The reactivity of this ELISA with other I $\kappa$ B isoforms has not been established.
- This ELISA does not react with mouse and rat I $\kappa$ B $\alpha$ .
- The data presented in Figure 3 indicate that I $\kappa$ B $\alpha$  [pSer<sup>32</sup>] ELISA recognizes *E. coli*-derived recombinant I $\kappa$ B $\alpha$  phosphorylated *in vitro* by recombinant IKK $\alpha$  subunit. The total I $\kappa$ B $\alpha$  assay reacts with both phosphorylated and non-phosphorylated I $\kappa$ B $\alpha$ .

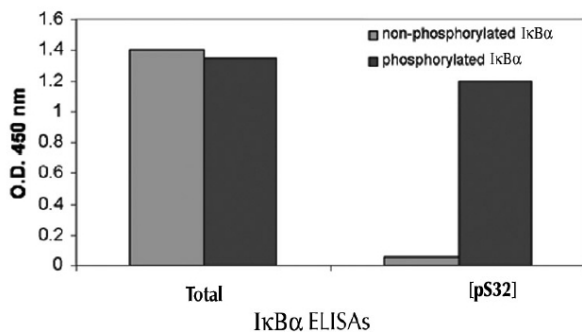


Figure 3

The specificity of this assay for I $\kappa$ B $\alpha$  phosphorylated at serine 32 was confirmed by peptide competition. The data presented in Figure 4 show that only the phospho-peptide containing the phosphorylated serine 32 could block the ELISA signal in Jurkat cell lysate treated with TNF- $\alpha$ . The same sequence containing non-phosphorylated serine at position 32 did not block the signal.

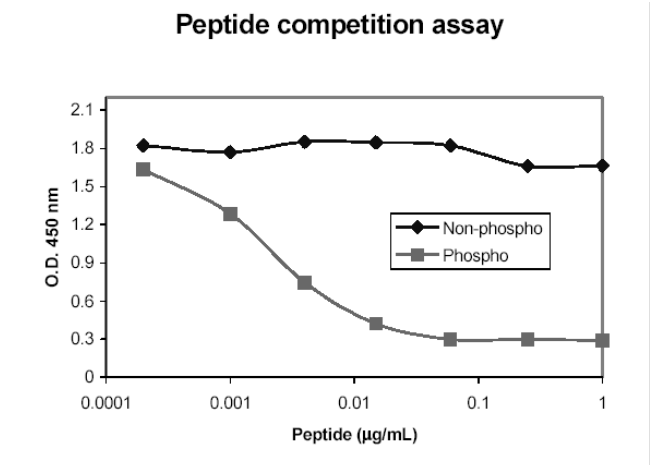
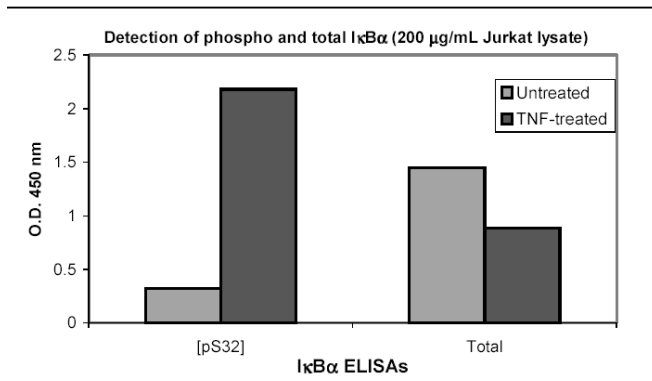


Figure 4 – Peptide Competition

TNF- $\alpha$  treatment of viable cells is known to initiate signals, which stimulate the phosphorylation of I $\kappa$ B $\alpha$  at serine residues 32 and 36. This phosphorylation marks I $\kappa$ B $\alpha$  for ubiquitination and subsequent degradation by the 26S proteasome. The data presented in Figure 5 demonstrate that the level of I $\kappa$ B $\alpha$  [pSer<sup>32</sup>] in lysates made from Jurkat cells treated with TNF- $\alpha$  is increased compared to non-treated controls, determined with the Phospho-I $\kappa$ B $\alpha$  [pSer<sup>32</sup>] ELISA. The data presented also demonstrate that TNF- $\alpha$  treatment causes a reduction in the level of Total I $\kappa$ B $\alpha$ , determined with I $\kappa$ B $\alpha$  ELISA (Prod. No. CS0620), which is attributable to the TNF- $\alpha$ -stimulated degradation of I $\kappa$ B $\alpha$ .



**Figure 5**

6. Ma, M.H., et al., The proteasome inhibitor PS-341 markedly enhances sensitivity of multiple myeloma tumor cells to chemotherapeutic agents., *Clin. Cancer Res.* **9**, 1136-1144 (2003).
7. Malek, S., et al., I $\kappa$ B $\alpha$  functions through direct contacts with the nuclear localization signals and the DNA binding sequences of NF $\kappa$ B., *J. Biol. Chem.* **273**, 25427-25435 (1998).
8. Tanaka K., et al., Control of I $\kappa$ B $\alpha$  proteolysis by the ubiquitin-proteasome pathway., *Biochimie*, **83**, 351-356 (2001).

Triton is a registered trademark of Dow Chemical.

AH/PHC 09/04

### Reference

1. Adams, J., Proteasome inhibition in cancer: development of PS-341., *Semin. Oncol.* **28**, 613-619 (2001).
2. Berenson, J.R., et al., The role of nuclear factor- $\kappa$ B in the biology and treatment of multiple myeloma., *Semin. Oncol.* **28**, 626-633 (2001).
3. Ghosh, S. and M. Karin, Missing pieces in the NF- $\kappa$ B puzzle., *Cell*, **109** Suppl:S81-96 (2002).
4. Israel, A., The IKK complex: an integrator of all signals that activate NF $\kappa$ B? *Trends Cell Biol.* **10**, 129-133 (2000).
5. Joyce, D., et al., NF- $\kappa$ B and cell cycle regulation: the cyclin connection., *Cytokine Growth Factor Rev.* **12**, 73-90 (2001).

Sigma brand products are sold through Sigma-Aldrich, Inc.

Sigma-Aldrich, Inc. warrants that its products conform to the information contained in this and other Sigma-Aldrich publications. Purchaser must determine the suitability of the product(s) for their particular use. Additional terms and conditions may apply. Please see reverse side of the invoice or packing slip.