TECHNICAL BULLETIN

cAMP-Glo™ Assay

Instructions for use of Products
V1501, V1502 and V1503

Revised 12/13
TB357

cAMP-Glo™ Assay

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1. Description

The cAMP-Glo™ Assay[a–f] is a homogeneous, bioluminescent and high-throughput assay to measure cAMP levels in cells. The cAMP-Glo™ Assay monitors cAMP production in cells in response to the effects of an agonist or test compound on G protein-coupled receptors (GPCRs). GPCRs that couple with adenylate cyclase will increase or decrease intracellular cAMP. The assay is based on the principle that cyclic AMP (cAMP) stimulates protein kinase A (PKA) holoenzyme activity, decreasing available ATP and leading to decreased light production in a coupled luciferase reaction (Figure 1).

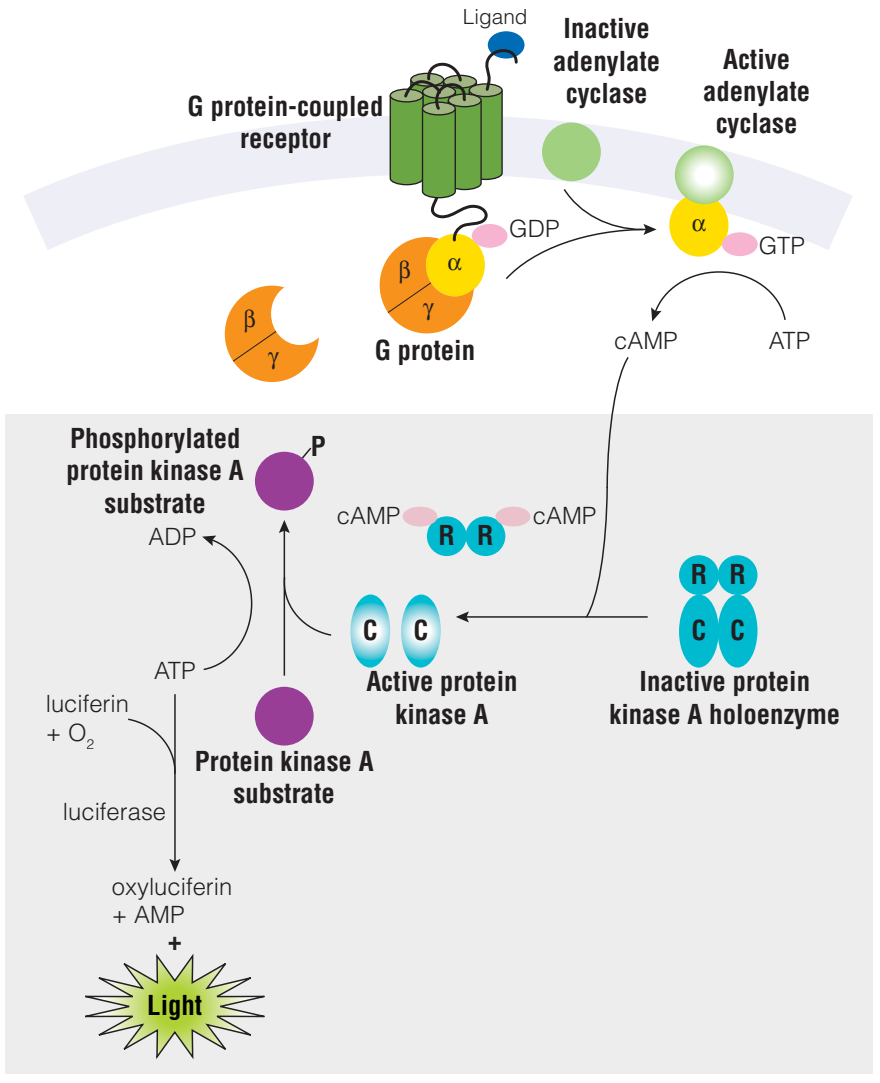

Figure 1. Schematic diagram of cAMP production in cells and the cAMP-Glo™ Assay. Binding of an extracellular ligand to its receptor alters the conformation of the associated heterotrimeric G protein, causing dissociation of the G_{α} and $G_{\beta\gamma}$ subunits and initiating a cascade of cellular events (1). The alpha subunit is categorized into one of several groups: α_s, $\alpha_{i/o}$, α_q, and $\alpha_{12/13}$ (1,2). $G\alpha_s$ activates adenylate cyclase, while $G\alpha_{i/o}$ inhibits adenylate cyclase activity. The cAMP-Glo™ Assay is depicted in the shaded box. As the concentration of cAMP increases, cAMP binds to protein kinase A, and the regulatory subunits undergo a conformational change to release the catalytic subunits. The free catalytic subunits then catalyze the transfer of the terminal phosphate of ATP to a protein kinase A substrate, consuming ATP in the process. The level of remaining ATP is determined using the luciferase-based Kinase-Glo® Reagent. Luminescence is inversely proportional to cAMP levels. Thus, as cAMP concentration increases, luminescence decreases.

PKA is composed of two regulatory and two catalytic subunits and remains in an inactive form in the absence of cAMP. As the concentration of cAMP increases, cAMP binds to two sites on the regulatory subunits, which then undergo a conformational change to release the catalytic subunits. The free catalytic subunits are active and catalyze the transfer of the terminal phosphate of ATP to a PKA substrate. The decrease in ATP concentration can be monitored as light output using luciferase in a reaction that requires ATP to produce light. Thus, there is a reciprocal relationship between cAMP concentration and the light output observed (Figure 2).

The cAMP-Glo™ Assay can be performed in 96-, 384- or 1536-well plates. The cells are induced with a test compound for an appropriate period of time to modulate cAMP levels. After induction, cells are lysed to release cAMP, then the cAMP Detection Solution, which contains protein kinase A, is added. The Kinase-Glo® Reagent is then added to terminate the PKA reaction and detect the remaining ATP via a luciferase reaction. Plates are read using a microplate-reading luminometer (Figure 3). Luminescence can be correlated to the cAMP concentrations by using a cAMP standard curve. The half-life for the luminescent signal is greater than 4 hours. This extended signal half-life eliminates the need for luminometers with reagent injectors and allows batch-mode processing of multiple plates.

Note: The cAMP concentration is based on the volume of cells + compound without reaction or cAMP-Glo™ Lysis Buffer added.

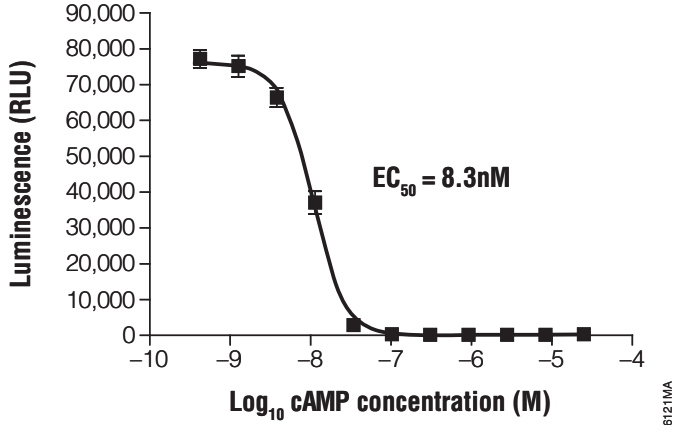

Figure 2. Titration of purified cAMP. Reactions were assembled with the indicated concentrations of purified cAMP in a low-volume 384-well plate. The cAMP-Glo™ Assay was performed as described in Section 5.A. Data were collected using a plate-reading luminometer (FLUOstar OPTIMA, BMG Labtech). Each point represents eight data points; the error bars represent the standard deviation. Data analysis was performed with GraphPad Prism® software, version 4.02, for Windows® using a sigmoidal dose-response (variable slope) equation.

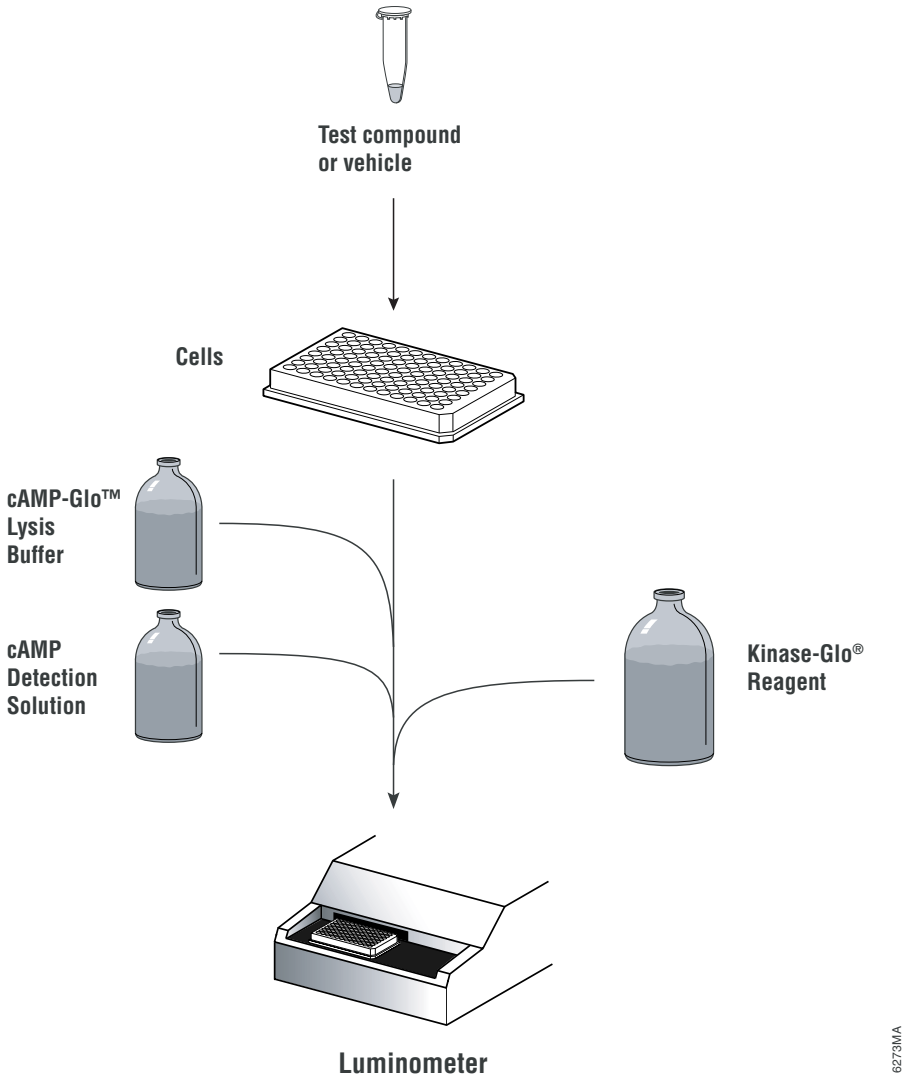

Figure 3. Schematic diagram showing cAMP-Glo™ Assay reagent preparation and protocol.

2. Product Components and Storage Conditions

Product	Size	Cat.#
cAMP-Glo™ Assay	300 assays (384-well plate)	V1501

The system contains sufficient reagents for 300 assays in a 384-well plate format. Includes:

- 100µl cAMP, 1mM
- 5ml cAMP-Glo™ Lysis Buffer
- 8ml cAMP-Glo™ Reaction Buffer
- 16µl Protein Kinase A
- 1 bottle Kinase-Glo® Substrate (lyophilized)
- 10ml Kinase-Glo® Buffer

Product	Size	Cat.#
cAMP-Glo™ Assay	3,000 assays (384-well plate)	V1502

The system is composed of two components: cAMP-Glo™ Assay (Cat.# V1504) and the Kinase-Glo® Luminescent Kinase Assay (Cat.# V6713) and contains sufficient reagents for 3,000 assays in a 384-well plate format. Includes:

- 100µl cAMP, 1mM
- 25ml cAMP-Glo™ Lysis Buffer
- 50ml cAMP-Glo™ Reaction Buffer
- 160µl Protein Kinase A
- 1 bottle Kinase-Glo® Substrate (lyophilized)
- 100ml Kinase-Glo® Buffer

Product	Size	Cat.#
cAMP-Glo™ Assay	30,000 assays (384-well plate)	V1503

The system is composed of three components: cAMP-Glo™ Assay (Cat.# V1505), Protein Kinase A (Cat.# V1506) and the Kinase-Glo® Luminescent Kinase Assay (Cat.# V6714) and contains sufficient reagents for 30,000 assays in a 384-well plate format. Includes:

- 500µl cAMP, 1mM
- 250ml cAMP-Glo™ Lysis Buffer
- 500ml cAMP-Glo™ Reaction Buffer
- 10 × 160µl Protein Kinase A
- 10 bottles Kinase-Glo® Substrate (lyophilized)
- 10 × 100ml Kinase-Glo® Buffer

Storage Conditions: Store the system at –20°C. Before use, all components should be thawed completely at room temperature except for the Protein Kinase A, which should be kept on ice when not at –20°C. Once thawed, all components should be thoroughly mixed before use. Once prepared, the cAMP Detection Solution (cAMP-Glo™ Reaction Buffer with Protein Kinase A) should not be frozen. Once prepared, the Kinase-Glo® Reagent should be dispensed into aliquots and stored at –20°C. See the product label for expiration date.

3. Cell Handling and Treatment

The cAMP-Glo™ Assay can be performed with adherent, suspension or frozen cells using the recommendations below. Adherent cells are incubated overnight to allow the cells to attach to the growth matrix prior to the cAMP-Glo™ Assays. Suspension cells and frozen cells do not require an overnight incubation and can be used in the assay on the same day that they are prepared. The recommendations for cell number and culture conditions given in this manual were developed for HEK293 cells. For other adherent cells lines or for suspension or frozen cells, the number of cells and culture conditions may need to be optimized. Use a known agonist or antagonist to determine the optimal conditions that result in the largest effect on cAMP levels.

The cAMP-Glo™ Assay involves treating cells with an agonist or test compound to modulate cAMP levels. Treat the cells with agonist or test compound in induction buffer [Krebs Ringer bicarbonate buffer, 1X phosphate-buffered saline (PBS) or serum-free medium with 500µM isobutyl-1-methylxanthine (IBMX) and 100µM 4-(3-butoxy-4-methoxy-benzyl) imidazolidone (Ro 20-1724); IBMX and Ro 20-1724 are broad-range phosphodiesterase inhibitors to inhibit cAMP hydrolysis]. Induce the cells for an appropriate period of time, depending upon the nature of ligand and receptor. Performance of the cAMP-Glo™ Assay is not affected by the presence of up to 5% acetone or 5% DMSO (two common compound vehicles).

Perform a cAMP standard curve for each set of experiments. We recommend performing the cAMP standard curve in a plate separate from assays with treated cells. However, if you include the cAMP standards in the same plate with the cells, do not add cells to the wells reserved for the cAMP standard curve (Section 4.B) when preparing plates of cells.

Materials to be Supplied by the User
(Solution compositions are provided in Section 8.A.)

- induction buffer [Krebs Ringer buffer, 1X phosphate-buffered saline (PBS) or serum-free medium containing 500µM isobutyl-1-methylxanthine (IBMX, Sigma-Aldrich Cat.# I7018) and 100µM 4-(3-butoxy-4-methoxy-benzyl) imidazolidone (Ro 20-1724, Sigma Aldrich Cat.# B8279)], at room temperature
- poly-D-lysine-coated, white, clear-bottom plates (BD BioCoat™ plates), for adherent cells. **Do not** use black plates or clear plates.
- white, clear-bottom tissue culture plates, for suspension and frozen cells. **Do not** use black plates or clear plates.
- multichannel pipette or automated pipetting station
- 2X test compound of interest prepared in induction buffer

Adherent Cells

To prepare adherent cells, grow cells in flasks or dishes, and wash with 1X PBS. Trypsinize cells, and transfer the cell-containing culture medium to a 50ml conical centrifuge tube. Centrifuge the tube at 1,500 × *g* for 10 minutes. Resuspend the cell pellet in medium, and count the number of cells using a hemacytometer. Dispense the appropriate volume containing the recommended number of cells (Table 1) into each well of the assay plate, and allow cells to incubate overnight. On day 2, remove the medium, and add 1X test compound of interest in induction buffer to the cells to initiate induction. The volume of 1X test compound added to each well is shown in Table 1. Proceed to Section 4.

Note: We grow cells in poly-D-lysine-coated, white, clear-bottom plates (BD BioCoat™ plates) with complete medium overnight in a tissue culture cell incubator.

Table 1. Recommended Cell Densities and Test Compound Volumes for Adherent Cells.

	Cell Density (cells/well)	Volume of Cells Per Well	Volume of 1X Test Compound Per Well
96-well plate	2,500–10,000	50–100µl	20µl
384-well plate	2,500–5,000	20–50µl	7.5µl

Suspension Cells

To prepare suspension cells for the cAMP-Glo™ Assay, grow cells in flasks or dishes. Add trypsin, and after trypsinization, transfer the cell-containing culture medium to 50ml conical centrifuge tubes, and centrifuge tubes at 1,500 × *g* for 10 minutes. Resuspend the cell pellet in induction buffer, and count the number of cells using a hemacytometer. Dispense the appropriate volume containing the recommended number of cells (Table 2) into each well of the assay plate. Add 2X test compound of interest in induction buffer to the cells to initiate induction. The volume of 2X test compound added to each well is shown in Table 2. Proceed to Section 4.

Table 2. Recommended Cell Densities and Test Compound Volumes for Suspension Cells.

	Cell Density (cells/well)	Volume of Cells Per Well	Volume of 2X Test Compound Per Well
96-well plate	10,000–20,000	10µl	10µl
384-well plate	5,000–10,000	3.75µl	3.75µl
low-volume 384-well or 1536-well plate	1,000–2,000	0.5µl	0.5µl

3. Cell Handling and Treatment (continued)

Frozen Cells

To prepare frozen cells for the cAMP-Glo™ Assay, freeze cells in complete medium with or without an appropriate concentration of DMSO at or below –70°C until ready to perform the cAMP-Glo™ Assay. To perform the cAMP-Glo™ Assay, thaw the cells, then centrifuge the cells at 1,500 × *g* for 10 minutes. Resuspend the cell pellet in induction buffer. Count the cells using a hemacytometer. Dispense the appropriate volume containing the recommended number of cells into each well (Table 3), and add 2X test compound of interest in induction buffer to the cells to initiate induction. The volume of 2X test compound added to each well is shown in Table 3. Proceed to Section 4.

Table 3. Recommended Cell Densities and Test Compound Volumes for Frozen Cells.

	Cell Density (cells/well)	Volume of Cells Per Well	Volume of 2X Test Compound Per Well
96-well plate	20,000–50,000	10µl	10µl
384-well plate	10,000–20,000	3.75µl	3.75µl

4. Preparing for the cAMP-Glo™ Assay

Prior to performing the cAMP-Glo™ Assay, treat cells with an agonist or test compound in induction buffer to modulate cAMP levels (Section 3). Be sure to perform a cAMP standard curve for each set of experiments (Section 4.B).

Materials to be Supplied by the User

(Solution compositions are provided in Section 8.A.)

- induction buffer (Krebs Ringer bicarbonate buffer, 1X phosphate-buffered saline (PBS) or serum-free medium containing 500µM IBMX and 100µM Ro 20-1724), at room temperature
- 96-well, 384-well or 1536-well plate
- multichannel pipette or automated pipetting station

4.A. Reagent Preparation

All components except Protein Kinase A should be completely thawed at room temperature before use. Keep Protein Kinase A on ice when not at –20°C. For your experiments, calculate the required volumes of each reagent, and increase or decrease the volumes appropriately.

1. Prepare the cAMP solution as follows. Vortex to mix.

Component	Volume
induction buffer	250µl
1mM cAMP	1.0µl

Note: The final cAMP concentration of this cAMP solution is 4.0µM.

2. Transfer the entire volume of Kinase-Glo® Buffer into the amber bottle containing the Kinase-Glo® Substrate to reconstitute the lyophilized substrate. This forms the Kinase-Glo® Reagent. Mix by gently vortexing, swirling or inverting the contents to obtain a homogeneous solution. The Kinase Glo® Substrate should go into solution easily, in less than 1 minute.

   Note: The Kinase-Glo® Reagent should be used on the same day that it is prepared or dispensed into single-use aliquots and stored at –20°C.

4.B. Generating a cAMP Standard Curve

The cAMP standards can be prepared in a separate 96-well and/or 384-well plates. These standards will be transferred to the plate in which the cAMP standard curve will be performed. We recommend assaying each cAMP standard concentration in triplicate. Figure 2 shows representative data from a cAMP standard curve.

Note: The cAMP concentration is based on the volume of cells + compound without reaction or cAMP-Glo™ Lysis Buffer added.

The luminesence output of the assay is affected by the presence of cells so it is not possible to directly compare the raw RLU values of the samples with those of the cAMP standards. Instead, calculate the change in RLU (ΔRLU) for the standards and the samples as follows:

1. For each standard concentration: ΔRLU = RLU (0nM) – RLU (XnM). Plot ΔRLU (Y-axis) versus cAMP concentration in nM (X-axis). Perform a regression analysis. Figure 4 shows an example of a plotted standard curve.
2. For each sample: ΔRLU = RLU (untreated sample) – RLU (treated sample). Using this ΔRLU value and the linear equation generated from the standard curve, calculate the cAMP concentration.

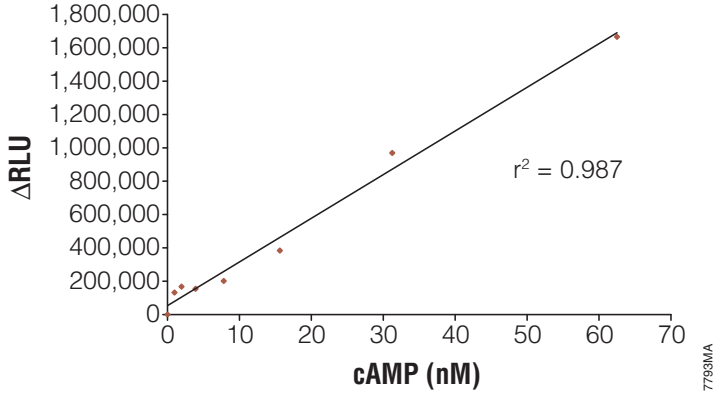

Figure 4. Titration of purified cAMP. Reactions were assembled with the indicated concentrations of purified cAMP in a white standard 384-well plate. The cAMP-Glo™ Assay was performed as described in Section 5.A. Data were collected using a plate-reading luminometer (FLUOstar OPTIMA, BMG Labtech). Each point represents the average of four data points. Data analysis was performed with Excel using linear regression.

4.B. Generating a cAMP Standard Curve (continued)

1. Add 100µl of induction buffer to wells A2 through A12 of a 96-well plate.
2. Add 200µl of the cAMP solution prepared in Section 4.A to well A1.
3. Perform a serial twofold dilution by transferring 100µl from well A1 to well A2 in column 2 with a pipette, pipetting to mix. Transfer 100µl to well A3. Repeat for wells A4 through A11. See Figure 5. Discard the extra 100µl from well A11. Do not add cAMP solution to the no-cAMP control reactions in well A12.

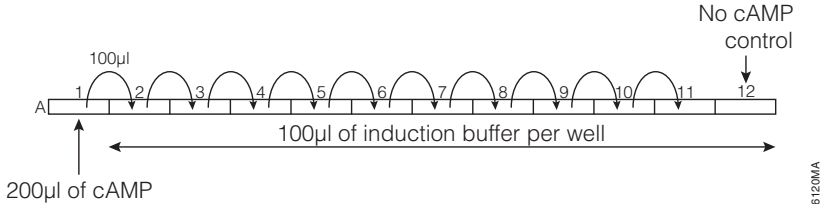

Figure 5. Dilution scheme for preparation of cAMP standards.

	96-well	384-well	low-volume 384-well or 1536-well
4. Transfer the indicated volume of each cAMP standard from the 96-well plate to wells reserved for the cAMP standard curve. Proceed immediately to the assay protocol (Section 5.A).	20µl	7.5µl	1.0µl

5. cAMP-Glo™ Assay Protocols

5.A. Performing the cAMP-Glo™ Assay

Prior to performing the cAMP-Glo™ Assay, prepare the cells, reagents and cAMP standards as described in Sections 3 and 4. This example protocol describes the use of adherent cells; to use frozen or suspension cells, add the 2X agonist to cells resuspended in induction buffer. Incubate the cells with the appropriate ligand for the desired time. Calculate the volume of cAMP-Glo™ Lysis Buffer, cAMP-Glo™ Reaction Buffer and Kinase-Glo® Reagent required for your experiments, and allow that volume to reach room temperature before use. Return the remaining cAMP-Glo™ Lysis Buffer, cAMP-Glo™ Reaction Buffer and Kinase-Glo® Reagent to a –20°C freezer.

	96-well	384-well	low-volume 384-well or 1536-well
1. Add cAMP-Glo™ Lysis Buffer to all wells, including the cAMP standards. Incubate the plate with shaking at room temperature for 15 minutes.	20µl	7.5µl	1.0µl

Note: We recommend viewing the cells with a microscope to be sure that cells have lysed. If cells have not lysed after the 15-minute incubation, allow the cells to incubate at room temperature for up to 30 minutes. Cell lysis should be complete after 30 minutes.

2. For your experiments, calculate the required volume of cAMP Detection Solution. Prepare the cAMP Detection Solution as described below, increasing or decreasing the volumes of Protein Kinase A and cAMP-Glo™ Reaction Buffer appropriately. Mix by inversion; do not vortex.

   96-well plates: Add 2.5µl of Protein Kinase A to 1.0ml of cAMP-Glo™ Reaction Buffer.

   384-well plates: Add 3.5µl of Protein Kinase A to 1.0ml of cAMP-Glo™ Reaction Buffer.

   low-volume, 384-well and 1536-well plates: Add 5.0µl of Protein Kinase A to 1.0ml of cAMP-Glo™ Reaction Buffer.

   Notes:

   1. Add Protein Kinase A to cAMP-Glo™ Reaction Buffer immediately before use, then return the Protein Kinase A to the –20°C freezer. Prepare only the volume of cAMP Detection Solution needed for the experiment; once prepared, the cAMP Detection Solution should not be frozen.
   2. The volume of Protein Kinase A added to prepare the cAMP Detection Solution varies, depending on the plate used to perform the cAMP-Glo™ Assay. **Be sure to prepare the cAMP Detection Solution using the recommendation for the appropriate plate.**

	96-well	384-well	low-volume 384-well or 1536-well
3. Add the indicated volume of cAMP-Glo™ Detection Solution to all wells, and mix the plate by shaking for 30–60 seconds. Incubate the plate at room temperature for 20 minutes.	40µl	15µl	2.0µl
4. Add the indicated volume of room-temperature Kinase-Glo® Reagent to all wells. Mix the plate by shaking for 30–60 seconds, and incubate at room temperature for 10 minutes.	80µl	30µl	4.0µl

5. Measure the luminescence with a plate-reading luminometer.

5.B. Determining EC_{50} Values

We developed this protocol to determine the EC_{50} value for forskolin using the cAMP-Glo™ Assay. This example protocol can be adapted to determine the EC_{50} value of any agonist in cells that express the target receptor for that agonist. Resuspend the agonist in induction buffer (Section 3). This protocol describes the use of suspension cells; to use adherent cells, add the 1X agonist directly to the cells adhered to the plate. Guidelines for preparing cells and the appropriate volume of 1X test compound for adherent cells or 2X test compound for suspension and frozen cells are given in Section 3.

Representative data showing titration with forskolin are shown in Figure 6. The EC_{50} value for forskolin, 4.5µM, is similar to EC_{50} values published in the literature (3,4).

Prior to performing the cAMP-Glo™ Assay, prepare the cells, reagents and cAMP standards as described in Sections 3 and 4. Incubate the cells with the appropriate ligand for the desired time. Allow the cAMP-Glo™ Reaction Buffer to reach room temperature before use. Calculate the volume of cAMP-Glo™ Lysis Buffer and Kinase-Glo® Reagent required for your experiments, and allow that volume of cAMP-Glo™ Lysis Buffer and Kinase-Glo® Reagent to reach room temperature before use. Return the remaining cAMP-Glo™ Lysis Buffer and Kinase-Glo® Reagent to a –20°C freezer.

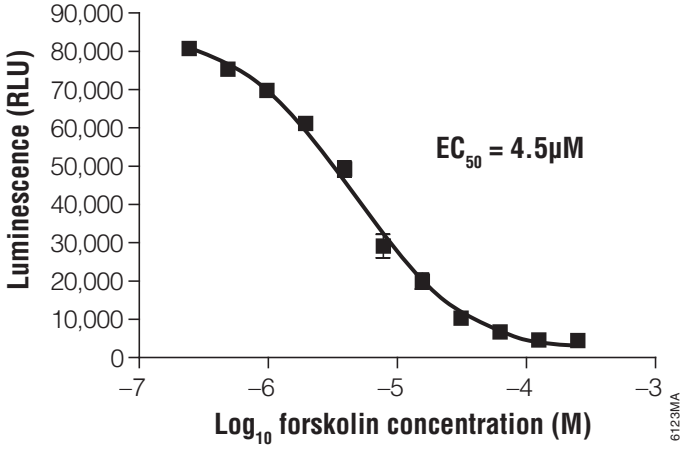

Figure 6. Example titration of forskolin using the suspension cell line D293. In a white, clear-bottom, 384-well plate, 5,000 D293 cells were exposed to the indicated concentration of forskolin. The cAMP-Glo™ Assay protocol was performed as described in Section 5.B. Each point represents eight data points; the error bars represent the standard deviation. Data analysis was performed with GraphPad Prism® software,version 4.02, for Windows® using a sigmoidal dose-response (variable slope) equation.

Preparation of Test Compound

When preparing the test compound, use a stock solution at an appropriate concentration in the proper solvent.

1. Prepare a 2X forskolin stock solution (500µM) in induction buffer; mix well.

   Note: The final concentration of forskolin is 250µM in wells of column 1 of the assay plate. For other agonists, we recommend a starting concentration of 250µM, but the test compound concentration can be adjusted by the users, depending upon the potency of ligand.

2. Add 50µl of induction buffer to wells A2 through A12 of a V-bottom, 96-well plate.

3. Add 100µl of the 2X forskolin stock solution prepared in Step 1 to well A1.

4. Perform a serial twofold dilution of forskolin by transferring 50µl from well A1 to well A2 with a pipette, pipetting to mix. Transfer 50µl to well A3; mix. Repeat for wells A4 through A11. See Figure 7. Discard the extra 50µl from well A11. Do not add forskolin to the no-forskolin control reactions in well A12.

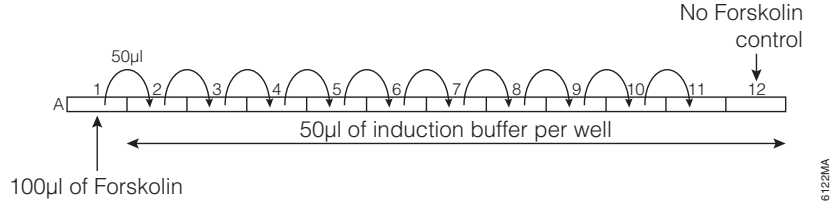

Figure 7. Dilution scheme for the agonist forskolin.

Assay Protocol

	96-well	384-well	low-volume 384-well or 1536-well
1. Transfer the indicated volume of the various concentrations of forskolin to the assay plate. Add an equal volume of a cell suspension containing the recommended number of cells to the same plate (Section 3). Mix the plate by shaking for 30–60 seconds. Incubate the plate at room temperature for 15 minutes to allow cells and forskolin to interact.	10µl	3.75µl	0.5µl
2. Add the indicated volume of Lysis Buffer to all wells, including the cAMP standards. Incubate the plate with shaking at room temperature for 15 minutes.	20µl	7.5µl	1.0µl

5.B. Determining EC_{50} Values (continued)

Note: We recommend viewing the cells with a microscope to be sure that cells have lysed. If cells have not lysed after the 15-minute incubation, allow the cells to incubate at room temperature for up to 30 minutes. Cell lysis should be complete after 30 minutes.

3. For your experiments, calculate the required volume of cAMP Detection Solution. Prepare the cAMP Detection Solution as described below, increasing or decreasing the volumes of Protein Kinase A and cAMP-Glo™ Reaction Buffer appropriately. Mix by inversion; do not vortex.

   96-well plates: Add 2.5µl of Protein Kinase A to 1.0ml of cAMP-Glo™ Reaction Buffer.

   384-well plates: Add 3.5µl of Protein Kinase A to 1.0ml of cAMP-Glo™ Reaction Buffer.

   low-volume, 384-well and 1536-well plates: Add 5.0µl of Protein Kinase A to 1.0ml of cAMP-Glo™ Reaction Buffer.

   Notes:

   1. Add Protein Kinase A to the cAMP-Glo™ Reaction Buffer immediately before use, then return the Protein Kinase A to the –20°C freezer. Prepare only the volume of cAMP Detection Solution needed for the experiment because, once prepared, the cAMP Detection Solution should not be frozen.
   2. The volume of Protein Kinase A added to prepare the cAMP Detection Solution varies, depending on the plate used to perform the cAMP-Glo™ Assay. **Be sure to prepare the cAMP Detection Solution using the recommendation for the appropriate plate.**

	96-well	384-well	low-volume 384-well or 1536-well
4. Add the indicated volume of cAMP Detection Solution to all wells, and mix by shaking for 30–60 seconds. Incubate the plate at room temperature for 20 minutes.	40µl	15µl	2.0µl
5. Add the indicated volume of room-temperature Kinase-Glo® Reagent to all wells. Mix plate by shaking for 30–60 seconds, and incubate the plate at room temperature for 10 minutes.	80µl	30µl	4.0µl

6. Measure luminescence in each well using a plate-reading luminometer.

5.C. Determining IC_{50} Values

We developed this protocol to determine the IC_{50} value of SCH23390, a dopamine D1 receptor antagonist, in stably transfected HEK293 cells expressing the dopamine D1 receptor. Experiments were performed with 5,000 D1 receptor-expressing HEK293 cells resuspended in induction buffer per well. The antagonist SCH23390 was titrated in the presence of 100nM of dopamine D1 receptor-specific agonist, SKF38393 (100nM is the EC_{80} concentration). Alprenolol, a b2 adrenergic receptor antagonist, was used as a negative control. This protocol can be adapted to determine the IC_{50} value of antagonists in cells that express the appropriate target receptor. This example protocol describes the use of adherent cells; to use frozen or suspension cells, add the 2X agonist to cells resuspended in induction buffer. Guidelines for preparing cells are given in Section 3. The IC_{50} value determined for SCH23390 was 9nM, which is similar to the published IC_{50} value (5). Representative results using HEK293 cells as suspension cells are shown in Figure 8.

Prior to performing the cAMP-Glo™ Assay, prepare the reagents as described in Section 4. Allow the cAMP-Glo™ Reaction Buffer to reach room temperature before use. Calculate the volume of cAMP-Glo™ Lysis Buffer and Kinase-Glo® Reagent required for your experiments, and allow that volume of cAMP-Glo™ Lysis Buffer and Kinase-Glo® Reagent to reach room temperature before use. Return the remaining cAMP-Glo™ Lysis Buffer and Kinase-Glo® Reagent to a –20°C freezer.

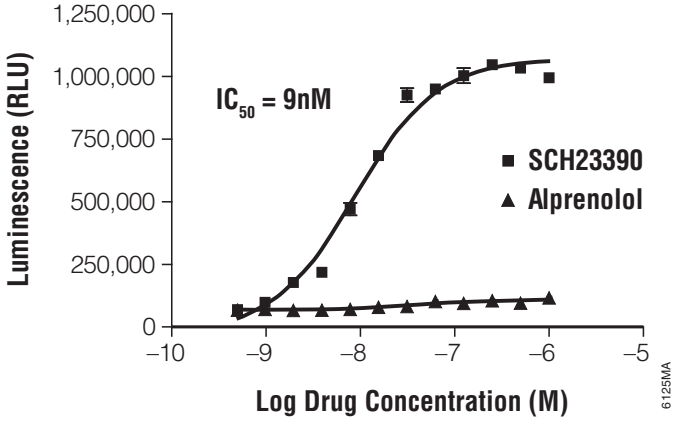

Figure 8. Determining the IC_{50} value of SCH23390. D1 receptor-expressing HEK293 cells were suspended in induction buffer, and 5,000 cells were added to each well of a white, clear-bottom, 96-well plate. Cells were treated with the indicated amount of antagonist SCH23390 in the presence of 100nM agonist SKF38393. In the negative control reactions, SCH23390 was replaced with alprenolol. The cAMP-Glo™ Assay was performed as described in Section 5.C. Each point represents three data points; the error bars represent the standard deviation. Data analysis was performed with GraphPad Prism® software, version 4.02, for Windows® using a sigmoidal dose-response (variable slope) equation.

5.C. Determining IC_{50} Values (continued)

Preparation of Test Compound

When preparing the test compound, use a stock solution at an appropriate concentration in the proper solvent.

1. Prepare induction buffer containing a final concentration of 100nM of SKF38393.
2. Prepare a 1X (1µM) solution of SCH23390 in induction buffer with 100nM SKF38393, and mix well.
3. Add 50µl of induction buffer with 100nM SKF38393 to wells A2 through A12 of a V-bottom, 96-well plate.
4. Add 100µl of 1µM SCH23390 solution to well A1.
5. Prepare a twofold dilution of antagonist SCH23390 by transferring 50µl from well A1 to well A2 with a pipette, pipetting to mix. Transfer 50µl to well A3. Repeat for wells A4 through A11. See Figure 9. Discard the extra 50µl from well A11. Do not add SCH23390 to the no-antagonist control reactions in well A12.

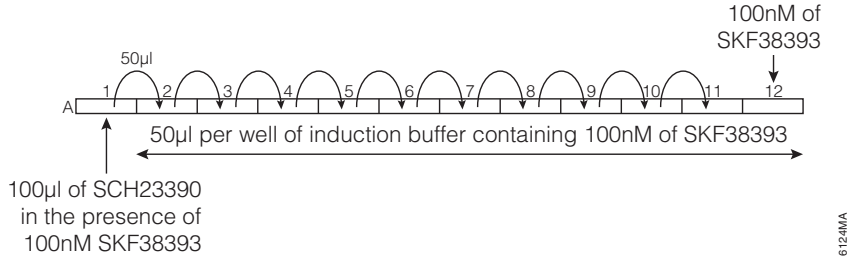

Figure 9. Dilution scheme for the antagonist SCH23390.

Assay Protocol

	96-well	384-well	low-volume 384-well or 1536-well
1. Transfer the indicated volume of the various concentrations of the antagonist SCH23390 to the assay plate containing adherent cells prepared as described in Section 3. Mix the plate by shaking for 30–60 seconds. Incubate the cells with the antagonist for 20 minutes at room temperature.	20µl	7.5µl	1.0µl
2. Add the indicated volume of cAMP-Glo™ Lysis Buffer to all wells, including the cAMP standards. Incubate the plate with shaking at room temperature for 15 minutes.	20µl	7.5µl	1.0µl

Note: We recommend viewing the cells with a microscope to be sure that cells have lysed. If cells have not lysed after the 15-minute incubation, allow the cells to incubate at room temperature for up to 30 minutes. Cell lysis should be complete after 30 minutes.

3. For your experiments, calculate the required volume of cAMP Detection Solution. Prepare the cAMP Detection Solution as described below, increasing or decreasing the volumes of Protein Kinase A and cAMP-Glo™ Reaction Buffer appropriately. Mix by inversion; do not vortex.

   96-well plates: Add 2.5µl of Protein Kinase A to 1.0ml of cAMP-Glo™ Reaction Buffer.

   384-well plates: Add 3.5µl of Protein Kinase A to 1.0ml of cAMP-Glo™ Reaction Buffer.

   low-volume, 384-well and 1536-well plates: Add 5.0µl of Protein Kinase A to 1.0ml of cAMP-Glo™ Reaction Buffer.

   Notes:

   1. Add Protein Kinase A to the cAMP-Glo™ Reaction Buffer immediately before use, then return the Protein Kinase A to the –20°C freezer. Prepare only the volume of cAMP Detection Solution needed for the experiment because, once prepared, the cAMP Detection Solution should not be frozen.
   2. The volume of Protein Kinase A added to prepare the cAMP Detection Solution varies, depending on the plate used to perform the cAMP-Glo™ Assay. **Be sure to prepare the cAMP Detection Solution using the recommendation for the appropriate plate.**

	96-well	**384-well**	**low-volume 384-well or 1536-well**
4. Add the indicated volume of cAMP Detection Solution to all wells, and mix by shaking for 30–60 seconds. Incubate the plate at room temperature for 20 minutes.	40µl	15.0µl	2.0µl
5. Add the indicated volume of room-temperature Kinase-Glo® Reagent to all wells. Mix by shaking for 30–60 seconds. Incubate the plate at room temperature for 10 minutes.	80µl	30µl	4.0µl

6. Measure luminescence in each well using a plate-reading luminometer.

5.D. Determining Z´ Factor

This protocol allows you to determine the Z´ factor (5) for the cAMP-Glo™ Assay in a cell-free system. This protocol also can be used for cell-based systems, using the number of cells recommended in Section 3. Induce the cells with the agonist of interest.

Representative data using cAMP are shown in Figure 10.

Prior to performing the cAMP-Glo™ Assay, allow the cAMP-Glo™ Reaction Buffer to reach room temperature. Calculate the volume of cAMP-Glo™ Lysis Buffer and Kinase-Glo® Reagent required for your experiments, and allow that volume of cAMP-Glo™ Lysis Buffer and Kinase-Glo® Reagent to reach room temperature before use. Return the remaining cAMP-Glo™ Lysis Buffer and Kinase-Glo® Reagent to a –20°C freezer.

Reagent Preparation

All components except Protein Kinase A should be completely thawed at room temperature before use. Keep Protein Kinase A on ice. For your experiments, calculate the required volumes of each reagent, and increase or decrease the volumes appropriately.

1. Prepare the 100nM cAMP solution as follows. Vortex to mix.

Component	Volume for a 96-Well Plate	Volume for a 384-Well Plate	Volume for a Low-Volume 384-Well or 1536-Well Plate
Induction Buffer	1,000µl	1,600µl	500µl
cAMP, 1mM	0.4µl	0.64µl	0.2µl

2. Transfer the entire volume of Kinase-Glo® Buffer into the amber bottle containing the Kinase-Glo® Substrate to reconstitute the lyophilized substrate. This forms the Kinase-Glo® Reagent. Mix by gently vortexing, swirling or inverting the contents to obtain a homogeneous solution. The Kinase Glo® Substrate should go into solution easily, in less than 1 minute.

   Note: The Kinase-Glo® Reagent should be used on the same day that it is prepared or dispensed into single-use aliquots and stored at –20°C.

Assay Protocol

	96-well	384-well	low-volume 384-well or 1536-well
1. Prepare the no-cAMP reactions by adding the indicated volume of induction buffer to each well in half of the plate.	20µl	7.5µl	1.0µl

	96-well	384-well	low-volume 384-well or 1536-well
2. Prepare the cAMP reactions by adding the indicated volume of 100nM cAMP solution to wells in the other half of the plate.	20µl	7.5µl	1.0µl
3. Add the indicated volume of cAMP-Glo™ Lysis Buffer to all wells, including the no-cAMP reactions. Incubate the plate with shaking at room temperature for 15 minutes.	20µl	7.5µl	1.0µl

4. For your experiments, calculate the required volume of cAMP Detection Solution. Prepare the cAMP Detection Solution as described below, increasing or decreasing the volumes of Protein Kinase A and cAMP-Glo™ Reaction Buffer appropriately. Mix by inversion; do not vortex.

   96-well plates: Add 2.5µl of Protein Kinase A to 1.0ml of cAMP-Glo™ Reaction Buffer.

   384-well plates: Add 3.5µl of Protein Kinase A to 1.0ml of cAMP-Glo™ Reaction Buffer.

   low-volume, 384-well and 1536-well plates: Add 5.0µl of Protein Kinase A to 1.0ml of cAMP-Glo™ Reaction Buffer.

   Notes:

   1. Add Protein Kinase A to the cAMP-Glo™ Reaction Buffer immediately before use. Prepare only the volume of cAMP Detection Solution needed for the experiment because, once prepared, the cAMP Detection Solution should not be frozen.
   2. The volume of Protein Kinase A added to prepare the cAMP Detection Solution varies, depending on the plate used to perform the cAMP-Glo™ Assay. **Be sure to prepare the cAMP Detection Solution using the recommendation for the appropriate plate.**

	96-well	384-well	low-volume 384-well or 1536-well
5. Add the indicated volume of cAMP-Glo™ Detection Solution to all wells, and mix plate by shaking for 30–60 seconds. Incubate the plate at room temperature for 20 minutes.	40µl	15µl	2.0µl

5.D. Determining Z´ Factor (continued)

	96-well	384-well	low-volume 384-well or 1536-well
6. Add the indicated volume of room-temperature Kinase-Glo® Reagent to all wells. Mix the plate by shaking for 30–60 seconds, and incubate at room temperature for 10 minutes.	80µl	30µl	4.0µl

7. Measure luminescence with a plate-reading luminometer.

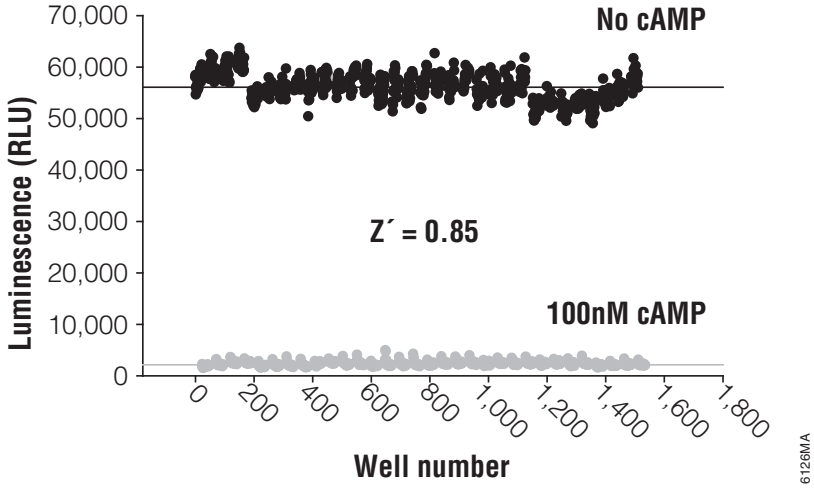

Figure 10. A scatter plot to determine the Z´ factor of the cAMP-Glo™ Assay. The cAMP-Glo™ Assay was performed in a 1536-well plate as described in Section 5.D using the Deerac Fluidics® Equator™ HTS non-contact dispenser. Luminescence was measured using the PHERAstar high-end microplate reader (BMG Labtech). Data analysis was performed using Sigma Plot/Sigma Stat, version 9.0 for Windows®.

The Z´ factor was calculated using the formula:

$$Z' = 1 - \frac{[(3 \times \text{std. dev. of experimental wells}) + (3 \times \text{std. dev. of control wells})]}{(\text{mean of experimental wells}) - (\text{mean of control wells})}$$

6. Troubleshooting

For questions not addressed here, please contact your local Promega branch office or distributor. Contact information available at: **www.promega.com**. E-mail: **techserv@promega.com**

Symptoms	Causes and Comments
High luminescent signal	ATP contamination in one or more reaction components. Use clean laboratory space and pipettes that are free of ATP or other nucleotides. Use aerosol-resistant pipette tips. Decontaminate work surfaces by wiping with detergent solution or ethanol and rinsing with clean water. Rinse pipettes and other labware with distilled water at least three times. For automated dispensing systems, replace any components that have been used to dispense ATP-containing solutions.
	Protein Kinase A was not added to the cAMP Detection Solution. Be sure to prepare the cAMP Detection Solution as directed.
	Protein Kinase A was inactivated. Store Protein Kinase A at –20°C, and keep on ice while preparing the cAMP Detection Solution.
	Cells are not healthy or do not express a necessary receptor. Be sure the cells express necessary receptors and are from a healthy, subconfluent culture that can produce cAMP.
	Adherent cells were dislodged from the plate during the PBS wash. Grow cells in white, clear-bottom, poly-D-lysine-coated plates to minimize dissociation.
	The test compound inhibits Protein Kinase A. During a screen of the LOPAC library (Sigma), which contains 1,280 compounds, we experienced less than 0.5% false hits, with only two of the compounds being protein kinase A inhibitors.
	To test for Protein Kinase A inhibition, assemble two reactions with cAMP. Add test compound to one reaction but not the other. Compare luminescence from these reactions. An increase in luminescence in the presence of the test compound is an indication of Protein Kinase A inhibition.

6. Troubleshooting (continued)

Symptoms	Causes and Comments
Low luminescent signal	Use only white, clear-bottom plates. Do not use black plates or clear plates.
	Improper storage of the Kinase-Glo® Reagent. Store the Kinase-Glo® Reagent in aliquots at –20°C, and avoid repeated freeze-thaw cycles of this reagent.
	Be sure to add cAMP-Glo™ Lysis Buffer to all wells of the assay plate, including the cAMP standards and no-cAMP reactions.
	The cAMP-Glo™ Lysis Buffer was stored at room temperature for too long. On the day of the assay, equilibrate the required volume of cAMP-Glo™ Lysis Buffer to room temperature, and return the remaining cAMP-Glo™ Lysis Buffer to a –20°C freezer for storage.
	The agonist or antagonist was a cAMP analog.
	The test compound inhibited the luciferase reaction. To identify luciferase inhibitors, perform the cAMP-Glo™ Assay with the test compound, but omit the Protein Kinase A. Compare luminescence to that from a reaction with Protein Kinase A. Low light output in the absence of Protein Kinase A indicates inhibition of luciferase.
Unexpected inhibition of the cAMP-Glo™ Assay by test compound	Luciferase inhibition. To identify luciferase inhibitors, perform the cAMP-Glo™ Assay with the test compound, but omit the Protein Kinase A. Compare luminescence to that from a reaction with Protein Kinase A. Low light output in the absence of Protein Kinase A indicates inhibition of luciferase.
	Protein Kinase A inhibition. During a screen of the LOPAC library (Sigma), which contains 1,280 compounds, we experienced less than 0.5% false hits, with only two of the compounds being Protein Kinase A inhibitors. To test for Protein Kinase A inhibition, assemble two reactions with cAMP. Add test compound to one reaction but not the other. Compare luminescence from these reactions. An increase in luminescence in the presence of the test compound is an indication of Protein Kinase A inhibition.

Symptoms	Causes and Comments
Unexpected inhibition of the cAMP-Glo™ Assay by test compound (continued)	Inhibition by an organic solvent. Minimize the solvent concentration, or use a different solvent to dissolve the test compound. Perform control reactions that contain solvent but no test compound to test the effect of the solvent on assay performance. Performance of the cAMP-Glo™ Assay is not affected by the presence of up to 5% acetone or 5% DMSO.

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8. Appendix

8.A. Composition of Buffers and Solutions

100mM IBMX

Dissolve 22.2mg/ml 3-isobutyl-1-methylxanthine (IBMX) in 100% DMSO.

induction buffer

Krebs Ringer buffer, serum-free medium or 1X PBS containing 100µM Ro 20-1724 [4-(3-butoxy-4-methoxy-benzyl) imidazolidone] and 500µM IBMX (3-isobutyl-1-methylxanthine).

Krebs Ringer buffer (pH 7.5)

1.8g	D-glucose
0.047g	$MgCl_2$
0.34g	KCl
7.0g	NaCl
0.1g	Na_2HPO_4
0.18g	NaH_2PO_4
1.26g	$NaHCO_3$

Dissolve in 900ml of deionized water. Adjust the pH to 7.5. Add deionized water to a final volume of 1 liter. Filter-sterilize.

PBS buffer, 10X

11.5g	Na_2HPO_4
2g	KH_2PO_4
80g	NaCl
2g	KCl

Dissolve in 1 liter of sterile, deionized water. The pH of 1X PBS will be 7.4.

100mM Ro 20-1724

Dissolve 27.84mg/ml [4-(3-butoxy-4-methoxy-benzyl) imidazolidone] (Ro 20-1724) in 100% DMSO.

8.B. Related Products

Product	Size	Cat.#
Kinase-Glo® Luminescent Kinase Assay*	10ml	V6711
Kinase-Glo® Plus Luminescent Kinase Assay*	10ml	V3771
Kinase-Glo® Max Luminescent Kinase Assay*	10ml	V6071
GloMax® 96 Microplate Luminometer	each	E6501
GloMax® 96 Microplate Luminometer w/Single Injector	each	E6511
GloMax® 96 Microplate Luminometer w/Dual Injectors	each	E6521

*Additional sizes available.

[a]Patent Pending.

[b]U.S. Pat. Nos. 7,083,911, 7,452,663 and 7,732,128, European Pat. No. 1383914 and Japanese Pat. Nos. 4125600 and 4275715.

[c]U.S. Pat. No. 7,700,310, European Pat. No. 1546374 and other patents pending.

[d]U.S. Pat. No. 7,741,067, Japanese Pat. No. 4485470 and other patents pending.

[e]U.S. Pat. Nos. 6,602,677, 7,241,584 and 8,030,017, European Pat. No. 1131441, Japanese Pat. Nos. 4537573 and 4520084 and other patents 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.

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