

Automation of Homogeneous Proximity assays for Detection of Erk1/2 or Smad3 Phosphorylation

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Abstract

Cell signaling is generally initiated by cell surface receptor ligand binding events resulting in activation of serine/threonine receptor kinases. The concomitant phosphorylation of cytoplasmic signaling molecules begins a signaling cascade. The transforming growth factor- β (TGF- β) superfamily consist of a range of proteins involved in a wide array of biological processes such as cell growth, differentiation, and development. TGF- β signaling occurs with the cell through the Smad family of transcriptional activators. Smad 2 and Smad 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. ERK 1 and 2 (extracellular signal-regulated kinase 1 and 2) have been shown to be regulated by both receptor tyrosine kinase receptors (RTKs) and G protein-coupled receptors (GPCRs) activation. Erk1/2 have been shown to have a regulatory role in the Smad signaling pathway as well. 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. 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.

AlphaLISA[®] Assay Principle

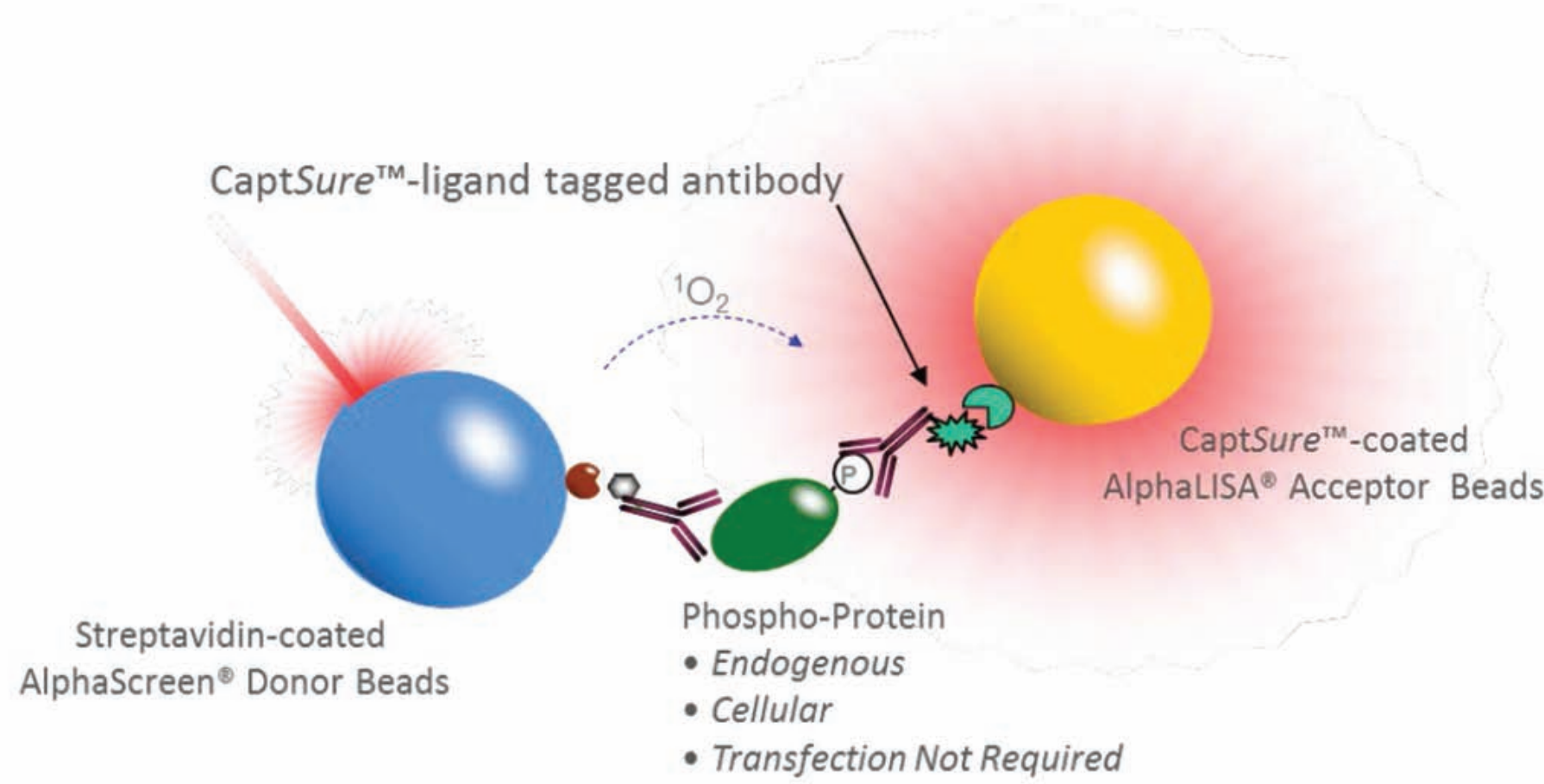


Figure 1. Assay schematic for AlphaLISA homogeneous proximity assay principle for the detection of phosphorylated proteins. The AlphaLISA[®] SureFire[™] Ultra[™] assay kits utilize Alpha beads that are each coated to specifically capture the assay antibodies. The Donor bead is coated with streptavidin 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 of light emission at 615 nm.

BioTek[®] Instrumentation



Figure 2 – Cytation[™]5. 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.



Figure 3 – MultiFlo[™] FX Microplate Dispenser. MultiFlo FX Microplate Dispenser offers up to four reagents dispensed in parallel with one compact instrument. The instrument was used to dispense assay specific reagents to the 384-well assay plates.

Materials and Methods

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).

Reagents

AlphaLISA[®] SureFire[™] Ultra phospho-ERK1/2 (No. ALSU-PERK-A500) and phospho-Smad3 Kits (No. ALSU-PSM3-A500) were from Perkin Elmer (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 (AlphaLISA)	
Mode	Alpha
Gain	120
Delay after plate movement	0 msec
Excitation time	80 msec
Delay after excitation	120 msec
Integration time	160 μ sec
Read height	8.00 mm

Table 1. Cytation 5 AlphaLISA reading parameters used in Gen5 Data Analysis software.

Materials and Methods Cont.

AlphaLISA control lysate assay

- p-Erk1/2 and p-Smad3 control lysates were prepared 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 μ L each, to a 384-well AlphaPlate.
- Acceptor Mix was prepared as per the manufacturers recommendation: Activation Buffer was diluted 25-fold in Reaction buffer and Acceptor beads 50-fold in Reaction buffer and 5 μ L 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 min. at RT.
- Donor Mix was prepared as per the manufacturers recommendation: Donor beads were diluted 50-fold in Dilution buffer and 5 μ L added to each well.
- For the p-Erk1/2 assay the plate was incubated for a min. of 2 hrs. at RT, or up to overnight.
- For the p-Smad assay the plate was incubated for a min. of an additional 60 min., or up to overnight, following addition of the Donor Mix.
- Following the final incubation period the plate was read on the microplate readers.

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 $^{\circ}$ C, 5% CO₂ in a humidified incubator.
- Cells were harvested at ~ 80-90% confluency and quadruplicates wells were seeded with 80 μ L of cells at the appropriate cells 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 μ L FBS-free media for ~2 hrs. 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, including a zero data point, was prepared for the agonists EGF and TGB-beta 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 μ L of media was removed, leaving 15 μ L of residual media, cells were treated with 5 μ L of the EGF dilution series prepared at 4x the final concentration (f.c.) and allowed to incubate for 10 min. @ 37 $^{\circ}$ C, 5% CO₂ in a humidified incubator.
- For the p-Smad3 assay 40 μ L of media was removed, leaving a 40 μ L residual, cells were treated with 20 μ L of the TGF-beta dilution series prepared at 3x the f.c., and allowed to incubate for 60 min. @ 37 $^{\circ}$ C, 5% CO₂ in a humidified incubator.
- Following incubation, all media was removed and cells were lysed with 10 μ L 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 manufacturers recommendations, with the following modifications, for both p-Erk1/2 and p-Smad3 assays.
- An 11-pt.,1:3 serial dilution, including a zero data point, of the inhibitors AG1478 (EGF-R inhibitor) and SB432542 (TGF-beta-R inhibitor) 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 μ L of media was removed, leaving 10 μ L of residual media, cells were treated with 5 μ L of the AG1478 dilution series prepared at 3x the f.c. and allowed to incubate for 60 min. @ 37 $^{\circ}$ C, 5% CO₂ in a humidified incubator.
- For the p-Smad3 assay 60 μ L of media was removed, leaving a 20 μ L residual, cells were treated with 20 μ L of the SB432542 dilution series prepared at 2x the f.c., and allowed to incubate for 60 min. @ 37 $^{\circ}$ C, 5% CO₂ in a humidified incubator.
- Following incubation with the appropriate inhibitor the EC₅₀ of the appropriate agonist was added to all wells as described above for agonist titrations, 5 μ L of 4x or 20 μ L 3x for EGF or TGF-beta, respectively, and incubated for the appropriate time.
- Following incubation, all media was removed and cells were lysed with 10 μ L 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.

Results and Discussion

AlphaLISA Control Lysate Standard Curve

- An 11-point, 1:3 serial dilution using control lysates was prepared for each assay.
- The data can be fit using a second order polynomial (quadratic) (Figure 4).

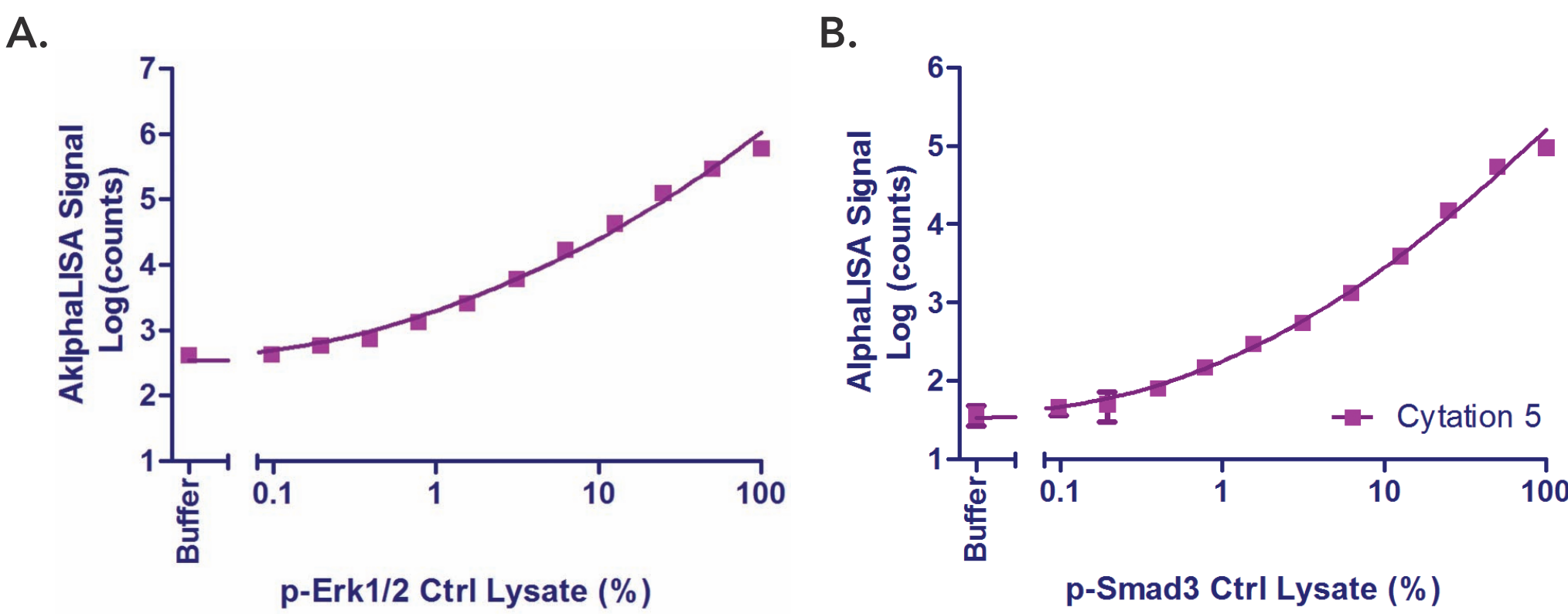


Figure 4. Control Lysate Standard Curves. A) AlphaLISA p-Erk1/2 Assay. An 11-point dilution series of the p-Erk1/2 positive control lysate was prepared ranging from 100 – 0 percent and assayed in quadruplicate. **B) AlphaLISA p-Smad3 Assay.** An 11-point dilution series of the p-Smad3 positive control was prepared ranging from 100 – 0 percent and assayed in quadruplicate.

- High S/B were detected; p-Erk1/2 lysates S/B= 1,432, p-Smad3 lysate S/B= 2,522.

Results and Discussion Cont.

AlphaLISA Agonist Titration

- An 11-point, 1:3 serial dilution of agonist was prepared for each assay.
- The data can be fit using a Hill Slope model (Figure 5).

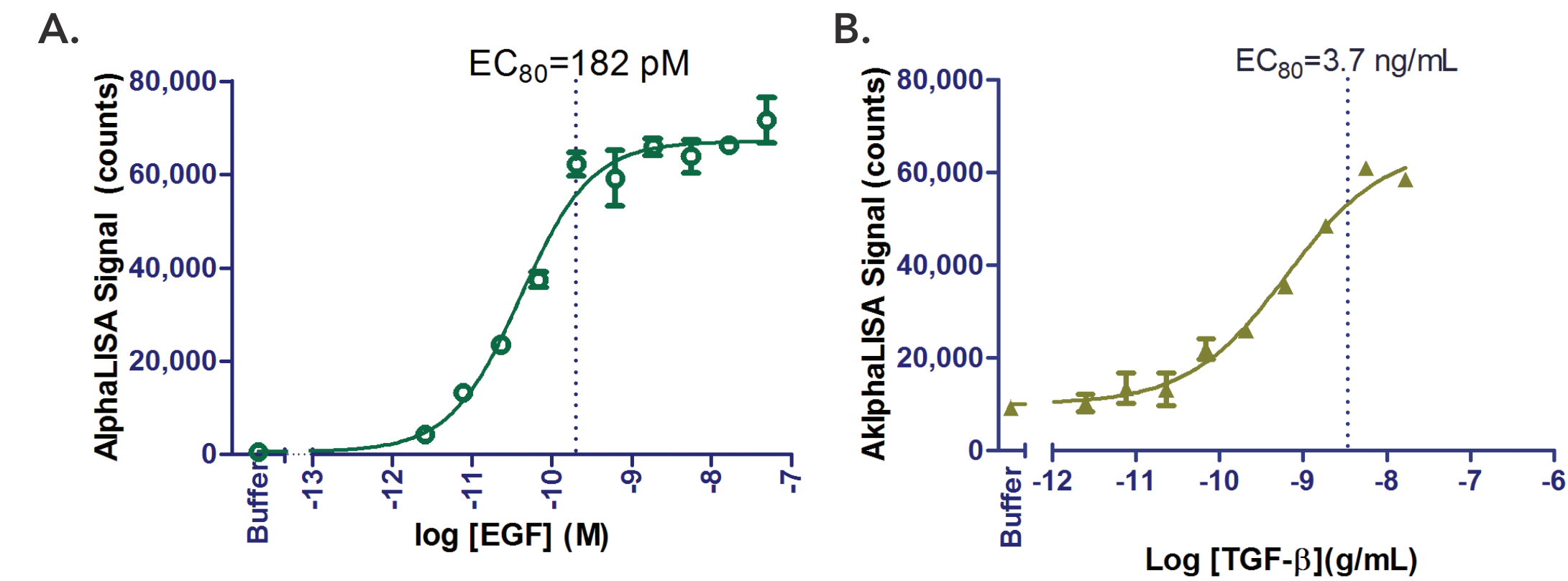


Figure 5. Agonist Titration Curves. A) AlphaLISA p-Erk1/2 Assay. An 11-point dilution series of the agonist EGF was prepared ranging from 50 – 0 nM and assayed in quadruplicate. **B) AlphaLISA p-Smad3 Assay.** An 11-point dilution series of the TGF-beta was prepared ranging from 50 – 0 ng/mL and assayed in quadruplicate.

- The AlphaLISA assay showed excellent dynamic range and correlation between replicates for both assays (Figure 5).
- The agonist dose response curves yielded EC₉₀ values of 182 pM and 3.7 ng/mL for p-Erk1/2 and p-Smad3, respectively.
- The EC₈₀ determinants were used for the inhibition studies.
- The EC₅₀ value of 40 pM EGF for EGFR stimulation of HEK293 cells correlates well with previous data provided by the manufacturer (39 pM).

AlphaLISA Inhibitor Titration

- An 11-point, 1:3 serial dilution of inhibitor was prepared for each assay
- The data can be fit using a Hill Slope model (Figure 6).

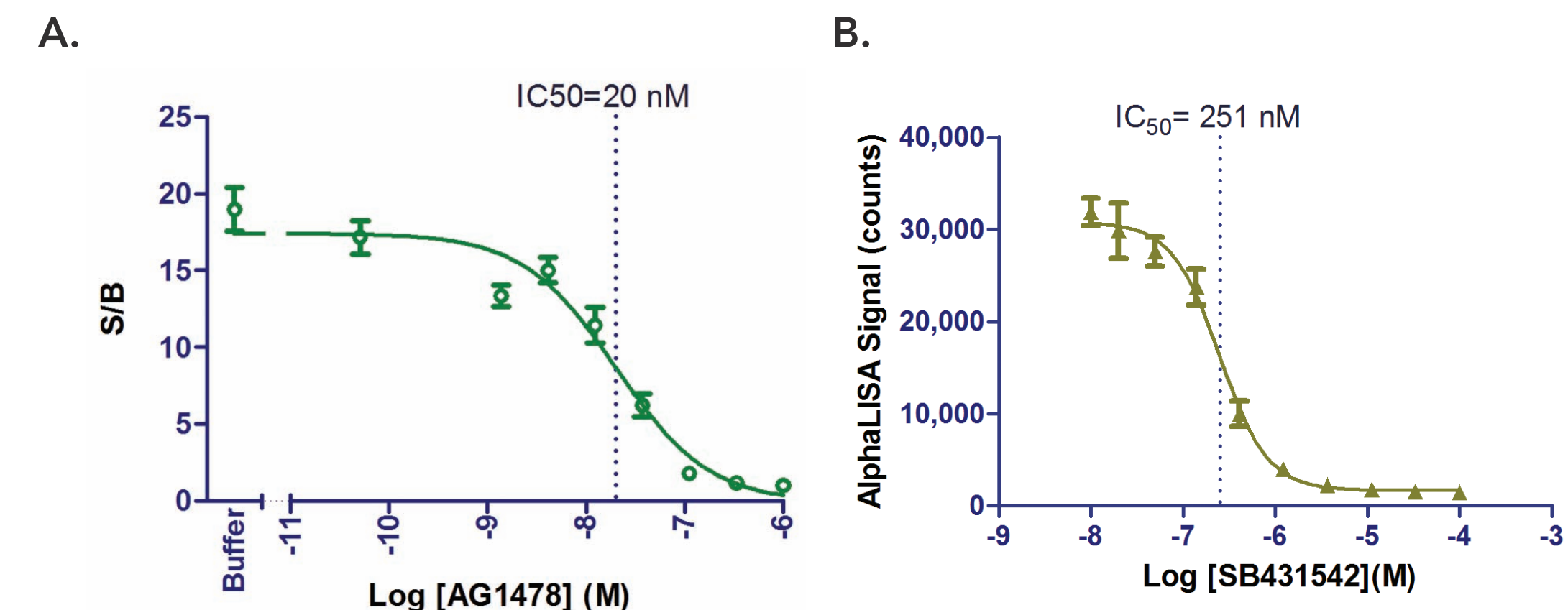


Figure 6. Agonist Titration Curves. A) AlphaLISA p-Erk1/2 Assay. An 11-point dilution series of the potent EGFR tyrosine kinase inhibitor AG1478 was prepared ranging from 1.0 – 0 μ M and assayed in quadruplicate. **B) AlphaLISA p-Smad3 Assay.** An 11-point dilution series of the selective ALK family of kinases inhibitor SB431542 was prepared ranging from 100 – 0 μ M and assayed in quadruplicate.

- An 11-point, 1:3 serial dilution of a potent selective kinase inhibitor was prepared for each assay.
- The potent EGFR tyrosine kinase inhibitor AG1478 was determined to have an IC₅₀ of 20 nM, consistent with previously reported values¹.
- SB431542 is a potent and selective inhibitor of transforming growth factor-beta(TGF- β) type 1 receptor activin receptor-like kinase ALK5, and its relatives ALK4 and ALK7.
- The dose response curve and IC₅₀ of 251 nM correlate well with previously reported data₂.

Conclusions

- The assay was performed, in its 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 signal transduction.
- Cytation 5 equipped with the Alpha-specific laser generates higher signals than conventional tungsten light sources allowing for more rapid analysis times suitable for HTS operation.

¹Levitzki, A. and A. Gazit (1995). Tyrosine Kinase Inhibition: An Approach to Drug Development. Science. **267** 1782. ²Inman et al (2002) SB-431542 is a potent and specific inhibitor of transforming growth factor- β superfamily type I activin receptor-like kinase (ALK) receptors ALK4, ALK5, and ALK7. Mol. Pharmacol. **62** 65.