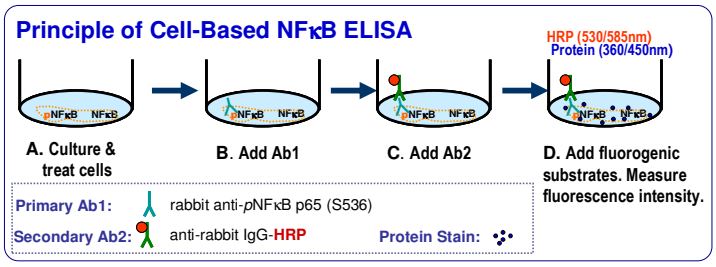
EnzyFluo™ NFκB Phosphorylation Assay Kit (ENFκB-100)

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. 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

New and improved. Total assay time reduced from the standard 21 hours to 6.5 hours (hands-on time 2.5 hrs).

Simple and convenient. Cells are directly cultured in 96-well plates. No cell lysis necessary.

Accurate and high-throughput. Protein phosphorylation is normalized to total cellular protein in the same well, greatly minimizing well-to-well variations. Can be readily automated as a high-throughput 96-well plate assay for thousands of samples per day.

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

Stock Wash Buffer:	25 mL	**Blocking Buffer:**	25 mL
Protein Stain:	6 mL	**Dye Reagent:**	120 µL
Ab1:	10 µL	**Ab2 (gR-HRP):**	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. We recommend the use of a multi-channel pipette. Use separate reservoirs for each reagent. Prior to Assay, prepare *1x Wash Buffer* by diluting Stock Wash Buffer 40-fold with dH_2O, e.g. mix 10 mL Stock Wash Buffer and 390 mL dH_2O.

   Important: reserve 6 mL *1x Wash Buffer* for Detection Step 2 below.
2. It is recommended that samples be assayed in triplicate or higher.
3. Two different blanks are necessary. For each *plate* include a Protein Blank (no cells) in triplicate. For each *sample* include a Sample Blank (cells w/ only Ab2) in triplicate. The blanks are used to determine background fluorescence for total protein and pNFκB respectively.

A. Culture and Treat Cells

1. Seed 100 µL of 1-3 × 10^4 adherent cells (or 4-10 × 10^4 suspension cells) into each well of a black 96-well culture plate.

   Add 100 µL of culture media without cells into three wells for the Protein Blank. Incubate overnight at 37°C in a cell culture incubator.

   Note: The cell number to be used depends on the cell line and NFκB phosphorylation status.
2. Treat the cells as desired (e.g. with ligands or drugs).
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. Simply 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°C and carefully remove as much media as possible without disturbing the cell pellet (repeat this for suspension cells with each wash step below). 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°C.
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 1 min with gentle shaking.
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.
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 Antibodies (Ab1)

1. Prepare 55 µL of primary antibody Ab1 Mixture for each well by mixing Ab1 into Blocking Buffer in a 1:625 dilution.
2. Remove the Wash Buffer from all assay wells. Add 50 µL of the Blocking Buffer to the Sample Blank wells and 50 µL of Ab1 Mixture to the Sample wells. Cover plate and incubate for 90 min at room temperature or overnight at 2-8°C with gentle shaking.
3. Remove the Ab1 Mixture and wash the cells 3 times with 150 µL of 1× Wash Buffer. Each wash step should be performed for 1 min with gentle shaking.

C. Add Secondary Antibodies (Ab2)

1. Prepare 55 µL of secondary antibody Ab2 Mixture for each well by mixing Ab2 into Blocking Buffer in a 1:625 dilution.
2. Remove Wash Buffer and add 50 µL of the Ab2 Mixture to all assay wells. Cover plate and incubate for 90 min at room temperature with gentle shaking.

D. Detection

1. Remove the Ab2 Mixture from each well and thoroughly wash the cells 5 times with 150 µL of 1× Wash Buffer. Each wash step should be performed for 1 min with gentle shaking.
2. Immediately before use, prepare HRP Substrate by mixing 60 µL Dye Reagent with 6 mL 1x Wash Buffer and 6 µL 3% H_2O_2 (for partial plate assay, adjust the volumes accordingly). Remove the Wash Buffer from the plate and add 50 µL of mixed 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 5 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 pNFκB fluorescence intensities at 530/585nm for the Sample Blank wells ($F_{BLK\ pNFκB}$) and Sample wells ($F_{SAMPLE\ pNFκB}$). Also calculate the mean protein fluorescence intensities at 360/450nm for the Protein Blank (no cells) well ($F_{BLK\ Prot}$) and Sample wells ($F_{SAMPLE\ Prot}$). Subtract the mean pNFκB fluorescence of the Sample Blank wells from the pNFκB fluorescence of the Sample wells to yield ΔF values for the pNFκB ($\Delta F_{pNFκB}$). Subtract the mean protein fluorescence of the Protein Blank (no cells) well from the protein fluorescence value of the Sample wells to yield ΔF values for the total protein (ΔF_{PROT}).

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

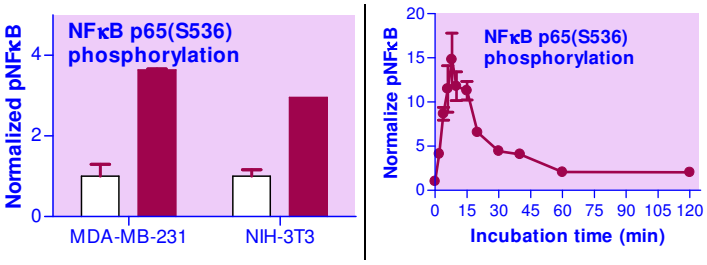
Normalized phosphorylated NFκB is calculated as,

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

where $(\Delta F_{pNFκB} / \Delta F_{Prot})_o$ 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 # 323381); 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 $\lambda_{ex/em}$ = 530/585 nm and at $\lambda_{ex/em}$ =360/450 nm.



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.