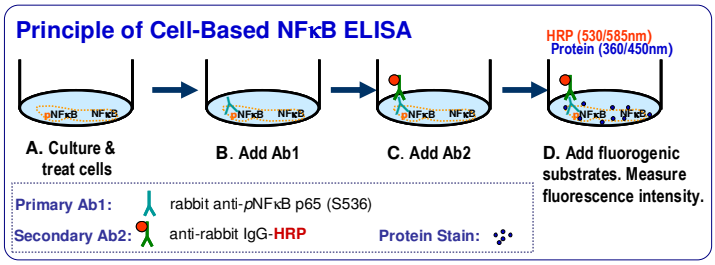
EnzyFluo™ NFκB Phosphorylation Assay Kit (ENFkB-100)

Fluorimetric Cell-Based Assay for p65/RelA (S536) Phosphorylation Status

DESCRIPTION

Nuclear factor-kappa B (NFκB) is a transcription factor that plays a central role in many physiological processes, e.g. inflammation, tumorigenesis, and apoptosis. NFκB is activated by a wide variety of stimuli, including inflammatory cytokines such as TNF-α. NFκB is a dimer composed of members of the Rel family of proteins: p65/RelA, c-Rel, RelB, NFκB1/p50, and NFκB2/p52. Phosphorylation of p65/RelA at Ser-536 results in decreased nuclear export and enhanced p65/RelA-dependent transcription.

BioAssay Systems' cell-based ELISA measures phosphorylated p65(S536) (pNFκB) in whole cells and normalizes the signal to the total protein content. The antibody was raised against a peptide corresponding to residues surrounding Ser536 of human p65/RelA and cross-reacts with murine p65/RelA. This simple and efficient assay eliminates the need for cell lysate preparation and can be used to study NFκB regulation in short-term and long-term assays.



KEY FEATURES

Simple and convenient. No cell lysis necessary, cells can be cultured for several days.

APPLICATIONS

Determination of NFκB p65 (S536) phosphorylation status in whole cells.
Evaluation of direct and indirect modulation of NFκB p65 phosphorylation.
Species tested: human, mouse.

KIT CONTENTS

10× Wash Buffer:	25 mL	**Blocking Buffer:**	25 mL
Protein Stain:	6 mL	**HRP Substrate:**	6 mL
pNFkB-Ab1:	10 µL	**HRP-Ab2**	10 µL

Storage conditions: This kit is shipped on ice. Upon delivery, store all reagents at -20°C. Shelf life of 6 months after receipt.

Precautions: reagents are for research use only. Normal precautions for laboratory reagents should be exercised while using the reagents. Please refer to material safety data sheet for detailed information.

ASSAY PROCEDURE

Important:

1. To avoid cross contamination, change pipette tips between additions of each reagent or sample. Use separate reservoirs for each reagent. Prior to the assay, dilute 10× Wash Buffer in dH_2O to prepare 250 mL 1× Wash Buffer.
2. It is recommended that assays be run in duplicate. Include in every experiment two "Sample Blank" wells with no cells but with the same volume of culture medium. The Sample Blank wells are treated the same way as with the "Sample Wells" for background fluorescence determination.

A. Culture and Treat Cells

1. Seed 100 µL containing ~10,000 adherent cells into each well of a black clear flat-bottom 96-well plate.

   Note: The cell number to be used depends on the cell line and pNFKB phosphorylation status.

2. Treat the cells as desired (e.g. drugs or cytokines).
3. Prepare formaldehyde solutions (*warning*: formaldehyde is toxic. Use chemical hood and wear appropriate gloves and eye protection):

   For adherent cells, prepare 4% formaldehyde by mixing 1.3 mL of 37% formaldehyde and 10.7 mL of 1× Wash buffer. Fix cells in each well by replacing the medium with 100 µL of 4% formaldehyde.

   For suspension cells, prepare 8% formaldehyde by mixing 2.6 mL of 37% formaldehyde and 9.4 mL of 1× Wash buffer. Centrifuge the plate at 500*g* for 15 min at 4℃ and carefully remove as much media as possible without disturbing the cell pellet. Fix the cells in each well by adding 100 µL of 8% formaldehyde to cell pellet.

   Cover the plate and incubate for 20 min at room temperature. Alternatively, the plate containing the fixed cells can be sealed and stored for up to 2 weeks at 2-8℃.
4. Remove the formaldehyde solution and wash the cells 3 times with 150 µL of 1× Wash Buffer. Each wash step should be performed for 5 min with gentle shaking. For suspension cells centrifuge the plate at 500*g* for 15 min at 4℃ before removing the formaldehyde solution. *Note, for all following wash and treatment steps, suspension cells need to be centrifuged before removal of any liquid.*
5. Prepare Quench Buffer by mixing 2.2 mL of 3% H_2O_2 and 8.8 mL of 1× Wash Buffer.

   Remove the Wash Buffer and add 100 µL of Quench Buffer to each assay well. Cover plate and incubate for 20 min at room temperature.
6. Remove the Quench Buffer and wash the cells 3 times with 150 µL of 1× Wash Buffer. Each wash step should be performed for 5 min with gentle shaking.
7. Remove the Wash Buffer, and add 100 µL of Blocking Buffer. Cover plate and incubate for 1 hr at room temperature.

B. Add Primary Antibody (Ab1)

1. Add 100 µL of Blocking Buffer to the pNFκB-Ab1 tube and mix well. Prepare enough primary antibody pNFκB-Ab1 for each well by mixing 1 µL diluted pNFκB-Ab1 and 55 µL Blocking Buffer. Unused pNFκB-Ab1 can be stored at -20°C for up to 45 days.
2. Remove the Blocking Buffer from all assay wells. Add 50 µL of pNFκB-Ab1 to all Sample and Sample Blank wells. Cover plate and incubate for 3 hrs at room temperature or overnight at 2-8℃ with gentle shaking.
3. Remove the Ab1 and wash the cells 3 times with 150 µL of 1× Wash Buffer. Each wash step should be performed for 5 min with gentle shaking.

C. Add Secondary Antibody (Ab2)

1. Immediately before use, add 100 µL of Blocking Buffer to the HRP-Ab2 and mix well. Prepare enough secondary antibody for each well, by mixing 1 µL diluted HRP-Ab2 and 55 µL Blocking Buffer. Unused HRP-Ab2 can be stored at -20°C for up to 45 days.
2. Remove Wash Buffer and add 50 µL of the HRP-Ab2 Mixture to all Sample and Sample Blank wells. Cover plate and incubate for 1 hr at room temperature with gentle shaking.

D. Detection

1. Remove the HRP-Ab2 from each well and wash the cells 5 times with 150 µL of 1× Wash Buffer. Each wash step should be performed for 5 min with gentle shaking.
2. Immediately before use, add 6 µL 3% H_2O_2 to the provided 6 mL HRP Substrate (for partial plate assay, adjust the volumes accordingly). Remove the Wash Buffer from the plate and add 50 µL of reconstituted HRP Substrate to each well. Incubate for 30 min at room temperature in the dark.
3. Add 50 µL of Protein Stain to each well and incubate for an additional 3 min at room temperature in the dark.

4. Read the plate at $\lambda_{ex/em}$ = 530/585 nm for phosphorylated NFκB (pNFκB) and at $\lambda_{ex/em}$ =360/450 nm for total protein.

CALCULATION

Calculate the mean fluorescence intensities for the Sample Blank ("BLK") wells and "SAMPLE" wells. Subtract the mean fluorescence of the Sample Blank wells from the fluorescence value of the Sample well to yield ΔF values for the phosphorylated NFκB (ΔF_{pNFkB}) at 530/585nm and for the total Protein (ΔF_{PROT}) at 360/450nm.

$$\Delta\bar{F}_{pNFkB} = \bar{F}^{SAMPLE}_{pNFkB} - \bar{F}^{BLK}_{pNFkB}; \quad \Delta\bar{F}_{Prot} = \bar{F}^{SAMPLE}_{Prot} - \bar{F}^{BLK}_{Prot}$$

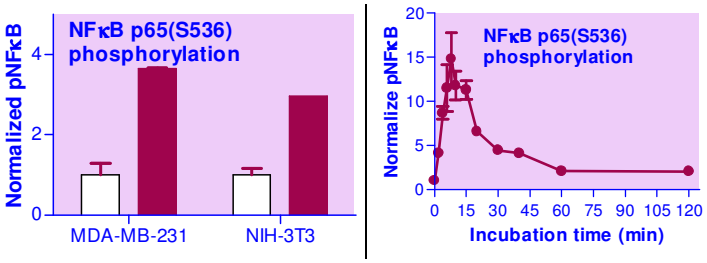
Normalized phosphorylated NFκB is calculated as,

$$\text{Normalized } \mathbf{pNF\kappa B} = \frac{\Delta\bar{F}_{pNFkB} / \Delta\bar{F}_{Prot}}{(\Delta\bar{F}_{pNFkB} / \Delta\bar{F}_{Prot})_o}$$

where $(\Delta F_{pERK} / \Delta F_{PROT})_o$ is the control reference value (e.g. time zero in kinetic studies or untreated wells in drug potency studies.)

MATERIALS REQUIRED BUT NOT PROVIDED

37% formaldehyde (Sigma, cat # F8775); 3% H_2O_2 (Sigma, cat # H1009); black cell culture 96-well plate: available separately at BioAssay System (cat# P96BCC) or at VWR (cat# 82050-748); plate sealers: available separately at BioAssay Systems (cat# AB96SL) or at Sigma (cat# A5596); deionized or distilled water; pipetting devices; cell culture incubators; centrifuge tubes; fluorescence plate reader capable of reading at $\lambda_{ex/em}$ = 530/585nm and at $\lambda_{ex/em}$ =360/450nm.



Left: Phosphorylation of NFκB p65(S536) in human breast cancer cell line MDA-MB-231 and murine NIH-3T3 fibroblast cells after stimulation with human TNF-α. ***Right***: Kinetics of NFκB p65(S536) phosphorylation in MDA-MB-231 cells after TNF-α stimulation.

LITERATURE

1. Neumann M and Naumann M (2007). Beyond IkappaBs: alternative regulation of NF-kappaB activity. FASEB J. 21(11):2642-54
2. Jiang X et al (2003). The NF-kappa B activation in lymphotoxin beta receptor signaling depends on the phosphorylation of p65 at serine 536. J Biol Chem. 278(2):919-26.
3. Gutierrez H et al (2008). Nuclear factor kappa B signaling either stimulates or inhibits neurite growth depending on the phosphorylation status of p65/RelA. J Neurosci. 28(33):8246-56.

EXAMPLE OF A 96-WELL ASSAY PLATE LAY-OUT