STARTUP GUIDE

AlphaLISA *SureFire Ultra* Assay Optimization

Introduction

Protein phosphorylation is a dynamic process, tightly controlled by kinase and phosphatase cascades, which regulates many cellular processes. Monitoring phosphorylation events in a cellular model is a useful approach to detect the activity of compounds, and to provide evidence of their mechanism of action or target modulation. AlphaLISA® *SureFire® Ultra*™ assays provide a robust and reliable method to quantify a targeted phosphorylation event in cell-based experiments. To obtain the most useful results from AlphaLISA *SureFire Ultra*, a thorough investigation of the assay conditions must be tested to observe the best response from the modulator and cell line chosen. Multiple parameters often need to be optimized in the first set of experiments, some of which are more important to optimize initially to obtain a sufficient assay window for further study. This guide defines an approach that can help accomplish that goal and outlines further possible optimization of cellular and immunoassay parameters to ensure the best possible results are obtained.

The assays as described in the AlphaLISA *SureFire Ultra* kit manuals can be performed as either two-plate transfer protocols, or as a single-plate assay. We recommend beginning with the two-plate protocol at the initial assay optimization stage.

Assay Optimization

STEP ONE

Perform a Multi-Variable Experiment to Find Initial Conditions That Give a Sufficient Stimulated:Basal Response Ratio.

This workflow illustrates how to test the three variables of cell seeding density, serum starvation, and stimulation time in one experimental protocol. These parameters can be critical when setting up an assay for the first time. In addition, this experiment may be duplicated so that a second plate can be kept for an extra day in culture to test the influence of recovery time after cell seeding, which can sometimes be important in obtaining the best cellular response. Achieving an initial signal above basal facilitates subsequent assay optimization.

To begin (Day 0), plate three columns of three different densities (10K cells/well, 25K cells/well, 50K cells/well) in a 96-well plate in 100 µL/well culture medium containing 10% serum. For the figures presented in this guide, we used A431 cells in DMEM culture medium. Incubate the cells at 37 °C in 5% CO_2 overnight. The plated cells can be incubated longer (up to three days) to ensure the cells adhere properly and to allow basal phosphorylation levels to reach their minimum value. *For suspension cells, the cells can be plated directly into HBSS or serum-free culture medium on Day 1.*

To begin the assay (Day 1), carefully aspirate the media from the wells and replace with 0%, 1%, or full (10%) serum-containing media for three hours (alternatively, the aspiration step can be omitted for the 10% serum conditions, keeping the 10% serum-containing seeding medium). The duration of serum starvation can also be optimized by testing one hour, three hours, or overnight starvation periods.

Cell stimulation can be carried out at room temperature (on the bench), or in a cell culture incubator (37 °C, 5% CO_2). Add a single dose of agonist (selected to give a maximal response) for two stimulation times (in this case, 20 minutes and five minutes). To add agonist, remove culture medium from the plate and replace with serum-free HBSS containing the agonist. For these studies, agonist was first added to rows A-C, then to rows D-F after waiting 15 minutes. After stimulation, lyse cells by either aspirating the media and adding 1X AlphaLISA *SureFire Ultra* Lysis Buffer (50 µL/well), or alternatively directly add 1/5th volume of 5X AlphaLISA *SureFire Ultra* Cell Lysis Buffer to the well. After 10 minutes of shaking (350 rpm), transfer 10 µL of cell lysate to a 384-well AlphaPlate™ and perform the AlphaLISA *SureFire Ultra* immunoassay. The shaking step is important to ensure well-to-well consistency of cell lysis and, hence, assay results.

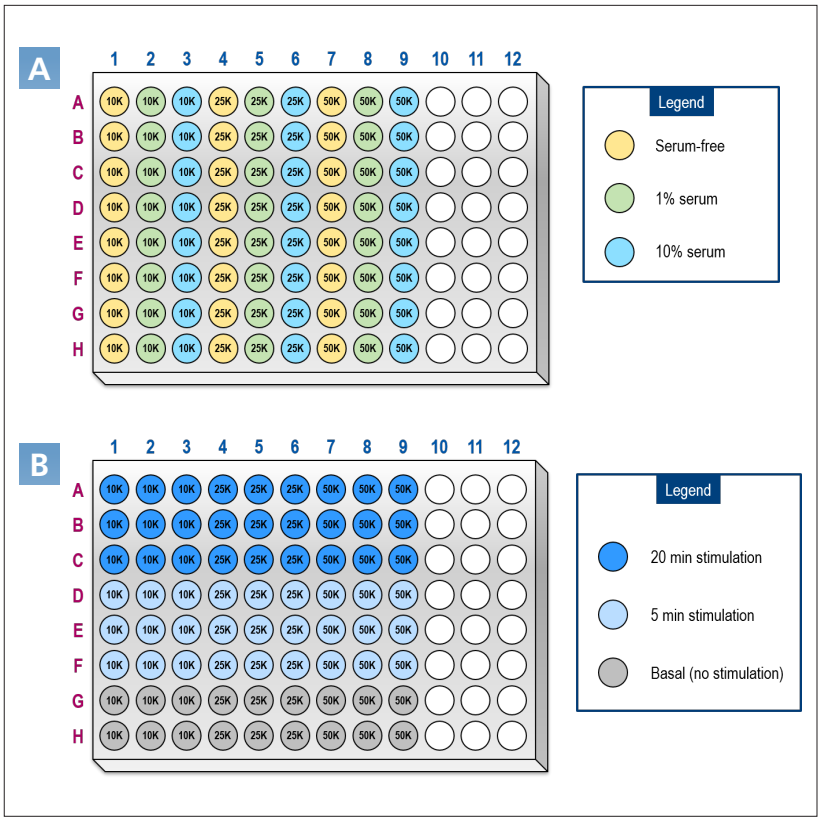

Figure 1. **Cell density/serum conditions optimization.** A) Platemap indicating the cell seeding densities and serum conditions. B) Platemap indicating the agonist stimulation conditions and time.

1444	1358	1831	4934	4284	4645	9507	8806	8054
1332	1763	1739	5376	4657	5490	9059	11077	14630
1892	1623	1614	4581	4273	5177	9313	10766	11003
2119	1449	1823	5977	5302	5285	13446	10523	12603
1485	1475	1579	5494	4281	5427	14399	8167	10834
1883	1601	1699	5434	4622	4546	13439	11334	9555
372	302	325	311	359	332	432	567	474
341	319	330	345	646	382	465	604	458

		Serum Conditions				
		0%	1%	10%		
Agonist Stimulation Time	20 min	1556	1581	1728	10K/well	Agonist Stimulation Time
	5 min	1829	1508	1700		
	Basal (No Stim)	357	311	328		
	20 min	4964	4405	5104	25K/well	
	5 min	5635	4735	5086		
	Basal (No Stim)	328	503	357		
	20 min	9293	10216	11229	50K/well	
	5 min	13761	10008	10997		
	Basal (No Stim)	449	586	466		

Table 1. Sample raw data (left) and averaged data (right) from experiment 1 (pSTAT3 assay). The AlphaLISA *SureFire Ultra* assay kit for phospho-STAT3 (Tyr705) (PerkinElmer #ALSU-PST3-A500) was run using A431 cells stimulated with 100 nM EGF. Platemap as indicated in Figure 1. Data is representative to what one can expect to see when carrying out Step #1. The assay conditions that gave the highest stimulated:basal ratio were 50,000 cells/well serum-starved (0% serum) for three hours, then stimulated with agonist for five minutes. In this example, the various serum conditions did not largely impact the basal phospho-STAT3 levels, so any of the serum conditions would be adequate.

STEP TWO

Optimization of Agonist Treatment Time Using Optimized Cell Culture Conditions.

The next step involves optimizing the agonist treatment time. Typically, cellular phosphorylation response to stimulation reaches some maximum level after ~ 2-30 minutes, or up to two hours later, depending on the pathway, target, cell type and stimulus used. Phosphorylation then decreases (or is sustained in some cases). For this reason, it is important to determine the optimal stimulation time for the cellular model used. Using optimized cell density and serum conditions from Step #1, test the agonist at various treatment times. Plate the cells at the density that gave the highest response ratio in Step #1 (Day 0) and treat under appropriate serum conditions (Day 1). Aspirate culture media and stimulate the cells with a single concentration of agonist (50 µL/well) in HBSS, selected to give an expected maximal response. Be sure to add the agonist to the wells for the longest treatment time first to enable lysing the entire plate at the same time. From these data, select a time-course using the condition that gives the largest assay window for further work.

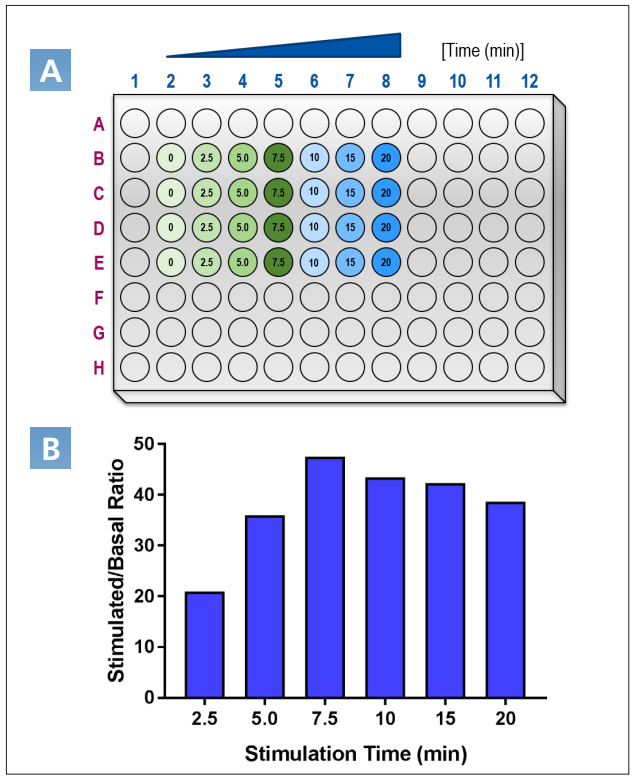

Figure 2. **Agonist time-course optimization.** A) Platemap and B) example data. 100 nM EGF was used to stimulate for 2.5 to 20 minutes A431 cells seeded at 50,000 cells/well, that had been starved for three hours in serum-free media. The AlphaLISA signal was measured for all stimulation samples as well as the unstimulated (basal) control (time=0), and the ratio of stimulated/unstimulated was calculated. A 7.5-minute stimulation yielded the largest window.

STEP THREE

Perform an Agonist Concentration Dose-Response Curve Using The Optimized Agonist Treatment Time.

With the agonist treatment time and cell density optimized, the next step is to identify the optimal agonist concentration that should be used in antagonist assays. For this purpose, a dose-response assay of the agonist should be carried out with the previous cell conditions and agonist treatment time from Steps #1 and #2. The resulting data should yield a dose-response curve from which the agonist concentrations yielding 50% of the maximal response (EC_{50}) and 90% of the maximal response (EC_{90}) can be determined. A reference agonist concentration between the EC_{50} and EC_{90} values should be selected to stimulate the cells to give at the largest assay window with the best sensitivity in antagonist assays.

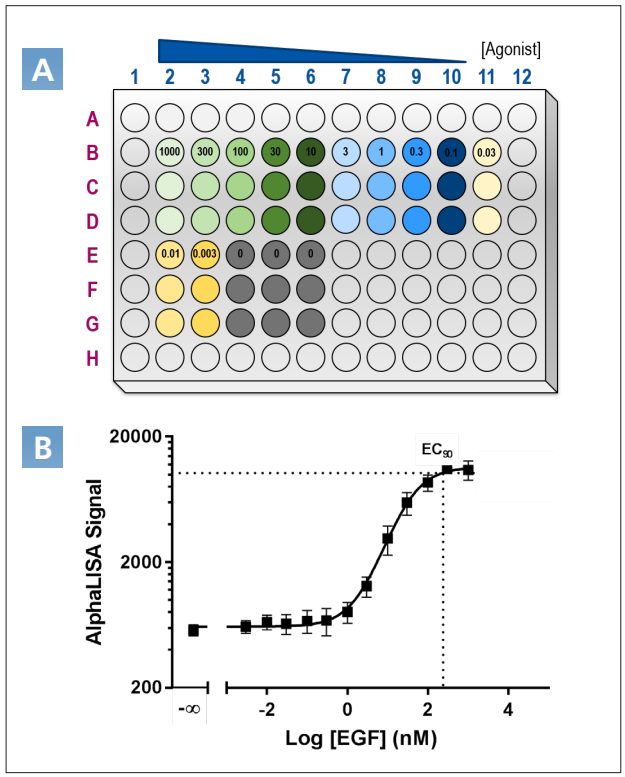

Figure 3. **Agonist dose-response optimization.** A) Platemap and B) example data. 3 pM - 1 µM EGF was used to stimulate serum-starved A431 cells for 7.5 minutes (50,000 cells/well, serum-free media for three hours). The dose-response curve was generated, showing 240 nM EGF gives near complete stimulation of the cells.

STEP FOUR

Perform an Antagonist Dose-Response Curve Using the Optimal Agonist Stimulation Conditions.

With the agonist concentration, cell density, and timing selected, antagonist testing should be carried out to determine how inhibitors of the system perform. We suggest using a well-studied antagonist where inhibition testing has been demonstrated on the chosen target, and if available, that has been demonstrated as being active on the chosen cellular model. Testing more than one antagonist builds confidence in the cell model and treatment conditions. Cell plating was carried out as in the previous tests, with culture medium aspirated and antagonist added in serum-free media for 30 minutes prior to the agonist stimulation.

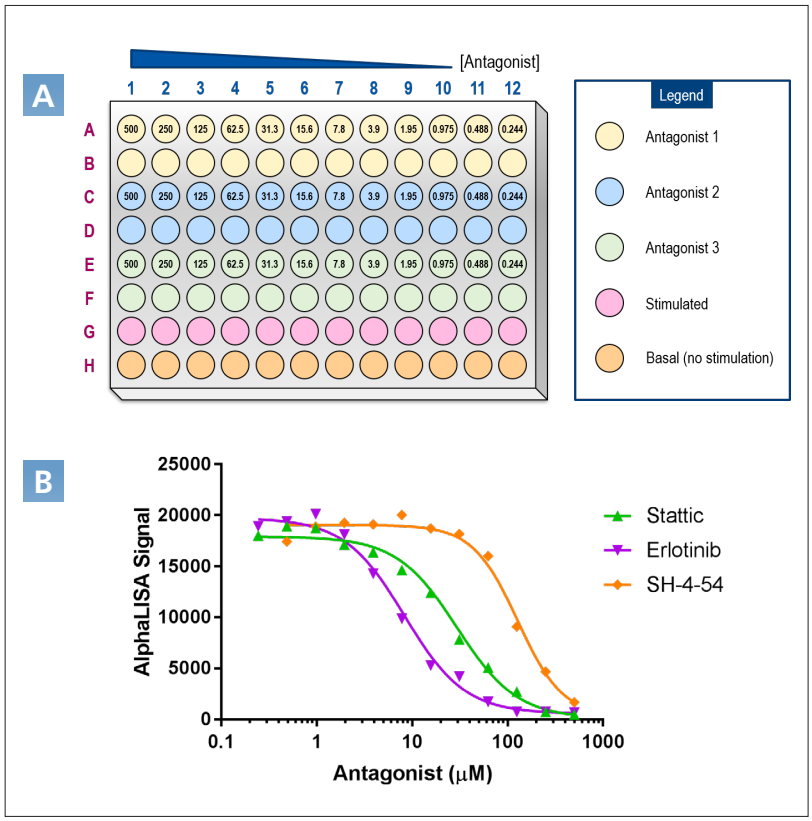

Figure 4. **Antagonist dose-response optimization.** A) Platemap and B) example data. A431 cells (50,000 cells/well) were serum-starved for three hours then treated with antagonist in serum-free media and incubated for 30 minutes. EGF (240 nM final) was then added to the cells and incubated for 7.5 minutes at room temperature. The media was aspirated, and cells were lysed by adding 50 µL AlphaLISA *SureFire Ultra* Lysis buffer and shaking for 10 minutes. All three antagonists show a dose-response effect in the AlphaLISA *SureFire Ultra* phospho-STAT3 (Tyr705) kit.

STEP FIVE

Immunoassay Incubation Time Optimization.

While the standard protocol has been designed to generate excellent results, it is sometimes possible to enhance assay performance by extending the AlphaLISA *SureFire Ultra* assay incubation times. Using optimized conditions from Steps #1-4, perform an assay to include a basal condition (no stimulation) and stimulated control (agonist only). For the data shown for Step #5, cell lysates were generated and the immunoassay was performed by varying the incubation time after the addition of the Donor Bead mix (one hour, two hours, and overnight).

A

Add 10 µL of cell lysate sample

Add 5 µL Acceptor Bead Mix

Incubate 1 hr at 23 °C

Add 5 µL Donor Bead Mix

Incubate 1 hr to overnight at 23 °C

Read with Alpha capable plate reader

B

Acceptor Bead Mix	60 Wells
Reaction Buffer 1	141 µL
Reaction Buffer 2	141 µL
Activation Buffer (25X)	12 µL
Acceptor Beads (50X)	6 µL
Total Volume	**300 µL**

Donor Bead Mix	60 Wells
Dilution Buffer 1	294 µL
Donor Beads (50X)	6 µL
Total Volume	**300 µL**

C

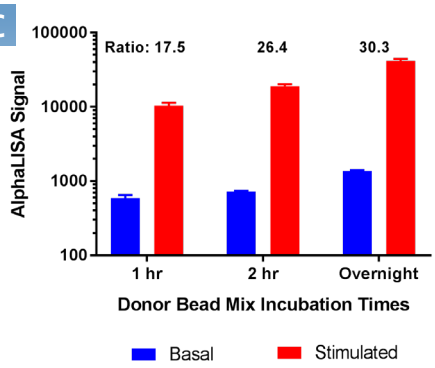

Figure 5. **Incubation time optimization. A) Protocol, B) reagent volumes, and C) example data.** Serum-starved A431 cells were treated with and without agonist (240 nM EGF). The cell lysates were pooled and tested using the AlphaLISA *SureFire Ultra* phospho-STAT3 (Tyr705) kit with the following incubation paradigms: one hour Acceptor Bead mix incubation followed by either an one hour, two hour, or overnight Donor Bead mix incubation. In this example, the stimulated:basal ratio increases with increasing Donor Bead incubation time.

Quantification

Positive control lysates are provided in AlphaLISA *SureFire Ultra* kits and should be run in parallel with unknown samples as a control of assay performance. This control is useful to have in to identify cases in which the selected cell type does not present a response to the assay being used. A dilution series of the positive control lysate can be assayed to determine the signal response obtained from an Alpha-capable microplate reader. Given that phosphoproteins are very labile by nature, it is not suggested to use the positive control lysate for absolute quantification. Proper storage of the positive control lysate (i.e. -20 °C when lyophilized and -80 °C in single-use aliquots for up to three months after reconstitution) is necessary to prevent degradation. With a well-optimized assay, the relative AlphaLISA signal can be used to determine an IC_{50}/EC_{50} if the response of the controls (stimulated and basal) are consistent. Normalization of the AlphaLISA *SureFire Ultra* signal response can be performed by measuring the total cell lysate protein levels using a method such as the Bradford assay. AlphaLISA *SureFire Ultra* Lysis Buffer is compatible with the Bradford spectrophotometric assay.

Another method to normalize the AlphaLISA *SureFire Ultra* signal response relies on the measurement of the total (i.e. both phosphorylated and non-phosphorylated) protein or of a housekeeping protein, such as cofilin. To achieve this, an aliquot of the prepared cell lysate can be transferred in parallel to a second microplate well, and tested with an AlphaLISA *SureFire Ultra* total protein assay (in this example the Total STAT3 assay kit would be used), or with the AlphaLISA *SureFire Ultra* Total Cofilin assay kit.

Multiplexing

To normalize AlphaLISA *SureFire Ultra* results, one can measure the total (phosphorylated and non-phosphorylated) protein from the same assay well. This can be done by using Alpha Acceptor Beads that utilize Terbium, which emit a signal at 545 nm, and can therefore be discriminated from the AlphaLISA signal at 615 nm using adequate filters and optics. Alpha *SureFire Ultra* Multiplex kits contain all reagents necessary to detect a specific phosphoprotein and its total protein counterpart. Terbium *SureFire Ultra* Multiplex kits include reagents that can be combined with those of an AlphaLISA *SureFire Ultra* kit to measure either two phosphoprotein targets or one phosphoprotein target and total cofilin as a control housekeeping protein. Additional details on Alpha *SureFire Ultra* Multiplex assay are available from our website.

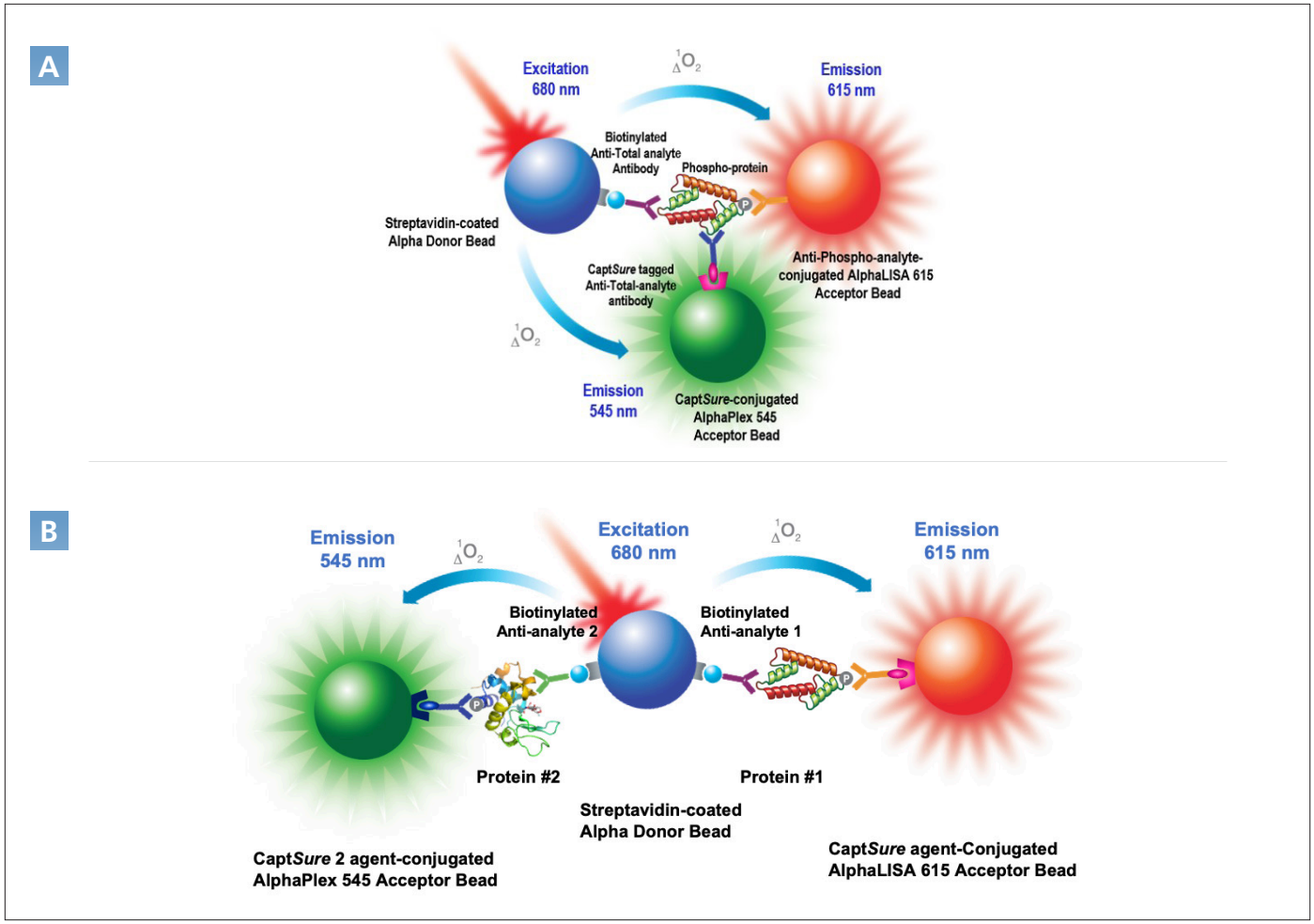

Figure 6. **Configuration of Alpha *SureFire Ultra* Multiplex assays.** A) Using the integrated Alpha *SureFire Ultra* Multiplex (MPSU) kits, or B) combining a Terbium *SureFire Ultra* (TBSU) kit with an AlphaLISA *SureFire Ultra* (ALSU) kit.

Summary of Possible Optimization Parameters

The table below lists the variables that one can consider when performing an AlphaLISA *SureFire Ultra* assay optimization. While most of the factors were mentioned or tested in this guide, this list contains helpful strategies and other caveats to consider when optimizing a specific assay.

As a reminder, the typical criteria that should guide you through your assay optimization when testing the impact of the listed parameters will be:

- S/B, CV, Z', pharmacology (EC_{50}, IC_{50}, % efficacy, …)
- Intra-plate variation
- Inter-plate variation
- Day-to-day variation

Please be aware that absolute signal variation can vary from day to day, or from experiment to experiment, when working with Alpha technology (due to differences in temperature when measuring the plate, pre-lighting before reading the plate, non-optimal reagent storage conditions, etc.). Data should always be analyzed primarily in terms of relative variations, compared to a control situation (e.g., non-stimulated cells).

We also recommend including measurement of the kit's control lysates in every experiment, for unforeseen troubleshooting. Running control lysates will allow PerkinElmer to support you better and faster in such situations.

CELLULAR OPTIMIZATIONS		
	CONDITION	**REASON**
MANDATORY	**Cellular Model**	Levels of expression of the target protein, as well as basal level of phosphorylation and amplitude of response to stimuli, may vary from cell type to cell type. It may be advantageous to try a different cell type if the response is less than desired.
	Cell Seeding Density	Typically, 10,000 to 70,000 cells per well are used in 96-well plates. Cell density can impact the quantity of analyte but can also modulate basal levels of phosphorylation.
	Cell Stimulation Time	The rise in protein phosphorylation can be elusive and using a sub-optimal stimulation time can have a negative impact on S/B and assay quality. Be aware that the optimal stimulation time of different phosphorylation events will often vary, even if working with the same cell type.
	Agonist Concentration	Using too low or too high concentration of agonist can reduce the S/B and overall assay quality. Agonist concentration can also impact the kinetics of the response; hence, these two factors should ideally be tested together.
	Agonist Type	Some agonists can be partial (less than 100% efficacy) or biased. It is important to select an agonist that aligns with the scope of the project pursued, to make sure the data reflects the relevant biological question.
	Shaking to Lyse Cells	Once lysis buffer is added, shaking the plate to produce a homogenous cell lysate is important for well-to-well reproducibility. This is less important when all the assay is run in the same 384-well plate from cell seeding to plate reading.
RECOMMENDED	**Recovery Time Before Stimulation**	The basal level of phosphorylation and ability to respond to stimuli can be impacted by the time cells grow in the well after seeding. Optimal time in culture for some cells/pathways can vary from four hours to up to four days.
	Serum Starvation	Basal or unstimulated phosphorylation can be decreased by removing growth factors or other signaling molecules from the media. Incubating cells with no (0%) or low (1-5%) serum-containing media can sometimes reduce background phosphorylation. Long-term serum starvation (> four hours) can also have a detrimental effect on some cellular pathways. It is important to select a consistent yet non-apoptotic-inducing starvation condition. This needs to be optimized on a case-by-case basis.
	Antagonist or Inhibitor Treatment Time	Generally, a five-minute pre-incubation is sufficient to block cell surface receptors, and 30 minutes - two hours to block intracellular kinases.
	DMSO Tolerance	DMSO should normally cause no issue up to 0.5 to 2% DMSO depending on the cell type and incubation times. It is advantageous to check the desired DMSO concentration in the final optimized assay.
	Choice of Agonists and Antagonists	It can be helpful to use well-studied agonist and antagonists as comparisons to test compounds, to provide performance relativity. Depending on the cell type and conditions, the absolute IC_{50}/EC_{50} can vary. It is also not necessarily expected that IC_{50}/EC_{50} values be identical to the ones obtained in other types of assays, as the pathway examined and the assay conditions may lead to different maximal and half-maximal responses.
OPTIONAL	**Temperature When Stimulating Cells**	In most cases, stimulating cells at room temperature (23 °C) is suitable, but stimulation at 37 °C can be performed for specific applications.
	Adherent vs. Suspension Cell Types	AlphaLISA *SureFire Ultra* kits work well with either cell type. Resuspending cells in HBSS for suspension cells in particular before stimulation can be validated if desired for easier automation.

CELLULAR OPTIMIZATIONS *Continued*		
	CONDITION	**REASON**
HINTS/REMINDERS	**Phosphatase Inhibitors**	Phosphatase inhibitors are included in the *SureFire Ultra* Lysis Buffer.
	Protease Inhibitors	The *SureFire Ultra* Lysis Buffer does not contain protease inhibitors, as they typically are not needed when working with commonly cultured cell types. However, protease inhibitors can be added for specific applications, such as working with cells very rich in proteases or working with tissue lysates. Protease inhibitors do not interfere with the AlphaLISA technology when used at standard concentrations.
	Medium in Which Cells are Stimulated	Stimulation can be performed in culture medium or HBSS. Some culture media, such as RPMI, contain extremely high biotin concentrations (820 nM biotin), which can compete for the binding of the biotinylated antibody by streptavidin Donor Beads used in the kit. Therefore, cells stimulated in high biotin-containing media should be washed before cell lysis, or stimulated in other culture media or HBSS. Also, some media contain unspecified growth factors or interfering agents that may affect the stimulation or assay window.
	Reoptimizing an AlphaScreen *SureFire* Assay to an AlphaLISA *SureFire Ultra* Assay	AlphaLISA *SureFire Ultra* is more sensitive than AlphaScreen *SureFire*; therefore, less cellular material is required. A general guideline is to reduce the number of cells per well used in the assay by 1/3, however, it is best to validate this parameter for each target..

IMMUNOASSAY OPTIMIZATIONS		
	CONDITION	**REASON**
MANDATORY	**Activation Buffer Solubilization**	Activation buffer components will precipitate at 4 °C, and it is very important to be sure that all components are well-solubilized before use. To ensure performance, Activation buffer can be stored permanently at room temperature.
	Activation Buffer Volume	Activation buffer concentration has been optimized for best assay performance. If deviations from the recommended protocol change the final percentage of Activation buffer in the immunoassay, assay performance may suffer.
	Assay Linearity	Linearity in the immunoassay largely depends on the cellular lysate concentration used in the assay, which is a factor of cells and lysis buffer quantities utilized. The ideal cellular lysate concentration should be such that the quantity of analyte to detect falls within the sensitive range of the assay. In extreme cases, using too much cellular material can result in the saturation of the detection reagents (e.g., the Hook effect, where more sample leads to less signal).
RECOMMENDED	**Plate Type**	AlphaPlates are advantageous for assay reproducibility, as they minimize well-to-well crosstalk effects and generally lead to better assay reproducibility compared to white plates. ProxiPlates™ can be used when working with smaller volumes, as the sample is closer to the detector for increased signal detection.
	Lysis Buffer	The AlphaLISA *SureFire Ultra* Lysis Buffer is a mild detergent-containing buffer, formulated to avoid release of genomic DNA from the cells and clogged pipette tips. For specific cell types and protein targets, other cell lysis buffers can be investigated, which sometimes can result in a more complete release of target protein.
	Volume of Lysis Buffer	To enrich protein levels, decreasing the volume of lysis buffer can be tested. A 96-well plate can be lysed in 25-100 µL lysis buffer.
OPTIONAL	**Choice of Phosphoprotein Target**	Basal phosphorylation levels and amplitude of response will vary depending on the particular signaling pathway. For example, ERK is downstream of MEK1; therefore, higher S/B ratio is expected when detecting phospho-ERK compared to phospho-MEK.
	Dispensing Steps	Adding the Acceptor and Donor Beads at the same time will reduce sensitivity and is not recommended. For best sensitivity, it is recommended to incubate the sample with the Acceptor Bead mix for at least one hour before adding the Donor Bead mix.
	Immunoassay Incubation Time	The standard assay incubation time is one hour with Acceptor Beads followed by one hour with Donor Beads. This is sufficient for most applications. If greater sensitivity is desired, increasing the first incubation time (up to four hours), and the second incubation (up to overnight in the dark) can be tested.

IMMUNOASSAY OPTIMIZATIONS *Continued*

	CONDITION	REASON
HINTS/REMINDERS	**Assay Consistency and Reproducibility**	Alpha technology is dependent upon a number conditions including the specific plate reader and ambient temperature. Steps should be taken to ensure consistency in using the same plate reader and maintaining room temperature (22-24 °C) for experiments. This will help improve the comparability between experiments.
	Altering Bead Concentrations	The immunoassay reagent concentrations have been optimized in the standard protocol; changing the bead amounts is not recommended. Deviating from the recommended concentrations could result in reduced kit performance compared to the lot-specific kit performance provided in each kit's certificate of analysis.
	Peptide Competition	The antibodies used in the kits have been carefully selected to generate a signal that is specific for the desired target. However, in some situations additional evidence for the specificity of signal detection may be desired. In such situations, using peptides as competitors in the assay can be very useful to show specificity of the AlphaLISA signal. A phospho-peptide recapitulating the sequence containing the phosphorylated site can be used to demonstrate that the AlphaLISA signal obtained from unknown samples can be competed by such a peptide. Conversely, negative control peptides, such as a non-phosphorylated version of the target peptide, or an unrelated phosphopeptide, can be used as well. Peptide competition data can be seen in the manuals of the following AlphaScreen *SureFire* kits: Phospho-p53 (Ser15); Phospho-p53 (Ser392); Phospho-p70S6K (Thr389); Phospho-p70S6K (Thr421/Ser424); Phospho-Topoisomerase IIα (Ser1106); Phospho-Topoisomerase IIα (Thr1343).

For more details please visit www.perkinelmer.com/SureFireFAQ or contact your local PerkinElmer Filed Application Specialist.

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