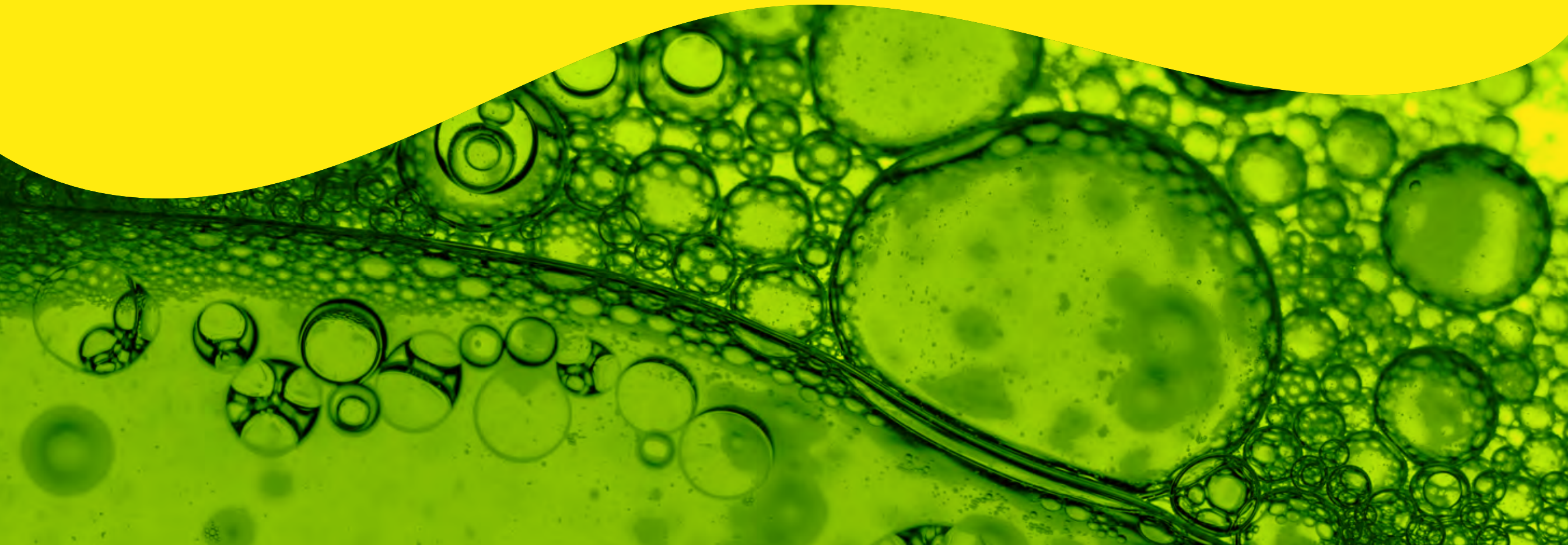


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cAMP

Guide to optimizing antagonists of $G\alpha_i/o$



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 GPCR signaling, the most suitable assay conditions are dependent on the GPCR coupling ($G\alpha_s$ or $G\alpha_i$) as well as the pharmacological characteristics of the compound being studied (agonist or antagonist). The optimal assay conditions will also hinge on the particular cell line used for the assay. 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 different manuals provide the GPCR community with the most up to date guidelines for the optimization of $G\alpha_s$ and $G\alpha_i/o$ GPCR assays.

Background

Cyclic AMP (cyclic adenosine 3',5'-monophosphate, or simply cAMP) is one of the most important GPCR intracellular mediators of $G\alpha_i$ coupled GPCRs. Agonists of $G\alpha_i$ negatively regulate of adenylate cyclase, decreasing cAMP production. Antagonists of $G\alpha_i$ release that negatively regulation.

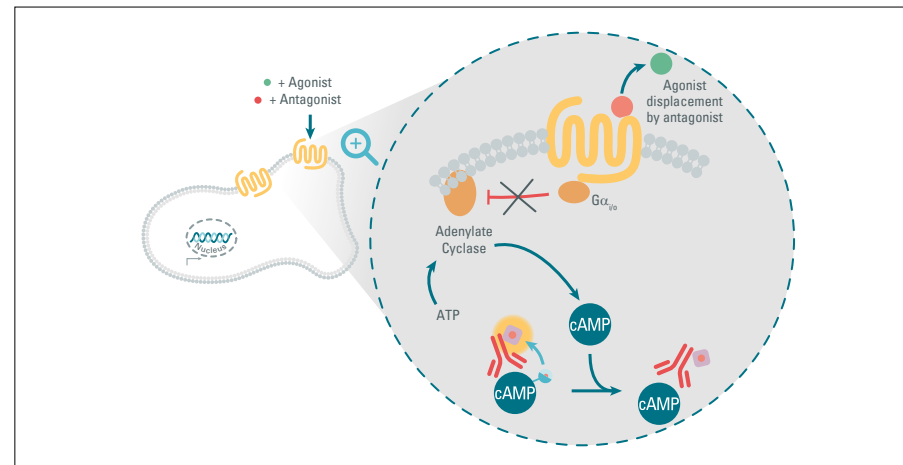
To counteract the degradation of cAMP to AMP by the action of phosphodiesterase (PDE) enzymes, a PDE pan-inhibitor (IBMX) is recommended to ensure proper cAMP accumulation and its subsequent measurement.

The cAMP kits developed by Revvity Bioassays are specifically intended for the direct quantitative determination of cyclic AMP. All kits are built on the same exact format.

The HTRF cAMP G_i assay is based on a competition between native cAMP produced by cells and cAMP labeled with the cryptate for binding to a d2 labeled antibody. The specific signal (i.e. energy transfer signal) is inversely proportional to the concentration of cAMP in standard or sample.

Three kits are available to better serve your needs when quantifying cAMP accumulation in response to G_i coupled GPCR activation:

cAMP- G_i Kits	
1,000 tests	# 62AM9PEB
20,000 tests	# 62AM9PEC
100,000 tests	# 62AM9PEJ



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G_i antagonist assay optimization

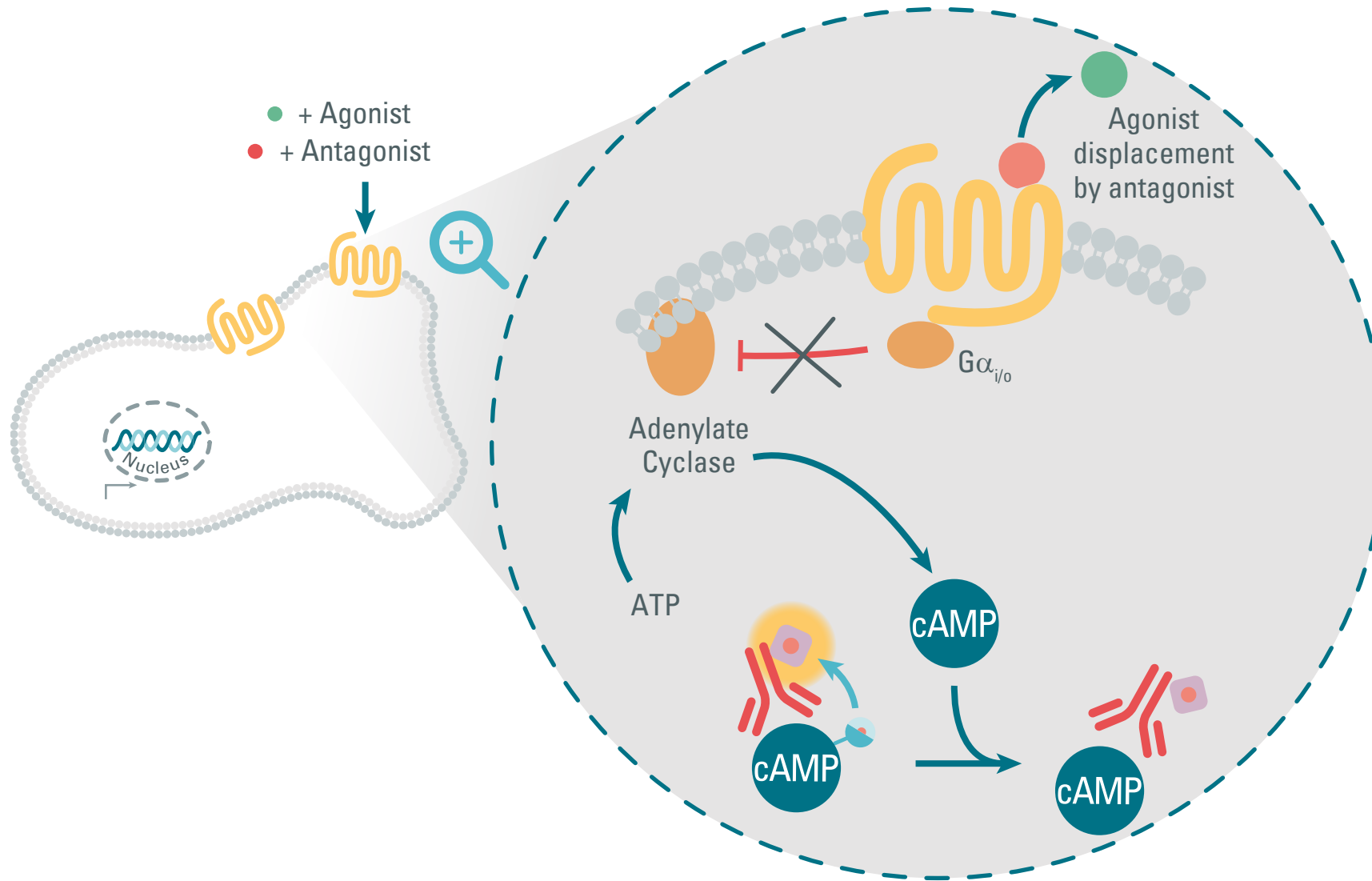
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Gi/o pathway A Gi antagonist prevents the negative regulation of adenylate cyclase by an agonist. In the presence of a Gi antagonist, Forskolin is free to positively stimulate adenylate cyclase, resulting in an increase in cAMP production.

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REAGENTS AND OPTIMIZATION WORKFLOW

Reagents

Reagent preparation

Phosphodiesterase inhibitors

PDEs are represented by a large super-family of enzymes whose role is to hydrolyze cAMP into AMP, and cGMP into GMP. It should be emphasized that while not all PDEs lead to the specific hydrolysis of cAMP, the non-selective PDE inhibitor IBMX is often the preferred chemical used in most cAMP accumulation assays. The use of a Pan-PDE inhibitor ensures that the hydrolysis effect of most PDEs will be inhibited, which in turn guarantees high levels of cAMP accumulation in the cell.

IBMX stock solution is offered as spare reagents in DMSO at a concentration of 500 nM (40 µl size Cat # 62AMXADA / 500 µl size Cat # 62AMXADC).

Ideally, the IBMX concentration to be used in the assay should be optimized, and a procedure for this optimization is provided in the Phosphodiesterase inhibitor section of the manual (Page 27).

Alternatively, a recommended concentration of 0.5 mM in the stimulation buffer can be selected, and should be enough to study cAMP accumulation in most cell types.

Choice of stimulation buffer

Stimulation buffer included in the kits is used to prepare the cells and all the chemicals and compounds needed in the assay. Note that Revvity's cAMP kits are also compatible with all suitable nutrient media (DMEM, HAM-F12, HBSS, note that PBS is not recommended) and any of them can be used in the assay as an alternative to the supplied stimulation buffer.

The stimulation buffer should be supplemented with IBMX, as previously described.

Example: To prepare 10 mL of complete stimulation buffer, add 10 µl of IBMX stock solution at 500 mM to 9.990 ml of buffer or medium. Adding the IBMX just before use leads to improved assay performance.

Standards

The standard vial contains a known concentration of cAMP. Further dilutions of this standard can be used to establish a standard curve. Using the standard curve, the fluorescence units from the assay can be converted into concentrations of cAMP. It is recommended to use the same stimulation as that used for the cell based assay to quantify cAMP released by the cells.

Example: If DMEM + 0.5 mM IBMX were used to prepare the cells, then the same solution should also be used to reconstitute the standard and then dilute it.

Adenylyl cyclase activator

Forskolin activates the adenylyl cyclase enzyme and increases the intracellular level of cAMP. It is used as a positive control for Gs applications (biological models validation, maximal intracellular cAMP modulation determination). It is highly used for Gi coupled receptor study as a pre-activation step to reveal a cAMP inhibition upon cell stimulation.

The forskolin stock solution is provided in DMSO at 10 mM (40 µl size Cat # 62AMYADA / 800 µl size Cat # 62AMYADC).

For reconstitution, refer to the package insert of the kit.

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REAGENTS AND OPTIMIZATION WORKFLOW

Optimization workflow

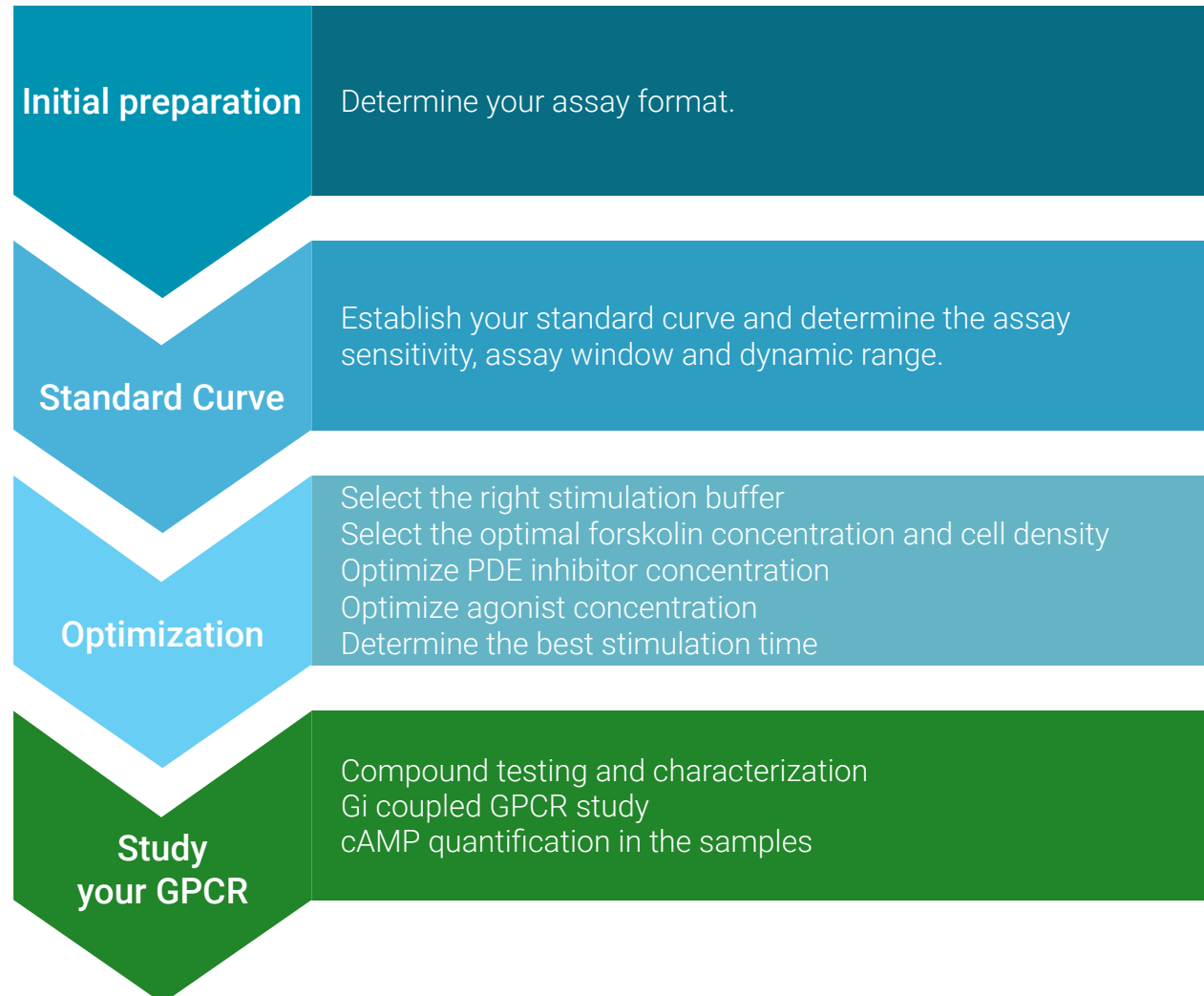


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CELL PREPARATION

Before you begin

Cryopreserved cells have already been successfully employed in a number of IP1 studies using Revvity reagents. Advances in cell culture technologies have increasingly enabled researchers to rely on freshly cultured and collected cells. Cryopreserved vs freshly maintained cells will be discussed in this manual.

The question of adherent cells versus suspension cells is one of the first decisions a researcher must make when optimizing an IP1 assay.

- Cells presenting an adherent phenotype are typically cultured in plates overnight prior to being assayed.
- Cells with a non-adherent phenotype are typically dispensed and tested in suspension immediately following plating.

Your decision regarding adherent cells versus suspension cells should be made on the basis of biology expectations and ease of assaying. It is possible to assay either suspension cells or adherent cells, and some guidelines for both protocols are provided below.

Finally, the IP-One assay can be performed in :

- 96-well plates
- HTRF 96-well Iv plate (ref 66PL96001)
- 96-well half area plates
- 384-well plates
- 384-well plates (low volume plates)

The choice of plate format will depend on the preferred protocol, desired throughput, and considerations of reagent consumption.

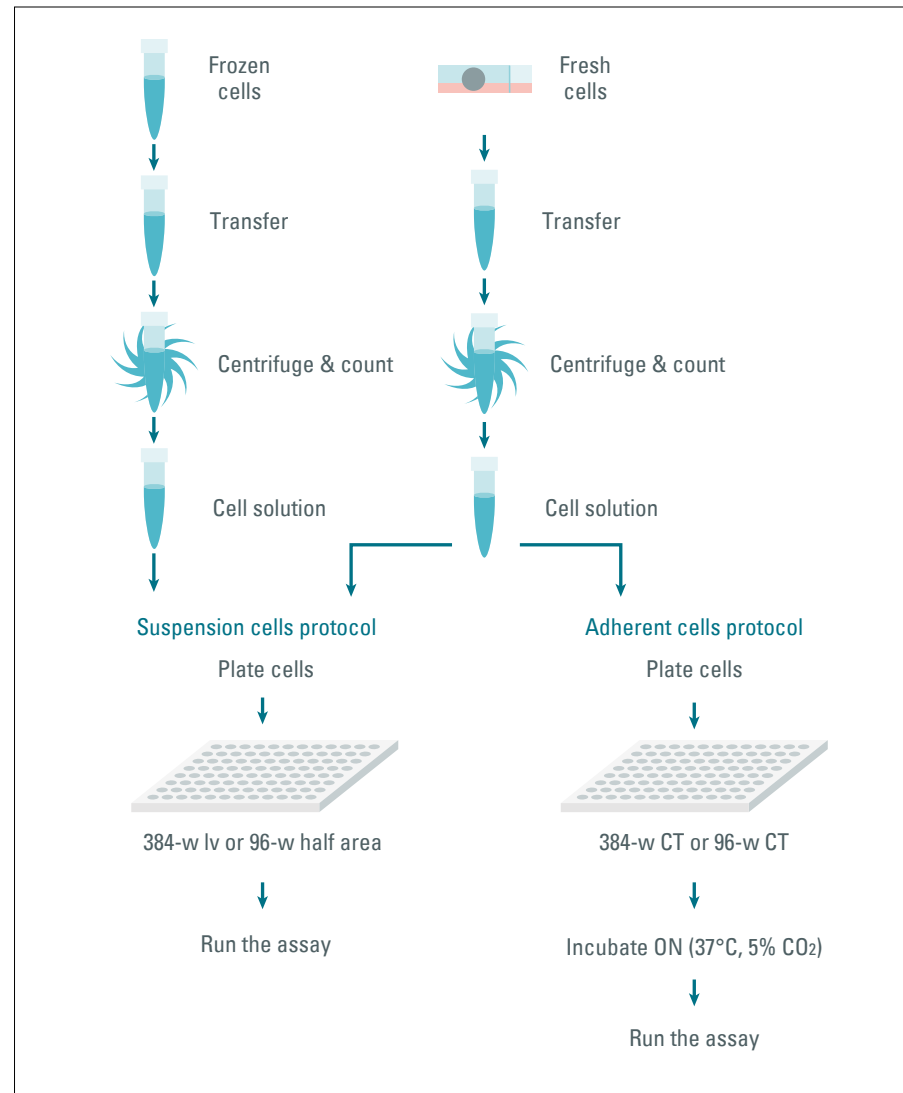


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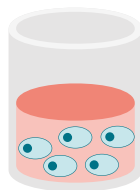
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CELL PREPARATION

Freshly harvested cells

Suspension protocol (for biologically adherent cells)

- Cells from T175 cm² flasks should be near 80% confluency before collection.
- Remove culture medium and gently rinse flask using PBS.
- Add cell dissociating solution (5 ml for a T175 cm² flask), incubate the plate at 37 °C and 5% CO₂ for 5–10 min or until cells are dislodged.
- Add 5 ml of PBS to a final volume of 10 mL and pipette up and down until cells are homogeneously dispersed in the solution.
- Spin the cell suspension at 340 g for 3 min.
- Calculate the volume of stimulation buffer needed to re-suspend the cell pellet, achieving an optimal cell density.
- Always use the stimulation buffer, as previously discussed, when re-suspending the cells. Cell density should be optimized. Refer to the later sections of this manual for recommendations regarding cell density optimization.
- The cells are ready to be dispensed into the assay plate. Refer to table on next page for recommended dispensing volumes.
- Run the assay.



Adherent protocol

- Cells from T175 cm² flasks should be near 80% confluency before collection.
- Remove culture medium and gently rinse flask using PBS.
- Add cell dissociating solution (5 ml for a T175 cm² flask), incubate the plate at 37 °C and 5% CO₂ for 5–10 min or until cells are dislodged.
- Add 5 ml of PBS to a final volume of 10 mL and pipette up and down until cells are homogeneously dispersed in solution.
- Spin the cell suspension at 340 g for 3 min.
- Calculate the volume of complete growth medium needed to re-suspend the cell pellet, achieving an optimal cell density.
- Cell density should be optimized. Refer to the later sections of this manual for recommendations regarding cell density optimization.
- The use of cell-culture treated well-plates or Poly-d-Lysine coated plates is preferable.
- The cells are ready to be dispensed into the assay plate. Refer to table on next page for recommended dispensing volumes.
- Incubate overnight at 37°C, 5% CO₂.
- Flip the plate to remove the cell supernatant.
- Run the assay.



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CELL PREPARATION

Cryopreserved cells

- Thaw frozen cells rapidly in a water bath at 37°C until completely thawed.
- Transfer cells into a vial and add 10 ml PBS.
- Centrifuge 3 min at 340 rcf (g).
- Discard supernatant and re-suspend the pellet in stimulation buffer.
- Determine cell concentration and viability.
- Dilute the cells in the stimulation buffer to reach the cell concentration required for the experiment.
- Dispense the cells into the plates (refer to the table below under “suspension protocol” for the recommended volumes).
- Run the assay.



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CELL PREPARATION

Plate formats and volumes

Dispensing volumes for each step of each protocol are summarized in the table below.

		 ADHERENT CELLS PROTOCOL			 SUSPENSION CELLS PROTOCOL			
CELL BASED ASSAY OR STANDARD CURVE		384-w CT white	96-w CT white	1536-w white	384-w lv white	HTRF 96- w lv plate	96-w half area white	
	Plate							
Stimulation step	Cells or standard (4X)	10 µl*	50 µl*	2.5 µl	5 µl	5 µl	25 µl	
	Cell incubation step	Overnight at 37°C, 5% CO ₂			No cell incubation			
	Compounds (2.5X) or Stimulation buffer	8 µl	40 µl	2 µl	4 µl	4 µl	20 µl	
	Agonist incubation step	Necessary time at 37°C			Necessary time at 37°C			
	Forskolin (10X)	2 µL	10 µL	0.5 µl	1 µl	1 µl	5 µl	
	Forskolin incubation step	Necessary time at 37°C,			Necessary time at 37°C			

*If the cell supernatant is removed, add stimulation buffer in the recommended volume.

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G α Q AGONIST ASSAY OPTIMIZATION

Dispensing formats

Adherent and suspension protocols have different dispensing volume requirements. A user who has opted for an adherent protocol removes the cell-culture media from the plate before proceeding to cell stimulation, and essentially starts the day with a liquid-free plate. On the other hand, a user who has selected a suspension protocol starts the day with cells in suspension in a volume of liquid. The concentration of agonist to be used in the assay must be optimized first, in the absence of antagonist. The first dispensing step is a buffer

addition that mimics the antagonist addition. In later stages when the agonist concentration has been properly optimized, the buffer will be replaced with the antagonist. Untreated cells, where the agonist is substituted for by an equivalent volume of buffer, are a useful control. From that control, the basal levels of cAMP production can be assessed. For each protocol, the recommended dispensing volumes must be reviewed.

Suspension protocol

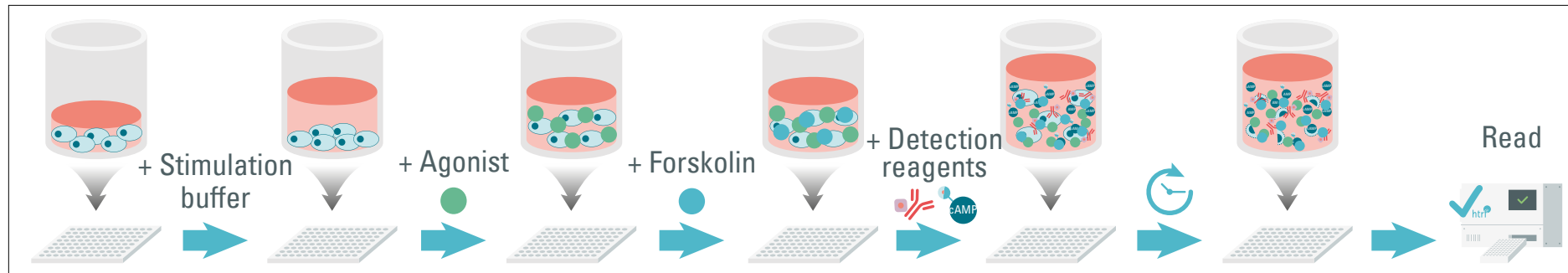


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G α Q AGONIST ASSAY OPTIMIZATION

Dispensing formats

CELL BASED ASSAY OR STANDARD CURVE		SUSPENSION CELLS PROTOCOL			
	Plate	1536-w white	384-w lv white	HTRF 96-w lv white	96-w Half area white
Stimulation step	Cells or standard (4X)	2.5 μ l	5 μ l	5 μ l	25 μ l
	Stimulation Buffer	1 μ l	2 μ l	2 μ l	10 μ l
	Incubation step	Necessary time at 37°C			
	Agonist (5 X) or stimulation buffer	1 μ l	2 μ l	2 μ l	10 μ l
	Incubate agonist with cells	Necessary time at 37°C			
	Forskolin (10X)	0.5 μ l	1 μ l	1 μ l	5 μ l
	Incubate forskolin with cells	Necessary time at 37°C (empirical incubation is about 45min)			
Detection step	cAMP-Cryptate	2.5 μ l	5 μ l	5 μ l	25 μ l
	Anti-cAMP-d2	2.5 μ l	5 μ l	5 μ l	25 μ l
Final assay volume		10 μl	20 μl	20 μl	100 μl

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Adherent protocol

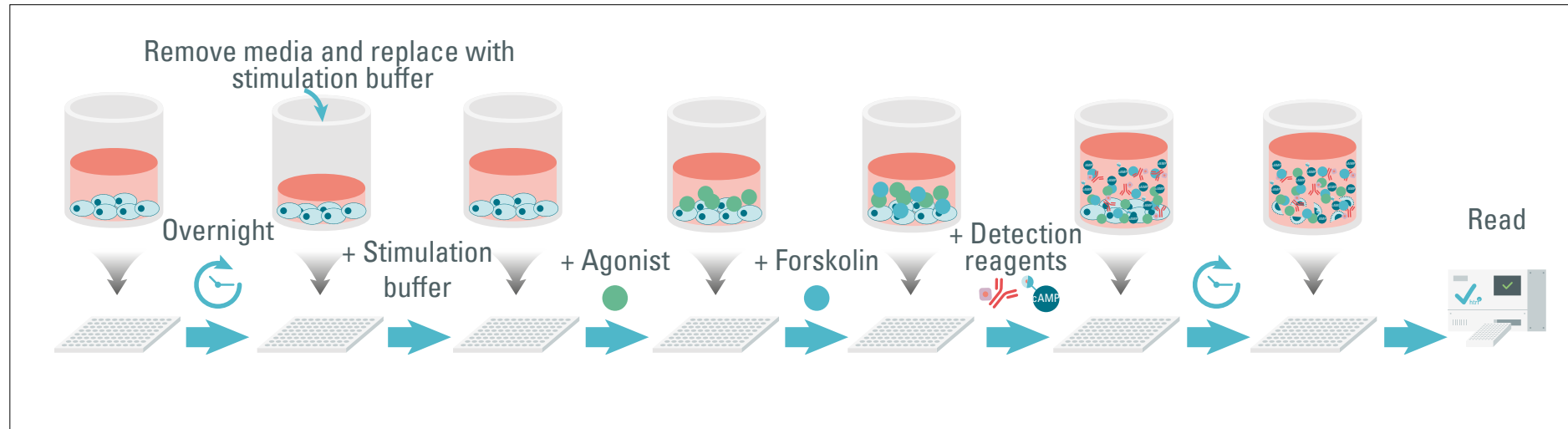


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G α Q AGONIST ASSAY OPTIMIZATION

Dispensing formats

CELL BASED ASSAY OR STANDARD CURVE		ADHERENT CELLS PROTOCOL	
	Plate	384-w CT white	96-w CT white
Stimulation step	Cells or standard (4X)	10 μ l	50 μ l
	Cell incubation step	overnight at 37°C, 5% CO ₂	
	Stimulation buffer	4 μ l	20 μ l
	Incubation (mimics antagonist incubation time)	Necessary time at 37°C	
	Agonist (5X) or Stimulation buffer	4 μ l	20 μ l
	Incubate agonist with cells	Necessary time at 37°C	
	Forskolin (10X)	2 μ l	10 μ l
	Incubate forskolin with cells	Necessary time at 37°C (Empirical: 45min)	
Detection step	cAMP-Cryptate	10 μ l	50 μ l
	Anti-cAMP-d2	10 μ l	50 μ l
Final assay volume		40 μl	200 μl

Choosing between the adherent or suspension protocols depends on the biological model and the receptor studied. The adherent protocol could be more physiologically relevant in some cases, while the suspension protocol is more rapid and miniaturizable. It is possible to run a simple side by side experiment with your internal cell model on a reference agonist to test the two protocols, and then choose the protocol which is best adapted to your experiments.

Hereafter is a side by side experiment comparing the suspension and adherent protocols. In this experiment, CHO cells stably expressing the Gi coupled receptor Delta Opioid are stimulated with the agonist SNC-162. Different protocols may lead to differences in maximal efficacy while potency remains the same.

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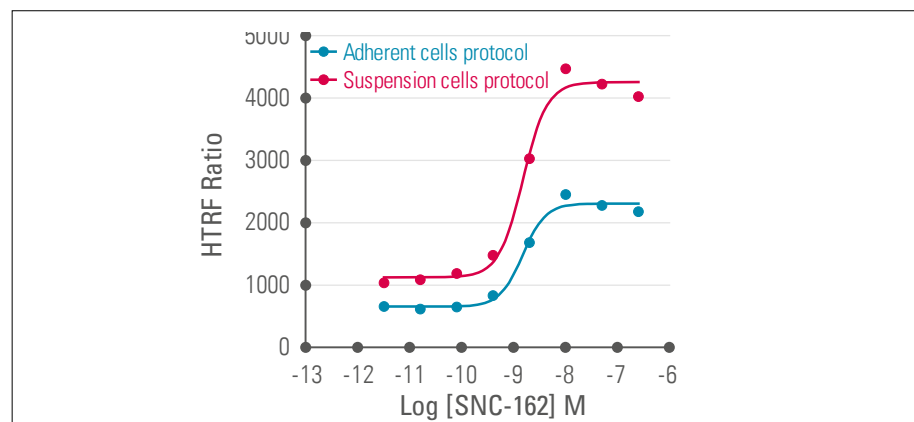
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G α Q AGONIST ASSAY OPTIMIZATION

Dispensing formats

The following table describes the main protocols used for the experiment:

	CELL BASED ASSAY	ADHERENT CELLS PROTOCOL	SUSPENSION CELLS PROTOCOL
	Plate	384-w CT white	384-w Iv white
	Cells	10 μ l	5 μ l
	Incubation	Overnight at 37°C, 5% CO ₂	No incubation
	Stimulation buffer	4 μ l	2 μ l
Stimulation step	Incubation (mimics antagonist incubation time)	45 min at 37°C	
	SNC-162	4 μ l	2 μ l
	Incubate agonist	45 min at 37°C	
	Forskolin	2 μ l	1 μ l
	Incubate Forskolin	45 min at 37°C	
Detection step	cAMP-Cryptate	10 μ l	5 μ l
	Anti-cAMP-d2	10 μ l	5 μ l
Final assay volume		40 μl	20 μl



Type of protocol	Adherent	Suspension
S/B	3.5	4.0
EC ₅₀ SNC-162 (nM)	1.5	1.5

Side by side comparison between suspension cell protocol and adherent cells protocol: CHO cells stably expressing the Delta opioid receptor (DOR) were dispensed at a density of 6,000 cells /well. Serial dilutions of SNC-162 were added and incubated for 45 min. Forskolin was added and incubated for 45 min. The HTRF® Ratio data was fitted to a sigmoidal dose response and SNC-162 potencies extracted.

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GαQ AGONIST ASSAY OPTIMIZATION

Standard curve

The standard curve will be extremely useful in assessing the assay sensitivity (IC_{50}), the maximum assay window, and the assay dynamic range (IC_{10} - IC_{90})

Establishing the standard curve

Refer to the most recent package insert accompanying your test kit for complete instructions on standard reconstitution and dilutions. In all cases, the stimulation buffer must be used to reconstitute the standard and dilute it.

Curve analysis

The curve is analyzed by plotting the Ratio as a function of the [cAMP] of the different standards expressed as a logarithm. Graphpad Prism can be used to analyze the curve. The preferred fit is the log(inhibitor) vs. response -- Variable slope. Several parameters can be derived from the fit:

Maximum assay window & IC_{50}

The standard curve assay window is obtained by dividing the Ratio obtained in the absence of cAMP by the Ratio obtained by testing the highest concentrated cAMP standard. It represents the highest achievable assay window it is possible to obtain from the system, and will always be higher than the assay window recorded when cells are added to the system.

$$\text{Assay window} = \frac{\text{Max HTRF}^{\circledR} \text{ Ratio (No cAMP Std)}}{\text{Min HTRF}^{\circledR} \text{ Ratio (Max cAMP Std)}}$$

The IC_{50} value is the concentration of cAMP at which 50% of the initial signal is lost. In other words at IC_{50} , the concentration of cAMP present in the assay is such that half the initial FRET produced by the cAMP-Cryptate/Anti-cAMP-d2 has been competed off. The IC_{50} is a measurement of how efficient and responsive the system is at recording changes in cAMP concentrations.

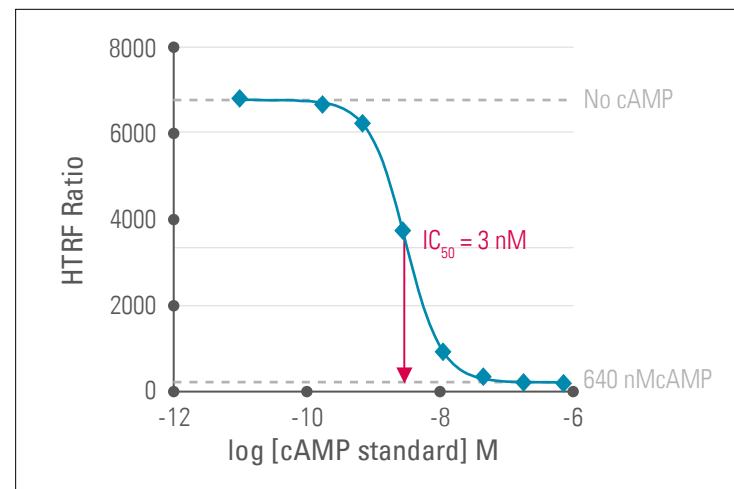
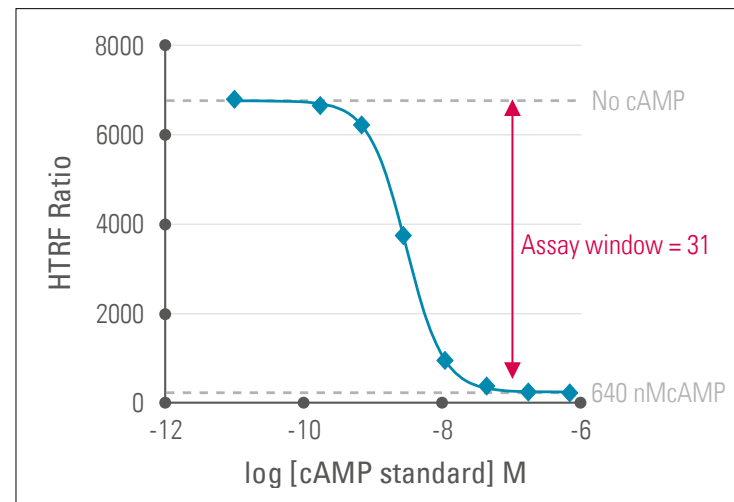


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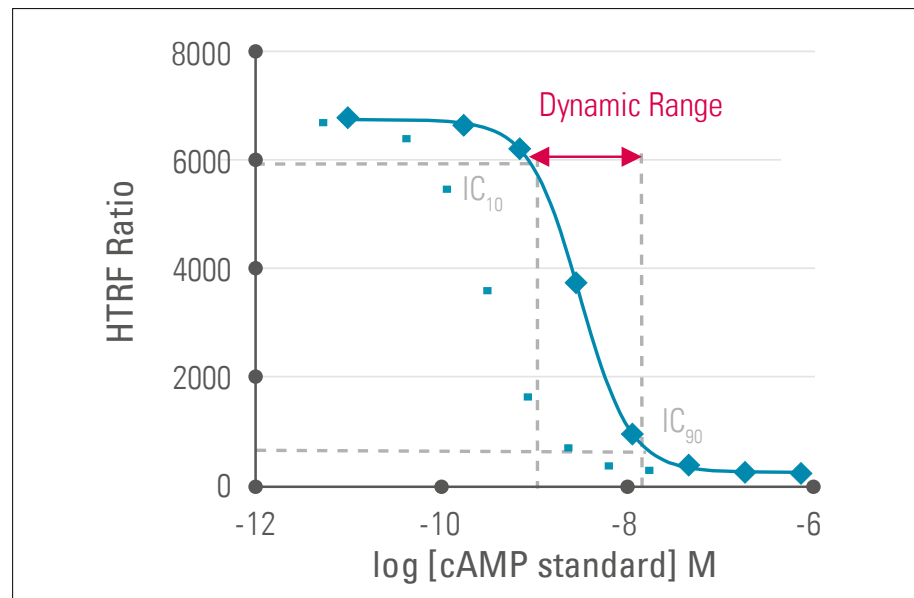
G α Q AGONIST ASSAY OPTIMIZATION

Standard curve

Dynamic range

The IC_{10} - IC_{90} determines the assay dynamic range. This range plays a central role in assay optimization. As will be explained later in this manual, it is of primary importance that the signal recorded when assaying cells lies within the assay linear range of the standard curve. Failure to restrict Ratio values to the assay dynamic range will result in significant potency inaccuracies when assaying compounds on cells.

Using Graphpad Prism, determine the standard curve IC_{10} and IC_{90} values, and record the corresponding Ratio and [cAMP].



The assay dynamic range indicates the concentration of cAMP the assay can accurately quantify. Samples with cAMP concentrations above IC_{90} or below IC_{10} cannot be reliably estimated. Similarly, samples with Ratio values below IC_{90} and above IC_{10} cannot be reliably quantified.

LINEAR RANGE	HTRF [®] RATIO	[cAMP] nM
IC_{10}	6124	0.76
IC_{90}	870	12.1

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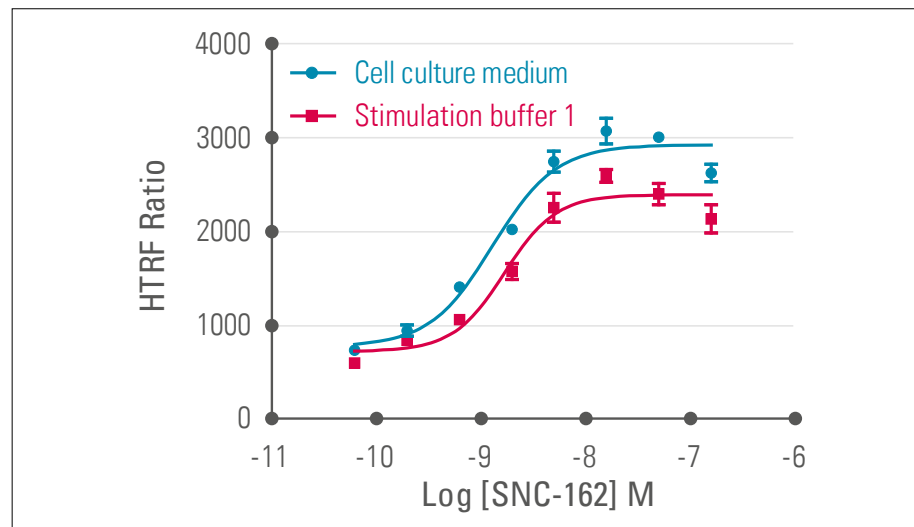
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G α Q AGONIST ASSAY OPTIMIZATION

Choice of stimulation buffer

It is recommended to use the stimulation buffer provided in the kit (stimulation buffer 1) supplemented with IBMX for stimulation below 2H. For stimulation times above 2H, use cell culture medium (i.e RPMI or DMEM,...) supplemented with IBMX. In fact, it is possible to run the assay in the cellular culture media without affecting the HTRF signal or detection.

The following data demonstrates that the results are the same in this model using Delta opioid receptor stably expressing CHO cells.



Stimulation buffer supplemented with IBMX: using either the stimulation buffer provided or cell culture medium such as F-12 is possible when running the cAMP assay. CHO cells stably expressing delta receptor were stimulated with the SNC-162 and then forskolin diluted in the stimulation buffer provided in the kit or F-12 stimulation buffer supplemented with 0.5mM of IBMX. The agonist stimulation step was stopped after 45 min by the addition of detection reagents.

	EC ₅₀ (nM)	S/B
Stimulation Buffer 1	1.7	4.0
Cell culture medium (F12 Medium)	1.4	4.1

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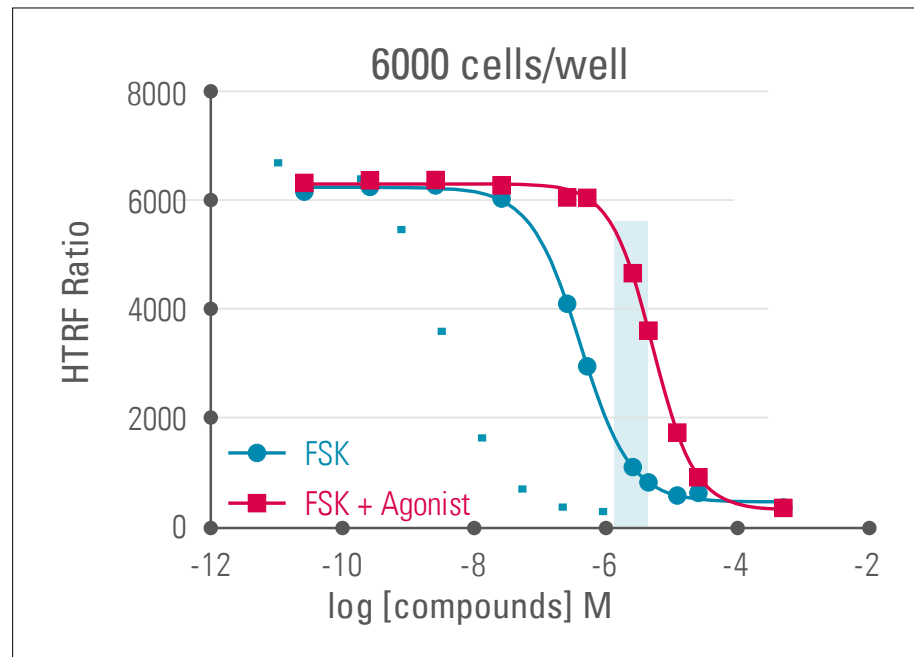
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G α Q AGONIST ASSAY OPTIMIZATION

Forskolin concentration optimization

The preferred optimization method is to assay the concentration-effect of a full agonist over a range of cell densities treated with increasing doses of forskolin (FSK).

A first step consists in optimizing the forskolin concentration and the associated optimal cell density. A simple way to optimize this is to do the experiment for each cellular density, assaying a full dose response of the forskolin alone (basal) or pretreated with a maximal dose of the agonist (treated cells). The Ratio treated cells / basal gives the maximal assay window which could be obtained for each condition.



Forskolin concentration optimization: CHO stably expressing Delta opioid receptor cells were treated with a full dose response of forskolin alone or pretreated with a maximal dose of SNC-162 (100 nM) for 45 min before forskolin addition. In both conditions forskolin incubation time was 45 min. This experiment was performed for 4 cellular densities from 2000 to 8000 cells / well. The results show the maximal assay window (Ratio treated cells / Ratio basal) obtained for each condition. The best compromise is 6000 cells/well treated with a dose of forskolin between 2.5 and 5 μ M.

[FSK] (μ M)	Assay window (Ratio treated cells / Ratio basal)			
	2 kcells/w	4 kcells/w	6 kcells/w	8 kcells/w
0	1	1	1	1
0.025	1	1	1	1.1
0.25	1.1	1.3	1.5	1.9
0.51	1.3	1.6	2	2.7
2.5	2.2	3.2	4.2	4.5
5	2.5	3.6	4.3	4.4
12	2.5	2.8	2.9	2.7
25	1.7	1.5	1.4	1.4
500	1.9	1	1	0.9

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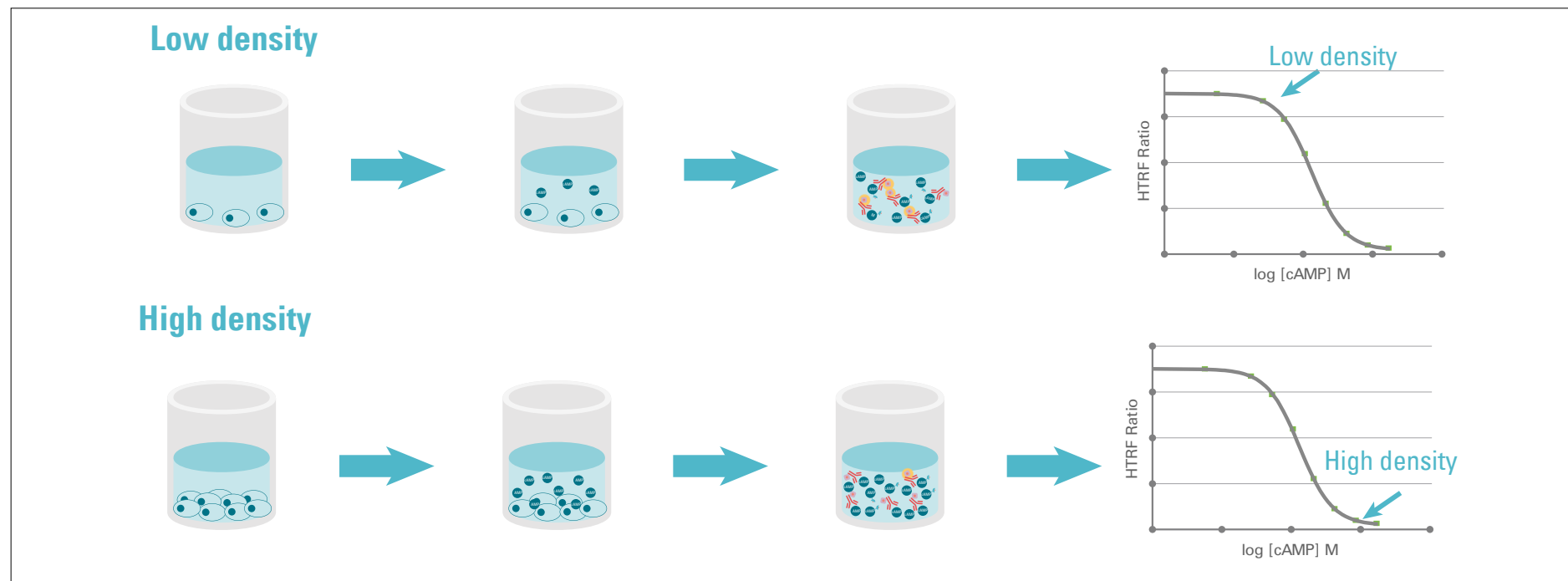
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G α Q AGONIST ASSAY OPTIMIZATION

Cell density

Optimizing the cell density is the key to obtaining the best possible results. Too low a cell density may not result in enough cAMP production for the assay to record (after treatment with forskolin). Too high a cell density may give a dramatical decrease in the assay window.



The assay performance must be evaluated for a range of cell densities. The preferred optimization method is to assay the concentration-effect of a full agonist over a range of cell densities. Note that a full dose response is preferred to assaying a single concentration of agonist at this point.

Organizing the standard curve graph and the cell density graph side by side reveals at a glance which cell densities are within the assay range. Cell densities "in range" are those for which the reference agonist dose response lies within IC_{90} - IC_{10} of the standard curve.

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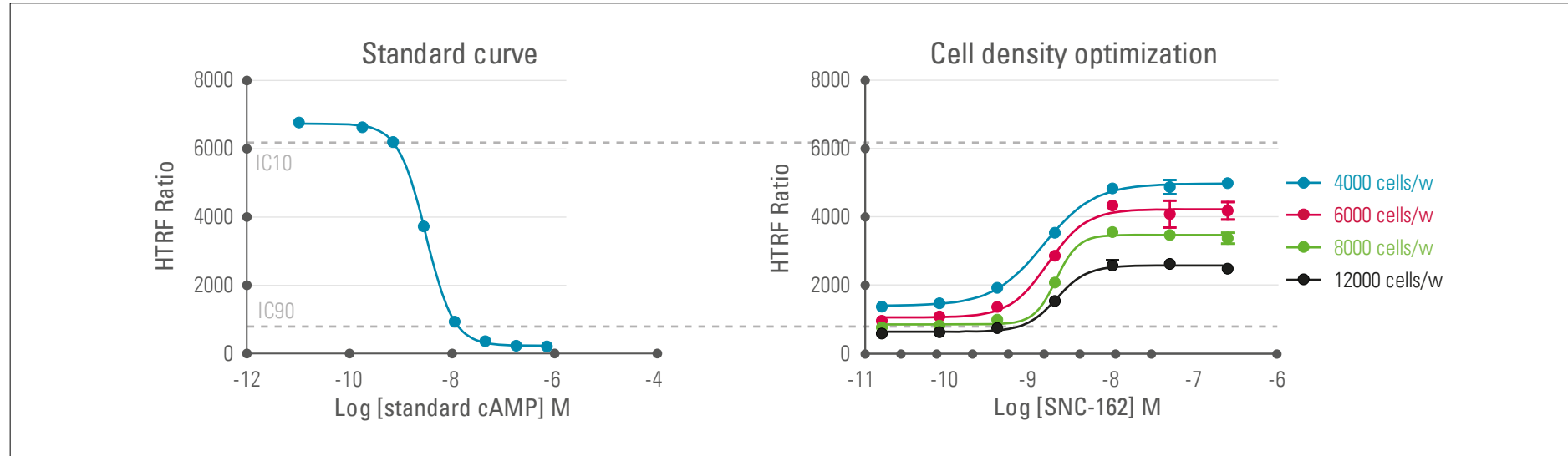
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GαQ AGONIST ASSAY OPTIMIZATION

Cell density



CELLS	4,000 CELLS/W	6,000 CELLS/W	8,000 CELLS/W	12,000 CELLS/W
Assay window	3.7	4.5	4.4	4.3
EC ₅₀ SNC-162 (nM)	1.4	1.6	2.0	2.0

Dose response curves for CHO cells stably expressing the delta opioid receptor (DOR) treated with SNC-162, a reference agonist. 5 µl of cells were dispensed into a 384-lv white plate and treated 45 min at 37°C with the agonist and then stimulated with 5µM of forskolin (5µM) for 45 min at 37°C. 5 µl of each detection reagent were then added for detection. Reading was done on a

Pherastar lamp reader after 1H incubation time at 22°C. A cell density of 6,000 cells per well was optimal. This density maximizes the assay window while ensuring the signal remains within the standard curve dynamic range. The highlighted cell densities in the table (in blue) represent the cell densities in which the quantification of cAMP is out of the dynamic range of the assay.

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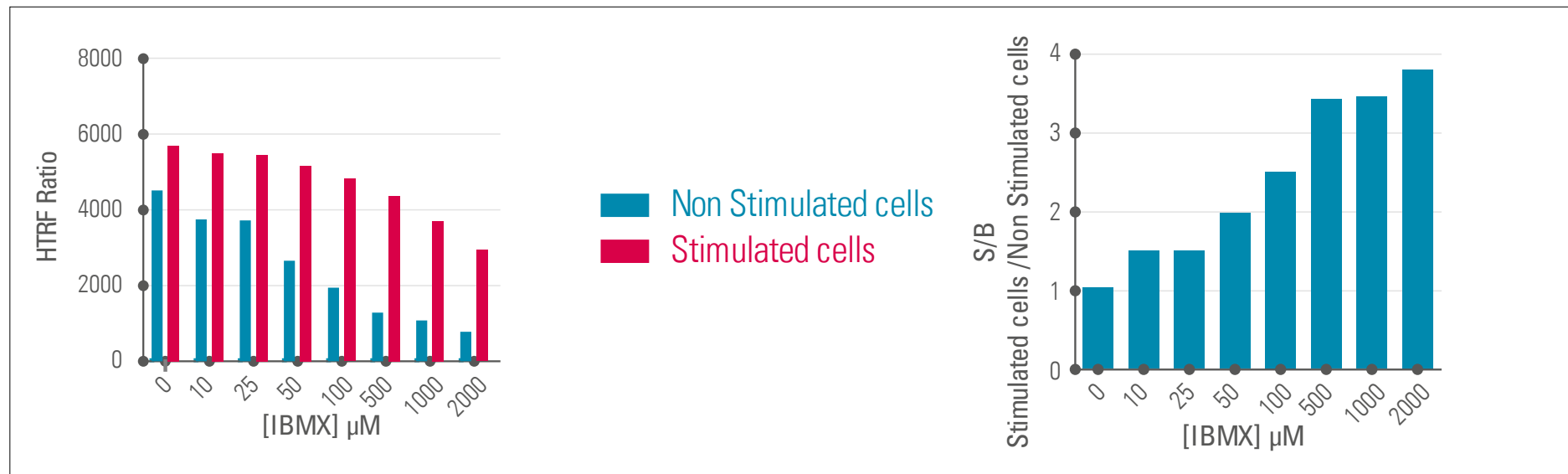
G α Q AGONIST ASSAY OPTIMIZATION

Phosphodiesterase (PDE) inhibitors

IBMX is by far the most widely-used non-specific inhibitor of cAMP and cGMP phosphodiesterases with EC₅₀ in the range of 7 to 50 μ M. By inhibiting PDEs, IBMX guarantees an accumulation of cAMP.

Alternatively, an empirical concentration of 0.5 mM in the stimulation buffer can be selected and should be enough to study cAMP accumulation in most cell types. If necessary, the IBMX concentration to be used in the assay could be further optimized.

A typical optimization step consists in preparing different stimulation buffers with increasing IBMX concentrations and assaying the cells using a known reference agonist. The optimal IBMX concentration is that for which the assay window has been maximized.



PDE inhibitor concentration optimization: Several different concentrations of IBMX were added to the stimulation buffer. CHO cells stably expressing delta opioid receptor were treated by the delta opioid agonist SNC-162 at 200 nM

and dispensed at 6000 cells/well into a 384-lv white plate. 500 μ M is the optimal IBMX concentration because it both maximizes the assay signal and ensures a robust accumulation of cAMP in the assay.

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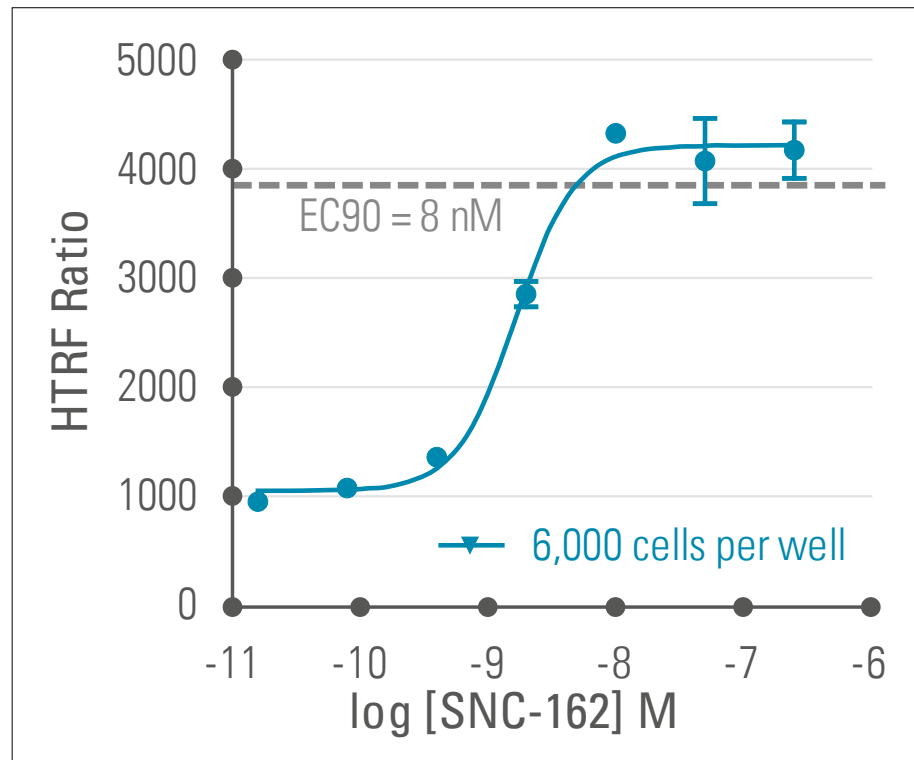
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GαQ AGONIST ASSAY OPTIMIZATION

Optimized condition for antagonist testing

In our DOR cell line case study, the optimal cell density is 6,000 cells per well. This cell density maximizes assay window while producing cAMP quantities within the boundaries of the standard curve. The graph corresponding to the optimal density of 6,000 cells per well is shown here.

Next identify the agonist EC90. The EC90 is the concentration of agonist that leads to a 90% increase in signal. In this case, the EC90 of Isoproterenol is 8-10nM



Dose response curve for HEK293 cells stimulated with SNC-162, a delta opioid receptor and reference agonist: The agonist EC90 will be the concentration inhibiting 90% of the maximum cAMP response and the concentration of agonist to be tested in the presence of the antagonist.

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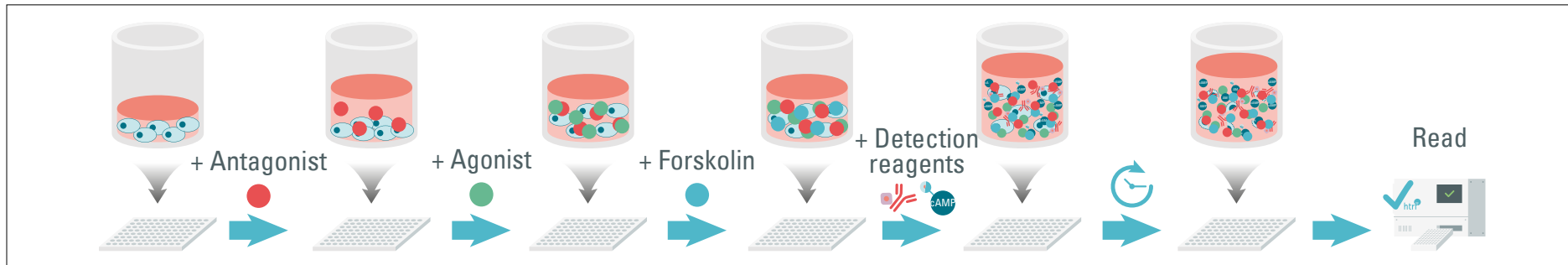
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GaQ ANTAGONIST ASSAY OPTIMIZATION

Dispensing formats

Antagonist dilutions are prepared 7x the desired final concentration and added before the agonist at EC₉₀. Untreated cells, where the antagonist is substituted for an equivalent volume of buffer, are a useful control. From the control, the level of IP1 upon stimulation with the agonist at EC₉₀ can be assessed.

Suspension protocol



CELL BASED ASSAY OR STANDARD CURVE		SUSPENSION CELLS PROTOCOL			
	Plate	1536-w white	384-w lv white	HTRF 96-w lv white	96-w Half area white
Stimulation step	Cells or standard (4X)	2.5 µl	5 µl	5 µl	25 µl
	Antagonist (5X) or Stim Buffer	1 µl	2 µl	2 µl	10 µl
	Incubate antagonist with cells	Necessary time at 37°C			
	Agonist @EC90 (5X)	1 µl	2 µl	2 µl	10 µl
	Incubate agonist with cells	Necessary time at 37°C			
	Forskolin (10X)	0.5 µl	1 µl	1 µl	5 µl
	Incubate forskolin with cells	Necessary time at 37°C (empirical incubation is about 45 min)			
Detection step	cAMP-cryptate	2.5 µl	5 µl	5 µl	25 µl
	Anti-cAMP-d2	2.5 µl	5 µl	5 µl	25 µl
Final assay volume		10 µl	20 µl	20 µl	100 µl

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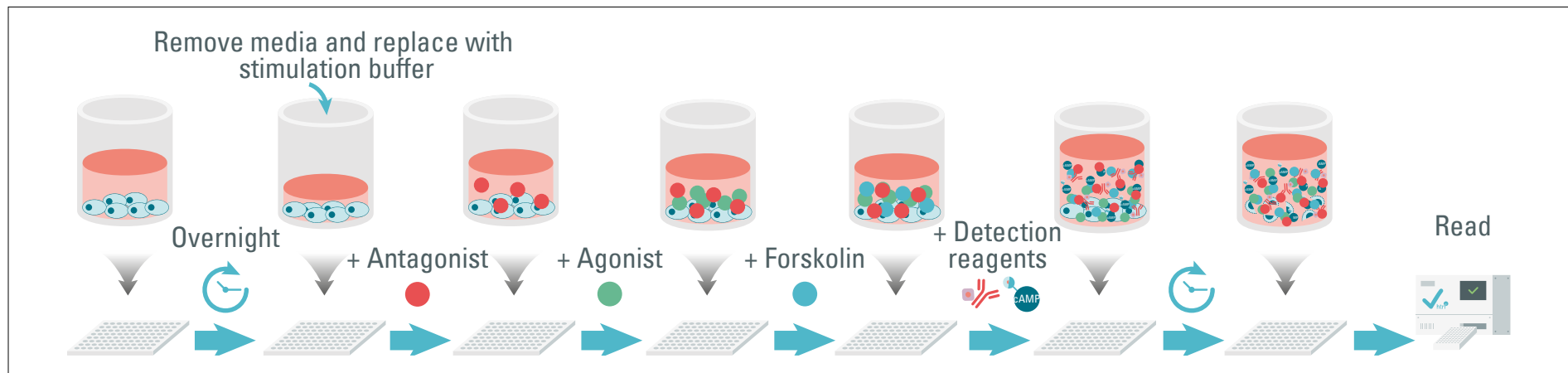
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GaQ ANTAGONIST ASSAY OPTIMIZATION

Dispensing formats

Adherent protocol



	CELL BASED ASSAY OR STANDARD CURVE	ADHERENT CELLS PROTOCOL	
	Plate	384-w CT white	96-w CT white
	Cells or standard (4X)	10 µl	50 µl
	Cell incubation step	overnight at 37°C, 5% CO ₂	
Stimulation step	Antagonist (5X) or Stimulation Buffer	4 µl	20 µl
	Incubate antagonist with cells	Necessary time at 37°C	
	Agonist @EC90 (5X)	4 µl	20 µl
	Incubate agonist with cells	Necessary time at 37°C	
	Forskolin (10X)	2 µl	10 µl
	Incubate forskolin with cells	Necessary time at 37°C	
Detection step	cAMP-cryptate	10 µl	50 µl
	Anti-cAMP-d2	10 µl	50 µl
	Final assay volume	40 µl	200 µl

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GaQ ANTAGONIST ASSAY OPTIMIZATION

Stimulation time

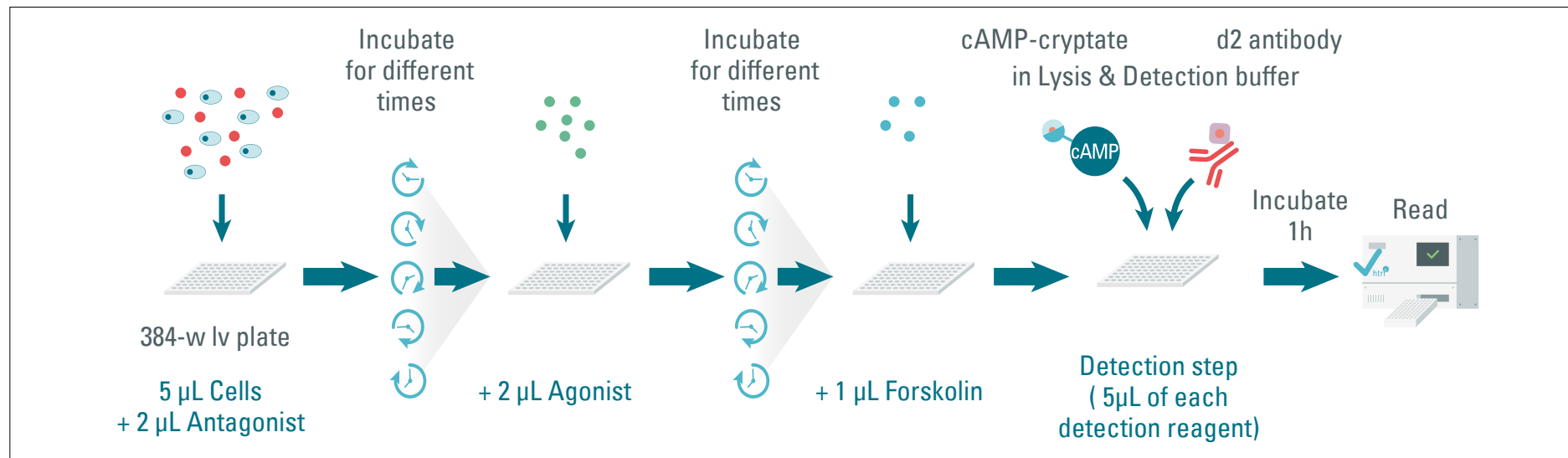
The antagonist must be fully pre-equilibrated prior to adding the agonist. We suggest testing different antagonist incubation times.

The agonist must also be fully equilibrated prior to adding the detection reagents. We suggest optimizing the agonist incubation time as well.

The diagram below illustrates the process of optimizing agonist stimulation time. Cells and compounds are incubated for different lengths of time. The addition of detection reagents diluted in the lysis buffer marks the end of the stimulation time.

For agonists, a time-dependent decrease in EC50 is often observed, at least initially. The ideal incubation time is when no evolution of EC50 can be observed in time.

Forskolin stimulation time optimization is recommended. An empirical time of 45 min is recommended for enough stimulation, but it should be checked for your own cellular model.



Note that first time users may proceed directly to optimizing their agonist stimulation time in the absence of the antagonist. Cells in the presence of the pre-equilibrated antagonist are stimulated with the agonist for the optimal duration previously identified. This convenient method is only recommended

when a rapid answer regarding the antagonistic nature of the compound is sought. For accurate pharmacology, proceed to optimizing the agonist stimulation time in the presence of the pre-equilibrated antagonist.

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GaQ ANTAGONIST ASSAY OPTIMIZATION

Antagonist dose response

In this example, an EC90 concentration of SNC-162 was used to stimulate cells pre-incubated with either Naltrindole and Naloxone, 2 known antagonists of the Delta opioid receptor.

	NALTRINDOLE	NALOXONE
IC ₅₀ (nM)	1.4	956

Antagonist characterization (Delta opioid receptor): CHO cells stably expressing the Delta opioid receptor are dispensed at 6000 cells/well. Compounds were added and incubated 45 min at 37°C. The agonist SNC-162 was added at 10nM (EC90), and incubated 45 min at 37°C. Forskolin was added at 5 µM and incubated 45 min at 37°C. Dose-response curves were plotted to obtain the IC50 values. The Delta opioid receptor antagonists Naltrindole and Naloxone show different ranking, as described in the literature.

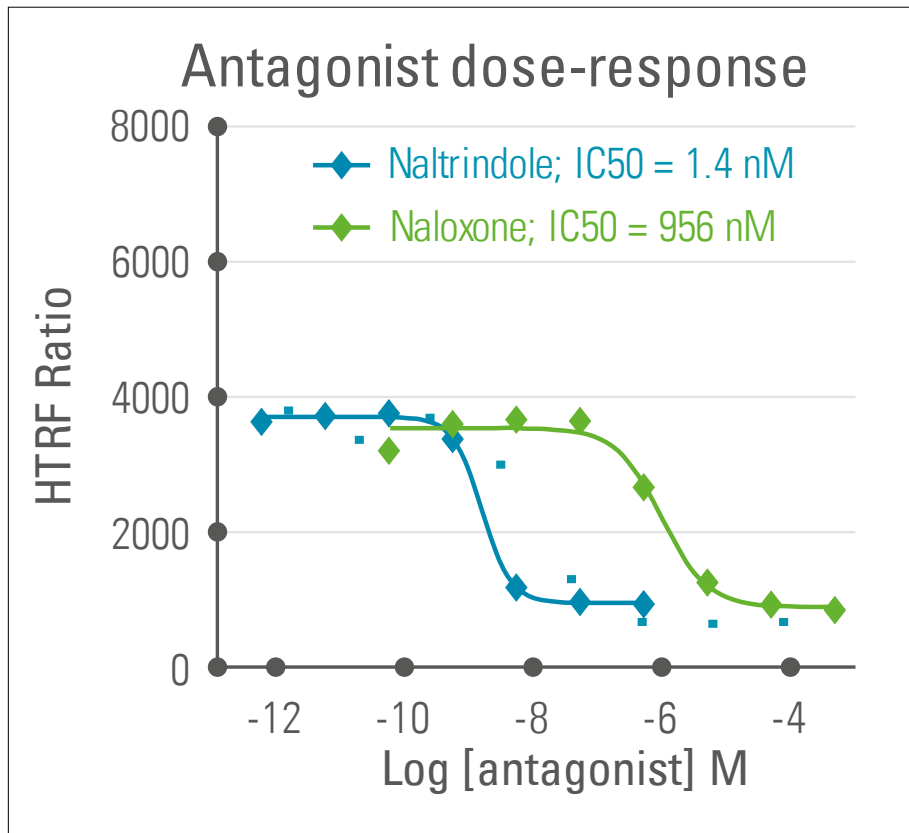


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DETECTION

Choosing the Right Plate Reader

Choosing the suitable microplate reader ensures you'll get an optimal readout. A multimodal reader gives your research the ideal flexibility and expands the field of possibilities by measuring a wide range of technologies.

Revvity offers multimode plate readers that are certified for use with HTRF technology. Certification involves rigorous testing of plate reader with HTRF technology to ensure optimal readout. HTRF assays are also compatible with multimode readers from other providers as well.



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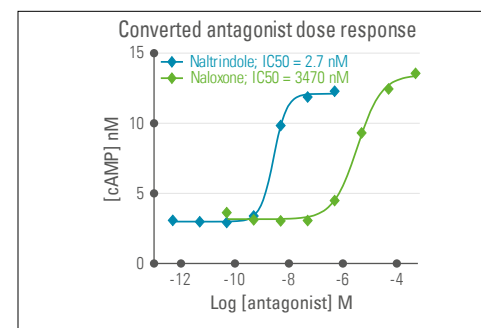
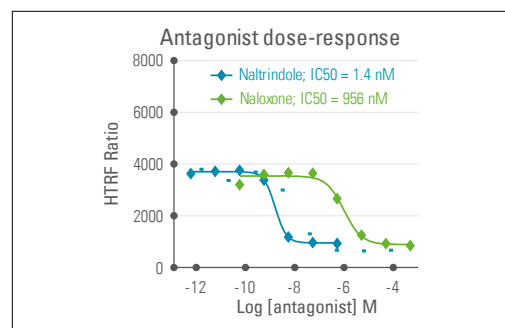
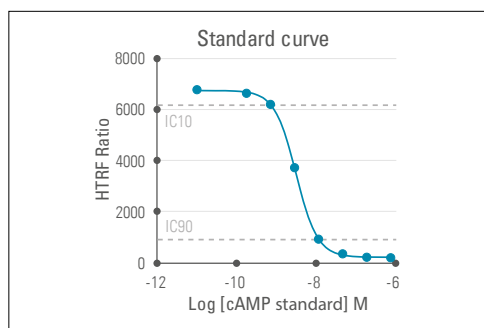


DATA REDUCTION AND ANALYSIS

Data reduction and analysis

The fluorescence resonance energy transfer ratio (665 nm/620 nm) measurements could be converted to cAMP concentration-response curves to determine the IC50 values of the antagonist. Conversion from Ratio to cAMP

concentration can be obtained by using the standard curve. The conversion from Ratio to concentration of cAMP will produce a dose response, revealing the compound as an antagonist. From the stimulation dose response, the IC50 can be determined.



Data conversion

STANDARD CURVE	
Log [cAMPstd] M	HTRF Ratio
-11.00	6781
-9.8	6643
-9.2	6211
-8.6	3735
-8.0	944
-7.4	367
-6.7	235
-6.1	214

CELL BASED ASSAY			
Log [Naltrindole] M	HTRF Ratio	Log [] M	HTRF Ratio
-12.3	2993	-9.3	3593
-11.3	3107	-8.3	3659
-10.3	3033	-7.3	3637
-9.3	2876	-6.3	2654
-8.3	1045	-5.3	1251
-7.3	841	-4.3	920
-6.3	773	-3.3	844



CELL BASED ASSAY			
Log [Naltrindole] M		Log [Naloxone]M	
-12.3	3.1	-9.3	3.1
-11.3	3.0	-8.3	3.0
-10.3	2.9	-7.3	3.1
-9.3	3.4	-6.3	4.5
-8.3	9.2	-5.3	9.3
-7.3	11.9	-4.3	12.5
-6.3	12.3	-3.3	13.6

Data conversion into cAMP concentration. CHO stably expressing the delta opioid receptor were treated with the Naltrindole and Naloxone antagonists. In parallel, a cAMP standard curve was generated. The antagonist dose response curve initially expressed in HTRF® Ratio was plotted against the

standard curve to extrapolate the corresponding cAMP concentration. A new sigmoidal dose response curve was then fitted using log [compound] against [cAMP], using appropriate software (e.g. Graphpad Prism). The potency of the compounds was determined and it remains in the same range of concentration

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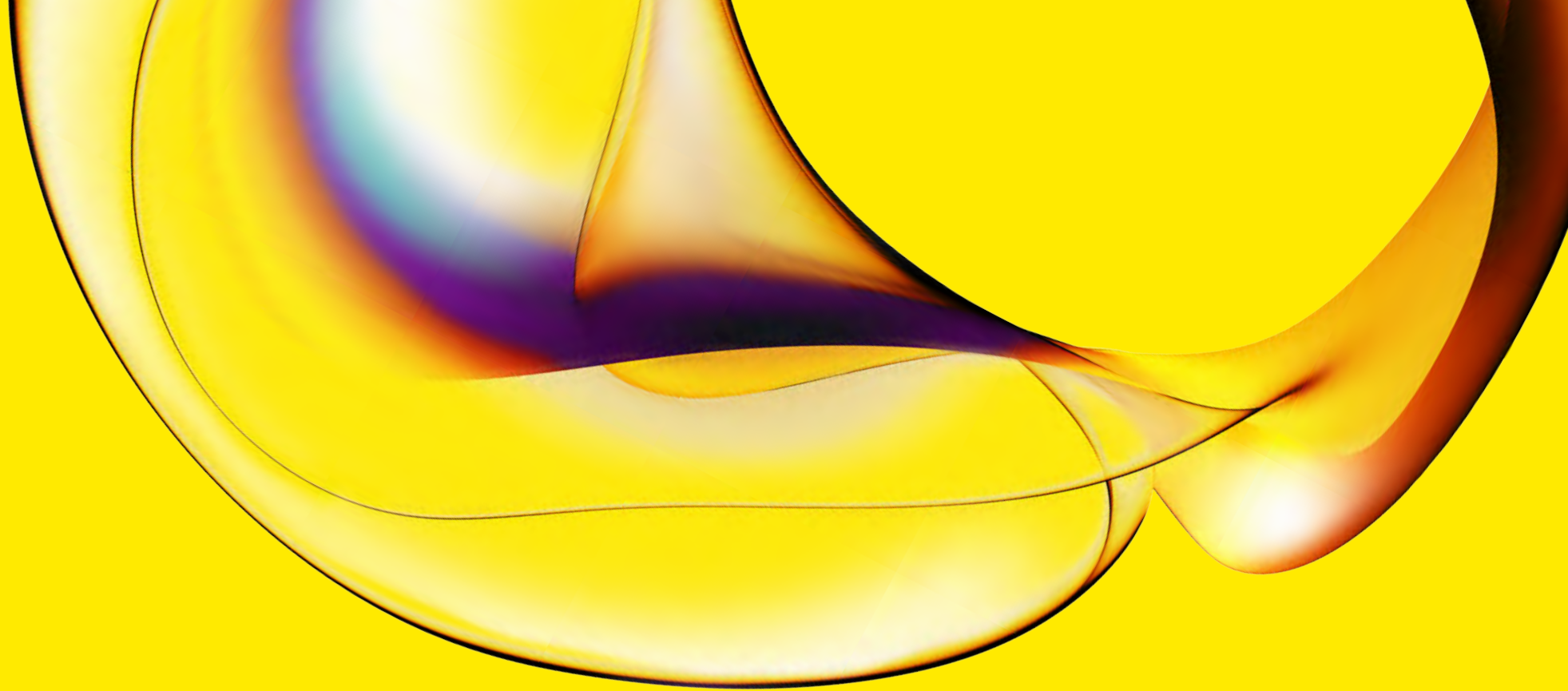
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