Product Manual

# EFLUXX-ID® Gold multidrug resistance assay Kit

## Catalog No. ENZ-51030-K100

For flow cytometry (100 assays)

For fluorescence microscopy (100 assays)

**NOTE**: This version contains a change to procedure for microscopy assay.

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# Product Manual

Please read entire booklet before proceeding with the assay.

Carefully note the handling and storage conditions of each kit component.

Please contact Enzo Life Sciences Technical Support if necessary.

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## INTRODUCTION

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.

Enzo Life Sciences' EFLUXX-ID® Gold Multidrug Resistance Assay Kit 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 kit includes EFLUXX-ID® Gold Detection Reagent as a major component. Being a substrate for three main ABC transporter proteins, this reagent serves as an indicator of these proteins' activity in the cell. The proprietary AM- ester form of the EFLUXX-ID® Gold 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 EFLUXX-ID® 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 EFLUXX-ID® Gold 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.

The activity of a particular MDR transporter which is reflected by intensity of intracellular fluorescence can also be qualitatively imaged by microscopy assay.

## MATERIALS SUPPLIED & STORAGE

All reagents are shipped on blue ice (-20°C). Upon receipt, the kit should be stored upright at ≤-20°C, protected from light. When stored properly, these reagents are stable for one year upon receipt. **Avoid repeated freezing and thawing.**

Reagents provided in the kit are sufficient for 100 flow cytometry assays or 100 fluorescence microscopy assays using live cells (adherent or in suspension).

| Reagent | Quantity |
|---|---|
| EFLUXX-ID® Gold Detection Reagent | 2 vials |
| MDR1 Inhibitor (Verapamil) | 300 nmoles |
| MRP Inhibitor (MK-571) | 750 nmoles |
| BCRP Inhibitor (Novobiocin) | 1.5 µmoles |
| Propidium Iodide | 500 µL |

## OTHER MATERIALS NEEDED

1. $CO_2$ incubator (37°C), tissue culture plasticware
2. Water bath or thermostated dry block for 37°C incubation
3. Standard flow cytometer equipped with a blue laser (488nm)
4. Calibrated, adjustable precision pipetters, preferably with disposable plastic tips
5. 5 mL round bottom polystyrene tubes for holding cells during staining and assay procedure
6. Water bath or thermostated dry block

7. Adjustable speed centrifuge with swinging buckets
8. Anhydrous DMSO
9. Complete growth medium without Phenol Red (*e.g.*, Dulbecco's Modified Eagle medium, D-MEM)
10. PBS (optional, for washing procedures)
11. Standard fluorescence microscope
12. Glass microscope slides (*e.g.*, Nunc™ Lab-Tek™ II Chamber Slide™ System, 4-well)
13. Glass cover slips of appropriate size

## SAFETY WARNINGS & PRECAUTIONS

**FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.**

- 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.
- Observe good laboratory practices. Gloves, lab coat, and protective eyewear should always be worn. Never pipet by mouth. Do not eat, drink or smoke in the laboratory areas. All blood components and biological materials should be treated as potentially hazardous and handled as such. They should be disposed of in accordance with established safety procedures.
- To avoid photobleaching, perform all manipulations in low light environments or protected from light by other means.

## METHODS AND PROCEDURES

***NOTE**: PLEASE READ THE ENTIRE PROCEDURE BEFORE STARTING. Allow all reagents to thaw at room temperature before starting with the procedures. Upon thawing, gently hand-mix or vortex the reagents prior to use to ensure a homogenous solution. Briefly centrifuge the vials at the time of first use, as well as for all subsequent uses, to gather the contents at the bottom of the tube.*

### A. Reagent Preparation

**EFLUXX-ID® Gold Dye Stock Solution**

As needed, resuspend the contents of each vial of EFLUXX-ID® Gold Detection Reagent in 60 µL anhydrous DMSO. Vortex gently or slowly rotate the tube to dissolve. Store the solution at –20°C. The reconstituted reagent is stable for at least 6 months when stored as recommended.

1. **5 mM Verapamil (MDR1 Inhibitor)**

   Resuspend the MDR1 Inhibitor (Verapamil) in 60 µL anhydrous DMSO. Vortex gently or slowly rotate the tube to dissolve. Store the solution at -20°C. The reconstituted reagent is stable for at least 6 months when stored as recommended.

2. **10 mM MK-571 (MRP Inhibitor)**

   Resuspend the MRP Inhibitor (MK-571) in 75 µL anhydrous DMSO. Vortex gently or slowly rotate the tube to dissolve. Store the solution at -20°C. The reconstituted reagent is stable for at least 6 months when stored as recommended.

3. **50 mM Novobiocin (BCRP Inhibitor)**

   Resuspend the BCRP Inhibitor (Novobiocin) in 30 µL anhydrous DMSO. Vortex gently or slowly rotate the tube to dissolve. Store the solution at -20°C. The reconstituted reagent is stable for 6 months when stored as recommended.

## B. Flow Cytometry Assay

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.

***IMPORTANT**: 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.*

1. Grow cell line of choice in the appropriate medium. Select drugs may interfere with dye efflux. Therefore, the cells should be kept in drug-free medium for at least one week. Anti-microbial agent may be included in the medium, since they do not interfere with multi- drug resistance proteins. Medium should be replaced one day before the assay. Adherent cells should be dislodged from the plates using standard methods and used in suspension for the assay.
2. Count cells using a hemacytometer. For one sample test, four assays should be performed in triplicates (with three different inhibitors and without inhibitor). Approximately 2-5 x $10^5$ cells are required for each assay. Prepare a cell suspension (at least 250 µL per assay) containing 1-2 x $10^6$ cells/ml in pre-warmed (37°C) complete indicator-free medium.
3. For each sample to be assayed, prepare four sets of tubes (in triplicate). Include one tube for the unstained cell control.
4. Immediately prior to use, prepare intermediate dilutions of all 3 inhibitors by mixing appropriate volumes of inhibitor stock solutions (from step A2, A3 and A4) and pre-warmed (37°C) complete indicator-free medium using the volumes specified in the following table (**Table 1**).

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

**Table 1. Dilution of Inhibitors**

| Inhibitor | Volume of Inhibitor/Concentration | Volume of Medium |
|---|---|---|
| MDR1 Inhibitor *(white cap)* | 16 µL, 5mM | 1 mL |
| MRP Inhibitor *(yellow cap)* | 20 µL, 10mM | 1 mL |
| BCRP Inhibitor *(purple cap)* | 8 µL, 50mM | 1 mL |

5. Add 125 µL of diluted inhibitor into separate tubes as indicated in the following table (**Table 2**). For control, add 125 µL pre-warmed (37°C) complete indicator-free medium containing 5% DMSO.

6. Add 250 µL of cell suspension into each tube (see **Table 2**). Gently mix the contents of each tube by pipetting (avoid introducing bubbles) and incubate all the tubes at 37°C for 5 min.

**Table 2. Assay Reagent Volumes**

| Tube Numbers | Volume of Dilute Inhibitor (from step B-4) | Volume of Cell Suspension | Volume of Dilute EFLUXX-ID® Gold Dye |
|---|---|---|---|
| 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 Controls) | 125 µL Medium/DMSO | 250 µL | 125 µL |
| 13 (Unstained Control) | 250 µL Medium/DMSO | 250 µL | — |

7. Dilute the EFLUXX-ID® Gold dye stock solution (from step A-1) by combining 16 µL of the stock solution and 2 mL of pre-warmed (37°C), complete indicator-free medium. Mix well by vortexing the tube gently.

   ***IMPORTANT**: Dilution of the EFLUXX-ID® Gold dye stock solution should be done just prior to use as it is susceptible to hydrolysis in aqueous solution.*

8. Begin the assay by adding 125 µL of the freshly diluted EFLUXX-ID® Gold dye solution (from step B-7) into each tube (except the tube labeled for unstained cells) (see **Table 2**). Gently mix cell suspensions by pipetting (avoid introducing bubbles) and incubate all the tubes at 37°C for 30 min.

9. After 25 min of incubation, 5 µL of the provided stock solution of Propidium Iodide can be added to each tube for cell viability monitoring.

10. Perform flow cytometry measurements immediately after reaction. The cellular orange fluorescence signal of EFLUXX-ID® Gold Detection Reagent should be measured using a flow cytometer in all tubes in the living (PI negative) cell population with identical equipment settings.

***Notes**: If immediate analysis on a flow cytometer is not possible, or if the number of samples is over 30, stop the staining /incubation reaction by rapid centrifugation (200xg, 1 min). Discard the supernatant and re-suspend the cells in 0.5 mL of ice cold complete indicator-free medium or 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 hours, waiting to be analyzed on a flow cytometer.*

11. Flow Cytometry Measurement of Samples

11.1 Within the flow cytometry software, set an FSC-SSC dot plot, an FSC-FL3 dot plot, and an FL2 histogram plot. For better separation of the different cell populations, using a log scale is recommended for the fluorescence channels (FL2 and FL3).

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

11.3 Set a gate (R1) on the FSC-SSC dot plot, selecting the cell population of interest but excluding cell debris (**Figure 1A**). Display the cells selected by the R1 gate in an FSC-FL3 dot plot format.

***IMPORTANT**: Compensation correction may be needed to avoid overlap between orange and red fluorescent signals (see **Appendix C**)*

**Recommended Controls for Compensation Correction**

- Unstained untreated cells
- Inhibitor (verapamil is recommended) treated cells stained with EFLUXX-ID® Gold Detection Reagent ("Orange" cells)
- Untreated cells stained with Propidium Iodide only ("Red" cells)

11.4 Set a second gate (R2) in the FSC-FL3 window to exclude PI-positive cells from analysis (**Figure 1B**). To avoid errors originated from the spillover of orange fluorescence of the EFLUXX-ID® Gold Detection Reagent, set the R2 border as high as possible. Display R2-gated events in the FL2 histogram plot format.

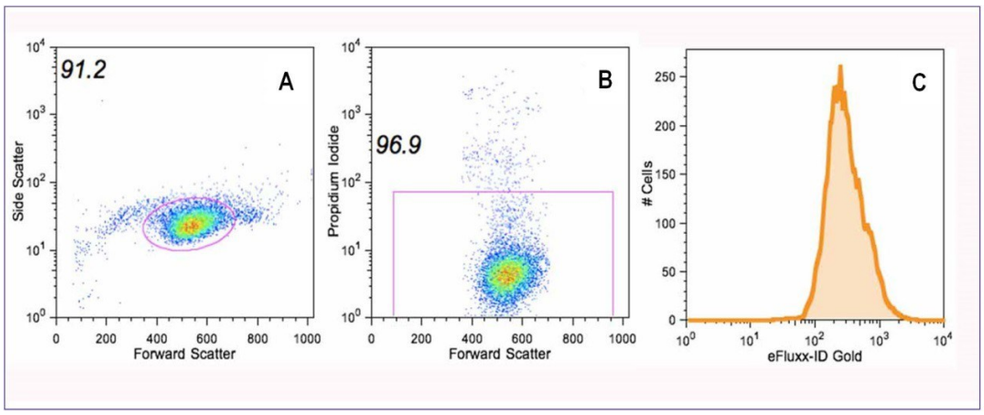


**Figure 1**. Flow cytometry measurements of the samples. Setting the parameters: Gate out the debris (Panel A), gate PI-negative events (Panel B), and set PMTs for the FL2 fluorescence channel (Panel C)

11.5 Run tube no. 1 and adjust the PMT amplification for FL2 so that the peak of the histogram is located between the second and third decades on the FL2 histogram channel (**Figure 1C**).

11.6 Save settings in a properly designated template file (*e.g.* "MDR settings"). We recommend the use of the 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.

## C. Microscopy Assay

1. Grow cell line of choice in the appropriate medium. Always make sure that cells are healthy and in the log phase of growth before using them for an experiment. Grow cells on coverslips or tissue culture treated slides such that cells will reached 80% ~ 100% level of confluence overnight before the experiment.

   ***NOTE**: Cells should be healthy and not overcrowded as results of the experiments will depend significantly on the cells' condition.*

2. Immediately prior to use, prepare intermediate dilutions of all 3 inhibitors by mixing appropriate volumes of inhibitor stock solutions (from step A2, A3 and A4) and pre-warmed complete indicator-free medium using the volumes specified in the following table.

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

**Table 3. Dilution of Inhibitors**

| Inhibitor | Volume of Inhibitor/Concentration | Volume of Medium |
|---|---|---|
| MDR1 Inhibitor | 2 µL, 5 mM | 450 µL |
| MRP Inhibitor | 2.5 µL, 10 mM | 450 µL |
| BCRP Inhibitor | 1 µL, 50 mM | 450 µL |
| DMSO | 2.5 µL | 450 µL |

3. Drain cultural medium in the chamber of cells and add 450 µL of each inhibitor into separate chamber of cells. Use DMSO as non-inhibitor control.
4. Dilute the EFLUXX-ID® Red dye stock solution (from step A1) by combining 10 µL of the stock solution and 190 µL of pre-warmed complete indicator-free medium. Mix well by vortexing the tube gently and add 50 µL of the diluted dye into each chamber of cells, so that the dye is 200 times diluted in final concentration.

   ***NOTE**: Be careful during dispensing procedure to minimize dislodging cells from the slides.*

5. Incubate at 37 °C for 30min.

6. Carefully wash the cells with 500 µL of PBS. Remove excess PBS and place coverslip on microscope slide.
7. Analyze the stained cells by wide-field fluorescence or confocal microscopy (60X magnification is recommended). Use appropriate filter set (for example filters compatible with Texas Red and TRITC) for imaging the signal.

## CALCULATION OF FLOW CYTOMETRY RESULTS

a. Calculate the mean FL2 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

b. 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 (see the **Troubleshooting Guide**).

c. 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.

# APPENDICES

## A. Spectral Characteristics of EFLUXX-ID® Gold Detection Reagent

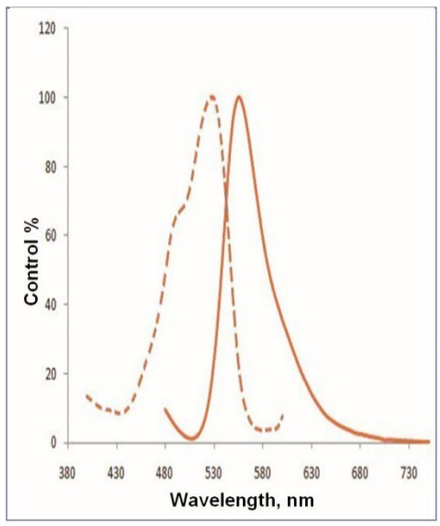


**Figure 2**. The absorption and emission peaks of E FLUXX-ID® Gold detection dye are 530 nm and 555 nm, respectively. It can be well excited with an argon ion laser at 488 nm, and detected in the FL2 channel of most bench flow cytometers.

## B. Technical Hints

1. 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.
2. Shear stress can be harmful to the cells. Do not vortex cell suspensions. Always mix them with gentle pipetting. Avoid forming excessive bubbles.
3. 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 avoid forming excessive bubbles.
4. 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.

5. The EFLUXX-ID® Gold Multidrug Resistance Assay Kit has been validated in various cell lines expressing multidrug resistance proteins that are summarized in **Table 4**.

**Table 4: Cell Lines Expressing Multidrug Resistance**

| Cell Line (Reference) | MDR1 | MRP | BCRP |
|---|---|---|---|
| CHO K1 (6) | + | + | + |
| HeLa (6) | - | - | - |
| A549 (7) | - | + | + |
| HCT-8 (8, 9) | + | + | - |
| Hep-G2 (10) | + | - | - |
| Jurkat (11) | - | + | - |
| U-2 OS (12) | + | + | + |
| U-2 OS RFP (12) | + | + | + |

## C. Compensation and Correction

Fluorescence of EFLUXX-ID® Gold Detection reagent will be detected in the FL2 channel. Dead (non-viable) cells will be detected in the FL3 channel. To avoid overlap between orange and red fluorescent signals, the following compensation procedure should be performed.

1. Run the unstained untreated sample first. Generate a FSC versus SSC dot plot and gate out cell debris.
2. Generate a log FL2 (X-axis) versus a log FL3 (Y-axis) dot plot. Adjust the PMT voltages for both channels so that the signals from the unstained cells falls within the first log decade scale of FL2 and FL3 axes.
3. Run single stained “Red” positive control and adjust the FL2 - %FL3 compensation until the orange fluorescence signal falls into the first decade of the log FL2 scale.

4. Repeat the compensation procedure with the "Orange" single stained positive control and adjust the FL3 - %FL2 compensation until the red fluorescence signal falls into the first decade of the log FL3 scale.

***Note**: It is important to use the brightest positive single stained samples (inhibitor-treated) for proper compensation correction to allow negative cells to be distinguished from slightly positive (dim) cells.*

## D. Results

### FLOW CYTOMETRY

1. The theoretical range of the MAF values are between 0 and 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.
2. In drug-selected cell lines exhibiting extremely high expression levels of ABC transporter proteins, the MAF values can be as high as 95-98.
3. Typical results of the assay are presented in **Figure 3**.

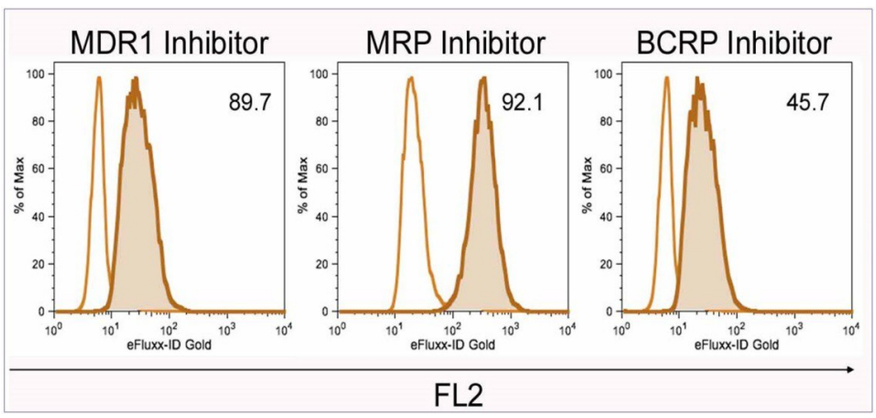


**Figure 3:** Typical results of the multidrug resistance assay. CHO K1 cells were incubated with EFLUXX-ID® Gold Detection Reagent with and without specific inhibitors according to the kit protocol. Resulting fluorescence was measured using flow cytometry. Tinted histograms show fluorescence of inhibitor-treated 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 right corners are MAF scores (multidrug resistance activity factors) — quantitative characteristics of multidrug resistance.

## MICROSCOPY

The proprietary AM-ester form of the EFLUXX-ID® Gold 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 EFLUXX-ID® Gold 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, including untreated cells, will demonstrate lower fluorescence, comparing to cells with impaired ABC transporter proteins, such as the cells treated with inhibitors. Three inhibitors provided in the kit are specific to the three important multidrug resistance proteins: MDR1, MRP and BCRP, respectively. The activity of a particular multidrug resistant protein is reflected by difference of fluorescence in the presence and absence of inhibitors. Typical result from CHO-K1 cell is shown in **Figure 4**.

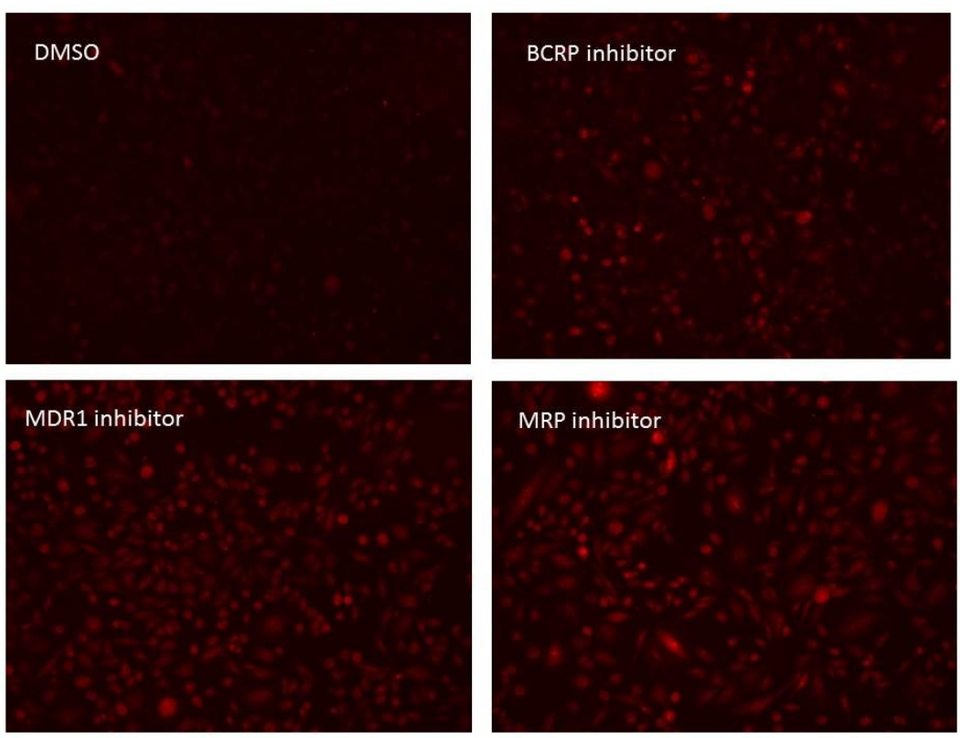


**Figure 4.** CHO-K1 cells were stained with EFLUXX-ID® Gold Detection Reagent after being cultured in full media (with DMSO control), BCRP inhibitor, MDR1 inhibitor or MRP inhibitor for a half hour. Cells treated with different ABC transporter inhibitor demonstrated different degree of increasing of intracellular fluorescence.

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## TROUBLESHOOTING GUIDE

| Problem | Potential Cause | Suggestion |
|---|---|---|
| Cells do not exhibit fluorescence after incubation with the detection reagent. | Cell viability is low. | Cells should be in log growth phase.<br>Cell samples cannot be kept longer than 6 hours before the assay.<br>Do not use fixatives, azides or other preservatives. Avoid shear stress, do not vortex, and avoid bubbling. |
| | Very low concentration of the EFLUXX-ID® Gold 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.<br>Quality of the reagents is compromised. | Use a positive control cell type expressing corresponding ABC transporter. Check storage, stability and freshness of the reconstituted reagents. |
| Lower than expected fluorescence increases after incubation with inhibitor. | Overcompensation of the FL2 signal | Change the values of compensation correction using single stained positive samples. Follow the recommendations in **Appendix C**. |
| Percentage of dead (PI positive) cells is too high. | Wrong compensation correction. | Change the values of compensation correction using single stained positive samples. Follow the recommendations in **Appendix C.** |
| 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 cells (irreproducible results) | Inadequate incubation condition. | Always use a water bath (not incubator).<br>Ensure temperature of water bath is 37°C. |
| | Cell viability is low. | Cells should be in log growth phase.<br>Cell samples cannot be kept longer than 6 hours before the assay.<br>Do not use fixatives, azides or other preservatives.<br>Avoid shear stress, do not vortex, and avoid bubbling. |

## NOTES

## NOTES

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