Vybrant™ Multidrug Resistance Assay Kit (V-13180)

Quick Facts

Storage upon receipt:

- –20°C
- Desiccate
- Protect from light

Note: Prepare the working solution of calcein AM just prior to use.

Introduction

Multidrug resistance (MDR) is a complex group of biological processes of great interest to researchers in clinical and experimental oncology.[1-3] The MDR phenotype is characterized by development of tumor cell resistance to structurally and functionally dissimilar anticancer drugs. MDR is often associated with over-expression of two transmembrane transporters, the ATP-dependent 170 kDa P-glycoprotein (Pgp) encoded by the MDR1 gene [4] and the 190 kDa MDR2-encoded multidrug resistance protein (MRP).[5] The drug cross-resistance profiles of cells that overexpress these two proteins are similar but not identical, and both Pgp and MRP can overcome the passive diffusion-driven influx of cytotoxic drugs by actively pumping these compounds out of cells. This results in decreased intracellular concentrations of potentially therapeutic agents, including anthracyclins, actinomycin D, epipodophyllotoxins, vinca alkaloids and various hydrophobic peptides.[6] Pgp-mediated MDR can be inhibited by noncytotoxic agents such as the calcium channel blocker verapamil and the competitive Pgp drug-binding inhibitor cyclosporin A. The means to rapidly screen and identify novel agents that can reverse or circumvent MDR is of great interest to both researchers and clinicians.

The Vybrant™ Multidrug Resistance Assay Kit, based on the fluorescence microplate method developed by Tiberghien and Loor,[7] provides a rapid and simple method for large-scale screening of MDR inhibitors. This assay utilizes the fluorogenic dye calcein acetoxymethyl ester (calcein AM) as a substrate for efflux activity of Pgp.[8] Calcein AM is a nonfluorescent, highly lipid soluble dye that can rapidly penetrate the plasma membrane of normal cells. Once inside the cell, ester bonds are cleaved by endogenous esterases, transforming calcein AM into hydrophilic and intensely fluorescent calcein. Calcein is well retained in the cytosol and, unlike other fluorescent Pgp substrates such as BCECF AM or fura-2 AM, its fluorescence is neither pH nor calcium dependent.

MDR cells expressing high levels of Pgp rapidly extrude nonfluorescent calcein AM from the plasma membrane, reducing accumulation of fluorescent calcein in the cytosol (Figure 1).[9-12] The degree of inhibition of Pgp activity can be quantitated by measuring the increase in intracellular calcein fluorescence. This assay is designed for use with fluorescence microplate readers and is particularly useful for large-scale, rapid and sensitive screening of candidate Pgp inhibitors in MDR cell lines. Calcein's absorbance maximum of 494 nm and emission maximum of 517 nm are ideally suited for detection by instruments equipped with standard fluorescein filters. In addition to calcein AM, two reference inhibitors of Pgp activity are supplied in this kit: verapamil, a calcium channel blocker that inhibits Pgp activity noncompetitively, and cyclosporin A, a competitive inhibitor of Pgp–drug binding.[4]

Materials

Kit Contents

- **Calcein AM** (Component A), MW 995, 10 vials, each containing 50 µL of 1 mM calcein AM in anhydrous DMSO
- **Cyclosporin A** (Component B) MW 1203, 250 µg
- **Verapamil hydrochloride** (Component C) MW 491, 10 mg

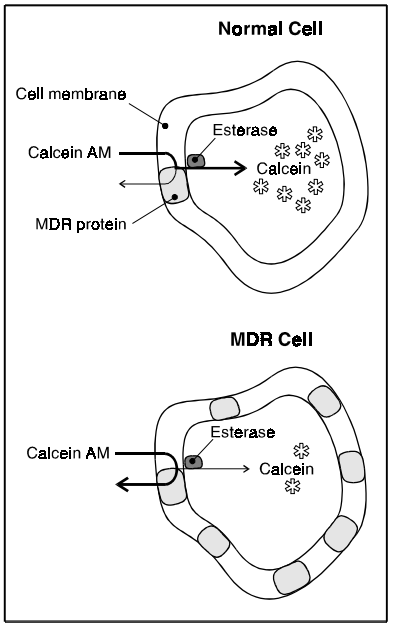

Figure 1. *Principle of the Vybrant Multidrug Resistance Assay. In normal cells, nonfluorescent calcein AM readily diffuses across the cell membrane. Fluorescent calcein accumulates in the cytoplasm after cleavage of calcein AM by endogenous esterases. In MDR cells, overexpression of MDR transporter proteins increases extrusion of calcein AM from the cell membrane before it can be hydrolyzed by esterases, thus reducing accumulation of calcein.*

Storage and Handling

Kit reagents should be stored at -20°C, sealed, desiccated and protected from light. Allow reagents to warm to room temperature and centrifuge briefly before opening. Before refreezing, seal all stock solutions tightly. Calcein AM is susceptible to hydrolysis when exposed to moisture. Prepare aqueous working solutions containing calcein AM immediately prior to use, and use within about one day. Verapamil and cyclosporin A stock solutions prepared in anhydrous ethanol should be stored at -20°C.

When used at the concentration suggested in the protocol below, this kit provides sufficient calcein AM to perform about 10,000 assays using a fluorescence microplate reader.

Reagent Preparation

Calcein AM

In the protocol presented below, calcein AM is used at a final assay concentration of 0.25 µM. Assays should always be run in duplicate; the total number of assays to be run in an experiment will determine the volume of the 1 mM calcein AM stock solution (Component A) to be diluted. Depending on the level of Pgp overexpression in the MDR cell type being assayed, it may be necessary to use higher concentrations of calcein AM in each assay.

1.1 Determine the number of assays to be performed; each assay will require 50 µL of a 1 µM calcein working solution.

1.2 Calculate the volume of 1 mM calcein AM stock solution that will be required to make a 1000-fold dilution sufficient for the number of assays to be run. For example, 1 µL of 1 mM calcein AM added to 999 µL PBS will provide sufficient calcein AM for 20 assays. *Do not prepare the working solution of calcein AM until just prior to use as it is susceptible to hydrolysis in aqueous solution.*

Pgp Inhibitors

For use of the included reference inhibitors, prepare verapamil and cyclosporin A stock solutions at 10 mg/mL in absolute ethanol. Store at -20°C, protected from light.

2.1 Add 25 µL absolute ethanol to the vial containing 250 µg cyclosporin A (Component B). Mix thoroughly until dissolved.

2.2 Add 1 mL absolute ethanol to the vial containing 10 mg verapamil (Component C). Mix thoroughly until dissolved.

2.3 Prepare a series of dilutions for each of the above reference inhibitor stock solutions in PBS ranging in concentration from 0.04 µg/mL to 120 µg/mL. Each assay to be tested for inhibitor effects will require 50 µL of diluted inhibitor stock solution.

Multidrug Resistance Assay

The protocol below outlines the method of Tiberghien and Loor.[7] The methodology presented was developed using Par-P388 and MDR-P388 monocytic leukemia and human Par-CEM and MDR-CEM lymphocytic leukemia cells. In this assay, the effects of modulators on Pgp activity were determined by measuring calcein fluorescence in both MDR and parental cell lines. Researchers are encouraged to consult this citation and related references for details about preparing and working with specific MDR cell lines and for discussions of other MDR phenotypes and their substrates.[5,7,13-15] Because calcein AM is also a substrate for MDR2-encoded MRP, researchers are encouraged to consult Lautier *et al*.,[5] for a discussion of substrates, inhibitors and modulators specific for MRP activity.

Suggested Protocol

3.1 Add 50 µL of reference or experimental Pgp modulators/inhibitors to 96-well microplate wells from prepared dilutions ranging in concentration from 0.04 µg/mL to 120 µg/mL (step 2.3). This will result in final substrate inhibitor concentrations ranging from 0.01 µg/mL to 30 µg/mL in each assay. Include 50 µL of PBS as a control.

3.2 Add ~5×10^5 cells in 100 µL volumes of tissue culture medium to each well.

3.3 Mix well and incubate at 37°C for 15 minutes.

3.4 Dilute sufficient 1 mM calcein AM (Component A) to 1 µM in PBS to have at least 50 µL for each assay. Add 50 µL of 1 µM calcein AM to each microplate well (final concentration of 0.25 µM).

3.5 Mix and incubate at 37°C for 15 minutes.

3.6 Centrifuge the microplates for 5 minutes at 200 × g, remove the supernatant and resuspend the cells in 200 µL cold (4°C) tissue culture medium.

3.7 Repeat the washing step 3.6 two more times, ending with the cells suspended in 200 µL cold tissue culture medium.

3.8 Measure the calcein retention as calcein-specific fluorescence with filters appropriate for fluorescein. The absorption maximum for calcein is 494 nm, the emission maximum is 517 nm.

Analysis

The effectiveness of inhibitors on Pgp efflux activity may be expressed as the amount of drug that can give 50% inhibition of calcein AM efflux (EC_{50}). Calculate calcein retention in parental cells and MDR cells for each concentration of inhibitor tested:

$$\text{calcein retention}_{\text{par}} = \frac{\text{fluorescence of treated parental cells}}{\text{fluorescence of untreated parental cells}} \times 100$$

$$\text{calcein retention}_{\text{MDR}} = \frac{\text{fluorescence of treated MDR cells}}{\text{fluorescence of untreated parental cells}} \times 100$$

Plot dose–response curves for parental cells and MDR cells for each inhibitor used. Modulator concentrations required in the MDR cells to achieve 50% of the calcein-specific fluorescence measured for similarly treated parental cells may be determined from the dose-response curves and defined as EC_{50}.

References

1. Cancer Res 54, 666 (1994); **2.** Proc Natl Acad Sci USA 91, 3497 (1994); **3.** Ann Rev Biochem 62, 385 (1993); **4.** Biochim Biophys Acta 1288, 37 (1996); **5.** Biochem Pharmacol 52, 967 (1991); **6.** J Biol Chem 271, 12897 (1996); **7.** Anti-Cancer Drugs 7, 568 (1996); **8.** The use of calcein AM assay for analysis of MDR is covered under US patents 5,872,014 and 6,277,655 B1, and this kit is provided under liscense from Solvo Biotechnology, Inc.; **9.** J Biol Chem s271, 13668 (1996); **10.** FEBS Lett 368, 385 (1995); **11.** Biochim Biophys Acta 1191, 384 (1994); **12.** J Biol Chem 268, 21493 (1993); **13.** Anti-Cancer Drugs 7, 257 (1996); **14.** Anti-Cancer Drugs 6, 669 (1995); **15.** Exp Cell Res 212, 291 (1994).

Product List

Current prices may be obtained from our Web site or from our Customer Service Department.

Cat #	Product Name	Unit Size
V-13180	Vybrant™ Multidrug Resistance Assay Kit	1 kit

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