APPLICATION NOTE

A Practical Approach to Cell Signaling Pathway Analysis

From sensitive western blots to quantitative assays of multi-target pathways using AlphaScreen® *SureFire*® assays

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Introduction

Cellular kinase signal transduction pathways are involved in the regulation of many important cellular processes such as cell survival, differentiation and apoptosis. Kinase signaling networks are typically characterized by multiple kinases arranged in cascades containing nodes with feedback loops, and crosstalk between pathways. One of the most widely studied pathways is the phosphoinositide-3 kinase (PI3K) pathway (Figure 1), which contains the downstream kinases Akt and mTOR (mammalian target of rapamycin). Genetic mutations resulting in abnormal activation of the PI3K pathway are found in a high number of human cancers. Therefore, inhibitors targeting PI3K and other components in the pathway are potential drugs for cancer therapy.

Akt, also known as protein kinase B, is a central effector in the PI3K pathway. A common mechanism for controlling the activity of a kinase is phosphorylation on serine, threonine, or tyrosine residues. Akt is activated by phosphorylation on threonine residue T308 by PDK1, and on serine S473 by the mTOR Complex 2 (mTORC2). Upon activation, Akt phosphorylates several downstream targets culminating in the regulation of cell survival and metabolism. In this study, we stimulated MCF7 cells with insulin-like growth factor 1 (IGF-1) to activate the PI3K/Akt pathway. Conversely, the pathway inhibitors wortmannin and rapamycin were used to block the activity of PDK1 and mTOR Complex 1 (mTORC1), respectively.

Numerous assay technologies have been developed for studying kinase signaling pathways, and for screening compound libraries in search of agents to modify kinase activities. Depending on the stage of the investigation and the antibody reagents available to the researcher, both western blot and AlphaScreen® *SureFire*® assays can yield valuable information. Western blots are widely used for studies of cellular kinases, but these assays suffer from limited sensitivity and throughput. We demonstrate here that an ultra-sensitive western blot substrate (Western Lightning *Ultra*) increases detection sensitivity 5-10 fold, enabling much more robust detection of phosphorylated targets in cellular extracts.

PerkinElmer products used in this study:

AlphaScreen® *SureFire*® assay kits
(Akt pathway, p-PDK1, GAPDH)
AlphaScreen® Protein A detection kit
Western Lightning *Ultra* chemiluminescent substrate
Western Lightning Plus ECL
ViewPlate®-96 TC
ProxiPlate™-384 Plus
PolyScreen® PVDF Transfer Membranes
EnVision® Multilabel Plate Reader
JANUS® Automated Workstation

PerkinElmer®
For the Better

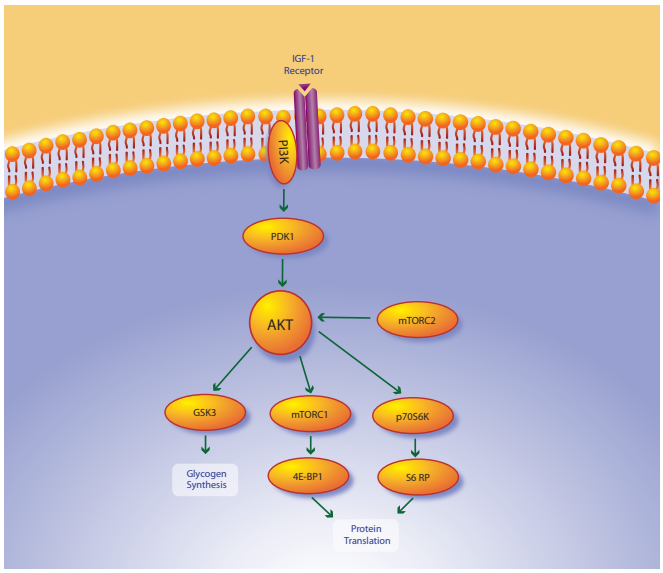


Figure 1. The PI3K/Akt cellular signaling pathway

We further compared the detection of multiple phosphorylated targets using AlphaScreen® *SureFire*® assays of cellular kinase activity. Our data indicates that AlphaScreen® *SureFire*® assays for several targets in the PI3 signaling pathway give results comparable in sensitivity to ultra-sensitive western blots, but with higher throughput and a much less time-consuming protocol. Modulation of kinase pathways generally affects multiple kinases in the pathway. AlphaScreen® *SureFire*® assays are well-suited for kinase pathway mapping, since the assay requires only 4 µL of cellular lysate per assay point.

The results of cell-based assays can be affected by variability in the number of cells sampled per assay point. A common method for controlling for variability is to normalize the assay signal to a cellular protein whose level does not change as a function of the assay. We have validated two targets for normalization of the PI3K/Akt pathway. The first is the metabolic enzyme GAPDH, and the second is the total Akt present in the cell.

AlphaScreen® *SureFire*® technology

AlphaScreen® *SureFire*® cellular kinase assays measure endogenous levels of phosphorylated cellular proteins involved in various signaling pathways. The assay is a homogeneous, bead-based technology that utilizes two antibodies in a sandwich assay format. One antibody recognizes a specific phospho-epitope on the analyte, while the other antibody is directed towards another, non-phosphorylated, epitope on a distal part of the analyte. One of the antibodies binds to a Donor bead and the other to an Acceptor bead, so that in the presence of a phosphoprotein the two beads are drawn into close proximity. Excitation of the Donor bead with laser light with a wavelength of 680 nm results in energy transfer to the Acceptor bead yielding an emission signal at 520-620 nm.

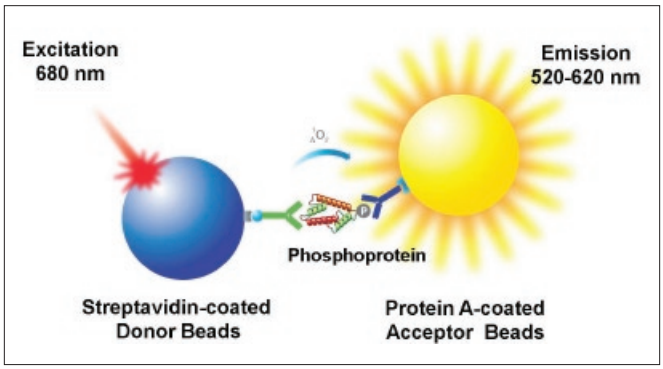


Figure 2. AlphaScreen® *SureFire*® assay principle

Cell culture

MCF7 cells were cultured in T75 flasks in EMEM supplemented with 10% FBS. Cells were maintained in culture at 20-70% confluency and typically were passed twice per week.

Western Lightning *Ultra* chemiluminescent substrate

The high sensitivity and broad dynamic range of the Western Lightning *Ultra* substrate make it ideal for investigation of cell signaling pathways, where concentration of targets of interest can vary widely.

Cell lysate preparation

Flasks of confluent cells were stimulated for 20 minutes at room temperature with 200 nM IGF-1 in complete medium. The medium was removed and the cells were lysed with 5 mL of lysis buffer for 10 minutes with gentle shaking. The lysate was centrifuged to remove solid cell debris and the supernatant was collected and frozen in aliquots at -80 °C. Pathway inhibition was performed by treating the cells with a combination of 20 µM wortmannin and 200 nM rapamycin in complete medium for 2 hours at 37 °C, 5 % CO_2. Cells were serum-starved by incubation in base medium for 3 hours at 37 °C, 5 % CO_2.

Western blot analysis

Protein concentration in MCF7 cell lysates was determined by the BCA protein assay with bovine serum albumin as the standard. Western blot analysis was performed by resolving cell lysates loaded at 20 µL/lane on NuPAGE® Novex® polyacrylamide mini gels followed by transfer of the proteins to PVDF membranes. The membranes were probed with the same primary antibodies to phospho-proteins contained in the AlphaScreen® *SureFire*® kits, followed by exposure to anti-species HRP-conjugated secondary antibodies. The membranes were treated with the chemiluminescent substrate for 1 minute, and the image was acquired on a Kodak Image Station 440 CF imager.

AlphaScreen® *SureFire*® assay protocol

A 4 µL aliquot of lysate was added to the well of a ProxiPlate. A 5 µL mixture of Reaction buffer, Activation buffer, and AlphaScreen Protein A Acceptor beads (40:10:1) was added and the plate was incubated at room temperature for 2 hours. Two µL of AlphaScreen streptavidin-coated Donor beads in AlphaScreen® *SureFire*® Dilution buffer (20:1) was added and the plate was incubated for an additional 2 hours. The plate was then read on an EnVision® Multilabel Plate Reader using the factory defined AlphaScreen settings.

Pathway mapping workflow

The workflow shown in Figure 3 illustrates an efficient and cost-effective approach to cell signaling pathway analysis. By freezing multiple aliquots of the stimulated cell lysate at -80 °C, a first-pass analysis of one phosphorylated target can be followed by a more extensive analysis using the full range of AlphaScreen® *SureFire*® assays available for a given pathway. Confirmatory western blots can then be performed on the same samples only as needed.

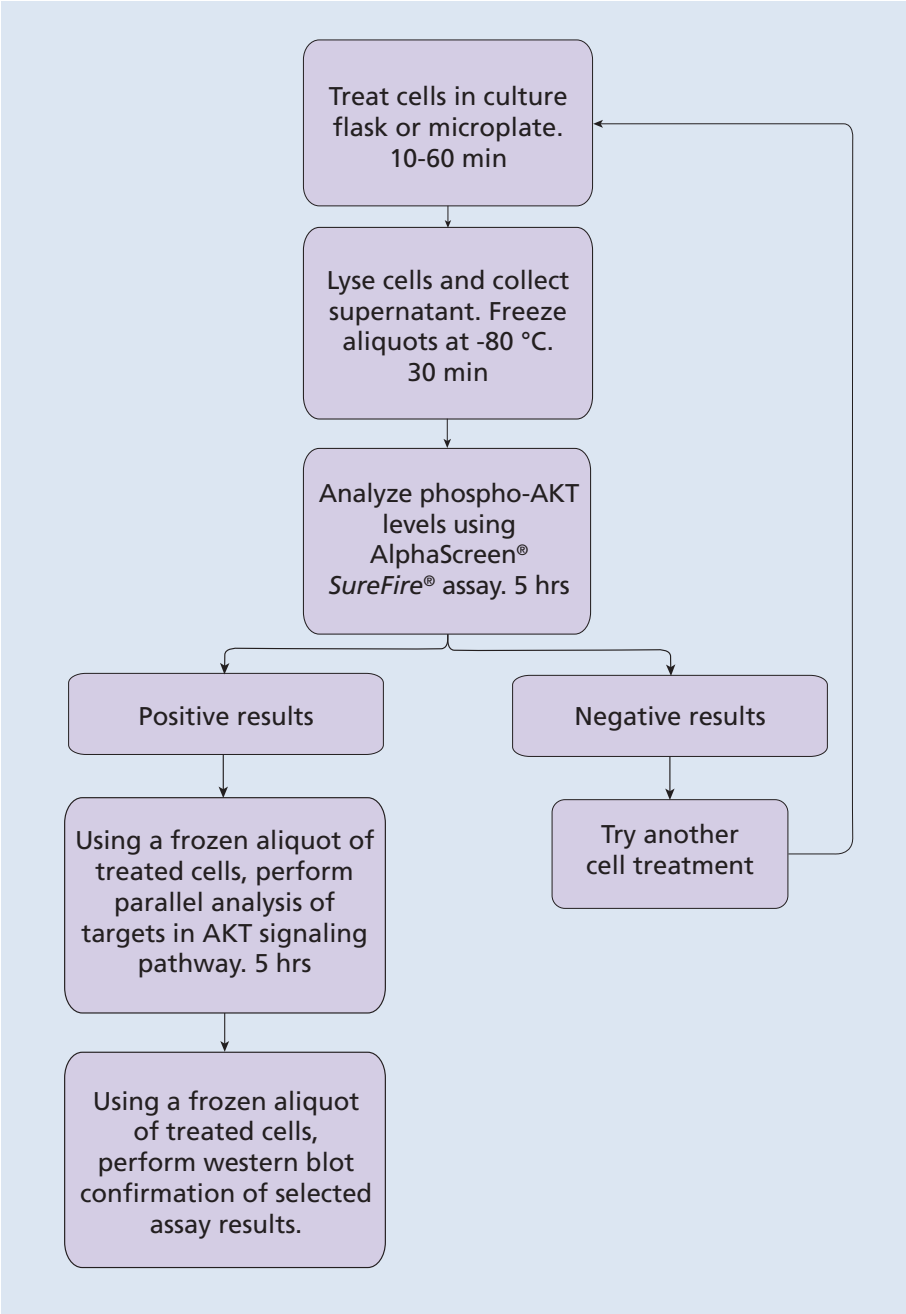
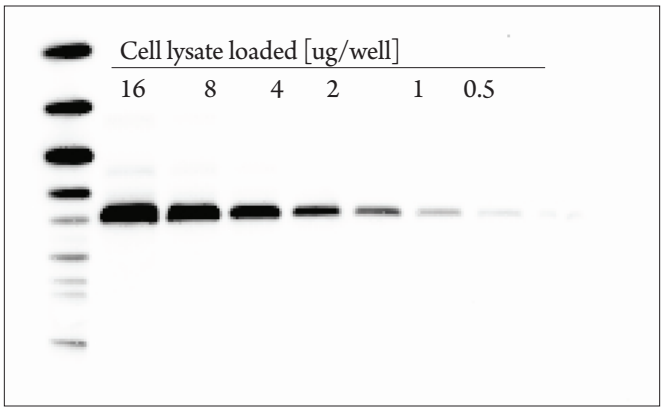


Figure 3. A workflow for efficient analysis of cell signaling pathways.

Western Lightning *Ultra*



Western Lightning *Plus*

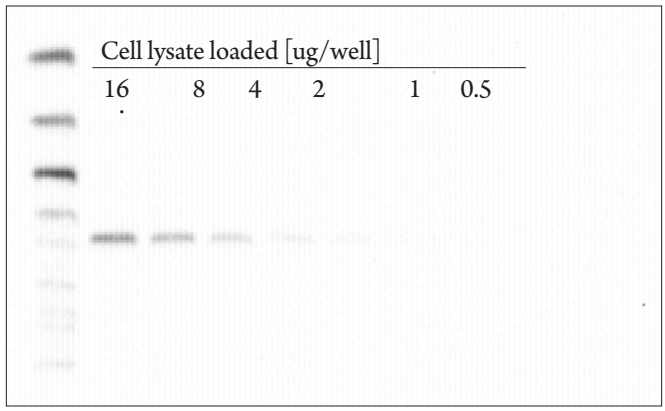


Figure 4. Sensitivity of detection using Western Lightning *Ultra* compared to Western Lightning *Plus*.

Results

Ultra-sensitive western blot

Two western blots were run using the same aliquot of lysate from cells stimulated with IGF-1. Two-fold serial dilutions of lysate were performed to determine assay sensitivity. The membranes were probed with antibody to total Akt, and detected with either Western Lightning *Ultra* or Western Lightning *Plus*. The results shown in Figure 4 demonstrate that 5-10 fold greater sensitivity is obtained with Western Lightning *Ultra* as the substrate.

Western blot sensitivity compared to AlphaScreen® *SureFire*®

The sensitivity of western blot assays using Western Lightning *Ultra* was compared to the sensitivity of AlphaScreen® *SureFire*® assays in detecting seven phosphorylated proteins in the PI3K/Akt signaling pathway. The components tested ranged from PDK1 at the beginning of the pathway to the final downstream targets, p-4E-BP1 and p-S6 RP, and included both phosphorylation sites on Akt. Serial dilutions of lysates of IGF-1 stimulated cells show comparable sensitivity of detection by both assay technologies as presented in Figure 5 (next page).

PI3K/Akt pathway mapping: basal vs. stimulated cells

AlphaScreen® *SureFire*® assays were used to measure the level of phosphorylated substrates in the PI3K/Akt pathway compared to their basal level in cells stimulated with IGF-1. As shown in Figure 6, four members of the pathway, p-Akt (T308), p-Akt (S473), p-p70 S6K and p-S6 RP exhibited a stimulated response from 2-20 fold greater than their basal level. The other three pathway components did not show stimulation significantly above their basal level.

PI3K/Akt pathway mapping: basal vs. serum starved vs. inhibited cells

The targets that had shown significant basal levels as described above were further studied by either serum starving the cells or treating them with pathway inhibitors. Inhibition was performed using a combination of two inhibitors, wortmannin and rapamycin, to ensure maximal inhibition. As observed in Figure 7, serum starvation reduces the signal for these targets except p-S6 RP. Pathway inhibition reduced the signal to a greater extent than serum starvation for all of the targets.

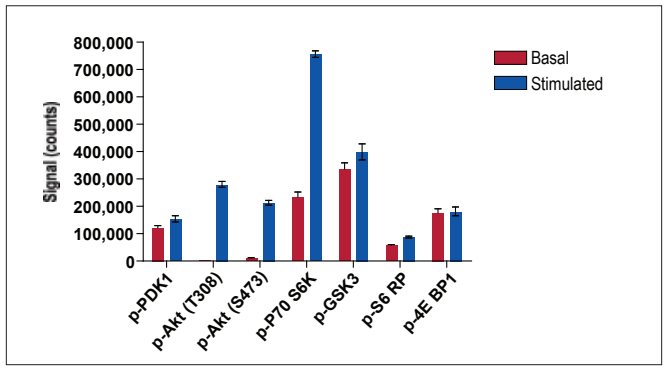


Figure 6. Comparison of basal vs. stimulated level of phospho-proteins in the PI3K/Akt pathway in cells stimulated with IGF-1.

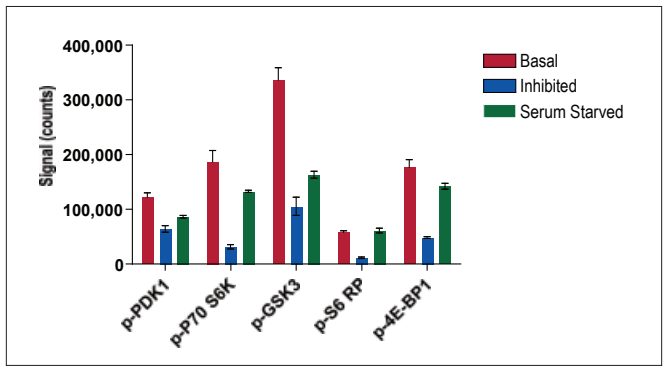


Figure 7. Effect of serum starvation or pathway inhibition on MCF7 cells.

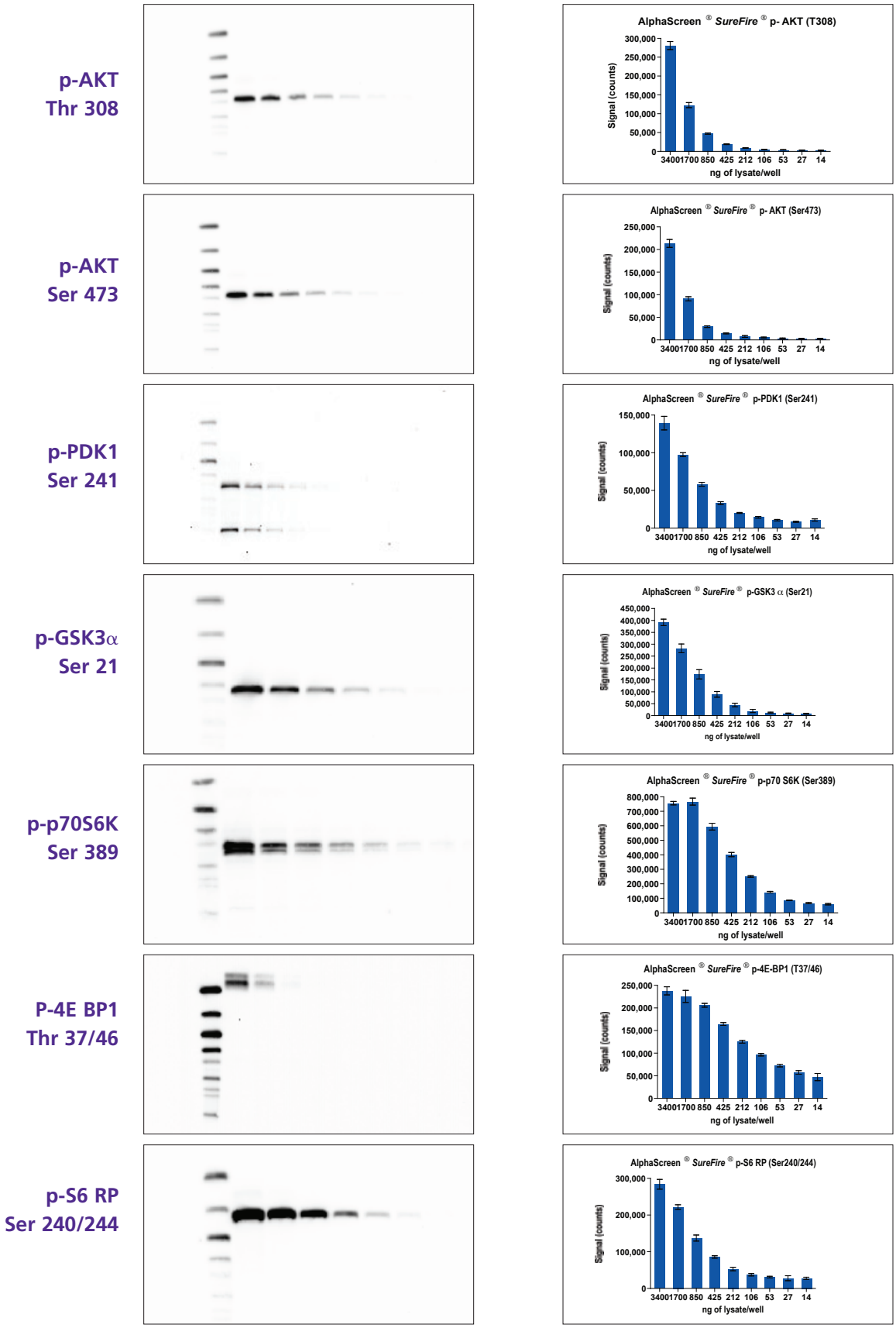


Figure 5. Sensitivity of detection of ultra-sensitive western blot compared to AlphaScreen® SureFire® in detecting targets in the PI3K/Akt pathway.

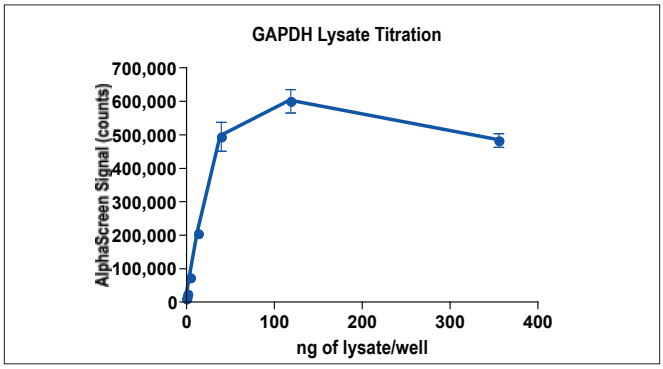


Figure 8. GAPDH signal for a dilution series of MCF7 cellular lysate.

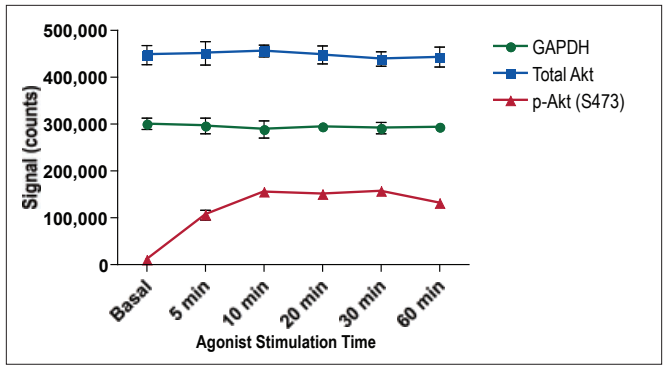


Figure 9. Time course of detection of GAPH, total Akt, and p-Akt.

Assay normalization – GAPDH and total Akt

In order to normalize AlphaScreen® *SureFire*® assays to account for well-to-well variability in cell number or daily fluctuations in the cellular metabolic state, two approaches were examined. The first was to use the level of the metabolic enzyme GAPDH for normalization, and the second to normalize against total Akt. Prior to running the GAPDH assay we found it is necessary to dilute the lysate so that the concentration of GAPDH is below the "hook point" of the assay. At the "hook point" of the assay binding to either the Donor or Acceptor bead is saturated, and the maximal assay signal is generated. Above this point the beads are oversaturated with analyte, which inhibits their association and causes a progressive signal decrease. Figure 8 shows the GAPDH signal obtained from a serial dilution of lysate indicating that at least a fifty-fold dilution is necessary for MCF7 cells.

To verify that GAPDH and total Akt were suitable analytes for normalization, MCF7 cells were stimulated with IGF-1 for a range of time points from 5 minutes to one hour. Lysates were prepared for each time point and were assayed for GAPDH, total Akt and p-Akt (S473). The results plotted in Figure 9 show that the level of p-Akt (S473) varies over time, whereas, GAPDH and total Akt levels remain constant.

Summary

Ultra-sensitive western blot analysis has been compared to AlphaScreen® *SureFire*® assays for detecting multiple analytes in the PI3K/Akt cellular kinase pathway. Western blot using Western Lightning *Ultra* substrate detects phosphorylated targets in cell lysates 5-10 fold better than standard western blot reagents. This provides greater dynamic range, which helps in quantitative comparisons of phosphorylation levels. AlphaScreen® *SureFire*® assays show comparable detection sensitivity. The homogeneous format of AlphaScreen® *SureFire*® assays offers higher throughput with a much simpler protocol.

The two assay technologies have complementary features that make each the preferred method at different stages in the assay development and execution process. The table below summarizes the most important characteristics of each technology. We recommend using western blot when beginning assay development, and then moving to AlphaScreen® *SureFire*® when higher throughput is required. Western blot can verify that the desired target is present in sufficient quantity, and confirms the target identity on the basis of the observed molecular weight. The homogenous, no-wash assay format for AlphaScreen® *SureFire*® makes it much more suitable for automation, pathway mapping, and processing large numbers of samples. Taken together, these two research tools provide a robust solution for analyzing cellular kinase pathways.

Range	Ultra-sensitive western blot	AlphaScreen® *SureFire* ®
Basis of specificity	Requires only one specific antibody, so good for initial investigations. Assay specificity confirmed using target gel motility.	Assay kit uses two specific antibodies to ensure assay specificity.
Relative sensitivity	5-10X higher than standardWestern blot	Comparable to ultra-sensitive Western blot
Quantification	Relative quantification based on apparent intensity of gel bands	Quantification relative to standard curve or untreated samples, with CV≤5%. Can normalize to targets such as total Akt or GAPDH.
Sample throughput	Up to 10-20 gel lanes	Up to 100 samples in parallel (in a 384-well microplate)
Protocol steps and overall time	20 steps, 4-5 hrs hands-on 8-24 hrs overall	7 steps, 1-1.5 hrs hands-on 5-6 hrs overall
Number of targets that can be analyzed per sample	Limited by number of gel lanes	Analysis of up at 10 targets in the same signaling pathway

Materials Used

Material	Vendor	Catalog Number
MCF7 cell line	ATCC	HTB-22
EMEM	ATCC	30-2003
Fetal bovine serum	HyClone	SH 30071.03
AlphaScreen® *SureFire*® (Akt Signaling pathway) kit	PerkinElmer	TGRSP2S500
AlphaScreen® *SureFire*® IgG (Protein A) detection kit	PerkinElmer	6760617M
AlphaScreen® *SureFire*® p-PDK1 kit	PerkinElmer	TGRPS500
AlphaScreen® *SureFire*® GAPDH kit	PerkinElmer	TGRGDS500
ViewPlate®-96 TC	PerkinElmer	6005181
ProxiPlate™-384 Plus	PerkinElmer	6008289
Western Lightning *Ultra*	PerkinElmer	NEL1120
Western Lightning Plus ECL	PerkinElmer	NEL1030
PolyScreen® PVDF Transfer Membranes	PerkinElmer	NEF1003
Goat anti-rabbit HRP	PerkinElmer	NEF8120
Goat ant-mouse HRP	PerkinElmer	NEF8220
BCA™ Protein Assay Kit	ThermoFisher	23227
XCell SureLock™ Mini-Cell & XCell™ Blot Module	Invitrogen	EI0002
NuPAGE® Novex® Bis-Tris Mini Gels	Invitrogen	NP00321
Long R3 IGF-1	Sigma	I1271
Wortmannin	Calbiochem	681675
Rapamycin	Calbiochem	553210

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