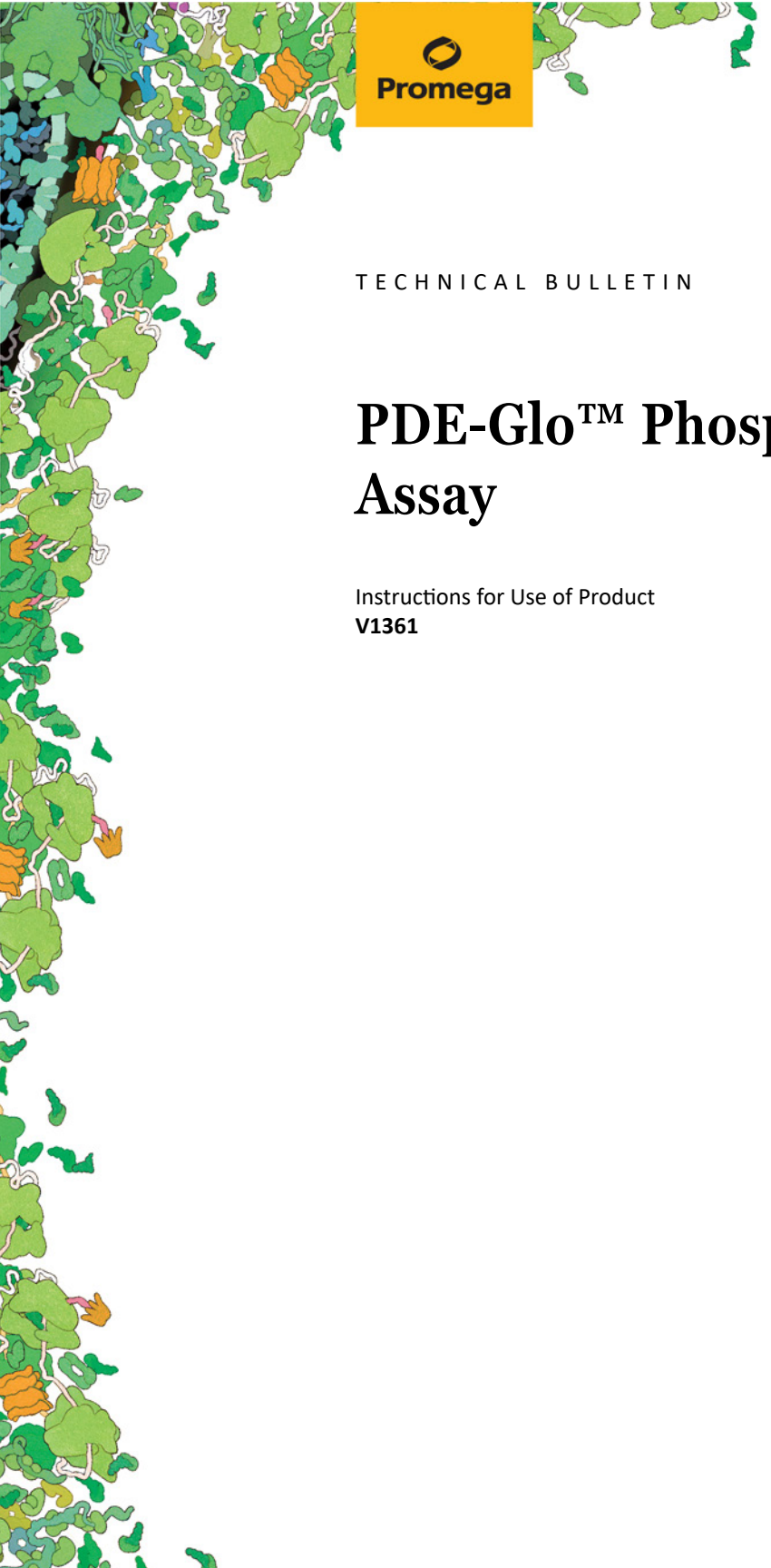

TECHNICAL BULLETIN

PDE-Glo™ Phosphodiesterase Assay

Instructions for Use of Product
V1361

Revised 6/21
TB353

Promega

PDE-Glo™ Phosphodiesterase Assay

All technical literature is available at: www.promega.com/protocols/
Visit the web site to verify that you are using the most current version of this Technical Bulletin.
E-mail Promega Technical Services if you have questions on use of this system: techserv@promega.com

1. Description

The PDE-Glo™ Phosphodiesterase Assay[a] is a luminescent, high-throughput screening (HTS) method for measuring cyclic nucleotide phosphodiesterase activity from purified sources. Cyclic nucleotide phosphodiesterases (PDEs) are involved in a myriad of cellular processes due to their ability to hydrolyze, and thus control, the levels of the second messenger signaling molecules cAMP and cGMP (1). PDEs have been implicated in a variety of diseases (1–6) such as erectile disfunction, asthma, chronic obstructive pulmonary disease (COPD, 3), learning disorders, depression and memory functions (7,8), neurodegenerative diseases, autoimmune disorders, heart failure and myocardial infarction (9,10). The availability of selective inhibitors for PDEs has facilitated their use as tools to study cyclic nucleotide signaling and paved the way to investigate the role of PDEs in cellular and tissue pathologies. The PDE-Glo™ Phosphodiesterase Assay allows lead candidates to be identified from compound libraries. The assay is designed for 384-well plates, but assay volumes can easily be scaled for 96- or 1536-well plates. The PDE-Glo™ Phosphodiesterase Assay is optimized to work with both cAMP- and cGMP-dependent phosphodiesterases. The total time required for the assay from start to finish is less than 1 hour after the PDE reaction is complete.

An overview of the PDE-Glo™ Phosphodiesterase Assay is shown in Figure 1. The assay involves incubating a purified preparation of phosphodiesterase with a cyclic nucleotide (cAMP or cGMP). Following this PDE reaction, the PDE-Glo™ Termination Buffer and PDE Detection Solution, which contains ATP, protein kinase A (PKA) and a PKA substrate, are added. The cyclic nucleotide remaining after the PDE reaction drives a kinase reaction that leads to a reduction in ATP levels. The amount of ATP consumed by this reaction depends on the amount of cyclic nucleotide present (Figure 2).

1. Description (continued)

Following a brief incubation, Kinase-Glo® Reagent is added to the reaction, and after a 10-minute incubation, the plate is read using a plate-reading luminometer. The luminescent signal produced is directly related to the amount of ATP remaining and correlates with phosphodiesterase activity. 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 3 shows that the PDE-Glo™ Phosphodiesterase Assay is very sensitive to cAMP concentrations relative to cGMP. For best results when screening for inhibitors of PDEs, we recommend using 1µM cAMP or 10µM cGMP in PDE reactions, since higher concentrations of cyclic nucleotides will require more PDE enzyme to be added or longer incubation times for the protein kinase reaction. Figure 3 shows a schematic diagram of the PDE-Glo™ Phosphodiesterase Assay protocol.

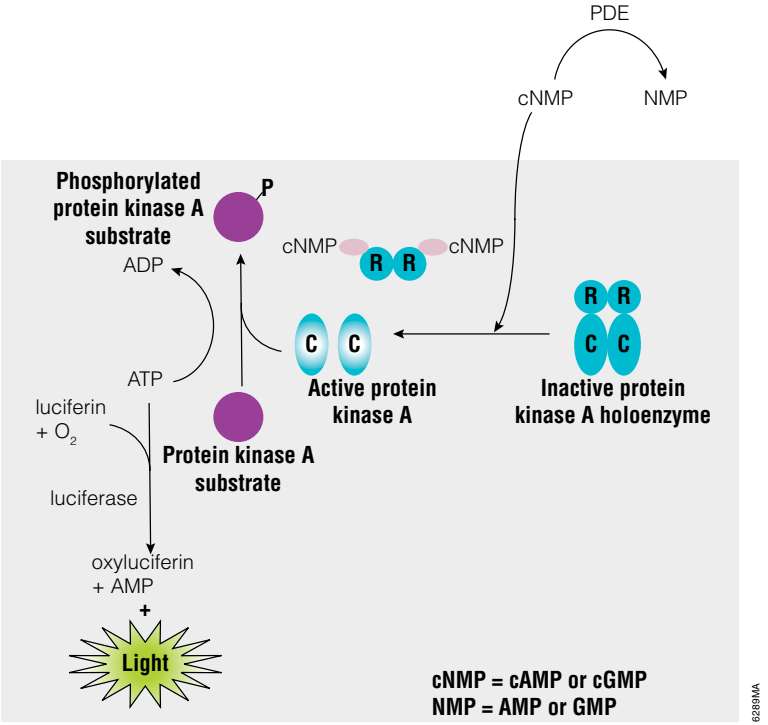

Figure 1. The PDE-Glo™ Phosphodiesterase Assay. As PDE hydrolyzes cAMP or cGMP to AMP or GMP, respectively, the concentration of cNMP decreases. In the PDE-Glo™ Phosphodiesterase Assay, depicted by the shaded box, cNMP binds to the inactive protein kinase A holoenzyme, and the regulatory subunits undergo 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. As PDE hydrolyzes cNMP, less protein kinase A is activated and more ATP is available for the luciferase reaction. As a result, luminescence increases. Thus, luminescence is directly proportional to the remaining ATP levels, which is directly proportional to PDE levels.

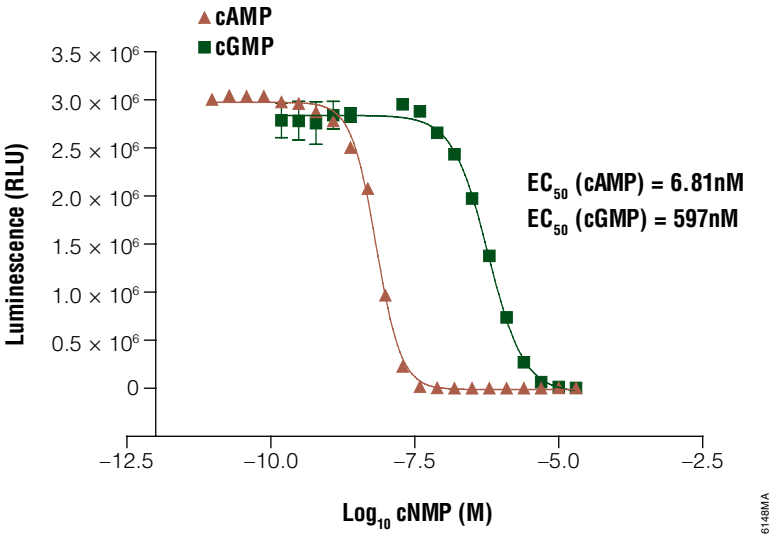

Figure 2. cAMP and cGMP dose response curves. PDE-Glo™ Phosphodiesterase Assays were performed in a white, 96-well plate using the indicated amount of cAMP or cGMP. PDE reactions were mixed and incubated for 20 minutes at room temperature. Following the kinase reaction, an equal volume of Kinase Glo® reagent was added to each well, and reactions were mixed and incubated for an additional 10 minutes at room temperature. Luminescence was measured using a Veritas™ microplate luminometer (Turner Biosystems). All data points are the average of two determinations.

1. Description (continued)

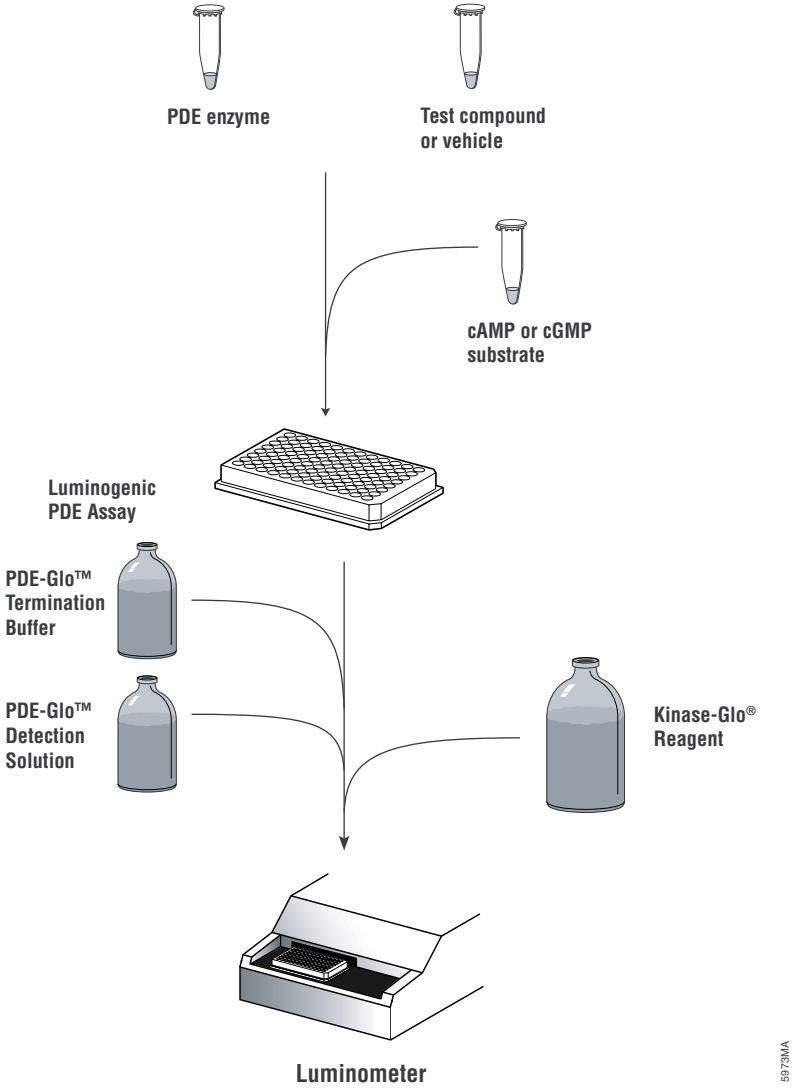

Figure 3. Schematic diagram of the PDE-Glo™ Phosphodiesterase Assay protocol.

2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
PDE-Glo™ Phosphodiesterase Assay	**1,000 assays**	**V1361**

The kit contains sufficient reagents for 1,000 reactions in a 384-well plate format. Includes:

- 100µl cAMP, 1mM
- 500µl cGMP, 1mM
- 1.2ml PDE-Glo™ Reaction Buffer, 5X
- 600µl PDE-Glo™ Detection Buffer, 5X
- 600µl PDE-Glo™ Termination Buffer, 5X
- 20µl Protein Kinase A
- 1 bottle Kinase-Glo® Substrate
- 10ml Kinase-Glo® Buffer

Storage Conditions: Store the system at –10°C to –30°C. See product label for expiration date.

3. PDE-Glo™ Phosphodiesterase Assay Protocol

The PDE-Glo™ Phosphodiesterase Assay is compatible with 96-, 384- and 1536-well plate formats. The PDE-Glo™ Phosphodiesterase Assay protocol begins by assembling a PDE reaction using cAMP or cGMP as a substrate at a final concentration of 1µM and 10µM, respectively. Following completion of the PDE reaction, PDE-Glo™ Termination Buffer containing the PDE inhibitor IBMX (3-isobutyl-1-methylxanthine, not supplied) is added to inhibit most PDEs; additional inhibitors may need to be added to this buffer. The PDE-Glo™ Detection Solution is added, then the Kinase-Glo® Reagent is added. Luminescence is measured using a plate-reading luminometer. The light output is proportional to PDE activity in the reaction.

Materials to be Supplied by the User
(Solution compositions are provided in Section 6.A.)

- 100mM 3-isobutyl-1-methylxanthine (IBMX, Sigma-Aldrich Cat.# I5879) in 100% DMSO
- additional PDE inhibitors, if desired
- white, opaque, polystyrene, nontreated, flat-bottom 96-, 384- or 1536-well plates
  Do not use treated plates, black plates or clear plates.
- luminometer or charge-coupled device (CCD) capable of reading multiwell plates
- multichannel pipette or automated pipeting station
- purified active PDE enzyme(s)

Note: To assay the PDE type I isoform add Ca^{2+} and calmodulin (CaM) to the PDE-Glo™ Reaction Buffer prior to assembling the PDE reaction.

3.A. Reagent Preparation

The reagent volumes for individual wells of a 96-, 384- or 1536-well plate are given in Table 1.

Table 1. PDE-Glo™ Phosphodiesterase Assay Reagent Volumes for 96-, 384- and 1536-Well Plates.

	Volume per Assay (µl)		
Reagent	**96-Well Plate**	**384-Well Plate**	**1536-Well Plate**
active PDE in 1X PDE-Glo™ Reaction Buffer	12.5	2.5	1.0
2µM cAMP or 20µM cGMP solution	12.5	2.5	1.0
PDE-Glo™ Termination Buffer	12.5	2.5	1.0
PDE-Glo™ Detection Solution	12.5	2.5	1.0
Kinase-Glo® Reagent	50	10	4.0

The following instructions describe the preparation of 1ml of each reagent. For your experiments, calculate the required volumes of each reagent, and increase or decrease the volumes appropriately. Mix all reagents well before use.

1. Prepare the 1X PDE-Glo™ Reaction Buffer as follows:

Component	Volume
PDE-Glo™ Reaction Buffer, 5X	200µl
NANOpure® water at room temperature	800µl

2. Prepare the 2µM cAMP or 20µM cGMP solution as follows:

Component	Volume for Reactions Using cAMP as a Substrate	Volume for Reactions Using cGMP as a Substrate
PDE-Glo™ Reaction Buffer, 5X	200µl	200µl
1mM cAMP	2µl	—
1mM cGMP	—	20µl
NANOpure® water at room temperature	798µl	780µl

3. Prepare the PDE-Glo™ Termination Buffer as follows:

Component	Volume
PDE-Glo™ Termination Buffer, 5X	200µl
100mM IBMX	20µl
NANOpure® water at room temperature	780µl

4. 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.

3.B. Titration of Cyclic Nucleotide Phosphodiesterase

We recommend performing a PDE titration to empirically determine the optimal amount of PDE for screening and the optimal PDE reaction time and to determine whether the enzyme preparation contains any components that negatively affect assay performance.

A sample PDE titration protocol is provided for purified, active, recombinant bovine cGMP-binding cGMP-specific PDE (PDE V) and PDE from bovine brain (using cAMP as a substrate). This protocol describes the setup of the PDE-Glo™ Phosphodiesterase Assay in quadruplicate (four reactions per sample). The following protocol is designed to use one-fourth of a low-volume, 384-well plate (rows A through D). The suggested layout of control and test samples in a 384-well plate format is shown in Figure 4. The PDE-Glo™ Phosphodiesterase Assay can also be performed in 96-well and 1536-well plate formats. Reagent volumes for individual wells of a 96-, 384- or 1536-well plate are given in Table 1.

At several steps in the protocols, you must mix the plates well. For reactions in a 96-well plate, we recommend shaking the plate for 60 seconds to mix the reaction components and obtain consistent results. However, in some 384-well plates, particularly low-volume 384-well plates, and 1536-well plates, the reaction volumes are too low and shaking to mix is not efficient. For these plates, the speed of reagent delivery may be sufficient to mix the reactions as long as the components are delivered to the bottom of the well. Alternatively, you may centrifuge the plate to force the reaction components to the bottom of the well.

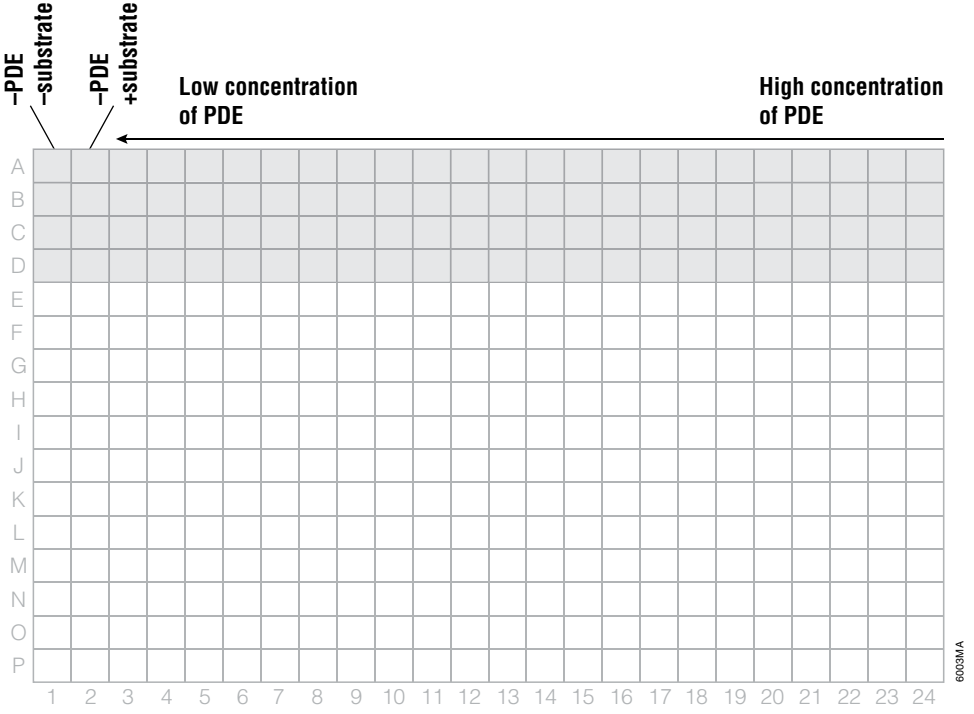

Figure 4. Suggested plate layout for the titration of cyclic nucleotide phosphodiesterase (PDE).

The performance of the PDE-Glo™ Phosphodiesterase Assay is not affected by the presence of up to 2% DMSO, a common compound vehicle, in reactions with PDE isoforms that tolerate this concentration of DMSO.

Representative titration results are shown in Figures 5, 6 and 7.

Reagent Preparation

1. Prepare the 1X PDE-Glo™ Reaction Buffer, 2µM cAMP or 20µM cGMP solution, PDE-Glo™ Termination Buffer and Kinase-Glo® Reagent as described in Section 3.A. Calculate the volume of Kinase-Glo® Reagent required for your experiments, and allow that volume of Kinase-Glo® Reagent to reach room temperature before use. Return the remaining Kinase-Glo® Reagent to a –20°C freezer.

Enzyme Preparation

1. Dilute purified active PDE to the desired concentration in 1X PDE-Glo™ Reaction Buffer and mix well. Keep on ice.
2. Add the volume of 1X PDE-Glo™ Reaction Buffer indicated below to each well in columns 1 through 23 of rows A through D of the plate.

96-well plate	384-well plate	1536-well plate
12.5µl	2.5µl	1.0µl

3. Add the volume of diluted PDE indicated below to each well of column 24 of rows A through D.

96-well plate	384-well plate	1536-well plate
37.5µl	7.5µl	3.0µl

4. Perform a serial dilution of the enzyme by transferring the volume of PDE indicated below from column 24 to column 23 with a multichannel pipette, pipetting to mix. Transfer the indicated volume from column 23 to column 22 and mix. Repeat for columns 21 through 3, moving from right to left. Discard the final aliquot from column 3. Do not add PDE to reactions in columns 1 and 2. Proceed immediately to the assay protocol.

96-well plate	384-well plate	1536-well plate
25µl	5.0µl	2.0µl

Notes:

1. Each dilution results in a 2:3 dilution.
2. Reactions in columns 1 and 2 are the no-PDE control reactions. Reactions in column 1 contain no substrate (cAMP or cGMP) and should give high luminescence readings; reactions in column 2 contain substrate (cAMP or cGMP) and should give low readings.

3.B. Titration of Cyclic Nucleotide Phosphodiesterase (continued)

Assay Protocol

1. To initiate the PDE reaction, add the volume of 2µM cAMP or 20µM cGMP solution indicated below to wells in columns 2 through 24; do not add cGMP or cAMP substrate to column 1. Mix well and incubate at room temperature for the desired time.

96-well plate	384-well plate	1536-well plate
12.5µl	2.5µl	1.0µl

2. Add the volume of PDE-Glo™ Termination Buffer indicated below to all reactions. Mix well.

96-well plate	384-well plate	1536-well plate
12.5µl	2.5µl	1.0µl

3. Prepare the PDE-Glo™ Detection Solution as follows. Mix well.

Component	Volume
PDE-Glo™ Detection Buffer, 5X	200µl
Protein Kinase A	8µl
NANOpure® water	792µl

Note: Prepare the PDE-Glo™ Detection Solution immediately before use. Once the PDE-Glo™ Detection Solution is prepared, immediately return the Protein Kinase A to –20°C.

4. Add the volume of PDE-Glo™ Detection Solution indicated below to all reactions. Mix well and incubate for 20 minutes at room temperature.

96-well plate	384-well plate	1536-well plate
12.5µl	2.5µl	1.0µl

5. Add the volume of Kinase-Glo® Reagent indicated below to all reactions. Mix well and incubate for 10 minutes at room temperature. Measure luminescence with a plate-reading luminometer.

96-well plate	384-well plate	1536-well plate
50µl	10µl	4.0µl

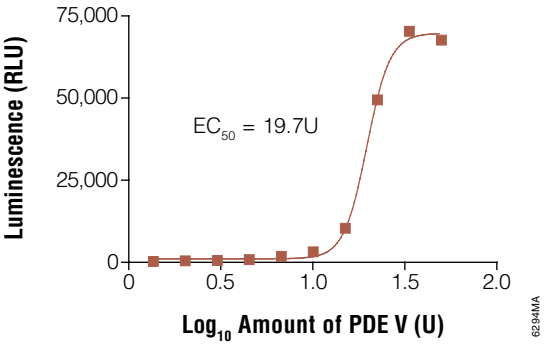

Figure 5. Titration of PDE Type V. The PDE-Glo™ Phosphodiesterase Assay was performed using the CyBi®-Well 384/1536 pipetting system (CyBio) in a standard 384-well plate with 10µM cGMP as the substrate and the indicated amount of PDE V (Calbiochem Cat.# 524715, lot number D34976) as described in Section 3.B. Reactions were incubated for 90 minutes at room temperature. Luminescence was measured using the PHERAstar high-end plate reader (BMG Labtech). Data analysis was performed with GraphPad Prism®, version 4.02, for Windows® using a sigmoidal dose-response (variable slope) equation. Each point represents an average of four replicates.

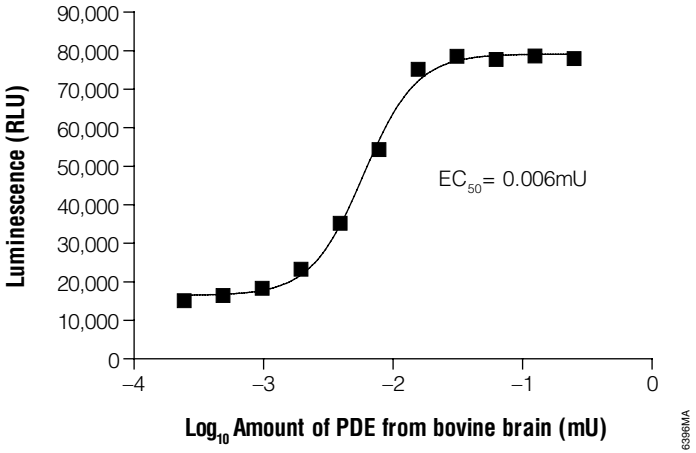

Figure 6. Titration of 3´,5´-cyclic-nucleotide-specific phosphodiesterase from bovine brain (Sigma-Aldrich Cat.# P9529). The PDE-Glo™ Phosphodiesterase Assay was performed using the Deerac Fluidics® Equator™ HTS dispenser in a standard 384-well plate with 5µM cGMP as the substrate and the indicated amount of PDE from bovine brain. Assays were performed as described in Section 3.B except that reactions were incubated for 15 minutes at room temperature. Luminescence was measured using the PHERAstar high-end plate reader (BMG Labtech). Data analysis was performed with GraphPad Prism® software, version 4.02, for Windows® using a sigmoidal dose-response (variable slope) equation. Each point represents an average of four replicates.

3.B. Titration of Cyclic Nucleotide Phosphodiesterase (continued)

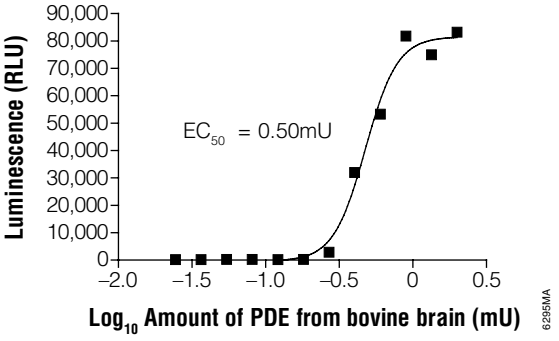

Figure 7. Titration of 3´,5´-cyclic-nucleotide-specific phosphodiesterase from bovine brain (Sigma-Aldrich Cat.# P9529). The PDE-Glo™ Phosphodiesterase Assay was performed using the CyBi®-Well 384/1536 pipetting system (CyBio) in a low-volume, 384-well plate with 1µM cAMP as the substrate and the indicated amount of PDE from bovine brain as described in Section 3.B except that reactions were incubated for 5 minutes at room temperature. Luminescence was measured using the Tecan Safire2™ plate reader. Data analysis was performed with GraphPad Prism® software, version 4.02, for Windows® using a sigmoidal dose-response (variable slope) equation. Each point represents an average of four replicates.

3.C. Z´ Factor Protocol

The following protocol is designed for one 384-well plate. The PDE-Glo™ Phosphodiesterase Assay can also be performed in 96-well and 1536-well plate formats. Reagent volumes for individual wells of a 96-, 384- or 1536-well plate are given in Table 1. Representative results are shown in Figure 8.

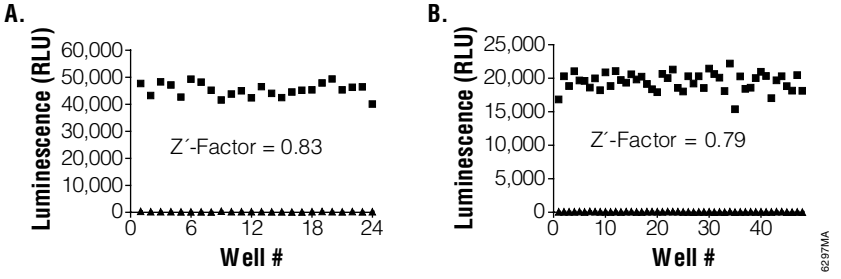

Figure 8. Z´-factor determination using PDE from bovine brain in low-volume, 384-well and 1536-well formats. The PDE-Glo™ Phosphodiesterase Assay was performed with the CyBi®-Well 384/1536 pipetting system (CyBio) in a low-volume, 384-well plate (**Panel A**) and a 1536-well plate (**Panel B**) using 1µM cAMP substrate per reaction and 1mU/reaction of PDE from bovine brain (low-volume 384-well plates) or 0.4mU/reaction of PDE from bovine brain (1536-well plates). Reactions were incubated for 5 minutes at room temperature. Luminescence was measured using the PHERAstar high-end plate reader (BMG Labtech).

Reagent Preparation

The following instructions describe the preparation of 1ml of each reagent. For your experiments, calculate the required volumes of each reagent, and increase or decrease the volumes appropriately. Mix all reagents well before use.

1. Prepare the 1X PDE-Glo™ Reaction Buffer, 2µM cAMP or 20µM cGMP solution, PDE-Glo™ Termination Buffer and Kinase-Glo® Reagent as described in Section 3.A. Calculate the volume of Kinase-Glo® Reagent required for your experiments, and allow that volume of Kinase-Glo® Reagent to reach room temperature before use. Return the remaining Kinase-Glo® Reagent to a –20°C freezer.
2. Dilute PDE in 1X PDE-Glo™ Reaction Buffer as follows:

Component	Volume
PDE-Glo™ Reaction Buffer, 5X	200µl
PDE enzyme[1]	Xµl
NANOpure® water at room temperature	to a final volume of 1.0ml

[1]Choose the amount of PDE from the PDE titration (Section 3.B) that results in approximately 80% of the maximum assay signal. Using less PDE is possible; however, the dynamic range of the assay will be narrower.

! Prepare freshly diluted PDE enzyme immediately before use for each set of experiments.

3.C. Z´ Factor Protocol (continued)

Assay Protocol

1. Add the volume of PDE in 1X PDE-Glo™ Reaction Buffer indicated below to each well of the plate.

96-well plate	384-well plate	1536-well plate
12.5µl	2.5µl	1.0µl

2. Add the volume of 1X PDE-Glo™ Reaction Buffer indicated below to each well in rows A through H. Add an equal volume (indicated below) of 2µM cAMP or 20µM cGMP solution to each well in rows I through P to initiate the PDE reaction. Mix well and incubate at room temperature for the desired reaction time.

96-well plate	384-well plate	1536-well plate
12.5µl	2.5µl	1.0µl

Note: Reactions in rows A through H contain no substrate (cAMP or cGMP) and should give high luminescence readings; reactions in rows I through P contain substrate (cAMP or cGMP) and should give low readings.

3. Add the volume of PDE-Glo™ Termination Buffer indicated below to all reactions. Mix well.

96-well plate	384-well plate	1536-well plate
12.5µl	2.5µl	1.0µl

4. Prepare the PDE-Glo™ Detection Solution as follows. Mix well.

Component	Volume
PDE-Glo™ Detection Buffer, 5X	200µl
Protein Kinase A	8µl
NANOpure® water at room temperature	792µl

Note: Prepare the PDE-Glo™ Detection Solution immediately before use. Once the PDE-Glo™ Detection Solution is prepared, immediately return the Protein Kinase A to –20°C.

5. Add the volume of PDE-Glo™ Detection Solution indicated below to all reactions. Mix well and incubate for 20 minutes at room temperature.

96-well plate	384-well plate	1536-well plate
12.5µl	2.5µl	1.0µl

6. Add the volume of Kinase-Glo® Reagent indicated below to all reactions. Mix well and incubate for 10 minutes at room temperature. Measure luminescence with a plate-reading luminometer.

96-well plate	384-well plate	1536-well plate
50µl	10µl	4.0µl

3.D. IC_{50} Determination

This example protocol describes the determination of the IC_{50} value of Zaprinast (Sigma-Aldrich Cat.# Z0878), an inhibitor of phosphodiesterase V and VI, using purified active recombinant bovine cGMP-binding cGMP-specific PDE (PDE V, Calbiochem EMD Cat.# 524715). This protocol describes the setup of the PDE-Glo™ Phosphodiesterase Assay in quadruplicate (4 reactions per sample). The following assay protocol is designed to use one-fourth of a low-volume, 384-well plate (rows A through D). The suggested plate layout of control reactions and reactions with and without inhibitor in a 384-well plate format is shown in Figure 9.

Representative titration results are shown in Figures 10 and 11.

The performance of the PDE-Glo™ Phosphodiesterase Assay is not affected by the presence of up to 2% DMSO, a common compound vehicle, in reactions with PDE isoforms that tolerate this concentration of DMSO.

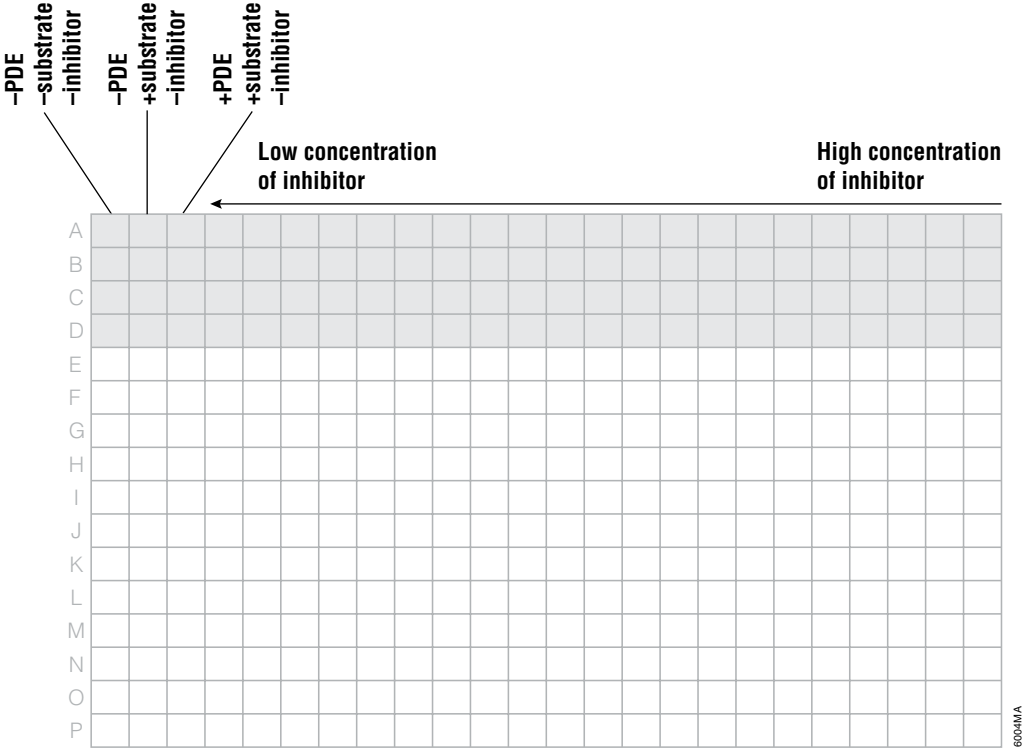

Figure 9. Suggested plate layout for IC_{50} determination.

3.D. IC_{50} Determination (continued)

Reagent Preparation

The following instructions describe the preparation of 1ml of each reagent. For your experiments, calculate the required volumes of each reagent, and increase or decrease the volumes appropriately.

1. Prepare the 1X PDE-Glo™ Reaction Buffer, 2µM cAMP or 20µM cGMP solution, PDE-Glo™ Termination Buffer and Kinase-Glo® Reagent as described in Section 3.A. Calculate the volume of Kinase-Glo® Reagent required for your experiments, and allow that volume of Kinase-Glo® Reagent to reach room temperature before use. Return the remaining Kinase-Glo® Reagent to a –20°C freezer.
2. Prepare PDE in 1X PDE-Glo™ Reaction Buffer as follows:

Component	Volume
PDE-Glo™ Reaction Buffer, 5X	200µl
PDE enzyme[1]	Xµl
NANOpure® water at room temperature	to a final volume of 1.0ml

[1]Choose the amount of PDE from the PDE titration (Section 3.B) that results in approximately 80% of the maximum assay signal. Using less PDE is possible; however, the dynamic range of the assay will be narrower.

! Prepare freshly diluted PDE enzyme immediately before use for each set of experiments.

Inhibitor Preparation

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

1. Add the volume of 1X PDE-Glo™ Reaction Buffer indicated below to each well in columns 1 through 23 of rows A through D of a 384-well plate.

96-well plate	384-well plate	1536-well plate
5.0µl	1.0µl	0.40µl

2. Prepare 100µl of a 5X inhibitor solution in 1X PDE-Glo™ Reaction Buffer and mix well.
3. Add the volume of the 5X inhibitor solution indicated below to each well in column 24 of rows A through D.

96-well plate	384-well plate	1536-well plate
10.0µl	2.0µl	0.80µl

4. Perform a serial dilution of the inhibitor by transferring the volume indicated below from wells in column 24 to wells in column 23 with a multichannel pipette, pipetting to mix. Transfer the indicated volume from wells in column 23 to wells in column 22 and mix. Repeat for columns 21 through 4, moving from right to left. Discard the final aliquot from column 4. Do not add inhibitor to columns 1, 2 and 3. Proceed immediately to the assay protocol.

96-well plate	384-well plate	1536-well plate
5.0µl	1.0µl	0.4µl

Notes:

1. All dilutions are 1:2 dilutions.
2. Reactions in columns 1 and 2 are the no-inhibitor control reactions. Reactions in column 1 contain no-substrate (cAMP or cGMP) control reactions and should give high luminescence readings. Reactions in column 2 contain substrate (cAMP or cGMP) and act as positive control reactions for PDE activity, so luminescence should be low. Reactions in column 3 are the PDE positive control reactions. These reactions do not contain inhibitor and should have high luminescence; however, the luminescence should not be as high as that from the reactions in column 1.

Assay Protocol

1. Add the volume of PDE diluted in 1X PDE-Glo™ Reaction Buffer indicated below to each reaction in rows A through D of columns 3–24. Add an equal volume of 1X PDE-Glo™ Reaction Buffer to each reaction in rows A through D of columns 1 and 2. Mix the plate, and pre-incubate at room temperature for 5 minutes or for other time periods as desired to allow PDE to interact with the inhibitor.

96-well plate	384-well plate	1536-well plate
7.5µl	1.5µl	0.6µl

2. To initiate the PDE reaction, add an equal volume (indicated below) of 2µM cAMP or 20µM cGMP solution to each reaction in rows A through D; do not add cGMP or cAMP substrate to reactions in column 1. Mix well and incubate at room temperature for the desired reaction time.

96-well plate	384-well plate	1536-well plate
12.5µl	2.5µl	1.0µl

3. Add the volume of PDE-Glo™ Termination Buffer indicated below to each reaction. Mix well.

96-well plate	384-well plate	1536-well plate
12.5µl	2.5µl	1.0µl

3.D. IC_{50} Determination (continued)

Assay Protocol (continued)

4. Prepare the PDE-Glo™ Detection Solution as follows. Mix well.

Component	Volume
PDE-Glo™ Detection Buffer, 5X	200µl
Protein Kinase A	8µl
NANOpure® water at room temperature	792µl

Note: Prepare the PDE-Glo™ Detection Solution immediately before use. Once the PDE-Glo™ Detection Solution is prepared, immediately return the Protein Kinase A to –20°C.

5. Add the volume of PDE-Glo™ Detection Solution indicated below to each reaction. Mix well and incubate for 20 minutes at room temperature.

96-well plate	384-well plate	1536-well plate
12.5µl	2.5µl	1.0µl

6. Add the volume of Kinase-Glo® Reagent indicated below to each reaction. Mix well and incubate for 10 minutes at room temperature. Measure luminescence with a plate-reading luminometer.

96-well plate	384-well plate	1536-well plate
50µl	10µl	4.0µl

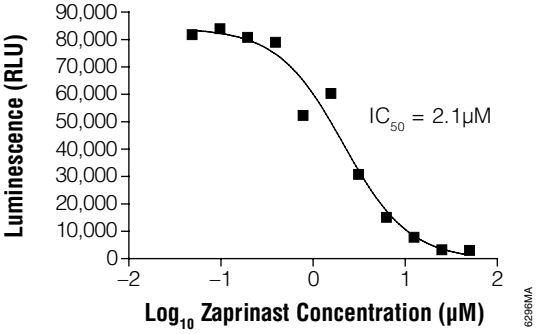

Figure 10. Titration of a PDE inhibitor using PDE V. The PDE-Glo™ Phosphodiesterase Assay was performed with the CyBi®-Well 384/1536 pipetting system (CyBio) in a standard 384-well plate using 30 units of PDE V (Calbiochem Cat.# 524715, lot number D34976), 10µM cGMP substrate and the indicated amount of the PDE inhibitor Zaprinast. Zaprinast and PDE V were mixed and pre-incubated at room temperature for 5 minutes. Substrate was added, and the reactions were incubated for 90 minutes at room temperature. Luminescence was measured using the PHERAstar high-end plate reader (BMG Labtech). Data analysis was performed with GraphPad Prism®, version 4.02, for Windows® using a sigmoidal dose-response (variable slope) equation. Each point represents an average of four replicates per concentration.

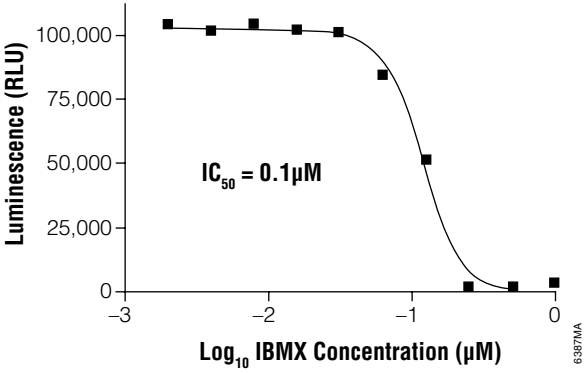

Figure 11. Titration of a PDE inhibitor using bovine brain PDE. The PDE-Glo™ Phosphodiesterase Assay was performed with the CyBi®-Well 384/1536 pipetting system (CyBio) in a low-volume 384-well plate using 1 milliunit of PDE from bovine brain, 1µM cAMP substrate and the indicated amount of the PDE inhibitor 3-isobutyl-1-methyl-xanthine (IBMX). The IBMX and PDE bovine brain were pre-incubated together for 5 minutes. The substrate was added, and the reactions were incubated for an additional 5 minutes at room temperature. Luminescence was measured using the Tecan Safire2™ plate reader. Data analysis was performed with GraphPad Prism®, version 4.02, for Windows® using a sigmoidal dose-response (variable slope) equation. Each point represents an average of two replicates per concentration.

4. 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
Unexpected 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. 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.
	Enzyme contamination of the no-PDE control reactions. Choose a control preparation known to be free of PDE activity. Avoid contact between inactive control and active PDE preparations.
	Protein Kinase A inhibition. To test for Protein Kinase A inhibition, assemble two no-PDE control reactions with cAMP or cGMP: one with test compound and one without. The control reaction with test compound will give low luminescent signal if there is no inhibition of Protein Kinase A.
Unexpected low luminescent signal	Use only white, opaque luminometer plates. Do not use black plates or clear plates.
	Low PDE activity in enzyme preparation. • Avoid multiple freeze-thaw cycles of the PDE enzyme. • Thaw PDE preparation immediately before use. • Avoid extended incubations of PDE on ice or at room temperature, which may lead to enzyme inactivation. • Incubate PDE reaction at the desired reaction temperature; we recommend a range of room temperature to 37°C. Stronger signals are typically generated at 37°C.
	Increase the PDE reaction time.
	Improper storage of Kinase-Glo® Reagent. Store the Kinase-Glo® Reagent at –20°C. Avoid multiple freeze-thaw cycles.

Symptoms	Causes and Comments
Unexpected inhibition of the PDE-Glo™ Phosphodiesterase Assay by test compound	Luciferase inhibition. Screen compounds using multiple PDE isoforms. Inhibition of only a subset of isoforms indicates that the test compounds are not luciferase inhibitors. To test for luciferase inhibition, screen compounds by assembling control reactions in PDE-Glo™ Termination Buffer with no Protein Kinase A. These reactions should give high luminescent signal if there is no inhibition of luciferase.
	Protein Kinase A inhibition. To test for inhibition, assemble two no-PDE control reactions with cAMP or cGMP: one with test compound and one without. The control reaction with test compound will give low luminescent signal if there is no inhibition of Protein Kinase A.
	Inhibition by an organic solvent. Minimize the solvent concentration, or use a different solvent to dissolve test compound. Perform control reactions that contain solvent but no test compound to test the effect of the solvent on assay performance.

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6. Appendix

6.A. Composition of Buffers and Solutions

PDE-Glo™ Reaction Buffer, 5X

200mM Tris-HCl (pH 7.5)
50mM $MgCl_2$
0.5mg/ml BSA

100mM IBMX

22.2mg 3-isobutyl-1-methylxanthine

Dissolve 3-isobutyl-1-methylxanthine (IBMX) in 1ml of 100% DMSO.

6.B. Related Products

Product	Size	Cat.#
cAMP-Glo™ Assay	300 assays	V1501
	3,000 assays	V1502
	30,000 assays	V1503
Kinase-Glo® Luminescent Kinase Assay	10ml	V6711
	10 × 10ml	V6712
	100ml	V6713
	10 × 100ml	V6714
Kinase-Glo® Plus Luminescent Kinase Assay	10ml	V3771
	10 × 10ml	V3772
	100ml	V3773
	10 × 100ml	V3774
GloMax® Navigator System	1 each	GM2000
GloMax® Navigator System w/Dual Injectors	1 each	GM2010
GloMax® Discover System	1 each	GM3000
GloMax® Explorer System	1 each	GM3500

6.C. Summary of Changes

The following changes were made to the 6/21 revision of this document:

1. Removed the discontinued Cat.# V1362.
2. Revised Section 6.B.
3. Updated the cover page.

[a]Patent Pending.