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Ultra-Sensitive Detection of Akt Kinase Activity Using AlphaScreen™

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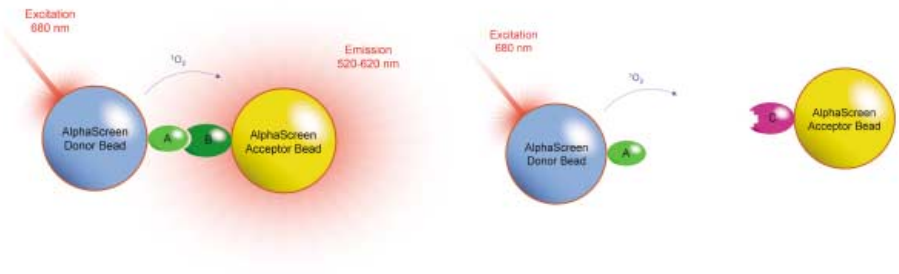
Abstract

AlphaScreen technology has been validated for use in measuring the activity of a variety of tyrosine- and serine/threonine-kinases. Specific kits have been developed for tyrosine kinase assays based on phosphotyrosine antibodies, including P-tyr-100 from CST. Kits are now being developed to address serine/threonine kinases, based on anti-phosphothreonine antibodies as well as antibodies against the phosphorylated substrates of specific kinases. One example of the latter approach is the development of a kit for Akt kinase, an enzyme in the PI3-kinase pathway. Upon activation of PI3-kinase, PIP3 is produced which activates PDK1. PDK1, in turn, activates Akt by phosphorylation at the Thr308 residue. Akt then phosphorylates various substrates including GSK-3. Using a peptide derived from the GSK-3 phosphorylation site, we have produced a very sensitive assay to measure the activity of Akt. Data demonstrating the utility of this assay in high throughput screening of Akt inhibitors will be presented.

1 Principles of AlphaScreen

When biological interactions bring the Donor and Acceptor beads into close proximity, reactive oxygen, generated by irradiation of the Donor beads, initiates a luminescence/fluorescence cascade in the Acceptor beads. This process leads to a highly amplified signal with light output in the 520-620 nm range.

When the Acceptor and Donor beads are not in proximity, the reactive oxygen decays and only a very low background signal is generated.



2 Introduction

Akt plays a central role in mediating critical cellular responses including cell growth and survival, angiogenesis, and transcriptional regulation. This protein kinase is activated by insulin and various growth and survival factors and functions in a wortmannin-sensitive pathway involving PI3 kinase. Akt promotes cell survival by inhibiting apoptosis by means of its ability to phosphorylate and inactivate several targets. Akt phosphorylates substrates only at Ser/Thr in a conserved motif characterized by Arg at positions -5 and -3.

One of the many Akt substrates is GSK-3 (glycogen synthase kinase-3). GSK-3 is a ubiquitously expressed Ser/Thr protein kinase that phosphorylates and inactivates glycogen synthase. GSK-3 is a critical downstream element of the PI3-kinase Akt cell survival pathway, and its activity can be inhibited upon phosphorylation by Akt at Ser21 of GSK-3α or Ser9 of GSK-3β.

Using the AlphaScreen technology we have developed an assay to measure the activity of Akt protein kinase using GSK-3 as the substrate. The assay format is presented in figure 1.

3 Material and Methods

Material

- The following material was provided by Cell Signaling Technology
  - 2 x 500 µl Akt1/PKB protein kinase (# 9274) at 10,000 units/ml (1 mg/ml)
  - 4 x 120 µl GSK-3 fusion protein (# 9278) at 1 mg/ml
  - 55 µl (0.1 mg) Phospho-GSK-3α/β antibody (# 9331B) at 1.8 mg/ml
  - 90 µl (0.1 mg) Phospho-(Ser/Thr) Akt Substrate antibody (# 9611B) at 1.1 mg/ml
- The following material was provided by PerkinElmer Life Sciences
  - Protein A-Acceptor beads at 5 mg/ml
  - Streptavidin-Donor beads at 5 mg/ml
  - Biotin-GSK-3 at 4 µM
  - Staurosporine (Sigma # S-4400) at 5 mM
  - ATP at 10 mM
  - All buffer components

Methods

- Buffers composition
  - Assay buffer: 25 mM Tris/HCl pH 7.5 containing 10 mM $MgCl_2$, 2 mM DTT, 100 µM Na_3VO_4 and 0.1% Tween-20
  - Detection buffer: 25 mM Tris/HCl pH 7.5 containing 200 mM NaCl, 100 mM EDTA and 0.3% BSA
- Assay

  The reagents were prepared as follows:
  - Akt protein kinase @ 10,000 U/ml: dilute to 0.5 U/ml and 0.1 U/ml with assay buffer (2.5 U and 0.5 U final)
  - Biotin-GSK-3 @ 4 µM: dilute to 150 nM with assay buffer (30 nM final)
  - Staurosporine @ 10 mM: dilute to 30 µM with assay buffer (10 µM final)
  - ATP @ 10 mM: dilute to 300 µM with b-substrate dilution (100 µM final)
  - Protein A Acceptor beads @ 5 mg/ml: dilute to 50 µg/ml in detection buffer (20 mg/ml final)
  - Streptavidin Donor beads @ 5 mg/ml: dilute to 50 µg/ml in Acceptor beads dilution(20 µg/ml final)
  - Anti-phospho-GSK-3α/β @ 8 µM: dilute to 2.5 nM in beads dilution (1 nM final)

  The assay was performed in 15 µl for a final reaction volume of 25 µl using white opaque 384 well plates. The reagents were added as follows:
  - 5 µl enzyme
  - 5 µl inhibitor or assay buffer
  - incubate 15 minutes at RT
  - 5 µl biotin-GSK-3 / ATP mix
  - incubate 1 hour at RT
  - 10 µl Antibody /Acceptor beads /Donor beads solution (pre-mix 1 hour at RT)
  - incubate 1 hour and read on AlphaQuest HTS microplate analyzer

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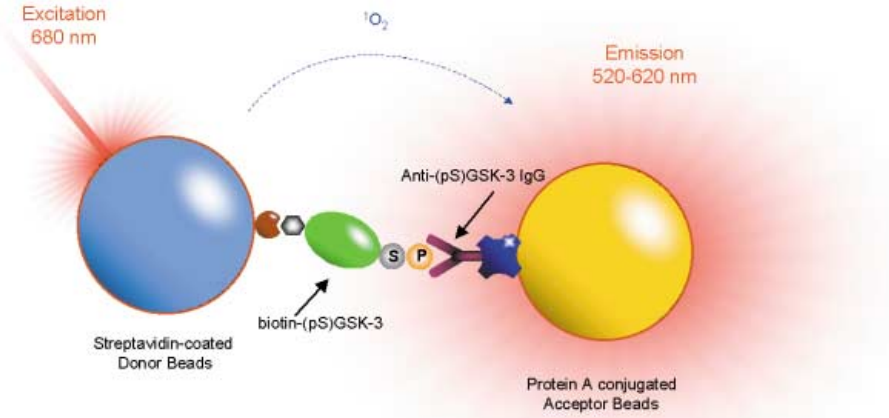
AlphaScreen Assay Format

Figure 1. Upon Akt activity, the AlphaScreen signal is generated when phosphorylated biotin-GSK-3 is simultaneously captured by Streptavidin-Donor beads and anti-phospho-GSK-3 antibodies bound to protein A-coated Acceptor beads.



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Titration of Biotin-GSK-3

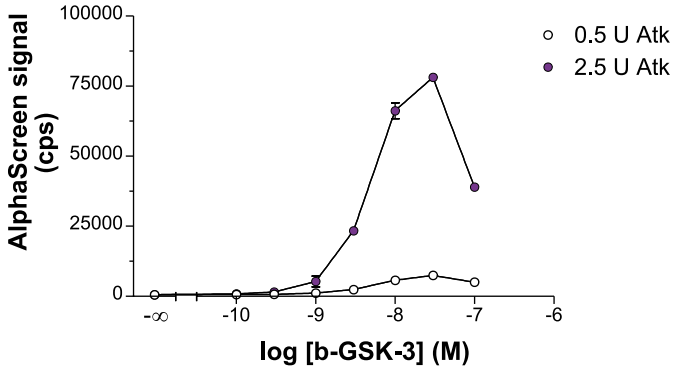

Figure 2. Biotin GSK-3 was titrated from 0.1 to 100 nM in the presence of either 0.5U or 2.5U AKT. A significant signal (Signal:Background = 3) was detectable using as low as 0.3 nM GSK-3 and 2.5 U Akt. For both enzyme concentrations, maximum signal was reached using 30 nM biotin-GSK-3.

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Enzyme Titration

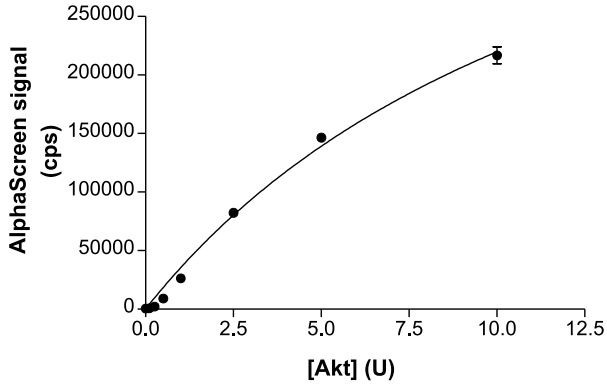

Figure 3. Akt was titrated from 0.1U to 10U in the presence of 30 nM GSK-3 and 100 µM ATP. As little as 0.25U enzyme generated 3000 cps (S:B = 5) whereas 10U produced 220,000 cps (S:B > 350).

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Time Course of Biotin-GSK-3 Phosphorylation by Akt

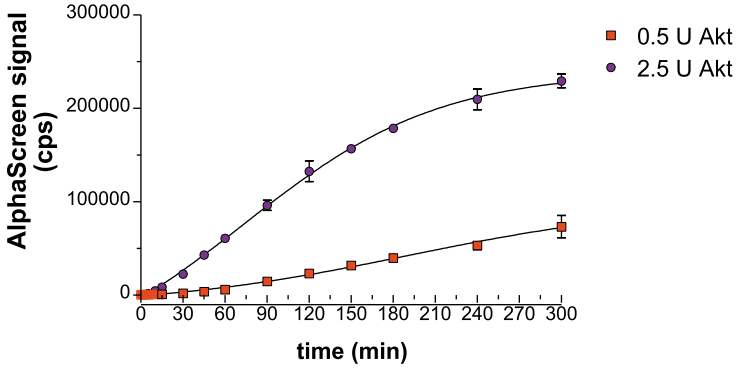

Figure 4. Time course of biotin-GSK-3 phosphorylation by Akt (30 nM) was performed with either 0.5U or 2.5U Akt. For both enzyme concentrations, reaction was linear for at least 2 hours.

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ATP Titration

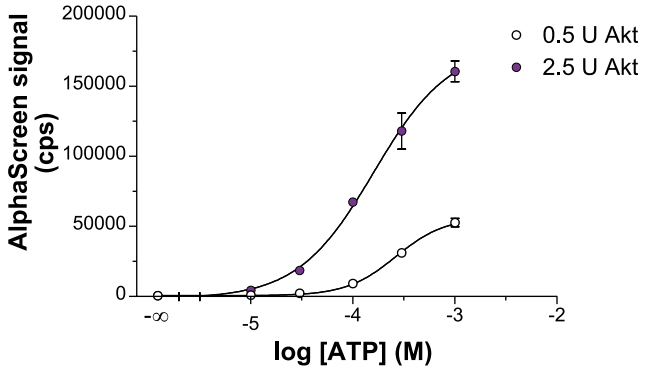

Figure 5. ATP was titrated from 10 µM to 1 mM at either 0.5U or 2.5U Akt. An EC_{50} value of 150 µM was found at both enzyme concentrations.

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Inhibition of Akt Activity by Staurosporine

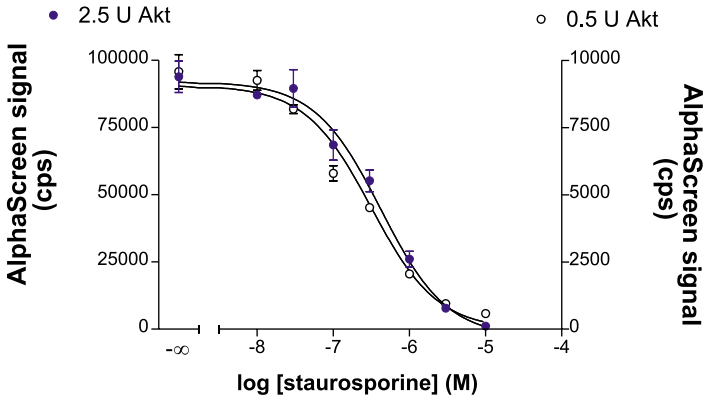

Figure 6. Akt activity was inhibited in a dose-dependent manner by staurosporine (10 nM to 10 µM). IC_{50} values of 420 nM and 180 nM were measured at 2.5 and 0.5 U of Akt respectively.

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DMSO Tolerance

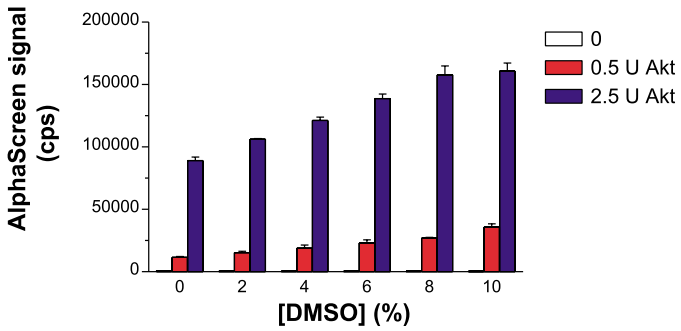

Figure 7. Increasing concentrations of DMSO (0 –10%) were used to assess the impact of that solvent on the assay performance. Increasing DMSO concentration up to 10% improved the assay performance. The 50% signal increase observed at 10% DMSO could be attributed to either an enhanced reagent solubility or an increase of enzyme activity.

Conclusion

AlphaScreen was used to develop a robust and sensitive assay to measure GSK-3 phosphorylation by Akt. In the presence of an optimal concentration of GSK-3 (30 nM), as little as 0.5 U produces a signal of 7300 cps (S:B >10). Increasing the concentration of enzyme to 2.5U increases the signal to 78,000 cps (S:B = 144). Time course studies of substrate phosphorylation show that the assay is linear for at least 2 hours. We have measured an EC_{50} value of 150 µM for ATP in the presence of 2.5U Akt. Using the latter conditions, the IC_{50} of the universal kinase inhibitor staurosporine was 400 nM. Staurosporine used at 10 µM inhibited the enzyme activity by more than 95%. The assay tolerates DMSO which has been shown to improve the signal appreciably. These results taken together show that AlphaScreen provides a robust platform suitable for screening Akt inhibitors.

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