APPLICATION NOTE

Alpha Terbium *SureFire Ultra* multiplex: Simultaneous dual phosphoprotein target analysis.

Authors

Antony Sheehan
Michael Crouch
Andrea Brown

TGR BioSciences
Adelaide, Australia

Vincent Dupriez

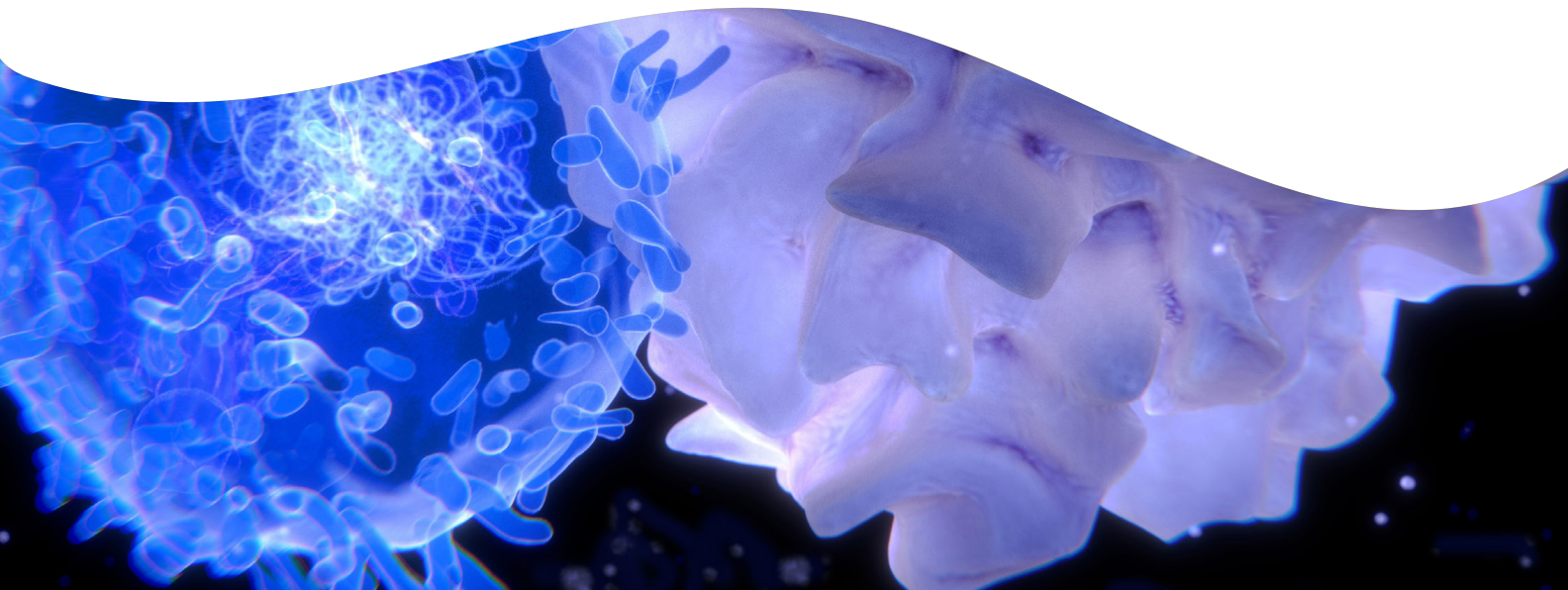
Revvity, Inc.

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

Introduction

The evaluation of pharmaceutical compound efficacy and safety in regulating cell behavior can involve the study of multiple signal transduction pathways. Measurement of more than one cellular target can provide greater confidence of positive compound hits on the cellular target of interest, through examination of an unrelated pathway or an upstream target that should be unaffected by the drug. More specifically, protein phosphorylation is a very useful readout of cellular activation or inhibition, and these pathways are commonly targeted for therapeutic modulation of disease. Examples include modulation of cell surface receptors, such as GPCRs or receptor tyrosine kinases, by antibodies or small molecules, and of intracellular kinases by cell permeable drugs.

There are several available assay technologies that can be used to look at protein phosphorylation for drug development and other research applications. Of these, AlphaLISA™ *SureFire*® *Ultra*™ provides a highly sensitive, fully homogeneous option for the analysis of both small and large sample numbers for a large selection of signaling pathways. Typically, different wells within a single assay plate are used to measure different targets by using aliquots from the same sample, to give multi-target analysis on a single assay plate. However, in many cases it would be advantageous to measure more than one target within a single well. Combining this capability with the benefits of a homogenous assay and the ability to fully automate the assay would be very valuable to screening programs.



For this reason, we have developed the Alpha *SureFire Ultra* Multiplex assay, the only phosphoprotein multiplexing detection platform available that is truly homogeneous and high throughput. While the initial assay introduction included just a few kits for measuring phospho- and total ERK or AKT proteins, the newest additions to the group of multiplexing reagents allow for individual phospho-targets to be selected from a panel of Europium and Terbium-based individual reagents and combined as needed. This pick-and-pack capability allows scientists to choose dual targets of interest for easy implementation in the lab.

Alpha *SureFire Ultra* Multiplex assays are divided into two categories (1) Multiplex *SureFire Ultra* (MPSU) kits and (2) Terbium *SureFire Ultra* (TBSU) kits. The MPSU kits are complete kits for phospho and total detection of the same target, while the TBSU kits measure a single phosphoprotein target and are intended to be combined with a standard AlphaLISA *SureFire Ultra* kit (ALSU) to assess the second target. The TBSU kits allow for a pick-and-pack combination of any two phosphoprotein targets in the assay lists, providing measurement of two separate targets with ultimate flexibility of selection. The TBSU kits will report the target of interest through the Terbium (Tb) Acceptor beads, while the ALSU kit reports via the Europium (Eu) AlphaLISA Acceptor beads.

Alpha *SureFire Ultra* Multiplex technology measures two targets in a single well of an assay plate. The measurements of the two targets are discriminated through the use of two types of Alpha Acceptor beads, based on the use of Europium (Eu) and Terbium (Tb) which emit light at distinct wavelengths of 615 nm and 545 nm, respectively. Antibodies in the kits are tagged specifically to bind to the correct Acceptor bead, and produce a signal proportional to the level of the target being measured.

For multiplexed detection using the combination of an AlphaLISA *SureFire Ultra* kit (ALSU) and a Terbium *SureFire Ultra* kit (TBSU), two different phosphoproteins are measured in the same well of an assay plate. In this case, the reagents of both the ALSU kit and the TBSU kit are combined, as instructed in the TBSU kit manual. The target of the ALSU kit will be reported through the Europium Acceptor bead, and the target of the TBSU kit through the Terbium Acceptor bead. Thus, two pairs of antibodies are present in the ALSU + TBSU kit combination, and the antibodies bind to their respective Acceptor beads via highly specific association with two distinct antibody immobilization agents, CaptSure™ and CaptSure2™. The Donor bead will bind the biotinylated antibodies of both targets. This assay principle is illustrated in Figure 1.

In this application note we describe the implementation of multiplexing with the TBSU/ALSU kit combination and provide some example data to show assay performance. An extensive analysis of the MPSU kit setup and data can be found in separate publications on the Revvity website.[1,2]

Material and methods

Cell culture

96-well plates used for all cell culture were Revvity tissue culture-treated, black, clear bottom ViewPlates (#6005182).

HeLa cells, a human cervix adenocarcinoma cell line, were plated into 96-well plates at 150K cells/mL in MEM (Gibco, #11095-080) with 10% FBS (Gibco, #10099-141) (200 µL/well, i.e. 30,000 cells/well). Cells were grown overnight, and then serum starved for two hours. Where pathway inhibitors were studied, these were added to cells for two hours, prior to agonist stimulation. Medium was then removed, and cells lysed with 100 µL of 1X *SureFire Ultra* Lysis Buffer.

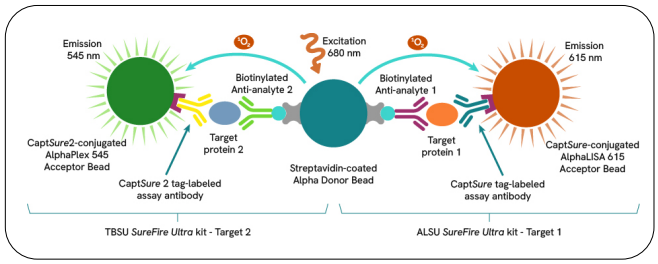


Figure 1. Multiplexed Alpha measurement using Terbium *SureFire Ultra* (TBSU) and AlphaLISA *SureFire Ultra* (ALSU) kits in combination.

A431 cells, a human epidermoid carcinoma cell line, were grown in DMEM (Gibco, #11960-044) with 10% FBS, seeded at 200K cells/mL in 96-well plates (200 µL/well, i.e. 40,000 cells/well), and grown overnight. Cells were then serum starved, and treated with pathway inhibitors as required for two hours. Cells were then stimulated as indicated with agonists, medium removed, and cells lysed with 100 µL of 1X *SureFire Ultra* Lysis Buffer.

MCF7 cells, a human breast adenocarcinoma cell line, were plated in 96-well plates at 200K cells/mL in MEM with 10% FBS (200 µL/well, i.e. 40,000 cells/well), and grown

overnight. Cells were then serum starved for two hours in the presence or absence of pathway inhibitors. Cells were then treated with agonists as indicated, and medium removed and cells lysed with 100 µL of 1X *SureFire Ultra* Lysis Buffer.

Stimulators used include anisomcyin (Sigma, #A9789), TNFα (R&D Systems, #210-TA-010), and EGF (Merck, #324831). Inhibitors used include AG1478 (Merck, #658548), wortmannin (Merck, #681676), and rapamycin (Merck, #553210).

Alpha *SureFire* immunoassays

The reagents used in this application note are available from Revvity and are detailed in the tables in Figure 2B. All kits are available in the 100 (High Volume, 96-well Immunoassay format), 500, 10000 or 50000 (384-well Immunoassays format) data points sizes, but only the catalog number for the 500 data points kits are listed. For each assay, 10 µL of cell lysate were transferred to a 384-well AlphaPlate (Revvity, #6005350). These plates are light-gray, in order to minimize cross-talk issues in luminescent applications.

The set-up of an Alpha *SureFire Ultra* Multiplex assay with ALSU and TBSU starts with setting up for a standard AlphaLISA SureFire Ultra assay, by preparing the Acceptor Mix as indicated in the kit protocol. To this Acceptor Mix, the components from the Terbium *SureFire Ultra* kits are added for the second target (antibody-containing Reaction Buffers and Tb Acceptor beads), completing the Multiplex Acceptor Mix preparation. The TBSU reagents are provided as concentrated solutions, avoiding any significant dilution of the ALSU reagents.

The Donor Mix is subsequently made up with Donor beads. The Donor Beads are provided in both the ALSU and TBSU kits and are combined to reach a concentration that is double the concentration normally used in a single ALSU assay reaction. This is done in order to generate an optimal level of signal in the multiplexed assay. The remainder of the assay procedure is carried out in the same way a standard AlphaLISA *SureFire Ultra* assay is. The protocol for an Alpha *SureFire Ultra* Multiplex assay is detailed in Figure 3.

Instrumentation

All assays were read on a standard Revvity EnVision™ 2104 multimode plate reader equipped with Alpha capability and with two detectors. For AlphaLISA *SureFire Ultra* single target reading, the standard alpha settings were used (AlphaScreen™ single mirror barcode #444, catalogue #2101-4010, AlphaScreen 570/100 filter barcode #244, catalogue #2100-5710). The filters and mirrors used to collect the duoplex data are shown in Table 1. Each duoplex determination was carried out in simultaneous duplex reading mode, where the entire plate was only read one time, with simultaneous measurement of Terbium and Europium. Instrument set up was duplicated as previously described.[1,2]

Data analysis

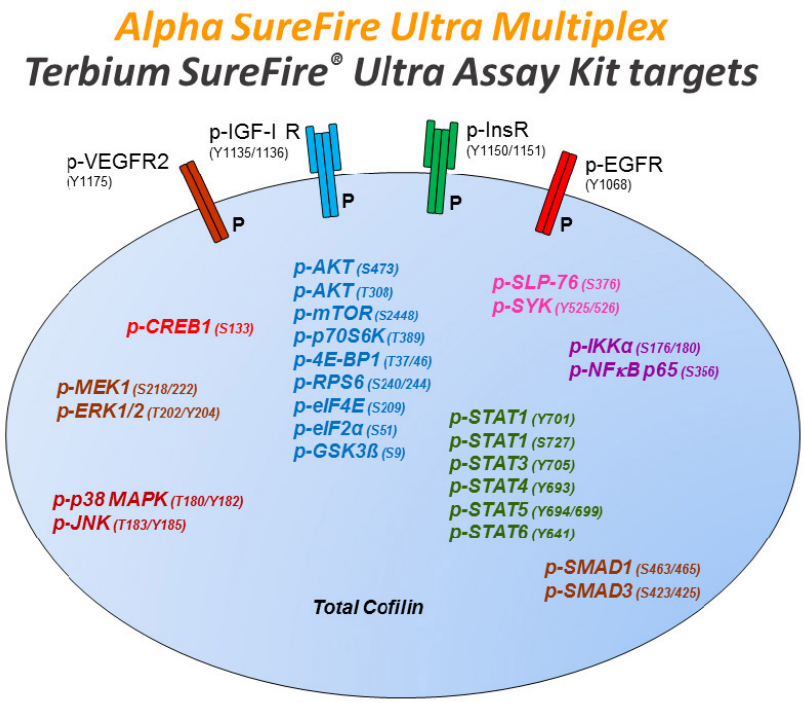
Spectral overlap correction (SOC) factors were established for each target pair as previously described,[1,2] using the ALSU and TBSU reagents. In summary, a serial dilution of a portion of the samples generated in each experiment was prepared using 1X AlphaLISA *SureFire Ultra* lysis buffer, and three concentrations (high, medium and low) of sample were used to calculate SOC factor values for both Eu and Tb data. The SOC were calculated as being x% for the overlap of the Tb signal into the Eu channel, and y% for the overlap of the Eu signal into the Tb channel. The average of the SOC factor values calculated using the high, medium and low sample concentrations was then calculated. Then, for each multiplexed experiment performed, these averaged SOC factors were used to correct the Eu counts, by subtracting from it x% of the corresponding Tb signal, and for correcting the Tb counts by subtracting from it y% of the corresponding Eu signal. The Alpha signals shown in the figures are the SOC-corrected Alpha values.

Agonist and compound inhibitor titration curves were plotted and EC_{50} or IC_{50} values calculated using GraphPad Prism® software according to a nonlinear regression using the four-parameter logistic equation sigmoidal dose-response curve with variable slope. Data shown are the average of triplicate measurements.

Table 1. Filters and mirror modules used on EnVision multimode plate reader.

Reading mode	EnVision mirror module	EnVision filter
Simultaneous Duplex	*AlphaPlex Dual Tb/Eu* Barcode 653-(#2102-5900)	**Terbium** *Resorufine/Amplex Red* Barcode 124 (#2102-5570) **Europium** *Europium* Barcode 203 (#2100-5090)

A

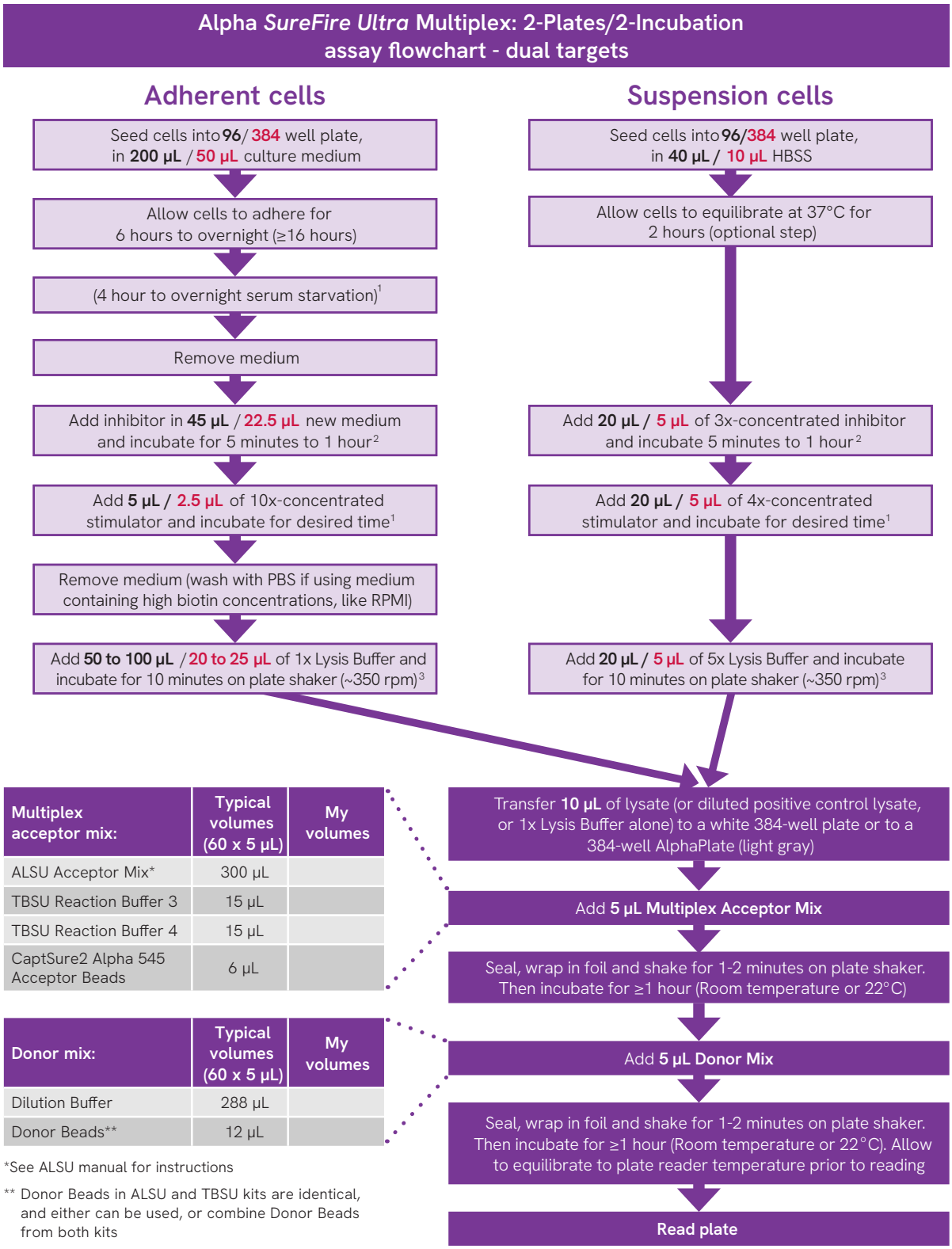


B

Target	ALSU kit	TBSU kit
Phospho-Akt1/2/3 (Ser473)	ALSU-PAKT-B500	TBSU-PAKT-B500
Phospho-CREB-1 (Ser133)	ALSU-PCREB-A500	TBSU-PCREB-A500
Phospho-EGF Receptor (Tyr1068)	ALSU-PEGFR-A500	TBSU-PEGFR-A500
Phospho-ERK1/2 (T202/Y204)	ALSU-PERK-A500	TBSU-PERK-A500
Phospho-IGF1 Receptor β (Tyr1135/1136)	ALSU-PIGFR-B500	TBSU-PIGFR-B500
Phospho-JNK1/2/3 (Thr183/Tyr185)	ALSU-PJNK-A500	TBSU-PJNK-A500

Target	ALSU kit	TBSU kit
Phospho-MEK1 (Ser218/222)	ALSU-PMEK1-A500	TBSU-PMEK1-A500
Phospho-NFκB p65 (Ser536)	ALSU-PNFKB-A500	TBSU-PNFKB-A500
Phospho-p38 MAPK (Thr180/Tyr182)	ALSU-PP38-B500	TBSU-PP38-B500
Phospho-p70S6K (Thr389)	ALSU-PP70-A500	TBSU-PP70-A500
Phospho-STAT1 (Ser727)	ALSU-PST1-B500	TBSU-PST1-B500
Phospho-STAT3 (Tyr705)	ALSU-PST3-A500	TBSU-PST3-A500
Phospho-STAT5 (Tyr694/699)	ALSU-PST5-B500	TBSU-PST5-B500

Figure 2. **Phosphoprotein Targets.** (A) Panel 1 TBSU targets available for combining with AlphaLISA *SureFire Ultra* assay kits for pick-and-pack multiplexing of phosphoprotein targets. Further assays will be developed and a complete list of targets can be found at www.Revvity.com/category/alpha-*SureFire*-kits (B) Reagents used in this application note to assess phosphoproteins.



Multiplex acceptor mix:	Typical volumes (60 x 5 µL)	My volumes
ALSU Acceptor Mix*	300 µL	
TBSU Reaction Buffer 3	15 µL	
TBSU Reaction Buffer 4	15 µL	
CaptSure2 Alpha 545 Acceptor Beads	6 µL	

Donor mix:	Typical volumes (60 x 5 µL)	My volumes
Dilution Buffer	288 µL	
Donor Beads**	12 µL	

*See ALSU manual for instructions

** Donor Beads in ALSU and TBSU kits are identical, and either can be used, or combine Donor Beads from both kits

[1]Depending on cell type and pathway analyzed.

[2]Depending on type of inhibitor used: 5 minutes is generally enough for receptor antagonists; more time is needed to block intracellular targets.

[3]May stop and freeze lysates at -20°C if desired. If doing this, re-shake after thawing to ensure homogeneity of lysate before pipetting.

Figure 3. Protocol for multiplexed measurement of two phosphoproteins using the combination of an AlphaLISA *SureFire Ultra* (ALSU) assay kit and a Terbium *SureFire Ultra* (TBSU) assay kit.

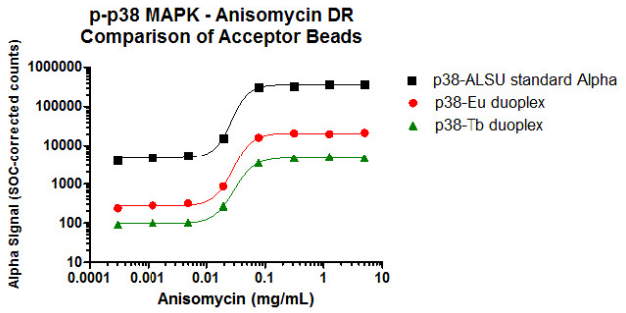
Results

Determination of relative data congruity across different alpha output settings and acceptor beads

HeLa cells were treated with anisomycin at the indicated concentrations for 30 minutes at 37 °C, medium removed, and cells lysed with 1X *SureFire Ultra* Lysis buffer. Replicate samples were then analysed for levels of p38MAPK phosphorylation either as a single target (AlphaLISA *SureFire Ultra* kit, standard alpha reading) or in duoplex mode (Eu or Tb). As shown in Figure 4, the dose-response relationship of standard measurement of p-p38MAPK (ALSU kit alone) has an identical profile to that seen in duoplex mode from either the Eu or Tb channel. The levels of counts were reduced in the selective Eu and Tb duoplex channels, but this is expected due to the necessity to selectively filter the bead-specific wavelength light. Furthermore, as indicated by the calculated EC_{50} values for anisomycin action, the dose-response curves are qualitatively identical. The signal to background was slightly lower for the Tb curve, due to the lower signal intensity in this channel, giving a higher relative contribution to the instrument background signal.

Multiplex analysis of protein phosphorylation in HeLa cells

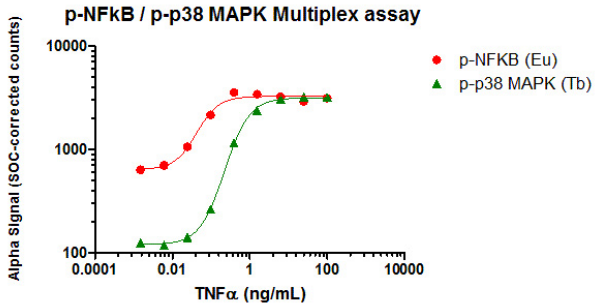
To assess the ability of the AlphaLISA *SureFire Ultra* and the Terbium *SureFire Ultra* kits to detect changes on phosphorylation, HeLa cells were treated with either TNFα or anisomycin for 30 minutes, medium was removed and cells were lysed with 1X *SureFire Ultra* lysis buffer. A Multiplex Acceptor mix was made by the combination of an AlphaLISA *SureFire Ultra* (ALSU) assay kit and a Terbium *SureFire Ultra* (TBSU) assay kit, for two separate targets. Samples (10 µL) of lysates were pipetted into an AlphaPlate 384, 5 µL of Multiplex Acceptor mix added to each, and the plate incubated for one hour. Donor Mix (5 µL) was then added to each well, and after an hour the plate was read on an EnVision multimode plate reader in Alpha duoplex mode for both Eu and Tb, to provide readout of phosporylation levels of both targets. The results of these experiments are shown in Figure 5. In Figure 5A p-NFkB was assessed with ALSU, while p-p38MAPK was assessed using TBSU; Figure 5B shows p-CREB (ALSU) and p-ERK (TBSU); Figure 5C shows p-JNK (ALSU) and p-p38MAPK (TBSU); Figure 5D shows p-p70S6K (ALSU) and p-STAT1(Ser727) (TBSU).



	P38-Ultra	P38-Eu	P38-Tb
EC_{50} (µg/mL Anisomycin)	26.8	28.5	29.6
S/B (TOP/Bottom)	110.2	87.5	58.5

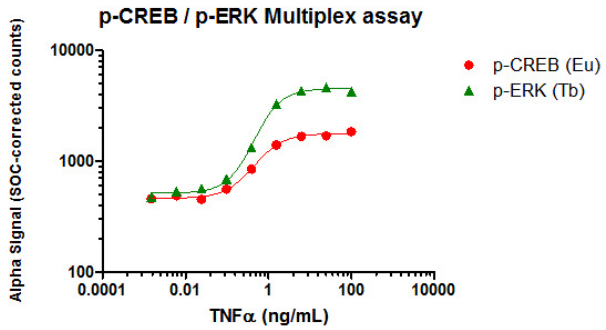
Figure 4. **Determination of relative data**. Cells were treated with anisomycin and levels of p38MAPK phosphorylation were measured using either a single target (ALSU standard Alpha), or in duoplex mode (Eu or Tb). For duoplex readings, the output of the other target from the alternative Acceptor bead has been omitted in this figure.

A



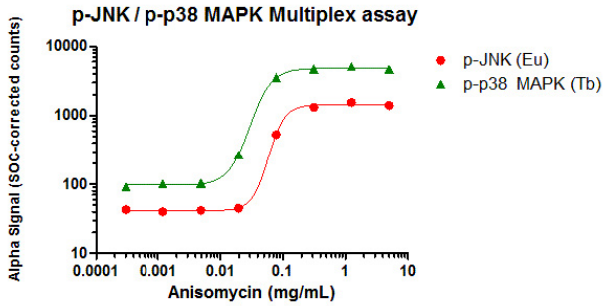
	p-NFkB (Eu)	p-P38 (Tb)
EC_{50} (ng/mL TNFα)	0.042	0.22
S/B (TOP/Bottom)	5.1	26.4

B



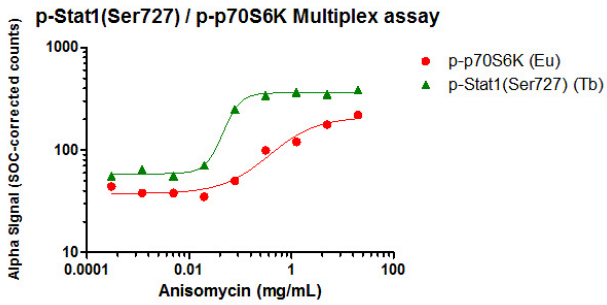
	p-CREB (Eu)	p-ERK (Tb)
EC_{50} (ng/mL TNFα)	0.43	0.46
S/B (TOP/Bottom)	4.4	10.1

C



	p-JNK (Eu)	p-p38MAPK (Tb)
EC_{50} (µg/mL Anisomycin)	59	30
S/B (TOP/Bottom)	36.6	51.3

D



	p-p70S6K (Eu)	p-STAT1 (Tb)
EC_{50} (µg/mL Anisomycin)	369	45.6
S/B (TOP/Bottom)	5.6	7.2

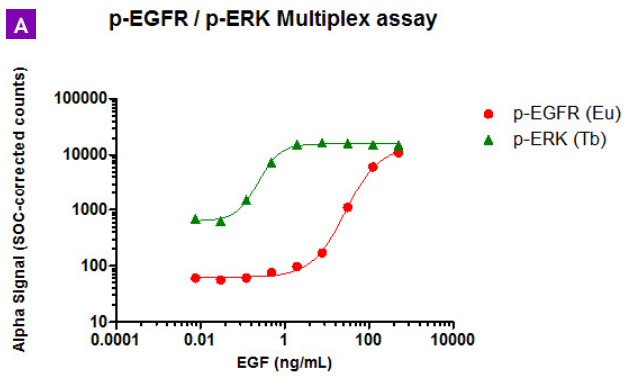
Figure 5. **Multiplex anaylsis of protein phosphorylation in HeLa cells.** (A) NFκB using ALSU and p38MAPK using TBSU; (B) CREB using ALSU and ERK using TBSU; (C) JNK using ALSU and p38MAPK using TBSU; (D) p70S6K using ALSU and STAT1(Ser727) using TBSU.

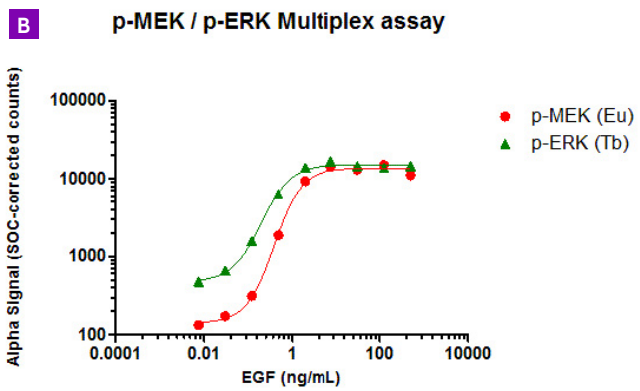
Multiplex analysis of protein phosphorylation in A431 cells

Next, A431 cells were stimulated with EGF for 10 minutes, medium removed, and cells lysed with 1X *SureFire Ultra* lysis buffer. A Multiplex Acceptor mix was made by the combination of an AlphaLISA *SureFire Ultra* (ALSU) assay kit and a Terbium *SureFire Ultra* (TBSU) assay kit, for two separate targets. Samples (10 µL) of lysates were pipetted into an AlphaPlate 384, 5 µL of Multiplex Acceptor mix added to each, and the plate incubated for one hour. Donor Mix (5 µL) was then added to each well, and after an hour the plate was read on an EnVision multimode plate reader in Alpha duoplex mode for both Eu and Tb, to provide readout of phosporylation levels of both targets.

The results of these experiments are shown in Figure 6. In Figure 6A p-EGFR was assessed with ALSU, while p-ERK was assessed using TBSU; Figure 6B shows p-MEK1 (ALSU) and p-ERK (TBSU).

The effectiveness of multiplex analysis when phosphorylation is inhibited was next assessed. A431 cells were treated with the EGF receptor inhibitor AG1478 at the various concentrations indicated for two hours and then stimulated with 1 ng/ml of EGF for 10 minutes. Figure 7 illustrates the ability of this multiplexed approach to detect decreased levels of phosphorylation.

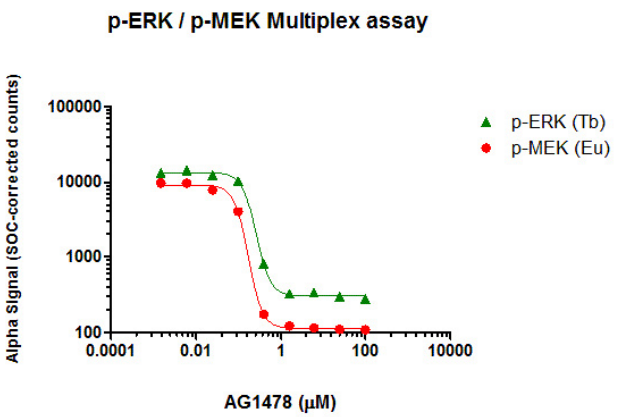




	p-EGFR (Eu)	p-ERK (Tb)
EC_{50} (ng/mL EGF)	28	0.23
S/B (TOP/Bottom)	165	34

	p-MEK1 (Eu)	p-ERK (Tb)
EC_{50} (ng/mL EGF)	0.39	0.20
S/B (TOP/Bottom)	71	26

Figure 6. **Multiplex anaylsis of protein phosphorylation in A431 cells.** (A) EGFR using ALSU and ERK using TBSU; (B) MEK1 using ALSU and ERK using TBSU.



	p-MEK1 (Eu)	p-ERK (Tb)
IC_{50} (µM AG418)	0.26	0.17
S/B (TOP/Bottom)	102	50

Figure 7. **Multiplex analysis of MEK1 and ERK phosphorylation in A431 cells after treatement with inhibitor.** After treatment with inhibitor followed by stimulation, levels of phosphorylated MEK1 were measured using a standard ALSU assay kit while levels of phosphorylated ERK were simultaneously measured using the TBSU assay kit.

Multiplex analysis of EGF-induced time-course changes in p-EGFR, p-MEK, p-STAT3 and p-STAT5 in A431 cells

A431 cells were treated with EGF (500 ng/mL) for the various time points indicated. Medium was then removed and cells lysed with 1X *SureFire Ultra* lysis buffer. Multiplex assays were made by the combination of an AlphaLISA *SureFire Ultra* (ALSU) assay kit and a Terbium *SureFire Ultra* (TBSU) assay kit, for two separate targets. Samples (10 µL) of lysates were pipetted into AlphaPlates 384, 5 µL of Multiplex Acceptor mixes added to each, and the plates incubated for one hour. Donor Mix (5 µL) was then added to each well, and after an hour the plates were read on an EnVision in Alpha duoplex mode for both Eu and Tb, to provide readout of levels of each phosphoprotein. The results of this experiment can be found in Figure 8.

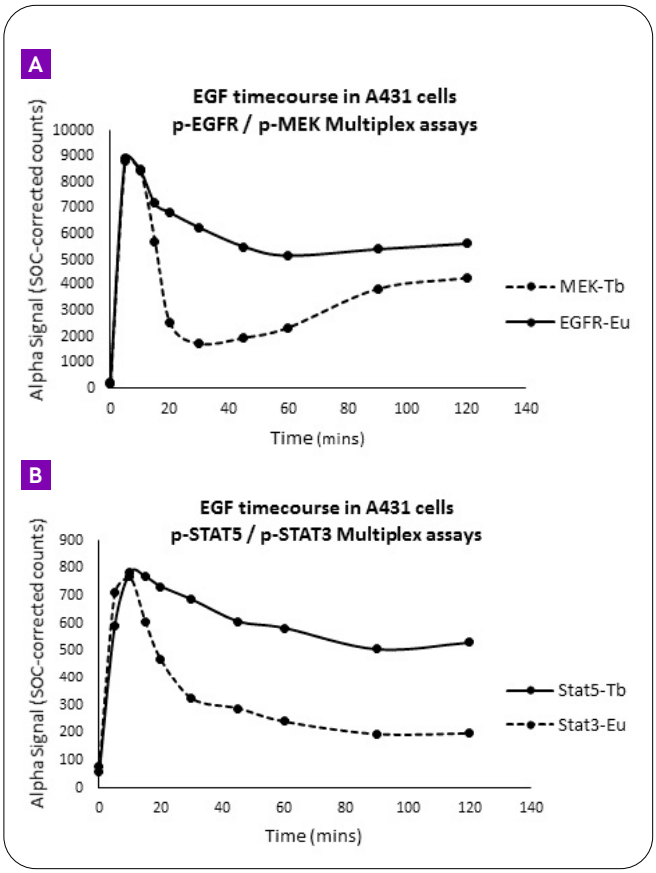
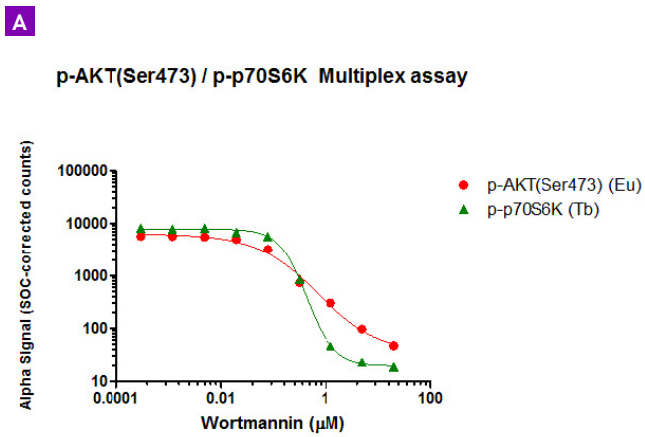


Figure 8. **Multiplex analysis of EGF-induced time-course changes in protein phosphorylation in A431 cells.** A431 cells were treated with 500 ng/mL of EGF for vaious time points. (A) p-EGFR assessed by ALSU and p-MEK assessed by TBSU, (B) p-STAT3 assessed by ALSU and p-STAT5 assessed by TBSU.

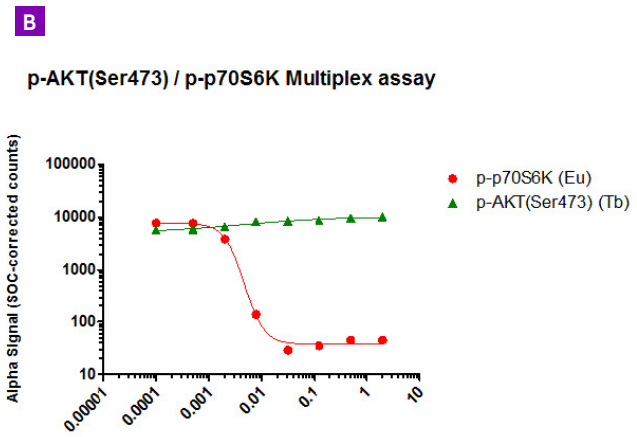
Multiplex analysis of protein phosphorylation in MCF-7 cells

MCF-7 cells were treated with either the PI 3-kinase inhibitor wortmannin or the mTOR inhibitor rapamycin at the various concentrations indicated for two hours, then stimulated with insulin (2.5 µg/mL) for 30 minutes. Medium was then removed and cells lysed with 1X *SureFire Ultra* lysis buffer. Multiplex assays were made by the combination of an AlphaLISA *SureFire Ultra* (ALSU) assay kit and a Terbium *SureFire Ultra* (TBSU) assay kit, for two separate targets. Samples (10 µL) of lysates were pipetted into AlphaPlates 384, 5 µL of Multiplex Acceptor mixes added to each, and the plates incubated

for one hour. Donor Mix (5 µL) was then added to each well, and after an hour the plates were read on an EnVision in Alpha duoplex mode for both Eu and Tb, to provide readout of levels of each phosphoprotein. The results of this experiment can be found in Figure 9. In Figure 9A cells were treated with the PI 3-kinase inhibitor wortmannin and AKT 1/2/3 (Ser473) phosphorylation was assessed using ALSU while p70S6K (Thr389) phosphorylation was assessed using TBSU. In Figure 9B cells were treated with the mTOR inhibitor rapamycin and p70S6K (Thr389) phosphorylation was assessed using ALSU while AKT 1/2/3 (Ser473) phosphorylation was assessed using TBSU.



	p-AKT(S473) (Eu)	p-p70S6K (Tb)
IC_{50} (µM Wortmannin)	0.71	0.43
S/B (TOP/Bottom)	108	420



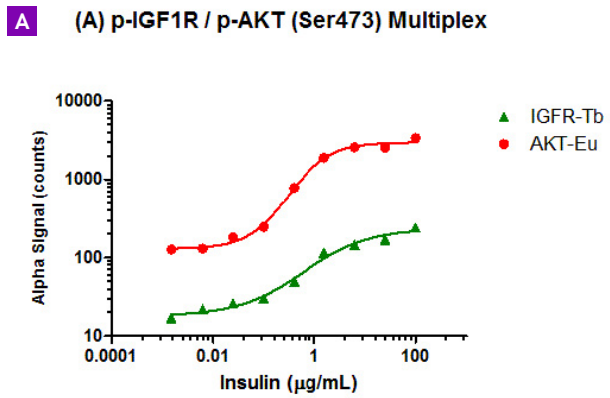
	p-AKT(S473) (Eu)	p-p70S6K (Tb)
IC_{50} (µM Rapamycin)	n.a	0.0047
S/B (TOP/Bottom)	n.a	262

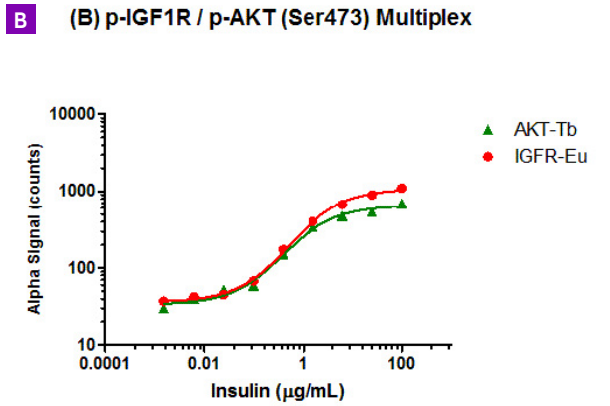
Figure 9. **Multiplex analysis of protein phosphorylation in MCF-7 cells.** (A) Cells were treated with PI-3 kinase inhibitor wortmannin, p-AKT 1/2/3 (Ser473) levels were measured by ALSU and p-p70S6k (Thr389) levels measured by TBSU; (B) Cells were treated with mTOR inhibitor rapamycin, p-p70S6k (Thr389) levels were measured by ALSU and p-AKT 1/2/3 (Ser473) levels measured by TBSU.

Specific selection of assay kit readouts can provide optimal assay windows for low abundance targets

A431 cells were treated for five minutes with insulin at the concentrations shown, medium removed, and cells lysed with 1X *SureFire Ultra* Lysis buffer. Samples of each lysate were then analyzed for duoplex detection of AKT (Ser473) and IGF-I receptor phosphorylation, either in the Tb or Eu channels or the reverse orientation, using respective TBSU and ALSU assay kits. Figure 10 shows the results of this experiment. In Figure 10A, AKT (Ser473) phosphorylation is assessed by ALSU while IGF-1 receptor phosphorylation is assessed by TBSU. Figure 10B shows the same samples being assayed in the reverse orientation, with IGF-I receptor phosphorylation assayed by ALSU, and AKT

(Ser473) phosphorylation by TBSU. It can be seen that optimal analysis of these targets was best achieved when the lower-abundance IGF-I receptor was assayed in the Eu channel using the ALSU kit for that target in combination with p-AKT (Ser473) measurement using its TBSU kit.





	p-AKT(S473) (Eu)	IGFR (Tb)
EC_{50} (µg/mL Insulin)	0.30	0.60
S/B (TOP/Bottom)	16.3	11.5

	p-AKT(S473) (Tb)	IGFR (Eu)
EC_{50} (µg/mL Insulin)	0.39	0.52
S/B (TOP/Bottom)	15.6	24.6

Figure 10. **Multiplex analysis of AKT (Ser473) and IGF-I receptor phosphorylation in A431 cells** shows that specific selection of assay kit readouts can provide optimal assay windows for low abundance targets. (A) p-AKT (Ser473) assessed by ALSU and p-IGFR assessed by TBSU; (B) p-IGFR assessed by ALSU and AKT (Ser473) assessed by TBSU.

Discussion

The data presented here show that the Alpha *SureFire Ultra* Multiplex system provides excellent assay windows and sensitivity for the simultaneous measurement of two phosphoprotein targets per sample, combined with the ease and throughput of a homogeneous assay format. Importantly, the targets to be measured in combination can be selected as desired by the scientist, rather than being pre-defined off-site. The combination of the Alpha Terbium *SureFire Ultra* (TBSU) kits available at the launch of this technology (panel 1) with formerly existing AlphaLISA *SureFire Ultra* (ALSU) kits already makes more than 1000 possible target combinations.

As a general rule, we recommend comparing the two possible orientations of the assay (i.e. each target on the Eu and Tb side vs the other target on the Tb and Eu side). But from our experience, and due to the lower signal intensity in the Tb channel, the most abundant target should be tested on the Tb side. Alternatively, it can be decided to put the most important target (for example the one to be inhibited by the test compounds) on the Eu side to benefit from the strongest signal intensity, and to use the less intense Tb channel for the control target. Such analysis of a low abundance target is shown in Figure 10, where the measurement of the p-IGF-1 receptor is more effectively measured in the Eu channel, and the counter screen of AKT (Ser473) phosphorylation is analysed in the Tb channel.

While the TBSU + ALSU Multiplex kit combinations are designed principally to compare two phosphorylation events, the Total Cofilin assay TBSU kit has been developed to allow for sample loading control for a phospho target measured in the Eu channel. The ALSU Total protein kits are generally not suitable for combining with a TBSU kit in multiplex, due to incompatibilities in the antibody orientations. It should also be noted that we do not recommend combining the total GAPDH ALSU assays with the TBSU kits, as the extremely high concentration of GAPDH in many cell lysates generally requires differential sample dilution.

As most phospho targets in the ALSU range can be combined with a TBSU target, this opens up the potential for further levels of plexing as required by the researcher. For example, in side-by-side wells of an assay plate, a TBSU target can be combined with different duoplex partners from the ALSU range (or vice versa). This can allow a very broad level of pathway analysis for the scientist, which can be decided at the time of the experiment. Such flexibility of assay set-up and target selection is unsurpassed in the industry, and should provide researchers with a much improved capability of discovery.

References

1. Nelson, Lindsay; Hurt, Steve; Dupriez, Vincent; Sheehan, Antony; Crouch, Michael (2016). Alpha *SureFire Ultra* Multiplex Technology. Revvity. Application Note.
2. Nelson, Lindsay (2017). Multiplex Assays with Alpha *SureFire Ultra* Multiplex Technology using the EnVision Multilabel Plate Reader. Revvity. Technical Note.

Revvity, Inc.
940 Winter Street
Waltham, MA 02451 USA
www.revvity.com