ab204534
MDR Assay Kit – flow cytometry (green)

Instructions for Use

For functional detection and profiling of multidrug resistant phenotypes in live cells (both suspension and adherent).

This product is for research use only and is not intended for diagnostic use.

Version 4 Last Updated 16 October 2019

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1. BACKGROUND

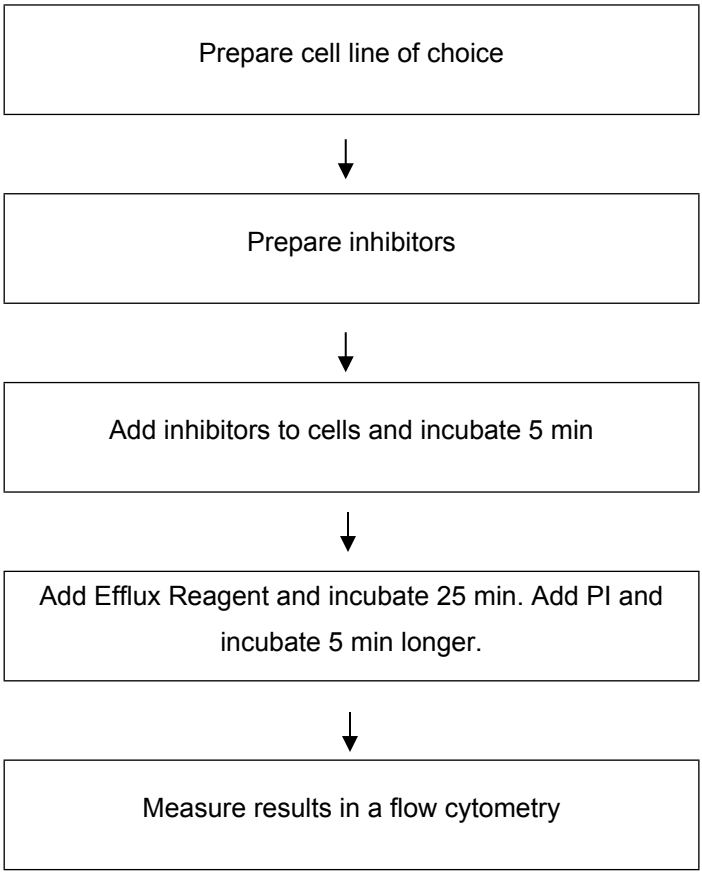
Abcam's MDR Assay Kit - flow cytometry (green) (ab204534) is designed for functional detection and profiling of multidrug resistant phenotypes in live cells (both suspension and adherent).

The kit provides a fast, sensitive and quantitative method for monitoring the function and expression of the three clinically most important multidrug resistance proteins: MDR1 (P-glycoprotein), MRP1/2 and BCRP. The major component of the kit is an Efflux Green Detection Reagent, a substrate for three main ABC transporter proteins which serves as an indicator of these proteins' activity in the cell. The proprietary AM-ester form of the Efflux Green Detection Reagent is a hydrophobic non-fluorescent compound that readily penetrates the cell membrane and is subsequently hydrolyzed inside of the cells by intracellular esterases. Unless the Efflux Reagent is pumped out of the cell, the esterase cleaved dye is trapped inside the cell. The fluorescence signal of the dye generated within the cells thus depends upon the activity of the ABC transporters. The cells with highly active transporters will demonstrate lower fluorescence because of the active efflux of the reagent from the cell. Application of specific inhibitors of the various ABC transporter proteins, included in the kit, allows differentiation between the three common types of pumps. The activity of a particular MDR transporter is defined by the difference between the amount of the dye accumulated in the presence and in the absence of the inhibitors, respectively.

The flow cytometry assay is based on determining fluorescence intensities of the tested cells after a short *in vitro* incubation of cell suspension with the Efflux Green Detection Reagent in the presence or absence of specific ABC transporter inhibitors. The results of the test can be quantified by calculating the MDR activity factor (MAF) values, which allow comparison of multidrug resistance between different samples or cell lines.

Multidrug resistance relates to resistance of tumor cells to a whole range of chemotherapy drugs with different structures and cellular targets. The phenomenon of multidrug resistance (MDR) is a well-known problem in oncology and thus needs profound consideration in cancer treatment. One of the underlying molecular rationales for MDR is the up regulation of a family of transmembrane ATP binding cassette (ABC) transporter proteins that present in practically all living organisms. These proteins cause chemotherapy resistance in cancer by actively extruding a wide variety of therapeutic compounds from the malignant cells. The same ABC transporters play an important protective function against toxic compounds in a variety of cells and tissues and at blood-tissue barriers.

2. ASSAY SUMMARY



3. PRECAUTIONS

Please read these instructions carefully prior to beginning the assay.

All kit components have been formulated and quality control tested to function successfully as a kit. Modifications to the kit components or procedures may result in loss of performance.

Some components of this kit may contain hazardous substances. Reagents can be harmful if ingested or absorbed through the skin and may cause irritation to the eyes. They should be treated as possible mutagens, should be handled with care and disposed of properly.

4. STORAGE AND STABILITY

All reagents are shipped on dry ice. Upon receipt, the kit should be stored upright and protected from light at -80°C. Kit has a storage time of 1 year from receipt, providing components have not been reconstituted.

Refer to list of materials supplied for storage conditions of individual components. Observe the storage conditions for individual prepared components in Materials Supplied section.

Aliquot components in working volumes before storing at the recommended temperature.

To avoid photobleaching, perform all manipulations in low light environments or protected from light by other means.

5. LIMITATIONS

- Assay kit intended for research use only. Not for use in diagnostic procedures.
- Do not mix or substitute reagents or materials from other kit lots or vendors. Kits are QC tested as a set of components and performance cannot be guaranteed if utilized separately or substituted.

6. MATERIALS SUPPLIED

Item	Amount	Storage Condition (Before Preparation)	Storage Condition (After Preparation)
Efflux Green Detection Reagent	1 vial	-20ºC	-80ºC
Novobiocin (BCRP Inhibitor)	1.5 µmol	-20ºC	-80ºC
Verapamil (MDR1 Inhibitor)	300 nmol	-20ºC	-80ºC
MK-571 (MRP Inhibitor)	750 nmol	-20ºC	-80ºC
Propidium Iodide	500 µL	-20ºC	-80ºC

7. MATERIALS REQUIRED, NOT SUPPLIED

These materials are not included in the kit, but will be required to successfully utilize this assay:

- CO_2 incubator (37°C), tissue culture plasticware
- PBS (optional, for washing procedure)
- Complete growth medium without Phenol Red (e.g. Dulbecco's Modified Eagle Medium D-MEM)
- Heat block or water bath
- Calibrated, adjustable precision pipettes, preferably with disposable plastic tips
- 5 mL round bottom polystyrene tubes for holding cells during staining and assay procedure
- Adjustable speed centrifuge with swinging buckets
- Anhydrous DMSO
- Standard flow cytometer – equipped with a blue laser (488 nm)

8. TECHNICAL HINTS

- **This kit is sold based on number of tests. A 'test' simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions.**
- Selected components in this kit are supplied in surplus amount to account for additional dilutions, evaporation, or instrumentation settings where higher volumes are required. They should be disposed of in accordance with established safety procedures.
- Multidrug resistance assay is a functional test that requires living cells in good condition. The cells should always be kept in the appropriate incubation buffer, containing all the essential components. Do not use fixatives, azide or other preservatives.
- Shear stress can be harmful to the cells. Do not vortex cell suspensions. Always mix them with gentle pipetting. Avoid forming excessive bubbles.
- Cells in suspension sediment very rapidly and have to be mixed prior to any procedure (counting, aliquotting, running the samples on a flow cytometer). Mix cells by gentle pipetting and forming excessive bubbles.
- Cell suspensions at the recommended concentrations will normally result in 100-300 events/sec flow rate. Keeping the flow rate below 600 events/sec is recommended.

9. REAGENT PREPARATION

- Briefly centrifuge small vials at low speed prior to opening.

9.1 **Efflux Green Detection Reagent Stock Solution:**

Resuspend the Efflux Green Detection Reagent in 300 µL anhydrous DMSO. Vortex gently or slowly rotate the tube to dissolve. Equilibrate to room temperature before use, Aliquot Detection Reagent so that you have enough volume to perform the desired number of assays. Store at -20°C. Use within 6 months.

9.2 **Verapamil (MDR1 Inhibitor):**

Resuspend the MDR1 Inhibitor (Verapamil) in 60 µL DMSO to create a 5 mM stock solution. Vortex gently or slowly rotate the tube to dissolve. Aliquot inhibitor so that you have enough volume to perform the desired number of assays. Store at -20°C. Use within 6 months.

9.3 **MK-571 (MRP Inhibitor):**

Resuspend the MK-571 (MRP Inhibitor) in 75 µL DMSO to create a 10 mM stock solution. Vortex gently or slowly rotate the tube to dissolve. Aliquot inhibitor so that you have enough volume to perform the desired number of assays. Store at -20°C. Use within 6 months.

9.4 **Novobiocin (BCRP Inhibitor)**

Resuspend the BCRP Inhibitor (Novobiocin) in 30 µL DMSO to create a 50 mM stock solution. . Vortex gently or slowly rotate the tube to dissolve. Aliquot inhibitor so that you have enough volume to perform the desired number of assays. Store at -20°C. Use within 6 months.

10. SAMPLE PREPARATION

- Cells should be maintained via standard tissue culture practices. Always make sure that cells are healthy and in the log phase of growth before using them for an experiment.
- Because membrane transport mediated by ABC transporters is a complex process that is highly dependent on physiological conditions of cell populations and intracellular ATP status it is important to use living cells in good condition. ATP depletion will decrease the activity of the membrane transporters.

10.1 Grow cell line of choice in the appropriate medium.

Some drugs may interfere with dye efflux; therefore, cells should be kept in a drug-free medium for at least one week. Anti-microbial agent may be included in the medium, since they do not interfere with multidrug resistance proteins.

10.2 Replace medium one day before the assay.

Adherent cells should be dislodged from the plates using standard methods and used in suspension for the assay.

10.3 Count cells using a hemacytometer:

Initial recommendation = 2 – 5 x 10^5 cells/assay. Prepare a cell suspension containing 1 – 2 x 10^6 cells/mL in pre-warmed (37°C) complete indicator-free medium.

For one sample test, four assays should be performed in triplicate with and without three different inhibitors.

11. INHIBITOR PREPARATION

11.1 For each sample to be assayed, prepare four set of tubes (in triplicate). Include one tube for the unstained cell control.

11.2 Immediately prior to use, prepare intermediate dilutions of all three inhibitors by mixing the appropriate volumes of inhibitor stock solutions and pre-warmed (37°C) complete indicator-free medium using the volumes specified in the following table:

Inhibitor	Volume of inhibitor / concentration	Volume of medium
MDR1 Inhibitor	16 µL (5 mM)	1 mL
MRP Inhibitor	20 µL (10 Mm)	1 mL
BCRP Inhibitor	8 µL (50 mM)	1 mL

NOTE: *Prepare the dilutions of the inhibitors immediately prior to use as they are susceptible to hydrolysis in aqueous solution.*

12. ASSAY PROCEDURE

- **Equilibrate all materials and prepared reagents to room temperature prior to use.**
- **It is recommended to assay all samples in triplicate.**

12.1 Set up Reaction tubes:

12.2 Add 125 µL of inhibitor into separate tubes as indicated in the following table. For control, add 125 µL pre-warmed (37°C) complete indicator-free medium containing 5% DMSO.

Tube Number.	Dilute Inhibitor (from step 11.2)	Vol cell suspension	Vol dilute Efflux Green Detection Reagent
1-3	125 µL MDR1 Inhibitor	250 µL	125 µL
4-6	125 µL MRP Inhibitor	250 µL	125 µL
7-9	125 µL BCRP Inhibitor	250 µL	125 µL
10-12 (stained control)	125 µL medium/DMSO	250 µL	125 µL
13 (unstained control)	250 µL medium/DMSO	250 µL	0

12.3 Add 250 µL of cell suspension into each tube. Gently mix the contents of each tube by pipetting (avoid introducing bubbles) and incubate all the tubes at 37°C for 5 minutes.

12.4 Dilute the Efflux Green Detection Reagent Stock Solution by combining 40 µL of the stock solution and 2 mL of pre-warmed (37°C) complete indicator-free medium. Mix well by vortexing the tube gently.

NOTE: *Dilution of Efflux Green Detection Reagent Stock Solution should be done just prior to use as it is susceptible to hydrolysis in aqueous solution.*

12.5 Begin the assay by adding 125 µL of the freshly diluted Efflux Green Detection Reagent into each tube, except the tube labeled for unstained cells. Gently mix cell suspensions by pipetting, avoid introducing bubble, and incubate all the tubes at 37°C for 30 minutes.

12.6 After 25 minutes of incubation, add 5 µL of the provided stock solution of Propidium Iodide to each tube to monitor cell viability.

12.7 Perform flow cytometry measurements immediately after reaction.

12.8 If immediate measurements are not possible, or if the number of samples is over 30, stop the reaction by rapid centrifugation (1 minute, 200 x *g*). Discard the supernatant and re-suspend the cells in 5 mL of ice cold complete indicator-free medium of PBS containing dilute Propidium Iodide (50 µL of the provided stock solution of Propidium Iodide in 5 mL of medium or PBS). These samples can be stored at 4°C for several days.

13. FLOW CYTOMETRY DETECTION

- **The cellular green fluorescence signal of Efflux Green Detection Reagent should be measured using a flow cytometer in all tubes in the living (PI negative) cell population with identical equipment settings.**

13.1 Within the flow cytometry software, set an FSC-SSC dot plot, an FSC-FL3 dot plot, and an FL1 histogram plot. For better separation of the different cell populations, using a log scale is recommended for the fluorescence channels (FL1 and FL3).

13.2 Run unstained cells and adjust both forward and side scatter PMT amplifications to display all the cell subsets on the FSC-SSC plot.

13.3 Set a gate (R1) on the FSC-SSC plot, selecting the cell population of interest but excluding cell debris. Display the cells selected by the R1 gate in an FSC-FL3 dot plot format.

13.4 Set a second gate (R2) in the FSC-FL3 window to exclude PI-positive cells from analysis. To avoid errors originated from the spillover of green fluorescence of the Efflux Green Detection Reagent, set the R2 border as high as possible. Display R2 gated events in the FL1 histogram plot format.

13.5 Run tube no. 1 and adjust the PMT amplification for FL1 so that the peak of the histogram is located between the second and third decades on the FL1 histogram channel.

13.6 Save settings in a properly designed template file. We recommend same or similar settings whenever possible. You may need to readjust slightly the PMT amplifications and/or the gate locations after an initial test run.

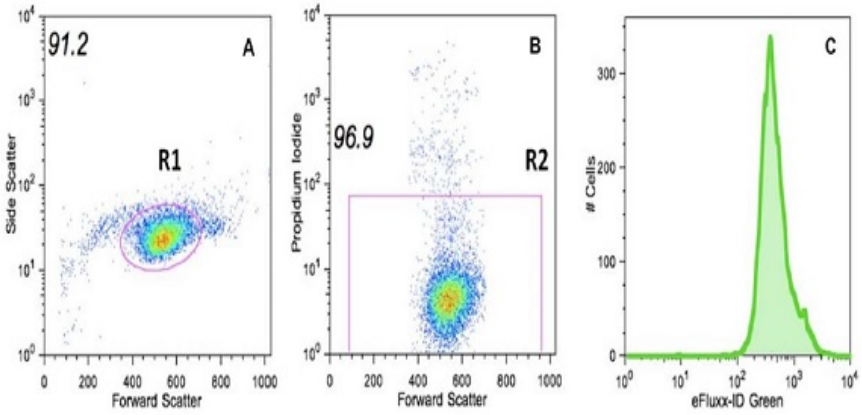


Figure 1. Flow cytometry measurements of the samples. Setting the parameters: Gate out the debris (panel A), gate PI-negative events (panel B) and set PMTs for the FL1 fluorescence channel (panel C).

14. CALCULATION OF RESULTS

14.1 Calculate the mean fluorescence intensity (MFI) values for each triplicate set of measurements:

- **FMDR1** from tubes no. 1, 2 and 3
- **FMRP** from tubes no. 4, 5 and 6
- **FBCRP** from tubes no. 7, 8 and 9
- **F0** from tubes no. 10, 11 and 12

If differences between parallel sets of measurements are <10%, use all three values to calculate the mean.

If one value is extreme (a difference of >10%), disregard the unreliable data, and calculate the mean from the other two. If all MFI values differ by >10%, redo the analysis.

14.2 Calculate the multidrug resistance activity factor (MAF) for each transporter, using the following formulas:

MAFMDR1 = 100 × (FMDR1 - F0)/FMDR1

MAFMRP = 100 × (FMRP - F0)/FMRP

MAFBCRP = 100 × (FBCRP - F0)/FBCRP

In extreme cases (without MDR1, MRP or BCRP activity), the MFI values corresponding to inhibitor-treated cells can be smaller than the MFI value of non-inhibitor-treated cells. In such cases, corresponding MAF values should be regarded as zero.

15. TYPICAL DATA

- The theoretical range of the MAF values are between 0 – 100. Studies comparing MAF values with clinical response to a chemotherapeutic treatment suggest that a specimen with an MAF value of <20 can be regarded as multidrug resistance negative, while MAF values >25 are indicative of multidrug resistance positive specimens.
- In drug-selected cell lines exhibiting extremely high expression levels of ABC transporter proteins, the MAF values can be as high as 95-98.
- This product has been validated in various cell lines expressing multidrug resistance proteins that are summarized in the table.

Cell line	MDR1	MRP	BCRP
CHO K1	+	+	+
HeLa	-	-	-
A549	-	+	+
HCT-8	+	+	-
HepG2	+	-	-
Jurkat	-	+	-
U-2OS	+	+	+
U-2OS RFP	+	+	+

- Typical results are shown in Figure 2.

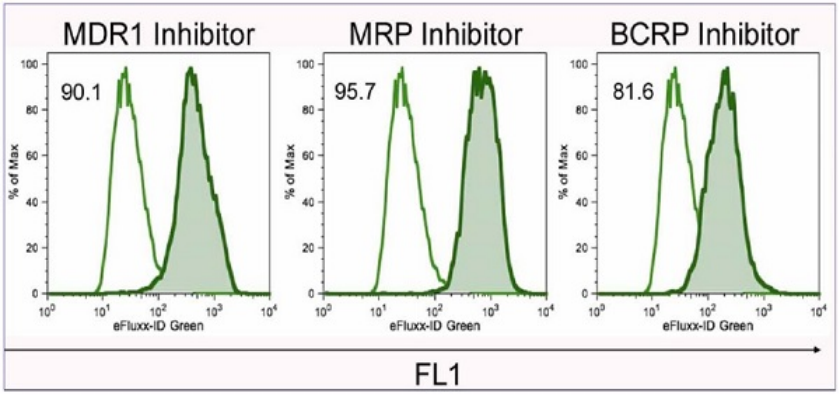


Figure 2. CHO K1 cells were incubated with Efflux Green Detection Reagent with and without specific inhibitors according to the kit protocol. Resulting fluorescence was measured using flow cytometry. Tinted histograms show fluorescence of inhibitor-treated samples and non-tinted histograms show fluorescence of untreated cells. The difference in fluorescence is indicative of a corresponding protein activity. The numbers in the upper left corners are MAF scores (multidrug resistance activity factors)-quantitative characteristics of multidrug resistance.

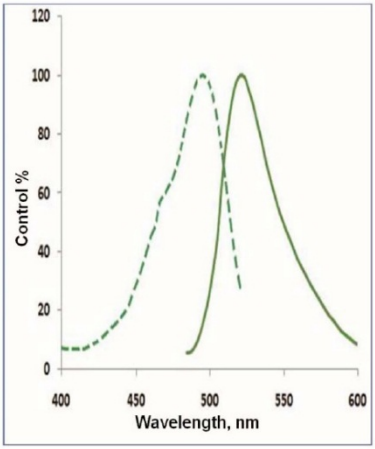


Figure 3. The absorption and emission peaks of Efflux Green Detection Reagent are Ex/Em = 490nm/ 514nm, respectively. It can be well excited with an argon ion laser at 488nm, and detected in the FL1 channel of most bench flow cytometers.

16. TROUBLESHOOTING

Problem	Cause	Solution
Cells do not exhibit fluorescence after incubation with the detection reagent	Cell viability is low	Cells should be in log growth phase. Cell samples cannot be kept longer than 6 hours before the assay. Do not use fixatives, azides or other preservatives. Avoid shear stress, do not vortex, and avoid bubbling
	Very low concentration of the Efflux Green Detection Reagent	Check the concentration of the reagent
Fluorescence does not increase after incubation with inhibitor	Cells do not express any MDR1, MRP1/2 or BCRP	Use a positive control cell type expressing corresponding ABC transporter
	Quality of the reagents is compromised	Check storage, stability and freshness of the reconstituted reagents
There are differences in MFI (mean fluorescence intensity) values between cell lines.	Detection reagent accumulation may be influenced by cell size, endogenous esterase activity, etc	Using the inhibitors and calculating the MAF values eliminate these differences
Inconsistent fluorescence shift using the same cell (irreproducible results)	Inadequate incubation condition	Always use a water bath or heat block (not incubator). Ensure temperature of water bath is 37°C
	Cell viability is low	Cells should be in log growth phase. Cell samples cannot be kept longer than 6 hours before the assay. Do not use fixatives, azides or other preservatives. Avoid shear stress, do not vortex, and avoid bubbling

17. NOTES

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