FunctionELISA™

IκBα

For the detection and analysis of IκBα phosphorylation

(version B1)

Catalog Nos. 48005 & 48505

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Background

The transcription factor NFκB is implicated in the regulation of many genes that code for mediators of the immune, acute phase and inflammatory responses[1]. NFκB is composed of homo- and heterodimeric complexes of members of the Rel (NFκB) family. There are five subunits of the NFκB family in mammals: p50, p65 (RelA), c-Rel, p52 and RelB[2]. These proteins share a conserved 300 amino acid sequence in the N-terminal region, known as the Rel homology domain, that mediates DNA binding, protein dimerization and nuclear localization. This domain is also a target of the IκB inhibitors, which include IκBα, IκBβ, IκBγ, Bcl-3, p105 and p100[3]. In the majority of cells, NFκB exists in an inactive form in the cytoplasm, bound to the inhibitory IκB proteins[4]. IκB proteins are phosphorylated by the IκB kinase complex, which consists of at least three proteins: IKK1/α, IKK2/β and IKK3/γ[4-7]. External stimuli, such as tumor necrosis factor (TNF) or other cytokines, initiate a signal transduction cascade that leads to the activation of the IκB kinase complex. This complex specifically phosphorylates IκBα on Serine-32 and Serine-36, which leads to ubiquitination of IκBα and its subsequent degradation by the 26S proteosome. Degradation of IκBα results in unmasking of the nuclear localization signal of NFκB dimers, which subsequently translocate to the nucleus and activate target genes[8, 9]. Thus, analysis of the phosphorylation state of IκBα provides a correlation to the activity of the IKK complex as well as the activation and nuclear localization of NFκB.

Overview

FunctionELISA™ Kits provide a useful tool to capture and quantifiably measure the amount of a specific protein in a sample. They utilize the "Sandwich ELISA" method for detecting a protein in a sample, which uses two antibodies that each recognize a distinct epitope on the protein. An ELISA plate is coated with the first antibody, called the Capture Antibody, which is used to capture the protein from the sample. The second antibody, called the Detecting Antibody, is used to detect the protein bound by the Capture Antibody. An HRP-conjugated Secondary Antibody is then used to quantitate the amount of bound Detecting Antibody (Figure 1). By using Capture Antibody that recognizes a phosphorylation site on a protein, one can specifically detect phosphorylated protein. Because many proteins involved in signaling cascades are regulated by phosphorylation, this assay proves useful in monitoring and analyzing the phosphorylation state of a given protein. Compared to Western blots, FunctionELISA provides a quicker, more quantifiable way to monitor a protein's phosphorylation state.

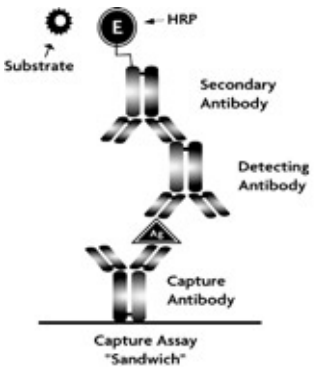


Figure 1: Sandwich ELISA schematic.

Advantages

- Multiple samples can be analyzed in low-volume, high-throughput experiments
- Analysis is complete in just hours
- Quantitative nature of the assay enables direct measurement of changes in phosphorylation

Example Experiment

To monitor the phosphorylation state of IκBα, Jurkat cells were grown to 2 x 10^6 cells/ml and treated with 1 nM TNF-α. The cells were harvested at various time points, lysed and Western blot and the FunctionELISA IκBα Kit were used to monitor and quantify IκBα phosphorylation using 100 µg of lysate per well. TNF-α-induced phosphorylation of IκBα was very rapid, peaking at around 10 minutes and disappearing by 30 minutes (see Figure 2).

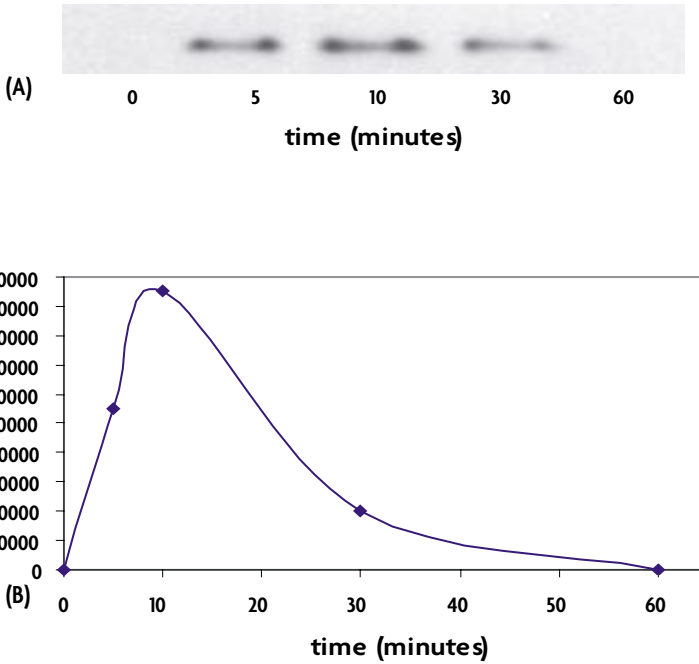


Figure 2: Induction of IκBα phosphorylation with TNF-α in Jurkat cells. Jurkat cells, grown to a density of 2 x 10^6 cells/ml, were treated with 1 nM TNF-α. The cells were harvested at various indicated time points, and cell lysates were tested in Western analysis (Fig. 2A) using an anti-Phospho-IκBα antibody (Active Motif Cat. No. 40904) and in a FunctionELISA IκBα assay (Fig. 2B).

Kit Components and Storage

Reagents	Quantity 1 plate / 5 plates	Composition	Storage
Detecting Antibody (200 µg/ml)	24 µl / 120 µl	PBS, 0.1% sodium azide 0.2% gelatin	4°C for 1 year
HRP-conjugated Secondary Antibody	12 µl / 60 µl	PBS, 0.1% sodium azide	4°C for 1 year
Lysis Buffer	10 ml / 50 ml	20 mM Hepes, pH 7.5 400 mM NaCl 20% glycerol 1% Triton X-100 0.5% NP-40 0.1 mM EDTA 10 mM NaF 10 µM Na_2MoO_4 1 mM $NaVO_3$ 10 mM PNPP 10 mM β-glycerophosphate	4°C for 6 months
100X Protease Inhibitor	100 µl / 500 µl	Proprietary Mix	-20°C for 1 year
1M DTT	100 µl / 500 µl	solution in H_2O	-20°C for 1 year
BSA powder	0.3 g / 1.5 g		4°C for 1 year
20X Wash Buffer	25 ml / 125 ml	20X PBS, pH 7.4 1% Tween-20 4 mM Sodium Orthovanadate	4°C for 6 months
HRP Substrate Solution A	2.5 ml / 12.5 ml		4°C for 6 months
HRP Substrate Solution B	2.5 ml / 12.5 ml		4°C for 6 months
FunctionELISA IκBα plate	1 plate / 5 plates	12 x 8-microwell strips w/ holder	4°C for 1 year
Plate sealer	1 roll / 5 rolls		
Phospho-IκBα Control (20 ng/ml)	1 ml / 5 ml	Blocking Buffer	-20°C for 6 months

Note: The included buffers and reagents have been optimized for use with this kit. Substitution with other reagents may not give optimal results.

Additional Materials Required

Multi-channel pipettor
Multi-channel pipettor reservoirs
Orbital shaker
Microplate luminometer
Distilled water (dH_2O)
Phosphate buffered saline (PBS, pH 7.4)

Preparation of Reagents

Complete Lysis Buffer

An excess of Lysis Buffer is provided for preparing cell extracts for the assay. Prepare the amount of Complete Lysis Buffer required for the assay by adding 1 µl DTT and 10 µl Protease Inhibitor Cocktail per ml of Lysis Buffer.

Note: Some protease inhibitors lose activity within 24 hours of dilution. Therefore, we recommend using the Complete Lysis Buffer immediately for cell lysis. The remaining amount should be discarded if not used on the same day.

Wash Buffer

1X Wash Buffer may be stored at 4°C. If you do not plan on using the entire ELISA plate at once, we recommend preparing only enough Wash Buffer to meet the needs of the experiment.

- Dilute 20X Wash Buffer to 1X with dH_2O. The following chart can be used as a guide.

1X Wash Buffer	20X Wash Buffer	dH_2O	Sufficient for
25 ml	1.25 ml	23.75 ml	5 rxns
50 ml	2.5 ml	47.5 ml	10 rxns
100 ml	5 ml	95 ml	20 rxns
250 ml	12.5 ml	237.5 ml	48 rxns
500 ml	25 ml	475 ml	96 rxns

Blocking Buffer

Prepare Blocking Buffer by dissolving 0.3 g BSA in 30 ml 1X Wash Buffer in a sterile bottle.

Note: Sodium azide has not been included as it inhibits Horseradish Peroxidase. To prevent microbial growth, store Blocking Buffer at 4°C and reduce exposure to contaminants. Dissolve the BSA in 1X Wash Buffer only when ready to use.

Preparation of Whole-Cell Extract

This procedure can be used for a confluent cell layer of 25 cm^2 (60 mm dish). The yield is approximately 0.3 mg of total cellular protein.

1. Treat the cells as required for activation.
2. Wash the cells 2X with 8 ml ice-cold PBS (10 mM phosphate buffer, pH 7.4, 150 mM NaCl).
3. Collect the cells in 3 ml ice-cold PBS. Use a cell scraper for adherent cells.
4. Centrifuge the cells for 10 min at 1,000 rpm at 4°C.

5. Discard the supernatant and add a volume of Complete Lysis Buffer equivalent to the volume of the cell pellet. Pipette gently to mix.

   Note: Preparing such a concentrated lysate will aid in the capture of phosphorylated proteins, whose concentration is very low.

6. Transfer into a 1.5 ml microcentrifuge tube.
7. Incubate on ice for 30 minutes.
8. Centrifuge for 20 min at 14,000 x *g* (full speed in a microcentrifuge) at 4°C.
9. Collect the supernatant at 4°C, aliquot and store at –80°C. Avoid freeze/thaw cycles.
10. Measure the protein content by a Bradford-based assay.

IκBα Assay

IκBα protein is phosphorylated in response to stimulation of the NFκB pathway, and then broken down very rapidly. Thus, capture and detection of this transient form of IκBα is very difficult, as is the long-term storage of extracts. Because of these limitations, this kit's positive control is a synthetic antigen generated by coupling a phospho-peptide to full-length IκBα protein. The conjugated phospho-peptide is the same one used to generate the monoclonal Capture Antibody. The Phospho-IκBα Control is stable and can be used to generate a standard curve.

Determine the appropriate number of microwell strips required for testing your samples, controls and blanks in duplicate. If less than 8 wells in a strip are to be used, cover the unused wells with a portion of the plate sealer before you perform the assay. The contents of these wells are stable at room temperature and, if kept dry, can be used later for a separate assay. Store the unused strips in the aluminum pouch at 4°C. Use the strip holder for the assay. Prepare the Washing and Blocking Buffers as described in the section **Preparation of Reagents**. Multi-channel pipettor reservoirs may be used for dispensing the various buffers and Substrate Solutions into the wells being used.

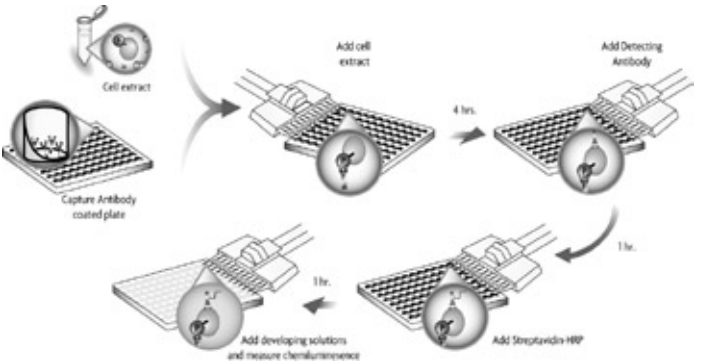


Figure 3: Flow Chart of the FunctionELISA™ IκBα Process.

Protocol

PLEASE READ THE ENTIRE PROTOCOL BEFORE STARTING!

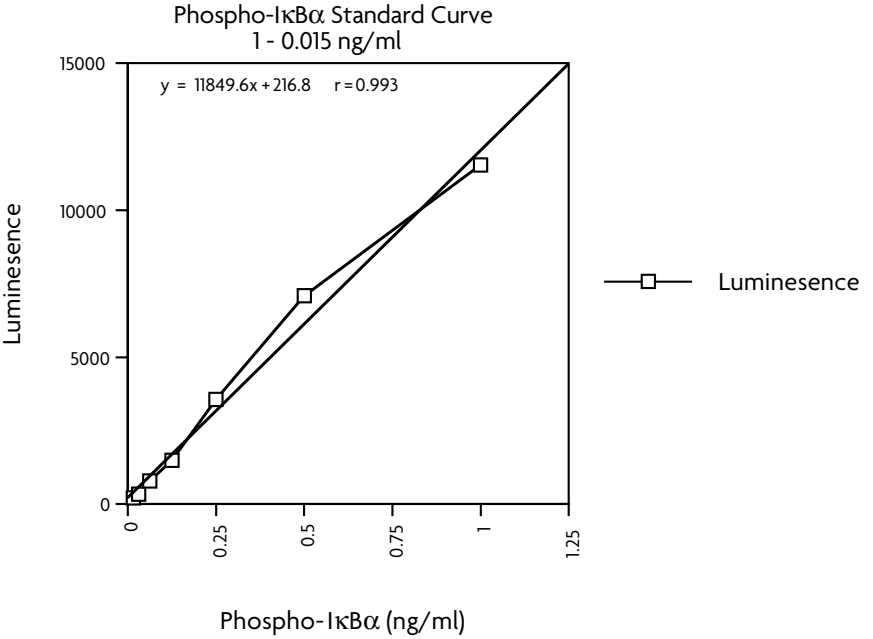
Step 1: Preparation of Phospho-IκBα Standard Curve

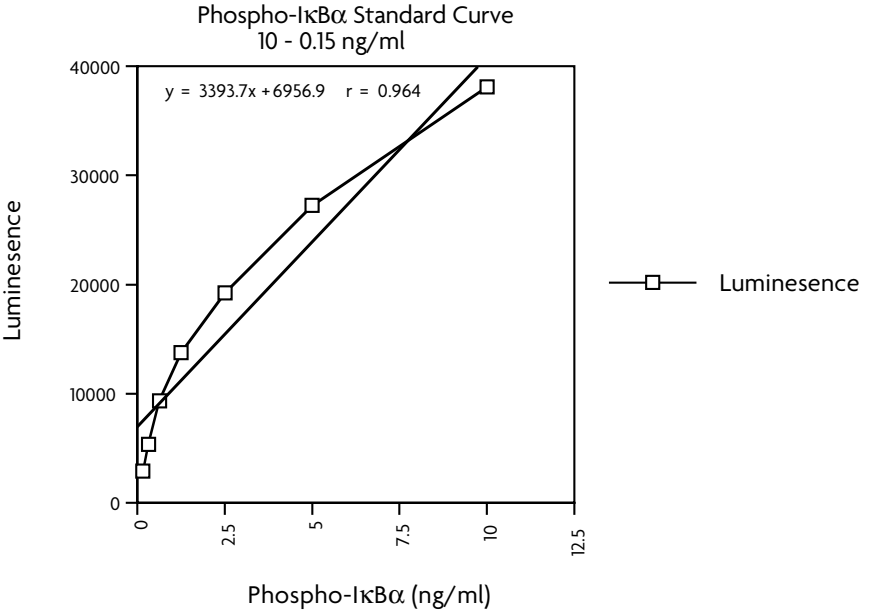
1. Set up a Phospho-IκBα Standard Curve in duplicate using the following concentrations: 10, 5, 2.5, 1.25, 0.63, 0.32, 0.15 and 0.0 ng/ml. (See table below for suggested layout.)

   Note: For higher sensitivity, set up one or both Phospho-IκBα Standard Curve(s) using a 10-fold dilution of the 20 ng/ml Phospho-IκBα Control in Blocking Buffer to 2 ng/ml (final standard curve concentrations of 1, 0.5, 0.25, 0.125, 0.063, 0.032, 0.015 and 0.0 ng/ml).

2. Add 100 µl of Blocking Buffer to wells A1 through H1 and A2 through H2.
3. Pipette 100 µl Phospho-IκBα Control solution (20 ng/ml) into wells A1 and A2.
4. Mix wells A1 and A2 by pipetting.
5. Transfer 100 µl from well A1 to B1 and A2 to B2.
6. Mix wells B1 and B2 by pipetting.
7. Transfer 100 µl from well B1 to C1 and B2 to C2.
8. Continue this procedure to wells G1 and G2. After mixing, discard 100 µl of solution from wells G1 and G2.
9. Wells H1 and H2 are blanks and should contain only 100 µl of Blocking Buffer.

	1	2	3	4	5	6	7	8	9	10	11	12
A	10 (1) ng/ml	10 (1) ng/ml	–	–	–	–	–	–	–	–	–	–
B	5(0.5) ng/ml	5(0.5) ng/ml	–	–	–	–	–	–	–	–	–	–
C	2.5 (0.25) ng/ml	2.5 (0.25) ng/ml	–	–	–	–	–	–	–	–	–	–
D	1.25 (0.125) ng/ml	1.25 (0.125) ng/ml	–	–	–	–	–	–	–	–	–	–
E	0.63 (0.063) ng/ml	0.63 (0.063) ng/ml	–	–	–	–	–	–	–	–	–	–
F	0.32 (0.032) ng/ml	0.32 (0.032) ng/ml	–	–	–	–	–	–	–	–	–	–
G	0.15 (0.015) ng/ml	0.15 (0.015) ng/ml	–	–	–	–	–	–	–	–	–	–
H	Blank	Blank	–	–	–	–	–	–	–	–	–	–


Phospho-IκBα Standard Curve
1 - 0.015 ng/ml
y = 11849.6x + 216.8 r = 0.993
Luminesence
15000
10000
5000
0
0
0.25
0.5
0.75
1
1.25
Phospho-IκBα (ng/ml)
Luminesence


Phospho-IκBα Standard Curve
10 - 0.15 ng/ml
y = 3393.7x + 6956.9 r = 0.964
Luminesence
40000
30000
20000
10000
0
0
2.5
5
7.5
10
12.5
Phospho-IκBα (ng/ml)
Luminesence

Step 2: Binding of IκBα

1. **Sample wells:** Dilute concentrated cell lysates to 1 mg/ml in Blocking Buffer. Pipette 100 µl lysate into each well.
2. Cover the plate with plate sealer and incubate at 4°C for 4 hours.
3. Remove cell lysate from wells and wash four times (4X) with 250 µl Wash Buffer. After final wash, tap plate upside down several times to remove any residual Wash Buffer.

Step 3: Binding of IκBα-Detecting Antibody

1. Dilute Detecting Antibody 1:250 in Blocking Buffer. For one entire plate, use 20 µl Detecting Antibody in 5 ml Blocking Buffer.
2. Add 50 µl diluted Detecting Antibody to each well.
3. Cover the plate and incubate at room temperature for 1 hour.
4. Remove Detecting Antibody and wash wells four times (4X) with 250 µl Wash Buffer. After final wash, tap plate upside down several times to remove any residual Wash Buffer.

Step 4: Binding of Secondary Antibody

1. Dilute Secondary Antibody 1:1000 in Blocking Buffer. For one entire plate, use 5 µl Secondary Antibody in 5 ml Blocking Buffer.
2. Add 50 µl diluted Secondary Antibody to each well.
3. Cover the plate and incubate at room temperature for 1 hour.
4. While Secondary Antibody is reacting, mix equal amounts of HRP Substrate Solution A and HRP Substrate Solution B. For one entire plate, combine 2.5 ml of each solution. Mix and allow temperature to equilibrate to room temperature.
5. Remove Secondary Antibody solution and wash thoroughly five times (5X) with 250 µl Wash Buffer to remove any unbound conjugate. Let plate sit for 1 minute and then tap plate upside down several times to remove any residual Wash Buffer.

Step 5: Chemiluminescent Detection

1. Add 50 µl Substrate Solution mixture (See Step 4, No.4) per well.
2. Use a 96-well luminometer to measure each well and take readings for 2-second and for 20-second intervals.

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Appendix

Section A. Troubleshooting

PROBLEM	POSSIBLE CAUSE	RECOMMENDATION
No signal or weak signal in all wells	Omission of key reagent	Check that all reagents have been added in the correct order
	Substrate or conjugate is no longer active	Test conjugate and substrate for activity
	Enzyme inhibitor present	Sodium azide will inhibit the peroxidase reaction, follow our recommendations when preparing buffers
	Plate reader settings not optimal	Verify the wavelength and filter settings of the plate reader
	Incorrect assay temperature	Bring substrate to room temperature
	Inadequate volume of Substrate Solution	Check to make sure that correct volume is delivered by pipette
High background in all wells	Concentration of antibodies too high	Increase antibody dilutions
	Inadequate washing	Ensure all wells are filled with Wash Buffer and follow washing recommendations
Uneven signal development	Incomplete washing of wells	Ensure all wells are filled with Wash Buffer and follow washing recommendations
	Well cross-contamination	Follow washing recommendations
High background in sample wells	Too much cell extract per well	Decrease amount of cell extract to 50 µg/well
	Concentration of antibodies too high	Perform antibody titration to determine optimal working concentration. Start using 1:1000 for Detecting Antibody and 1:2000 for the Secondary Antibody. The sensitivity of the assay will be decreased
No signal or weak signal in sample wells only	Not enough cell extract per well	Increase amount of cell extract to 150 µg/well
	IκBα is poorly activated or is inactivated	Perform a time course for IκBα activation in the studied cell line
	Cell extracts are not from rat, mouse or human origin	Perform study with a rat, mouse or human model

Section B. Related Products

NFκB PRODUCTS	Application	Format	Catalog No.
TransAM™			
NFκB p65	Transcription Factor Assay	96-well plate	40096
NFκB p50	Transcription Factor Assay	96-well plate	41096
Cell extracts			
Unstimulated HeLa whole-cell extract		125 µg	40050
IL-1α-stimulated HeLa whole-cell extract		125 µg	40100
TNF-α-stimulated HeLa whole-cell extract		125 µg	40200
TNF-α-stimulated HeLa nuclear extract		125 µg	40210
Nuclear Extract Kit		100 rxns	40010
Antibodies			
A20 mAb	WB, IP	100 µg	40901
Phosphorylated-Akt1 mAb	WB, IP	100 µg	40902
IκBα mAb	WB, IP	100 µg	40903
Phosphorylated-IκBα mAb	WB, IP	100 µg	40904
IKKα mAb	WB	100 µg	40905
IKKβ mAb (Clone 10AG2)	WB	100 µg	40906
IKKβ mAb (Clone 10A9B6)	IP	100 µg	40907
IKKγ mAb	WB	100 µg	40908
IKKi/IKKε mAb	WB	100 µg	40950
IKKi/IKKε pAb	WB	100 µg	40957
TBK1 mAb	WB	100 µg	40909
IRAK-1 pAb	WB, IP	100 µg	40910
IRAK-2 pAb	WB	100 µg	40912
MyD88 pAb	WB	100 µg	40914
NFκB (p65) mAb	WB	100 µg	40916
RANK mAb	WB	100 µg	40917
TAB1 mAb	WB	100 µg	40918
TRAF2 mAb	WB	100 µg	40919
TRAF5 mAb	WB	100 µg	40920
TRAF6 mAb	WB	100 µg	40921
TLR2 pAb	WB	100 µg	40981
TLR3 mAb	WB	100 µg	40952
TLR6 mAb	WB, FC	100 µg	40954
TLR8 mAb	WB, FC	100 µg	40955
TLR9 mAb	WB, FC	100 µg	40956
TRAIL mAb	WB, FC, E	100 µg	40966

Technical Services

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