Live-Cell Assay to Interrogate GPCRs by Monitoring cAMP Levels using a Bioluminescent Readout

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Abstract

Monitoring GPCR responses to both agonists and antagonists represent a major focus as targets of both academic research and drug discovery efforts. GPCR signaling can elicit several second messenger molecules, one of which, cAMP, is engaged in several physiologically relevant cellular signaling events.

Here we demonstrate assay performance in a 384-well format as well as the pharmacology of several compounds. The assay provides increased signal-to-background ratio and low-volume, low-cost miniaturization capability in comparison to other existing assay formats for high-throughput applications.

Introduction

G-protein coupled, seven-transmembrane (7-TM) receptors (GPCRs) represent large, diverse gene families found in fungi, plants and animals. The GPCR family of receptors are involved primarily with internalizing extracellular environmental signals via interaction with intracellular G-proteins. GPCR ligands and stimuli range from peptide and non-peptide molecules to chemokines, nucleosides, growth factors and light. The involvement of GPCRs in many critical cellular signaling responses has resulted in considerable interest as a pharmacologically important protein family. Monitoring the response of GPCRs to a variety of agonist and antagonists is currently being investigated by academic researchers and in drug discovery efforts.

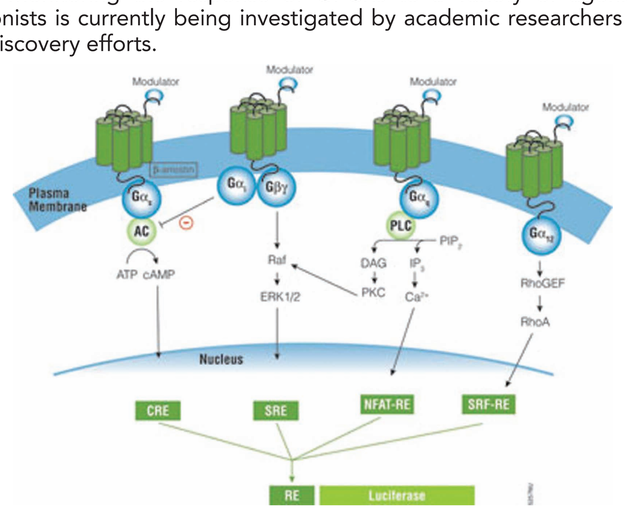

Figure 1 – GPCR signaling. Upon stimulation, G_S-coupled receptors activate adenylate cyclase (AC) with concomitant increase in intracellular cAMP levels.

Historically, cAMP quantification relied on a competitive assay consisting of labeled cAMP in competition with cAMP from a cell lysate with a colorimetric readout. More recently, increased sensitivity was achieved by several methods including TR-FRET and bead-based assays. However, these methods are considered end-point assays in that they require cell lysis prior to detection of intracellular cAMP levels.

Luminescent reporter genes have been shown to provide increased sensitivity with a wide dynamic range and are easily adaptable to a high density microplate format amenable to automation. A recently developed biosensor protein expressed from a genetically modified form of luciferase is capable of modulation of its luminescence activity dependent on reversible allosteric interaction with ligand allowing live-cell, real-time monitoring of cAMP kinetics. The ability to either stably or transiently transform the biosensor into a preferred cell line allows investigation of both endogenous or over-expressed GPCR targets. Here we used the GloSensor™ cAMP Assay consisting of GloSensor cAMP HEK293 Cell Line stably ransfected with pGloSensor-L9 cAMP Plasmid in conjunction with GloSensor cAMP Reagent.

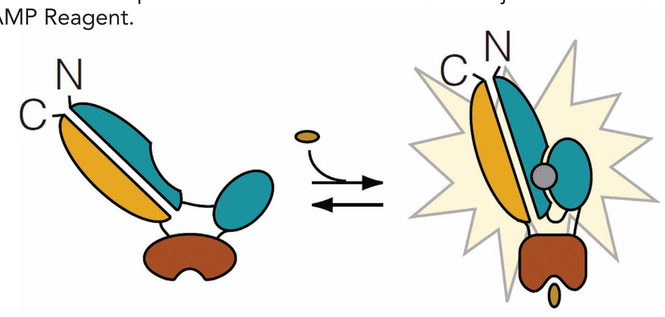

Figure 2 – Bioluminescent sensor allows detection of molecular processes in live cells through reversible allosteric modulation of luminescence activity during ligand binding. Binding of ligand, in this case cAMP, results in a closed conformation of the biomolecule resulting in increased luciferase activity.

BioTek Instrumentation

Figure 3 – Synergy™ H4 with Hybrid Technology™ is a patent-pending multi-mode detector ideal for research and drug discovery applications when having to choose between flexibility and performance is not an option. The monochromator-based optics provide a high level of flexibility; any wavelength can be used from the low UV to the near infrared. The quadruple grating optical system, equipped with a variable bandpass selection system, is ideal for spectral scanning applications. The filter-based optics use dichroic mirrors for enhanced performance. The instrument also contains a dedicated optical system, separate from the fluorescence optics, for high-performance luminescence detection.

Cell Culture Procedure

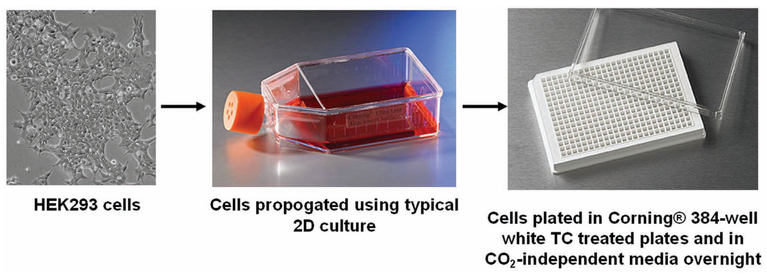

Figure 4 – Cell culture conditions for GloSensor cAMP HEK293L9-2 cell line.

GloSensor Assay Procedure

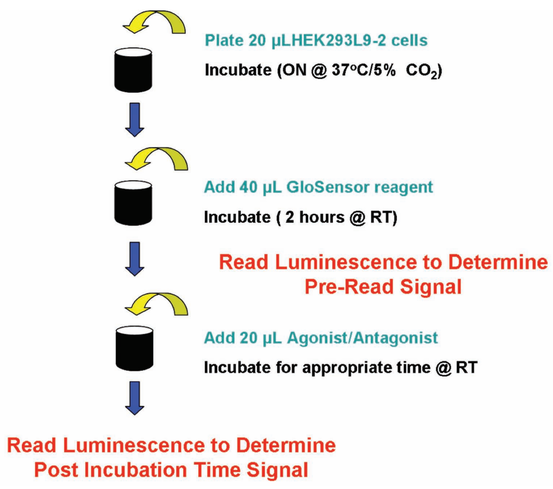

Figure 5 – GloSensor cAMP Assay workflow.

Fold Induction Calculation

- Pre-read data was used to normalize well-to-well variation due to plating variability and edge effects
- Post incubation time data points were divided by the pre-read value for each well
- Replicate data point ratios for each condition were then averaged
- Fold response was calculated by dividing the signal from wells containing compound by the signal from wells containing no compound (basal signal)

Cell Concentration Determination

Cell Titration

- GloSensor cAMP HEK293L9-2 cells were grown in culture to ~90% confluency per manufacturer's technical manual in DMEM media supplemented with 10% FBS and Hygromycin B
- Cells were plated in 384-well microplate density concentrations ranging from 1,000-10,000 cells/well in quadruplicate
- Plates were incubated overnight at 37°C, 5% CO_2
- GloSensor Reagent was prepared in CO_2-independent media supplemented with 10% FBS and added to each well followed by incubation at room temperature for 2 hours
- The plate was pre-read on the Synergy H4 plate reader to generate baseline values
- Forskolin was added at either a 0 or 10 µM 1X final concentration, and luminescence was read on Synergy H4 plate reader in kinetic mode
- Optimal cell number was determined from calculated fold induction values

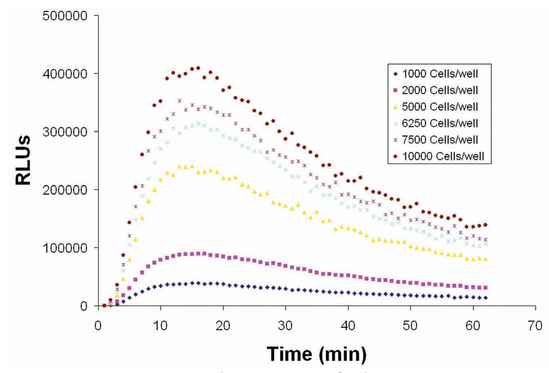

Figure 6 – Luminescence signal response of GloSensor cAMP HEK293L9-2 cell line to 10 µM Forskolin.

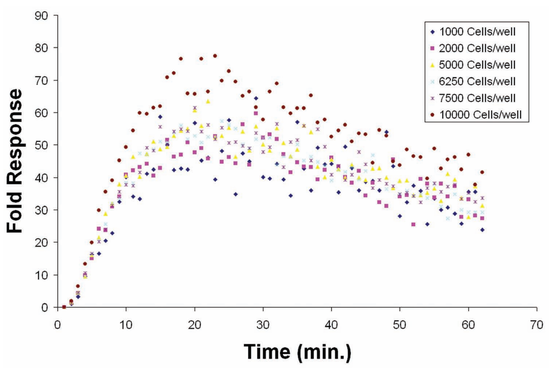

Figure 7 – Fold response of GloSensor cAMP HEK293L9-2 cell line when subject to 10 µM Forskolin vs. 0 µM control well at the indicated cell number/well.

- Signal intensity depicted in Figure 6 indicated sufficient signal generated from as little as 1,000 cells/well
- As shown in Figure 7 there is an ~40-fold induction sustained from ~12-30 minutes at a cell concentration of 1,000 cells/well indicative of an excellent S/N ratio.
- Further experiments preformed with 1,000 cells/well

β2 Adrenergic Receptor Agonist Optimization

β2 Adrenergic Receptor Agonist Incubation Time

- Cells were prepared and plated as described above at a density of 1,000 cells/well
- Plates were incubated overnight at 37°C, 5% CO_2 and GloSensor reagent added as described above
- The plate was pre-read as described above
- Agonists were added at positive control concentrations of 10 µM for isoproterenol and sabutamol, and 1 µM formoterol and a negative control concentration of 0 µM
- Luminescence was read on Synergy H4 plate reader in kinetic mode to determine optimal incubation time

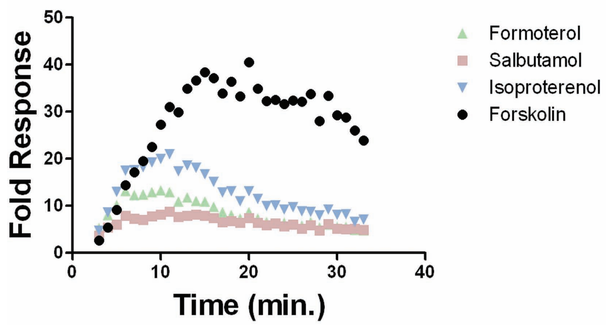

Figure 8 – Kinetic data depicting fold response of GloSensor cAMP HEK293L9-2 cell line when subjected to the indicated compound.

β2 Adrenergic Receptor Agonist Optimization (Continued)

β2 Adrenergic Receptor Agonist Titration

- Cells were prepared and plated as described above at a density of 1,000 cells/well
- Plates were incubated overnight at 37°C, 5% CO_2 and GloSensor reagent added as described above
- The plate was pre-read as described above
- An 11-point titration was performed for each compound, including a zero compound point
- The plate was read on the Synergy H4 at optimal incubation time

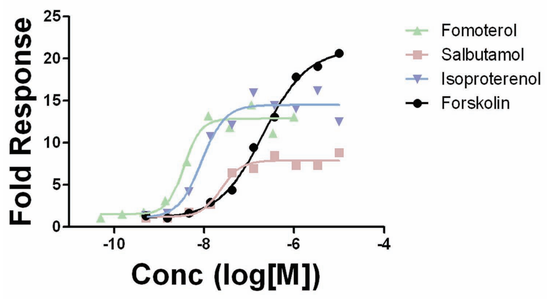

Figure 9 – Fold response of GloSensor cAMP HEK293L9-2 cell line when subject to an 11-point titration of the indicated compound.

Compound	EC50 (nM)	EC80 (nM)
Formoterol	3.6	6.5
Salbutamol	24	48
Isoproterenol	8.8	20
Forskolin	210	980

Table 1 – EC_{50} and EC_{80} concentrations of agonists as determined from dose response curves in Figure 9.

- It is apparent from Figure 8 that the optimal incubation time was between 10-15 minutes post-incubation period following agonist addition
- Dose response curves and IC_{50} values for the test compounds shown in Table 1 agree with values previously reported in assay literature

β2 Adrenergic Receptor Antagonist Optimization

β2 Adrenergic Receptor Antagonist Incubation Time

- Cells were prepared and plated as described above at a density of 1,000 cells/well
- Plates were incubated overnight at 37°C, 5-10% CO_2 and GloSensor reagent added as described above
- The plate was preread as described above
- Antagonist was added at 10 µM and 0 µM and incubated for 5, 10, 15 and 30 minutes prior to addition of the EC_{80} concentration of isoproterenol
- Luminescence was read on Synergy H4 plate reader to determine optimal antagonist incubation time

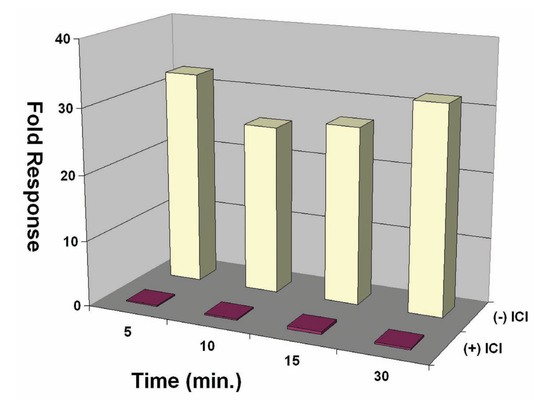

Figure 10 – Fold response of GloSensor cAMP HEK293L9-2 cell line when subjected to 10 µM ICI 118551.

β2 Adrenergic Receptor Antagonist Titration

- Cells were prepared and plated as described above at a density of 1,000 cells/well
- Plates were incubated overnight at 37°C, 5-10% CO_2 and GloSensor reagent added as described above
- The plate was pre-read as described above
- An 9-point antagonist titration was performed, including a zero compound point and EC_{80} concentration of isoproterenol was added following the optimal antagonist incubation time
- The plate was read on the Synergy H4 at the previously determined optimal agonist incubation time

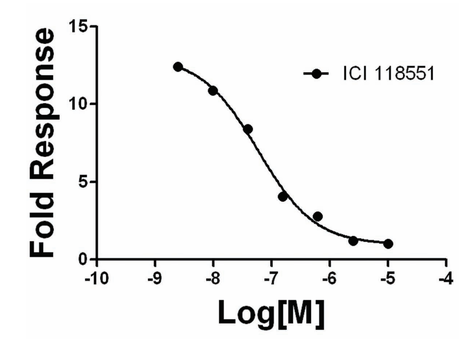

Figure 11 – Fold response of GloSensor cAMP HEK293L9-2 cell line when subject to varying concentrations of ICI 118551.

- A nearly complete inhibition of signal was seen within 5 minutes post addition of antagonist as indicated in Figure 10
- An incubation time of 10 minutes was used for the antagonist prior to addition of the EC_{80} concentration of isoproterenol
- The antagonist titration curve and IC_{50} concentration of 59 nM determined from the dose response curve was similar to values reported in assay literature

Conclusions

1. The combination of the stably transfected GloSensor cAMP HEK293L9-2 cell line and GloSensor technology allow for easy analysis of β2 Adrenergic receptor activity
2. The live cell capability of the assay allows for easy optimization through the use of kinetic reads
3. The Synergy H4 is able to easily quantitate the luminescent signal from basal and stimulated assay wells
4. The combination of assay and instrumentation provide an ideal solution for high-throughput detection of GPCR signal transduction