ab126580

Total Akt-1 and phospho S473 Flow Cytometry Panel

Instructions for Use

For measuring the total and phosphorylated levels (S473) of Akt-1 protein in human and mouse cell lines using flow cytometry.

This product is for research use only and is not intended for diagnostic use.

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1. Introduction

**Principle:** ab126580 is a panel of antibodies that measure the protein levels of Akt-1 and phosphorylation of Akt-1 at serine residue 473. The assay combines the power of single cell analysis obtained with flow cytometry and the specificity of antibody-based immunostaining to quantify protein and phosphorylation levels in cultured cells. Cells are harvested and fixed/permeabilized in suspension, targets of interest are detected indirectly with highly specific, well-characterized monoclonal antibodies that are then labeled with fluorescent antibodies.

**Background:**

Akt is a serine, threonine protein kinase critical in cellular metabolism, glucose uptake, protein synthesis, cell proliferation, growth, apoptosis, survival, angiogenesis, migration and invasion. It acts downstream of the phosphatidylinositol 3 kinase (PI3K) and it mediates the effects of several growth factors such as platelet-derived growth factor, epidermal growth factor and insulin growth factor. It is activated by phosphorylation at Ser-473, Thr-308 and Tyr-474 and when active it phosphorylates transcription factors (FOXO1), kinases (GSK-3, Raf-1, ASK, Chk1) and other signaling proteins (Bad, MDM2). There are three Akt isoforms (Akt1, Akt2 and Akt3) which share 80% sequence identity also known as PKBo,
PKBβ and PKBγ. Akt has been shown to have a role in metabolism, apoptosis and proliferation and therefore it has been proposed to be the candidate “Warburg Kinase”.

Akt is the most frequently activated oncoprotein in human cancers. As a target for cancer therapy it has gained interest in highly chemoresistant tumors. Defects in AKT1 are a cause of susceptibility to breast cancer (BC) [MIM:114480], colorectal cancer (CRC) [MIM:114500] and with susceptibility to ovarian cancer [MIM:604370]. Akt is also intensively studied in in-vitro and in-vivo models of type II diabetes. Akt also has been proposed to play a role in diseases mediated by macrophage innate immunity such as rheumatoid arthritis, atherosclerosis, diabetes, obesity, and osteoporosis and hence could become a future therapeutic target in this areas..

This flow cytometry panel generates quantitative data with specificity similar to Western blotting, but with much greater quantitative precision. This method rapidly fixes the cells in-situ, stabilizing the in-vivo levels of proteins, and thus essentially eliminates changes during sample handling, such as preparation of protein extracts.
2. Assay Summary

Harvest cells as a single cell suspension.

Fix cells with 4% paraformaldehyde for 15 minutes and pellet.

Permeabilize the cells with cold methanol. Incubate for 30 minutes at -20°C then pellet and wash.

Block cells with 1X blocking buffer for 15 minutes and pellet

Add primary antibodies diluted in 1X blocking buffer, incubate for 1 hour and wash.

Add secondary antibodies diluted in 1X blocking buffer, incubate for 1 hour, wash and read with flow cytometer.
3. Kit Contents

- 10X Phosphate Buffered Saline (PBS) 100 mL
- 400X Tween – 20 (20% solution) 4 mL
- Blocking Solution 10 mL
- 50X Antibody Cocktail 110 µL
  (Mouse Anti-Akt1 and Rabbit Anti-Akt1 phoso S473 Antibody)

4. Storage and Handling

Upon receipt spin down the contents of the antibody cocktail vial. Store all components upright at 4°C. This kit is stable for at least 6 months from receipt.

5. Additional Materials Required

- Flow cytometer
- Microfuge
- Aspirator
- 20% paraformaldehyde
• Methanol
• Goat anti-mouse detection antibodies labeled with fluorochrome(s) suitable for available flow cytometer (e.g. Abcam, GAM IgG – H&L DyLight® 488 Cat# ab96871)
• Goat anti-rabbit detection antibodies labeled with fluorochrome(s) suitable for available flow cytometer (e.g. Abcam, GAR IgG – H&L DyLight® 650 Cat#ab96898 or ab96902)
• Nanopure water or equivalent
• Optional for high throughput manipulations: Filter 96-well plates with pore sizes smaller than the cell line in use

6. Preparation of Reagents

6.1 Cool methanol to -20°C.
6.2 Prepare 1X PBS by diluting 100 mL of 10X PBS in 900 mL Nanopure water or equivalent. Mix well. Store at room temperature. Cool a small portion for sample preparation on ice.
6.3 Immediately prior to use prepare sufficient 1X Wash Buffer = 0.05% Tween-20, 1% Blocking solution in PBS. Any excess should be stored at 4°C for no longer than 24 hours.
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6.4 Immediately prior to use prepare 1X blocking buffer = 0.05% Tween-20, 10% Blocking Solution in PBS. Any excess should be stored at 4C for no more than 24 hours.

7. Sample Preparation

7.1 Cell culture and treatment conditions are dictated by the experiment at hand. As a general guideline, it is advisable to analyze at least 10,000 events (cells) on the flow cytometer per sample/data point. Therefore at least four to ten times that many cells should be collected per data point to ensure sufficient material at the end of the staining.

7.2 For suspension cells, generate a single cell solution by pipetting up and down

7.3 For adherent cells, fully dissociate cells (e.g. trypsin) into single cell suspension. Passaging the cell line the day before the experiment onto a fresh culture plate may help improve single cell dissociation on the day of the experiment.

7.4 Maintain cells resuspended in the culture treatment media, at approximately $1 \times 10^6$ cells per mL.
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7.5 Overlay 20% paraformaldehyde on the cell suspension so that the final concentration is 4%, gently mix by inverting the tube and incubate at room temperature for 15 minutes.

7.6 Pellet cells at 350 - 500 x g for 5 minutes (depending on cell size) and decant supernatant. **Note:** *paraformaldehyde is toxic: handle with care and dispose of according to local requirements*

7.7 Dislodge the pellet by gently tapping the bottom of the tube and resuspend the cells in a small volume of cold 1X PBS (1x10^6 per 0.1 mL).

7.8 Add 9X volumes of methanol (final concentration is 90% methanol) and store at -20°C for a minimum of 30 minutes. Cells may be kept frozen for at least 1 month. It is recommended to store cells aliquoted at 1x10^5 per vial/assay tube.

**8. Assay Procedure**

Note: Enough reagents are provided for 50 tests in a 100 µL volume or 100 tests in a 50 µL volume. Always include negative controls as follows (1) – primary/+secondary and (2) – primary/-secondary.

8.1 Pellet cells at 350 – 500 x g for 5 minutes (depending on cell size) and aspirate supernatant.
8.2 Dislodge the pellet by gently tapping the bottom of the tube and wash twice by centrifugation with 1mL of wash buffer per assay tube.

8.3 After final wash, aspirate supernatant, dislodge the pellet by gently tapping the bottom of the tube and add 50 µL of 1X Blocking buffer per tube (optimal concentration is 2x10^4 cells per µL). Mix by gently inverting the tube and incubate at room temperature for 15 minutes.

8.4 Prepare 50µL per assay tube of 2X Primary Antibody Cocktail Solution in 1X Blocking buffer (1:25 dilution) so that it can overlay the 50 µL cell suspension for a final 1X antibody cocktail solution. Incubate at room temperature for at least 1 hour.

8.5 Pellet cells at 350 - 500 x g for 5 minutes (depending on cell size) and aspirate supernatant.

8.6 Dislodge the pellet by gently tapping the bottom of the tube and wash twice by centrifugation with 1mL of 1X Wash per assay tube.

8.7 Prepare 100uL of a Goat Anti-Mouse and Goat Anti-Rabbit fluorochrome labeled cocktail solution in 1X Blocking buffer per assay tube.

8.8 After final wash, dislodge the pellet by gently tapping the bottom of the tube and add 100µL of detector solution per assay tube. Incubate for at least 1 hour at room temperature in the dark.
8.9 Pellet cells at 350 – 500 x g for 5 minutes (depending on cell size) and aspirate supernatant.

8.10 Dislodge the pellet by gently tapping the bottom of the tube and wash twice by centrifugation with 1mL of 1X Wash per assay tube.

8.11 After final wash, dislodge the pellet by gently tapping the bottom of the tube and add 100μL of PBS to each assay tube.

9. Data Analysis

Specific methods depend on the available flow cytometer. It is important to appropriately establish forward and side scatter gates to exclude debris and cellular aggregates from analysis. Certain treatments may generate subpopulations of cells that are apparent from the forward/side scatter plots. Under these circumstances it is recommended to adequately gate the subpopulation of interest before capturing events. If the histogram does not generate a perfect normal distribution, use the median measurement to prevent artifacts from skewing the data.
10. **Assay Performance and Specificity**

Assay specificity was demonstrated by using NIH3T3 cells grown in RPMI media with 10% FCS, exposed to serum starvation overnight, followed by treatment with PDGF recombinant protein or vehicle control. Figure 1 shows flow cytometry results of this cell culture model. Note human MCF-7 cells were shown to be similarly reactive when stimulated with PDGF (data not shown).

![Flow cytometry results](image1)

**Figure 1. Antibody specificity demonstrated by Flow cytometry.** Non induced NIH3T3 (red) and PDGF induced (blue) were targeted with the antibody cocktail against Akt1 and Akt1 (phospho S473). Background
fluorescence (black) was determined with a no-primary antibody control. After background subtraction, the PDGF induced cell line shows a 7 fold increase in the levels of phosphorylated Akt1 in comparison to a 1.2 increase in the levels of Akt1 protein.

Confidence in antibody specificity is critical to Flow data interpretation. Therefore, the antibodies in this kit were also tested for specificity by fluorescence immunocytochemistry and western blot using the same aforementioned cell culture model. The graphs below show induction of Akt phosphorylation at residue 473 both by ICC and WB using the antibodies included in this panel.

**Figure 2. Antibody specificity demonstrated by immunocytochemistry.**
ICC was carried out on NIH3T3 cells treated with PDGF (Left) or vehicle (right) with anti-Akt1 phospho S473 and anti-Akt1 using all buffer reagents as supplied in this kit. Labeling was carried out with a polyclonal antibody GAR-594 and GAM-488 respectively. The PDGF induced cells (left) show a significant induction of Akt phosphorylation at residue S473 in comparison to the non-induced control (right).
Figure 3. Validation of antibodies by WB. Western blot was run on a 10-20% gradient acrylamide gel. Samples were loaded as follows from left to right: (1) 50ng of Human recombinant AKT1 protein (tagged) Cat# ab62279, (2) 25ug of non-induced NIH3T3 cell extract and (3) 25ug of PDGF induced NIH3T3 cell extract. Membrane Blocking was carried out with 5% Milk+50mM Tris+0.05% Tween-20 pH 7.4, primary antibodies (Akt-1, left, and Akt-1 phosphoS473, right) were incubated overnight in 5% BSA+50mM+0.05% Tween-20 pH 7.4 and secondary antibodies were incubated for 2 hours in 5% Milk+50mM Tris+0.05% Tween-20 pH 7.4.
## 11. Troubleshooting

<table>
<thead>
<tr>
<th>Problem</th>
<th>Cause</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low Signal</td>
<td>Signal not correctly compensated</td>
<td>Check positive single color control is set up correctly on flow cytometer and gated/compensated correctly to capture all events</td>
</tr>
<tr>
<td></td>
<td>Insufficient secondary antibody</td>
<td>Increase concentration of antibody</td>
</tr>
<tr>
<td></td>
<td>Lasers not aligned</td>
<td>Run flow check beads and adjust alignment if necessary</td>
</tr>
<tr>
<td></td>
<td>Secondary antibody is not compatible with primaries</td>
<td>Use secondary goat antibodies raised against mouse and rabbit for this kit</td>
</tr>
<tr>
<td>High side scatter background</td>
<td>Cells lysed</td>
<td>Ideally samples should be freshly prepared. Do not vortex or shake the sample at any stage. Do not exceed 500 x g for centrifugation</td>
</tr>
<tr>
<td></td>
<td>Bacterial contamination</td>
<td>Ensure sample is not contaminated</td>
</tr>
<tr>
<td>Low event rate</td>
<td>Low number of cells</td>
<td>Run 1x10⁶ cells/mL</td>
</tr>
<tr>
<td></td>
<td>Cells clumped</td>
<td>Ensure a single cell suspension. Sieve clumps (30 μL nylon mesh)</td>
</tr>
<tr>
<td>High event rate</td>
<td>High number of cells/mL</td>
<td>Dilute between 1x10⁵ cells/mL and 1x10⁶ cells/mL</td>
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