Automation of a Bioluminescent Live-Cell cAMP Assay for the Pharmacological Evaluation of GPCRs

Peter Brescia, BioTek Instruments, Inc., Winooski, VT

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 ß$_2$-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 EC_{50}/IC_{50} precision.

Key Words:

Live-cell Assay

GPCR

cAMP

GloSensor Automation

GloSensor

Luminescence

Hybrid Microplate Reader

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.

Previously, the utility of the GloSensor™ cAMP Assay (www.promega.com/glosensor) to interrogate GPCRs by monitoring intracellular cAMP levels has been described[1]. The assay uses a recently developed biosensor protein expressed from a genetically modified form of luciferase and is capable of modulation of its luminescence activity dependent on reversible allosteric interaction with ligand allowing live-cell, real-time monitoring of cAMP kinetics[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.

Materials and Methods

Cell Culture Procedures and Reagent Preparation

Cell cultures were maintained and expanded as previously described[1]. Briefly, GloSensor cAMP HEK293/L9 Cell Line (human embryonic kidney cells stably transfected with pGloSensor L9 cAMP Plasmid) were a gift of Promega Corporation (Promega, Madison, WI). HEK293/L9 cells were grown and stored as per the manufacturer's protocol. Either Trypsin-EDTA 0.05% or TrypLETM Express was used for cell harvest and passage. Cells were maintained at < 90% confluency and passaged no more than 20 times. All cell culture reagents above were purchased from Invitrogen/Gibco (Carlsbad, CA) unless otherwise noted. Cells were allowed to adhere for >18 hours in a 37°C incubator with 5% CO_2 prior to use. Reagents were prepared as previously described[1].

BioTek Instruments, Inc.
P.O. Box 998, Highland Park,
Winooski, Vermont 05404-0998 USA
Tel: 888-451-5171
Outside the USA: 802-655-4740
E-mail: **customercare@biotek.com**
www.biotek.com

Automated Workflows

The procedures were modified to include the use of automated liquid handling instrumentation. Briefly, HEK293/L9 cells were harvested and plated in CO_2-independent medium in 384-well microtiter plates following the workflow depicted in Figure 3. Cells were plated at the appropriate cell number per microwell using a MultiFlo™ Microplate Dispenser (BioTek Instruments, Inc., Winooski, VT) in a final volume of 20 µL using a 5 µL dispense cassette, covered and incubated over night at 37°C with 5% CO_2. The equilibration medium, that includes the GloSensor cAMP Reagent, was prepared as previously described1. The MultiFlo was used for the addition of 40 µL of equilibration medium per assay well resulting in a 2% v/v reagent final assay concentration.

The assay plate was incubated and pre-read as previously described. Following the pre-read measurement, 20 µL total volume of compound delivery of receptor agonist, antagonists or direct activator of endogenous adenylate cyclase was accomplished using a Precision™ Microplate Pipetting System for a total assay volume of 80 µL. Serial dilutions for dose response curves were prepared in a 96-well assay plate by first manually loading the top concentration into column one. The required volume of diluent was then added to the remainder of the plate using the bulk reagent dispenser on the Precision. The top concentration was then serial diluted across the plate using the 8-channel pipettor on the Precision. The dilution series was transferred to the previously prepared 384-well assay plate in quadruplicate using the 8-channel pipettor. For all assays the concentration of DMSO was normalized to 0.75%.

After the appropriate incubation period(s) at room temperature the luminescence was read to determine post incubation signal and fold induction calculated as described below. For assays investigating antagonists, 10 µL of antagonist was added and incubated for the appropriate time. This was followed by the addition of 10 µL of agonist a second incubation period as depicted below in Figure 1. Both additions relied on the liquid handling capabilities of the Precision.

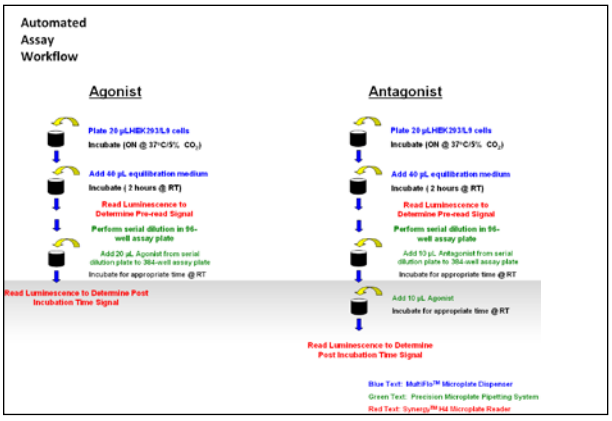

Figure 1. GloSensor cAMP Assay workflows to investigate either GPCR agonists or antagonists.

$ß_2$-Adrenergic Receptor Agonist Titration

HEK293/L9 cells and equilibration medium were added and the plate was pre-read as described above. A 1:3 serial dilution of each compound was performed on the Precision starting from either 10 µM for salbutamol, isoproterenol and forskolin or 1 µM for formoterol resulting in an 11-point titration, including a zero compound point. The compound titrations were then added to the 384-well assay microplate in quadruplicate using the Precision. The plate was read 12 minute after loading of the first compound concentration on the plate. The Gen5™ protocol was programmed to read the plate in paired columns mimicking the plate loading profile and to coordinate timing with the Precision loading scheme. For example, if rows A and B columns 23 and 24 were loaded first in quadruplicate then those wells were read in the first protocol read step.

$ß_2$-Adrenergic Receptor Antagonist Titration

HEK293/L9 cells and equilibration medium were added and the plate was pre-read as described above. A 1:4 serial dilution of ICI 118,551 HCl was performed on the Precision starting from 10 µM resulting in a 7-point titration, including a zero compound point. Each concentration was transferred to the 384-well assay microplate in quadruplicate using the Precision. Following a 10 minute incubation period from the time the first antagonist concentration was loaded the EC_{80} concentration of agonist was then added to the plate. The plate was read after the previously determined optimal agonist incubation period using a similar Gen5 protocol as the agonist titration experiment.

Z'-factor Determination

HEK293/L9 cells and equilibration medium were added using the MultiFlo Microplate Dispenser and wells pre-read as described above. ICI 118,551 HCl at concentrations of either 0 or the IC_{100}, 10 µM, was added to 48 replicate wells at each concentration, to create a positive and negative control, respectively. After the appropriate antagonist incubation period, the agonist isoproterenol at the EC_{80} concentration of 20 nM was added. The plate was read after the previously determined optimal agonist incubation period. The Z'-factor was determined from the luminescence values of the positive and negative control.

Instrument settings and Data Analysis

Synergy™ H4 Settings

All data were collected on the Synergy H4 Hybrid Multi-mode Microplate Reader in luminescence detection mode using endpoint read type. An optimal integration time used for all experiments was previously determined to be 0.3 seconds[1]. There was a 100 millisecond delay after plate movement and the dynamic range was set to extended. Luminescence was read at a sensitivity of 200 with a vertical probe offset of 1.00 mm.

Data Acquisition and Analysis

Data acquisition and analysis utilized Gen5 Data Analysis software (BioTek Instruments, Inc., Winooski, VT), Excel (Microsoft, Redmond, CA) and Prism (GraphPad Software, La Jolla, CA).

Fold Induction Calculation

The post incubation time data points were divided by the pre-read values for each well except where noted. Replicate data point ratios for each condition were then averaged. Fold response was calculated by dividing the signal ratio from wells containing compound by the signal ratio from wells containing no compound (basal signal).

Results and Discussion

ß$_2$-Adrenergic Receptor Agonist Titration

Dose response curves generated using either automated or manual liquid handling methods are depicted in Figure 2. EC_{50} values determined from dose response curves of various compounds generated using automated methods agree well with data collected in experiments using manual pipetting techniques (Table 1). It is apparent from Figure 2 that fold response differs between the two methods. It is likely that the increase in fold response seen in the automated assay resulted from the use of HEK293/L9 cells that had been passaged only once. This is in contrast to those used in the manual method that was performed with cells which had undergone numerous passages, ~10-12, prior to use.

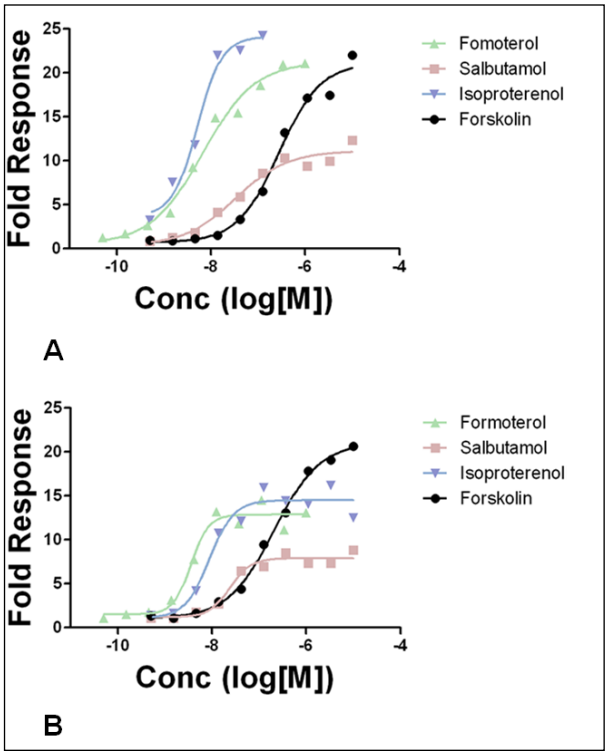

Figure 2. Comparisons between (A) automated and (B) manual assay methods. Dose response curves represented as fold response of GloSensor cAMP HEK293/L9 cell line when subjected to an 11-point agonist titration of the indicated compounds.

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

Table 1. Comparison of agonist EC_{50} concentrations calculated from dose response curves using either automated or manual assay methods. Forskolin dose response was included as an assay control.

ß$_2$-Adrenergic Receptor Antagonist Titration

The antagonist dose response curve comparing automated and manual liquid handling methods are depicted in Figure 3. The dose response curves indicate that both methods result in nearly identical responses when the GloSensor cAMP cell line is subjected to the antagonist ICI 118.551. The calculated IC_{50} values from the titration data agree as indicated in Table 2.

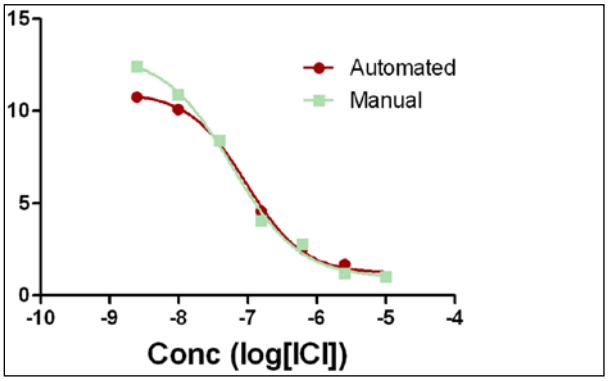

Figure 3. Fold response of GloSensor cAMP HEK293/L9 cell line when subjected to varying concentration of ICI 118,551 HCl.

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

Table 2. Comparison of antagonist IC_{50} concentrations calculated from dose response curves using either automated or manual assay methods.

Z′-factor Determination

The Z′-factor was calculated from 48 replicate measurements using 20 nM (EC_{80}) isoproterenol stimulation with and without 10 µM (IC100) antagonist ICI 118,551 HCl. The results in Figure 4 show excellent assay robustness (Z ≈ 0.82).

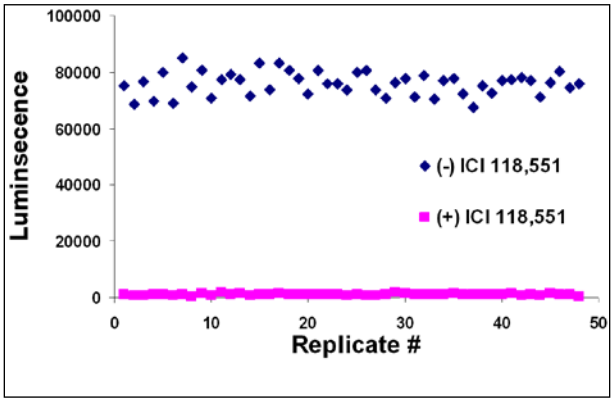

Figure 4. Z'-factor calculation.

Conclusion

The GloSensor cAMP Assay can be performed using simple inexpensive automated methods. The high sensitivity and low background achieved using manual assay methods were reproduced with significantly higher throughput and ease-of-use. Pharmacology of known agonists and antagonists generated using a Multi-Flo Microplate Dispenser for cell and GloSensor cAMP Reagent dispensing and Precision Microplate Pipetting System for serial dilution and compound transfers agreed with those generated using manual methods. An increased Z'-factor determination when compared to manual methods indicated the automated processes provide for an increase in assay performance with the additional benefit of increased throughput.

References

1. Brescia, P., Larson, B., Stecha, P., Binkowski, B., Cosby, N., and Banks, P. *Live-Cell Assay to Interrogate GPCRs by Monitoring cAMP Levels using a Bioluminescent Readout*, Winooski, (VT), BioTek Instruments, Inc., Sept. 2010, Application Note.

2. Fan F, Binkowski B, Butler B, Stecha P, Lewis M, and Wood K. (2008) Novel Genetically Encoded Biosensors Using Firefly Luciferase. ACS Chem. Biol. 3(6):346-51.