APPLICATION NOTE

cAMP assay provides flexibility and stable pharmacology.

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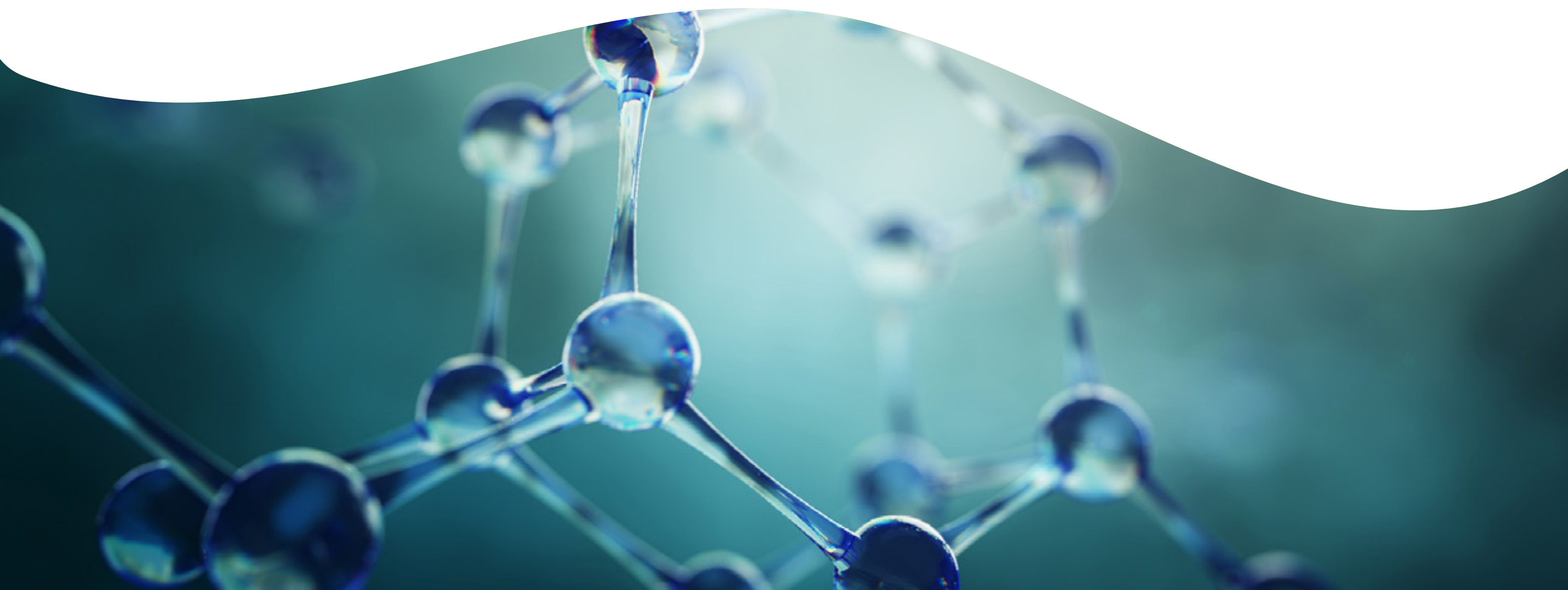
Introduction

G protein-coupled receptors (GPCRs) are the largest and most diverse protein family in the human genome with over 800 members identified to date. They play a role in cellular and physiological processes including cell proliferation, differentiation, neurotransmission, development, and apoptosis.[1] GPCRs are cell surface transmembrane receptors that catalyze the activation of G-proteins. They 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 adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP). Acting as a second messenger, cAMP is used for intracellular signal transduction in a variety of biological processes including cell growth and differentiation, gene transcription, and protein expression.[2] GPCR activity for the subunits Gαs and Gαi is commonly assessed by measuring levels of intracellular cAMP upon stimulation by agonists. Abnormal GPCR activity is related to many disease states such as cancer and therefore GPCRs have been an important class of pharmaceutical drug targets.[3] Multiple assay formats have been developed to promote further research and support pharmacological efforts. By measuring the level of cAMP generated, one can determine the pharmacological potency of different agonists and antagonists. While there are a large number of assays available on the market to detect and quantify cAMP levels in cells, the ideal assay is a homogenous, non-radioactive assay that allows for sensitive and reproducible detection.



AlphaScreen® technology allows for the quantitative detection of molecules of interest in a homogeneous, no-wash format and can be readily applied to GPCR research. In the cAMP AlphaScreen assay illustrated in Figure 1, a biotinylated-cAMP tracer binds to Streptavidin-coated Donor beads and interacts with the anti-cAMP antibody conjugated to AlphaScreen Acceptor beads. When no source of endogenous cAMP is present, the beads come into close proximity. The excitation of the Donor beads triggers the release of singlet oxygen molecules which diffuse to the Acceptor beads, resulting in light emission at 520 - 620 nm. Competition for the anti-cAMP antibody on the Acceptor beads by endogenous cAMP results in a decreased signal due to a separation of the Donor and Acceptor beads.

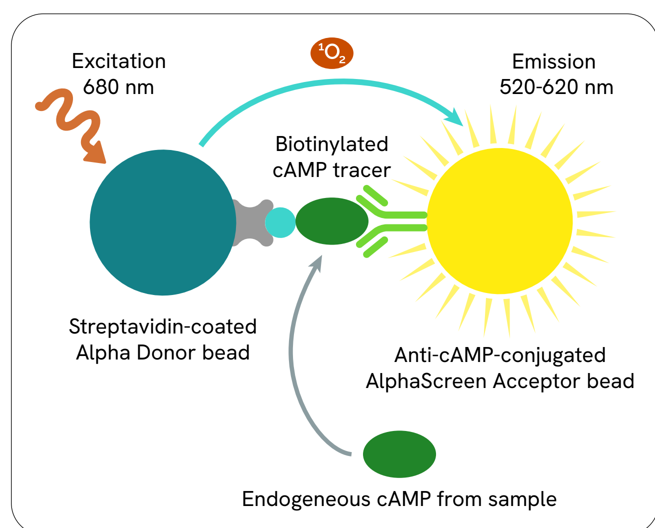


Figure 1: AlphaScreen cAMP assay principle.

Reagents

- AlphaScreen cAMP kit (Revvity #6760635)
- DPBS (1X) without Ca^{2+} & Mg^{2+} (Invitrogen #14190)
- HEPES Buffer (1M) pH 7.5 (Teknova #H1035)
- Hank's Balanced Salt Solution (ThermoFisher #14025-092)
- BSA protease-free (Sigma #A8577)
- IBMX (Sigma #I5879)
- Forskolin (Tocris #1099)
- DMSO (Sigma# D8418)
- Cell Dissociation Solution, enzyme free (Sigma #C5914)
- AlphaPlate™-384, light gray microplates (Revvity #6005350)
- TopSeal®-A Plus (Revvity #6050185)
- Microplate lid, black (Revvity #6000027)
- CHO-K1 MC4 (Melanocortin 4) cell line (Revvity #ES-191-C)
- Culture Media: Ham's F12 (ThermoFisher #11765-054) + 10% FBS (ThermoFisher #26140-079)
- Geneticin G418, for cell line selection (ThermoFisher #10131-027)
- α-MSH MC4 Agonist (Tocris #2584)
- SHU 9119 MC4 Antagonist (Tocris #3420)

Adjustable dynamic range protocol

The "Adjustable Dynamic Range" protocol presented in Figure 2 enables stable overnight incubations and allows users to adjust the dynamic range and sensitivity of the AlphaScreen cAMP detection kit. Assay volume (25 µL total), plate type (384-well AlphaPlate), cell handling, and drug treatment steps were performed as described in the AlphaScreen cAMP manual. No changes were made to the Acceptor and Donor bead final concentration (20 µg/mL). Donor bead incubations were performed in subdued lab lighting and assay plates were sealed with TopSeal-A for longer incubations to prevent evaporation. Eliminating the pre-incubation of the biotinylated tracer with Streptavidin-coated Donor beads produces a higher signal-to-background ratio and generates stable pharmacology (EC_{50} or IC_{50}) independent of the final incubation time.

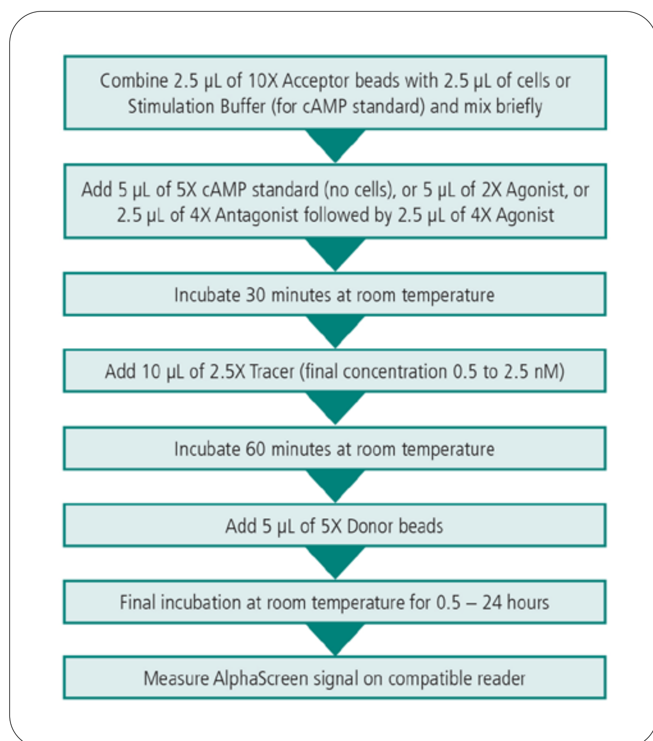


Figure 2: Adjustable Dynamic Range protocol for cAMP AlphaScreen assay. Acceptor beads, cells, cAMP standard and drug treatments were prepared in Stimulation Buffer. Biotinylated-cAMP Tracer and Streptavidin-coated Donor beads were prepared in 1X Immunoassay Buffer. Working concentrations and volumes for the step-wise detection step were adjusted as noted. Cell density is determined through forskolin experiments as outlined in the AlphaScreen cAMP manual.

Data collection and analysis

The AlphaScreen cAMP assay was measured using a Revvity EnVision® Multilabel plate reader using default values for AlphaScreen detection. cAMP standard curves were included for each experiment, increasing the highest cAMP standard concentration for tracer optimization experiments. Curves were plotted in GraphPad Prism according to nonlinear regression fitting using the four-parameter logistic equation (sigmoidal dose-response curve with variable slope) and $1/Y^2$ data weighting. Cell-based validation results were calculated in fmoles of cAMP produced per well as interpolated from the standard curve using the AlphaScreen signal and total assay volume. The biologically relevant pharmacological value for an agonist or antagonist was determined using fmoles of cAMP produced.

Tracer concentration determines assay sensitivity

Step-wise addition of the detection reagents lowered the concentration of the biotinylated-cAMP tracer required in the assay (original protocol uses 25 nM final tracer). Optimization was necessary to achieve the desired level of sensitivity and avoid excess biotinylated-cAMP in the reaction. The cAMP standard was used to determine a condition that yielded an IC_{50} value comparable to the original protocol. Figure 3 demonstrates that assay sensitivity can be adjusted by altering the final concentration of biotinylated-cAMP tracer. Table 1 contains additional data generated during optimization.

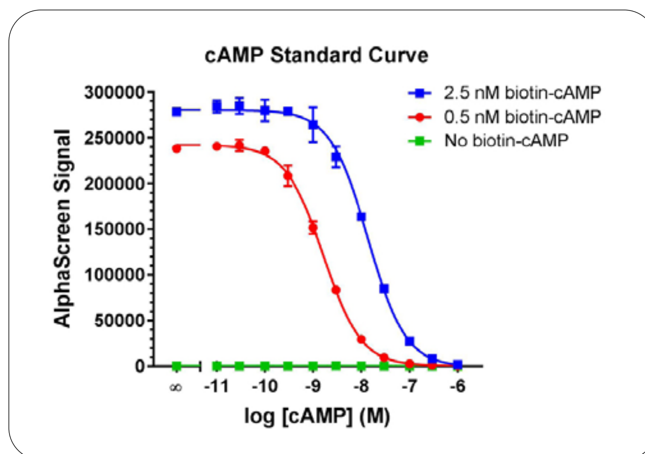


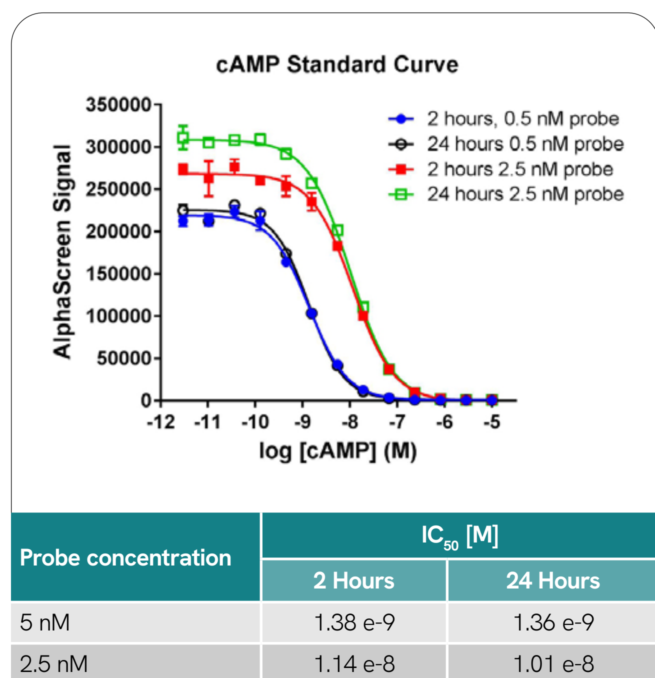
Figure 3: Optimization of biotinylated-cAMP tracer concentration using the Adjustable Dynamic Range protocol. cAMP standard curves are shown for select tracer concentrations (two hour final incubation time). Assay sensitivity correlated with the concentration of tagged tracer as expected; increasing tracer required more cAMP to compete for binding.

Table 1: Optimization of biotinylated-cAMP tracer concentration using the Adjustable Dynamic Range protocol. Dynamic range covers ~two logs and Signal-to-Background was ~three-fold higher than original protocol (data not shown).

Tracer concentration	IC_{50} [M]	IC_{10} [M]	IC_{90} [M]	Hill slope	Signal-to-background
5 nM	3.42e-08	5.64e-09	2.07e-07	-1.219	808.3
2.5 nM	1.65e-08	2.89e-09	9.44e-08	-1.260	807.9
1 nM	4.47e-09	7.11e-10	2.81e-08	-1.195	779.1
0.5 nM	1.82e-09	2.73e-10	1.21e-08	-1.159	701.5

Stable pharmacology is independent of incubation time using the adjustable dynamic range protocol

Data consistency over the course of a screening campaign and reliable assay performance are critical features required in order to compare pharmacology results. The Adjustable Dynamic Range protocol provides the benefit of reaching equilibrium of the detection reagents in a shorter period of time, and provides stability of EC_{50} or IC_{50} values. Final incubation times of 0.5 to 24 hours were tested for the cAMP standard curve; select results are shown in Figure 4. Replicate plates were required for each time point because AlphaScreen technology cannot be re-read given the nature of the singlet oxygen reaction on the Alpha beads.



Probe concentration	IC_{50} [M]	
	2 Hours	24 Hours
5 nM	1.38 e-9	1.36 e-9
2.5 nM	1.14 e-8	1.01 e-8

Figure 4: Stable pharmacology over time. Data were generated for time points from 0.5 hours to 24 hours; select times from a representative experiment are shown. IC_{50} determination at each biotinylated-cAMP tracer concentration was constant, independent of incubation time. The calculated IC_{50} was not affected by the slight AlphaScreen signal increase seen with the higher 2.5 nM tracer concentration.

Sensitivity of the adjustable dynamic range protocol with respect to cell number

Forskolin dose-response curves were generated at different cell densities in order to establish the optimal cell density for cAMP assays. Forskolin acts directly on adenylyl cyclase to produce cAMP, independently of receptor activation, and represents the maximum cAMP levels achievable. Data were collected in CHO-K1 cells expressing MC4 receptor using a range of biotinylated-cAMP tracer concentrations (Figure 5). The ability to rapidly determine optimal pairings of cell density and tagged tracer concentration provides flexibility for screening efforts and added sensitivity (increased cell density can be used with low cAMP producing cell lines and yield excellent results).

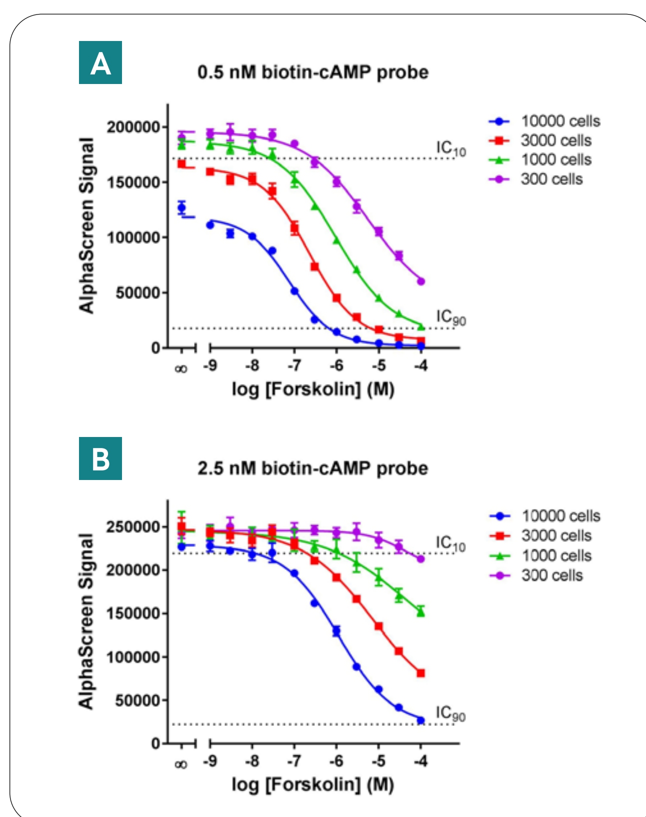


Figure 5: Forskolin dose-response curves determine appropriate cell density for screening. Values for IC_{10} and IC_{90} determined from a cAMP standard curve (dotted lines) represent the apparent linear range of the assay. The optimal cell concentration for subsequent experiments was chosen based on the proportion of the dose-response curve falling within this range. Optimal pairings selected from this experiment were 2,000 cells with 0.5 nM tracer, and 10,000 cells with 2.5 nM tracer.

Validation of the adjustable dynamic range protocol in a cell-based assay

The Adjustable Dynamic Range protocol was next validated in a cell-based model. Data were generated for both agonists and antagonists in CHO-K1 cells expressing MC4 receptor. α-MSH is an endogenous MC4 receptor agonist. Figure 6 shows data from two optimal cell density and tracer concentration pairs. The interpolated concentration of cAMP produced per well with α-MSH stimulation correlates with cell density as expected. The biologically relevant EC_{50} of the agonist is the same for all four conditions tested as seen in Table 2. The ~EC_{60} of α-MSH based on amount of cAMP produced per well was used to stimulate the cells for antagonist experiments with SHU 9119; results shown in Figure 7 and Table 2. In both sets of experiments, replicate plates at two final incubation times demonstrate the stability of pharmacology and reproducibility of the Adjustable Dynamic Range protocol.

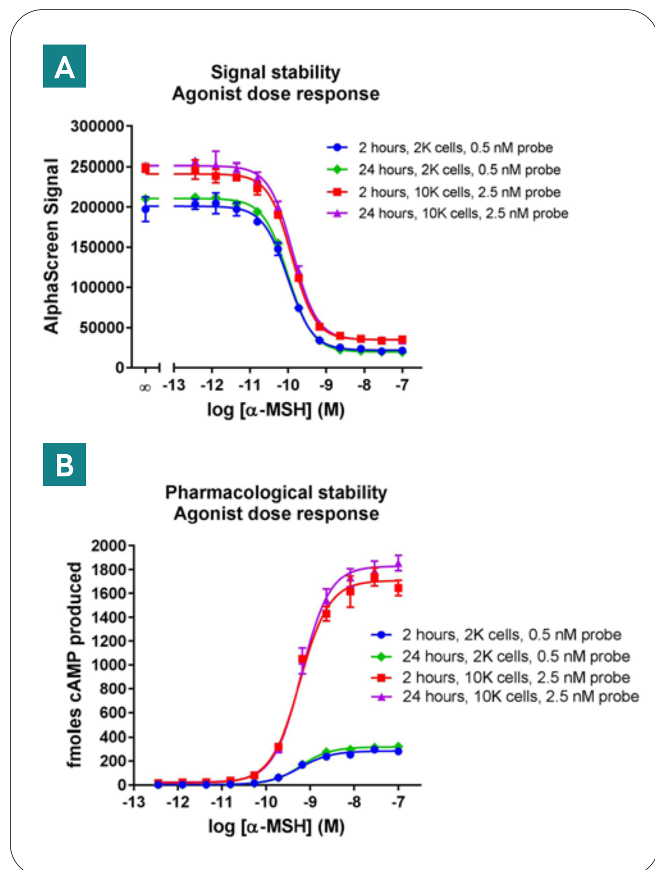


Figure 6: Stability of the cell-based AlphaScreen cAMP assay. Data shown at the optimal pairing of cell density and tracer concentration listed. Final incubation times of 2 hours vs. 24 hours demonstrate signal stability. (A) α-MSH dose-response plotted against AlphaScreen signal. (B) α-MSH results converted to fmoles of cAMP produced per well.

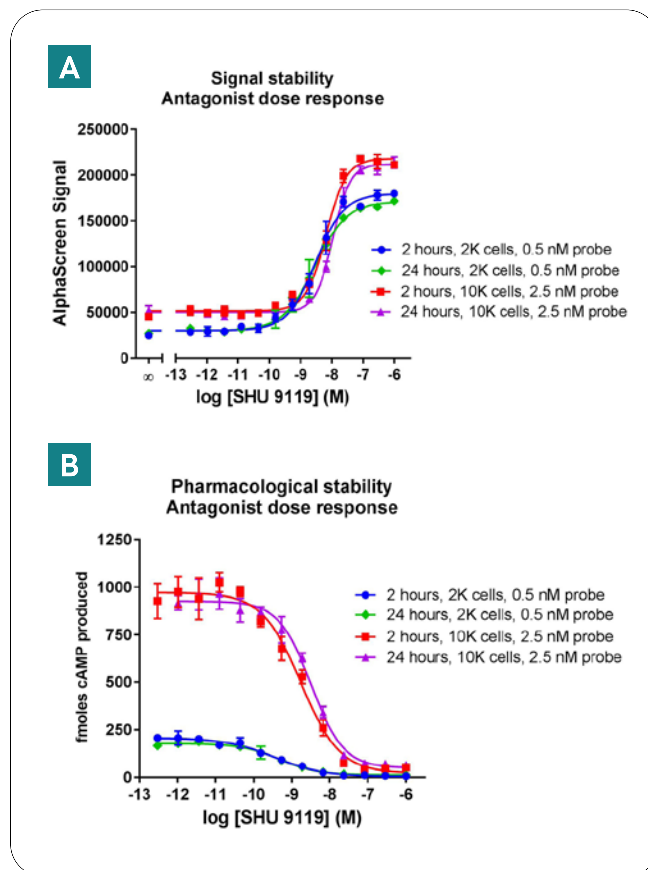


Figure 7: Stability of the cell-based AlphaScreen cAMP assay. Data shown at the optimal pairing of cell density and tracer concentration listed. Final incubation times of 2 hours vs. 24 hours demonstrate signal stability. (A and B) 1 nM α-MSH agonist stimulation and co-treatment of antagonist dose-response plotted against AlphaScreen signal (A) or fmoles of cAMP (B) produced.

Table 2: Pharmacological Results. Optimal pairing of cell density and tracer concentration listed. Final incubation times of 2 hours vs. 24 hours. For antagonist assay, 1 nM α-MSH agonist was used for stimulation of the MC4 receptor.

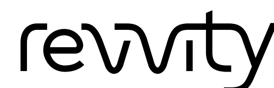
	Cells/[Probe]	AlphaScreen signal		fmoles cAMP	
		α-MSH IC_{50} [M]		α-MSH EC_{50} [M]	
		2 hours	24 hours	2 hours	24 hours
Agonist	2K / 0.5 nM	9.80 e-11	9.89 e-11	5.73 e-10	6.13 e-10
	10K / 2.5 nM	1.25 e-10	1.33 e-10	5.85 e-10	6.37 e-10
		SHU 9119 EC_{50} [M]		SHU 9119 IC_{50} [M]	
Antagonist	2K / 0.5 nM	2.99 e-9	2.85 e-9	3.56 e-10	4.65 e-10
	10K / 2.5 nM	6.53 e-9	1.07 e-8	1.75 e-9	3.24 e-9

Summary

The Adjustable Dynamic Range protocol presented here provides stable assay signal and pharmacology over time as evidenced by the results of the cAMP standard curves and cell-based assays. Optimization for your specific cell line of interest should be performed to determine the optimal pairing of cell density and tracer concentration that produce the sensitivity required for screening.

References

1. Gutierrez, AN, et al. (2018). GPCRs: Emerging anti-cancer drug targets. *Cellular Signaling*, Vol 41: 65-74.
2. Yan, K, et al. (2016). The cyclic AMP signaling pathway: Exploring targets for successful drug discovery (Review). *Molecular Medicine Reports*, Vol 13: 3715-3723.
3. Hauser, AS, et al. (2017). Trends in GPCR drug discovery: new agents, targets and indications. *Nature Reviews Drug Discovery*, Vol 16: 829-842.



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