Multidrug Resistance Direct Dye Efflux Assay

Cat. No. ECM910

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Introduction

The phenomenon of resistance of tumors to chemically unrelated anticancer drugs, termed multidrug resistance, represents the most formidable challenge to the field of oncology. Multidrug resistance can be present at the time of diagnosis, or can be acquired after initial treatment and remission of a cancer. Although multiple mechanisms mediate multidrug resistance, the first mediator of multidrug resistance to be characterized at the molecular level was MDR1, also known as P-glycoprotein (Pgp) and ABCB1 (Gottesman *et al*., 2002). MDR1 mediates resistance to various classes of chemotherapeutic agents, including vinca alkaloids (vinblastine and vincristine), anthracyclines, paclitaxel and etoposide, by actively pumping the drugs from the cytosol and plasma membrane into the extracellular space. The molecular structure of MDR1 consists of 12 transmembrane domains that form a drug-binding pore, and two cytoplasmic ATP-binding cassettes. At least nine proteins related to MDR1 have been characterized to date and shown to mediate efflux of small molecules from cells (Gottesman *et al*., 2002). Two of these MDR1 relatives, multidrug-resistance-associated protein 1 (MRP1, or ABCC1) and breast cancer resistance protein (BCRP, or ABCG2), have also been demonstrated to mediate multidrug resistance in tumor cells. These proteins belong to a larger family of ABC (ATP-binding cassette) proteins that function as transporters of ions, nutrients, and peptides.

The clinical importance of MDR1-mediated multidrug resistance has been best characterized in acute myelogenous leukaemia (Gottesman *et al*., 2002). The role of MDR1 in solid tumors has been more difficult to discern, due to variations in methods of detection of MDR1 in tissues. Multiple efforts have been made to standardize methods for MDR1 detection using flow cytometry, immunohistochemistry and in situ hybridization (Beck *et al.*, 1996). It has been estimated that at least 50% of human cancers express the MDR1 phenotype. *In vivo* imaging of MDR1-mediated efflux with the radiological MDR1 substrate, ^{99m}Tc (technetium)-sestamibi, indicates that MDR1 is active in several cancer types.

MDR1 activity is also observed in various cell types in normal tissues. Brain microvascular endothelial cells express MDR1, which contributes to the blood-brain barrier. It was proposed that expression of MDR1 in hematopoietic stem cells, intestine, and reproductive tissues (testicular endothelium and placental syncytiotrophoblast) protects these cells from the detrimental effects of xenobiotics. MDR1 tissue distribution suggests that it has a role in cholesterol and steroid metabolism. Several subsets of immune cells also express MDR1 (Gottesman *et al*., 2002). MRP1 is widely expressed, and has physiological significance in transporting anionic xenobiotics and metabolites in lung, placenta, choroid plexus in the brain, and Sertoli cells in testes.

Assessment of activity of MDR1, MRP1 and BCRP in cultured cells has been facilitated by the observation that several fluorescent small molecules, such as $DiOC_2(3)$, rhodamine 123, and calcein AM, serve as substrates for MDR1 and its relatives (Figure 1). $DiOC_2(3)$ is highly specific for MDR1, and is not transported by the related multidrug resistance protein, MRP1 (Minderman *et al*., 1996; Table 1). Rhodamine 123 is effluxed by MDR1 and to a lesser extent by MRP1, and thus serves as a more broad indicator of total cellular efflux activity. Another member of the ABC family, breast cancer resistance protein (BCRP), weakly transports $DiOC_2(3)$, but does not transport Rhodamine 123 (Minderman *et al.*, 2002; Table 1). Efflux of dyes can be inhibited by nonfluorescent transport substrates such as vinblastine (Figure 1). Dye efflux assays have proven to be instrumental in screening for compounds that inhibit activity of MDR1 and are thus likely to overcome MDR1-mediated multidrug resistance. In addition, in trials of MDR1 inhibitors *in vivo*, dye efflux analysis of $CD56^+$ cells isolated from patients after treatment with MDR1 inhibitors permits assessment of intracellular levels of the inhibitor (Robey *et al.*, 1999).

The Multidrug Resistance Direct Dye Efflux Assay Kit includes two of the best characterized and most commonly used multidrug resistance ABC transporter substrates, $DiOC_2(3)$ and rhodamine 123. The kit enables researchers to directly assess the functional activity of the MDR1, MRP1 and BCRP membrane pumps in living cells under physiologic conditions by directly measuring the relative fluorescence of cell populations that actively extrude fluorescent multidrug resistance transport substrates.

Application

The Multidrug Resistance Direct Dye Efflux Activity Kit is designed to directly measure the functional activity of MDR1, MRP1 and BCRP by assaying for the ability of the cell to extrude fluorescent transport substrates of these proteins. The kit can be used to measure relative amounts of MDR1, MRP and BCRP activity between different cell populations. In addition, dye efflux assays can be used to assess the ability of unlabeled small molecules to serve as transport substrates for MDR1, MRP1 and BCRP and the ability of candidate inhibitors to block the function of these proteins.

Two conveniently formulated solutions of MDR1, MRP1 and BCRP substrate dyes, $DiOC_2(3)$ Solution and Rhodamine 123 Solution, are included in the kit to give the investigator flexibility in the substrate specificity of the efflux probe. In addition, a solution of a nonfluorescent substrate of MDR1 and MRP1, Vinblastine Solution, is provided to serve as a competitive inhibitor of dye efflux. DMSO is included as a diluent control for the Vinblastine Solution. Sterile, concentrated RPMI-1640 and BSA are provided as components for buffer for the loading, efflux and washing steps of the procedure. A sterile, concentrated solution of gentamicin, which has been shown to not be a substrate for MDR1 (Mechetner and Roninson, 1992), is supplied as an optional antibiotic for the buffer. Propidium iodide solution is included to identify dead cells and exclude them from the analysis.

The extent of efflux of fluorescent dyes is best analyzed by flow cytometry. The assay can also be incorporated with immunostaining experiments for multicolor analysis of cell surface marker expression and MDR1, MRP1 and BCRP function. Efflux mediated by MDR1, MRP1 and BCRP can also be quantified by analysis under a fluorescence microscope or on a fluorometric plate reader.

The efflux activity of MDR1 and its relatives is highly temperature sensitive. MDR1 functions optimally near 37ºC, but is effectively inactive at 4ºC. MDR1-expressing cells preloaded with MDR1 fluorescent substrates retain the dye and consequently have high fluorescence when incubated at 4ºC (Figure 1, 4ºC, top panels). Conversely, cells incubated at 37ºC more readily efflux the dye and show reduced fluorescence (Figure 1, 37ºC + DMSO, middle panels). The kit also includes a specific inhibitor vinblastine, which is also a substrate for MDR1 and competitively blocks efflux of $DiOC_2(3)$ and Rhodamine 123. Inclusion of excess vinblastine in the efflux reaction at 37ºC therefore results in high fluorescence (Figure 1, 37ºC + vinblastine, bottom panels).

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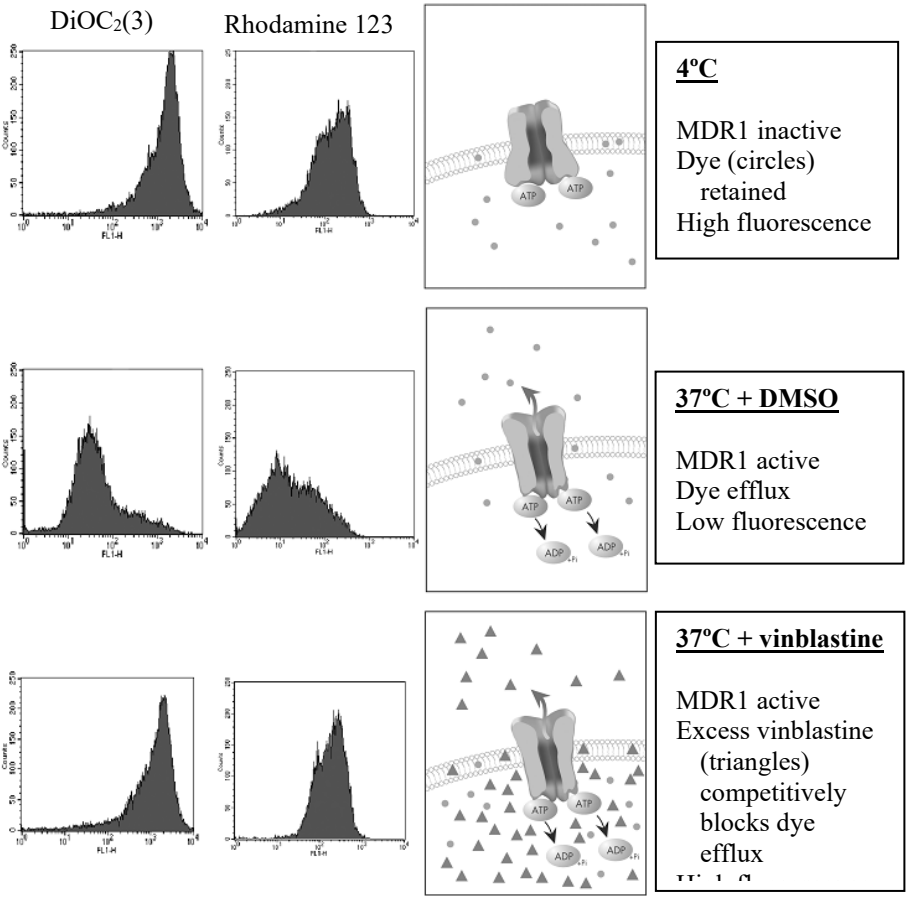

Figure 1. MDR1-overexpressing K562/I-S9 cells were loaded with $DiOC_2(3)$ or rhodamine 123, and incubated at 4ºC, or at 37ºC in the presence or absence of vinblastine. After washing, cells were analyzed by flow cytometry. Cells incubated at 4ºC exhibit high fluorescence, as MDR1 is inactive at low temperatures and the cells retain the dye. Cells incubated at 37ºC with DMSO (diluent control) have low fluorescence, as MDR1 is active and cells efflux the dye. Cells incubated at 37ºC in the presence of vinblastine, a specific MDR1 inhibitor, have high fluorescence, as vinblastine competes with the dye for efflux by MDR1.

	Rhodamine 123	$DiOC_2(3)$	Inhibition by vinblastine
MDR1/Pgp	+++	+++	+++
MRP-1	++	-	+++
BCRP	-	+	-

Table 1. Relative abilities of multidrug resistance mediators MDR1/Pgp, MRP-1 and BCRP to efflux rhodamine 123 and $DiOC_2(3)$, and their susceptibility to inhibition by vinblastine.

Kit Components

1. Sterile 5x RPMI-1640 (Part No. 90302) – One bottle containing 200 ml of sterile 5x concentrated RPMI-1640.
2. Sterile 30% BSA (Part No. 90303) – One bottle containing 35 ml sterile-filtered 30% bovine serum albumin (BSA) in PBS.
3. Gentamicin Solution, 1000x (Part No. 90353) – One vial containing 1 ml sterile-filtered 50 mg/ml gentamicin.
4. $DiOC_2(3)$ Solution (Part No. 90299) – One vial containing 50 µl of $DiOC_2(3)$ (3,3'-diethyloxacarbocyanine iodide) at 1 mg/ml in DMSO.
5. Rhodamine 123 Solution (Part No. 90300) - One vial containing 0.5 ml of rhodamine 123 at 1 mg/ml in DMSO.
6. Vinblastine Solution (Part No. 90301) – One vial containing 150 µl of 22 mM vinblastine in DMSO.
7. DMSO (Part No. 90294) – One vial containing 150 µl DMSO.
8. Propidium Iodide Stock Solution (Part No. 90296) – One vial containing 1 ml of 50 µg/ml propidium iodide in PBS.

Materials Not Supplied

- Cell line for testing
- Hemocytometer
- Sterile water, tissue culture grade
- Sterile 1 liter bottle
- 37ºC water bath with a rack to hold test tubes of choice
- Ice and ice bucket
- Centrifuge capable of 200 x g. A refrigerated centrifuge is recommended but not required
- Extra wash buffer (RPMI-1640 or other medium, containing 1% BSA or 2% fetal calf serum), if further staining with an antibody is incorporated into the assay
- Flow cytometer or fluorescence plate reader
- (Optional) Black-walled 96-well plates, for analysis of samples using fluorescence plate reader.

Storage

Kit components should be stored at 2-8°C upon arrival. Product is stable for 1 year after receipt. $DiOC_2(3)$ Solution, Rhodamine 123 Solution, Vinblastine Solution, and DMSO will freeze at 2-8°C, and can be thawed and refrozen at least 5 times. Protect $DiOC_2(3)$, Rhodamine 123 and Propidium Iodide Solutions from light.

Precautions

Propidium Iodide, Gentamicin, Vinblastine, Rhodamine 123 and $DiOC_2(3)$ may have toxic effects if handled improperly. DMSO is absorbed through the skin and can facilitate internalization of toxic agents. Use caution when handling each of these components. Standard precautions include wearing gloves and a lab coat. Please refer to the MSDS at www.chemicon.com for further information.

Preparation of Reagents

1. Cold Efflux Buffer – In a sterile field, mix the contents of the 200 ml sterile 5x RPMI-1640 bottle (200 ml) and 800 ml sterile water (tissue culture grade) in a sterile 1 liter bottle. With a sterile pipet, add 34.5 ml of the sterile 30% BSA to obtain sterile efflux buffer. If desired, to maintain sterility, add 1 ml of Gentamicin Solution, 1000x, and mix. Chill on ice for at least 2 h before using in the assay. Open the container only in a sterile environment. Store unused portion at 4ºC for up to 12 months. Discard if contamination is evident. Approximately 10 ml Efflux Buffer total is required for each test.

2. Warmed Efflux Buffer – Remove an aliquot of Cold Efflux Buffer and warm to 37ºC at least one hour before use in the assay; keep in water bath at 37ºC until use.

3. $DiOC_2(3)$ Loading Buffer – On the day of the assay, dilute $DiOC_2(3)$ Solution 1:1000 in the desired amount of Cold Efflux Buffer. A volume of 250 µl per test is required. Invert 5-10 times to mix. Keep on ice until adding to cells. Protect solutions containing $DiOC_2(3)$ from prolonged exposure to light.

4. Rhodamine 123 Loading Buffer – On the day of the assay, dilute Rhodamine 123 Solution 1:100 in the desired amount Cold Efflux Buffer. A volume of 250 µl per test is required. Invert 5-10 times to mix. Keep on ice until adding to cells. Protect solutions containing Rhodamine 123 from prolonged exposure to light.

   Note: *The user has the choice of using either $DiOC_2(3)$ or Rhodamine 123, or both as two separate experimental points, as an efflux probe in a given experiment, depending on the application of the user.*

5. Warmed Efflux Buffer containing 22 µM Vinblastine – On the day of the assay, dilute Vinblastine Solution 1:1000 in Warmed Efflux Buffer. For tests to assess vinblastine inhibition of dye efflux, a volume of 1 ml per test is required. Keep at 37ºC until use.

6. Warmed Efflux Buffer containing DMSO – On the day of the assay, dilute DMSO 1:1000 in Warmed Efflux Buffer. For tests to assess dye efflux in the absence of vinblastine, a volume of 1 ml per test is required. Keep at 37ºC until use

7. Propidium Iodide Buffer – Dilute Propidium Iodide Stock Solution 1:50 in Cold Efflux Buffer before use in flow cytometry. A volume of 0.5 ml per test is required. Protect solutions of Propidium Iodide from prolonged exposure to light.

8. (Optional) Cold efflux buffer or other antibody staining buffer containing 0.01% sodium azide. Needed if staining with an antibody will be performed after the efflux assay.

Amounts of reagents required per test:

	treatment			
item	**4ºC**	**37ºC + DMSO**	**37ºC + vinblastine**	**37ºC + experimental compound**
cells	2.5 x 10^5 or more	2.5 x 10^5 or more	2.5 x 10^5 or more	2.5 x 10^5 or more
$DiOC_2(3)$ or Rhodamine 123 Loading Buffer	0.25 ml	0.25 ml	0.25 ml	0.25 ml
Cold Efflux Buffer (for washing)	7.5 ml	7.5 ml	7.5 ml	7.5 ml
Cold Efflux Buffer (for efflux step)	1 ml			
Warmed Efflux Buffer + DMSO		1 ml		
Warmed Efflux Buffer + vinblastine			1 ml	
Warmed Efflux Buffer + experimental compound				1 ml
Propidium Iodide Buffer	0.5 ml	0.5 ml	0.5 ml	0.5 ml

Assay Protocol

Note: *Because membrane transport mediated by MDR1, MRP1 and BCRP is a complex process that is highly dependent on multiple factors, such as physiological conditions of the target cell population, intracellular ATP status, the degree of expression of MDR1, MRP1 and BCRP, and fluorescent dye concentration, some parameters may need to be adjusted for each experimental series. At the same time, it is essential that all temperature conditions and media formulations strictly correspond to the underlying protocol.*

1. Grow cell line of choice in its appropriate medium. The cells should be kept in media lacking multidrug resistance substrates for at least one week (selective drugs may interfere with dye efflux). Antimicrobial agents appear not to interfere with multidrug resistance ABC transporter function and may be included in the media. Media should be replaced one day before the assay. **Approximately 2.5 x 10^5 cells are required for each test.** Adherent cells should be dislodged from plates by standard methods, and used in the assay in suspension.
2. Count cells.
3. Calculate the total number of cells required for the number of tests to be performed, and take volume necessary to get the desired number of cells.
4. Centrifuge cells at 200 x g for 5 min. Discard the supernatant and retain the cell pellet.
5. Resuspend the cell pellet at 1 x 10^6 cells/ml in cold $DiOC_2(3)$ or Rhodamine 123 Loading Buffer.

   Note: *At this step, cells intended to be used in separate tests can be loaded with the fluorescent dye of choice in one tube. Minimize light exposure of dye-containing samples by handling quickly when in ambient light, and performing incubations in containers that offer protection from light.*
6. For loading with $DiOC_2(3)$, incubate for 15 min on ice. For loading with Rhodamine 123, incubate for 30 min to 2 h on ice.
7. Centrifuge cells at 200 x g for 5 min. Remove supernatant. Resuspend pellet in 2.5 ml Cold Efflux Buffer per 10^6 cells.
8. Repeat step 7.
9. At this point, distribute resuspended cells into different tubes for each different treatment. For an initial confirmation of specificity of efflux, use 3 tubes, each containing 625 µl (2.5 x 10^5 cells).

Note: *An initial characterization of the time course of dye efflux by each individual cell type is strongly recommended. For time course experiments, the quantity of cells undergoing the same treatment can initially be put in the same tube. For example, for* ***n*** *time points, put* ***n*** *x 2.5 x* 10^5 *cells(****n*** *x 625 µl at the cell density given in step 8) into one tube for each treatment.*

10. Centrifuge cells at 200 x g for 5 min. Remove supernatant.

11. Resuspend cells in the following media, at 1 ml per test containing 2.5 x 10^5 cells:

    A. 37ºC-Warmed Efflux Buffer containing DMSO

    B. 37ºC-Warmed Efflux Buffer containing vinblastine

    C. Ice-Cold Efflux Buffer

    Note*: For a time course experiment with* ***n*** *time points as described in step 9, resuspend cells for each treatment in a volume of* ***n*** *x 1 ml of the given media.*

12. Immediately transfer tubes **A** and **B** to a 37ºC water bath. Do not perform incubations in an incubator, which does not have adequate temperature control. Keep tube **C** on ice.

13. Incubate for the desired time period.

    Note: *For time course experiments, remove 1 ml aliquots from the incubation tube, add to Cold Efflux Buffer as in step 14, and immediately return the incubation tube to its proper incubation temperature to continue the time course. Cell types with high levels of MDR1 expression (e.g. KB-8-5-11 or KBV-1 cell lines) tend to efflux dyes within 15 min. Cell types expressing lower amounts or endogenous MDR1 (e.g. normal lymphocytes or hematopoietic stem cells) will require 30 min to 3 h to efflux dyes.*

14. Add 5 ml Cold Efflux Buffer per test, and immediately put the tube on ice.

    Note: *Low temperatures stop the efflux reaction. For time course experiments, the earlier time points may be kept on ice at this point until all of the samples are collected.*

15. Centrifuge at 200 x g for 5 min in a refrigerated centrifuge at 4ºC. Remove supernatant.

16. Resuspend cells in 1 ml per test (2.5 x 10^5 cells) Cold Efflux Buffer.

17. Centrifuge at 200 x g for 5 min in a refrigerated centrifuge at 4ºC. Remove supernatant.

18. (Optional) If antibody staining of cells subjected to the efflux assay is desired, use a PE-conjugated antibody and perform the staining using ice-cold buffers after the efflux phase is completed

19. For analysis by flow cytometry, resuspend cells in 0.5 ml per test Cold Propidium Iodide Buffer.

    For analysis in a fluorescence plate reader, resuspend cells in 0.25 ml per test Cold Efflux Buffer.

20. Maintain on ice until analysis by flow cytometry or fluorometry. Cells may be kept on ice for several hours. Longer times are not recommended, as prolonged exposure to the dyes and vinblastine can be toxic to the cells.

21. Analyze by flow cytometry, with $DiOC_2(3)$ and rhodamine 123 on FL1, PE (if employed in step 18) on FL2, and PI on FL3. Collect 2500-10,000 events.

    Alternatively, cell suspensions can be dispensed into the wells of a black-walled 96-well plate and measured in a fluorescence plate reader at an excitation wavelength of 485 nm and an emission wavelength of 530 nm.

Additional Usage Information

A reference cell line expressing MDR1 is necessary for studying MDR1-mediated dye efflux. Several MDR1-overexpressing cell lines have been described. The first is the KB line, with MDR1 expression from lowest to highest KB parental<KB8<KB-8-5<KB-8-5-11<KB-V1 (Akiyama *et al*., 1985; Shen *et al*., 1986). A second set is the MCF7 series (Mechetner *et al*., 1998). In addition, peripheral blood lymphocytes and Caco-2 human intestinal epithelial cells express MDR1 and actively efflux $DiOC_2(3)$ and rhodamine 123.

The time course of efflux of a dye depends on the level of expression of MDR1, MRP1 and BCRP in the target cell. Cell types expressing lower numbers of MDR1, MRP1 or BCRP molecules per cell require longer incubation times to complete efflux. It is recommended that the user perform an initial time course experiment to determine the optimum time for efflux for each cell type to be examined.

Troubleshooting

Problem	Cause	Remedy
Cells are not fluorescent after loading and storage at 4ºC	Inefficient loading	Load with dye for longer time Load at 4ºC, as cells loaded at higher temperatures will concomitantly efflux the dye.
Cells do not efflux dye after 37ºC incubation without vinblastine	Reaction at temperature below 37ºC	Warm efflux buffer before adding to cells Ensure temperature of water bath is at 37ºC
	Cells express little or no MDR1, MRP1 or BCRP	Verify MDR1 expression; use a reference MDR1-positive cell line For cells expressing low amounts of MDR1, MRP1 or BCRP, perform efflux at 37ºC for longer time (up to 5 hours)

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