

APPLICATION NOTE

Live cell G_i - and G_s -coupled GPCR second messenger signaling on the FLIPR Tetra System

Introduction

In this application protocol we demonstrate the use of the modified luminescent firefly luciferase-based Promega GloSensor™ cAMP Assay on the FLIPR Tetra® High Throughput Cellular Screening System to enable detection of cAMP-mediated G_s - and G_i -coupled GPCR activity in kinetic mode. With this assay, these GPCR subtypes can now be evaluated in a live cell assay measuring changes in intracellular cAMP concentration, the relevant second messenger mechanism.

Detection of G_s - and G_i -coupled GPCR second messenger signal activity has been traditionally accomplished using assays such as radioactive binding or endpoint cAMP assays that require cell lysis. Such assays measure activity at a single time point in the cellular response and do not provide kinetic information. Another option utilizes forced-coupling of G_s - and G_i - GPCRs to $G_{\alpha 16}$ followed by fluorescence detection of calcium flux upon agonist receptor activation. Again, this assay is sub-optimal as it does not signal through the biorelevant cAMP pathway.

We demonstrate endogenous receptor activity in CHO-K₁ and HEK 293 cell lines stably expressing the GloSensor plasmid. In addition, stably transfected G_s - and G_i -coupled receptor activity is measured in cell lines transiently transfected with GloSensor plasmid. Combined with the GloSensor cAMP Assay, the FLIPR Tetra System delivers a flexible and complete solution for kinetic screening of the major classes of GPCR subtypes.

As seen in Figure 1, the GloSensor cAMP Assay was created by fusion of a cAMP binding domain to the wild-type N- and C-termini of native firefly luciferase. In

the absence of cAMP, the genetically modified luciferase containing the cAMP binding domain is in the inactive state. Upon binding to cAMP, conformational changes in the cAMP binding domain likely determine the increased luminescence in the activated state that can be detected in living cells on the FLIPR Tetra System. Both forms of luciferase are represented in the presence of luciferase substrate. For more information about the GloSensor cAMP assay, refer to the Technical Manual (Cat# TM076, Promega). Flexible G_i - and G_s -coupled GPCR assay design is possible using a combination of plasmids for transient or stable transfections. Four options using either cell lines available from Promega or target cell lines available in the laboratory are listed below:

Flexible GloSensor cAMP assay design

- Stable receptor and stable GloSensor cAMP assay
- Transient receptor and stable GloSensor cAMP assay

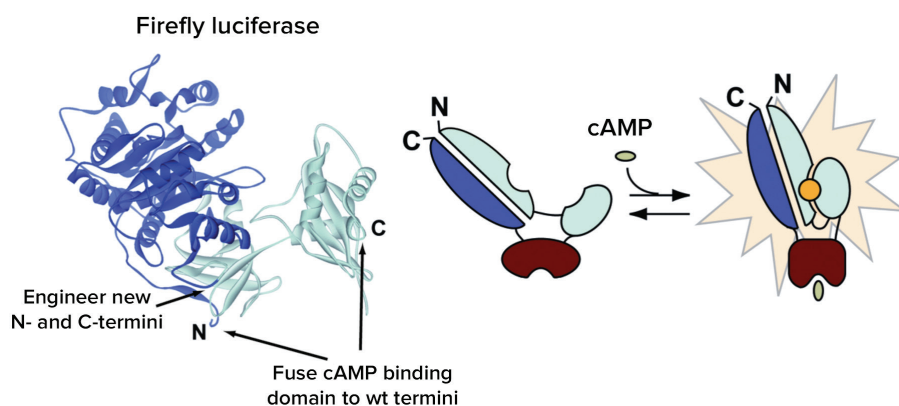


Figure 1. Intracellular biosensor of the GloSensor cAMP Assay. Figure courtesy of Promega Corporation.

Benefits

- Flexible assay development using GloSensor cAMP cell lines and GloSensor cAMP plasmids
- Complete solution for kinetic screening of the major classes of GPCR subtypes
- Scalable assay throughput for multiple microplate formats with easy integration with automation

- Stable receptor and transient GloSensor cAMP assay
- Transient receptor and transient GloSensor cAMP assay

About the FLIPR Tetra System:

- Flexible ICCD camera provides luminescent detection for luciferase assay as well as fluorescence detection
- Kinetic cAMP luminescent signal measurements in live cells enabled by the ICCD camera in the FLIPR Tetra System
- Scalable assay throughput: 96-, 384- and 1536-well plate formats, easily integrated with automation

Parameter	Setting
ICCD Camera Mode	Luminescence
Gain	280,000
Gate	100%
No of Reads	60–180
Exposure time*	1 sec.
Read time interval	10 sec.
Pipettor head	384-well with black tips
Aspirate volume	7.5 μ L
Dispense height	20 μ L
Dispense speed	30 μ L/sec.

* Up to 9 sec. exposures may be taken if luminescent signal intensity is lower. Conditions should be optimized.

Table 1. FLIPR Tetra System setup parameters.

Materials

cAMP cell lines and plasmids

- GloSensor cAMP HEK 293 stable cell line (Promega Corporation, Cat. #E1261)
- GloSensor cAMP 23F CHO K1-stable cell line (Promega R&D)
- Rat Y2R/GloSensor cAMP 23F CHO-K1 double stable cell line (Promega R&D)
- GloSensor cAMP assay plasmid pGlosensor-22FcAMP (Promega, Cat. #E2301)
- Dopamine D4 stable HEK 293T cell line (Multispan Inc., Cat. #C1338)

Cell culture reagents

- HEK 293 cell growth medium: 90% DMEM (Life Technologies, Cat. #11995-065), 10% FBS (Hyclone, Cat. #SH.30071), 200 μ g/mL hygromycin B (Sigma, Cat. #H3274)
- GloSensor cAMP-23F CHO-K1 cell growth medium: 90% F-12 Medium (Life Technologies, Cat. #11765), 10% FBS, and 200 mg/mL hygromycin B)
- Glo Sensor cAMP-23F/Y2R CHO-K1 double stable cell growth medium: 90% F-12 Medium (Life Technologies, Cat. #11765), 10% FBS, and 200 μ g/mL hygromycin B), and 500 μ g/mL G418 (Life Technologies, Cat. #10131027)

Assay reagents

- Plating medium: 90% CO₂-independent medium (Life Technologies, Cat. #18045), 10% FBS
- HEPES buffer: Re-suspend HEPES in deionized water to 10 mM; adjust pH to 7.5 using KOH

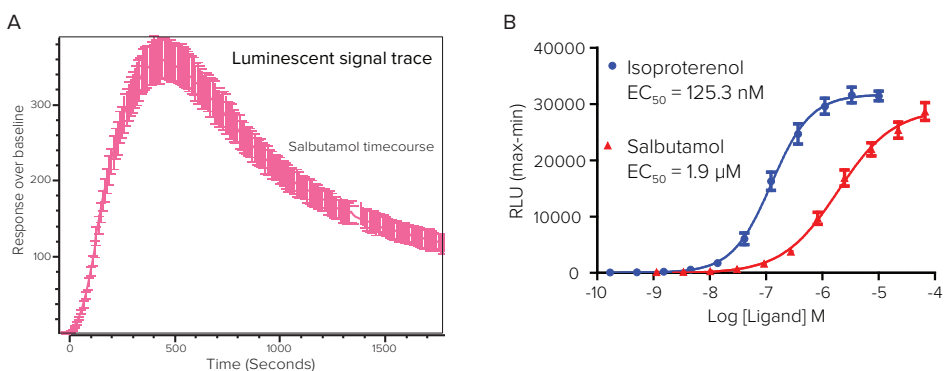


Figure 2. Stable GloSensor cAMP-23F HEK 293 cells. Stable GloSensor cAMP-23F HEK 293 cell line is used to demonstrate the GloSensor cAMP assay on the FLIPR Tetra system. The ICCD camera detects luminescent signal over a period of 30 minutes from modified firefly luciferase. **(A)** FLIPR ScreenWorks average signal trace of cAMP HEK 293 endogenous G_s-mediated endogenous β 2 Adrenoceptor response to salbutamol stimulation. The trace illustrates approximately a 350-fold increase over baseline signal. **(B)** Full agonism of HEK 293 cell line endogenous β 2 Adrenergic receptor in response to isoproterenol and partial receptor agonism by salbutamol.

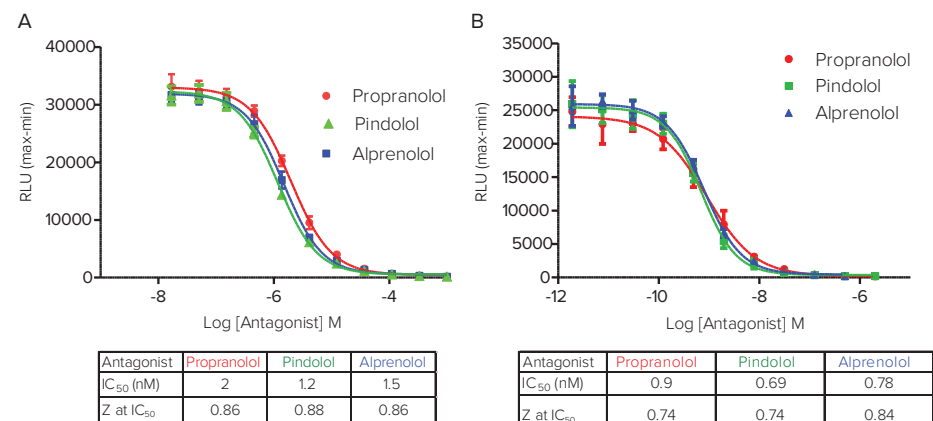


Figure 3. Antagonism of isoproterenol and salbutamol. Antagonism of isoproterenol and salbutamol response by three known inhibitors. **(A)** Inhibition of EC₉₀ isoproterenol response. **(B)** Inhibition of EC₉₀ salbutamol response. The robust signal window enables Z factors > 0.7.

- GloSensor cAMP Reagent stock solution: Re-suspend 250 mg GloSensor cAMP Reagent (Promega, Cat. #E1291) in 8.17 mL of HEPES buffer; store at -70°C in single-use aliquots
- Equilibration medium: Plating medium (above) and 2% or 5% GloSensor cAMP Reagent; optimization is necessary
- Compounds: (-)-isoproterenol (Sigma, Cat. #I6504); salbutamol (Sigma, Cat. #S8260); propranolol (Sigma, Cat. #P0884); pindolol (Sigma, Cat. #P0778); alprenolol (Sigma, Cat. #A8676); dopamine (Sigma, Cat. # H8502). Peptide YY(3-36), (Tocris Cat. #1618); all compound stock solutions made at 10 mM and further diluted in HBSS + 20 mM HEPES

Methods

Stable GloSensor cAMP cell line assay method

Step 1: Cells were plated overnight in 384-well black wall clear bottom plates (Corning, Cat. #3712).

Step 2: Two hours prior to assay, culture media was removed and cells were incubated for 1 hour at 37°C in 5% CO₂ and 1 hour at room temperature in 30 µL/well equilibration media containing GloSensor cAMP reagent.

- HEK cell lines were incubated in equilibration media containing 2% GloSensor cAMP reagent.
- CHO-K1 cell lines were incubated 5% GloSensor cAMP reagent.

Step 3: 5X compound added by the FLIPR Tetra System during kinetic read.

Step 4: Exposures taken every 10 or 30 seconds for 10–25 minutes.

Step 5: Generally, G_i-coupled receptor assays require longer overall read times due to slower kinetics. Setup parameters for the FLIPR Tetra instrument are seen in Table 1.

Step 6: Data were exported from FLIPR ScreenWorks® Software to Graphpad Prism 5 for analysis.

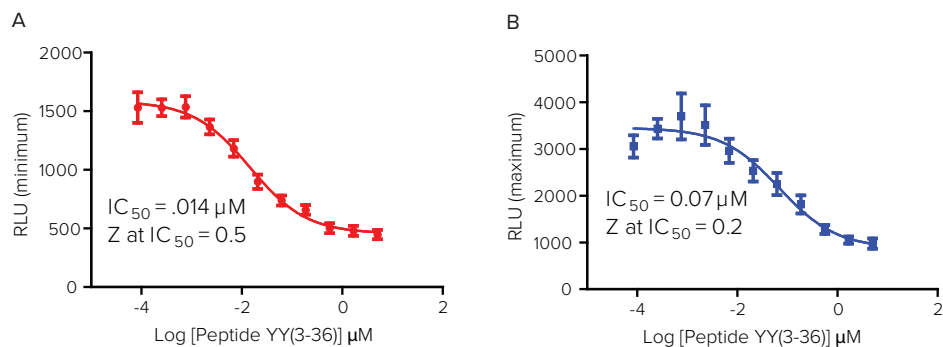


Figure 4. G_i-coupled agonsim results. G_i-coupled receptor agonism results in a reduction in signal correlated with reduction in cAMP inside the cell. Baseline increase in cAMP activity was induced by the addition of forskolin. Using stable P2Y receptor in CHO-K1 cells, we compared results upon addition of forskolin either before or after the agonist. **(A)** 10 µM forskolin addition followed 15 minutes later by addition of agonist Peptide YY(3-36) on the FLIPR Tetra System. **(B)** Peptide YY(3-36) was added 15 minutes prior to addition of 10 µM forskolin. In addition to assay flexibility, both methods illustrate a reduction in signal related to inhibited cAMP production.

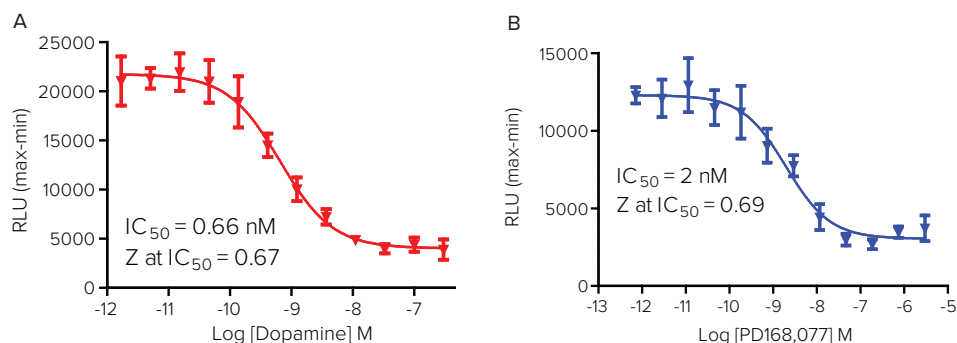


Figure 5. HEK 293 cells over expressing G_i-coupled D4 receptor. HEK 293 cells over expressing G_i-coupled dopamine D4 receptor from Multispan, Inc. were transiently transfected with GloSensor cAMP-22F plasmid. Ligand was added on-line to the wells, followed by 5 minute incubation. Continuing the assay, FLIPR Tetra System added 10 µM forskolin to stimulate cAMP production in the cell. **(A)** Inhibition of forskolin-mediated cAMP production by Dopamine. **(B)** Inhibition of forskolin-mediated cAMP production by the Dopamine D4 receptor specific compound, PD168,077.

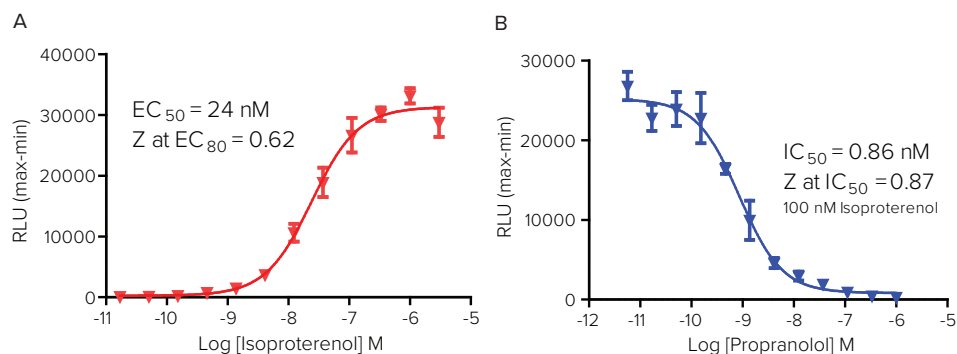


Figure 6. Transient transfection. Transient transfection of GloSensor cAMP-22F and endogenous G_s-coupled cAMP response in HEK 293 cells. **(A)** Response to isoproterenol and **(B)** inhibition of the response to 100 nM isoproterenol by propranolol. Results are comparable to those obtained from the experiment performed with the stable GloSensor HEK-22F cell line shown in Figure 2.

Transient GloSensor cAMP transfection method in both a stably transfected GPCR receptor cell line and an endogenous receptor cell line

Step 1: Culture cells without selection antibiotics at 37°C and 5% CO₂ overnight in flask so that the cells are about 70-80% confluent.

Step 2: Dilute the pGloSensor cAMP plasmid to 20 ng/mL in Opti-Mem- reduced serum media to make 1-µg DNA/mL cells in Step 4. (Life Technologies, Cat. #31985).

Step 3: Add Fugene HD transfection reagent mixture (Roche, Cat. #0409705001) at a 3:1 ratio per one µg DNA and mix by gentle pipetting followed by an incubation of 15 minutes at room temperature into microplate. Reagent is at a 3:1 ratio (µL) : DNA (µG).

Step 4: Add the DNA complex to a tube containing recently lifted cells at 0.48 million per mL (for seeding at 12000 cells/25µL/well for HEK-D4; this is cell line-dependent).

Step 5: Depending on the cell line, plate cells at 8000–12000 cells/well in 25 µL/well and incubate at 37°C and 5% CO₂ for two days.

Step 6: On the day of the assay, gently remove the culture media and add 30 µL 2–6% f/v GloSensor cAMP Reagent in Equilibration medium.

Step 7: Incubate for 1 hour at 37°C and an additional hour at room temperature.

Step 8: Following the FLIPR Tetra System setup parameters in Table 1, add 6X compound during kinetic read.

Conclusion

Use of the FLIPR Tetra System with GloSensor cAMP assay enables kinetic measurement of G_i- and G_s-coupled receptor signaling not possible using endpoint assays on standard plate readers.

We have shown assay development flexibility using GloSensor cAMP cell lines and GloSensor cAMP plasmids transfected in endogenous as well as stably transfected receptor cell lines.

Acknowledgement

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