

Simultaneous detection of total and phosphorylated protein in cellular kinase assays using Alpha SureFire Ultra Multiplex technology.

Introduction

Cellular kinase signal transduction pathways are involved in the regulation of many important cellular processes such as cell survival, differentiation, and apoptosis. Kinase signaling networks are typically characterized by multiple kinases arranged in cascades containing nodes with feedback loops and crosstalk between pathways. Numerous assay technologies have been developed for studying kinase signaling pathways, and for screening compound libraries in search of agents to modify receptors or kinase activities. The quality of these assays can be impacted by data variability due to cell seeding inhomogeneity or from compound toxicity. A common method for controlling for variability is to normalize the assay signal to a cellular protein whose level does not change as a function of the treatment. Alpha SureFire® Ultra™ Multiplex Phospho and Total assays utilize two types of Alpha Acceptor beads to simultaneously measure two signals from each assay well to easily normalize the assay. Here, we demonstrate the benefit and utility of normalizing assay signal of phosphorylated protein levels to total protein levels in two different cellular models: ERK 1/2 phosphorylation in human melanoma A375 cells and AKT 1/2/3 phosphorylation in mouse myoblast C2C12 cells.

For research purpose only. Not for use in diagnostic procedures.

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Alpha SureFire Ultra Multiplex assay kits provide the dual measurement of a phosphoprotein from cells, combined with the measurement of either the total amount of the same protein or of another phosphorylation event. This dual measurement is carried out in the same assay plate well from a single sample of cell lysate, and is achieved by the use of two types of Alpha Acceptor beads that emit at distinct wavelengths (AlphaPlex® Terbium beads: 545 nm and AlphaLISA® (Europium) beads: 615 nm).

A schematic of the Alpha SureFire Ultra Multiplex Phospho and Total assay format is shown in Figure 1. The Alpha 615 Acceptor bead is directly-conjugated with an antibody to the phosphorylated site on the target protein. The Alpha 545 Acceptor bead is coated with the CaptSure™ agent, which binds the CaptSure-tagged anti-total target protein antibody. The Alpha Donor bead binds the biotinylated anti-total target protein antibody. The protocol is homogeneous, can be performed with or without a transfer step, and provides rapid and sensitive detection of phosphorylated and total protein in less than three hours.

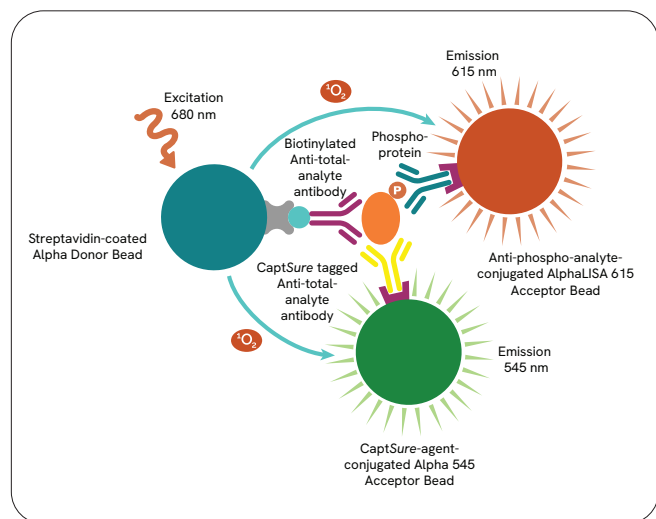


Figure 1: Alpha SureFire Ultra Multiplex assay schematic.

Materials and methods

Reagents

Recombinant phospho-ERK1 (#7741-5) (pERK) and Recombinant phospho-AKT1 (#7701-5) (pAKT) were purchased from BioVision. A375 human skin malignant melanoma cell line (ATCC® CRL- 1619™) and C2C12 mouse myoblast muscle cell line (ATCC® CRL-1772™) cell lines were purchased from ATCC. Wortmannin (#1232), LY294002 (#1130), U0126 (#1144) and PD 98059 (#1213) were purchased from Tocris. Human recombinant IGF-1 (#291-G1-200) was purchased from R&D systems. Alpha SureFire Ultra Multiplex kits were from Revvity: Phospho AKT 1/2/3 (Ser473) 615 (Eu) + Total AKT 1 545 (Tb) (#MPSU-PTAKT-K500) and Phospho ERK 1/2 (Thr202/Tyr204) 615 (Eu) + Total ERK 1/2 545 (Tb) (# MPSU-PTERK-K500)

Standard Curve Experiments

Recombinant human pERK and pAKT proteins were serially diluted in 1X SureFire Ultra lysis buffer. 10 µL of each protein dilution was engaged in the Alpha SureFire Ultra Multiplex assay as described below. Lower Detection Limit (LDL) was calculated by interpolating the Alpha counts corresponding to the average of the buffer wells signal + 3-fold its standard deviation on the dose-response curve, to find the lowest concentration of analyte that can be significantly detected.

Cell-based Experiments

Both A375 and C2C12 cell lines were cultured in DMEM (ATCC® 30-2002™) supplemented with 10% FBS (10437-028, Thermo Fisher) and handled according to ATCC guidelines. For cell-based assays, 25 µL of cells were seeded at the stated cell density in a 384-well Revvity ViewPlate (white, clear bottom, cat #6007480) and allowed to adhere overnight at 37 °C in 5% CO₂. All compounds were serially diluted in 100% DMSO then transferred to assay buffer (HBSS, HEPES, 0.1% BSA) at 2X or 3X final compound concentration. Final DMSO concentration during inhibitor treatment was 1%. For A375 cells, culture media was removed and 10 µL of assay buffer was added to each well, then cells were treated with 10 µL of the stated concentration of inhibitor (10 µL of assay buffer containing the 2X compound concentration were added per well) for one hour at room temperature. For C2C12 cells, media was removed, 10 µL of assay buffer was added to each well, then cells were treated for one hour with the stated concentrations of inhibitor at room temperature (10 µL of assay buffer containing the 2X compound concentration were added per well) then cells were stimulated with 12 nM of human

recombinant IGF-1 for 20 minutes at room temperature (addition of 10 μL per well of 3X IGF-1 solutions in assay buffer). For both A375 and C2C12 experiments, assay buffer was removed immediately following incubations and then 10 μL of 1X lysis buffer were added to the wells.

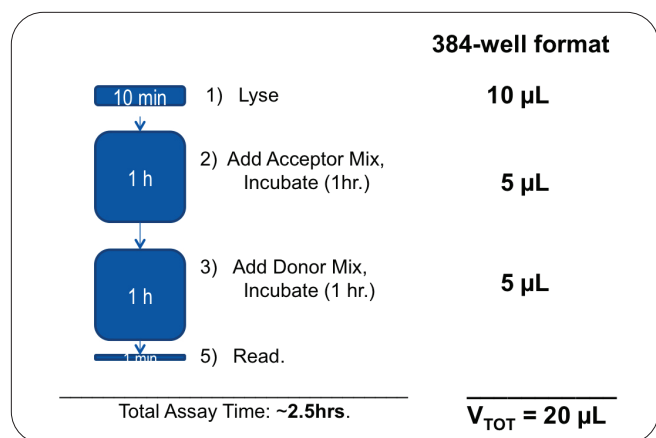


Figure 2: Alpha SureFire Ultra Multiplex workflow.

Alpha SureFire Ultra Multiplex Assays

An overview of the assay workflow is shown in Figure 2. Briefly, cells in the 384-well ViewPlate were lysed in 10 μL of 1X SureFire Ultra lysis buffer, incubated for 10 minutes with gentle shaking, followed by the addition of 5 μL of the acceptor mix (i.e., Alpha615 and Alpha545 acceptor Beads together with Activation Buffer and the biotinylated and CaptSure-labeled antibodies diluted as per the kit's protocol) for a one-hour incubation. 5 μL of the donor mix (Streptavidin Donor beads diluted 1:50 in dilution buffer as per the kit's protocol) were added for an additional one-hour incubation. A white BackSeal (cat #6005199) was placed on the back of the ViewPlate before reading the assay signal.

Instrumentation

All assays were read on a standard EnVision™ model 2104 multimode plate reader equipped with AlphaScreen® capability and with two detectors. The filters and mirrors used to collect the data are shown in Table 1. In sequential mode, the assay plate was excited a total of two times, whereby the entire plate was read measuring terbium emission signal first, and the entire plate was read a second time, measuring the europium emission signal. In simultaneous duplex reading mode, the entire plate was only read one time, with simultaneous measurement of terbium and europium with each of the two instrument detectors. For singleplex reads, only terbium or europium signal was read using the stated

filters. For the full details of the instrument protocol setup, please refer to the document "EnVision Set-Up and Data Correction Guide for using Alpha SureFire Ultra Multiplex Technology."

Table 1: Filters and mirror modules used on the EnVision.

Reading Mode	EnVision Mirror Module	EnVision Filter
Sequential Duplex	AlphaScreen single Barcode 444 (#2101-4010)	Terbium Resorufine/ Amplex Red Barcode 124 (#2102-5570) Europium Europium Barcode 203 (#2100-5090)
Simultaneous Duplex	AlphaPlex Dual Tb/Eu Barcode 653 (#2101-5900)	Same as sequential
SinglePlex Europium	Same as sequential	Europium Barcode 203 (#2100-5090)
SinglePlex Terbium	Same as sequential	Resorufine/ Amplex Red Barcode 124 (#2102-5570)

Spectral Overlap Correction

While minor, there are overlaps between the two emission spectra of terbium and europium chelates, which may result in small percentage of the europium signal measured in the assay to be coming from the terbium chelate and small percentage of the terbium signal measured in the assay to be coming from the europium chelate. This percentage of overlap will vary slightly from instrument to instrument but will be relatively small overall (typically less than 5%).

Nevertheless, we recommend setting up assay controls to calculate the spectral overlap correction factor (SOC), which can then be applied to the measured assay signal to remove the extraneous counts from each channel. For all of the data shown, the calculated SOC factor for each channel was applied to the raw data. Applying the SOC had very little effect on calculated assay sensitivity and compound IC_{50} s. More information on how to calculate SOC can be found in the document "EnVision Set-Up and Data Correction Guide for using Alpha SureFire® Ultra™ Multiplex Technology."

Results

Assay Sensitivity: Sequential vs. Simultaneous Reading Modes

The fastest and most efficient way to duplex Alpha SureFire Ultra Multiplex is to read the two channels simultaneously. In some cases, such as the presence of only one detector in the instrument used, the plate has to be read sequentially. In Figures 3 and 4, we compared the performance of the ERK and AKT kits read in both modes, using human recombinant proteins. As shown, the kits display similar assay sensitivity and S/B whether read sequentially or simultaneously. Using the top and bottom values from the europium data (pERK and pAKT) in Figures 3 and 4, Z' values were calculated and

determined to be acceptable whether reading in sequential or simultaneous mode. Z' values were 0.63 in sequential mode and 0.84 in simultaneous mode (pERK) and 0.81 in sequential mode and 0.74 in simultaneous mode (pAKT). Reading sequentially does result in higher overall counts, though as a consequence the plate read time is slightly longer. This difference in signal intensity is fully expected as it is a consequence of the need to use more restrictive mirrors and filters for wavelength separation when reading in simultaneous mode. For the remainder of the experiments performed during this study, simultaneous read mode was used to generate the presented data.

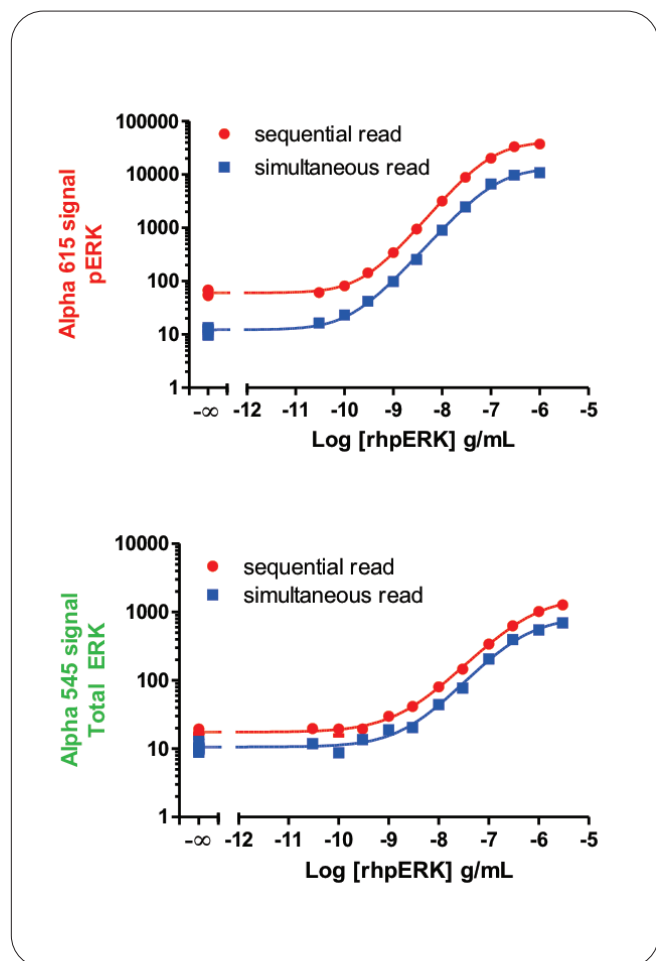


Figure 3: Detection of phosphorylated and total ERK protein in sequential or simultaneous duplexing modes. For phosphorylated ERK detection (Alpha 615, top graph): S/B was 613 in sequential read mode and 920 in simultaneous read mode. Lower detection limit (LDL) was 135 pg/mL in sequential mode and 102 pg/mL in simultaneous mode. For total ERK detection (Alpha 545, bottom graph): S/B was 74 in sequential mode and 70 in simultaneous mode. LDL was 0.7 ng/mL in sequential mode and 1.9 ng/mL in simultaneous mode. Each concentration was read in triplicate wells, and the buffer condition was read from 12 wells.

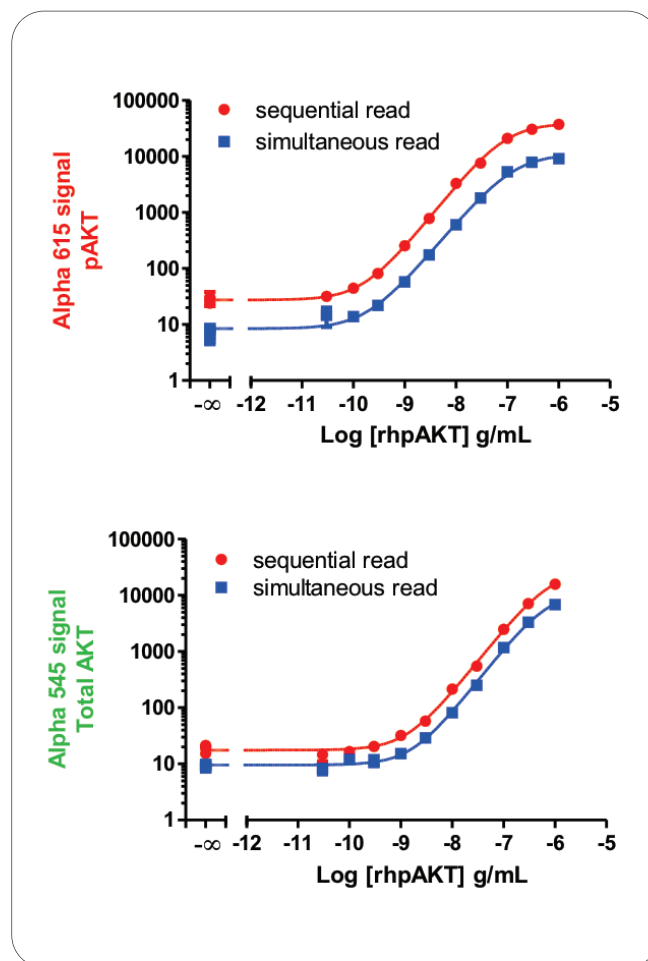


Figure 4: Detection of phosphorylated and total AKT protein in sequential or simultaneous duplexing modes. For phosphorylated AKT detection (Alpha 615, top graph): S/B was 1359 in sequential read mode and 1227 in simultaneous read mode. Lower detection limit (LDL) was 106 pg/mL in sequential mode and 135 pg/mL in simultaneous mode. For total AKT detection (Alpha 545, bottom graph): S/B was 834 in sequential mode and 750 in simultaneous mode. LDL was 0.85 ng/mL in sequential mode and 0.94 ng/mL in simultaneous mode. Each concentration was read in triplicate wells, and the buffer condition was read from 12 wells.

Assay Sensitivity: Duplex vs. Singleplex

One of the concerns about duplexing immunoassays may be loss in sensitivity due to an additional set of antibodies recognizing the target of interest. In order to determine if the detection assay sensitivity was affected by duplexing the detection of both total and phosphorylated protein, standard curves were performed with recombinant protein using detection reagents for either phosphorylated OR total protein (Singleplex) or using both detection reagents (Duplex). Results are shown in Figures 5 (ERK) and 6 (AKT). As shown we saw little differences in sensitivity whether only one set or both sets of detection reagents were used to detect recombinant protein.

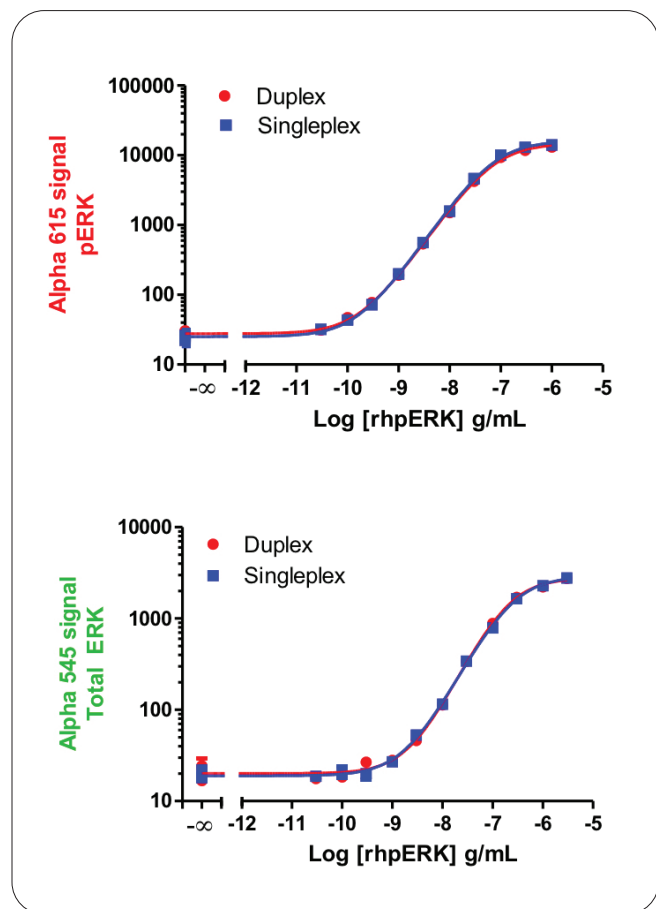


Figure 5: Detection of phosphorylated and total ERK protein in duplex or singleplex modes. For phosphorylated ERK detection (Alpha 615, top graph): Lower detection limit (LDL) was 63 pg/mL in duplex mode and 105 pg/mL in singleplex mode. For total ERK detection (Alpha 545, bottom graph): LDL was 1.89 ng/mL in duplex mode and 2.45 ng/mL in singleplex mode. Each concentration was read in triplicate wells, and the buffer condition was read from 12 wells.

Assay Normalization

One of the benefits of reading both total and phosphorylated assays simultaneously is the ability to control assay variability with normalization. In this manner, signal from the phosphorylated protein can be normalized to the signal from the total amount of protein present in each well. One concern around this might be that if too much of the total protein is present in the cell, it might saturate the capture reagents in the assay. In this case, the amount of signal in the terbium channel might not change as cell number changes. To test this, we performed a cell number titration of the cell lines of interest to monitor the total ERK and total AKT values (Figure 7) using only the terbium detection reagents and two different cell lines: human melanoma A375 cells in which pERK is constitutively active, and mouse myoblast C2C12, which were stimulated with insulin growth factor 1 (IGF-1).

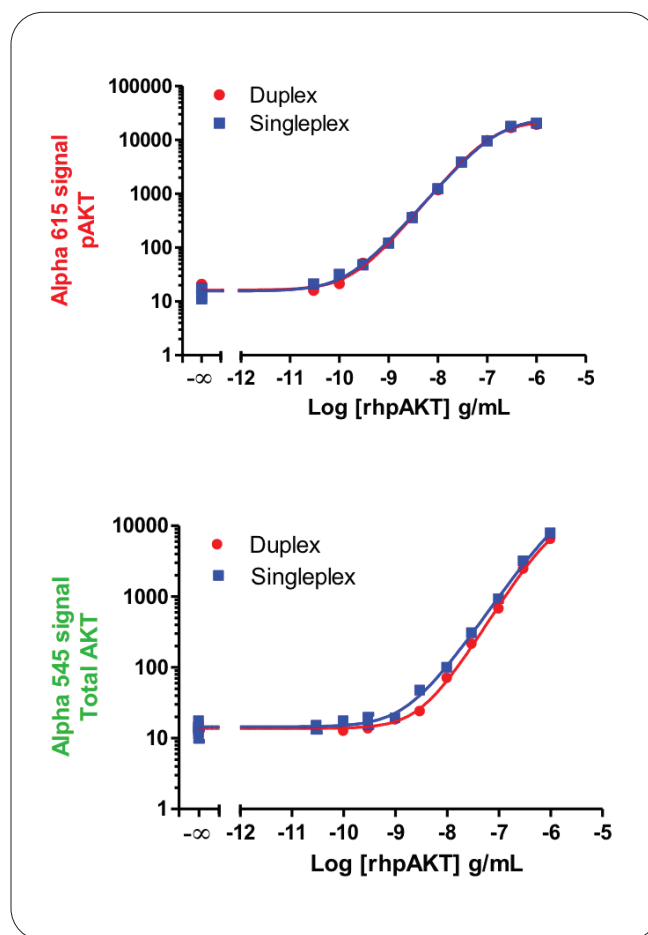


Figure 6: Detection of phosphorylated and total AKT protein in duplex or singleplex modes. For phosphorylated AKT detection (Alpha 615, top graph): Lower detection limit (LDL) was 123 pg/mL in duplex mode and 95 pg/mL in singleplex mode. For total AKT detection (Alpha 545, bottom graph): LDL was 2.45 ng/mL in duplex mode and 1.23 ng/mL in singleplex mode. Each concentration was read in triplicate wells, and the buffer condition was read from 12 wells.

As shown in Figure 7, as cell number increases a point is reached where the alpha signal does not increase anymore in proportion to the cell number increase. For both cell lines tested, the detection reagents saturated above 48,000 cells per well, meaning that above this cell number the total ERK and total AKT can no longer be used for normalization. In order to be sure of the validity of the normalization, we recommend performing phosphorylated to total protein normalization only in situations where the total detection reagents are not saturated (well below the assay hook point).

Data Normalization of Inhibitor Testing

In order to test the effect of data normalization, two known inhibitors were tested on A375 cells (MEK inhibitors U0126 and PD 98059) and on C2C12 cells (PI3K inhibitors

wortmannin and LY294002) for their ability to inhibit the production of pERK and pAKT respectively (Figures 8 and 9, top panels). The non-normalized data demonstrates that while the phosphorylated protein is being modulated by the inhibitors, the total protein is staying relatively constant. Next, the data generated for pERK and pAKT (generated in the europium channel) was then normalized to the counts obtained for total ERK and total AKT (generated in the Terbium channel). Normalized inhibition curves for pERK and pAKT are shown in Figures 8 and 9 (bottom panels), respectively. Compound IC_{50} s showed little shift after data normalization, but overall the signal variability improved after normalization, especially at low compound concentrations, particularly with the C2C12 cells (Figure 9).

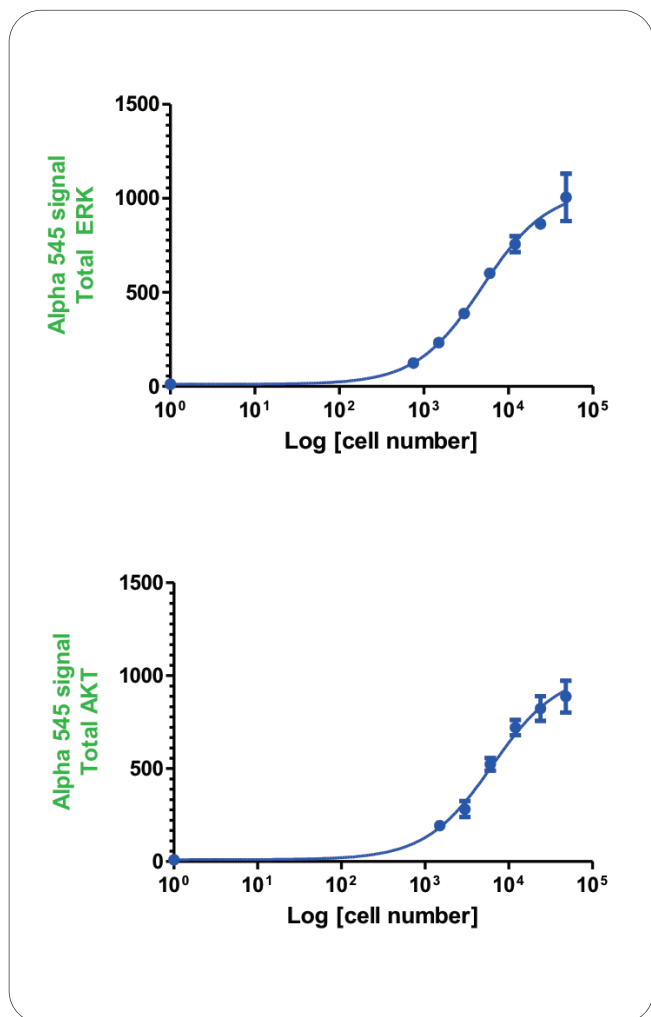


Figure 7: Cell number titration of A375 (ERK) and C2C12 (AKT) cell lines. Serial dilutions of A375 cells (Total ERK, top graph) and C2C12 cells (Total AKT, bottom graph) were tested with the Terbium detection reagents from the Alpha SureFire Ultra Multiplex kit.

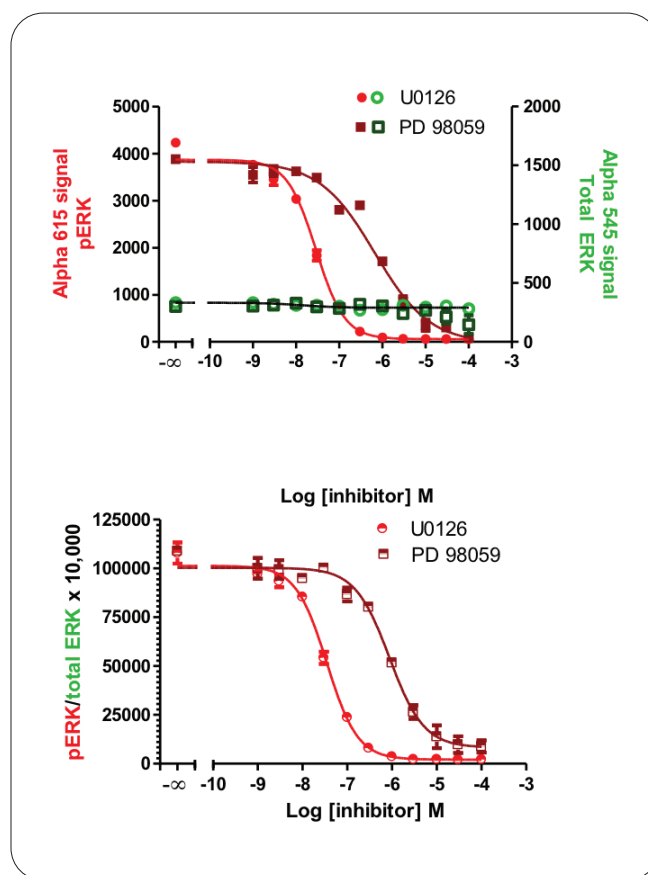


Figure 8: Measuring pERK inhibition in A375 cells. 3000 A375 cells were treated with stated compounds for 1 hour at room temperature before cell lysis and pERK detection. Top panel: Data obtained for each inhibitor in the 615 channel (red curves) for detection of pERK and in the 545 channel (green curves) for detection of total ERK. IC_{50} s were 28.7 nM for U0126 and 0.66 μ M for PD 98058. Bottom panel: Data obtained from normalizing the counts in the 615 nm channel to the 545 nm channel (pERK/total ERK). IC_{50} s were 31 nM for U0126 and 0.91 μ M for PD 98059. Each concentration was read in triplicate wells, and the buffer condition was read from 12 wells.

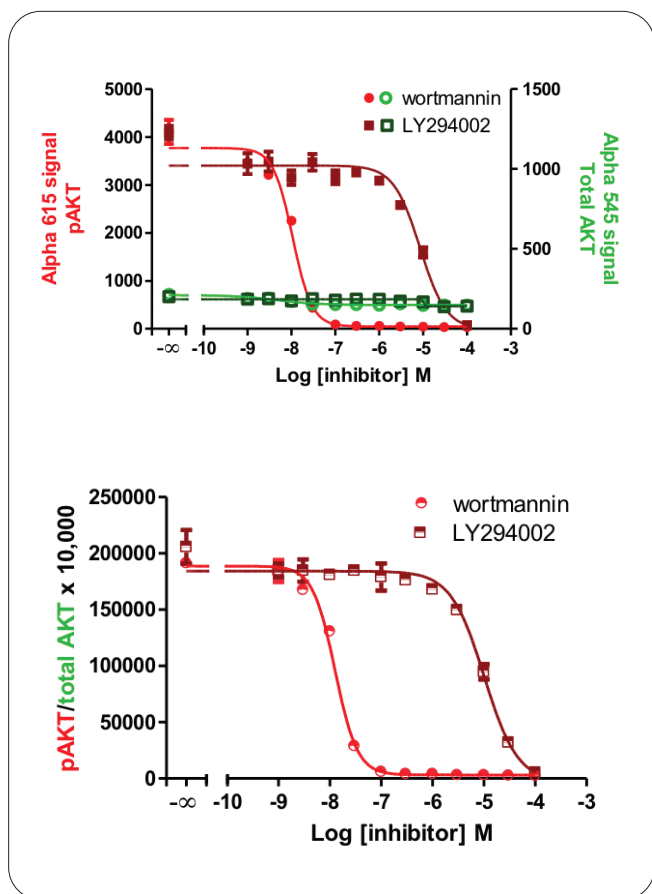


Figure 9: Measuring pAKT inhibition in C2C12 cells. 12,000 C2C12 cells were treated with stated compounds for 1 hour at room temperature before stimulation with IGF for 20 minutes prior to cell lysis and pAKT detection. Top panel: Data obtained for each inhibitor in the 615 channel (red curves) for detection of p-AKT and in the 545 channel (green curves) for detection of total AKT. IC_{50} s were 10.6 nM for wortmannin and 8.5 μ M for LY294002. Bottom panel: Data obtained from normalizing the counts in the 615 nm channel to the 545 nm channel (pAKT/total AKT). IC_{50} s were 12.6 nM for wortmannin and 10.1 μ M for LY294002. Each concentration was read in triplicate wells, and the buffer condition was read from 12 wells.

Data normalization: Improving Z'

Z' analysis is an important measure of assay performance and suitability for high throughput screening campaigns. A high Z' value (typically >0.6) is usually required in order to be sure signal responses generated by unknown compounds are statistically relevant. In cell based assays, variability in cell numbers from well-to-well usually reduce assay Z'. In Alpha SureFire Ultra Multiplex assays, differences in cell numbers can be observed by differences in the total ERK or total AKT values obtained in the terbium channel. Normalization of pERK and pAKT values in the Europium channel to values obtained in the terbium channel for total ERK and total AKT should remove assay variability originating from differences in cell number. Indeed, in Figures 10 and 11, data normalization of the values used for analysis for pERK and pAKT improved the Z' significantly.

Data Normalization: Eliminating False Positives

As shown in Figures 10 and 11, improvements seen in Z' calculations using normalized values are most likely due to subtle differences in cell number that result in slightly reduced phosphorylated protein response if cell numbers are too low. Another cause for concern during screening campaigns is compound toxicity, whereby the decreased cellular response can be due to a large portion of the cellular population being killed by the test compound, mimicking the effects of high inhibition. Most often, this results in a false positive whereby the compound causing cell death would be selected for further testing as a potential "hit." In order to determine if data normalization was sufficient to detect false positives, compound toxicity was induced in a subset of wells that also contained varied compound potencies. Using Alpha SureFire Ultra Multiplex, both phosphorylated and total protein signal was measured. Results are shown in Figures 12 (ERK) and 13 (AKT).

In both experiments, wells that were spiked with a toxic level of detergent (wells outlined in black) displayed lower overall pERK and pAKT levels similar to potent compound treatment, but also lower overall total ERK and total AKT levels, indicating cell death. When the values obtained in the europium channel for phosphorylated protein are normalized to values obtained in the terbium channel for total protein, the wells containing dead cells no longer appear to be as potent as real inhibitors.

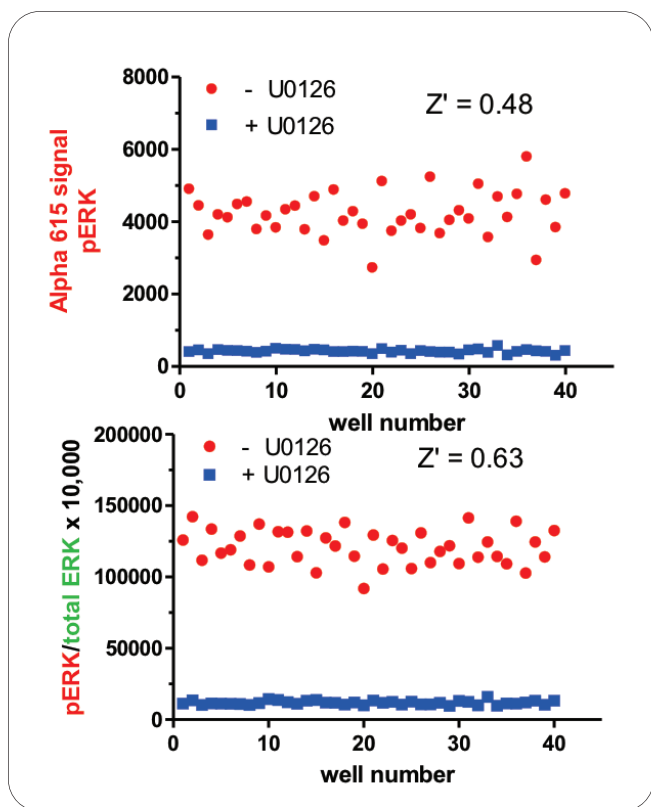


Figure 10: Z' analysis with and without data normalization for pERK assay. Forty wells each were either treated with 1% DMSO only (red circles) or 200 nM of U0126 for one hour. Top panel: Data obtained in the 615 channel. Bottom panel: Data obtained from normalizing the counts in the 615 nm channel to the 545 nm channel (pERK/total ERK).

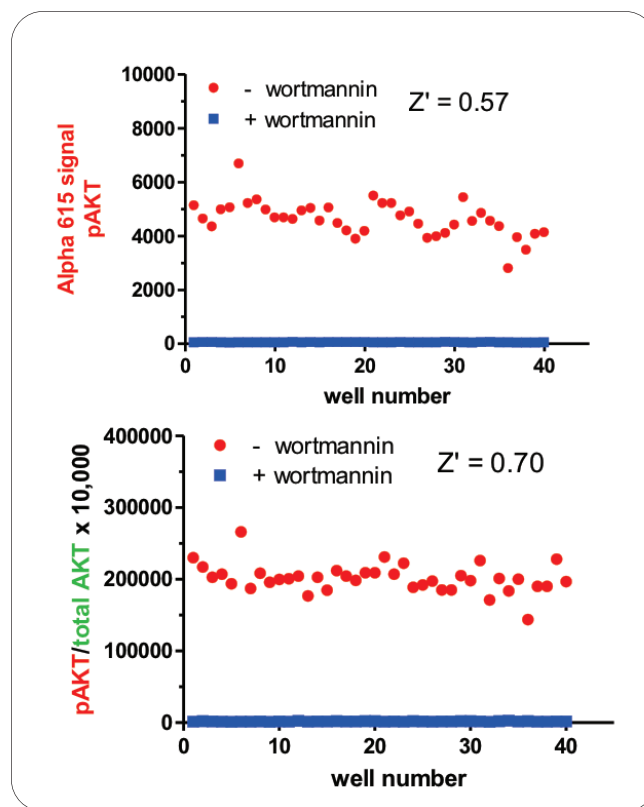


Figure 11: Z' analysis with and without data normalization for pAKT assay. Forty wells each were either treated with 1% DMSO only (red circles) or 200 nM of wortmannin for one hour prior to stimulation with 12 nM IGF. Top panel: Data obtained in the 615 channel. Bottom panel: Data obtained from normalizing the counts in the 615 nm channel to the 545 nm channel (pAKT/total AKT).



Figure 12: Compound toxicity with data normalization for pERK assay. 96 wells of 3000 A375 cells were treated with varied concentrations of U0126. Triton X-100 at a final concentration of 0.1% was spiked into wells highlighted with black borders prior to compound treatment.

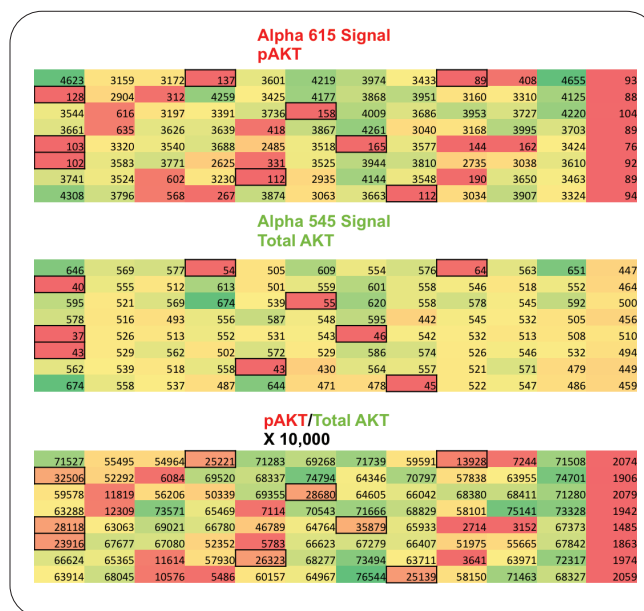


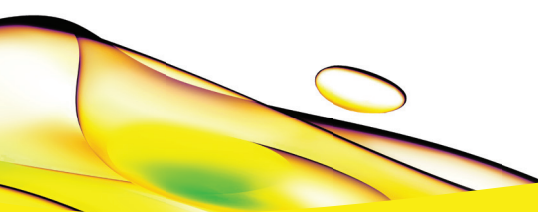
Figure 13: Compound toxicity with data normalization for pAKT assay. 96 wells of 12,000 C2C12 cells were treated with varied concentrations of LY294002 prior to stimulation with 12 nM IGF. Triton X-100 at a final concentration of 0.03% was spiked into wells highlighted with black borders prior to compound treatment.

Conclusion

Alpha SureFire Ultra Multiplex Phospho and Total assays, together with the EnVision Multimode Plate Reader, provide a robust platform to screen kinase modulators by measuring both total and phosphorylated-targets from the same well, simultaneously and homogenously, in less than three hours. Assay quality is improved further by normalization of phosphorylated protein levels to total protein levels, thereby reducing the number of false positives in high throughput screening campaigns saving both reagents, and less re-testing needed.



EnVision Plate Reader



revvity