Automated, High Throughput Methods for the Quantification of Endogenous Cellular Kinase Activity using HTRF Technology

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Introduction

Cellular kinases play an important role in relaying signals from activated receptors residing at the cell membrane to the interior of the cell, through signal transduction. The cellular processes in which they are associated include angiogenesis, cell growth, cell migration, and apoptosis. Overexpression of the kinase, or constitutive activity, has also been linked to a number of disease states, including vascular disease, bone disorders, and multiple forms of cancer. Therefore, cellular kinases continue to be an important target for small molecule and large molecule drug development. The ability to screen compounds or antibodies for their antagonistic effects on these signaling molecules, in a rapid and robust way, is critical to current efforts in this area.

Here we describe two methods using HTRF technology to quantify cellular kinase activity without overexpression of kinase or its substrate. Two specific kinases were included in the study:

1. Vascular Endothelial Growth Factor Receptor 2 (VEGFR2), one of multiple subtypes of the receptor tyrosine kinase VEGFR, is involved in mediation of almost all cellular responses to VEGF.

2. Signal Transducer and Activator of Transcription 3 (STAT3), acts as a transcription activator, and is phosphorylated by receptor-associated kinases in response to cytokines and growth factors.

Detection of phosphorylated kinase involves a sandwich immunoassay which includes an anti-phospho specific antibody coupled with an anti-total antibody labeled with either donor or acceptor fluors. Activation of the appropriate signaling cascade causes an increase in HTRF signal, whereas inhibition will demonstrate the opposite effect.

Automation of the STAT3 assay component dispense steps were performed in a high throughput, low-volume 384-well (LV384) format using a non-contact dispenser. The VEGFR2 kinase reaction and lysis step, in contrast, were performed in 96-well format.

Following a transfer step, the remaining portion of the assay procedure was performed once again in LV384 format. This process was easily completed using a liquid handler and combination washer/dispenser. Simultaneous, rapid detection of the fluorescent signal from the donor and acceptor molecules, in the aforementioned LV384-well format, was accomplished through the combination of a high performance Xenon flash lamp and dual matched PMTs incorporated into a novel HTS microplate reader. Optimization, validation, and pharmacology data demonstrate how the combination of assay chemistry, automation, and detection technologies create a robust method for high throughput screening of potential modulators of these important cell signaling pathways.

HTRF® Cellular Kinase Assays

The HTRF phospho-STAT3 (Tyr705) and phospho-VEGFR2 (Tyr1175) assays are designed for detecting and studying activated kinases directly in whole cells. Upon receptor activation, the kinases are activated, leading to kinase phosphorylation. After the lysis of the cell membrane, phosphorylated kinase can be detected upon the addition of two monoclonal antibodies: an anti-total 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 kinase, 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 no 665 nm signal is detected.
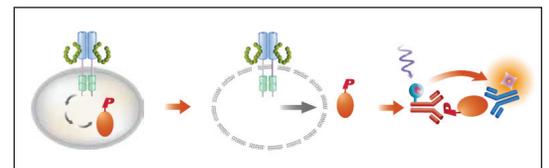

Figure 1. HTRF® phospho-STAT3 (Tyr705) and phospho-VEGFR2 (Tyr1175) Assay Process.

Key Words:

HTRF

Cellular Kinase

Cell Signaling

STAT3

VEGFR2

HUVEC

A431

HTS

Materials and Methods

Materials

Cells and Reagents

A431 cells (Catalog No. 85090402) were purchased from Sigma-Aldrich Corporation (Saint Louis, MO). Dulbecco's Modified Eagle Medium(DMEM), (Catalog No. 11995-065), Penicillin-Streptomycin, liquid (Catalog No. 15140-148), and Fetal Bovine Serum (FBS), Catalog No. 10437-028) was purchased from Life Technologies (Carlsbad, CA). Cryopreserved Human Umbilical Vein Endothelial Cells (HUVEC) (Catalog No. CC-2517, Lot 152470), EBM® (Endothelial Base Medium) (Catalog No. CC-3121), and EGM® SingleQuots® (Catalog # CC-4133) were donated by Lonza (Walkersville, MD). Human Serum Albumin (HSA), 25% Solution (Catalog No. 1500233) was purchased from Baxter (Deerfield, IL). HTRF® Phospho-STAT3 (Tyr705) (Catalog No. 62AT3PEG) and Phospho-VEGFR2 (Tyr1175) assay kits were provided by Cisbio Bioassays (Codolet, FR). Hank's Balanced Salt Solution (HBSS) was purchased from Mediatech, Inc. (Manassas, VA). Recombinant Human Epidermal Growth Factor (EGF) (Catalog No. cyt-217) and Recombinant Human Vascular Endothelial Growth Factor (VEGF) (Catalog No. cyt-241) were purchased from ProSpec-Tany Technogene Ltd. (Ness Ziona, Israel). SD 1008 (Catalog No. 3035), Stattic (Catalog No. 2798), Cryptotanshinone (Catalog No. 3713), Ki 8751 (Catalog No. 2542), DMH4 (Catalog No. 4471), and Human VEGFR2 Antibody (Ab) (Catalog No. AF357) were purchased from R&D Systems (Minneapolis, MN). 384 Well, Low Volume, Non-treated plates (Catalog No. 3674) were donated by Corning Life Sciences (Kennebunk, ME).

Instrumentation

MultiFlo™ Microplate Dispenser

The dispenser offers fast, accurate plate dispensing capabilities through its two peristaltic and two syringe pumps, with volumes ranging from 1-3000 µL. The instrument was used to dispense cells, agonist, lysis buffer, and antibody mixes in volumes as low as 4 µL.

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 EC_{80} agonist and HTRF assay components.

Precision™ Microplate Pipetting System

The Precision™ combines an 8-channel pipetting head and an 8-channel bulk reagent dispenser in one instrument. The instrument was used to titrate and dilute inhibitors, as well as transfer diluted inhibitors to the 96-well cell plate, and a portion of the lysate to the LV384-well plate in the VEGFR2 assay procedure.

Synergy™ NEO HTS Multi-Mode Microplate Reader

The reader combines a filter-based and monochromator-based detection system in one unit. The HTRF certified reader uses a filter-based system, high performance Xenon flash lamp, as well as dual matched PMTs to simultaneously detect the 665 nm and 620 nm fluorescent emissions from the assay chemistry.
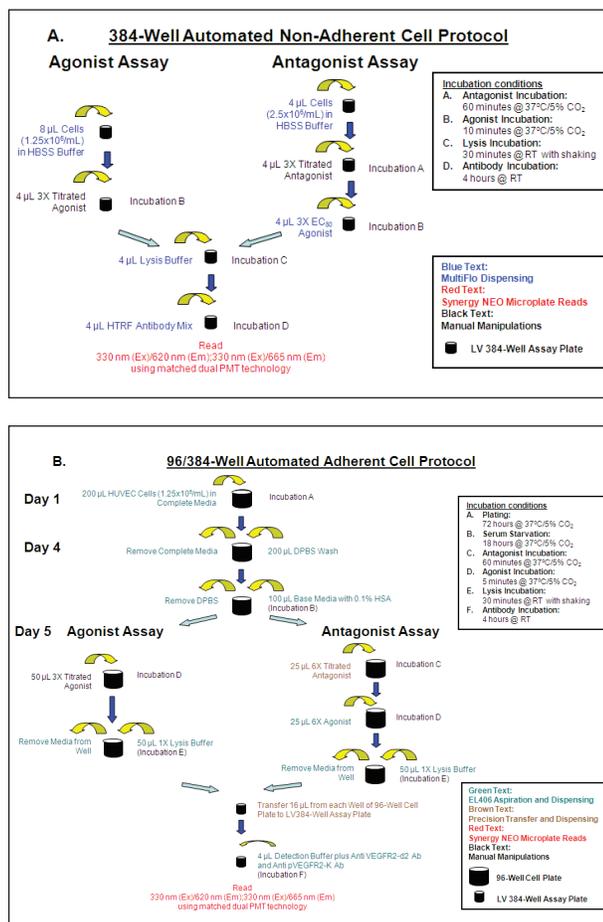
Automated Assay Procedures

Figure 2. **Automated phospho-kinase assay workflows. A.** phosphor-STAT3 Assay; **B.** phospho-VEGFR2 Assay. HTRF signal detection was carried out using the Xenon Flash Lamp of the Synergy NEO with the following settings: Delay after plate movement: 0 msec; Delay after excitation: 150 µsec; Integration time: 500 µsec; Read height: 8.0 mm.

Methods

Cell Propagation

A431 cells were propagated in complete medium, as described above. The cells were serum-starved 18 hours prior to running the assay by trypsinizing the cells from the flask, removing the complete medium, and replacing with serum-free medium (SFM) (DMEM plus 0.1% HSA). HUVECs were propagated in EBM plus EGM SingleQuots.

Cell Plating
Following the serum starvation period, A431 cells were removed from the flask and resuspended to a concentration of 2.5×10^6 cells/mL in HBSS buffer and plated in a 4 μL volume. HUVECs were trypsinized from the flask and resuspended to a concentration of 1.25×10^5 cells/mL in EBM. The cells were then plated in a 200 μL volume.

Ligand/Compound Preparation
EGF and VEGF were resuspended in Milli-Q water to a concentration of 100 μg/mL and then diluted appropriately in HBSS buffer (STAT3 assay) or EBM (VEGFR2 assay). Compounds were resuspended in 100% DMSO at a concentration of 10 mM, and then titrated in the same solvent. VEGFR2 Ab was resuspended in sterile PBS and titrated in EBM. The compounds and antibody were further diluted in HBSS buffer or medium as previously described to create the final assay concentrations.

Automated Assay Procedures

Assay Optimization Experiments
Optimization of assay parameters is essential to ensure the generation of robust, reliable experimental data. When monitoring the modulation of kinase signaling pathways, typical parameters include cell concentration, agonist stimulation time, and serum starvation length. Due to the fact that primary HUVECs were also incorporated into the VEGFR2 assay, post-cell plating incubation time was also optimized for this assay. Each condition tested was analyzed for its effect on the difference in signal between wells containing stimulated and unstimulated cells (assay window).

Assay Validation
Z'-factor, agonist, and antagonist titration experiments were performed to validate the optimized phospho-STAT3 and phospho-VEGFR2 automated assay procedures. Serial 1:3 EGF titrations were created in HBSS buffer. Concentrations tested ranged from 5000 – 0 nM. Serial 1:2 VEGF titrations were created in SFM. Concentrations tested ranged from 100 – 0 ng/mL. The concentrations yielding the largest stimulation, 600 nM EGF and 100 ng/mL VEGF, were then used as the positive controls in Z'-factor experiments to measure assay robustness, with wells containing no agonist being used as the negative control. The Z'-factor[1] value takes into account the difference in signal between a positive and negative control, as well as the variation in the signal amongst replicates. A scale of 0-1 is incorporated, with values ≥ 0.5 being indicative of an excellent assay. Finally, pharmacology validations were performed with the two automated assays. Three known STAT3 pathway inhibitors, SD 1008, Stattic, and Cryptotanshinone were used with the STAT3 assay. 11-point titrations were created using a 1:2 dilution scheme, starting at a 1X concentration of 100 μM. Two known VEGFR2 small molecule inhibitors, Ki 8751 and DMH4, as well as a VEGFR2 neutralizing antibody were also incorporated with the VEGFR2 assay. 5-point titrations were created using a 1:10 dilution scheme, starting at a 1X concentration of 1000 nM (small molecule) or 10 μg/mL (antibody).

Results and Discussion

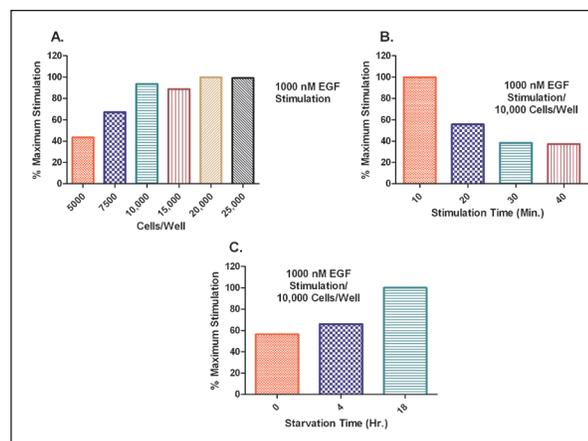#### Phospho-STAT3 Assay Optimization

Figure 3. phospho-STAT3 Assay optimization results. **A.** Cell concentration analysis (5,000-25,000 cells/well tested). **B.** Signaling pathway stimulation (10-40 minute stimulations). **C.** Serum starvation (0, 4 hour, or overnight starvations).

The results shown in Figure 3A demonstrate that cell concentrations above 10,000 cells/well yield no appreciable increase in assay window. Therefore 10,000 cells/well was chosen to lower cell propagation time and cost. Furthermore, 3B illustrates that rapid stimulation of the signaling pathway is seen at 37°C, following agonist addition. A 10 minute post agonist addition incubation time was then used for subsequent experiments. Finally, it is apparent from the data shown in Figure 3C that serum starving the A431 cells prior to assay performance serves to lower the basal level of STAT3 signaling pathway activity, thereby creating a larger assay window between stimulated and unstimulated cells.
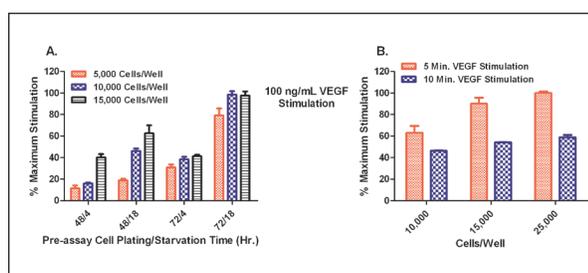
Phospho-VEGFR2 Assay Optimization

Figure 4. phospho-VEGFR2 Assay optimization results. **A.** Cell plating and serum starvation time analysis (2 and 3 day plating/4 hour and overnight starvations). **B.** Cell concentration and stimulation time test (5 and 10 minute stimulations/10,000-25,000 cells/well).

The data in Figure 4A demonstrate that allowing the cells to recover from plating and propagate for a period of three days, followed by an overnight serum starvation provided the largest assay window for each of the three cell concentrations tested (5,000-15,000 cells/well). Further optimization verified that higher cell concentrations, in addition to a minimized stimulation period, provided larger differences between stimulated and unstimulated cells (Figure 4B). The final conditions chosen included a 3 day plating and overnight (18 hour) serum starvation of 25,000 cells/well, followed by a 5 minute VEGF stimulation time.

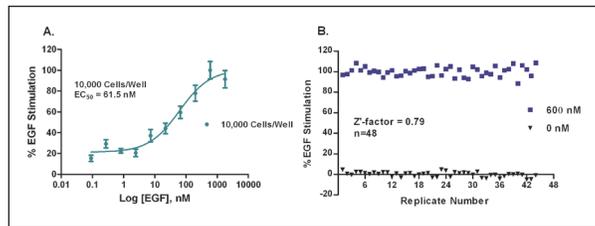#### Phospho-STAT3 Automated Assay Validation

Figure 5. phospho-STAT3 EGF titration and Z'-factor results. **A.** Data shown represents the average and standard deviation of four replicates tested at each EGF concentration. **B.** Assay run in agonist mode as previously described. Forty-four replicates of 600 nM and 0 nM EGF used as positive and negative control, respectively.

A Z' value of 0.79 was generated which, as explained previously, is indicative of an excellent, robust assay. The EC_{50} value of 61.5 nM for EGF stimulation of the STAT3 signaling pathway in A431 cells agrees with the value previously generated by the assay manufacturer of 180 nM. An EC_{80} value of approximately 200 nM was then used for subsequent antagonist testing.
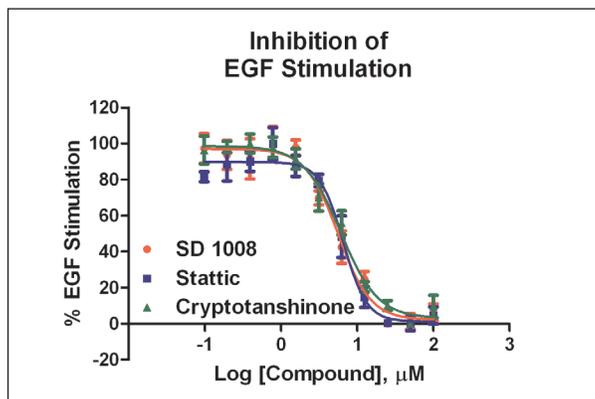

Figure 6. phospho-STAT3 Assay compound inhibition curves. Percent EGF stimulation plotted for each concentration of inhibitor tested.

Table 1. STAT3 Inhibitor IC_{50} Values (µM)		
Compound	**Generated IC_{50} Value**	**Literature IC_{50} Value**
SD 1008	5.6	Inhibition seen at 10 µM in as little as 30 minutes[2]
Stattic	6.6	5.1[3]
Cryptotanshinone	6.1	4.6[4]

Results from the antagonist dose response test were plotted using a Non-linear regression four-parameter curve fit. IC_{50} values were generated from each curve. The values were seen to be in close agreement to published literature values (Table 1), demonstrating the ability of the automated assay to produce accurate results when testing other unknown STAT3 pathway inhibitors.

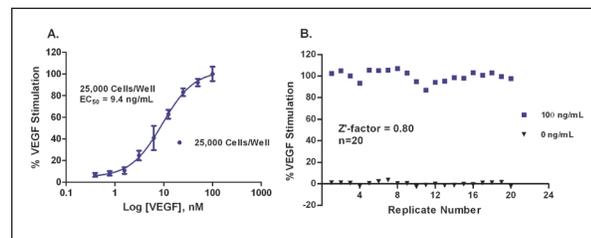#### Phospho-VEGFR2 Automated Assay Validation

Figure 7. phospho-VEGFR2 Assay validation results. **A.** Data shown represents the average and standard deviation of two replicates tested at each VEGF concentration. **B.** Assay run in agonist mode as previously described. Twenty replicates of 100 and 0 ng/mL VEGF used as positive and negative control, respectively.

The Z' value of 0.80 and similarity between the generated EC_{50} value for VEGF and that generated previously with HUVECs (Table 2), once again demonstrates the robustness of the automated process and ability to generated accurate results.
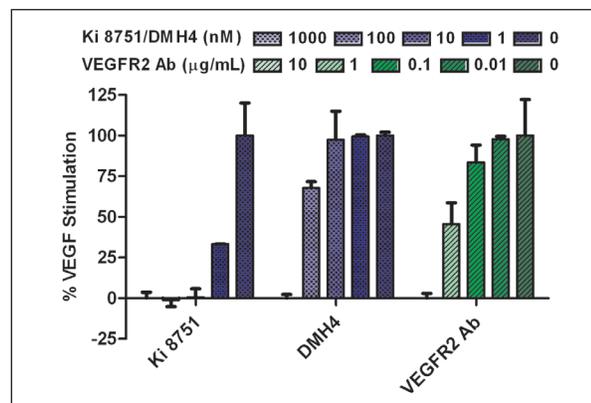

Figure 8. phospho-STAT3 Assay compound inhibition curves. Percent VEGF stimulation plotted for each concentration of inhibitor tested. Data shown represents average and standard deviation for n=2 replicates.

Table 2. VEGFR2 Ligand EC_{50}/Inhibitor IC_{50} Values		
Ligand/Inhibitor	Observed Value	Literature Value
Human VEGF (EC_{50})	9.4 ng/mL	1-6 ng/mL[5]
Ki 8751 (IC_{50})	< 1 nM	0.9 nM[6]
DMH4 (IC_{50})	100<IC_{50}<1000 nM	161 nM[7]
VEGFR2 Antibody (IC_{50})	0.1< IC_{50}<1 µg/mL	0.05-0.25 µg/mL[8]

Results from each inhibitor titration were plotted on a bar graph to show percent VEGF stimulation. IC_{50} values were estimated from each data set. The values once again agreed with published literature values (Table 2), demonstrating that the automated assay could produce accurate results with both small and large molecule inhibitors of VEGFR2 phosphorylation.

Conclusions

The HTRF® phospho-STAT3 and phospho-VEGFR2 assays afford sensitive, functional cell-based formats for the assessment of kinase signaling pathway modulation. Each assay procedure can be easily automated in 384-well or 96 to 384-well format using the aspiration and non-contact dispensing capabilities of the EL406 or MultiFlo. The Xenon-based excitation and filter-based detection system of the Synergy NEO microplate reader is also able to simultaneously quantify the emitted signals from the donor and acceptor fluors, meaning that high throughput detection of test plates can be easily accomplished. Finally, the combination of assay chemistry, as well as liquid handling and detection instrumentation, create an ideal solution for analysis of STAT3 and VEGFR2 pathway activators, in addition to small and large molecule inhibitors.

References

1. Zhang. et al.: A Simple Statistical Parameter for Use in Evaluation and Validation of High Throughput Screening Assays. J Biomol Screen 1999 4(2): 67-73.

2. Duan. et al.: 8-Benzyl-4-oxo-8-azabicyclo[3.2.1]oct-2-ene-6,7-dicarboxylic Acid (SD-1008), a Novel Janus Kinase 2 Inhibitor, Increases Chemotherapy Sensitivity in Human Ovarian Cancer Cells. Mol Pharmacol 2007 72(5): 1137-45.

3. Schust. et al.: Stattic: A Small-Molecule Inhibitor of STAT3 Activation and Dimerization. Chem Biol 2006 13(11): 1235-42.

4. Shin. et al.: Cryptotanshinone Inhibits Constitutive Signal Transducer and Activator of Transcription 3 Function through Blocking the Dimerization in DU145 Prostate Cancer Cells. Cancer Res 2009 69(1): 193-202.

5. Conn. et al.: Purification of a glycoprotein vascular endothelial cell mitogen from a rat glioma-derived cell line. Proc Natl Acad Sci U.S.A. 1990 87(4): 1323-1327.

6. Kubo. et al.: Novel potent orally active selective VEGFR-2 tyrosine kinase inhibitors: synthesis, structure-activity relationships, and antitumor activities of N-phenyl-N'-{4-(4-quinolyloxy)phenyl}ureas. J Med Chem 2005 48(5): 1359-1366.

7. Hao. et al.: In vivo structure-activity relationship study of dorsomorphin analogues identifies selective VEGF and BMP inhibitors. ACS Chem Biol 2010 5(2): 245-253.

8. Ferrara. et al.: The Biology of Vascular Endothelial Growth Factor. Endocr Rev 1997 18(1): 4-25.

Acknowledgements

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