High throughput cell-based screening of receptor activated protein kinases

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INTRODUCTION

Screening of protein kinases with compound libraries is traditionally done using isolated enzymes and measuring phosphorylation of substrate peptides in vitro. While sensitive, these methods give no information on effects of compounds on systems in living cells, on activity of enzymes on endogenous cellular proteins, nor or on compound bioavailability in a cellular environment. Additionally, studies of receptor-activation of kinases requires a cellular system. For these reasons, screening the effects of compounds on endogenous protein kinases in cells is preferable.

To address this, and to provide a cell-based screening technology that is amenable to HTS, we have developed the *SureFire*™ platform of protein phosphorylation assays. Currently, assay kits available include those for phosphorylated ERK, p38 MAPK, JNK, MEK, AKT, p70S6K, and Stat-3. Therefore, these assays allow for readout from multiple receptor and signal transduction pathways, notably the MAP kinases, PI 3-kinase and cytokine signalling pathways.

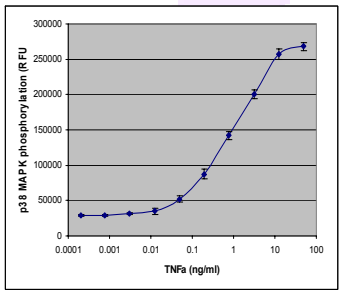
Of importance is that these assays are homogeneous, in that no washing steps are required, providing for a robotics compatible assay protocol. This is due to the assays utilizing the AlphaScreen® system (PerkinElmer®), a highly sensitive proximity based technology which we have used as the basis of assays of endogenous cellular proteins.

RESULTS

p38 MAP Kinase

U937 cells grown in RPMI with 10% serum were centrifuged and resuspended in RPMI with 0.1% BSA without serum at 1x10^7 cells per ml. After 90 min, cells were stimulated with TNFα for 20min or left unstimulated. Cells were then lysed by the addition to the medium of a 1/5th volume of 5X Lysis buffer. To a sample of this lysate was added a 1/5th volume of Activation Buffer, and 6ul transferred to a ProxiPlate™. To this lysate was added 6ul of Reaction Buffer containing AlphaScreen® beads (1:60 v/v), and the plate was incubated in the dark for 2 hours and then read on a EnVision™ Alpha™.
Results are the Mean+/-SEm of 3 replicates.

Phospho- p38 MAP Kinase



ERK

Bradykinin Receptor-2 transfected CHO cells were grown to confluence in a 96 well plate, starved overnight and then activated for 10 min with bradykinin or left unstimulated. Cells were lysed with 1X Lysis buffer (made from 5X Lysis buffer) and a sample transferred to a replicate plate. A 1/5th final volume of Activation Buffer was added, a sample of this added to a ProxiPlate™ and then Reaction Buffer with AlphaScreen® beads added. The plate was incubated in the dark for 2 hours and then read on an EnVision Alpha™.
Results are the Mean+/-SEm of 3 replicates at each point.

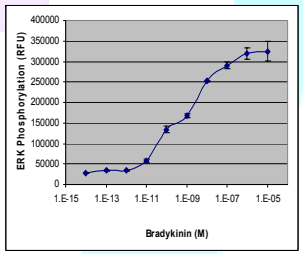
MEK1

NIH-3T3 cells were grown to confluence, starved overnight and then activated for 10 min with serum or left unstimulated. Cells were lysed with 50ul of 1X Lysis buffer plus Activation Buffer combination, and a 5ul sample transferred to a ProxiPlate™ . Reaction Buffer with AlphaScreen® beads was then added and the plate incubated for 4 hours and then read on an EnVision Alpha™.
Results are the Mean +/- SEm of 3 replicates.

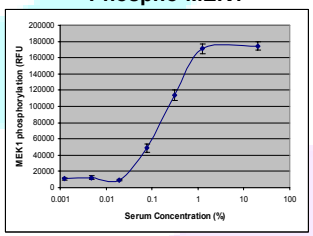
JNK

HEK-293 cells were grown to confluence and then stimulated with sorbitol or left unstimulated. After 30 min, media was removed and cells lysed with 1X Lysis buffer (made from 5X Lysis buffer) and a sample transferred to a replicate plate. A 1/5th final volume of Activation Buffer was added, a sample of this added to a ProxiPlate™ and then Reaction Buffer with AlphaScreen® beads added. The plate was incubated in the dark for 4 hours and then read on an EnVision Alpha™. Results are the Mean+/-SEm of 3 replicates at each point.

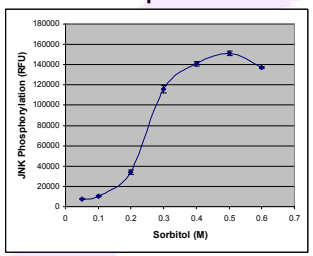
Phospho-ERK



Phospho-MEK1



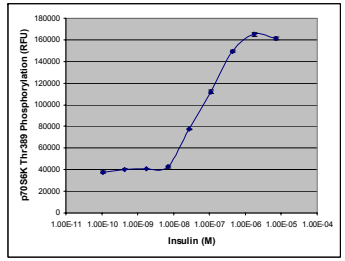
Phospho-JNK



p70 S6 Kinase (Thr389)

MCF-7 cells were grown to confluence in a 96 well plate, starved overnight and then activated for 30 min with insulin or left unstimulated. Cells were lysed with 1X Lysis buffer (made from 5X Lysis buffer) and a sample transferred to a replicate plate. A 1/5th final volume of Activation Buffer was added, a sample of this added to a ProxiPlate™ and then Reaction Buffer with AlphaScreen® beads added. The plate was incubated in the dark for 2 hours and then read on an EnVision Alpha™.
Results are the Mean+/-SEm of 3 replicates.

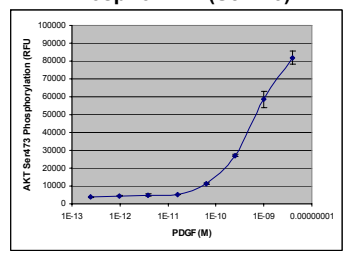
Phospho-p70 S6 kinase (Thr389)



AKT (Ser473)

NIH-3T3 cells were grown to confluence, starved overnight and then activated for 30 min with PDGF or left unstimulated. Cells were lysed with 1X Lysis buffer plus Activation Buffer combination, and a 5ul sample transferred to a ProxiPlate™ . Reaction Buffer with AlphaScreen® Acceptor beads was then added and after 2 hours Dilution buffer with Donor beads added. The plate was incubated a further 2 hours and then read on an EnVision Alpha™.
Results are the Mean +/- SEm of 3 replicates.

Phospho-AKT (Ser473)



CONCLUSIONS

The *SureFire*™ assays of phosphorylation of cellular signalling proteins provide a new tool for the rapid detection of bioactive molecules using a biologically relevant system, that of living cells. As the technology is sensitive enough to detect phosphorylation of endogenous cellular proteins, activation of these pathways by endogenous or cloned receptors can be carried out. Therefore, screening of both receptor modulators as well as small molecule inhbitors of signal transduction pathways can be carried out. Due to the homogeneneous nature of the assays, which utilize AlphaScreen® technology, HTS programs can be carried out using these assays using standard liquid handling robotics. Current assays allow the assessment of the major MAP kinases, the PI 3-kinase pathway activated protein kinases AKT and p70 S6K, and of Stat-3.
This assay portfolio will be added to in the future to provide an even broader spectrum of assays for the discovery of pharmaceutical agents and for basic research applications.

Contact Information

The *SureFire*™ Cellular Kinase assay kits are formulated for the HTS marketplace for screening large sample numbers and robotic operation, as well as the research laboratory. Further information about kits, prices and protocols can be obtained from the TGR BioSciences Pty Ltd and PerkinElmer Customer Service centres:

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