Promega

Technical Manual

AMP-Glo™ Assay

INSTRUCTIONS FOR USE OF PRODUCTS V5011, V5012 AND V5013.

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Part# TM384

AMP-Glo™ Assay

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1. Description

The AMP-Glo™ Assay is a homogenous assay that generates light signal from any reaction that produces AMP as a reaction product. This versatile system can assay multiple types of enzymes, such as cyclic AMP-specific phosphodiesterases (1), aminoacyl-tRNA synthetases (2), DNA ligases (3) and ubiquitin ligases (4) or enzymes modulated by AMP (5,6). The AMP-Glo™ Assay is designed to quantitatively monitor the concentration of AMP in a biochemical reaction in a high-throughput format. The stable luminescent signal of the assay eliminates the need for an injector-equipped luminometer and allows batch-mode processing of multiple plates. The assay can be used to determine the AMP produced either in the presence or absence of ATP as a substrate.

The assay contains two reagents: one to terminate the enzymatic reaction and simultaneously remove ATP and convert AMP produced into ADP, and a second reagent that converts ADP to ATP and ATP to light output using the luciferin/luciferase reaction (Figure 1). The AMP-Glo™ Assay can be performed in 96-, 384-, low-volume 384- and 1536-well plates.

For enzymatic reactions that use ATP as a substrate and produce AMP as a product (2,4), the assay is designed to terminate the enzymatic reaction and remove the remaining ATP in one step followed by the conversion of AMP to ATP and detection of the generated ATP using the luciferin/luciferase reaction in a second step.

The assay also can be used to monitor AMP produced in biochemical reactions catalyzed by enzymes that do not use ATP as a substrate, such as cAMP-phosphodiesterases (PDE) and bacterial DNA ligases.

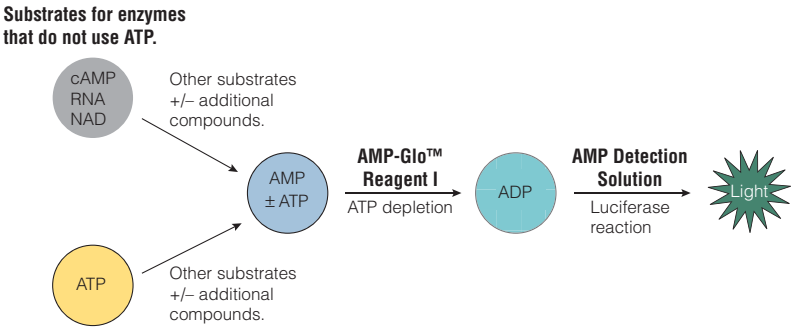


Figure 1. AMP-Glo™ Assay principle. The AMP-Glo™ Assay can be used to detect activity of enzymes that catalyze any reaction that produces AMP as a reaction product, including enzymes that do not use ATP as a substrate (e.g., cAMP-specific PDE, polyA-deadenylases, ribonucleases, bacterial DNA ligase) as well as enzymes that use ATP as a substrate (e.g., ubiquitin ligase, aminoacyl tRNA synthetase, eukaryotic DNA ligase, succinyl CoA synthetase). After completing the enzymatic reaction, adding AMP-Glo™ Reagent I terminates the reaction, removes any remaining ATP, and converts AMP to ADP. Adding AMP Detection Solution drives the conversion of ADP to ATP and the detection of ATP through the luciferase reaction. The amount of AMP produced by the reaction is proportional to the light measured and can be extrapolated using a standard curve.

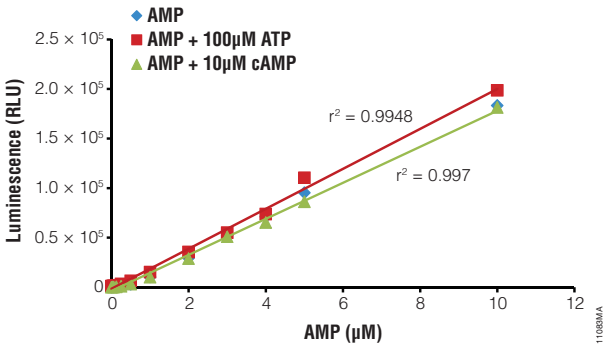


Figure 2. Titration of purified AMP. Reactions were assembled with the indicated concentrations of pure AMP in a low-volume, 384-well plate. AMP was titrated alone, with 100µM ATP or with 10µM cAMP. The AMP-Glo™ Assay was performed as described in Section 4.A. Data were collected using a plate-reading luminometer. Each point represents the average of four separate reactions, and error bars represent standard deviations. Data analysis was performed using Excel®, Microsoft Office Software version 2007.

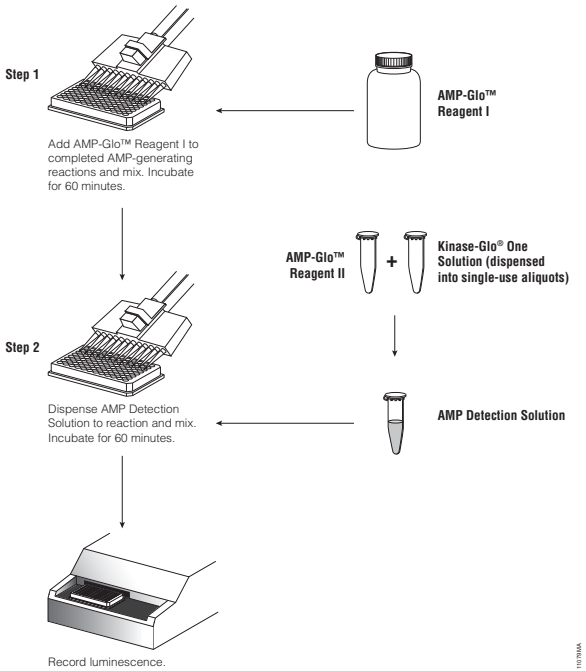


Figure 3. Overview of the AMP-Glo™ Assay Protocol.

2. Product Components and Storage Conditions

Product	Size	Cat.#
AMP-Glo™ Assay	1,000 assays	V5011

The system contains sufficient reagents for 1,000 assays in a low-volume 384-well plate format using, 5µl of enzyme reaction, 5µl of AMP-Glo™ I Reagent, and 10µl of AMP Detection Solution. The system also can be used in standard 96-well plates (25µl: 25µl: 50µl) for a total of 200 assays. Includes:

- 100µl AMP, 10mM
- 500µl Ultra Pure ATP, 10mM
- 5ml AMP-Glo™ Reagent I
- 100µl AMP-Glo™ Reagent II
- 10ml Kinase-Glo® One Solution

Product	Size	Cat.#
AMP-Glo™ Assay	10,000 assays	V5012

The system contains sufficient reagents for 10,000 assays in a low-volume 384-well plate format using, 5µl of enzyme reaction, 5µl of AMP-Glo™ I Reagent, and 10µl of AMP Detection Solution. The system also can be used in standard 96-well plates (25µl: 25µl: 50µl) for a total of 2,000 assays. Includes:

- 1ml AMP, 10mM
- 5ml Ultra Pure ATP, 10mM
- 50ml AMP-Glo™ Reagent I
- 1ml AMP-Glo™ Reagent II
- 100ml Kinase-Glo® One Solution

Product	Size	Cat.#
AMP-Glo™ Assay	50,000 assays	V5013

The system contains sufficient reagents for 50,000 assays in a low-volume 384-well plate format using, 5µl of enzyme reaction, 5µl of AMP-Glo™ I Reagent, and 10µl of AMP Detection Solution. The system also can be used in standard 96-well plates (25µl: 25µl: 50µl) for a total of 10,000 assays. Includes:

- 5ml AMP, 10mM
- 5 × 5ml Ultra Pure ATP, 10mM
- 250ml AMP-Glo™ Reagent I
- 5ml AMP-Glo™ Reagent II
- 500ml Kinase-Glo® One Solution

Storage Conditions: Store the system at –30 to –10°C. Before use, thaw all components completely at room temperature except for the AMP-Glo™ Reagent II, which should be kept on ice after thawing. Once thawed, mix all components thoroughly before use. Once prepared, the Kinase-Glo® One Solution should be dispensed into aliquots and stored at –20°C. See the product label for expiration date.

3. Setting Up the AMP-Glo™ Assay

Note: We strongly recommend performing an AMP standard curve for each set of experiments (see Section 3.D).

3.A. Materials to Be Supplied by the User

- enzyme reaction buffers: used for enzyme dilution and for substrate/compound dilution
- test compounds of interest prepared in reaction buffer
- white plate with solid bottom (do not use black plates or clear plates.)
- pipettes: multichannel pipettes or automated pipetting station
- luminometer: be sure the luminometer has the proper software program, sufficient sensitivity, and broad linear range such as the GloMax® luminometers

3.B. Guideline Volumes for the AMP-Glo™ Assay

The AMP-Glo™ Assay can be performed with different plate formats; the recommendations below show the guideline volume setup per well (Table 1). Volume size between Step 1 and Step 2 also may be changed if you follow the volume guideline and the volume ratio for Reagent I and AMP Detection Solution is kept at 1:2.

Table 1. General Assay Volume Format for AMP-Glo™ Assay.

Step	Description	96-Well Plate	Low-Volume 96- Well or 384-Well Plate	Low-volume 384-Well or 1536-Well Plate
1	Enzyme +/– Compounds	12.5µl	5µl	2.5µl or less
2	2X Substrate (s)	12.5µl	5µl	2.5µl or less
3	AMP-Glo™ Reagent I	25µl	10µl	5µl or less
4	AMP Detection Solution	50µl	20µl	10µl or less

3.C. Reagent Preparation

All components except AMP-Glo™ Reagent II should be completely thawed at room temperature before use. Keep AMP-Glo™ Reagent II on ice when not at –20°C. For your experiments, calculate the required volumes of each reagent, and increase or decrease the volumes appropriately. Here we give an example for how to prepare 1ml of solution. You should always use your own reaction buffer with appropriate substrates and cofactors when generating your standard curve and calculate the volume of each reagent to be used in the assay before preparing the reagents. The following volumes are suggested for the dilution series described in Section 3.D.

AMP Dilution Buffer with or without 100µM ATP or 10µM cAMP (2.5ml total volume)

Component	Volume
enzyme reaction buffer	2,475µl
10mM Ultra Pure ATP (or 1mM cAMP)	25µl

10µM AMP Solution (1ml total volume)

Component	Volume
AMP Dilution Buffer (with 10mM Ultra Pure ATP or 1mM cAMP)	999µl
10mM AMP	1µl
Briefly mix solution using a vortex mixer.	

AMP Detection Solution (1ml total volume)

Keep Kinase-Glo® One Solution on ice. The Kinase-Glo® One-Solution can be dispensed into single-use aliquots and stored at –20°C. **One hour before mixing** place an aliquot of Kinase-Glo® at room temperature to equilibrate, freeze the remaining solution.

Note: Add AMP-Glo™ Reagent II to Kinase-Glo® One solution immediately before use. Prepare only enough AMP Detection Solution required for the experiment.

Component	Volume
AMP-Glo™ Reagent II	10µl
Kinase-Glo® One Solution	1ml

3.D. Generating an AMP Standard Curve

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

1. Add 100µl of AMP Dilution Buffer to wells A2 through A12 of a 96-well plate.

   Note: Depending on the requirements of your system you may use AMP Dilution Buffer with ATP, cAMP, other appropriate substrate or nothing added.

2. Add 200µl of the AMP Solution prepared to well A1.

3. Perform a serial twofold dilution by transferring 100µl from well A1 to well A2 in column 2 and 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 AMP to the no-AMP control reactions in well A12.

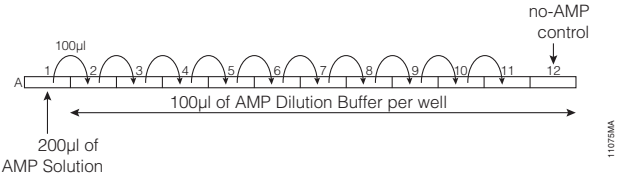


Figure 4. Dilution scheme for standard curve.

4. Transfer the indicated volume of each AMP standard from the 96-well plate to the wells reserved for the AMP standard curve on your assay plate. Proceed immediately to the assay protocol (Section 4.A).

   96-well assay plate: transfer 25µl of standard
   384-well assay plate: transfer 10µl of standard
   low-volume 384-well or 1536-well assay plate: transfer 5µl or less

3.E. Analysis of Standard Curve Data

The luminescence output of the assay is proportional to the concentration of AMP in the standard curve so that luminescence readout can be directly compared to those AMP concentrations generated in the sample.

1. For the AMP standards, calculate the difference in relative luminescence units (ΔRLU) between the RLU at each concentration and the RLU of the no-AMP control. Plot the ΔRLU for each standard on the Y axis against each corresponding AMP concentration on the X axis. This will generate the standard AMP curve used to calculate the AMP concentration in the unknown sample.
2. Calculate ΔRLU for the unknown sample with and without enzyme in the test reaction, and use the ΔRLU to calculate AMP concentration from the standard curve as described above.

4. AMP-Glo™ Assay Protocols

4.A. Performing the AMP-Glo™ Assay

Before performing the AMP-Glo™ Assay, calculate the volume of AMP-Glo™ Reagent I and Kinase-Glo® One Solution required for your experiments. You will need these reagents to reach room temperature before use. Prepare reagents (Section 3.C), and AMP standards as described in Section 3.D Return the remaining AMP-Glo™ Reagents and Kinase-Glo® One Solution to –30°C to –10°C.

1. Calculate the volume of AMP Detection Solution required. Prepare the AMP Detection Solution as described in Section 3.B., adjusting volumes as needed based on your experimental needs. Mix all reagents gently by inverting the tubes. Do not vortex.
2. Set up the AMP-generating reactions according to the guidelines recommended in Table 1. Incubate for desired length of time at desired temperature.
3. Add the indicated volume of AMP-Glo™ Reagent I to all wells, and mix the plate by shaking for 1–2 minutes. Incubate the plate at room temperature for 60 minutes.

   96-well assay plate: 25µl
   384-well assay plate: 10µl
   low-volume 384-well or 1536-well assay plate: 5µl or less

   Notes:

   Ensure that reagents are fully mixed and at the bottom of the wells by briefly centrifuging the plate.

   For low-volume 384-well or 1536-well plates, you can start with less than 5µl for the enzyme/substrate/compound mixture and add an equal volume of AMP-Glo™ Reagent I to maintain the 1:1 ratio.

4. Add the indicated volume of room-temperature equilibrated AMP Detection Solution to all wells. Mix the plate by shaking for 1–2 minutes, and incubate at room temperature for 60 minutes.

   96-well assay plate: 50µl
   384-well assay plate: 20µl
   low-volume 384-well or 1536-well assay plate: 10µl or less

   Note: Ensure that reagents are fully mixed and at the bottom of the wells by briefly centrifuging the plate.

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

4.B. Enzyme Titration (with or without ATP as a substrate)

We developed this protocol to determine the EC_{50} value of any enzyme or enzyme activity that uses ATP or other non-ATP substrate such as NAD or cAMP. This protocol describes running the assay using low-volume 384-well plates. Guidelines for preparing buffers and the appropriate volume of test compounds are given in Section 3.B and 3.C. Representative data show titration with four different biochemical reactions, including reactions with ATP as a cofactor and reactions without ATP (e.g., PDE482).

Preparation of Reagents

Assay Components

Before performing the AMP-Glo™ Assay, prepare reagents and/or AMP standards as described in Sections 3.C and 3.D. Calculate the volume of AMP-Glo™ Reagent I, Reagent II and Kinase-Glo® One Solution required for your experiments, and allow AMP-Glo™ Reagent I, Reagent II, and Kinase-Glo® One Solution to reach room temperature before use. Return the remaining AMP-Glo™ reagents and Kinase-Glo® One Solution to –30°C to –10°C.

Enzyme Dilution Buffer (1X reaction buffer)

Your enzyme reaction buffer should contain 0.1mg/ml BSA or other protein carrier and 0.5–1mM DTT.

Enzyme

Prepared in enzyme reaction buffer (100µl)

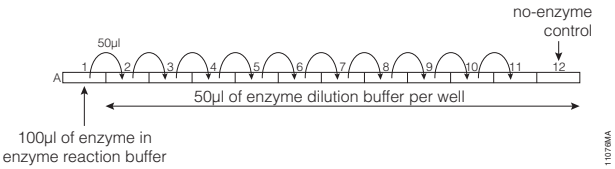
Note: The final concentration of ATP in the reaction is 100µM. For cAMP or NAD+, the final concentration in the reaction is 10µM.

2X substrates in enzyme reaction buffer (1ml total volume)

Component	Volume
10mM Ultra Pure ATP	20µl
1mM cAMP or 1mM NAD+	20µl
Other Substrate(s)	Xµl
Final volume of	1ml

Enzyme Titration (1/2 dilution factor)

1. Add 50µl of enzyme dilution buffer to wells A2 through A12 of a 96-well plate.
2. Add 100µl of the enzyme in enzyme reaction buffer.
3. Perform a twofold serial dilution of enzyme, transferring 50µl from A1 to A2 and pipetting to mix. Transfer 50µl from well A2 to well A3; mix well. Repeat for wells A4 through A11 (Figure 6). Discard the extra 50µl from ell



A11. Do not add enzyme to control reactions in A12.

Figure 6. Enzyme titration dilution scheme.

Assay Protocol

Here we provide a protocol with suggested volumes. Assay volumes can be adjusted as long as recommended ratios of components are maintained (Table 1).

1. Pipet the indicated volume of 2X substrate(s) in enzyme reaction buffer to the solid-white assay plate.

   96-well assay plate: 12.5µl
   384-well assay plate: 5µl
   low-volume 384-well or 1536-well assay plate: 2.5µl or less

2. Start the enzyme reaction by transferring the indicated volume of each enzyme titration from the 96-well plate to wells with 2X substrate in 1X reaction buffer.

   96-well assay plate: 12.5µl
   384-well assay plate: 5µl
   low-volume 384-well or 1536-well assay plate: 2.5µl or less

3. Mix the plate by shaking for 1–2 minutes. Incubate the plate at room temperature (or at the desired temperature) for the desired time.

   Note: Ensure that reagents are fully mixed and at the bottom of the wells by briefly centrifuging the plate.

4. Add the indicated volume of room-temperature AMP-Glo™ Reagent I to all wells of the assay plate. Mix the plate by shaking for 1–2 minutes, and incubate the plate at room temperature for 60 minutes.

   96-well assay plate: 25µl
   384-well assay plate: 10µl
   low-volume 384-well or 1536-well assay plate: 5.0µl or less

Notes:

Ensure that reagents are fully mixed and at the bottom of the wells by briefly centrifuging the plate.

For the low-volume 384-well or 1536-well plates, you can start with a lower volume than 5µl for the enzyme/substrate/compound mixture and add an equal volume of AMP-Glo™ Reagent I to maintain the 1:1 ratio.

5. Add the indicated volume of room-temperature adjusted AMP Detection Solution to all wells. Mix the plate by shaking for 1–2 minutes, and incubate the plate at room temperature for 60 minutes.

   96-well assay plate: 50µl
   384-well assay plate: 20µl
   low-volume 384-well or 1536-well assay plate: 10.0µl or less

   Note: Ensure that reagents are fully mixed and at the bottom of the wells by briefly centrifuging the plate.

6. Measure the luminescence with a plate-reading luminometer. Determine the EC_{50} using GraphPad Prism® software or another appropriate statistical package.

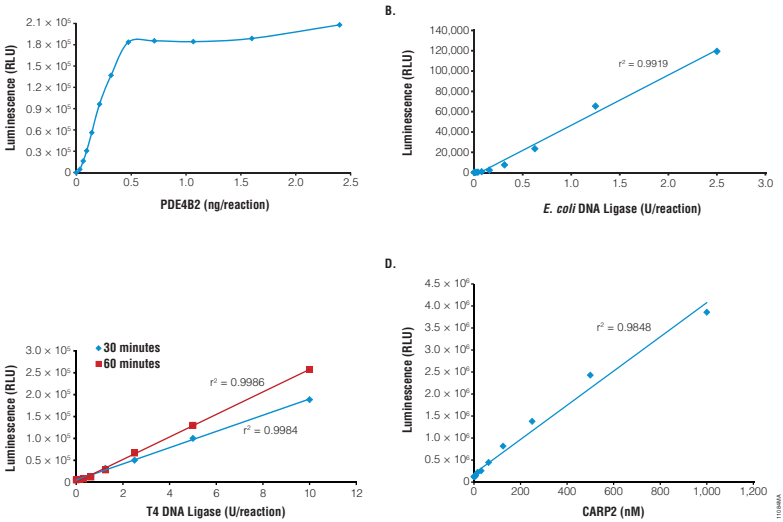


Figure 5. Titration of various enzymes. Panel A. PDE4B2 was titrated with 10µM cAMP. The enzyme reaction proceeded for 60 minutes. **Panel B**. DNA ligase (*E. coli*) reaction was performed in the presence of 10µM oliogos and 10µM NAD for 10 minutes. **Panel C.** T4 DNA ligase reaction proceeded for 30 minutes in the presence of 0.5µg pBR322 DNA and 100µM ATP. **Panel D.** Ubiquitin ligase reaction (CARP2) was carried out in the presence of 100µM ATP for two hours. All reactions were incubated at room temperature in a white, solid low-volume, 384-well plate. The AMP-Glo™ Assay was performed as described in Section 4.A using varying enzyme concentrations. Each point is an average of two experiments, and the error bars represent the standard deviations. Data analysis was performed with Microsoft Excel® 2007 program and GraphPad Prism® software,version 5, for Windows® using a sigmoidal dose-response (variable slope) equation.

4.C. Determining IC_{50} Values

We developed this protocol to determine the IC_{50} value for cAMP-specific PDEs, such as cilostamide for cAMP-specific PDE3B or with other PDE inhibitors. The IC_{50} value determined for cilostamide for cAMP-dependent PDE3B was 2.6µM, which is similar to the published IC_{50} value (5).

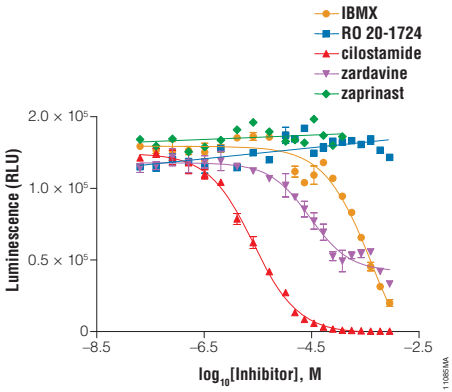


Figure 7. Determination of IC_{50} for various PDE inhibitors. The PDE reaction was performed with 20µM cAMP as a substrate in a solid white, low-volume 384-well plate. The PDE3B reaction was incubated 60 minutes at room temperature (23ºC). The AMP-Glo™ Assay was performed as described in Section 4.C. Each point represents the average of three trials; 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 Reagents

Before performing the AMP-Glo™ Assay, prepare reagents and/or AMP standards as described in Sections 3.C and 3.D. Calculate the volume of AMP-Glo™ Reagent I, Reagent II, and Kinase-Glo® One Solution required for your experiments, and allow AMP-Glo™ Reagent I, Reagent II, and Kinase-Glo® One Solution to reach room temperature before use. Return the remaining the AMP-Glo™ reagents and Kinase-Glo® One Solution to –30°C to –10°C.

Test Compound Dilution Buffer

Enzyme reaction buffer with same amount of solvent.

Note: 1X reaction buffer should contain 0.1mg/ml BSA or other protein carrier with 0.5–1mM DTT.

Enzyme

Prepared in enzyme reaction buffer.

2.5X substrates in enzyme reaction buffer (1ml total volume)	
Component	**Volume**
1mM cAMP	50µl (final concentration 50µM cAMP)
Final volume of	1ml

Preparation of Test Compound

When preparing the test compound, use a stock solution at an appropriate concentration in the proper solvent. (For solvent guidelines see Troubleshooting Section "Low luminescent signal" Section 5).

1. Prepare a 250µl of 5X (450µM) solution of test compound in enzyme reaction buffer and mix well.
2. Add 100µl of test compound dilution buffer to wells A2 through A12 of a 96-well plate.
3. Add 200µl of 5X compound solution to well A1.
4. Prepare a twofold serial dilution of test compound by transferring 100µl from well A1 to well A2, pipetting to mix. Transfer 100µl to well A3. Repeat for wells A4 through A11. See Figure 8. Discard the extra 100µl from well A11. Do not add any compound to the control reactions in well A12.

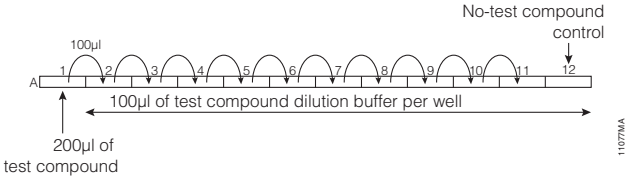


Figure 8. Dilution scheme for test compound.

Assay Protocol

This protocol can be adapted to determine the IC_{50} value for any inhibitor of enzymes that produce AMP as a product.

Here we provide a protocol with suggested volumes. Assay volumes can be adjusted as long as recommended ratios of components are maintained (Table 1).

1. Pipet the indicated volume of the 5X test compound in enzyme reaction buffer into the solid white assay plate:

   96-well assay plate: 5µl
   384-well assay plate: 2µl
   low-volume 384-well or 1536-well assay plate: 1µl or less

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

2. Transfer the indicated volume of enzyme solution to the assay plate, mix well and incubate for 5–10 minutes at room temperature.

   96-well assay plate: 10µl
   384-well assay plate: 4µl
   low-volume 384-well or 1536-well assay plate: 2µl or less

   Note: Ensure that reagents are fully mixed and at the bottom of the wells by briefly centrifuging the plate.

3. Start the enzyme reaction by transferring the indicated volume of 2.5X substrate in 1X reaction buffer to the assay plate with enzyme and inhibitor.

   96-well assay plate: 10µl
   384-well assay plate: 4µl
   low volume 384-well or 1536-well assay plate: 2µl or less

4. Mix the plate by shaking for 1–2 minutes. Incubate the plate at room temperature (or at the desired temperature) for the desired time.

   Note: Ensure that reagents are fully mixed and at the bottom of the wells by briefly centrifuging the plate.

5. Add the indicated volume of room temperature-adjusted AMP-Glo™ Reagent I to all wells. Mix the plate by shaking for 1–2 minutes, and incubate at room temperature for 60 minutes.

   96-well assay plate: 25µl
   384-well assay plate: 10µl
   low-volume 384-well or 1536-well assay plate: 5µl

   Notes:

   Ensure that reagents are fully mixed and at the bottom of the wells by briefly centrifuging the plate.

   For low-volume, 384-well or 1536-well plates, you can begin with a lower volume than 5µl for the enzyme/substrate/compound mixture and add an equal volume of AMP-Glo™ Reagent I to maintain the 1:1 ratio.

   This initial reaction, which produces the AMP, should be equilibrated to room temperature, since variations in temperature will affect the activity of the luciferin/luciferase reaction.

6. Add the indicated volume of room temperature-adjusted AMP Detection Solution to all wells. Mix the plate by shaking for 1–2 minutes. Incubate the plate at room temperature for 60 minutes.

   96-well assay plate: 50µl
   384-well assay plate: 20µl
   low-volume 384-well or 1536-well assay plate: 10µl

   Note: Ensure that reagents are fully mixed and at the bottom of the wells by briefly centrifuging the plate.

7. Measure the luminescence with a plate-reading luminometer. Determine the EC_{50} using GraphPad Prism® software or other statistical software.

4.D. Determining Z´ Factor

This protocol allows you to determine the Z´ factor (5) for the AMP-Glo™ Assay in a reaction system with or without ATP as a substrate. Representative data using AMP with or without 100µM ATP are shown in Figure 9.

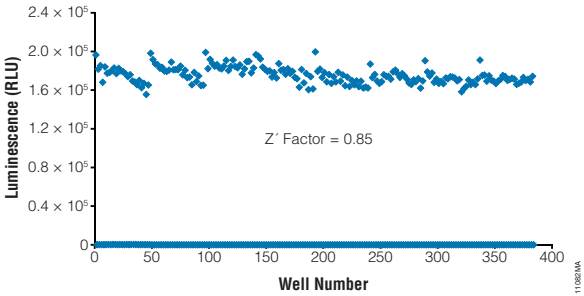


Figure 9. A scatter plot to determine the Z´ factor of the AMP-Glo™ Assay
PDE4B2 reaction at 0.7ng/reaction was run in the presence of 20µM cAMP for 60 minutes at room temperature in a low-volume 384-well plate. The AMP-Glo™ Assay was performed as described in Section 4.D using the Deerac Fluidics® Equator™ HTS noncontact dispenser. Luminescence was measured using the PHERAstar high-end multiwell plate reader (BMG Labtech). Data analysis was performed using Sigma Plot/Sigma Stat, version 9.0 for Windows®.

Reagent Preparation

Before performing the AMP-Glo™ Assay, prepare reagents and/or AMP standards as described in Sections 3.C and 3.D. Calculate the volume of AMP-Glo™ Reagent I, Reagent II, and Kinase-Glo® One Solution required for your experiments, and allow AMP-Glo™ Reagent I, Reagent II and Kinase-Glo® One Solution to reach room temperature before use. Return the remaining AMP-Glo™ reagents and Kinase-Glo® One Solution to –30°C to –10°C.

Prepare a **1.5µM AMP solution** in either enzyme reaction buffer or distilled water, and 1µM AMP with or without 100µM ATP. Mix using a vortex mixer.

1. Set up the "no-AMP" reactions by transferring the indicated volume of either 1X reaction buffer or distilled water with or without 100µM ATP to each well in half of the plate.

   96-well assay plate: 25µl
   384-well assay plate: 10µl
   low-volume 384-well or 1536-well assay plate: 5µl or less

4.D. Determining Z´ Factor (continued)

2. Set up the AMP reactions by adding the indicated volume of the 1.5µM AMP solution in either enzyme reaction buffer or distilled water with or without 100µM ATP to the wells in the other half of the plate.

   96-well assay plate: 25µl
   384-well assay plate: 10µl
   low-volume 384-well or 1536-well assay plate: 5µl or less

3. Add the indicated volume of AMP-Glo™ Reagent I to all wells and mix the plate by shaking for 1–2 minutes. Incubate the plate at room temperature for 60 minutes.

   96-well assay plate: 25µl
   384-well assay plate: 10µl
   low-volume 384-well or 1536-well assay plate: 5µl or less

   Note: Ensure that reagents are fully mixed and at the bottom of the wells by briefly centrifuging the plate..

4. Add the indicated volume of room temperature-adjusted AMP Detection Solution to all wells. Mix the plate by shaking for 1–2 minutes, and incubate the plate at room temperature for 60 minutes.

   96-well assay plate: 50µl
   384-well assay plate: 20µl
   low-volume 384-well or 1536-well assay plate: 10µl or less

   Note: Ensure that reagents are fully mixed and at the bottom of the wells by briefly centrifuging the plate..

5. Measure the luminescence with a plate-reading luminometer. Calculate Z´ Factor value using GraphPad Prism® software or other appropriate statistical package.

5. 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
Higher luminescent signal than expected	You may have ATP with ADP contamination in one or more reaction component. •To address this issue, use the Ultra Pure ATP provided in the kit. Be sure Ultra Pure ATP is handled properly: Avoid multiple freeze-thaw cycles; do not use expired components. •Use clean laboratory space and pipettes that are free of ADP and other nucleotides. Use aerosol-resistant pipette tips. Decontaminate surfaces by wiping with detergent solution or ethanol, and rinse 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.
	Removal of ATP by AMP-Glo™ Reagent I was incomplete. •Be sure to prepare and store the AMP-Glo™ Reagent I as described in Sections 2 and 3.C. •Ensure that reagents are fully mixed and at the bottom of the wells by briefly centrifuging the plate.
	The test compound inhibits AMP-Glo™ Reagent I. During a screen of the LOPAC library (Sigma), which contains 1,280 compounds, we experienced less than 0.2% false hits, with only two of the compounds inhibiting AMP-Glo™ Reagent I. To test for test compound inhibition of AMP-Glo™ Reagent I, assemble two reactions with 100µM ATP. Add the test compound to one of the reactions but not the other. Compare the luminescence from these reactions. A higher luminescence in the presence of the test compound indicates that it may inhibit AMP-Glo™ Reagent I.
Low luminescent signal	Plate color. We recommend using only white, solid-bottom plates. Do not use black or clear plates.

Troubleshooting (continued)

Symptoms	Causes and Comments
Low luminescent signal (continued)	Improper storage of reagents. Store the Kinase-Glo® One Solution and AMP Detection Solution as described in Sections 2 and 3.C. Dispense the Kinase-Glo® One Solution into single-use aliquots and store them at –20°C. Mix reagents well before use and avoid repeated freeze-thaw cycles of reagents.
	Test compound inhibited AMP Detection Solution or ATP generating reaction. •To test for inhibition of the AMP Detection Solution, assemble reaction sets with AMP (10µM) with and without test compound and compare luminescence. A decrease or no luminescence in the presence of the test compound is an indication of inhibition of AMP Detection Solution. Compare luminescence to that from a reaction with AMP without the test compound. Low light output indicates inhibition of luciferase or AMP Detection Solution. •To test for luciferase inhibition, perform the AMP-Glo™ Assay with the test compound directly with the AMP-Glo™ Detection System using 10µM ATP (omit the AMP-Glo™ Reagent I). Add an equal volume of Kinase-Glo® One Solution to each reaction set. Low light output in the presence of the test compound indicates luciferase 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 assess the effect of the solvent on assay performance. Performance of the AMP-Glo™ Assay is not affected by the presence of up to 5% acetone or 5% DMSO.

6. References

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2. Guo, M. *et al.* (2010) New functions of aminoacyl-tRNA synthetases beyond translation. *Nature Rev. Mol. Cell. Biol.* **11**, 668–74.

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4. Robinson, P.A. and Ardley, H.C. (2011) Ubiquitin-protein ligases. *J. Cell Sci.* **117**, 5191–4.

5. Galperin, M.Y. and Koonin, E.V. (1997) A diverse superfamily of enzymes with ATP-dependent carboxylate-amine/thiol ligase activity. *Protein Science* **6**, 2639-43.

6. Steinberg, G.R. and Kemp, B.E. (2009) AMPK in health and disease. *Phyisol. Rev.* **89**, 1025–78.

7. Related Products

Product	Size	Cat.#
ADP-Glo™ Kinase Assay	1,000 assays	V9101
ADP-Glo™ Max Assay	1,000 assays	V7001
Kinase-Glo® Luminescent Kinase Assay	10ml	V6711
Kinase-Glo® Plus Luminescent Kinase Assay	10ml	V3771
Kinase-Glo® Max Luminescent Kinase Assay	10ml	V6071

Product	Size	Cat.#
GloMax® 96 Multiwell plate Luminometer	1 each	E6501
GloMax® 96 Multiwell plate Luminometer with single injector	1 each	E6511
GloMax® 96 Multiwell plate Luminometer with dual injectors	1 each	E6521
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

[a]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.

[b]Licensed from Lonza Nottingham Ltd. under U.S. Pat. Nos. 6,599,711 and 6,911,319 and other pending and issued patents.