AlphaLISA *SureFire Ultra*

Guidelines to successful optimization of cell signaling phospho-/total protein assays

INTRODUCTION

Protein phosphorylation is a dynamic process, tightly controlled by kinase and phosphatase cascades, which regulates many cellular processes and has been identified as an underlying mechanism for a variety of diseases. Assessing phosphorylation events in a cellular model is particularly pertinent during development of therapeutic drugs as it can offer insights into the activity of compounds under study and provide evidence of their mechanism of action or target modulation.

AlphaLISA™ (Amplified Luminescent Proximity Homogeneous Assay) is a no-wash, bead-based assay technology used to study biomolecular interactions in a microplate format. AlphaLISA™ *SureFire® Ultra™* assays, a subset of the larger AlphaLISA superfamily, 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.

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General description

AlphaLISA™ *SureFire® Ultra*™ assay kits allow the rapid, sensitive, and quantitative detection of phosphoproteins from cells. The AlphaLISA *SureFire Ultra* assay kits are optimized for enhanced signal-to-noise windows. Alpha technology eliminates the need for laborious techniques, such as Western blotting or conventional ELISA. It is a homogeneous assay with no washing steps and this, combined with the short incubation times and excellent reproducibility (lower CV%), make it highly amenable to robotic automation.

AlphaLISA assays require two bead types: Donor beads and Acceptor beads. In AlphaLISA *SureFire Ultra*, the Donor bead is coated with streptavidin to capture the biotinylated antibody while the Acceptor bead is coated with a proprietary "CaptSure™" agent that immobilizes the other antibody which is labeled with a CaptSure™ tag. Donor beads contain a photosensitizer, phthalocyanine, which converts ambient oxygen to an excited and reactive form of O2, singlet oxygen, upon illumination at 680 nm. Singlet oxygen can diffuse approximately 200 nm in solution. In the presence of phosphorylated protein, the two antibodies bring the Donor and Acceptor beads in close proximity, whereby the singlet oxygen transfers energy to excite the Acceptor bead, enabling the generation of a luminescent Alpha signal. The amount of light emission is directly proportional to the quantity of phosphoprotein present in the sample. It should also be noted that the emission wavelength of the AlphaLISA Acceptor beads falls outside of hemoglobin's absorption spectrum, making it a tool suitable for working with tissue samples in complex matrices (tissue lysates, plasma, etc.).

In addition to AlphaLISA *SureFire Ultra* kits detecting phosphorylated proteins, there is a subset of AlphaLISA *SureFire Ultra* kits that allow for the detection of the "total" analyte (i.e. non-phosphorylated as well as phosphorylated), offering an ideal way to normalize for positive or negative modulation of protein expression in cellular contexts. This is especially useful for (but not limited to) PROTAC and molecular glue-based therapeutic modalities.

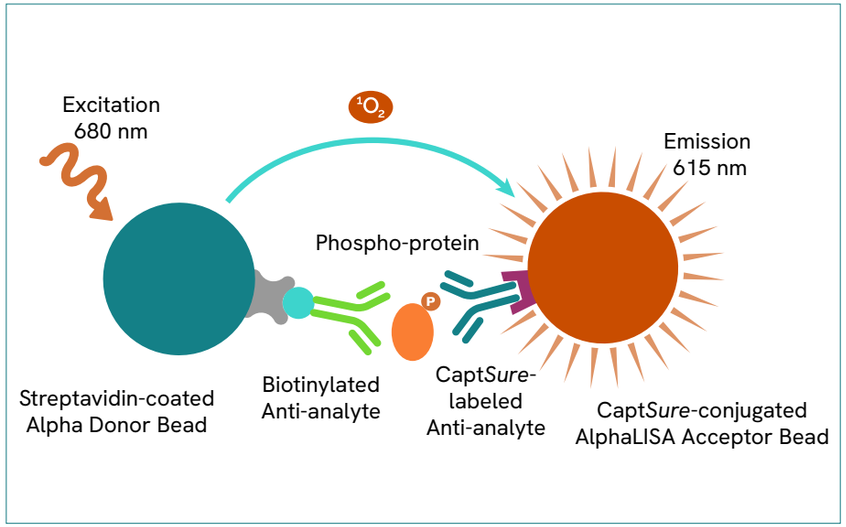

Figure 1: AlphaLISA SureFire® Ultra™ assay principle

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Multiplexing

In addition to the single-target AlphaLISA *SureFire Ultra* kits, Revvity also offers Multiplex assays, divided into two categories:

Multiplex *SureFire Ultra* (MPSU) kits are complete kits for phospho and total detection of the same target. Assessing the total protein level (phosphorylated and non-phosphorylated) is essential in distinguishing whether up- or down-regulation of phosphorylation is due to changes in that protein's expression levels or to cell viability. This misinterpretation of assay results can potentially lead to higher false positive and false negative rates, or incorrect classification of a compound's mechanism of action (MoA). The assay principle for MPSU kits is shown schematically below.

Terbium *SureFire Ultra* (TBSU) kits measure a single cell signaling 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 mix-and-match combination of any two targets in the assay lists, providing measurement of two separate targets with flexibility. The TBSU kits will report the target of interest through the Terbium (Tb) Acceptor beads (545 nm emission), while the ALSU kit reports via the Europium (Eu) AlphaLISA Acceptor beads (615 nm emission).

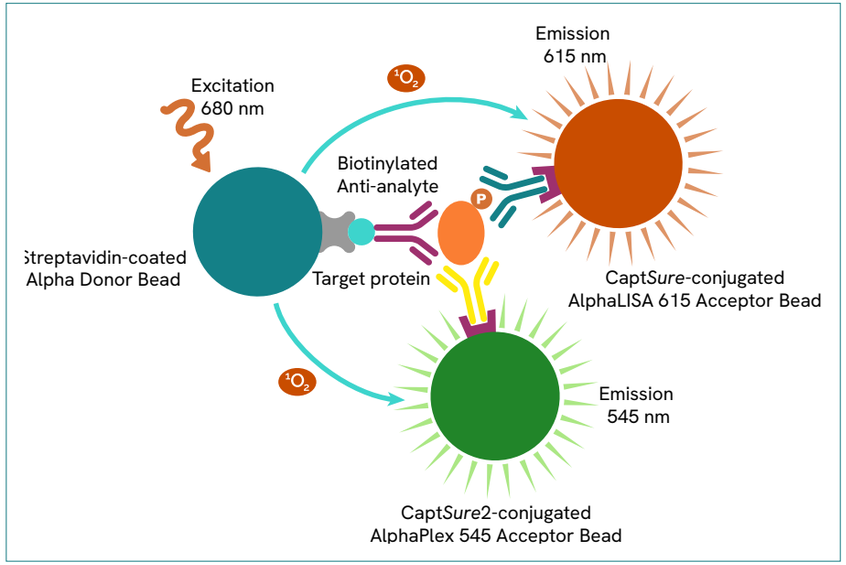

Figure 2: Multiplex SureFire Ultra (MPSU) assay principle

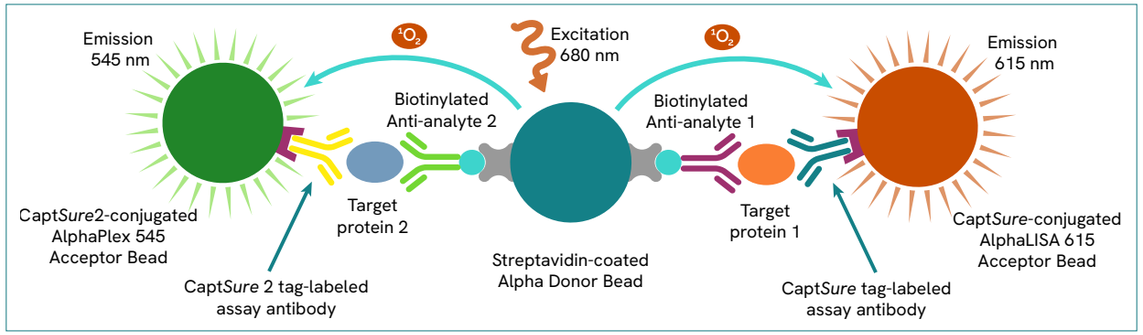

Figure 3: Terbium SureFire Ultra (TBSU) assay principle

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Main features

- Cell-based endpoint assay, demonstrated applications on recombinant proteins, cell lines, primary cells, stem cells, tissues, plasma, and blood
- Homogeneous (no wash) assay - mix components in well and read in microplate-based assay format (96-, 384-, and 1536-well amenable)
- Well suited for target identification through high-throughput screening and preclinical studies

FAQ: Sample types

AlphaLISA is amenable to complex matrices and a multitude of sample types including recombinant proteins, plasma, serum, supernatants, cell lysates, tissue homogenates[1], tumour[2] homogenates, PBMC[3], and whole blood. Learn more about sample compatibility in the application note entitled "Applicability of AlphaLISA Technology to a Wide Spectrum of Complex Biological Samples".

Key advantages

- User-friendly reaction volumes
- Lower background signal (due to the use of CaptSure™ technology for antibody specificity)
- High signal-to-background
- High sensitivity
- Compatible with the use of spiked antibodies (i.e., studies using biotherapeutic antibodies, antibody blockers, etc.)
- Best format for tissue sample analysis (due to sensitivity and red-shifted emission of AlphaLISA acceptor bead chemistry)

Formats

ALSU, MPSU, and TBSU kits are provided in the following formats:

Kit size	Recommended plate format	Final Assay Volume
500 assay points	384-well, or 96-well low volume plate	20 µL
10,000 assay points	384-well, or 96-well low volume plate	20 µL
50,000 assay points	384-well, or 96-well low volume plate	20 µL
High volume (100 assay points)	96-well 1/2 AreaPlate	60 µL

[1] Zanese et al. (2020) Alpha technology: A powerful tool to detect mouse brain intracellular signaling events. Journal of Neuroscience Methods 332 (2020) 108543

[2] Coulson et al. (2017) The angiotensin receptor blocker, Losartan, inhibits mammary tumor development and progression to invasive carcinoma. Oncotarget, 2017, Vol. 8 : 18640-18656

[3] Saroz et al. (2019) Cannabinoid Receptor 2 (CB2) Signals via G-alpha-s and Induces IL-6 and IL-10 Cytokine Secretion in Human Primary Leukocytes. ACS Pharmacol. Transl. Sci. 2019, 2, 414−428

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Assay workflow

The full experimental setup leading up to the assay can be summarized in the below main steps. Certain aspects of the protocol may vary depending on whether the cells are adherent or not, as well as for high-throughput applications.

1. Cell seeding.

Cells can be seeded into 96- or 384-well plates. Optimization of cell density is crucial in this step.

2. Cell stimulation.

Cells are treated with agonists / antagonists of receptors, or stimulators / inhibitors of signal transduction pathways to induce or inhibit protein phosphorylation, respectively.

3. Lysate preparation.

Cells are lysed using 1X Lysis Buffer provided in the kit on a plate shaker at ~350 rpm for 10 minutes.

4. Alpha *SureFire Ultra* Assay.

Acceptor mix is added to the cell lysates, followed by incubation at room temperature. The Donor mix is then added followed by a second incubation at room temperature and in the dark, and reading of the plates in an Alpha Technology-compatible plate reader, such as Revvity's VICTOR™ Nivo™, EnSight™, EnSpire™, and EnVision™ Plate Readers.

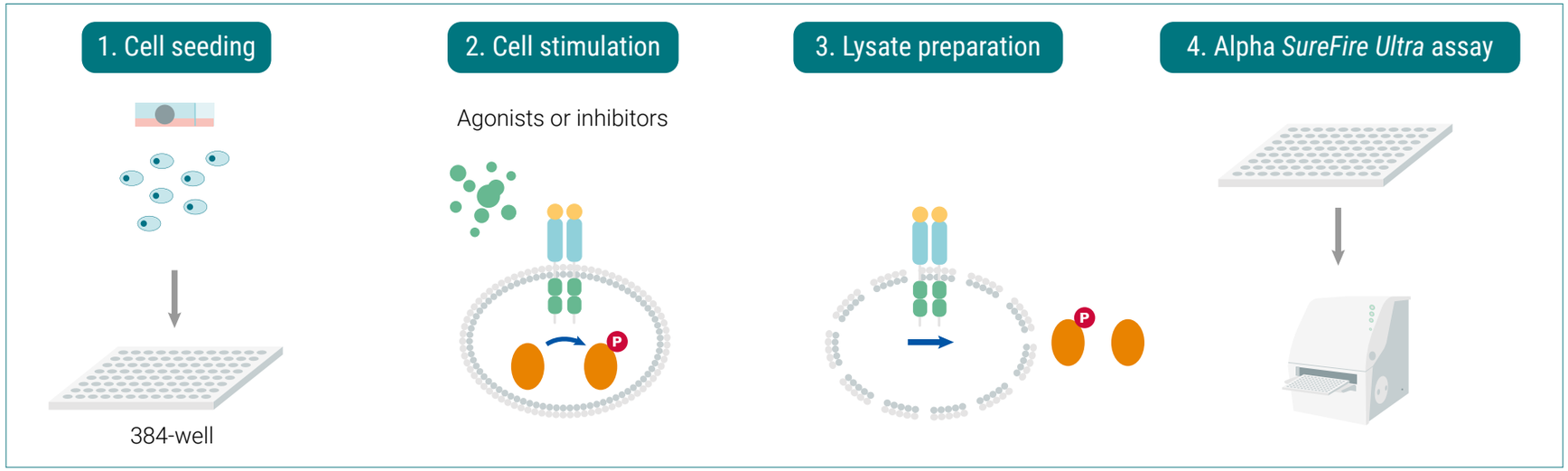

Figure 4: AlphaLISA™ SureFire® Ultra™ assay workflow

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2-Plate and 1-plate protocols

Two protocol variations exist, depending on the application and type of cell.

The 2-plate protocol separates cell culture and treatment from the detection step. Cells, either in suspension or adherent, are plated, treated, and lysed in a cell culture microplate, then 10 µl of cell lysate are transferred to the detection microplate. This protocol allows for visualization of the cells under a microscope before lysing and permits testing of multiple aliquots from a single treated well in different *SureFire Ultra* assays in parallel, generating more information at once.

The 1-plate protocol combines all steps in a single plate. The 1-plate protocol can be used with adherent or suspension cells and avoids a plate transfer step compared to the 2-plate protocol; this can make the workflow easier for scientists working in high throughput screening.

Table of volumes	2-Plate protocol			1-Plate protocol		
		Adherent cells	Suspension cells		Adherent cells	Suspension cells
Culture medium/HBSS	Culture plate	**200**/**50** µL	**40**/**10** µL	Culture and Detection plate	20 µL	4 µL
Inhibitor		**45**/**22.5** µL	**20**/**5** µL		5µL	2µL
Stimulator		**5**/**2.5** µL	**20**/**5**µL		5µL	2µL
Lysis Buffer		**50-100**/**20-25** µL	**20**/**5** µL		10 µL	2µL
Lysate transfer	Detection plate	10 µL	10 µL		-	-
Acceptor Mix		5µL	5µL		5µL	5µL
Donor Mix		5µl	5µL		5µL	5µL

Summary table. Volume suggested.
Dark blue text is for 96-well plate and light blue text is for 384-well plate.
Please refer to the flowcharts in next pages for full details.

The two protocols are detailed below.

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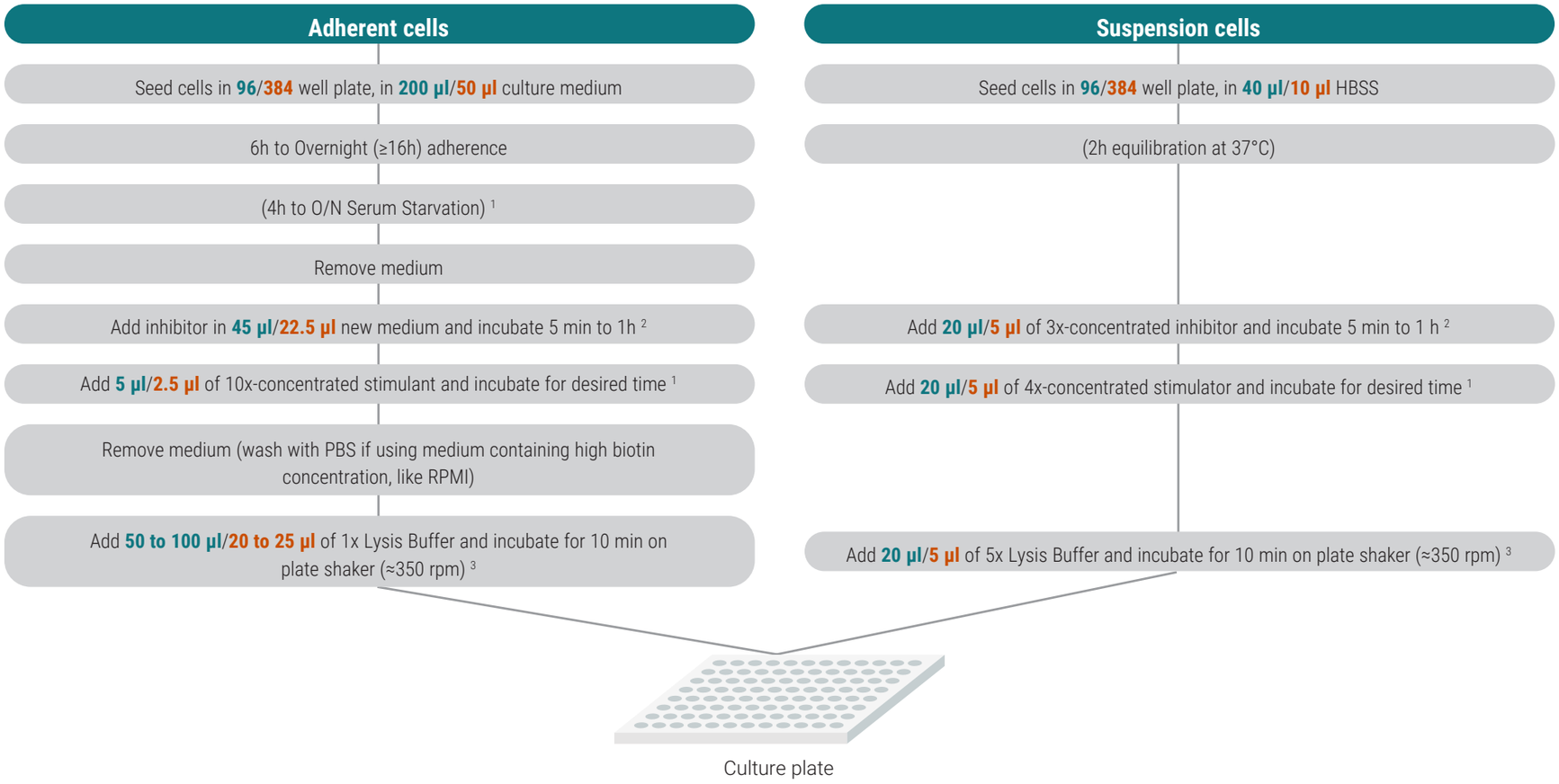
2-plate / 2 incubation assay flowchart



[1] Depending on cell type and pathway analyzed

[2] Depending on type of inhibitor used: 5 min 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.

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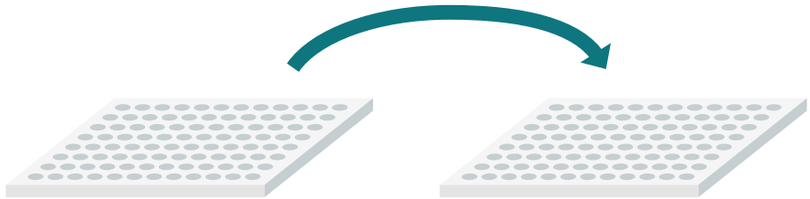
2-plate / 2 incubation assay flowchart



Culture Plate Detection Plate

Transfer 10 µl of lysate (or diluted positive control lysate, or Lysis buffer alone) to a white 384-well plate

Add 5 µL of Acceptor mix

Acceptor mix	Typical volume (60 x 5 µL)
Reaction Buffer 1	141 µL
Reaction Buffer 2	141 µl
Activation Buffer	12 µL
Acceptor Beads	6 µL

Seal and shake 1-2 minutes on plate shaker, then incubate ≥1 h (RT° or 22°C)

Add 5 µL of Donor mix

Donor mix	Typical volume (60 x 5 µL)
Dilution Buffer	294 µL
Donor Beads	6 µl

Seal, wrap in foil, and shake 1-2 minutes on plate shaker, then incubate ≥1 h, up to overnight (RT° or 22°C)
Allow to equilibrate to plate reader temperature prior to reading.

Read plate.

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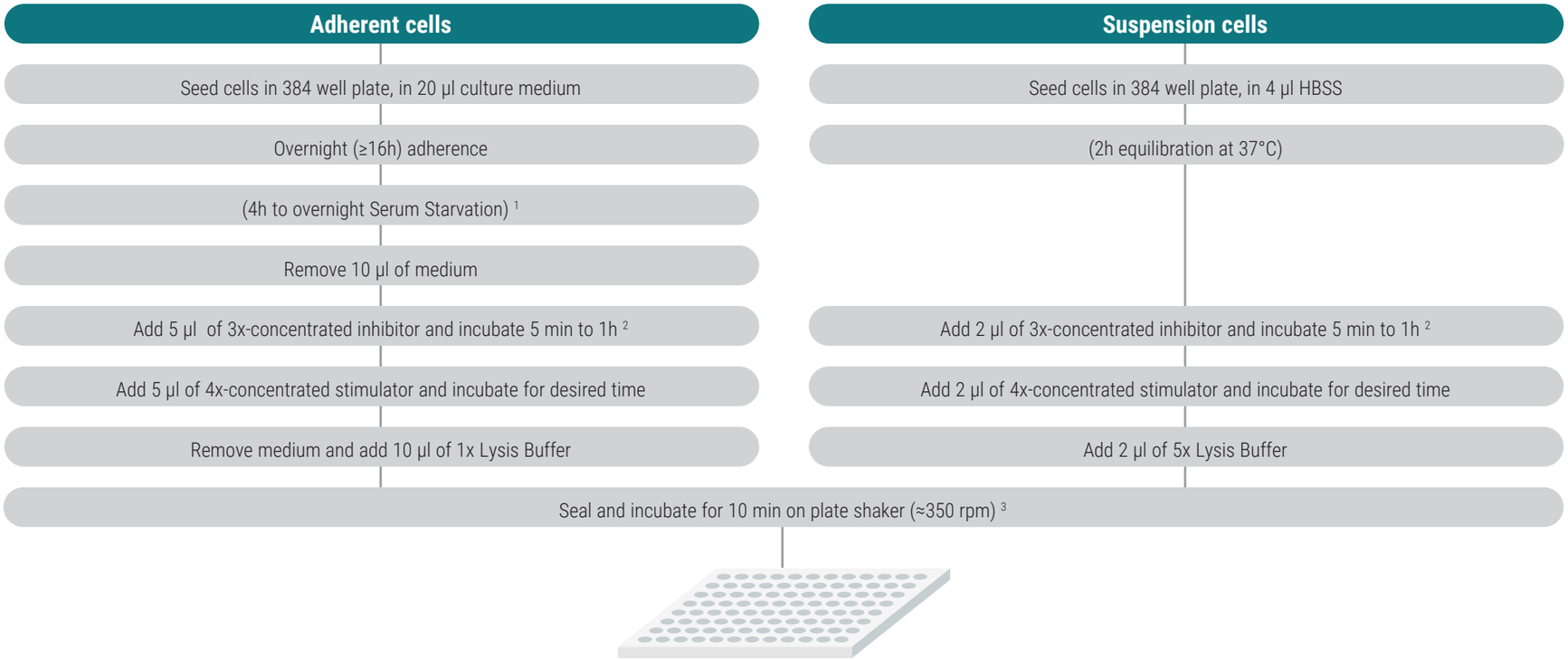
1-plate / 2 incubation assay flowchart



Culture and detection plate

[1] Depending on cell type and pathway analyzed

[2] Depending on type of inhibitor used: 5 min 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.

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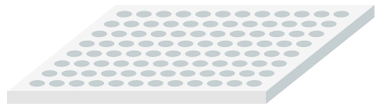
1-plate / 2 incubation assay flowchart



Culture and Detection Plate

In control wells, add 10 µl positive control lysate dilution or lysis buffer alone.

Add 5 µL of Acceptor mix

Seal and shake 1-2 minutes on plate shaker, then incubate ≥1 h (RT° or 22°C)

Add 5 µL of Donor mix

Seal, wrap in foil, and shake 1-2 minutes on plate shaker, then incubate ≥1 h, up to overnight (RT° or 22°C)
Allow to equilibrate to plate reader temperature prior to reading.

Read plate.

Acceptor mix	Typical volume (60 x 5 µL)
Reaction Buffer 1	141 µL
Reaction Buffer 2	141 µl
Activation Buffer	12 µL
Acceptor Beads	6 µL

Donor mix	Typical volume (60 x 5 µL)
Dilution Buffer	294 µL
Donor Beads	6 µl

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Assay reagents

AlphaLISA *SureFire Ultra* kits contain the following reagents:

Buffers

- Lysis Buffer (5X) - *Ultra*[1]

Lysis Buffer (5X) - *Ultra* is a proprietary mixture of buffers, detergents, and generic phosphatase inhibitors (Orthovanadate, Pyrophosphate, and sodium fluoride), optimized for lysis of a broad range of cells without the excessive release of nuclear DNA. It does not contain protease inhibitors. Additives can be supplemented to the Lysis Buffer if required for particular cell types and may include excipients such as protease inhibitors or extra detergents. These will need to be tested on a case-by-case basis[1].

- Activation Buffer – *Ultra*[2]
- Dilution Buffer - *Ultra*

Detection antibodies

- Reaction Buffer 1 – *Ultra*
- Reaction Buffer 2 – *Ultra*

Detection beads

- AlphaLISA™ CaptSure™ Acceptor beads (2 mg/mL in PBS plus 0.05% Proclin-300)
- Alpha Streptavidin Donor beads (2 mg/mL in PBS plus 0.05% Proclin-300)

Positive control lysate

- Lyophilized control lysate

Alpha Streptavidin Donor beads are mildly light-sensitive. All steps using the Donor beads should be performed under subdued laboratory lighting (< 100 lux). Alternatively, green filters (LEE 090 filters (preferred) or Roscolux filters #389 from Rosco, or the equivalent) can be applied to light fixtures and windows. The Donor beads should NOT be used under red/orange light as can be found in photographic work darkrooms because red light (680 nm) excites the beads. All other assay reagents can be used under normal light conditions.

All of the above reagents are provided and should be stored at 4°C. The positive control lysate can be stored at -20°C when lyophilized and -80°C in single-use aliquots for up to three months after reconstitution.

[1] Some kits contain assay specific Lysis Buffer B (5X)-*Ultra* or Lysis Buffer C (5X)-*Ultra*. Lysis Buffer B (5X) and Lysis Buffer C (5X) are assay specific and should not be interchanged.

[2] Some kits contain assay specific Activation Buffer B-*Ultra* or Activation Buffer C-*Ultra* are assay specific and should not be interchanged.

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Assay plates

We recommend use of the following types of plates for ALSU assays:

Plate name	Part number	Description
Optiplate-384 White Opaque assay plate	6007290	Can be used for 1- or 2- plate protocol depending on cell culture conditions
AlphaPlate-384 Light Gray Opaque assay plate	6005350	Same as above but optimal if cross-talk needs to be reduced
CulturPlate-384 White Opaque, Sterile, TC-Treated	6007680	Plates for both tissue culture and AlphaLISA detection
Viewplate-96/384, White with clear bottom, Sterile, TC-Treated	6005181 / 6007480	Clear bottomed plate offering ability to view cells by microscopy. Should be used with a white adhesive seal (Revvity # 6005199) stuck to the bottom of the plate before reading
ProxiPlate-384 Plus White, TC treated	6008238	384-well shallow volume plate (<20 µl final volume)
1/2 Area OptiPlate-96	6002290	96-well format using same volume as in 384-well
AlphaPlate-96 light gray, 1/2 Area	6002350	Same as above but optimal if cross-talk needs to be reduced
SpectraPlate-96 Clear, sterile TC-treated plate	6005650	Used to seed and stimulate cells before lysis, then transfer lysate to desired detection plate
HTRF 96-well low volume white plate	66PL96005	96-well format using same volume as in 384-well (≤ 20 µl final volume)

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Biological controls and negative control

Biological controls

The positive control lysates are prepared from various cell types, which have been cultured and prepared to optimize the activation of the intracellular pathway of interest The lysate is intended to be run in parallel with unknown samples for use as an assay positive control only This control is useful in identifying 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, with Lysis Buffer (1X), can be assayed to determine the signal response obtained from an Alpha-capable microplate reader. In most kits, the undiluted control lysate is so concentrated that it will be hooking the assay. Therefore, we recommend testing at least 2x and 5x diluted control lysates as they may generate a higher level of signal than the undiluted control lysate. Positive control lysates should not be used for the absolute quantification of a particular protein or phosphorylated target (see section “Quantification”).

Negative control

Lysis buffer (1X) should be tested as the negative control (level of Alpha signal in the absence of the target protein).

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Cell density, serum starvation and stimulation time optimization

The three variables of cell seeding density, serum starvation, and stimulation time can be critical when setting up an assay for the first time. We describe a process of optimizing these three parameters in one experiment, which can 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. This can sometimes be important in obtaining the best cellular response.

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, allow basal phosphorylation levels to reach their minimum value, and are at a proper confluency. 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 5 minutes). To add the agonist, remove culture medium from the plate and replace with serum-free HBSS containing the agonist. In the example shown below, the agonist was first added to rows A-C, then to rows D-F after waiting 15 minutes.

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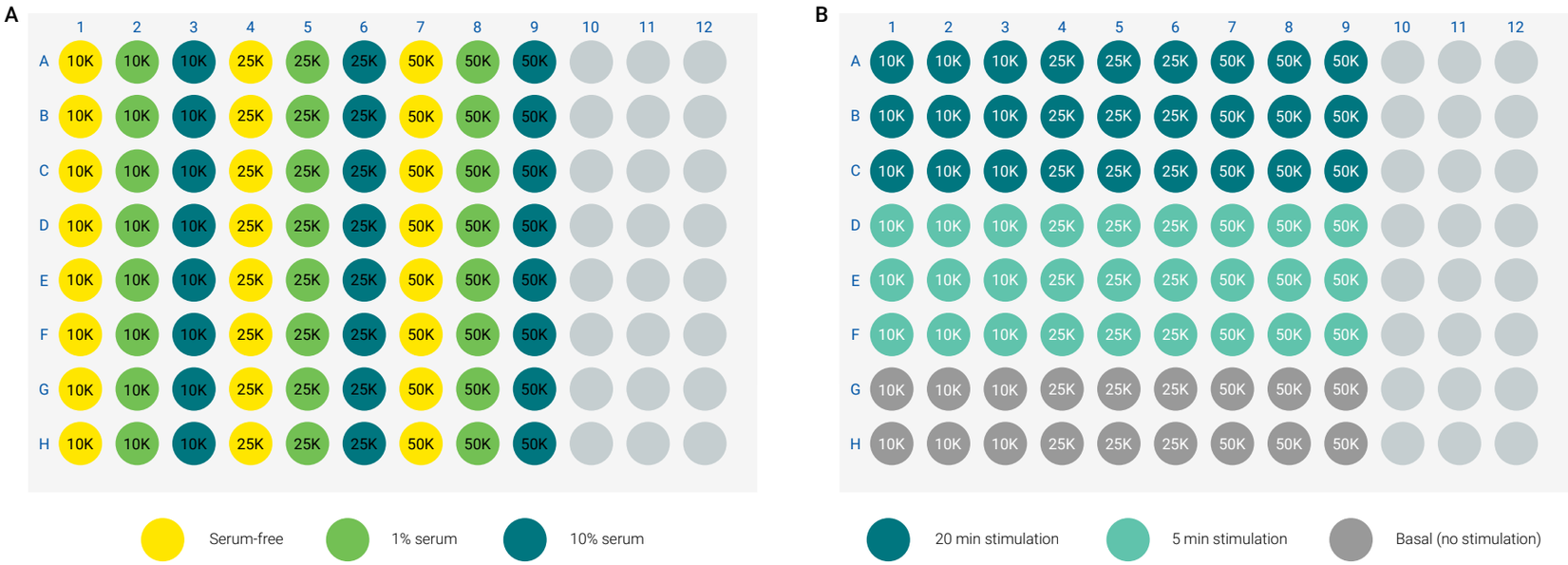

Figure 5: Cell density/serum conditions optimization. A) Platemap indicating the cell seeding densities and serum conditions. B) Platemap indicating the agonist stimulation conditions and time.

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ASSAY OPTIMIZATION

Cell density, serum starvation and stimulation time optimization

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* Lysis Buffer to the well. After 10 minutes of shaking (350 rpm), transfer 10 µL of cell lysate to a 384-well assay plate 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.

	1	2	3	4	5	6	7	8	9
A	1444	1358	1831	4934	4284	4645	9507	8806	8054
B	1332	1763	1739	5376	4657	5490	9059	11077	14630
C	1892	1623	1614	4581	4273	5177	9313	10766	11003
D	2119	1449	1823	5977	5302	5285	13446	10523	12603
E	1485	1475	1579	5494	4281	5427	14399	8167	10834
F	1883	1601	1699	5434	4622	4546	13439	11334	9555
G	372	302	325	311	359	332	432	567	474
H	341	319	330	345	646	382	465	604	458

		Description			
		0%	1%	10%	
Agonist Stimulation Time	20 min	1556	1581	1728	10K/well
	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	

Cell Number Per Well

Figure 6: Sample raw data (left) and averaged data (right) from experiment 1 (pSTAT3 assay). The AlphaLISA SureFire Ultra assay kit for phospho-STAT3 (Tyr705) (Revvity#ALSU-PST3-A500) was run using A431 cells stimulated with 100 nM EGF. Platemap as indicated in Figure 5. 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.

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A note on cell media

Stimulation can be performed in culture medium or HBSS. Some culture media, such as RPMI, contain extremely high biotin concentrations, 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.

Biotin content in commonly used cell culture media.

Medium	Biotin content (nM)
FreeStyle CHO Expression Medium	7200
BME Eagle	4100
RPMI 1640	820
McCoy's	820
MEM a	410
Iscove's MDM	53
Grace's Insect Medium	41
DMEM	0
DMEM/F12	14.3
HAM's F12	29.9
HAT	0
EMEM	0
FBS	192

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Optimization of cell treatment time

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 as determined above, 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), 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.

A note on lysis buffer volumes

To enrich protein levels, decreasing the volume of lysis buffer can be tested to concentrate the lysate. A 96-well plate can be lysed in 25-100 µL lysis buffer. A 384-well plate can be lysed in 10-50 µl lysis buffer.

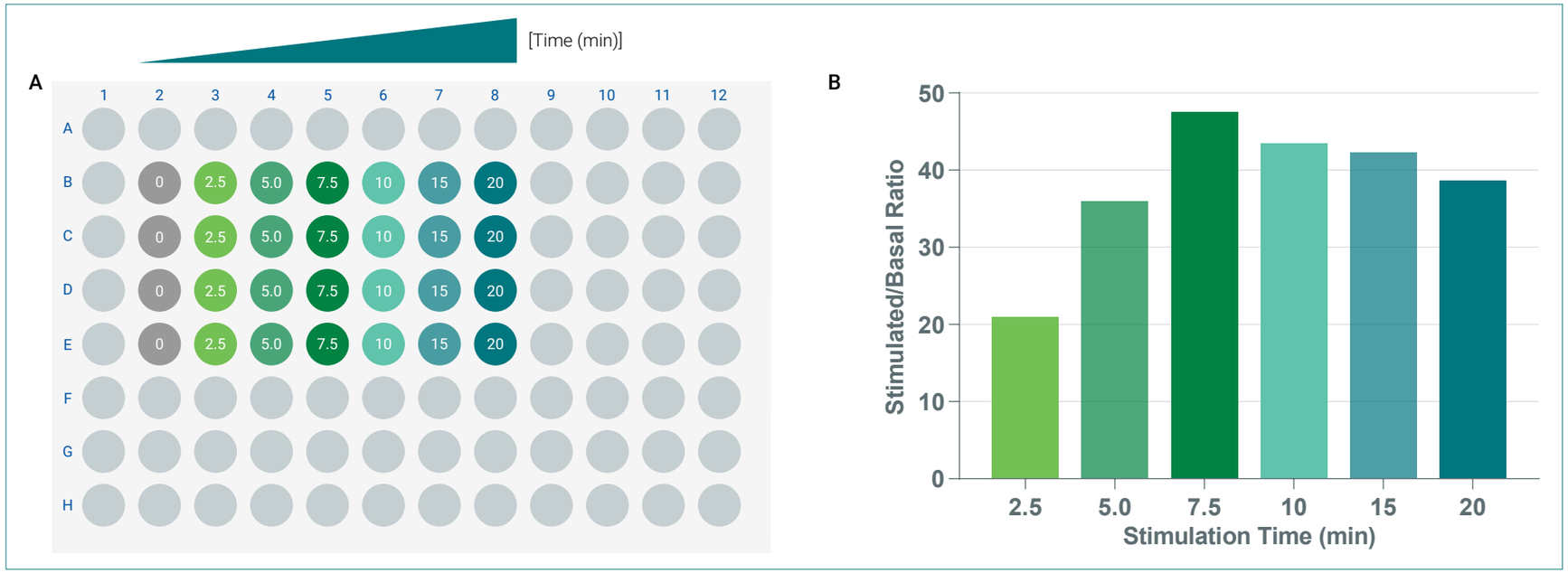

Figure 7: Treatment time-course optimization. A) Plate map and B) example data. 100 nM EGF was used to stimulate for 2.5 to 20 minutes A431 cells seeded at 50,000 cells/well, that had been starved for three hours in serum-free media. The AlphaLISA signal was measured for all stimulation samples as well as the unstimulated (basal) control (time=0), and the ratio of stimulated:unstimulated was calculated. A 7.5-minute stimulation yielded the largest window.

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Immunoassay incubation time and signal acquisition

Immunoassay incubation time

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 determined in the fashion described above, perform an assay to include a basal condition (no stimulation) and stimulated control (agonist only). An example of the process is shown below, where 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).

Signal acquisition

AlphaLISA *SureFire Ultra* kits can be read on Revvity plate readers using the standard AlphaScreen settings. Please refer to the instructions of the Nexus, EnVision, EnSpire, EnSight, or VICTOR Nivo for the filters and settings to be used. The standard Alpha protocol found by default in these readers can be used with the AlphaLISA *SureFire Ultra* kits.

Please refer to the Application note entitled "Multiplex Assays with Alpha SureFire Ultra Multiplex Technology using the EnVision Multilabel Plate Reader" to access information about Multiplex *SureFire Ultra* kits signal reading.

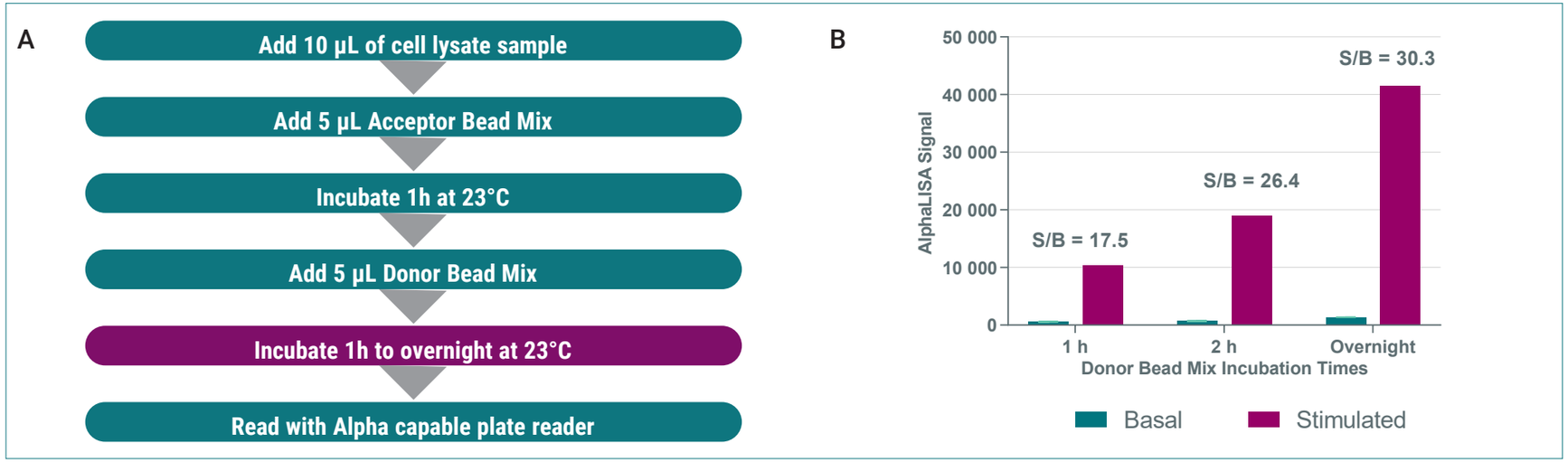

Figure 8: Incubation time optimization. A) Protocol, B) example data. Serum-starved A431 cells were treated with and without agonist (240 nM EGF). The cell lysates were pooled and tested using the AlphaLISA SureFire Ultra phospho-STAT3 (Tyr705) kit with the following incubation paradigms: one hour Acceptor bead mix incubation followed by either a one hour, two hour, or overnight Donor bead mix incubation. In this example, the stimulated:basal ratio increases with increasing Donor bead incubation time.

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ASSAY OPTIMIZATION

Quantification

Given that phosphoproteins are very labile by nature, it is not suggested to use the positive control lysate for absolute quantification. With a well-optimized assay, the relative AlphaLISA signal can be used to determine an IC50/EC50 and the fold-induction or inhibition . Normalization of the AlphaLISA *SureFire Ultra* signal response can be performed by measuring the total cell lysate protein concentration using a method such as the Bradford assay. The 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 or GAPDH. 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 ERK1/2 assay kit would be used), or with the AlphaLISA *SureFire Ultra* Total Cofilin assay kit.

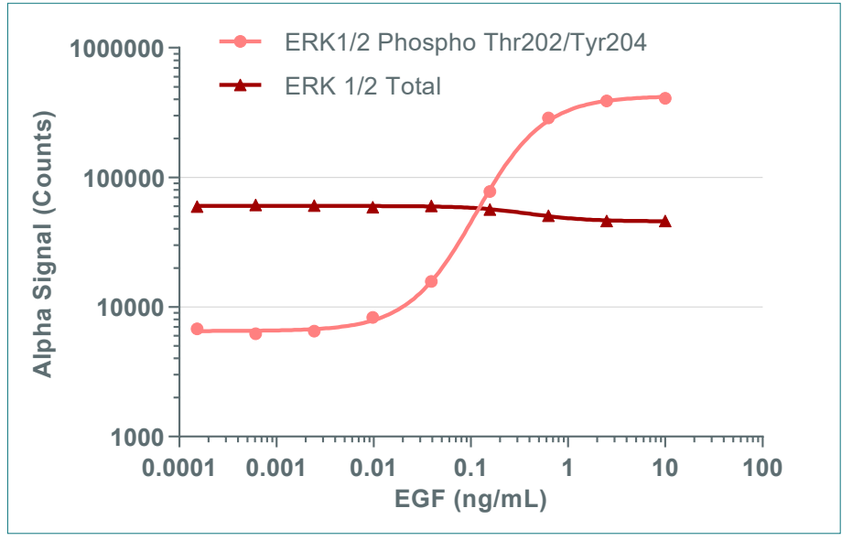

Figure 9: Parallel measurement of phospho and total ERK 1/2 in presence of EGF ligand. A431 cells were plated overnight at 40K cells/well in a 96-well plate in media containing 10% FBS. Cells were serum starved for 2 hours then treated for 10 minutes with varying concentrations of EGF ligand. Two aliquots of lysates were transferred in separate wells for every treated condition and evaluated for phospho (Thr202/Tyr204) and Total ERK 1/2 in parallel with the corresponding SureFire Ultra assay kits.

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ASSAY OPTIMIZATION

Multiplex assay normalization

The Alpha *SureFire Ultra* Multiplex (MPSU) assay kits enable 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: 545 nm and AlphaLISA™ Europium beads: 615 nm) as represented on Figure 2 above.

The same parameters that have been detailed earlier for optimization on the AlphaLISA *SureFire Ultra* assay including cell density, serum starvation and stimulation time will also be carefully tested on the Alpha *SureFire Ultra* Multiplex assay.

However, it is noteworthy that assay normalization consistency using the Alpha *SureFire Ultra* Multiplex assay will be dictated by a careful cell number optimization to ensure both phospho and total assays are in the dynamic range of the assay, then enabling a reliable normalization interpretation.

Performing phospho-specific and total protein assays simultaneously, and properly analyzing the data, enables you to accurately identify compounds that modulate the phosphorylation of specific proteins as well as elucidate the MoA by which it acts.

The data shown below illustrate the types of scenarios that can be encountered when measuring phospho and total signal in a properly optimized assay.

Further normalization can be performed by analyzing the level of housekeeping proteins, such as cofilin or GAPDH, either as ALSU assays run in parallel on aliquots of lysates or by combining the TBSU Cofilin assay kit with one of the ALSU assay kits to generate data from a single well.

The Alpha *SureFire Ultra* Multiplex assays can also be combined with the ATPlite 1step luminescence assay providing a fast and simple measurement of intracellular ATP for assessing cell viability and monitoring cytotoxic effects of the drug compounds. Please refer to the application note entitled "Simultaneous detection of drug efficacy and toxicity by combining HTRF, AlphaLISA, or AlphaLISA *SureFire Ultra* with ATPlite".

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ASSAY OPTIMIZATION

Multiplex assay normalization

- Phosphorylation of EGFR upon stimulation with the EGF ligand on A431 cells that will trigger an increase in EGFR phosphorylation at Tyr1068 without changing the expression level of the protein as highlighted by the total signal remaining constant.

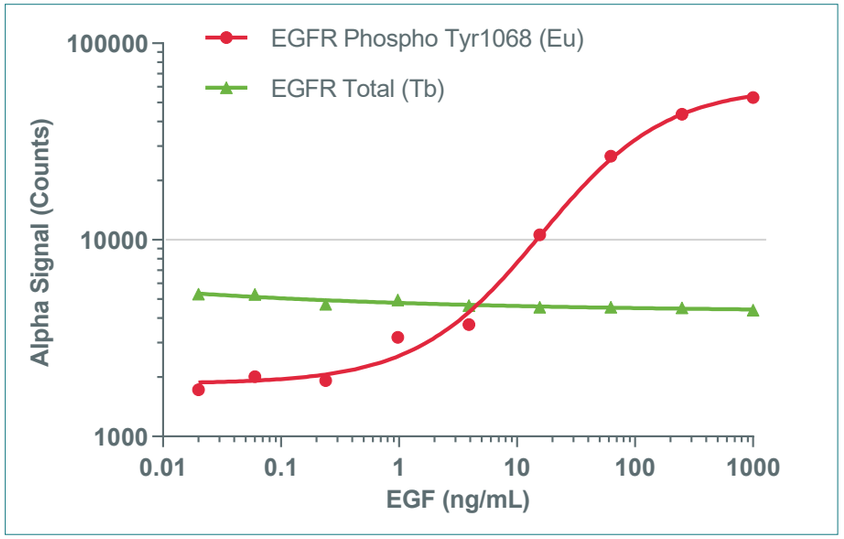

Figure 10: Simultaneous measurement of phospho and total EGFR in presence of EGF ligand. A431 cells were plated overnight at 40K cells/well in a 96-well plate in media containing 10% FBS. Cells were serum starved for 2 hours then treated for 10 minutes with varying concentrations of EGF ligand. Lysates were evaluated for phospho (Tyr1068) and total EGFR in the same well with the SureFire Ultra Multiplex assay kit.

- Phosphorylation of p53 upon treatment with doxorubicin on U2OS cells that will induce an increase in p53 phosphorylation at Ser15 and a simultaneous increase of the p53 protein as shown by the total signal increase.

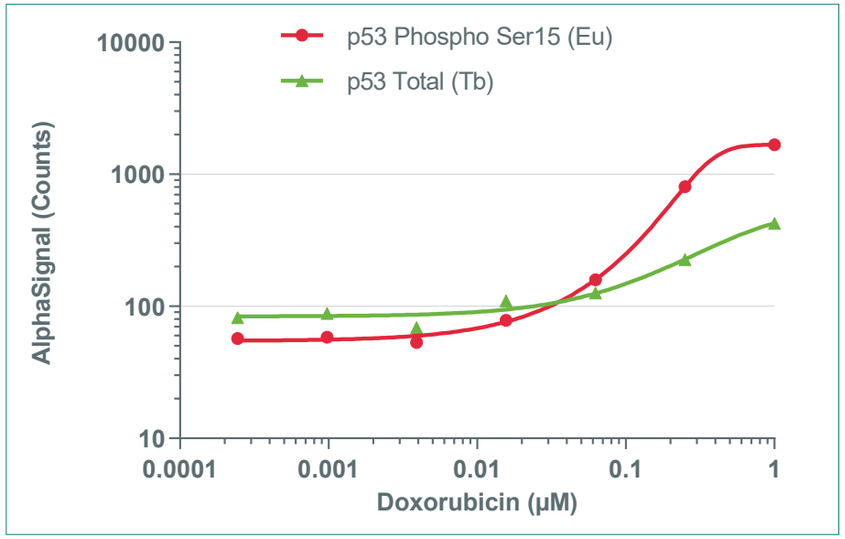

Figure 11: Simultaneous measurement of phospho and total p53 in presence of Doxorubicin compound. U2OS cells were plated overnight at 10K cells/well in a 96-well plate in media containing 10% FBS. Cells were treated for 18 hours with varying concentrations of Doxorubicin. Lysates were evaluated for phospho (Ser15) and Total p53 in the same well with the SureFire Ultra Multiplex assay kit.

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ASSAY OPTIMIZATION

Multiplex assay normalization

- De-phosphorylation of Rb at Ser780 following Palbociclib treatment . At the same time, you observe a slight decrease of the total protein level.

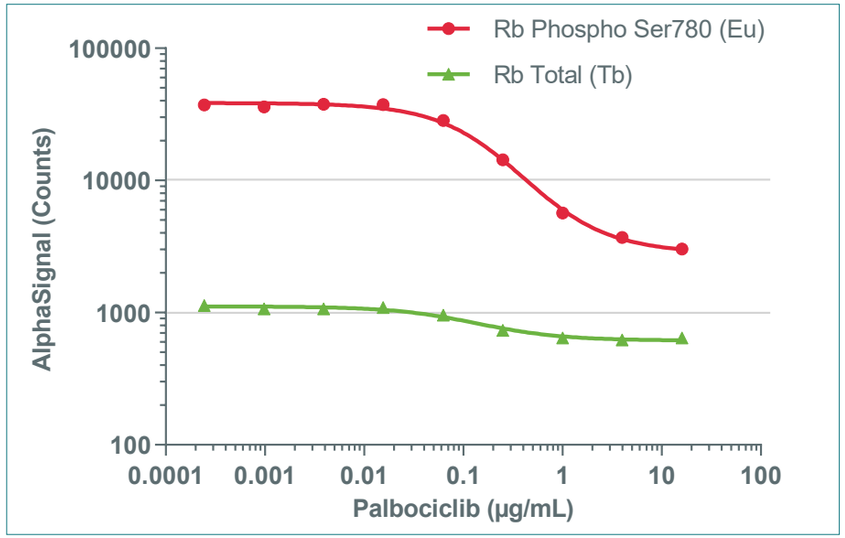

Figure 12: Simultaneous measurement of phospho and total Rb in presence of Palbociclib compound. THP-1 cells were plated at 2K cells/well in a 24-well plate and treated for 24 hours with varying concentrations of Palbociclib. Lysates were evaluated for phospho (Ser780) and total Rb in the same well with the SureFire Ultra Multiplex assay kit.

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SAMPLE DATA

Agonist dose-response

To identify the optimal agonist concentration, a dose-response assay of the agonist should be carried out with the cell conditions and agonist treatment time optimized as described earlier. 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 in antagonist assays to get the best sensitivity.

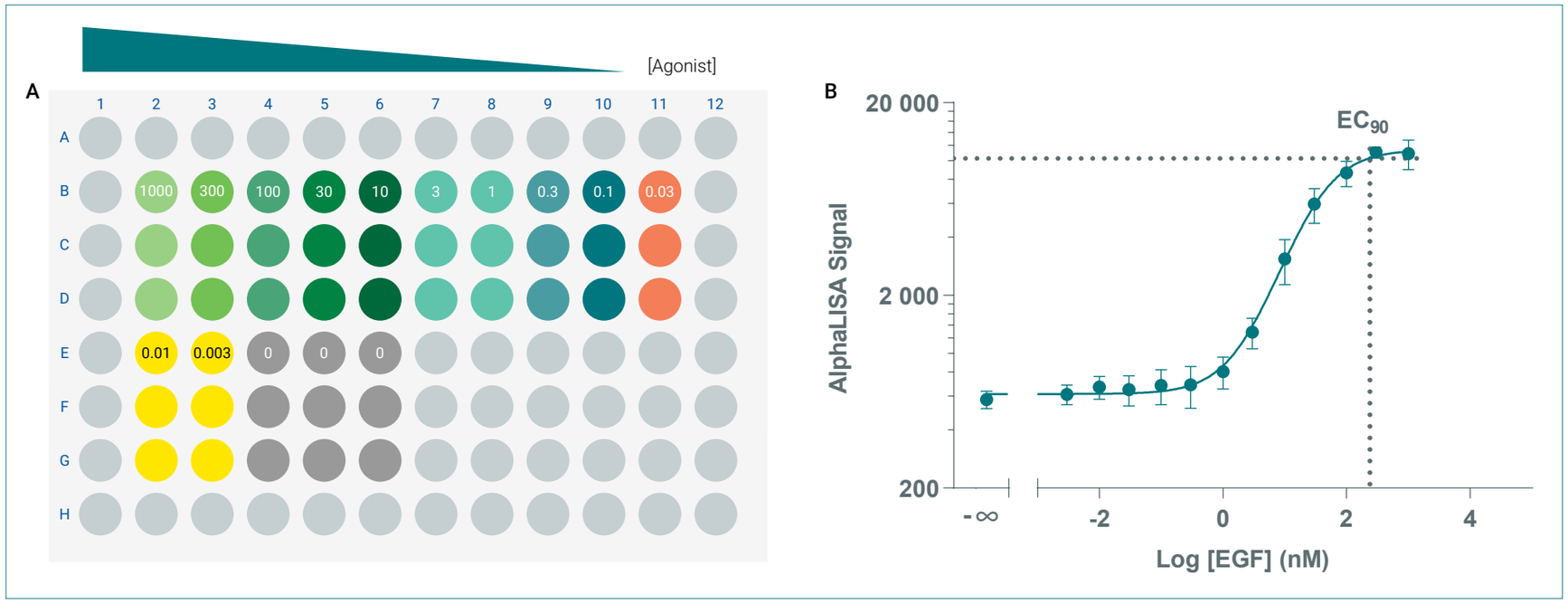

Figure 13: Agonist dose-response optimization. A) Plate map and B) example data. 3 pM - 1 µM EGF was used to stimulate serum-starved A431 cells for 7.5 minutes (50K cells/well, serum-free media for three hours). The dose-response curve was generated, showing 240 nM EGF gives near complete stimulation of the cells.

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SAMPLE DATA

Antagonist dose-response

Once agonist concentration, cell density, and treatment times are optimized, antagonist testing can 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 possible, 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. In the below example, cell plating was carried out as described previously, with the culture medium aspirated and the antagonist added in serum-free media for 30 minutes prior to the agonist stimulation.

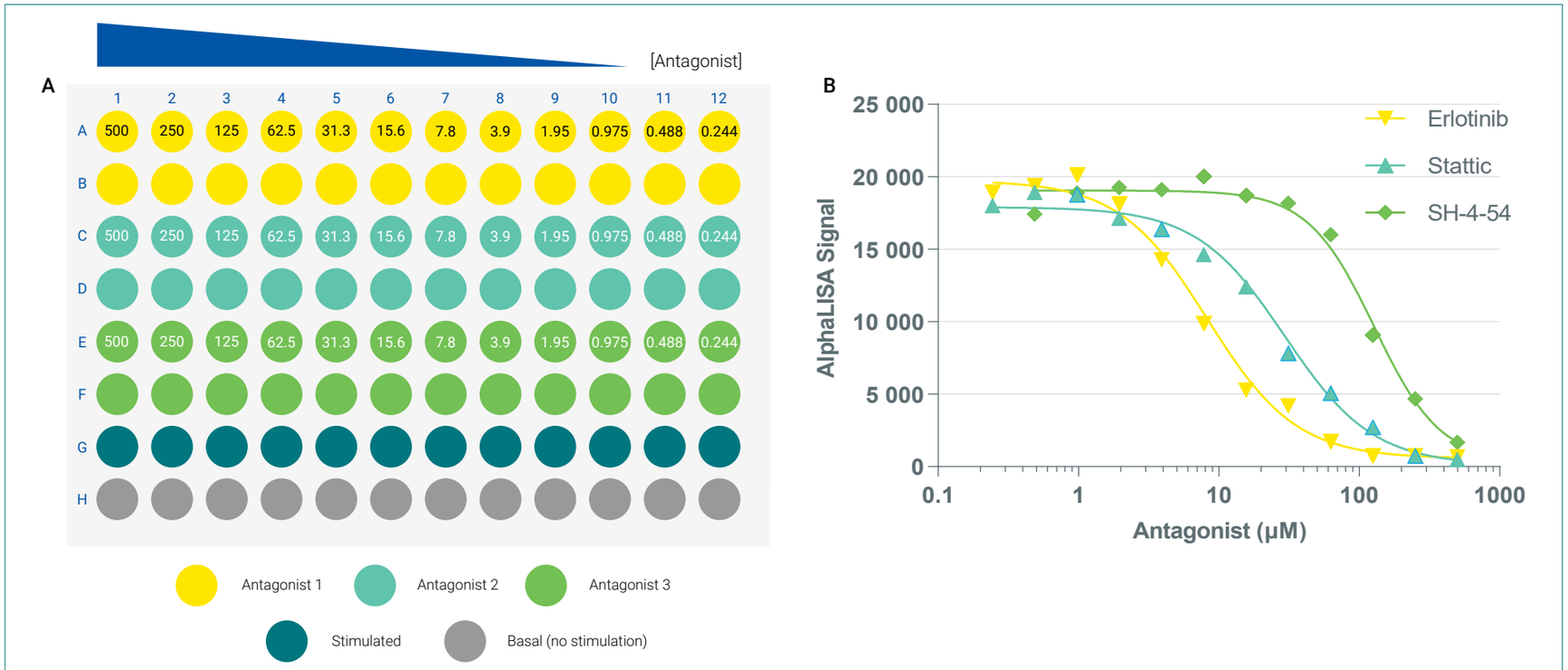

Figure 14: Antagonist dose-response optimization. A) Platemap and B) example data. A431 cells (50K cells/well) were serum-starved for three hours then treated with antagonist in serum-free media and incubated for 30 minutes. EGF (240 nM final) was then added to the cells and incubated for 7.5 minutes 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.

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SAMPLE DATA

Screening of antibody-containing samples

Extracts of cells from tissue culture, or from tissue itself, can contain antibodies. These antibodies can interfere with some 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.

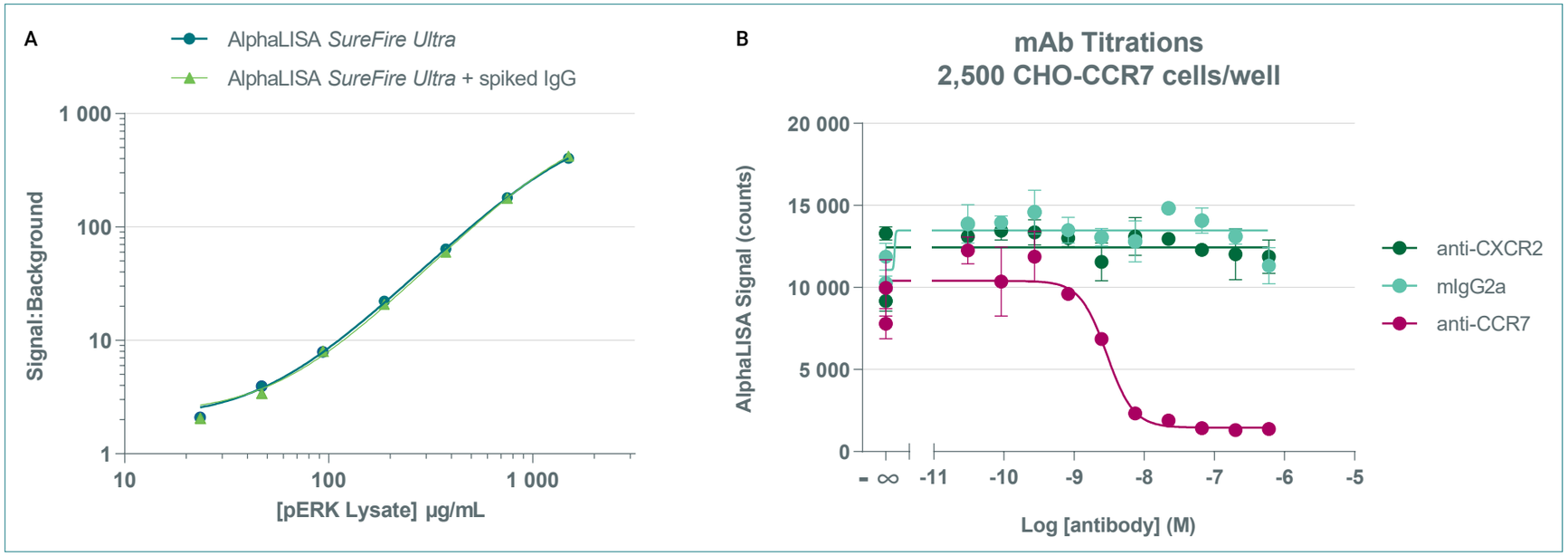

Figure 15: Measurement of phospho-ERK in the presence of extraneous antibodies. A) Lysates of EGF-activated A431 cells were serially diluted in the absence or presence of non-specific extraneous rabbit antibodies (10 µg/mL). Samples were then analyzed for phospho-ERK (T202/Y204) with an AlphaLISA SureFire Ultra phospho-ERK kit (Revvity # ALSU-PERK-A500). Data are presented as the signal obtained for the lysate divided by the signal obtained for lysis buffer alone. B) Detection of the inhibitory effect of a CCR7 blocking antibody (1h pre-incubation with cells) on CHO cells expressing the CCR7 receptor (Revvity #ES-140-C ; 2 500 cells/well) treated for 10 min with 10 nM of reference agonist CCL19 / Mip3β unrelated anti-CXCR2, and isotype control mIgG2a were used as negative controls..

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SAMPLE DATA

Multi-target analysis of phosphorylated cellular targets

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 three phosphoprotein targets, and total AKT, performed side-by-side from aliquots of individual culture wells of Rapamycin inhibitor-treated MCF-7 prior to Insulin stimulation.

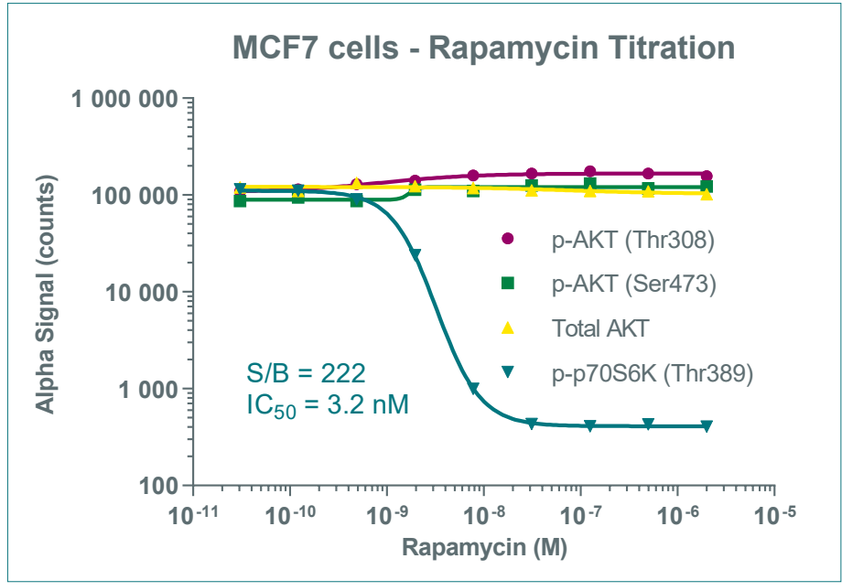

Figure 16: MCF-7 cells were plated overnight at 200K/mL in 200 µL MEM + 10% FCS. Cells were then treated for 2 hours with varying concentrations of rapamycin in MEM +1% FBS, and then stimulated for 30 min with 2.5 µg/mL insulin (n = 3 for each condition). Each well was lysed in 100 µL SureFire Ultra lysis buffer, and 10 µL aliquots of each lysate were assayed in parallel using the four AlphaLISA SureFire Ultra kits indicated.

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OPTIMIZATION PARAMETERS

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 factors 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, experiment to experiment, and reader to reader, 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 Revvity to support you better and faster in such situations.

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OPTIMIZATION PARAMETERS

Cellular optimization

	CONDITION	REASON
Mandatory	Cellular Model	Expression levels 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. Keep in mind that in homogenous immunoassays, too much of the analyte can result in a reduced level of signal (hooking), such that in cases of low signal , both lower and higher cell densities need to be tested.
	Cell Stimulation Time	The kinetics of protein phosphorylation can have a quick increase followed by a quick decrease, so it is important not to miss the optimal stimulation time, i.e., using a sub-optimal stimulation time can have a negative impact on S/B and assay quality. 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 can be helpful for well-to-well reproducibility.
	Lysis Buffer selection	Some kits use different lysis buffers (A, B, or C). Each assay has been optimized to work with its specific lysis buffer provided in the kit. If running several assays in parallel from the same cell lysate, please check which lysis buffer is compatible with which kit. Lysis buffer from other suppliers can be compatible with the *SureFire* assay – to be checked on a case-by case basis. RIPA lysis buffer is generally not compatible with *SureFire* assays, unless the sample is diluted further in the *SureFire* lysis buffer.
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 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 5-minute pre-incubation is sufficient to block cell surface receptors, and 30 minutes - 2 hours to block intracellular kinases.
	DMSO Tolerance	DMSO should normally cause no issues up to concentrations of 2%, depending on 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-characterized agonist and antagonist compounds as control test compounds, to provide performance relativity. Depending on the cell type and conditions, the absolute IC50/EC50 may vary, as the pathway examined and assay conditions may lead to different maximal and half-maximal responses.

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OPTIMIZATION PARAMETERS

Cellular optimization

	CONDITION	REASON
Optional	Temperature When Stimulating Cells	Cells must be stimulated in optimum conditions i.e. in incubator at 37 degrees and 5% CO_2, not on the bench.
	Adherent vs. Suspension Cell Types	AlphaLISA *SureFire Ultra* kits work well with both cell types. Washing cells can be helpful in removing potential matrix effects, if needed.
Hints/Reminders	Phosphatase Inhibitors	Phosphatase inhibitors are included in the *SureFire Ultra* Lysis Buffer (Orthovanadate, Pyrophosphate, and sodium fluoride). Additional phosphatase inhibitors may be added if necessary, but would require validation.
	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. The following protease inhibitors are known to be tolerated by Alpha technology: Sigma Cat.No. P2714 or Roche cOmplete™ *ULTRA* Tablets Mini, EDTA-free, EASYpack Protease Inhibitor Cocktail Cat. No. 05 892 791 001. Other cases may exist, please inquire.
	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, leading to a decrease of the assay sensitivity. 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.

"For more details please visit www.revvity.com, or contact your local Revvity sales specialist or field application specialist."

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OPTIMIZATION PARAMETERS

Immunoassay optimization

	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 may be advantageous for assay reproducibility, as they minimize well-to-well crosstalk effects and may 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. Color of the Lysis Buffer doesn't affect performance.
	Lysis Buffer[1]	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. A 384-well plate can be lysed in 10-50 µL lysis buffer.
	Activation Buffer selection	Each assay has been optimized to work with its specific activation buffer. Activation buffers A, B, and C are not interchangeable.
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.

[1] Some kits contain assay specific Lysis Buffer B (5X)-Ultra or Lysis Buffer C (5X)-Ultra. Lysis Buffer B (5X) and Lysis Buffer C (5X) are assay specific and should not be interchanged.

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OPTIMIZATION PARAMETERS

Immunoassay optimization

	CONDITION	REASON
Hints/Reminders	Assay Consistency and Reproducibility	Alpha technology is dependent upon a number of 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. AlphaLISA *SureFire Ultra* kits are developed and manufactured such that each kit meets stringent quality criteria to ensure robust and reproducible assay windows within and between experiments, making the platform an optimal research tool from basic research through screening campaigns and translational studies as well as quality control of manufactured biotherapeutics.
	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.
	Light Sensitivity	The SA Donor beads, both the working solutions and the stock parent tube, are mildly light sensitive and should be handled with care. A typical light condition suitable for handling Donor beads is in a tissue culture or fume hood with the hood lights off, but the lights in the room can remain on.
	Specificity	The antibodies used in the kits have been carefully selected, taking into account the target and species selectivity information from the providers of the antibodies, to generate a signal that is specific for the desired target. The specificity of the kit results from the specificity of both antibodies (i.e., a signal can be generated only when the two antibodies recognize the targets), resulting in very specific assays. However, in some situations additional evidence for the specificity of signal detection may be desired. In such situations using recombinant phosphorylated proteins, siRNAs specific to the target, or knock-out cell lines using, for example, CRISPR technology, or using peptides as competitors in the assay can be very useful to show specificity of the AlphaLISA signal. In the case of peptide competition, 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 phospho-peptide, can be used as well.

For more details please visit www.revvity.com

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APPENDIX

Sample calculations for AlphaLISA *Surefire Ultra* assay (single-target assay example)

	1	2	3	4	5	6	7	8	9	10	11	12
A		100%			40,000		40,000					
C		50%			20,000		20,000					
B		25%			10,000		10,000					
D		12.50%			5,000		5,000					
E		6.25%			2,500		2,500					
F		0% (lysis buffer)			1,250		1,250					
G												
H												

Concentration of Positive Control Lysate (provided in the kit, serially diluted with 1X Lysis Buffer)
Equivalent cells per datapoint (in 10ul lysate) without therapeutic treatment
Equivalent cells per datapoint (in 10ul lysate) with therapeutic treatment

Number of wells = 48

Scale up to accommodate for dead volume; *make enough for 60 wells*

Reaction components per well

- 10ul of lysate
- 5ul of Acceptor Mix
- 5ul of Donor Mix

Mix	Component	ul (60 wells)	ul (my # of assay points)
Acceptor	Reaction Buffer 1	141	= (141 x # wells) / 60
	Reaction Buffer 2	141	= (141 x # wells) / 60
	Activation Buffer	12	= (12 x # wells) / 60
	Acceptor Beads	6	= (6 x # wells) / 60
Donor	Dilution Buffer	294	= (294 x # wells) / 60
	Donor Beads	6	= (6 x # wells) / 60

For a number of assay points not represented in this table, calculate using equation found in this table

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APPENDIX

Sample calculations for AlphaLISA *Surefire Ultra* assay (single-target assay example)

For 60 assay points

Mix	Component	Vol (ul)
Acceptor	Reaction Buffer 1	141
	Reaction Buffer 2	141
	Activation Buffer	12
	Acceptor Beads	6
Donor	Dilution Buffer	294
	Donor Beads	6

Number of assay points (wells)		60	80	100	120	140	200	240	260	300	400
Mix	Component	Vol (ul)	Vol (ul)	Vol (ul)	Vol (ul)	Vol (ul)	Vol (ul)	Vol (ul)	Vol (ul)	Vol (ul)	Vol (ul)
Acceptor	Reaction Buffer 1	141	188	235	282	329	470	564	611	705	940
	Reaction Buffer 2	141	188	235	282	329	470	564	611	705	940
	Activation Buffer	12	16	20	24	28	40	48	52	60	80
	Acceptor Beads	6	8	10	12	14	20	24	26	30	40
Donor	Dilution Buffer	294	392	490	588	686	980	1176	1274	1470	1960
	Donor Beads	6	8	10	12	14	20	24	26	30	40

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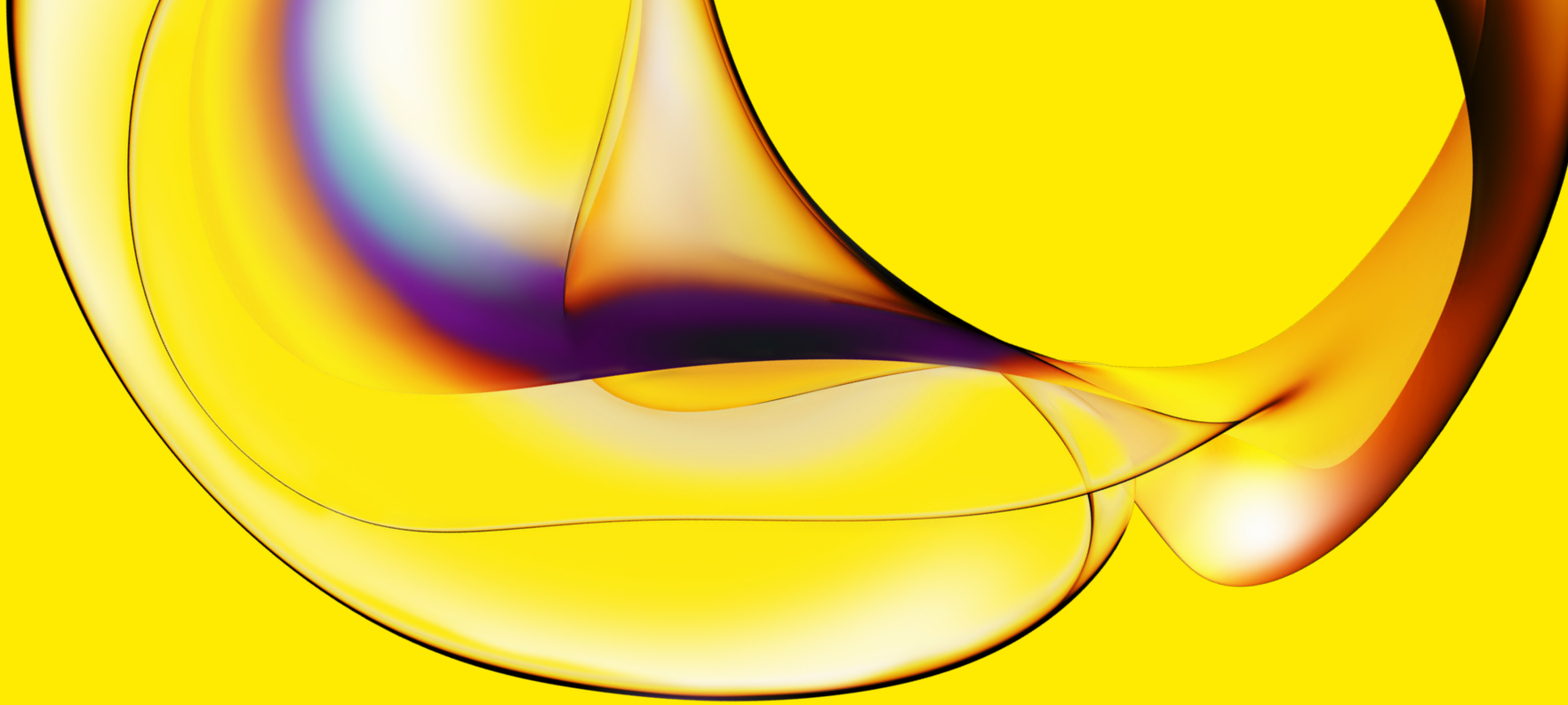

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