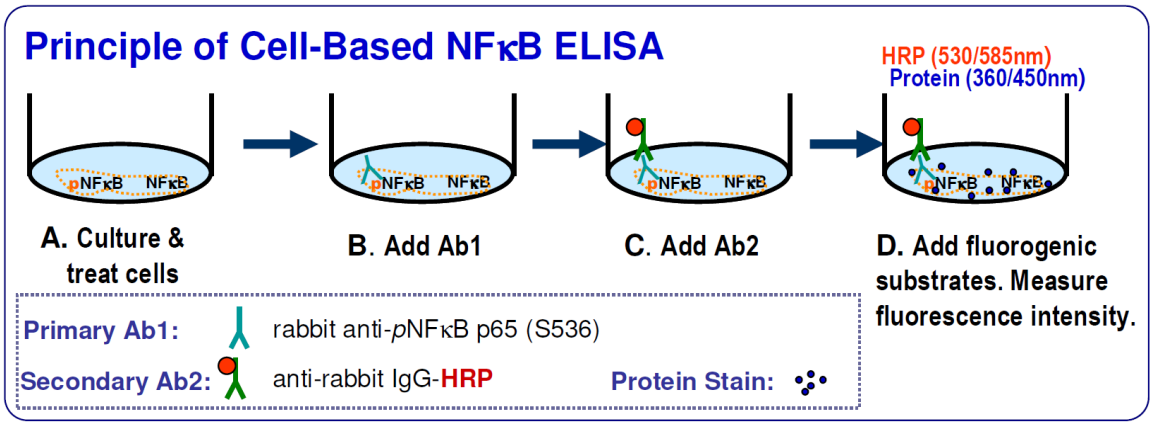
NFkB Phosphorylation Assay Kit (Fluorometric)

LS-K271-100 (100 Tests) • **Store at -20°C**

Introduction

Nuclear factor-kappa B (NFkB) is a transcription factor that plays a central role in many physiological processes, e.g. inflammation, tumorigenesis, and apoptosis. NFkB is activated by a wide variety of stimuli, including inflammatory cytokines such as TNF-α. NFkB is a dimer composed of members of the Rel family of proteins: p65/RelA, c-Rel, RelB, NFkB1/p50, and NFkB2/p52. Phosphorylation of p65/RelA at Ser-536 results in decreased nuclear export and enhanced p65/RelA-dependent transcription. This cell-based ELISA measures phosphorylated p65(S536) (pNFkB) 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 NFkB regulation in short-term and long-term assays.



Key Features

- Simple and convenient. No cell lysis necessary, cells can be cultured for several days. Total protein and pNFkB can be measured in the same sample.

Applications

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

Components

Component	K271-100
	100 Tests
10x Wash Buffer	25 mL
Protein Stain	6 mL
pNFkB-Ab1	10 µL
Blocking Buffer	25 mL
HRP Substrate	6 mL
HRP-Ab2	10 µL

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Materials Not Supplied

37% formaldehyde (Sigma, cat# F8775); 3% H_2O_2 (Sigma, cat# 323381); black cell culture 96-well plate (VWR, cat# 82050-748); plate sealers (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.

Storage

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

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, dilute 10× Wash Buffer in dH_2O to prepare 250 mL 1× Wash Buffer.
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 pNFkB respectively.

Procedure

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 NFkB 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 500g 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 pNFkB -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.

D. Add Secondary Antibodies (Ab2)

1. Prepare 55 µL of secondary antibody Ab2 Mixture for each well by mixing HRP-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.

E. 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, 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 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 NFkB (pNFkB) and at $\lambda_{ex/em}$ =360/450 nm for total protein.

Calculations

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 NFkB (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 NFkB is calculated as,

$$\text{Normalized pNF}\kappa\text{B} = \frac{\Delta\bar{F}_{p\text{NFkB}} / \Delta\bar{F}_{\text{Prot}}}{\left(\Delta\bar{F}_{p\text{NFkB}} / \Delta\bar{F}_{\text{Prot}}\right)_o}$$

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

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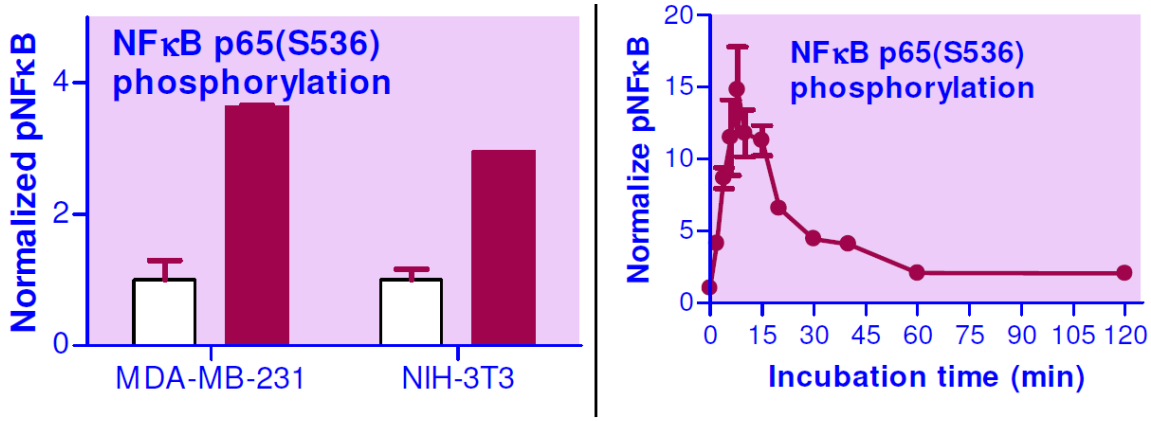
Sample Data



Left: Phosphorylation of NFkB p65(S536) in human breast cancer cell line MDAMB-231 and murine NIH-3T3 fibroblast cells after stimulation with human TNF-α.

Right: Kinetics of NFkB p65(S536) phosphorylation in MDA-MB-231 cells after TNF-α stimulation.

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