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Rapid multi-parameter optimization of AlphaScreen® *SureFire®* cellular kinase assays using the JANUS® automated workstation

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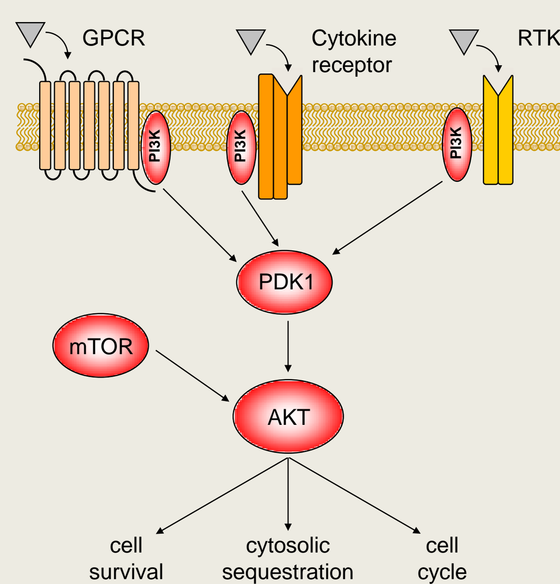
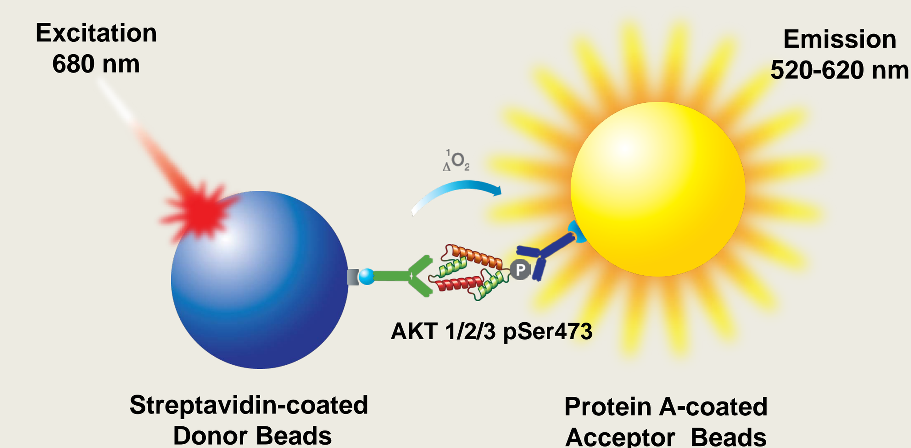
1 Abstract

AlphaScreen® *SureFire®* assays measure endogenous levels of phosphorylated proteins in a cell-based assay format. AlphaScreen is a homogenous bead-based technology that is an alternative to ELISA assays, and is easily automated since no wash steps are required. These assays are available to measure the activity of a range of intracellular kinase pathways. In this work we studied the phosphorylation of several targets in the PI3K/AKT/mTOR pathway, which is a key signaling cascade in regulating protein translation and cell growth. Screening kinase activities in cell-based assays such as SureFire offers advantages over the more traditional biochemical approach of using a purified recombinant enzyme to phosphorylate a substrate, since a cell-based assay gives information on a compound's activity in a more biological context. In particular, only compounds that are cell membrane permeable will show activity at intracellular targets. The initial development and optimization of cell-based kinase assays is highly dependent on optimal cell culture conditions, and often requires multiple parameters to be optimized in the first set of experiments. Certain parameters are more important to optimize initially in order to obtain sufficient assay window for further study. We have developed a strategy for quickly accomplishing that goal. In the course of this work we have utilized the JANUS automated workstation to perform the assays. The necessity of automated liquid handling in high throughput screening is generally recognized. We have found that automating AlphaScreen® *SureFire®* assays during the early development is also very helpful. In addition to saving labor, the assurance of precision and reproducibility when using the JANUS is a significant benefit in multi-parameter optimization. We will present representative data generated during the development of AlphaScreen® *SureFire®* assays for AKT phosphorylation in the PI3K pathway, and also the JANUS automation protocol and the key liquid handling parameters.

2 Introduction



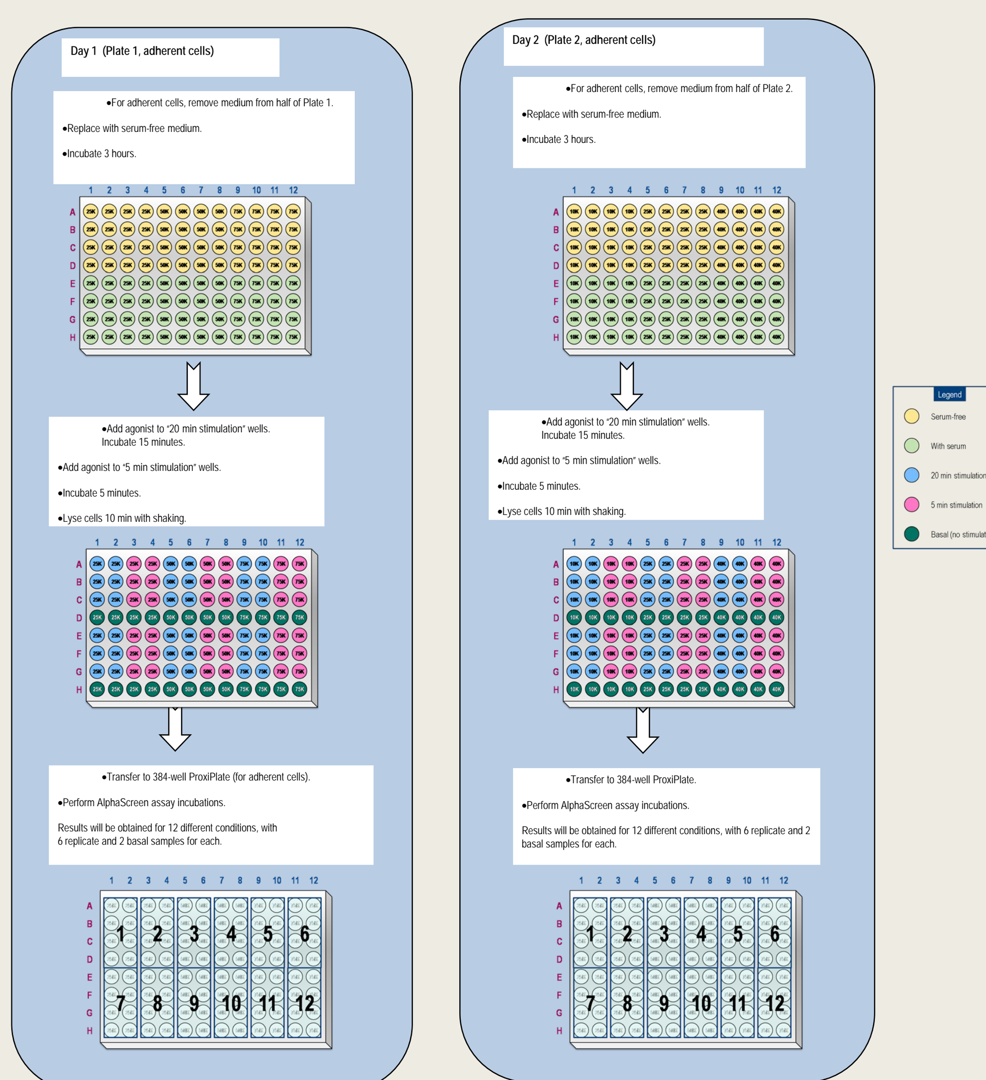
PI3K Pathway.
Different cell surface receptor agonists (GPCR, cytokines and growth factors) can activate the PI3K pathway. PDK1 and mTOR are upstream kinases known to modulate Akt functions including cytosolic sequestration, cell survival and cell cycle.



AlphaScreen® *SureFire®* AKT 1/2/3 pSer473.
Akt 1/2/3 isoforms are detected using a combination of antibodies to detect the general form of the protein and the phosphorylated Ser473 residue. In the presence of AKT 1/2/3 pSer473 both protein A coated Acceptor and Streptavidin-Donor beads are brought into proximity and the AlphaScreen signal is produced.

3 Materials & Methods

Material	Vendor	Catalog number
C2C12 Mouse Myoblast Cell Line	ATCC	CRL-1772
DMEM with L-Glutamine	ATCC	30-2002
Fetal Bovine Serum	HyClone	SH 30071.03
AlphaScreen® *SureFire®*		
p-Akt 1/2/3 (Ser473) Kit	PerkinElmer	TGRA4S10K
AlphaScreen IgG (Protein A)		
Detection Kit	PerkinElmer	6760617M
Insulin	Sigma	I5500
Long R3 IGF-1	Sigma	I-1271
Wortmannin	Sigma	W1628
LY 294002	Calbiochem	440202
ViewPlate-96 TC	PerkinElmer	6005181
ProxiPlate™-384 Plus	PerkinElmer	6008289



Protocol for Multi Parameter Optimization
C2C12 cells plated for 1 day (overnight, left panel) or 2 days (right panel), were stimulated with 100 nM insulin in the presence or without serum , for either 5 or 20 minutes. Basal counts were measured in absence of insulin.

4 Automated Liquid Handling Workflow

1. Add agonist to assay plate and incubate for 5 and 20 minutes
2. Aspirate media containing agonist
3. Dispense and aspirate 1X PBS to wash cells free of phenol red
4. Add SureFire lysis buffer and shake plate for 10 minutes
5. Transfer 4 µL of lysate to a ProxiPlate
6. Add reaction buffer/activation buffer Acceptor Bead mixture
7. Incubate for 2 hours and add Donor Bead/dilution buffer mixture
8. Incubate for 2 hours and read on the EnVision

Key Assay Liquid Handling Steps
All liquid handling steps were performed using JANUS automated workstation equipped with both an 8-tip Verispan™ VersaTip® arm and a Modular Dispense Technology™ (MDT) arm equipped with a 96-channel I200 head. Steps 7 and 8 were done with dimmed lights.

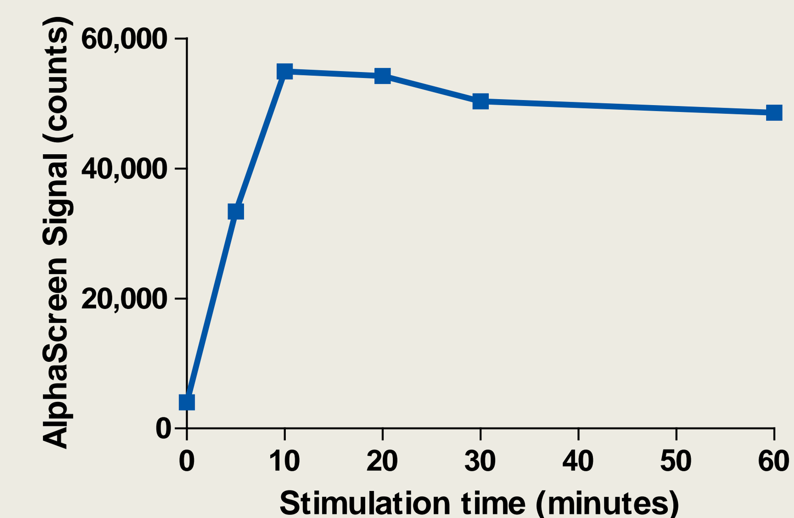
5 Multi Parameter Optimization

1 day protocol	cells/well	10,000		25,000		40,000	
	stimulation time (min)	20	5	20	5	20	5
w/o serum	stimulated signal (S)	26721 ± 5291	20838 ± 1898	34671 ± 2354	23313 ± 2758	50463 ± 3591	33222 ± 3530
	basal signal (B)	1928 ± 379	1924 ± 260	1964 ± 68	2200 ± 277	2398 ± 167	2218 ± 37
	S:B	13.9 (1)	10.8 (2)	17.7 (3)	10.6 (4)	21.0 (5)	15.0 (6)
	Z'	0.35	0.69	0.79	0.60	0.78	0.68
serum	stimulated signal (S)	34635 ± 2827	29265 ± 3991	59888 ± 7642	36870 ± 5384	75676 ± 5992	22385 ± 2373
	basal signal (B)	1900 ± 198	2166 ± 184	2578 ± 444	2350 ± 8	2720 ± 57	2390 ± 93
	S:B	18.2 (7)	13.5 (8)	23.2 (9)	15.7 (10)	27.8 (11)	9.4 (12)
	Z'	0.72	0.54	0.58	0.53	0.75	0.63

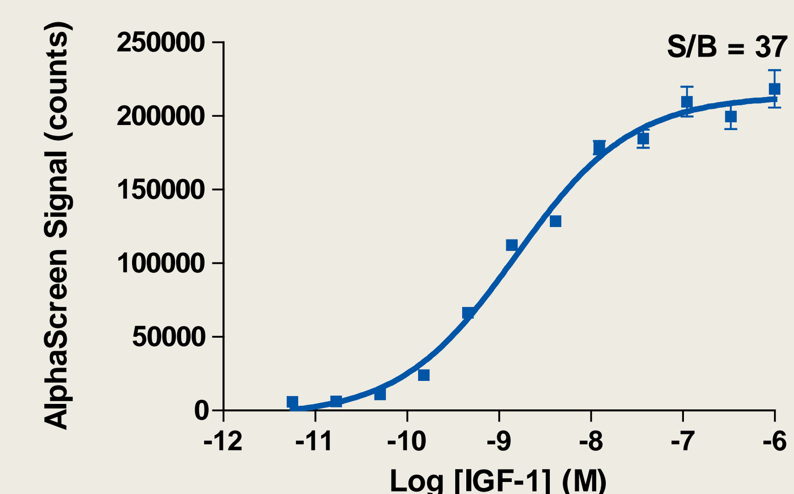
2 day protocol	cells/well	25,000		50,000		75,000	
	stimulation time (min)	20	5	20	5	20	5
w/o serum	stimulated signal (S)	29813 ± 3330	19992 ± 1535	47913 ± 5142	26005 ± 3208	62123 ± 6755	33429 ± 4453
	basal signal (B)	1756 ± 11	1708 ± 62	1880 ± 288	1868 ± 62	2096 ± 260	2292 ± 11
	S:B	17.0 (1)	11.7 (2)	25.5 (3)	13.9 (4)	29.6 (5)	14.6 (6)
	Z'	0.66	0.76	0.66	0.62	0.66	0.60
serum	stimulated signal (S)	36219 ± 3659	16755 ± 1518	67211 ± 7014	21272 ± 2591	86411 ± 4795	20326 ± 3513
	basal signal (B)	2046 ± 105	1802 ± 42	2378 ± 376	2170 ± 37	2628 ± 62	2198 ± 150
	S:B	17.7 (7)	9.3 (8)	28.3 (9)	9.8 (10)	32.9 (11)	9.2 (12)
	Z'	0.67	0.69	0.66	0.59	0.83	0.39

Results of the Multi Parameter Optimization
C2C12 cells plated for 1 day (overnight) or 2 days, were stimulated with 1 nM insulin in the presence or without serum , for either 5 or 20 minutes. Basal and stimulated counts were measured in absence or in the presence of 100 nM insulin respectively. Yellow circles indicate best assay conditions obtained with both protocols (combination of high S:B and Z' values).

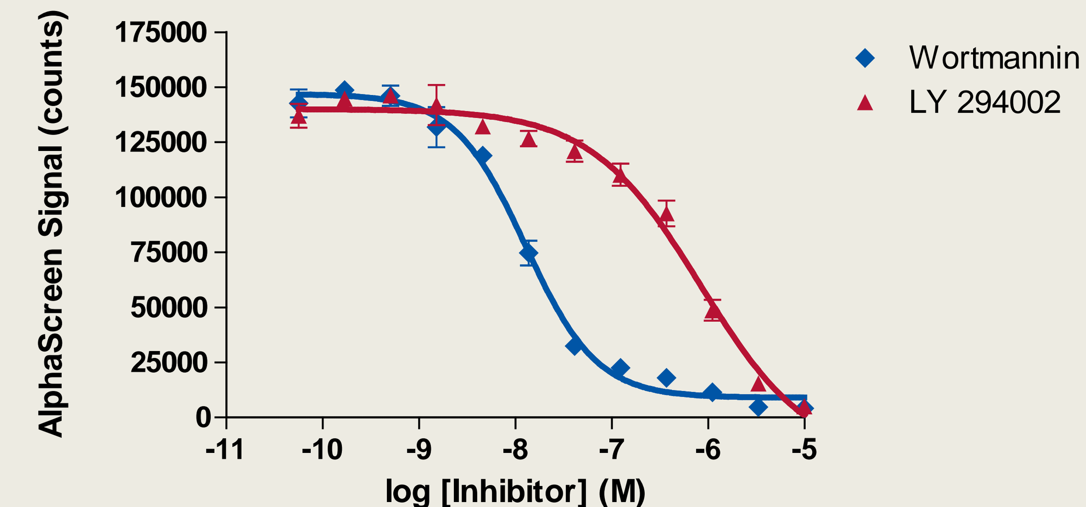
6 Pharmacological Validation



Time-Course of Insulin Stimulation.
100 nM Insulin was used to stimulate 30,000 cells. Phosphorylation of Akt on Ser473 peaked at about ten minutes and remained relatively stable for up to 1 hour.



IGF-1 Dose-Response Curves.
Dose-response curves for IGF-1. IFG-1 induced Akt phosphorylation with an EC_{50} value of 1.5 nM.



Inhibition of IGF-1 induced Akt phosphorylation.
The PI3K inhibitors Wortmannin and LY 294002 inhibited Akt phosphorylation with IC_{50} values of approximately 10 and 500 nM respectively. C2C12 were stimulated with IGF-1 used at its EC_{50} concentration.

7 Summary

A multi parameter optimization process was used to optimize the detection of cellular AKT pSer473 using the AlphaScreen® *Surefire®* AKT 1/2/3 Ser473 kit.

- The JANUS automated workstation was used to perform the assay development during the course of this work.
- In addition to saving labor, the assurance of precision and reproducibility achieved when using the JANUS is a significant benefit in multi-parameter optimization.

Pharmacological validation of the AKT 1/2/3 Ser473 assay was performed with known activators and inhibitors of the PI3K/AKT pathway.

- Both IFG-1 and Insulin stimulated Akt phosphor-ylation.
- Both PI3K inhibitors Wortmannin and LY 294002 inhibited Akt phosphorylati
- on induced via IGF-1 stimulation of C2C12 cells.

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