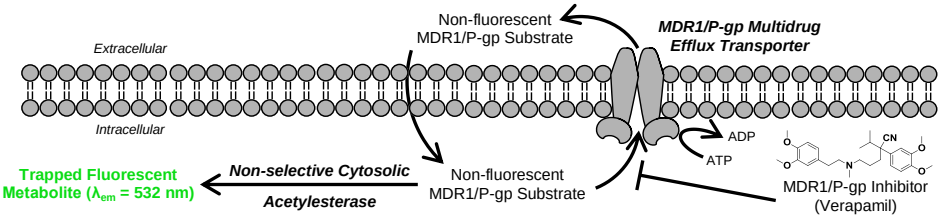
Multidrug Efflux Transporter (MDR1/P-gp) Ligand Screening Kit

4/16

(Catalog # K507-100; 100 Assays; Store at -20°C)

I. Introduction:

P-glycoprotein (P-gp, Multidrug Resistance Protein 1 (MDR1), EC 3.6.3.44) is a member of the ATP-binding cassette (ABC) ATPase superfamily of transmembrane transporter proteins. P-gp has an extremely broad substrate specificity and is capable of transporting a vast array of neutral and anionic lipophilic molecules. P-gp strongly affects the oral absorption, tissue distribution and excretion of many drugs and prevents certain lipophilic drugs from penetrating the blood brain barrier. Overexpression of P-gp confers tumor cells with resistance to chemically and pharmacologically distinct chemotherapeutic drugs (such as doxorubicin, vincristine and paclitaxel) by actively pumping them out of cells. Induction of P-gp expression is a frequent cause of treatment failure and tumor-targeted delivery of P-gp inhibitors is being investigated as a strategy for overcoming chemotherapy resistance. BioVision's MDR1/P-gp Ligand Screening Kit is designed for rapidly screening test compounds for modulation of efflux transporter activity in MDR1-expressing cell lines. The assay uses a lipophilic non-fluorescent P-gp substrate that readily diffuses through the plasma membrane, where it is hydrolyzed to an active fluorophore by cytosolic esterases. The resulting hydrophilic fluorophore is neither membrane permeable nor a substrate for P-gp, hence it remains trapped inside the cell. In MDR1-expressing cell lines, the lipophilic pro-fluorophore is continuously extruded from the cytosol by P-gp, leading to a low intracellular fluorescence. Inhibition of P-gp-mediated efflux by a test compound leads to increased intracellular fluorescence. Specific transporter activity is quantified by comparison with fluorescence accumulated in the presence and absence of a saturating concentration of the included selective P-gp inhibitor. The assay is highly sensitive, has a simple no-wash protocol and is high-throughput adaptable. The kit contains a complete set of reagents sufficient for performing 100 reactions in a 96-well plate format.



II. Applications:

- Screening and characterization of drugs and new chemical entities for inhibition of native/recombinant MDR1/P-gp efflux transporter.
- Identification/characterization of cells and cell lines with a high level of MDR1-mediated efflux (*i.e.* chemotherapy-resistant).

III. Sample Type:

- Cells expressing high levels of MDR1/P-gp (*e.g.* cancer cell line with cytotoxic drug-resistant phenotype)

IV. Kit Contents:

Components	K507-100	Cap Code	Part Number
Efflux Assay Buffer	50 ml	NM	K507-100-1
Fluorogenic P-gp Substrate	1 vial	Amber	K507-100-2
P-gp Inhibitor (Verapamil)	1 vial	Orange	K507-100-3

V. User Supplied Reagents and Equipment:

- Cell line for testing: cells with high levels of endogenous MDR1/P-gp (*e.g.* A549/ADR, KB-V1 or MES-SA/MX2 drug-resistant cancer cell lines) or heterologous cells stably transfected with human MDR1 (*e.g.* MDCKII-MDR1 cells) are recommended
- Appropriate cell culture medium and 5% CO_2 cell culture incubator
- Multiwell fluorescence microplate reader
- Precision multi-channel pipette and reagent reservoir
- Sterile anhydrous (reagent grade) DMSO
- White-walled 96-well plates with clear flat bottom

VI. Storage Conditions and Reagent Preparation:

Store kit at -20°C and protect from light. Briefly centrifuge all small vials prior to opening. Open all of the reagents under sterile conditions (*e.g.* a cell culture hood) only. Read entire protocol before performing the assay procedure.

- **Efflux Assay Buffer:** Allow to thaw to room temperature under sterile conditions. Store at 4°C.
- **Fluorogenic P-gp Substrate:** Reconstitute with 55 µl anhydrous DMSO and vortex thoroughly to obtain a 400X stock solution. Aliquot the stock solution as desired and store aliquots at -20°C, protected from light. Avoid repeated freeze/thaw cycles.
- **P-gp Inhibitor (Verapamil):** Reconstitute with 110 µl anhydrous DMSO and vortex until fully dissolved to obtain a 100X stock solution. Store at -20°C, stable for at least 4 freeze/thaw cycles.

VII. Multidrug Efflux Transporter (MDR1/P-gp) Ligand Screening Protocol:

The procedure described below is for a 96-well plate format but may be adapted to other formats by scaling the reagent volumes and cell density according to the desired plate size. To ensure assay consistency, we recommend that each treatment condition (including no inhibition and maximal inhibition controls) be performed in duplicate or triplicate wells.

1. **Cell Culture and Seeding:** If using an adherent cell line, split cells one day prior to assay and seed approximately 3-5 x 10^4 cells/well in a white-walled 96-well plate (with clear bottom) using 200 µl appropriate culture media/well. Grow cells overnight in a 5% CO_2 atmosphere 37°C incubator (cell monolayer should be ≈80-90% confluent for optimal assay). For suspension cell lines, pellet cells by

centrifugation, replace standard growth medium with serum-free, phenol red-free medium and plate approximately 1-2 x 10^5 cells/well using 100 µl medium/well in a white-walled, clear bottom 96-well plate.

2. **Efflux Assay Reaction and Test Compound Preparation:**
   - **a.** Pre-warm Efflux Assay Buffer to 37°C. For adherent cells, gently aspirate culture medium, wash the cells once with 100 µl Efflux Assay Buffer to ensure complete removal of medium and add 100 µl fresh Efflux Assay Buffer to each well before returning cells to incubator. For suspension cells, no wash step is needed if cells have been plated in 100 µl serum-free, phenol red-free medium/well.
   - **b.** Dissolve test compounds in proper solvent(s) to produce stock solutions. For each test compound, prepare a 4X solution of each desired test concentration by diluting stock solutions in Efflux Assay Buffer. To determine IC_{50} values for test compounds, 4X test compound solutions should be prepared in a range of concentrations in order to generate a multi-point dose-response curve (the concentration of organic solvent should be the same for all test compound dilutions). Final organic solvent concentration should be minimized to avoid impacting cell health or P-gp efflux pump activity. We recommend that the 4X test compound solutions contain 4% DMSO (1% final concentration), as DMSO has been shown to have little effect on P-gp activity at a concentration of ≤2% (v/v).
   - **c.** Prepare a 4X maximal inhibition control solution by adding 20 µl of the 100X verapamil stock to 480 µl Efflux Assay Buffer. The maximal inhibition control (Verapamil, 100 µM final concentration) serves as a definition of 100% inhibition of P-gp mediated efflux. Prepare a 4X control solution (no inhibition) by adding 20 µl of anhydrous DMSO to 480 µl Efflux Assay Buffer (1% DMSO at final concentration). Add 50 µl of either 4X test compound solution, 4X maximal inhibition control solution or 4X no inhibition control solution to each well. Each plate should contain its own maximal inhibition and no inhibition control wells.
   - **d.** Prepare 4X solution of Fluorogenic P-gp Substrate by adding 50 µl of the 400X stock solution to 4950 µl pre-warmed Efflux Assay Buffer. This preparation is sufficient for 100 reaction wells, but can be scaled depending upon the number of reactions to be performed. The 4X Fluorogenic P-gp Substrate working solution should be made fresh prior to use.
   - **e.** Add 50 µl of 4X Fluorogenic P-gp Substrate to each well (for a final reaction volume of 200 µl per well) and incubate the plate at 37°C in a 5% CO_2 atmosphere, protected from light for 30 min.
3. **Measurement:** After 30 min incubation, measure the fluorescence intensity (Ex/Em = 488/532 nm) of all of the wells in end-point mode using the 'bottom read' function of the spectrofluorometer.
4. **Calculation:** For each test compound (TC) well, quantify the relative inhibition of P-gp mediated substrate efflux using the equation below, where $F_{vehicle}$ is the fluorescence intensity of the no inhibition control condition (solvent control), F_{max} is the defined maximal inhibition control condition (100 µM verapamil) and F_{TC} is the fluorescence intensity of a test compound at the given concentration:

$$\% \text{ Activity} = 100 - \left(\frac{F_{TC} - F_{vehicle}}{F_{max} - F_{vehicle}} \times 100\right)$$

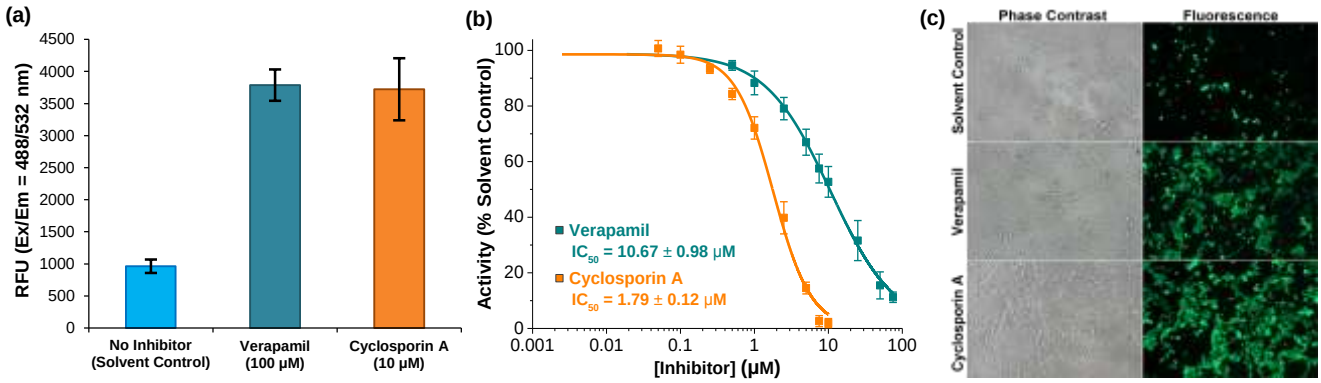


Figure: (a) Intracellular accumulation of fluorogenic MDR1 substrate hydrolysis product in the presence and absence of the MDR1 inhibitors verapamil and cyclosporin A. Fluorescence was measured 30 min after addition of MDR1 substrate. **(b)** Dose-response curves for MDR1 inhibition by verapamil and cyclosporin A. Percent activity was calculated for each concentration by comparison to transporter activity in the presence of 100 µM verapamil (positive inhibition control) and vehicle (negative inhibition control). **(c)** Fluorescence microscopy showing increased intracellular fluorescence in the presence of verapamil (middle right panel) and cyclosporin A (lower right panel). Cells were cultured overnight on a clear-bottom 96-well plate and exposed to MDR1 substrate for 30 min following 30 min pre-incubation with either vehicle (1% DMSO), verapamil (100 µM) or cyclosporin A (10 µM). Brightfield and fluorescence images were obtained with a Nikon TE2000 inverted microscope using a 10X Plan Fluor objective. All assays were performed according to the kit protocol using MES-SA/MX2 cells (ATCC CRL-2274).

VIII. RELATED PRODUCTS:

Microsome Isolation Kit (K249)
Cytochrome P450 Reductase Activity Kit (K700)
Cytochrome P450 3A4 Activity Assay Kit (K701)
Cytochrome P450 3A4 Inhibitor Screening Kit (K702)
Cytochrome P450 2D6 Activity Assay Kit (K703)
Cytochrome P450 2D6 Inhibitor Screening Kit (K704)
UGT Activity Assay / Ligand Screening Kit (K692)
Loperamide Hydrochloride (2683)
Cytochrome P450 2C19 Activity Assay Kit (K848)
Cytochrome P450 2C19 Inhibitor Screening Kit (K849)
Cytochrome P450 1A2 Activity Assay Kit (K893)
Cytochrome P450 1A2 Inhibitor Screening Kit (K894)
Cytochrome P450 2C9 Activity Assay Kit (K895)
Cytochrome P450 2C9 Inhibitor Screening Kit (K896)
Cyclosporin A (1522)
Elacridar (2792)