Monitor GPCR Modulation of Cellular cAMP with an HTS, Bioluminescence-Based Assay

ABSTRACT The cAMP-Glo™ Assay is a bioluminescence assay for monitoring changes in intracellular cAMP concentrations with high sensitivity (30 fmol ± 5 SEM cAMP/well) and reproducibility (Z´ >0.8). This assay can be used to measure cAMP concentration in a single-tube or high-throughput-screening (HTS) format and is less prone to compound interference than fluorescence-based assays. We have used the cAMP-Glo™ Assay to generate EC_{50} values for agonists and IC_{50} values for antagonists that modulate GPCRs that alter adenylate cyclase activity ($G\alpha_s$, and $G\alpha_i$). The cAMP-Glo™ Assay is compatible with adherent, suspension, and frozen cells, offering flexibility and convenience to the researcher, and the cAMP-Glo™ Assay can be used to determine cAMP concentration in tissue extracts.

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INTRODUCTION

G-Protein-Coupled Receptors (GPCRs) represent one of the largest receptor families in the human genome, and nearly half of all prescription drugs are targeted toward GPCRs (1). Since these drugs target only 40–50% of well characterized GPCRs, many more GPCRs remain to be investigated. Thus, there is intense interest in developing novel ligands for orphan receptors both as potential drugs as well as pharmacological tools for understanding cellular physiology.

Binding of extracellular ligands to GPCRs alters the conformation of the associated heterotrimeric G proteins and causes their dissociation into their Gα and Gβγ complex proteins, and initiates a cascade of cellular events (2). The interaction of an activated receptor with a heterotrimeric G protein catalyzes the exchange of guanosine diphosphate (GDP) for guanosine triphosphate (GTP) and subsequent dissociation of the Gα-GTP complex from the Gβγ complex enabling both Gα-GTP and Gβγ dimers to interact with a variety of downstream effectors (2). While the $G\alpha_s$ activates the effector adenylate cyclase with a resulting increase in cAMP concentration, $G\alpha_{i/o}$ inhibits its activity, resulting in a decrease in intracellular cAMP concentration.

In addition to ligand-binding-based assays and measurements of guanine nucleotide exchange, functional activity-based assays of receptors have attracted attention. Functional activity assays use either reporter-based assays or assays that monitor the intracellular concentrations of the messengers cAMP or calcium (3–5). Reporter-based assays offer the advantage of wide linearity and sensitivity that allow them to detect weak agonists or allosteric modulators (6,7). However, because of the long incubation times required they are limited by false-positive results, interactions with other signaling pathways and desensitization of receptors. Because of the signal amplification in the pathway, weak agonists can appear as potent agonists. To address these issues, other functional assays have emerged (8). Here we describe the cAMP-Glo™ Assay[a,b] for monitoring modulation of GPCRs. This assay can be used to monitor agonists and antagonists that modulate adenylate cyclase activity by measuring the level of intracellular cAMP.

PRINCIPLE OF THE ASSAY

cAMP-dependent protein kinase (PKA) is activated by very low concentrations of cAMP, and thus PKA activation can be altered by changing the concentration of cAMP. PKA is composed of two catalytic subunits and two regulatory subunits in an inactive heterotetrameric form. In the presence of cAMP, the catalytic and regulatory subunits dissociate into two free, enzymatically active catalytic subunits and two regulatory subunits. Since modulation of $G\alpha_s$- and $G\alpha_i$-protein-coupled receptors leads to alteration in intracellular cAMP concentration, monitoring the activation of PKA using a kinase assay can be used to assess the functional activity of the GPCR (Figure 1). To demonstrate the utility of this strategy using a luminescence kinase assay (9), we generated an EC_{50} value for cAMP activation of PKA (10,11). Our assay generated an EC_{50} value of 8.3 nM, which compares favorably with values obtained using other methods (10,11) and with what is reported in the literature (Figure 2; 12). The signal-to-noise ratios in the presence of 20 nM or 100 nM cAMP are 91 and 254, respectively, indicating that the assay can detect extre-mely low concentrations of cAMP. The Z´-factor values, statistical measures of reproducibility and robustness (13), are 0.8 or higher (Table 1) in both 384-well and 1536-well plates.

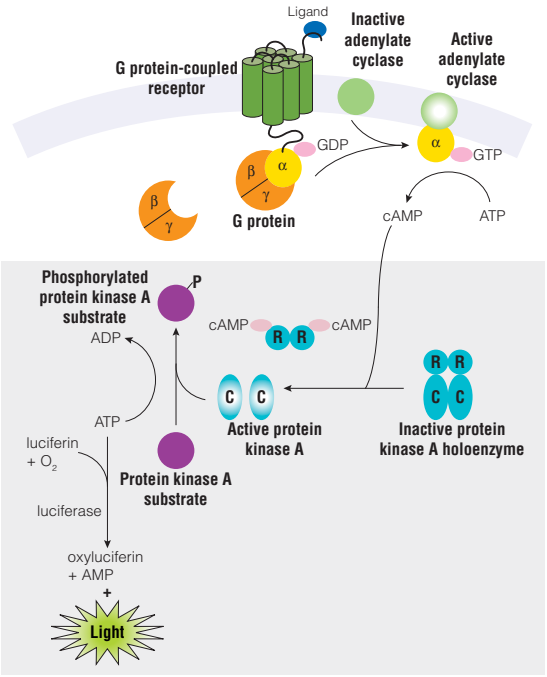

Figure 1. Schematic presentation of cAMP-Glo™ Assay principle. Ligand binding to its GPCR triggers dissociation of the heterotrimeric G protein to Gα and Gβγ complex and exchange of GTP for Gα-GDP. The Gα-GTP interacts with adenylate cyclase (AC), resulting in either activation ($G\alpha_s$) or inhibition ($G\alpha_i$) of AC activity. Changes in intracellular cAMP modulate the activity of the tetrameric, inactive cAMP-dependent protein kinase (PKA) into the active free catalytic subunit and the regulatory subunit dimer. The activation of PKA can be monitored by the decrease in ATP substrate in the kinase reaction, and then the remaining ATP can be quantified in a luciferase reaction. The amount of light detected is inversely proportional to the intracellular cAMP concentration. Thus, activation of $G\alpha_s$-coupled receptors generates low RLU, and inhibition of those receptors results in high RLU. In contrast, activation of $G\alpha_i$-coupled receptors generates an increase in RLU, and inhibition of those receptors results in a decrease in RLU.

TITRATION OF FORSKOLIN

Since forskolin activates adenylate cyclases independently of receptors and G proteins, we tested its effect on the intracellular cAMP concentration. Incubation of varying forskolin concentrations with HEK 293 cells for 15 minutes resulted in increased cAMP concentrations with an EC_{50} value of 4.38 µM (Figure 3), similar to the published value of 2.44 µM (8). Z´-factor values of 0.7 or higher were obtained, confirming the reproducibility of the assay (Table 1).

ASSAY PERFORMANCE WITH $G\alpha_s$-PROTEIN-COUPLED RECEPTORS

To validate the cAMP-Glo™ Assay for use with $G\alpha_s$-protein-coupled receptors, we generated D293 cells expressing a dopamine (D1) receptor. As shown in Figure 4, Panel A, we tested different concentrations of agonists of the D1 receptor and generated a dose response curve and the EC_{50} values for each of the agonists tested. We obtained an EC_{50} of 35.7 nM for dopamine, 78.1 nM for SKF38393, 89.3 nM for apomorphine and 23.4 nM for SKF82958.

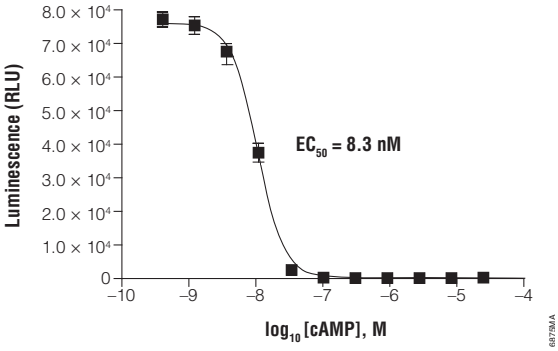

Figure 2. Effect of cAMP concentration on activation of PKA. Increasing cAMP concentration results in activation of PKA. The increase in PKA activity is inversely proportional to the amount of relative luminescence units (RLU). The PKA reaction was performed as described in reference 18; PKA activity was detected using the Kinase-Glo® Reagent (18). Data represent means ± SEM (n = 3).

These values agree with published values for these agonists using a competition radioimmunoassay with ^{125}I-labeled cAMP (14). We also tested the response of D1 receptors to D1 antagonists by incubating cells with various concentrations of antagonist in the presence of a fixed concentration of agonist (100 nM dopamine). The response curve of antagonist was dose-dependent, and an IC_{50} value of 10 nM was obtained for SCH23390 (Figure 4, Panel B), which is similar to the reported IC_{50} value for this compound using a competitive radiolabel binding assay (15). As expected, no inhibition was observed with alprenolol, a known β-adrenergic receptor antagonist. Z´-factor values of 0.8 or higher were obtained in 96-well and 384-well plates, and a Z´-factor value of 0.63 was obtained using 1536-well plates, underscoring the high quality of the assay (Table 1).

Table 1. Fold Induction and Z´-Factor Values for Solutions Containing cAMP[1] (0, 20 or 100 nM) and for DRD1 Cells in Medium Containing Dopamine[2] (0 or 100 nM) or Forskolin (0, 5 or 100 µM).

Compound Added (Concentration)	Fold Induction	Z´-Factor Value
cAMP (20 nM)	91	0.84
cAMP (100 nM)	254	0.85
dopamine (100 nM)	25.5	0.86
dopamine[3] (100 nM)	15	0.63
forskolin (5 µM)	8	0.70
forskolin (100 µM)	26.6	0.88

[1]cAMP was added at 0, 20 or 100 nM concentration to a reaction containing PKA holoenzyme, ATP, peptide substrate, and buffer and incubated for 20 minutes followed by the addition of Kinase-Glo® Reagent. Activation of PKA was monitored as described in reference 18. Fold induction was calculated as RLU signal generated in control (vehicle only) divided by RLU obtained in the presence of 20 or 100 nM cAMP.

[2]Cells stably expressing DRD1 were plated at 5,000 cells/well in 384-well plates and incubated with either dopamine or forskolin and processed for measurement of cAMP accumulation using the cAMP-Glo™ Assay.

[3]Cells stably transfected with DRD1 were plated at 2,500 cells/well and incubated with or without dopamine in 1536-well plates. Fold induction was calculated as RLU signal generated in control (vehicle only) divided by RLU obtained in the presence of dopamine or forskolin. Z´-factor values were calculated as described in reference 17.

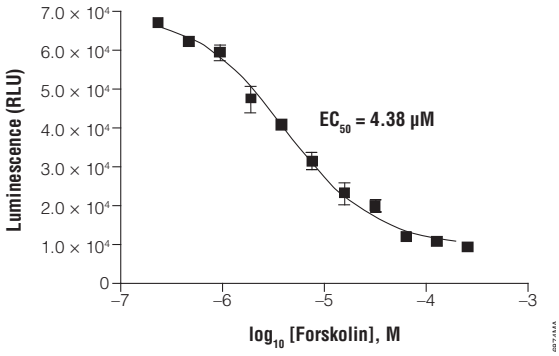

Figure 3. Forskolin titration using HEK 293 cells. HEK 293 cells (5,000/well) in a 384-well plate were incubated with various concentrations of forskolin and processed for cAMP detection as described in reference 18. The data shown represent means ± SEM (n = 3).

Since these drugs target only 40–50% of well characterized GPCRs, many more GPCRs remain to be investigated.

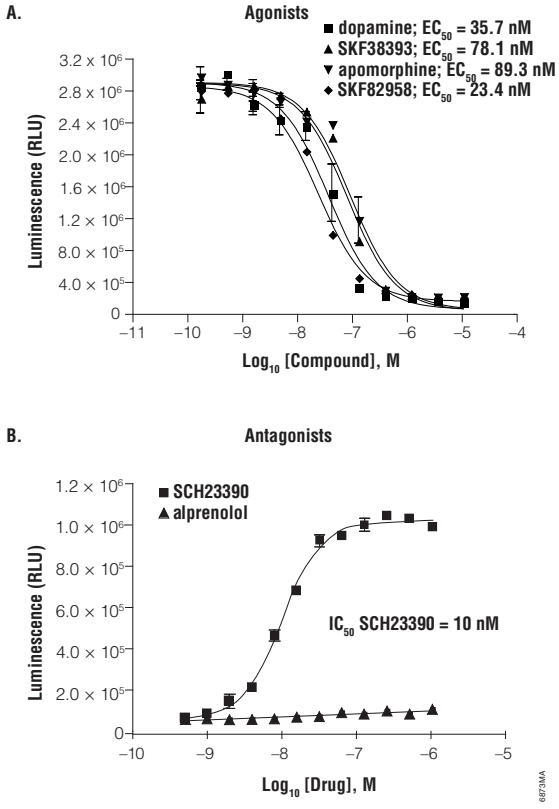

Figure 4. Titration of D1 receptor agonists and antagonists. Panel A. Agonists. D293 cells stably expressing the dopamine D1 receptor (DRD1; 5,000/well) were cultured in 384-well plates overnight to 80% confluency. Medium was removed and induction buffer containing various concentrations of agonists was added and incubated for additional 30 minutes. Lysis buffer was then added, and cAMP concentration was monitored as described in reference 18. The data shown represent means ± SEM (n = 3). **Panel B.** Antagonists. D293 cells stably expressing the dopamine D1 receptor (5,000/well) were incubated and processed as in reference 18. Induction buffer contained 100 nM of dopamine and various concentrations of the antagonist (SCH23390) or control compound alprenolol. The data shown represent means ± SEM (n = 3).

ASSAY PERFORMANCE WITH $G\alpha_i$-PROTEIN-COUPLED RECEPTORS

D293 cells stably expressing the D2 receptor were tested for their response to selective D2 agonists such as quinpirole by incubating them with 10 µM forskolin and increasing concentrations of agonist for 15 minutes at 37 °C. As shown in Figure 5, Panel A, we obtained an EC_{50} for quinpirole of 0.57 nM, which is similar to the reported value in the literature using radiolabeled quinpirole binding to D2 receptors in rat brain (16). Similar experimental design was used to test antagonists, except that cells were incubated with 10 µM forskolin and 10 nM of the D2 agonist quinpirole and with increasing concentrations of the antagonist raclopride. In this experiment, we monitored the functional activity of antagonists by reversing the effect of fixed concentration of the agonist quinpirole on cAMP increase by forskolin. Thus true antagonists should increase intracellular cAMP concentration as the dose is increased. As shown in Figure 5, Panel B, we have obtained an IC_{50} value of 0.92 nM for raclopride, D2 receptor antagonist, which is similar to values reported in the literature using a competitive radiolabel assay (17).

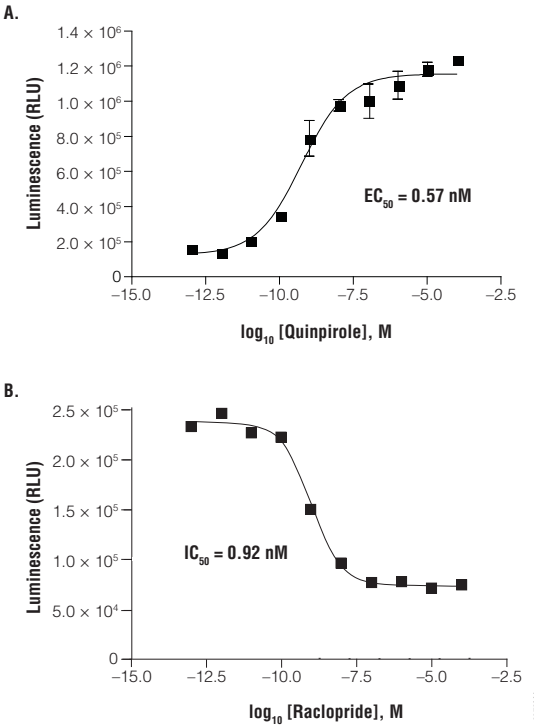

Figure 5. Titration of D2 receptor agonists and antagonists. Panel A. Titration of dopamine D2 receptor agonist quinpirole. Dopamine D2 (DRD2) receptor stably transfected D293 cells (5,000/well) were induced with induction buffer containing 10 µM forskolin and increasing concentrations of the D2 agonist quinpirole followed by lysis and cAMP detection as described (18). The data shown represent means ± SEM (n = 3). **Panel B.** Titration of dopamine D2 Receptor Antagonist. D293 cells stably transfected with DRD2 (5, 000 cells/well) were induced with induction buffer containing 10 µM forskolin and 10 nM quinpirole and various concentrations of raclopride. Cells were processed as described in reference 18. The data shown represent means ± SEM (n = 3).

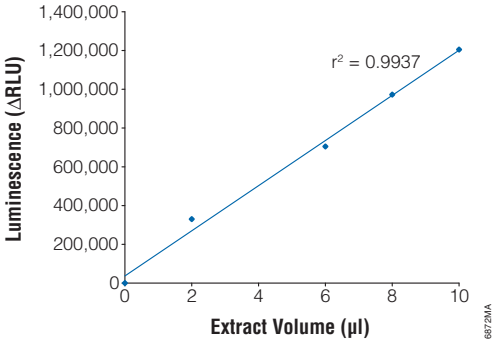

Figure 6. cAMP-Glo™ Assay in tissue extracts. A 2 ml/gm rat brain extract was homogenized in cAMP-Glo™ Lysis Buffer containing 0.5 mM IBMX, 100 µM Ro20-1724 and then heated for 5 minutes at 70 °C. Samples of 0, 2, 4, 6, 8 and10 µl were tested by the addition of an equal volume of Krebs Ringer buffer followed by the addition of cAMP-Glo™ reaction buffer. The reaction was incubated for 20 minutes at room temperature and then an equal volume of Kinase-Glo® Reagent was added. Relative luminescence was read after 10 minutes, and ΔRLU was calculated by subtracting each value for each sample from that obtained with lysis buffer only. ΔRLU = RLU (0 µl)–RLU (sample).

The cAMP-Glo™ Assay was also tested with $G\alpha_s$- and $G\alpha_i$-protein-coupled receptors using adherent, suspension, and frozen cells, and similar values for EC_{50} for agonists and IC_{50} for antagonists were obtained (data not shown), offering convenience and flexibility to the user.

MEASUREMENT OF cAMP IN TISSUE EXTRACTS

Rat brain was homogenized in lysis buffer containing phosphodiesterase inhibitors, heated at 70 °C for 5 minutes, and cAMP concentration was determined using serially diluted extract. Results of a typical experiment using rat brain extract are shown in Figure 6. This protocol was used successfully with other rat tissues including liver and heart (data not shown).

CONCLUSION

The luminescent cAMP-Glo™ Assay is robust and easy to use for monitoring changes in cellular or tissue cAMP concentrations. The assay can be formatted to any size experiments, from single tubes to high-density plates, offering flexibility and convenience, which are highly desirable features in HTS applications.

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PROTOCOL

- *cAMP-Glo™ Assay Technical Bulletin*, #TB357, Promega Corporation
  www.promega.com/tbs/tb357/tb357.html

ORDERING INFORMATION

Product	Size	Cat.#
cAMP-Glo™ Assay	300 assays	V1501
	3,000 assays	V1502
	30,000 assays	V1503

(a)Patent Pending.

(b)The method of recombinant expression of *Coleoptera* luciferase is covered by U.S. Pat. Nos. 5,583,024, 5,674,713 and 5,700,673.

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