Determination of Endogenous Cellular Kinase Activity through the Use of an Automated, High Throughput AlphaScreen®-Based Method

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Introduction

Cellular kinases are a key component in numerous cell signaling pathways. They play a role in relaying messages from receptors positioned at the cell membrane to other locations within the cell. The processes in which they are a part of are crucial for the development and life cycle of a cell and organism. These include, but are not limited to angiogenesis, cell growth, cell migration, and apoptosis. Over expression, or constitutive kinase activity, has also been linked to a number of disease states including vascular disease, bone disorders, and multiple forms of cancer. Because of this, cellular kinases including receptor tyrosine kinases and protein kinases, continue to be one of the most highly screened targets in small and large molecule drug development. The ability to perform screens on compounds or antibodies to determine their antagonistic effects on these signaling molecules in a rapid and robust fashion is therefore critical to current efforts in this area.

Here we describe a cell-based method using AlphaScreen® SureFire® technology to quantify the phosphorylation of Signal Transducer and Activator of Transcription 3 (STAT3). Following pathway stimulation by cytokines or growth factors, STAT3 is phosphorylated by receptor-associated kinases. Translocation to the nucleus then follows where the protein acts as a transcription activator. Detection of phosphorylated kinase involves the incorporation of AlphaScreen donor and acceptor beads, in addition to antibodies specific for either the STAT3 protein or the Tyr705 phosphorylation site. Activation of the appropriate signaling cascade causes an increase in emitted luminescent signal between 520-620 nm, whereas antagonism will demonstrate the opposite effect.

The assay was automated using both an adherent and non-adherent cell method. All dispense steps were accomplished using non-contact dispensing and the incorporation of a 96-pin wash manifold for the adherent cell model. Rapid quantification of the luminescent signal from the acceptor bead, in LV384-well format, was accomplished through the combination of high performance laser-based excitation and filter-based PMT detection incorporated into a novel HTS microplate reader.

AlphaScreen® SureFire® STAT3 (pTyr705) Assay
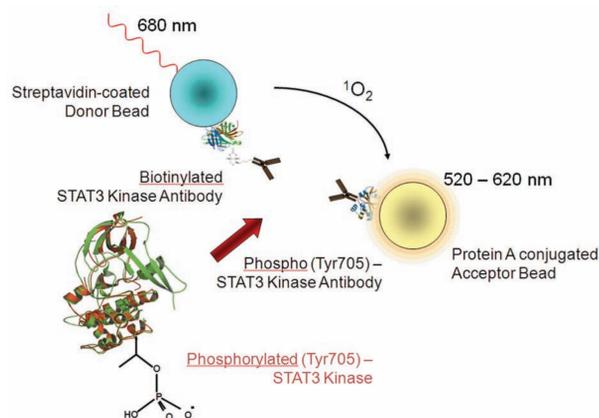
Figure 1 – Sandwich antibody complexes are formed in the presence of analyte (pSTAT3). The complex is then captured by AlphaScreen donor and acceptor beads, bringing them into close proximity. The excitation of the donor bead at 680 nm provokes the release of singlet oxygen molecules that triggers a cascade of energy transfer in the acceptor beads, resulting in the emission of light at 520-620 nm.

Materials and Methods

Materials

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) were purchased from Life Technologies (Carlsbad, CA). Human Serum Albumin (HSA), 25% Solution (Catalog No. 1500233) was purchased from Baxter (Deerfield, IL). AlphaScreen SureFire STAT3 (p-Tyr705) Assay Kit - 10,000 Points (Catalog No. TGRS3S10K) was purchased from Perkin Elmer (Waltham, MA). Hank's Balanced Salt Solution (HBSS) was purchased from Mediatech, Inc. (Manassas, VA). Recombinant Human Epidermal Growth Factor (EGF) (Catalog No. cyt-217) was purchased from ProSpec-Tany Technogene Ltd., (Ness Ziona, Israel). SD 1008 (Catalog No. 3035), Stattic (Catalog No. 2798), and Cryptotanshinone (Catalog No. 3713) were purchased from R&D Systems (Minneapolis, MN). 384 Well, Low Volume, Non-treated plates (Catalog No. 3674), 384 Well, Low Volume, TC treated plates (3826), and Breathable sealing tape (Catalog No. 3345) were donated by Corning Life Sciences (Kennebunk, ME).

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).

Cell Plating: For the adherent assay, cells were diluted to 1.0×10^6 cells/mL and plated in a 10 µL volume. The plates were covered with breathable sealing tape to prevent evaporation and incubated at 37°C/5% CO_2 for the serum starvation period. For the non-adherent cell assay, 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.

**Ligand/Compound Preparation:** EGF was resuspended in Milli-Q water at a concentration of 16.07 µM. The ligand was then titrated using 1:3 dilutions in either SFM (adherent cell protocol) or HBSS buffer (non-adherent cell protocol). Compounds were resuspended in 100% DMSO at a concentration of 10 mM. They were then titrated in DMSO using 1:2 dilutions. The compounds were further diluted 1:25 in either SFM or HBSS buffer to create the final 4X concentration.

BioTek Instrumentation

MultiFlo™ Microplate Dispenser. The dispenser offers fast, accurate plate dispensing capabilities through its two peristaltic and two syringe pumps, with volumes ranging from 0.5-3000 µL. The instrument was used to dispense cells, agonist, modified lysis buffer (non-adherent cell assay), and acceptor and donor bead mixes in volumes as low as 2 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 0.5-3000 µL/well. The instrument was used in the adherent cell assay to remove media and compounds, wash the cells with Hank's Balanced Salt Solution (HBSS), and add a 2 µL volume of modified lysis buffer.

Synergy™ NEO HTS Multi-Mode Microplate Reader. The reader combines a filter- and monochromator-based detection system in one unit. A dedicated high performance laser and filter-based PMT detection system are used for excitation of the donor beads and quantification of the emitted luminescent signal.
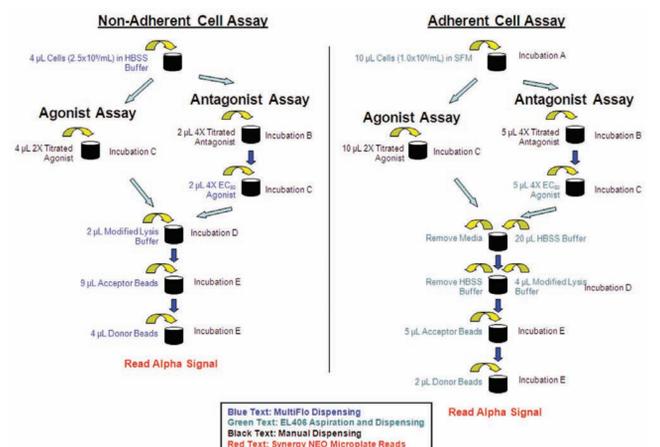
Figure 2 – Automated assay workflows. Incubation conditions: A. Serum Starvation - 18 hours @ 37°C/5% CO_2; B. Antagonist Incubation - 60 minutes @ 37°C/5% CO_2; C. Agonist Incubation - 10 minutes @ 37°C/5% CO_2; D. Lysis Incubation - 30 minutes @ RT with shaking; E. AlphaScreen Bead Incubation - 2 minutes @ RT with shaking followed by 2 hours @ RT. AlphaScreen signal detection was carried out using the Synergy NEO with the following settings: Delay after plate movement: 0 msec; Excitation time: 80 msec; Delay after excitation: 120 msec; Integration time: 160 msec; Read height: 8.0 nm; Gain 200.

Assay Optimization – Cell Lysis

Initial experiments revealed ineffective cell lysis, which negatively impacted data quality. A modified lysis buffer, which incorporates the Activation buffer into the lysis buffer, is recommended by the manufacturer for cells that are more difficult to lyse. A test of the two lysis buffers was conducted using three different cell concentrations by evaluating the signal ratio of cells stimulated with high and low concentrations of EGF. The non-adherent cell protocol was used for the evaluation.
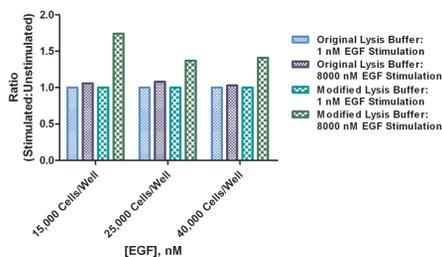

Figure 3 – Variable lysis buffer evaluation results. Ratios for signal from stimulated (8000 nM EGF) and unstimulated (1 nM EGF) cells, lysed with the original or modified lysis buffer.

The results shown in Figure 3 demonstrate that the modified lysis buffer, containing Activation buffer, provides a more complete cell lysis. A greater concentration of phosphorylated kinase is able to interact with the donor and acceptor beads, creating a higher signal from wells containing stimulated cells. It can also be seen that wells containing lower numbers of cells (15,000 cells/well) provide a larger signal ratio than wells containing higher cell concentrations. The larger number of cells may have a negative impact on the results from the assay. This hypothesis was tested in the subsequent cell concentration evaluation.

Assay Optimization – Cell Concentration

Three cell concentrations (5,000, 10,000, and 15,000 cells/well) were tested with the non-adherent cell assay to determine if larger signal ratios between stimulated and unstimulated cells could be achieved. The adherent cell assay was also performed using the same concentrations. The protocol used for this assay format calls for removal of all media before the addition of lysis buffer. It was thought that by adding an undiluted lysis buffer to the cells, a more robust assay could be achieved.
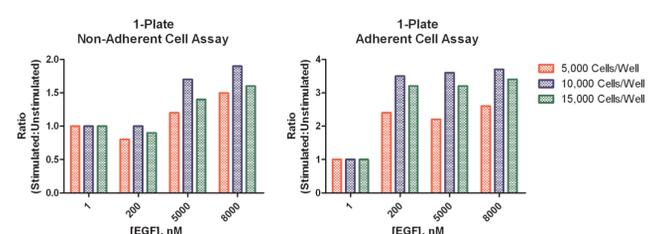

Figure 4 – Cell concentration optimization ratios. Alpha signal ratios for cells stimulated with 8,000, 5,000, 200, or 1 nM EGF. Cells were lysed with the modified lysis buffer.

The highest ratios of signal from stimulated and unstimulated cells were generated using a concentration of 10,000 cells/well. This was true for both assay formats. Results also demonstrated that higher ratios were attained with the adherent cell assay format. This was most likely due to the addition of lysis buffer directly to a well containing little or no current volume. Due to this finding, both assay formats would be carried forward for further testing, using a cell concentration of 10,000 cells/well.

Automated Assay Validation

Z'-factor

A Z'-factor experiment was performed to validate the optimized automated assay procedures. 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. Therefore, the measurement is an excellent estimation of data quality generated from subsequent experiments using the two assay formats.
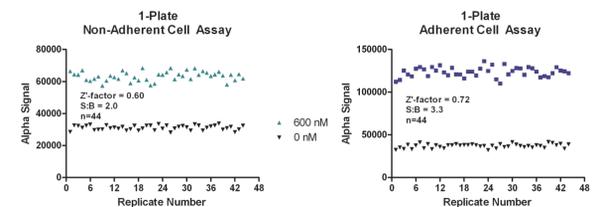

Figure 5 – Z'-factor validation data. The assay was run in agonist mode, with EGF being used as the control agonist. Forty-four replicates of 600 nM and 0 nM EGF were used as the positive and negative control, respectively. Cells were prepared, and assays run as previously described.

Z' values of 0.60 and 0.72 were generated with the non-adherent and adherent cell assays, respectively. These values, as explained previously, are indicative of excellent, robust assays using both formats tested.

Agonist Titration

The assays were further validated by creating agonist dose response curves with EGF. Serial 1:3 titrations were created in HBSS buffer or SFM, as previously described. Concentrations tested ranged from 8,000 – 0 nM.
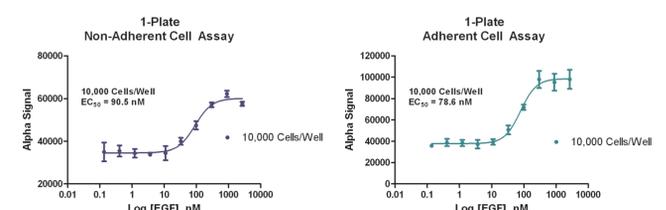

Figure 6 – EGF agonist titration results. Data shown represents the average and standard deviation of four replicates tested at each EGF concentration.

The EC_{50} values of 90.5 and 78.6 nM for EGF stimulation of the STAT3 signaling pathway in A431 cells agree with the value of 180 nM previously generated by the assay manufacturer. An EC_{80} value of approximately 200 nM was generated from the EC_{50} value and hill slope from each curve. This concentration of EGF was used for subsequent antagonist testing.

Antagonist Evaluation

A pharmacology validation was performed using the three known STAT3 pathway inhibitors, SD 1008, Stattic, and Cryptotanshinone. 11-point titrations were created using a 1:2 dilution scheme, starting at a 1X concentration of 100 µM. Following the inhibitor incubation period, EGF was added at a final 1X EC_{80} concentration.
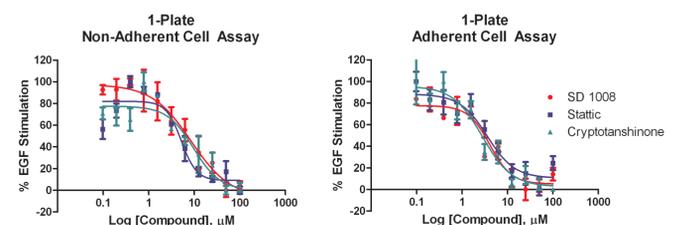
Figure 7 – Compound inhibition curves. Percent EGF stimulation plotted for each concentration of inhibitor tested. Data shown represents average and standard deviation for n=4 replicates.

STAT3 Inhibitor IC_{50} Values (µM)			
	Generated IC_{50} Value		Literature IC_{50} Value
Compound	Non-Adherent Cell Assay	Adherent Cell Assay	
SD 1008	8.6	4.1	Inhibition seen at 10 µM in as little as 30 minutes[2]
Stattic	5.2	3.6	5.1[3]
Cryptotanshinone	9.7	2.8	4.6[4]

Table 1 – STAT3 inhibitor IC_{50} values.

Inhibition curves and IC_{50} values were generated for each compound with the non-adherent and adherent cell assay methods. Results from each assay format were then compared to published literature values. Close agreement between data sets demonstrates the ability of each assay format to produce accurate results when testing other unknown STAT3 pathway inhibitors.

Conclusions

- The AlphaScreen SureFire assay format affords a sensitive, functional cell-based format for the assessment of kinase signaling pathway modulation.

- The adherent cell protocol provides a more robust assay platform with a larger assay window and higher Z' values.

- Each assay procedure can be easily automated in 384-well format using the aspiration and non-contact dispensing capabilities of the EL406 or MultiFlo.

- The laser-based excitation and filter-based detection system of the Synergy NEO microplate reader is able to easily and efficiently quantify the emitted signal from the AlphaScreen Acceptor beads.

- The combination of liquid handling and detection instrumentation create an ideal solution for analysis of STAT3 pathway activators and inhibitors using Alpha technology.

[1]Zhang, et al.: A Simple Statistical Parameter for Use in Evaluation and Validation of High Throughput Screening Assays. Journal of Biomolecular Screening 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. Molecular Pharmacology 2007 72(5): 1137-45. [3]Schust. et al.: Stattic: A Small-Molecule Inhibitor of STAT3 Activation and Dimerization. Chemistry and Biology 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 Research 2009 69(1): 193-202. AlphaScreen and SureFire are registered trademarks of PerkinElmer, Inc.