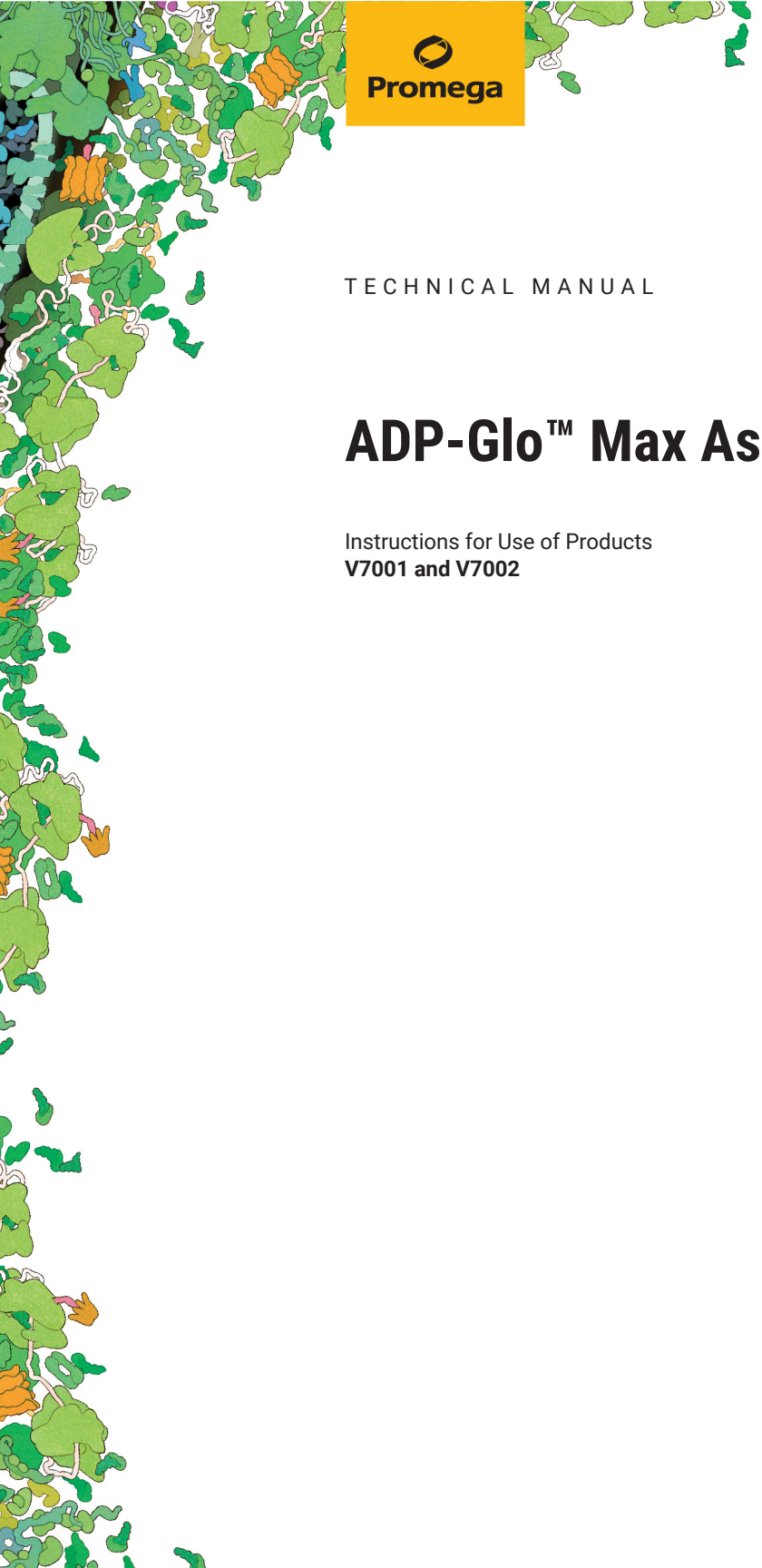


TECHNICAL MANUAL

ADP-Glo™ Max Assay

Instructions for Use of Products
V7001 and V7002

Revised 7/25
TM343

ADP-Glo™ Max Assay

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

Promega

1. Description

The ADP-Glo™ Max Assay[a,b] is a luminescent ADP detection assay that provides a universal, homogeneous, high-throughput screening method to measure ATPase or kinase activity by quantifying the amount of ADP produced in a reaction. The ADP-Glo™ Max Assay can be used to monitor the activity of virtually any ADP-generating enzyme (e.g., kinase or ATPase) when higher ATP concentration is required (up to 5mM). The ADP-Glo™ Max Assay is performed in a multiwell plate and can detect kinase or ATPase activity in a reaction volume as low as 5µl.

The assay is performed in two steps: first, after the completion of the ADP-producing reaction, an equal volume of ADP-Glo™ Reagent is added to terminate the reaction and deplete the remaining ATP. Second, the ADP-Glo™ Max Detection Reagent is added to simultaneously convert ADP to ATP, and the latter is converted to light using a luciferase/luciferin reaction (Figures 1 and 2). Luminescence is read using a luminometer and is correlated to ADP concentrations by using an ATP-to-ADP conversion curve (Figure 3). This assay is sensitive enough to detect very low amounts of ADP and can detect generated ADP in a reaction containing up to 5mM ATP (Figure 3). The luminescent signal generated is proportional to the ADP concentration produced and is correlated with the kinase or ATPase activity (Figure 4). Because of its high sensitivity, the ADP-Glo™ Max Assay can be used with essentially any ATPase and especially those with low enzyme turnover. Therefore, the assay is ideal for high-throughput screening because even the use of a small amount of enzyme that doesn't convert much ATP to ADP still results in a high Z´-factor value (Figures 5 and 6).

The ADP-Glo™ Max Assay relies on the properties of a proprietary thermostable luciferase (Ultra-Glo™ Recombinant Luciferase) that is formulated to generate a stable "glow-type" luminescent signal and improve performance across a wide range of assay conditions. The signal, which is produced by the luciferase reaction initiated by adding the ADP-Glo™ Max Detection Reagent, is stable for more than 3 hours (data not shown). This extended stability allows batch-mode processing of multiple plates. In addition, the unique combination of Ultra-Glo™ Recombinant Luciferase and proprietary formulations of the ADP-Glo™ Reagent and ADP-Glo™ Max Detection Buffer result in luminescence that is much less susceptible to interference from library compounds than other luciferase- or fluorescence-based assays (1,2). In addition to providing biochemical values (e.g., K_m of ATP or EC_{50} and IC_{50} for activity modulators) comparable to those reported in the literature (Figures 5 and 6), the ADP-Glo™ Max Assay can be used to study the mode of action of inhibitors at physiological levels (mM) of ATP (e.g., distinguish between ATP-competitive and noncompetitive inhibitors).

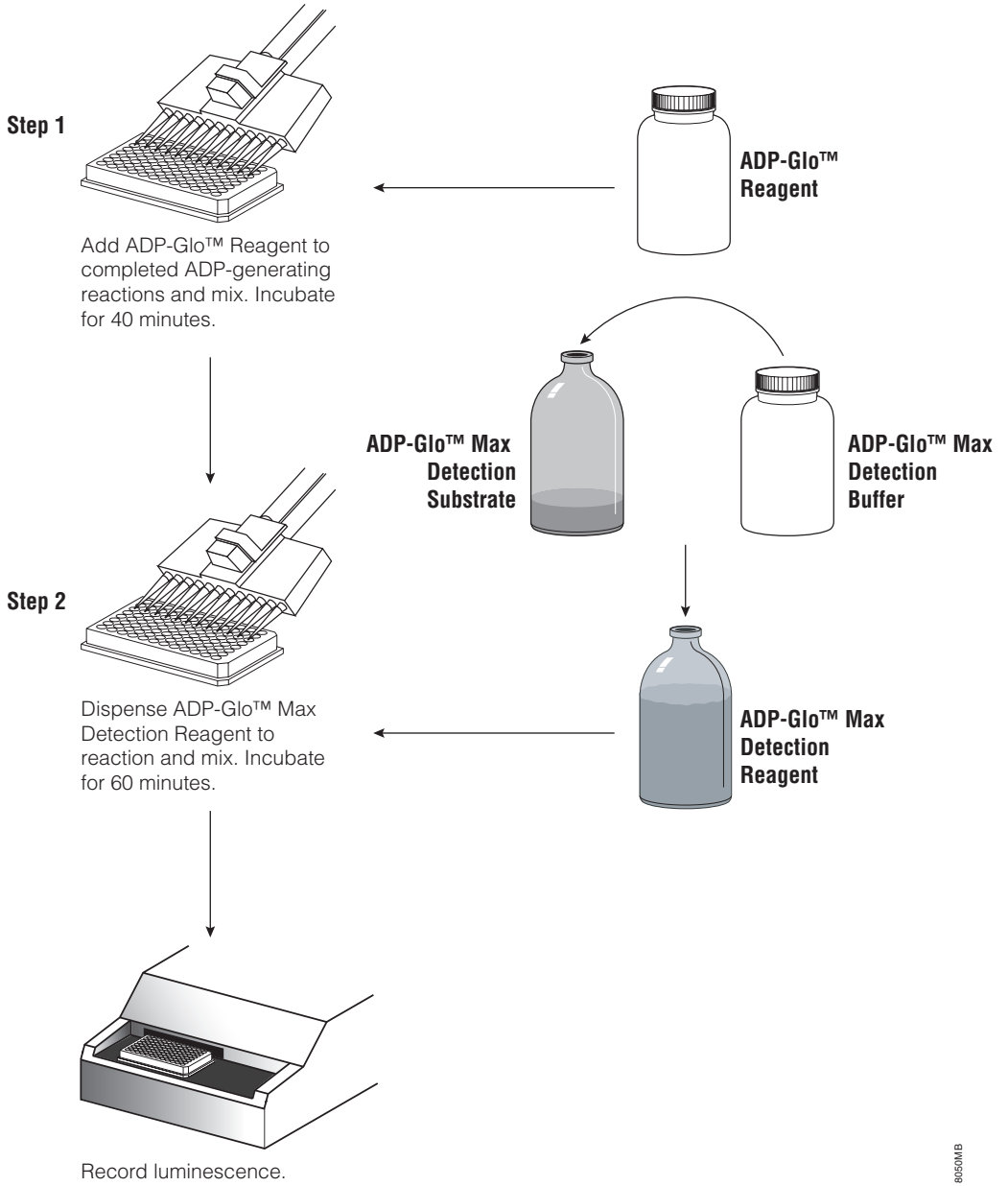


Figure 1. Overview of the ADP-Glo™ Max Assay procedure.

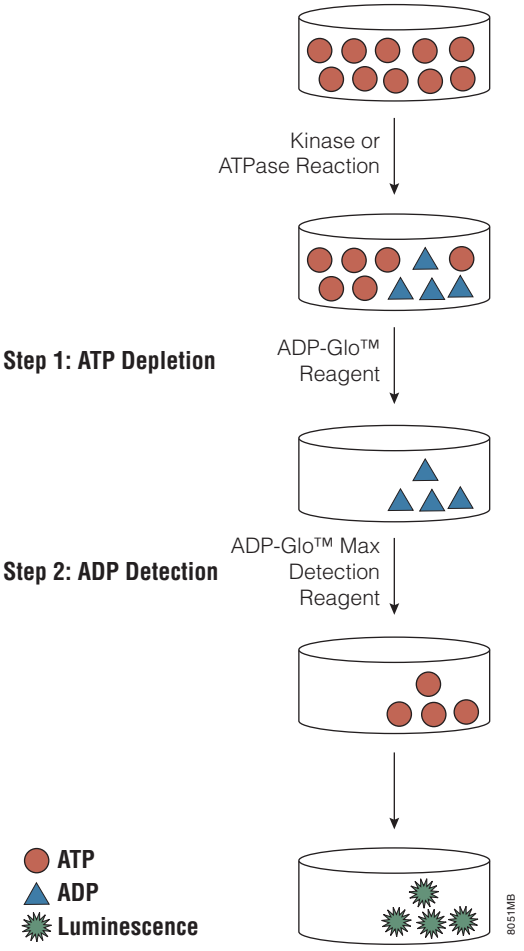
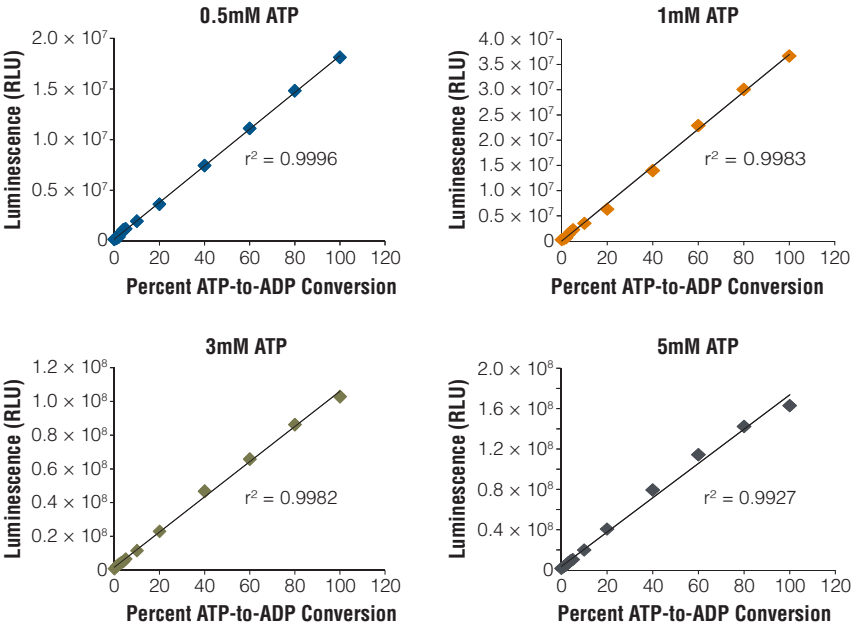


Figure 2. Principle of the ADP-Glo™ Max Assay. The assay is performed in two steps: 1) after the kinase or ATPase reaction, ADP-Glo™ Reagent is added to terminate the enzymatic reaction and to deplete the remaining ATP; and 2) the ADP-Glo™ Max Detection Reagent is added to convert ADP to ATP and allow the newly synthesized ATP to be measured using a luciferase/luciferin reaction. The light generated correlates with the amount of ADP generated in the kinase or ATPase assay, which is indicative of kinase or ATPase activity.

A.



B.

Signal-to-Background Ratios

[ATP + ADP]	Percent ADP in an ATP + ADP Mixture											
	100	80	60	40	20	10	5	4	3	2	1	0
0.5mM	114	94	70	47	23	12	8	7	5	3	2	1
1mM	138	113	86	53	24	13	8	7	5	2	2	1
3mM	113	95	72	51	25	13	7	6	5	4	3	1
5mM	102	89	71	50	25	12	7	6	5	3	2	1

9013MA

Figure 3. Sensitivity and linearity of the ADP-Glo™ Max Assay. Four ranges of ATP-to-ADP conversion curves were prepared as described in Section 3.B in a solid white 96-well plate using 25µl of reaction buffer A (Section 8), 25µl of ADP-Glo™ Reagent and 50µl of ADP-Glo™ Max Detection Reagent added at room temperature according to the protocol described in Section 4.B. Luminescence was recorded using a GloMax® 96 Microplate Luminometer. Values represent the mean of two replicates. **Panel A**. There is a linear relationship between the luminescent signal and the amount of ADP in the reaction buffer in all ATP +ADP concentration ranges used. **Panel B**. The ADP-Glo™ Max Assay is highly sensitive as demonstrated by the high signal-to-background ratios. The assay also generates high Z´-factor values (>0.7) even at low percent conversions (Table 1). Luminescence was measured 1 hour after adding ADP-Glo™ Max Detection Reagent. To monitor the signal stability, the luminescence measurement was repeated several times at one-hour intervals. The signal is stable over a period of 5 hours with ~20% increase after 3 hours, while the signal-to-background ratio does not change (data not shown). RLU = relative light units.

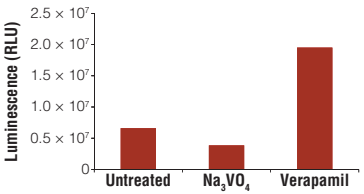
Promega

Table 1. Assay Performance Parameters at the Lowest ADP Percentages in the 5mM Series of ATP+ADP.

	Percent ADP in ATP + ADP Mixture			
	5%	4%	3%	2%
Signal-to-Background Ratio*	16.4	13.2	9.3	6.7
Signal-to-Noise Ratio*	145	115	78	53
Z´-Factor Value*	0.83	0.82	0.82	0.78

*Values produced from data generated using 96 and 128 replicates for the 0% controls and other conversions, respectively.

A.



B.

	Pgp Activity	
	Basal	Verapamil
Luminescence	2,761,090	15,658,717
Percent Conversion	1.4	7.8
Total ADP (pmol)	1,720	9,755
ADP (pmol/µg Pgp/minute)	4.3	24.4

C.

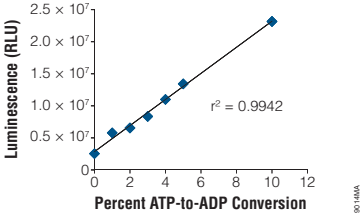


Figure 4. Stimulation of P-glycoprotein (Pgp) ATPase activity by verapamil. Untreated, 100µM sodium vanadate (Na_3VO_4) and 200µM verapamil-treated Pgp reactions were performed according to the protocol described in Section 5.B using 10µg of Pgp membranes and 1X Pgp reaction buffer (Section 8) in the presence of 5mM ATP. Luminescence was read on a GloMax® 96 Microplate Luminometer (**Panel A**). ADP produced by Pgp membranes in the presence or absence of verapamil (**Panel B**) was calculated using the 5mM ATP-to-ADP conversion curve performed at the same time (**Panel C**). Basal and stimulated Pgp ATPase activities were calculated by subtracting the values obtained in the Na_3VO_4 sample from the untreated sample (basal) or from the verapamil-treated sample (verapamil), respectively.

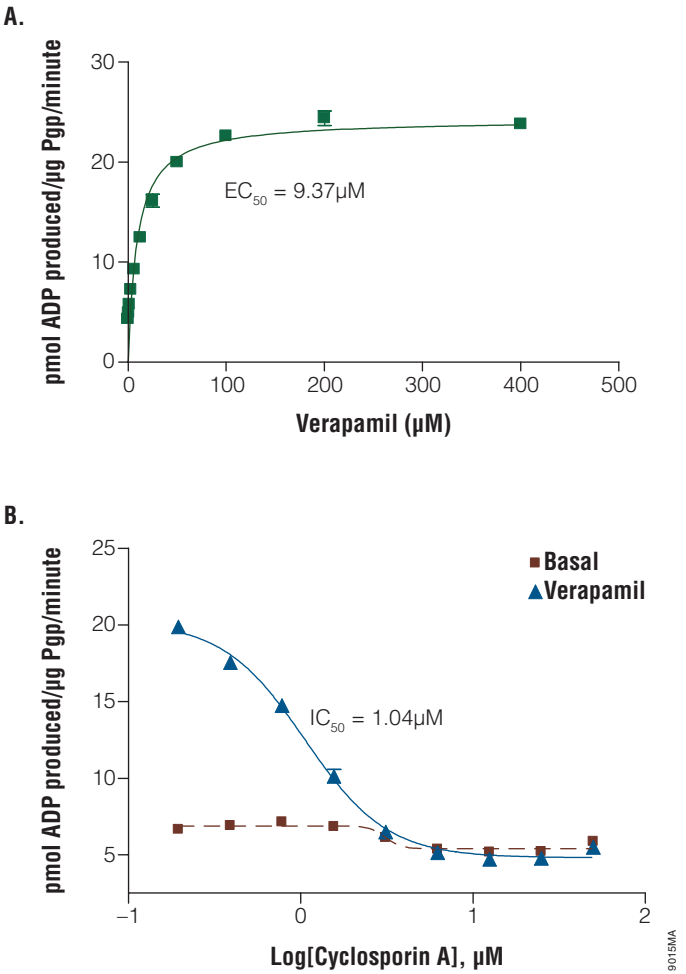
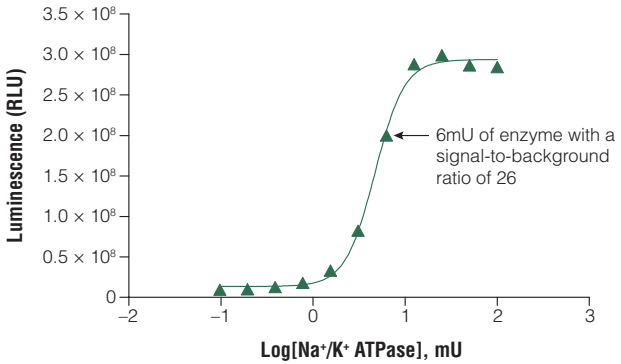
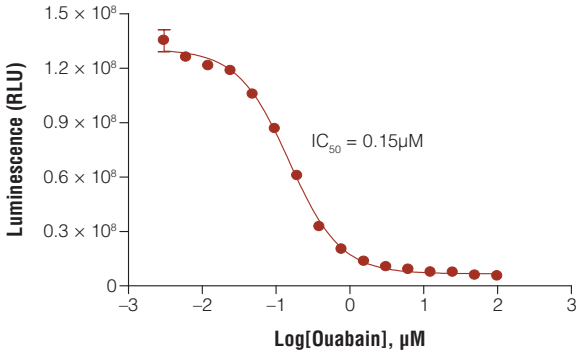


Figure 5. Effects of test compounds on basal and drug-stimulated Pgp ATPase activities. Panel A. The titration of the Pgp activator, verapamil, was performed in solid white, flat-bottom 96-well plates in a total volume of 25µl, and the range of verapamil concentrations indicated. **Panel B**. Cyclosporin A (Sigma-Aldrich Cat.# 30024) was tested at a range of concentrations for its capacity to inhibit Pgp basal and verapamil-stimulated ATPase activity. Pgp reactions were performed according to the protocol described in Section 5.B using 10µg of Pgp membranes and 1X Pgp reaction buffer in the presence of 5mM ATP. Luminescence was read on a GloMax® 96 Microplate Luminometer. Data points are the average of two determinations, and error bars represent the standard deviation. EC_{50} and IC_{50} values determined using the ADP-Glo™ Max Assay compare favorably to the values reported for these compounds in the literature (3–5). Curve fitting was performed using GraphPad Prism® software. Data analysis and calculation of the amount of ADP produced were performed as described in Figure 4 using a 5mM ATP-to-ADP conversion curve and subtracting the Na_3VO_4 values.

A.



B.



C.

Na⁺/K⁺ ATPase (mU/reaction)	100	50	25	12.5	6.3	3.1	1.6	0.8	0.4	0.2	0.1	0
Average RLU	2.85 × 10⁸	2.86 × 10⁸	2.99 × 10⁸	2.88 × 10⁸	200 × 10⁸	8.2 × 10⁷	3.3 × 10⁷	1.8 × 10⁷	1.3 × 10⁷	1.0 × 10⁷	9.4 × 10⁶	7.4 × 10⁶
Signal-to-Background Ratio	38.6	38.9	40.6	39.1	27.1	11.2	4.5	2.4	1.7	1.4	1.3	1.0

9016MA

Figure 6. Demonstration of ADP-Glo™ Max Assay performance using Na⁺/K⁺ ATPase. To define the amount of enzyme needed for subsequent assays, a titration of the Na⁺/K⁺ ATPase was performed in 25µl Na⁺/K⁺ ATPase Reaction Buffer (**Panel A**) according to Section 4. The Na⁺/K⁺ ATPase reaction was performed in the buffer supplemented with 2.5mM ATP at 37°C for 15 minutes. **Panel B**. Ouabain (Sigma-Aldrich Cat.# 03125) was tested at a range of concentrations for its capacity to inhibit the Na⁺/K⁺ ATPase activity using 6mU enzyme, because it was determined that this amount generates an amount of ADP in the linear range of the assay with a signal-to-background ratio (SB) of 26 (**Panel C**). ADP was detected according to Section 4.B using the ADP-Glo™ Max Assay (25µl:25µl:50µl ratio). Curve fitting was performed using GraphPad Prism® sigmoidal dose-response (variable slope) software. These data demonstrate the high performance of the assay when using low amounts of enzyme. Data points represent the average of duplicate wells.

2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
ADP-Glo™ Max Assay	**1,000 assays**	**V7001**

The system is sufficient for 1,000 assays if performed in 384-well plates using 5µl, 5µl and 10µl of an ATPase or kinase reaction, ADP-Glo™ Reagent and ADP-Glo™ Max Detection Reagent, respectively, per sample. The system also can be used in 96-well plates using 25µl:25µl:50µl for a total of 200 assays. Includes:

- 5ml ADP-Glo™ Reagent
- 10ml ADP-Glo™ Max Detection Buffer
- 1 vial ADP-Glo™ Max Detection Substrate (Lyophilized)
- 250µl Ultra Pure ATP, 100mM
- 2 x 500µl ADP, 10mM

PRODUCT	SIZE	CAT.#
ADP-Glo™ Max Assay	**10,000 assays**	**V7002**

The system is sufficient for 10,000 assays if performed in 384-well plates using 5µl, 5µl and 10µl of an ATPase or kinase reaction, ADP-Glo™ Reagent and ADP-Glo™ Max Detection Reagent, respectively, per sample. The system also can be used in 96-well plates using 25µl:25µl:50µl for a total of 2,000 assays. Includes:

- 50ml ADP-Glo™ Reagent
- 100ml ADP-Glo™ Max Detection Buffer
- 1 vial ADP-Glo™ Max Detection Substrate (Lyophilized)
- 2.5ml Ultra Pure ATP, 100mM
- 5ml ADP, 10mM

Storage Conditions: Store the system at –30°C to –10°C. Before use, thaw all components completely at room temperature. Once thawed, mix all components thoroughly before use. Because ATP is naturally prone to hydrolysis after freeze/thaw cycles dispense into single-use aliquots and store at –30°C to –10°C. Once prepared, dispense, ADP-Glo™ Max Detection Reagent (ADP-Glo™ Max Detection Buffer + Substrate) into aliquots and store at –30°C to –10°C. ADP-Glo™ Max Detection Buffer may form a precipitate when thawed. See Section 3.A for a protocol to dissolve any precipitate. For convenience, ADP-Glo™ Reagent and ADP-Glo™ Max Detection Reagent may be kept at room temperature (22°C) for 24 hours without loss of signal.

3. Before You Begin the ADP-Glo™ Max Assay

Materials to Be Supplied by the User

- solid **white**, multiwell plate (do not use black plates)
- multichannel pipette or automated pipetting station
- ADP-producing enzyme (e.g., ATPase or kinase)
- luminometer capable of reading multiwell plates
- plate shaker

Promega

3.A. Preparing the ADP-Glo™ Max Detection Reagent

ADP-Glo™ Max Detection Buffer Preparation

The ADP-Glo™ Max Detection Buffer may contain a precipitate depending on conditions used for storage and handling. There is no observed change in performance of the ADP-Glo™ Max Assay if the buffer contains a precipitate. However, to avoid clogging the pipette tips, the precipitate may be removed or solubilized according to the following steps.

1. Thaw ADP-Glo™ Max Detection Buffer at room temperature, and observe for the presence of precipitate.
2. If a precipitate is present, incubate the ADP-Glo™ Max Detection Buffer at 37°C with constant swirling for 15 minutes to dissolve the precipitate. Alternatively, remove the precipitate from the ADP-Glo™ Max Detection Buffer by carefully pipetting the supernatant from the bottle.

ADP-Glo™ Max Detection Reagent Preparation

1. Equilibrate the ADP-Glo™ Max Detection Buffer and ADP-Glo™ Max Detection Substrate to room temperature before use.
2. Transfer the entire volume of ADP-Glo™ Max Detection Buffer into the amber bottle containing ADP-Glo™ Max Detection Substrate to reconstitute the lyophilized substrate. This forms the ADP-Glo™ Max Detection Reagent.
3. Mix by gently vortexing, swirling or inverting the contents to obtain a homogeneous solution. The ADP-Glo™ Max Detection Substrate should go into solution in less than one minute.
4. The ADP-Glo™ Max Detection Reagent should be used immediately or dispensed into aliquots and stored at –30°C to –10°C. We have shown that the reconstituted reagent remains stable with no loss of signal after several freeze-thaw cycles.

3.B. Generating a Standard Curve for Conversion of ATP to ADP

ADP-Glo™ Max Assay can detect small changes in ATP-to-ADP conversion. Whether assaying a low-activity ATPase with low ATP turnover rate or using a small amount of enzyme that produces a small amount of ADP, a high signal-to-background ratio is obtained with the ADP-Glo™ Max Assay (Figure 3). The ADP-Glo™ Max Assay also produces high Z´-factor values (>0.7), even at low ATP-to-ADP conversions. An example of assay performance parameters, incluing Z´-factor values obtained with a 5mM ATP + ADP series are presented in Table 1. Z´ factors greater than 0.5 indicate excellent assay quality (6).

To estimate the amount of ADP produced in the ATPase reaction, we recommend creating a standard curve that represents the luminescence corresponding to the conversion of ATP to ADP (the ATP-to-ADP conversion curve) based on the ATP concentration used in the ATPase reaction. These conversion curves represent the amounts of ATP and ADP available in a reaction at the specified conversion percentage (Table 2). The standard samples used to generate an ATP-to-ADP conversion curve are created by combining the appropriate volumes of ATP and ADP stock solutions (Table 3). The sum of the ATP and ADP concentrations is denoted as "ATP + ADP" in this manual.

Note: Here we describe preparation of percent conversion series for four different concentrations of ATP + ADP (5, 3, 1, and 0.5mM). We recommend performing a conversion curve for the concentration of ATP that will be used in the ATPase or kinase reaction.

Table 2. Percent Conversion of ATP to ADP Represented by the Standard Curve.

%ADP	100	80	60	40	20	10	5	4	3	2	1	0
%ATP	0	20	40	60	80	90	95	96	97	98	99	100

1. Prepare 1.5ml of 5mM ATP and 600µl of 5mM ADP by diluting the supplied Ultra Pure ATP and ADP in suitable 1X reaction buffer (see $MgCl_2$ requirements in Section 4.A).

Use only the provided Ultra Pure ATP when performing the ADP-Glo™ Max Assay. Other sources of ATP may contain ADP that could result in higher background signals.

2. Prepare a dilution series of ATP + ADP. Add 40µl of 1X reaction buffer to wells B1–B12, 60µl to wells C1–C12 and 90µl to wells D1–D12 of a 96-well plate. This is your conversion curves preparative plate.
3. Combine the 5mM ATP and 5mM ADP solutions prepared in Step 1 in wells A1–A12 as indicated in Table 3 to simulate the ATP and ADP concentrations at each percent conversion (see Table 2). Mix well. **This is the 5mM series.**

Table 3. Preparation of the 5mM Series of ATP + ADP Standards.

Well Number	**A1**	**A2**	**A3**	**A4**	**A5**	**A6**	**A7**	**A8**	**A9**	**A10**	**A11**	**A12**
5mM ADP (µl)	150	120	90	60	30	15	7.5	6	4.5	3	1.5	0
5mM ATP (µl)	0	30	60	90	120	135	142.5	144	145.5	147	148.5	150

3.B. Generating a Standard Curve for Conversion of ATP to ADP (continued)

4. Dilute the samples in wells A1–A12 by transferring 60µl of the sample in well A1 to well B1, 60µl from well A2 to well B2, etc. Mix well. **This is the 3mM series.**
5. Dilute the samples in wells B1–B12 by transferring 30µl of the sample in well B1 to well C1, 30µl from well B2 to well C2, etc. Mix well. **This is the 1mM series.**
6. Dilute the samples in wells A1–A12 by transferring 10µl of the sample in well A1 to well D1, 10µl from well A2 to well D2, etc. Mix well. **This is the 0.5mM series.**
7. Transfer the amount needed (5 or 25µl) from the desired ATP + ADP series of the conversion curve preparative plate into wells of a new assay plate or the assay plate where the ATPase or kinase reactions are present.
8. Follow the ADP-Glo™ Max Assay protocol described in Section 4.B, starting at Step 2.

4. ADP-Glo™ Max Assay Protocols

Prior to performing the ADP-Glo™ Max Assay, prepare the reagents and ATP + ADP standards as described in Section 3. Calculate the volume of ADP-Glo™ Max Assay Reagents required for your experiments, and allow that volume to reach room temperature before use. Return the remaining ADP-Glo™ Reagent and ADP-Glo™ Max Detection Reagent to –30°C to –10°C. For information about optimizing the enzyme concentrations, refer to Section 4.C. The ADP-Glo™ Reagent and the ADP-Glo™ Max Detection Reagent may be kept at room temperature (22°C) for 24 hours without loss of signal.

4.A. Reaction Buffer Considerations

The ADP-Glo™ Max Assay is designed for use with 1X reaction buffer A (see Section 8 for composition) in the presence of the kinase or ATPase enzyme, ATP and substrate. Other buffers are used with different ATPases, and their compositions are listed in Section 8.

If other reaction buffer compositions are preferred, make sure that the $MgCl_2$ concentration is at least 5mM after adding the ADP-Glo™ Reagent to the enzyme reaction.

4.B. ADP-Glo™ Max Assay Protocol

The ADP-Glo™ Max Assay is performed in two steps once the ADP-producing reaction is complete as outlined in Figure 1. For 96-well plates, we recommend 25µl of ATPase or kinase reaction, 25µl of ADP-Glo™ Reagent and 50µl of ADP-Glo™ Max Detection Reagent for a total volume of 100µl. For 384-well plates, volumes are reduced fivefold to 5µl of reaction, 5µl of ADP-Glo™ Reagent and 10µl of ADP-Glo™ Max Detection Reagent. Other volumes may be used, provided the 1:1:2 ratio of enzyme reaction volume to ADP-Glo™ Reagent volume to ADP-Glo™ Max Detection Reagent volume is maintained. The ADP-Glo™ Max Assay Protocol for 96-well plates is outlined below.

Use the Ultra Pure ATP provided with the ADP-Glo™ Max Assay when setting up standard curves and running enzyme reactions.

1. Perform a 25µl kinase or ATPase reaction using 1X reaction buffer (e.g., 1X reaction buffer A).

   Note: If the enzymatic reaction was not run at room temperature, equilibrate the plate to room temperature before adding the ADP-Glo™ Reagent.

2. Add 25µl of ADP-Glo™ Reagent to stop the enzyme reaction and deplete the unconsumed ATP, leaving ADP intact.

   Notes:

   a. The ADP-Glo™ Reagent is effective in terminating the enzyme reaction; therefore, there is no need to add an inhibitor to terminate the ATPase or kinase reaction. However, although not recommended, if the protocol is modified to add any termination reagent, do not use a magnesium-chelating agent such as EDTA because the ADP-Glo™ Assay requires the presence of magnesium.

   b. The final Mg^{2+} concentration must be at least 5mM.

3. Incubate at room temperature for 40 minutes.

4. Add 50µl of ADP-Glo™ Max Detection Reagent to convert ADP to ATP and introduce luciferase and luciferin to detect ATP.

5. Incubate at room temperature for 60 minutes.

6. Measure the luminescence with a plate-reading luminometer or charge-coupled device (CCD) camera.

   Note: Instrument settings depend on the manufacturer. An integration time of 0.25–1 second per well should serve as a guideline. The long half-life of the ADP-Glo™ Max Assay signal allows plates to be left longer at room temperature before reading if desired.

4.C. Optimizing Reaction Conditions

For optimal performance when using the ADP-Glo™ Max Assay, optimize the amounts of enzyme and ATP used in the reaction. If these amounts have been determined, proceed to Section 4.B.

Notes:

a. Use only the provided Ultra Pure ATP when performing the ADP-Glo™ Max Assay. Other sources of ATP may contain ADP that could result in higher background signals.

b. If the enzymatic reaction was not run at room temperature, we recommend equilibrating the plate to room temperature before adding the ADP-Glo™ Reagent.

Determining the Optimal Amount of Enzyme

1. Make serial twofold dilutions of ATPase or kinase in 1X reaction buffer across the plate using the desired amount of ATP (up to 5mM). Mix the plate, and incubate at the desired temperature for the desired amount of time.
2. At the same time as the enzyme titration reactions, set up an ATP-to-ADP conversion curve at the same ATP + ADP concentration series as described in Section 3.B. This helps in determining the percent ATP-to-ADP conversion generated by each amount of enzyme.

Note: If the enzymatic reaction was not run at room temperature, we recommend equilibrating the plate to room temperature before adding the ADP-Glo™ Reagent to ensure uniform temperature across the plate during the ADP-Glo™ Assay.

3. Follow the ADP-Glo™ Max Assay protocol described in Section 4.B, starting at Step 2.
4. Record luminescence.

   Note: The optimal amount of enzyme to use in subsequent compound screens and IC_{50} determinations is the amount that produces luminescence within the linear range of the enzyme titration curve and generates an adequate signal-to-background ratio.

Because the ADP-Glo™ Max Assay is very sensitive, it can detect a very small amount of ADP with a high signal-to-background ratio. A small amount of enzyme that produces low conversion of ATP to ADP is sufficient for use with the ADP-Glo™ Max Assay due to the high signal-to-background ratio generated. Figures 5 and 6 show that the amount of ADP produced with a small amount of ATPases (Pgp membranes or Na^+/K^+ ATPase) results in a high signal-to-background ratio.

5. Using ADP-Glo™ Max Assay to Determine ATPase Activity

Example ATPase Activity Assay: P-Glycoprotein (Pgp) Assay

P-glycoprotein (Pgp), also known as MDR1 and ABCB1, is a 170kDa integral plasma membrane protein that functions as an ATP-dependent drug efflux pump and plays an important role in multidrug resistance and certain adverse drug-drug interactions. Compounds that interact with Pgp can be identified as stimulators or inhibitors of its ATPase activity. Compounds that are substrates for transport by Pgp typically stimulate its ATPase activity (4). Because Pgp requires a high ATP concentration (5mM) during the reaction, the ADP-Glo™ Max Assay can be used to detect its ATPase activity with high sensitivity so that lesser amounts of membrane preparations are used.

The impact of a test compound on Pgp ATPase activity is examined by comparing untreated samples and samples treated with a test compound to a Na_3VO_4 (sodium orthovanadate)-treated control. Na_3VO_4 is a selective inhibitor of Pgp, and samples treated with Na_3VO_4 have no Pgp ATPase activity (4). In the absence of Na_3VO_4, basal and drug-stimulated Pgp ATPase activities can be detected. ADP production in the presence of Na_3VO_4 is attributed to minor non-Pgp ATPase activities present in the membrane preparation (Figure 4).

- The difference in luminescent signal between Na_3VO_4-treated samples and untreated samples represents the basal Pgp ATPase activity.
- The difference in luminescent signal between Na_3VO_4-treated samples and samples treated with the test compound represents Pgp ATPase activity in the presence of the test compound.
- By comparing basal activity to test compound-treated activities, the compounds can be ranked as stimulating (e.g., verapamil), inhibiting, (e.g., cyclosporin A) or having no effect on basal Pgp ATPase activity.

Another use for the assay is to examine effects of compounds on drug-stimulated Pgp ATPase activity. This approach is primarily used to characterize inhibitors of Pgp ATPase activity. Test compounds are applied to reactions that include a stimulatory drug such as verapamil. Reduction of drug-stimulated ATPase activity by a compound indicates it is a Pgp ATPase inhibitor. Figure 5 shows cyclosporin A inhibition of verapamil-stimulated Pgp ATPase activity and shows an IC_{50} value of 1.04µM.

5.A. Before You Begin the ATPase Activity Assay

Materials Required for an ATPase Activity Assay

ATPase Source: In this example we use Pgp membranes as our source of Pgp. We thawed the Pgp membranes rapidly at 37°C, then immediately placed them on ice until ready for use. The membranes were diluted to 1mg/ml in 1X Pgp reaction buffer; we used 10µl of Pgp membranes (10µg) per reaction.

Stimulator: In this example we used verapamil. Verapamil stock solution was diluted to 0.5mM in 1X Pgp reaction buffer

Inhibitor: In this example, we used **Na_3VO_4**. The Na_3VO_4 stock solution was diluted to 0.25mM in 1X Pgp reaction buffer.

ATP: We then diluted the Ultra Pure ATP stock solution to 25mM in 1X Pgp reaction buffer.

Test Compound (TC): We prepared a 2.5X concentration of other test compounds in 1X Pgp reaction buffer.

Conversion Curve Samples: We set up an ATP-to-ADP conversion curve in 1X Pgp reaction buffer at the 5mM ATP + ADP concentration series as described in Section 3.B. This helps determine the amount of ADP in picomoles generated by each amount of Pgp membranes.

5.B. Example Protocol for a Pgp ATPase Activity Assay

The following protocol is an example; the actual volumes used can be adjusted as needed. The volume of the ADP-Glo™ Reagent used should equal the volume of the enzyme reaction, and the volume of the ADP-Glo™ Max Detection Reagent should be double the amount of the reaction. The volumes provided are intended for a 96-well plate (25µl:25µl:50µl ratio); to perform the assay in a 384-well plate, decrease the volumes fivefold.

1. Add 10µl of 1X Pgp reaction buffer to wells labeled "NT" (untreated).
2. Add 10µl of 0.25mM Na_3VO_4 in 1X Pgp reaction buffer to wells labeled "Na_3VO_4".
3. Add 10µl of 0.5mM verapamil in 1X Pgp reaction buffer to wells labeled "ver".
4. Add 10µl of 2.5X concentrated test compounds to wells labeled "TC".
5. Add 10µl of diluted Pgp membranes in 1X Pgp reaction buffer to each well. Incubate at 37°C for about 5 minutes (e.g., float plate in 37°C water bath or place on 37°C heat block).
6. Initiate reactions by adding 5µl of 25mM ATP to all wells. At this point, each Pgp reaction contains 5mM ATP.

   Notes:

   Use only the provided Ultra Pure ATP when performing the ADP-Glo™ Max Assay. Other sources of ATP may contain ADP that could result in high background.

   We recommend equilibrating the plate to room temperature before adding the ADP-Glo™ Reagent.

7. Mix briefly on a plate shaker or by gently tapping plate and incubate for 40 minutes at 37°C.
8. Follow the ADP-Glo™ Max Assay protocol described in Section 4.B, starting at Step 2.
9. Record luminescence.

   Note: Instrument settings depend on the manufacturer. An integration time of 0.25–1 second per well should serve as a guideline.

5.C. Effects of Test Compounds on Basal and Drug-Stimulated Pgp ATPase Activities (Pgp Example Protocols)

The following protocols are designed for a 96-well plate using a 25µl:25µl:50µl ratio. To perform the assay in a 384-well plate, reduce volumes fivefold. Other volumes may be used, provided the 1:1:2 ratio of kinase reaction volume to ADP-Glo™ Reagent volume to ADP-Glo™ Max Detection Reagent volume is maintained.

Determining EC_{50} Values of ATPase Stimulators (Pgp example protocol)

1. Add 10µl of 1X Pgp reaction buffer to each well in columns 2 though 11.
2. Add 10µl of 0.25mM Na_3VO_4 in 1X Pgp reaction buffer to wells labeled "Na_3VO_4" (column 12).
3. Add 20µl of the test compound diluted to 2.5X the highest concentration desired in 1X Pgp reaction buffer to the wells in column 1.
4. Transfer 10µl from the wells in column 1 to column 2. Mix well. Continue to make twofold serial dilutions across the plate, mixing well before each transfer. Discard the 10µl removed from column 10. Do **not** continue the dilution in wells in columns 11 and 12.
5. Add 10µl of diluted Pgp membranes in 1X Pgp reaction buffer to each well. Incubate at 37°C for about 5 minutes (e.g., float plate in 37°C water bath or place on 37°C heat block).
6. Initiate reactions by adding 5µl of 25mM ATP to all wells. At this point, each Pgp reaction contains 5mM ATP.
7. Mix briefly on a plate shaker or by gently tapping plate, and incubate for 40 minutes at 37°C.
8. Follow the ADP-Glo™ Max Assay protocol described in Section 4.B, starting at Step 2.
9. Record luminescence.

   Note: Instrument settings depend on the manufacturer. An integration time of 0.25–1 second per well should serve as a guideline.

5.C. Effects of Test Compounds on Basal and Drug-Stimulated Pgp ATPase Activities (Pgp example protocols; continued)

Determining IC_{50} Values of ATPase Inhibitors (Pgp example protocol)

The following protocol is designed for determining IC_{50} value for the inhibition of verapamil-stimulated Pgp activity. In order to determine the IC_{50} value for the inhibition of basal Pgp activity, verapamil should not be included in the 1X Pgp reaction buffer.

1. Add 10µl of 1X Pgp reaction buffer containing 0.5mM verapamil to each well in columns 2 though 11.
2. Add 10µl of 0.25mM Na_3VO_4 in 1X Pgp reaction buffer to wells labeled "Na_3VO_4" (column 12).
3. Add 20µl of the test compound diluted to 2.5X the highest concentration desired in 1X Pgp reaction buffer containing 0.5mM verapamil to the wells in column 1.
4. Transfer 10µl from the wells in column 1 to column 2. Mix well. Continue to make twofold serial dilutions across the plate, mixing well before each transfer. Discard the 10µl removed from the column 10. Do **not** continue the dilution in wells in columns 11 and 12.
5. Add 10µl of diluted Pgp membranes in 1X Pgp reaction buffer to each well. Incubate at 37°C for about 5 minutes (e.g., float plate in 37°C water bath or place on 37°C heat block).
6. Initiate reactions by adding 5µl of 25mM ATP to all wells. At this point, each Pgp reaction contains 5mM ATP.
7. Mix briefly on a plate shaker or by gently tapping plate, and incubate for 40 minutes at 37°C.

   Notes:

   Use only the provided Ultra Pure ATP when performing the ADP-Glo™ Max Assay. Other sources of ATP may contain ADP that could result in high background.

   We recommend equilibrating the plate to room temperature before adding the ADP-Glo™ Reagent.
8. Follow the ADP-Glo™ Max Assay protocol described in Section 4.B, starting at Step 2.
9. Record luminescence.

   Note: Instrument settings depend on the manufacturer. An integration time of 0.25–1 second per well should serve as a guideline.

6. General Considerations

Temperature: The intensity and stability of the luminescent signal from the ADP-Glo™ Max Assay depends on the rate of the luciferase reaction. Environmental factors that affect the rate of the luciferase reaction will result in a change in the intensity of light output and stability of the luminescent signal. Temperature is one factor that affects the rate of this enzymatic assay and thus the light output. For consistent results, equilibrate assay plates and reagent to room temperature before adding the ADP-Glo™ Max Assay reagents. Insufficient equilibration may result in a temperature gradient between the wells in the center and at the edge of the plate and therefore variability across the plate.

Solvents and Other Chemicals: The chemical environment in which the first or second step of the ADP-Glo™ Max Assay is performed will affect the enzymatic rates and thus luminescence intensity. We recommend a pH of 7–8 for the kinase or ATPase buffer. Some vehicles used to resuspend the various test compounds or reagents used in the kinase or ATPase reaction buffer may interfere with the luciferase reaction and thus affect the light output of the assay. Various chemicals were shown to be compatible with or tolerated by the ADP-Glo™ Max Assay (Table 4). Interference with the luciferase reaction can be detected by assaying a parallel set of control wells without enzyme or enzyme substrate and using ADP as a substrate.

Table 4. Solvents and Chemicals Compatible with the ADP-Glo™ Max Assay.

Compound	Maximum Concentration Used
NaCl	200mM
β-mercaptoethanol (BME)	10mM
$CaCl_2$	2.5mM
calmodulin	5µM
DTT	10mM
$MgCl_2$	50mM
$MnCl_2$	5mM
DMSO	5%
sodium orthovanadate	500µM
Tergitol-NP-9	0.1%
Tween®-20	0.2%
Triton®X-100	0.2%

6. General Considerations (continued)

Plate Recommendations: We recommend using standard solid white, multiwell plates suitable for luminescence measurements (e.g., Corning® Cat. # 3912, 3674 or 3673). Please contact your luminometer instrument manufacturer to find out which plates will work best for your particular instrument.

Inhibitors of Luciferase and/or other ADP-Glo™ Max Assay Reagents: Test compounds that inhibit only the enzyme will result in lower luminescence compared to vehicle-only controls and are easily distinguishable from compounds that inhibit only luciferase activity, which decrease luminescence of a no-enzyme control with ADP. Test compounds that inhibit other components of the assay alone or together with the kinase, however, might increase, decrease or have no effect on luminescence, depending on the level of inhibition of the kinase or ATPase tested, luciferase and other enzyme components of the assay. Test compounds that inhibit luciferase may result in false hits. However, the unique combination of Ultra-Glo™ Recombinant Luciferase and the proprietary buffer compositions of the ADP-Glo™ Max Assay reagents significantly reduce the number of false hits (1,2).

7. References

1. Auld, D.S. *et al*. (2009) A basis for reduced chemical library inhibition of firefly luciferase obtained from directed evolution. *J. Med. Chem*. **52**, 1450–8.
2. Tanega, C. *et al*. (2009) Comparison of bioluminescent kinase assays using substrate depletion and product formation. *ASSAY Drug Dev. Tech*. **7**, 606–14.
3. Ambudkar, S.V. *et al*. (1999) Biochemical, cellular and pharmacological aspects of the multidrug transporter. *Annu. Rev. Pharmacol. Toxicol*. **39**, 361–98.
4. Boulton, D.W. *et al*. (2002) In vitro P-glycoprotein affinity for atypical and conventional antipsychotics. *Life Sciences* **71**, 163–9.
5. Litman, T. *et al*. (2003) Pumping of drugs by P-glycoprotein: A two step process? *J. Pharm. Exp. Ther*. **3F07**, 846–853.
6. Zhang, J.H. *et al*. (1999) A simple statistical parameter for use in evaluation and validation of high throughput screening assays. *J. Biomol. Screen*. **4**, 67–73.

8. Composition of Buffers and Solutions

5X Kinase Reaction Buffer A

200mM	Tris (pH 7.5)
100mM	$MgCl_2$
0.5mg/ml	BSA

5X Pgp Reaction Buffer

250mM	Tris-MES (pH 6.8)
50mM	$MgCl_2$
10mM	EGTA
250mM	KCl
25mM	sodium azide
10mM	DTT

5X Na^+/K^+ ATPase Reaction Buffer

100mM	Tris (pH 7.8)
2.8mM	EDTA
100mM	$MgCl_2$
15mM	KCl
665mM	NaCl

9. Related Products

Product	Size	Cat.#
ADP-Glo™ Kinase Assay	1,000 assays	V9101
	10,000 assays	V9102
	100,000 assays	V9103

10. Summary of Changes

The following changes were made to the 7/25 revision of this document:

1. Removed expired patent statements and added a patent statement.
2. Updated the fonts and cover image.
3. Removed discontinued Cat.# V3601 and Cat.# E6501.
4. Notes were changed from bullets or numerical listings to alphabetical listings.

Promega

[a]U.S. Pat. No. 8,183,007 and other patents and patents pending.

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