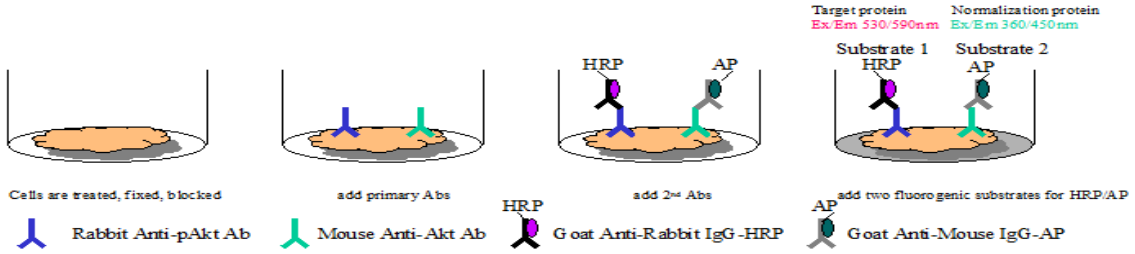
Phospho-Akt (Ser473) Detection Kit A (Cell-based)

(Cat# CEF010-Akt; 100 Fluorimetric assays; store kit at -20°C)

Introduction

Akt plays an essential role in cell survival, growth, migration, proliferation, polarity, and metabolism (lipid and glucose). Akt is activated through receptor tyrosine kinase pathways. Thr308 and Ser473 phosphorylation are necessary and sufficient for the full activation of Akt. Quantification of intracellular proteins and phosphorylation events is extremely important for biomedical research. The plate-based immunoassay has become a popular alternative method for rapid protein detection.

The ABS_Bio™ Phospho-Akt (Ser473) Fluorimetric Detection Kit provides a Cell-Based ELISA format allows two target cellular proteins, or events, to be analyzed simultaneously in the same well. Cells are grown in 96-well plates and treated with the appropriate conditions, such as inhibitors or ligand stimulation. The cells are then fixed and permeabilized in the wells. This is followed by incubation with two primary antibodies derived from different species: a phospho-specific antibody (pAkt-Ser473) and a normalization antibody (Akt) that recognizes the total protein regardless of its phosphorylation status. Species-specific secondary antibodies labeled with horseradish peroxidase (HRP) and alkaline phosphatase (AP), and spectrally distinct fluorogenic substrates for each enzyme, are used for detection. The fluorescence of the phosphorylated protein is normalized to that of the total protein in each well to correct for well-to-well variations. The kit provides reagents sufficient for 100 assays. Cell-Based ELISAs are amenable to high-throughput applications and may prove a valuable addition to kinase inhibitor screening strategies.



Kit Components

Rabbit Anti-pAkt(Ser473) Ab1:	10 µL	Goat Anti-rabbit IgG-HRP Ab2:	10 µL
Mouse Anti-Akt(pan) Ab1:	10 µL	Goat Anti-mouse IgG-AP Ab2:	10 µL
HRP substrate:	6 mL	AP substrate:	6 mL
Wash Buffer (20x):	25 mL	Blocking Buffer:	25 mL

Storage and Handling: shipping on icepack. Store all of the components at -20°C.
Required materials (not provided): 37% formaldehyde (sigma, #F8775) & 3% H_2O_2 (sigma, #323381), Tissue culture Black clear-bottom Corning 96 well plate (Sigma, cat# CLS3603).

Protocol

Reagent Preparation

1. Wash Buffer: 20x dilute Wash Buffer in dH_2O to prepare 1x Wash Buffer.
2. Prepare Fixing Buffer: Add 0.65 mL 37% formaldehyde to 5.35 mL of 1x Wash Buffer.
3. Prepare Quenching Buffer: Add 1.3 mL 3% H_2O_2 into 5.7 mL of 1x Washer Buffer.
4. Primary Antibody Mixture: Immediately before use, add 10 µL of the each primary antibodies (pAkt/Akt) in 5.5 mL of Blocking Buffer for one plate (for partial plate assay, adjust the volumes accordingly).
5. Secondary Antibody Mixture: Immediately before use, add 10 µL of the each secondary antibodies (HRP/AP) in 5.5 mL of Blocking Buffer for one plate (for partial plate assay, adjust the volumes accordingly).
6. HRP substrate solution: Immediately before use, add 6 µL 3% H_2O_2 to 6 mL HRP substrate bottle (for partial plate assay, adjust the volumes accordingly).

A. Culture, Stimulate, Fix, and Block Cells

1. Seed 100 µL of 10,000 - 20,000 adherent cells into each well of the black clear-bottom 96-well microplate and incubate overnight at 37° C in a cell culture incubator. It is recommended that samples be assayed in triplicate, cell and reagents control wells are necessary.

Note: The cell number used is dependent upon the cell line and the relative amount of target protein. Optimal cell numbers should be determined by each laboratory for each assay.

2. Grow and treat the cells as desired.

3. Fix cells by replacing medium with 50 µL of fixing solution. Add the plate covers and incubate for 20 minutes in a fume hood at room temperature.
4. Remove Fixing solution and wash the cells 3 times with 150 µL of Wash Buffer.
Each wash step should be performed for 5 minutes with gentle shaking.
5. Remove Wash Buffer and add 50 µL prepared Quenching Buffer to each well and incubate at room temperature for 10 minutes.
5. Remove Quenching Buffer and wash 3 times with wash buffer.
6. Remove Wash Buffer and add 100 µL of Blocking Buffer. Add the plate covers and incubate for 60 minutes at room temperature.

B. Binding of Primary and Secondary Antibodies

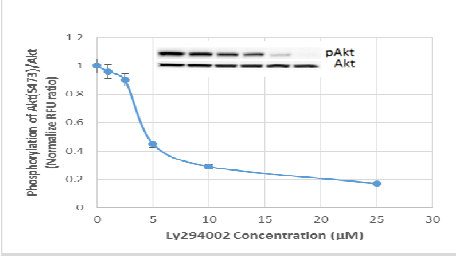
1. Remove Blocking Buffer and add 50 µL of the Primary Antibody Mixture to each well. Cover with plate sealer and incubate at room temperature for 1-2 hours with gentle shaking.
Note: Depending on the experimental design, some wells may be incubated with Primary Antibody Mixture and some with only the Blocking Buffer as the negative controls (secondary antibody only).
2. Remove the Primary Antibody Mixture and wash the cells 3 times with 150µL of Wash Buffer.
3. Add 50 µL of the Secondary Antibody Mixture to each well. Cover with plate sealer and incubate for 1 hour with gentle shaking at room temperature.

C. Fluorogenic Detection

1. Remove the Secondary Antibody Mixture from each well and wash the cells 5 times with 150 µL of Wash Buffer.
2. Remove the Wash Buffer from the plate and add 50 µL of HRP Substrate to each well. Incubate for 20 minutes at room temperature. Protect the plates from direct light. A pink or rosy color should develop in the wells.
3. Add 50 µL of AP Substrate to each well.
4. Read the plates using a fluorescence plate reader with excitation at 530 nm and emission at 590 nm for pAkt; then read with excitation at 360 nm and emission at 450 nm for total Akt.

D. Calculation.

Control wells with no primary antibody (secondary antibody only) should be included in each experiment. The fluorescence (RFUs) from these wells is the background fluorescence and is subtracted from all sample wells. If normalization is desired, the fluorescence at 590 nm derived from the target protein pAkt in each well is normalized to the fluorescence at 450 nm derived from normalization protein total Akt. The normalized duplicate readings for each sample are then averaged.



Dose response of Akt phosphorylation inhibition. Human cancer cell MDA-MB-231 were seeded at 1.5 x10^4 cells/well in 96-well plate cultured overnight and treated for 1 h with the PI3-kinase inhibitor LY294002 at the indicated doses. Phosphorylation of Akt on Ser473 was determined and normalized to total Akt in the same well using Phospho-Akt (Ser473) Detection Kit (**Cat# CEF010-Akt**). Values represent the mean ± SD. Western blot analysis of total and phosphorylation Akt using the antibodies supplied in this kit is shown for comparison (insert of Fig).

References

1. Yu K. et al. Cancer Biology & Therapy. 7:307-15 (2008).
2. He X. et al. Cancer Letters. 301:168-76 (2011).
3. Samadi N. et al. Oncogene. 28:1028-39 (2009).

Related Products:

Phospho-AMPK Detection Kit A (CEF050-AMPK)
Phospho-EGFR Detection Kit A (CEF020-EGFR)
Phospho-ERK1/2 Detection Kit A (CEF030-ERK)
Phospho-Stat Detection Kit A (CEF060-Stat)
Phospho-Akt(Ser473) Detection Kit B (CEC010-Akt)
Phospho-Akt(Thr308) Detection Kit A (CEF011-Akt)
Phospho-p38 MAPK Detection Kit A(CEF100-MAPK)
Phospho-NFkB p65 Detection Kit A (CEF090-NFkB)
Phospho-SMAD1 Detection Kit A (CEF120-SMAD1)
Phospho-CREB Detection Kit A (CEF140-CREB)
Phospho-Akt(Ser473) Detection Kit C (SEF010-Akt)
Phospho-Akt(Ser473) Detection Kit D (SEC010-Akt)