

Abstract

GPCR responses to extracellular signaling events remain a major focus of both academic research and drug discovery efforts as pharmacological targets. Hit to lead applications typically require the pharmacological evaluation of hits from screening campaigns where their dose-response is quantified. This secondary screening usually incorporates a functional assay where the GPCR is expressed in a cell line of relevance. Here we demonstrate the automation of the workflow for the assessment of agonist and antagonist activity for the b2-adrenergic receptor endogenously expressed in HEK293 cells using a stably transfected bioluminescent protein that binds cAMP. Data obtained using automated methods were consistent with data generated when using manual methods including data quality and EC₅₀/IC₅₀ precision.

Introduction

G-protein coupled receptors (GPCRs) remain one of the most druggable targets. The function of GPCRs is to sense the extracellular environment and communicate some specific aspect of that environment to intracellular machinery. The topology of the GPCR ensures that small molecule compounds or other signals do not have to traverse the cell membrane barrier in order to reach the site of action. Conversely, when targeting intracellular enzyme targets, such as protein kinases, significant medicinal chemistry effort is spent trying to get compounds into the cells. Studies involving GPCRs typically focus on measuring the functional responses of the receptors to various compounds and determining the pharmacology of agonists and antagonists. The ability to make these observations in an *in-vitro*, live-cell assay remains a highly sought after alternative to the more typical lytic, end-point assays.

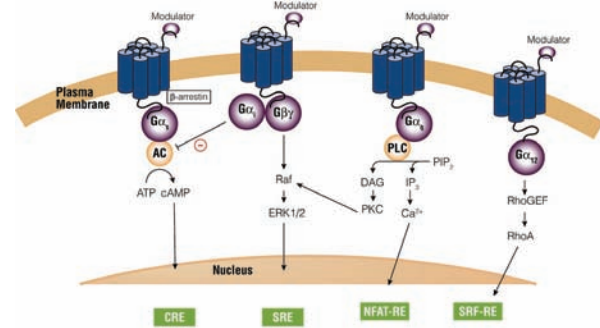


Figure 1 – GPCR signaling. Upon stimulation, G_s-coupled receptors activate adenylyl cyclase (AC) with concomitant increase in intracellular cAMP levels.

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 (Figure 2). Here we demonstrate the automation of the GloSensor™ cAMP Assay, particularly for multi-dose point secondary screening efforts of hit compounds from a primary screening campaign, which are necessary to construct dose-response curves and to determine potency. Assay performance as well as the pharmacology of several compounds is investigated and compared to those obtained when manual methods were employed.

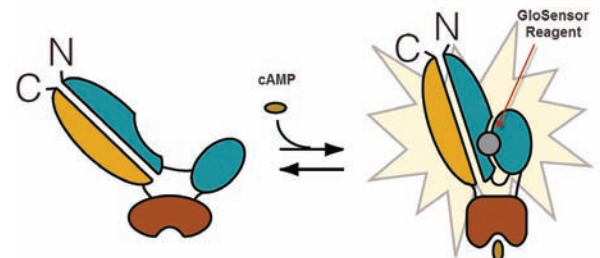


Figure 2 – Bioluminescent sensor allows detection of molecular processes in live cells through reversible allosteric modulation of luminescence activity during ligand binding. Binding of ligand, in this case cAMP, results in a closed conformation of the biomolecule resulting in increased luciferase activity.

BioTek Instrumentation



Figure 3 – MultiFlo™ Microplate Dispenser.



Figure 4 – Precision™ Microplate Pipetting System.



Figure 5 – Synergy™ H4 Microplate Reader.

Cell Culture Procedure

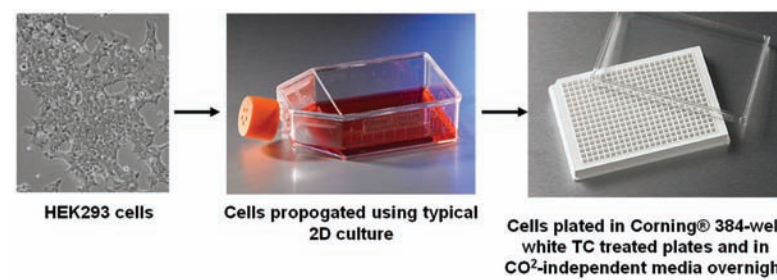


Figure 6 – Cell culture conditions for GloSensor cAMP HEK293/L9 cell line.

GloSensor cAMP HEK293/L9 cells were grown in culture to ~90% confluency as per manufacturers technical manual in DMEM media supplemented with 10% FBS and Hygromycin B.

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)

GloSensor Assay Procedure

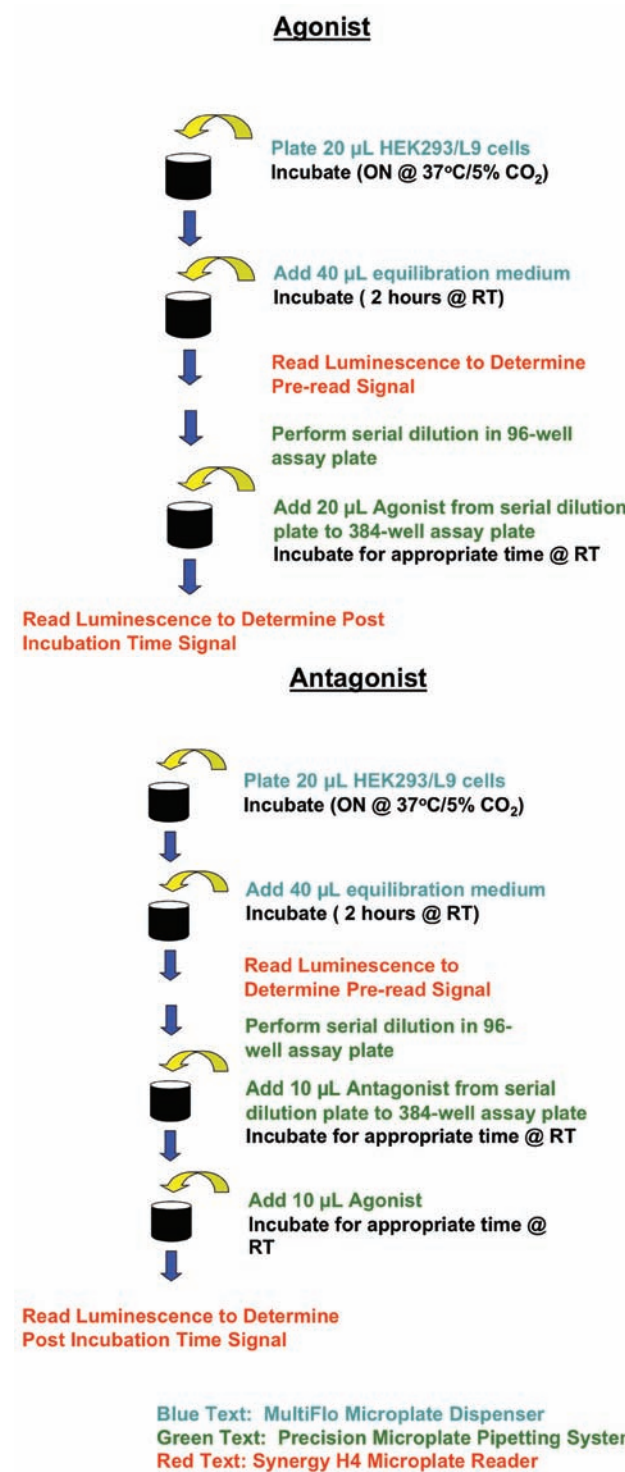


Figure 7 – GloSensor cAMP Assay workflow.

β2 Adrenergic Receptor Agonist Dose Response

- Cells were prepared as described above and plated at a density of 1,000 cells/well in a volume of 20 µL using the MultiFlo Microplate Dispenser
- Plates were incubated overnight at 37°C, 5% CO₂
- Equilibration medium containing 4% v/v GloSensor cAMP Reagent in a volume of 40 µL/well was added using the MultiFlo
- The plate was pre-read on the Synergy H4
- An 11-point serial dilution was performed for each compound including a zero compound point and 20 µL transferred from the 96-well serial dilution plate to the 384-well assay plate using the Precision
- The plate was read on the Synergy H4 at the optimal incubation time

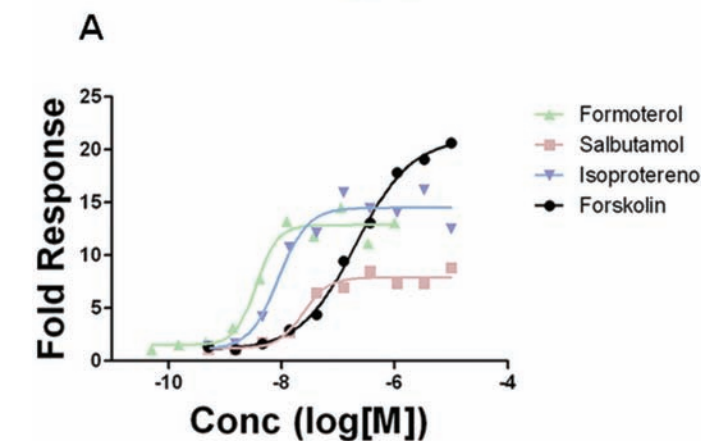
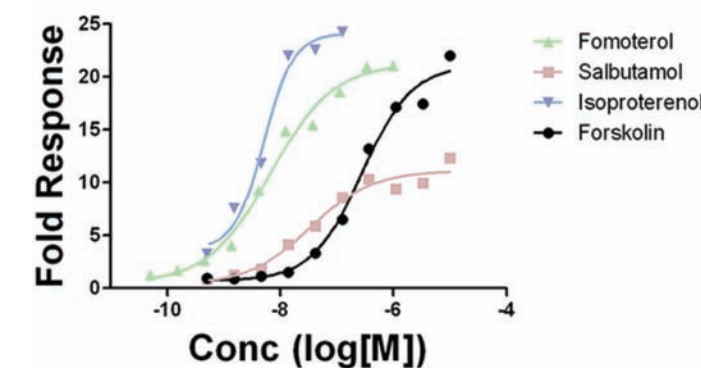


Figure 8 – Fold response of GloSensor cAMP HEK293/L9 cell line when subject to an 11-point titration of the indicated compound using either A) automated or B) manual methods.

β2 Adrenergic Receptor Agonist EC₅₀ Comparison

Compound	Manual (nM)	Automated (nM)
Formoterol	3.6	6.7
Salbutamol	24	33
Isoproterenol	8.8	5.1
Forskolin	210	280

Table 1 – EC₅₀ concentration of agonists as determined from dose response curves in Figure 9.

- Fold response curves shown in Figure 9 differs between the two methods likely due to passage number between methods, 1 vs. ~10 passages for automated vs. manual, respectively
- Calculated IC₅₀ values for the test compounds shown in Table 1 agree between the two methods and are consistent with values previously reported in assay literature

β2 Adrenergic Receptor Antagonist Dose Response

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% CO₂ and GloSensor cAMP 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 EC₈₀ concentration of isoproterenol was added following a 10 minute antagonist incubation period
- The plate was read on the Synergy H4 following a 12 minute agonist incubation period

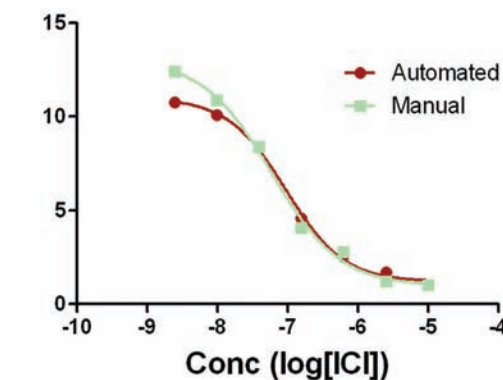


Figure 9 – Fold response of GloSensor cAMP HEK293/L9 cell line when subject to varying concentrations of ICI 118,551.

Compound	Manual (nM)	Automated (nM)
ICI 118,551	95	59

Table 2 – Comparison of antagonist IC₅₀ concentrations calculated from dose response curves using either automated or manual assay methods.

The dose response curves depicted in Figure 10 indicate both methods result in nearly identical responses to the antagonist ICI 118,551 and are consistent with assay literature

Z'-factor Determination

The Z'-factor was calculated from 48 replicate measurements using 20 nM (EC₈₀) isoproterenol stimulation with and without 10 µM (IC₁₀₀) antagonist ICI 118,551.

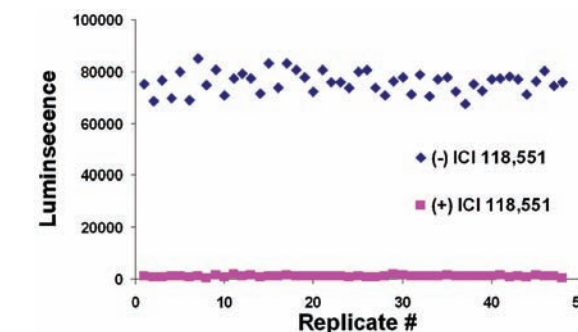


Figure 10 – Z'-factor calculation.

The results in Figure 10 show excellent assay robustness (Z'≈ 0.82)

Conclusions

1. The GloSensor cAMP Assay can be performed using simple, inexpensive automated methods.
2. The high sensitivity and low background achieved with manual methods were reproduced with higher throughput and ease-of-use with automated methods.
3. Pharmacology of known compounds generated using the MultiFlo Microplate Dispenser for cell and GloSensor cAMP Reagent dispensing and Precision Microplate Pipetting System for serial dilution and compound transfer agree with those generated using manual methods.
4. An increased Z'-factor determination when compared to manual methods provides for an increase in assay performance as well as the additional benefit of increased throughput.