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ProductInformation

Multi-Drug Resistance Quantitation Kit

Product Number **MDRQ1**
Storage Temperature –20 °C

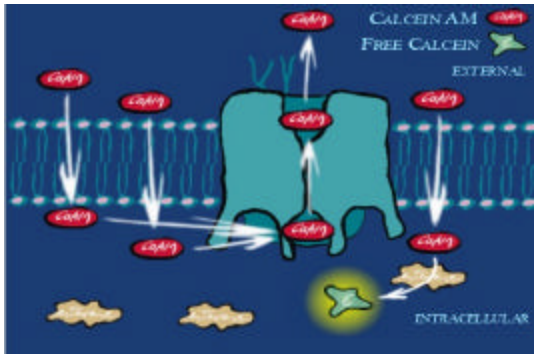
TECHNICAL BULLETIN

Product Description

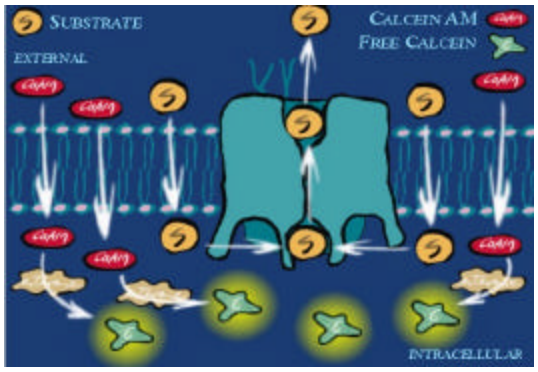
The Multi-Drug Resistance Quantitation Kit is designed for functional quantitative measurement of drug resistance in live cells. The procedure is fast, sensitive, and quantitative, and measures the drug transport activity of two subfamilies of multidrug resistance proteins: MDR1 or P-glycoprotein (MDR1/Pgp) and multidrug resistance-associated proteins (MRP1). MDR1/Pgp and MRP1 are ATP-dependent trans-membrane proteins that remove hydrophobic xenobiotic compounds (typically environmental toxins) from the cell. The kit utilizes calcein-AM, a non-fluorescent hydrophobic compound that enters all cells nonspecifcally. Calcein-AM is an excellent substrate for targeted extrusion by multi-drug transporters. If MDR1/Pgp and MRP1 are active, the hydrophobic calcein-AM will be removed intact before it can be hydrolyzed. If MDR1/Pgp and MRP1 are not active, enzymatic cleavage of the calcein-AM by endogenous esterases results in the fluorescent hydrophilic free-acid, calcein, which is retained within the cytoplasm. Normal, drug sensitive cells will fluoresce when exposed to calcein-AM. The degree of fluorescence observed in test cells is inversely proportional to MDR1/Pgp and MRP1 activity (Figures 1A and 1B).

Quantitation of this fluorescence is possible through the development of the MDR Activity Factor (MAF). The dye efflux activity of the MDR transporter is measured as the difference between the amount of the dye accumulated in the presence and absence of inhibitors. The fluorescence measurement in the presence of Inhibitor 1 constitutes the maximal potential fluorescence with the given cell population when the multidrug transporters are rendered nonfunctional. This represents a standardization method, which eliminates unknown cell type-specific variables that influence cellular calcein accumulation, such as esterase activity, cell size, etc. This, in turn, allows for intra- and interlaboratory comparison of test results and MAF values.

Figures 1A and 1B.
Principle of the Calcein Assay



1A: Low intracellular dye accumulation and fluorescence is observed in MDR-expressing cells



1B: Interference of MDR proteins with inhibitors or the presence of excess substrate prevents dye extrusion resulting in higher intracellular accumulation of the dye and fluorescence.

The transport activity of MDR1/Pgp and MRP1 can be easily distinguished with the inhibitors included in this kit. Inhibitor 1 blocks both MDR1/Pgp- and MRP1-mediated dye efflux, which provides the maximum dye accumulation used for standardization. Inhibitor 2 selectively blocks the activity of MRP1. Separate measures for both transporters can be determined using the simple calculations given in the Data Collection/MAF Calculation section.

The kit has been optimized for use in flow cytometry, but can be readily adapted for use in other cell-based assay formats such as fluorescence microscopy, spectrophotometry, or 96 well plate assays. If these applications are utilized it is necessary to consider the following:

- Heterogeneous cell populations accumulate calcein at different rates, which cannot be resolved by fluorometry (cuvette or plate reader).
- Homogeneous cell population can be easily tested in the above-mentioned formats.
- For consistency and reproducibility, adequate mixing of cell suspensions and temperature control are necessary.
- Adherent and suspension cells take up the fluorescent indicator at different rates.
- Protocol optimization for other formats will be necessary.

MDR Overview

As the name implies cell membrane transporter proteins are transmembrane proteins involved in moving compounds in either direction across the plasma membrane. These transporters are part of a large family of ATP binding cassette (ABC) proteins found in all organisms from bacteria to human. Multidrug transporter proteins form a diverse subset within this superfamily and are distinguished by their broad substrate specificity. MDR transporters include many proteins with important disease phenotypes. Some well known examples include cystic fibrosis (mutations in cystic fibrosis conductance regulator or CFTR), Dubin Johnson Syndrome (mutations in MDR2 or cMOAT), and retinitis pigmentosa (mutations in ABCR).

At least three molecular elements characterize these transporters:

- transmembrane domains or TMD
- nucleotide binding domains or NBD (ABC)
- conserved peptide sequence motifs (Walker A, Walker B, and ABC-signature motifs)

ABC transporters have been classified into three major subfamilies. B subfamily members include MDR1/Pgp, Pgp3/MDR3, and BSEP/sPgp or sister Pgp. The C subfamily has many members, MRP 1 through 6 and CFTR among them. G subfamily members include MXR/BCRP and ABC8. Links to several helpful databases on nomenclature and classification can be found at the Sigma-Aldrich web site at: www.sigmaaldrich.com/Area_of_Interest/Drug_Discovery/MultiDrug_Resistance.html

MDR1-Pgp and MRP1 are two multidrug transporters with a primary role of cell protection by actively removing hydrophobic xenobiotic compounds and endogenous toxic metabolites from cells. This activity is ATP dependent. The specific localization of different MDR transporters implies a complex protective system of transport proteins. MDR1 has been located in the blood-brain barrier and is implicated in protecting the brain from toxic compounds. In human hepatocytes MDR1 tends to be expressed at the canalicular membrane while MRP1 is more prevalent at the basolateral membrane interface.

The broad range of substrates for these proteins takes on particular significance in chemotherapy. Tumor cells with high MDR1/Pgp and MRP1 activity are multidrug resistant, although other mechanisms may also contribute to this phenomenon. In this bulletin multidrug resistance refers specifically to the cell membrane tranporter-associated form. Major substrates of MDR 1/Pgp include Vinca alkaloids, anthracyclines, etc. The unusually wide range of substrates of MDR transporters has been clearly documented in a large volume of experimental data. In general, selection with one cytotoxic compound in culture (*e.g.* doxorubicin), or as an unintended consequence in chemotherapy, leads to cross-resistance to other cytotoxic compounds with unrelated structures and a concomitant increase in the expression of MDR proteins.

Reagents
Reagents supplied are sufficient for 20 determinations run in triplicate.

- MDR Fluorescent Cytoplasmic Indicator, Calcein-AM-based lyophilized powder, Product Code M2566 — 1 vial
- MDR Inhibitor 1 Inhibitor of MDR1/Pgp- and MRP1-mediated dye efflux, Product Code M2066 — 1 vial
- MDR Inhibitor 2 Inhibitor of MRP1-mediated dye efflux, Product Code M2316 — 1 vial
- 10× Reaction Buffer Product Code M2191 — 66 ml
- DMSO, Product Code D0941 Volume sufficient for reconstitution of M2566, MDR Fluorescent Cytoplasmic Indicator. — 550 µl
- Propidium Iodide Product Code P2868 — 1 vial
- Sodium Bicarbonate (0.23 g) Product Code S4821 — 1 bottle

Equipment and Reagents Required But Not Provided

- EDTA for blood collection
- ACCUSPIN™ System-Histopaque®-1077, Product Code A6929
  or
  Histopaque, Product Code 10771 or H8889
- 15 ml conical centrifuge tubes
- Deionized water
- Flow cytometer equipped with an argon laser and relative fluorescence data output option; multiwell plates and plate reader; fluorescent microscope; or spectrophotometer
- Laboratory centrifuge
- Microcentrifuge
- Water bath or thermostated dry block for 37 °C microtube incubations
- 1.5 ml microcentrifuge tubes
- Pipettors
- Vortex mixer
- Timer

Precautions and Disclaimer
This product is for R&D use only, not for drug, household, or other uses. Please consult the Material Safety Data Sheet for information regarding hazards and safe handling practices. It is not for compound testing use on cell lines.

Preparation Instructions

- 1× Reaction Buffer - Thaw 10× Reaction Buffer, Product Code M2191 slowly at 2–8 °C or at ambient temperature. A slight precipitate is normal and will dissolve on dilution. To 60 ml of 10× buffer add 500 ml of distilled water and the contents (0.23 g) of the sodium bicarbonate bottle, Product Code S4821. Adjust pH to 7.4 using 0.1 N NaOH or 0.1 N HCl. Bring the final volume to 600 ml with distilled water.
- MDR Fluorescent Cytoplasmic Indicator, Product Code M2566: Dissolve in 550 µl of DMSO, D0941.
- MDR Inhibitor 1, Product Code M2066: Dissolve in 340 µl of 1× Reaction Buffer, M2191.
- MDR Inhibitor 2, Product Code M2316: Reconstitute in 370 µl of deionized water.
- Propidium Iodide, Product Code P2868: dissolve contents in 1 ml of deionized water with vortexing.

Storage/Stability
Note: This kit is stored at –20 °C and shipped accordingly. After the first thawing not all components need to be returned to –20 °C, but may be stored as follows:

- MDR Fluorescent Cytoplasmic Indicator, Product Code M2566: Store at –20 °C. Store stock solution at 2–8 °C for 6 months or in frozen aliquots at –20 °C for one year. Protect from light.
- MDR Inhibitor 1, Product Code M2066: Store at –20 °C. Store stock solution at 2–8 °C for 6 months or in frozen aliquots at –20 °C for one year. Protect from light
- MDR Inhibitor 2, Product Code M2316: Store at –20 °C. Store stock solution at 2–8 °C for 6 months or in frozen aliquots at –20 °C for one year. Protect from light.
- 10× Reaction Buffer, Product Code M2191: Store at 2–8 °C. Store 1× Reaction Buffer at 2–8 °C for 6 months.
- DMSO, Product Code D0941: Store at room temperature.
- Propidium Iodide Product Code P2868: Store at –20 °C. Store solution at 4 °C for up to 12 months. Protect from light.

Procedure

Sample Preparation

The assay can be performed on cultured cells, peripheral blood, or bone marrow samples.

A. Cell Culture

1. A minimum of 2–5 × 10^6 viable cells in a log growth phase is preferable. Samples not in log phase may have significant depletion of endogenous ATP. Membrane transporter activity tends to be low in cells depleted of ATP.
2. Establish viable cell density/count by manual or automated method.
3. Dilute as needed using culture medium or buffer to obtain a concentration of ~2.5–6.25 × 10^5 cells/ml. An 8 ml total sample volume is required.
4. Proceed to Assay section.

B. Peripheral Blood or Bone Marrow Samples

1. Use EDTA as the anti-coagulant for peripheral blood or bone marrow samples. Heparin and other anticoagulants may interfere.
2. Primary samples should be tested within 6 hours of sampling. Samples older than 6 hours may have significant depletion of endogenous ATP.
3. Mix 3 ml of 1× Reaction Buffer (pH 7.4) and 3 ml of sample.
4. Add sample solution to an ACCUSPIN System-Histopaque-1077 tube and go to step 6.

   or

   Add 3 ml of Histopaque, 10771 or H8889, to 15 ml conical centrifuge tube.
5. Gently add sample solution to overlay the Histopaque. Do not allow to mix.
6. Centrifuge at 400 × *g* for 30 minutes at room temperature.
7. After centrifugation, remove the layer above the interphase (opaque layer containing the mononuclear cells) using a Pasteur pipette and discard. Do not disturb the opaque interphase that contains the mononuclear cells.
8. With a Pasteur pipette, carefully transfer the opaque interface to a clean, conical centrifuge tube.
9. Add to this tube 5 ml of 1× Reaction Buffer and mix gently by inversion.
10. Centrifuge at 300 × *g* for 10 minutes.
11. Aspirate the supernatant and discard.
12. Repeat Steps 9, 10, and 11 once.
13. Resuspend cells in 1 ml of 1× Reaction Buffer and mix by gentle trituration with Pasteur pipette.
14. Establish viable cell density/count by manual or automated method.
15. Dilute using medium or buffer as needed to obtain a concentration of approximately 2.5–6.25 × 10^5 cells/ml. An 8 ml total sample volume is required.
16. Proceed to Assay section.

Assay

Each sample requires 3 sets of tubes in triplicate for a total of 9 tubes. Label reaction tubes by numbering according to Table 1.

Table 1.
Suggested assay setup per sample

	1	2	3	4	5	6	7	8	9
Inhibitor 1	x	x	x						
Inhibitor 2				x	x	x			
Baseline							x	x	x

1. Dilute 25 µl of MDR Fluorescent Cytoplasmic Indicator solution with 2.0 ml of 1× Reaction Buffer. Vortex 1 minute.
2. Add 50 µl of Propidium Iodide solution to 5 ml of 1× Reaction Buffer. Vortex minimum 10 seconds.
3. Add 800 µl of cell suspension to each of the 9 sample tubes. Do not vortex and avoid bubbles.
4. Add 5 µl of MDR Inhibitor 1 solution to each of tubes 1-3. Add 5 µl of MDR Inhibitor 2 solution to each of tubes 4-6. Mix well by gentle agitation, avoid bubbles. Incubate all 9 tubes at 37 °C for 5 minutes.
5. Following incubation, add 200 µl of MDR Fluorescent Cytoplasmic Indicator solution (step 1) to each of the 9 tubes. Addition of indicator and mixing process should occur within 20 seconds for all tubes. Mix by gentle agitation. Incubate immediately for exactly 10 minutes at 37 °C. Due to critical incubation times, it is recommended that no more that 2 samples or 18 tubes be run at the same time.
6. Stop reaction at exactly 10 minutes by rapid centrifugation for 1 minute using low speed (~2,000 × *g*), rapid acceleration and deceleration (≤15 seconds).
7. Discard supernatant; resuspend pellet in 0.5 ml of Propidium Iodide solution (step 2).
8. For best results, read samples immediately. Samples can, however, be stored at 2–8 °C up to 24 hours with minimal interference.

Flow Cytometry Instrument Settings

It is recommended that PMT settings and an established window of analysis be stored in template files for future reference. MAF values are independent of PMT settings, whenever the acquisition occurs within the linear range of the equipment. However, the use of the same, or at least similar, setting for the PMT amplifications is recommended whenever possible.

1. Set an FL1 histogram window, and FSC-SSC dot plot, an FSC-FL3 dot plot, and a second FL1 histogram window. For better separation of the different cell populations, using a log scale for the fluorescence channels (FL1 and FL3) is recommended.
2. Run tube 1 (or equivalent), and adjust the PMT amplification for FL1 so that the peak of the histogram is located around the border of the 3rd or 4th decade on the FL1 histogram channel. See Figure 2 for illustration of optimal FL1 PMT amplification.
3. Adjust both forward and side scatter PMT amplification so that all cells are visible on the FSC-SSC dot-plot. (Figure 3).
4. Adjust FL3 PMT so that dead or dying cells are clearly separated from the main cell population (Figure 4). If no PI positive cells are observed, adjust FL3 PMT amplification in order to see the main cell population immediately above the FSC-axis, but clearly separated from it. Maintain these same PMT settings throughout the measurement of all 9 tubes. Do not readjust.
5. Place a wide region (R1) excluding only very dim FL1 events against the vertical axis in the FL1 histogram window (Figure 5A). The R1 gate should include all calcein positive cells (maximally fluorescent, normally drug-sensitive cells and phenotypically drug-sensitive cells in the presence of dual inhibitor, MDR Inhibitor 1, for MDR1/Pgp and MRP1, illustrated in Figure 2) as well as MDR-expressing cells that display a spectrum of dye extrusion activities and a positive, but dimmer, fluorescence.
6. Set a second region (R2) on the FSC-SSC dot plot (Figure 5B), selecting the cell population of interest, but excluding cell debris. Note that blast cells of hematological malignancies are usually larger that normal cells. Histopaque separation results in a single population.
7. The third region (R3) should be set on the FSC-FL3 so that FL3 (PI) positive cells are excluded from analysis (Figure 5C). Since Calcein is a bright fluorophore with a very broad spectrum, its fluorescence may spill over the FL3 channel. To avoid underestimation of PI negative live cells, set a wide inclusive region for R3 excluding only the PI bright cells (dead cells).
8. Validate R1 for FSC-SSC dot plot, R1 and R2 for the FSC-FL3 dot plot, R1, R2, and R3 for the second FL1 histogram window.
9. Apply sequential gating strategy for the analysis. See Figure 5D for a typical FL1 histogram of multi-drug resistant cells with sequential gating applied (tubes 7, 8, and 9; baseline FL1 fluorescence).
10. Save settings in a template file for future reference for similar biological samples. Use the same settings for all samples within one assay.

Data Collection/ MAF Calculations

1. Run all samples counting 10,000 events on the second FL1 histogram, applying the gating described above (R1, R2, and R3). Save raw data for each tube.
2. Determine the mean fluorescence intensity (MFI) values on the second FL1 histogram gated on R1, R2, and R3.
   Note: Do not use channel numbers. If your system cannot provide the absolute fluorescence values, follow the instructions given in the Trouble Shooting Guide at the end of this document.
3. Calculate the mean of the MFI's for parallel measurements:
   F_{max} from tubes 1 – 3
   F_{MRP} from tubes 4 – 6
   F_o from tubes 7 – 9
   If differences between triplicates are <10% use all three values to calculate the mean. If one value is extreme it may be disregarded. Calculate mean from remaining two values. If 2 or more values show >10% difference, review protocol, sample prep, and settings, then repeat assay.
4. Calculate the total multi-drug resistance activity factor, MAF_T, according to the following equation:
   $MAF_T = 100 \times (F_{max} - F_o) / F_{max}$
   In extreme cases (no MDR1/Pgp or MRP1 activity detected) F_{MRP} can be slightly smaller than F_o. If this occurs assign $MAF_{MRP} = 0$.
5. The MRP1-related MAF_{MRP} should be calculated by the following equation:
   $MAF_{MRP} = 100 (F_{MRP} - F_o) / F_{max}$
   In extreme cases (no MRP1 activity detected) F_{MRP} can be slightly smaller than F_o. If this occurs assign $MAF_{MRP} = 0$.
6. Calculate the MDR1-related MAF_{MDR} by subtracting MAF_{MRP} from MAF_T:
   $MAF_{MDR} = MAF_T - MAF_{MRP}$
   In extreme cases (very high MRP1 activity, but no MDR1 activity), MAF_T can be slightly smaller than MAF_{MRP}. If this occurs, assign $MAF_{MDR} = 0$.

Results

The theoretical value of MAF ranges between 0 and 100. In the case of hematological malignancies, the MAF_T values in the tumor cell population are usually found between 0 and 50, but in extreme cases values can be as high as 70. Studies comparing the MAF_T values with the clinical response to the chemotherapeutic treatment suggested that the specimen with a MAF_T value <20 can be regarded as MDR negative, while MAF_T value <25 is indicative of positive MDR activity.

The MAF_T found in normal peripheral blood mononuclear cells are in the range of 0–20, while in drug selected cell lines exhibiting extremely high levels of MDR expression, the MAF_T value can be as high as 95-98.

References

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LPG,LM,MAM 12/05-1

Appendix

Troubleshooting Guide

Problem	Recommended Solutions
Cell Viability	Assay requires viable tumor cells that are not seriously depleted of energy stores. Peripheral blood that is more that 6 hours old or cells that are not in a log growth phase may show incorrect MAF values without affecting the ratio of PI positive and negative cells. Cells should be maintained in an appropriate buffer containing all essential components. Do not use fixatives, azides, or other preservatives. Shear stress can also be harmful. Do not vortex. Avoid bubbling. Cells in suspension sediment very rapidly and must be gently, but thoroughly mixed.
Fibrinous Samples	Bone marrow and other types of tissue may contain fibrin or other solid debris. Samples can be filtered through a large pore nylon mesh prior to processing the sample. The presence of fibrin and other debris may interfere with cell separation process.
Insufficient Cells	For the purposes of flow cytometry, 3 ml of whole blood or bone marrow usually provides enough cells to prepare the required 8 ml suspension at the suggested concentration. If the cell count is lower it is possible to perform that assay by lowering the counts that determine the end of the acquisition (*i.e.* 10,000 events in the gated F1 histogram). Results will not be optimal because the signal/noise ratio is decreased, risking the proper evaluation of the assay. Low cell counts may be the result of poor separation of mononuclear cells on the Histopaque cushion, of insufficient mixing of cells following centrifugation or sedimentation, or decanting a portion of the cells with the supernatant.
Flow Rate	Cell numbers in the kit are also optimized for measurement in flow cytometry. Cell suspensions at the recommended concentration will normally result in the 100-300 events/sec flow rate suitable for analysis. Keeping the flow rate below 600 events/sec is recommended.
Differences in MFI Values	Calcein accumulation may be influenced by several factors (i.e. cell size, endogenous esterase activity, etc.). Even by using the same MDR-set for FL1 PMT amplification, cells of different samples may not display the same MFI values. Internal standardization using the inhibitors and calculations of MAF values eliminates these differences.
MAF Values Abnormally Low	A value of 0 for MAF is theoretically possible. In one analysis of 40 normal donors of both peripheral blood and bone marrow, a mean MAF value of 11 was obtained. If suspiciously low MAF values are obtained, check storage, stability, and freshness of reconstituted reagents.
MAF Values Abnormally High	It is important to note that the highest theoretical value of 100 for MAF is never attained because there is always some degree of background fluorescence in the cells on the FL1 channel. Drug-selected cell lines (e.g. KBV-1), expressing $\sim 5 \times 10^5$ copies of Pgp, exhibit an MAF_T value of 95-99. During analysis of >200 leukemic samples there were no MAF_T values greater than 70.
Fluorescence Values and Equipment	Older flow cytometers cannot provide absolute fluorescence values. If that is the case, the fluorescence intensities are expressed in channel numbers. Since this scale is logarithmic the exponent of channel numbers should be taken to replace the MFI values for the calculation of F_{max}, F_{MRP}, and F_o. The same MAF values will be obtained as by using the MFI's.

Figure 2.

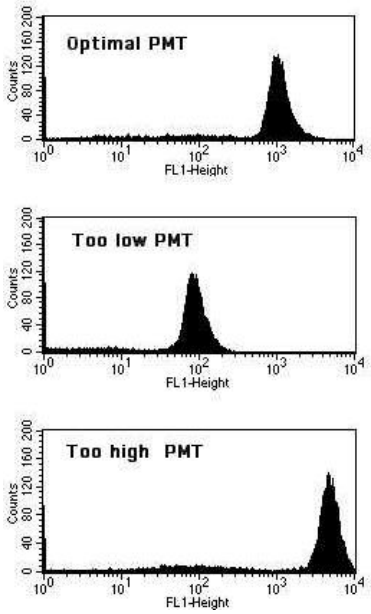
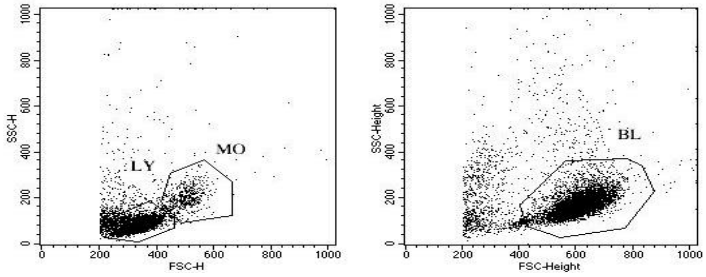


Illustration of FL1 histograms at various PMT settings for a cell sample incubated with a dual inhibitor for MDR-1/Pgp-1 and MRP-1 (Inhibitor 1) (corresponding to sample tubes 1-3 in assay protocol). This sample represents the maximal fluorescence obtainable with the given biological material in this assay. Optimal PMT setting should place this population near the top of the scale to allow for clear resolution of cells with high MDR activities and dim fluorescence.

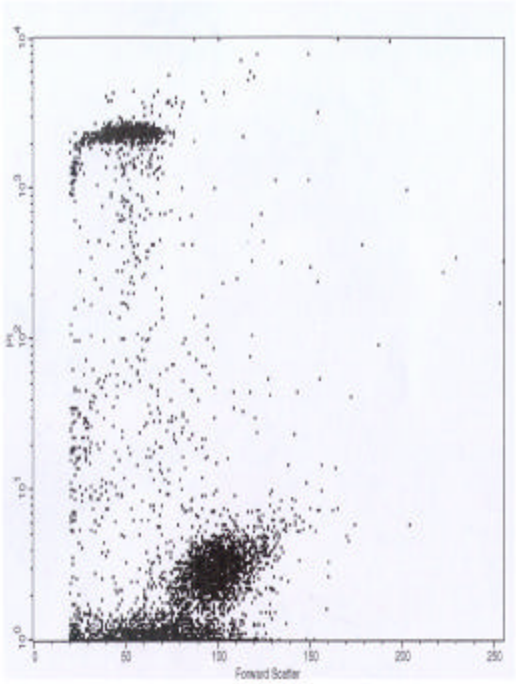
Figures 3A and 3B.
Adjustment of Forward and Side Scatter PMT



3A: Mononuclear cells from a Histopaque separated peripheral blood sample of a healthy sample (LY: lymphocytes; MO: monocytes).

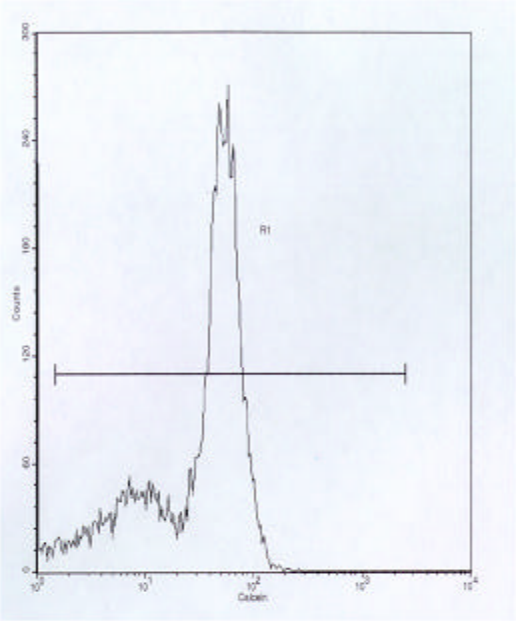
3B: Histopaque separated peripheral blood sample from a sample containing acute myeloid leukemia cells (BL: blast cells).

Figure 4.
Adjustment of FL3 PMT

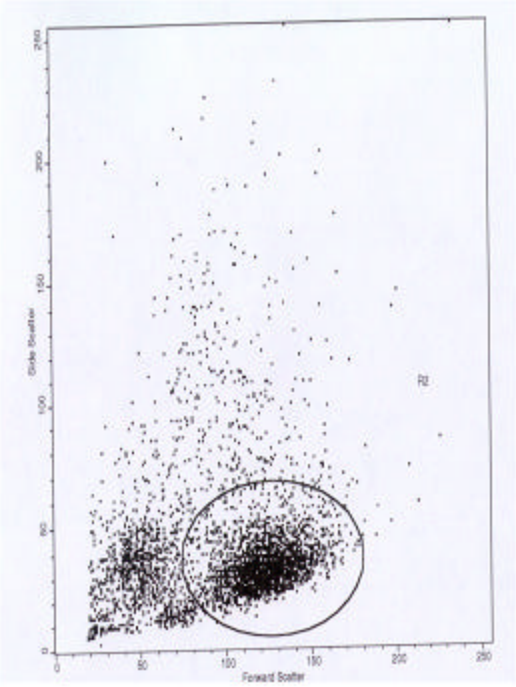


Note: No gating was applied in the FSC-SSC dot plot to demonstrate the presence of dead, PI positive cells.

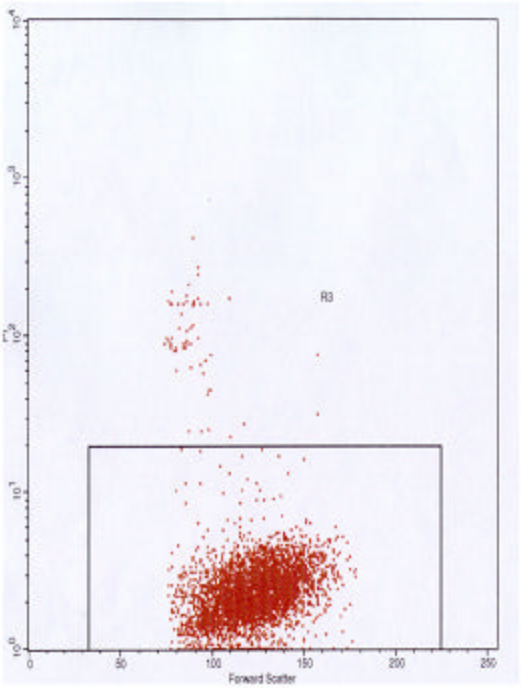
Figures 5A - 5D
Sequential Gating Strategy Demonstrated on a Peripheral Blood Sample taken from an Individual Subject with Acute Myeloid Leukemia



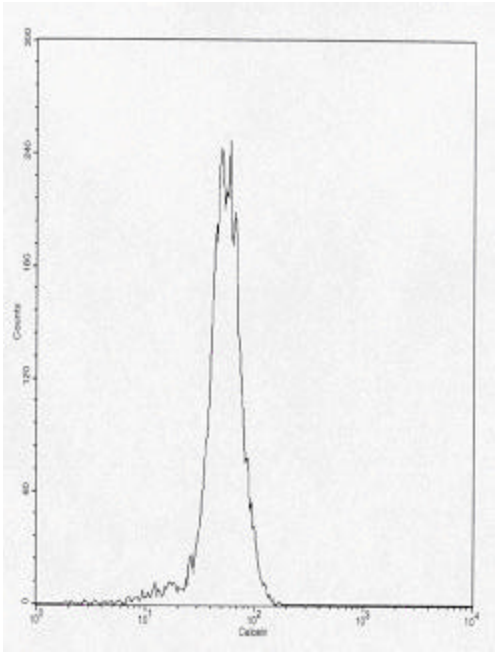
5A: Typical calcein histogram of a sample in the absence of any inhibitor (corresponding to sample tubes 7 – 9 in assay protocol). Lower fluorescence or dye accumulation compared to Figure 2 Top Panel is indicative of MDR activity.



5B: The cell population of interest (*e.g.* blast cells) is selected by a second region in the FSC-SSC dot-plot.



5C: PI negative, live cells are selected with a third region set in the FL-3 (PI fluorescence) and FSC dot-plot.



5D: Typical FL-1 (Calcein fluorescence) histogram of a multi-drug resistant cell sample with the application of R1, R2 and R3 gates to include only live cells of interest.