AlphaLISA® *SureFire® Ultra*™ and Alpha *SureFire® Ultra*™ Multiplex Assays: Rapid homogeneous detection systems for cellular signalling pathways

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1 Next-generation kinase screening

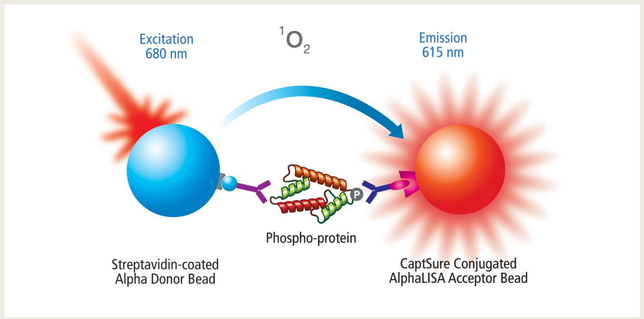
To address the ever-increasing demands for assay sensitivity and multiplexing, and the need to screen complex samples such as tissue extracts and antibody-containing samples, we have developed the next-generation *SureFire* kinase assays: AlphaLISA® *SureFire® Ultra*™ *and* Alpha *SureFire® Ultra*™ *Multiplex Assays*.

Incorporating the new Capt*Sure*™ technology, these assays provide unsurpassed sensitivity and assay flexibility for all screening requirements, including tissue extracts (e.g. animal models) and antibody-containing samples (e.g. biotherapeutics).

Key Features

- Mix-and-Read = no washing steps
- Enhanced assay sensitivity and signal-to-noise ratios
- No interference by sample antibodies = screen any sample type including from tissues or biotherapeutic antibody samples
- AlphaLISA bead means maximized assay performance for all sample types
- Multiplexing (Alpha *SureFire Ultra* Multiplex Assay)

AlphaLISA *SureFire Ultra*

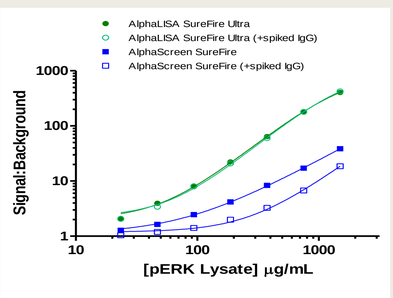


AlphaLISA *SureFire Ultra* detects phospho-proteins in cell lysates.

Each assay kit antibody is tagged to specifically link to either the Donor or Acceptor bead. Streptavidin-coated Donor beads bind the biotinylated antibody, and the Capt*Sure*™-coated Acceptor bead binds the other antibody via its Capt*Sure* tag. The result is maximized sensitivity and no interference by extraneous antibodies.

2 Screening of antibody-containing samples with AlphaLISA *SureFire Ultra*

Extracts of cells from tissue culture, or from tissue itself, can contain antibodies. These antibodies can interfere with some assays, such as AlphaScreen *SureFire* assays. The AlphaLISA *SureFire Ultra* assays, however, show no sensitivity to interference from antibodies. In addition, the AlphaLISA beads convey benefits for minimizing sample interference.



Measurement of p-ERK in the presence of extraneous antibodies. Lysates of EGF-activated A431 cells were serially diluted in the absence or presence of non-specific extraneous rabbit antibodies (10 µg/mL). Samples were then analysed for phospho-ERK (T202/Y204) with either a standard AlphaScreen *SureFire* kit (PerkinElmer cat# TGRES500) or an AlphaLISA *SureFire Ultra* p-ERK kit (PerkinElmer cat# ALSU-PERK-A500). Data are presented as the signal obtained for the lysate divided by the signal obtained for lysis buffer alone.

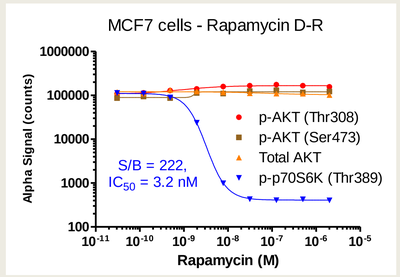
AlphaLISA *SureFire Ultra* kits already available:

- p-4E-BP1 (Thr37/46)
- p-Akt1/2/3 (Thr308)
- p-Akt1/2/3 (Ser473)
- p-CREB (Ser133)
- p-EGF Receptor (Tyr1068)
- p-ERK1/2 (Thr202/Tyr204)
- p-eIF2α (Ser51)
- p-eIF4E (Ser209)
- p-IGF-1 Receptor β (Tyr1135/1136)
- p-IKKα (Ser176/180)
- p-Insulin Receptor β (Tyr1150/1151)
- p-NF-κB p65 (Ser536)
- p-p38 MAPK (Thr180/Tyr182)
- p-p70 S6K (Thr389)
- p-SMAD1 (Ser463/465)
- p-SMAD3 (Ser423/425)
- p-STAT3 (Tyr705)
- p-STAT5 (Tyr694/699)
- p-VEGF Receptor 2 (Tyr1175)
- ERK1/2 Total
- Akt1 Total
- GAPDH Total
- p38 MAPK Total

Further kits in development

3 Multi-target analysis of phosphorylated cellular targets with AlphaLISA *SureFire Ultra*

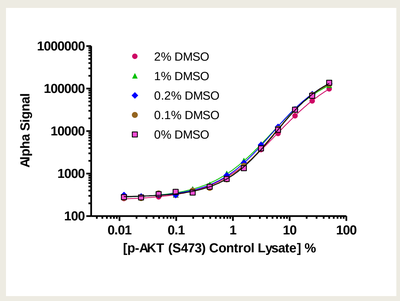
The ability to measure multiple different cellular targets on a single assay plate, with no wash steps, provides the potential for full automation of HTS pathway analysis. We show here the measurement of 3 phosphoprotein targets, and total AKT, performed side-by-side from aliquots of individual culture wells of insulin-stimulated MCF-7 cells.



MCF-7 cells were plated overnight at 200K/mL in 200 µL MEM + 10% FCS. Cells were then treated for 2 hours with varying concentrations of rapamycin in MEM +1% FBS, and then stimulated for 30 min with 2.5 µg/mL insulin (n = 3 for each condition). Each well was lysed in 100 µL *SureFire Ultra* lysis buffer, and 10 µL aliquots of each lysate were assayed in parallel using the four AlphaLISA *SureFire Ultra* kits indicated.

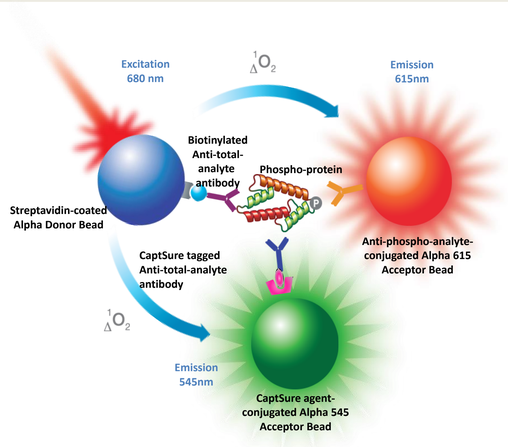
4 DMSO tolerance of AlphaLISA *SureFire Ultra*

Measurement of p-AKT (Ser473) in the presence of various concentrations of DMSO. Lysates of insulin-activated MCF-7 cells were serially diluted in the absence or presence of various concentrations of DMSO. Samples were then analysed for p-AKT (Ser473) with an AlphaLISA *SureFire Ultra* p-AKT (Ser473) kit (PerkinElmer cat# ALSU-PAKT-B500).
There was little or no effect of DMSO on the assay up to 2% DMSO.

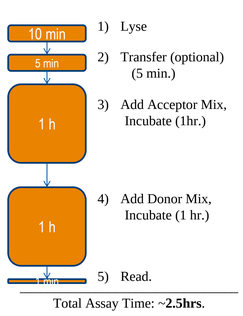


5 Alpha *SureFire Ultra* Multiplex Assay: Multiple target analysis in a single well

The Alpha *SureFire® Ultra*™ Multiplex assay kits provide the dual measurement of phosphoproteins from cells, combined with the measurement of the total amount of the same protein. 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: 545nm and AlphaLISA® (Europium) beads: 615nm). Currently, kits for AKT and ERK are available.



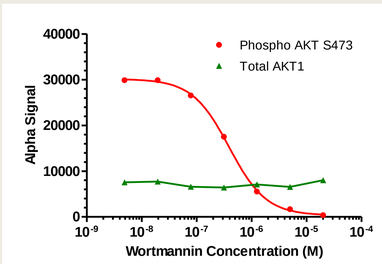
Quick and easy protocol:



	384-well format	96-well format
1) Lyse		
2) Transfer (optional) (5 min.)	10 µL	30 µL
3) Add Acceptor Mix, Incubate (1hr.)	5 µL	10 µL
4) Add Donor Mix, Incubate (1 hr.)	5 µL	10 µL
	V_{TOT} = 20 µL	V_{TOT} = 60 µL
5) Read.		

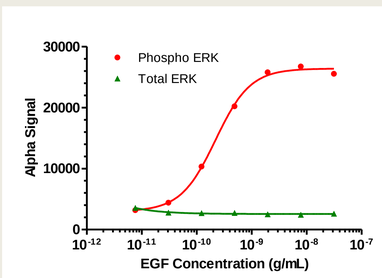
Total Assay Time: ~**2.5hrs.**

6 Alpha *SureFire Ultra* Multiplex Assay: Simultaneous measurement of p-AKT (Ser473) and Total AKT in each well



MCF-7 cells were plated overnight at 200K/mL in 200 µL MEM + 10% FCS. Cells were then treated for 2 hours with varying concentrations of wortmannin in MEM +1% FBS, and then stimulated for 30 min with 2.5µg/mL insulin (n = 3 for each condition). Each well was lysed in 100 µL *SureFire Ultra* lysis buffer, and 10 µL aliquots of each lysate were assayed simultaneously for p-AKT 1/2/3 (Ser473) and total AKT1 using the Alpha *SureFire Ultra* Multiplex kit.

7 Alpha *SureFire Ultra* Multiplex Assay: Simultaneous measurement of p-ERK and Total ERK in each well



A431 cells were plated overnight at 200K/mL in 200 µL MEM + 10% FCS. Cells were then serum starved for 2 hours, and then stimulated for 10 min with various concentrations of EGF (n = 3 for each condition). Each well was lysed in 100 µL *SureFire Ultra* lysis buffer, and 10 µL aliquots of each lysate were assayed simultaneously for p-ERK and total ERK using the Alpha *SureFire Ultra* Multiplex kit.

8 Summary

The measurement of cellular activation status frequently involves the study of protein phosphorylation, as these pathways can provide a detailed understanding of pharmaceutical compound effectiveness. In some cases, samples can contain antibodies, such as therapeutic antibody testing, or extracts of tissue specimens. Assays must therefore be compatible for such samples and, ideally, be a rapid mix-and-read format with high sensitivity.

To address these assay requirements, we have developed AlphaLISA® *SureFire® Ultra*™ and Alpha *SureFire® Ultra*™ Multiplex assays. These mix-and-read assays utilize the highly sensitive Alpha bead-based proximity detection system, coupled with Capt*Sure*™ immobilization technology. As there are no wash steps, these assays can be fully automated, and provide application to small and large screening requirements, including complex samples such as sera and biotherapeutics.

AlphaLISA® *SureFire® Ultra*™ assays have been optimized to measure key phosphoprotein targets in cellular signal transduction pathways. These assays have greatly enhanced signal windows, providing extremely high assay sensitivity.

Alpha *SureFire® Ultra*™ Multiplex assay utilizes two types of Alpha Acceptor beads, emitting light at 615 nm (Europium) and at 545 nm (Terbium), allowing the simultaneous measurement of two signals from each assay well, with an AlphaPlex™ compatible reader. We have recently released kits that allow the simultaneous measurement from the same well of phospho and total ERK, and phospho (Ser473) and total AKT. These kits, therefore, allow for immediate normalization of phospho signal to the level of total protein.

The new AlphaLISA® *SureFire® Ultra*™ and Alpha *SureFire® Ultra*™ Multiplex assays, therefore, provide new and highly optimized platforms for cellular analysis in all sample types, including those containing antibodies.

Further assays for both platforms will be available over the next 12 months.

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