revvity

cAMP

Guide to optimizing antagonists 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 (Gαs or Gαi) 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/o 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.

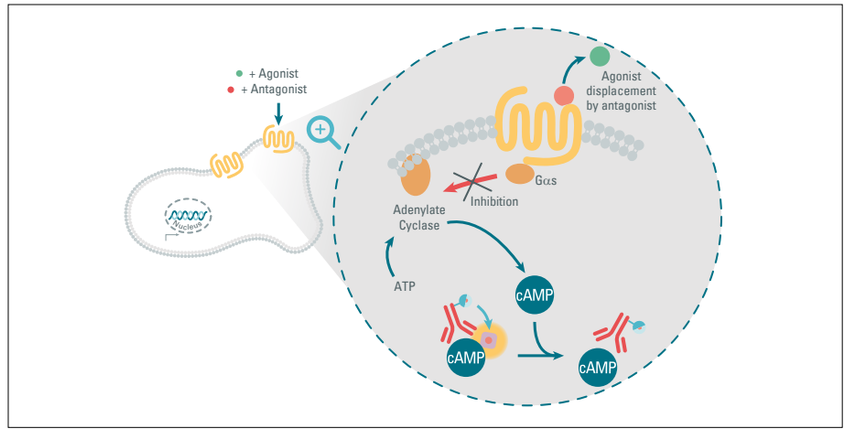
To counteract the degradation of cAMP to AMP by the action of phosphodiesterase (PDE) enzymes, a PDE pan-inhibitor (IBMX) is recommended to ensure proper cAMP accumulation and its subsequent measurement.

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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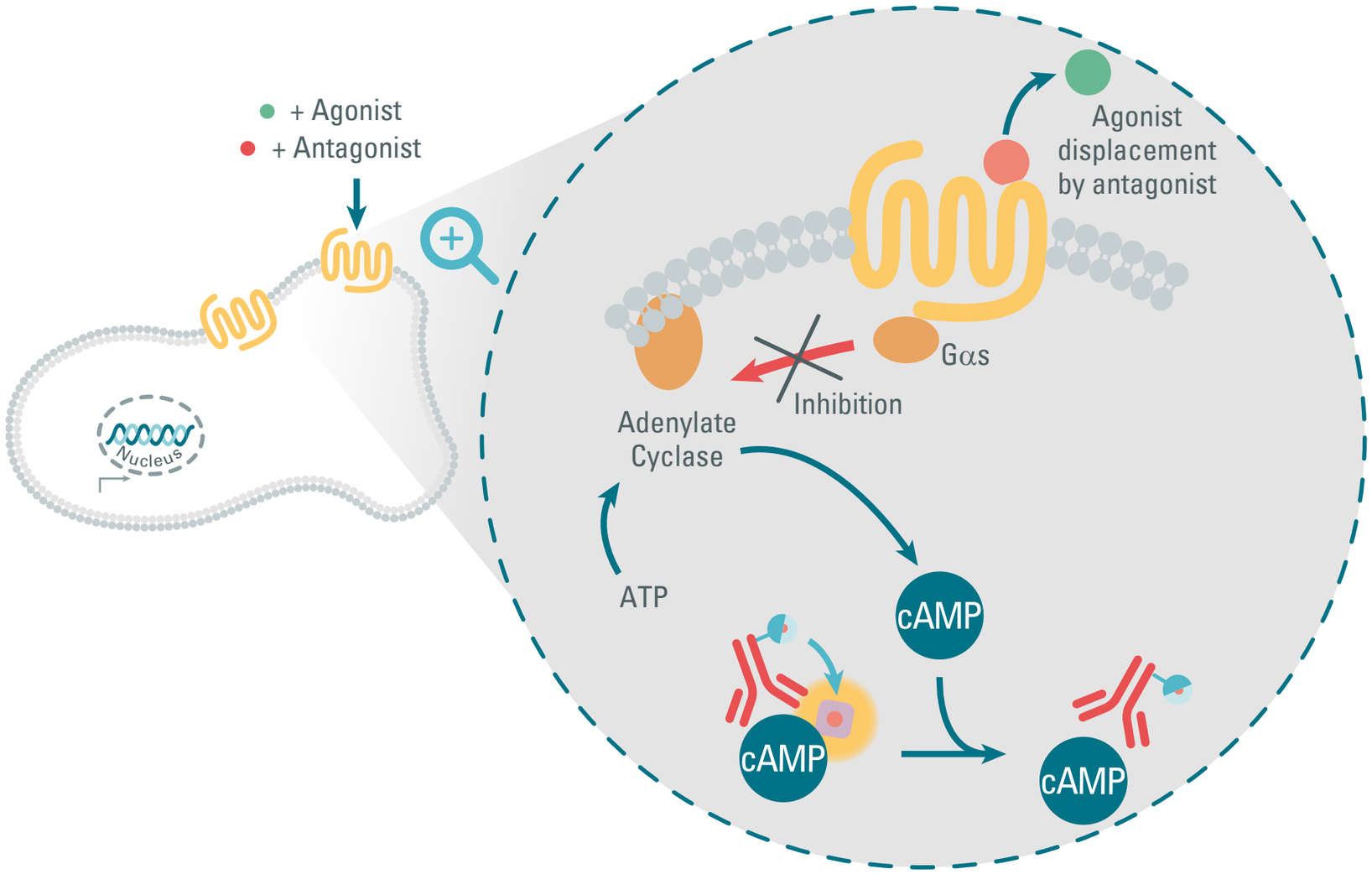

Gαs pathway *An antagonist prevents the activation of the GPCR by the agonist and thus prevents the activation of adenylate cyclase and the production of cAMP that would have been otherwise observed.*

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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 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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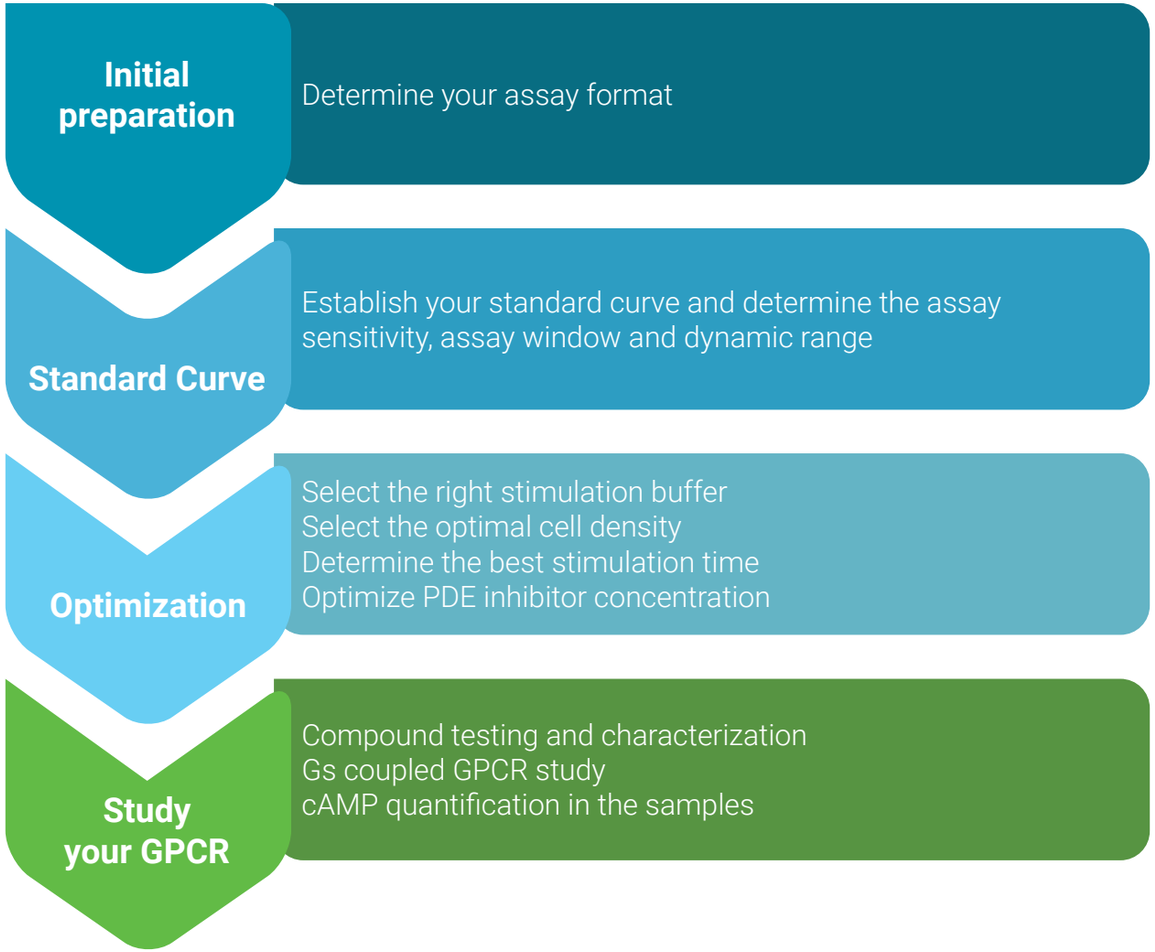
REAGENTS AND OPTIMIZATION WORKFLOW

Optimization Workflow



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CELL PREPARATION
Before You Begin

Cryopreserved cells have already been successfully employed in a number of 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.

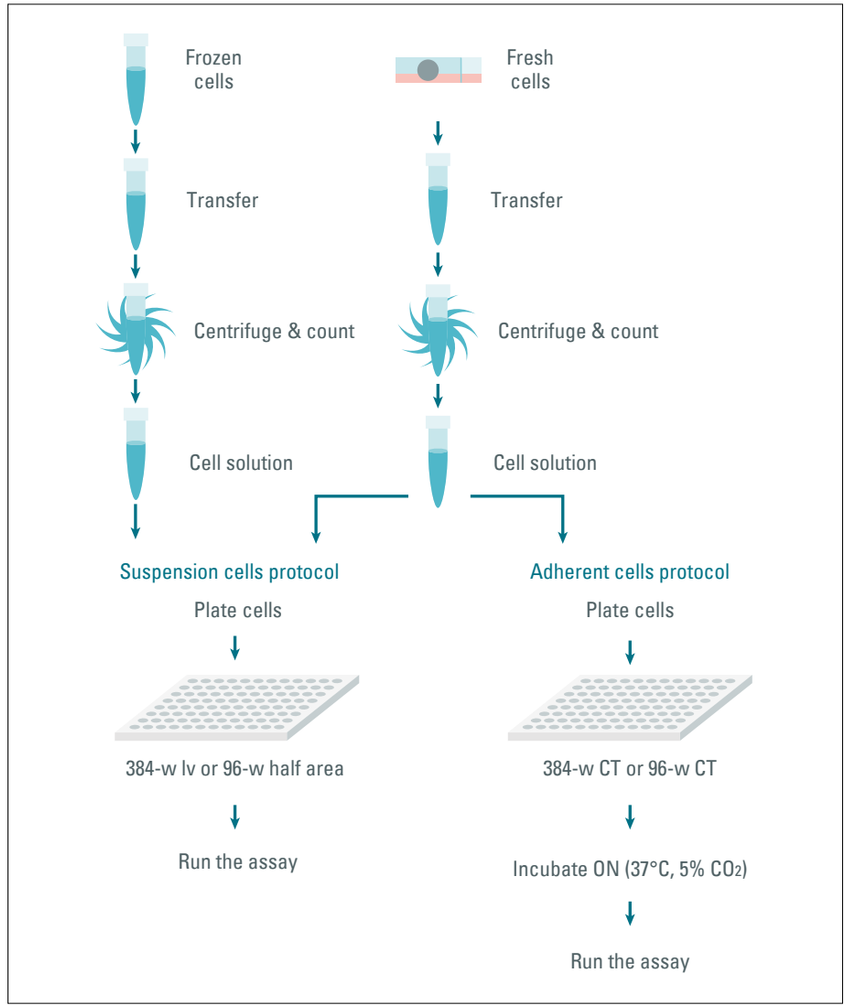
Cells with a non-adherent phenotype are typically dispensed and tested in suspension immediately following plating.

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 lv plates

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



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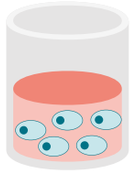
CELL PREPARATION
Freshly Harvested Cells

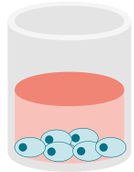
Suspension protocol (for biologically adherent cells)

- 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 cm^2 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 cm^2 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	96-w half area white
	Cells or standard (4X)	10 µl*	50 µl*	2.5 µl	5 µl	25 µl
	Cell incubation step	Overnight at 37°C, 5% CO_2		No cell incubation		
	Compounds or Stimulation Buffer	10 µl	50 µl	2.5 µl	5 µl	25 µl

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

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GαS AGONIST ASSAY OPTIMIZATION

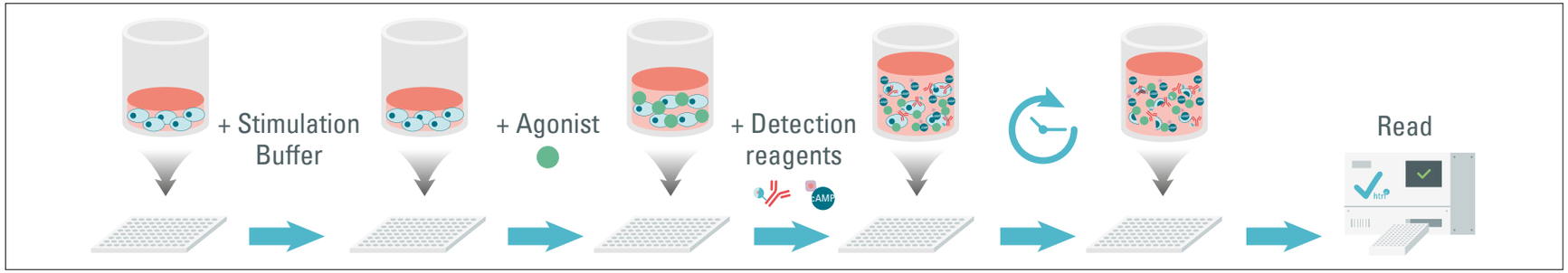
Dispensing Formats

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.

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 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
	Stimulation Buffer 1X	1.5 µl	3 µl	3 µl	15 µl
	Agonist (5X)	1 µl	2 µl	2 µl	10 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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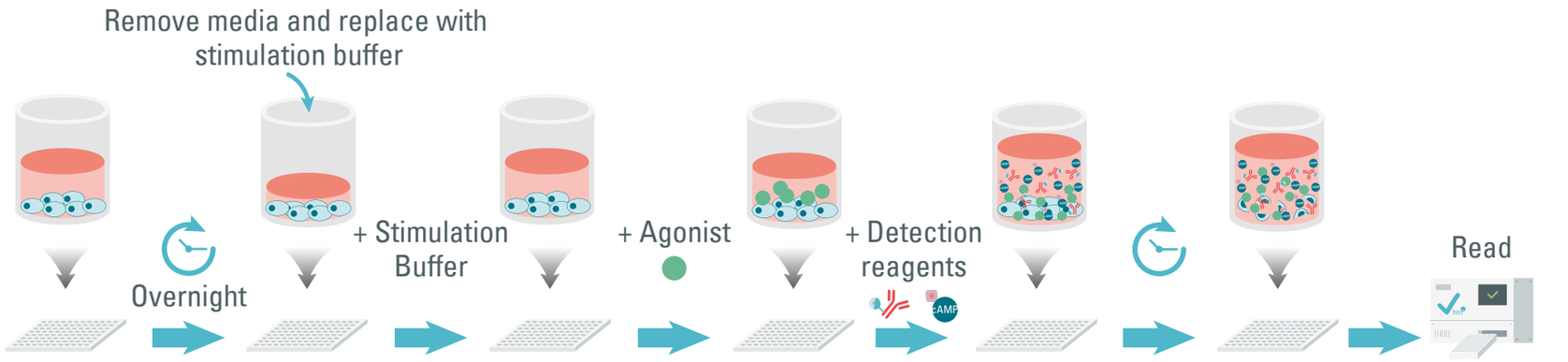
GαS AGONIST ASSAY OPTIMIZATION
Dispensing Formats

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	
	Stimulation buffer 1X	6 µl	30 µl
	Incubation	Necessary time at 37°C	
	Agonist (5X)	4 µl	20 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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GαS 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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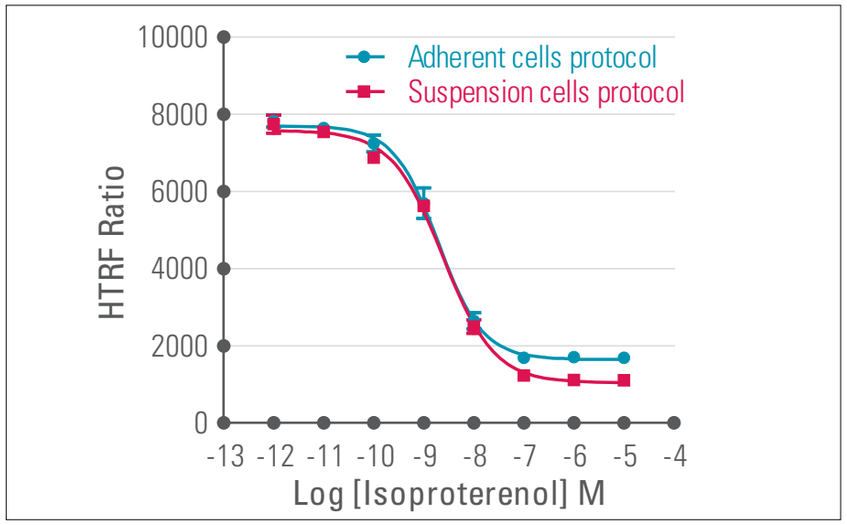
GαS AGONIST ASSAY OPTIMIZATION
Dispensing Formats

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

	CELL BASED ASSAY	ADHERENT CELLS PROTOCOL	SUSPENSION CELLS PROTOCOL
Stimulation step	Plate	384-w CT white	384-w lv white
	Cells	10 µl	5 µl
	Incubation	overnight at 37°C, 5% CO_2	No incubation
	Stimulation buffer 1X	6 µl	3 µl
	Isoproterenol (5X)	4 µl	2 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 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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GαS 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

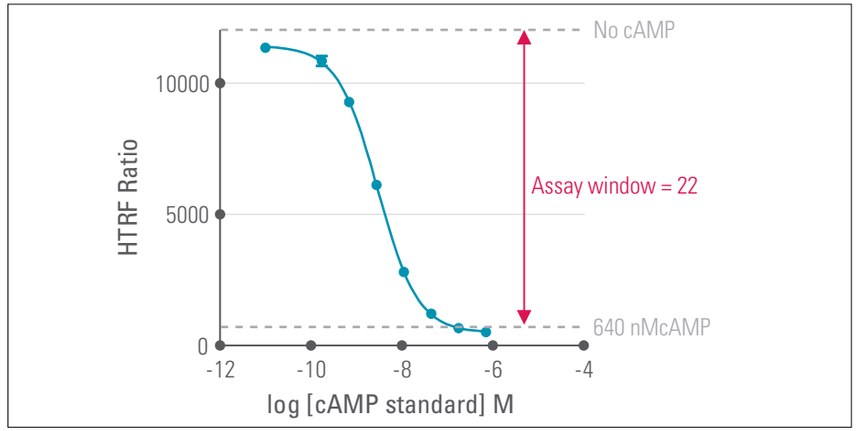
The curve is analyzed by plotting the Ratio as a function of the [cAMP] of the different standards expressed as a logarithm. Graphpad Prism can be used to analyze the curve. The preferred fit is the log(inhibitor) vs. response – Variable slope. Several parameters can be derived from the fit:

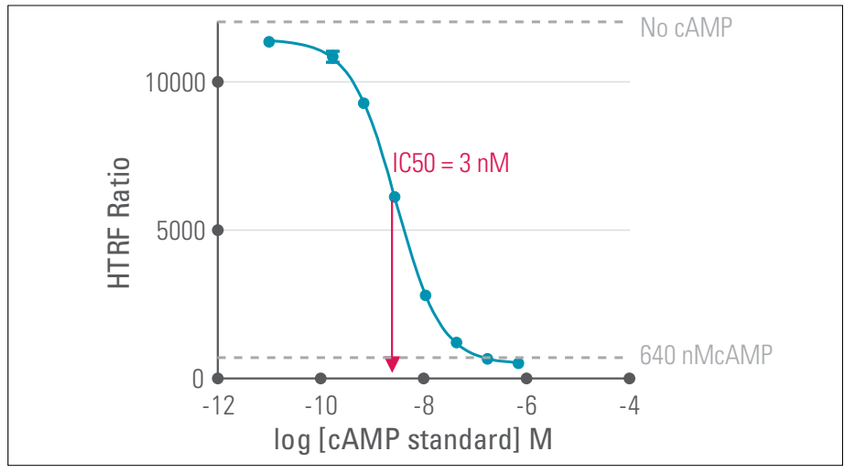
Maximum assay window & IC_{50}

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.

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

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 IC50 is a measurement of how efficient and responsive the system is at recording changes in cAMP concentrations.





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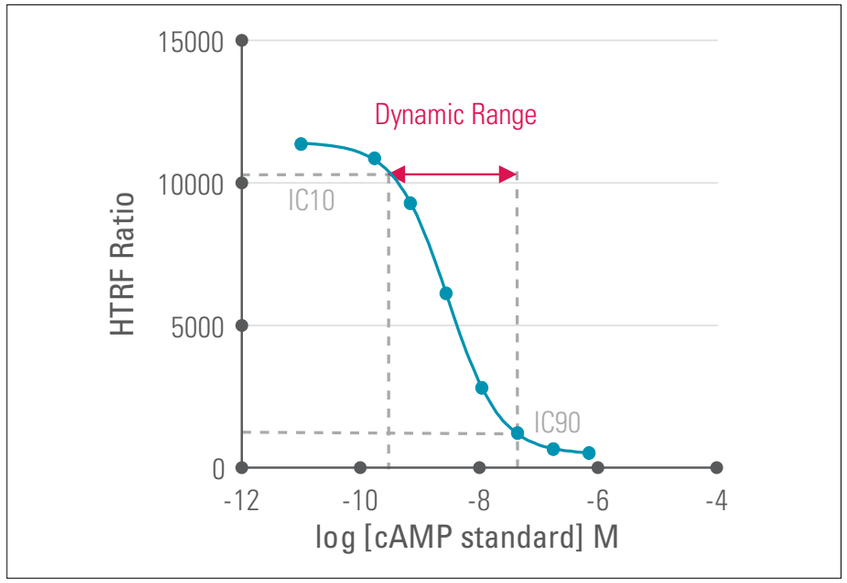
GαS AGONIST ASSAY OPTIMIZATION

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}	10315	0.32
IC_{90}	1298	37.4

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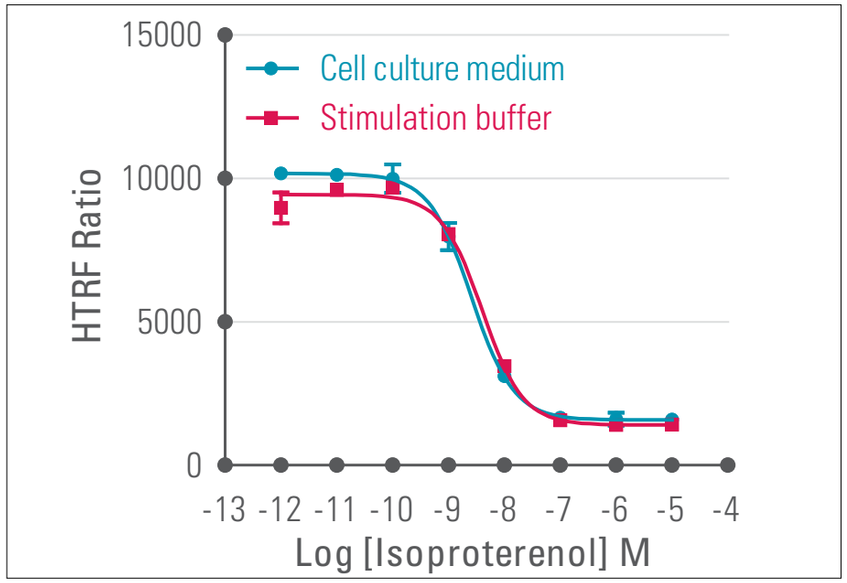
GαS AGONIST ASSAY OPTIMIZATION

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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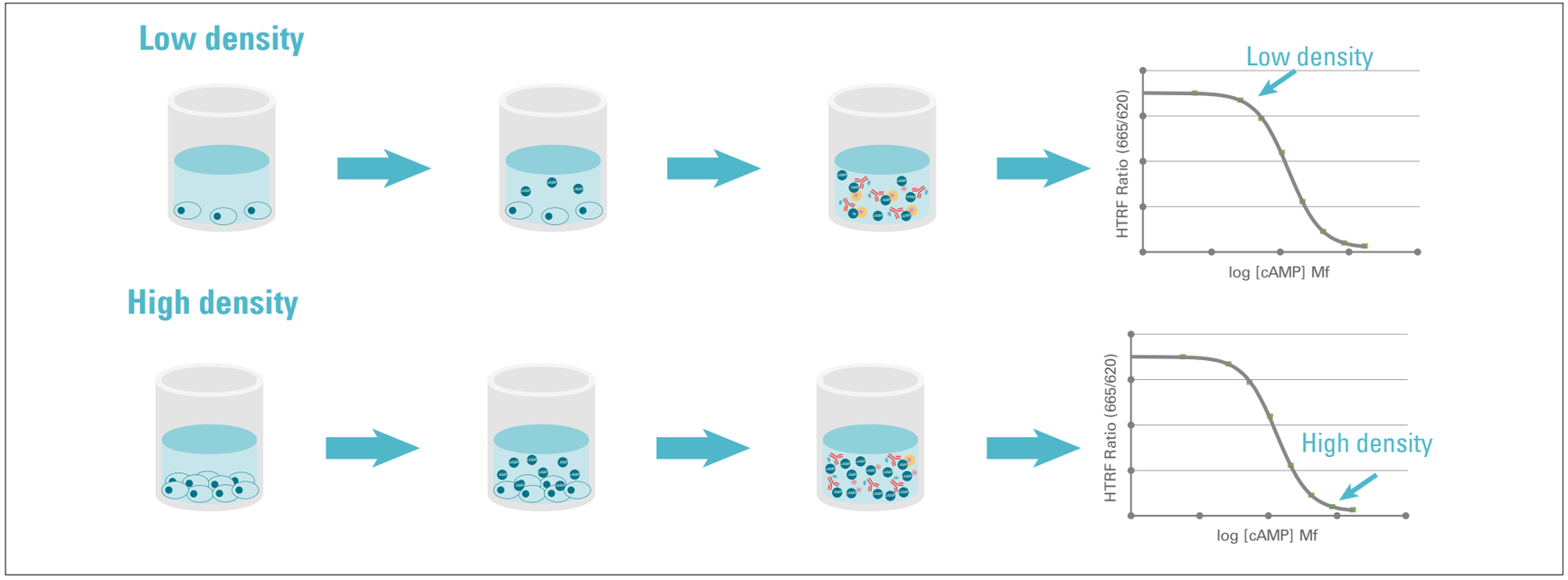
GαS AGONIST ASSAY OPTIMIZATION
Cell Density

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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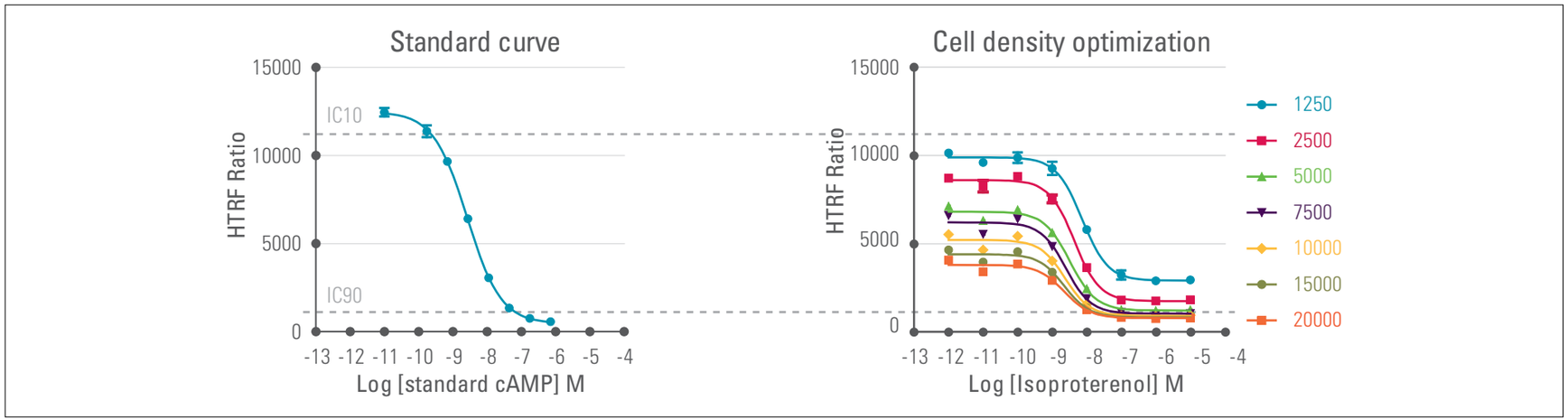
GαS AGONIST ASSAY OPTIMIZATION
Cell Density



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} isoproterenol (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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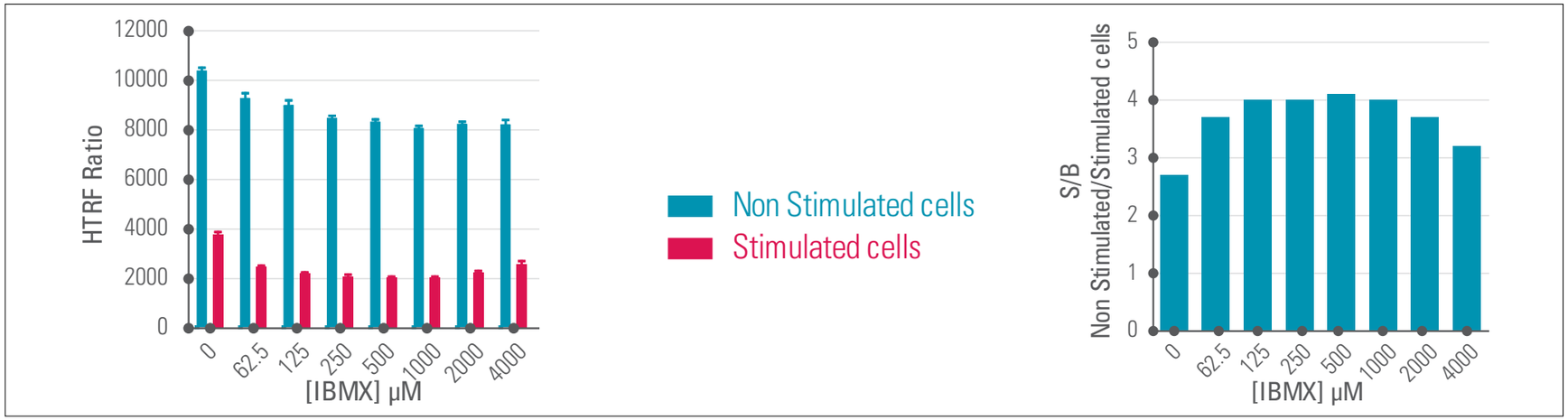
GαS AGONIST ASSAY OPTIMIZATION

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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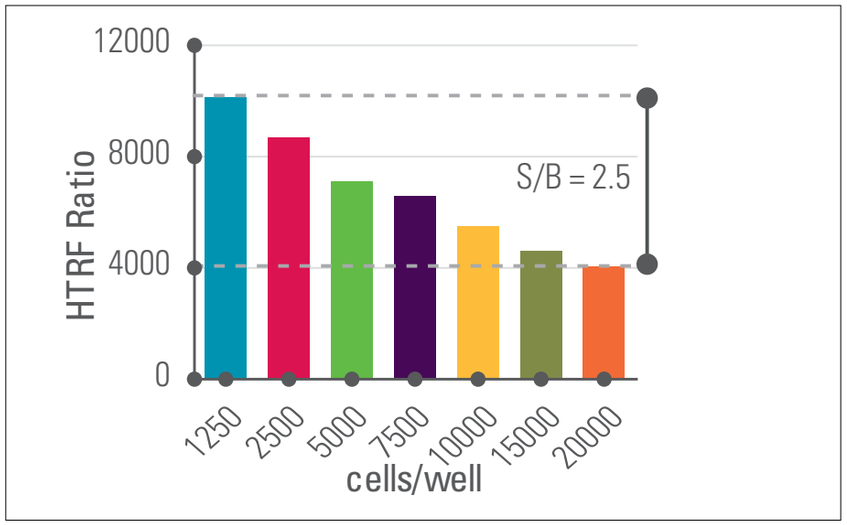
GαS AGONIST ASSAY OPTIMIZATION
Constitutive Activity

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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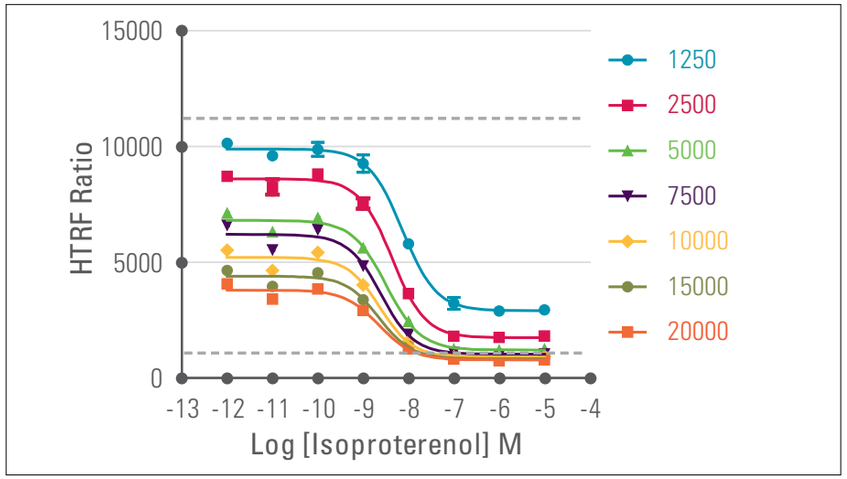
GαS AGONIST ASSAY OPTIMIZATION
Constitutive Activity



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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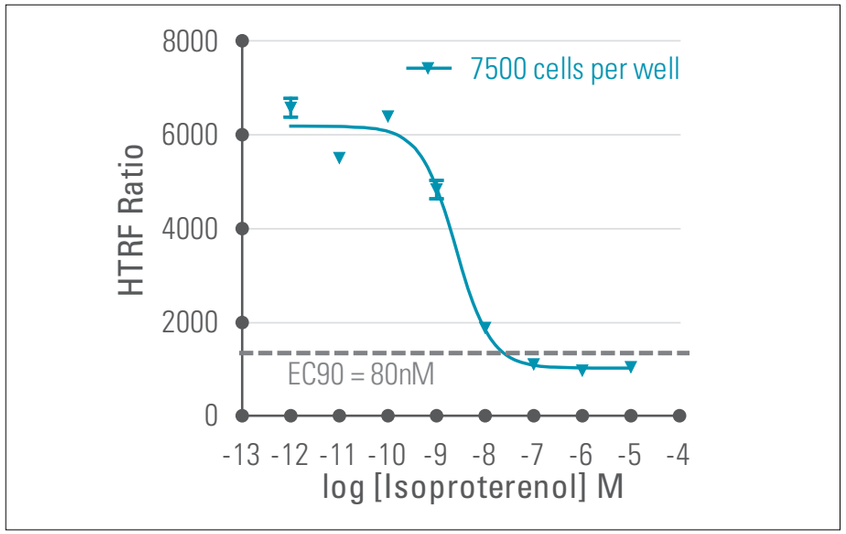
GαS AGONIST ASSAY OPTIMIZATION

Optimized Conditions For Agonist Testing

The optimal cell density is 7,500 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 7,500 cells per well is shown here.

Next identify the agonist EC90. The EC90 is the concentration of agonist that leads to a 90% reduction in signal. In this case, the EC_{90} of Isoproterenol is 80nM



Dose response curve for HEK293 cells stimulated with isoproterenol, a β2 adrenergic receptor and reference agonist: The agonist EC_{90} will be the concentration producing 90% of the maximum cAMP response and the concentration of agonist to be tested in the presence of the antagonist.

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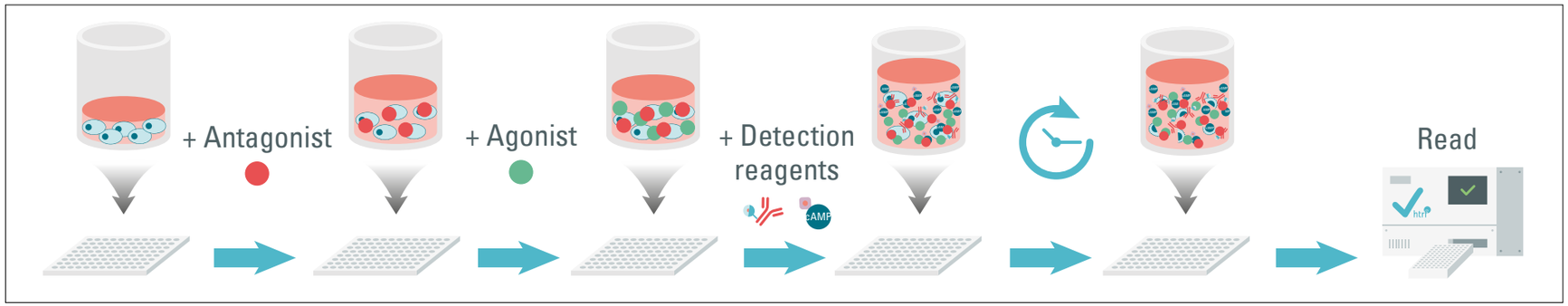
GαS AGONIST ASSAY OPTIMIZATION
Dispensing Formats

Antagonists dilutions are prepared at 3.33X the desired final concentration and added before the agonist at EC_{90}. Untreated cells, where the antagonist 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
	Antagonist (3.33x) or Stim Buffer	1.5 µl	3 µl	3 µl	15 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			
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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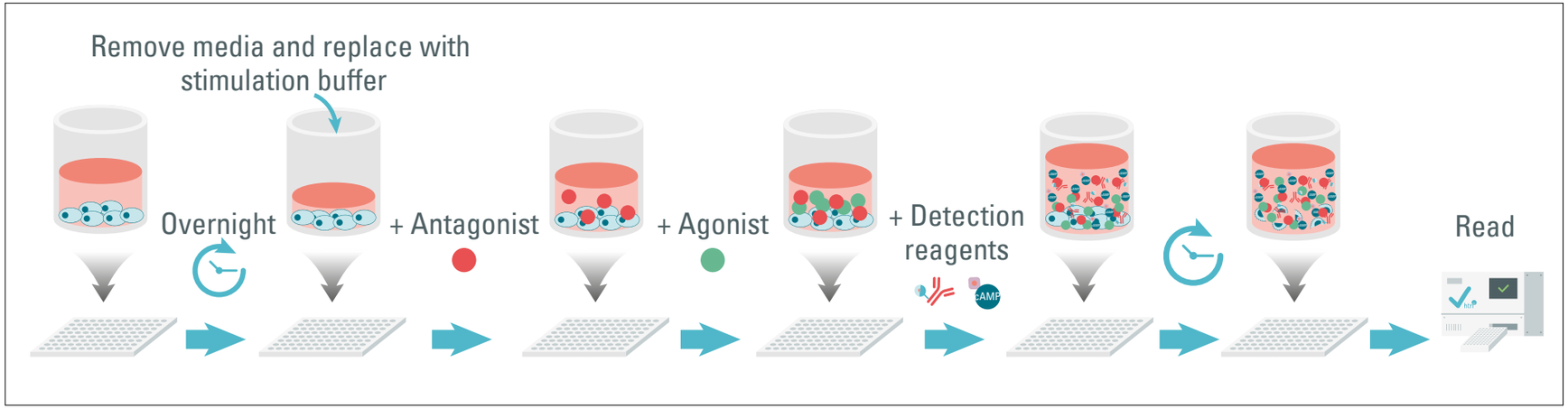
GαS AGONIST ASSAY OPTIMIZATION
Dispensing Formats

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	
	Antagonist (3.33X) or Stimulation Buffer	6 µl	30 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	
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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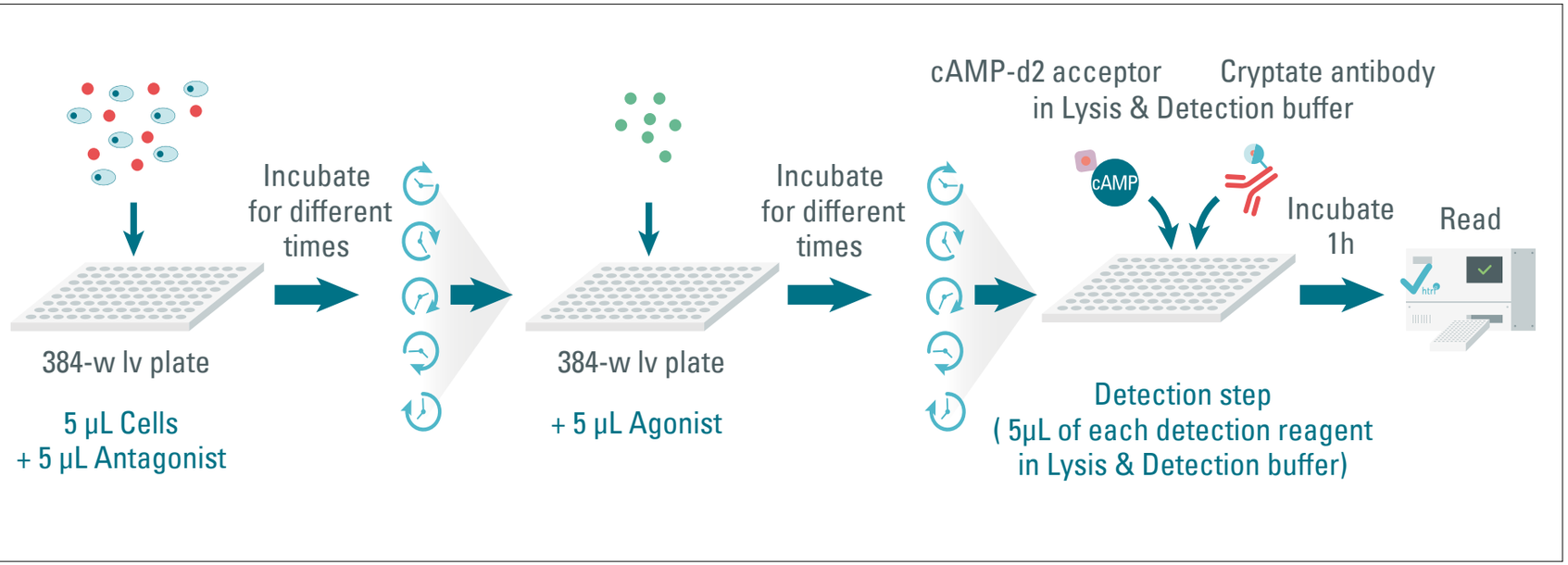
GαS AGONIST ASSAY OPTIMIZATION
Stimulation Time

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

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



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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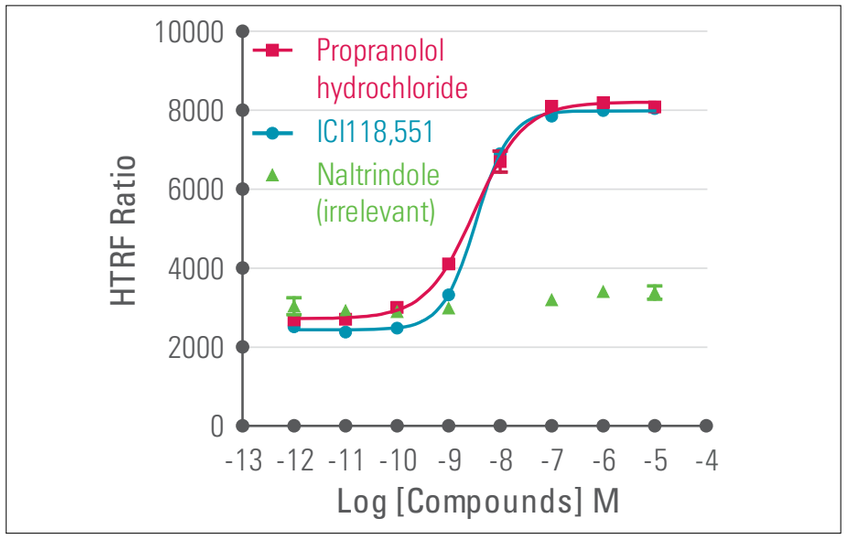
GαS AGONIST ASSAY OPTIMIZATION

Antagonist Dose Response

In this example, an EC_{90} concentration of isoproterenol was used to stimulate cells pre-incubated with either Propanolol hydrochloride or LcL118,551, 2 known antagonists of the Beta 2 adrenergic receptor.



	ICL118,551	PROPRANOLOL	NALTRINDOLE (DELTA OPIOID ANTAGONIST)
IC_{50} (nM)	3.4	3.3	none

Antagonist screening (Beta2 AR receptor): HEK293 cells were dispensed at 7,500 cells/well. Tested compounds were added and incubated 45 min at 37°C. The agonist isoproterenol were added at 80 nM (EC_{90}), and incubated 45 min at 37°C. Dose-response curves were plotted to obtain the TC50 values of the antagonists Beta2 adrenergic receptor antagonists ICI118,551 and propranolol have the same potency. while naltrindole was used as a negative control.

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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.

EnVision® Nexus™

Provides lightning speed and superior sensitivity across all established detection technologies with advanced options for ultimate performance. It is the next generation of high-throughput screening, ideal for your most demanding assays.

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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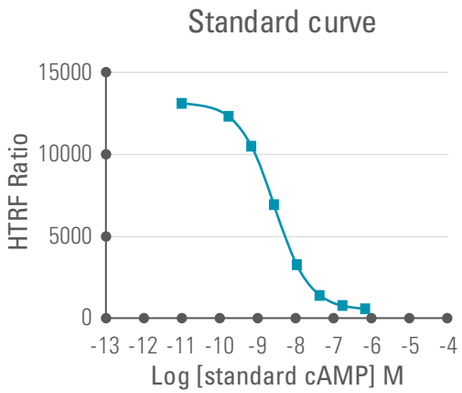
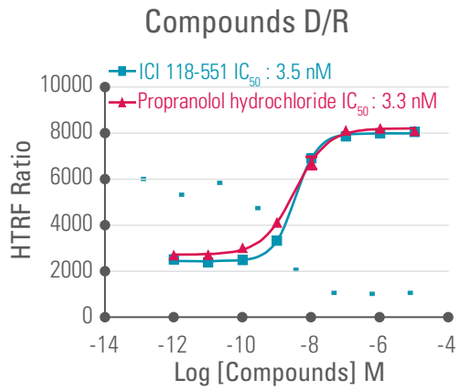
DATA REDUCTION AND ANALYSIS

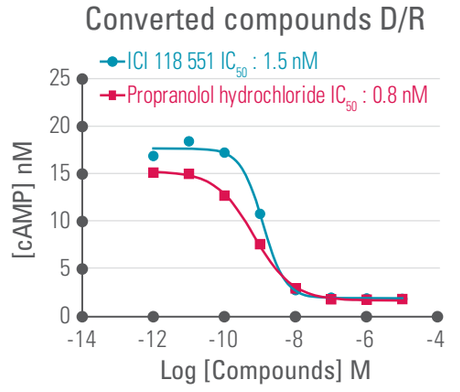
Data Reduction And Analysis

The fluorescence resonance energy transfer ratio (665 nm/620 nm) measurements could be converted into cAMP concentration-response curves to determine the IC_{50} 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 an inhibition dose response, revealing the compound as an antagonist. From the inhibition dose response, the IC_{50} can be determined.





Data conversion



STANDARD CURVE	
log [cAMP]	HTRF Ratio
-12	13098
-11	12306
-10	10479
-9	6923
-8	3258
-7	1384
-6	770
-5	566

CELL BASED		
Log [Antagonist] M	HTRF Ratio	
	Propranolol	ICI-118 551
-12	2690	2510
-11	2705	2377
-10	3002	2479
-9	4103	3321
-8	6698	6892
-7	8099	7849
-6	8185	7986
-5	8082	8034

CELL BASED		
Log [Antagonist] M	[cAMP] nM	
	Propranolol	ICI-118 551
-12	15.16	16.91
-11	15.02	18.44
-10	12.73	17.25
-9	7.63	10.83
-8	2.98	2.79
-7	1.85	2.02
-6	1.79	1.92
-5	1.86	1.89

Antagonist mode optimization (Beta2 AR receptor). HEK293 cells at 7,500 cells/well (optimal cellular concentration situated in the dynamic range of the kit) were treated with a serial concentration of the isoproterenol, a beta 2 adrenergic receptor full-agonist. The EC_{80}-EC_{90} is determined to choose the optimal agonist concentration to use for the antagonist mode experiment. Fitting the serial dilution of the antagonist allows IC_{50} calculation of the compounds (potency).

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