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

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AKT is a serine/threonine protein kinase that plays a key role in cellular processes. 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.

Key Words:

Cellular Kinase Signaling
HUVEC
AKT
HTRF
Cell Signaling
Kinase
Automation
Detection

Introduction

The serine/threonine kinase AKT, also known as protein kinase B (PKB), mediates cell survival, therefore inhibiting apoptosis, or cellular death. It also regulates many cellular functions such as cell proliferation and differentiation, cellular migration, glucose and intermediary metabolism, and transcription. Additionally, AKT induces protein synthesis pathways, and is therefore a key signaling protein in the cellular pathways that lead to skeletal muscle hypertrophy, tumor development and general tissue growth. Its implication for cancer research and that of other disease states makes AKT an important target for drug discovery campaigns.

AKT is a key downstream intracellular convergence point for many cell signaling pathways. These diverse signaling pathways are activated by 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). AKT phosphorylation is linked through PI-3 kinase activity. AKT possesses a PH binding domain for binding PIP3, the product of PI-3 kinase phosphorylation of PIP2. By binding PIP3, AKT becomes correctively positioned at the membrane for phosphorylation by its activating kinases. One of these, the mammalian target of rapamycin complex 2 (mTORC2), phosphorylates AKT at its serine 473 residue as a first step to AKT phosphorylation by PDK1. Activated AKT can then go on to activate or deactivate its myriad substrates via its own kinase activity[1].

Here we demonstrate a homogeneous HTRF® phospho-AKT (Ser473) assay from CisBio US (Bedford, Massachusetts) for measuring AKT phosphorylation at serine residue 473 (Ser473) using primary human umbilical vein epithelial cells (HUVEC). Primary cells are neither altered nor transformed, for less risk of functional or phenotypical changes compared to other cell lines. Endothelial cells, such as these, provide robust and biologically relevant data for angiogenesis and cancer development, macromolecule and cell adhesion and transport, clotting, cell signaling pathway analysis, and other pharmacological studies. HUVECs, lining the umbilical vein, serve as the selective barrier between circulating blood and the underlying smooth muscle, and as they closely simulate in vivo environments, they are well suited for in vitro cellular research. Additionally, cells from multiple donors can be analyzed, allowing for a good estimation of physiological responses across human populations. Validation and pharmacology data demonstrate that the combination of assay, instrumentation, and cells are sufficiently sensitive to detect endogenous phosphorylation of this key signal transduction pathway.

Materials and Methods

Materials

Cryopreserved HUVEC (Catalog # CC-2517, Lot 152470), EBM® (Endothelial Base Medium) (Catalog # CC-3121), and EGM® SingleQuots (Catalog # CC-4133) were attained from Lonza (Walkersville, Maryland). Albumin (Human), USP, 25% Solution (Catalog # 1500233) was purchased from Baxter (Deerfield, IL). Collagen, Type 1 (Catalog # 354236) was purchased from BD Biosciences (Bedford, MA). Recombinant Human VEGF 165 (Catalog # 293-VE-010/CF) was purchased from R&D Systems (Minneapolis, MN). PI-103 Hydrochloride (Catalog # 2930) and LY294002 Hydrochloride (Catalog # 1130) were purchased from Tocris Bioscience (Ellisville, MO). The HTRF Phospho-AKT (Ser473) kit (Catalog # 64AKSPEG) was attained from Cisbio US (Bedford, MA).

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96-Well Clear, Flat Bottom, Polystyrene TC-Treated Microplates (Catalog # 3598) and 384-Well Low Volume, White, Round Bottom, Polystyrene Non-Treated Microplates (Catalog # 3674) were attained from Corning Life Sciences (Kennebunk, ME).

The EL406™ Microplate Washer Dispenser, and Precision™ Automated Microplate Pipetting System, as well as the Synergy™ H4 Hybrid Multi-Mode Microplate Reader were donated from BioTek Instruments (Winooski, VT).

Methods

Cisbio HTRF® phospho-AKT (Ser473) Assay Principle

The phospho-AKT (Ser473) assay is designed to detect and study activated AKT directly in whole cells, and is based on a sandwich immunoassay principle. Upon receptor activation, the kinases are subsequently activated, thus phosphorylating AKT kinase. After cell membrane lysis, an anti-kinase d2 labeled monoclonal antibody (Anti AKT-d2) and an anti-phospho-kinase Eu(III)-cryptate labeled monoclonal antibody (Anti pAKT-K) are introduced. Upon Eu(III)-cryptate excitation, phosphorylated AKT transfers energy to the d2 molecule, and emission is detected at 655 nm. In the absence of phosphorylated AKT, no energy is transferred, and emission from the Eu(III)-cryptate is detected at 620 nm.

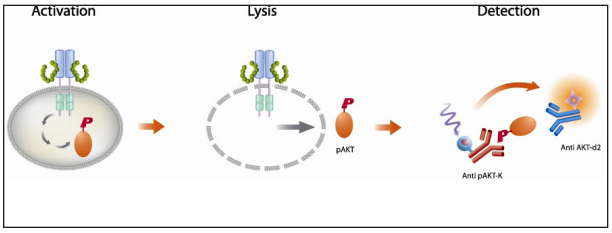

Figure 1. HTRF phospho-AKT (Ser473) Assay.

Instrumentation

The Precision was used for all compound serial titrations and transfers, as well as 96-well to 384-well lysate transfers. Cell, agonist EC_{80} and HTRF assay component dispensing, media removal and plate washing were performed with the EL406™. Fluorescent Eu(III)-cryptate and d2 signals were detected using the HTRF-certified Synergy™ H4.

Cell Processing

Cryopreserved HUVEC were thawed, resuspended in complete Clonetics® EGM Endothelial Cell Growth Medium, and propagated until the cells reached 60-70% confluency, in culture flasks. Cell concentrations of 6.5 x 10^4 cells/mL were then plated in 200 µL aliquots into clear, tissue culture-treated 96-well microplates and incubated at 37°C/5% CO_2 for 40 hours. The complete media was removed, the cells were washed with 200 µL DPBS wash, 100 µL base EBM media with 0.1% human serum albumin was added, and the cells were again incubated for four hours.

Assay Procedure

Agonist Assay: 50 µL of 3X titrated agonist was added to the 96-well plate, and incubated for 10 minutes at 37°C/5% CO_2. Media was removed from the well, 50 µL of 1X lysis buffer was added, and the plate was incubated for 20 minutes at room temperature on a plate shaker set at 750-1000RPM. Following incubation, 16 µL aliquots were transferred from the 96-well cell plate to an LV384-well assay plate. 4 µL of detection buffer containing the Anti AKT-d2 and Anti pAKT-K antibodies were then added to the well, the plate was covered, and incubated at room temperature for 4 hours. The fluorescent signal was then read using: 330 nm (Ex)/620 (Em)/665 (Em).

Antagonist Assay: 25 µL of 6X titrated antagonist was added to the 96-well plate, and incubated for 60 minutes at 37°C/5% CO_2. 25 µL of 6X agonist (EC_{80}) was added to the 96-well plate, and incubated for 10 minutes at 37°C/5% CO_2. The remainder of the procedure is as described for the agonist assay.

Instrument Detection Component		BioTek Catalog Number
Excitation Filter #1	330/80 nm	7082263
Emission Filter #1	620/10 nm	7082265
Emission Filter #2	665/8 nm	7082266
Dichroic	365 nm Cutoff	7138365

Optimized Instrument Settings			
Light Source	Xenon Flash Lamp	Delay After Plate Movement	0 mS
Measurements per Datapoint	40	Lamp Energy	High
Delay Before Collecting Data	100 µS	Data Collection Time	300 µS

Table 1. Synergy™ H4 Instrument Settings.

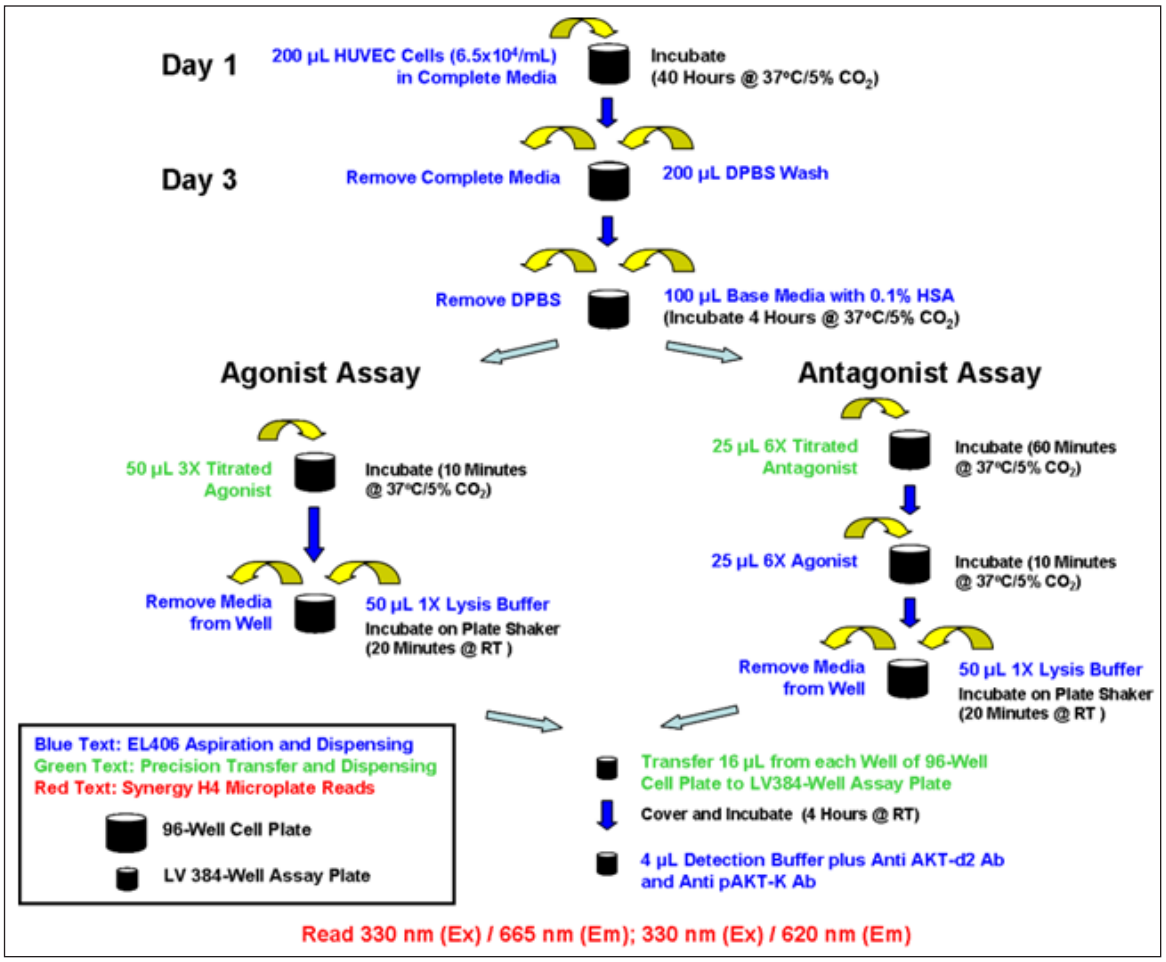

Figure 2. HTRF® phospho-AKT (Ser473) Automated Assay Protocol.

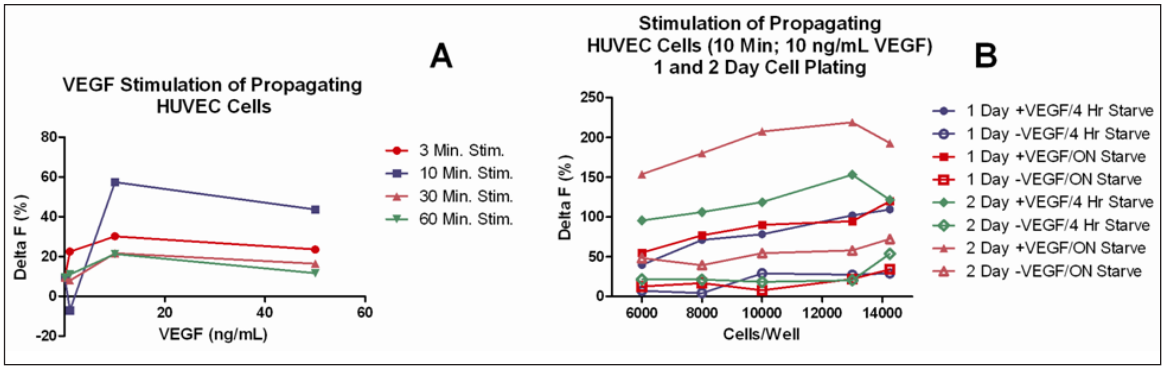

Figure 3. HTRF phospho-AKT Assay Optimization Results.

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 (ΔF) calculation. This value was determined using the following formula:

ΔF = ((Ratio(Cell Containing Well) – Ratio(Negative Control))/Ratio(Negative Control))

Results and Discussion

Assay Optimization Conditions

HUVEC were stimulated with VEGF, using concentrations of 0, 1, 10, and 50 ng/mL. Stimulation times used were 3, 10, 30, and 60 minutes. Per the HTRF and ΔF ratio values, and as seen in Figure 3A, it is apparent that the maximum VEGF stimulation occurs at a concentration of 10 ng/mL and a total of 10 minutes.

The aforementioned process was repeated using sixteen and forty-hour cell incubations in complete media, and four hour and overnight incubations using serum starvation media (EBM media with 0.1% HSA). The optimal 10 ng/mL VEGF concentration and 10-minute stimulation incubation were applied, and HTRF and ΔF ratio values were once again calculated. Per Figure 3B, it is apparent that the ΔF ratio for wells containing VEGF-stimulated cells increases with longer plating times and higher cell concentrations. Only at the highest cell concentrations and using a 2-day plating, do the ΔF ratios decrease. It can also be seen that unstimulated well ΔF ratios are stable for the first three conditions tested. Additionally, the ΔF ratios increase 2-3 fold using a two-day plating and overnight serum starvation media incubation.

Since stimulated cell ΔF ratios increase with longer plating times, and ΔF ratios do not increase for a two-day plating with four hour serum starvation media incubation, the optimal conditions for greatest fold stimulation are plating 13,000 cells/well, incubating them in complete media for 40 hours followed by a serum starved media incubation of four hours. Two-day plating and overnight incubation in serum starvation media were excluded as the ΔF ratio for unstimulated cells increases dramatically, reducing VEGF stimulation.

The conditions determined in the assay optimization were carried forth in the remaining validation experiments.

Automated Assay Validation

A Z'-Factor assay was performed to validate the automated procedure. The assay was run in antagonist mode, with 25 µL PI-103 (6X) added to each well as the control antagonist. After a 60 minute incubation at 37°C/5% CO_2, 25 µL VEGF, 60 ng/mL (6X) was added to each well as the control agonist, and the microplate was incubated for an additional 10 minutes. Forty replicates of 0 µM or 10 µM PI-103 (1X) were used as the positive and negative control, respectively. The remainder of the antagonist procedure was carried out as previously described.

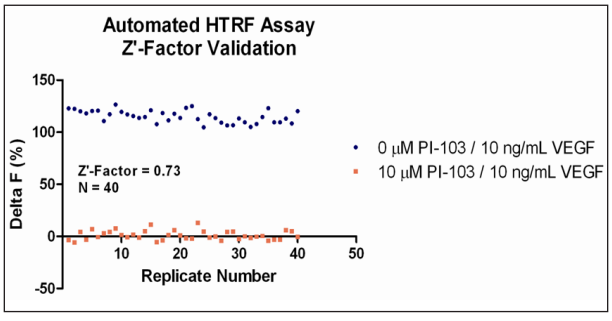

Figure 4. Automated Z'-Factor Assay Validation Data

As seen in Figure 4, the calculated Z'-factor equals 0.73, which correlates to excellent assay robustness as values greater than 0.5 are indicative of an excellent assay (2).

Assay Pharmacology Validation

The assay was further validated by creating an agonist dose response curve with VEGF. The assay was performed with 50 µL of VEGF concentrations ranging from 40 – 0 ng/mL added to the wells, and the remainder of the agonist procedure being performed as previously described.

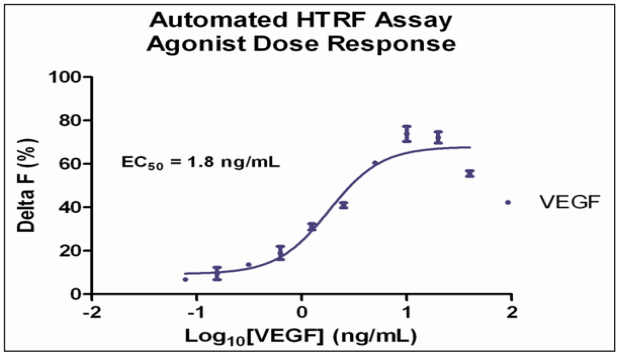

Figure 5. VEGF Agonist Validation

The EC_{50} value of 1.8 ng/mL (Figure 5) compares favorably to published EC_{50} values ranging from 1-6 ng/mL (3).

Antagonist dose response curves were also generated using PI-103 and LY294002 with concentrations of 10-0 µM and 100-0 µM, respectively. VEGF, at a 6X EC80 concentration of 60 ng/mL, was used as the agonist for the experiment.

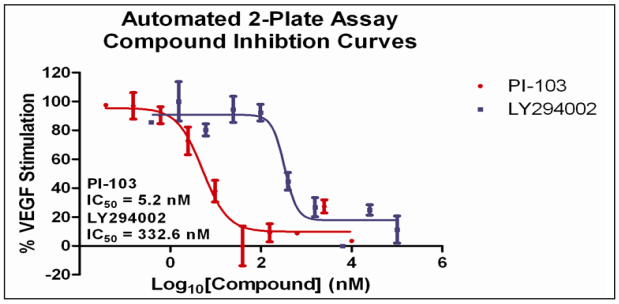

Figure 6. Antagonist Validation

Calculated IC_{50} values were 5.2 nM and 332.6 nM for PI-103 and LY294002, respectively (Figure 6). These values compare favorably to published IC_{50} values of 11 nM (4) for PI-103 and 310 and 730 nM for LY294002 (5,6).

Summary

Primary HUVEC provide an excellent method to measure compound effects on important signaling pathways, providing increased data relevance compared to immortalized cell lines. The HTRF phospho-AKT (Ser473) assay effectively monitors PI-3 kinase and AKT cell signaling pathways in these cells. The assay may be automated using a 2-microplate protocol where pathway stimulation or inhibition is carried out in a 96-well microplate, then cell lysate aliquots are transferred to a 384-well microplate for assaying. The Synergy™ H4 reader detects signal changes caused by endogenous kinase phosphorylation events. Automation of the assay provides excellent robustness, and appropriate inhibition pharmacology is evident at the level of PI-3 kinase.

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