NFκB p65 (Total/Phospho) ELISA kit

Catalog Number: ADI-EKS-446A

Table of Contents

Assay Principle

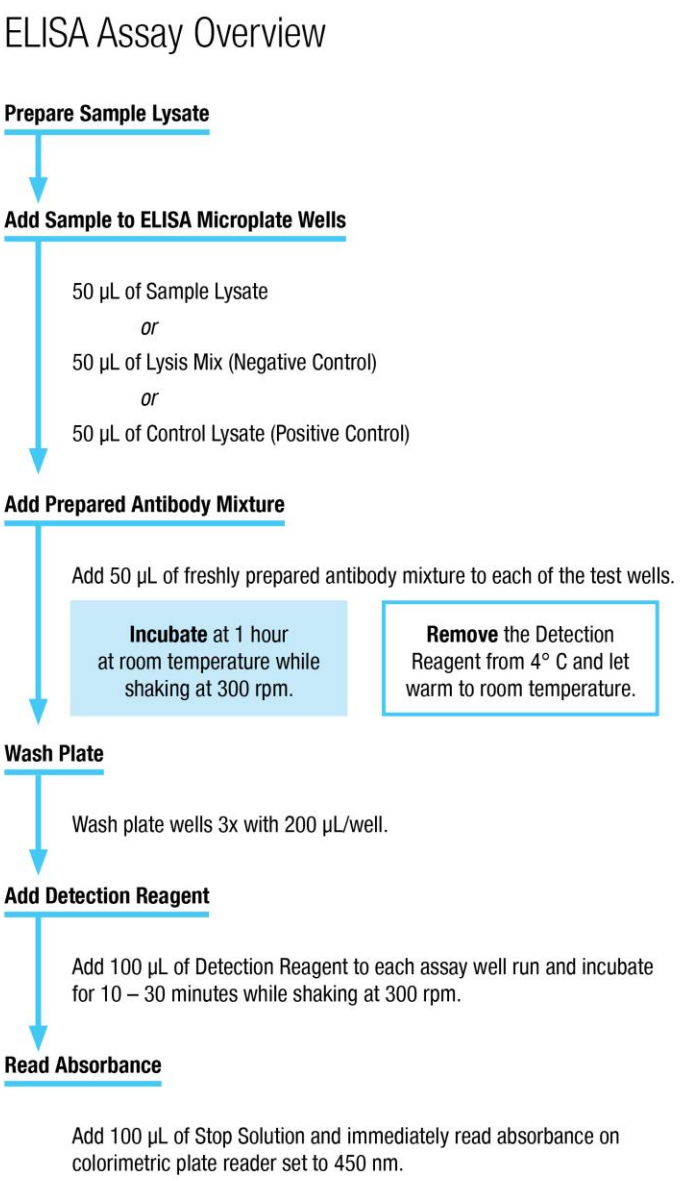
This ELISA uses the traditional sandwich ELISA format, but with a major difference. We allows for greater flexibility, ease of use, and reduced assay time by allowing the target analyte to bind to both of the two sandwich ELISA antibodies in solution as the capture antibody binds to the plate through a proprietary mechanism. This allows for both the sample and the assay reagents to be added to the assay microplate at the same time. Unbound assay reagents and nonspecific sample components are washed away just as in a traditional sandwich ELISA, while the specific analyte is detected though a colorimetric detection reagent. The whole process can take just over 60 minutes to complete.

In addition to the ease that the 1-hour/1-wash ELISA provides, it also adds a layer of flexibility not readily accessible with traditional sandwich ELISAs. As the antibodies are not precoated in the wells, several different targets can be analyzed simultaneously in the same plate in different wells. Simply add the sample lysate to the plate wells and add different antibody cocktails to the different wells. It has never been easier to analyze both total and phosphorylated MAP Kinase family members or across pathways (e.g., ERK and AKT) in the same plate.

Kit Components

PART#	COMPONENT	SIZE
80-2823	Microtiter plate	96 wells
80-2824	Cell Lysis Buffer (5X)	10 mL
80-2826	Wash Buffer (10X)	15 mL
80-2819	phospho-NFkB p65 (Ser536) Capture Antibody Reagent	1.5 mL
80-2820	phospho-NFkB p65 (Ser536) Detector Antibody Reagent	1.5 mL
80-2821	NFkB p65 (Total) Capture Antibody Reagent	1.5 mL
80-2822	NFkB p65 (Total) Detection Antibody Reagent	1.5 mL
80-2825	Enhancer Solution	1 mL
80-2827	TMB Substrate	12 mL
80-2828	Stop Solution	12 mL
80-2829	Positive Control Cell Lysate	250 µL
30-0012	Plate Sealer	1 ea

Workflow


ELISA Assay Overview
Prepare Sample Lysate
Add Sample to ELISA Microplate Wells
50 µL of Sample Lysate
or
50 µL of Lysis Mix (Negative Control)
or
50 µL of Control Lysate (Positive Control)
Add Prepared Antibody Mixture
Add 50 µL of freshly prepared antibody mixture to each of the test wells.
Incubate at 1 hour at room temperature while shaking at 300 rpm.
Remove the Detection Reagent from 4° C and let warm to room temperature.
Wash Plate
Wash plate wells 3x with 200 µL/well.
Add Detection Reagent
Add 100 µL of Detection Reagent to each assay well run and incubate for 10 – 30 minutes while shaking at 300 rpm.
Read Absorbance
Add 100 µL of Stop Solution and immediately read absorbance on colorimetric plate reader set to 450 nm.

Assay Preparation

Note: Avoid vortexing the capture antibody or the detector antibody reagent, as vigorous mixing can damage some antibodies.

REAGENT	INSTRUCTIONS
Cell Lysis Mix (5X)	Prepare Cell Lysis Mix (5X) by diluting Enhancer Solution 10-fold in Cell Lysis Mix (5X) (e.g., mix 900 µL Cell Lysis Buffer (5X) and 100 µL Enhancer Solution). Cell Lysis Mix (5X) is used to directly lyse cells in cell culture medium. Prepare immediately prior to use. Discard unused Cell Lysis Mix (5X). Control Cell Lysate can be used to give an indication of the expected signal range for a given assay.
Enhancer Solution	Supplied as a concentrate. Precipitation will occur during storage at 4°C, which is normal. To redissolve, warm to 37°C and mix by inversion prior to use.
Cell Lysis Mix (1X)	Prepare immediately prior to use. Discard unused Cell Lysis Mix (1X). Cell Lysis Mix (1X) is used to directly lyse cells when the medium has been aspirated.

	Prepare Cell Lysis Mix (1X) by diluting Cell Lysis Mix (5X) prepared (as instructed above) 5-fold in ddH_2O (e.g., mix 200 µL 5X Lysis Buffer and 800 µL ddH_2O).
Capture Antibody Reagent + Detection Antibody Reagent	Prior to each experiment, prepare by adding an equal volume of Capture Antibody Reagent and Detection Antibody Reagent, and mixing by inversion. Prepare enough to use 50 µL/well (e.g., for a single 8-well strip, prepare 400 µL Antibody Mix).
Positive Control Cell Lysate	The control is supplied lyophilized, and should be reconstituted with 250 µL of ddH_2O. If required, control lysates can be further diluted with Cell Lysis Buffer Mix (1X). When reconstituted, aliquot and store at –20°C for up to 1 month.
Wash Buffer (10X)	Dilute Wash Buffer (10X) to 1X with ddH_2O (e.g., mix 1 mL of Wash Buffer (10X) and 9 mL of water). Prepare at least 600 µL of Wash Buffer (1X) per well to allow for the 3 washes.
Detection Reagent	Allow Detection Reagent to warm to room temperature prior to use for optimal assay performance.

Assay Protocols

Method 1: Prior sample preparation protocol

Use this method when samples are grown, treated/stimulated, and lysed prior to the assay.

Sample preparation

1. For adherent cultured cells, remove any media from the cells and gently wash cells with PBS.
    a. For cells cultured in 96-well microplates, lyse the cells with 100 µL of freshly prepared Cell Lysis Buffer Mix (1X).

       Lysis volume should be adjusted depending on the desired lysate concentration. Lysates in the range of 0.1–0 .5 mg/mL protein are usually sufficient. However, preparing more concentrated lysates can help with the detection of low abundance analytes.

    b. Shake cells (~300 rpm) at room temp for 10 minutes.
2. For nonadherent cells, centrifuge the cells, gently remove the media while leaving the cells undisturbed. It is recommended, but not required, to wash the cells in PBS. Resuspend the cell pellet at an appropriate density in HBSS containing 5% FBS. A cell density that yields cellular lysate at a protein concentration of 0.1–0.5 mg/mL is suitable for many cell lines.

   Alternatively, resuspend cells in cell culture medium if necessary for the cells.

3. Return cells to a 37°C incubator for 1–2 hours. For certain pathways, this can allow handling-mediated pathway activation to subside. This step is optional, and depends on the activation status of your cells following resuspension.

4. At the completion of the treatment, lyse cells with 20% final volume of Cell Lysis Mix (5X), with shaking (~300 rpm) at room temp for 10 minutes (e.g., for 40 µL of cells, use 10 µL of Cell Lysis Mix (5X).
5. Alternatively cells can be harvested by centrifugation and lysed with Cell Lysis Mix (1X).

Assay Protocol

Remove Detection Reagent from refrigerator and allow to equilibrate to room temperature.

1. Determine and remove the desired number of microplate strips needed for the experiment including the Positive Control Cell Lysate and negative control. Return unused microplate well strips to storage pouch with desiccant and seal.
2. Add negative control, positive control, and sample lysate to assay wells
    a. Add 50 µL/well of prepared sample lysate (as described above) to be tested to each of the ELISA assay wells.
    b. Add 50 µL/well of Cell Lysis Mix (1X) (negative control) and 50 µL/well of Positive Control Cell Lysate to separate wells for assay controls. The negative control can also act as the blank when the plate is read.
3. Add 50 µL/well of prepared Antibody Mix to each of the testing wells. Cover the microplate with adhesive seal and incubate for 1 hour at room temperature on a microplate shaker (~300 rpm).
4. Wash wells with 200 µL/well of Wash Buffer (1X) (repeat 3 times).
5. After final wash, completely remove any remaining wash solution from wells by inverting on a paper towel.
6. Add 100 µL of the Detection Reagent to each of the wells.
7. Incubate for 10–30 minutes with shaking at 300 rpm. Watch color development as high abundance targets/samples will take significantly less time than lower abundant targets.

8. 6. Stop the reaction by adding 100 µL of Stop Solution to each well.
9. 7. Read the plate by measuring the absorbance of the samples using a colorimetric (spectrophotometric) plate reader set at 450 nm.

Plate should be read within 1 hour of adding the Stop Solution.

Method 2: All-in-one-well protocol

This protocol allows for the preparation of cellular lysates and their subsequent analysis in the assay microplate. This protocol avoids lysate transfer steps.

Sample preparation

1. Harvest cells by centrifugation, and resuspend at an appropriate density in HBSS containing 5% FBS. A cell density that yields 10,000–25,000 cells/well (in 20 µL volume) is suitable for the analysis of many cell lines.

   Alternatively, resuspend cells in cell culture medium if necessary for the cells, using Method 1.
2. Determine the desired number of assay plate strips needed. Remove unused strips from frame and return to storage pouch and with desiccant seal.
3. Rinse wells for use in the assay 2 times with sterile H_2O to remove preservatives.
4. Add 20 µL cells/well to the assay microplate.
5. Incubate cells at 37°C for 1–2 hours. For certain pathways, this can allow handling-mediated pathway activation to subside. This step is optional, and depends on the activation status of your cells following resuspension.
6. Add 20 µL treatment/well to cells (e.g., 2X agonists and/or antagonists) for the desired time period. The final volume in wells prior to lysis should be 40 µL.
7. Directly add 10 µL Cell Lysis Mix (5X) (not prediluted) to each of the wells after desired treatment time with shaking (~300 rpm) at room temperature for 10 minutes. This results in cell lysis.

Assay Protocol

Remove Detection Reagent from +4°C and allow to equilibrate to room temperature.

1. Add 50 µL/well of Cell Lysis Mix (1X) (negative control) and 50 µL/ well Positive Control Cell Lysate (positive control) to separate assay wells for controls.
2. Add 50 µL/well of prepared Antibody Cocktail to each of the testing wells. Cover the microplate with adhesive seal and incubate for 1 hour at room temperature on a microplate shaker (~300 rpm).
3. Wash wells with 200 µL/well of Wash Buffer (1X) (repeat 3 times). After final wash, completely remove any remaining wash solution from wells.
4. Add 100 µL of the Detection Reagent to each of the assay wells. Incubate for 10–30 minutes with shaking at 300 rpm. Watch color development as high abundance targets/samples will take significantly less time than lower abundant targets.
5. Stop the reaction by adding 100 µL of Stop Solution to each well.
6. Read the plate by measuring the absorbance of the samples using a colorimetric (spectrophotometric) plate reader set at 450 nm. Plate should be read within 1 hour of adding the stop solution.

Data Analysis

- To analyze the data, calculate the averaged counts for untreated and treated cells. It is recommended to run the assay at least in duplicate wells (n = 2) to calculate a response, but triplicate is strongly advised.
- Dose response and dose inhibition curves can be fitted to 4 parameter nonlinear regression equations. These types of regression analyses output key parameters such as EC50 (or IC50), Min and Max signals, and Hillslope factors.
- Ensure that samples readings are within the linear range of the assay. This can vary based on reader performance, and analyte concentration. If a lysate sample generates a signal outside the linear range, the lysate samples should be diluted with Cell Lysis Mix (1X) and re-assayed.

Procedure Limitations

1. This ELISA kit is for Research Use Only. Not for use in diagnostic procedures.
2. Do not use the kit reagents beyond the expiry stated on the label.
3. Variations in general operator-related procedures, such as pipetting, washing, and incubation times, can cause variation in the final signal.
4. The assay is designed to work for the detection of endogenous cellular proteins across a wide variety of cell lines. However, until each cell line in particular is tested, the possibility of the presence of interfering factors cannot be excluded.
5. Users should ensure that their cell line has measurable levels of the pathway of interest. Expression levels of signaling proteins in different cell types vary widely.

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