

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 a 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 and instrument 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 developed using the Synergy[™] H4 Hybrid Multi-Mode Microplate Reader. Optimization and validation experiments demonstrate how the combination of assay, cells, and instrument can provide an easy to use method to examine the function of this important signal transduction pathway.

BioTek Instrumentation

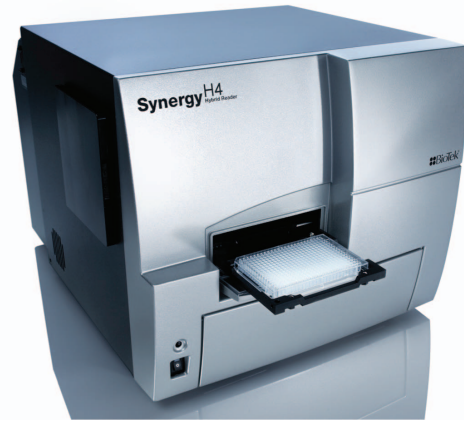


Figure 1 – Synergy H4 Hybrid Multi-Mode Microplate Reader.

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.

Reader Setup		BioTek Catalog Number	
Instrument	Detection Component		
Excitation Filter 1	330/80 nm	7082263	
Emission Filter 1	620/10 nm	7082265	
Emission Filter 2	665/7.5 nm	7082266	
Dichroic Mirror	365 nm Cutoff	7138365	
Optimized Instrument Settings			
Delay Before Collecting Data	100 µSeconds	Data Collection Time	300 µSeconds
Light Source	Xenon Flash Lamp	Delay after Plate Movement	0 mSeconds
Measurements per Data Point	20	Lamp Energy	High

Table 1 – Synergy H4 HTRF Reader Settings.

VEGF-AKT Signal Transduction

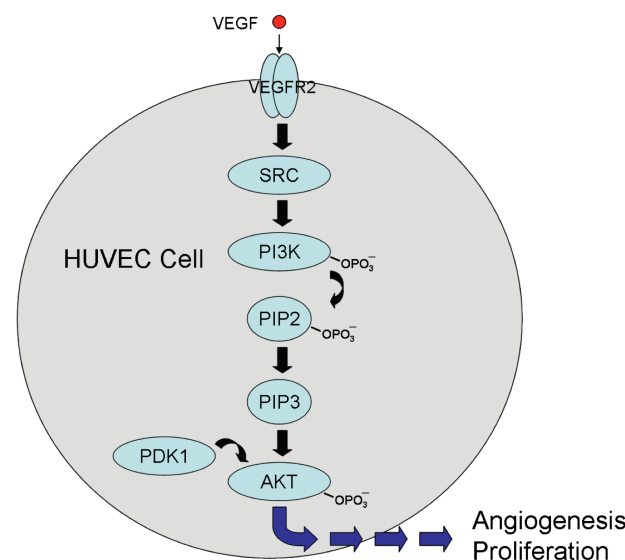


Figure 2 – VEGF-induced activation of AKT serving as a model for constitutive activity common to some cancers resulting in uncontrolled cell proliferation.

HTRF phospho-AKT (Ser473) Assay

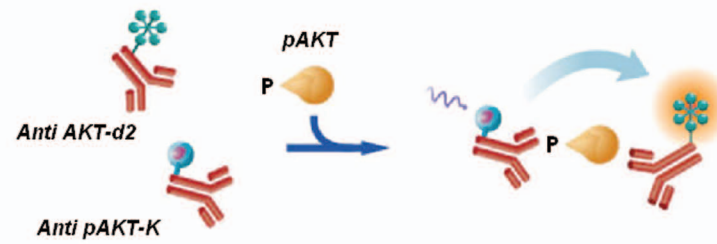


Figure 3 – Representation of HTRF phospho-AKT (Ser473) Assay.

Based on homogeneous and robust HTRF assays, the 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 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 665 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.

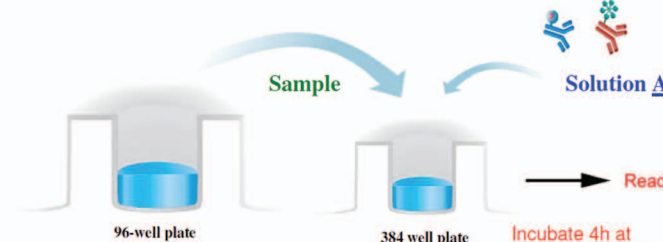


Figure 4 – Representation of 2 plate assay process incorporating 96- and 384-well plates.

Cells are plated, stimulated and lysed in the same 96-well culture plate. Lysates are then transferred to the assay plate for the detection of phosphorylated AKT by HTRF reagents. Using a pre-optimized protocol, the assay is amenable to low-volume 384-well format, and ideal for use with primary cells.

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

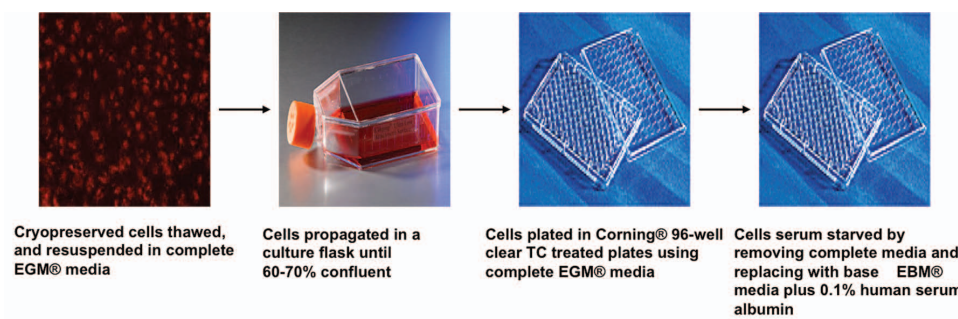


Figure 5 – HUVEC cell preparation process.

Optimized Assay Procedure

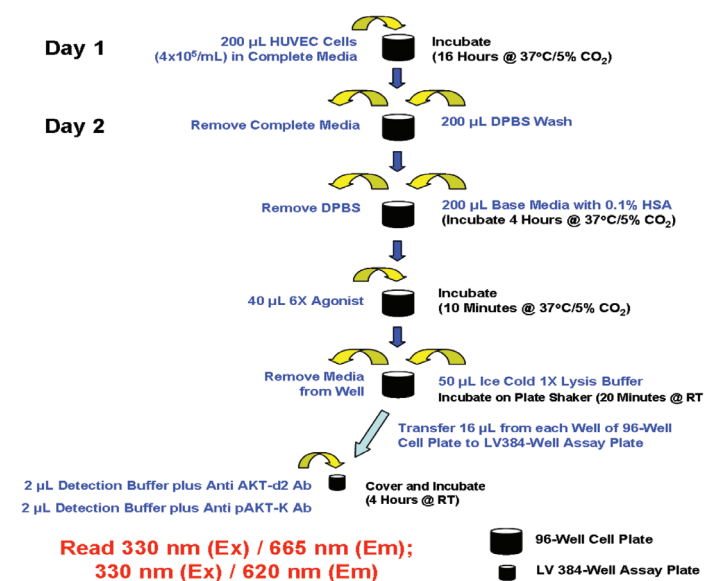


Figure 6 – HTRF phospho-AKT (Ser473) Assay Protocol.

HTRF Data Reduction

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

$$\text{Ratio} = ((665 \text{ em}/620 \text{ em}) \times 10,000)$$

A negative control was also run on the plate containing 1X lysis buffer and Solution A and B, 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:

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

Assay Optimization: VEGF Concentration-Stimulation Time

Cryopreserved HUVEC cells (Lonza Catalog Number CC-2517, Lot 152470) were thawed and propagated for one day in complete media. Cells were then plated into 96-well clear tissue culture treated cell culture plates, and incubated at 37°C/5% CO₂ overnight. The complete media was then removed, and replaced with serum starvation media for 4 hours. The cells were then stimulated using VEGF at concentrations ranging from 50-0 ng/mL (1X). Stimulation times of 3, 10, 30, or 60 minutes were used. At the conclusion of the stimulation period, the media was removed, ice cold lysis buffer was added, and the plate was shaken for 20 minutes. Following the lysis incubation time, lysate was transferred into low-volume 384-well plates. Anti AKT-d2 (Solution A) and Anti pAKT-K antibody (Solution B) mixtures were then added to the lysate. The plate was then covered, incubated for 4 hours at RT, and the fluorescent 665 nm and 620 nm signals were quantified.

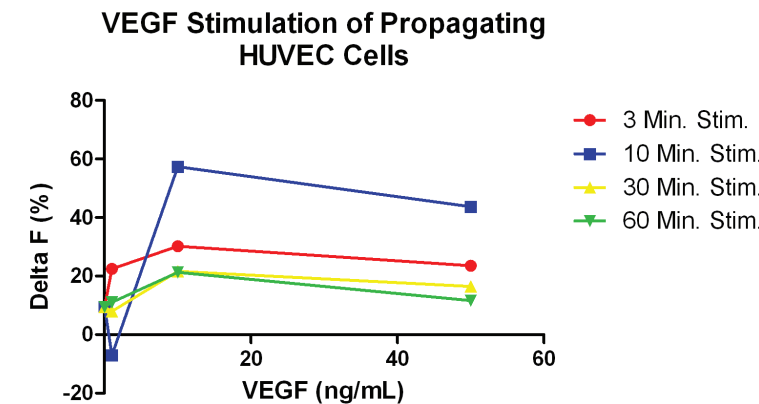


Figure 7 – VEGF stimulation of primary HUVEC cells.

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 the cell concentration, plating, and starvation times.

Assay Optimization: Cell Concentration, Plating and Starvation Times

Cryopreserved HUVEC cells were thawed and propagated until the cells reached 60-70% confluency. Cells were then plated into 96-well clear tissue culture treated cell culture plates and incubated at 37°C/5% CO₂ for 16 or 40 hours. The complete media was then removed, and replaced with serum starvation media for either 4 hours or overnight. The cells were then stimulated using VEGF at concentration of 10 ng/mL (1X) for 10 minutes. At the conclusion of the stimulation period, the cells were lysed, transferred to the low-volume 384-well plate, and had the Anti AKT-d2 (Solution A) and Anti pAKT-K antibody (Solution B) mixtures added as previously described. The plate was once again covered, incubated for 4 hours at RT, and the fluorescent 665 nm and 620 nm signals were quantified.

Stimulation of Propagating HUVEC Cells (10 Min; 10 ng/mL VEGF) 1 and 2 Day Cell Plating

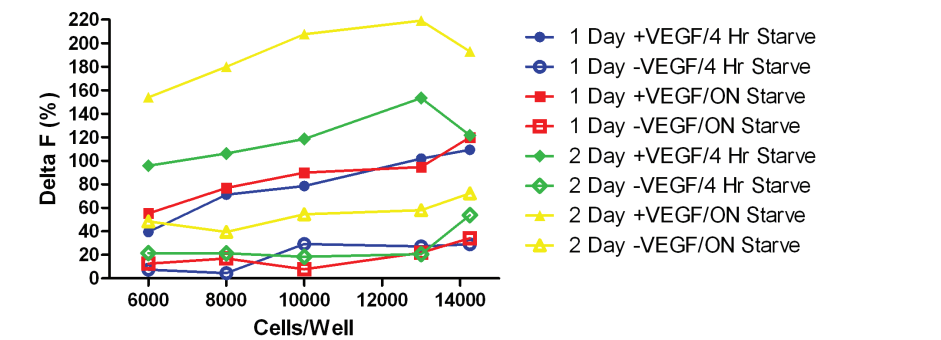


Figure 8 – VEGF stimulation (10 ng/mL; 10 minute stimulation time) using variable plating and starvation times.

The HTRF and ΔF ratio values were once again calculated as previously described.

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.

Further tests, using the optimized condition listed above, will look to confirm this finding, as well as look at more detailed pharmacology from agonists and antagonists of this pathway.

Conclusions

- Primary HUVEC cells provide an excellent way to measure the effects of compounds on important signaling pathways, providing more relevant data when compared to other currently used cancer cell lines.
- The HTRF phospho-AKT (Ser473) Assay provides the ability to monitor activity of the VEGF receptor cell signaling pathway in primary HUVEC cells.
- The filter-based detection system of the Synergy H4 is sensitive enough to accurately detect the change in signal caused by endogenous kinase phosphorylation events.
- The combination of cells, assay, and instrument provide a unique capability to measure the activity of important kinase signaling pathways.