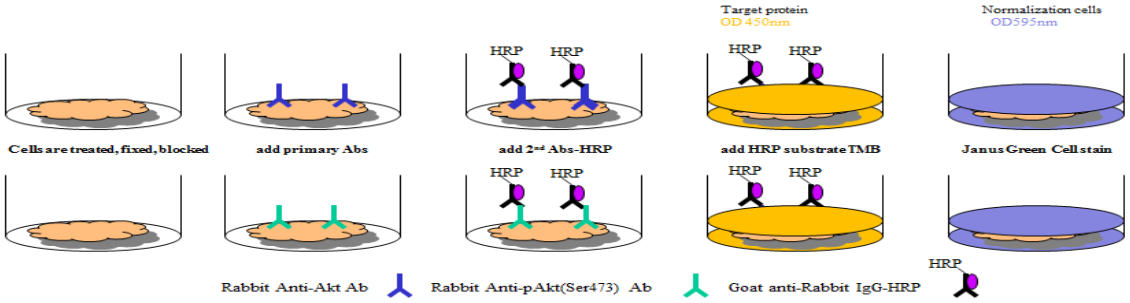
Phospho-Akt (Ser473) Detection Kit B (Cell-based)

(Cat# CEC010-Akt; 100 Colorimetric assays; store kit at -20+4℃)

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™ Cell-Based Phospho-Akt (Ser473) Colorimetric Detection Kit is a very rapid, convenient and sensitive assay kit enable simultaneous detection of both total and phospho-Akt (Ser473) in cells, eliminating the need for lysate and protein preparation. 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 parallel incubation with two primary antibodies: a phospho-specific antibody (pAkt-Ser473) and an anti-Akt antibody, after washing away unbound antibody, HRP-conjugated secondary antibody was added, after washing step, the TMB substrate was used for color developing. Therefor the relative amount of phospho-specific and total Akt protein are determined using target-specific primary antibody and HRP conjugated secondary antibody detection agent. Following the colorimetric measurement of HRP activity, the Janus Green cell staining is used for counting cell number to normalize the well-to-well different. 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
Rabbit Anti-Akt(pan) Ab1:	10 µL	TMB solution:	6 mL
Janus Green stain solution:	6 mL	Blocking Buffer:	25 mL
Wash Buffer (20x):	25 mL	Stop solution:	12 mL

Storage and Handling: shipping on icepack. Store all of the antibodies at -20℃, other components at 4℃. Required materials (not provided): 37% formaldehyde (sigma, #F8775) & 3% H_2O_2 (sigma, #323381), Tissue culture 96 well plate (Sigma, cat# CLS3628).

Protocol

Reagents 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 primary antibodies (pAkt (Ser473) or 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 secondary antibodies (HRP) in 5.5 mL of Blocking Buffer for one plate (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 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 culture 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

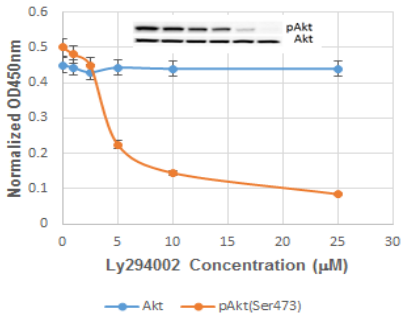
1. Remove Blocking Buffer and add 50 µL of the Primary Antibody Mixture to each well. Cover with plate sealer and incubate at room temperature for 2 hours with gentle shaking or overnight at 4℃.
Note: Depending on the experimental design, some wells may be incubated with Primary Antibody Mixture and 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 150 µL 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. Colorimetric 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 TMB solution to each well. Incubate for 10-20 minutes. Protect the plates from direct light.
3. Stop the reaction by add 50 µL of Stop solution to each well when the blue color has been achieved.
4. Read the plates using a plate reader at 450 nm.
5. Cell staining (optional): Remove the reaction mixture and wash plate twice with 150 µL of dH_2O.
6. Remove dH_2O and add 50 µL of Janus Green cell stain solution. Incubate for 5 minutes at RT.
7. Remove stain solution and wash 3-5 times with 150 µL of dH_2O until all excess stain is removed.
8. Remove dH_2O and add 50 µL of stop solution, incubate for 10 minutes with shaking.
9. Read the plates using a plate reader at 595 nm.

D. Calculation.

Control wells with no primary antibody (secondary antibody only) should be included in each experiment. The OD450nm from these wells is the background value and is subtracted from all sample wells. If normalization is desired, the OD450 nm derived from the target protein pAkt (or Akt) in each well is normalized to the OD595 nm derived from cell numbers. The normalized duplicate readings for each sample are then averaged. For assessing target protein (Ser473 or total Akt) modification with treatment, calculate the fold change as a ration of the treated and no treated modified protein OD450nm values.



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(Ser473) and total Akt were determined and normalized to cell numbers in the same well using Phospho-Akt (Ser473) Detection Kit (**Cat# CEC010-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-Akt(Thr308) Detection Kit A (CEF011-Akt)	**Phospho-Akt(Ser473) Detection Kit A (CEF010-Akt)**
Phospho-Akt(Ser473) Detection Kit C (SEF010-Akt)	**Phospho-Akt(Ser473) Detection Kit D (SEC010-Akt)**