Cell signaling

Guide to optimizing your phospho-/total protein assays

PURPOSE AND BACKGROUND

Purpose

The proper optimization of assay conditions is essential to ensure you obtain the best reagent use and performance. In the case of Cell Signaling, the most suitable assay conditions are dependent on the signaling pathway being investigated as well on the pharmacological characteristics of the compound being studied (activator or inhibitor). The optimal assay conditions will also hinge on the particular cellular model used for the assay (adherent or suspension cells, immortalized cell line or primary cells, tumor, tissue…). For each setup, a number of optimization steps can be implemented that will ensure the best, most accurate results are obtained.

Drawing on hundreds of data sets and cases established by Revvity's scientists over the years, our manual provides the scientific community with the most up to date guidelines for the optimization of Cell Signaling assays.

Background

Intracellular signaling pathways, also called signal transduction cascades, play a key role in sensing and integrating diverse external stimuli (growth factor, hormone, cytokine, stress…) to generate biological responses such as changes in enzyme activity, gene expression or cytoskeletal remodeling, finally leading to cellular processes (proliferation, migration, differentiation, nutrient metabolism…).

The stimuli are detected by a cell surface receptor which transmits the initial signal inside the cell. There, it is then multiplied by a transduction cascade mediated by the phosphorylation of multiple protein substrates.

The phosphorylation reaction is a post-translational modification, mediated by kinases, that controls the activity of proteins involved in signal transduction. It is therefore an appropriate readout of the activation status of a pathway of interest.

The Cell Signaling kits developed by Revvity are specifically intended for the direct measurement of proteins (phosphorylated and/or total) within cells. All kits are built on the same precise format and protocol.

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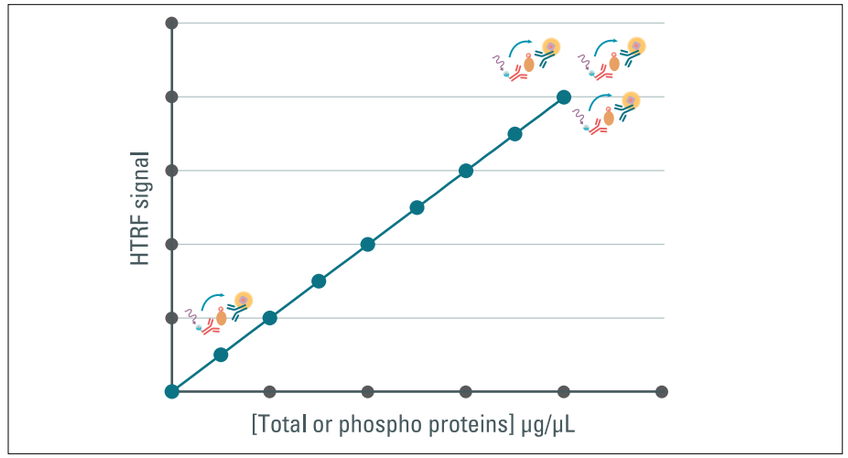
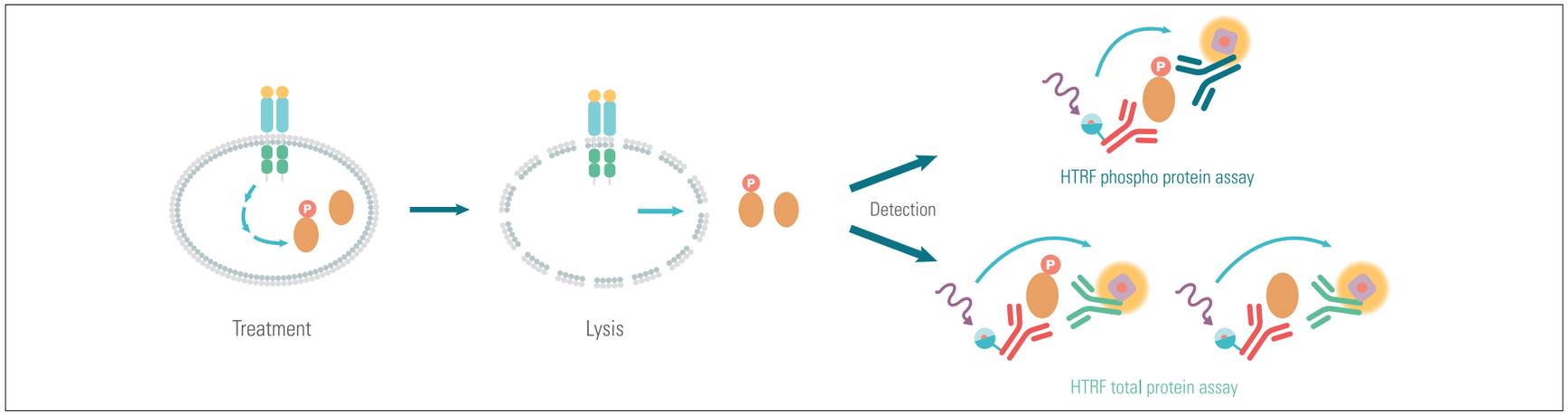
ASSAY DESCRIPTION
Assay principle

- Following treatment, cells receive a lysis treatment leading to the release of intracellular proteins, both phosphorylated or unphosphorylated, from the cell's compartment.
- Released proteins are detected by addition of the reagents.
- All HTRF phospho and total assays are based on a sandwich immunoassay format:
  - Phospho specific and total antibodies, labelled with Cryptate or d2, are used for the quantification of phosphorylated proteins
  - Two different antibodies, labelled with Cryptate or d2, are used for the quantification of total proteins.
- Due to the sandwich format of HTRF phospho and total assays, the HTRF signals increase proportionally to the concentration of the protein of interest.





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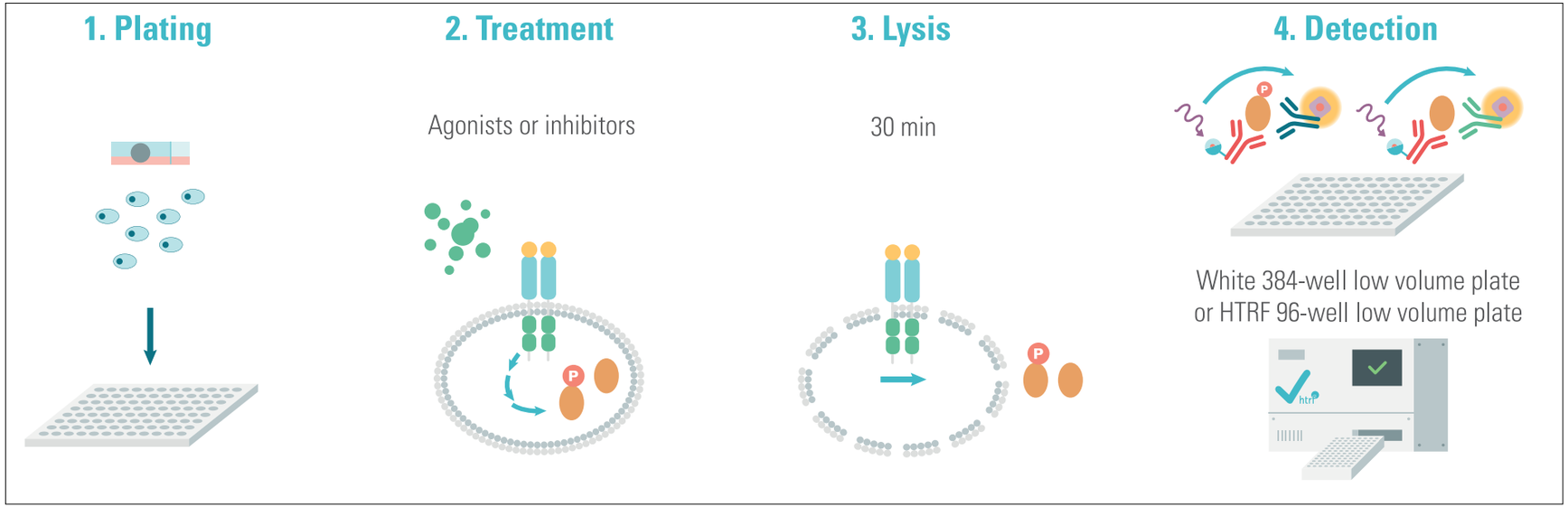
ASSAY DESCRIPTION
Assay workflow

A cell signaling experiment has 4 steps:

- Cell culture: cells are usually plated in 96 well plates. Note that other types of cell culture vessel can also be used (*see p13*)
- Cell stimulation using agonists, antagonists or any other class of modulators
- Cell lysis, usually performed for 30min at RT
- Detection: following addition of HTRF reagents, the detection step is performed on an HTRF compatible plate reader

4 MAIN STEPS shared by all HTRF phospho and total assay protocols



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ASSAY DESCRIPTION
Assay reagents

All HTRF phospho and total assays are frozen*.

All HTRF phospho and total assays are offered in 500 and 10,000 tests.
Some references are also available in 96 tests.

All reagents needed are supplied in each kit.

Please refer to our website for the references of commercial HTRF compatible microplates.

Note that HTRF phospho and total lysis buffers are also available separately.

The HTRF reagents provided (500 tests) are:

- HTRF Detection antibodies**:
  Cryptate-antibody (20X) (red cap vial x1)
  d2-antibody (20X) (blue cap vial x1)
- Buffers:
  Blocking reagent (100X) (purple cap vial x1): contains anti-phosphatases
  Lysis buffer (4X) (transparent cap vial x4): each kit includes an optimal lysis buffer giving the best assay performance (www.revvity.com/htrf-alternative-ripa). However a lysis buffer compatibility sheet is provided for some applications that would require the same lysate, such as analyses of multiple parameters (www.revvity.com/htrf-lysis-buffer-compatibility)
  Detection buffer (orange cap vial x2)
- Positive control:
  Positive control lysate (green cap vial)
- Package insert
- Low Volume microplate is included in 96 phospho-total assays.

* except the lyophilized phospho-ERK assay

** The concentration of each detection antibody has been carefully optimized. Please do not change their dilution, since this will impair the performances of assays.

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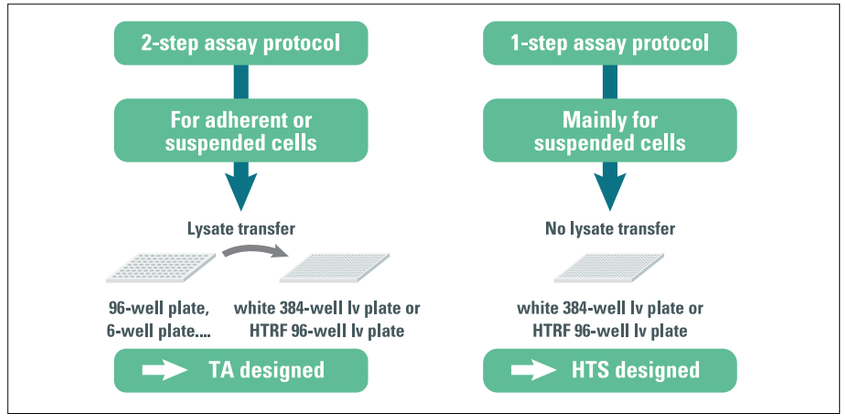
ASSAY FORMATS
Two-step and one-step protocols

The two-step protocol dissociates cell culture & treatment from the detection step. Cells, either suspended or adherent, are plated, treated and lysed in a cell culture microplate, then 16µl of cell lysate are transfered to the detection microplate. Because it is much more physiological than the one-step, this protocol should be preferred for adherent cells.

The two-step protocol is mainly designed for the therapeutic area.

The one-step protocol combines all steps in a single plate. Since adherent cells need to be in contact with the plastic surface for proper growth and functioning, a one step protocol should be avoided when the experimental conditions involve adherent cells.

The one-step protocol is mainly designed for scientists working in High Throughput Screening.



This table gives the volumes required at each step of the two different protocols.

TABLE OF VOLUMES	TWO-STEP PROTOCOL			ONE-STEP PROTOCOL	
		Adherent cells	Suspended cells		Suspended cells
Cells	Culture plate	50 µL	25 µL	Detection plate	8 µL
Compound		50 µL	5 µL		4 µL
Harvest medium		yes	no		no
Supplemented lysis buffer		50 µL(1)	10 µL(2)		4 µL(2)
Lysate transfer	Detection plate	16 µL	16 µL		-
HTRF detection reagents		4 µL	4 µL		4 µL

(1) Lysis buffer 1X supplemented with blocking reagent (1X)
(2) Lysis buffer 4X supplemented with blocking reagent (4X)

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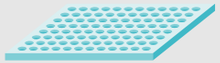
ASSAY FORMATS
Cell culture vessels

The standard protocol provided in the HTRF phospho-total package insert describes the cell seeding step in a clear-bottom 96 well microplate.

Scientists looking to scale-up their lysate can refer to the table below for optimal seeding densities. This is of particular interest if multiple phospho and total proteins need to be analyzed from the same lysate.

The table below provides cell density adjustments, as well as cell culture medium volumes in different types of vessels.

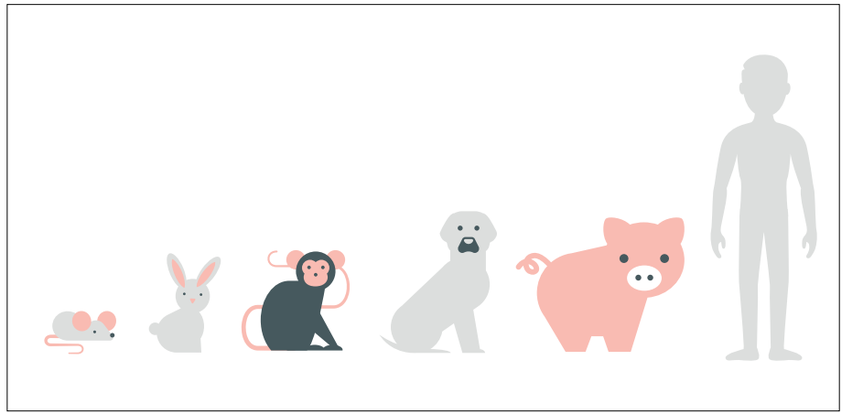
(For more information, please refer to this technical note).

	CELL CULTURE VESSEL		
	Microplate	**Cell seeding density ($x10^3$ cells/well)**	**Culture medium (µL)**
	96 wells	25-100	50
	24 wells	140-550	500
	12 wells	290-1150	1000
	6 wells	700-2800	2500
	Flask 75cm2	6000-24000	10000

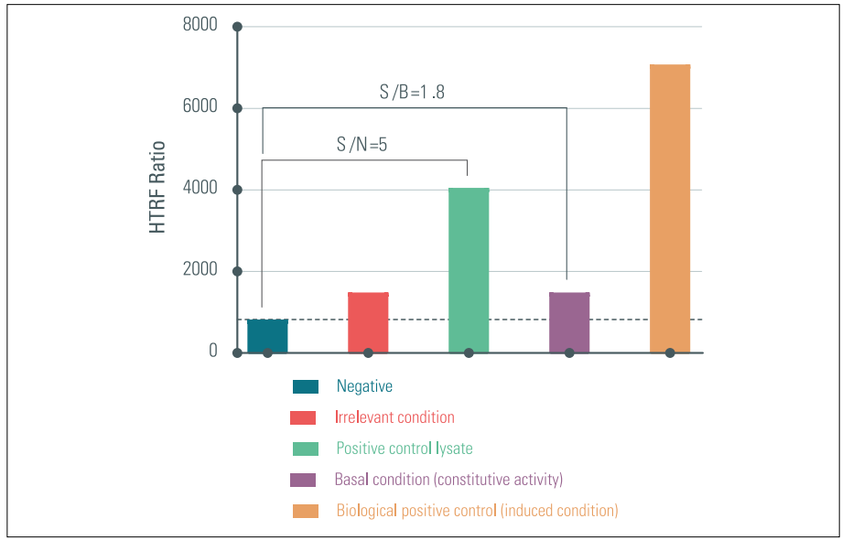
ASSAY OPTIMIZATION
Biological controls

Start by checking the assay is compatible with the species of the cell line used (e.g while the HTRF phospho p53 assay is compatible with human cell lines only, the Phospho-ERK kit is compatible with cell lines of human, mouse, rat, bovine or even dog origins. Find here our dedicated species compatibility sheet.

- The positive control lysate included in each kit is used as an internal control to validate proper HTRF antibody dilution and the settings of the HTRF microplate reader. The Signal over Negative (S/N) should be above 2.
- The untreated or vehicle-treated cells represent the basal or constitutive expression (HTRF total protein assay) or phosphorylation level (HTRF phospho protein assay) of the readout of interest.
- The biological positive control corresponds to cells that have been treated with a compound of known efficacy **to modulate the pathway of interest**.
- Optionally, Irrelevant controls can also be included to **validate the specificity** of the biological response, i.e. compounds known not to activate the pathway.



Representative HTRF Ratio of the recommended controls



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ASSAY OPTIMIZATION

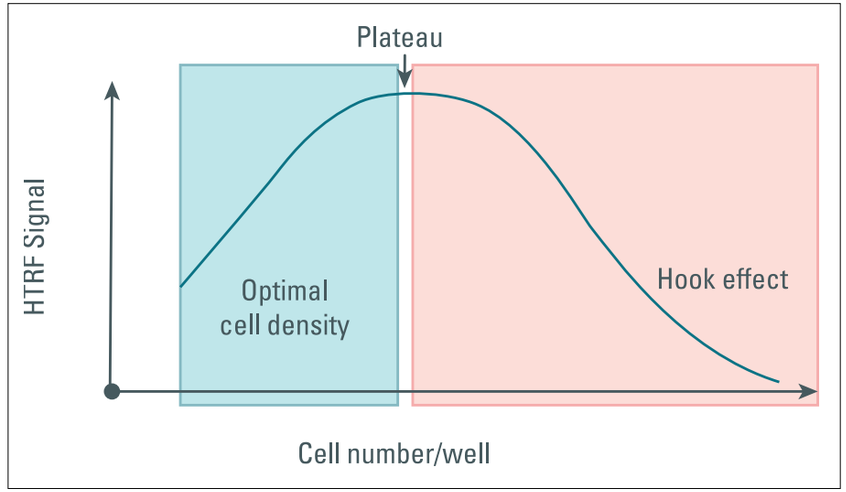
Cell density optimization

The HTRF signal is directly proportional to the amount of phosphorylated and total proteins resulting from the treatment.

Like any sandwich immunoassay, the signal increases proportionally to the analyte being quantified until a plateau is reached. If the amount of phosphorylated proteins is significantly higher than the detection reagents present in the well, the signal goes down.

This situation is known as the hook effect.

The cell density must be carefully optimized for each HTRF phospho or total kit, each cell-based model, or even each experimental condition in order to avoid the hook effect.



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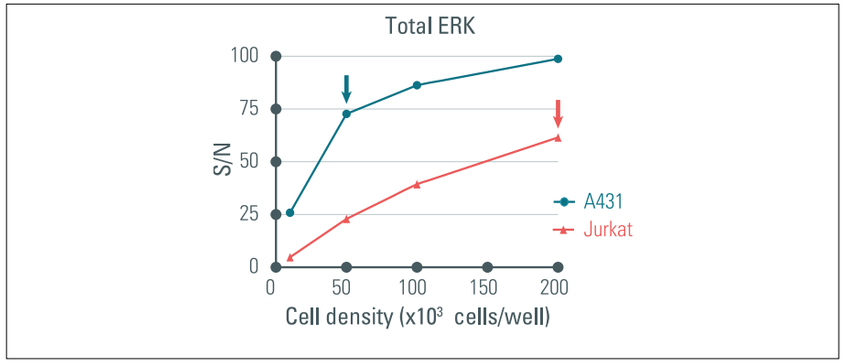
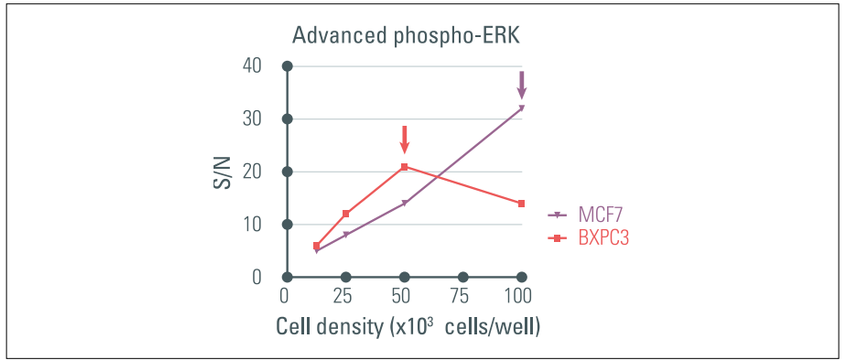
ASSAY OPTIMIZATION
Cell density optimization

For a Total ERK assay, whereas 50,000 cells per well (seeded in a 96 well plate) is the maximum amount of cells for the A431 cell model, up to 200,000 cells per well is fine when addressing the ERK expression level in Jurkat cells

Similarly, while working at 100,000 MCF7 cells per well is fine for the advanced phospho-ERK kit, less than 50,000 BXPC3 cells are required.

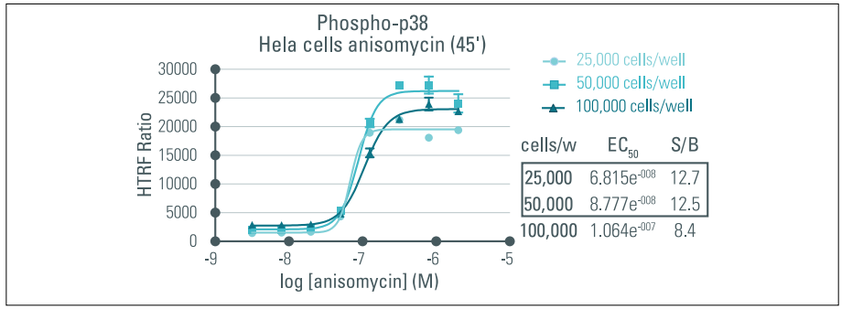


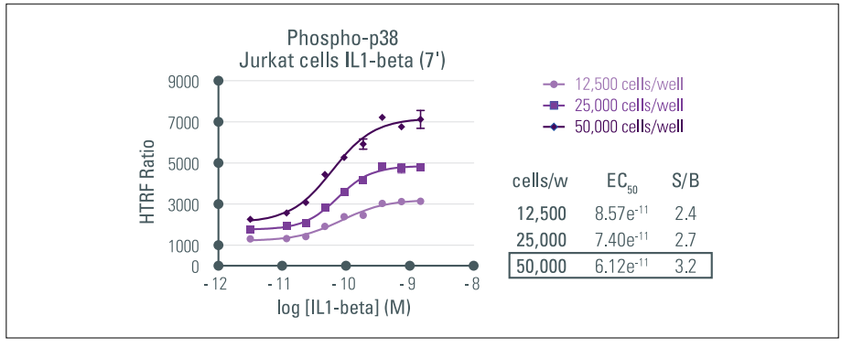


Improper cell density optimization may impair IC_{50} determination.

As illustrated here, investigation of p38 phosphorylation upon anisomycin treatment in Hela cells is optimal at 50,000 cells per well. Note than EC_{50} is comparable between 25 and 50,000 cells and tends to be degraded at 100,000 cells, as is the pharmacological assay window (S/B).

For IL1β-treated Jurkat cells, HTRF is linear between 12,000 and 50,000 cells per well and EC_{50} remains similar for all cell densities tested.





Plating → **Treatment** → **Lysis** → **Detection**

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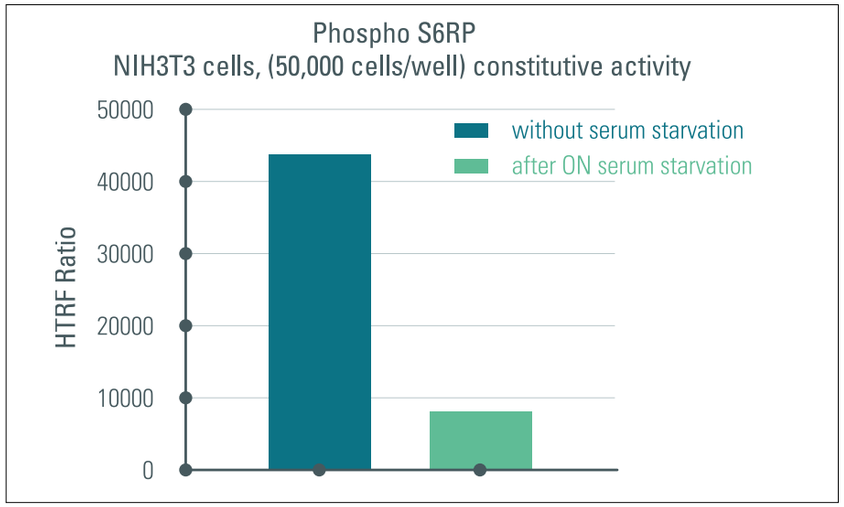
ASSAY OPTIMIZATION
Cell treatment

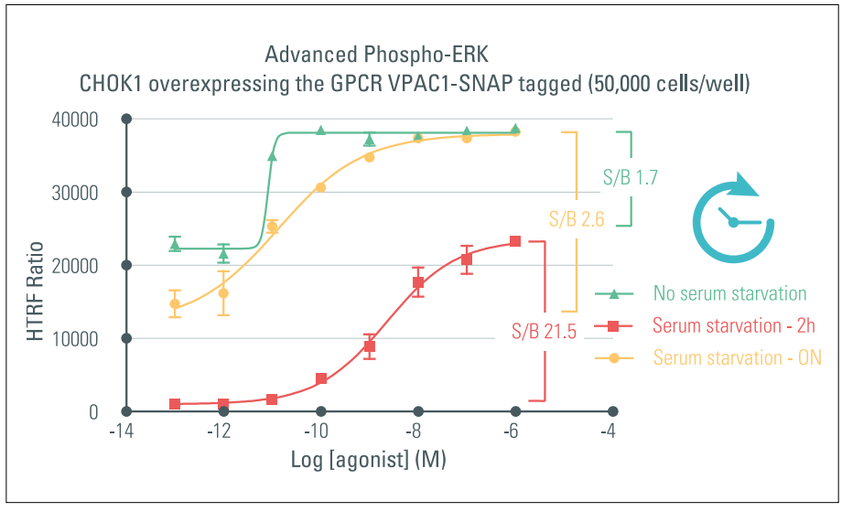
STARVATION STEP

Depending on the signaling pathway to be investigated, a starvation step can be beneficial to reduce a constitutive phosphorylation of the protein of interest. Most of the time, eliminating serum from cell culture medium constitutes an efficient way of starving the cells, as evidenced by the example from a phospho S6RP assay.

However for some cellular models, the presence of serum is essential to ensure appropriate cell spreading or growth. In such cases, reducing serum to 0.5%, or up to 2% serum can be an alternative.

In addition, the duration of serum starvation should be optimized. For instance, phosphorylation of ERK upon agonist stimulation is optimal after 2 hours of serum starvation.





Plating → **Treatment** → Lysis → Detection

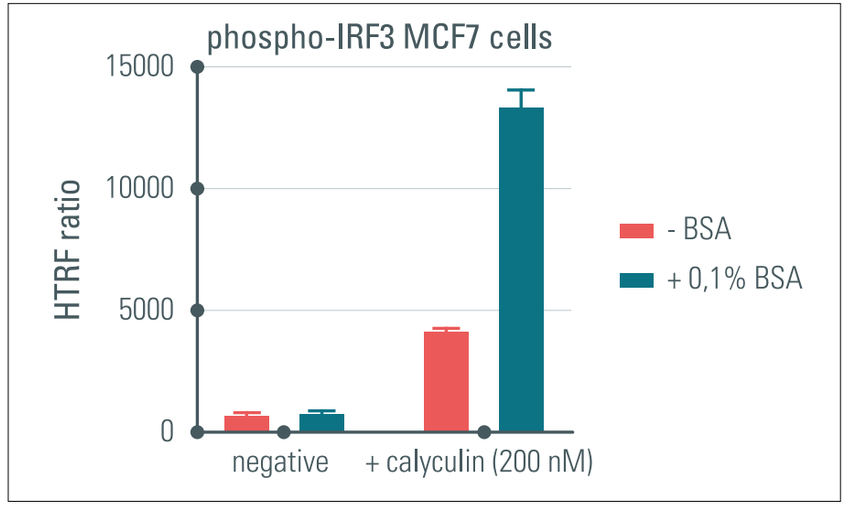
ASSAY OPTIMIZATION
Cell treatment

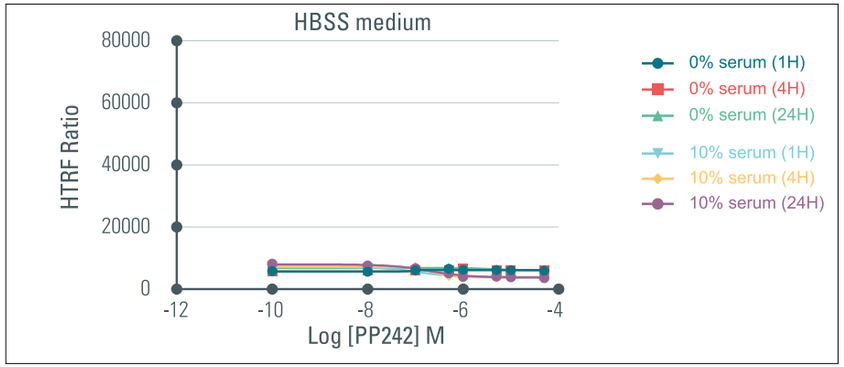
DEFINE OPTIMAL STIMULATION MEDIUM FOR CELLS AND COMPOUNDS

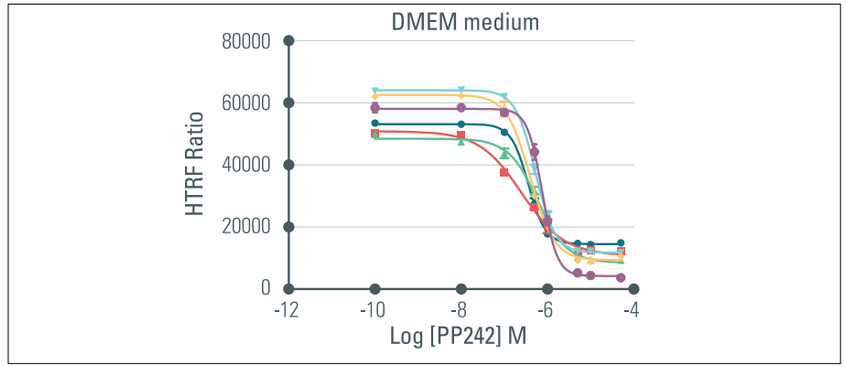
Compounds are usually diluted in cell culture medium, with or without 10% FCS.

Since some compounds tend to stick to the plastic vial, supplementation of cell culture medium with 0.1% BSA is highly recommended, especially when a starvation step is necessary. For instance, Calyculin dilution necessitates BSA supplementation to induce IRF3 phosphorylation in MCF-7 cells.

For suspended cells, with either the two-step or the one-step protocol, the utilization of cell culture medium without phenol red is recommended. HBSS or a similar buffer can be used to dilute cells and compounds. Remember that exceeding 30 minutes of incubation may impair cell health and consequently the biological response. This is pointed out on phospho-4EBP1, where 25,000 Jurkat cells/well were seeded for 1, 4 or 24 hours in HBSS or DMEM in the presence or not of serum, prior to a further 3 hour incubation with increasing doses of the inhibitor PP242. Whereas Jurkat cells in DMEM display a satisfactory inhibition curve, HBSS medium does not.







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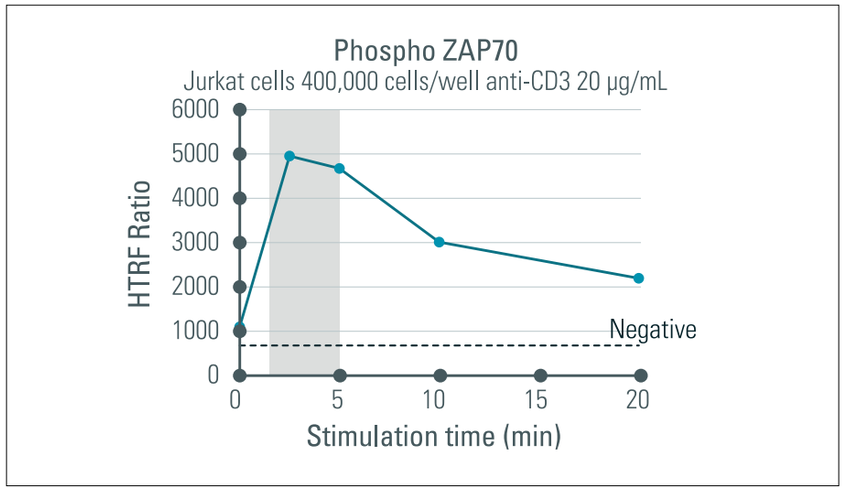
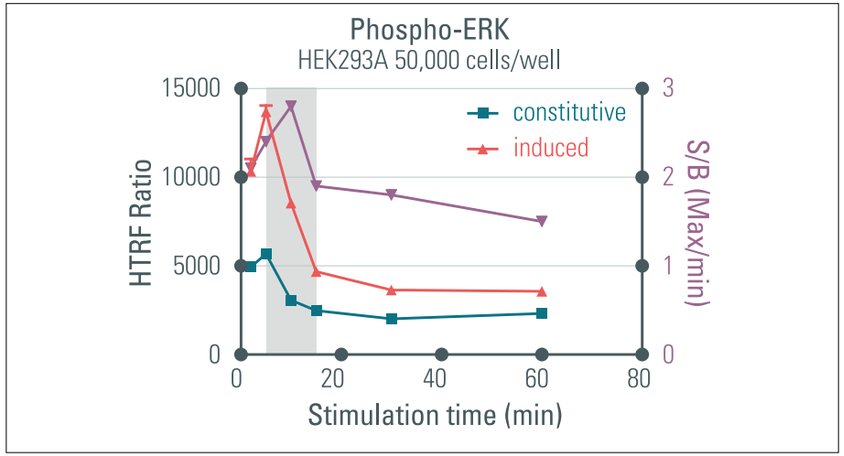
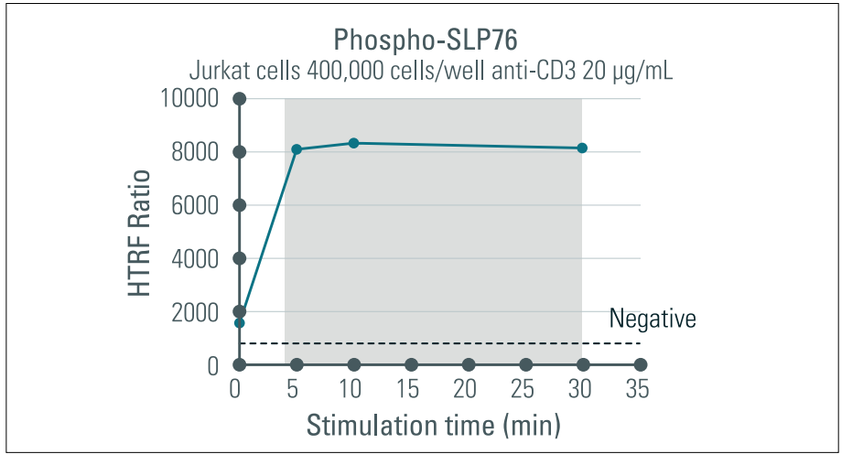
ASSAY OPTIMIZATION
Cell treatment

TIME-COURSE OF COMPOUND TREATMENT

As shown below, the phosphorylation of ZAP70 or ERK is very transient with a maximum signal reached at 2'30 and 10' respectively, whereas phosphorylation of SLP76 remains stable from 5' up to 30'. Therefore it is mandatory to perform a time-course experiment to make sure you work in the optimal temporal window, which is shown in the grey area on the graphs.







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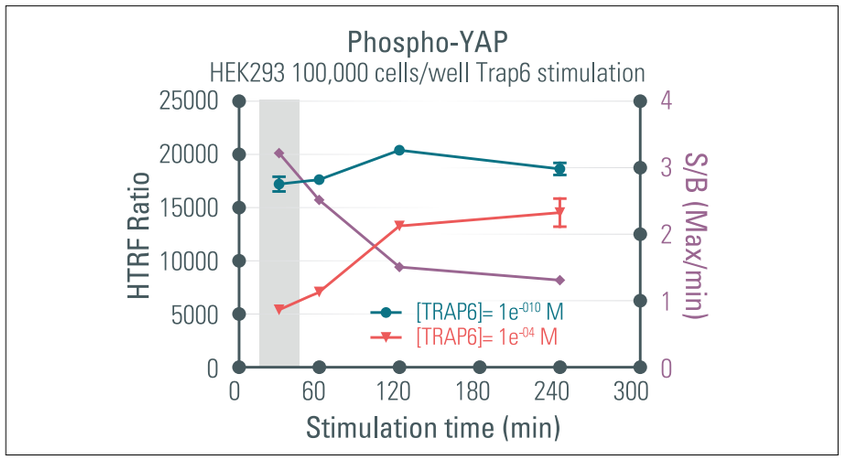
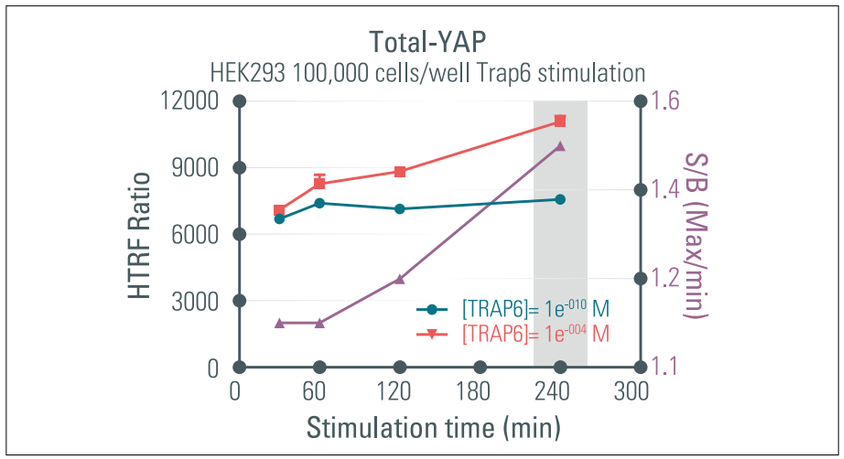
ASSAY OPTIMIZATION
Cell treatment

In addition, the temporal window for assessing the phosphorylation state and the expression level of a protein might be different. For example, TRAP6 induces a significant response on YAP phosphorylation around 30' whereas the TRAP6 counter-effect on the YAP expression level can be observed after 3 hours of incubation.





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ASSAY OPTIMIZATION
Lysis step

Optimization of the lysis buffer volume (only for the two-step adherent protocol). Refer to the kit's package insert for the exact preparation procedure.

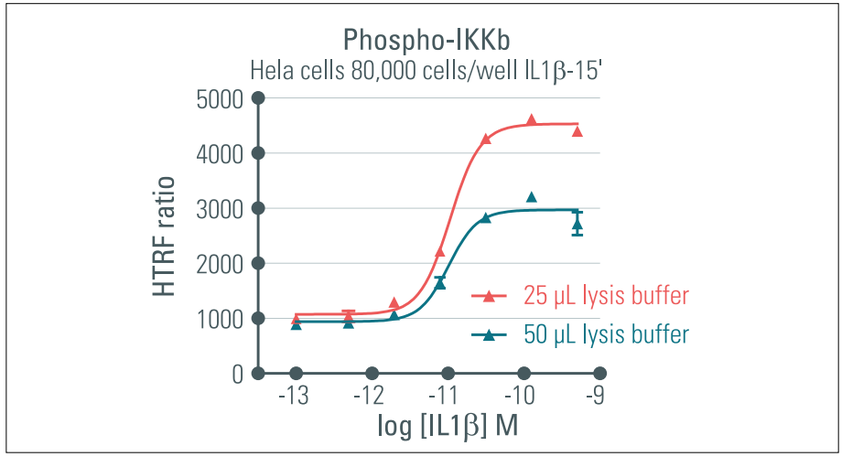
Optimal lysis buffer volumes are recommended in the table below:

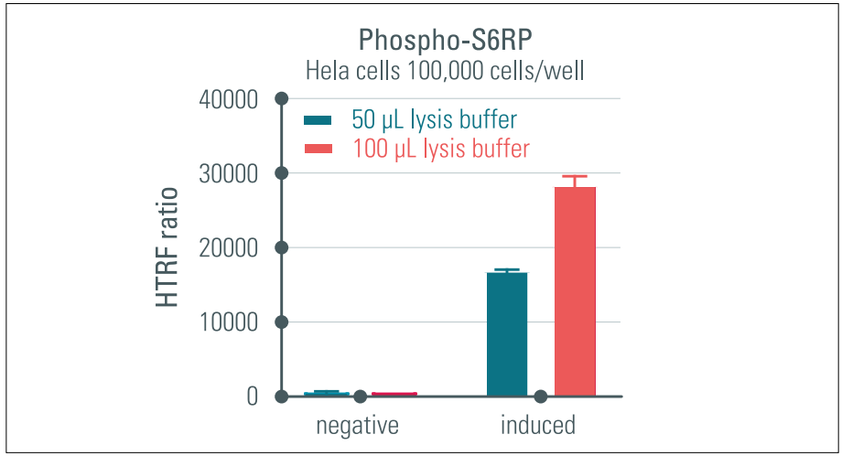
CELL CULTURE VESSEL		
CELL CULTURE PLASTICS	**CELL SEEDING DENSITY (X10³ CELLS/WELL)**	**LYSIS BUFFER (µL)**
96 wells	25-100	50
24 wells	140-550	250
12 wells	290-1150	500
6 wells	700-2800	1500
Flask 75cm2	6000-24000	12000

Note that the lysis step is usually performed for 30' at RT.

Increased incubation time may lead to high lysate viscosity, making the liquid much more difficult to pipette, and thus may degrade result accuracy.

However if higher sensitivity is needed, the lysis buffer volume can be reduced as reported for phospho-IKKb, where 25 µL of lysis buffer improved the assay window. Lysis buffer volume can also be increased - see results for phospho-S6RP for which 100 µL of lysis buffer delivered a better performance compared to 50 µL.





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ASSAY OPTIMIZATION
Incubation step and signal acquisition

INCUBATION STEP

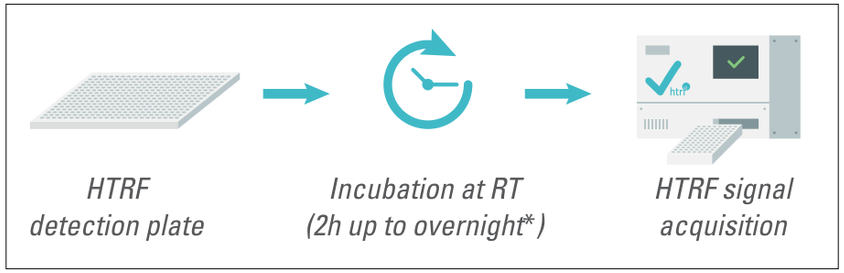
- The incubation step is performed at room temperature with a sealer.
- The incubation time has been optimized for each assay*. In some cases, the optimal performance is reached after only 2, 3 or 4 hours. Otherwise, an overnight incubation is required to get the best sensitivity. However, it is generally possible to read the plate earlier to obtain preliminary results.

*Please refer to each kit package insert for the optimal incubation time.

SIGNAL ACQUISITION

HTRF signal remains stable over time.

Plates can be read multiple times up to 24 hours of incubation (and even up to 48 hours for most assays) without degrading data quality.



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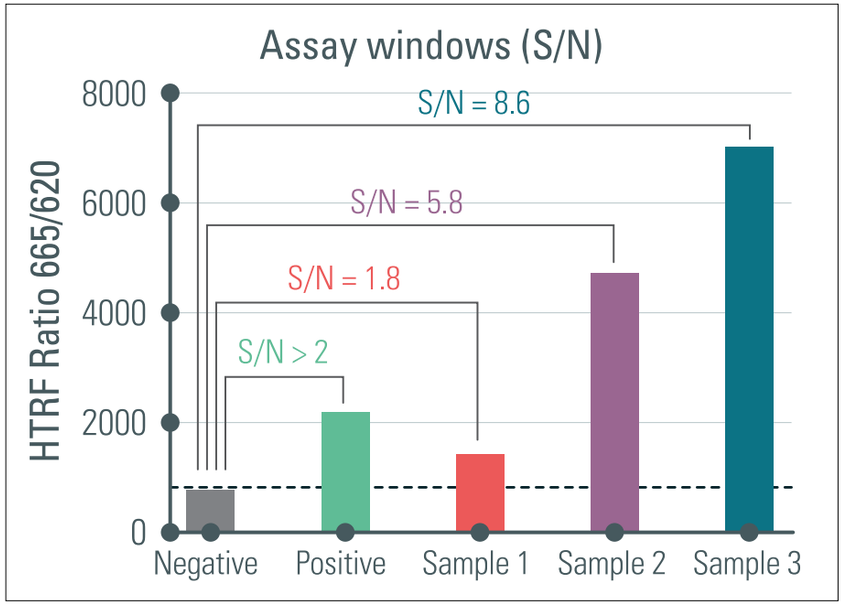
DATA INTERPRETATION

Assay window (S/N)

- The HTRF Ratio of the negative control corresponds to the non-specific signal of the assay. It enables the calculation of the assay window or "signal to negative" (S/N), using the following formula:
  $S/N = HTRF\ Ratio_{positive\ or\ sample} / HTRF\ Ratio_{negative}$
- The S/N of the positive control must be > 2.
- The S/N of your samples may be compared (< or >) to that of the positive control.
- HTRF signal is considered as significant if the S/N is > 1.2.



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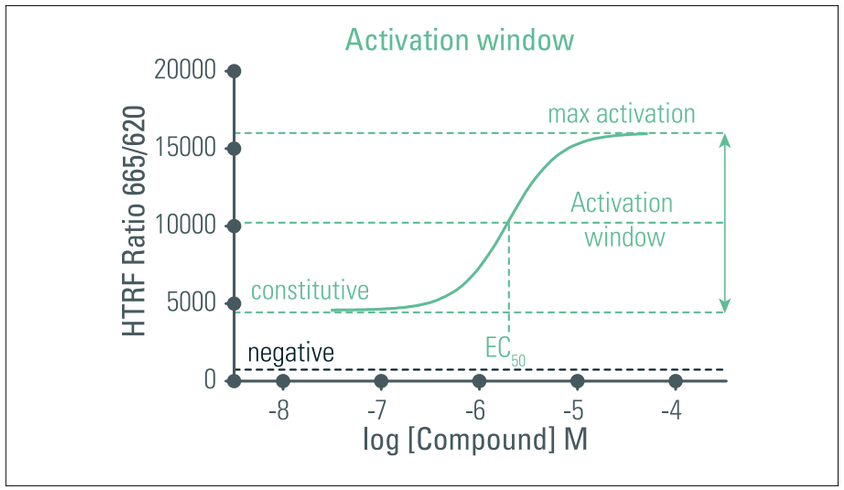
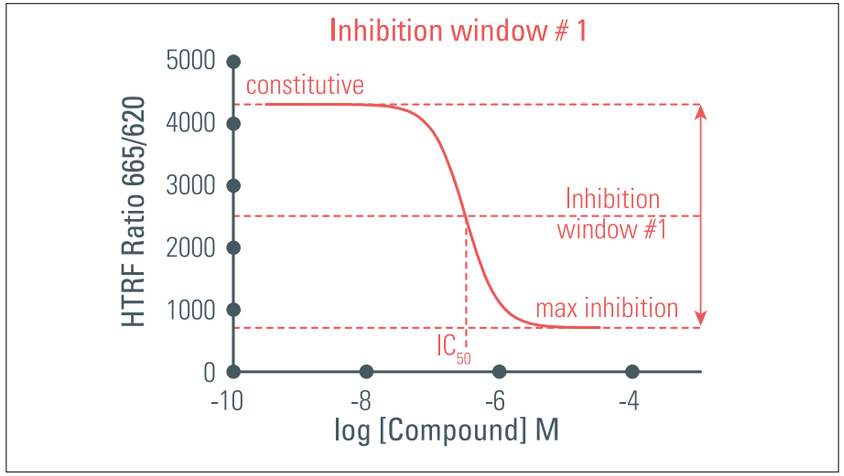
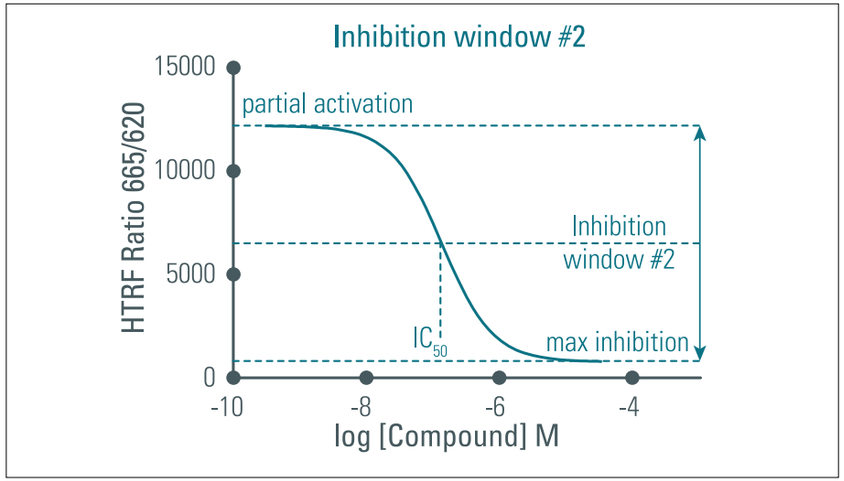
DATA INTERPRETATION

Pharmacological study of compounds

- Dose-response experiments are performed by treating cells with increasing concentrations of compounds (activators or inhibitors).
- A non-treated condition must be included to determine the constitutive level of phosphorylation or expression of the protein.
- To determine EC_{50}/IC_{50} value (half maximal effective/inhibitory concentration), a sigmoidal dose-response curve is drawn up by plotting HTRF Ratio versus the log of compound concentration (M).
- The pharmacological window is calculated by dividing the HTRF Ratio of the top of the curve by that of the bottom of the curve, as described below:







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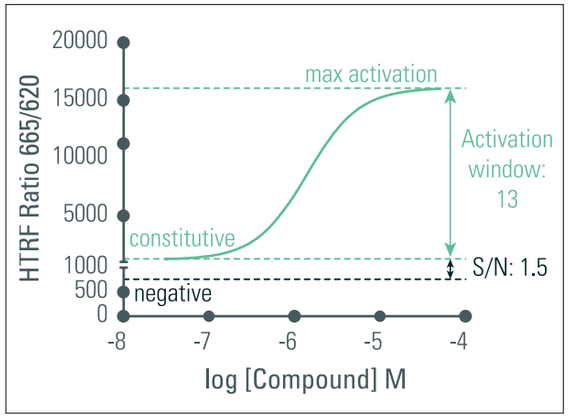
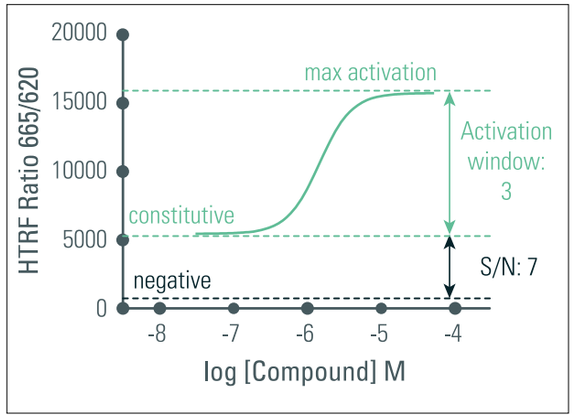
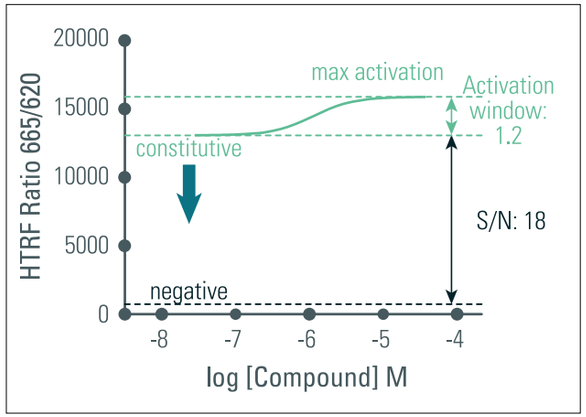
CASE STUDIES
Activation experiments







Low or no constitutive level

- The constitutive level of the phospho- or total protein of interest is undetectable or very low (1 < S/N < 2).
- The compound induces a large dose-dependent increase of the signal, leading to a comfortable activation window (> 3).

This is the perfect scenario to identify and characterize activator compounds.

No optimization is required.

Medium constitutive level

- The constitutive phosphorylation or expression level of the protein is clearly detectable (S/N > 3).
- The compound is still able to induce a response, but the activation window is often reduced compared to that obtained without the constitutive level.

In this case, it is also possible to analyze the effect of activator compounds without any optimization.

However, if the activation window is not comfortable enough (< 3), the constitutive level may be decreased by testing different experimental conditions (cf. next paragraph on the right).

High constitutive level

- The phospho- or total protein has a high constitutive level close to its maximum.
- The effect of the drug is not significant or very low (activation window < 2).

This kind of result doesn't mean that the HTRF assay doesn't work. The biology is simply not adapted to study activators.

The first option is to reduce the constitutive level by testing the following parameters:

- a starvation step (e.g. without serum or glucose) for the appropriate time period.
- a cell density optimization (generally by reducing the number of cells).

The second option is to work with another cell line with a lower constitutive level.

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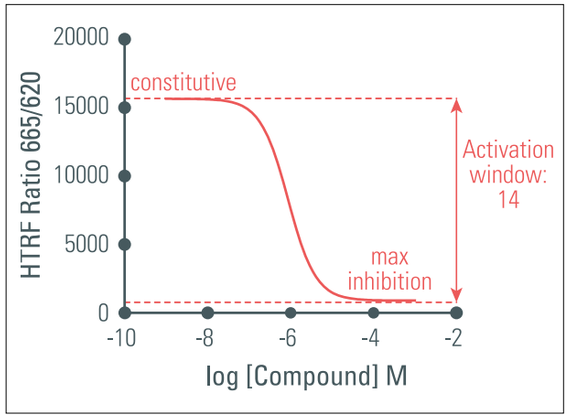
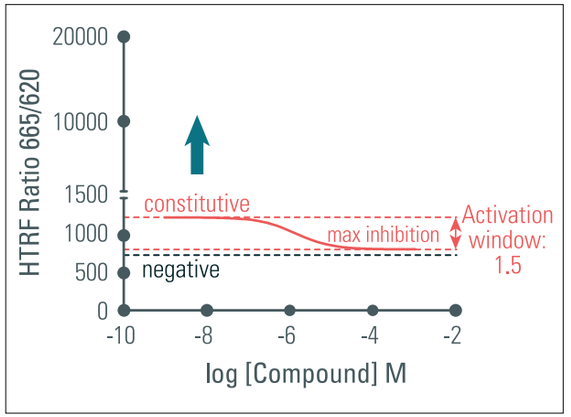
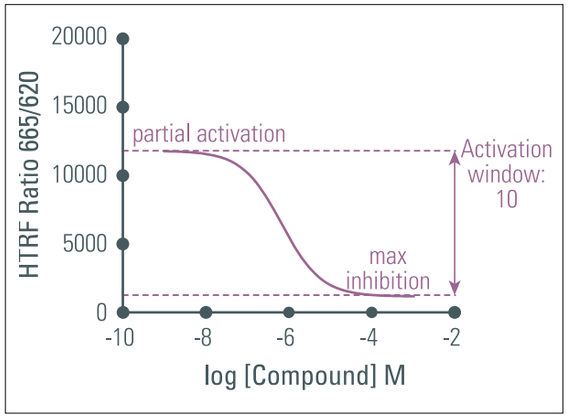
CASE STUDIES
Inhibition experiments







High or medium constitutive level

- The constitutive level of the phospho- or total protein of interest is already sufficient to work with.
- The compound induces a good dose-dependent decrease of the signal, giving a comfortable inhibition window (> 3).

This is the best situation to study the effect of inhibitor compounds.

There is no need to stimulate cells with an activator following treatment with the inhibitor. No optimization is required.

Low or no constitutive level

- The constitutive phospho- or total level is undetectable or very low (1 < S/N < 2).
- Inhibitor compounds cannot be identified/ characterized, or the inhibition window is too low (< 2) to correctly interpret the effect of the molecule.

This kind of data doesn't mean that the HTRF assay doesn't work. The biological model used in these conditions is just not suitable for studying inhibitor drugs.

The first option is to increase the constitutive level by optimizing the cell density (most of the time by increasing the number of cells).

The second option is to choose another cell line model in which the constitutive level of the protein is higher.

The third option is to stimulate cells with an activator of the pathway of interest (using a dose between the EC_{50} and EC_{80} values) after treatment with the inhibitor. It enables the study of the effect of inhibitors on the partially activated protein.

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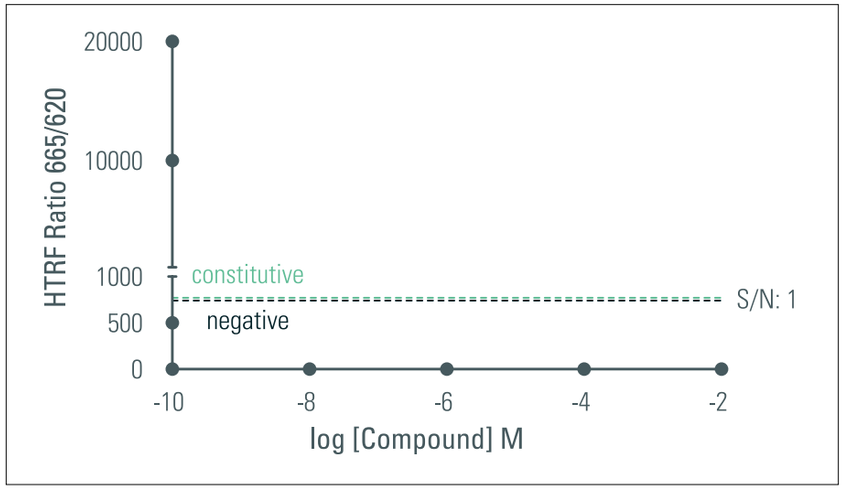
CASE STUDIES
No signal detection



- The constitutive level of the phospho- or total protein of interest is undetectable (S/N = 1).
- The stimulation of cells with activator compounds does not induce any significant increase in the HTRF signal.

Such results do not mean that the HTRF assay doesn't work. Different reasons can explain negative results:

1. *The signaling pathway being investigated is not functional due to a poor cell viability or an inappropriate cell density.*
   - Check cell health under a microscope before any experiment and optimize the cell density for each assay.
2. *The incubation time with compounds or the concentrations tested are not optimal, or the compounds are not bioactive.*
   - Perform a time-course study and dose-response experiments for each compound. Include a reference compound already described in the literature with a well-known effect on the protein.
3. *The biological model selected is not appropriate to study the protein of interest.*
   - Test another cellular model already described in the literature.
4. *The HTRF assay is not compatible with the species of the cellular model used.*
   - Check species compatibility.

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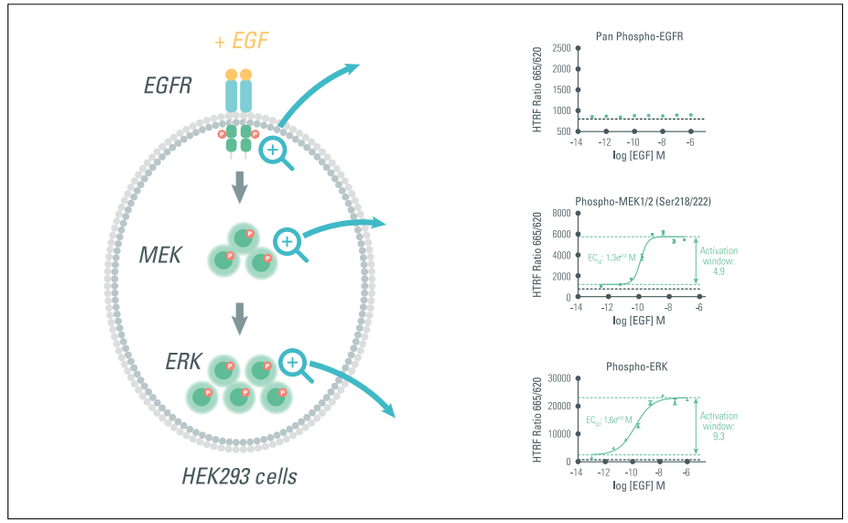
CASE STUDIES
No signal detection

If the phospho- or total protein of interest is still not detected using the HTRF assay, it is important to test the same samples using another method (e.g. Western Blot).
If it is still not detected, this means that the expression or phosphorylation level of the protein of interest in the conditions tested is too low and thus undetectable.

If the protein initially selected is used as a readout of a signaling pathway of interest, another alternative is to analyze a downstream protein in the same cascade.

Because the initial signal is amplified throughout the transduction pathway, it is likely that downstream readouts will be more easily detected.

On the left is a representative example of signal amplification in the EGFR/MEK/ERK cascade: following EGF treatment, the phosphorylation of the cell surface receptor EGFR is undetectable, while the phosphorylation of downstream kinases MEK and ERK are well detected with similar EC_{50} values. The best activation window is obtained with the phospho-ERK assay, since this protein is the most downstream substrate in the cascade.



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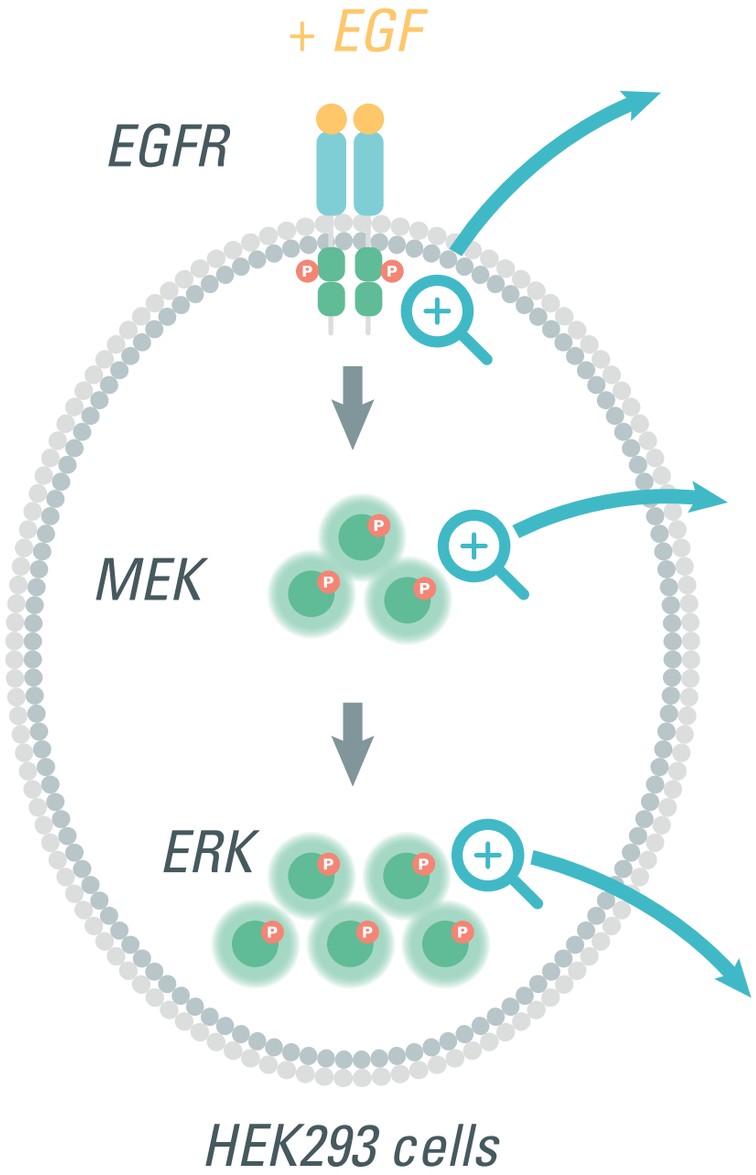

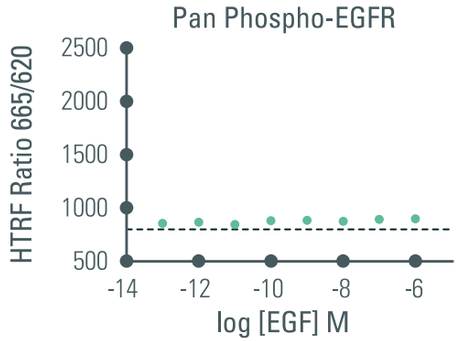

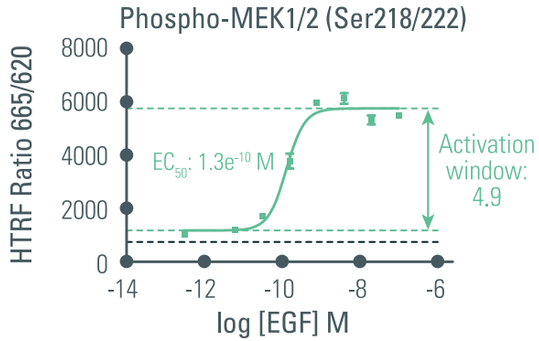

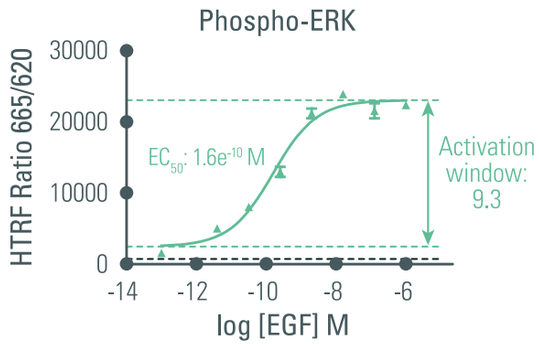

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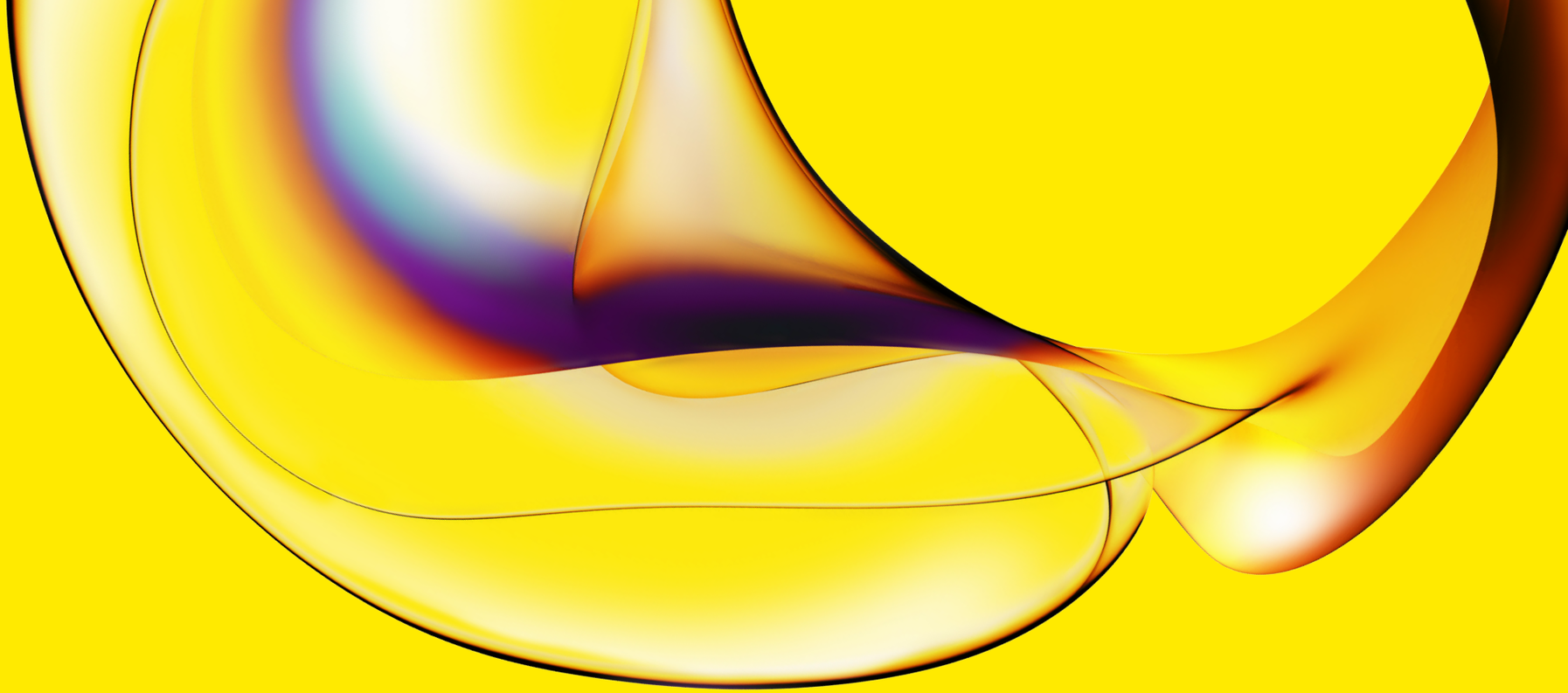

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