TECHNICAL MANUAL

GloSensor™ cAMP Assay

Instructions for use of Products
E1171, E1261, E1290, E1291 and E2301

Revised 5/13
TM076

Promega

GloSensor™ cAMP Assay

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1. Description

G-protein coupled, seven-transmembrane (7-TM) receptors (GPCRs) represent a major class of drug targets, and the prevalence of these receptors in physiologically important signaling events is well known. The GloSensor™ cAMP Assay provides an extremely sensitive and easy-to-use format for the interrogation of overexpressed or endogenous GPCRs that signal via changes in the intracellular concentration of cAMP. The assay uses genetically encoded biosensor variants with cAMP binding domains fused to mutant forms of *Photinus pyralis* luciferase (1–3). Upon binding to cAMP, conformational changes occur that promote large increases in light output (1–3). Following pre-equilibration with substrate, cells transiently or stably expressing a biosensor variant can be used to assay GPCR function using a live-cell, non-lytic assay format (Figure 1), enabling facile kinetic measurements of cAMP accumulation or turnover in living cells. Moreover, the assay offers a broad dynamic range, showing up to 500-fold changes in light output. Extreme sensitivity allows

detection of G_i-coupled receptor activation or inverse agonist activity in the absence of artificial stimulation by compounds such as forskolin.

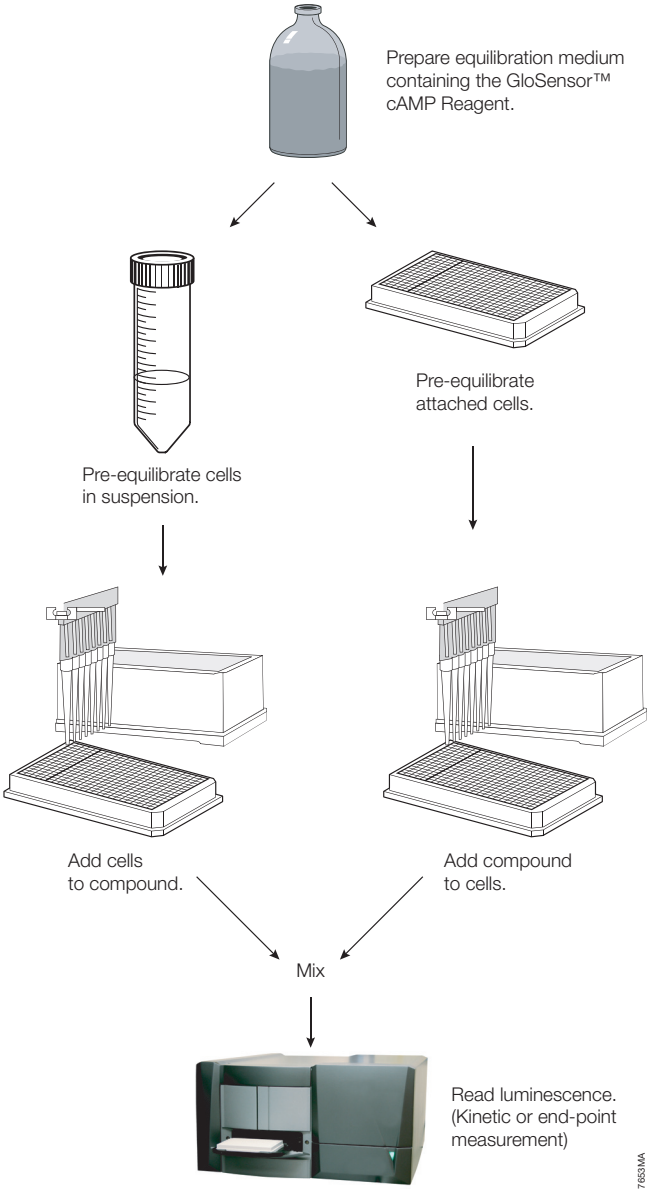

Figure 1. An overview of the GloSensor™ cAMP Assay. See Sections 4.B and 4.C, respectively, for specific information on performing kinetic or end-point measurements in 96- or 384-well formats.

2. Product Components and Storage Conditions

Product	Size	Cat.#
GloSensor™ cAMP HEK293 Cell Line[a,f–i]	2 vials	E1261

Each vial contains 2 × 10^6 cells in freezing medium (80% DMEM medium, 15% fetal bovine serum, 5% DMSO).

Product	Size	Cat.#
pGloSensor™-22F cAMP Plasmid[a–f]	20µg	E2301
pGloSensor™-20F cAMP Plasmid[a–f]	20µg	E1171

Product	Size	Cat.#
GloSensor™ cAMP Reagent	(25mg)	E1290

Product	Size	Cat.#
GloSensor™ cAMP Reagent	(250mg)	E1291

Cells are shipped frozen. If cells are not frozen upon arrival, contact Promega Customer Service immediately.

Store frozen cells at temperatures equal to or lower than –140°C (mechanical deep freeze or vapor-phase liquid nitrogen). Thaw and propagate cells when you are ready to use them.

Warning: Do not store cryotubes in the liquid phase of liquid nitrogen. Storage in the liquid phase may trap liquid nitrogen inside the vial and lead to pressure buildup, resulting in possible explosion or biohazard release. Use appropriate safety procedures when handling and disposing of the cryotubes.

Storage Conditions: Store the pGloSensor™-22F and pGloSensor™-20F cAMP Plasmids at –20°C. Store the GloSensor™ cAMP Reagent at –70°C. Store the resuspended GloSensor™ cAMP Reagent at –70°C in single-use aliquots.

Product Warranty

Promega warrants that cells will be viable upon shipment from Promega for a period of thirty days, provided they have been properly stored and handled during this period.

Handling the GloSensor™ cAMP HEK293 Cells Upon Arrival

We strongly recommend that you propagate the cells as soon as possible after receipt using the procedures provided in Section 5.B. This will ensure the optimal cell viability and assay performance. The second vial of cells is supplied as an immediate backup and is not intended for long-term storage.

2. Product Components and Storage Conditions (continued)

Cell Line Stability

Cells may undergo genotypic changes, resulting in reduced responsiveness over time under normal cell culture conditions. Genetic instability is a biological phenomenon that occurs in many stably transfected cell lines. Thus, it is critical to prepare an adequate number of frozen stocks at early passages for future or prolonged use (Section 5.B). We strongly recommend assaying the response to 10µM forskolin in each experiment to determine if the response of the cell line is diminishing.

3. Before You Begin

3.A. General Considerations for GPCR Assays

The following protocols have been used successfully at Promega to obtain concentration response curves for both agonists and antagonists of overexpressed and endogenous GPCRs. In each case, cells must be pre-equilibrated with the GloSensor™ cAMP Reagent prior to the addition of compounds. Detailed protocols for use of transient or stable biosensor expression can be found in Sections 4 and 5, respectively. Use these protocols as a starting point for assay design and optimization.

G_s-Coupled Receptors

Add varying concentrations of agonist and acquire kinetic or end-point luminescence measurements. In general, we have seen maximal changes in light output within 2–10 minutes following addition of saturating concentrations of agonist, depending on assay temperature. In the absence of phosphodiesterase (PDE) inhibitors, induced signals will decrease depending on factors such as receptor desensitization and PDE activity. However, signals can remain significantly above background hours after agonist addition. Inclusion of PDE inhibitors can stabilize signals near maximal values of fold induction for extended periods of time.

If measuring antagonist activity, first determine the EC_{80} concentration of the agonist that will be used in the assay as described above. Once done, pre-incubate with varying concentrations of antagonist for 5–10 minutes, followed by addition of an EC_{80} concentration of agonist to all wells. Acquire kinetic or end-point measurements of luminescence for 10–20 minutes post-agonist addition.

G_i-Coupled Receptors

Pre-incubate with varying concentrations of agonist for 5–10 minutes. Add a fixed concentration of forskolin to all wells; the optimal concentration of forskolin for maximal signal-to-background ratio (S/B) of agonist is determined empirically (doses between 0.1–10µM are typical, depending on the cell line). Acquire kinetic or end-point measurements of luminescence for 15–30 minutes post-forskolin addition.

For overexpressed receptors, we have been able to detect a significant decrease in the basal level of light output in the absence of added forskolin. Similarly, we have been able to detect the activity of inverse agonists of overexpressed G_s- and G_i-coupled receptors in the absence of added forskolin. For updates and results, see the GloSensor™ Technology web site: **www.promega.com/glosensor**

3.B. Recommendations on Choice of GloSensor™ Plasmid

Two versions of the biosensor exist for use in the GloSensor™ cAMP Assay (Figure 2). Following cell-free expression in vitro, the version encoded by the pGloSensor™-22F cAMP construct (22F) shows an increased EC_{50} for activation together with increased S/B ratio at cAMP saturation relative to the version encoded by the pGloSensor™-20F cAMP construct (20F). In general, we have observed similar relationships between the two constructs when their performance is compared in living cells.

For G_s-coupled receptors, the 22F construct has shown markedly increased S/B and an enhanced ability to discriminate between the efficacy of full and partial agonists (Figure 4) compared to the 20F construct, likely due to cAMP saturation effects associated with the 20F construct. For G_i-coupled receptors, the 22F construct has shown increased S/B in the presence or absence of added forskolin; saturation effects can hinder the 20F construct in the presence of forskolin in select experimental systems (Figure 6).

The 20F construct performs well in HEK293 cells at 37°C. Luminescence from the 22F construct can be more difficult to detect at physiologic temperatures. In general, increases in assay temperature lead to decreased levels of both basal and induced light output. Increases in assay temperatures have been associated with increases in S/B for the 20F construct. In addition, cAMP saturation effects for the 20F construct appear to be less pronounced above room temperature, perhaps owing to decreased basal levels of cAMP. Performance of the 20F and 22F constructs at 37°C need to be determined empirically across multiple cell types.

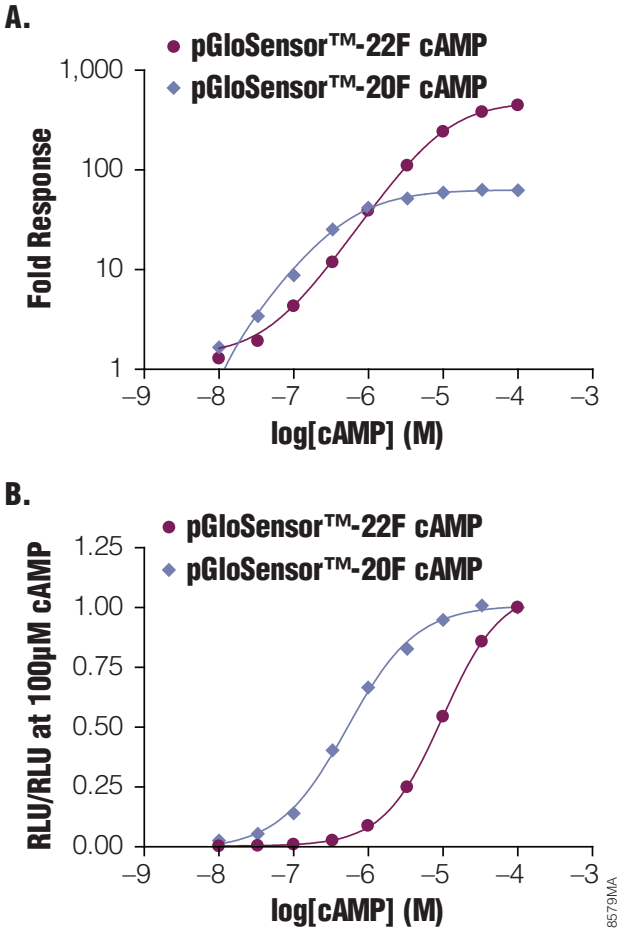

Figure 2. Cell-free expression of GloSensor™ cAMP biosensor variants and incubation with varying concentrations of cAMP in vitro. The 22F construct shows an increased signal-to-background ratio (**Panel A**) and EC_{50} value for activation (**Panel B**), whereas both constructs show similar stepwise increases in fold response and sensitivity at low concentrations of cAMP. Fold response (**Panel A**) was calculated relative to a control sample containing vehicle alone. Sample number = 3 per dose.

3.C. Materials to Be Supplied By the User

Reagents

- DMEM (Invitrogen Cat.# 11995-065)
- fetal bovine serum (FBS; Hyclone Cat.# SH30071)
- FuGENE® HD transfection reagent (Promega Cat.# E2311)
- Opti-MEM® I reduced-serum medium (Invitrogen Cat.# 31985)
- DMSO (Sigma-Aldrich Cat.# D2438)
- hygromycin B (Invitrogen Cat.# 10687-010)
- phosphate-buffered saline, Ca^{2+}-, Mg^{2+}-free (PBS; Invitrogen Cat.# 14190-144)
- 0.05% trypsin-EDTA (Invitrogen Cat.# 25300)
- CO_2-independent medium (Invitrogen Cat.# 18045)
- HEPES (Sigma-Aldrich Cat.# H4034)
- **optional:** For CHO cells, F12 medium (Invitrogen Cat.# 11765)

Supplies and Equipment

- tissue culture-treated, solid white, 96-well assay plate (Costar Cat.# 3917)
- tissue culture-treated, solid white, 384-well assay plate (Costar Cat.# 3704)
- 15ml conical tubes
- centrifuge capable of 250 × *g*
- cryogenic vials
- tissue culture flasks
- class II biological safety cabinet
- hemacytometer
- humidified 37°C, 5–10% CO_2 incubator
- inverted microscope
- luminometer

3.D. Preparation of Buffers and Media

Be sure to prepare any buffers or media that come in contact with the GloSensor™ cAMP HEK293 Cell Line using standard cell culture techniques under sterile conditions (i.e., in a tissue culture hood).

growth medium

90%	DMEM medium
10%	fetal bovine serum

freezing medium

80%	DMEM medium
15%	fetal bovine serum
5%	DMSO

growth medium + hygromycin B

90%	DMEM medium
10%	fetal bovine serum
200µg/ml	hygromycin B

plating medium (CO_2-independent medium + 10% FBS)

90%	CO_2-independent medium
10%	fetal bovine serum solution

3.D. Preparation of Buffers and Media (continued)

equilibration medium

88%	CO_2-independent medium
10%	fetal bovine serum
2%	GloSensor™ cAMP Reagent stock solution

Prepare only enough equilibration medium for experiments performed within a single day. We do not recommend storing the equilibration medium once the GloSensor™ cAMP Reagent stock solution has been added.

HEPES buffer

Resuspend HEPES in deionized water to 10mM; adjust pH to 7.5 using KOH.

GloSensor™ cAMP Reagent stock solution

To resuspend the GloSensor™ cAMP Reagent, add 817µl of HEPES buffer to a vial containing 25mg of GloSensor™ cAMP Reagent (Cat.# E1290), or add 8.17ml of HEPES buffer to a vial containing 250mg of GloSensor™ cAMP Reagent (Cat.# E1291). Store the GloSensor™ cAMP Reagent stock solution at –70°C in single-use aliquots.

4. pGloSensor™-22F and pGloSensor™-20F cAMP Plasmids

4.A. Plasmid Descriptions

The GenBank® accession number for the pGloSensor™-22F cAMP Plasmid is GU174434. The GenBank® accession number for the pGloSensor™-20F cAMP Plasmid is EU770615.

The pGloSensor™-22F and pGloSensor™-20F cAMP Plasmids allow transient expression of biosensor variants in mammalian cells. To date, we have validated transient expression in the following cell lines: HEK293, CHO, HeLa, NIH3T3 and U2OS. Alternatively, clones stably expressing a biosensor variant can be selected by treating cells with 200µg/ml hygromycin. Please refer to Section 3.B for recommendations on the choice of biosensor variant and assay format.

We offer two variants of the biosensor: pGloSensor™-22F and pGloSensor™-20F cAMP Plasmid. We recommend the pGloSensor™-22F cAMP Plasmid as the first choice for most applications.

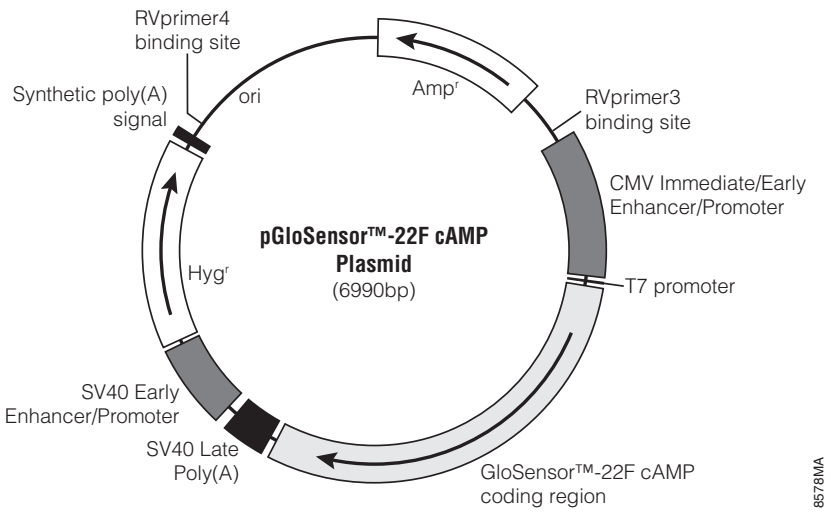

Figure 3. pGloSensor™-22F cAMP Plasmid map. Note that this map also represents the pGloSensor™-20F cAMP Plasmid.

pGloSensor™-22F and -20F cAMP Plasmid Sequence Reference Points:

synthetic β-lactamase (Amp^r) coding region	5–865
synthetic poly (A) signal/transcriptional pause site	970–1123
Reporter vector primer 3 (RVprimer3) binding region	1072–1091
CMV immediate early enhancer/promoter	1137–1878
T7 RNA polymerase promoter (–17 to +3)	1890–1909
GloSensor™-22F (or -20F) cAMP coding region	1922–4027
SV40 late poly(A) signal	4060–4281
SV40 early enhancer/promoter	4329–4747
synthetic hygromycin resistance (Hyg^r) coding region	4772–5809
synthetic poly(A) signal/transcriptional pause site	5833–5881
Reporter vector primer 4 (RVprimer4) binding region	5948–5967
ColE1-derived plasmid replication origin	6205–6241

4.B. Sample Protocol for 96-Well Format, Adherent Cells

The following protocol has been used successfully to transiently express biosensor variants in HEK293 cells using FuGENE® HD transfection reagent. This protocol may be used as a template for transfecting additional cell types. However, further optimization may be required for maximal performance.

Note: This protocol can also be used for transient transfection of CHO cells with either pGloSensor™-22F or -20F cAMP Plasmid. In Step 7, below, use CHO cells at 1.0×10^5 cells/ml. For CHO cells, use growth medium consisting of F12 medium + 10% FBS, Steps 4 and 7, below.

Cell Culture Preparation

The volumes listed in Steps 1–4 below are for propagation in a T75 flask. Scale volumes accordingly for flasks with different total surface area.

1. Harvest cells when the monolayer is at 50–90% confluence.
2. Wash cell monolayer using 10ml of PBS.
3. Add 2ml of 0.05% trypsin-EDTA prewarmed to 37ºC. Coat the surface of the flask evenly. Dislodge the cells from the flask surface by rocking and gently tapping the side of the flask. Once cells are dislodged, proceed immediately to Step 4.
4. Add 10ml of growth medium prewarmed to 37ºC (for CHO cells, use growth medium consisting of F12 + 10% FBS).
5. Transfer cell suspension to a conical tube. Mix gently, and dislodge cell aggregates by slowly pipetting. Determine cell number using a hemacytometer.
6. Pellet cells at 250 × *g* for 5 minutes at room temperature.
7. Aspirate supernatant and resuspend HEK293 cells at a density of 1.5×10^5 cells/ml in growth medium prewarmed to 37ºC (for CHO cells use growth medium consisting of F12 + 10% FBS).
8. Add 100µl (15,000 HEK293 cells) to the individual wells of a tissue culture-treated, 96-well flat bottom plate.
9. Place plates in a 37°C tissue culture incubator with 5–10% CO_2, overnight.

Transient Transfection using FuGENE® HD Transfection Reagent

This protocol is sufficient for 20 wells (100µl of medium per well prior to addition of FuGENE® HD transfection reagent/DNA complex).

1. Dilute the pGloSensor™-22F cAMP or pGloSensor™-20F cAMP Plasmid to a final concentration of 12.5ng/µl in Opti-MEM® I reduced-serum medium.
2. Add 6µl of FuGENE® HD transfection reagent to 160µl of diluted plasmid and mix carefully by gentle pipetting.
3. Incubate for 0–15 minutes at room temperature.

4. Add 8µl of complex per well of a 96-well plate and gently mix without disturbing the cell monolayer.

5. Incubate 20–24 hours in a 37°C tissue culture incubator with 5–10% CO_2.

Equilibration with GloSensor™ cAMP Reagent

1. Carefully remove the medium from the individual wells. To accomplish this, place the pipette tips at the side of the well to minimize disruption of the cell monolayer. Move quickly to Step 2.

2. Add 100µl of equilibration medium per well for a 96-well plate. Add medium to the side of each well; do not pipet directly onto the cell monolayer. The equilibration medium contains a 2% v/v dilution of the GloSensor™ cAMP Reagent stock solution.

3. Incubate for 2 hours at room temperature or until a steady-state basal signal is obtained. Incubation at higher temperatures can facilitate equilibration, but care must be taken to allow the entire plate to come to a uniform temperature prior to starting the assay.

Notes:

1. We have found equilibration medium with 2% v/v GloSensor™ cAMP Reagent stock solution to be optimal for a majority of cell types. However, if the basal level of luminescence is not significantly above the luminometer background, increased concentrations of substrate can promote increased levels of light output. For example, equilibration medium with 6% v/v GloSensor™ cAMP Reagent stock solution provides a significantly increased basal level of light output (up to 50-fold) from CHO cells transiently transfected with pGloSensor™-22F or -20F cAMP Plasmid following a two-hour pre-equilibration at room temperature. These conditions were used to assay the response of endogenous G_s-coupled, 7-TM receptors in CHO cells (Figure 5).

2. **Requirement for use of buffered medium.** If the plates will be out of the CO_2 incubator for extended periods of time (such as during a kinetic read), the medium must be buffered to avoid the deleterious pH changes associated with equilibration to atmospheric conditions. This can be achieved using a commercially available buffered medium (CO_2-independent medium, Section 3.C). Alternatively, buffering agents can be added to medium (4), although Promega has not independently validated this approach.

Compound Preparation

To obtain a concentration response curve, serially dilute the compound in storage solvent (aqueous solution or DMSO) to 100X stock solutions, followed by direct addition to the respective wells. Alternatively, serially dilute the compound in storage solvent to 1,000X stock solutions, followed by dilution to 10X aqueous stock solutions and delivery to the respective wells.

Controls

We recommend the inclusion of both positive and negative controls in each experiment. A suitable positive control is treatment with 10µM forskolin. A suitable negative control is treatment with vehicle alone.

Luminescence Measurements

The GloSensor™ cAMP Assay is compatible with a wide range of instrumentation, including luminometers commonly used for reporter gene assays (GloMax®, GloMax®-Multi luminometers; see Section 7.C). Section 3.A provides general guidelines on the timing of compound additions and assay measurements for G_s- or G_i-coupled, 7-TM receptors.

End-Point Analysis at Room Temperature

1. Take a pre-read measurement prior to compound addition. Although this step is not required, normalization of data to a pre-read measurement can increase data quality by removing the well-to-well variability associated with transient transfection and differing total cell numbers. We have found integration times of 0.1–1 second to be sufficient for most luminometers.

2. Add 1µl of 100X compound stock solution or 10µl of a 10X compound stock solution per well using a multichannel pipet. Gently mix without disturbing the cell monolayer. We have found no deleterious effects associated with running assays using a 1% final DMSO concentration.

3. Measure luminescence. See Section 3.A for recommended times for measurement after compound addition, depending on assay format.

Kinetic Analysis

Note: Most luminometers operate above room temperature, especially in kinetic modes of operation. Therefore, it is important to allow the plate to pre-equilibrate to the steady-state operating temperature of the instrument prior to compound addition. This can typically be done by acquiring pre-read kinetic measurements for 15–20 minutes, where the basal level of luminescence can be monitored until a steady-state value is reached.

1. Equilibrate the plate to the steady-state operating temperature of the luminometer as described above.

2. Remove the plate from the instrument and quickly add compounds from 10X or 100X stock solutions using a multichannel pipet. Quickly return the plate to the instrument and begin taking measurements. Alternatively, use a luminometer with injectors to deliver compound(s) following the manufacturer's recommendations.

Note: If performing experiments at 37°C, it may be beneficial to increase the total volume to 200µl per well (making the appropriate changes to compound stock solutions) and to include distilled water in the spaces between wells to buffer any temperature changes associated with removing the plate from the instrument. If present, cooling effects will be apparent as sharp increases in the kinetic traces of wells receiving vehicle alone (negative controls).

Expected Results

1. See Figures 4, 5 and 6 for representative data of end-point measurements using HEK293 and CHO cells in a 96-well format.
2. See Figure 7 for representative data of kinetic measurements using HEK293 cells in a 96-well format.

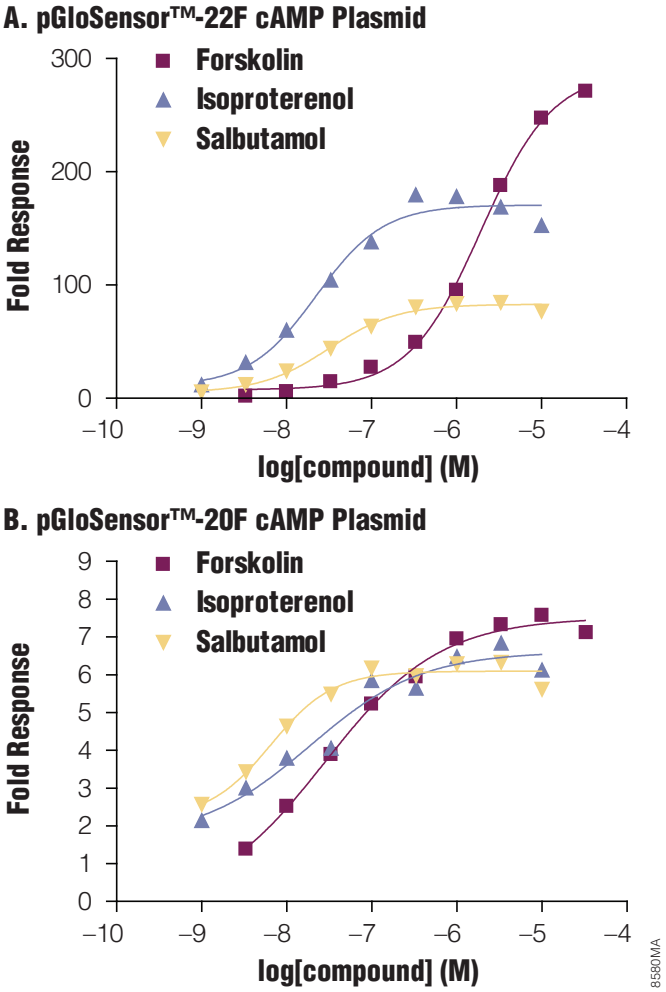

Figure 4. Performance comparison of GloSensor™ biosensor variants following activation of an endogenous G_s-coupled, 7-TM receptor in HEK293 cells. HEK293 cells were transiently transfected with pGloSensor™-22F cAMP Plasmid (**Panel A**) or pGloSensor™-20F cAMP Plasmid (**Panel B**) and assayed following the protocol outlined in Section 4.B. Luminescence was measured 10 minutes after addition of varying concentrations of the respective compounds, and this value was divided by a pre-read measurement taken prior to compound delivery to determine fold response. This experiment was performed in the absence of phosphodiesterase inhibitors. Isoproterenol is a full β2-adrenergic receptor agonist; salbutamol is a partial β2-adrenergic receptor agonist; forskolin is an activator of endogenous adenylate cyclase. Sample number = 1 per dose.

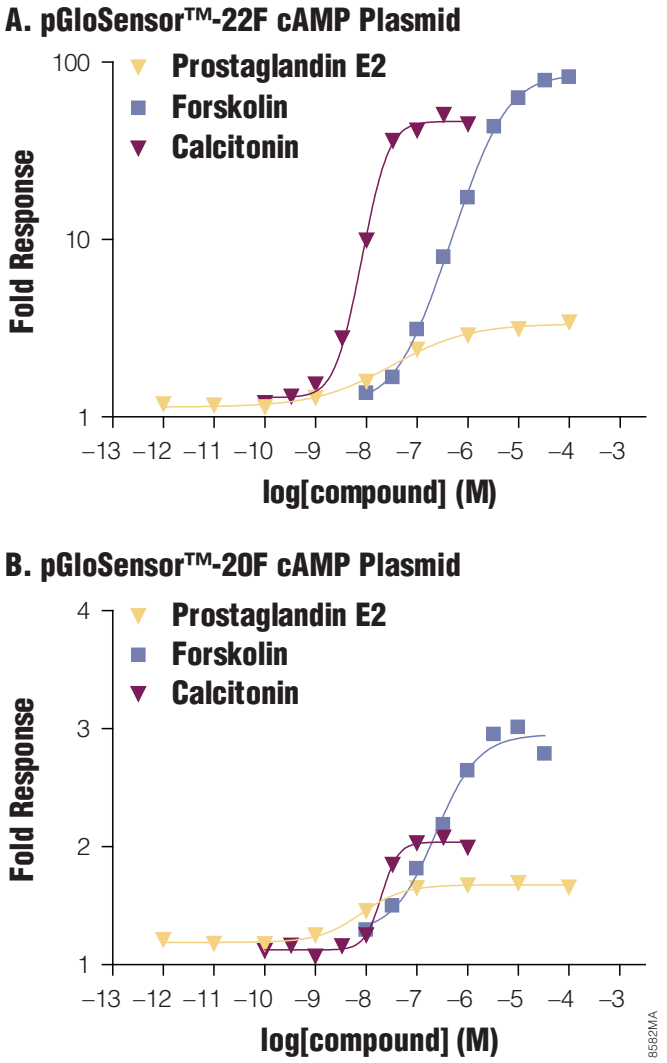

Figure 5. Performance comparison of GloSensor™ biosensor variants following activation of endogenous G_s-coupled, 7-TM receptors in CHO cells. CHO cells were transiently transfected with pGloSensor™-22F cAMP Plasmid (**Panel A**) or pGloSensor™-20F cAMP Plasmid (**Panel B**), then assayed following a modification of the protocol outlined in Section 4.B. For these experiments, the equilibration medium was made with a 6% v/v dilution of GloSensor™ cAMP Reagent stock solution and F12 medium was substituted for DMEM. Luminescence was measured 30 minutes after addition of varying concentrations of the compounds, and this value was divided by a pre-read measurement taken prior to compound delivery to determine fold response. This experiment was performed in the absence of phosphodiesterase inhibitors. Sample number = 1 per dose.

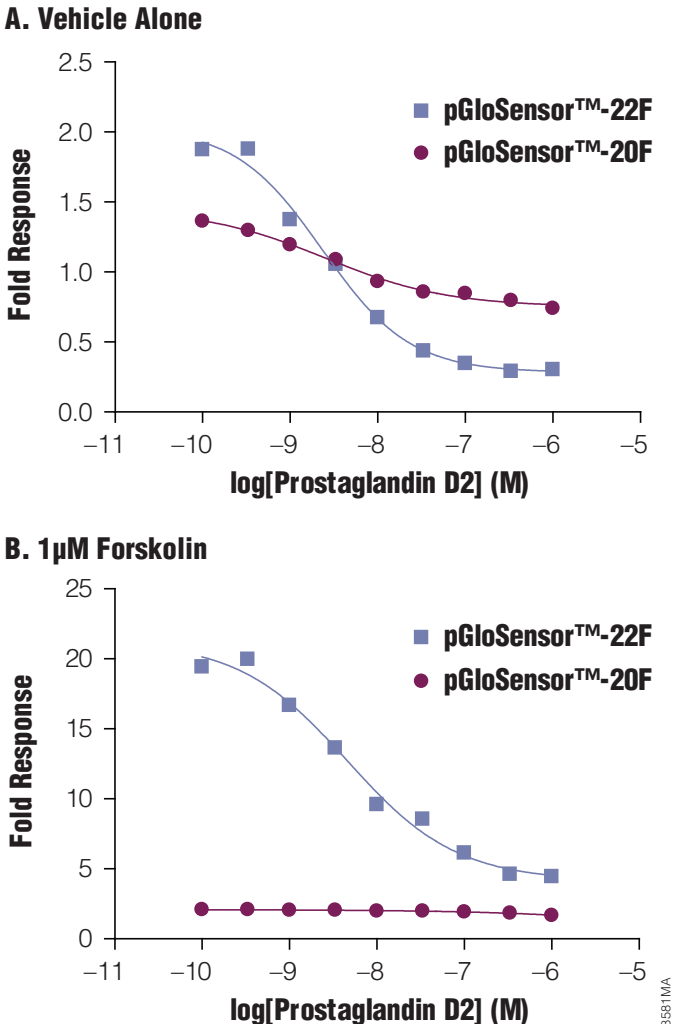

Figure 6. Performance comparison of GloSensor™ biosensor variants following activation of an overexpressed G_i-coupled, 7-TM receptor in HEK293T cells. HEK293T cells stably expressing the DP2/GPR44 receptor (Multispan, Inc.) were transiently transfected and assayed following the protocol outlined in Section 4.B. Cells were pretreated with varying concentrations of prostaglandin D2 agonist for five minutes prior to the addition of either vehicle alone (**Panel A**) or 1µM forskolin (**Panel B**). Luminescence was measured 30 minutes after forskolin addition, and this value was divided by a pre-read measurement taken prior to compound delivery to determine fold response. This experiment was performed in the absence of phosphodiesterase inhibitors. Sample number = 1 per dose.

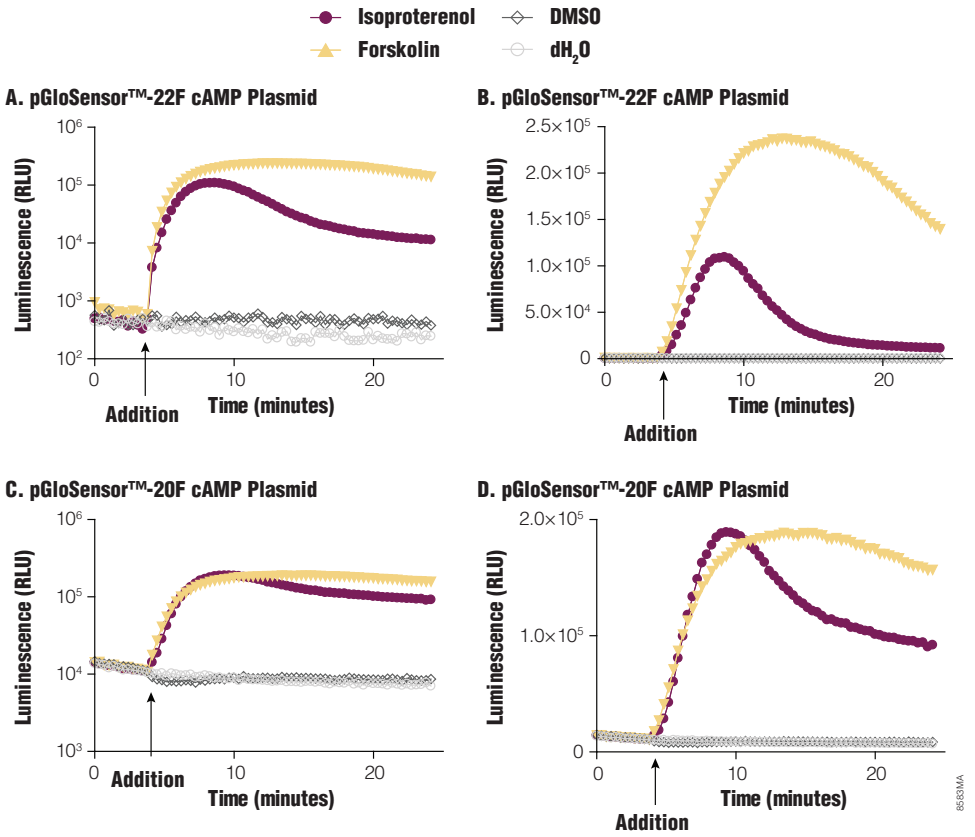

Figure 7. Performance comparison of GloSensor™ biosensor variant kinetic measurements taken at 28°C. HEK293 cells transiently transfected with the pGloSensor™-22F cAMP Plasmid (**Panels A** and **B**) or the pGloSensor™-20F cAMP Plasmid (**Panels C** and **D**) and assayed following the protocol outlined in Section 4.B. Following pre-equilibration to the steady-state operating temperature of the luminometer, 10µM of isoproterenol (β2-adrenergic receptor agonist) or 10µM forskolin (direct activator of adenylate cyclase) were added at the indicated time points. Kinetic traces for the pGloSensor™-22F cAMP Plasmid-transfected cells were then plotted on log (**Panel A**) and linear (**Panel B**) scales. Kinetic traces for the pGloSensor™-20F cAMP Plasmid-transfected cells plotted on log (**Panel C**) and linear (**Panel D**) scales.

4.C. Sample Protocol for Cells in Suspension in Various Plate Formats

This protocol has been used successfully for bulk transient transfection of biosensor variants in HEK293 cells using FuGENE® HD transfection reagent followed by incubation in equilibration medium. This protocol can be used to deliver a variety of total cell numbers to 96-, 384- or 1536-well formats for subsequent analysis and may be used as a template for transfecting additional cell types, where further optimization may be required to achieve maximal performance.

1. Add 1.5 × 10^6 HEK293 cells to a new T75 tissue culture flask. Incubate for 20–24 hours in a 37°C tissue culture incubator with 5–10% CO_2.
2. Dilute the pGloSensor™-22F cAMP or pGloSensor™-20F cAMP Plasmid to a final concentration of 12.5ng/µl in Opti-MEM® I serum-reduced medium (Section 3.C).
3. Add 24µl of FuGENE® HD transfection reagent to 640µl of diluted plasmid and mix carefully by pipetting.
4. Incubate for 0–15 minutes at room temperature.
5. Add 8.3ml of growth medium (DMEM + 10% FBS) to the solution in Step 3. Mix carefully by pipetting.
6. Remove existing medium from flask and replace with the solution from Step 5. **Note:** Pipet the replacement solution onto the side of the flask, taking care to not pipet directly onto the cell monolayer.
7. Incubate 20–24 hours in a 37°C tissue culture incubator with 5–10% CO_2.
8. Wash cell monolayer with 10ml PBS.
9. Add 2ml 0.05% trypsin-EDTA prewarmed to 37°C. Coat the surface of the flask evenly. Dislodge the cells from the flask surface by rocking and gently tapping the side of the flask. Once cells are dislodged, proceed immediately to Step 10.
10. Add 10ml of growth medium (CO_2-independent medium + 10% FBS).
11. Transfer cell suspension to a conical tube. Mix gently and dislodge cell aggregates by pipetting slowly onto the side of the tube. Determine cell number using a hemacytometer.
12. Pellet cells at 250 × g for 5 minutes at room temperature.
13. Resuspend cells to the desired cell number per unit volume using equilibration medium. For example, resuspend to 2.5 × 10^5 cells/ml to deliver 5,000 cells/20µl.

4.C. Sample Protocol for Cells in Suspension in Various Plate Formats (continued)

Equilibration with GloSensor™ cAMP Reagent

Incubate cells with equilibration medium for 2 hours at room temperature. Incubation at higher temperatures can facilitate equilibration, but care must be taken to allow the cell suspension to come to a uniform temperature prior to starting the assay. Gently rock the cell suspension or invert the tube approximately every 15 minutes to prevent settling of cells.

Transfer to 96-, 384- or 1536-Well Plates

Transfer the desired number of cells to 96-, 384- or 1536-well plates. The minimum number of cells needed to give a basal signal significantly above the luminometer background will be a function of both cell type and luminometer sensitivity. Increasing concentrations of substrate can promote increases in the basal levels of luminescence for certain cell types (Section 4.B).

Compound Preparation

Please see Section 4.B.

Note: The concentration of stock solutions will depend on the ability to accurately deliver small volumes to the total volume of cell suspension per well. It is important to consider the aqueous solubility of compounds of interest when preparing stock solutions.

Controls and Luminescence Measurements

Please see Section 4.B.

Expected Results

See Figure 8 for representative data using HEK293 cells in 384-well format.

5. GloSensor™ cAMP HEK293 Cell Line

5.A. Description

The GloSensor™ cAMP HEK293 Cell Line stably expresses the biosensor variant encoded by the pGloSensor™-20F cAMP Plasmid.

5.B. Thawing, Routine Maintenance and Storage

1. Rapidly thaw cells by placing them in a 37°C water bath with gentle agitation for 1–2 minutes.

   Note: Freezing medium may appear yellow immediately after thawing. This will not affect cell viability if these instructions are followed.

2. Decontaminate the vial by wiping it with 70% ethanol before opening it in a class II biological safety cabinet.

3. Slowly transfer the vial contents to 10ml of growth medium that has been prewarmed to 37°C in a sterile 15ml conical tube.

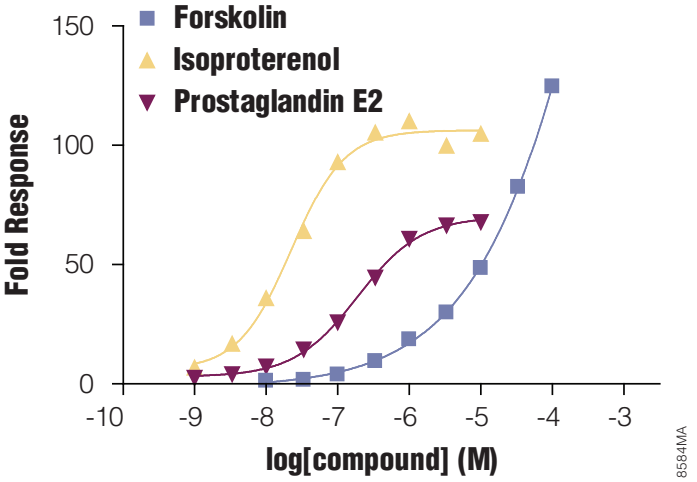

Figure 8. Performance of the pGloSensor™-22F biosensor variant after transient expression. HEK293 cells were transiently transfected with the pGloSensor™-22F biosensor and assayed in suspension in 384-well format following the protocol outlined in Section 4.C. Luminescence was measured 10 minutes after addition of varying concentrations of the respective compounds. This luminescence value was divided by the luminescence value from a well that received vehicle alone to determine fold response. This experiment was performed in the absence of phosphodiesterase inhibitors. Sample number = 1 per dose.

4. Centrifuge cells at 250 x *g* for 5 minutes at room temperature.
5. Aspirate the supernatant, and resuspend the cell pellet in 12ml of growth medium prewarmed to 37°C.
6. Transfer cells to a T75 flask. Incubate in a 37°C tissue culture incubator with 5–10% CO_2.
7. At the first cell passage, switch to growth medium + hygromycin B (Section 3.D).

It is important to freeze multiple aliquots of the cell line at this stage for repeated or prolonged use (see the following section).

5.B. Thawing, Routine Maintenance and Storage (continued)

Cell Propagation

Maintain cells between 10% and 90% confluency in a 37°C, 5–10% CO_2 tissue culture incubator. This will typically require passaging the culture twice a week. The approximate cell number for a 100% confluent monolayer for this cell line in a T75 flask is 1×10^7 cells. Medium formulations are provided in Section 3.D. The volumes listed in Steps 1–4 below are for propagation in a T75 flask.

1. When cells have reached the appropriate density, aspirate the medium from the flask.
2. Wash cell monolayer using 10ml of PBS.

The GloSensor™ cAMP HEK293 Cell Line is **not** strongly adherent. Pipet slowly to avoid dislodging cells from the flask surface. Do not pipet directly onto the cell monolayer.

3. Add 2ml of 0.05% trypsin-EDTA prewarmed to 37ºC. Coat the surface of the flask evenly. Dislodge cells from the flask surface by rocking and gently tapping the side of the flask. Once cells are dislodged, proceed immediately to Step 4.
4. Add 10ml of growth medium prewarmed to 37ºC.
5. Transfer cell suspension to a conical tube. Mix gently, and dislodge cell aggregates by slowly pipetting. Determine cell number using a hemacytometer.
6. Centrifuge cells at 250 × *g* for 5 minutes at room temperature.
7. Aspirate supernatant, and resuspend cells in growth medium + hygromycin B prewarmed to 37ºC.
8. Seed new flasks at the appropriate cell density: 1×10^6 cells in 10ml for a T75 flask and 2×10^6 cells in 20ml for a T150 flask.
9. Place flasks in a 37°C incubator with 5–10% CO_2.

Freezing Cells

1. Grow cells until 50% confluent. Replace growth medium + hygromycin B with growth medium only (no hygromycin B) the day before harvest.
2. Harvest cells as described in Section 4.B, Steps 1–6. Resuspend the cell pellet in freezing medium (Section 3.D) at a density of 2×10^6 cells/ml.
3. Dispense 1.0ml per cryogenic vial.
4. Freeze vials overnight at –80°C in a Styrofoam® box or similar slow-freeze container. Do not snap freeze the cells.
5. Transfer vials to a liquid nitrogen tank or a –140°C freezer for long-term storage.

5.C. Sample Protocol for 384-Well Format

Cell Culture Preparation

The volumes for Steps 1–4 below refer to a T75 tissue culture flask.

1. Harvest cells at 50–90% confluence.
2. Wash cell monolayer with 10ml of PBS.

The GloSensor™ cAMP HEK293 Cell Line is **not** strongly adherent. Pipet slowly to avoid dislodging cells from the flask surface. Do not pipet directly onto the cell monolayer.

3. Add 2ml of 0.05% trypsin-EDTA prewarmed to 37ºC. Coat the surface of the flask evenly. Dislodge cells from the flask surface by rocking and gently tapping the side of the flask. Once cells are dislodged, proceed immediately to Step 4.
4. Add 10ml of growth medium prewarmed to 37ºC.
5. Transfer cell suspension to a conical tube. Mix gently, and dislodge cell aggregates by slowly pipetting. Determine cell number using a hemacytometer.
6. Pellet cells at 250 × *g* for 5 minutes at room temperature.
7. Aspirate supernatant and resuspend cells to a density of 2.5 x 10^5 cells/ml in plating medium prewarmed to 37ºC.
8. Add 25µl (6,250 cells) to each well of a tissue culture-treated, 384-well plate.
9. Place plates in a 37°C tissue culture incubator with 5–10% CO_2, overnight.

Equilibration with GloSensor™ cAMP Reagent

1. Add 25µl of equilibration medium with 4% v/v dilution of the GloSensor™ cAMP Reagent per well.

   Note: This represents a 2% v/v final dilution of the GloSensor™ cAMP Reagent stock solution.

2. Incubate at room temperature for 2 hours or until a steady-state basal signal is obtained. Incubation at higher temperatures can facilitate equilibration, but take care to allow the entire plate to come to a uniform temperature prior to starting the assay.

5.C. Sample Protocol for 384-Well Format (continued)

Compound Preparation

Please see Section 4.B.

Note: The concentration of stock solutions will depend on the ability to accurately deliver small volumes to the total volume of solution per well. It is important to consider the aqueous solubility of compounds when preparing stock solutions.

Controls and Luminescence Measurements

Please see Section 4.B.

Expected Results

See Figure 9 for representative data of kinetic measurements using the GloSensor™ cAMP HEK293 Cell Line in 384-well format.

5.D. Additional Protocols Using the GloSensor™ cAMP HEK293 Cell Line

A modified version of the protocol listed in Section 4.B can be used to assay the GloSensor™ cAMP HEK293 Cell Line in 96-well format:

1. Day 1: Plate 20,000 cells/well.
2. Day 2: Perform the substrate equilibration, compound addition, etc., as described in Section 4.B.

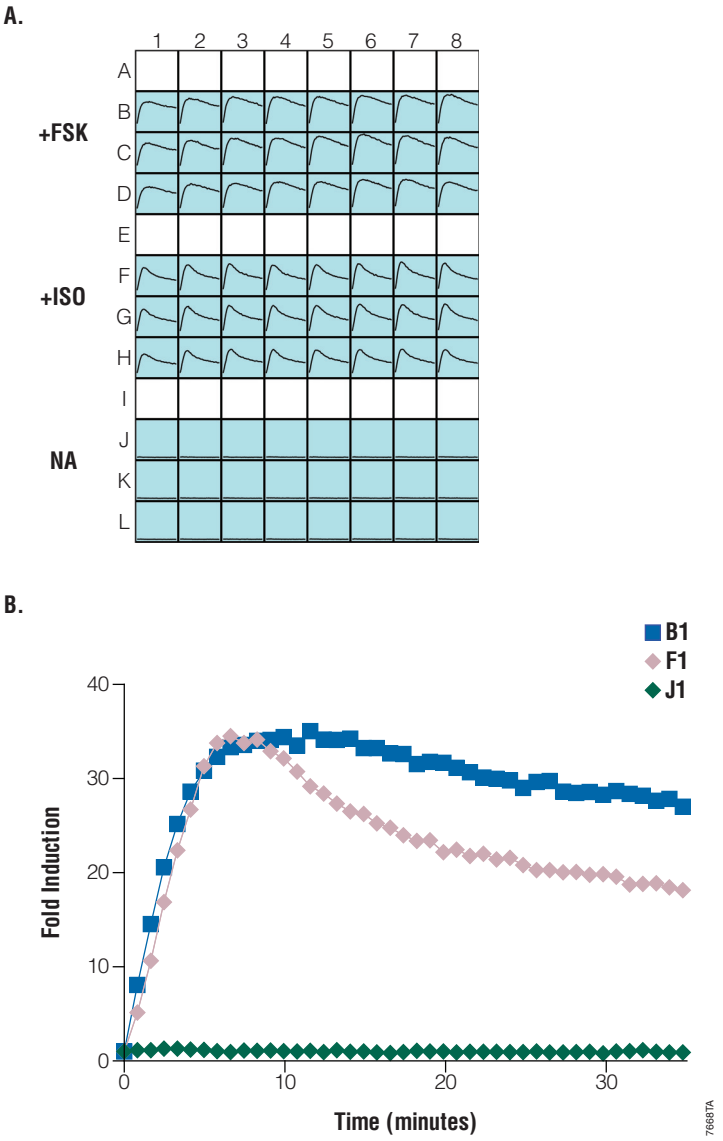

Figure 9. Kinetic response of the GloSensor™ cAMP HEK293 Cell Line. Luminescence was measured from cells treated with 10µM forskolin (FSK, direct activator of adenylate cyclase) or 10µM isoproterenol (ISO, a β2-adrenergic receptor agonist) or from nontreated cells (NA). **Panel A.** Kinetic traces from the individual wells of a 384-well plate. **Panel B.** Individual kinetic traces from wells B1, F1, and J1. This experiment was done in the absence of phosphodiesterase inhibitors following steps outlined in the Section 5.C. The steady-state temperature of the luminometer during the experiment was 28ºC, and a stable baseline signal was obtained following pre-incubation of the plate for 10 minutes prior to compound addition. For Panel A, Y-axis for each well: 0–193435 RLU; X-axis for each well: 0–34.8 minutes.

6. Troubleshooting

Symptoms	Causes and Comments
Following incubation with equilibration medium + 2% GloSensor™ cAMP Reagent stock solution, the basal luminescence level was at or near the luminometer background.	We have found that the basal level of luminescence of certain cell types can benefit from pre-equilibration with increased concentrations of substrate. For instance, CHO cells pre-equilibrated with a 6% v/v dilution of the GloSensor™ cAMP Reagent stock solution for two hours at room temperature have shown up to 50-fold increases in basal levels of light output. Overall, basal levels of luminescence will be influenced by factors such as permeability of substrate, activity of efflux pumps, transfection efficiency, steady-state expression levels of biosensor protein, basal levels of cAMP in the cell and sensitivity of the luminometer. Many of these factors will vary from cell type to cell type.
A basal level of luminescence above instrument background is detectable, but little or no change in light output is seen following the addition of compounds known to modulate intracellular levels of cAMP.	Repeat using the pGloSensor™-22F cAMP version of the biosensor. See Section 3.B.
For assays performed above room temperature, abrupt increases are seen in kinetic traces following removal of the assay plate from the luminometer and compound addition, even in wells left untreated or receiving vehicle alone.	Changes in the assay temperature promote changes in the overall levels of light output. In general, increases in temperature decrease basal and induced levels of light output and decreases in temperature increase light output.
	Care must be taken to avoid cooling effects associated with compound addition. This can be achieved by the use of luminometers with injectors, by increasing the total volume of medium added per well or by adding distilled water to the spaces between wells for experiments done in 96-well format.

Symptoms	Causes and Comments
A gradual decrease in signal is seen after placing a room temperature plate into the luminometer and initiating kinetic measurements, even in nontreated wells or wells receiving vehicle alone.	Changes in the assay temperature promote changes in the overall levels of light output, where changes in the basal level of cAMP in the cell may be a contributing factor. In general, increased temperatures decrease basal and induced levels of light output.
	Most luminometers operate above room temperature, especially in kinetic modes of operation. Therefore, it is important to allow the plate to pre-equilibrate to the steady-state operating temperature of the instrument prior to compound addition. This can typically be done by acquiring pre-read kinetic measurements for 15–20 minutes, where the basal level of luminescence can be monitored until a steady-state value is reached.

7. Appendix

7.A. References

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2. Binkowski, B.F., Fan, F. and Wood, K.V. (2009) Live-cell luminescent assays for GPCR studies. *Gen. Eng. Biotech.* **29**, 30–1.
3. Binkowski, B.F., Fan, F. and Wood, K.V. (2009) Engineered luciferases for molecular sensing in living cells. *Curr. Opin. Biotech.* **20**, 14–8.
4. Kimple, A.J. *et al.* (2009) Structural determinants of G-protein α subunit selectivity by regulator of G-protein signaling 2 (RGS2). *J. Biol. Chem.* **284**, 402–11.

7.B. GloSensor™ Technology Web Site

For the latest information on the GloSensor™ cAMP Assay and the GloSensor™ technology platform, including Frequently Asked Questions, noncommercial materials and more, visit:
www.promega.com/glosensor

7.C. Related Products

Product	Size	Cat.#
GloResponse™ NFAT-RE-*luc2P* HEK293 Cell Line	2 × 10^6 cells	E8510
GloResponse™ CRE-*luc2P* HEK293 Cell Line	2 × 10^6 cells	E8500
pGL4.29[*luc2P*/CRE/Hygro] Vector	20µg	E8471
pGL4.33[*luc*2P/SRE/Hygro] Vector	20µg	E1340
pGL4.34[*luc2P*/SRF-RE/Hygro] Vector	20µg	E1350
cAMP-Glo™ Assay*	300 assays	V1501
PDE-Glo™ Phosphodiesterase Assay*	1,000 assays	V1361
ADP-Glo™ Kinase Assay	1,000 assays	V9101
	10,000 assays	V9102
	100,000 assays	V9103
Kinase-Glo® Luminescent Kinase Assay*	10ml	V6711
Kinase-Glo® Plus Luminescent Kinase Assay*	10ml	V3771
Kinase-Glo® Max Luminescent Kinase Assay*	10ml	V6071

*Additional Sizes Available.

Luminometers

Product	Size	Cat.#
GloMax®-Multi+ Detection System with Instinct™ Software: Base Instrument with Shaking	each	E8032
GloMax®-Multi+ Detection System with Instinct™ Software: Base Instrument with Heating and Shaking	each	E9032
GloMax®-Multi+ Luminescence Module	each	E8041
GloMax®-Multi+ Fluorescence Module	each	E8051
GloMax®-Multi+ Visible Absorbance Module	each	E8061
GloMax®-Multi+ UV-Visible Absorbance Module	each	E9061
GloMax® 96 Microplate Luminometer	each	E6501
GloMax® 96 Microplate Luminometer w/Single Injector	each	E6511
GloMax® 96 Microplate Luminometer w/Dual Injectors	each	E6521
GloMax®-Multi Base Instrument	each	E7031
GloMax®-Multi Luminescence Module	each	E7041
GloMax®-Multi Fluorescence Module	each	E7051
GloMax®-Multi Absorbance Module	each	E7061
GloMax® 20/20 Luminometer	each	E5311
GloMax® 20/20 Luminometer w/Single Auto-Injector	each	E5321
GloMax® 20/20 Luminometer w/Dual Auto-Injector	each	E5331
Luminometer Plates	50 plates	Z3291

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[c]U.S. Pat. No. 8,008,006 and European Pat. No. 1341808.

[d]U.S. Pat. No. 7,728,118 and other patents pending.

[e]Patent Pending.

[f]The method of recombinant expression of *Coleoptera* luciferase is covered by U.S. Pat. Nos. 5,583,024, 5,674,713 and 5,700,673. A license (from Promega for research reagent products and from The Regents of the University of California for all other fields) is needed for any commercial sale of nucleic acid contained within or derived from this product.

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