APPLICATION NOTE

# Functional GPCR studies using AlphaScreen cAMP detection kit.


## Authors

Jen Carlstrom
Dawn Nida

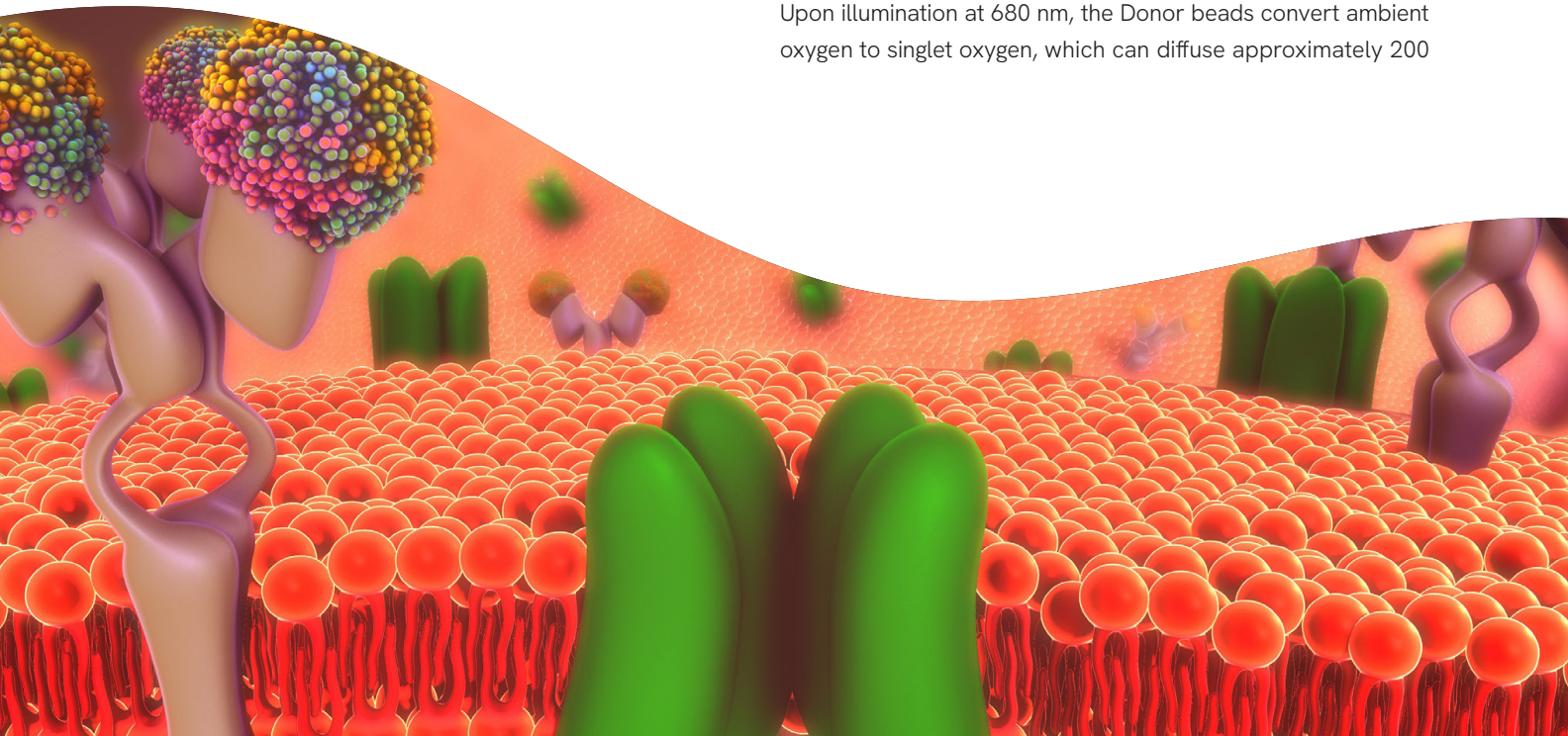
Revvity, Inc.


For research purposes only.
Not for use in diagnostic procedures.

## Introduction

G protein-coupled receptors (GPCRs) are cell surface transmembrane receptors that catalyze the activation of G-proteins. GPCRs couple to three main families of Gα subunits: Gαi/o, Gαs and Gαq. Gαs subunits activate adenylate cyclase, while Gαi subunits inhibit adenylate cyclase. Adenylate cyclase is an enzyme that catalyzes the conversion of ATP to cAMP. Therefore, GPCR activity is commonly assessed by measuring levels of the intracellular cAMP levels upon stimulation by agonists. Since GPCRs represent an important class of pharmaceutical drug targets, methods for detecting and quantifying cAMP are in high demand. There are a large number of assays available on the market to detect and quantify cAMP levels in cells. However, the ideal assay is a homogenous, non-radioactive assay that allows for sensitive and reproducible detection of cAMP.

Here, we present data showing performance of an AlphaScreen™ cAMP assay in a 384-well plate. The assay was evaluated for its ability to detect agonist or antagonist-induced cAMP responses in cells expressing either endogenous (Gαs-β-adrenergic) or recombinant receptors (Gαi-CXCR3; Gαi-CB1). The principle of the AlphaScreen cAMP assay is shown in Figure 1. In the AlphaScreen cAMP assay, a biotinylated-cAMP tracer molecule is captured by Streptavidin Donor beads and an anti-cAMP monoclonal antibody (mAb) is conjugated to the AlphaScreen Acceptor beads. When the anti-cAMP antibody binds to the biotinylated cAMP, the Donor and Acceptor beads are brought into close proximity. Upon illumination at 680 nm, the Donor beads convert ambient oxygen to singlet oxygen, which can diffuse approximately 200



nm in solution. If an Acceptor bead is within that distance, energy is transferred to the Acceptor bead, resulting in light production between 520 and 620 nm. In the absence of free cAMP (Figure 1, left panel), maximal signal is achieved. When cells are stimulated and produce free cAMP, it competes with the biotinylated-cAMP tracer for the binding to the Acceptor beads (Figure 1 right panel), causing a decrease in Alpha signal proportional to the concentration of cAMP produced.

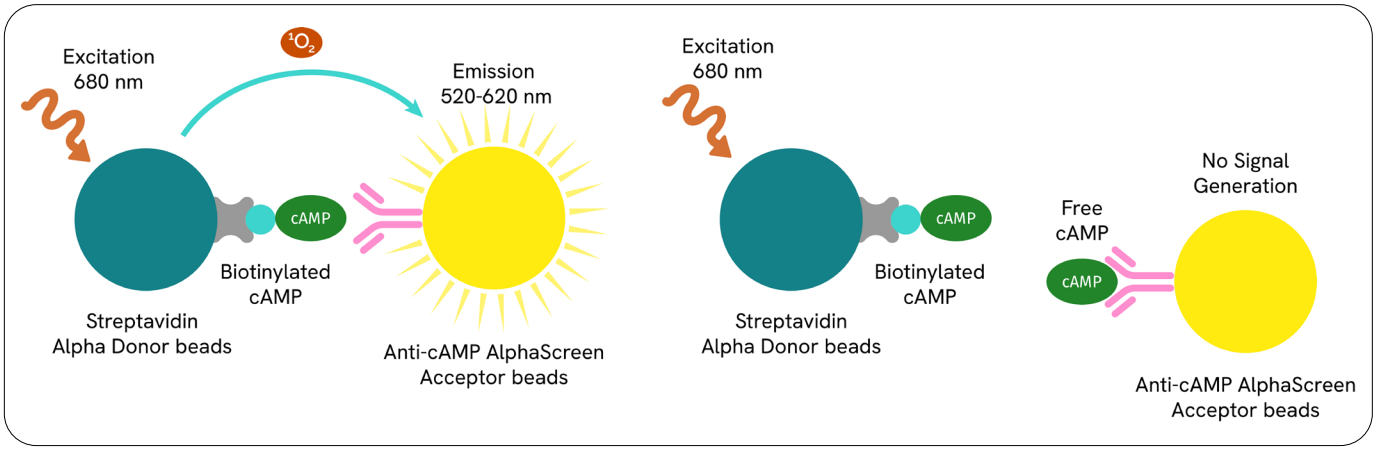



Figure 1: AlphaScreen cAMP assay principle.

## Materials and methods

### Instrumentation

All AlphaScreen measurements were performed on the Revvity EnVision™ multimode plate reader which provides fast, sensitive Alpha technology detection, in addition to fluorescence intensity, luminescence, absorbance, fluorescence polarization, and time-resolved fluorescence detection technologies. The system is based on hybrid technology, combining filters and a monochromator for enhanced flexibility. It incorporates proprietary Direct Double Optics™ technology for high speed and sensitivity in simultaneous filter-based readouts, such as FRET assays, and for TRF, lamp-based excitation is standard, with the option of a high energy laser for higher speed and sensitivity.

### Reagents

All assays were performed using the AlphaScreen cAMP kit (#6760635D). Cell lines used were SK-N-MC (ATCC #HTB-10), CHO-K1 expressing CB1 (#ES-110-C), and CHO-K1 cells expressing CXCR3 (ES-142-C). Compounds used for stimulation were AM 251 (Tocris #1117), BRL 37344 (Tocris #0948), CXCL9 (Peprotech #300-26), CXCL10 (Peprotech #300-42), CXCL11 (Peprotech #300-46), Forskolin (Calbiochem # 44270), Isoproterenol (Sigma #I6504), LY 320135 (Tocris #2387), Norepinephrine (Sigma #A0937), and WIN 55, 212-2 (Sigma #W102). All reagents were prepared and dispensed according to each manufacturer's recommendations. All assays were performed in OptiPlate™ -384 (#6007290) white opaque microplates following the protocol provided with the kit. The stimulation buffer used for all assays contained 1X HBSS, 5 mM HEPES, 0.5 mM IBMX and 0.1% BSA.

### AlphaScreen assays

The AlphaScreen cAMP assays were done according to the manual. The protocols used in this application note are outlined in Table 1.


Figure 2: EnVision multilabel plate reader.

Table 1: Protocols used for measuring cAMP upon stimulation of the GPCRs.

| cAMP standard curve | Gs agonist | Gs or Gi forskolin titration | Gi agonist | Gi antagonist |
|---|---|---|---|---|
| 5 µL 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 | 5 µL 2X Forskolin | 2.5 µL 4X Agonist | 2.5 µL 4X Antagonist |
| – | – | – | 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™) | | | | |

**Data analysis**

The data were analyzed using GraphPad software. The binding curves were generated using nonlinear regression (assuming one binding site and no non-specific binding), using a four-parameter logistic equation (sigmoidal dose-response curve with variable slope). The inhibition curves were generated using log (inhibitor) vs. response – variable slope (four parameters).

## Results and discussion

**cAMP standard curves**

cAMP standard curves were run using known concentrations of cAMP. The standard curves show sensitivity, dynamic range, and stability of the assay over time. The standard curve can also be used to convert the Alpha signal from a cellular assay into measured concentrations of cAMP. Figure 3 shows standard curves that have been incubated at 1 hour (according to the manual), or overnight (~24 hours). As shown in Figure 3, the $IC_{50}$ of the assay shifts slightly and the signal to background increases over time. Therefore, in order to accurately quantify the amount of cAMP in a sample, a standard curve should be run with each assay and incubated approximately the same amount of time as the sample.

**Agonist responses in SK-N-MC cells expressing endogenous β-adrenergic receptors**

GPCRs coupled to Gαs subunits stimulate adenylate cyclase and are expected to show an increase in cAMP levels upon agonist stimulation. The AlphaScreen cAMP kit was evaluated for characterizing the agonist response in SK-N-MC cells expressing endogenous Gαs-coupled β-adrenergic receptors.

Forskolin is a ubiquitous activator of adenylate cyclase. In order to determine the cell number giving the highest assay window within the assay dynamic range of the kit, a cross-titration of cell number and forskolin was performed (Figure 4). Four different cell numbers per well were tested. As the cell number increases, the basal level (no forskolin) of cAMP increases resulting in a decrease in the AlphaScreen signal. The highest signal to background ratio was seen for 3,000 cells per well. In addition, 3,000 cells per well provides a response that fits within the dynamic range (linear part) of the cAMP standard curve. Therefore, 3,000 cells per well were used for measuring agonist response in the SK-N-MC cells.

Agonist response curves were then performed using three well-known agonists of the β-adrenergic receptor: isoproterenol, norepinephrine, and BRL 37344 (Figure 5A). A standard curve run on the same plate was used to interpolate the fmoles of cAMP per well produced by each agonist. Although the measured AlphaScreen signal is a fast way to compare potencies of compounds, the interpolated fmoles of cAMP values make it easier to compare values between assay technologies. Figure 5B shows the agonists' responses as a function of fmoles of cAMP. The table shows the analyzed data for the agonist titrations using each method of analysis. Differences between the $IC_{50}$ reported by analyzing the AlphaScreen signal and the $EC_{50}$ reported by interpolating cAMP production can be explained by the nonlinear relationship between AlphaScreen signal and cAMP concentration. The $EC_{50}$ values reported by cAMP production represent the biologically relevant pharmacological value.

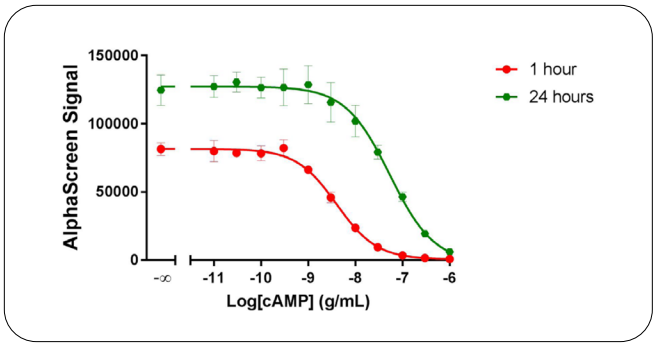



Figure 3: Standard cAMP curves in the AlphaScreen cAMP assay.

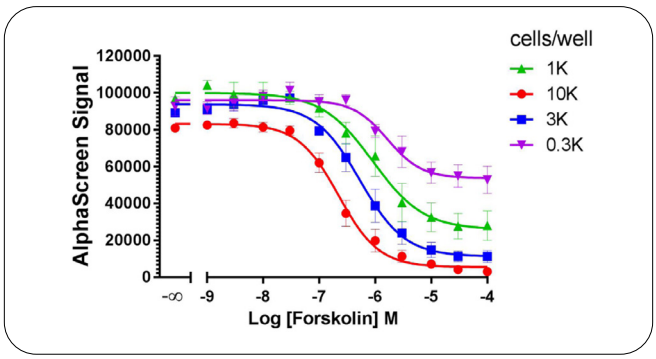
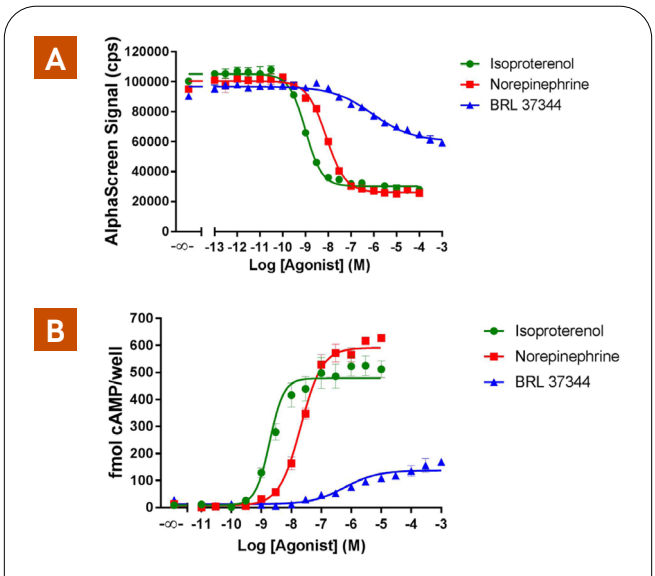



Figure 4: Forskolin titration of SK-N-MC cells.

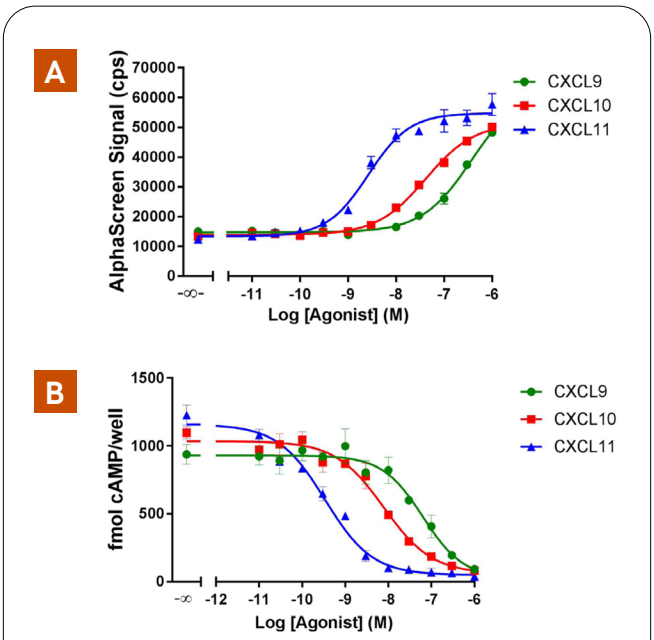



| | AlphaScreen signal | | cAMP production | |
|---|---|---|---|---|
| | S/B | $IC_{50}$ (nM) | S/B | $EC_{50}$ (nM) |
| Isoproterenol | 3.6 | 1.0 | 58 | 1.9 |
| Norepinephrine | 3.7 | 7.8 | 81 | 20 |
| BRL 37344 | 1.2 | 860 | 11 | 608 |

Figure 5: Agonist titration of SK-N-MC cells. A) Data shown plotting raw AlphaScreen signal against log agonist concentration. It is also recommended to convert data obtained into cAMP production values by interpolating from a standard curve run in parallel. B) Results derived from plotting cAMP production against log agonist concentration.

**Agonist responses in CHO cells expressing the Gαi-coupled human CXCR3 receptor**

The AlphaScreen cAMP assay was next evaluated for measuring agonist responses in a Gαi-couple receptor. CHO-hCXCR3 cells are CHO-K1 cells that express the human Chemokine CXCR3 receptor. CXCR3 is a Gαi-coupled receptor that inhibits adenylate cyclase upon agonist stimulation, and therefore, results in a decrease in cAMP levels. Assay conditions were again optimized by performing a cross-titration of cell density and forskolin, which allowed for determination of the optimal cell density. As with SK-N-MC cells, 3,000 cells per well was optimal (data not shown). In order to measure a decrease in cAMP levels upon agonist stimulation of the Gαi coupled receptor, the cells were first stimulated with forskolin to increase the cAMP levels in the cell. The $EC_{90}$ for forskolin titration in 3,000 cells per well was determined to be 30 µM for the CHO-hCXCR3 cells (data not shown). Agonist response curves were then performed in the presence of 30 µM forskolin using three known agonists of the hCXCR3 receptor (Figure 6).

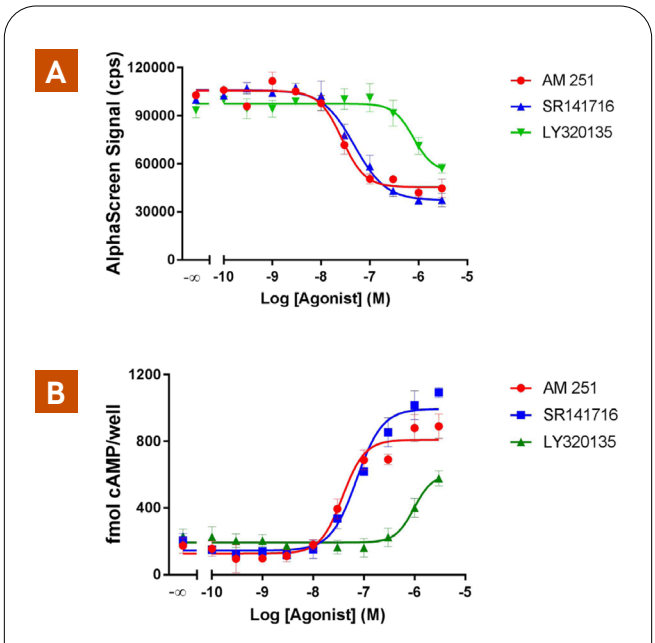



| | AlphaScreen signal | | cAMP production | |
|---|---|---|---|---|
| | S/B | $EC_{50}$ (nM) | S/B | $IC_{50}$ (nM) |
| CXCL9 | 4.3 | 384 | 37 | 64 |
| CXCL10 | 3.7 | 41 | 18 | 7.8 |
| CXCL11 | 4.1 | 2.6 | 24 | 0.34 |

Figure 6: Titration of agonists (CXCL9, CXCL10 and CXCL11) for CXCR3 receptor in the presence of 30 µM forskolin. A) Data shown plotting raw AlphaScreen signal against log agonist concentration. It is also recommended to convert data obtained into cAMP production values by interpolating from a standard curve run in parallel. B) Results derived from plotting cAMP production against log agonist concentration.

### Antagonist responses in CHO cells expressing the Gαi-coupled human CB1 receptor

The AlphaScreen cAMP kit was next used to characterize antagonist responses in a Gαi-coupled receptor. Antagonists are molecules that bind to the receptor but do not illicit a response. Instead, they can block the binding of an agonist and inhibit the agonist response. To measure antagonist responses, the Gαi-coupled receptor CB1 was chosen. CHO-CB1 cells are CHO-K1 cells that express the human Cannabinoid CB1 receptor. Again, a cross-titration of cell density and forskolin was performed. $EC_{90}$ for forskolin was determined (data not shown). The optimal cell density was 3,000 cells/well and the $EC_{90}$ for forskolin was 30 µM. Next, a concentration-response curve using the agonist WIN 55, 212-2 was conducted to determine a suitable agonist concentration to be used for antagonist assays (data not shown). The agonist concentration used for the antagonist assay was 1 µM WIN 55, 212-2. Antagonist concentration-response curves were then performed with a set of three known CB1 antagonists, AM 251, SR141716, and LY 320135 (Figure 7).

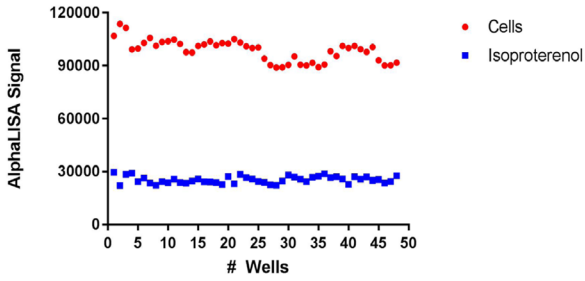



| | AlphaScreen signal | | cAMP production | |
|---|---|---|---|---|
| | S/B | $IC_{50}$ (nM) | S/B | $EC_{50}$ (nM) |
| AM 251 | 2.3 | 26.7 | 6.4 | 38 |
| SR141716 | 2.8 | 47.9 | 6.8 | 72 |
| LY 320135 | 1.8 | 786 | 3.1 | 974 |

Figure 7: Antagonist titration for CB1 receptor in the presence of 30 µM forskolin and 1 µM WIN 55,212-2. A) Data shown plotting raw AlphaScreen signal against log agonist concentration. It is also recommended to convert data into cAMP production values by interpolating from a standard curve run in parallel. B) Results derived from plotting cAMP production against log agonist concentration.

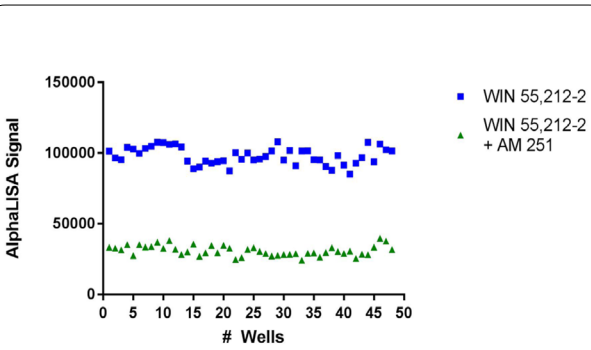
### Z′-Factor determination for agonist and antagonist assays

The robustness of the AlphaScreen cAMP assay in 384-well plate format was assessed by performing Z′-factor analysis using SK-N-MC for the agonist assay (Figure 8) and CHO-K1 CB1 for the antagonist assay (Figure 9). Z′-factor was determined for both the agonist and antagonist assays using the optimized assay conditions described previously. After one hour of incubation, high S/B ratios and Z′-factor values were obtained for both the agonist (S/B=3.9 and Z′ = 0.67) and antagonist assays (S/B= 3.2 and Z′ = 0.57). Percent CV values were acceptable for cell-based assays conducted manually. These data clearly show that the AlphaScreen cAMP cell-based assays are robust and well-suited for HTS.




| Agonist assay | Mean | SD | % CV | Z′ | S/B |
|---|---|---|---|---|---|
| Cells | 98594 | 6090 | 6.2% | 0.67 | 3.9 |
| Isoproterenol | 25383 | 1999 | 7.9% | | |

Figure 8: Agonist Assay using SK-N-MC cells in the presence or absence of 10 nM Isoproterenol.




| Antagonist assay | Mean | SD | % CV | Z′ | S/B |
|---|---|---|---|---|---|
| WIN 55, 212-2 | 97981 | 6042 | 6.2% | 0.57 | 3.2 |
| WIN 55, 212-2 + AM 251 | 30933 | 3626 | 11.7% | | |

Figure 9: Antagonist assay using CB1 receptor in the presence of 30 µM forskolin and 1 µM WIN 55, 212-2 and 1 µM AM 251.

## Conclusions

The AlphaScreen cAMP kit provides a cAMP assay with high sensitivity, a large signal window, and demonstrates multifaceted flexibility. This single kit with a simple assay protocol can be used for screening of Gαs and Gαi GPCRs as well as both endogenous and recombinant GPCRs. Here, we show that cell-based assays provide comparable assay pharmacology with the expected rank order of agonist or antagonist potency. The AlphaScreen cAMP technology was also shown to be robust and suitable for compound characterization in hit-to-lead programs. We demonstrated the suitability of the AlphaScreen cAMP assay kit for screening and the EnVision multimode plate reader by showing assay precision as calculated by Z′. Z′ was greater than 0.5 in both agonist stimulation and antagonist inhibition of response. In view of the complexity of these types of biomolecular interactions, this represents a highly robust system for the detection of agonist stimulation and antagonist inhibition of GPCRs.

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