TECHNICAL MANUAL

cAMP-Glo™ Max Assay

Instructions for use of Products
V1681, V1682 and V1683

Revised 11/13
TM347

Promega

cAMP-Glo™ Max Assay

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1. Description

The cAMP-Glo™ Max Assay[a–f] is a homogeneous, bioluminescent and high-throughput assay to measure cyclic AMP (cAMP) levels in cells. Compounds that modulate G protein-coupled receptors (GPCRs) coupled with adenylate cyclase typically alter intracellular cAMP levels. The cAMP-Glo™ Max Assay monitors cAMP levels in cells in response to the effect of agonists, antagonists or test compounds on GPCRs.

The cAMP-Glo™ Max Assay is based on the principle that cyclic AMP (cAMP) stimulates protein kinase A (PKA) holoenzyme activity; this stimulation depletes ATP in the reaction resulting in decreased light production in a coupled luciferase reaction (Figure 1).

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Printed in USA. Part# TM347
Revised 11/13

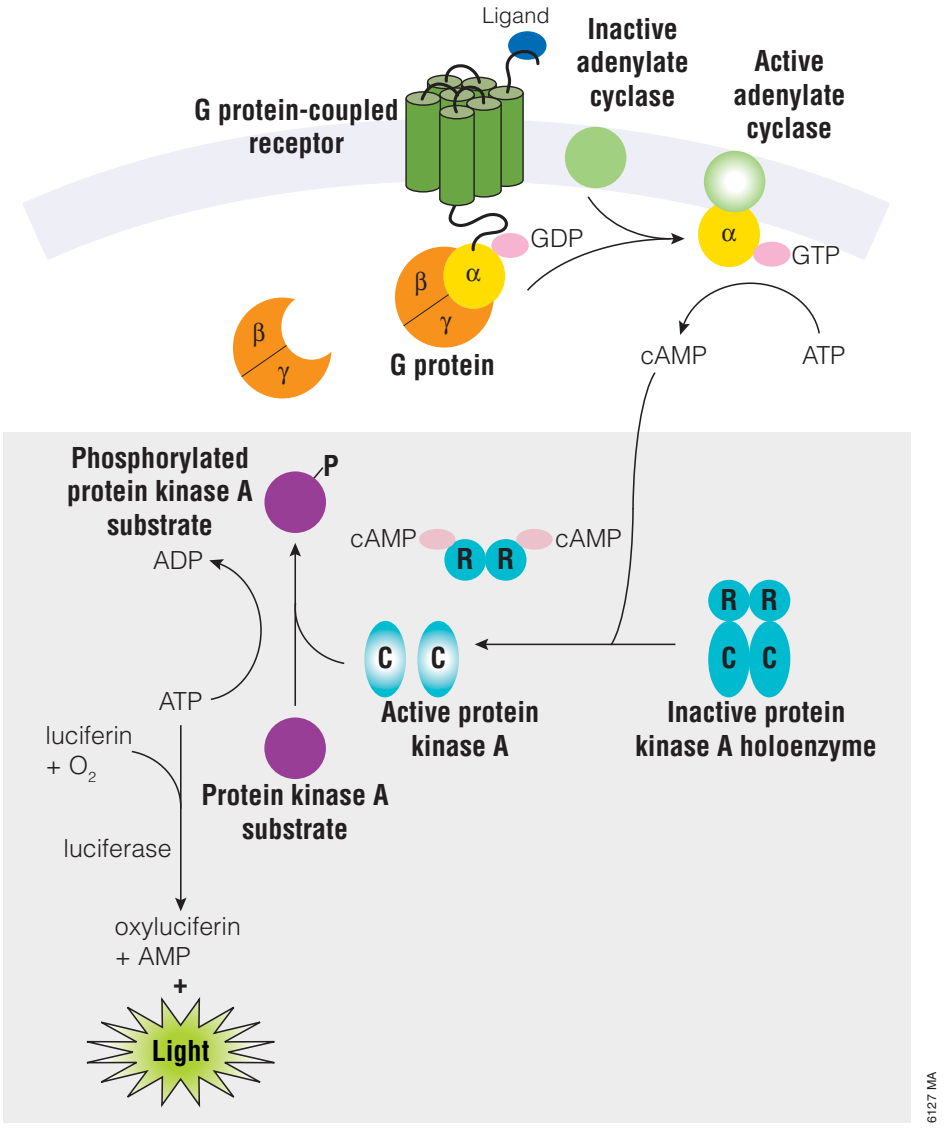

Figure 1. Schematic diagram of cAMP production in cells and the cAMP-Glo™ Max Assay. Binding of an extracellular ligand to its receptor alters the conformation of the associated heterotrimeric G protein, causing dissociation of the G_{α} and $G_{\beta\gamma}$ subunits and initiating a cascade of cellular 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™ Max 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.

cAMP-dependent Protein Kinase (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 regulatory subunits, which then undergo a conformational change to release the active catalytic subunits. The free catalytic subunits 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™ Max Assay can be performed in multiple plate formats including 96-, 384- and 1536-well plates. The cells are induced for an appropriate period of time to modulate cAMP levels. After induction the cells are lysed and cAMP release is detected by the addition of cAMP Detection Solution, which contains PKA. 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.

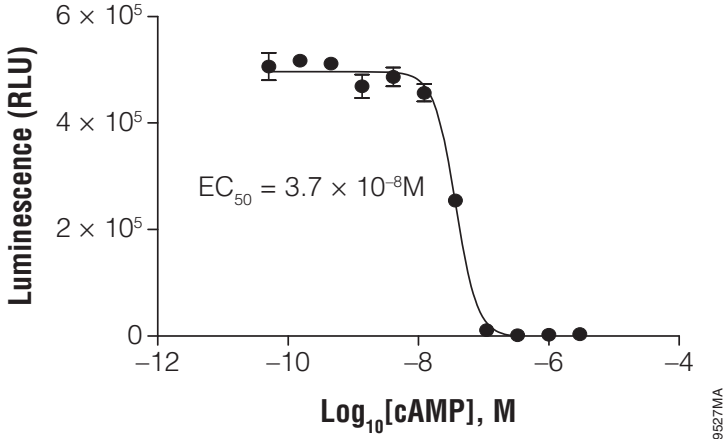

Figure 2. Titration of cAMP. Reactions were assembled with the indicated concentrations of cAMP in a low-volume 384-well plate. The cAMP-Glo™ Max Assay was performed as described in Section 5. Data were collected using a plate reading luminometer (Infinite® F500, TECAN). Each data point is the mean of eight replicates; the error bars represent the standard deviation. Data analysis was performed with GraphPad Prism® software, version 5.00.2, for Windows® using a sigmoidal dose-response (variable slope) equation.

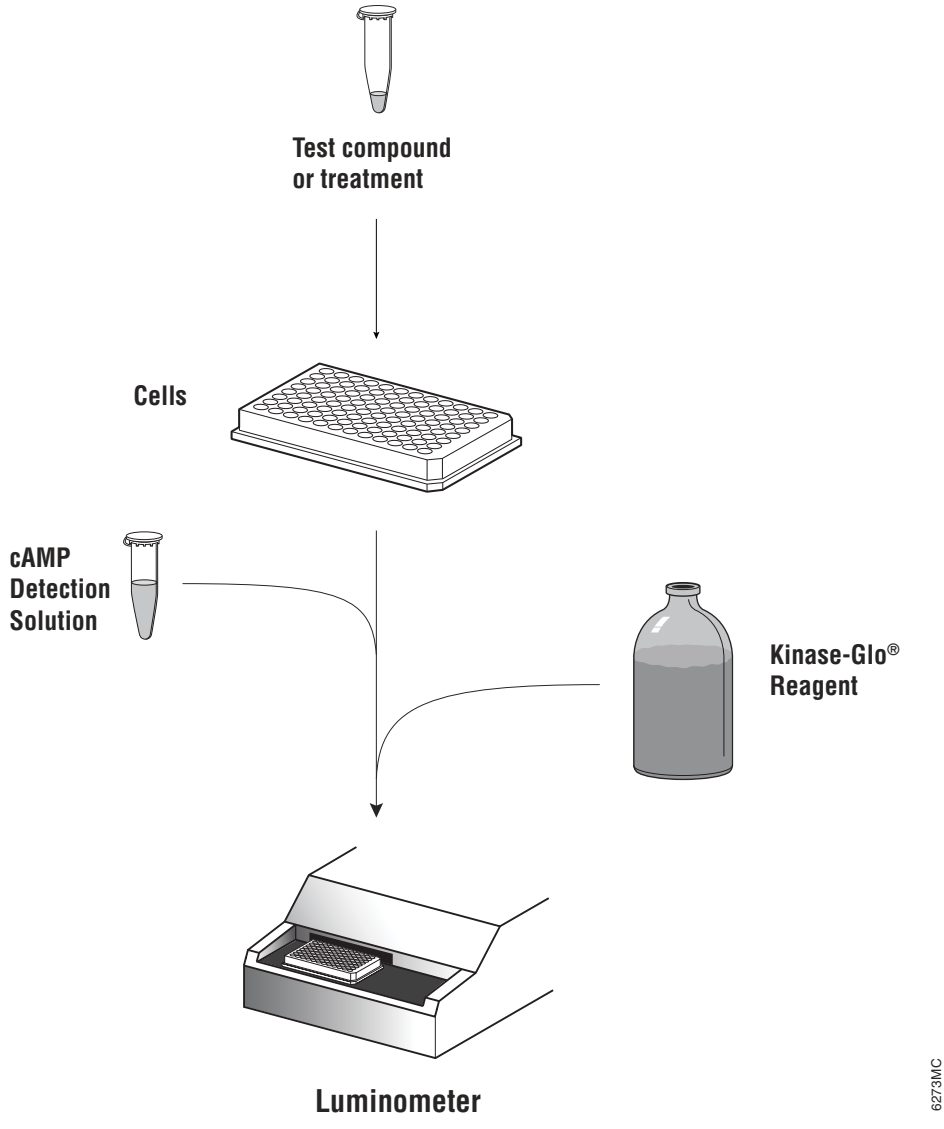

Figure 3. Schematic diagram of reagent preparation and protocol.

2. Product Components and Storage Conditions

Product	Size	Cat.#
cAMP-Glo™ Max Assay	2 plates	V1681

Each system contains sufficient reagents for 2 plates (96- or 384-well formats). Includes:

- 100µl cAMP, 1mM
- 1ml $MgCl_2$, 1M
- 2.5ml cAMP-Glo™ ONE-Buffer
- 20µl Protein Kinase A
- 1 bottle Kinase-Glo® Substrate (lyophilized)
- 10ml Kinase-Glo® Buffer

Product	Size	Cat.#
cAMP-Glo™ Max Assay	20 plates	V1682

Each system contains two packages, the cAMP-Glo™ Max Assay (Cat.# V1684) and the Kinase-Glo® Luminescent Kinase Assay (Cat.# V6713) and contains sufficient reagents for 20 plates (96- or 384-well formats). Includes:

- 500µl cAMP, 1mM
- 5ml $MgCl_2$, 1M
- 20ml cAMP-Glo™ ONE-Buffer
- 200µl Protein Kinase A
- 1 bottle Kinase-Glo® Substrate (lyophilized)
- 100ml Kinase-Glo® Buffer

Product	Size	Cat.#
cAMP-Glo™ Max Assay	10 × 20 plates	V1683

Each system contains two packages, the cAMP-Glo™ Max Assay (Cat.# V1685) and the Kinase-Glo® Luminescent Kinase Assay (Cat.# V6714) and contains sufficient reagents for 10 × 20 plates (96- or 384-well formats). Includes:

- 6 × 500µl cAMP, 1mM
- 4 × 5ml $MgCl_2$, 1M
- 10 × 20ml cAMP-Glo™ ONE Buffer
- 10 × 200µl 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. After thawing, mix all components thoroughly before use. Once prepared, the cAMP Detection Solution (cAMP-Glo™ ONE-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. Preparing Cells Prior to the Assay

The cAMP-Glo™ Max Assay can be performed with adherent, suspension or frozen cells using the following recommendations. Adherent cells are incubated overnight to allow the cells to attach to the growth matrix prior to the cAMP-Glo™ Max 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 (such as cell number per well) that result in the maximum effect on cAMP levels.

The cAMP-Glo™ Max Assay involves treating cells with an agonist or test compound to modulate cAMP levels. Treat the cells with agonist/antagonist or test compound in **Complete Induction Buffer** for an appropriate period of time, depending upon the nature of ligand and receptor. Performance of the cAMP-Glo™ Max Assay is not affected by the presence of up to 5% acetone or 5% DMSO, two commonly used vehicles for chemical compounds.

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

Be sure to add $MgCl_2$ to the Induction Buffer so that the final concentration after the addition of cAMP Detection Solution is ≥20mM of $MgCl_2$.

Adherent Cells

To prepare adherent cells, grow cells in flasks or culture dishes and wash with 1X PBS. Trypsinize cells to detach from the flask or dish, and transfer the cell-containing culture medium to a 15ml or 50ml conical centrifuge tube. Centrifuge the tube at 1,500 × *g* for 10 minutes (centrifugation speed and time may depend on the type of cells used). Resuspend the cell pellet in medium, and count the number of cells using a hemacytometer. Dispense a 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 **Complete 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.

Plate Type	Cell Density (cells/well)	Volume of Cells per Well	Volume of 1X Test Compound per Well
96-well	2,500–10,000	50–100µl	40µl
384-well	2,000–5,000	20–50µl	8.0µl

Suspension Cells

To prepare suspension cells for the cAMP-Glo™ Max Assay, grow cells in flasks or dishes. Add cell dissociation buffer (enzyme-free) or trypsin to detach cells (add enough medium with serum to stop trypsin action if using trypsin). Transfer the cell-containing culture medium to either a 15ml or 50ml conical centrifuge tube and centrifuge at 1,500 × *g* for 10 minutes at 22–25°C. Resuspend the cell pellet in **Induction Buffer (without $MgCl_2$/IBMX/Ro)**, and count the number of cells using a hemacytometer. Dispense an appropriate volume containing the recommended number of cells (Table 2) into each well of the assay plate. Add a test compound of interest in **Complete Induction Buffer (with $MgCl_2$/IBMX/Ro; Section 4)** to the cells to initiate induction. The volume of test compound added per well is shown in Table 2. Proceed to Section 4.

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

Plate Type	Cell Density (cells/well)	Volume of Cells per Well	Volume of 2X Test Compound per Well
96-well	5,000–20,000	20µl	20µl
384-well	1,000–10,000	4.0µl	4.0µl
low-volume 384-well or 1,536-well	500–2,000	2.0µl (or less)	2.0µl (or less; make equal to volume of cells per well)

Note: This is a guideline for the volume of cells and test compound. The volume of cells and test compound can be changed. However, the final volume shown in Table 1 is the guideline in volume for the cAMP-Glo™ Max Assay.

3. Preparing Cells Prior to the Assay (continued)

Frozen Cells

To perform the cAMP-Glo™ Max Assay, thaw the cells and centrifuge 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 **Complete 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.

Plate Type	Cell Density (cells/well)	Volume of Cells per Well	Volume of 2X Test Compound per Well
96-well	5,000–20,000	20µl	20µl
384-well	1,000–10,000	4.0µl	4.0µl
low-volume 384-well or 1,536-well	500–2,000	2.0µl (or less)	2.0µl (or less; make equal to volume of cells per well)

4. Treating Cells Prior to the Assay

Prior to performing the cAMP-Glo™ Max Assay, treat cells with an agonist/antagonist or test compound in **Complete Induction Buffer** to modulate cAMP levels (Section 3). Ensure that the magnesium concentration is sufficient to make 20mM after the addition of cAMP Detection Reagent. Be sure to prepare a cAMP standard curve for each set of experiments (Section 4.C).

Materials to Be Supplied by the User

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

- IBMX: isobutyl-1-methylxanthine (Sigma-Aldrich Cat.# I7018), 100mM in 100% DMSO. Store at –20°C.
- Ro 20-1724 [4-(3-butoxy-4-methoxy-benzyl) imidazolidone] (Sigma Aldrich Cat.# B8279), 100mM in 100% DMSO. Store at –20°C.
- induction buffer [Krebs Ringer buffer **or** 1X phosphate-buffered saline (PBS) **or** serum-free medium.]
- 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
- **test compounds of interest** prepared in **Complete Induction Buffer**
- **luminometer** with instrumentation that includes proper program

4.A. Important Treatment and Volume Guidelines for the cAMP-Glo™ Max Assay

The cAMP-Glo™ Max Assay provides cAMP-Glo™ ONE-Buffer for flexibility in assay volume between cells and test compounds. Volume sizes between Step 1 and Step 2 can be changed as long as the final concentration of the cAMP Detection Solution is 1X. The final step, addition of Kinase-Glo® Reagent, should be in a 1:1 ratio to the total volume from Steps 1 and 2. In other words, the volume of Kinase-Glo® Reagent added should equal the volume from Step 1 + Step 2.

Here is a sample procedure, using a 4:1 ratio of cells plus treatment or test compound volume to cAMP Detection Solution volume.

1. For a 384-well plate, add 8µl of cells plus treatment per well.
2. Add 2µl of cAMP Detection Solution. Incubate for 20 minutes at room temperature (22°C).
3. Add 10µl of Kinase-Glo® Reagent. Incubate 10 minutes at 22°C.

! For Step 3, temperatures higher or lower than 22°C will not give consistent results with Kinase-Glo® Reagent.

Note: Other ratios of cells and treatment to cAMP Detection Solution can be used. See Table 4 for additional ratios and volume recommendations for other plate sizes.

Table 4. General Assay Volume Guidelines for the cAMP-Glo™ Max Assay.

Step	Description	96-Well			384-Well			Low-Volume 384-Well or 1536-Well		
	Step1:Step 2	**4:1**	**3:2**	**1:1**	**4:1**	**3:2**	**1:1**	**4:1**	**3:2**	**1:1**
1	(Agonist/Antagonist) + Cells/cAMP	40µl	30µl	25µl	8µl	6µl	5µl	4µl	3µl	2.5µl
2	cAMP Detection Solution (**Concentration)**	10µl (**5X**)	20µl (**2.5X**)	25µl (**2X**)	2µl (**5X**)	4µl (**2.5X**)	5µl (**2X**)	1µl (**5X**)	2µl (**2.5X**)	2.5µl (**2X**)
3	Kinase-Glo® Reagent (add volume equal to volume of Step 1 + Step 2)	50µl			10µl			5µl		

4.B. Reagent Preparation

All components except Protein Kinase A should be thawed completely 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 shown here, appropriately. In this example we prepare 1ml of solution at a 4:1 ratio of cell treatment volume to cAMP Detection Solution volume.

Induction Buffer (for cells): Use Krebs Ringer buffer **or** 1X phosphate-buffered saline (PBS) **or** serum-free medium.

Complete Induction Buffer (for test compounds or treatments): First add IBMX to 500µM and Ro 20-1724 to 100µM in Induction Buffer containing cells and test compound. Then add $MgCl_2$ to a final concentration of ≥20mM after addition of the cAMP Detection Solution.

Concentrated cAMP Standard Solution (for standard curve; prepare 1ml):

Dilute the 1mM cAMP solution with NANOpure® or distilled water to make a 0.1mM cAMP solution. Combine 974µl of Induction Buffer (no IBMX or Ro) with 1.25µl of 0.1mM cAMP and 25µl of 1M $MgCl_2$. Vortex to mix.

Note: The final concentration of cAMP solution prepared above is 125nM. When used as described in subsequent sections, the final concentration of the top standard after addition of cAMP Detection Solution is 100nM. 100nM is used in the calculations because it allows direct comparison to the sample concentration.

Note: To use standard curve for determining cAMP produced from cells, we strongly recommend using **Complete Induction Buffer**.

cAMP Detection Solution: Add 10µl of Protein Kinase A per 1ml of cAMP-Glo™ ONE-Buffer to make cAMP Detection Solution.

Notes:

1. Add Protein Kinase A to the cAMP-Glo™ ONE-Buffer immediately before use. Prepare only the volume of cAMP Detection Solution needed for the experiment; once prepared, the cAMP Detection Solution cannot be frozen or stored.
2. The volume of Protein Kinase A added to the cAMP-Glo™ ONE Buffer varies depending on the plate size used for the cAMP-Glo™ Max Assay.

Kinase-Glo® Reagent

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 gently, 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.C. Generating a cAMP-Glo™ Max Standard Curve

The cAMP-Glo™ Max standards can be prepared in 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.

1. Add 100µl of Induction Buffer to wells A2 through A12 of a 96-well plate.
2. Add 200µl of the cAMP Standard Solution prepared in Section 4.B 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 4. Discard the extra 100µl from well A11. Do not add cAMP Standard Solution to the no-cAMP well, A12.

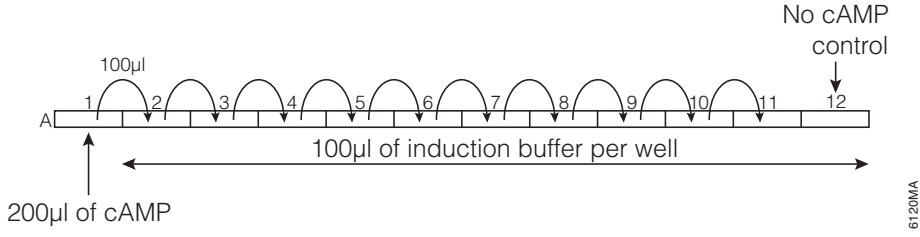

Figure 4. Dilution scheme for preparation of cAMP standards.

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).

96-Well	384-Well	Low-Volume 384- or 1536-Well
40µl	8µl	4µl or less

4.D. Calculating the Standard Curve

The luminescence output of the assay is affected by the number of cells per well, 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), and generate a linear regression graph for analysis using an Excel® program. Figure 5 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 (from Figure 5), calculate the cAMP concentration using the linear formula from the graph.

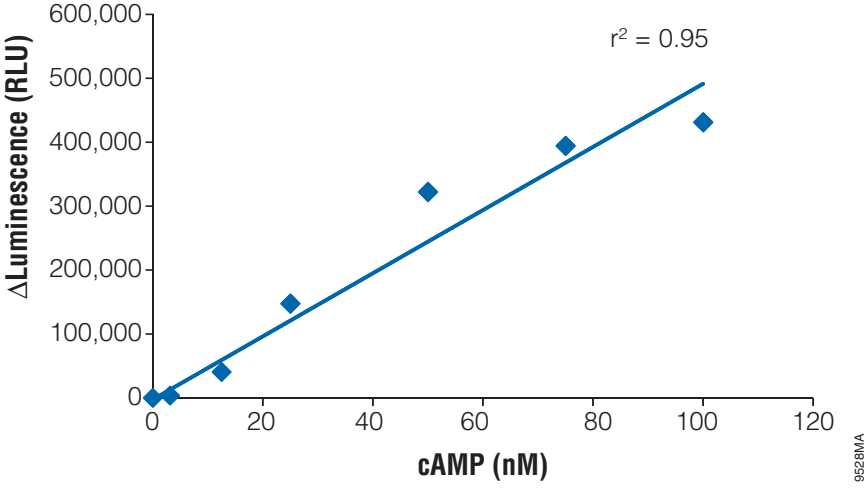

Figure 5. Titration of cAMP. Reactions were assembled with the indicated concentrations of cAMP in a white standard 384-well plate. The cAMP-Glo™ Max Assay was performed as described in Section 5. Data were collected using a plate-reading luminometer (GloMax®-Multi+ Detection System). Each point is the average of four data points. Data analysis was performed using Microsoft Excel® using linear regression.

5. cAMP-Glo™ Max Assay Protocols

5.A. Performing the cAMP-Glo™ Max Assay Using a 4:1 Ratio of Cells and Test Compound to cAMP Detection Solution

Prior to performing the cAMP-Glo™ Max Assay, prepare the cells, the reagents (Sections 3 and 4) and the cAMP standards as described in Section 4.C. Calculate the volume of cAMP-Glo™ ONE-Buffer and Kinase-Glo® Reagent required for your experiments, and allow that volume to reach room temperature before use. Return the remaining cAMP-Glo™ ONE-Buffer and Kinase-Glo® Reagent to –20°C.

1. For each experiment, calculate the required volume of cAMP Detection Solution. Prepare the cAMP Detection Solution as described in Section 4.B., increasing or decreasing the volumes of Protein Kinase A and cAMP-Glo™ ONE-Buffer appropriately. Mix by inversion; **do not vortex**.

2. Add the indicated volume of cAMP Detection Solution to all wells, and mix the plate by shaking for 1–2 minutes. Incubate the plate at room temperature (23°C) for 20 minutes.

96-Well	384-Well	Low-Volume 384-Well or 1536-Well
10µl	2µl	1.0µl or less

3. Add the indicated volume of room-temperature Kinase-Glo® Reagent to all wells. Mix the plate by shaking for 1–2 minutes, and incubate at room temperature for 10 minutes.

96-Well	384-Well	Low-Volume 384-Well or 1536-Well
50µl	10µl	5.0µl

   Note: For low-volume 384- or 1536-well plates, add a volume of testing solution equal to the volume of other reagents:

   Volume of Test Solution = Volume of cells + ligand + cAMP Detection Solution

4. Measure 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™ Max 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. Preparation of the cells in Induction Buffer and the number of cells per well are described in Sections 4.A and 4.B. This EC_{50} protocol is for use with suspension cells; to use adherent cells, add the 1X agonist directly to the cells after discarding the medium. Guidelines for preparing cells and the appropriate volume of test compounds are given in Tables 1–3 of Section 3.

Representative data for titration with forskolin is shown in Figure 6. The EC_{50} value for forskolin, 30nM, is similar to EC_{50} values published in the literature (3,4).

Prior to performing the cAMP-Glo™ Max Assay, prepare the cells, reagents and cAMP standards as described in Sections 3, 4 and 5. Incubate the cells with the appropriate ligand for the desired time. Calculate the volume of cAMP-Glo™ ONE-Buffer and Kinase-Glo® Reagent required for your experiments. Allow that volume of each reagent to reach room temperature before use. Return the remaining the cAMP-Glo™ ONE-Buffer and Kinase-Glo® Reagent to a –20°C freezer.

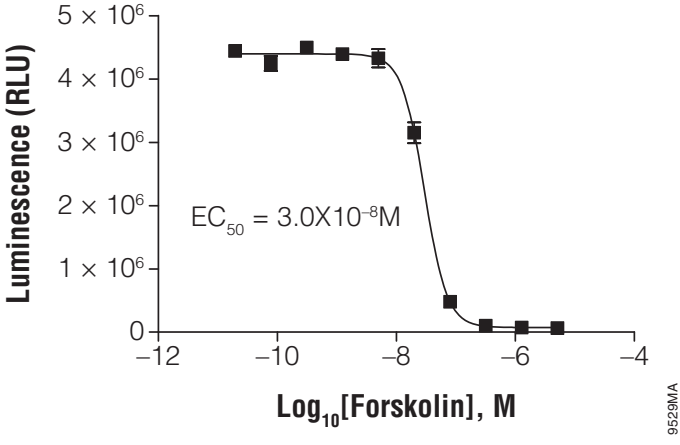

Figure 6. Titration of forskolin using the suspension cell line HEK293. In a white, clear-bottom, 384-well plate, 2,000 HEK293 cells were exposed to the indicated concentration of forskolin. The cAMP-Glo™ Max Assay protocol was performed as described in Section 5.A. Each point represents eight data points; 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. Here is an example using a 4:1 ratio of cells plus test compound to cAMP Detection Solution, described in Section 4.A.

1. Prepare a 2X forskolin stock solution in the **Complete Induction Buffer** (with $MgCl_2$/IBMX/Ro 20-1724, Section 5.A). Mix well.

   Note: The final concentration of forskolin is 30µM in wells with cells in column 1 of the assay plate. For other agonists, we recommend a starting concentration of 2X, but the test compound concentration can be adjusted by the user, depending upon the potency of ligand.

2. Add 100µl of the **Complete Induction Buffer** (with $MgCl_2$/IBMX/Ro 20-1724) to wells A2 through A12 of a 96-well plate.

3. Add 200µl of the 2X forskolin stock solution prepared in Step 1 to well A1.

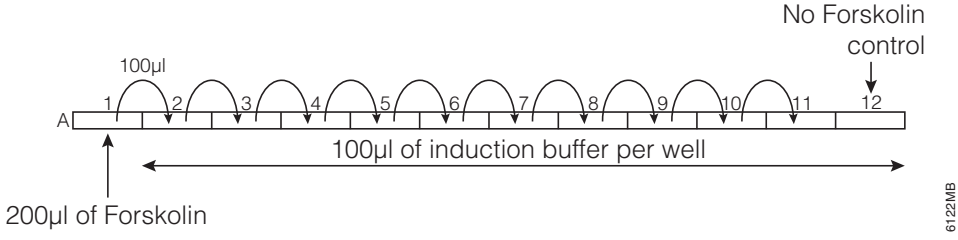

Figure 7. Dilution scheme for forskolin.

4. Perform a serial twofold dilution of forskolin by transferring 100µl from well A1 to well A2 with a pipette, pipetting to mix. Transfer 100µl from well A2 to well A3; mix well. Repeat for wells A4 through A11, Figure 7. Discard the extra 100µl from well A11. Do not add forskolin to the no-forskolin control reactions in well A12.

Assay Protocol (using a 4:1 ratio; see Sections 4.A and 4.B)

1. Transfer the indicated volume of the various concentrations of forskolin to the assay plate. Add an equal volume of the recommended number of suspension cells to the same plate (Section 4.B). Mix the plate by shaking for 1–2 minutes. Incubate the plate at room temperature for 15 minutes to allow cells and forskolin to interact.

96-Well	384-Well	Low-Volume 384-Well or 1536-Well
20µl	4µl	2µl

   Note: This protocol can be adapted to determine the EC_{50} value of any agonist in cells that express the target receptor for that agonist. Optimal incubation time should be determined for other agonists.

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

2. For each experiment, calculate the required volume of cAMP Detection Solution. Prepare the cAMP Detection Solution as described in Section 4.B. Mix by inversion; do not vortex.

3. Add the indicated volume of cAMP Detection Solution to all wells based on Table 1, Section 4.A, and mix the plate by shaking for 1–2 minutes. Incubate the plate at room temperature (23°C) for 20 minutes.

96-Well	384-Well	Low-Volume 384-Well or 1536-Well
10µl	2µl	1.0µl

4. Add the indicated volume of room-temperature Kinase-Glo® Reagent to all wells. Mix the plate by shaking for 1–2 minutes, and incubate at room temperature for 10 minutes.

96-Well	384-Well	Low-Volume 384-Well or 1536-Well
50µl	10µl	5.0µl

   Note: For low-volume 384- or 1536-well plates, add an equal volume of test solution:

   Volume of Test Solution = Volume of cells + ligand + cAMP Detection Solution

5. Measure luminescence with a plate-reading luminometer. Determine EC_{50} either by GraphPad Prism® or a similar software.

5.C. Determining IC_{50} Values

This sample protocol describes the use of suspension cells, with the addition of 2X antagonist to cells resuspended in Induction Buffer. To use adherent cells or frozen cells, see the guidelines for preparing cells and volumes in Section 3.

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 2,000 suspension D1 receptor-expressing HEK293 cells 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 β2 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. The IC_{50} value determined for SCH23390 was 10nM, which is similar to the published IC_{50} value (5). Representative results using DRD1-HEK293 suspension cells are shown in Figure 8.

Prior to performing the cAMP-Glo™ Max Assay, prepare the reagents as described in Section 3. Allow the cAMP-Glo™ ONE-Buffer to reach room temperature before use. Calculate the volume of Kinase-Glo® Reagent required for your experiments, and allow this volume of buffers and Kinase-Glo® Reagent to reach room temperature before use. Return the remaining cAMP-Glo™ ONE-Buffer and Kinase-Glo® Reagent to a –20°C freezer.

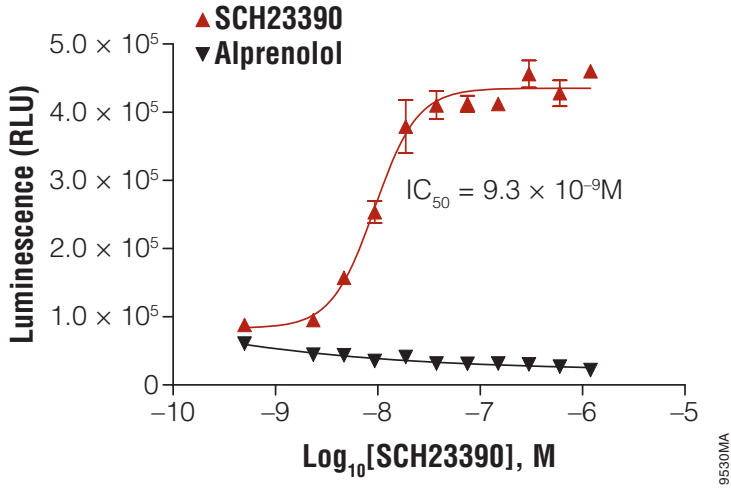

Figure 8. Determining the IC_{50} value of SCH23390 in D1 receptor-expressing HEK293 cells. Cells were resuspended in Induction Buffer and 2,000 cells were added to each well of a white solid 384-well plate. Cells were treated with the indicated amount of antagonist, SCH23390, in the presence of 100nM agonist, SKF38393. In the negative control reactions, SCH23390 was replaced with alprenolol. The cAMP-Glo™ Max Assay was performed as described in Section 5.A. Each point represents the average of three data points; the error bars show 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 **Complete Induction Buffer** (Section 5.A, Step 1) containing a final concentration of 2X (100nM) of SKF38393.
2. Prepare 500µl of a 2X (1.2µM) solution of SCH23390 in **Complete Induction Buffer** with 200nM SKF38393. Mix well.
3. Add 100µl of **Complete Induction Buffer** containing 200nM SKF38393 to wells A2 through A12 of a 96-well plate.
4. Add 200µl of 2X 1.2µM SCH23390 stock solution to well A1.

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

5. Prepare a twofold dilution of antagonist SCH23390 by transferring 100µl from well A1 to well A2 with a pipette, pipetting to mix. Transfer 100µl to well A3. Repeat for wells A4 through A11. See Figure 9. Discard the extra 100µl from well A11. Do not add SCH23390 to the no-antagonist control reactions in well A12.

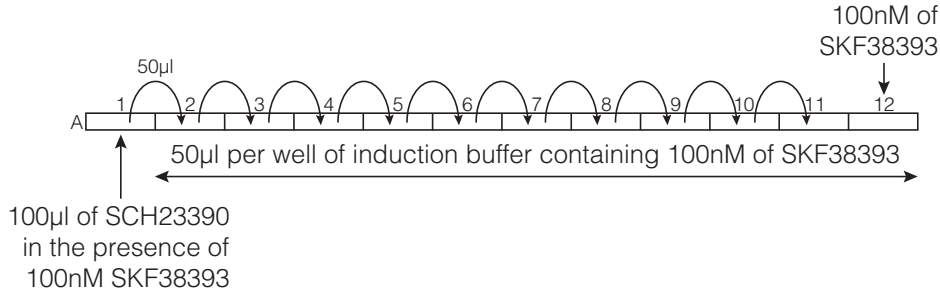

Figure 9. Dilution scheme for the antagonist SCH23390.

Assay Protocol Using a 4:1 Ratio

1. Transfer the indicated volume of the various concentrations of the antagonist SCH23390 to the assay plate. Add an equal volume of a suspension cell culture containing the recommended number of cells to the same plate (Section 3). Mix the plate by shaking for 1–2 minutes. Incubate the plate at room temperature for 20–30 minutes to allow cells and SCH23390 to interact.

96-Well	384-Well	Low-Volume 384-Well or 1536-Well
20µl	4µl	2.0µl

2. For experiments, calculate the required volume of cAMP Detection Solution. Prepare the cAMP Detection Solution as described in Section 4.B. Mix by inversion; do not vortex.

3. Add the indicated volume of cAMP Detection Solution to all wells based on Table 1, Section 4.A, and mix the plate by shaking for 1–2 minutes. Incubate the plate at room temperature (23°C) for 20 minutes.

96-Well	384-Well	Low-Volume 384-Well or 1536-Well
10µl	2µl	1.0µl

4. Add the indicated volume of room-temperature Kinase-Glo® Reagent to all wells. Mix the plate by shaking for 1–2 minutes, and incubate at room temperature for 10 minutes.

96-Well	384-Well	Low-Volume 384-Well or 1536-Well
50µl	10µl	5.0µl

Note: For low-volume 384- or 1536-well plates, add a volume of test solution that is equal to the volume of the other reagents:

Volume of test solution = Volume of cells + ligand + cAMP Detection Solution

5. Measure luminescence with a plate-reading luminometer. Determine the EC_{50} value using GraphPad Prism® or a similar software.

5.D. Determining Z´ Factor

This protocol allows you to determine the Z´ factor (6) for the cAMP-Glo™ Max 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.

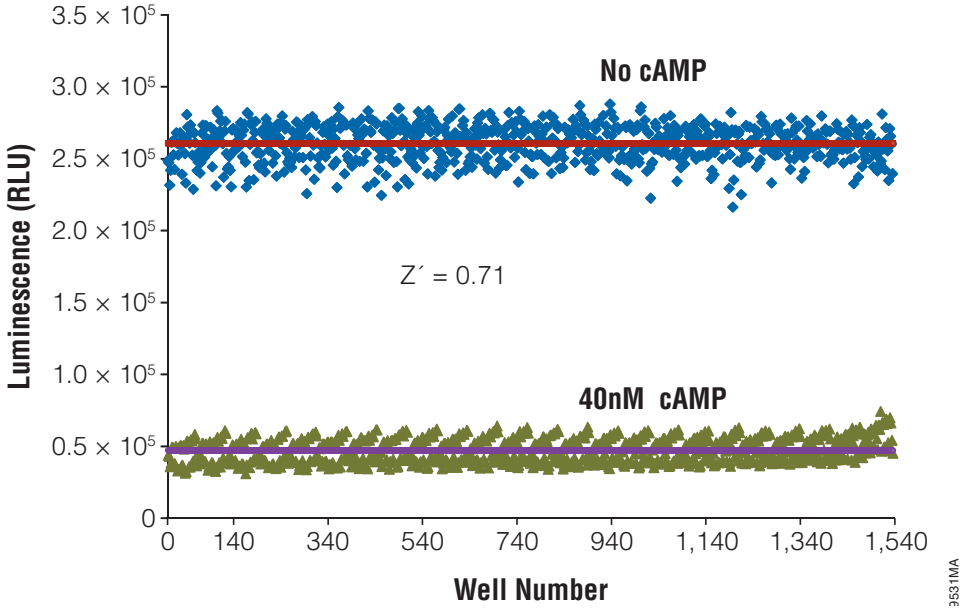

Figure 10. A scatter plot to determine the Z´ factor of the cAMP-Glo™ Max Assay. The cAMP-Glo™ Max Assay was performed in a 1536-well plate as described in Section 5.D using the Deerac Fluidics® Equator™ HTS noncontact dispenser. Luminescence was measured using the PHERAstar high-end microplate reader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

5.D. Determining Z´ Factor (continued)

Prior to performing the cAMP-Glo™ Max Assay, prepare the reagents as described in Section 4.B. Allow the cAMP-Glo™ ONE-Buffer to reach room temperature before use. Calculate the volume of Kinase-Glo® Reagent required for your experiments, and allow it to reach room temperature before use. Return the remaining cAMP-Glo™ ONE-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 or at –20°C. Calculate the required volumes of each reagent for your experiments and increase or decrease the volumes appropriately.

1. Prepare the 40nM cAMP solution as in Section 4.B (modify the cAMP concentration from 100nM). Vortex to mix.
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 Using a 4:1 Ratio

1. Prepare the no-cAMP reactions by adding the indicated volume of induction buffer to each well in half of the plate.

96-Well	384-Well	Low-Volume 384-Well or 1536-Well
40µl	8.0µl	4.0µl

2. Prepare the cAMP reactions by adding the indicated volume of 40nM cAMP solution to wells in the other half of the plate.

96-Well	384-Well	Low-Volume 384-Well or 1536-Well
40µl	8.0µl	4.0µl

Assay Protocol (continued) Using a 4:1 Ratio

3. Add the indicated volume of cAMP Detection Solution to all wells based on Table 1, Section 4.A, and mix the plate by shaking for 1–2 minutes. Incubate the plate at room temperature (23°C) for 20 minutes.

96-Well	384-Well	Low-Volume 384-Well or 1536-Well
10µl	2.0µl	1.0µl

4. Add the indicated volume of room-temperature Kinase-Glo® Reagent to all wells. Mix the plate by shaking for 1–2 minutes, and incubate at room temperature for 10 minutes.

96-Well	384-Well	Low-Volume 384-Well or 1536-Well
10µl	2.0µl	1.0µl

   Note: For low-volume 384- or 1536-well plates, add a volume of test solution that is equal to the volume of the other reagents:

   Volume of test solution = Volume of cells + ligand + cAMP Detection Solution

5. Measure the luminescence with a plate-reading luminometer. Determine the EC_{50} value using GraphPad Prism® or a similar software.

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 $MgCl_2$ to the Complete Induction Buffer to a final concentration of 20mM with respect to the cAMP Detection Solution, Section 4.B.
	The cAMP-Glo™ ONE-Buffer was stored at room temperature too long. On the day of the assay, equilibrate the required volume of cAMP-Glo™ ONE-Buffer to room temperature, and return the remaining 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™ Max 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™ Max Assay by test compound	Luciferase inhibition. To identify luciferase inhibitors, perform the cAMP-Glo™ Max 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.

6. Troubleshooting (continued)

Symptoms	Causes and Comments
Unexpected inhibition of the cAMP-Glo™ Max Assay by test compound (continued)	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.
	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™ Max Assay is not affected by the presence of up to 5% acetone or 5% DMSO.

7. References

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4. Gabriel, D. *et al*. (2003) High throughput screening technologies for direct cyclic AMP measurement. *Assay Drug Dev. Technol.* **1**, 291–303.
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6. Zhang, J. *et al.* (1999) A simple statistical parameter for use in evaluation and validation of high throughput screening assays. *J. Biomol. Screening* **4**, 67–73.

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 7.0. Add deionized water to a final volume of 1 liter. Filter to 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
GloSensor™ cAMP HEK293 Cell Line	2 vials	E1261
pGloSensor™-22F cAMP Plasmid	20µg	E2301
pGloSensor™-20F cAMP Plasmid	20µg	E1171
GloSensor™ cAMP Reagent*	25mg	E1290
ADP-Glo™ Kinase Assay*	1,000 assays	V9101
ADP-Glo™ Max Assay*	1,000 assays	V7001

*Additional sizes available.

8.B. Related Products (continued)

Luminometers

Product	Size	Cat.#
GloMax®-Multi+ Detection System with Instinct® Software: Base Instrument with Shaking	1 each	E8032
GloMax®-Multi+ Detection System with Instinct® Software: Base Instrument with Heating and Shaking	1 each	E9032
GloMax® 20/20 Luminometer	1 each	E5311
GloMax® 20/20 Luminometer with Single Auto-Injector	1 each	E5321
GloMax® 20/20 Luminometer with Dual Auto-Injector	1 each	E5331
GloMax® 96 Microplate Luminometer	1 each	E6501
GloMax® 96 Microplate Luminometer w/Single Injector	1 each	E6511
GloMax® 96 Microplate Luminometer w/Dual Injectors	1 each	E6521

[a]Patent Pending.

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

[c]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.

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

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

[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