MANUAL

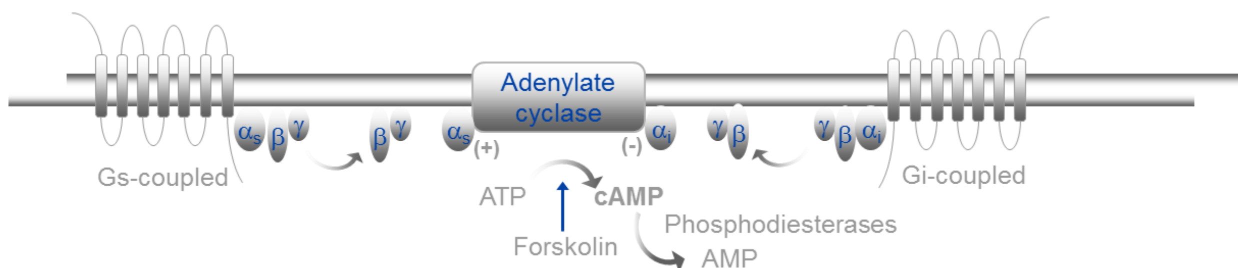
AlphaScreen™ cAMP User Manual and Assay Development Guide

Catalog numbers: 6760635D, 6760635M, 6760635R

Research Use Only.

1. Introduction

The AlphaScreen™ cAMP kit is intended for the quantitative determination of 3',5'-cyclic adenosine monophosphate (cAMP) in cell lysate and cellular membrane samples. cAMP is one of the most important second messengers, mediating diverse physiological responses of neurotransmitters, hormones, and drugs. Intracellular concentration of cAMP is tightly regulated by two membrane-bound enzymes, adenylyl cyclases and phosphodiesterases, as illustrated below. Adenylyl cyclases promote the synthesis of cAMP from adenosine triphosphate (ATP), while phosphodiesterases degrade cAMP to AMP. The activity of adenylyl cyclases is controlled through various G-protein-coupled receptors (GPCRs), via their interaction with one of two distinct GTP binding protein classes, G_s and G_i. These G proteins are heterotrimeric molecules composed of the subunit G_α (s or i), G_β and G_γ. Agonist activation of GPCRs leads to the dissociation of the trimer into –G_α-GTP on one side and the $G_{\beta\gamma}$ dimer on the other side. Upon dissociation, $G_{\alpha s}$ is primarily involved in adenylyl cyclase stimulation, leading to the production of cAMP, whereas $G_{\alpha i}$ is inhibitory, leading to a decrease in cAMP synthesis. The measurement of intracellular cAMP is thus an ideal method for measuring the effect of test compounds on GPCR-mediated adenylyl cyclase activation or inhibition.



2. Provided reagents

Kit Components	6760635D 1,000 points*	6760635M 10,000 points*	6760635R 50,000 points*
cAMP standard, 50 µM	1 vial, 1 mL	1 vial, 1 mL	1 vial, 1 mL
Biotin-cAMP tracer, solid**	1 vial, 10 nmol	1 vial, 10 nmol	1 vial, 65 nmol
Anti-cAMP AlphaScreen Acceptor Beads, 5 mg/mL in 1X PBS, 0.05% Proclin-300 pH 7.2	1 vial, 100 µL	1 vial, 1 mL	1 vial, 5 mL
Streptavidin Donor Beads, 5 mg/mL in 1X PBS, 0.05% Proclin-300 pH 7.2	1 vial, 100 µL	1 vial, 1 mL	1 vial, 5 mL
10X Immunoassay Buffer* reorder as AL000C or AL000F**	1 vial, 2 mL	1 bottle, 10 mL	1 bottle, 100 mL

Important note: For maximum recovery of products, centrifuge all original vials prior to removing the caps. Resuspend the beads by pipetting before use.

* When using the recommended protocols (25-µL assay in 384-well microplates).

** Tracer is supplied as a solid. Solid may not be visible deposited along the walls of the tube. For reconstitution instructions, see section 7.4.

***10X Immunoassay Buffer should be diluted to 1X fresh for each assay and only used for the preparation of the biotin-cAMP tracer and Streptavidin Donor Bead detection mix, as it contains detergents which will lyse cells in the assay (see section 8.5 for details)

3. Product information

Antibody/protein: The cAMP antibody is a rabbit monoclonal antibody highly specific for cyclic AMP. Its cross-reactivity with cGMP is < 0.005%.

Stability: This product is stable for at least **6 months** from the manufacturing date if used and stored under recommended conditions.

Storage Conditions: Store undiluted at 4°C protected from light. Freeze-thaw is not recommended and can cause the beads to form aggregates.

4. Assay principle

The AlphaScreen cAMP assay has been designed to measure levels of cAMP produced upon modulation of adenylate cyclase activity by GPCRs. The assay is based on the competition between endogenous cAMP and exogenously added biotinylated cAMP. The capture of cAMP is achieved by using a specific antibody conjugated to Acceptor beads. In the absence of free (endogenous) cAMP, maximal Alpha signal is achieved (Figure 1, left panel). Free cAMP produced by stimulated cells competes with the biotin-cAMP tracer for the binding to the anti-cAMP AlphaScreen Acceptor beads, causing a decrease in Alpha signal (Figure 1, right panel). The assay is efficient at measuring both agonist and antagonist activities on Gαi- and Gαs-coupled GPCRs. Gαs and Gαi subunits act through the cAMP pathway by respectively activating or inhibiting adenylate cyclase, an enzyme catalyzing the conversion of ATP to cAMP.

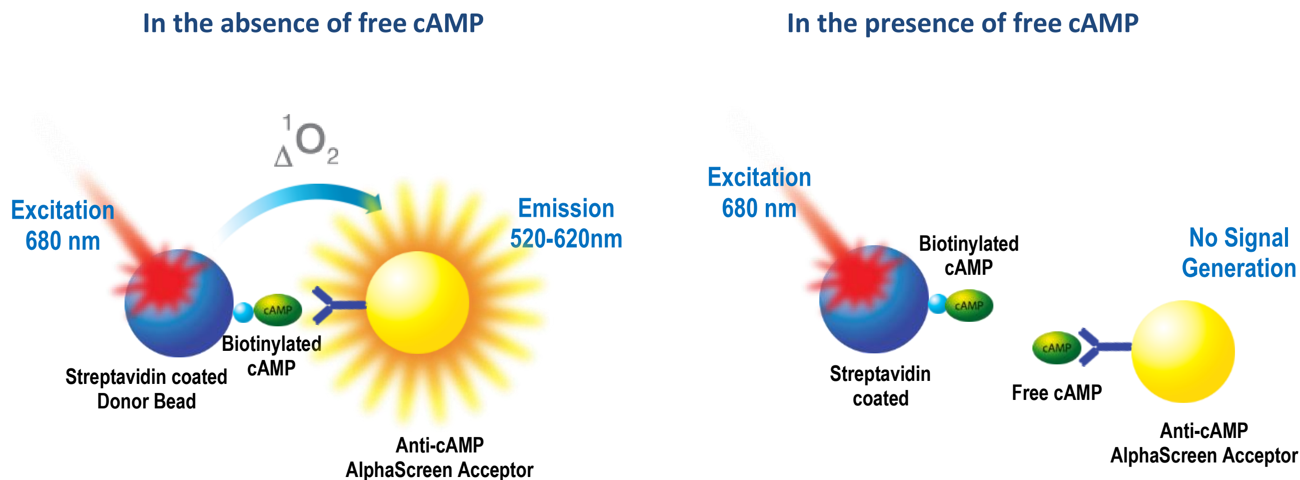


Figure 1. AlphaScreen cAMP assay principle

The current Assay Development Guidelines describe procedures to perform AlphaScreen cAMP assays in 384-well microplates in a total assay volume of 25 µL. Assays can also be performed in 96- and 1536-well plate formats, providing all assay component concentrations and volumes are scaled up or down proportionally. AlphaScreen cAMP assays can be performed using either cells (attached or in suspension) or cell membrane preparations.

5. Recommended reagents not supplied in the kit

Item	Recommended source	Product no.
DPBS (1X), without Ca & Mg	Invitrogen™	14190
Hank's Balanced Salt Solution (HBSS) (1X)	Invitrogen™	14025
HEPES Buffer Solution (1 M) pH 7.2 to 7.5	Teknova	H1035
BSA	Sigma®	A7284
IBMX	Sigma®	I5879
Forskolin	Calbiochem	344270
DMSO	Sigma®	D8418
Cell Dissociation Solution, enzyme free	Sigma®	C5914
OptiPlate™-384, white	revvity	6007290 (pack of 50)
AlphaPlate™-384, light gray	revvity	6005350 (pack of 50)
ProxiPlate™-384 Plus, white	revvity	6008280 (pack of 50)
AlphaPlate-384, light gray, Shallow Well	revvity	6008350 (pack of 50)
½ AreaPlate-96, white	revvity	6005560 (pack of 50)
OptiPlate-1536, white	revvity	6004290 (pack of 50)
TopSeal™-A PLUS	revvity	6050185 (pack of 100)
Microplate lid, black	revvity	6000023 (pack of 200)

6. Precautions

- AlphaScreen Donor beads are light sensitive and should be handled under subdued laboratory lighting. Lighting should be under 100 Lux or alternatively green filters (Roscolux Chroma Green #389) can be applied to light fixtures. Any incubation involving the Donor beads should be performed in the dark. Plates can be covered by a microplate lid (Revvity cat. #6000023) to minimize the effect of light.
- The small volumes of the assay are prone to evaporation. It is recommended that microplates be covered with TopSeal-A Plus adhesive sealing film to reduce evaporation during incubation. Microplates can be read with the TopSeal-A Plus in place.
- Beads should be stored in the dark at 4°C. It is recommended not to store reagents containing BSA for longer than 1 day at 4°C.

7. Reagent Preparation

7.1 IBMX

When stimulating cells, it is recommended to add a phosphodiesterase inhibitor, in order to prevent cAMP degradation into AMP by cellular phosphodiesterases, and to lead to some cAMP accumulation, which improves the quality of the assay. Typically, 3-isobutyl-1-methylxanthine (IBMX) is used as a broad phosphodiesterase inhibitor. IBMX presents some structure homologies with cAMP, but the antibody used in the assay is very specific for cAMP and, at the IBMX concentration used, the assay will not be impacted by the presence of IBMX, as shown in Figure 2 below. Alternatively, more specific phosphodiesterase inhbitors, such as rolipram, which inhibits the cAMP specific phosphodiesterase-4 could be used in place of IBMX. No interference was observed when using up to 1 mM of rolipram in the stimulation buffer.

Dissolve 100 mg in 900 µL DMSO to give a 500 mM stock solution. Aliquot and store at -20°C; use as required.

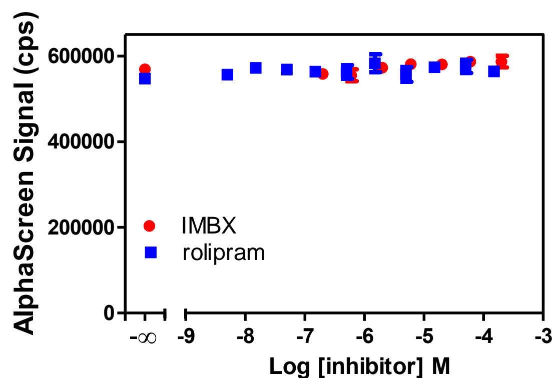


Figure 2. AlphaScreen cAMP assay tested in the presence of common phosphosdiesterase inhibitors. AlphaScreen cAMP assay components were incubated with increasing concentrations of IBMX (red circles) or rolipram (blue squares). No decrease in AlphaScreen signal was observed, indicating these compounds are not recognized by the anti-cAMP antibody.

7.2 Stimulation Buffer

The recommended Stimulation Buffer for cell-based assays is **1X HBSS, 5 mM HEPES, 0.5 mM IBMX, 0.1% BSA**. Make fresh. (use protease-free BSA if using peptide or protein receptor agonists)

To prepare 20 mL of Stimulation Buffer, add the following to a tube:

- 19.81 mL of 1X HBSS (Invitrogen, cat. #14025-092)
- 100 µL of 1M HEPES, pH 7.5 (Teknova, cat. # H1035)
- 20 µL of 500 mM IBMX dissolved in DMSO (Sigma, cat. # I5879)
- 66.7 µL of 30% BSA solution (Sigma, cat. #D8418)

7.3 AlphaScreen Acceptor bead preparation in Stimulation Buffer

For acceptor bead standard curve mix: Prepare a 5X mix (100 µg/mL) of anti-cAMP AlphaScreen Acceptor beads in stimulation buffer by making a 1:50 dilution of the Acceptor bead stock solution.
Example: Add 5 µL of the Acceptor bead stock solution to 245 µL of Stimulation Buffer and mix gently.

For cell/acceptor bead suspension mix: Prepare a 10X mix (200 µg/mL) of anti-cAMP AlphaScreen acceptor beads in stimulation buffer by making a 1:25 dilution of the Acceptor bead stock solution.
Example: Add 10 µL of the Acceptor bead stock solution to 240 µL of Stimulation Buffer and mix gently.

For additional details of cell preparation, see Section 8.

7.4 Biotin-cAMP resuspension

Dissolve the biotinylated cAMP tracer in 1X PBS (Invitrogen, Cat# 14190) to make a 10 µM stock solution. Be sure the PBS is freshly opened and free of contaminants. Further dilute 10X in 1X PBS to obtain a working solution of 1 µM.

Example: For 10 nmol vial of biotin-cAMP, add 1 mL of 1X PBS to obtain a 10 µM solution. Add 10 µL of 10 µM stock solution to 90 µL of 1X PBS to obtain a 1 µM solution. Mix gently.

Reconstituted biotin-cAMP can be stored long term at either 10 µM or 1 µM at 4°C for up to 6 months.

7.5 Biotin-cAMP and Streptavidin Donor bead detection mix in Immunoassay buffer

Note: This step should be performed in subdued lighting (100 lux) or under green filters. Once diluted, the donor bead preparations should be kept away from light.

Prepare 1X Immunoassay buffer (Revvity AL000) by diluting the 10X stock.
Example: Add 0.5 mL of 10X stock to 4.5 mL of dH_2O

The detergent present in the 1X Immunoassay buffer is sufficient to lyse cells. If desired, alternate buffers can be used for biotin-cAMP/Streptavidin Donor bead Detection Mix preparation (and lysis), such as: 5 mM HEPES containing 0.1% BSA and 0.3% Tween-20, pH 7.4.

Prepare a 1.67X mix of biotin-cAMP (41.7 nM) and Streptavidin Donor beads (33.3 µg/mL) in 1X Immunoassay Buffer by diluting the 1 µM biotin-cAMP stock 1:24 and the Donor bead stock 1:150.
Example: Add 50 µL of biotin-cAMP and 8 µL of Streptavidin Donor beads to 1.142 mL of 1X Immunoassay Buffer and immediately vortex gently.

Prepare this mixture fresh and incubate 30 minutes in the dark at room temperature before adding to the assay plate, preferably during cell stimulation.

8. Cell Preparation

For best results, we recommend working with cells grown to ~70-90% confluency and showing at least 95% viability.

For cell suspension assays:
Remove growth media and briefly rinse with 1X PBS without calcium and magnesium. Add 37°C warmed cell dissociation solution (Sigma, cat# C5914) and incubate at 37°C for ~5 minutes to help detach the cells. Collect cells and centrifuge for 5 minutes at 275 x g. Remove supernatant and resuspend the cell pellet in 1X PBS. Determine cell concentration. Re-centrifuge for 5 minutes at 275 x g and remove the supernatant. Resuspend the cells in Stimulation Buffer to a final concentration of 10,000 cells/µL (10 x 10^6 cells/mL). ***Prepare fresh prior to assay.***

When using ready to use frozen cAMPZEN cells (www.revvity.com): thaw (or let cells recover in culture medium when indicated) as recommenced, centrifuge and resuspend the cells in Stimulation Buffer.

For adherent cell assays:
Plate cells at desired density using preferred growth media at least 12-18 h prior to assay start. Before running assay, it is recommended to wash cells 2-3 times in Stimulation Buffer or similar (HBSS + 0.1% BSA, pH 7.4) and incubate cells in Stimulation Buffer for at least 20 minutes at 37°C prior to stimulation. See Section 18 for more details on adherent cell assays.

9. Assay development workflow

AlphaScreen cAMP assays are developed and optimized following the assay development workflow described in the table below.

By default, a standard stimulation time of 30 min at room temperature is used for all cell types, but this may be optimized if desired in specific situations.

Step	$G_{\alpha s}$-coupled Receptor Assay	$G_{\alpha i}$-coupled Receptor Assay	Purpose
1	cAMP standard curve	cAMP standard curve	To determine the sensitivity (IC_{50} value) and dynamic range (IC_{10} – IC_{90}) of the cAMP assay and to extrapolate the amount of cAMP produced by the cell in the assay.
2	Known full-agonist or forskolin concentration-response experiment at different cell densities	Forskolin concentration-response experiment at different cell densities.	To define the optimal cell density giving the highest assay window while staying within the assay dynamic range. For $G_{\alpha i}$ assays: define the $EC_{80\text{-}90}$ concentration of forskolin to be used for the agonist assay.
3	Agonist concentration-response	Agonist concentration-response (using $EC_{80\text{-}90}$ forskolin).	To check pharmacology (agonist potency and efficacy, etc.) and to determine the $EC_{50\text{-}90}$ concentration of selected agonist to be used for the antagonist assay.
4	Antagonist concentration-response (using $EC_{50\text{-}90}$ agonist).	Antagonist concentration-response (using $EC_{80\text{-}90}$ forskolin + $EC_{50\text{-}90}$ agonist).	To determine antagonist pharmacology: potencies, surmountable antagonism or not, etc...

10. Sample assay protocols for a 384-well plate (total assay volume of 25 µL)

In the protocols described in the table below, both the cells and tested compounds must be prepared in Stimulation Buffer (including 0.5 mM IBMX). Immunoassay Buffer must be used only for the preparation of biotin-cAMP tracer and Streptavidin Donor Bead detection mix as it contains detergent and will lyse cells.

Important note: These protocols differ slightly from the instructions listed on the Certificate of Analysis (CoA). The protocol listed on the CoA is recommended for QC purposes only and should be used to verify all kit components are working satisfactorily.

cAMP standard curve	Gs Agonist	Gs Antagonist	Gi Forskolin titration	Gi Agonist	Gi Antagonist
5 µL Acceptor Bead standard curve mix	5 µL cells/ Acceptor Beads suspension mix	5 µL cells/ Acceptor Beads suspension mix	5 µL cells/ Acceptor Beads suspension mix	5 µL cells/ Acceptor Beads suspension mix	5 µL cells/ Acceptor Beads suspension mix
5 µL cAMP standard	5 µL 2X Agonist	2.5 µL 4X Antagonist	5 µL 2X Forskolin	2.5 µL 4X Agonist	2.5 µL 4X Antagonist
-	-	2.5 µL 4X Agonist	-	2.5 µL 4X Forskolin	2.5 µL 4X Forskolin/Agonist
Incubate 30 min at room temperature*					
15 µL 1.67X biotin-cAMP/Streptavidin Donor Bead Detection Mix (pre-incubated 30 min at room temperature)					
Incubate 1 h at room temperature*					
Read on an Alpha-enabled Reader (EnSpire®, EnVision®, or EnSight®)					

*Cover plate with TopSeal-A Plus during incubations

NOTE: For 96 and 1536-well formats, adjust proportionally the volume of each assay component in order to maintain the volume ratios for the 384-well format. Do not modify the biotin-cAMP and/or the Donor and/or Acceptor bead final concentrations.

Final Assay Concentrations are:

- **20 µg/mL anti-cAMP AlphaScreen Acceptor beads**
- **25 nM biotin-cAMP**
- **20 µg/mL Streptavidin Donor beads**

11. Generating a cAMP standard curve

The cAMP standard curve allows determining assay sensitivity (IC_{50} value) and dynamic range (IC_{10} – IC_{90}). It also provides a means to translate the measured AlphaScreen signal into actual quantities of cAMP produced in cell- or membrane-based assays. It should always be prepared in the same manner and in the same matrix as the test samples for accurate determination of cAMP concentration in each well. For example, to be able to use the cAMP standard curve for interpolation of the measurements performed on cell lysates, the cAMP standard curve should be serially diluted in stimulation buffer. In case cells, at the cell density used, would have a significant matrix effect, a lysate from cells having the lowest cAMP level possible (like unstimulated cells) should be added to the stimulation buffer used for cAMP standard dilution. See section 11.3 below which demonstrates the importance of standard curve preparation matching the matrices of the sample being tested.

11.1 Reagent preparation

11.1.1 Stimulation Buffer

Prepare 20 mL, as described in Section 7.2.

11.1.2 cAMP standard serial dilutions (5X) in Stimulation Buffer

Prepare the **5X cAMP standard serial solutions** from the 50 µM cAMP standard supplied with the kit by making half-log dilutions in Stimulation Buffer, as indicated in the table below. Include a “no cAMP” control (dilution #12). Final cAMP concentration in the assay will range from 1 µM to 10 pM. **Prepare fresh prior to assay**.

Dilution	[Final] (M)	[5X] (M)	Volume of dilution	Stimulation Buffer
1	1×10^{-6}	5×10^{-6}	10 µL of 50 µM cAMP	90 µL
2	3×10^{-7}	1.5×10^{-6}	30 µL of 1	70 µL
3	1×10^{-7}	5×10^{-7}	30 µL of 2	60 µL
4	3×10^{-8}	1.5×10^{-7}	30 µL of 3	70 µL
5	1×10^{-8}	5×10^{-8}	30 µL of 4	60 µL
6	3×10^{-9}	1.5×10^{-8}	30 µL of 5	70 µL
7	1×10^{-9}	5×10^{-9}	30 µL of 6	60 µL
8	3×10^{-10}	1.5×10^{-9}	30 µL of 7	70 µL
9	1×10^{-10}	5×10^{-10}	30 µL of 8	60 µL
10	3×10^{-11}	1.5×10^{-10}	30 µL of 9	70 µL
11	1×10^{-11}	5×10^{-11}	30 µL of 10	60 µL
12 (ctrl)	0	0	-	70 µL

11.1.3 AlphaScreen Acceptor bead preparation in Stimulation Buffer

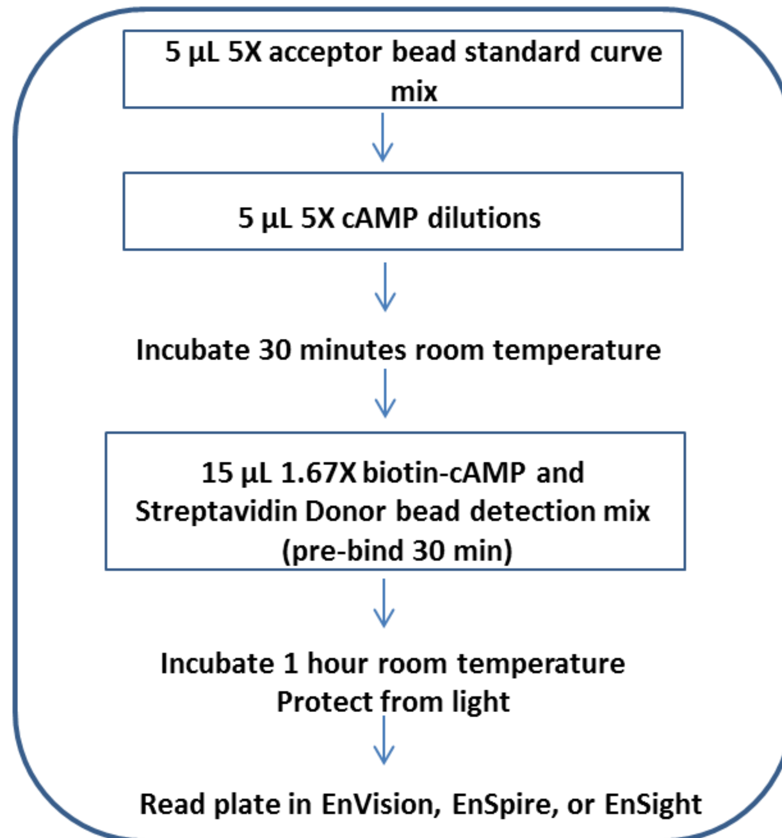
Prepare as described in Section 7.3.

11.1.4 Biotin-cAMP and Streptavidin Donor bead detection mix in Immunoassay buffer

Prepare as described in Section 7.5.

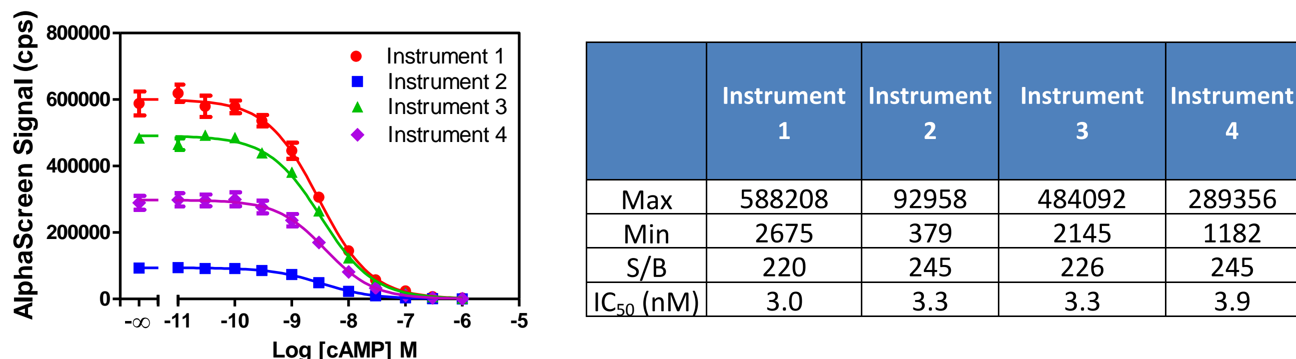
11.1.5 Assay Flowchart

To a white, opaque OptiPlate-384 microplate, add in triplicate wells:



11.2 Representative cAMP standard curves

Representative AlphaScreen cAMP standard curves obtained on different instruments are shown in Figure 3.



	Instrument 1	Instrument 2	Instrument 3	Instrument 4
Max	588208	92958	484092	289356
Min	2675	379	2145	1182
S/B	220	245	226	245
IC_{50} (nM)	3.0	3.3	3.3	3.9

Figure 3. Representative AlphaScreen cAMP standard curves obtained on different Alpha-capable plate readers. Assay reagents were incubated in a white opaque OptiPlate™-384 microplate for 1 hour at room temperature. **Left panel**: representative standard curves; **Right panel**: summary table of values obtained from standard curves.

Observations:

While total AlphaScreen signal will vary from instrument to instrument, assay sensitivity, dynamic range, and S/B should be comparable regardless of overall total counts.

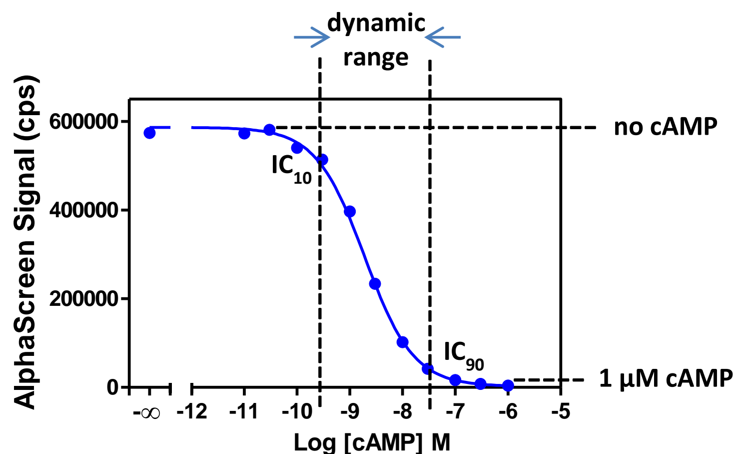


Figure 4. Illustration of dynamic range determination

As expected for a competitive immunoassay, the AlphaScreen cAMP standard curve exhibits a sigmoidal relationship between the log of the cAMP concentrations and the Alpha signal (Figure 4). This type of curve is best fit using a four-parameter logistic equation, with weighting of $1/Y^2$, which optimizes accuracy and precision over the maximum usable calibration range.

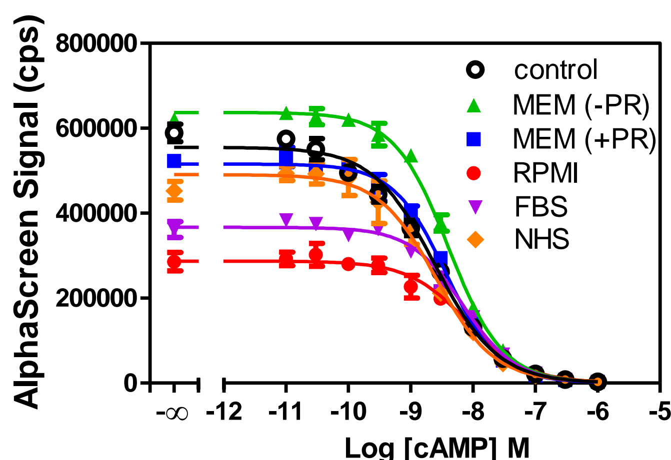
Since cAMP standard curves are non-linear, it is common among users to consider as dynamic range only the apparent “linear” portion. Forskolin and agonist are prepared in Stimulation Buffer as a single 4X working solution. We recommend adding both forskolin and agonist for cell stimulation at concentrations producing 90%

of their maximal effect (EC_{90} concentrations) in order to obtain a maximal assay window while minimizing inter-day data variability of the cAMP standard curve (section of the standard curve between the IC_{20} to IC_{80}). However, because data are fitted by non-linear regression analysis, there is no need to restrict the assay dynamic range to the "linear" portion of the standard curve.

In fact, the use of a four-parameter logistic equation allows extending significantly the assay dynamic range. For this reason, we operationally defined the assay dynamic range throughout this document as the section of the standard curve located between the IC_{10} to IC_{90}.

11.3 Effect of sample matrices on standard curve

As discussed in section 11.1 above, it is important to prepare a standard curve in the same manner as the samples to be tested. This is to ensure that the Alpha counts generated by the sample can be translated into actual quantities of cAMP produced. In Figure 5 below, standard curves were prepared with the addition of different matrices to represent different sample types.



	Control	MEM (-PR)	MEM (+PR)	RPMI	FBS	NHS
Max counts	588854	620869	522907	285898	361738	405090
Min counts	3047	3461	2764	3852	3266	4404
S/B	193.3	179.4	189.2	74.2	110.8	92.0
IC_{50} (nM)	2.5	4	3.7	8.2	6.9	2.6

Figure 5. Matrix effect on AlphaScreen cAMP standard curves. Top panel: Standard curves performed in the presence of 5 µL stimulation buffer (control, open black circles), MEM media lacking phenol red (MEM (-PR), green triangles), MEM media containing phenol red (MEM (+PR), blue squares), RPMI media (RPMI, red circles), fetal bovine serum (FBS, purple inverted triangles), and normal human serum (NHS, orange diamonds). **Bottom panel**: summary table of values obtained from standard curves in top panel.

Observations:

Different sample matrices will affect overall AlphaScreen counts and assay window (S/B). In order to accurately quantitate the amount of cAMP being produced it is important to use the same conditions for the standard curve that represent the sample type.

12. Determination of optimal cell density for Gαs- and Gαi-coupled receptors

Like in any immunoassay, determination of the optimal cell density is a key step of AlphaScreen cAMP assay development. Selection of an inappropriate cell density can result in:

- Decreased assay window (S/B ratio) due to either high "minimal" signal at high forskolin concentrations (typically due to the use of a too low cell density) or low "maximal" signal (typically due to the use of a too high cell density, which increases basal cAMP levels).
- Distortion in EC_{50} and IC_{50} values, due to the abnormal compression of the upper or lower part of the cellular response when an inappropriate cell number is used

Forskolin dose-response curves are generated at different cell densities in order to establish the optimal cell density to be used in cAMP assays. Forskolin acts directly on the adenylyl cyclase to produce cAMP, independently of receptor activation. Due to the competitive nature of the assay, a **decrease** in Alpha signal is observed following cell stimulation with forskolin. The amount of cAMP produced at a saturating forskolin concentration generally represents the maximum amount of cAMP that can be produced by the cells.

At the optimal cell density, the forskolin dose-response curve typically gives the highest assay window (S/B ratio) while staying within the assay dynamic range. We recommend performing forskolin dose-response curves using 500 to 10,000 cells per well in a 25-µL assay.

For $G_{\alpha s}$-coupled receptors, the optimal cell density can also be determined by performing dose- response experiments with a full agonist, rather than forskolin.

Assays for $G_{\alpha i}$-coupled receptors typically require forskolin stimulation in order to be able to measure the inhibition of adenylate cyclase activity induced by an agonist. The forskolin dose-response curve at the optimal cell density allows determining the forskolin concentration that will be used for stimulating cAMP production in agonist and antagonist assays for $G_{\alpha i}$-coupled receptors (see Section 16 and 17). For most cell types, a concentration around 10 µM (generally 5 to 20 µM) can be used.

12.1 Reagent preparation

12.1.1 IBMX

Prepare as described in Section 7.1.

12.1.2 Forskolin

Dissolve 5 mg forskolin in 244 µL of Ethanol to make up the required 50 mM stock solution. Aliquot and store at -20°C.

12.1.3 Stimulation Buffer

Prepare 20 mL, as described in Section 7.2.

12.1.4 Cell dilutions

Prepare a 1:1 mix of cell suspension prepared in Section 8 and 10X Acceptor bead mix prepared in Section 7.3

An example of different dilution schemes is shown in the table below, where 250 µL of each mix can be prepared (enough for one 12 pt. curve, in triplicate).

Cells/well (final)	10,000 cells/µL solution (µL)	Stimulation Buffer (µL)	10X Acceptor bead working solution (µL)
10000	50	75	125
3000	15	110	125
1000	5	120	125
300	15*	110	125
0	0	125	125

*In order to avoid large variations from well to well, for 300 cells/well preparation, we first recommend pre-diluting the 10,000 cells/µL solution to 1,000 cells/µL, and taking 15 µL from this pre-dilution to prepare the acceptor bead working solution

12.1.5 Forskolin dilutions (2X) in Stimulation Buffer

- Thaw the 50 mM forskolin stock solution (see Section 10.1.2 for stock solution preparation).
- Prepare a 500 µM working dilution by adding 5 µL of the 50 mM stock solution to 495 µL Stimulation Buffer
- Make serial dilutions from the 500 µM forskolin working dilution in Stimulation Buffer to obtain 2X intermediate solutions ranging from 2 X 10^{-4} to 2 X 10^{-9} M in half-log intervals, as indicated in the table below. Include a “no forskolin” control (dilution #12). **Prepare fresh prior to assay.**

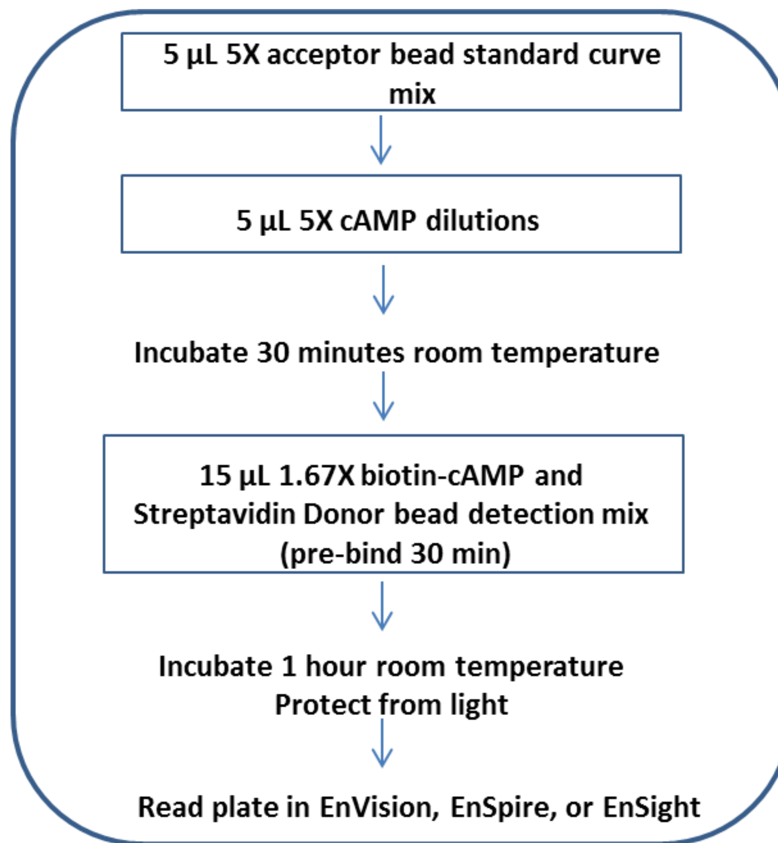
Dilution	[Final] (M)	[2X] (M)	Volume of dilution	Stimulation Buffer
1	1 X 10^{-4}	2 X 10^{-4}	160 µL of 500 µM	240 µL
2	3 X 10^{-5}	6 X 10^{-5}	120 µL of 1	280 µL
3	1 X 10^{-5}	2 X 10^{-5}	120 µL of 2	240 µL
4	3 X 10^{-6}	6 X 10^{-6}	120 µL of 3	280 µL
5	1 X 10^{-6}	2 X 10^{-6}	120 µL of 4	240 µL
6	3 X 10^{-7}	6 X 10^{-7}	120 µL of 5	280 µL
7	1 X 10^{-7}	2 X 10^{-7}	120 µL of 6	240 µL
8	3 X 10^{-8}	6 X 10^{-8}	120 µL of 7	280 µL
9	1 X 10^{-8}	2 X 10^{-8}	120 µL of 8	240 µL
10	3 X 10^{-9}	6 X 10^{-9}	120 µL of 9	280 µL
11	1 X 10^{-9}	2 X 10^{-9}	120 µL of 10	240 µL
12 (ctrl)	0	0	-	280 µL

12.1.6 Biotin-cAMP and Streptavidin Donor bead detection mix in Immunoassay buffer

Prepare as described in Section 7.5.

12.1.7 Assay Flowchart

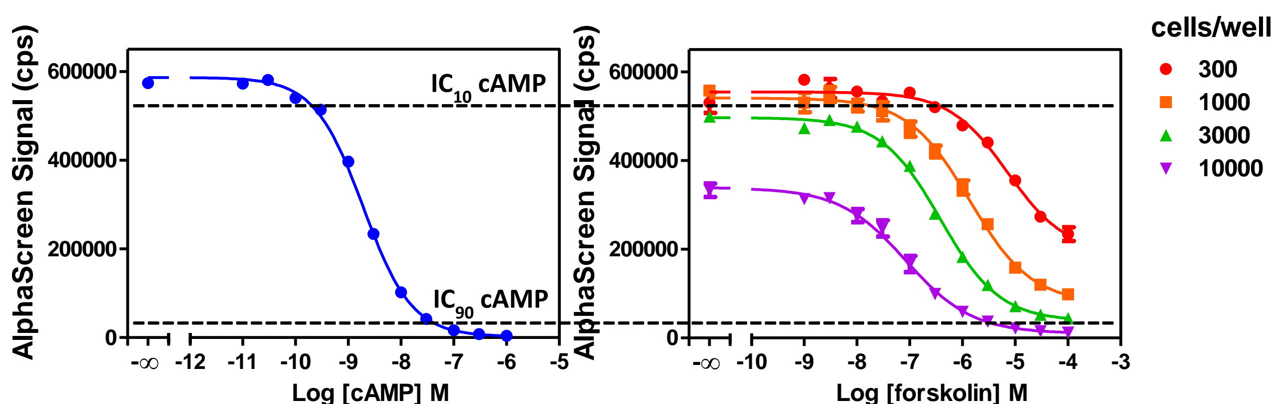
To a white, opaque OptiPlate-384, add in triplicate:



12.2 Interpreting forskolin and cell cross-titration data

12.2.1 Optimal cell density

AlphaScreen signal is plotted against the logarithmic values of forskolin concentrations for each cell density tested. The forskolin dose-response curves obtained are related to the cAMP standard curve performed in parallel in order to establish which cell number provides a response that fits in the dynamic range of the cAMP standard curve (operationally defined as IC_{10} – IC_{90}). This typically corresponds to the cell density giving the highest S/B ratio calculated using the maximal signal (untreated cells) and the minimal signal obtained with a saturating concentration of forskolin (fully activated cells), as shown in Figure 6.



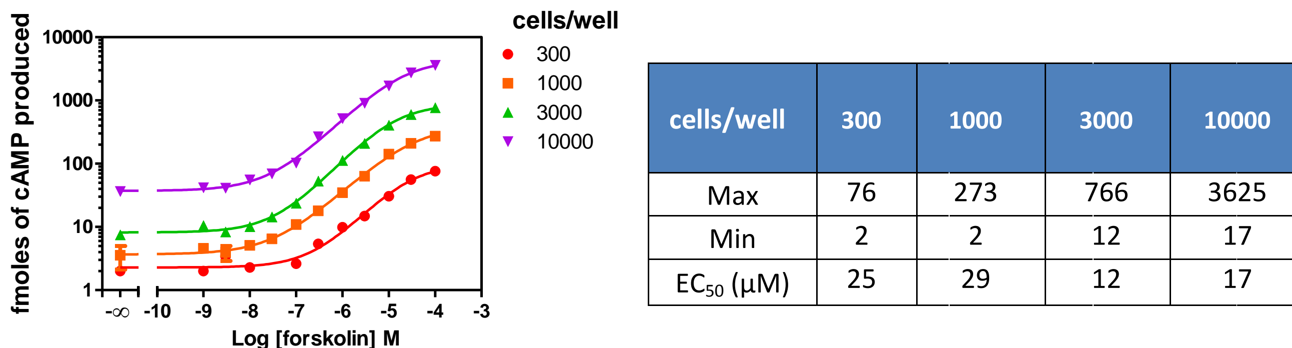
cells/well	300	1000	3000	10000
Max	530611	557576	499286	332863
Min	234134	98098	44333	12367
S/B	2.3	5.7	11.3	26.9
EC_{50} (µM)	7.3	1.4	0.4	0.1

Figure 6. Determination of optimal cell density. Top left panel: cAMP standard curve; **Top right panel**: cell and forskolin cross-titration. **Bottom panel**: summary table of values obtained from forskolin cross-titration curves. CHO-K1 cells expressing the 5HT1a serotonin receptor are used for this example.

Observations:

From the above example, the preferred cell concentration for subsequent experiments (ex. agonist effect determinations) would be 3,000 cells/well as the majority of the forskolin dose-response curve at this cell concentration falls within the linear region of the cAMP standard curve

It is also recommended to convert the AlphaScreen data into cAMP production values by interpolating from a standard curve run in parallel (to achieve this, cAMP concentrations are usually expressed as fmoles per well). Then, forskolin dose-response curves at different cell densities are re-plotted in terms of cAMP data, as shown in Figure 7.



cells/well	300	1000	3000	10000
Max	76	273	766	3625
Min	2	2	12	17
EC_{50} (µM)	25	29	12	17

Figure 7. Forskolin dose-response curves at different cell densities. Data from Figure 6 (right panel) were re-plotted as fmoles of cAMP produced per well by interpolating from a cAMP standard curve run in parallel (Figure 6, left panel).

Observations:

EC_{50} values for forskolin (10-30 µM) are comparable regardless of the cell density used. This is a clear indication that at all cell densities tested the AlphaScreen cAMP assay are reasonable not out of the assay dynamic range.

It is to be noticed that S/B and EC50 values differ whether they are calculated from the raw alpha counts, or from the cAMP concentration converted values. This is not unexpected, as in the dynamic range of the standard curve the alpha counts vary more or less linearly in proportion to the log of the cAMP concentration (standard curve is a log-linear graph), i.e. this is not a true linear relationship (where linearity would be observed for a log/log or linear/linear graph). The "true" biologically relevant pharmacological value is obtained from the cAMP concentration converted curve.

13. Gαs-coupled receptor stimulation/Agonist dose-response curve

Agonist stimulation of cells or membranes expressing $G_{\alpha s}$-coupled receptors **increases cAMP production**. As a result, a concentration-dependent **decrease** in Alpha signal is observed. Agonist potency (EC_{50} value) is determined by performing a dose-response curve at the optimal cell density.

13.1 Reagent preparation

13.1.1 Stimulation Buffer

Prepare 20 mL, as described in Section 7.2.

13.1.2 Agonist dilutions (2X) in Stimulation Buffer

Agonist dilutions are prepared in Stimulation Buffer as 2X intermediate solutions. Ideally, the dilution series should cover concentrations that are 3 log units higher and lower than the expected agonist EC_{50} value. Include 11 half-log agonist dilutions and a "no agonist" control.

13.1.3 Acceptor bead and Cell suspension mix

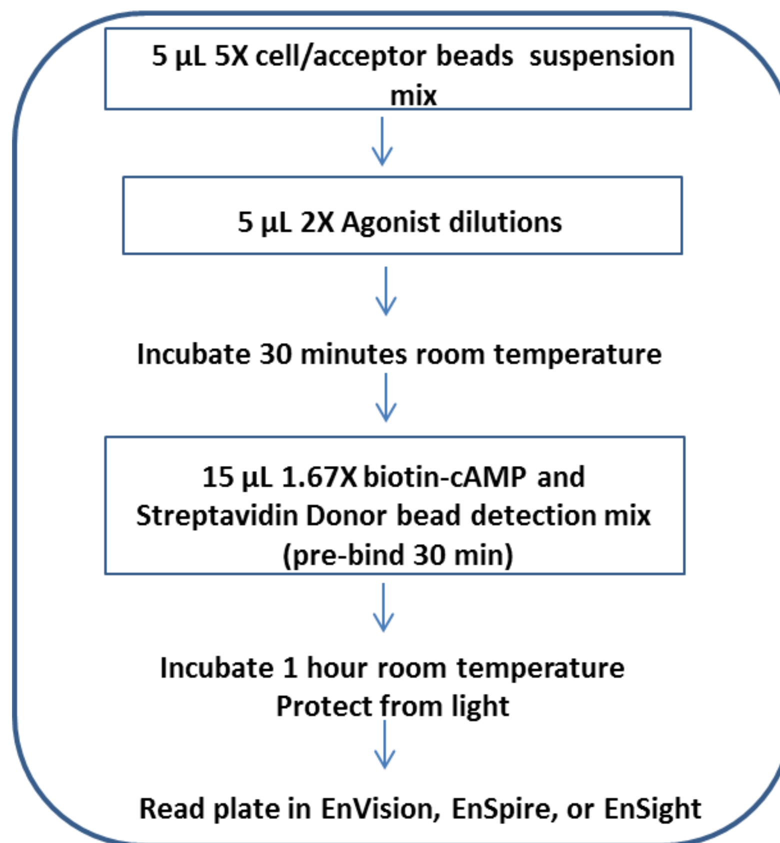
Prepare a 1:1 mix of cells prepared in Section 8 and 10X Acceptor bead mix prepared in Section 7.3 using the appropriate amount of cells for the selected cell density.

13.1.4 Biotin-cAMP and Streptavidin Donor bead detection mix in Immunoassay Buffer

Prepare as described in Section 7.5

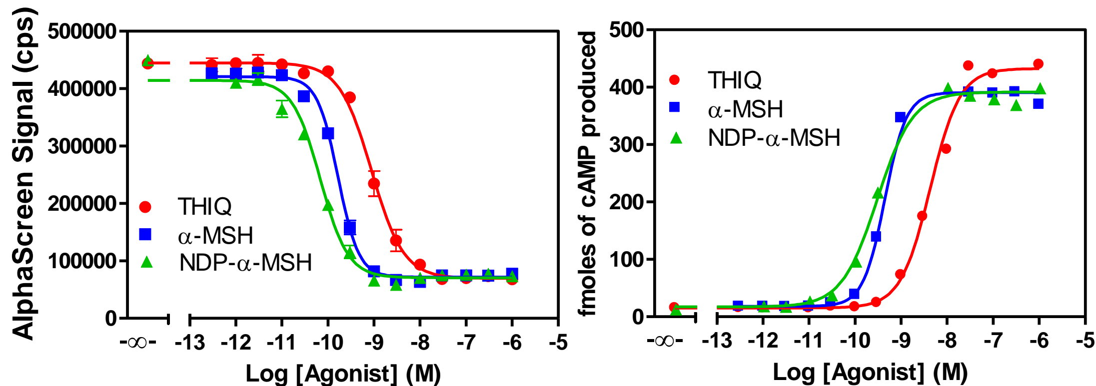
13.1.5 Assay flowchart

To a white, opaque OptiPlate-384, add in triplicate:



13.2 Example $G_{\alpha s}$ agonist dose-response data

AlphaScreen signal is plotted against the logarithmic values of agonist concentrations (Figure 8, left panel). Signal can then be transformed to the amount of cAMP produced using a cAMP standard curve run in parallel. The amount of cAMP produced can then be re-plotted against the logarithmic values of agonist concentrations
(Figure 8, right panel). The example included in this section is based on the stimulation of $G_{\alpha s}$-coupled melanocortin 4 (MC4) receptor expressed in CHO-K1 cells. Three different agonists were tested on 3000 cells per well.



	AlphaScreen Signal			fmoles cAMP		
	THIQ	α-MSH	NDP-α-MSH	THIQ	α-MSH	NDP-α-MSH
Max	443939	426865	449675	439	391	400
Min	68147	77911	73336	15	18	20
EC_{50} (nM)	0.88	0.17	0.07	4.3	0.44	0.3

Figure 8. $G_{\alpha s}$ agonist dose-response curve. Top Left panel: Alpha signal plotted against the logarithmic values of agonist concentrations; **top right panel:** Data re-plotted as fmoles of cAMP per well as interpolated from a cAMP standard cure run in parallel (data not shown). Compounds tested included THIQ (red curve, circles), α-MSH (blue curve, squares), and NDP-α-MSH (green curve, triangles). **Bottom panel**: summary table of values obtained for agonist dose-response curves

Observations:
EC_{50} values can be calculated from both types of curves, either as the concentration of a $G_{\alpha s}$ agonist that reduces the AlphaScreen signal by 50% (Figure 8, left panel) or as the agonist concentration generating 50% of the maximal response in the interpolated cAMP values curves (Figure 8, right panel). Some differences between EC_{50} values based on Alpha signal or cAMP production data are expected and explained by the non-linear relationship between Alpha signal and cAMP concentration. The "true" biologically relevant pharmacological value is obtained from the cAMP concentration converted curve.

13.3 Reference compounds used for Example $G_{\alpha s}$ (MC4) agonist assays

Ligand	Recommended source	Product no.
THIQ Stock prepared at 10 mM in dH_20	Tocris	3032
α-MSH Stock prepared at 10 mM in dH_20	Tocris	2584
NDP-α-MSH Stock prepared at 100 mM in dH_20	Tocris	3013

14. Determination of Gαs agonist concentration for antagonist assay

The presence of an agonist is required to screen for antagonists of $G_{\alpha s}$-coupled receptors. The optimal concentration for the agonist can be determined as follows:

- **In HTS for antagonist of $G_{\alpha s}$-GPCRs**, the assay requires the highest signal difference between agonist-activated cells and cells co-treated with antagonist and a fixed concentration of agonist. Such a maximal signal difference (assay window) is typically obtained using the **agonist at its EC_{90} value (based on Alpha signal).** Using the agonist at a concentration producing 90% (EC_{90}) of the maximum agonist activation, allows obtaining maximal assay window while minimizing inter-day data variability.
- **For ligand characterization, the preferred agonist concentration is usually located within its $EC_{50\text{-}80}$ value based on cAMP data**. The $EC_{50\text{-}80}$ value based on cAMP production is typically close to the EC_{90} value based on Alpha data and as such, it can also be used in HTS assays and will give a very good signal difference between agonist- and agonist/antagonist-activated cells. The optimal reference agonist concentration is a balance between optimizing the assay window and antagonist IC_{50} values. In some cellular systems, with a good cAMP response, one may prefer to use the agonist EC_{50} concentration to get optimal antagonist IC_{50} values. In other cellular systems, having a lower cAMP response, one may prefer using a reference agonist EC_{80} concentration (or even higher) in order to get a sufficient assay windows.

15. Gαs-coupled receptor stimulation/Antagonist dose-response curve

Antagonist stimulation of cells or membranes expressing $G_{\alpha s}$-coupled receptors results in a **blockade of the agonist-induced cAMP production**. As a result, less intracellular cAMP is produced and an **increase** in Alpha signal is observed, compared to cells treated with the agonist alone.

Antagonist potency (IC_{50} value) is determined by performing a dose-response curve at the optimal cell density using an agonist at a fixed concentration.

15.1 Reagent preparation

15.1.1 Stimulation Buffer

Prepare 20 mL, as described in Section 7.2.

15.1.2 Antagonists dilutions (4X) in Stimulation Buffer

Antagonist dilutions are prepared in Stimulation Buffer as 4X intermediate solutions. Ideally, the dilution series should cover concentrations that are 3 log units higher and lower than the expected IC_{50} value. Include 11 half-log antagonist dilutions and a "no antagonist" control.

15.1.3 Agonist working solution (4X) in Stimulation Buffer

The agonist is prepared in Stimulation Buffer as a 4X working solution

15.1.4 Acceptor bead and Cell suspension mix

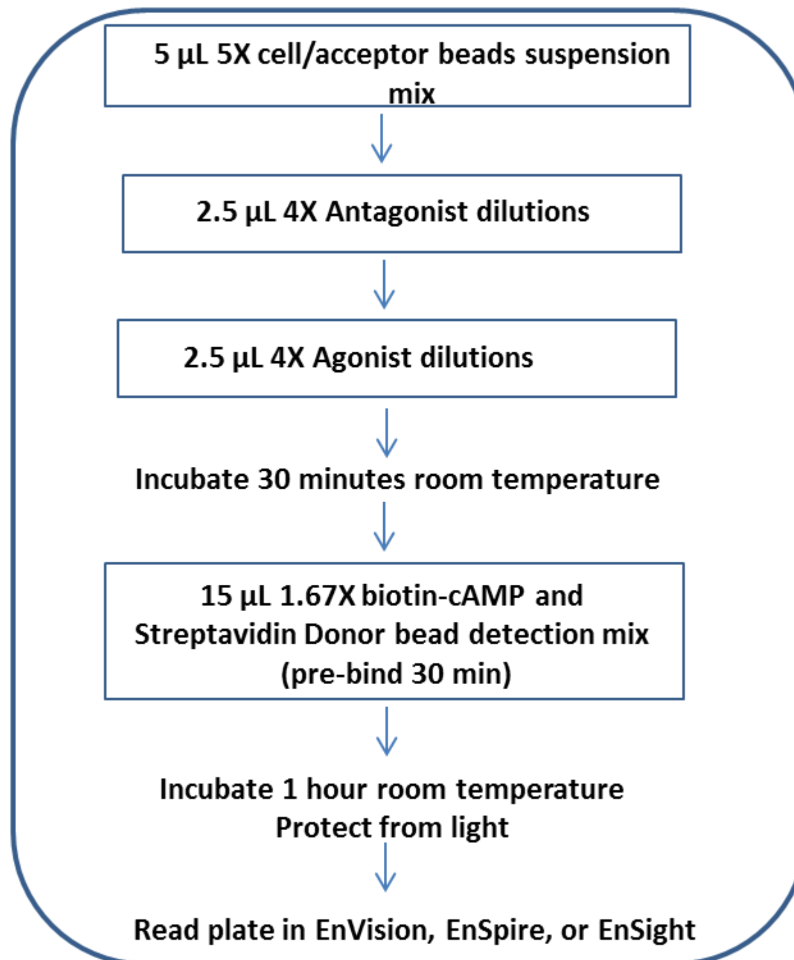
Prepare a 1:1 mix of cells prepared in Section 8 and 10X Acceptor bead mix prepared in Section 7.3 using the appropriate amount of cells for the selected cell density.

15.1.5 Biotin-cAMP and Streptavidin Donor bead detection mix in Immunoassay Buffer

Prepare as described in Section 7.5

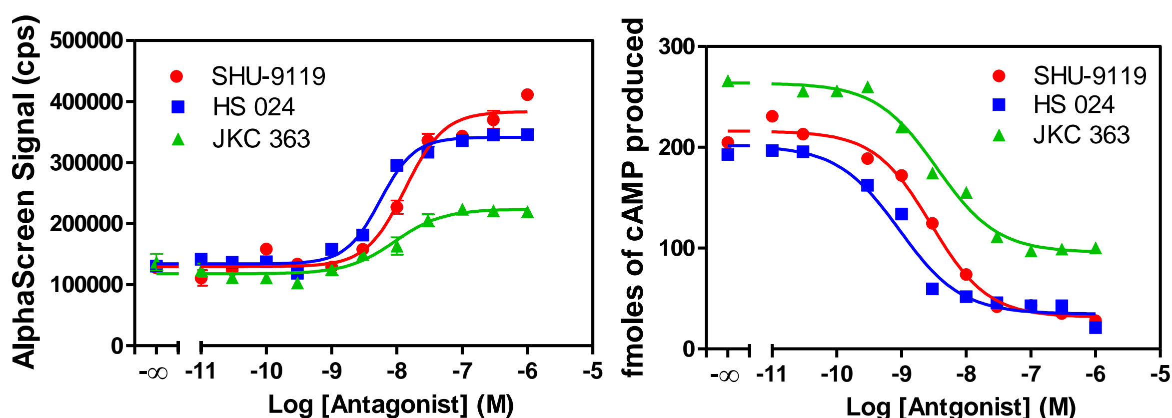
15.1.6 Assay flowchart

To a white, opaque OptiPlate-384, add in triplicate:



15.2 Example $G_{\alpha s}$ antagonist dose-response data

The AlphaScreen signal is plotted against the logarithmic values of antagonist concentrations (Figure 9, left panel). Signal can then be transformed to the amount of cAMP produced using a cAMP standard curve run in parallel (not shown). The amount of cAMP produced can then be re-plotted against the logarithmic values of antagonist concentrations (Figure 9, right panel). In the example included below, MC4 receptor expressing CHO-K1 cells were co-treated with the 0.3 nM of NDP-α-MSH and three different antagonists using 3000 cells/well.



	AlphaScreen Signal			fmoles cAMP		
	SHU 9119	HS 024	JKC 363	SHU 9119	HS 024	JKC 363
Max	411513	346153	219753	213	195	256
Min	127825	130501	126592	28	21	100
EC_{50} (nM)	13.4	5.6	9.3	2.8	0.9	3.5

Figure 9. Gαs antagonist dose-response curve. Top Left panel: Alpha signal plotted against the logarithmic values of antagonist concentrations; **Top right panel:** Data re-plotted as fmoles of cAMP per well as interpolated from a cAMP standard cure run in parallel (data not shown). Compounds tested included SHU-9119 (red curve, circles), HS 024 (blue curve, squares), and JKC 363 (green curve, triangles). **Bottom panel**: summary table of values obtained for antagonist dose-response curves.

Observations:

IC_{50} values can be calculated from both types of curves, either as the concentration of a $G_{\alpha s}$ antagonist that reduces the maximal Alpha response of the $G_{\alpha s}$ agonist by 50% (Figure 9, left panel) or as the antagonist concentration inhibiting 50% of the response in the interpolated cAMP values curve (Figure 9, right panel). Some differences between IC_{50} values based on Alpha or cAMP production data are expected and explained by the non-linear relationship between Alpha signal and cAMP concentration. It is to be noted that JKC-363, used here as an antagonist, does not seem to fully inhibit the MC4 agonist response, as the other 2 antagonists used here can do. This is explained by some partial agonist activity of JKC-363, as already reported by Doghman *et al*. (2004) Endocrinology 145:541–547.

15.3 Reference compounds used for Example $G_{\alpha s}$ (MC4) antagonist assays

Ligand	Recommended source	Product no.
SHU-9119 Stock prepared at 10 mM in dH_20	Tocris	3420
HS 024 Stock prepared at 10 mM in dH_20	Tocris	1832
JKC 363 Stock prepared at 10 mM in dH_20	Tocris	3426

16. Gαi-coupled receptor stimulation/Agonist dose-response curve

Agonist stimulation of cells expressing $G_{\alpha i}$-coupled receptors decreases cAMP production. In order to detect this agonist-induced reduction in cAMP levels, cells are stimulated with forskolin to increase the intracellular cAMP concentration. In the presence of an agonist, **the forskolin-induced cAMP production is decreased**. As a result, a $G_{\alpha i}$-coupled receptor agonist concentration-dependent **increase** in Alpha signal is observed compared to cells treated only with forskolin.

Agonist potency (EC_{50} value) is determined by performing a dose-response curve at the optimal cell density in the presence of a fixed forskolin concentration (see below).

16.1 Determination of forskolin concentration for Gαi-GPCR assays

cAMP assays for $G_{\alpha i}$-coupled receptors require forskolin stimulation. The optimal forskolin concentration to be used in those assays can be determined as follows:

- **In high-throughput screening (HTS) for agonists of $G_{\alpha i}$-GPCRs**, the assay requires the highest signal difference between forskolin-activated cells and cells co-stimulated with forskolin and a fixed concentration of agonist. Such a maximal signal difference (assay window) is typically obtained using forskolin at the $EC_{80\text{-}90}$ value (concentration that reduces by 80 or 90% the maximal Alpha response). When screening for antagonist of $G_{\alpha i}$-GPCRs, forskolin is also used at its $EC_{80\text{-}90}$ value, as well as the reference agonist also used at its $EC_{80\text{-}90}$ concentration.
- **For ligand characterization, the preferred forskolin concentration is its EC_{50} value based on cAMP data.** The EC_{50} value based on cAMP production is preferred in order to let enough room for inhibition by the Gαi proteins (i.e. if using too much forskolin, EC_{50} values of the agonists would be increased). The forskolin EC_{50} value based on cAMP production is typically close to the EC_{90} value based on Alpha data and as such, it can also be used in HTS assays and will give a very good signal difference between forskolin- and forskolin/agonist-treated cells (agonist screening) or forskolin/ agonist- and forskolin/agonist/antagonist-treated cells (antagonist screening). Higher forskolin values can be used but the higher the forskolin concentration selected for activating the cell system, the lower the sensitivity of the cAMP assay to weak agonists (i.e. increased EC_{50} values). The optimal forskolin concentration is a balance between optimizing the assay window and agonist EC_{50} values.

16.2 Reagent preparation

16.2.1 Stimulation Buffer

Prepare 20 mL, as described in Section 7.2.

16.2.2 Forskolin working solution (4X) in Stimulation Buffer

Forskolin is prepared in Stimulation Buffer as a 4X working solution. We recommend adding the forskolin at a concentration producing 90% (EC_{90}) of the maximum forskolin activation, which allows obtaining maximal assay window while minimizing inter-day data variability.

Prepare a 500 µM forskolin working dilution by adding 5 µL of the 50 mM stock solution (see Section 16.1) to 495 µL stimulation buffer. Further dilute to obtain a solution at 4X the desired final value.

16.2.3 Agonist dilutions (4X) in Stimulation Buffer

Agonist dilutions are prepared in Stimulation Buffer as 4X intermediate solutions. Ideally, the dilution series should cover concentrations that are 3 log units higher and lower than the expected agonist EC_{50} value. Include 11 half-log agonist dilutions and a "no agonist" control.

16.2.4 Acceptor bead and Cell suspension mix

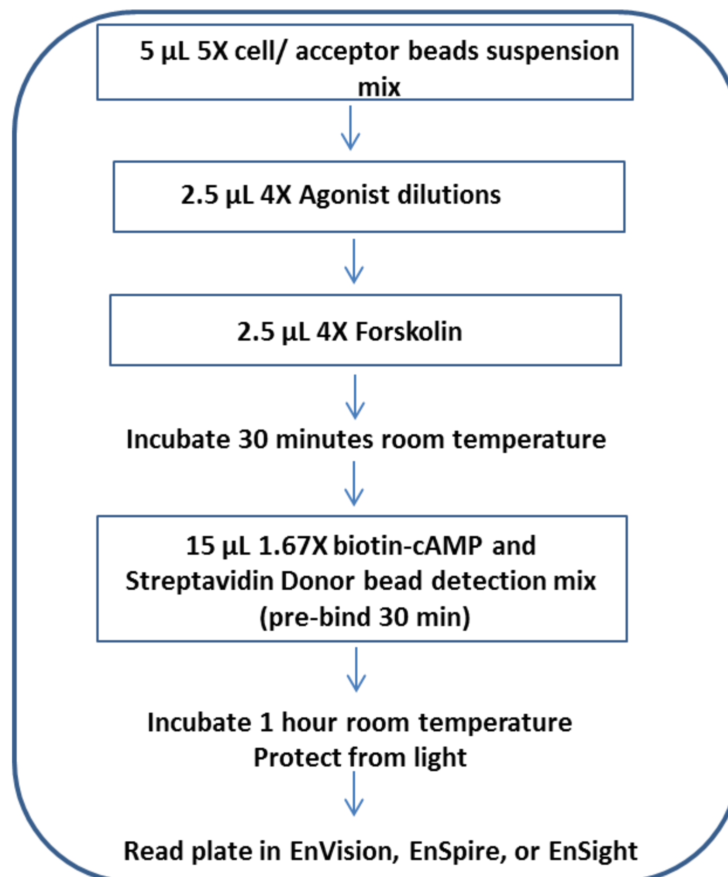
Prepare a 1:1 mix of cells prepared in Section 8 and 10X Acceptor bead mix prepared in Section 7.3 using the appropriate amount of cells for the selected cell density.

16.2.5 Biotin-cAMP and Streptavidin Donor bead detection mix in Immunoassay Buffer

Prepare as described in Section 7.5

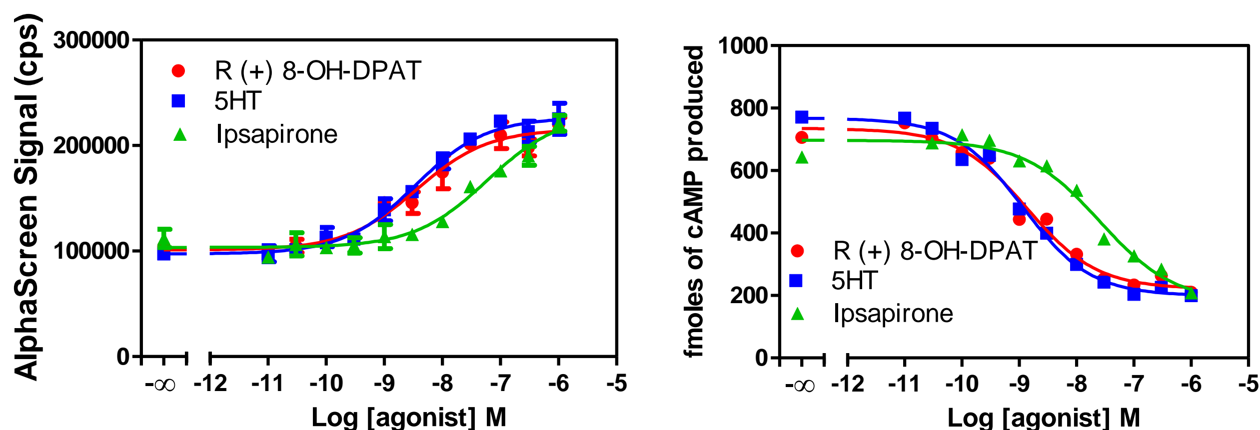
16.2.6 Assay flowchart

To a white, opaque OptiPlate-384, add in triplicate:



16.3 Gαi agonist dose-response data

AlphaScreen signal is plotted against the logarithmic values of agonist concentrations (Figure 10, left panel). Signal can then be transformed to the amount of cAMP produced using a cAMP standard curve run in parallel (not shown). The amount of cAMP produced can then be re-plotted against the logarithmic values of antagonist concentrations (Figure 10, right panel). In the example included below, serotonin (5HT1a) receptors expressed in CHO-K1 cells were stimulated with three different agonists in the presence of the 10 µM forskolin. Cells were tested at a density of 3000 cells/well.



	AlphaScreen Signal			fmoles cAMP		
	R-8OH-DPAT	5HT	Ipsapirone	R-8OH-DPAT	5HT	Ipsapirone
Max	220036	225324	221109	753	768	715
Min	104364	101086	94398	211	199	208
EC_{50} (nM)	3.6	3.4	59	1.4	1.1	25

Figure 10. Gαi agonist dose-response curve. Top left panel: Alpha signal plotted against the logarithmic values of antagonist concentrations; **Top right panel:** Data re-plotted as fmoles of cAMP per well as interpolated from a cAMP standard cure run in parallel (data not shown). Compounds tested included R(+)-8-OH-DPAT (red curve, circles), 5HT (blue curve, squares), and Ipsapirone (green curve, triangles). **Bottom panel**: summary table of values obtained for agonist dose-response curves

Observations:

EC_{50} values can be calculated from both types of curves, either as the concentration of a $G_{\alpha i}$ agonist that produces 50% of the maximal Alpha response in the presence of forskolin (Figure 10, left panel) or as the agonist concentration generating 50% of inhibition of the cAMP production (Figure 10, right panel). Some differences between EC_{50} values based on Alpha or cAMP production data are expected and explained by the non-linear relationship between Alpha signal and cAMP concentration. The "true" biologically relevant pharmacological value is obtained from the cAMP concentration converted curve.

16.4 References of compounds used for Gαi (5HT1a) agonist assays

Ligand	Recommended source	Product no.
8-hydroxy-DPAT ((R+)-8-OH-DPAT), Stock prepared at 10 mM in dH_20 (antagonist assays) or DMSO (agonist assay)	Tocris	0529
8-hydroxytryptamine (5HT) Stock prepared at 10 mM in DMSO	Tocris	3547
Ipsapirone Stock prepared at 10 mM in DMSO	Tocris	1869

17. Gαi-coupled receptor stimulation/Antagonist dose-response curve

Antagonist treatment of cells expressing $G_{\alpha i}$-coupled receptors results in a **blockade of the agonist- induced cAMP reduction**. As a result, a concentration-dependent **decrease** in Alpha signal is observed compared to cells co-treated with forskolin and agonist.

Antagonist potency (IC_{50} value) is determined by performing a dose-response curve at the optimal cell density using forskolin and a fixed concentration of a reference agonist. The choice of the reference agonist concentration to be used obeys to the same way of thinking as the choice of the optimal forskolin concentration (section 16.1) and is usually around the EC_{90} concentration (AlphaScreen data) or EC_{80} concentration (cAMP data) in order to get a sufficient assay window.

17.1 Reagent preparation

17.1.1 Stimulation Buffer

Prepare 20 mL, as described in Section 7.2.

17.1.2 Antagonist dilutions (4X) in Stimulation Buffer

Antagonist dilutions are prepared in Stimulation Buffer as 4X intermediate solutions. Ideally, the dilution series should cover concentrations that are 3 log units higher and lower than the expected antagonist IC_{50} value. Include 11 half-log antagonist dilutions and a "no antagonist" control.

17.1.3 Combined forskolin/Agonist working solution (4X) in Stimulation Buffer

Forskolin and agonist are prepared in Stimulation Buffer as a single 4X working solution. We recommend adding both forskolin and agonist for cell stimulation at concentrations producing 90% of their maximal effect (EC_{90} concentrations) in order to obtain a maximal assay window while minimizing inter-day data variability

17.1.4 Acceptor bead and cell suspension mix

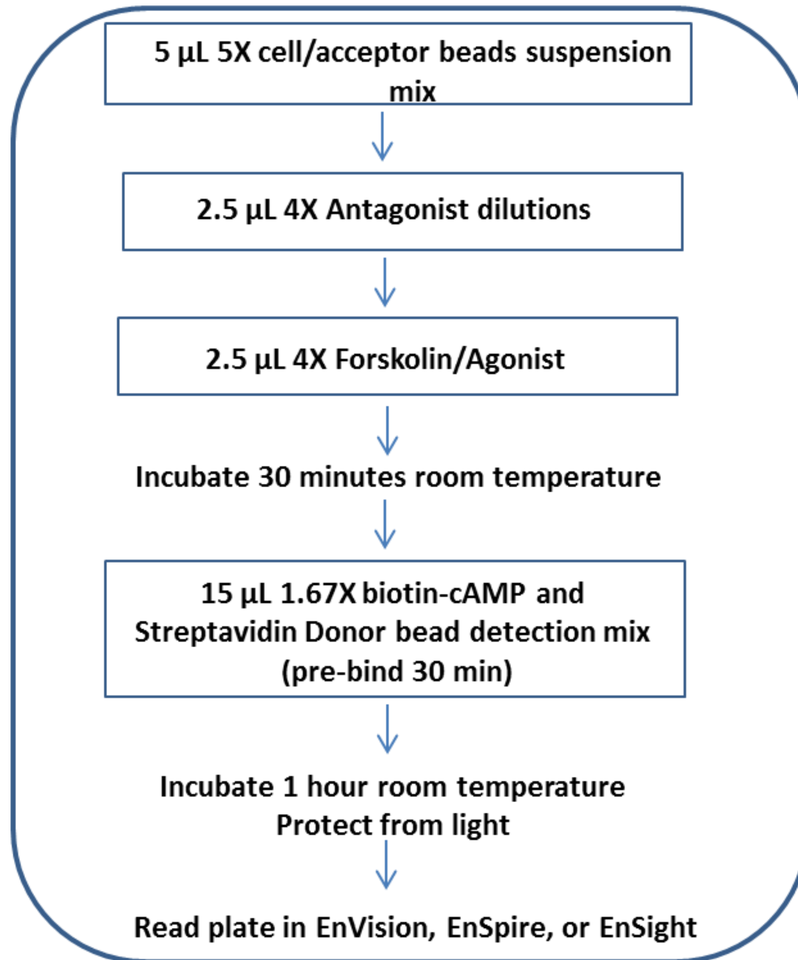
Prepare a 1:1 mix of cells prepared in Section 8 and 10X Acceptor bead mix prepared in Section 7.3 using the appropriate amount of cells for the selected cell density.

17.1.5 Biotin-cAMP and Streptavidin Donor bead detection mix in Immunoassay Buffer

Prepare as described in Section 7.5.

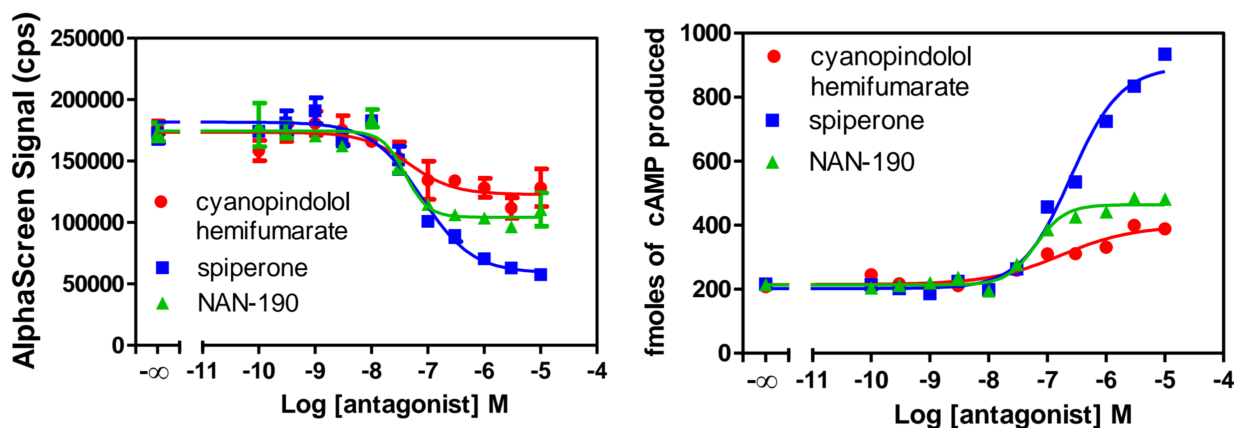
17.1.6 Assay flowchart

To a white, opaque OptiPlate-384, add in triplicate:



17.2 Example $G_{\alpha i}$ antagonist dose-response data

The Alpha signal is plotted against the logarithmic values of antagonist concentrations (Figure 11, left panel). Signal can then be transformed to the amount of cAMP produced using a cAMP standard curve run in parallel (not shown). The amount of cAMP produced can then be re-plotted against the logarithmic values of antagonist concentrations (Figure 11, right panel). In the example included below, serotonin (5HT1a) receptors expressed in CHO-K1 cells were incubated with the 10 µM forskolin, 100 nM R(+)-8-OH-DPAT and three different antagonists.



	AlphaScreen Signal			fmoles cAMP		
	Cyanopindolol hemifumarate	Spiperone	NAN-190	Cyanopindolol hemifumarate	Spiperone	NAN-190
Max	177338	172910	173762	388	934	482
Min	128326	57512	110583	208	216	215
EC_{50} (nM)	45.0	78.0	39.0	171	249	64

Figure 11. Gαi antagonist dose-response curve. Top left panel: Alpha signal plotted against the logarithmic values of antagonist concentrations; **Top right panel:** Data re-plotted as fmoles of cAMP per well as interpolated from a cAMP standard cure run in parallel (data not shown). Compounds tested included cyanopindolol hemifumarate (red curve, circles), spiperone (blue curve, squares), and NAN-190 (green curve, triangles). **Bottom panel**: summary table of values obtained for antagonist dose-response curves

Observations:

IC50 values can be calculated from both types of curves, either as the concentration of a Gαi antagonist that reduces the maximal Alpha response of the Gαi agonist by 50% in the presence of both forskolin and agonist (Figure 11, left panel) or as the antagonist concentration inhibiting 50% of the response in the interpolated cAMP values curve (Figure 11, right panel). Some differences between IC50 values based on Alpha or cAMP production data are expected and explained by the non-linear relationship between Alpha signal and cAMP concentration. The "true" biologically relevant pharmacological value is obtained from the cAMP concentration converted curve. It is to be noted that both cyanopindolol hemifumarate and NAN-190 have been reported as having partial agonist activity at the 5-HT1A receptor, which explains that they did not lead to a full inhibition of the receptor as spiperone did.

17.3 Reference compounds used for Example $G_{\alpha i}$ (5HT1a) antagonist assays

Ligand	Recommended source	Product no.
Cyanopindolol hemifumarate Stock prepared at 10 mM in DMSO	Tocris	0993
Spiperone Stock prepared at 100 mM DMSO	Tocris	0995
NAN-190 Stock prepared at 10 mM in DMSO	Tocris	0553

18. Adherent Cell Assays

Adherent cells assays may also be performed using the AlphaScreen cAMP kit.

18.1 Reagent preparation

18.1.1 Stimulation Buffer

Prepare 20 mL, as described in Section 7.2.

18.1.2 Compound Preparation

All compounds (forskolin, agonists, and antagonists) should be prepared in the same manner as described in Sections 12-17.

18.1.3 Acceptor bead preparation

Prepare a 5X mix (100 µg/mL) of anti-cAMP AlphaScreen Acceptor beads in stimulation buffer by making a 1:50 dilution of the Acceptor bead stock solution.

Example: Add 5 µL of the Acceptor bead stock solution to 245 µL of Stimulation Buffer and mix gently.

Note- This is the same concentration of Acceptor beads used for the cAMP standard curve.

18.1.4 Biotin-cAMP and Streptavidin Donor bead detection mix in Immunoassay buffer

Prepare as indicated in Section 7.5.

18.1.5 Cell preparation

See Section 8 for cell preparation details.

Stimulation buffer should be completely removed from the wells before adding the assay components, otherwise the volumes of the remaining additions will have to be scaled down (and the concentrations of each addition scaled up accordingly).

18.1.6 Assay Overview

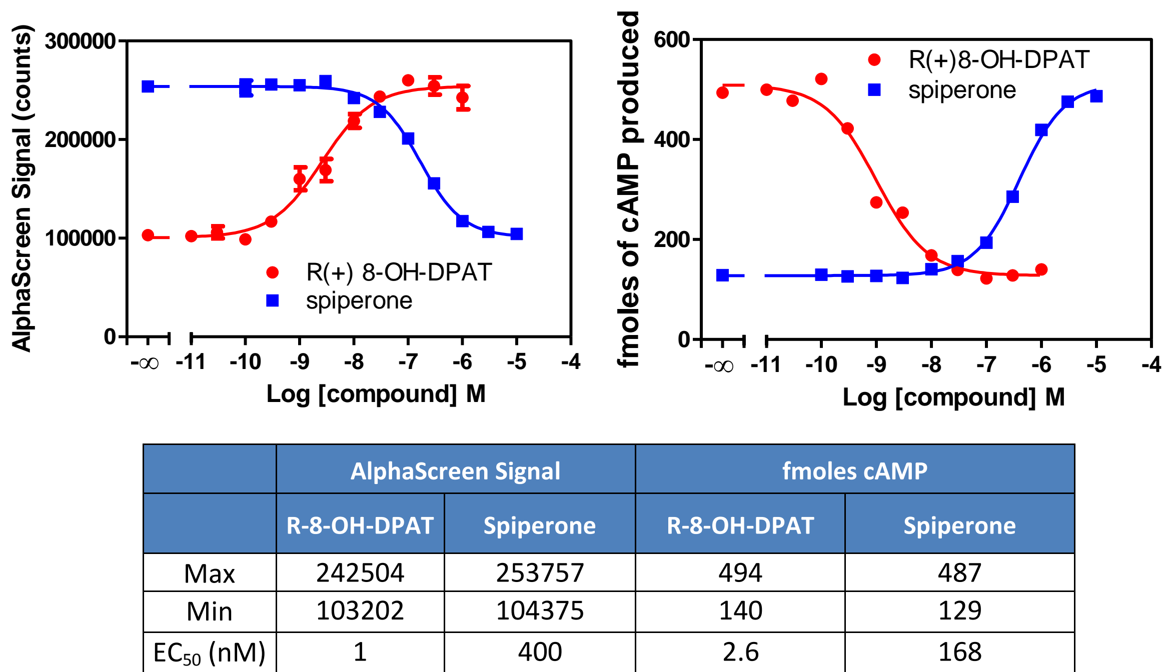
Depending on the type of assay desired, to a white, opaque OptiPlate-384, add in triplicate:

cAMP standard curve	Gs Agonist	Gs Antagonist	Gi Forskolin titration	Gi Agonist	Gi Antagonist
5 µL 5X Acceptor Beads preparation	5 µL 5X Acceptor Beads mix	5 µL 5X Acceptor Beads mix	5 µL 5X Acceptor Beads mix	5 µL 5X Acceptor Beads mix	5 µL 5X Acceptor Beads mix
5 µL cAMP standard	5 µL 2X Agonist	2.5 µL 4X Antagonist	5 µL 2X Forskolin	2.5 µL 4X Agonist	2.5 µL 4X Antagonist
-	-	2.5 µL 4X Agonist	-	2.5 µL 4X Forskolin	2.5 µL 4X Forskolin/Agonist
Incubate 30 min at room temperature*					
15 µL 1.67X biotin-cAMP/Streptavidin Donor Bead Detection Mix (pre-incubate 30 min at room temperature)					
Incubate 1 h at room temperature*					
Read on an Alpha-enabled Reader (EnSpire®, EnVision®, or EnSight®)					

*Cover plate with TopSeal-A Plus during incubations

18.2 Adherent assay data using Gαi receptor

Experiments with 5HT1a serotonin receptor expressing CHO-K1 cells described in Sections 16 and 17 were repeated using the adherent assay protocol described above. AlphaScreen signal is plotted against the logarithmic values of agonist/antagonist titrations (Figure 12, left panel), and signal converted to fmoles of cAMP (Figure 12, right panel).



	AlphaScreen Signal		fmoles cAMP	
	R-8-OH-DPAT	Spiperone	R-8-OH-DPAT	Spiperone
Max	242504	253757	494	487
Min	103202	104375	140	129
EC_{50} (nM)	1	400	2.6	168

Figure 12. Gαi dose-response curves using adherent cells. Serotonin (5HT1a) receptor expressing CHO-KI cells **Top left panel:** Agonist (R(+)-8-OH-DPAT, red curve, circles) and antagonist (spiperone, blue curve, squares) dose-response curves using 3000 cells/well in an adherent assay protocol. **Top right panel:** Values from left curve converted into fmoles cAMP. **Bottom panel:** summary table of values obtained for agonist and antagonist dose-response curves

Observations:

In this case, the adherent cell protocol generated similar assay windows and IC_{50}/EC_{50} values for compounds tested in the suspension protocol. The situation may vary, depending on the cell type and receptor used in the assay.

19. Assays on G_s receptors membrane preparations

For some membrane systems, the AlphaScreen cAMP assay allows detecting cAMP in cellular membrane preparations when the Stimulation Buffer contains the appropriate supplements (e.g. ATP, GDP, GTP and $MgCl_2$). We recommend titrating membranes and all supplemented components for optimizing the performance of membrane-based assays. Below is a sample protocol.

19.1 Reagent preparation

19.1.1 Stimulation Buffer

Stimulation buffer for membrane assays: 25 mM $MgCl_2$, 375 mM NaCl, 250 µM ATP, 2.5 µM GDP, 2.5 nM GTP prepared in H_2O

19.1.2 Compound Preparation

All compounds should be prepared in the same manner as described in Sections 12- 17, with the exception of using the stimulation buffer noted in section 19.1.1 above.

19.1.3 Acceptor bead and membrane preparation mix

Prepare a 1:1 mix of membranes (see table below) and 10X Acceptor bead mix prepared in Section 7.3, with the exception of using the stimulation buffer noted in section 19.1.1 above.

An example of different dilution schemes is shown in the table below, where 250 µL of each mix can be prepared (enough for one 12 pt. curve, in triplicate).

µg membrane/well (final)	2 µg/µl Membrane stock (µL)	Membrane Stimulation Buffer (µL)	10X Acceptor bead working solution (µL)
5	125	0	125
2.5	62.5	62.5	125
1.25	31.25	93.75	125
0	0	125	125

19.1.4 Biotin-cAMP and Streptavidin Donor bead detection mix in Immunoassay buffer

Prepare as indicated in Section 7.5.

19.1.5 Assay Overview

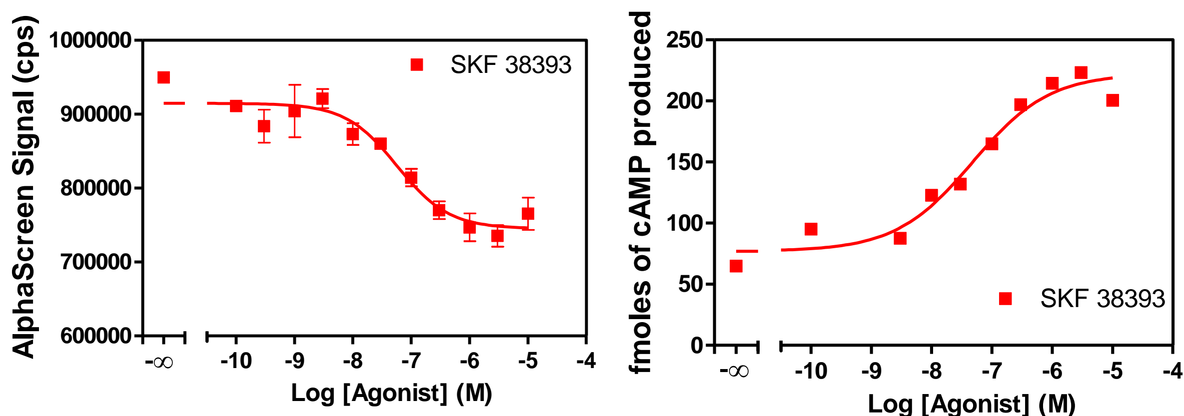
To a white, opaque OptiPlate-384, add in triplicate:

cAMP standard curve	Gs Agonist	Gs Antagonist
5 µL 5X Acceptor Beads	**5 µL 5X Acceptor Beads/membrane preparation mix**	**5 µL 5X Acceptor Beads/membrane preparation mix**
5 µL cAMP standard	**5 µL 2X Agonist**	**2.5 µL 4X Antagonist**
-	-	**2.5 µL 4X Agonist**
Incubate 30 min at room temperature, while shaking*		
15 µL 1.67X biotin-cAMP/Streptavidin Donor Bead Detection Mix (pre-incubate 30 min at room temperature)		
Incubate 1 h at room temperature*		
Read on an Alpha-enabled Reader (EnSpire®, EnVision®, or EnSight®)		

*Cover plate with TopSeal-A Plus during incubations

19.2 Example $G_{\alpha s}$ agonist dose-response data using membrane preparations

AlphaScreen signal is plotted against the logarithmic values of agonist concentrations (Figure 13, left panel). Signal can then be transformed to the amount of cAMP produced using a cAMP standard curve run in parallel. The amount of cAMP produced can then be re-plotted against the logarithmic values of agonist concentrations
(Figure 13, right panel). The example included in this section is based on the stimulation of a membrane preparation of Gαs-coupled dopamine D1 receptors expressed in L cells (Revvity cat#6110513400UA) with the compound SKF38393 (Tocris cat#0922, stock prepared at 25 mM in dH_2O).

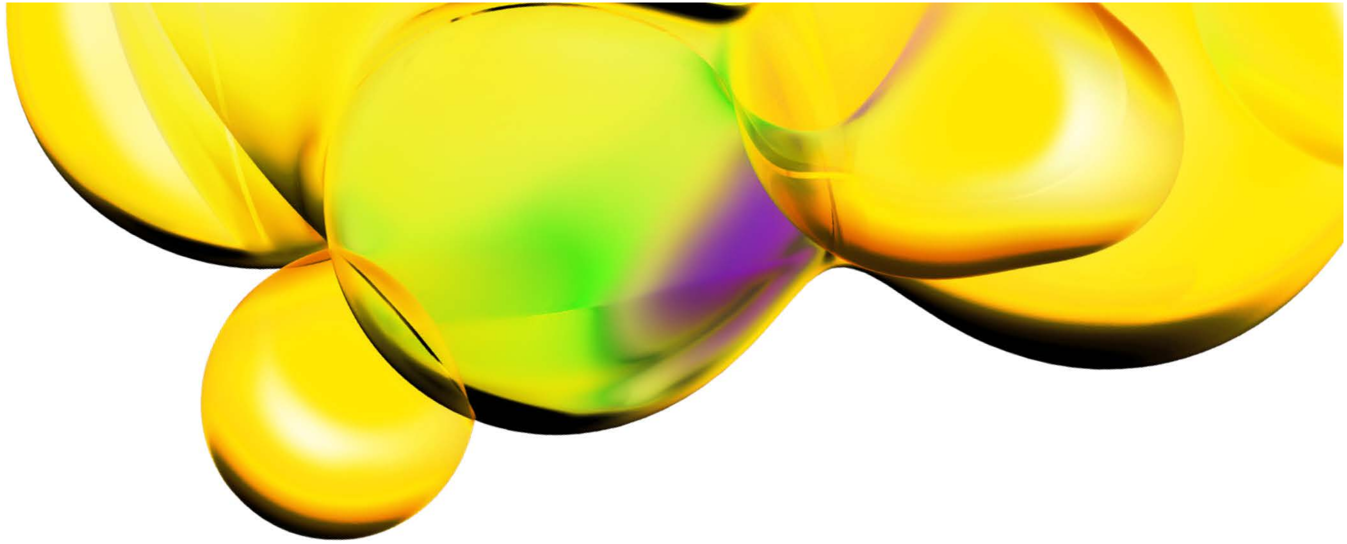


	AlphaScreen Signal	fmol cAMP
Max	949878	201
Min	765377	65
EC_{50} (nM)	57	47

Figure 13. Gαs dose-response curves using membrane preparations. Top left panel: Alpha signal plotted against the logarithmic values of agonist concentrations; **Top right panel:** Alpha signal (left panel) re-plotted in terms of cAMP data by interpolating from a cAMP standard curve run in parallel. Assay used 1.5 µg of dopamine D1 membrane preparations and the compound SKF38393. **Bottom panel**: summary table of values obtained for agonist dose-response curves

Observations:

The AlphaScreen cAMP assay is able to provide robust assay windows using frozen membrane preparations



The information provided in this document is for reference purposes only and may not be all-inclusive. Revvity, Inc., its subsidiaries, and/or affiliates (collectively, "Revvity") do not assume liability for the accuracy or completeness of the information contained herein. Users should exercise caution when handling materials as they may present unknown hazards. Revvity shall not be liable for any damages or losses resulting from handling or contact with the product, as Revvity cannot control actual methods, volumes, or conditions of use. Users are responsible for ensuring the product's suitability for their specific application. REVVITY EXPRESSLY DISCLAIMS ALL WARRANTIES, INCLUDING WARRANTIES OF MERCHANTABILITY OR FITNESS FOR A PARTICULAR PURPOSE, REGARDLESS OF WHETHER ORAL OR WRITTEN, EXPRESS OR IMPLIED, ALLEGEDLY ARISING FROM ANY USAGE OF ANY TRADE OR ANY COURSE OF DEALING, IN CONNECTION WITH THE USE OF INFORMATION CONTAINED HEREIN OR THE PRODUCT ITSELF

www.revvity.com

revvity

Revvity, Inc.
940 Winter Street
Waltham, MA 02451 USA
www.revvity.com

For a complete listing of our global offices, visit www.revvity.com
Copyright ©2024, Revvity, Inc. All rights reserved.