Automation of a Homogeneous Proximity Assay for Detection of ERK1/2 or SMAD3 Phosphorylation

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Key Words:

Erk1/2

SMAD3

EGF

EGFR

TGF-β

Phosphoproteins

AlphaLISA®

SureFire™

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Introduction

The transforming growth factor-β (TGF-β) superfamily consists of a range of proteins involved in a wide variety of biological processes such as cell growth, differentiation, development and apoptosis. Members of this superfamily are encoded by 28 genes in the human genome and include TFG-β isoforms as well as activins and bone morphogenetic proteins (BMPs)[1]. Cell signaling is initiated by cell surface receptor ligand binding events resulting in the activation and subsequent formation of heterotrimeric complexes of type I and type II serine/threonine receptor kinases. The type II receptors have been shown to bind ligand and activate type I receptors via phosphorylation. TGF-β signaling occurs within the cell through the Smad family of transcriptional activators[1]. Smad family members fall into three subfamilies: receptor-activated Smads (R-smads), common mediator Smads (Co-Smads) and inhibitory Smads (I-Smads). The concomitant phosphorylation of the R-Smads by activated Type 1 receptors initiates association of phosphorylated R-Smad proteins with Co-Smad. Once associated, the R-Smad/Co-Smad complexes translocate to the nucleus where interaction occurs with a range of nuclear protein partners. I-Smads are induced by TGF-β family members exerting a negative feedback loop via competitive inhibition at the receptor level and marking the receptors for degradation[1]. R-Smad 2 and 3, present in the TGF-β/activin Smad pathway, are well studied phosphoproteins for their potential as drug targets for disorders such as cardiovascular, musculoskeletal, fibrosis and cancer.

Protein kinases are components of large signaling networks responsible for propagating extracellular stimuli via cell surface receptors to assist in regulating a wide range of cellular activities. Stimuli including growth factors, cytokines, hormones and heat stress can activate signaling via formation of heteromeric receptor complexes such as receptor tyrosine kinase receptors (RTKs) and G protein-coupled receptors (GPCRs) or epidermal growth factor receptors (EGFRs). Aberrant regulation of a number of mitogen-activated protein kinase (MAPK) associated pathways have been associated with diseases such as cancer, Alzheimer's and obesity, among others. Extracellular signal-regulated kinases 1 and 2 (ERK1/2) are members of the MAPK superfamily. ERK 1/2 have been shown to be regulated by both RTK and GPCR activation as well as playing a regulatory role in Smad signaling pathways.

Here we investigate the performance of two homogeneous high-throughput screening assays capable of screening both modulators of receptor activation (e.g. agonists and antagonists) as well as intracellularly acting agents, such as inhibitors of upstream events (Figure 1). The assays were coupled to automated processes for increased throughput. Smad3 or ERK1/2 phosphorylation was measured following endogenous receptor activation in HeLa or HEK293 cell lines, respectively. The pharmacology of known inhibitors was also investigated.

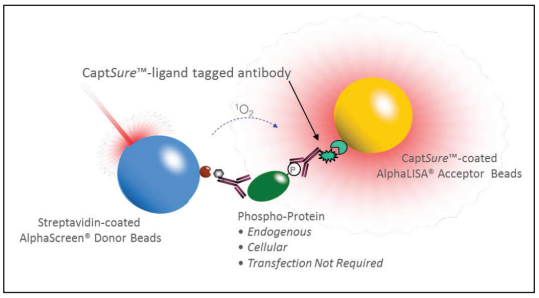

Figure 1. **Assay schematic for AlphaLISA® homogeneous proximity assay principle for the detection of phosphorylated proteins.** The AlphaLISA® SureFire® Ultra™ assay kits utilize Alpha beads that are each coated to specifically capture the assay antibodies. The Donor bead is coated with streptavidin to capture the biotinylated antibody. The Acceptor bead is coated with a proprietary "CaptSure™" agent that immobilizes the other assay antibody. Upon excitation, the AlphaLISA® donor bead generates singlet oxygen molecules. If the acceptor bead is in close proximity due to the creation of a sandwich immunoassay, the singlet oxygen molecules will trigger a cascade of energy transfer in the acceptor bead, resulting in light emission at 615 nm.

Instrumentation

MultiFlo™ FX Multi-Mode Dispenser

MultiFlo™ FX is an automated multi-mode reagent dispenser for 6- to 1536-well microplates offering BioTek's unique Parallel Dispense™ technology. Up to four independent reagents can be dispensed in parallel without potential carryover. The instrument was used to dispense assay specific reagents to the 384-well assay plates.

Cytation™ 5 Cell Imaging Multi-Mode Reader

Cytation 5 combines automated digital microscopy and conventional microplate detection in a configurable, upgradable platform. Cytation 5 includes both filter-based and monochromator-based optics for multi-mode versatility and offers laser-based excitation for Alpha assays.

Materials and Methods

Reagents

AlphaLISA® SureFire™ Ultra phospho-ERK1/2 (No. ALSU-PERK-A500) and phospho-Smad3 Kits (No. ALSU-PSM3-A500) were from PerkinElmer (Waltham, MA, USA)..

Assay Plates

AlphaLISA: CulturPlate™ -384 white, opaque 384-well (No.6007680) and AlphaPlate™ -384, grey, opaque, 384-well (No. 6005350) microplates were from PerkinElmer (Waltham, MA, USA).

Instrument Settings

The Cytation 5 Cell Imaging Multi-Mode Reader was used with the settings shown in Table 1.

Cytation™ 5 Read Parameters	
Mode	Alpha
Gain	120
Delay after plate movement	0 msec
Excitation time	80 msec
Delay after excitation	120 msec
Integration	160 µsec
Read height	8.00 mm

Table 1. Cytation 5 AlphaLISA® reading parameters used in Gen5™ Data Analysis Software.

AlphaLISA® Control Lysate Assay

p-ERK1/2 and p-Smad3 control lysates were prepared as 11-pt., 1:3 serial dilutions, including a zero percent control lysate, in lysis buffer that was prepared as per the manufacturers recommendation. Quadruplicate samples were then transferred, 10 µL each, to a 384-well AlphaPlate. Acceptor Mix was prepared as per the manufacturer's recommendation: Activation Buffer was diluted 25-fold in Reaction buffer and Acceptor beads 50-fold into the same Reaction buffer and 5 µL added to each well. For the p-ERK1/2 assay the plate was placed on an orbital shaker for the time needed to prepare the Donor Mix. For the p-Smad3 assay the plate was placed on an orbital shaker and allowed to incubate for 60 minutes at room temperature (RT). Donor Mix was prepared as per the manufacturer's recommendation: Donor beads were diluted 50-fold in Dilution buffer and 5 µL added to each well. For the p-ERK1/2 assay the plate was incubated for a minimum of 2 hours at RT, or up to overnight. For the p-Smad assay the plate was incubated for a minimum of an additional 60 minutes, or up to overnight, following addition of the Donor Mix. Following the final incubation period the plate was read on the microplate reader.

AlphaLISA Cell-based Assay

HEK293 or HeLa cells were cultured using standard tissue culture methods in Advanced DMEM medium supplemented with 10% FBS, 1x P/S/G @ 37 °C, 5% CO_2 in a humidified incubator. Cells were harvested at ~ 80-90% confluency and quadruplicate wells were seeded with 80 µL of cells at the appropriate cell density in 384-well CulturPlates. The cells were allowed to adhere overnight prior to performing the assay. For the p-ERK1/2 assay the cells were serum starved with 80 µL FBS-free media for ~2 hours prior to performing the assay.

Agonist Titrations

Agonist titrations were performed as per the manufacturers' recommendations, with the following modifications, for both p-ERK1/2 and p-Smad3 assays. An 11-pt., 1:3 serial dilution series, including a zero data point, was prepared for the agonists EGF and TGF-β for stimulation of HEK293 and HeLa cells, respectively. A no-cell control was also added for each experiment as well as control lysate prepared at 25%. Briefly, for the p-ERK1/2 assay, following serum starvation, 65 µL of media was removed, leaving 15 µL of residual media, cells were treated with 5 µL of the EGF dilution series prepared at 4x the final concentration (f.c.) and allowed to incubate for 10 minutes @ 37 °C, 5% CO_2 in a humidified incubator. For the p-Smad3 assay 40 µL of media was removed, leaving a 40 µL residual, cells were treated with 20 µL of the TGF-β dilution series prepared at 3x the f.c., and allowed to incubate for 60 minutes @ 37 °C, 5% CO_2 in a humidified incubator. Following incubation, all media was removed and cells were lysed with 10 µL 1x lysis buffer with shaking for 10 min. The AlphaLISA assays were performed as described above and the Alpha signal was read on a microplate reader.

Inhibitor Titrations

Inhibitor titrations were performed as per the manufacturer's recommendations, with the following modifications, for both p-ERK1/2 and p-Smad3 assays. An 11-pt., 1:3 serial dilution series, including a zero data point, of the inhibitors AG1478 (EGF-R inhibitor) and SB432542 (TGF-β -R inhibitor) was prepared for inhibition of HEK and HeLa stimulation, respectively. A no-cell control was also added for each experiment as well as control lysate prepared at 25%. Briefly, for the p-ERK1/2 assay, following serum starvation, 70 µL of media was removed, leaving 10 µL of residual media, cells were treated with 5 µL of the AG1478 dilution series prepared at 3x the f.c. and allowed to incubate for 60 min. At 37 °C, 5% CO_2 in a humidified incubator. For the p-Smad3 assay 60 µL of media was removed, leaving a 20 µL residual, cells were treated with 20 µL of the SB432542 dilution series prepared at 2x the f.c., and allowed to incubate for 60 minutes @ 37 °C, 5% CO_2 in a humidified incubator. Following incubation with the appropriate inhibitor the EC_{80} of the appropriate agonist was added to all wells as described above for agonist titrations, 5 µL of 4x or 20 µL 3x for EGF or TGF-β, respectively, and incubated for the appropriate time. Following incubation, all media was removed and cells were lysed with 10 µL 1x lysis buffer with shaking for 10 minutes. The AlphaLISA assays were performed as described above and the Alpha signal was read on a microplate reader.

Results and Discussion

AlphaLISA Control Lysate Assay

Positive control lysates for p-ERK1/2 and p-Smad3 provided from the kit manufacturer were used for optimization of Cytation 5 reader parameters (Table 1) and determination of the optimal control concentration for use when performing cell-based assays. As can be seen in figure 4, the data can be fit using a second order polynomial (quadratic) equation. High signal-to-background (S/B) were detected for both control lysates, S/B=1,432 and 2,522 for p-ERK1/2 and p-Smad3, respectively.

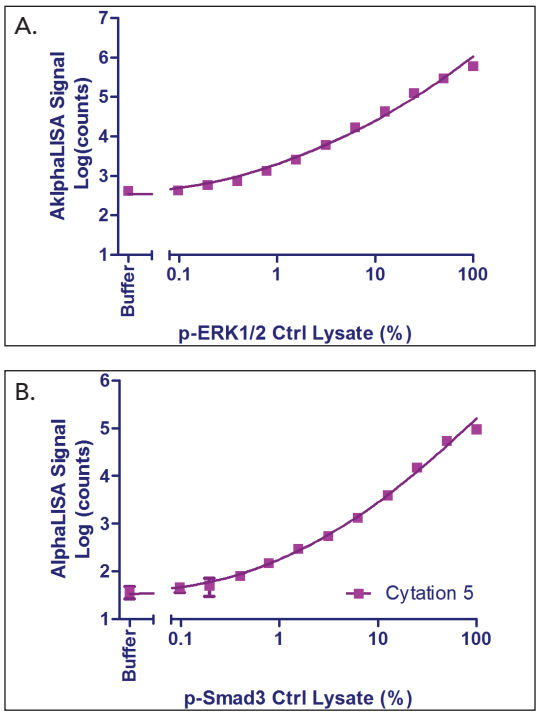

Figure 2. **Control Lysate Standard Curves. A)** AlphaLISA p-ERK1/2 Assay. **B)** AlphaLISA p-Smad3 Assay.

AlphaLISA Agonist Titration

Agonist dose response titrations of EGF and TGF-β were prepared for stimulation of HEK293 or HeLa cells, respectively. The cells were stimulated and the production of phosphorylated ERK1/2 and Smad3 was detected and plotted versus Alpha signal. The data can be fit using a Hill Slope model (Figure 3).

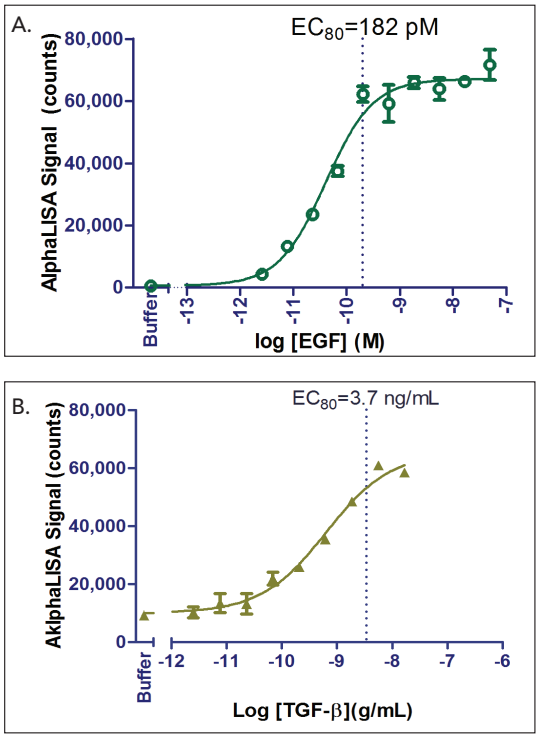

Figure 3. **Agonist Titration Curves. A)** AlphaLISA p-ERK1/2 Assay. **B)** AlphaLISA p-Smad3 Assay.

The AlphaLISA assay showed excellent dynamic range, covering nearly 4 decades, and good correlation between replicates for both assays (Figure 3). The agonist dose response curves yielded EC_{80} values of 182 pM and 3.7 ng/mL for p-ERK1/2 and p-Smad3, respectively. The EC_{80} determinants were subsequently used for inhibition studies. The EC_{50} value of 40 pM EGF for EGFR stimulation of HEK293 cells correlates well with previously generated data provided by the manufacturer (39 pM).

AlphaLISA Inhibitor Titration

Inhibitor dose response titrations of the potent selective kinase inhibitors AG1478 and SB432542 were prepared to evaluate inhibition of p-ERK1/2 and p-Smad3 phosphorylation, respectively. Following incubation with inhibitor, cells were stimulated with the EC_{80} of the appropriate agonist prior to detection of phosphorylated product. The data can be fit using a Hill Slope model as shown in figure 4.

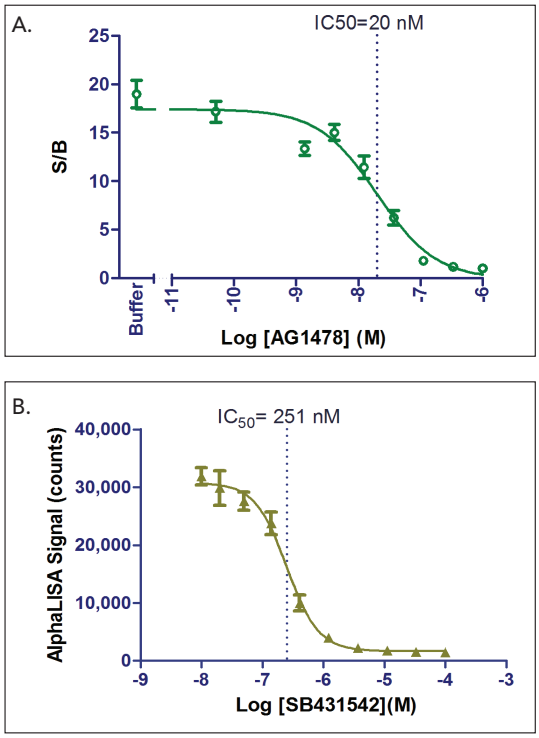

Figure 4. **Agonist Titration Curves. A)** AlphaLISA p-ERK1/2 Assay. **B)** AlphaLISA p-Smad3 Assay.

The potent EGFR tyrosine kinase inhibitor AG1478 was determined to have an IC_{50} of 20 nM, consistent with previously reported values[1] . SB431542 is a potent and selective inhibitor of TGF-β type 1 receptor activin receptor-like kinase ALK5, and its relatives ALK4 and ALK7. The dose response curve and IC_{50} of 251 nM correlate well with previously reported data[2].

Conclusion

The assays were performed in their entirety in a HTS compatible 384-well microplate format using automated liquid handling for cell seeding and reagent dispensing. The homogenous assay format allows for improved workflow as compared to the alternative 2-plate protocol requiring culturing and lysis in a 96-well format and transfer of lysate to the higher density 384-well assay plate. Demonstration of both agonist stimulation of kinase phosphorylation and inhibition of receptor signaling were shown for two independent pathways leading to the generation of p-ERK1/2 and p-Smad3. The combination of assay and instrumentation provide an ideal solution for high-throughput detection of these phosphorylation events.

References

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