FlowCellect™ Human T Cell MitoDamage Kit

100 Tests

Cat. No. FCCH100139

FOR RESEARCH USE ONLY
Not for use in diagnostic procedures.

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Application

Apoptosis plays a critical role in immune cell development, regulation and functioning of the immune system and is also a contributor to the pathology of many human diseases and disorders of immune function (1-3). Mitochondria and mitochondrial function are highly sensitive indicators of cell health and stress because of their critical role in maintaining crucial cellular energy balance, regulation of cell death processes such as apoptosis and as a primary site for production of free radicals and oxidative stress (4). Mitochondrial membrane potential (MMP) changes have been established to be universal and important in intrinsic pathways of immune system cell death as well as extrinsic pathways such as FAS mediated pathway of death. In addition, mitochondrial potential changes have been found to be important in granzyme B mediated cytotoxic killing pathways and caspase independent cell death in immune cell subpopulations (5-8).

Cellular energy produced during mitochondrial respiration is stored as an electrochemical gradient across the mitochondrial membrane and this accumulation of energy in healthy cells creates a mitochondrial trans-membrane potential, ($\Delta\Psi m$) that enables the cell to drive the synthesis of ATP. Loss of the mitochondrial inner transmembrane potential is often but not always, observed to be associated with the early stages of apoptosis (9-11). In recent years, the study of mitochondrial potential changes in immune cell populations during drug development and disease has become increasingly important due to increased number of drugs that cause mitochondrial stress and toxicity and the emerging importance of immune cell mitochondrial dysfunction in multiple diseases such as HIV, systemic lupus, surgical stress, autism, inflammation and sepsis, Alzheimer's and aging (2-8).

Multiparametric Flow cytometric analysis is an attractive method to obtain identification of T cell subpopulations along with cell health markers. The FlowCellect T cell MitoDamage Kit allows for the detection of T-lymphocytes and T-lymphocyte subsets in peripheral blood mononuclear cell samples and simultaneously provides information on the mitochondrial membrane potential status in a simple easy to use assay utilizing flow cytometry. The kit thus allows the determination of % o CD4 and CD8 T cells and the subset of these cells which have impaired mitochondrial membrane potential. When performed on the guava easyCyte 8HT system, it allows the determination of the count of population without using external bead sets. Multiparametric evaluation of T Cell subpopulations along with mitochondrial membrane potential changes is of great utility in studying impact of compounds on immune cell subpopulations in drug screening studies; it can provide a deeper understanding of the mechanistic machinery of immune cell apoptosis, mechanism of disease and immune dysfunction. Additionally such an assay can also be of great value in quality control of immune cell sub-populations

Test Principle

Millipore's FlowCellect™ T Human T cell MitoDamage Kit includes (1) Antibody Cocktail containing CD3-PECy5, CD4-PE and CD8-FITC antibodies (2) MitoSense Red (1,1',3,3,3',3' - Hexamethylindodicarbocyanine iodide), a fluorescent cationic dye that accumulates in the mitochondria and is responsive to mitochondrial potential changes and (3) 1X Assay buffer BA solution to perform the assays.

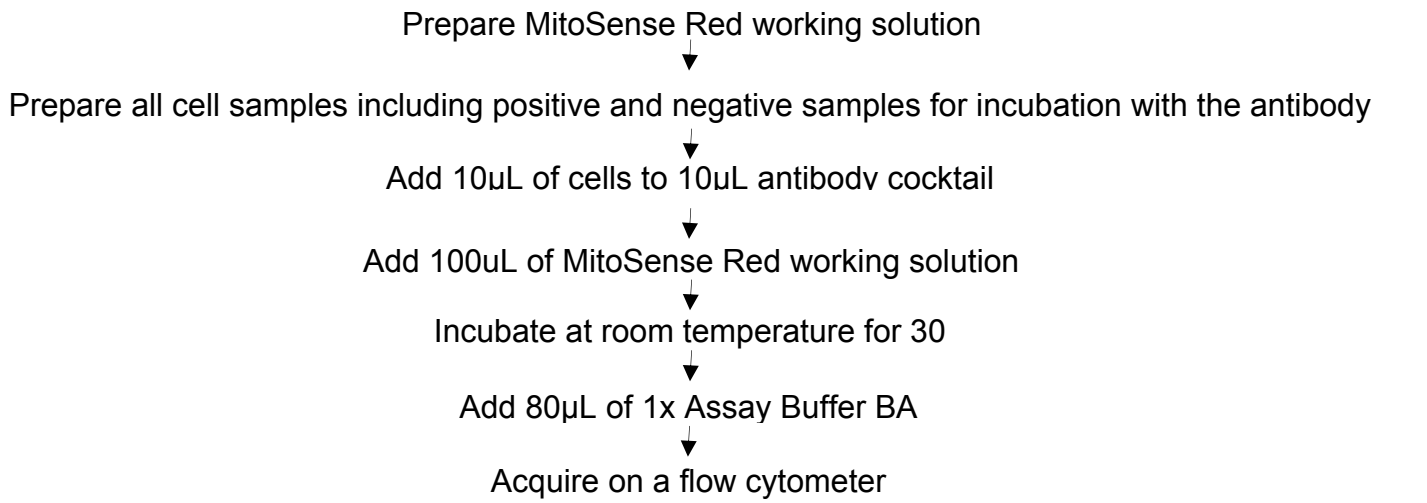
The CD3 antibody Anti-CD3, UCHT1 clone, reacts with the ε-chain of the CD3 part of the TCR/CD3 complex. CD3 is a pan-T marker expressed by normal and neoplastic T cells and uniquely allows the identification of all T cell lymphocytes. The Anti-Human CD4 Antibody, MT310 allows the identification of human helper/inducer CD4+ T cell (HLA Class II reactive) and recognizes a 55 kDa glycoprotein on the surface of CD4 T helper cells. Monocytes also express CD4 but at lower density, and have no co-expression of CD3 and hence can be distinguished away from CD4 T Cells when using this kit. The Anti-human CD8 antibody (Clone DK-25) allows the identification of CD8, a 68 kDa, disulfide linked transmembrane glycoprotein expressed by class I major histocompatibility complex restricted, mature suppressor/cytotoxic T cells, the great majority of cortical thymocytes and approximately 30% of medullary thymocytes. In addition a proportion of γδ T cells and NK cells express CD8. Inclusion of the Anti-CD3 antibody allows for the unique identification of the CD8 cytotoxic T Cells when using the kit.

MitoSense Red, a fluorescent dye for probing mitochondrial membrane potential is excitable by a red laser and fluoresces maximally at 650 nm (Red2 fluorescence on the guava easyCyte 8HT). Uninduced cells with intact mitochondrial membrane potential demonstrate high Red2 fluorescence while cells which have impaired mitochondrial membrane potential depict lower Red2 fluorescence.

The kit can thus distinguish multiple populations (1) CD4 T Helper cells and % of these cells which show intact mitochondrial membrane potential (2) % of CD4 T Helper cells with dissipated mitochondrial membrane potential (3) CD8 cytotoxic T Cells and the % of these cells that show intact mitochondrial membrane potential change and (4) CD8 Cytotoxic T cells and % of these cells that demonstrate dissipated membrane potential. The kit thus provides a complete picture of T cell mitochondrial perturbation status and its response for inducer treatment conditions or diseases. The entire assay can be performed in 30 min a simple no wash manner without loss of apoptotic cells when using PBMC's.

Sufficient reagents are provided for 100 tests. The kit includes all optimized fluorescently labeled antibodies, dyes and buffers necessary for cell preparation and analysis.

Flow chart for performing the FlowCellect™ T Cell MitoDamage Kit



Kit Components

- CD8-FITC/CD4-PE/CD3-PECy5 (Part No.4700-1355) One vial containing 1000 uL of cocktail.
- 1X Assay Buffer BA: (Part No. 4700-1360) One vial containing 50 mL.
- MitoSense Red Dye (Part No. 4300-0315) One vial containing 200µL of MitoSense Red Dye.

Materials Not Supplied

1. easyCyte HT System (guava® easyCyte 8HT or easyCyte 6HT-2L) with guavaSoft™Software or equivalent flow cytometry system with ability to detect green, red1 and red2 fluorescence
2. ViaCount™ reagent (Catalog No. 4000-0041) or ViaCount Flex reagent (Catalog No. 4700-0060)
3. PBMC Samples
4. Media for cell line of interest
5. Tissue culture instruments and supplies (including 37°C incubator, growth media, plates, detachment buffer, etc.)
6. Polypropylene tubes and or bottles for sample and buffer preparation and storage.
7. 0.5-mL microcentrifuge tubes (VWR Cat. No. 16466-036 or equivalent) for sample acquisition
8. 1.5-mL microcentrifuge tubes (VWR Cat. No. 16466-030 or equivalent)
9. 96-well microplate plates, round bottom (Falcon Cat. Nos. 353910 or 353918) or flat bottom (Falcon Cat. No. 353075 or 353915), or equivalent. Refer to the appropriate Guava System user's guide for other compatible microplates.
10. Pipettors with corresponding tips capable of accurately measuring 1 – 1000 µL

11. Tabletop centrifuge capable of exceeding x300G.
12. Vortex mixer
13. Milli-Q™ Distilled Water or DI water.
14. Reagent reservoirs, optional
15. Guava® Instrument Cleaning Fluid (ICF) (Cat. No. 4200-0140), optional
16. guava easyCheck Kit (Cat. No. 4500-0025), optional
17. 20% bleach solution

Precautions

- Wear proper laboratory attire (lab coat, gloves, safety glasses) when handling or using this product.
- The instructions provided have been designed to optimize the kit's performance. Deviation from the kit's instructions may result in suboptimal performance and may produce inaccurate data.
- Some assay components included in the kit may be harmful. Please refer to the MSDS sheet for specific information on hazardous materials.
- All fluorochrome conjugated antibodies and dyes are light sensitive and must be stored in the dark at 2-8°C.
- During storage and shipment, small volumes of product will occasionally become entrapped in the seal of the product vial. For maximum recovery of product, centrifuge vial briefly prior to removing cap.
- Avoid microbial contamination of the solution, which may cause erroneous results.
- Do not use reagents beyond their expiration date.

Storage

Upon receipt, all antibodies, dyes and buffers should be stored at 2-8°C.

Caution: *Fluorochrome conjugated antibodies should always be stored at 2-8°C. Any deviation in temperature for long periods of time may compromise the performance of the antibodies.*

Caution: *MitoSense Red Dye is highly hygroscopic and needs to be stored desiccated.*

Preparation of Reagents

1. Preparation of MitoSense Red Working Solution: Prepare a working solution by diluting the MitoSense Red Dye 1:50 in 1X Assay Buffer BA. Each sample to be tested requires 100 µL of the MitoSense Red Working Solution. MitoSense Red Working Solution must be made fresh each day of use.

   Note: MitoSense Red Dye is in DMSO and therefore solid at 2-8ºC. Allow the reagent to completely thaw prior to making the working solution.

   a. Dilute the MitoSense Red Dye stock solution with 1X Assay Buffer BA as suggested in the following table:

   Note: Quantities below are for one or more extra tests to allow for sufficient volume for the desired number of tests. Prepare only the number of tests needed for day of testing. Use within 1 hr of preparation.

	1 Test	10 Tests	25 Tests	100 Tests
MitoSense Red Dye	2 uL	20 uL	50 uL	200 uL
1X Assay Buffer BA	98 uL	980 uL	2450 uL	9800 uL

   b. The MitoSense Red Working Solution must be used the same day it is prepared. Store Working Solution at room temperature, protected from light until ready for use.
   c. Store the remaining MitoSense Red Dye Stock desiccated at 2-8 ºC

Before You Begin

This protocol was developed to allow direct determination of the percent of T cells and the subset of apoptotic T cells which have impaired mitochondrial membrane potential in PBMC's in cultures. The kit should give good staining with cell concentrations in the range of 2×10^5 and 3×10^4 cells/well (or 2×10^7 to 3×10^6 cells/mL). Millipore recommends using the ViaCount™ reagent to obtain accurate cell counts. Care should be taken to keep cell concentrations as constant as possible in all samples of an experiment.

Cells should be acquired shortly after the sample preparation had been completed. While some donors have been shown to yield stable results for up to 3 hours, the stability of individual donors may vary. This time variability is a consequence of using live, unfixed cells. You should determine the stability of results for your own cells.

Time considerations: The process of staining cells with the FlowCellect™ T Cell MitoDamage Kit takes approximately 45 minutes. Acquiring data on your guava system usually takes approximately 1 hour but can vary depending on your cell concentration. However, preparing cells for testing may require periodic maintenance and cultivation several days in advance. Once you cultivate the proper number of cells for your experiment, it may take an additional 15 minutes to 48 hours of culture with various reagents to induce activation.

Example Cell Staining Protocol

1. Prepare MitoSense Red Working Solution as described under Preparation of Reagents.
2. Prepare Peripheral Blood Mononuclear cell (PBMC) samples including positive and negative controls to cause changes in mitochondrial potential of the cells
3. Centrifuge and resuspend cells at 5 x 10^6 cells/mL in 1x Assay Buffer BA.
4. Pipette 10 µL of CD8-FITC/CD4-PE/CD3-PECy5 Cocktail into each well or tube.
   CAUTION: Put the stock bottle of CD8-FITC/CD4-PE/CD3-PECy5 Cocktail back into the refrigerator or on ice immediately after use. Do not allow the bottle of Cocktail to remain at elevated temperatures for extended times.
5. Add 10 uL of PBMC to each well or tube.
6. Mix the samples thoroughly by pipetting up and down.
7. Add 100 uL of MitoSense Red Working Solution to each well
8. Mix the samples thoroughly by pipetting up and down.
9. Incubate the samples for 30 minutes at room temperature (18 to 25ºC) in the dark.
10. Pipette 80 µL of 1X Assay Buffer BA directly into the wells/tubes to bring total sample volume to 200 µL.
    NOTE: If using a flow cytometer other than the easyCyte HT System, add 280uL of 1X Assay Buffer BA to bring the final volume to 400 µL.
11. Immediately mix the sample thoroughly by pipetting up and down.
12. Samples are ready for acquisition and analysis on a flow cytometer.
    NOTE: Batch your preparations to avoid over-incubation of samples. Samples must be acquired within 3 hours after preparation.

Sample Data

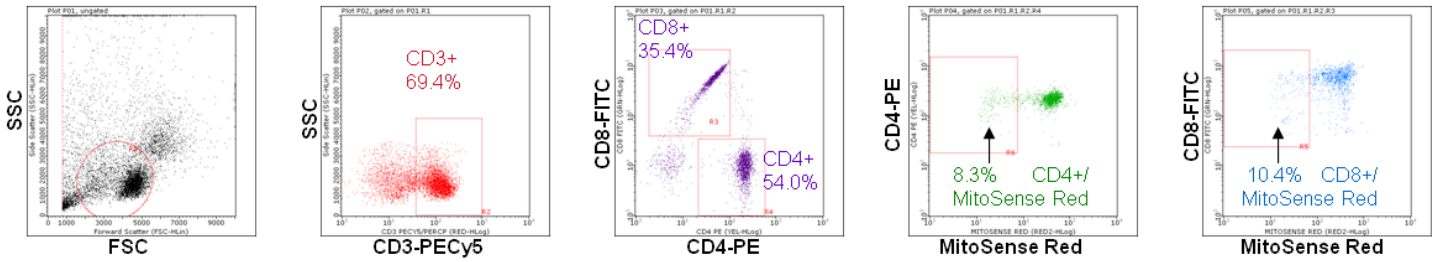


Figure 1 Display of Plots for Sample Acquisition: Set up of plots for data acquisition for samples treated with the T Cell MitoDamage Kit. Plot 1 provides the plot of FSC (Lin) vs. SSC which is typically used to gate the lymphocyte population. Plot 2 gates and counts the CD3 positive cells. Typically about 3000 CD3 + cells are acquired. Plot 3 separated the CD3 positive cells into CD4 T cell and CD8 T cell subcomponents. Plot 4 provides comparison of CD4 (red Channel) vs. MitoSense Red (red2 channel). Plot 5 provides the comparison of CD8 (green channel) and. MitoSense Red (red2 channel). A negative control sample should be used to set the MitoSense Red gates for both the CD4 and CD8 populations.

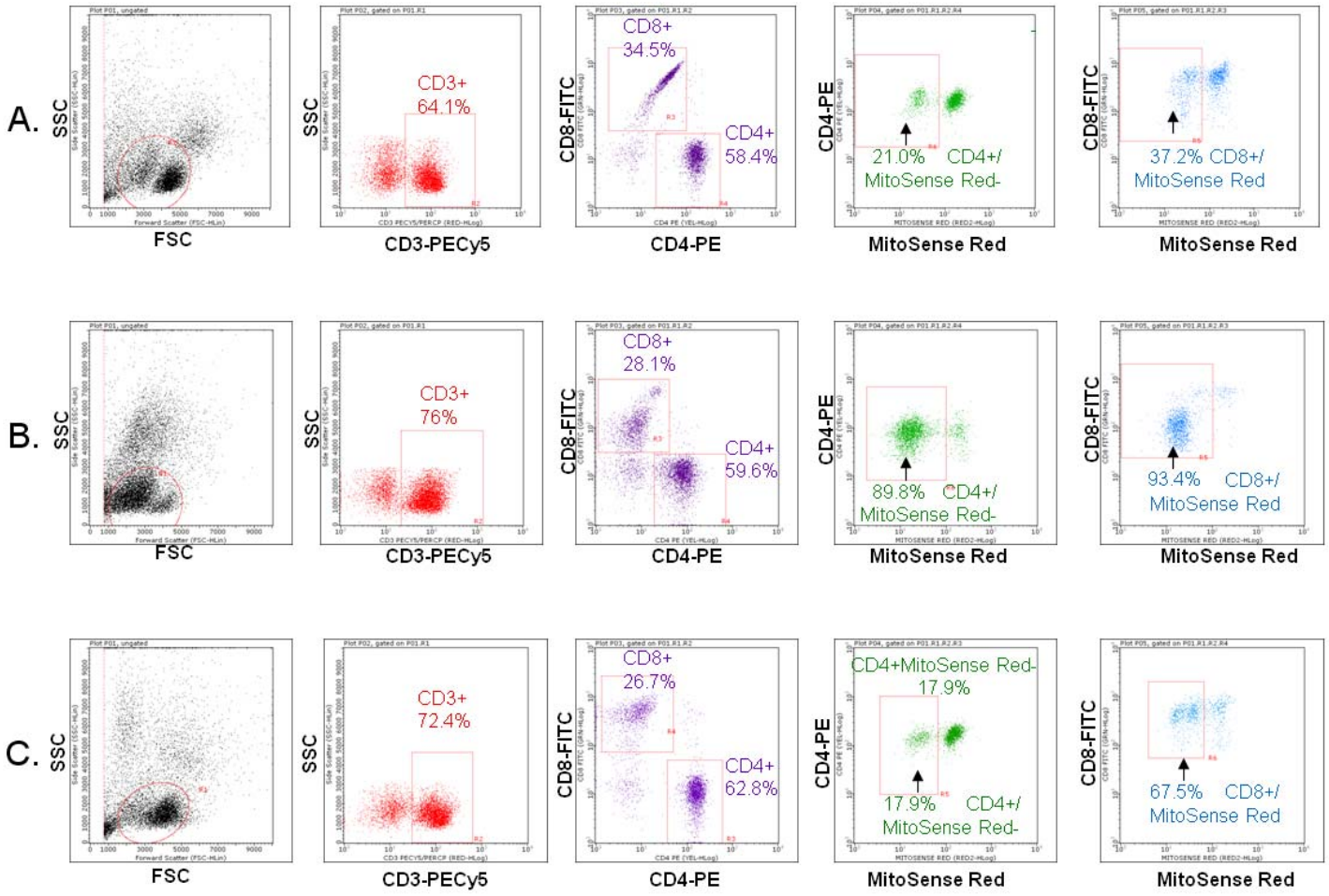


Figure 2 Analyzed Dual Parameter Data: Dot plots depicting PBMC treated overnight with A. 50uM *Diamide, B. 3.13uM Gambogic Acid, or C.0.5uM Staurosporine and* stained with the T Cell MitoDamage Kit. Plots show the percentage of positive cells for 1) CD3 T cells, 2) CD4 T cells and CD8 T cells 3) CD4 T cells and MitoSense Red +ve and –ve population, and 4) CD8 T cells and MitoSense Red +ve and –ve populations. Treatment with different inducers causes populations with diminished Red2 fluorescence to appear as shown in the CD4 vs. MitoSense Red (Red2) and CD8 vs. MitoSense Red plots. In all cases, the gating was set up on an unstimulated control sample and applied to the stimulated samples.

Technical Hints

- All kit reagents, CD8-FITC/CD4-PE/CD3-PECy5, MitoSense Red Dye, and 1X Assay Buffer BA, should be brought to room temperature prior to staining and washing.
- After removing the desired amount of MitoSense Red Dye, the reagent should be desiccated and placed back at 2-8ºC.
- For cellular staining and analysis to be most effective, make sure that test cells have good viability prior to use.
- The easyCyte HT System and FlowCellect™ Human T Cell MitoDamage Kit yield optimal results when the stained cell sample used for acquisition is between 2 x 10^7 to 3x 10^6 cells/mL cells/mL. To obtain the most accurate results, adjust the cell concentrations to within the recommended range.

Troubleshooting

Potential Problem	Experimental Suggestions
Acquisition rate decreases dramatically Instrument clogging Too many cells	• Cell concentration too high - Decrease the number of cells per microliter by diluting sample to 300 – 500 cells/uL. The Guava EasyCyte™ Plus or guava easyCyte HT systems gives the most accurate data when the flow rate is less 500 cells/uL. • Run a Clean and Rinse to clean out capillary. This procedure can be performed during or after an assay. This will wash away any material forming within the glass capillary walls.
Too few cells	• Ensure that cells are counted properly prior to beginning the experiment. The assay instructions are optimized to give you a range of cells between 100-500 cells/μL in the final sample volume so accurate population count results are obtained. A substantial decrease in cell numbers can lead to difficulty in adjusting settings.
Background staining and/or non-specific staining of cells	• Although the assay procedure has been optimized to function utilizing PBMC's, further antibody titrations may be necessary for some donors capture the ideal staining concentration. Non-specific staining and background may indicate that less antibody will need to be used during the staining procedure. • Although the assay procedure has been optimized so that compensation is not needed, some samples may have improved staining patterns if compensation is applied. The compensation can be performed after acquisition if needed.
Low level of staining of CD markers	• Although the assay procedure has been optimized to function utilizing PBMC's, every donor may respond differently. A lack of signal may indicate that excess antibody will need to be used during the staining procedure or that the staining time needs to be increase.
No downward shift in mitochondrial membrane potential.	• Cells may not have undergone a change in membrane potential. Positive controls should be included for each experiment to ensure accurate staining protocol. Treatments to induce a change in membrane potential in various cell lines include CCCP, valinomycin, and staurosporine.
Dim or Low level of staining of MitoSense Red	• Possible reagent degradation. Verify that the reagent has been stored desiccated and is not past its expiry date. • Live/uninduced control samples are recommended for each experiment. • Dim staining may be a sign that the cell concentration was too high and the concentration of reagents was insufficient to stain the cells. Repeat experiment using lower number of cells per well.
Poor resolution of stained populations.	• Poor resolution could indicate that the staining time was too short. Make sure that the cells were stained for 30 minutes at room temperature. • Increase the staining time to 45 minutes. • Wash the samples and resuspend in fresh Assay Buffer BA to increase separation.

Variability in day to day experiments	• If the FlowCellect Cell Activation Kit results are inconsistent, check that the samples were well mixed prior to acquisition. If using an easyCyte 8HT System, be sure that the mixing option has been selected in the Worklist file used to collect data. Cells may quickly settle in your samples and your results will be inaccurate unless the cells are mixed just prior to acquisition. • Monitor experimental cell cultures to ensure that cell viability and cell numbers being analyzed are consistent. Any drop in cell numbers or viability can influence experimental results. • If there appears to be day-to-day variation of the staining pattern, ensure the easyCyte HT System is working properly. Run the easyCheck Procedure using the easyCheck Kit (Part No 4500-0025) to verify proper instrument function and accuracy.

***For further support, please contact Millipore's Technical services at 1-800-645-5476*

References

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Related Kits

1. FlowCellect™ Human T Cell Apoptosis Kit (Catalog No. FCCH100138)
2. FlowCellect™ Human T Cell Activation Kit (Catalog No. FCCH100141)
3. FlowCellect™ Human CD8 T Cell Fas Kit (Catalog No. FCCH100140)
4. FlowCellect™ Human CD4 T Cell Fas Kit (Catalog No. FCCH100154)
5. FlowCellect™ Human B Cell Fas Kit (Catalog No. FCCH100137)
6. FlowCellect™ MitoPotential Red Kit (Catalog No. FCCH100105)
7. FlowCellect™ MitoDamage Kit (Catalog No. FCCH100106)
8. FlowCellect™ MitoLive Kit (Catalog No. FCCH100107)
9. FlowCellect™ Annexin Red Kit (Catalog No. FCCH100108)
10. FlowCellect™ MitoStress Kit (Catalog No. FCCH100109)
11. FlowCellect™ Cytochrome *c* Kit (Catalog No. FCCH100110)
12. Guava® EasyCyte™ MitoPotential™ Kit (Catalog No. 4500-0250)
13. Guava Nexin® Reagent (Catalog No. 4500-0450, 4500-0455)

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