CHEMICON®
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MultiDrugQuant™ Assay Kit

For the Functional Determination of Multidrug Resistance in Living Cells

Kit for 20 Tests Carried Out in Triplicate

Cat. No. ECM900

FOR RESEARCH USE ONLY
Not for use in diagnostic procedures
Not for compound testing use on cell lines

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Application

The MultiDrugQuant™ Assay Kit* is used for the functional determination of multidrug resistance in living tumor cells. The kit provides a fast, sensitive and quantitative method for measuring the drug transport activity of the clinically most important multidrug resistance proteins: MDR1 (P-glycoprotein) and MRP1. The assay is based on determining fluorescence intensities using a flow cytometer after a short *in vitro* incubation of the cell suspension with the dye calcein-acetoxymethyl ester (calcein AM), in the presence or absence of selective inhibitors of MDR1 and MRP1. This testing approach provides a separate measure of multidrug resistance for both MDR1 and MRP1. Since the assay includes an internal standardization, the results of the test, the MDR activity factor (MAF) values provide valuable opportunity for the intra-laboratory and inter-laboratory comparison of multidrug resistance of tumor cells. The MAF values obtained in the MultiDrugQuant™ Assay assist the user to monitor drug sensitivity of malignancy.

For Research Use Only; Not for use in diagnostic procedures; Not for compound testing use on cell lines.

*** Kit manufactured by Solvo® Biotechnology.**

Assay Principle

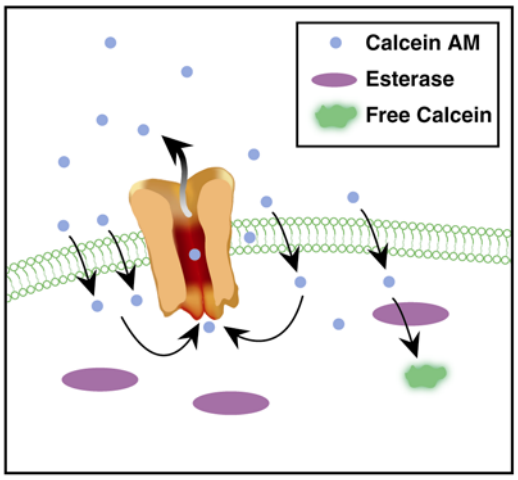
The MultiDrugQuant™ Assay Kit utilizes the fluorogenic dye calcein AM, which is a hydrophobic, non-fluorescent compound that readily penetrates the cell membrane. After entering the cell, calcein AM is rapidly hydrolyzed by endogenous esterases. As a result of the cleavage, highly fluorescent free acid derivative of the dye is formed. Since calcein free acid is a hydrophilic compound, it becomes trapped in the cytoplasm. Calcein is a bright green-fluorescent dye, which does not affect most cell functions, including cell viability. Another advantage of calcein is its relative insensitivity to changes of various cellular parameters, including intracellular pH, $Ca^{2+,}$ and Mg^{2+} concentrations.

The multidrug transporters extrude different hydrophobic compounds, including cytotoxic drugs from the cells, hindering the effectiveness of chemotherapy. Since calcein AM is an excellent substrate of both MDR1 and MRP1, whenever a multidrug transporter is active in the cell membrane, the dye uptake is limited by the outward transport of calcein AM. This results in a lower cellular accumulation of calcein free acid (*Figure 1, Panel A*). The more MDR proteins are active in the cell membrane, the less calcein is accumulated intracellularly. In MDR-expressing cells, the addition of an MDR inhibitor or an MDR substrate in excess dramatically alters the rate of calcein accumulation. Since the dye extrusion activity of the MDR transporter is blocked in the case, the inward net flux of calcein AM is increased; thus, a larger amount of free calcein is accumulated in the cells (*Figure 1, Panel B*). The drug-sensitive tumor cells without significant MDR activity accumulate free calcein at a high rate that is not affected by an MDR inhibitor or substrate.

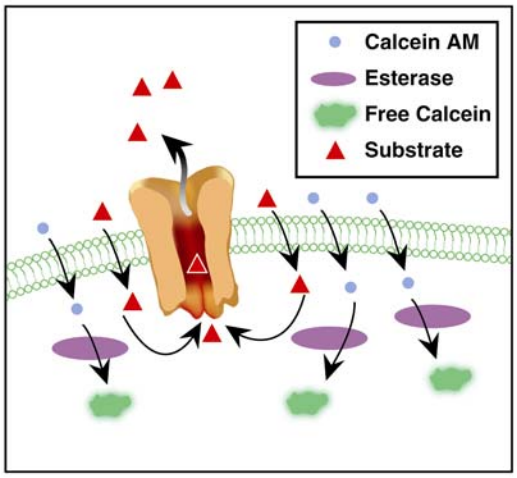
The dye extrusion activity of the multidrug transporter is reflected by the difference between the amount of the dye accumulated in the presence and absence of the inhibitors, respectively. When calculating the MAF values, this difference is normalized to the dye uptake measured in the presence of the inhibitor. By this approach, an internal standardization is included in the MultiDrugQuant™ Assay. Thus, the result of the test becomes independent from factors influencing the cellular accumulation of calcein other than the activity of the multidrug transporters. These variables include the differences in the cellular properties (membrane composition, intracellular esterase activity, cell size, cell surface, etc.); and the methodological differences (e.g. use of different equipment, amplification, and individual variables). Since the influence of these factors is diminished by the simple normalization approach mentioned above, the intra- and inter-laboratory comparison of MAF values is possible.

Using selective inhibitors, the transport activity of P-glycoprotein (MDR1) and MRP1 can be distinguished. The kit component, *Inhibitor 1*, blocks both MDR1- and MRP1-mediated dye extrusions providing a dye accumulation rate that can be used for standardization, while *Inhibitor 2* selectively blocks the activity of MRP1. After a short, simple calculation, separate measures of multidrug resistance for both MDR1 and MRP1 can be obtained. This information can help monitor drug sensitivity of different malignancies.

Figure 1. Principle of the Calcein Assay



A. Due to the transport activity of MDR proteins, low intracellular dye accumulation is observed in MDR-expressing cells.



B. Inhibition of the Calcein AM extrusion by MDR inhibitor or MDR substrate in excess results in higher intracellular accumulating of the dye.

Kit Components

Vial 1 Calcein AM, lyophilized powder to be reconstituted with DMSO;

Vial 2 Inhibitor 1 (an inhibitor for both MDR1 and MRP1-mediated dye extrusion), dry powder to be reconstituted with the Reaction Buffer;

Vial 3 Inhibitor 2 (a specific inhibitor of MRP1-mediated dye extrusion), dry powder to be reconstituted with the Reaction Buffer;

Vial 4 Propidium iodide (PI), dry powder to be dissolved in distilled water;

Vial 5 DMSO in sealed vial;

Vial 6 Reaction Buffer (10x) to be diluted with distilled water;

Vial 7 $NaHCO_3$ powder to add to the reaction buffer.

Materials Not Supplied

- Flow cytometer equipped with an argon laser and relative fluorescence data output option
- Laboratory centrifuge for cell separation e.g. on a Ficoll cushion
- Water bath or thermostated dry block for 37 °C incubation of microtubes
- Microfuge with 2000xg capacity for Eppendorf type microtubes
- 1.5 mL microcentrifuge tubes
- Pipettors
- Vortex mixer
- Stopwatch
- Vacutainer tube with EDTA anticoagulant
- Density gradient for separating mononuclear cells (e.g. Ficoll-Histopaque, Sigma Cat. No.: H.1077)
- Distilled water

Precautions

WARNING: *Working with human samples involves potential risk of infection. Handle samples and waste products accordingly. Wear protective clothing, gloves, and eyes/face protection. Use appropriate caution. Some of the components of the kit are highly toxic (inhibitor 1) and may cause cancer and heritable genetic damage (Propidium iodide, DMSO). They are also irritating to eyes, respiratory system, and skin. Other chemicals should also be handled as potential health hazards. Do not swallow or inhale any of them. Do not breathe dust. Avoid contact with your eyes or skin. In case of accidents, or if you feel unwell, seek medical attention immediately. In case of contact with eyes, rinse immediately with plenty of water and seek medical attention.*

Inhibitor 1

Toxic.

Toxic by inhalation, in contact with skin and if swallowed. Target organ(s): cardiovascular system. In case of accidents, or if you feel unwell, seek medical attention immediately (show the label where possible). Wear suitable protective clothing, gloves, and eye/face protection. Do not breathe dust. If swallowed, wash out mouth with water, provided the person is conscious. Call a physician immediately. If inhaled, remove the person to fresh air. If not breathing, give artificial respiration. If breathing is difficult, give oxygen. In case of skin contact, flush with copious amounts of water for at least 15 minutes. Remove contaminated clothing and shoes. Call a physician. In case of contact with eyes, flush with copious amounts of water for at least 15 minutes. Assure adequate flushing by separating the eyelids with fingers. Call a physician.

Inhibitor 2

No toxicity data available.

Inhibitor 2 should be handled as a potentially hazardous material. Only fully trained persons should handle this compound. Do not swallow. Wear gloves and mask when handling this compound. Avoid contact by all modes of exposure.

Dimethyl-Sulfoxide (DMSO)

Irritant.

Irritating to eyes, respiratory system, and skin. Combustible liquid. Readily absorbed through skin. Target organs are eyes, skin. In case of contact with eyes, rinse immediately with plenty of water and seek medical attention. Wear suitable protective clothing. Do not breathe vapor. If swallowed, wash out mouth with water provided the person is conscious. Call a physician. If inhaled, remove the person to fresh air. If not breathing, give artificial respiration. If breathing is difficult, give oxygen. In case of contact, immediately wash skin with soap and copious amounts of water. In case of contact, immediately flush eyes with copious amounts of water for at least 15 minutes.

Propidium Iodide

Toxic.

May cause heritable genetic damage. Irritating to eyes, respiratory system, and skin. In case of accident, or if you feel unwell, seek medical attention. (Show the label where possible). Wear suitable protective clothing, gloves, and eye/face protection. Do not breathe dust.

In case of contact with eyes, rinse immediately with plenty of water and seek medical attention. In case of contact, immediately wash skin with copious amounts of water for at least 15 minutes while removing contaminated clothing and shoes. If inhaled, remove to fresh air. If not breathing, give artificial respiration. If breathing is difficult, give oxygen. If swallowed, wash out mouth with water provided person is conscious. Call a physician. Wash contaminated clothing before reuse.

Storage

Reaction Buffer should be refrigerated, DMSO can be stored at room temperature, and other components of the kit should be stored at -20°C. After reconstitution, the reagents from vial # 1-3 are stable up to six months when stored at 4 °C, and up to one year when stored at -20 °C in aliquots. Propidium iodide stock solution should be stored at 4 °C, and is stable for one year. Aliquotation and desiccation are recommended. Protect the key kit components (vials 1-4) from light.

Preparation of Reagents

1. Reaction buffer (1x)- Dilute 66 mL of Reaction Buffer (10x) tenfold: add 500 mL distilled water, add the supplied amount of $NaHCO_3$ in Vial 7, adjust pH to 7.4 slowly with 0.1 M NaOH or HCl, adjust volume to 660 mL with distilled water.
2. Calcein AM stock solution- Add 550 µL DMSO to Vial 1, dissolve vial contents by thorough pipetting (8-10 times) and/or vortexing.
3. Inhibitor 1 stock solution- Reconstitute the contents of Vial 2 with 340µL buffer. Dissolve by vortexing.
4. Inhibitor 2 stock solution- Add 370 µL distilled water to Vial 3 and dissolve the powder by vortexing.
5. Propidium iodide stock solution- Dissolve propidium iodide powder (Vial 4) in 1 mL distilled water by vortexing.

Assay Instructions

The assay consists of four well-separated stages: Preparation of the samples, Reaction, Measurement, and Calculations. The entire assay on one or two samples can be completed within 2 hours. The volumes and number of tubes given below are calculated for one clinical specimen.

Preparation of Samples

The MultiDrugQuant™ Assay is a functional test that requires living cells ($2\text{-}5 \times 10^6$) in good condition, not depleted of intracellular energy stores.
ATP depletion tends to decrease the activity of the membrane transporters and may lead to inaccurate results. When the multidrug resistance is assayed in hematological malignancies, the following procedure is suggested for separation of the mononuclear cells from peripheral blood or bone marrow samples.

1. Use peripheral blood or bone marrow samples anticoagulated with EDTA. Since other anticoagulants like heparin may interfere with the activity of MDR proteins, the use of anticoagulants other than EDTA is not recommended. For higher clinical relevance, the MDR phenotype should preferably be investigated in bone marrow samples.
2. Samples should be processed within 6 hours after drawing. Samples stored beyond 6 hours may undergo serious ATP depletion, leading to inaccurate results.
3. Take 32 mL of refrigerated reaction buffer (1x), check pH, and readjust it to 7.4 with 0.1 M NaOH or HCl if necessary.

4. Put 3 mL of Ficoll-Histopaque solution into a 15 mL capped centrifuge tube (or a Becton Dickinson Leukoprep tube). Equilibrate to room temperature.
5. Dilute 3 mL of bone marrow or blood with an equal volume of reaction buffer (1x) at room temperature.
6. Transfer the sample carefully as an overlay to Ficoll-Histopaque cushion. Avoid mixing through careful overlaying.
7. Centrifuge the sample at 400xg for 30 min at room temperature.
8. Remove the mononuclear cell layer by careful pipetting, and transfer the cells into a new tube.
9. Dilute the interface with 5 mL of reaction buffer (1x) and mix well. Do not vortex, avoid bubbling.
10. Centrifuge at 300xg for 10 min.
11. Discard the supernatant. Add 5 mL of reaction buffer (1x) and mix well. Do not vortex and avoid bubbling.
12. Centrifuge at 300xfor 10 min.
13. Discard the supernatant. Re-suspend the cells in 1 mL reaction buffer (1x).
14. Measure the cell count by using a hemocytometer or hematology analyzer.
15. Prepare a cell suspension containing 2-5 $\times 10^6$ cells in 8 mL of reaction buffer (1x).

Reaction

Assaying one clinical sample involves three types of tubes measured in triplicates:

Table 1: One Clinical Sample Set-up (in triplicate):

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Inhibitor 1	+	+	+						
Inhibitor 2				+	+	+			
Basal							+	+	+

16. Dilute 25 µL of calcein AM stock solution with 2000 µL of reaction buffer (1x). Vortex the solution for 1 min.
17. Add 50 µL of PI stock solution to 5 mL of reaction buffer (1x). Vortex it.

18. Prepare 9 samples in numbered tubes each containing 800 µL of cell suspension. Do not vortex the cell suspension and avoid bubbling.

19. Add 5 µL Inhibitor 1 into tubes #1-3. Add 5 µL Inhibitor 2 into tubes # 4-6. Mix thoroughly with gentle pipetting. Avoid bubbling. Incubate all nine tubes for 5 min at 37 °C.

20. To begin the reaction, add 200 µL of calcein AM solution prepared as described above (under point 16) into all nine tubes. Mix by gentle pipetting and start stopwatch. Incubate the tubes at 37 °C for exactly 10 minutes. **Since timing is crucial for accurate measurements, the addition of calcein AM solution should take place within 20 seconds**. Do not attempt to start reaction with more than 2 clinical samples (18 tubes) at once. Otherwise, it is hard to comply with the exact incubation time. If you want to measure more clinical samples, apply at least a 2 min time shift before initiating the reaction.

21. After exactly 10 min of incubation, stop the reaction by rapid centrifugation (1 min). Setting of the microcentrifuge to relatively low speed (~2,000x), rapid acceleration and deceleration (15 sec!) are recommended. Discard the supernatant and re-suspend the cells in 0.5 mL buffer containing PI.

22. The *Measurement of Samples* should preferably be performed immediately after the *Reaction*. However, the processed samples can be stored at 4 °C for a maximum of 24 hours without interfering with the results.

Measurement of Samples

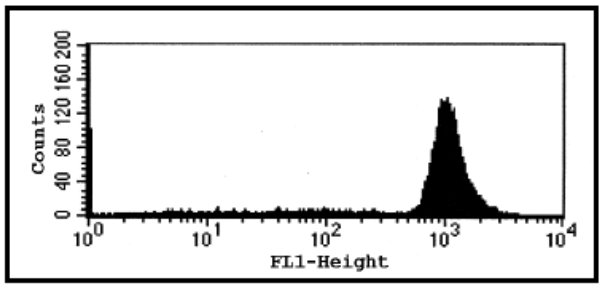
The cellular green fluorescence (FL1) of calcein should be measured in the living (i.e. PI negative) cell population of all 9 tubes in a flow cytometer with the same equipment settings. We recommend that you store PMT settings and the previously established window of analysis in template files (e.g. "MultiDrugQuant™-set" and "MultiDrugQuant™-image"). If you already have an established setting for the PMT amplifications and an analysis template file containing all the windows, regions and gating sequence, load these files, and skip points 23-31. Nevertheless, you may need to readjust slightly the PMT amplifications and/or the location of the regions after a test run of tube # 1 (or 2-3).

The MultiDrugQuant™ Assay includes an internal standardization, thus, MAF values are independent from the PMT settings, whenever the acquisition occurs within the linear range of the equipment. However, we recommend the use of the same or at least similar settings for the PMT amplifications, whenever possible.

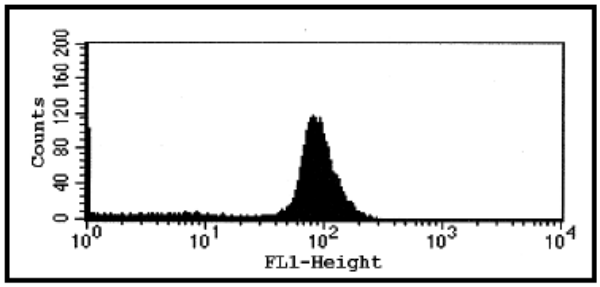
Setting of the PMT amplifications

23. Set an FL1 histogram window, an FSC-SSC dot plot, an FSC-FL3 dot plot, and a second FL1 histogram window. For better separation of the different cell populations, using log scale for the fluorescence channels (FL1 and FL3) is recommended.

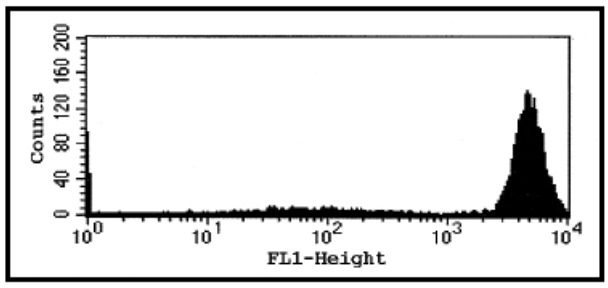
Figure 2. Setting of PMT amplification for FL1 channel.



A. Optimal adjustment.



B. Low PMT amplification.

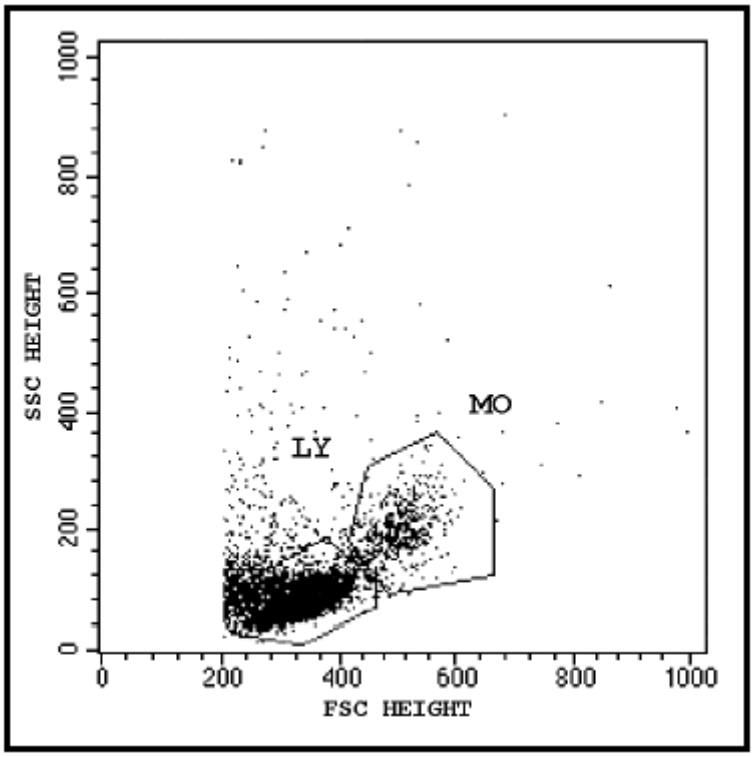


C. High PMT amplification.

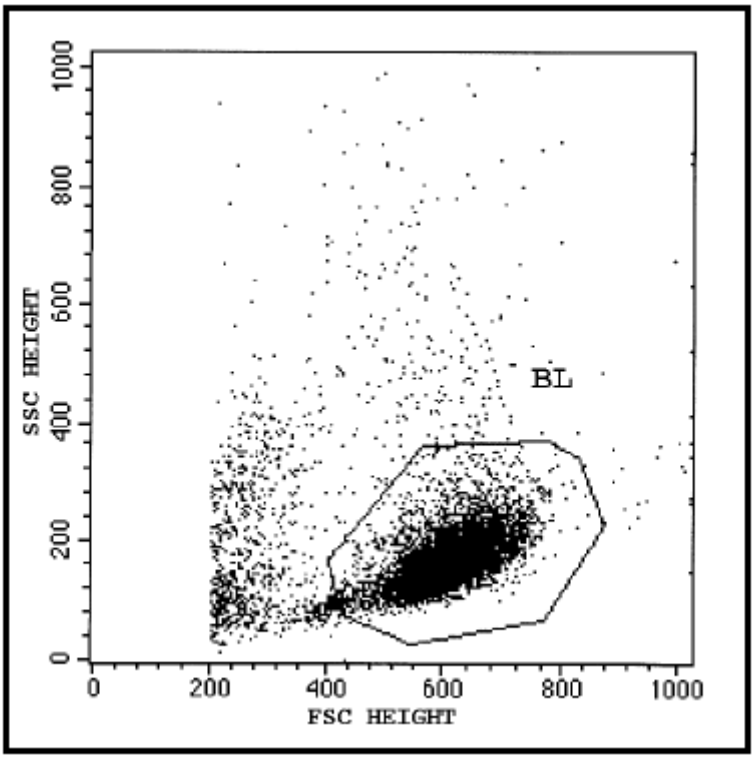
24. Run tube # 1 (or 2-3), and adjust the PMT amplification for FL1 so that the peak of the histogram is located around the border of the 3rd and 4th decade on the FL1 histogram channel *(Figure 2, Panel* A).

25. Adjust both forward and side scatter PMT amplifications so that all cell subsets are displayed on the FSC-SSC dot plot *(Figure* 3).

Figure 3. Adjustment of forward and side scatter PMT amplification.



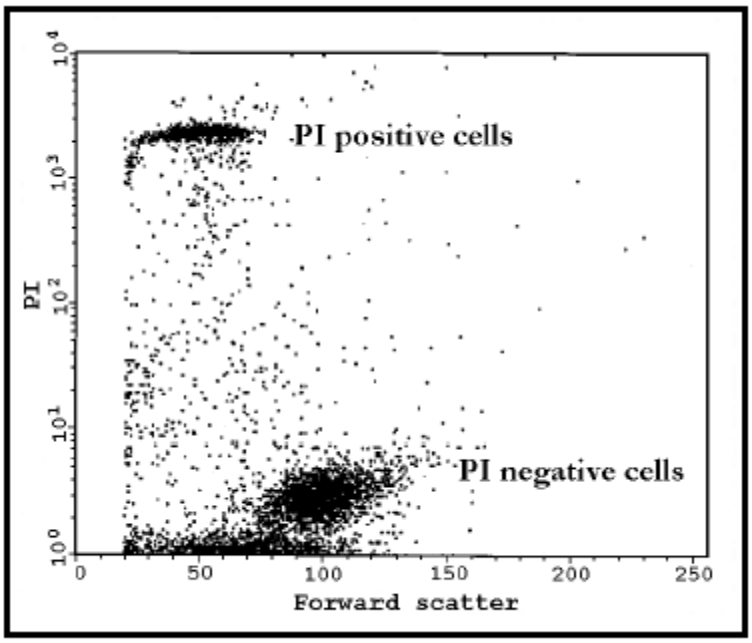
A. Mononuclear cells from Ficoll-separated peripheral blood sample obtained from a healthy subject (LY: lymphocytes; MO: monocytes).



B. Ficoll-separated peripheral blood sample from a patient with acute myeloid leukemia (BL: blast cells).

26. Place a region (R1) excluding FL1 dim events in the FL1-FSC dot plot. (Figure 5A).

Figure 4. Setting PMT amplification for FL3 channel*.



***Note:** No gating was applied for this plot, to demonstrate PI positive events.*

27. Set a second region (R2) on the FSC-SSC dot plot, selecting the cell population of interest, but excluding cell debris *(Figure 5*B). Note, that blast cells of hematological malignancies are usually larger than normal cells. Ficoll separation usually results in a single population.

28. The third region (R3) should be set on the FSC-FL3 so that FL3 (PI) positive cells are excluded from analysis *(Figure 5*C). Since calcein is a bright fluorophore with a very broad spectrum, its fluorescence may spill over the FL3 channel. To avoid errors originated from this, set the higher border of R3 as high as possible.

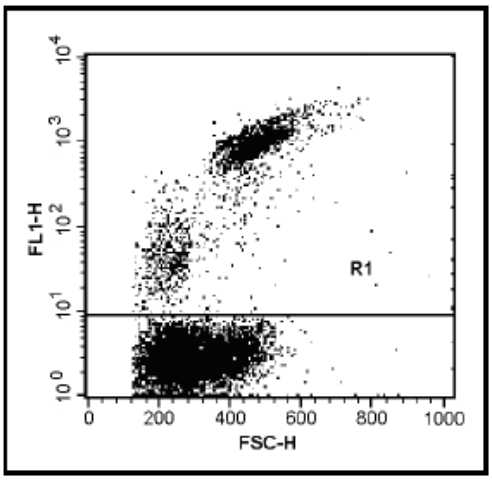
29. Apply sequential gating strategy for the analysis. Validate R1 for the FSC-SSC dot plot, R1&R2 for the FSC-FL3 dot plot, R1&R2&R3 for the second FL1 histogram window *(Figure 5*D).

30. Save settings in a template file (e.g. "MultiDrugQuant™-set").

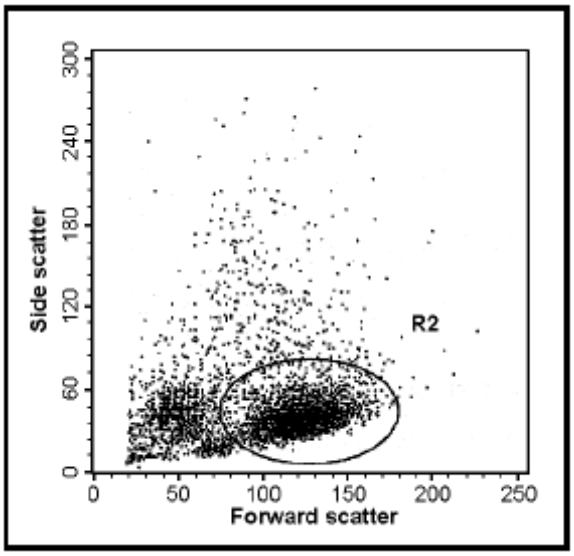
31. Run all 9 samples counting 10,000 events on the second FL1 histogram, applying the gating described above (R1&R2&R3). Save raw data for each tube.

32. Determine the mean fluorescence intensity (MFI) values on the second FL1 histogram window, applying the R1&R2&R3 gating. Do not use channel numbers. If your equipment cannot provide the absolute fluorescence values, follow the instructions described in the "Technical Hints" section

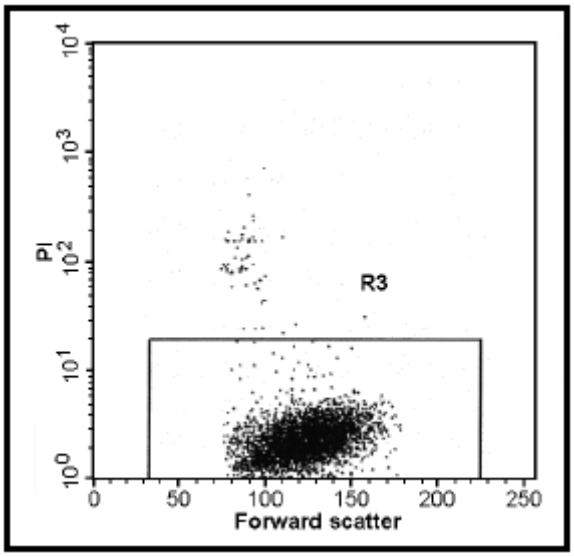
Figure 5. Sequential gating strategy demonstrated on a peripheral blood sample from a patient with acute myeloid leukemia.



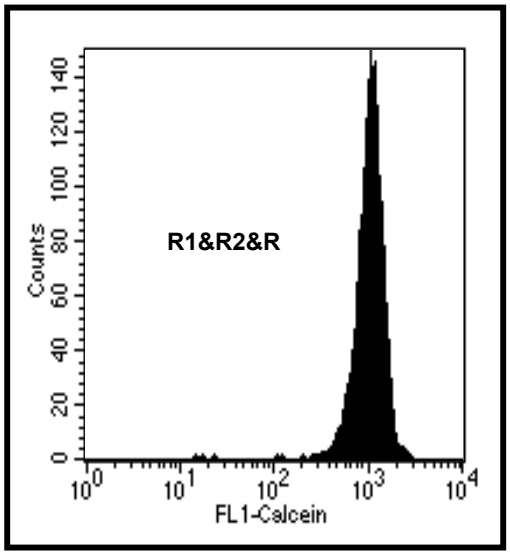
A. FL1 dim events are excluded by region (R!) in the FL1 histogram window.



B. The cell population of interest (e.g. blast cells) is selected by a second region (R2) on the FSC-SSC dot plot.



C. A third region (R3) is set on the FSC-FL3 dot plot to select the PI negative cells.



D. A histogram window contains only the living cells of interest when R1, R2, and R3 are validated.

Calculation of Results

1. Calculate mean of MFIs for parallel measurements:

   F_{max} from tubes # 1-3

   F_{MRP} from tubes # 4-6

   F_o from tubes # 7-9

2. If differences between parallels 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 with >10 %, redo the analysis.

3. Calculate the total multidrug resistance activity factor (MAF_T) according to the following equation:

   $$\mathbf{MAF_T = 100 \times (F_{max} - F_o)/F_{max}}$$

   In extreme cases (no MDR1 or MRP1 activity at all), F_{max} can be slightly smaller than F_o. Regard MAF_T as 0 in this case.

4. The MRP1-related multidrug resistance activity factor (MAF_{MRP}) should be calculated by the following equation:

   $$\mathbf{MAF_{MRP} = 100 \times (F_{MRP} - F_o)/F_{max}}$$

   In extreme cases (no MRP1 activity at all), F_{MRP} can be slightly smaller than F_o. Regard MAF_{MRP} as 0 then.

5. Calculate the MDR1-related multidrug resistance activity factor

   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 at all), MAF_T can be slightly smaller than MAF_{MRP}. Regard MAF_{MDR} as 0.

The theoretical value of the multidrug resistance activity factors 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 chemotherapeutical treatment suggested that the specimen with a MAF_T value <20 can be regarded as MDR negative, while MAF_T value >25 is indicative for positive MDR.

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

Additional Usage Information

The assay procedure involves an internal standardization. However, the quality of reagents, equipment, and settings can be tested as follows.

Prepare cell suspension of normal peripheral blood mononuclear cells as described under *Section 7*A. Perform the assay procedure only with 3 tubes, applying no inhibitor (as in the case of tubes #7-9 in the normal procedure). Readjust regions (especially on the FSC-SSC dot plot) to select the cell population of interest. Normal peripheral leukocytes are usually smaller than blast cells. Determine the mean fluorescence intensity (MFI) in FL1 channel, applying the gating described above. The values should fall in the 3rd or 4th decade on the FL1 histogram channel (see *Figure 2A*). Test your assay performance on a regular basis, as determined by the laboratory-specific quality control guidelines.

Technical Hints

Working with living cells

Flow cytometry laboratories performing leukemia analysis are mostly involved in structural studies, i.e. 2-3-4-color immunophenotyping of surface and intracellular antigens. Unlike these investigations, the MultiDrugQuant™ Assay is a functional test that requires living cells in good condition. Thus, some practical hints are provided here to facilitate your work.

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 suspension, mix them with gentle pipetting. Avoid bubbling.

Cells in suspension sediment very rapidly. Prior to any action with them (e.g. counting, distributing to microtubes for the reaction, or running the samples on the flow cytometer), mix the cell suspension by gentle pipetting (for 10-15 times).

Fibrinous sample

If bone marrow sample contains fibrin or other solid elements, it is recommended that it is filtered through a nylon mesh before sample processing. The application of such samples without filtering may result in inappropriate separation of mononuclear cells on the Ficoll cushion.

Non-fresh sample

The functional assay requires tumor cells with proper viability and not seriously depleted from internal energy stores. If a sample is stored beyond 6 hours, incorrect MAF values may be obtained due to ATP depletion that may influence the activity of multidrug transporters without affecting the ratio of PI positive and negative cells.

Insufficient number of cells

Starting from 3 mL of bone marrow or peripheral blood sample usually provides enough cells to prepare 8 mL of suspension containing 2-5x10^6 cells as given in the protocol above. However, if cell count is lower, it is still possible to perform the measurement by lowering the counts that determine the end of acquisition (i.e. 10 000 events in the gated FL1 histogram). This is not recommended, since signal/noise ratio is decreased, risking the proper evaluation of the assay. Low cell counts may result from inappropriate separation of mononuclear cells on the Ficoll cushion or insufficient re-suspension during washing or decanting a portion of the cells with supernatant.

Too many samples

As most of the available microfuges can accommodate 20-24 microtubes, simultaneous measurement of more than 2 samples (i.e. 2x9 tubes) is not recommended. If analysis of more samples is required, incubate them separately in groups of two. Attempting to pipette too many samples may become a limiting factor and the incubation periods of 5 and 10 minutes cannot be kept.

Functional assay

It is important to keep in mind that unlike in conventional immunophenotyping, this test investigates functional activity of living unfixed cells. This implies that acquisition should be performed preferably the same day. However, calcein loaded samples can be stored for up to 24 hours at 4 °C without significant change in MAF values. Do not store samples at room temperature for prolonged periods.

Too high/low flow rate

The cell number in the MultiDrugQuant™ Assay is also optimized for measurement with flow cytometer. If $2\text{-}5\times10^5$ /mL cell suspension is prepared as recommended in the "Assay Protocol" section, it will normally result in 100-300 events/sec flow rate suitable for analysis. Keeping the flow rate below 600 events/sec is recommended.

Difference in MFI values

Calcein accumulation may be influenced by some factors (e.g. cell size, difference in intrinsic esterase activity of the cells, 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 by using inhibitors and calculations of MAF values eliminates these differences.

Which cells to gate on

In some samples - particularly with high cell counts – a single population appears on the FSC-SSC dot plot that makes gating easy. In samples displaying multiple subsets, gating on larger (blast) cells is recommended. Alternatively, selective marker-positive populations (e.g. CD34+, or CD45dim cells) obtained during immunophenotyping may be back gated to the FSC-SSC dot plot and selected for MDR analysis.

What is pathological?

In normal peripheral blood samples, MAF_T values of mononuclear cells are in the range of 0-20. Values above 25 seem to be associated with clinically appreciable level of multidrug resistance in acute myeloid leukemia. However, it is important to emphasize that further studies are required to establish exact cutoff values in other malignancies.

Suspiciously low values

Theoretically it cannot be excluded that a zero MAF value is obtained. During analysis of 40 normal peripheral blood and bone marrow samples, a mean MAF value of 11 was obtained.

Suspiciously high values

It is important to note that the theoretically highest value of MAF (100) is never obtained, as there is always some background fluorescence of cells on the FL1 channel. Drug-selected cell lines (e.g. KBV-1), expressing about $5x10^5$ copies of P-glycoprotein, exhibit a MAF_T value of 95-99. During analysis of more than 200 leukemia samples no MAF_T value above 70 was found.

No absolute fluorescence values are provided by the equipment

Some of the older flow cytometers cannot provide absolute fluorescence values. The fluorescence intensities are expressed in channel numbers in that case. Since this scale is logarithmic, you should take any exponent of channel numbers (e.g. $10^{ch.num}$ or $e^{ch.num}$) to replace the MFI values for calculation of F_{max}, F_{MRP}, and F_o. The same MAF values will be obtained as by using MFIs.

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