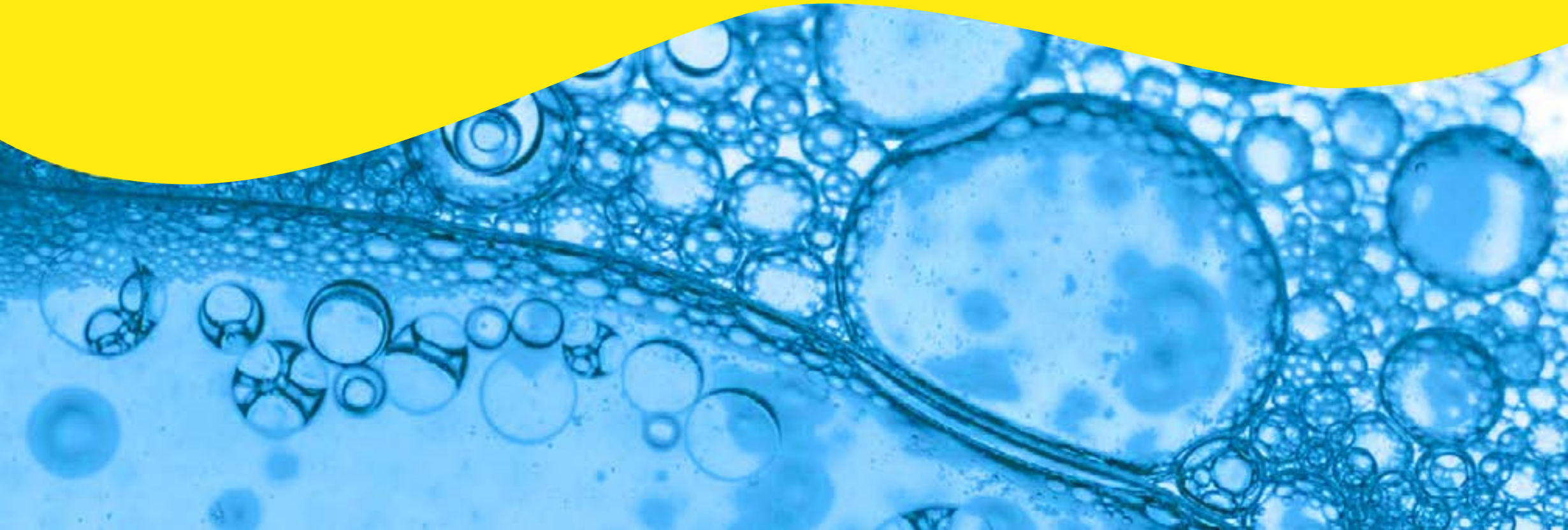
cAMP

Guide to optimizing agonists of Gαs



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 (Gs or Gi) 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 Gαs and Gαi GPCR assays.

Background

Cyclic AMP (cyclic adenosine 3',5'-monophosphate, or simply cAMP) is one of the most important GPCR intracellular mediators. In many cell types, cAMP production often results from the regulation of adenylate cyclase by the Gα subunit of heterotrimeric G-proteins. Gαs coupled GPCRs act to positively stimulate the activity of adenylate cyclase, resulting in an increase in cellular cAMP. Gαi coupled GPCRs lead to a negative regulation of adenylate cyclase, and thus to a decrease in cAMP production.

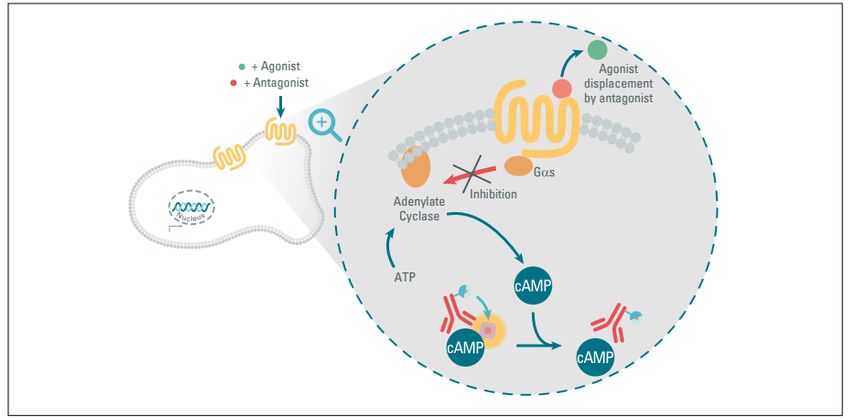
To counteract the degradation of cAMP to AMP by the action of phosphodiesterase (PDE) enzymes, a PDE pan-inhibitor (IBMX) is recommended to ensure proper cAMP accumulation and its subsequent measurement.

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 precise format.

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

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

	cAMP-GS DYNAMIC	cAMP-GS HIRANGE
1,000 tests	# 62AM4PEB	# 62AM6PEB
20,000 tests	# 62AM4PEC	# 62AM6PEC
100,000 tests	# 62AM4PEJ	# 62AM6PEJ



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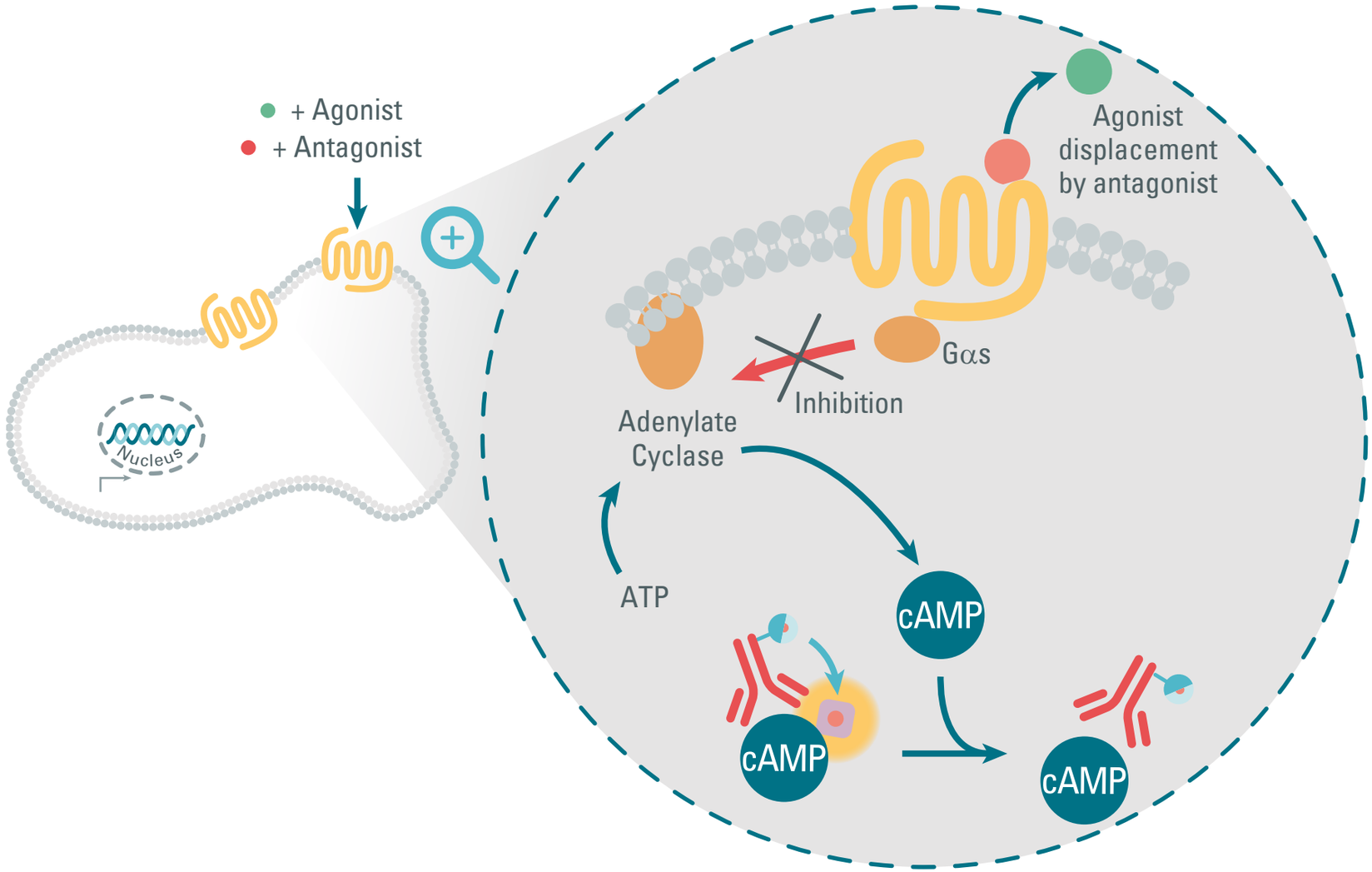

Gs pathway The positive stimulation of the adenylate cyclase results in an increase in cellular cAMP. After lysing the cells the kit enables to quantify this production.

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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 offred as spare reagents in DMSO at a concentration of 500nM (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 500mM 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 10mM (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 cAMP studies using Revvity reagents. Advances in cell culture technologies have increasingly enabled researchers to rely on freshly cultured and collected cells. Cryopreservation 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 a cAMP assay.

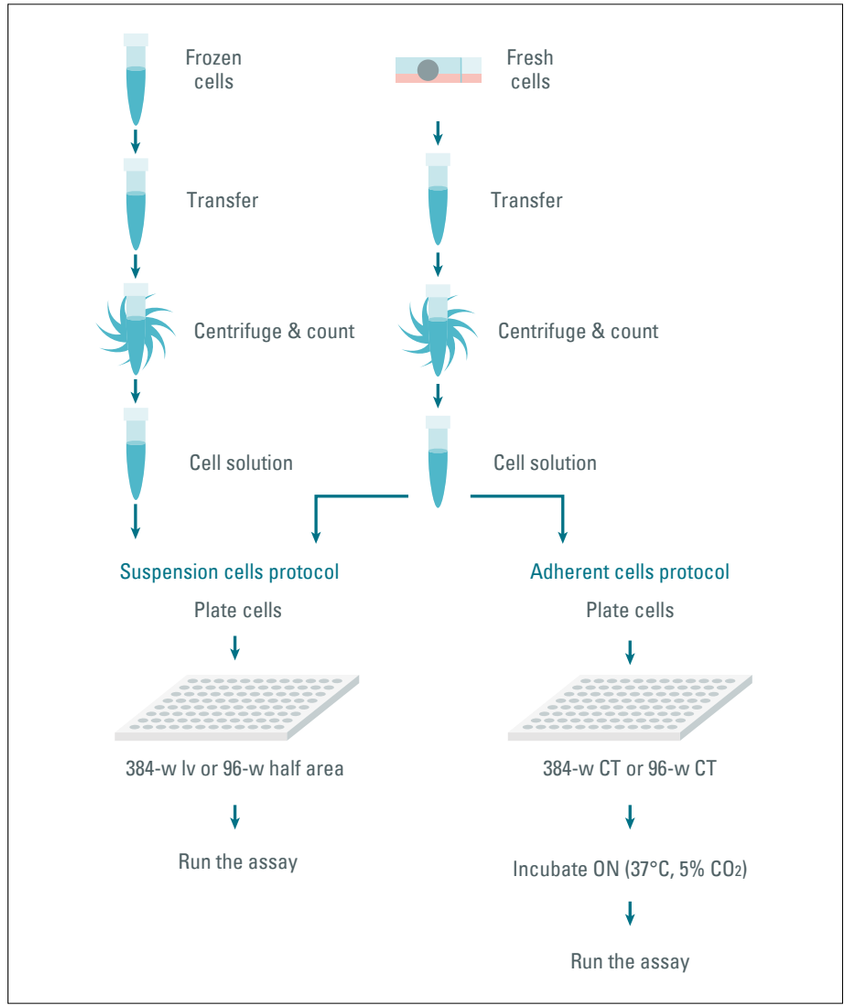
- Cells presenting an adherent phenotype are typically cultured in plates overnight prior to being assayed.
- Cells with a non-adherent phenotype are typically dispensed and tested in suspension immediately following plating.

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, all of the cAMP assays can be performed in:

- 96-well plates
- HTRF 96-well lv 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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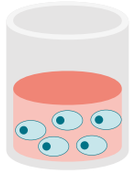
CELL PREPARATION
Freshly harvested cells

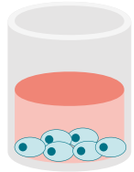
Suspension protocol (for biologically adherent cells)

- Cells from T175 cm^2 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% CO_2 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 cm^2 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% CO_2 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% CO_2.
- 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.

	CELL BASED ASSAY OR STANDARD CURVE	 ADHERENT CELLS PROTOCOL		 SUSPENSION CELLS PROTOCOL			
Stimulation step	Plate	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
	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 (2X) or buffer	10 µl	50 µl	2.5 µl	5 µl	5 µl	25 µl

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

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GαQ AGONIST ASSAY OPTIMIZATION

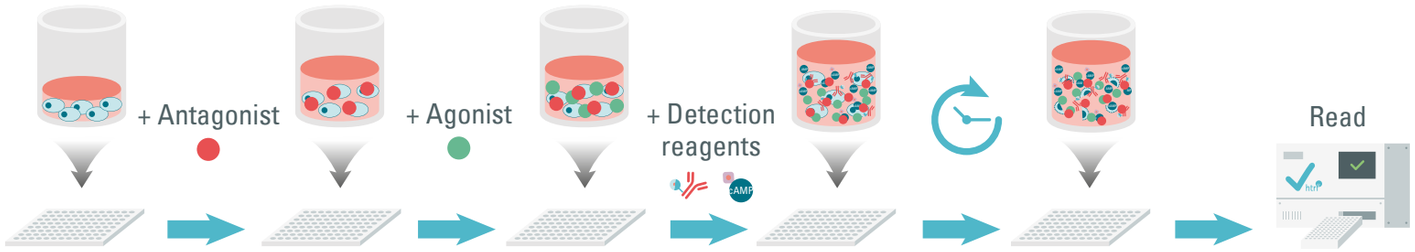
Dispensing formats

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 with 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.

For each protocol, the recommended dispensing volumes must be reviewed.

Untreated cells, where the agonist is substituted for an equivalent volume of buffer, are a useful control. From that control, the basal levels of cAMP production 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
	Agonist (2X) or buffer	2.5 µl	5 µl	5 µl	25 µl
	Incubate agonist with cells	Necessary time at 37°C			
Detection step	cAMP-d2	2.5 µl	5 µl	5 µl	25 µl
	Anti-cAMP-Cryptate	2.5 µl	5 µl	5 µl	25 µl
Final assay volume		10 µl	20 µl	20 µl	100 µl

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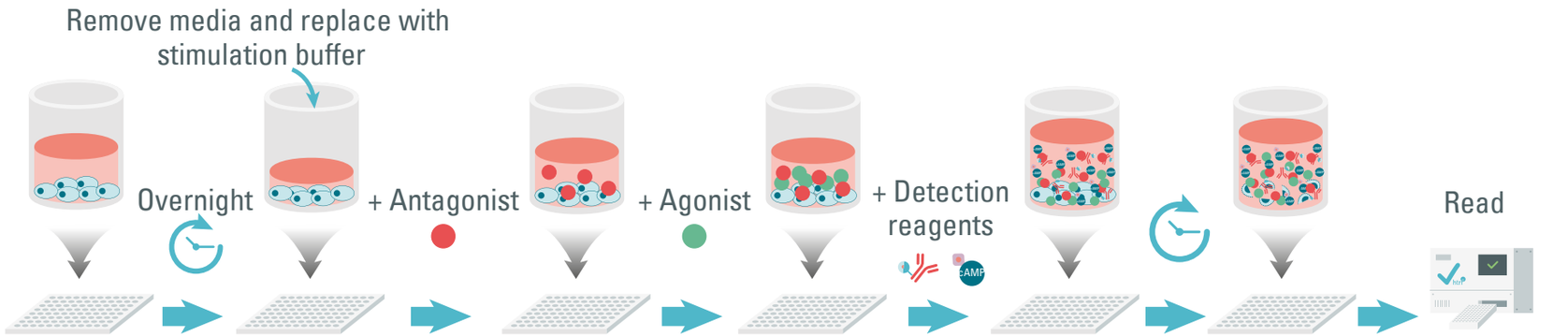
GαQ AGONIST ASSAY OPTIMIZATION

Dispensing formats

Adherent protocol



	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 µl	50 µl
	Cell incubation step	overnight at 37°C, 5% CO_2	
	Agonist (2X) or buffer	10 µl	50 µl
	Incubate agonist with cells	Necessary time at 37°C	
Detection step	cAMP-d2	10 µl	50 µl
	Anti-cAMP-Cryptate	10 µl	50 µl
Final assay volume		40 µl	200 µl

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GαQ AGONIST ASSAY OPTIMIZATION

Dispensing formats

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.

As an example, we describe hereafter a side by side comparison between the suspension cells protocol and the adherent cells protocol with a Gs receptor cellular model: the endogeneous β2 adrenergic receptor expressing HEK293 cells (biogically adherent). The example demonstrates cAMP detection ability using suspension and adherent cells protocols with the same potency for the well-known β2 adrenergic receptor agonist isoproterenol.

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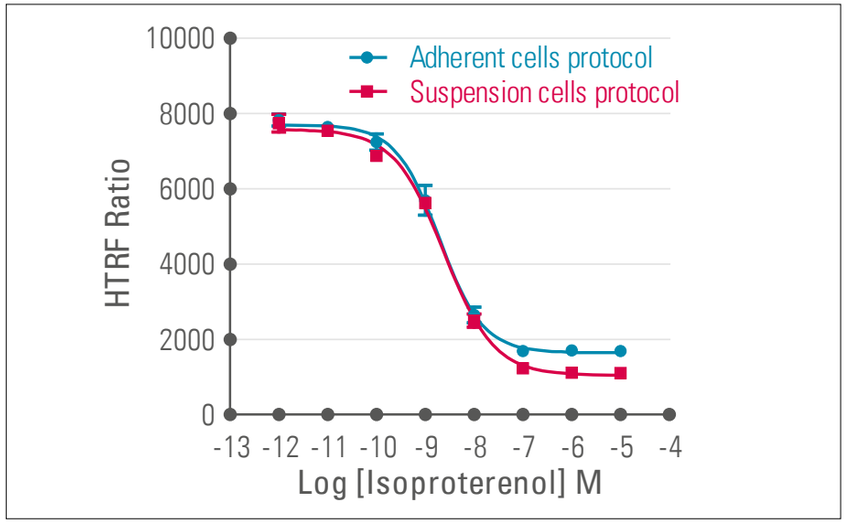
GαQ AGONIST ASSAY OPTIMIZATION

Dispensing formats

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

	CELL BASED ASSAY	ADHERENT CELLS PROTOCOL	SUSPENSION CELLS PROTOCOL
	Plate	384-w CT white	384-w lv
Stimulation step	Cells	10 µl	5 µl
	Incubation	Overnight at 37°C, 5% CO_2	No incubation
	Isoproterenol	10 µl	5 µl
	Incubate agonist	45 min at 37°C	45 min at 37°C
Detection step	cAMP-d2	10 µl	5 µl
	Anti-cAMP-Cryptate	10 µl	5 µl
Final assay volume		40 µl	20 µl



TYPE OF PROTOCOL	ADHERENT	SUSPENSION
S/B	4.7	7.0
EC_{50} isoproterenol (nM)	2.0	2.4

Side by side comparison between suspension cells protocol and adherent cells protocol: HEK293 cells expressing the endogenous β2 adrenergic receptor were dispensed at a density of 2,000 cells /well. Serial dilutions of the isoproterenol, a β2 AR agonist, were added and incubated for 45 min. The HTRF® Ratio data were plotted to trace the sigmoidal dose response, and to calculate the EC_{50} (potency) of the compound.

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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

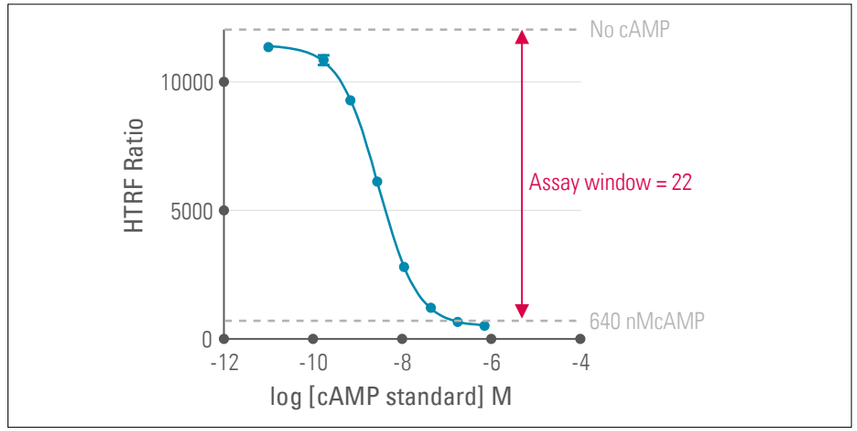
The curve is analyzed by plotting the Ratio as a function of the [cAMP] of the different standards expressed as a logarithm. Graphpad Prism can be used to analyze the curve. The preferred fit is the log(inhibitor) vs. response — Variable slope. Several parameters can be derived from the fit:

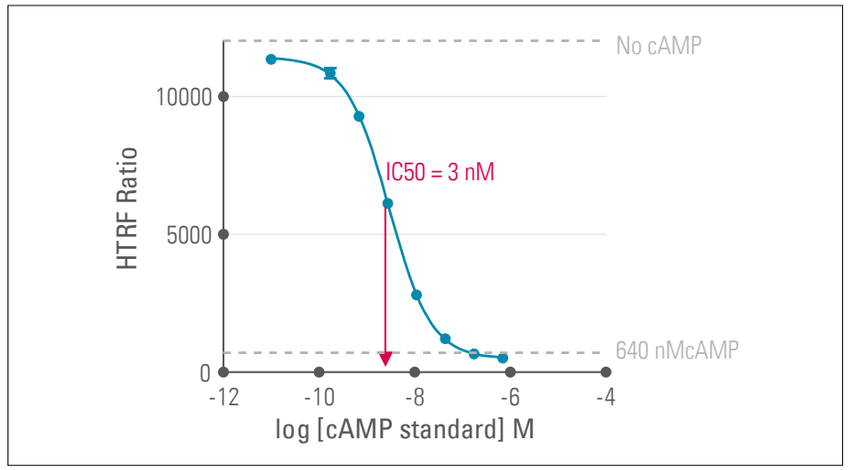
Maximum assay window & IC_{50}

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.

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

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-d2/Anti-cAMP-Eu cryptate 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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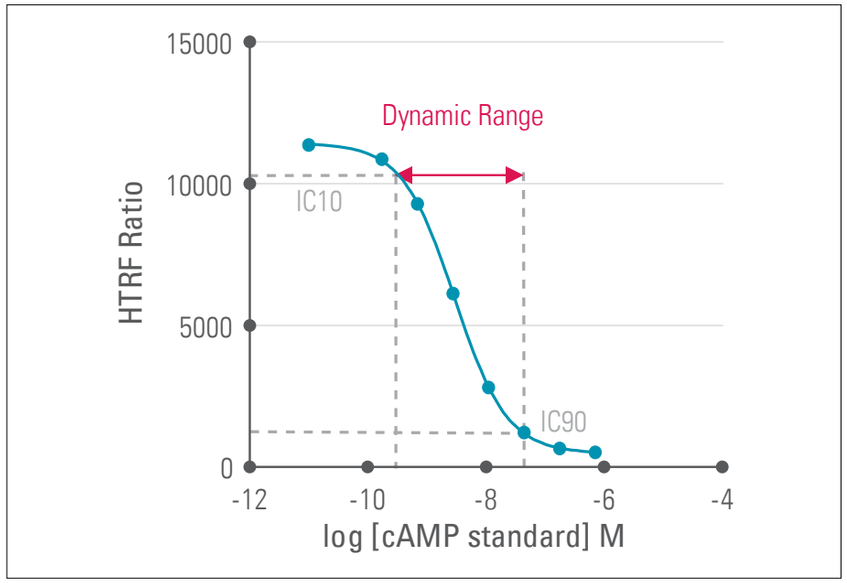
GαQ AGONIST ASSAY OPTIMIZATION

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]



LINEAR RANGE	HTRF® RATIO	[cAMP] NM
IC_{10}	10315	0.32
IC_{90}	1298	37.4

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.

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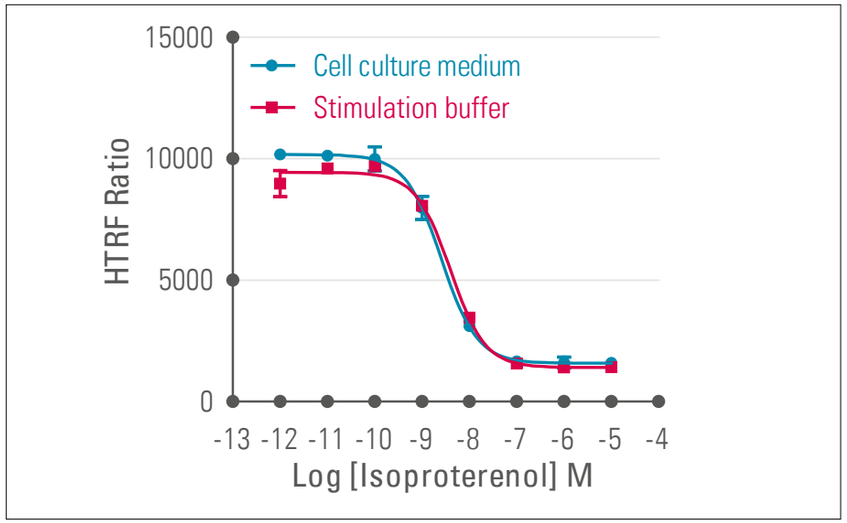
GαQ AGONIST ASSAY OPTIMIZATION

Choice of stimulation buffer

The optimal stimulation buffer can be checked for the cell model of interest. We recommend using the stimulation buffer provided in the kit (Stimulation Buffer 1) for stimulation times below 2h. For stimulation times above 2h, dilute test compounds in cell culture medium (such as RPMI or DMEM). 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 the endogenous beta2 adrenergic receptor expressing HEK293 cells.



	EC_{50} (NM)	S/B
Stimulation Buffer	3.9	6.7
Cell culture medium (supplemented DMEM)	2.6	6.4

Stimulation buffer supplemented with IBMX: using either the stimulation buffer provided or cell culture medium such as DMEM is possible when running the cAMP assay. HEK293 cells expressing endogenous β2 adrenergic receptor were stimulated with the isoproterenol diluted in the stimulation buffer provided in the kit or DMEM stimulation buffer supplemented with 0.5mM of IBMX. The agonist stimulation step was stopped after 45 min by the addition of detection reagents.

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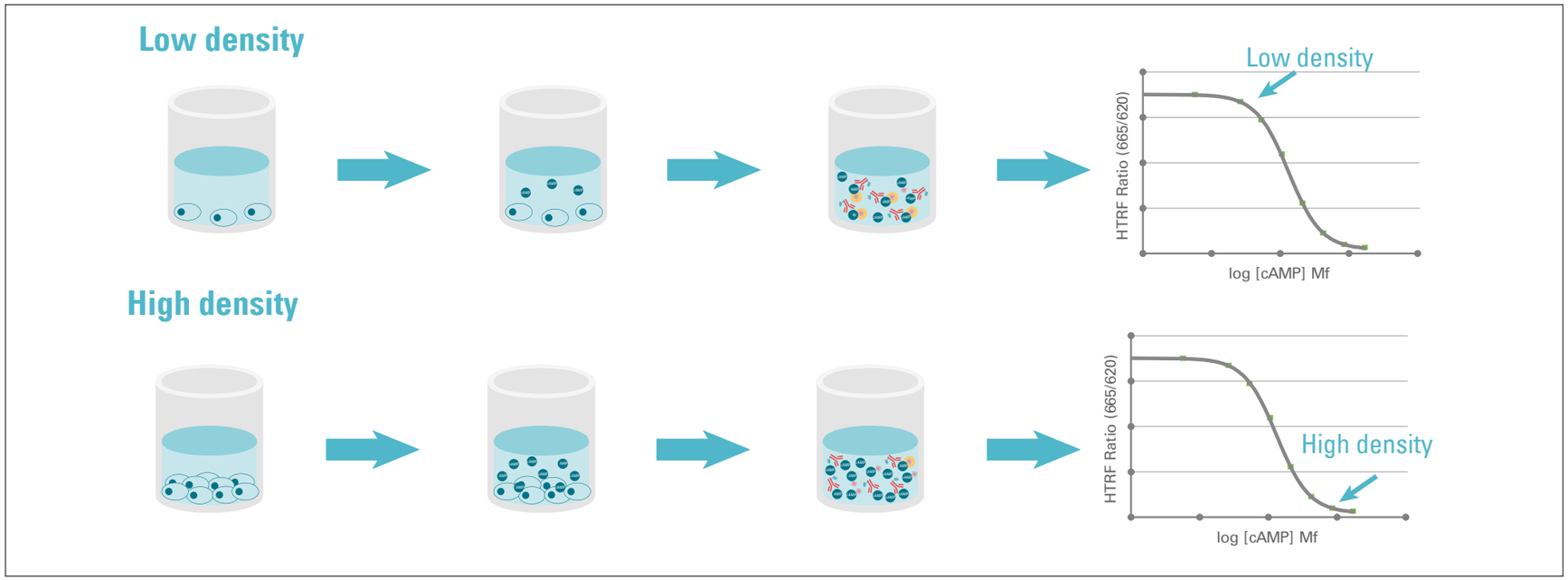
GαQ AGONIST ASSAY OPTIMIZATION

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. Too high a cell density may give a dramatic 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. Forskolin can be used when a reference agonist is not available.

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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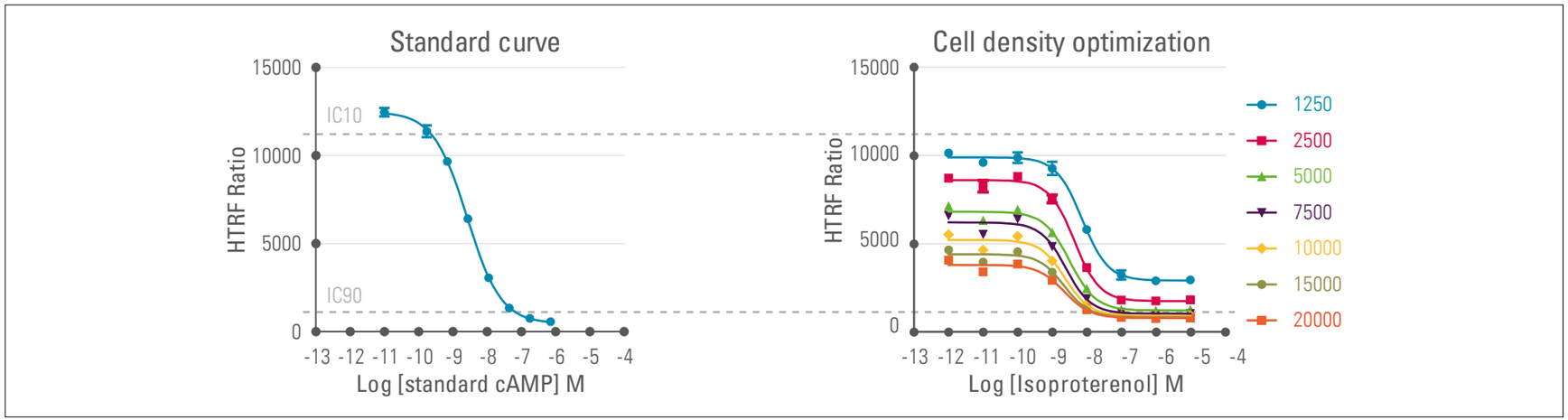
GαQ AGONIST ASSAY OPTIMIZATION

Cell density



CELLS	1,250 cells/w	2,500 cells/w	5,000 cells/w	7,500 cells/w	10,000 cells/w	15,000 cells/w	20,000 cells/w
Assay window	3.4	4.8	5.6	6.3	5.9	5.5	5.2
EC_{50} Acetylcholine (nM)	7.4	4.4	3.2	2.5	2.3	2.2	2.2

Dose response curves for HEK293 cells stimulated with isoproterenol, a β2 adrenergic receptor and reference agonist. 5 µl of cells were dispensed into a 384-lv white plate and stimulated 45 min at 37°C. 5 µl of each detection reagent were then added for detection. Reading was done with a Pherastar lamp reader after 1H incubation time at 22°C. Cell densities of 7,500 cells per well are 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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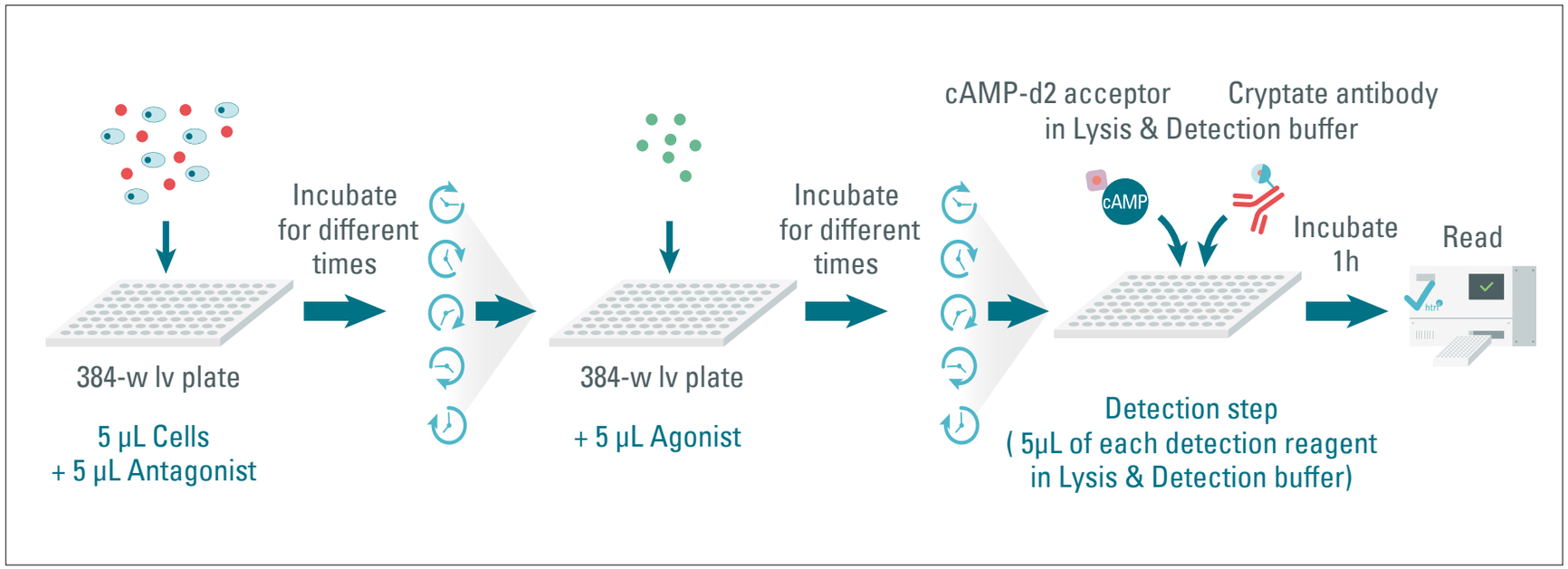
GαQ AGONIST ASSAY OPTIMIZATION

Stimulation time

Agonist stimulation time can significantly impact your results. Allowing for full agonist-receptor equilibrium can reveal patterns that would have otherwise been missed under hemi-equilibrium conditions. In particular, slow-associating agonists will require longer pre-incubation times. Remember that the kinetic action of agonists can differ significantly and cannot be determined a priori. Establishing a time course is a must.

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 EC_{50} is often observed, at least initially. The ideal incubation time is when no evolution of EC_{50} can be observed in time.



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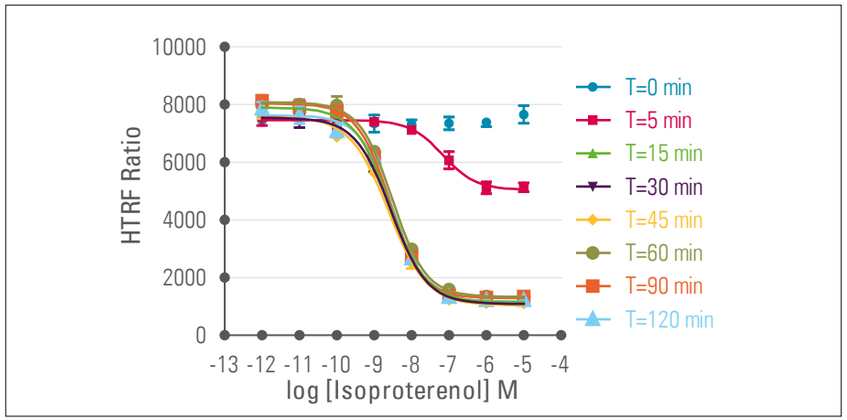
GαQ AGONIST ASSAY OPTIMIZATION

Stimulation time

An example of a stimulation time course is provided below.



	EC_{50} (nM)	S/B
0	0	1
5 min	68.5	1.5
15 min	2.7	6.6
30 min	2.4	6.7
45 min	3.1	7
60 min	2.6	6.1
90 min	3.1	6.1
120 min	2.9	6.5

Kinetic of cAMP accumulation after agonist treatment: HEK293 cells expressing endogeneous beta2 adrenergic receptor were stimulated at different times with the isoproterenol agonist. The results show that equilibrium was reached after 15 min, at which point the EC_{50} remains stable overtime. The suspension cells protocol was applied, where 5µl of cells at a density of 2000 cells/well were dispensed into a 384-lv plate. The isoproterenol was incubated from 5 min to 120 min. The reaction was stopped by adding the detection reagents diluted in the lysis & detection buffer to the well.

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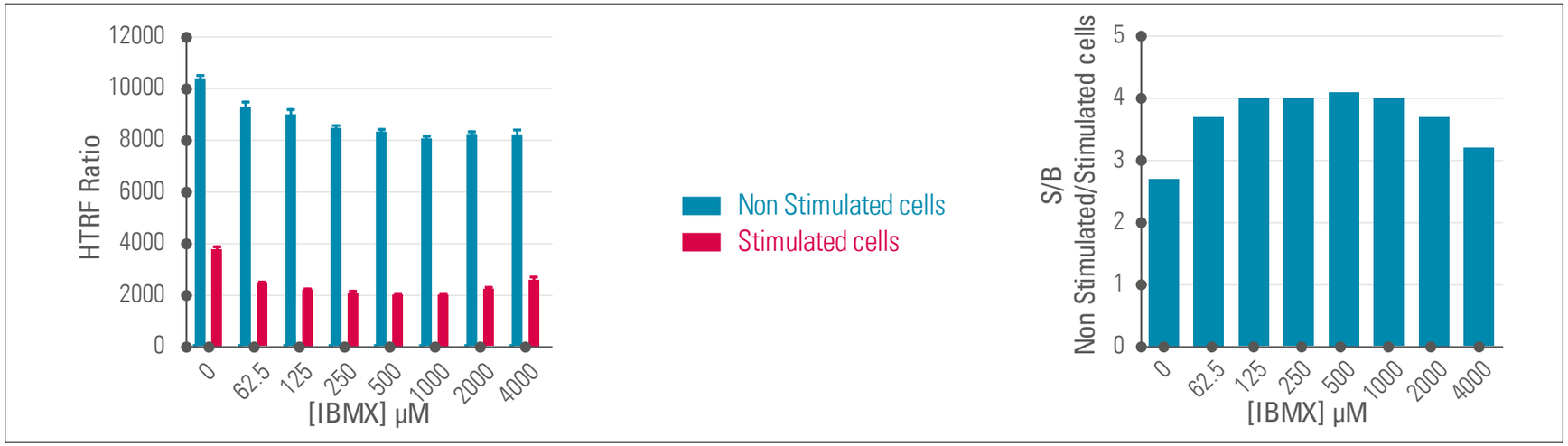
GαQ AGONIST ASSAY OPTIMIZATION

Phosphodiesterase (PDE) inhibitors

IBMX is by far the most widely-used non-specific inhibitor of cAMP and cGMP phosphodiesterases with IC_{50} in the range of 7 to 50 µM. 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. HEK293 cells were stimulated by the β2 adrenergic agonist Isoproterenol at 10 µM and dispensed at 3000 cells/well into a 384-lv white plate. 500 µM 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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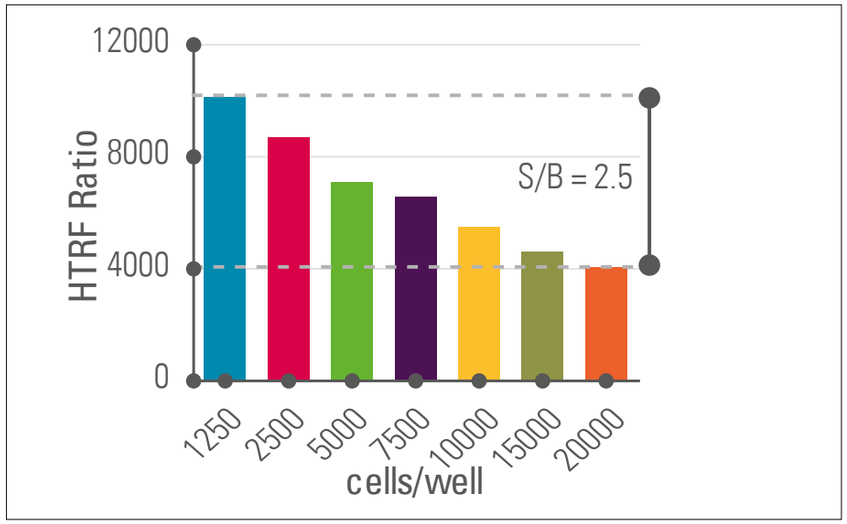
GαQ AGONIST ASSAY OPTIMIZATION

Constitutive activity

Constitutive activity is observed at both low and high GPCR expression levels and has been observed for Gαs, Gαi and Gαq coupled GPCR. For Gαs coupled GPCR, constitutive activity is the production of cAMP in the absence of any agonist stimulation. High constitutive activity negatively impacts the assay window.

Fortunately, the extent to which constitutive activity reduces an assay window can readily be assessed by following the steps described in "Cell density optimization".



Highlighting the constitutive activity of the Gs coupled receptors in HEK293 cells. The HTRF® ratio decreases when the cell density increases, a consequence of the cells being constitutively active. In this example, a 2.5 fold loss in assay signal is recorded.

In this example, increased cell densities lead to a decrease in Ratio, suggesting an increase in cAMP assayed. Since this occurs in the absence of agonist stimulation, it can be concluded that the receptor constitutively produced cAMP.

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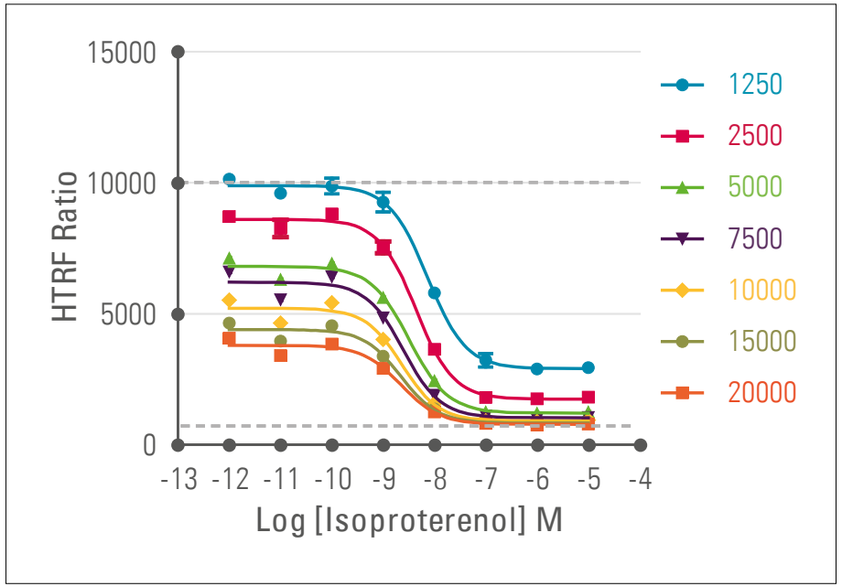
GαQ AGONIST ASSAY OPTIMIZATION

Constitutive activity



CELL/WELL	EC_{50} nM	S/B
1,250	7.4	3.4
2,500	4.4	4.8
5,000	3.3	5.6
7,500	2.5	6.3
10,000	2.3	5.8
15,000	2.2	5.5
20,000	2.6	5.2

Optimizing the test conditions in the case of constitutive activity.
The constitutive activity decreases the basal HTRF® signal in the absence of compounds (blue arrow). The assay window of the isoproterenol agonist dose response curve does not increase even if more cells are added to the well. The optimal condition in this example would be 7500 cells/well. The assay is also limited by the dynamic assay window (grey dashes).

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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 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.

EnVision®

Provides ultra-high throughput and maximum sensitivity across all detection technologies. It is ideal for complex assays to drive your scientific breakthroughs.

VICTOR® Nivo™

Packs all the latest major detection technologies into the industry's smallest benchtop footprint. The perfect microplate reader for everyday biochemical and cell-based assays.

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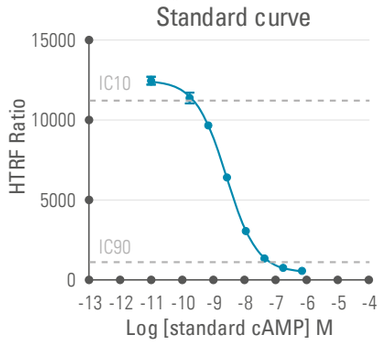
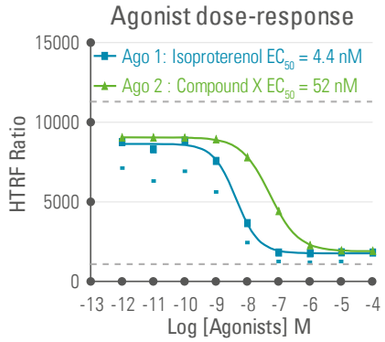
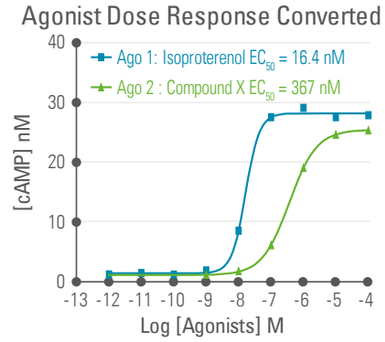
DATA REDUCTION AND ANALYSIS

Data reduction and analysis

The fluorescence resonance energy transfer ratio (665 nm/620 nm) measurements could be converted into cAMP concentration-response curves to determine the EC_{50} value of the compound tested. Conversion from Ratio to cAMP concentration can be obtained by using the standard curve. Note that this conversion will produce a stimulation sigmoid dose response, as would be expected for an agonist.







STANDARD CURVE	
log [cAMP]	HTRF® Ratio
-11.0	11354
-9.8	10851
-9.2	9281
-8.6	6123
-8.0	2806
-7.4	1217
-6.7	661
-6.1	518

CELL BASED		
Log [Agonist] M	HTRF® Ratio	
	Agonist 1	Agonist 2
-12	8706	9000
-11	8250	8999
-10	8805	8985
-9	7531	8875
-8	3646	7757
-7	1812	4394
-6	1755	2259
-5	1813	1937
-4	1800	1904

Data conversion

CELL BASED		
Log [Agonist] M	[cAMP] nM	
	Agonist 1	Agonist 2
-12	1.1	1.0
-11	1.3	1.0
-10	1.0	1.0
-9	1.8	1.0
-8	8.4	1.6
-7	27.5	6.0
-6	29.0	18.9
-5	27.4	24.5
-4	27.8	25.3

Data conversion into cAMP concentration. HEK293 cells expressing the endogenous β2 adrenergic receptor were treated with the isoproterenol agonist and another agonist called compound X. In parallel, a cAMP standard curve was generated. The agonists 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 the ranking remains the same after conversion.

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