A Homogeneous Assay to Quantify Endogenous AKT Phosphorylation in Human Umbilical Endothelial Cells

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Overview

AKT is a serine/threonine protein kinase that plays a role in key cellular processes. These include the cell cycle, metabolism, and angiogenesis. Activated AKT has been implicated in the proliferation and survival of cells, leading to tumor development. Because of these functions, AKT has become a popular target for drug discovery campaigns, due to the fact that AKT inhibitors may help to treat a number of cancers. Here we demonstrate an automated homogeneous assay to probe AKT phosphorylation at its serine 473 residue using endogenous levels of kinase expression within human primary HUVEC cells. Validation data demonstrate that the combination of assay, cells, and instrumentation are sensitive enough to detect endogenous phosphorylation of this important drug target.

Introduction

AKT is a serine/threonine protein kinase that plays a key role in multiple cellular processes such as glucose metabolism, cell proliferation, apoptosis, transcription and cell migration. AKT is involved in cellular survival pathways, by inhibiting apoptotic processes. AKT is also able to induce protein synthesis pathways, and is therefore a key signaling protein in the cellular pathways that lead to skeletal muscle hypertrophy, and general tissue growth. Since it can block apoptosis, and thereby promote cell survival, AKT has been implicated as a major factor in many types of cancer.

AKT is a key downstream intracellular point of convergence for a number of cellular signaling pathways. These diverse signaling pathways are activated by a variety of growth factors (including vascular endothelial growth factors (VEGFs), platelet-derived growth factor (PDGF), epidermal growth factor (EGF), and insulin-like growth factor 1 (IGF-1)). One or more of these signaling pathways may be abnormally activated in patients with many different types of cancer, resulting in deregulated cell proliferation, tumor angiogenesis, and abnormal cell metabolism.

Here we provide results for an HTRF® assay for measuring AKT phosphorylation at its serine(473) residue, using primary human umbilical vein epithelial (HUVEC) cells. Due to the increased need to generate the most biologically relevant data during drug discovery, primary cells are gaining in popularity for use in target based cellular assays. HUVEC cells are a robust primary endothelial cell type which is widely used for *in vitro* studies, including angiogenesis.

The assay was automated to provide an easy-to-use and reproducible process. Cells were plated and treated with compounds in 96-well format. Following lysis, aliquots were transferred into 384-well assay plates to complete the HTRF assay. Validation and pharmacology results demonstrate how the combination of assay, cells, and instrumentation can provide an easy to use method to examine the function of this important signal transduction pathway.

BioTek Instrumentation

Figure 1 – Synergy™ H4 Hybrid Multi-Mode Microplate Reader.

The Synergy H4 combines a filter-based and monochromator-based detection system. The HTRF certified reader uses the filter-based system and Xenon flash lamp to detect the 665 nm and 620 nm fluorescent emissions from this chemistry.

Figure 2 – EL406™ Combination Washer Dispenser.

The EL406 offers fast, accurate media removal and plate washing capabilities through its Dual-Action™ Manifold. It also offers reagent dispensing capabilities through the use of its peristaltic or syringe pumps, with volumes ranging from 1-3000 µL/well. The instrument was used for cell dispensing, media removal and plate washing, as well as to dispense EC80 compound and HTRF assay components.

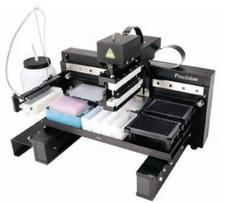

Figure 3 – Precision™ Microplate Sample Processor.

The Precision combines an 8-channel pipetting head and an 8-channel bulk reagent dispenser in one instrument. The instrument was used to serially titrate compounds across a 96-well polypropylene plate, transfer compounds to the 96-well cell plates, as well as transfer lysate aliquots from the 96-well cell plates to the 384-well assay plates.

VEGF-AKT Signal Transduction

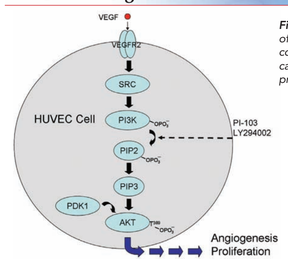

Figure 4 – VEGF-induced activation of AKT serving as a model for constitutive activity common to some cancers resulting in uncontrolled cell proliferation.

HTRF phospho-AKT (Ser473) Assay

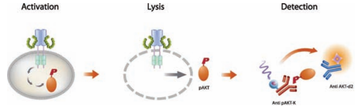

Figure 5 – Representation of HTRF phospho-AKT (Ser473) Assay.

Cisbio's HTRF phospho-Akt (Ser473) assay is designed for detecting and studying activated AKT directly in whole cells. Upon receptor activation, the kinases are activated, leading to the phosphorylation of AKT kinase. After the lysis of the cell membrane, phosphorylated AKT can be detected upon the addition of two monoclonal antibodies: an anti-kinase antibody labeled with d2 and an anti-phospho-kinase antibody labeled with Eu 3+-cryptate.

The assay is based on a sandwich immunoassay principle. In the presence of phosphorylated AKT, upon excitation of the Eu 3+-cryptate, energy is transferred to the d2 molecule, and emission at 655 nm is seen. In the absence of the phosphorylated kinase, no energy is transferred, and emission from the Eu 3+-cryptate is seen at 620 nm.

HUVEC Cells

HUVECs are endothelial cells lining the umbilical vein and serve as the selective barrier between circulating blood and the underlying smooth muscle. Endothelial cells are a more robust primary cell type in culture than other primary cells, and are commonly used for pharmacological studies such as angiogenesis and cancer development, macromolecule and cell adhesion and transport, clotting, and cell signaling pathway analysis.

In comparison to cancer cell lines commonly used by researchers, primary HUVECs provide a more biologically relevant tool for measuring cellular activities *in vitro* since they more closely simulate the *in vivo* environment. Primary cells are neither altered nor transformed, so there is much less risk of change in function and phenotype as seen in cell lines. In addition, cells from multiple donors can be analyzed, allowing for a better estimation of physiological responses across human populations.

Optimized HUVEC Cell Preparation

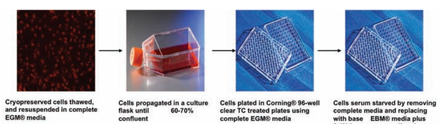

Figure 6 – HUVEC Cell Preparation Process.

Optimized Automated Assay Procedure

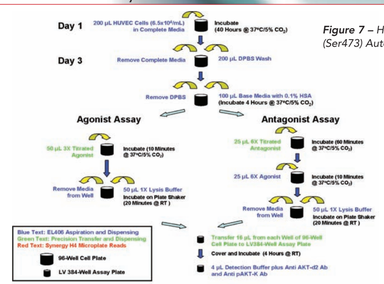

Figure 7 – HTRF phospho-AKT (Ser473) Automated Assay Protocol.

HTRF Data Reduction

The HTRF ratio for each cell containing well was calculated using the following formula:

Ratio = ((665 em/620 em)*10,000)

A negative control was also run on the plate containing 1X lysis buffer and the two HTRF antibodies, only. The data was normalized to eliminate plate to plate variations by determining the Delta F calculation. This value was determined using the following formula:

$$\Delta F = ((Ratio_{(Cell\ Containing\ Well)} - Ratio_{(Negative\ Control)})/Ratio_{(Negative\ Control)})$$

Assay Optimization

VEGF Concentration-Stimulation Time

Cryopreserved HUVEC cells (Lonza Catalog Number CC-2517, Lot 152470) were stimulated with VEGF, at concentrations ranging from 50 – 0 ng/mL (1X) for 3, 10, 30, or 60 minutes were used. The assay procedure was performed as previously described.

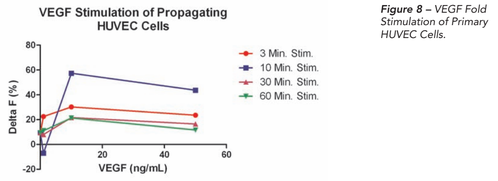

Figure 8 – VEGF Fold Stimulation of Primary HUVEC Cells.

By examining the data, it is apparent that the maximum stimulation from VEGF is seen when a concentration of 10 ng/mL is added to the wells, and the cells are stimulated for a total of 10 minutes. This combination was used to optimize cell concentration, plating, and starvation times.

CellConcentration/Plating/Starvation Time

HUVEC cells were plated into 96-well clear tissue culture treated cell culture plates, and incubated at 37°C/5% CO_2 for 16 or 40 hours. The complete media was then removed, and replaced with serum starvation media for either 4 hours or overnight. The remainder of the assay procedure is as previously described.

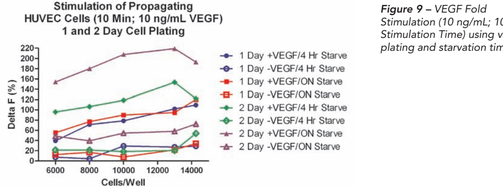

Figure 9 – VEGF Fold Stimulation (10 ng/mL; 10 Min. Stimulation Time) using variable plating and starvation times.

By examining the data, it is apparent that the ΔF ratio for wells containing cells stimulated by VEGF increase with longer plating times and higher cell concentrations. Only at the highest cell concentrations, using a 2 day plating, do the ΔF ratios decrease. It can also be seen that the ΔF ratios for unstimulated wells are stable for the first three conditions tested. Only using a 2 day plating, and an overnight serum starvation, do the ΔF ratios increase by 2-3 fold.

Therefore, due to the fact that the ΔF ratios for stimulated cells increase with a longer plating time, and the ΔF ratios do not increase up to a 2 day plating and 4 hour serum starvation, the condition giving the greatest fold stimulation would be plating 13,000 cells/well, maintaining the cells in the well for 40 hours, followed by a 4 hour serum starvation. The 2 day plating/overnight serum starvation is not chosen due to the fact that the ΔF ratios for the unstimulated cells increases dramatically, reducing the fold stimulation seen by the VEGF.

Automated Assay Validation

Z'-Factor Validation

A Z'-Factor assay was performed to validate the automated procedure. The assay was run in antagonist mode, with VEGF being used as the control agonist, and PI-103 the control antagonist. Forty replicates of 0 µM or 10 µM PI-103 were used as the positive and negative control, respectively.

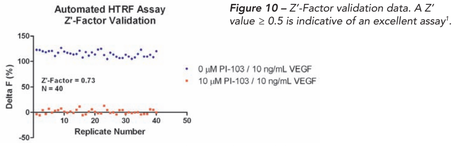

Figure 10 – Z'-Factor validation data. A Z' value ≥ 0.5 is indicative of an excellent assay[1].

Assay Pharmacology Validation

The assay was further validated by first creating an agonist dose response curve with VEGF. Concentrations tested ranged from 40 – 0 ng/mL. The EC_{50} value generated was 1.8 ng/mL. This value compares favorably to literature EC_{50} value ranges of 1-6 ng/mL[2].

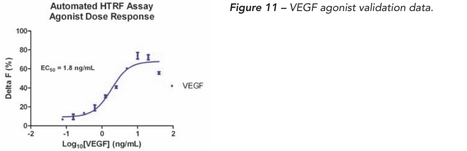

Figure 11 – VEGF agonist validation data.

Antagonist dose response curves were also generated with PI-103 and LY294002. Concentrations tested ranged from 10 – 0 µM for PI-103 and 100 – 0 µM for LY294002. The IC_{50} values generated were 5.2 nM and 332.6 nM, respectively. These values compare favorably to literature IC_{50} values of 11 nM[3] for PI-103 and 310 and 730 nM for LY294002[4, 5].

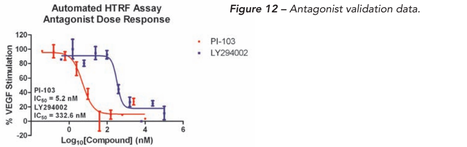

Figure 12 – Antagonist validation data.

Conclusions

1. The HTRF phosphor-Akt (Ser473) Assay allows for the monitoring the activity of the PI-3 Kinase / Akt pathway in primary HUVEC cells.
2. The assay can be automated using a 2-microplate protocol where stimulation or inhibition of the pathway is conducted in a 96-well plate, then a portion of cell lysate is transferred to a 384-well microplate for assaying.
3. Excellent robustness of the inhibition assay is demonstrated with a z' factor of 0.73
4. Appropriate inhibition pharmacology is evident at the level of PI-3 Kinase.

[1]Zhang J. *et al.*: A Simple Statistical Parameter for Use in Evaluation and Validation of High Throughput Screening Assays. *Journal for Biomolecular Screening* 1999 4(2): 67-73. • [2]Conn, G. *et al.*: Amino acid and cDNA sequences of a vascular endothelial cell mitogen that is homologous to platelet-derived growth factor *Proceedings of the National Acadamy of Sciences* USA 1990 87: 2628-2632. • [3]Fan, Q. *et al.*: A dual PI3 kinase/mTOR inhibitor reveals emergent efficacy in glioma *Cancer Cell* 2006 9(5): 341-349.
[4]Vlahos *et al.*: A Specific Inhibitor of Phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002). *J.Biol.Chem.* 1994 269: 5241. [5]Chasserot-Golaz *et al.*: Possible Involvement of Phosphatidyl-3-Kinase in Regulated Exocytosis: Studies in Chromaffin Cells with Inhibitor LY294002. *J. Neurochem.* 1998 70: 2347. • HTRF is a registered trademark of Cisbio Bioassays.