APPLICATION NOTE

cAMP AlphaScreen assay: a method for the pharmacological characterization and screening of $G_{\alpha i}$-coupled receptors in whole cells.

Authors

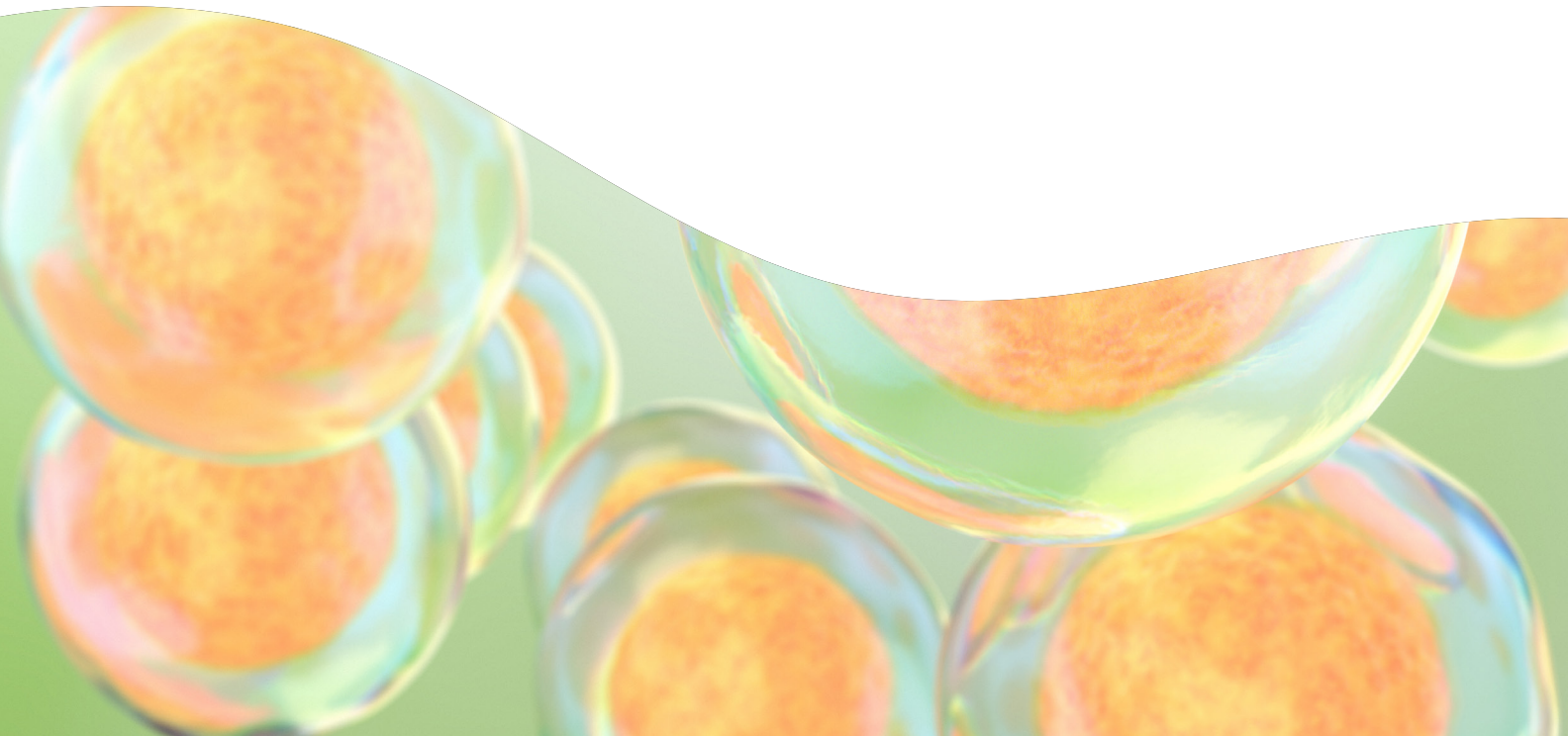
Nathalie Bouchard
Erik Robitaille
Dean Wenham

Revvity, Inc.

Introduction

G protein-coupled receptors (GPCRs) are a large family of cell surface transmembrane receptors that represent an extremely tractable class of drug targets. GPCRs are essentially membrane-associated enzymes that catalyze the activation of G-proteins.[1] GPCRs couple to three main families of G_{α} subunits: $G_{\alpha i/o}$, $G_{\alpha s}$ and $G_{\alpha q}$. The specificity of G_{α} subunit coupling is dependent upon the GPCR and its cellular environment. $G_{\alpha s}$ and $G_{\alpha i}$ subunits act through the cAMP pathway by respectively activating or inhibiting adenylate cyclase, an enzyme catalyzing the conversion of ATP to cAMP.[2]

To demonstrate the application of the AlphaScreen™-based cAMP detection technology for $G_{\alpha i}$-coupled receptors, the $G_{\alpha i}$-coupled 5-hydroxytryptamine-$_{1A}$ (5-HT_{1A}) serotonergic receptor[3] has been chosen as a model system. In this study, the utility of the AlphaScreen-based cAMP Assay kit for the pharmacological characterization and the potential high-throughput screening of $G_{\alpha i}$-coupled receptors has been demonstrated.



Principles of the AlphaScreen cAMP assay

The AlphaScreen cAMP assay has been designed to directly 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 biotin-cAMP (Figure 1). The capture of cAMP is achieved by using a specific antibody conjugated to Acceptor beads. The assay is efficient at measuring both agonist and antagonist activities on $G_{\alpha i}$- and $G_{\alpha s}$-coupled GPCRs. Cells are stimulated to either increase or decrease intracellular cAMP levels followed by a combined cell lysis / detection step. For $G_{\alpha i}$-coupled receptors, an elevation in intracellular cAMP is stimulated using forskolin, resulting in a decrease in AlphaScreen signal due to an inhibition of association between the beads. However, when forskolin stimulation occurs concurrently with the agonist stimulation of a $G_{\alpha i}$-coupled receptor, the resultant decreased signal evoked by forskolin alone is inhibited i.e. there is a signal increase relative to the forskolin alone treatment.

For further details on AlphaScreen technology, refer to application note entitled "Principles of AlphaScreen".

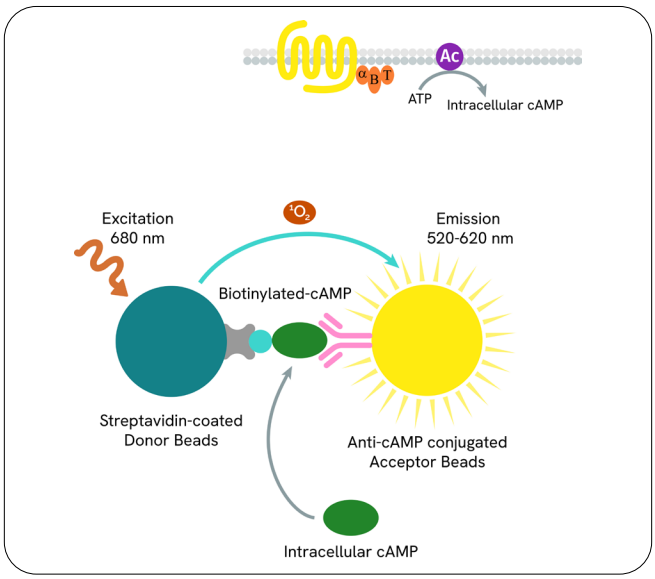


Figure 1: AlphaScreen Detection of cAMP

Materials and Methods

The AlphaScreen cAMP assay kit (cat. # 6760600M, 10 000 assay points) was used in this study. The kit is composed of biotinylated cAMP, Streptavidin-Donor beads, Anti-cAMP-Acceptor beads and control buffer.

Cell culture

CHO-5-HT_{1A} transfected cells were maintained in culture with MEM media supplemented with 10% FBS and 2 mM glutamine and passaged using 1:3 – 1:20 dilutions. Cells were detached using PBS:EDTA, centrifuged and resuspended in stimulation buffer immediately before use at 0.6 million cells/mL (3000 cells/well).

Cell-based assay

The following buffers were used in the study:

Stimulation buffer: HBSS containing 5 mM HEPES pH 7.4, 0.1% BSA and 0.5 mM IBMX.

Detection buffer: 5 mM HEPES pH 7.4, 0.1% BSA and 0.3% Tween-20.

All assays were performed in white, opaque 384-well plates (Corning, Cat. #3705) in a final volume of 25 µL.

Standard curves were performed in triplicates by the addition of 5 µL of stimulation buffer containing 75 µg/mL Acceptor beads (15 µg/mL final concentration) to the wells of a 384-well plate. 5 µL of cAMP at different concentrations ranging from 1 µM and 10 pM final concentration were added followed by 30 minutes incubation at room temperature in the dark. Finally, 15 µL of detection mix (16.7 nM of biotin-cAMP (10 nM final concentration) and 33.4 µg/mL of Streptavidin-Donor beads (20 µg/mL final concentration) pre-mixed for 30 minutes in detection buffer) was added to each well and incubated for 1 hour at RT in the dark. AlphaScreen signal was detected using an AlphaQuest™-HTS microplate analyzer.

In cell stimulation studies, 5 µL of the cell suspension (3000 cells/5 µL) containing Acceptor beads (15 µg/mL final concentration) were added to wells of a 384-well plate in triplicates. Stimulation was carried out by addition of 2.5 µL of a 5-HT_{1A} agonist and 2.5 µL of forskolin followed by a 30 minute incubation at RT in the dark before the detection step (as described above for the standard curve) was performed. For antagonist assays, cells were pre-incubated for 30 minutes with 2.5 µL of antagonist prior stimulation with 2.5 µL of a mix of forskolin and agonist solution. Precision assays were performed in the presence of 1% DMSO to simulate screening conditions. Culture media, HBSS and HEPES were from Invitrogen Canada (Burlington, ON) and agonists, antagonists and other reagents were obtained from Sigma-Aldrich Canada Ltd (Oakville, ON)

Data analysis

Data were analyzed using GraphPad Prism software4; the data points and vertical error bars on the graphs depict the mean ± S.D. of a representative experiment performed in triplicate. Each experiment was performed on at least three separate occasions. pK_b values were calculated from functional inhibition curves according to a derivation of the Cheng & Prusoff equation[5] specifically for the analysis of functional inhibition curves: $K_b = IC_{50}/(1+A/EC_{50})$[6] where A is the agonist concentration used, IC_{50} is the concentration of antagonist producing a 50% reduction in the response to agonist and EC_{50}, is the agonist concentration which causes a 50% maximal response. pA_2 values were calculated using Schild Analysis[7] of agonist dose-response curves performed in presence of varying concentrations of antagonist. Assay precision was calculated using the Z′ factor.[8]

Results and discussion

cAMP standard curves were performed to calculate the dynamic range of the assay and to estimate the quantity of cAMP produced by the cells. A typical standard curve is shown in Figure 2A where an IC_{50} of 4.4 nM was obtained with a dynamic range of between 0.3 and 30 nM. Key to the optimization of a cell-based assay is determining the optimum cell number to use per well. Figure 2B shows a typical cell optimization assay where cell number was titrated against a response to forskolin. As can be seen, as the cell number increased, the maximal basal AlphaScreen signal decreased as a consequence of the increasing quantity of basal cAMP. Further, the signal to background ratio was observed to reduce beyond 3000 cells per well. This together with the fact that the signal range for the forskolin concentration effect curve coincided with the more linear component of the cAMP standard curve suggested that 3000 cells per well represented an optimal cell number for this assay. Thus, the curve generated by 3000 cells per well was converted from AlphaScreen signal to pmol cAMP per well by interpolation from the standard curve. The resultant curve is shown in Figure 2C where an EC_{50} of 2.1 µM was obtained for forskolin stimulation.

The pharmacological profile for agonist stimulation was determined for the 5-HT_{1A} receptor. Agonist responses to this $G_{\alpha i}$-coupled receptor were studied following stimulation with 10 µM forskolin, a concentration that represents about 90% of the maximal forskolin response. Inhibition of the forskolin-induced cAMP response was observed with the

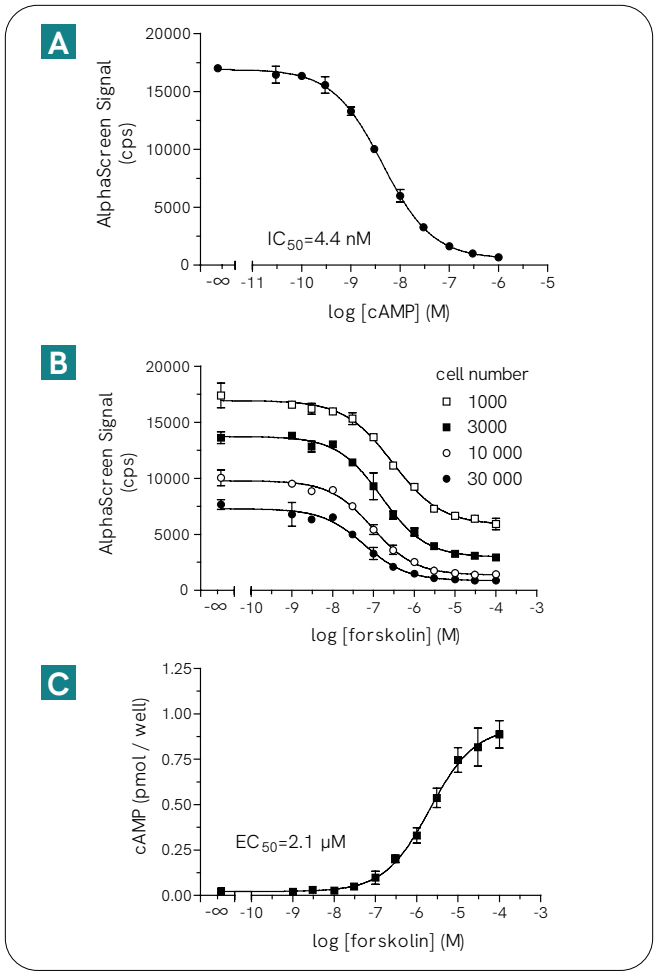


Figure 2: Standard curve and optimization of cell number. (A) Standard curve to cAMP used for interpolation of cAMP concentrations in cell based assay (B) Forskolin concentration effect curves performed over a range of CHO-5-HT_{1A} cells densities (C) Forskolin concentration effect curve for 3000 CHO-5-HT_{1A} cells/well converted to cAMP concentration using cAMP standard curve.

non-selective 5-HT receptor agonist 5-hydroxytryptamine (5-HT) as well as the selective 5-HT_{1A} agonist, R(+)8-OH-DPAT (Figure 3). Both of these full agonists demonstrated a similar efficacy and potency with a pEC_{50} of 7.8 ± 0.2 for 5-HT and 7.7 ± 0.2 for R(+)8-OH-DPAT. Buspirone acted as a partial agonist in this study where it demonstrated only 44.7% of the maximal response obtained by R(+)8-OH-DPAT and a reduced potency relative to the full agonists (pEC_{50} of 6.8 ± 0.2). The antagonist spiperone and the dopamine D_1 selective agonist SKF38393 had no effect on the forskolin-induced cAMP production (Figure 3). These data are summarized in Table 1 where it can be seen that the agonist pEC_{50} values obtained in this study are consistent with published literature.[9-16] Furthermore, the rank of order potency obtained by the agonists correlated well with published binding data for the 5-HT_{1A} receptor.[17]

Table 1: Comparison of pEC_{50} values for 5-HT agonist inhibition of forskolin-stimulated cAMP.

Agonists	pEC_{50}	Emax (%)	Published values for pEC_{50}
5-HT	7.8 ± 0.3	101.8 ± 10.7	7.6 ± 0.2
R(+)8-OH-DPAT	7.7 ± 0.2	100	7.9 ± 0.3
Buspirone	6.8 ± 0.2	44.7 ± 13.4	7.2 ± 0.3
Published pEC_{50} values are means ± S.D of references 9 to 16.			

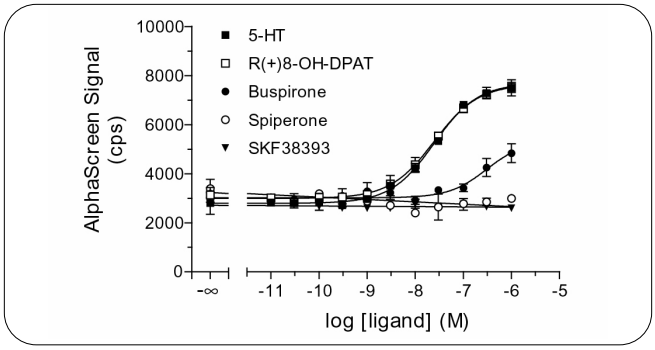


Figure 3: Pharmacological profile for agonist stimulation of cAMP CHO-5-HT_{1A} cells. Cells were stimulated with the 5-HT agonists 5-HT, R(+)8-OH-DPAT and buspirone, the antagonist spiperone and the dopamine receptor agonist, SKF38393 in the presence of 10 µM forskolin. Representative data are shown. pEC_{50} values are shown in Table 1.

The 5-HT_{1A} receptor was further characterized by performing an antagonist pharmacological profile using a functional inhibition curve approach. Cells were pre-incubated for 30 minutes with various concentrations of antagonist followed by stimulation with a cocktail containing 10 µM forskolin and 100 nM R(+)8-OH-DPAT. A representative set of inhibition curves are shown in Figure 4 where it can be seen that spiperone and NAN-190 competitively antagonized the effect of R(+)8-OH-DPAT in CHO-5-HT_{1A} cells with a similar potency to each other (IC_{50} of 7.9 ± 4.0 and 2.6 ± 1.6 nM, respectively). S(-)propranolol was less potent than spiperone and NAN-190 where its curve was right-shifted with an IC_{50} of 987.7 ± 615.6 nM. Both clozapine (D4, 5-HT_6 and 5-HT_7 subtype selective) and metergoline (5-HT_2 and 5-HT_{1d} subtype selective) demonstrated no antagonism in this system as expected. The antagonist IC_{50}'s are converted to pK_b values and compared to published data as shown in Table 2. Further quantitative antagonist studies were performed using Schild analysis (data not shown). Schild slope factors close to unity and pA_2 values of 8.8 ± 0.2 and 8.4 ± 0.2 for NAN-190 and spiperone, respectively were obtained (Table 2).

These data demonstrate that the affinities estimated for NAN-190 and spiperone were similar using either functional inhibition curves or Schild analysis indicating that the functional inhibition curve approach, a method used more routinely by screening scientists, is suitable for the estimation of antagonist affinity. Further, this data demonstrates the utility of AlphaScreen cAMP detection technology for quantitative pharmacological applications.

Table 2: Comparison of antagonist pharmacology in CHO-5-HT_{1A} cells.

Agonists	IC_{50} (nM)	pK_b	pA_2	Published values for pK_b
Spiperone	7.9 ± 4.0	8.9 ± 0.2	8.4 ± 0.2	7.6
NAN-190	2.6 ± 1.6	9.4 ± 0.3	8.8 ± 0.2	8.6 ± 0.2 (pA_2)
S(-)Propranolol	987.7 ± 615.6	6.8 ± 0.2	--	6.4 ± 0.1
Published pKb values are from references 9, 10, 13 and 15.				

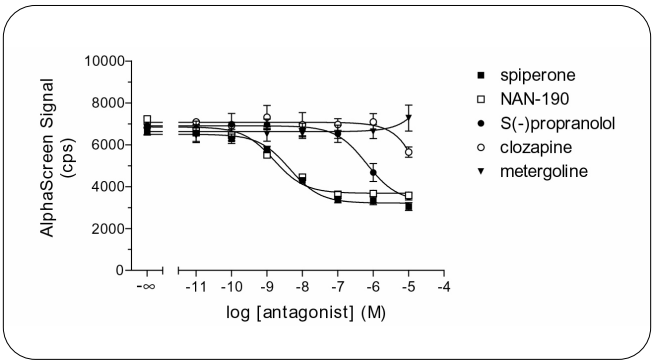


Figure 4: Pharmacological profile for antagonist inhibition of R(+)8-OH-DPAT stimulation of CHO-5-HT_{1A} cells. CHO-5-HT_{1A} cells were pre-incubated for 30 mins with the 5-HT_{1A} selective antagonists spiperone, NAN-190 and S(-)propranolol, the 5-$HT_{6/7}$ selective antagonist clozapine or the 5-HT_{1d}/5-HT_2 selective antagonist metergoline before stimulation with 100 nM R(+)8-OH-DPAT and 10 µM forskolin. Representative data are shown. IC_{50} and pK_b values are shown in Table 2.

It is crucial to measure the tolerance of any assay system to organic solvents such as DMSO, which is routinely used as carrier solvent for compound libraries. The effect of DMSO was studied on both the cAMP standard curve and on the cell stimulation step. Figure 5A demonstrates that increasing concentrations of DMSO up to a 5% final concentration in a standard curve resulted in a concentration-dependent decrease in the AlphaScreen signal where a 7, 13, 27 and 49%

decrease in the signal was observed compared to the control for 0.5, 1, 2 and 5% DMSO respectively. The EC_{50} for cAMP was unaffected by the DMSO concentration. In the cell stimulation experiments, the signal was relatively unaffected at DMSO concentrations up to 1% and a signal decrease of 9.5 and 26% was observed in the presence of 2 and 5% DMSO, respectively without any change in the potency of R(+)8-OH-DPAT to inhibit forskolin-stimulated cAMP production (Figure 5B).

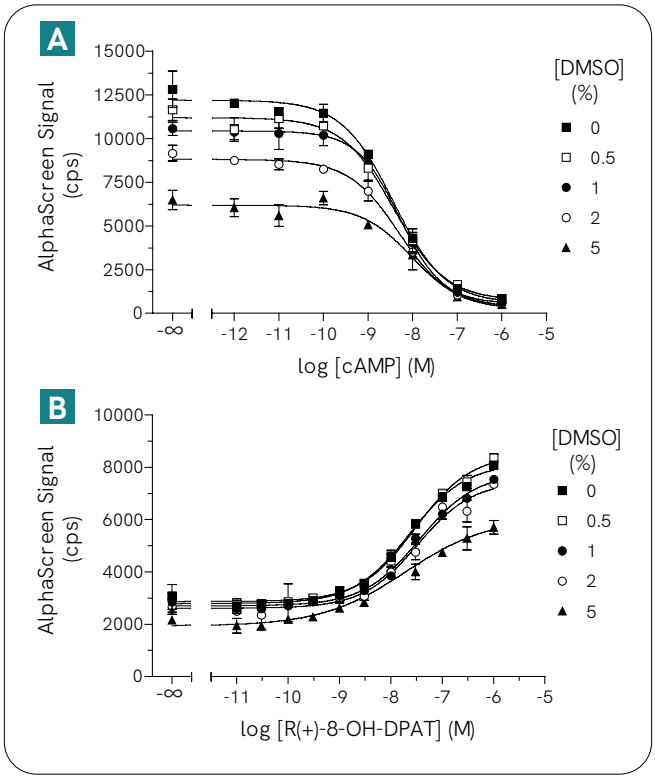


Figure 5: Effect of DMSO on cAMP standard curve and CHO-5-HT_{1A} agonist stimulation. (A) Acceptor beads and cAMP dilutions were incubated with increasing DMSO concentrations (0-12.5%; giving final concentrations of 0-5%) followed by the detection step. (B) In agonist stimulation experiments, stimulation was performed in presence of a final concentration of 0-5% DMSO. Representative data are shown.

The reproducibility and robustness of the AlphaScreen cAMP Assay were studied using replicate 24 wells for each cell-stimulated condition on two different plates between two different days. 1% DMSO was added to buffers to simulate screening conditions. Cells were incubated with buffer (basal level) or stimulated with forskolin (10 µM) only, forskolin and agonist R(+)8-OH-DPAT (100 nM) or finally, forskolin, agonist and 1 µM spiperone antagonist (Figure 6). The results demonstrated good intra- and inter-plate variability with coefficients of variation (CV's) varying from 4.3 to 11.6 for all the different stimulation conditions (Table 3). The Z' factor calculated for the forskolin stimulation, agonist stimulation and antagonist effect are shown in Table 4. Both forskolin stimulation and R(+)8-OH-DPAT stimulation produced Z' values that indicate a very robust assay suitable for the transfer to a high-throughput screen. The antagonist response showed a lower Z' value of approximately 0.3. Although this Z' was lower than that observed for the forskolin and R(+)8-OH-DPAT stimulations, it demonstrates that this assay system is still robust despite the relative complexity of this $G_{\alpha i}$ antagonist measurement.

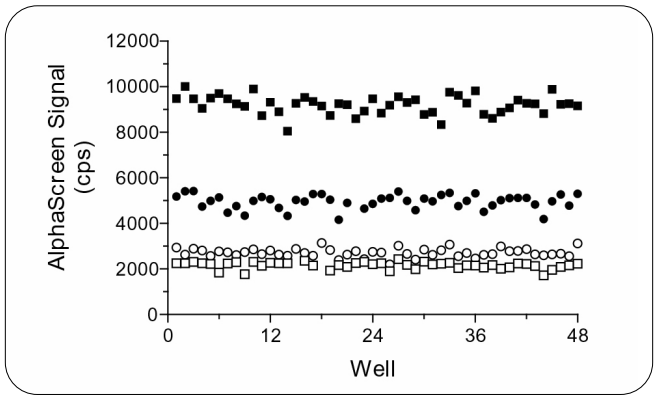


Figure 6: Variability/precision analysis. Cells were stimulated with buffer (■), 10 µM forskolin (❑), 10 µM forskolin + 100 nM R(+)8-OH-DPAT (●) and 10 µM forskolin + 100 nM R(+)8-OH-DPAT +1 µM spiperone (❍) in the presence of 1% DMSO. The data represents a total of 48 wells for each condition performed over 2 separate plates. Statistical data are represented in Tables 3 and 4.

Table 3: Intra and inter-plate variability between 2 days.

	Basal			Fsk			Fsk + R(+)8OH-DPAT			Fsk + R(+)8OH-DPAT + spiperone		
	mean	S.D.	%CV	mean	S.D.	%CV	mean	S.D.	%CV	mean	S.D.	%CV
Day 1 Plate 1	9233	429	4.6	2193	154	7.0	4912	348	7.1	2727	166	6.1
Day 1 Plate 2	9190	392	4.3	2138	150	7.0	5001	289	5.8	2713	213	7.8
Day 2 Plate 1	8951	540	6.0	2776	184	6.6	6319	388	6.1	3335	386	11.6
Mean values were calculated from 24 replicates.												

Table 4: Z′ data from variability experiments

	Basal / Fsk	Fsk / Fsk + R(+)8-OH-DPAT	Fsk + R(+)8-OH-DPAT / Fsk + R(+)8-OH-DPAT + spiperone
Day 1, plate 1	0.75	0.45	0.30
Day 1, plate 2	0.77	0.54	0.34
Day 2, plate 1	0.65	0.51	0.22
Mean ± S.D.	0.73 ± 0.05	0.50 ± 0.04	0.30 ± 0.05

Conclusion

The AlphaScreen cAMP Assay kit has been used to characterize the $G_{\alpha i}$-coupled 5-HT_{1A} receptor expressed in CHO cells and further, validated as a platform that can be used for the high-throughput screening of $G_{\alpha i}$-coupled receptors. The AlphaScreen cAMP Assay kit demonstrated excellent utility, where standard agonists and antagonists demonstrated the expected pharmacology. The use of functional inhibition curves for the determination of antagonist affinities yielded affinity constants consistent with published data. These data demonstrate suitability of the platform for compound characterization in hit-to-lead seeking programs. Further, where a more detailed study of antagonist pharmacology is required, the AlphaScreen cAMP Assay kit was highly applicable, where Schild analysis produced the expected competitive interactions and the pA_2 values were consistent with the inhibition curve data and published values.

Key to the success of a screening platform is its resistance to organic solvents such as DMSO as well as its precision in being able to distinguish active compounds from those with no activity. Here we have demonstrated the suitability of the AlphaScreen cAMP Assay kit for screening where DMSO tolerance up to 1% was observed and assay precision as calculated by Z′ was 0.5 for $G_{\alpha i}$-coupled agonist stimulation and 0.3 for antagonist inhibition of this response. In view of the complexity of the types biomolecular interactions occurring in this system as well as the fact that the precision analysis was performed in the presence of 1% DMSO, the AlphaScreen cAMP Assay kit represents a highly robust system for the detection of agonists and antagonists at $G_{\alpha i}$-coupled receptors.

Thus in conclusion, the AlphaScreen cAMP Assay kit demonstrates multifaceted flexibility for studying $G_{\alpha i}$-coupled receptors where everything from primary screening through to hit-to-lead characterization and in depth pharmacological studies can be performed.

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