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Datasheet/User Guide

Human, Mouse and Rat Phospho-AKT (Ser473) & Total AKT ELISA Kit

Catalog Number: E172-000

For Cell and Tissue lysates

Introduction

The Phospho-AKT (Ser473) and Total AKT 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 AKT 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-AKT and total AKT. An anti-pan AKT antibody has been coated onto a 96-well plate. Samples are pipetted into the wells and AKT present in a sample is bound to the wells by the immobilized antibody and the wells are washed. In select wells, rabbit anti-phospho-Akt (Ser473) antibody is added to detect phosphorylated AKT. In the remaining wells, rabbit anti-pan-Akt antibody is used to detect pan AKT. After washing away unbound antibody, HRP-conjugated anti-rabbit IgG 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 AKT (Ser473) or pan AKT 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-AKT Microplate	96 wells (12 x 8) coated with anti-Pan-AKT antibody	1 month at -20°C
Positive Control	1 vial lyophilized powder from NIH 3T3 cell lysate	1 week at -80°C
anti-Phospo AKT (Ser473) Detection Antibody	1 vial of rabbit anti-phospho-Akt (Ser473) (1 vial is enough to assay half of the microplate)	5 days at 4°C
Pan AKT Detection Antibody	1 vial of rabbit anti-Pan-Akt (1 vial is enough to assay half microplate)	5 days at 4°C
HRP-conjugated anti-Rabbit IgG	1 vial (25 µl) of 500X concentrated HRP conjugated anti-rabbit IgG	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

Cell lysates - Rinse cells with PBS, making sure to remove any remaining PBS before adding the lysis buffer. Solubilize cells at 4 x 10 cells/ml in 1x Lysis Buffer (we recommend adding protease and phosphatase inhibitors to lysis buffer prior to sample preparation). Pipette up and down to resuspend and incubate the lysates with shaking at 2 - 8°C for 30 minutes. Microcentrifuge at 13,000 rpm for 10 minutes at 2 - 8°C and transfer the supernatants into a clean test tube. Lysates should be used immediately or aliquoted and stored at -70°C. Avoid repeated freeze-thaw cycles. Thawed lysates should be kept on ice prior to use.

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 before use.
2. Assay Diluent B 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 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-Akt (Ser473) antibody: Briefly spin the vial of rabbit anti-phospho-Akt (Ser473). 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 4ºC for 5 days or at -80ºC 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 rabbit anti-pan-Akt antibody: Briefly spin the vial of rabbit anti-pan-Akt. 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 4ºC for 5 days or at -80ºC for one month). The concentrate should then be diluted 55-fold with 1X Assay Diluent.
7. 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 500X 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-Akt (Ser473) antibody (see Reagent Preparation step 6a) into the wells designated to detect phosphorylated protein. Add 100 µl of prepared 1X rabbit anti-Pan-Akt 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 7) to each well. 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

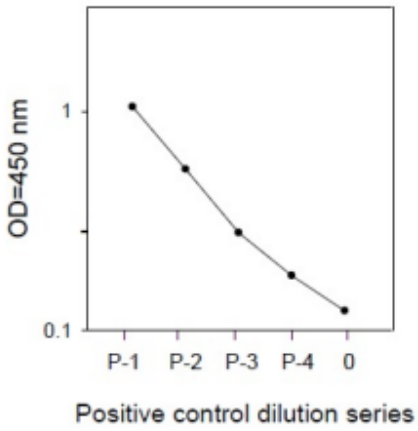
1. Prepare all reagents, samples and standards as instructed.
2. Add 100 µl positive control or sample to each well. Incubate 2.5 hours at room temperature or overnight at 4°C with gentle shaking.
3. Add 100 µl prepared detection antibody to each well. Incubate 1 hour at room temperature with gentle 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.

A. Positive Control

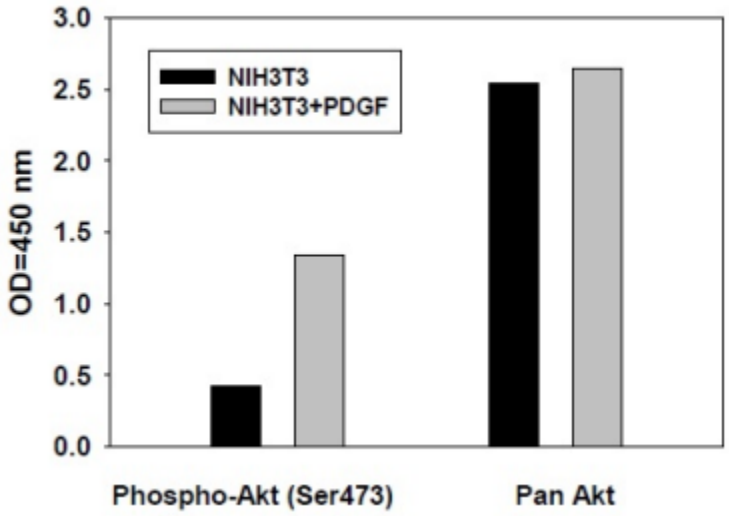
NIH3T3 cells were treated with PDGFBB for 10 min. Cells were solubilized at 4 x 10 7 cells/ml in Cell Lysate Buffer. Serial dilutions of lysates were analyzed in this ELISA.



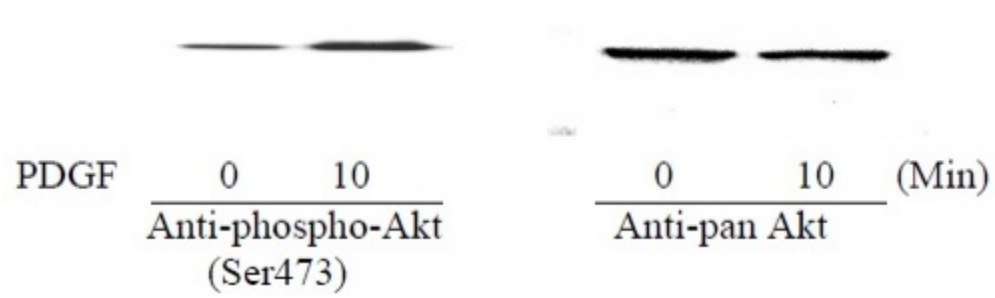
B. Recombinant Human PDGF Stimulation of NIH 3T3 Cells

NIH3T3 cells were untreated or treated with recombinant human PDGF for 10 min. Cell lysates were analyzed using this phospho ELISA and Western Blot.

ELISA



Western Blot Analysis



Specificity

The antibodies provided in this kit recognize human, mouse and rat Phospho-Akt (pSer473).

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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 -70ºC after reconstitution, others at 2-8 ºC. Keep substrate solution protected from light ◦Add stop solution to each well before reading plate ◦Ensure correct dilution

Warranty

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