

Live-Cell Biosensor Assay Used To Interrogate GPCRs: Comparative Analysis of Biosensor Variants

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Promega

Abstract

Functional, cell-based assays are essential to identify modulators of GPCRs, including those that signal via increases or decreases in cAMP. Previously, we demonstrated the automation of the GloSensor™ cAMP Assay from Promega, which utilizes a live-cell biosensor consisting of a fusion of a cAMP binding domain to a circularly permuted form of firefly luciferase. Upon binding to cAMP, conformational changes in the expressed sensor lead to increases in light output in the presence of substrate. Several variants of the biosensor exist with different affinities for cAMP, some offering increased sensitivity and others offering increased dynamic range. The sensor with the broadest dynamic range, 22F, has the lowest affinity for cAMP, where the basal signal for this construct can be difficult to detect under select conditions. Here we compare the performance of the highest and lowest affinity variants using automated methods and the highly-sensitive bottom reading capabilities of an HTS microplate reader. Sensitive detection of the 22F basal signal allows for use of this variant under a broad range of conditions, providing an assay with both superior dynamic range and sensitivity.

Introduction

G-protein coupled receptors (GPCRs) primarily function as sensors of the extracellular environment and communicators of changes in that environment to intracellular machinery¹. The transmembrane nature of GPCRs ensure that signaling compounds or other stimuli can propagate a response without physically breaching the cellular membrane (Figure 1). Considerable effort remains focused on measuring the functional responses of the receptors to various compounds and determining the pharmacology of agonists and antagonists. The obvious advantage of performing these types of studies in an *in-vitro*, live-cell assay over lytic, end-point assays has led to recent advances in cell-based GPCR assays.

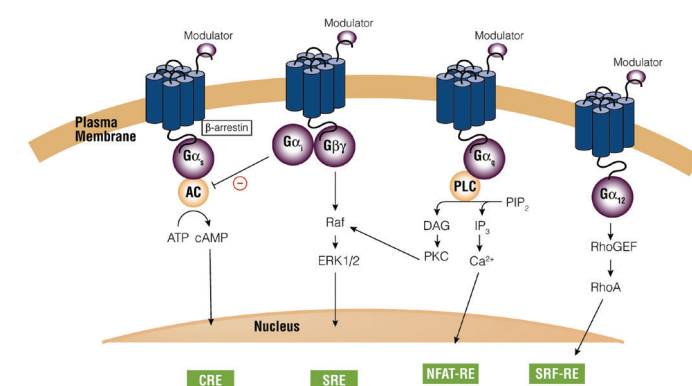


Figure 1 – GPCR signaling. Upon stimulation G_s-coupled receptors activate adenylate cyclase (AC) with concomitant increase in intracellular cAMP levels.

Luminescent reporter genes have been shown to provide increased sensitivity with a wide dynamic range and have been adapted to a high density microplate format using high-throughput automated methods. Several variants of a biosensor protein, expressed from genetically modified forms of luciferase, are capable of modulation of their luminescence activity dependent on reversible allosteric interaction with ligand allowing live-cell, real-time monitoring of cAMP kinetics (Figure 2)². Here we compare the automation of two GloSensor variants, having a high and low affinity for cAMP, using a high density, clear bottom microplate allowing for the ability to multiplex the assay. The use of clear bottom plates in conjunction with bottom reading can take advantage of both an increase in sensitivity due to proximity when using adherent cell lines as well as allowing simultaneous investigation using imaging methods. Assay performance as well as the pharmacology of several compounds is investigated allowing comparisons to be drawn between the two variants.

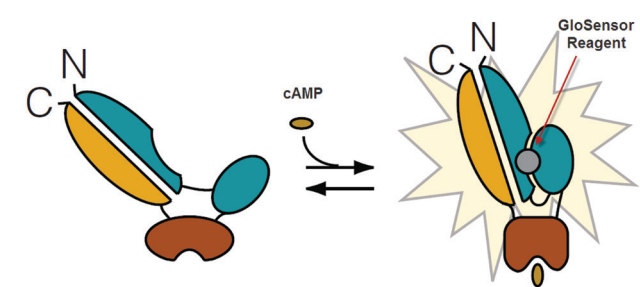


Figure 2 – Bioluminescent sensor allows detection of molecular processes in live cells through reversible allosteric modulation of luminescence activity during ligand binding. Binding of ligand, in this case cAMP, results in a closed conformation of the biomolecule resulting in increased luciferase activity.

BioTek Instrumentation



Figure 3 – MultiFlo™ Microplate Dispenser.

MultiFlo™ Microplate Dispenser offers up to four reagents dispensed in parallel with one compact instrument. The MultiFlo offers a choice of either peristaltic pump or microprocessor controlled syringe drive technologies. A wide array of plate types are accommodated from 6- to 1536-well formats as well as a broad volume range from 0.5 µL to 3 mL. BioTek's proprietary angled dispensing ensures compatibility with all dispense protocols including media exchanges with loosely adherent cell monolayers. The instrument was used to dispense cells, GloSensor Reagent and compounds to the 384-well assay plates.

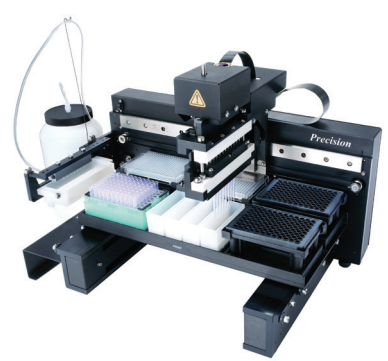


Figure 4 – Precision™ Microplate Pipetting System.

Precision™ is an affordable solution for automated 96-/384-well microplate liquid handling. The unique XY transport design provides effortless 96- to 384-well plate transfers with the same pipette mechanism. The instrument was used to transfer compounds from a 96- to a 384-well assay plate.



Figure 5 – Synergy™ NEO HTS Multi-Mode Microplate Reader.

The Synergy™ NEO is an HTS Multi-Mode Microplate Reader designed specifically for today's screening and core laboratories. Synergy NEO has all the features of a screening instrument, including multiple parallel detectors for fast measurements, laser-based excitation, fast plate stacker and high sensitivity on low volume assays. The Synergy NEO incorporates BioTek's unique patented Hybrid Technology™.

Cell Culture Procedure

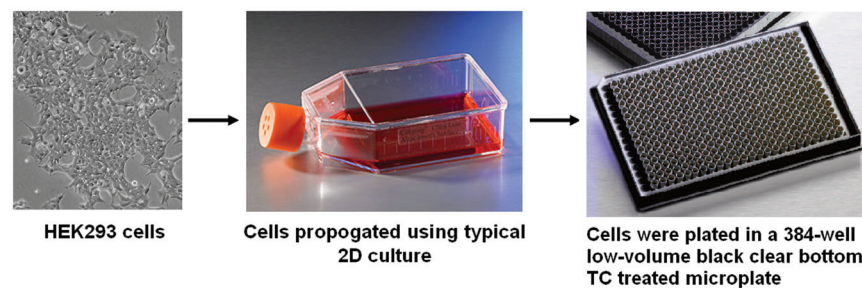


Figure 6 – Cell culture conditions for GloSensor cAMP HEK293 cell lines.

GloSensor cAMP HEK293-20F and -22F cells were grown in culture to ~90% confluency as per manufacturers technical manual in DMEM media supplemented with 10% FBS and Hygromycin B.

Cells were plated in low-volume, black, clear bottom microplates using CO₂-independent media overnight prior to performing assays.

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

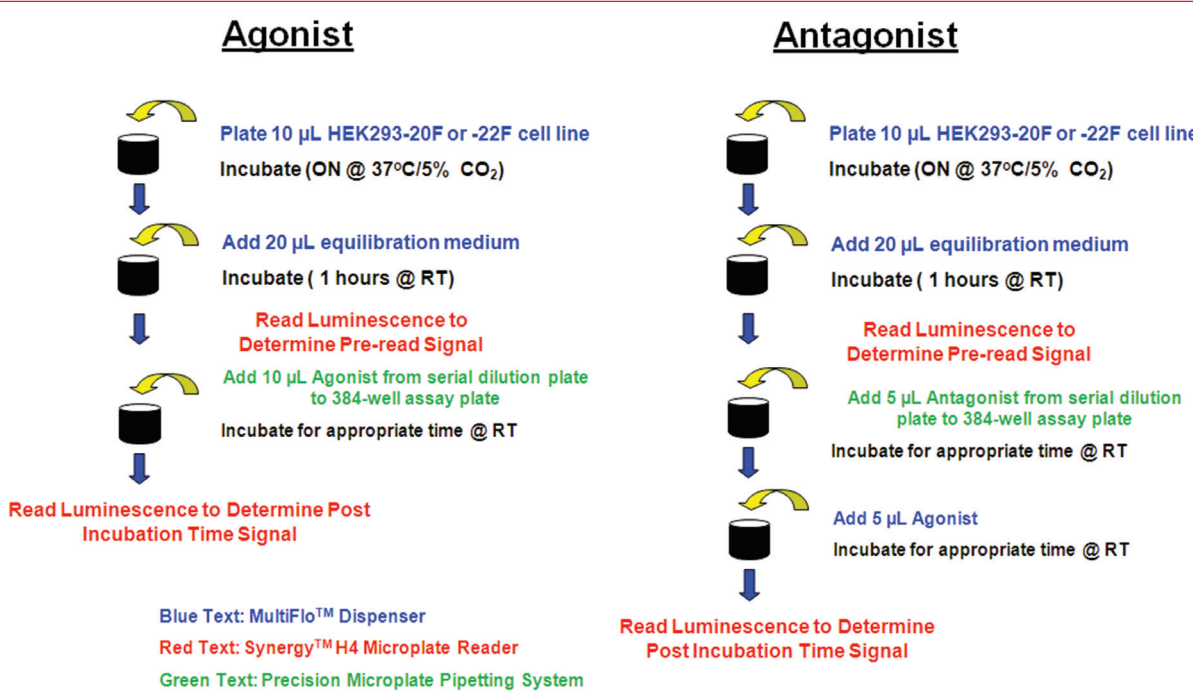


Figure 7 – GloSensor cAMP Assay workflow.

β2 Adrenergic Receptor Agonist Dose Response

- Cells were prepared as described previously and plated at a density of 2,000 cells/well in a volume of 10 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 20 µL/well was added using the MultiFlo.
- The plate was pre-read on the Synergy NEO HTS Multi-Mode Reader.
- An 7-point serial dilution was performed for each compound including a zero compound point and 10 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 NEO following a 12 minute incubation time.

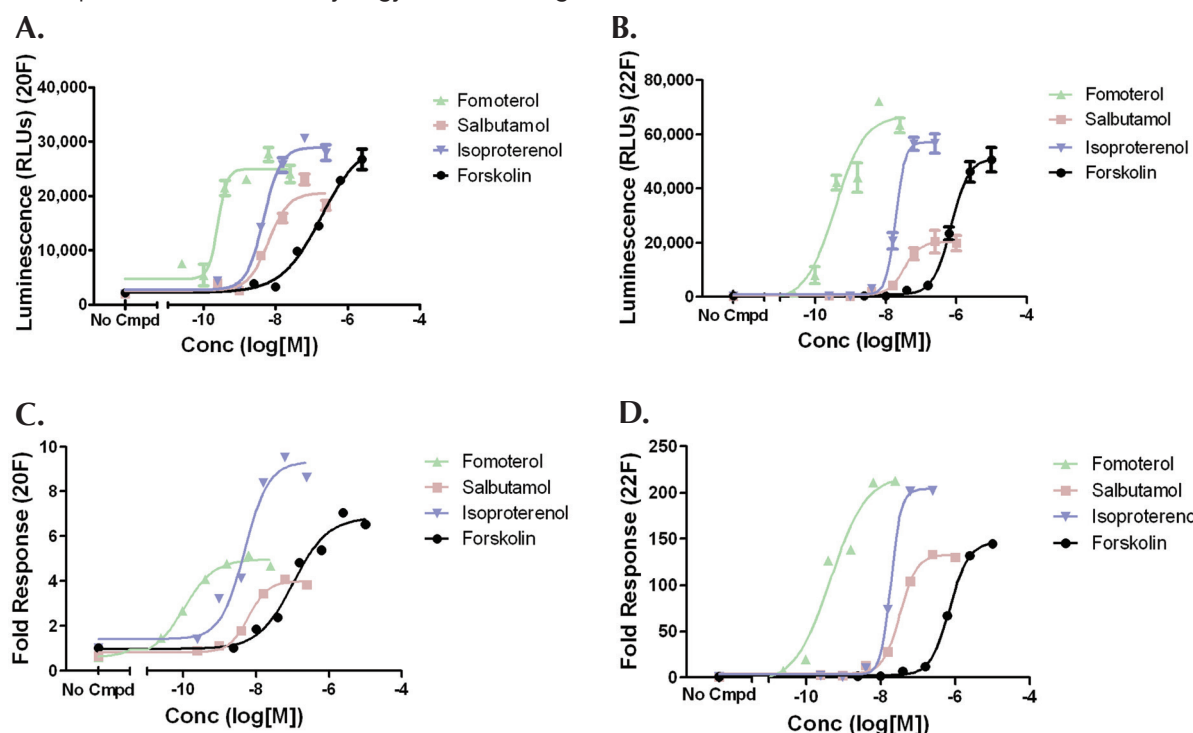


Figure 8 – Luminescence signal intensity and fold-induction of GloSensor cAMP HEK293-20F or -22F cell line when subject to a 7-point titration of the indicated compound: A) luminescent intensity of 20F variant, B) luminescent intensity of 22F variant, C) fold-induction of 20F variant, and D) fold-induction of 22F variant.

β2 Adrenergic Receptor Agonist EC₅₀ Comparison

Compound	20F (nM)	22F (nM)
Formoterol	0.1	0.45
Salbutamol	6.2	32
Isoproterenol	4.8	19
Forskolin	103	704

Table 1 – EC₅₀ concentration of agonists as determined from dose response curves in Figure 8.

- Calculated EC₅₀ values for the test compounds shown in Table 1 show good agreement between the two variants and are consistent with values previously reported in assay literature³.

β2 Adrenergic Receptor Antagonist Response

- Cells were prepared and plated as described previously at a density of 2,000 cells/well.
- Plates were incubated overnight at 37°C, 5-10% CO₂ and GloSensor cAMP Reagent added as described previously.
- The plate was pre-read as described previously and antagonist added.
- Agonist was added at the EC₈₀ concentrations of 0.37 or 0.82 nM formoterol for the 20F or 22F variant, respectively, following a 10 minute antagonist incubation period.
- The plate was read on the Synergy H4 following a 12 minute agonist incubation period.

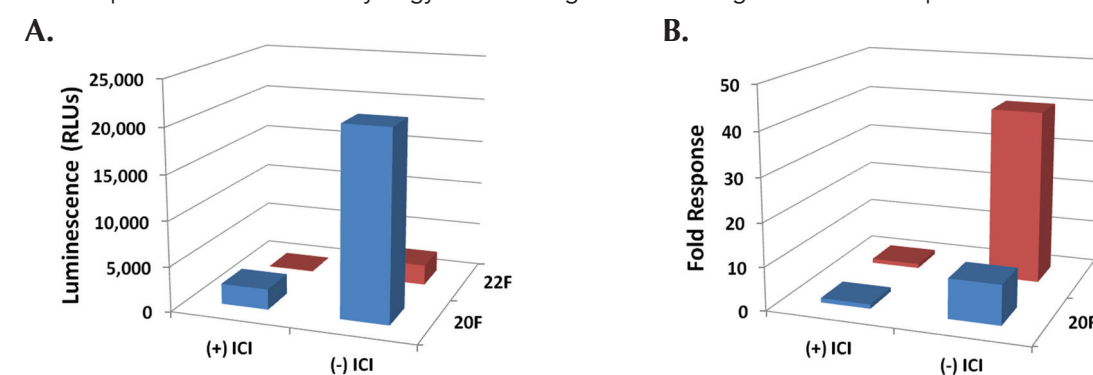


Figure 9 – Comparison of changes in A) luminescence signal and B) fold response of GloSensor cAMP HEK293 cell lines when subject to 10 µM ICI 118,551 antagonist and the corresponding EC₈₀ concentration of the agonist formoterol for each variant.

- The 22F variant shows lower induced luminescent intensity upon agonist addition as well as suppression of basal activity by addition of antagonist resulting in a significant increase in observed fold induction compared to the 20F variant when the assay is performed at room temperature.

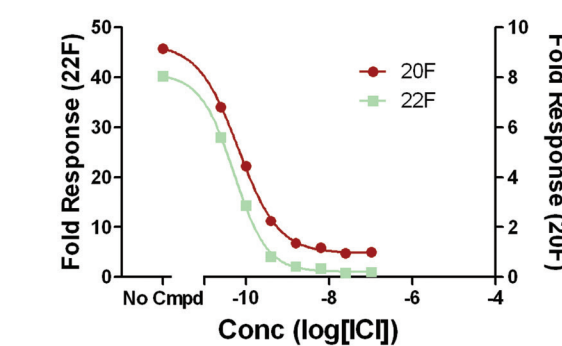


Figure 10 – Fold response of GloSensor cAMP HEK293 cell lines when subject to varying concentrations of the antagonist ICI 118,551.

- The dose response curves depicted in Figure 10 indicate both variants show nearly identical responses to the potent antagonist ICI 118,551 that are consistent with literature values showing K_i values less than 1 nM⁴ (Table 2).

Compound	20F (nM)	22F (nM)
ICI 118,151	0.06	0.05

Table 2 – Comparison of antagonist IC₅₀ concentrations calculated from dose response curves.

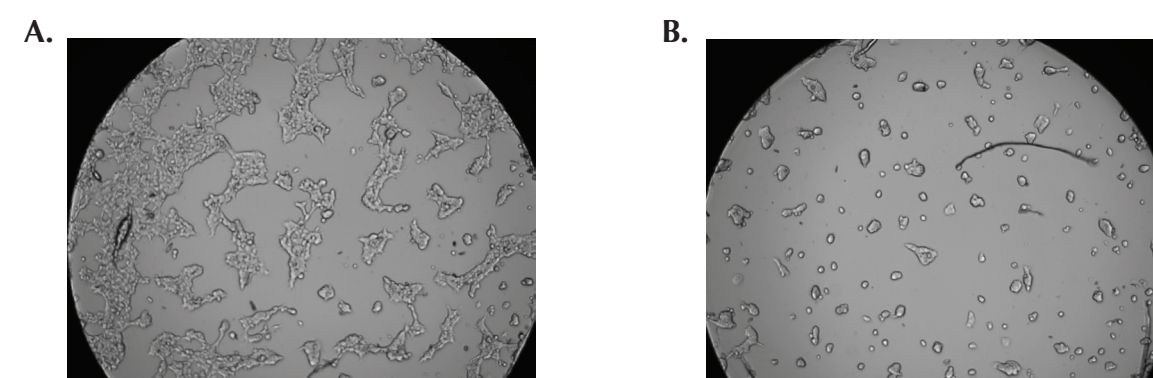


Figure 11 – Cell imaging: A) 5,000 cells/well and B) 2,000 cells/well. Cells were plated in CO₂ independent media in a 384-well low-volume plate and allowed to attach overnight prior to being assayed.

Z'-Factor Determination

- The Z'-factor was calculated from 48 replicates for each variant.
- The results show good assay robustness with Z'-factors of 0.69 and 0.67 for the 20F and 22F variants, respectively.

Conclusions

1. The GloSensor cAMP Assay can be performed using simple, inexpensive automated methods.
2. The highly sensitive bottom-reading capabilities of the Synergy NEO HTS Multi-mode Microplate Reader provided the ability to read both the low basal signal for the lowest affinity 22F biosensor variant as well as discriminate changes from the more sensitive, reduced dynamic range 20F variant.
3. A significant increase in signal-to-background was seen in the 22F variant when compared to the 20F variant following activation of an endogenous Gs-coupled receptor in HEK293 cells when assayed at room temperature.
4. The improved S/B ratio seen when using the 22F variant allowed for the use of a 384-well low-volume, black, clear bottom microplate plate suitable for HTS applications and imaging.

¹Bockaert J, Pin JP. (1999) Molecular tinkering of G protein-coupled receptors: an evolutionary success. *EMBO J.* 18(7):1723-9. ²Fan 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.

³Binkowski B. "RE: EC50/IC80 values for agonist/antagonist" E-mail to Peter Brescia. 20 September, 2010. ⁴McConaughy, M, et. al. (2004) Differences in β-Adrenergic Receptor Densities in Omental and Subcutaneous Adipose Tissue From Obese African American and Caucasian Women. *Metabolism.* 53(2):247-251.