



Promega

Technical Bulletin

Pgp-Glo™ Assay Systems

INSTRUCTIONS FOR USE OF PRODUCTS V3591 AND V3601.



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Pgp-Glo™ Assay Systems

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1. Description

The Pgp-Glo™ Assay Systems^(a,b) provide the necessary reagents for performing luminescent P-glycoprotein (Pgp) ATPase assays. Pgp, also known as MDRI 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.

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.

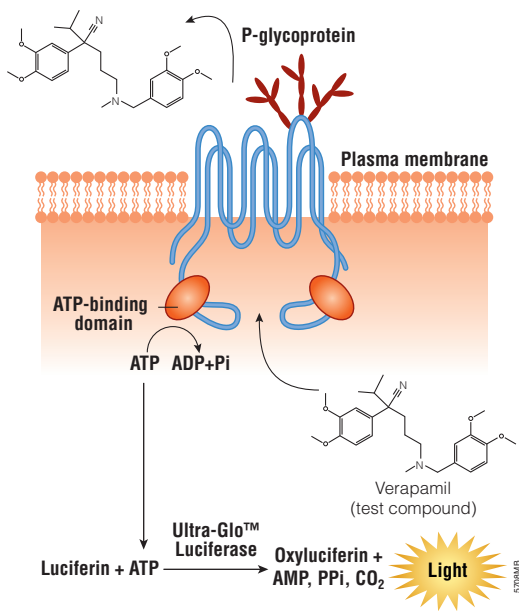


Figure 1. The Pgp-Glo™ Assay System. The Pgp-Glo™ Assay relies on the ATP-dependence of the light-generating reaction of firefly luciferase. After a pool of ATP is first exposed to the Pgp ATPase, ATP consumption is detected as a decrease in luminescence from a second reaction with a recombinant firefly 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 $5.4\mu\text{M}$ (1,2).

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 $\Delta\text{RLU}_{\text{basal}}$. 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 $\Delta\text{RLU}_{\text{TC}}$. For the experiment shown in Figure 2, the increase in Pgp ATPase activity caused by Verapamil ($\Delta\text{RLU}_{\text{TC}} / \Delta\text{RLU}_{\text{basal}}$) 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.

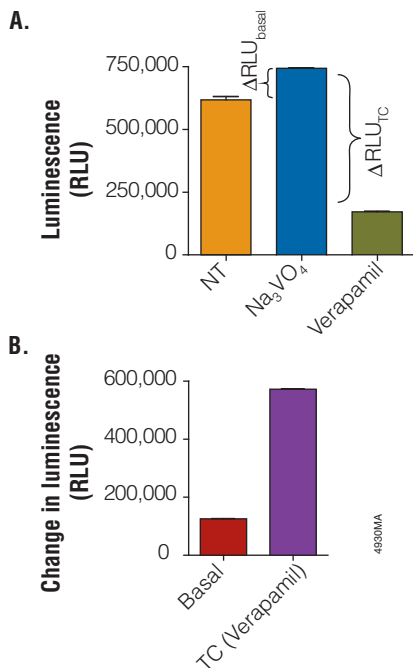


Figure 2. Stimulation of Pgp ATPase activity by Verapamil. Untreated (NT), 100 μ M Na₃VO₄- and 200 μ M Verapamil-treated Pgp reactions were performed according to the protocol described in Section 5. Luminescence was read on a BMG LABTECH Polarstar Optima luminometer (**Panel A**). The decrease in luminescence of NT samples compared to samples plus Na₃VO₄ (Δ RLU_{basal}) represents basal Pgp ATPase activity. The decrease in luminescence of Verapamil-treated samples (Δ RLU_{TC}) represents Verapamil-stimulated Pgp ATPase activity. Δ RLU_{basal} and Δ RLU_{TC} were replotted (**Panel B**) to illustrate the stimulation of Pgp ATPase activity by Verapamil.

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.

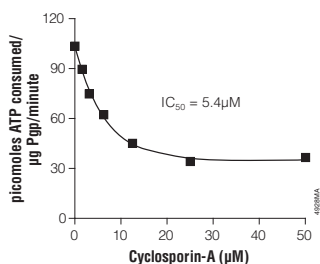


Figure 3. IC₅₀ measurements. Cyclosporin-A was tested at a range of concentrations for its capacity to inhibit 200µM Verapamil-stimulated Pgp ATPase activity. Luminescence was read on a BMG LABTECH Polarstar Optima luminometer, and data was analyzed as described by Section 6.B.

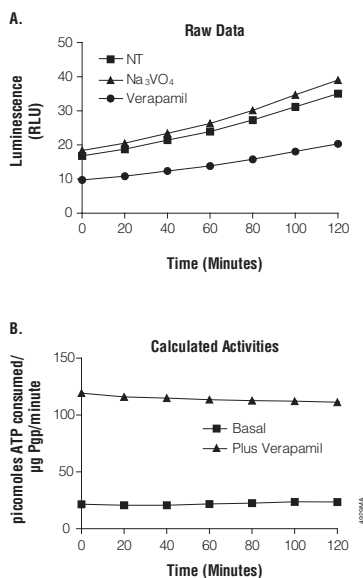


Figure 4. Stability of calculated activities. Untreated (NT), Verapamil-treated and Na₃VO₄-treated Pgp reactions were incubated for 40 minutes at 37°C in a 96-well plate. The reactions were stopped and luminescence initiated by addition of ATP Detection Reagent as described in Section 5. Following a 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.

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, Na₃VO₄, 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 allows for 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.

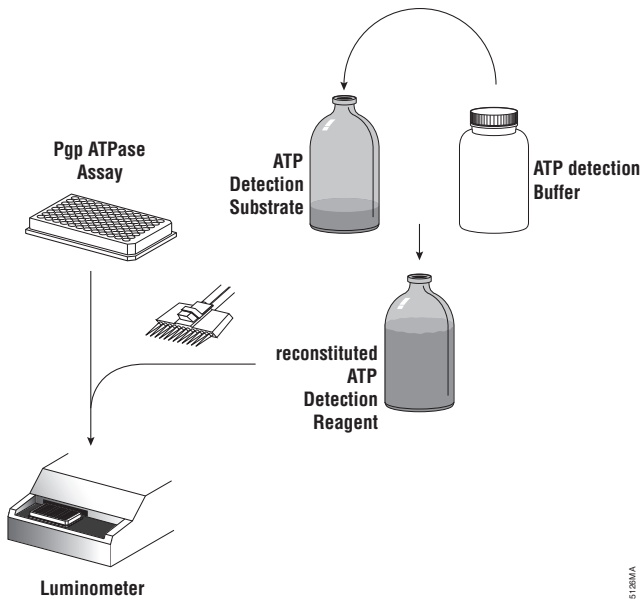


Figure 5. Flow diagram showing preparation and use of the reconstituted ATP Detection Reagent.

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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 Na₃VO₄ (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 Na₃VO₄ (sodium orthovanadate)

Storage Conditions: Store Recombinant Human Pgp Membranes at -70°C. The Pgp Membranes are stable for up to 5 freeze-thaw cycles. Since individual freeze-thaw conditions may vary, we recommend dispensing the Pgp 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. When stored and handled properly, this system is guaranteed for 6 months from the date of purchase.

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 Na₃VO₄-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 Na₃VO₄

Combine 5µl of 10mM Na₃VO₄ 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 RLUs to ATP concentrations.

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

1. Add 30 μ l of water to tubes for the 15mM, 7.5mM and 3.75mM standards. Add 30 μ l 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 30 μ l of the solution in that tube to the 7.5mM tube, mix well and then transfer 30 μ l from the 7.5mM tube to the 3.75mM tube. **Mix well** between transfers. Discard 30 μ l of the solution from the 3.75mM tube.
3. Add 20 μ l 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 20 μ l of Pgp Membrane (25 μ g) per reaction. For example, combine 50 μ l of Pgp Membranes with 150 μ l 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 ₃ VO 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 to convert RLUs to specific activity expressed in units of nanomoles ATP consumed/ μ g Pgp/minute (see Sections 6.A and 6.B).
- **NT:** No treatment control provides a measure of total, unregulated ATPase activity.
- **Na₃VO₄:** 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 EC₅₀ 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 Na₃VO₄ in Pgp-Glo™ Assay Buffer to wells labeled “Na₃VO₄”, 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 plate and incubate for 40 minutes at 37°C.
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 ($\text{RLU}_{\text{Na}_3\text{VO}_4}$) and untreated (NT) samples (RLU_{NT}) to determine $\Delta\text{RLU}_{\text{basal}}$ as follows:
$$\text{RLU}_{\text{Na}_3\text{VO}_4} - \text{RLU}_{\text{NT}} = \Delta\text{RLU}_{\text{basal}}$$
$$\Delta\text{RLU}_{\text{basal}}$$
 reflects basal Pgp ATPase activity.
2. Calculate the difference between the average luminescent signals from Na_3VO_4 -treated samples ($\text{RLU}_{\text{Na}_3\text{VO}_4}$) and test compound-treated samples (RLU_{TC}) to determine $\Delta\text{RLU}_{\text{TC}}$ as follows:
$$\text{RLU}_{\text{Na}_3\text{VO}_4} - \text{RLU}_{\text{TC}} = \Delta\text{RLU}_{\text{TC}}$$
$$\Delta\text{RLU}_{\text{TC}}$$
 reflects Pgp ATPase activity in the presence of a test compound.

Possible outcomes:

- If $\Delta\text{RLU}_{\text{TC}} > \Delta\text{RLU}_{\text{basal}}$ then the test compound is a stimulator of Pgp ATPase activity.
- If $\Delta\text{RLU}_{\text{TC}} = \Delta\text{RLU}_{\text{basal}}$ then the test compound has no effect on Pgp ATPase activity.
- If $\Delta\text{RLU}_{\text{TC}} < \Delta\text{RLU}_{\text{basal}}$ 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 $\Delta\text{RLU}_{\text{TC}}$ by $\Delta\text{RLU}_{\text{basal}}$.

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

6.A. Analysis without ATP Standards (continued)

Note: The effects of test compounds can be ascertained quickly by comparing the test compound samples to untreated and Na₃VO₄-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 Na₃VO₄-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

$$\frac{([ATP_{Na_3VO_4}] - [ATP_{NT}])}{(25\mu g \text{ Pgp} \times 40 \text{ minutes})} = \text{nmol ATP consumed}/\mu g \text{ Pgp}/\text{minute}$$

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

$$\frac{([ATP_{Na_3VO_4}] - [ATP_{TC}])}{(25\mu g \text{ Pgp} \times 40 \text{ minutes})} = \text{nmol ATP consumed}/\mu g \text{ Pgp}/\text{minute}$$

5. Fold stimulation by a test compound calculated as:

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

6. To calculate test-compound EC₅₀ or IC₅₀ values, plot Pgp specific activity versus test compound concentration. See Figures 3 and 7 for examples of IC₅₀ and EC₅₀ measurements. Figure 7 shows that the EC₅₀ 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).

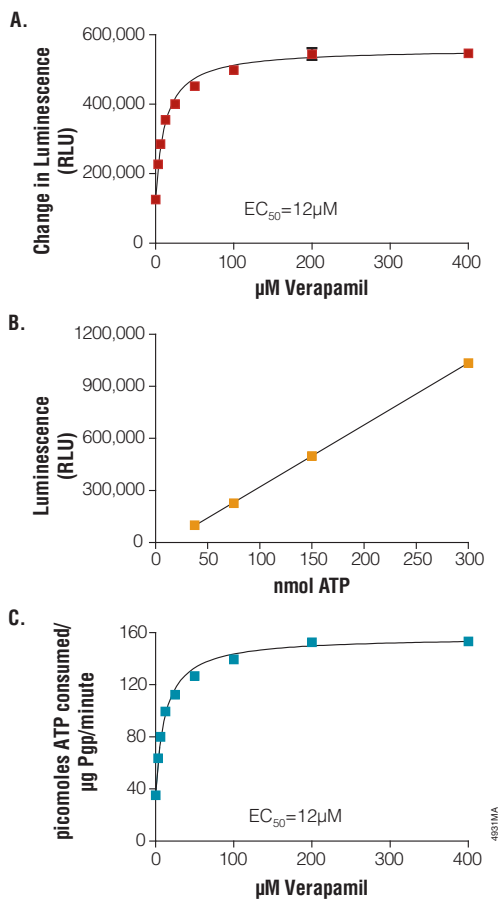


Figure 7. EC₅₀ measurements. Verapamil was tested at a range of concentrations for its capacity to stimulate Pgp ATPase activity. Luminescence was read on a BMG LABTECH Polarstar Optima luminometer. Data was analyzed in terms of RLU as described in Section 6.A (Panel A) and in terms of ATPase activity (Panel C) by comparison to the ATP standard curve (Panel B) as described in Section 6.B. Linear regression, curve fits and EC₅₀ 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 | <p>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).</p> <p>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 Na₃VO₄.</p> <p>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.</p> <p>Inactive Pgp. Avoid multiple Pgp freeze-thaw cycles; keep Pgp on ice until ready to use.</p> |
| Unexpected stimulation of Pgp ATPase activity by a test compound (false positive) | <p>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 Na₃VO₄ to a sample with Na₃VO₄ 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 Na₃VO₄ will emit less light than the sample with Na₃VO₄ alone.</p> |
| ATP standard curve is not linear; linear regression shows R ² <0.97 | <p>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.</p> |

| Symptoms | Causes and Comments |
|---|---|
| ATP standard curve is not linear; linear regression shows $R^2 < 0.97$ (continued) | 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 addition of the ATP Detection Reagent, should be 3.0, 1.5, 0.75 and 0.375mM. Note: For interpolating sample concentrations from a standard curve, a non-linear, 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

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2. Litman, T. *et al.* (2003) Pumping of drugs by P-glycoprotein: A two step process? *J. Pharm. Exp. Ther.* **3F07(3)**, 846-853.
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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 | V9700 |
| 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 | 10ml | V8751 |
| | 50ml | V8752 |
| P450-Glo™ CYP1B1 Assay | 10ml | V8761 |
| | 50ml | V8762 |
| P450-Glo™ CYP1A2 Assay | 10ml | V8771 |
| | 50ml | V8772 |
| P450-Glo™ CYP2C8 Assay | 10ml | V8781 |
| | 50ml | V8782 |
| P450-Glo™ CYP2C9 Assay | 10ml | V8791 |
| | 50ml | V8792 |
| P450-Glo™ CYP3A4 Assay | 10ml | V8801 |
| | 50ml | V8802 |
| P450-Glo™ CYP3A7 Assay | 10ml | V8811 |
| | 50ml | V8802 |
| P450-Glo™ CYP2C19 Assay | 10ml | V8881 |
| | 50ml | V8882 |
| P450-Glo™ CYP2D6 Assay | 10ml | V8891 |
| | 50ml | V8892 |
| NADPH Regeneration System | 1,000 Assays | V9510 |

MAO-Glo™ Assays

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

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 |

^(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) Patent Pending.

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All prices and specifications are subject to change without prior notice.

Product claims are subject to change. Please contact Promega Technical Services or access the Promega online catalog for the most up-to-date information on Promega products.