TECHNICAL BULLETIN

Pgp-Glo™ Assay Systems

Instructions for Use of Products
V3591 and V3601

Revised 11/15
TB341

Pgp-Glo™ Assay Systems

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1. Description

The Pgp-Glo™ Assay Systems[a] provide the necessary reagents for performing luminescent P-glycoprotein (Pgp) ATPase assays. 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 multi-drug 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 (1).

The Pgp-Glo™ Assay detects the effects of compounds on recombinant human Pgp in a cell membrane fraction. The assay relies on the ATP dependence of the light-generating reaction of firefly luciferase (Figure 1). ATP is first incubated with Pgp, then the Pgp ATPase reaction is stopped, and the remaining unmetabolized ATP is detected as a luciferase-generated luminescent signal. Pgp-dependent decreases in luminescence reflect ATP consumption by Pgp; thus the greater the decrease in signal, the higher the Pgp activity. Accordingly, samples containing compounds that stimulate the Pgp ATPase will have significantly lower signals than untreated samples.

1. Description (continued)

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 (1). In the absence of Na_3VO_4, basal and drug-stimulated Pgp ATPase activities can be detected. ATP consumption in the presence of Na_3VO_4 is attributed to minor non-Pgp ATPase activities present in the membrane preparation (Figure 2).

- 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 (TC) 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, inhibiting or having no effect on basal Pgp ATPase activity. A range of compound concentrations can also be tested to determine effective half-maximal concentrations.

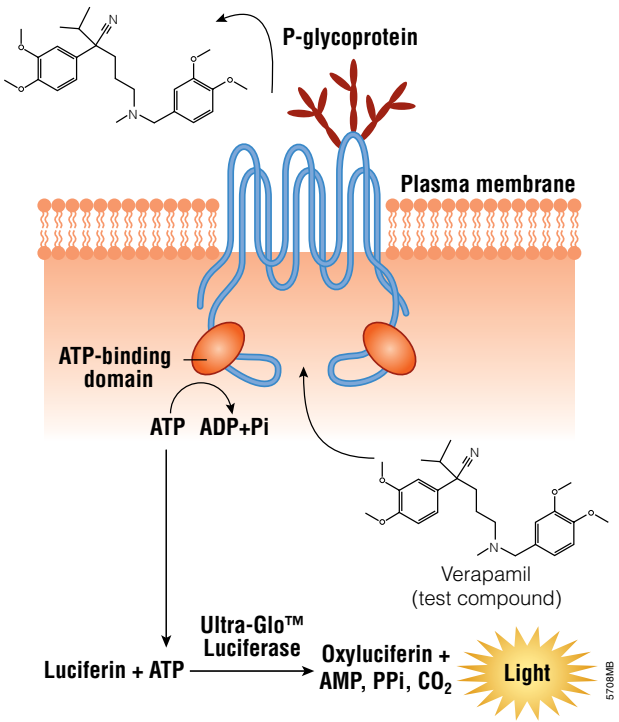

Figure 1. The Pgp-Glo™ Assay System. The Pgp-Glo™ Assay relies on the ATP-dependence of the light-generating reaction of firefly luciferase. After a pool of ATP is first exposed to the Pgp ATPase, ATP consumption is detected as a decrease in luminescence from a second reaction with a recombinant firefly luciferase (Ultra-Glo™ Luciferase).

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 3 shows cyclosporin inhibition of Verapamil-stimulated Pgp ATPase activity and shows an IC_{50} value of 5.4µM (1,2). **Note:** Verapamil is sometimes referred to as a Pgp inhibitor because as a substrate for transport it inhibits Pgp activity with other substrates by interfering with their transport in a competitive mode.

Figure 2 shows data generated with a test compound (TC), Verapamil, which is supplied as a positive control substrate with the Pgp-Glo™ Assay System. Verapamil is a substrate for transport by Pgp that stimulates Pgp ATPase activity. Note that there is a significant drop in the average relative light units (RLU) in the untreated samples (NT) as compared to Na_3VO_4-treated samples. This decrease reflects the consumption of ATP by the basal Pgp ATPase activity and is expressed as ΔRLU_{basal}. Verapamil causes a substantially larger drop in RLU compared to Na_3VO_4-treated samples. This decrease reflects the consumption of ATP by Verapamil-stimulated Pgp and is expressed as ΔRLU_{TC}. For the experiment shown in Figure 2, the increase in Pgp ATPase activity caused by Verapamil (ΔRLU_{TC}/ ΔRLU_{basal}) is 572,251/125,717 = 4.6-fold. This activity ratio is a measure of the effect of drug treatment on ATPase activity. Note in Figure 4 that the RLU increases slightly as the luciferase reaction continues, but the increase is the same for all conditions. Therefore the specific activities and the activity ratios remain stable.

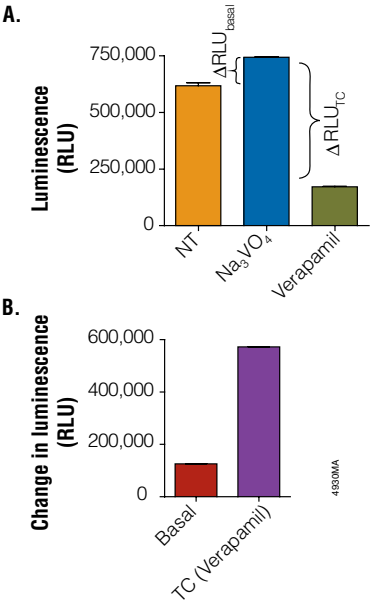

Figure 2. Stimulation of Pgp ATPase activity by Verapamil. Untreated (NT), 100µM Na_3VO_4- and 200µM Verapamil-treated Pgp reactions were performed according to the protocol described in Section 5. Luminescence was read on a BMG LABTECH Polarstar Optima luminometer (**Panel A**). The decrease in luminescence of NT samples compared to samples plus Na_3VO_4 (ΔRLU_{basal}) represents basal Pgp ATPase activity. The decrease in luminescence of Verapamil-treated samples (ΔRLU_{TC}) represents Verapamil-stimulated Pgp ATPase activity. ΔRLU_{basal} and ΔRLU_{TC} were replotted (**Panel B**) to illustrate the stimulation of Pgp ATPase activity by Verapamil.

1. Description (continued)

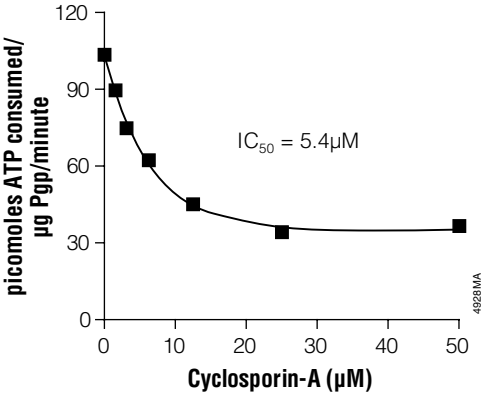

Figure 3. IC_{50} measurements. Cyclosporin-A was tested at a range of concentrations for its capacity to inhibit 200µM Verapamil-stimulated Pgp ATPase activity. Luminescence was read on a BMG LABTECH Polarstar Optima luminometer, and data was analyzed as described by Section 6.B.

The luminescence detection portion of the Pgp-Glo™ System is catalyzed by a stabilized mutant of the firefly luciferase enzyme that provides stable, glow-type luminescent signals that allow a wide window of time for reading samples. After an initial 20-minute signal-stabilization period, readings can be taken immediately or after a delay (up to two hours) to give the same results in terms of calculated Pgp ATPase activity (Figure 4). In contrast, the colorimetric signals from molybdate-based ATPase assays are quickly saturated. Thus the luminescent approach allows users more time to perform reactions and simplifies automation of the assay.

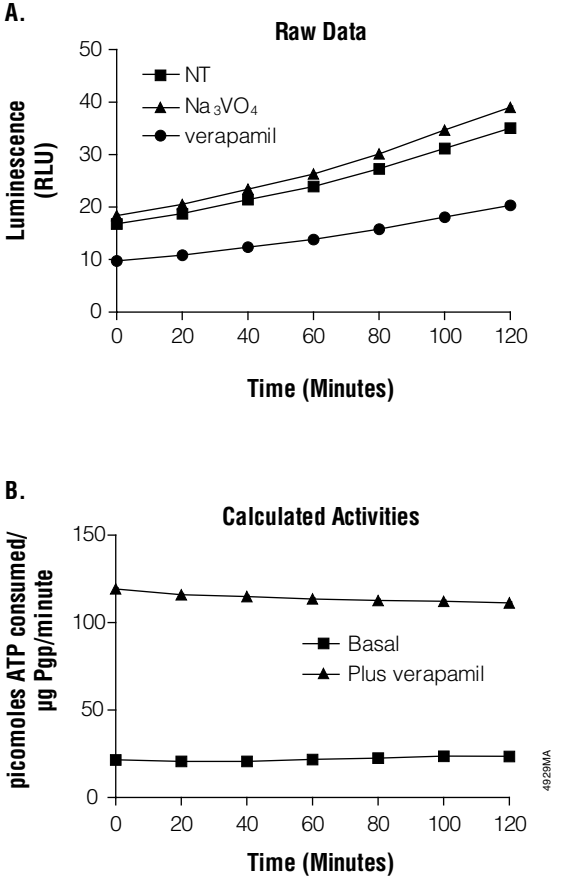

Figure 4. Stability of calculated activities. Untreated (NT), Verapamil-treated and Na_3VO_4-treated Pgp reactions were incubated for 40 minutes at 37°C in a 96-well plate. The reactions were stopped and luminescence initiated by addition of ATP Detection Reagent as described in Section 5. Following a 20-minute room temperature signal-stabilization period (Time 0), luminescence was read on a Dynex MLX Microtiter® Plate luminometer. Luminescent readings were taken at 20-minute intervals for two hours. Raw data (**Panel A**) was converted to ATPase activities (**Panel B**) by comparing the luminescence of samples at each time point to luminescence of ATP standards at the corresponding time point as described in Section 6.B.

1. Description (continued)

There are two forms of the Pgp-Glo™ Assay System available:

The Pgp-Glo™ Assay System includes all the reagents required to run the assay except the P-glycoprotein: A Pgp reaction buffer, MgATP, Verapamil, Na_3VO_4, and a lyophilized ATP detection reagent and its reconstitution buffer.

The Pgp-Glo™ Assay System with P-glycoprotein includes all the reagents provided in the Pgp-Glo™ System with the addition of Recombinant Human Pgp Membranes to provide a completely optimized kit.

Advantages of the Pgp-Glo™ Assay Systems include:
Complete System: The protocol and reagents of the Pgp-Glo™ Assay System have been tested for optimal performance.
Simplified Method: The simple protocol makes the assay amenable to high-throughput screening in multiwell plates.
Stable Activities: Glow-type signal enables the processing of multiple samples without concern for variability over time.
Mechanistic Assay: The Pgp-Glo™ Assay reveals the capacity of a compound to stimulate or inhibit Pgp ATPase activity.

Both inhibition and stimulation of Pgp ATPase activity are indicative of an interaction with Pgp (1). Stimulators of the ATPase activity are likely substrates for Pgp-mediated efflux that will also competitively inhibit transport of other Pgp substrates. Inhibitors of the ATPase activity will likely not be substrates for efflux but will inhibit efflux of other compounds (1). In contrast, a marker substrate-based Pgp transport assay can detect inhibited transport of a marker substrate by a test compound but will not reveal the mechanism.

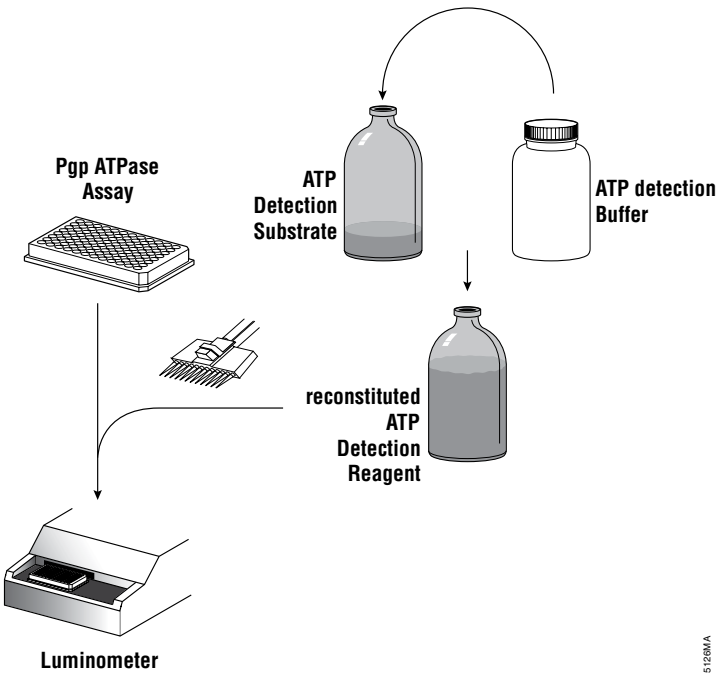

Figure 5. Flow diagram showing preparation and use of the reconstituted ATP Detection Reagent.

2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
Pgp-Glo™ Assay System with P-Glycoprotein	**10ml**	**V3601**

Each system contains sufficient reagents for 96 assays at 50µl per assay in 96-well plates. Includes:

- 500µl Recombinant Human Pgp Membranes
- 1 vial ATP Detection Substrate (lyophilized)
- 10ml ATP Detection Buffer
- 10ml Pgp-Glo™ Assay Buffer
- 1ml 50mM MgATP
- 100µl 10mM Verapamil
- 250µl 10mM Na_3VO_4 (sodium orthovanadate)

PRODUCT	SIZE	CAT.#
Pgp-Glo™ Assay System	**10ml**	**V3591**

Each system contains sufficient reagents for 192 assays at 50µl per assay in 96-well plates. Includes:

- 1 vial ATP Detection Substrate (lyophilized)
- 10ml ATP Detection Buffer
- 10ml Pgp-Glo™ Assay Buffer
- 1ml 50mM MgATP
- 100µl 10mM Verapamil
- 250µl 10mM Na_3VO_4 (sodium orthovanadate)

Storage Conditions: Store Recombinant Human Pgp Membranes at –70°C. The Membranes are stable for up to 5 freeze-thaw cycles. Since individual freeze-thaw conditions may vary, we recommend dispensing the Membranes into single-use aliquots for storage at –70°C. All other components can be stored at –70°C or –20°C, protected from light. The reconstituted ATP Detection Reagent can be stored at –20°C for up to 3 months, at 4°C for 1 week or at room temperature (approximately 23°C) for 24 hours without loss of activity. Avoid multiple freeze-thaw cycles of all components.

3. Assay Conditions

Pgp-Glo™ Assays are performed in two steps.

The Pgp Reaction: In this step a Recombinant Human Pgp Membrane fraction is incubated in Pgp-Glo™ Assay Buffer with a non-limiting concentration of ATP (5mM) for 40 minutes at 37°C. Untreated and Na_3VO_4-treated control samples are included in addition to Verapamil-treated samples (positive control). If an ATP standard curve is used, the ATP Standards are added to the plate at the end of this step.

The ATP Detection Reaction: ATP Detection Reagent (see Section 4 for preparation) is added to the Pgp reaction described above. The ATP Detection Reagent stops the Pgp reaction and measures the ATP remaining in the reaction mixture. This luciferase-based detection reaction provides a linear response to ATP concentration over the range of ATP concentrations found in the Pgp-Glo™ Assay. Thus any changes in signal directly reflect changes in ATP concentration.

4. Preparation of Buffers and Solutions

ATP Detection Reagent

1. Thaw the ATP Detection Buffer and equilibrate to room temperature. Mix briefly to resuspend any precipitate that may form after freezing. To prevent the Buffer from foaming, mix gently and do not vortex.
2. Transfer the entire contents of the 10ml bottle of ATP Detection Buffer to the amber bottle containing the lyophilized ATP Detection Substrate.
3. Mix by swirling or inverting several times to obtain a homogeneous solution of ATP Detection Reagent. To prevent the Reagent from foaming, mix gently and do not vortex.

0.5mM Verapamil

Combine 5µl of 10mM Verapamil with 95µl Pgp-Glo™ Assay Buffer.

0.25mM Na_3VO_4

Combine 5µl of 10mM Na_3VO_4 with 195µl Pgp-Glo™ Assay Buffer.

25mM MgATP

To prepare enough for a 96-sample assay, combine 0.5ml 50mM MgATP with 0.5ml Pgp-Glo™ Assay Buffer. For an assay with fewer samples, prepare proportionately less.

2.5X concentrated solutions of the test compounds of choice

Prepare 2.5X concentration of test compounds in Pgp-Glo™ Assay Buffer.

Note: If test compounds diluted from concentrated stocks are dissolved in a solvent other than Pgp ATPase Assay Buffer (e.g., DMSO), an equivalent amount of that solvent must be included in the controls and standards. Keep concentrations of organic solvents as low as possible (e.g., ≤1%) for best results.

Note: To evaluate the effect of a solvent, an additional set of controls and standards without solvent may be included for comparison to the set with solvent.

ATP Standards (optional)

The inclusion of standards facilitates the conversion of sample RLU to ATP concentrations.

Prepare a set of four MgATP standards at concentrations of 30mM, 15mM, 7.5mM and 3.75mM MgATP.

1. Add 30µl of water to tubes for the 15mM, 7.5mM and 3.75mM standards. Add 30µl of the 50mM MgATP solution to tubes for the 30mM and 15mM standards. Mix well.
2. Prepare a serial dilution of the solution in the 15mM tube by transferring 30µl of the solution in that tube to the 7.5mM tube, mix well and then transfer 30µl from the 7.5mM tube to the 3.75mM tube. **Mix well** between transfers. Discard 30µl of the solution from the 3.75mM tube.
3. Add 20µl of Pgp-Glo™ Assay Buffer to all four tubes for final concentrations of 30mM, 15mM, 7.5mM and 3.75mM MgATP. Ten-microliter aliquots of each standard will be added to the appropriate wells for the assay (Figure 6). At the end of the assay, after the ATP Detection Reagent is added, the final concentrations of the standards will be 3, 1.5, 0.75 and 0.375mM.

Note: The ATP standards are optional; it is not necessary to make the conversion from RLU to ATP concentration to measure fold changes in Pgp ATPase activity caused by a test compound or to measure the dose dependence of these changes.

Pgp Membranes

1. Thaw the Pgp Membranes rapidly at 37°C, then immediately place them on ice until ready for use. Dispense unused Pgp Membranes into single-use aliquots and store at –70°C.
2. Dilute the 5mg/ml Membranes to 1.25mg/ml with Pgp-Glo™ Assay Buffer. Dilute only enough Pgp Membranes for the experiment at hand. You will need 20µl of Pgp Membrane (25µg) per reaction. For example, combine 50µl of Pgp Membranes with 150µl Pgp-Glo™ Assay Buffer for a 10-sample assay.

5. Protocol

Materials To Be Supplied by the User

- **untreated white opaque multiwell plates**
  (e.g., 96-well, Corning/Costar® Cat.# 3912 or Thermo Labsystems Cat.# 9502887).

Note: Plain, untreated white polystyrene plates are preferred for the Pgp-Glo™ Assay. **Do not** use clear or black plates or plates that have been treated to support the binding of cells, proteins, nucleic acids or other biomolecules.

- luminometer or CCD camera imaging device capable of reading multiwell plates
- multichannel pipette or automated pipetting station (optional)
- multiwell plate shaker for mixing plates (optional)

Figure 6 shows an example 96-well plate layout appropriate for testing the effects of multiple compounds at a single concentration or for multiple concentrations of one or a few compounds. Note that the ATP standards are optional (see Section 6); it is not necessary to make the conversion from RLU to ATP concentration in order to measure fold changes in Pgp ATPase activity caused by a test compound or to measure the dose dependence of these changes.

	Controls and ATP Standards				Test Compounds							
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	0.375mM ATP Standard	→	→	→	TC1	→	→	→	TC9	→	→	→
B	0.75mM ATP Standard	→	→	→	TC2	→	→	→	TC10	→	→	→
C	1.5mM ATP Standard	→	→	→	TC3	→	→	→	TC11	→	→	→
D	3mM ATP Standard	→	→	→	TC4	→	→	→	TC12	→	→	→
E	NT	→	→	→	TC5	→	→	→	TC13	→	→	→
F	Na_3VO_4	→	→	→	TC6	→	→	→	TC14	→	→	→
G	Ver	→	→	→	TC7	→	→	→	TC15	→	→	→
H					TC8	→	→	→	TC16	→	→	→

Figure 6. Example 96-well plate layout. The layout includes the following samples:

- **ATP standards:** The averages of these values are used to plot a luminescent ATP standard curve and convert RLU to specific activity expressed in units of nanomoles ATP consumed/µg Pgp/minute (see Sections 6.A and 6.B).
- **NT:** No treatment control provides a measure of total, unregulated ATPase activity.
- **Na_3VO_4:** Provides a measure of Pgp-independent ATPase activity (background, nonPgp ATPases).
- **Ver:** Verapamil is a Pgp substrate that stimulates Pgp ATPase activity and serves here as a positive control for drug stimulation of Pgp ATPase activity.
- **TC1–TC16:** Experimental test compounds. Provide a measure of total ATPase activity in the presence of test compounds.

  Note: TC1–TC16 wells can be used to measure the EC_{50} of a few test compounds that affect Pgp activity by adding a range of test compound concentrations to these wells instead of multiple compounds at a single concentration.

5.A. Performing the Assay

1. Add 20µl of Pgp-Glo™ Assay Buffer to wells labeled "NT".
2. Add 20µl of 0.25mM Na_3VO_4 in Pgp-Glo™ Assay Buffer to wells labeled "Na_3VO_4", and "ATP standards".
3. Add 20µl of 0.5mM Verapamil in Pgp-Glo™ Assay Buffer to wells labeled "Ver".
4. Add 20µl of 2.5X concentrated test compounds to wells labeled "TC1–TC16".
5. Add 20µl of diluted Pgp Membranes 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 10µl of 25mM MgATP to all wells **EXCEPT** those for the ATP standards. At this point, each Pgp reaction contains 5mM ATP.
7. Mix briefly on a plate shaker or by gently tapping the plate. Incubate for 40 minutes at 37°C. **Note:** In cases where the Pgp membranes have relatively low ATPase activity, incubations longer than 40 minutes may be required (see Section 7).
8. Two minutes prior to the next step, add 10µl of the MgATP standards to the appropriate wells (add 30mM to 3mM wells, 15mM to 1.5mM wells, 7.5mM to 0.75mM wells and 3.75mM to 0.375mM wells; Figure 6).
9. Remove plate from 37°C heat source, then stop reactions, and initiate luminescence by adding 50µl of ATP Detection Reagent to all wells.
10. Mix briefly on a plate shaker or by gently tapping the plate, and then incubate plate at room temperature for 20 minutes to allow luminescent signal to develop.
11. Read luminescence on a plate-reading luminometer.

    Note: If the inhibitory effects of test compounds are being examined against a Verapamil-stimulated Pgp ATPase activity, then Verapamil is included in the TC1–TC16 wells. In this case, 10µl of Verapamil and 10µl of 5X concentrated test compound, both dissolved in Pgp-Glo™ Assay Buffer, are added to TC1–TC16 wells. If the stimulatory drug is something other than Verapamil, control samples including that drug must be added.

6. Analysis of Results

Luminescence from the Pgp-Glo™ Assay System changes in direct proportion to ATP concentration. Reactions where no ATP is consumed will generate the brightest signals. Reactions where ATP has been consumed will have relatively lower luminescent signals, reflecting lower ATP concentrations. For example, a sample that emits half the light emitted by a sample with 2.5mM ATP contains 1.25mM ATP because half of the starting ATP was consumed during the reaction. Within a given experiment, differences in sample luminescence are used to calculate the basal Pgp ATPase activity and the effect of a test compound on Pgp ATPase activity.

- The decreased luminescence of the untreated (NT) reactions relative to Na_3VO_4-treated reactions reflects basal Pgp ATPase activity.
- The decreased luminescence of reactions treated with the positive control drug Verapamil (Ver) relative to Na_3VO_4-treated reactions reflects Verapamil-stimulated Pgp ATPase activity.
- The luminescence of a test compound-treated reaction (TC) relative to that of the NT-treated reaction reflects the effect, if any, of that compound on Pgp ATPase activity. For example, a decrease in luminescence reflects a stimulated Pgp ATPase activity

Data Analysis

The following examples describe comparisons between basal and test compound-treated values.

6.A. Analysis without ATP Standards

1. Calculate the difference between the average luminescent signals from Na_3VO_4-treated samples (RLU_{Na3VO4}) and untreated (NT) samples (RLU_{NT}) to determine ΔRLU_{basal} as follows:

   $RLU_{Na3VO4} - RLU_{NT} = \Delta RLU_{basal}$.

   ΔRLU_{basal} reflects basal Pgp ATPase activity.

2. Calculate the difference between the average luminescent signals from Na_3VO_4-treated samples (RLU_{Na3VO4}) and test compound-treated samples (RLU_{TC}) to determine ΔRLU_{TC} as follows:

   $RLU_{Na3VO4} - RLU_{TC} = \Delta RLU_{TC}$.

   ΔRLU_{TC} reflects Pgp ATPase activity in the presence of a test compound.

Possible outcomes:

- If $\Delta RLU_{TC} > \Delta RLU_{basal}$, then the test compound is a stimulator of Pgp ATPase activity.
- If $\Delta RLU_{TC} = \Delta RLU_{basal}$, then the test compound has no effect on Pgp ATPase activity.
- If $\Delta RLU_{TC} < \Delta RLU_{basal}$, then the test compound is an inhibitor of Pgp ATPase activity.

To calculate the fold change in Pgp ATPase activity caused by a test compound, divide ΔRLU_{TC} by ΔRLU_{basal}.

For calculating test compound EC_{50} or IC_{50} values, plot ΔRLU_{TC} versus test compound concentration. Figure 7 shows that the EC_{50} value for Verapamil, calculated using this method (Section 6.A), is 12µM, which is similar to reported values (2).

Note: The effects of test compounds can be ascertained quickly by comparing the test compound samples to untreated and Na_3VO_4-treated controls as follows.

- Samples with test compounds that stimulate the Pgp ATPase can be identified as those with signals that are lower than untreated controls.
- Samples with test compounds that inhibit the Pgp ATPase can be identified as those with signals that are greater than untreated controls but equal to or less than Na_3VO_4-treated controls.
- Samples with test compounds that do not affect the Pgp ATPase can be identified as those with signals that are equal to untreated controls.

6.B. Analysis with ATP Standard Curve

If you wish to calculate the Pgp ATPase activity as a specific activity (i.e., in terms of ATP consumed rather than change in luminescence), you can do this using an ATP standard curve and calculating nanomoles ATP consumed/µg Pgp/minute. In this approach, RLU values are first converted to ATP concentrations by comparison to ATP standards.

The ATP concentrations of the Pgp reactions are determined by comparing RLU values from samples to 3.0, 1.5, 0.75 and 0.375mM ATP standards in 100µl final volume.

1. Perform linear regression analysis of the average luminescence values of each ATP standard to generate a standard curve. Plot ATP concentration on the X axis and RLU on the Y axis.
2. Interpolate ATP concentrations in control and test samples by comparing their RLU values to the standard curve. Figure 7, Panel B, shows a representative standard curve.
3. Multiply interpolated values by 100 to convert millimolar ATP concentrations to nanomoles ATP per reaction. For example, a 100µl reaction with 2.0mM ATP contains 200 nanomoles of ATP.
4. Calculate the average amount of ATP for each sample, and then use the averages to perform the following specific activity calculations:

Basal Pgp activity: Calculated as:

$$\frac{([ATP_{Na3VO4}] - [ATP_{NT}])}{(25\mu g\ Pgp \times 40\ minutes)} = \text{nmol ATP consumed/µg Pgp/minute}$$

Test Compound- (or Verapamil-) stimulated Pgp activity: Calculated as:

$$\frac{([ATP_{Na3VO4}] - [ATP_{TC}])}{(25\mu g\ Pgp \times 40\ minutes)} = \text{nmol ATP consumed/µg Pgp/minute}$$

5. Fold stimulation by a test compound calculated as:

$$\frac{\text{Test Compound stimulated activity}}{\text{Basal activity}}$$

6. To calculate test-compound EC_{50} or IC_{50} values, plot Pgp specific activity versus test compound concentration. See Figures 3 and 7 for examples of IC_{50} and EC_{50} measurements. Figure 7 shows that the EC_{50} for Verapamil calculated by this method is 12µM. This is the same as that measured using the method without standards (Section 6.A) and similar to reported values (3).

6.B. Analysis with ATP Standard Curve (continued)

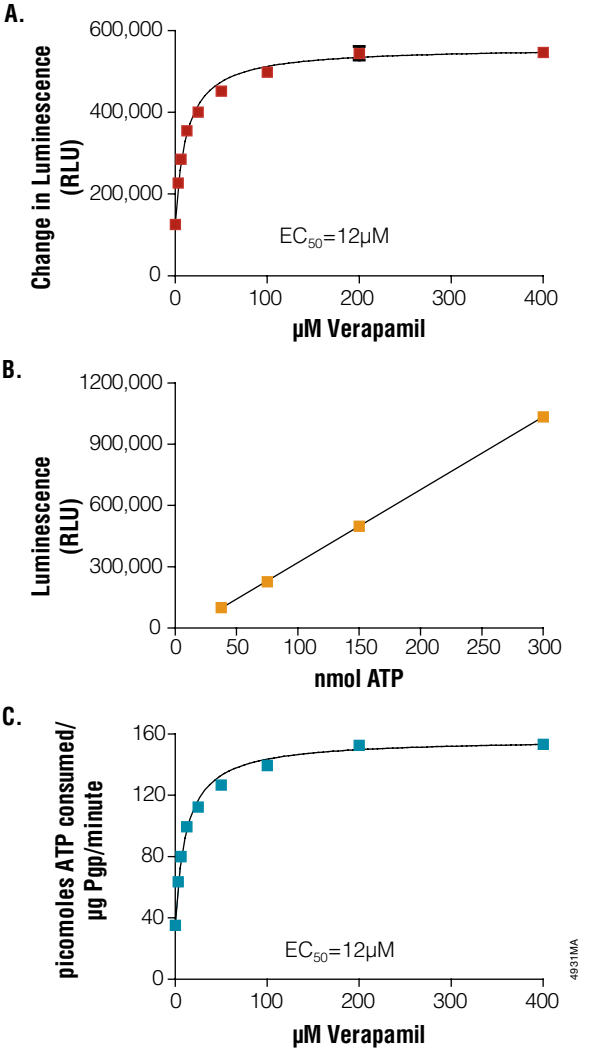

Figure 7. EC_{50} measurements. Verapamil was tested at a range of concentrations for its capacity to stimulate Pgp ATPase activity. Luminescence was read on a BMG LABTECH Polarstar Optima luminometer. Data was analyzed in terms of RLU as described in Section 6.A (**Panel A**) and in terms of ATPase activity (**Panel C**) by comparison to the ATP standard curve (**Panel B**) as described in Section 6.B. Linear regression, curve fits and EC_{50} calculations were performed with the GraphPad Prism® software program.

7. Troubleshooting

Symptoms	Causes and Comments
Lower than expected fold stimulation of Pgp ATPase activity by test compounds or verapamil	Use **untreated** plates. Certain multiwell plates with surface treatments, for example, for low protein binding, can cause a reduced fold stimulation of Pgp. Use untreated white opaque polystyrene plates only (e.g., 96-well, Corning/Costar® Cat# 3912 or Thermo Labsystems Cat# 9502887).
	Contaminating stimulatory or inhibitory compound. Contaminating compounds that cause an increase in basal or decrease in stimulated Pgp activity will reduce fold stimulation. Take care to avoid cross-contaminating samples with control or test compounds, especially verapamil and Na_3VO_4.
	Use Pgp-Glo™ Assay Buffer to dilute all assay components. Fold stimulation will be reduced if, during preparation of buffers and solutions, dilutions are made with water instead of Pgp-Glo™ Assay Buffer.
	Inactive Pgp. Avoid multiple Pgp freeze-thaw cycles; keep Pgp on ice until ready to use.
Very low or undetectable basal ATPase activity	The Pgp ATPase activity in Pgp membrane preparations varies somewhat from lot to lot. Certain lots of Pgp membranes supplied in the Pgp-Glo™ Assay System with P-glycoprotein (Cat.# V3601) may have relatively low basal ATPase activity. This may also be a factor when user-supplied Pgp membranes are used with the Pgp-Glo™ Assay System (Cat.# V3591). The detection of basal ATPase activity can be improved by increasing the Pgp ATPase assay incubation time from 40 minutes to 80 or 120 minutes depending on the Pgp ATPase activity level in the membrane prep.

7. Troubleshooting (continued)

Symptoms	Causes and Comments
Unexpected stimulation of Pgp ATPase activity by a test compound (false positive)	In rare cases, test compounds may inhibit luciferase. From a sample containing a test compound that is a luciferase inhibitor, luminescence would be reduced compared to the untreated control and therefore appear to represent an increased rate of ATP consumption; that is, stimulation of Pgp by the compound. To determine if a compound is a luciferase inhibitor, compare a sample plus the compound and Na_3VO_4 to a sample with Na_3VO_4 only. If the compound is not a luciferase inhibitor then luminescence from both samples will be the same. If the compound is a luciferase inhibitor, the sample containing the compound and Na_3VO_4 will emit less light than the sample with Na_3VO_4 alone.
ATP standard curve is not linear; linear regression shows R^2 <0.97	Brightness of standards exceeds linear range of luminometer. Adjust gain settings on instrument to accommodate brighter signals. Dilute samples to bring signals within linear range of instrument.
	An error was made when diluting the standards, resulting in higher than targeted ATP concentrations. Check that ATP dilutions were correct. The final ATP standard concentrations, after adding ATP Detection Reagent, should be 3.0, 1.5, 0.75 and 0.375mM. **Note:** For interpolating sample concentrations from a standard curve, a nonlinear, hyperbolic curve fit can be performed in place of a linear regression for an R^2 value that is closer to 1.0.

8. References

1. Ambudkar, S.V. *et al.* (1999) Biochemical, cellular and pharmacological aspects of the multidrug transporter. *Annu. Rev. Pharmacol. Toxicol.* **39**, 361–98.
2. Litman, T. *et al.* (2003) Pumping of drugs by P-glycoprotein: A two step process? *J. Pharm. Exp. Ther.* **3F07(3)**, 846–853.
3. Boulton, D.W. *et al.* (2002) In vitro P-glycoprotein affinity for atypical and conventional antipsychotics. *Life Sciences* **71**, 163–169.

9. Related Products

P450-Glo™ Screening Systems

Product	Size	Cat.#
P450-Glo™ CYP1A2 Screening System	1,000 assays	V9770
P450-Glo™ CYP2C9 Screening System	1,000 assays	V9790
P450-Glo™ CYP3A4 Screening System	1,000 assays	V9800
P450-Glo™ CYP2C19 Screening System	1,000 assays	V9880
P450-Glo™ CYP2D6 Screening System	1,000 assays	V9890

P450-Glo™ Assays

Product	Size	Cat.#
P450-Glo™ CYP1A1 Assay	50ml	V8752
P450-Glo™ CYP1B1 Assay	50ml	V8762
P450-Glo™ CYP1A2 Assay	50ml	V8772
P450-Glo™ CYP2C8 Assay	50ml	V8782
P450-Glo™ CYP2C9 Assay	50ml	V8792
P450-Glo™ CYP3A4 Assay	50ml	V8802
P450-Glo™ CYP3A7 Assay	50ml	V8802
P450-Glo™ CYP2C19 Assay	50ml	V8882
P450-Glo™ CYP2D6 Assay	50ml	V8892
P450-Glo™ CYP1A2 Induction/Inhibition Assay	50ml	V8422
P450-Glo™ CYP3A4 Assay with Luciferin-IPA	50ml	V9002
P450-Glo™ CYP3A4 Assay (Luciferin-PPXE) DMSO-Tolerant Assay	50ml	V8912
P450-Glo™ CYP3A4 Assay (Luciferin-PFBE) Cell-Based/Biochemical Assay	50ml	V8902

Also available in 10ml size.

NADPH Regeneration System

Product	Size	Cat.#
NADPH Regeneration System	1,000 assays	V9510

9. Related Products (continued)

MAO-Glo™ Assay

Product	Size	Cat.#
MAO-Glo™ Assay	200 assays	V1401
	1,000 assays	V1402

UGT-Glo™ Assays and Screening Systems

Product	Size	Cat.#
UGT-Glo™ Assay	200 assays	V2081
	1,000 assays	V2082
UGT-Glo™ UGT1A1 Screening System	200 assays	V2120
	1,000 assays	V2121
UGT-Glo™ UGT2B7 Screening System	200 assays	V2130
	1,000 assays	V2131

CellTiter-Glo® Luminescent Cell Viability Assays

Product	Size	Cat.#
CellTiter-Glo® Luminescent Cell Viability Assay	10ml	G7570
	10 × 10ml	G7571
	100ml	G7572
	10 × 100ml	G7573

Kinase-Glo® Plus Luminescent Kinase Assay

Product	Size	Cat.#
Kinase-Glo® Plus Luminescent Kinase Assay	10ml	V3771
	10 × 10ml	V3772
	100ml	V3773
	10 × 100ml	V3774

10. Summary of Changes

The following changes were made to the 11/15 revision of this document:

1. Patent and disclaimer statements were updated.
2. The document design was updated.

[a]Patent Pending.

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