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ProductInformation

IκBα ELISA, Human

Product Number **CS0620**
Storage Temperature 2-8 °C

Technical Bulletin

Product Description

IκBα ELISA is a solid phase sandwich Enzyme Linked-Immuno-Sorbent Assay (ELISA). for the quantitative determination of IκBα protein. A monoclonal antibody specific for IκBα (regardless of phosphorylation state) has been coated onto the wells of the multiwell plate strips provided. IκBα standard dilutions, control specimens, and unknown samples are pipetted into these wells. During the first incubation, the IκBα antigen binds to the immobilized (capture) antibody. After incubation, the excess reagents are washed away and Anti-IκBα specific for total IκBα is added to the wells. During the second incubation, this antibody serves as a detection antibody by binding to the immobilized IκBα. 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 IκBα present in the original specimen. The optical density measured at 450 nm in the multiwell plate reader is used to calculate the concentration of IκBα.

IκBα ELISA is designed to detect and quantify the level of IκBα protein, independent of its phosphorylation state. This ELISA can be used to detect IκBα in human cells. This kit is not recommended for detection of mouse and rat IκBα. This assay is intended for the detection of IκBα from human cell lysates and can be used to normalize IκBα content of samples when examining quantities of IκBα [pSer^{32}] phosphorylation using Sigma Phospho-IκBα [pSer^{32}] ELISA (Product No. CS0660).

IκBα (Inhibitor of Nuclear Factor (NF)-κBα) is a member of the IκB protein family with MW of 40 kDa. IκBα is ubiquitously expressed among mammals. Located at IκBα'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 IκB family, IκBα possesses ankyrin repeats approximately 33 amino acid residues in length, which mediate binding to the Rel homology region of NF-κB. The interaction of IκBα with NF-κB masks the nuclear localization sequence of NF-κB, preventing NF-κB translocation to the nucleus.

A variety of stimuli can activate gene expression by liberating NF-κB through the degradation of IκBα. These stimuli include the proinflammatory cytokines TNF-α and IL-1β, 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 IκBα are phosphorylated, and this serine phosphorylation provides a signal for IκBα E3 ligase, a protein complex composed of SKP-1, Cul-1, Roc1, and Fbw1. IκBα E3 ligase polyubiquitinates IκBα at lysine residues 21 and 22, and the polyubiquitinated IκBα is then targeted to the 26S proteasome for degradation. Liberated NF-κB 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 IκBα for degradation is phosphorylation of serines 32 and 36. This phosphorylation is catalyzed by a protein complex known as IKK (IκB kinase). IKK contains two kinase subunits designated IKKα (MW = 85 kDa) and IKKβ (MW = 87 kDa), and scaffold protein designated IKKγ/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-κB, IκBα controls immune and inflammatory responses, cell division, and apoptosis. Numerous disease states including arthritis, asthma, and inflammatory bowel disease are associated with loss of IκBα regulation. The importance of regulation of IκBα in cancer is underscored by the observation that multiple myelomas often possess polymorphisms at IκBα regulatory sites, and certain symptoms can be ameliorated using the proteasome inhibitor PS-341which favors the sequestration of NF-κBα in the cytoplasm.

Reagents

- **IκBα Standard, Lyophilized, 2 vials, Product No. I 0534,-** Full length recombinant IκBα expressed in *E. coli.* 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- IκBα Coated 96 well plate, 1EA, Product No. I 0159 -** A plate using break-apart strips coated with monoclonal antibody specific for full-length IκBα (regardless of phosphorylation state).
- **Anti- IκBα, 11 mL, Product. No. I 0284** A detection antibody, produced in rabbit. Contains sodium azide. Ready to use.
- **Anti-Rabbit IgG-HRP, Concentrate (100X), 1 vial, Product No. R 8528 -** 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 µL and 1,000 µL.
- 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 $Na_4P_2O_7$
2 mM Na_3VO_4
1% Triton® 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 µl 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 and expression of IκBα. For example, $1X10^8$ Jurkat cells grown in RPMI plus 10% FBS can be extracted in 1 mL of Cell Extraction Buffer. Under these conditions, use of 0.4-4.0 µL of the clarified cell extract diluted to a volume of 100 µL/well in Standard Diluent Buffer is sufficient for the detection of IκBα.
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 IκBα standard was prepared from, and calibrated against, the mass of a highly purified, full length, recombinant human IκBα protein expressed in *E. coli*.

1. Reconstitute IκBα 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 4000 pg/mL Use standard within 1 hour of reconstitution.
3. Prepare serial standard dilutions as follows

Tube #	Standard Buffer	Standard from tube #:	Final pg/mL
1	Reconstitute according to label instructions		4000 pg/mL
2	0.25 mL	0.25 mL (1)	2000 pg/mL
3	0.25 mL	0.25 mL (2)	1000 pg/mL
4	0.25 mL	0.25 mL (3)	500 pg/mL
5	0.25 mL	0.25 mL (4)	250 pg/mL
6	0.25 mL	0.25 mL (5)	125 pg/mL
7	0.25 mL	0.25 mL (6)	62.5 pg/mL
8	0.25 mL	-	0 pg/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 µL 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 µL	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

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 4th 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

IκBα ELISA Assay Summary

1) 100 µL of IκBα 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-IκBα
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 dilution 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

1st incubation
a Add 100 µL Standard Diluent to zero wells.
b Add 100 µL standards, samples or controls to the appropriate wells.
c Samples prepared in cell extraction buffer must be diluted 1:10 or greater in *Standard Diluent Buffer* (for example, 10 µL sample plus 90 µL buffer). *The dilutions should be optimized for each assay.*
d Cell culture supernatants or buffered solutions; dilute 1:2 in *Standard Diluent Buffer* (50 µL buffer + 50 µL sample).

e Tap gently on the plate to mix, cover with Plate Cover and incubate 2 hours at room temperature.
f Thoroughly aspirate or decant solution from wells and discard the liquid. Wash wells for a total of 4 times following washing instructions.

2nd incubation
a Add 100 µL Anti-IκBα detection antibody to all wells (except chromogen blanks).
b Tap gently on the plate to mix, cover with Plate Cover and incubate 1 hour at room temperature.
c Wash wells for a total of 4 times following washing instructions.
d After the final wash blot dry on a lint free paper towel to remove any remaining wash buffer.

3rd incubation
a Add 100 µL Anti-Rabbit IgG-HRP Working Solution to each well except the chromogen blanks.
b Cover with Plate Cover and incubate 30 minutes at room temperature.
c Wash wells for a total of 4 times following washing instructions.
d After the final wash blot dry on a lint free paper towel to remove any remaining wash buffer.

Substrate incubation
a Add 100 µL of Stabilized Chromogen into all wells. *The liquid in the wells will begin to turn blue.*
b Do not cover the plate
c 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
a Add 100 µL of Stop Solution to each well. This stops the reaction
b Tap gently to mix. *The solution will turn yellow.*

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

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

Results

1. The results may be calculated using any immunoassay software package
2. The four-parameter algorithm provides the best curve fit.
3. If the software program is not readily available, the concentrations of IκBα may be calculated manually.
4. Calculate the Average Net OD (average reading of 2 wells) for each standard dilution and samples as follows:
5. Average Net OD = Average Bound OD – Average Chromogen Blank OD
6. On graph paper plot the Average Net OD of standard dilutions against the standard concentration (pg/mL) of IκBα. Draw the best curve through these points to construct the standard curve.
7. The IκBα concentrations in unknown samples and controls can be determined by interpolation from the standard curve.
8. Multiply the values obtained for the samples by dilution factor of each sample.
9. Samples producing signals higher than the 4000 pg/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 IκBα (pg/mL)	Optical density (450 nm)
0	0.196
62.5	0.255
125	0.341
250	0.478
500	0.719
1000	1.076
2000	1.847
4000	3.213

Limitations

- Do not extrapolate the standard curve beyond the 4000 pg/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 IκBα in various matrices has not been investigated.

Performance characteristics

Sensitivity

The analytical sensitivity of this assay is <0.30 pg/mL of IκBα. 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 IκBα protein extractable from 2000 cells.

The sensitivity of this ELISA was compared to immunoblotting using known quantities of IκBα. The data presented in Figure 1 show that the sensitivity of the ELISA is approximately 2 times more sensitive than immunoblotting. The bands shown in the immunoblotting data were developed using rabbit anti-IκBα, an alkaline phosphatase conjugated anti-rabbit IgG, followed by chemiluminescent substrate.

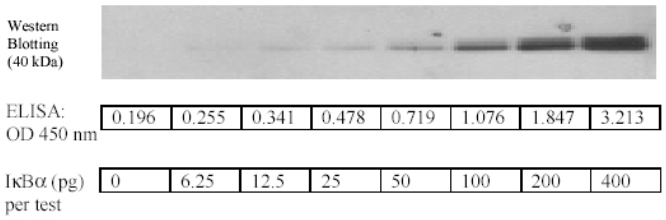


Figure 1 Detection of IκBα by ELISA vs. immunoblot

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 (pg/mL)	2990	1001	520
Standard Deviation (SD)	200	110	50
% Coefficient of Variation	6.97	11.0	9.6

2. Inter-Assay Precision

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

	Sample 1	Sample 2	Sample 3
Mean (pg/mL)	3110	1001	520
Standard Deviation (SD)	320	110	60
Coefficient of Variation %	10.21	10.59	11.0

Recovery

To evaluate recovery, IκBα was extracted from mouse 3T3L1 cells with cell lysate buffer and the extract adjusted to 100 µg/mL. Recombinant human IκBα was spiked into the extract at 3 different levels and the percent recoveries over endogenous levels were calculated. The average recovery was 95%.

Parallelism

Natural IκBα from human Jurkat cell lysates was serially diluted in *Standard Diluent Buffer.* The optical density of each dilution was plotted against the IκBα standard curve. Parallelism demonstrated in Figure 2 indicates that the standard accurately reflects IκBα content in samples.

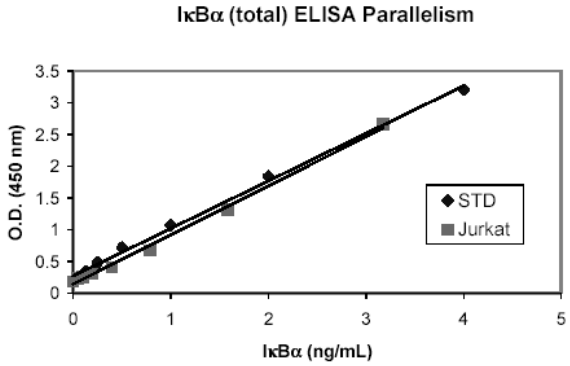


Figure 2 Parallelism: IκBα total

Linearity of Dilution

Jurkat cells were grown in tissue culture medium containing 10% fetal calf serum and lysed with Cell Extraction Buffer. This lysate was diluted with *Standard Diluent Buffer* over the range of the assay and measured for IκBα content. Linear regression analysis of sample values versus the expected concentration yielded a correlation coefficient of 0.99.

Dilution	Cell Lysate		
	Measured pg/mL	Expected pg/mL	% Expected
Neat	3774	3774	100
1:2	1945	1887	103
1:4	992	944	105
1:8	494	471	105
1:16	252	356	106

Specificity

- The IκBα ELISA is specific for measurement of total IκBα protein, irrespective of phosphorylation state.
- The reactivity of this ELISA with other IκB isoforms has not been established.
- This ELISA does not react with mouse and rat IκBα
  The data presented in Figure 3 show that the kit detects non-phosphorylated and phosphorylated *E. coli*-derived IκBα protein.

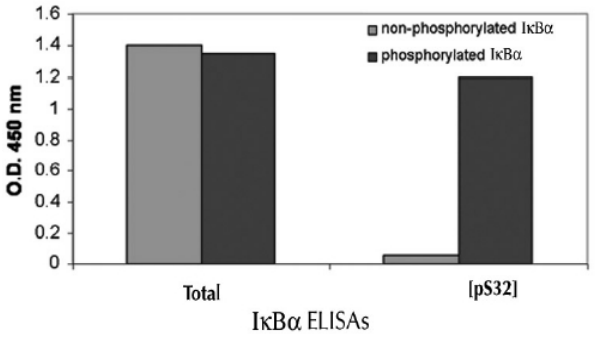
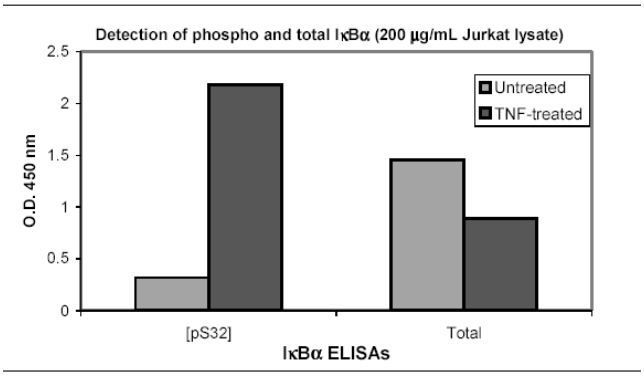


Figure 3

Figure 4 shows the results obtained from TNF-α-treated Jurkat cells. TNF-α treatment of viable cells is known to initiate signals, which stimulate the phosphorylation of IκBα at serine residues 32 and 36. This phosphorylation marks IκBα for ubiquitination and subsequent degradation by the 26S proteasome. The data presented in Figure 4 demonstrate that the level of IκBα [pSer^{32}] in lysates made from Jurkat cells treated with TNF-α is increased compared to non-treated controls, determined with the Phospho-IκBα [pSer^{32}] ELISA (Product No. CS0660).

The data presented also demonstrate that TNF-α treatment causes a reduction in the level of Total IκBα, determined with IκBα ELISA, which is attributable to the TNF-α-stimulated degradation of IκBα. This human IκBα ELISA was tested with 10 ng/mL of JNK, AKT, p38 MAPK, and ERK1 proteins. No cross-reactivity was observed.

Figure 4



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