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Datasheet/User Guide

Human, Mouse and Rat Phospho-NF-KB p65 (Ser536) & Total NF-KB p65 ELISA Kit

Catalog Number: E172-008

For Cell and Tissue lysates

Introduction

The Phospho-NF-KB p65 (Ser536) and Total NF-KB p65 ELISA kit is a very rapid, convenient and sensitive assay kit that can monitor the activation or function of important biological pathways in Human, Mouse and Rat cell lysates. By determining phosphorylated NF-kB P65 protein in your experimental model system, you can verify pathway activation in your cell lysates. You can simultaneously measure numerous different cell lysates without spending excess time and effort in performing a Western Blotting analysis.

This Sandwich ELISA kit is an in vitro enzyme-linked immunosorbent assay for the measurement of Human, Mouse and Rat phospho-NF-KB p65 and total NF-KB p65. An anti-pan NF-KB p65 antibody has been coated onto a 96-well plate. Samples are pipetted into the wells and NF-KB p65 present in a sample is bound to the wells by the immobilized antibody and the wells are washed. In select wells, rabbit anti-phospho-NF-KB p65 (Ser536) antibody is added to detect phosphorylated NF-KB p65. In the remaining wells, biotinylated anti-pan-NF-KB p65 antibody is used to detect pan NF-KB p65. After washing away unbound antibody, HRP-conjugated anti-rabbit IgG or HRP-Streptavidin is pipetted into the wells. The wells are again washed, a TMB substrate solution is added to the wells and color develops in proportion to the amount of NF-KB p65 (Ser536) or pan NF-KB p65 bound. The Stop Solution changes the color from blue to yellow, and the intensity of the color is measured at 450 nm.

Storage/Shelf Life

-20°C/6 months from date of receipt

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Reagents

Component	Size / Description	Storage/Stability After Preparation
anti-Pan-NF-KB p65 Microplate	96 wells (12 x 8) coated with anti-Pan-NF-KB p65 antibody	1 month at -20°C
Positive Control	1 vial lyophilized powder from Hela cell lysate	1 week at -80°C
anti-Phospho NF-KB p65 (Ser536) Detection Antibody	1 vial of rabbit anti-Phospho NF-KB p65 (Ser536) (1 vial is enough to assay half of the microplate)	5 days at 4°C
Pan NF-KB p65 Detection Antibody	1 vial of biotinylated anti-Pan-NF-KB p65 (1 vial is enough to assay half microplate)	5 days at 4°C
HRP-conjugated anti-Rabbit IgG	1 vial (25 µl) 1000X concentrated HRP conjugated anti-rabbit IgG	Do not store and reuse
HRP-Streptavidin	1 vial (200 µl) 100X concentrated HRP-conjugated Streptavidin	Do not store and reuse
Wash Buffer	25 ml 20X concentrated solution	1 month at 4°C
Assay Diluent B	15 ml 5X concentrated diluent buffer	1 month at 4°C
Lysis Buffer	5 ml 2X cell lysis buffer	1 month at 4°C
TMB One-Step Substrate Reagent	12 ml 3,3,5,5'-tetramethylbenzidine (TMB) in buffer solution	n/a
Stop Solution	8 ml 0.2 M sulfuric acid	n/a

Additional Materials Required (but not provided)

- Microplate reader capable of measuring absorbance at 450 nm.
- ELISA plate shaker or platform rocker.
- Precision pipettes to deliver 2 µl to 1 ml volumes.
- Adjustable 1-25 ml pipettes for reagent preparation.
- 100 ml and 1 liter graduated cylinders.
- Absorbent paper.
- Distilled or deionized water.
- Log-log graph paper or computer and software for ELISA data analysis.
- Tubes to prepare standard or sample dilutions.

Sample Preparation

For the initial experiment, we recommend a serial dilution, such as a 5-fold to 50-fold dilution, for your cell lysates with prepared Assay Diluent (see Reagent Preparation step 2) before use.

Note: The fold dilution of sample used depends on the abundance of phosphorylated proteins and should be determined empirically. More of the sample can be used if signals are too weak. If signals are too strong, the sample can be diluted further.

Reagent Preparation

1. Bring all reagents and samples to room temperature (18 - 25°C) before use.
2. 5X Assay Diluent should be diluted 5-fold with deionized or distilled water before use.
3. Cell lysate buffer should be diluted 2-fold with deionized or distilled water (for cell lysate and tissue lysate). We also recommend the addition of protease and phosphatase inhibitors (not included) to the lysis buffer prior to use.
4. Preparation of Positive Control: Briefly spin the Positive Control Vial. Add 400 µl of prepared 1X Assay Diluent into Positive Control. Gently mix the powder to allow it to dissolve thoroughly. If a precipitate is seen in the solution after mixing, this can be removed by a quick centrifuge of the positive control vial and then pipetting the supernatant only for the assay.
5. If the Wash Buffer Concentrate (20X) contains visible crystals, warm to room temperature and mix gently until dissolved. Dilute 25 ml of Wash Buffer Concentrate into 475 ml deionized or distilled water to yield 500 ml of 1X Wash Buffer.
6. Preparation of Detection Antibodies:
    a. Preparation of rabbit anti-phospho-NF-KB p65 (Ser536) antibody: Briefly spin the vial of rabbit anti-phospho-NF-KB p65 (Ser536). Add 100 µl of 1X Assay Diluent into the vial to prepare a phospho detection antibody concentrate. Pipette up and down to mix gently (the concentrate can be stored at 4°C for 5 days or at -80°C for one month). The concentrate should then be diluted 55-fold with 1X Assay Diluent and used in step 5 of the Assay Procedure.
    b. Preparation of biotinylated anti-pan-NF-KB p65 antibody: Briefly spin the vial of biotinylated anti-pan-NF-KB p65. Add 100 µl of 1X Assay Diluent into the vial to prepare a detection antibody concentrate. Pipette up and down to mix gently (the concentrate can be stored at 4°C for 5 days or at -80°C for one month). The concentrate should then be diluted 55-fold with 1X Assay Diluent.

7. Preparation of HRP-conjugated concentrates:
    a. Preparation of HRP-conjugated anti-rabbit IgG: Briefly spin the vial of HRP-conjugated anti-rabbit IgG concentrate before use. HRP-conjugated anti-rabbit IgG should be diluted 1000X with 1X Assay Diluent and used in step 7 of the Assay Procedure.
    b. Preparation of HRP-Streptavidin: Briefly spin the vial of HRP-Streptavidin concentrate before use. HRP-Streptavidin should be diluted 100X with 1X Assay Diluent and used in step 7 of the Assay Procedure.

Assay Procedure

1. Bring all reagents and samples to room temperature before use. It is strongly recommended to run all positive controls and samples in at least duplicate. It is also recommended to run the positive controls in singlet for each of the pan and phospho-specific antibodies.
2. Label removable 8-well strips as appropriate for your experiment.
3. Add 100 µl of positive control (see Reagent Preparation step 4) or sample into appropriate wells. Cover the wells and incubate for 2.5 hours at room temperature or overnight at 4ºC with gentle shaking.
4. Discard the solution and wash 4 times with 1X Wash Solution. Wash by filling each well with Wash Buffer (300 µl) using a multi-channel pipette or autowasher. Complete removal of liquid at each step is essential for good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.
5. Add 100 µl of prepared 1X rabbit anti-phospho-NF-KB p65 (Ser536) antibody (see Reagent Preparation step 6a) into the wells designated to detect phosphorylated protein. Add 100 µl of prepared 1X biotinylated anti-pan-NF-KB p65 antibody (see Reagent Preparation step 6b) to the remaining wells to detect pan protein. Incubate for 1 hour at room temperature with gentle shaking.
6. Discard the solution. Repeat the wash as in step 4.
7. Add 100 µl of prepared HRP-conjugated anti-rabbit IgG solution (see Reagent Preparation step 7a) to the wells corresponding with rabbit anti-phospho-NF-KB p65 (Ser536) to detect phosphorylated protein. To the remaining wells (corresponding with biotinylated anti-pan-NF-KB p65), add 100 µl of prepared HRP-Streptavidin solution (see Reagent Preparation step 7b) to detect pan protein. Incubate for 1 hour at room temperature with gentle shaking.

8. Discard the solution. Repeat the wash as in step 4.
9. Add 100 µl of TMB One-Step Substrate Reagent to each well. Incubate for 30 minutes at room temperature in the dark with gentle shaking.
10. Add 50 µl of Stop Solution to each well. Read at 450 nm immediately.

Assay Procedure Summary

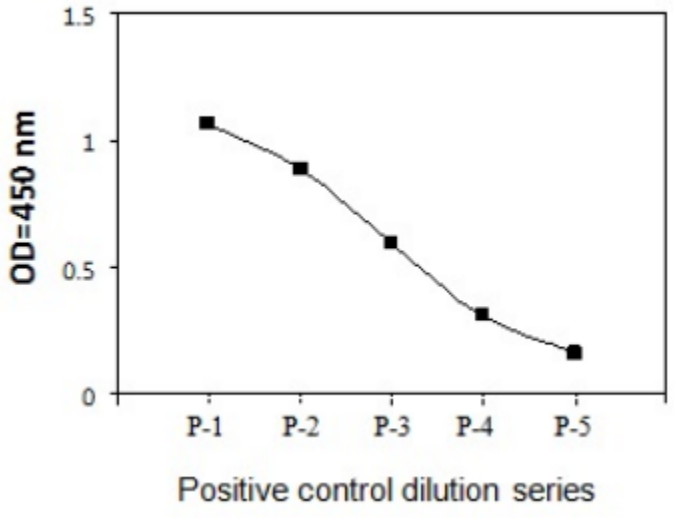
1. Prepare all reagents, samples and standards as instructed.
2. Add 100 µl positive control or sample to each well. Incubate 2.5 hours at room temperature or overnight at 4°C with gentle shaking.
3. Add 100 µl prepared detection antibody to each well. Incubate 1 hour at room temperature with gentle shaking.
4. Add 100 µl prepared HRP-conjugated solution. Incubate 1 hour at room temperature with gentle shaking.
5. Add 100 µl TMB One-Step Substrate Reagent to each well. Incubate 30 minutes at room temperature.
6. Add 50 µl Stop Solution to each well. Read at 450 nm immediately.

Typical Data

Calculate the mean absorbance for each sample. Then, subtract the average zero (blank) optical density from each sample mean and set of singlet positive controls.

Positive Control

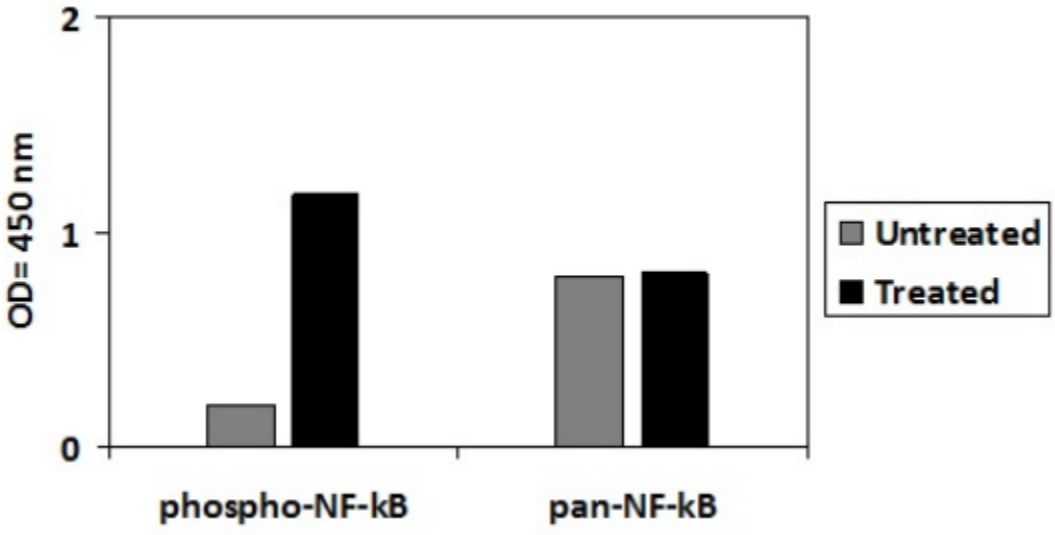
HeLa cells were treated with TNFa and Calyculin A for 5 minutes at 37°C. Cells were solubilized at 4 x 10^7 cells/ml in Cell Lysate Buffer. Serial dilutions of lysates were analyzed in this ELISA.



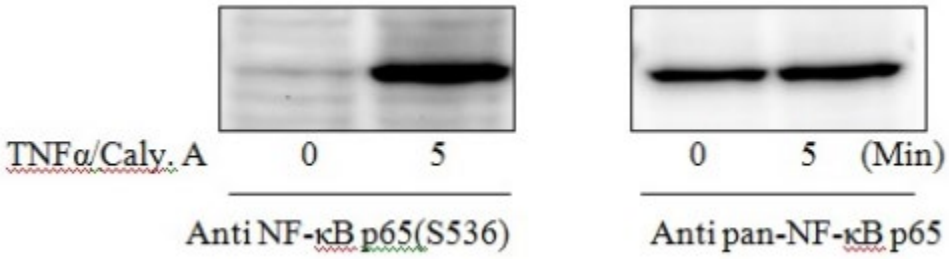
Calyculin A Stimulation of HeLa Cell Line

HeLa cells were untreated or treated with TNFa and 100nM Calyculin A for 5 minutes. Cell lysates were analyzed using this phospho ELISA and Western Blot.

ELISA



Western Blot Analysis



Specificity

The antibodies provided in this kit recognize Human NF-KB p65 phosphorylated at site Serine-536.

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Troubleshooting Guide

Problem	Potential Cause	Solution
High Signal in samples	◦Sample concentration is too high	◦Reduce sample concentration
Low Signal in samples	◦Sample concentration is too low ◦Improper preparation of detection antibody ◦Incubation times too brief ◦Inadequate reagent volumes or improper dilution	◦Increase sample concentration. Briefly spin down vials before opening. Dissolve the powder thoroughly ◦Ensure sufficient incubation time; assay procedure step 3 may be done overnight. Check pipettes and ensure correct preparation
Large CV	◦Inaccurate pipetting ◦Air bubbles in wells	◦Check pipettes ◦Carefully remove air bubbles
High Background	◦Plate is insufficiently washed ◦Contaminated wash buffer	◦Review protocol for proper wash steps. If using a plate washer, ensure all ports are unobstructed. ◦Make fresh wash buffer
Low Sensitivity	◦Improper storage of the kit ◦Stop Solution issue ◦Improper primary or secondary antibody dilution	◦Store the positive control at -70°C after reconstitution, others at 2-8 °C. Keep substrate solution protected from light ◦Add stop solution to each well before reading plate ◦Ensure correct dilution

Warranty

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