



Identify P-glycoprotein Substrates and Inhibitors with the Rapid, HTS Pgp-Glo™ Assay System

ABSTRACT The Pgp-Glo™ Assay Systems are luminescent P-glycoprotein (Pgp) ATPase assays. The Pgp-Glo™ Assay detects the effects of compounds on recombinant human Pgp in a cell membrane fraction. The assay couples the ATPase activity of Pgp to the ATP dependence of the light-generating reaction of firefly luciferase. The rate of ATP consumption by Pgp is inversely correlated with the light output of the luciferase reaction. The Pgp-Glo™ Assay is easily amenable to high-throughput screening and can complement cell-based Pgp studies.

Dongping Ma, M.S., and James J. Cali, Ph.D., Promega Corporation

INTRODUCTION

P-glycoprotein (Pgp, also known as MDR1 or ABCB1) is an ATP-dependent efflux pump for a wide range of hydrophobic compounds including drugs and other xenobiotics (1,2). The Pgp transporter is mainly found on the apical side of epithelial cells in the small intestine, liver, kidney and capillaries of the blood-brain barrier. Many compounds that enter cells by passive diffusion across the membrane or by active transport are pumped out of the cell by Pgp. This Pgp function is the basis of multidrug resistance in tumors that overexpress Pgp; because of the promiscuous transport activity of Pgp, many antitumor drugs fail to accumulate to cytotoxic levels. Also, the apical transport activity of Pgp limits drug uptake in the gut and brain and influences renal and hepatic drug clearance. Furthermore, coadministration of drugs that interact with Pgp as substrates, inhibitors or inducers can result in adverse drug-drug interactions (3). For all these reasons understanding how drugs and drug candidates will interact with Pgp is important.

A direct way to determine how a drug will interact with Pgp is to measure its effect on the rate of Pgp ATP hydrolysis (1,4). Pgp has a basal ATPase activity that may be affected by test compounds as follows: 1) compounds that do not interact with Pgp have no effect on the basal ATPase activity; 2) compounds that are substrates for transport by Pgp stimulate basal ATPase activity; 3) compounds that are transported slowly inhibit the stimulated ATPase activity measured in the presence of a second transported substrate; 4) inhibitors of the pump that are noncompetitive for transport inhibit both the basal and stimulated ATPase activity. A Pgp ATPase assay can therefore identify compounds that interact with Pgp and discern the nature of the interaction.

A common Pgp ATPase assay uses recombinant Pgp in a membrane fraction with colorimetric detection of the inorganic phosphate produced by ATP hydrolysis (5,6). However, there are disadvantages

to the colorimetric assay. Instability of reagents and instability of colorimetric signals make the assay difficult to automate or perform in a high-throughput mode. The Pgp-Glo™ Assay System^(a,b) (Cat.# V3591), a luminescent Pgp ATPase assay for identifying and characterizing Pgp substrates and modulators, overcomes these limitations. The Pgp-Glo™ Assay is a simple-to-perform, high-throughput assay for identifying Pgp substrates and inhibitors.

PGP-GLO™ ASSAY PRINCIPLE

The Pgp-Glo™ Assay couples the ATPase activity of Pgp to the ATP dependence of the light-generating reaction of firefly luciferase (Figure 1). The rate of ATP consumption by Pgp is inversely correlated with the light output of a luciferase reaction. For

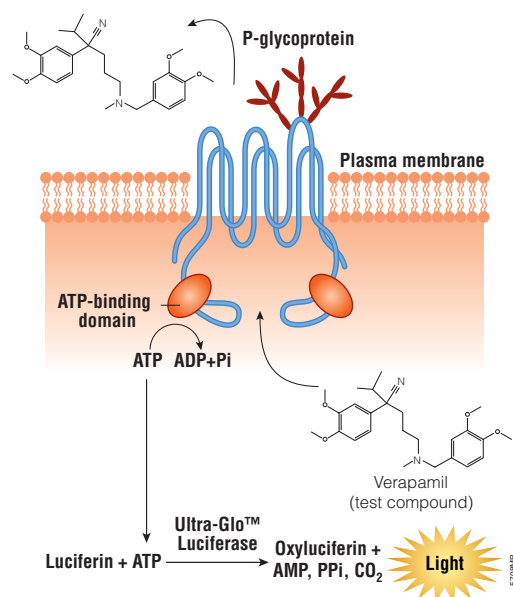


Figure 1. The Pgp-Glo™ Assay System. The Pgp-Glo™ Assay relies on the ATP-dependence of the light-generating reaction of firefly luciferase. After a pool of ATP is first exposed to the Pgp ATPase, ATP consumption is detected as a decrease in luminescence from a second reaction with a stabilized recombinant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

Pgp Activity

A direct way to determine how a drug will interact with Pgp is to measure its effect on the rate of Pgp ATP hydrolysis. A Pgp ATPase assay can identify compounds that interact with Pgp and discern the nature of the interaction.

The fold increase in ATP consumption stimulated by verapamil and detected using the Pgp-Glo™ Assay correlates well with previously published results.

the Pgp-Glo™ Assay, recombinant human Pgp is expressed in insect cells using the baculovirus system, and a membrane fraction is prepared. A sample of the Pgp membranes is incubated with a nonlimiting ATP concentration to give an ATPase rate at V_{max} . During the incubation some of the ATP is consumed by Pgp. A luciferase-based ATP Detection Reagent is then added to stop the Pgp activity and initiate a luciferase reaction that produces glow-type luminescence. Since the starting ATP concentration is in the linear range of the luciferase ATP curve, ATP consumption by Pgp will be seen as a decrease in luminescence compared to an inactive control. Samples treated with a drug or other test compound that stimulates the Pgp ATPase activity will show further decrease in luminescence compared to an untreated control. In this way substrates for transport by Pgp are identified as stimulators of the ATPase activity. Conversely, inhibitors of the ATPase activity are identified as compounds that minimize the decrease in luminescence seen for basal activity or a drug-stimulated activity.

PGP-GLO™ ASSAY RESULTS CORRELATE WELL WITH PUBLISHED LITERATURE

Figure 2 shows data from a Pgp-Glo™ experiment that examined the effect of the cardiac drug verapamil on Pgp ATPase activity. Verapamil is a known substrate for

transport by Pgp and activator of its ATPase activity (2). In Figure 2, Panel A, three conditions were tested: no treatment (NT), sodium orthovanadate treatment (Na_3VO_4), and verapamil treatment. Na_3VO_4 is an inhibitor of the Pgp ATPase, so this sample serves as a control where no Pgp-dependent ATP consumption occurs. In the untreated sample, Pgp consumes ATP at a basal rate, and this rate is compared to that of the Na_3VO_4 - and verapamil-treated samples. The untreated sample showed a decrease in luminescence compared to the Na_3VO_4 -treated sample, and that difference ($\Delta\text{RLU}_{\text{basal}}$) reflects the basal Pgp ATPase activity. The test compound (TC) verapamil caused a larger decrease in luminescence compared to basal levels, and that decrease ($\Delta\text{RLU}_{\text{TC}}$) reflects the verapamil-stimulated ATPase activ-

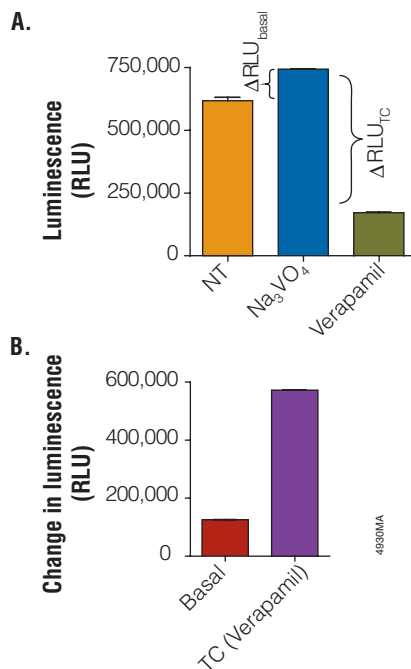


Figure 2. Stimulation of Pgp ATPase activity by verapamil. A recombinant Pgp membrane preparation was incubated for 40 minutes at 37°C with 5mM ATP alone (NT), or plus 100 μM Na_3VO_4 or the test compound verapamil (TC) at 200 μM . A luminogenic Ultra-Glo™ Luciferase-based ATP Detection Reagent was then added, and luminescence was read in relative light units (RLU) on a BMG LABTECH Polarstar Optima luminometer (**Panel A**). **Panel B** shows the difference in luminescence noted in Panel A between the Na_3VO_4 -treated samples and NT ($\Delta\text{RLU}_{\text{basal}}$) and the difference between the Na_3VO_4 -treated samples and verapamil-treated samples ($\Delta\text{RLU}_{\text{TC}}$).

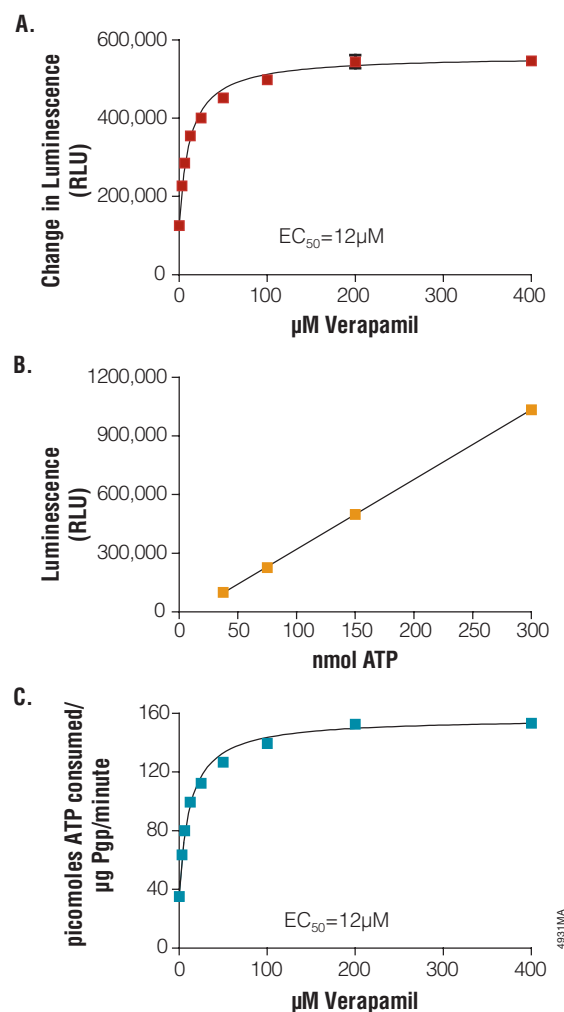


Figure 3. Interpolating ATPase activity from luminescence. Verapamil was tested for dose-dependent activation of Pgp ATPase activity as described in the legend to Figure 2. **Panel A**. Changes in luminescence represent the difference in RLU seen between Na_3VO_4 -treated samples and samples with the indicated concentrations of verapamil from 0 to 400 μM . EC_{50} is the concentration of verapamil that caused a half maximal effect. **Panel B**. A luminescent ATP standard curve generated with the Pgp-Glo™ ATP Detection Reagent. **Panel C**. The data from Panel A expressed in terms of ATPase activities that were calculated after interpolation of ATP concentrations from the luminescent ATP standard curve.

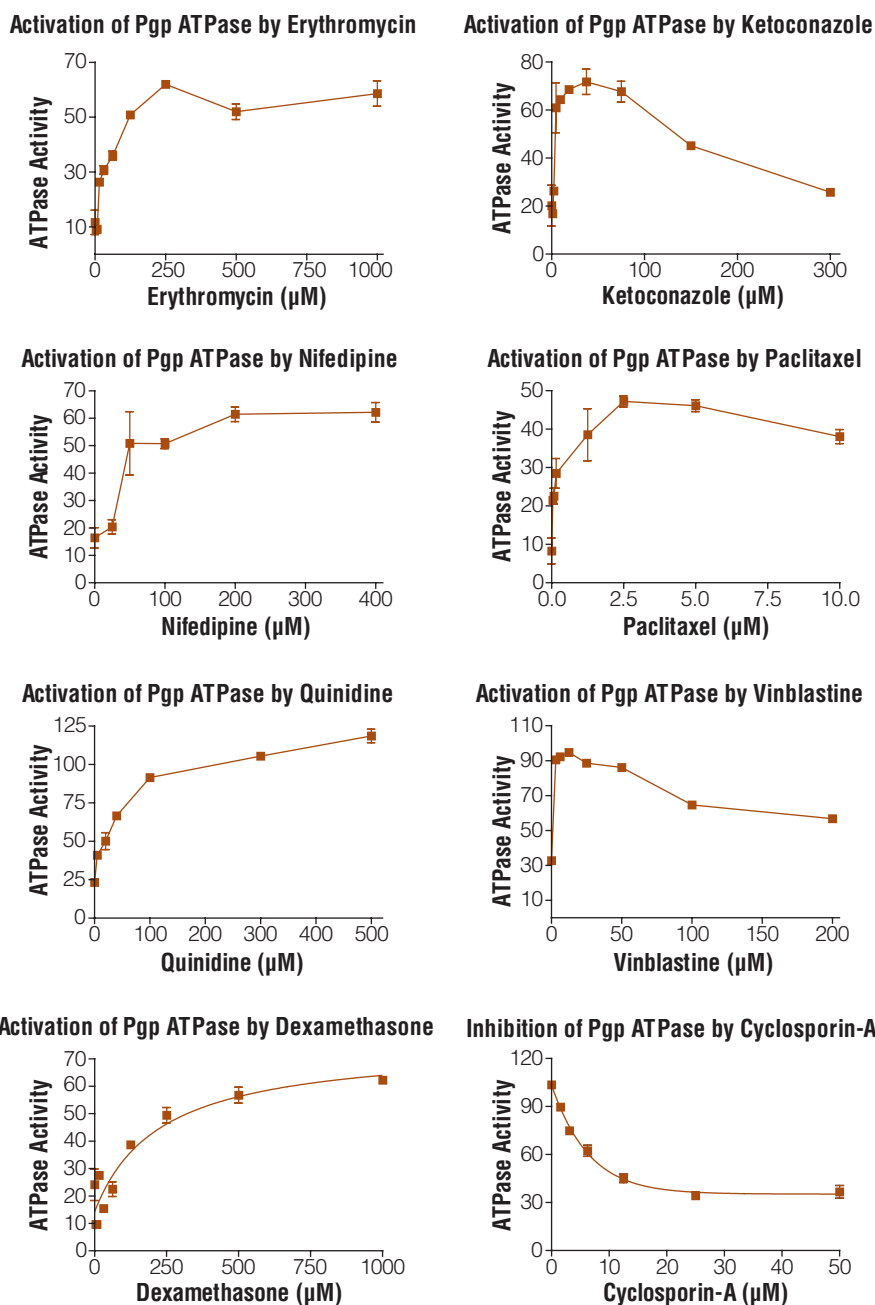


Figure 4. Dose-dependent modulation of Pgp ATPase activity by various drugs. Assays were performed with the indicated drug concentrations as described in the legend to Figure 3. The inhibitory effect of cyclosporin-A was measured against verapamil-stimulated Pgp ATPase activity (200 μM verapamil).

ity. In Figure 2, Panel B, absolute ΔRLU values from Figure 2, Panel A, were plotted to show the change in ATP consumption with verapamil, a 4.6-fold increase. The fold increase caused by verapamil, measured here with Pgp-Glo™ Assay correlates very well with a published value that used a conventional colorimetric ATPase assay method (5).

The dose dependency of the stimulatory effect of verapamil was also measured with the Pgp-Glo™ Assay (Figure 3). The magnitude of the change in luminescence relative to a Na_3VO_4 -treated control (ΔRLU) increased with increasing verapamil concentration to a

point of saturation. The half-maximal stimulatory effect (EC_{50}) was seen at 12 μM verapamil, a value that correlates very well with a previously published value (5).

To measure fold stimulation (Figure 2) and EC_{50} (Figure 3, Panel A) with the Pgp-Glo™ Assay, it was sufficient to express data in terms of luminescence in relative light units (RLU). To express the data as an ATPase specific activity (moles ATP consumed/unit Pgp/unit time), known quantities of ATP were combined with the Pgp-Glo™ Assay ATP Detection Reagent to generate a luminescent ATP standard curve (Figure 3, Panel B),

Pgp-Glo™ Assay
distinguishes between
Class I and Class II Pgp
substrates.

and ATP concentrations of samples were interpolated from the standard curve. Figure 3, Panel C, shows the verapamil concentration-dependent stimulation curve expressed as ATPase activity with a basal rate of 35pmol ATP consumed/ μ g Pgp/minute and the maximal stimulated rate of 160pmol ATP consumed/ μ g Pgp/minute.

The effects on ATPase activity of additional known Pgp substrates were tested using the Pgp-Glo™ Assay (Figure 4). There were two profiles to the dose-dependent ATPase activity curves representative of what have been termed class I and class II Pgp substrates (1,2). The ketoconazole, paclitaxel, vinblastine and cyclosporin-A curves conform to the class I profile where dose-dependent activation of ATPase activity is observed at low concentration and inhibition at higher concentrations. The erythromycin, nifedipine, quinidine and dexamethasone curves conform to the class II profile where a saturable, dose-dependent activation of ATPase activity is observed. Cyclosporin-A is known as a Pgp inhibitor, and inhibition was observed when it was applied to verapamil-stimulated Pgp (1,7).

The Pgp-Glo™ ATPase assay improves upon conventional recombinant membrane-based ATPase assays by providing a simplified protocol that is amenable to automated formats. The Pgp-Glo™ Assay can also replace or complement popular cell-based strategies. The Pgp-Glo™ recombinant membrane-based approach provides a means to eliminate labor intensive cell culture and also provides information about interacting compounds that may be difficult or impossible to extract from cell-based assays. For example, an indirect cell-based approach measures the impact of test compounds on a traceable fluorescent probe that is also a Pgp transport substrate (8). Compounds that enhance the retention of the probe are scored as Pgp ligands because they interfere with extrusion of the marker. The approach is simple and high-throughput, though indirect, and unlike the Pgp-Glo™ Assay, it cannot discriminate between noncompetitive inhibitors and competitive inhibitors that are themselves substrates for transport. With the Pgp-Glo™ Assay, competitive inhibitors that are substrates for transport score as activators of the ATPase activity, and noncompetitive inhibitors diminish the ATPase activity.

Cell-based vectorial transport assays directly track the movement of test compounds across a confluent monolayer of polarized cells (e.g., Caco-2 cells) grown on a permeable substratum with medium in the basolateral and apical compartments (9). The approach is direct and can provide important information about transport rates. However it is labor intensive; throughput is relatively low, and highly permeable, fast-diffusing compounds are

difficult to track. A fast-diffusing compound may be scored as an inhibitor of the transport of a second compound. However, if the fast-diffusing compound is transported by Pgp, that transport will be missed because the compound passively diffuses back into the cell as fast as it is pumped out, and a net change will not be observed (10). The Pgp-Glo™ Assay does identify fast-diffusing compounds that are substrates for transport by detecting stimulation of the ATPase activity. Conversely, the Pgp-Glo™ Assay does not miss low-permeability compounds that might be excluded from cells lacking an active uptake mechanism or closed vesicles that restrict access to Pgp binding or transport sites on the inner side of the plasma membrane. This is because a substantial portion of the recombinant Pgp membranes are open fragments and inside-out vesicles.

The choice of Pgp assay depends in part on the ultimate goals of the study. For rapid high-throughput screening for both Pgp transport substrates and inhibitors without the need for cell culture, the Pgp-Glo™ Assay is an excellent choice. This assay can stand alone or be used in parallel with cell-based assays to insure no Pgp ligands are missed or to more thoroughly characterize ligand mechanism of action.

REFERENCES

1. Ambudkar, S.V. *et al.* (1999) *Annu. Rev. Pharmacol. Toxicol.* **39**, 361–98.
2. Litman, T., Druley, T.E. and Bates, S.E. (2001) *Cell. Mol. Life Sci.* **58**, 931–59.
3. Glavinas, H. *et al.* (2004) *Curr. Drug Deliv.* **1**, 27–42.
4. Scarborough, G.A. (1995) *J. Bioenerg. Biomembr.* **27**, 37–41.
5. Sarkadi, B. *et al.* (1992) *J. Biol. Chem.* **267**, 4854–8.
6. Saheki, S., Takeda, A. and Shimazu, T. (1985) *Anal. Biochem.* **148**, 277–81.
7. Schmitt, A. *et al.* (2006) *J. Neural Transm.* **113**, 787–801.
8. Wang, E. *et al.* (2001) *Biochem. Biophys. Res. Com.* **289**, 580–5.
9. Feng, R.L. *et al.* (2002) *Drug Metab. Dispos.* **30**, 763–70.
10. Eytan, G.D. *et al.* (1996) *J. Biol. Chem.* **271**, 12897–902.

ORDERING INFORMATION

Product	Size	Cat.#
Pgp-Glo™ Assay System with P-glycoprotein	10ml	V3601
Pgp-Glo™ Assay System	10ml	V3591

^(a) Patent Pending.

^(b) 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.

Products may be covered by pending or issued patents or may have certain limitations. Please visit our Web site for more information.

Pgp-Glo and Ultra-Glo are trademarks of Promega Corporation.

The Pgp-Glo™ recombinant membrane-based approach provides a means to eliminate labor intensive cell culture and also provides information about interacting compounds that may be difficult or impossible to extract from cell-based assays.