FlowCellect™ Human T Cell Apoptosis Kit

100 Tests

Cat. No. FCCH100138

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. Apoptosis provides the mechanism for deletion of autoreactive T cells in the thymus,* low responsive B cells in the germinal center,' and of target cells attacked by cytotoxic T lymphocytes and natural killer cells (1-3). The process of apoptosis is accompanied by multiple morphological, biochemical and physiological changes in response to specific induction signals. Among these are externalization of phosphatidylserine (PS) to the cell surface, cleavage and degradation of specific cellular proteins, compaction and fragmentation of nuclear chromatin, and loss of membrane integrity (in late stages) (4-5)

The translocation of phosphatidylserine on the external surface of apoptotic cells is considered a critical marker of apoptosis. Early in the apoptotic process, the membrane phospholipid phosphatidylserine is translocated from the inner to the outer layer of the plasma membrane, thus exposing phosphatidylserine to the external environment of the cells. Annexin V is a 35-36kD calcium-dependent, phospholipid-binding protein with high affinity and specificity for phosphatidylserine and when fluorescently labeled can be used as a sensitive probe for the detection of apoptosis (4-5). Phosphatidyl serine level expression on the external surface of T cell subsets have been shown to be altered in a number of diseases such as HIV, Parkinson's, pneumonia, asthma and conditions such as septic shock, radiation treatment etc (6-13)

The FlowCellect T cell Apoptosis Kit allows for the detection of T-lymphocytes and T-lymphocyte subsets in peripheral blood mononuclear cell samples and simultaneously provides information on the apoptotic status of these cells as measured by Annexin V binding in a simple easy to use assay utilizing flow cytometry. The kit thus allows the determination of % of T cells and the subset of apoptotic T cells which are apoptotic/necrotic. 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 apoptotic status is of great utility in studying impact of compounds on immune cell subpopulations in drug screening studies; in the study of immunomodulatory drugs, and in immunotoxicity studies. It can provide an understanding of the mechanistic machinery of both normal and abnormal immune cell apoptosis; in addition it can provide insights into mechanism of disease and immune dysfunction and can also be of great value in quality control of immune cell sub-populations.

Test Principle

Millipore's FlowCellect™ T Human T cell Apoptosis Kit provides a simplified and rapid method to assess apoptosis in sub-populations of T cells. The kit includes (1) Antibody Cocktail containing CD3-PECy5, CD4-PE and CD8-FITC antibodies (2) recombinant Annexin V conjugated to a red sensitive dye CF647 to provide maximum sensitivity (3) 1X Assay buffer BA solution and (4) 1X Assay Buffer HSC to perform the assays.

The CD8FITC/CD4PE/CD3PECy5 cocktail consists of three anti-human antibodies and includes anti-human CD3, CD4 and CD8 antibodies which allow for T cell detection and identification. The CD3 antibody Anti-CD3, UCHT1, 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.

Annexin V is a calcium-dependent phospholipid binding protein with high affinity for phosphatidyl serine expressed on the cell membrane. Annexin V, CF647 is excitable by a red laser, fluoresces maximally at 670 nm and can be detected in the Red2 channel of dual laser cytometers. Live cells demonstrate little or no Annexin V, CF 647 binding and demonstrate minimal Red2 fluorescence. In apoptotic cells, due to the translocation and exposure of phosphatidylserine on the external cell surface, use of Annexin V, CF647 allows the detection of apoptotic cells and these cells demonstrate increased Red2 fluorescence. Late apoptotic and necrotic cells also demonstrate increased Annexin V CF 647 binding and increased Red2 fluorescence.

The FlowCellect Human T Cell Apoptosis kit can thus distinguish multiple populations (1) CD4 T Helper cells (2) % of CD4 T Helper cells which are apoptotic (3) CD8 cytotoxic T Cells and (4) % of CD8 cells which are apoptotic. The kit thus provides a complete picture of T cell apoptotic 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 Apoptosis Kit

Prepare Annexin V working solution

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Prepare all cell samples including positive and negative controls for incubation with the antibody cocktail.

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Add 10µL of cells to 10µL of antibody cocktail

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Incubate at room temperature for 20 minutes

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Add 100µL of Annexin V CF647 working solution

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Incubate at room temperature for 20 minutes

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Add 80µL of 1x Assay Buffer HSC

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Acquire on a flow cytometer

Kit Components

- CD8-FITC/CD4-PE/CD3-PECy5 (Part No.4700-1355) One vial containing 1000 uL of cocktail.
- Annexin V, CF647 Reagent (Part No. 4300-0325) One vial containing 500 µL Annexin V, CF647
- 1X Assay Buffer BA: (Part No. 4700-1360) One bottle containing 50 mL of buffer.
- 10X Assay Buffer HSC (Part No. 4700-1325) One bottle containing 10mL of buffer

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 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.*

Preparation of Reagents

1. Preparation of 1X Assay Buffer HSC: The Assay buffer is supplied as a 10X concentrate, which must be diluted to 1X with deionized water prior to use. Approximately 1 mL of 1X Assay Buffer HSC is required per test.
    a. Allow 10X Assay Buffer HSC to come to room temperature.
    b. Mix 1 part of 10X Assay Buffer HSC with 9 parts of deionized water. Mix thoroughly.

c. **Note:** Prepared 1X Assay Buffer HSC is stable up to one month if stored at 2-8ºC,

2. Preparation of Annexin Red Working Solution: Prepare Annexin Red working solution by diluting the Annexin V, CF647 stock solution 1:20 in 1X Assay Buffer HSC. Each sample to be tested requires 100 µL of the Annexin Red Working Solution. Annexin Red Working Solution must be made fresh each day of use.
   a. Dilute the stock solution with 1X Assay Buffer as suggested in Table 1. Vortex immediately after mixing the reagents.
   Note: Quantities below are for one or more extra tests to allow for sufficient volume for the desired number of tests.

Table 1: Preparation of Annexin Red Working Solution

	1 Test	10 Tests	25 Tests	100 Tests
Annexin V.CF647 Stock Reagent	5 µL	50 µL	125 µL	500 µL
1X Assay Buffer HSC	95 µL	950 µL	2375 µL	9500 µL

   b. The Annexin Red Working Solution must be used the same day it is prepared. Store at room temperature, protected from light until ready for use.

Before You Begin

This protocol was developed to allow direct determination of the percent of T cells subsets that have undergone apoptosis. The kit should give good staining with cell concentrations in the range of 2 x 10^5 and 2.5 x 10^4 cells/well (or 2 x 10^7 to 2.5x 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 Apoptosis Kit takes approximately 45 minutes. Acquiring data on your guava system usually takes approximately 1-2 hours 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 2 to 48 hours of culture with various reagents to induce activation.

Example Cell Staining Protocol

1. Prepare 1X Assay Buffer HSC and Annexin Red Working Solution as described under Preparation of Reagents.
2. Prepare Peripheral Blood Mononuclear cell PBMC samples including positive and negative controls to cause apoptosis 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 10uL of PBMC to each well or tube.
6. Mix the samples thoroughly by pipetting up and down.
7. Incubate the samples for 20 minutes at room temperature (18 to 25ºC) in the dark.
8. Add 100uL of Annexin V, CF647 Working Solution to each well or tube.
9. Mix the samples thoroughly by pipetting up and down.
10. Incubate the samples for 20 minutes at room temperature (18 to 25ºC) in the dark.
11. Pipette 80 µL of 1X Assay Buffer HSC 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 HSC to bring the final volume to 400 µL.
12. Immediately mix the sample thoroughly by pipetting up and down.
13. 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

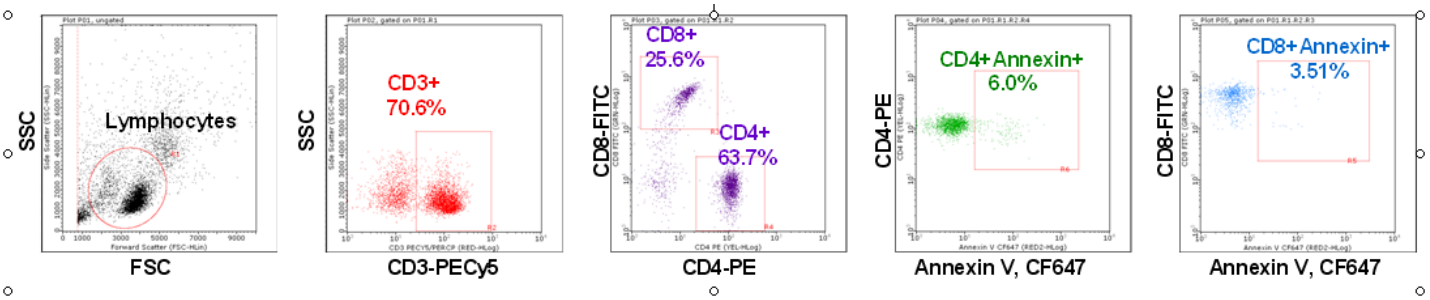

Figure 1 Display of Plots for Sample Acquisition: Set up of plots for data acquisition for samples treated with the T Cell Apoptosis Kit. Plot 1 provides the plot of FSC (Lin) vs. SSC (Lin) which is typically used to gate the lymphocyte population. Plot 2 gates and counts the CD3-PeCy5 (Red channel) positive cells. Typically 3000 CD3 + events are counted. Plot 3 separated the CD3 positive cells into CD4-PE (Yellow Channel) T cells and CD8-FITC (Green Channel) T cell subcomponents. Plot 4 provides comparison of CD4-PE (Yellow Channel) vs. Annexin V, CF647 (Red2 Channel). Plot 5 provides the comparison of CD8-FITC (Green Channel) and Annexin V, CF647 (Red2 Channel). A negative control sample should be used to set the Annexin gates for both the CD4 and CD8 populations.

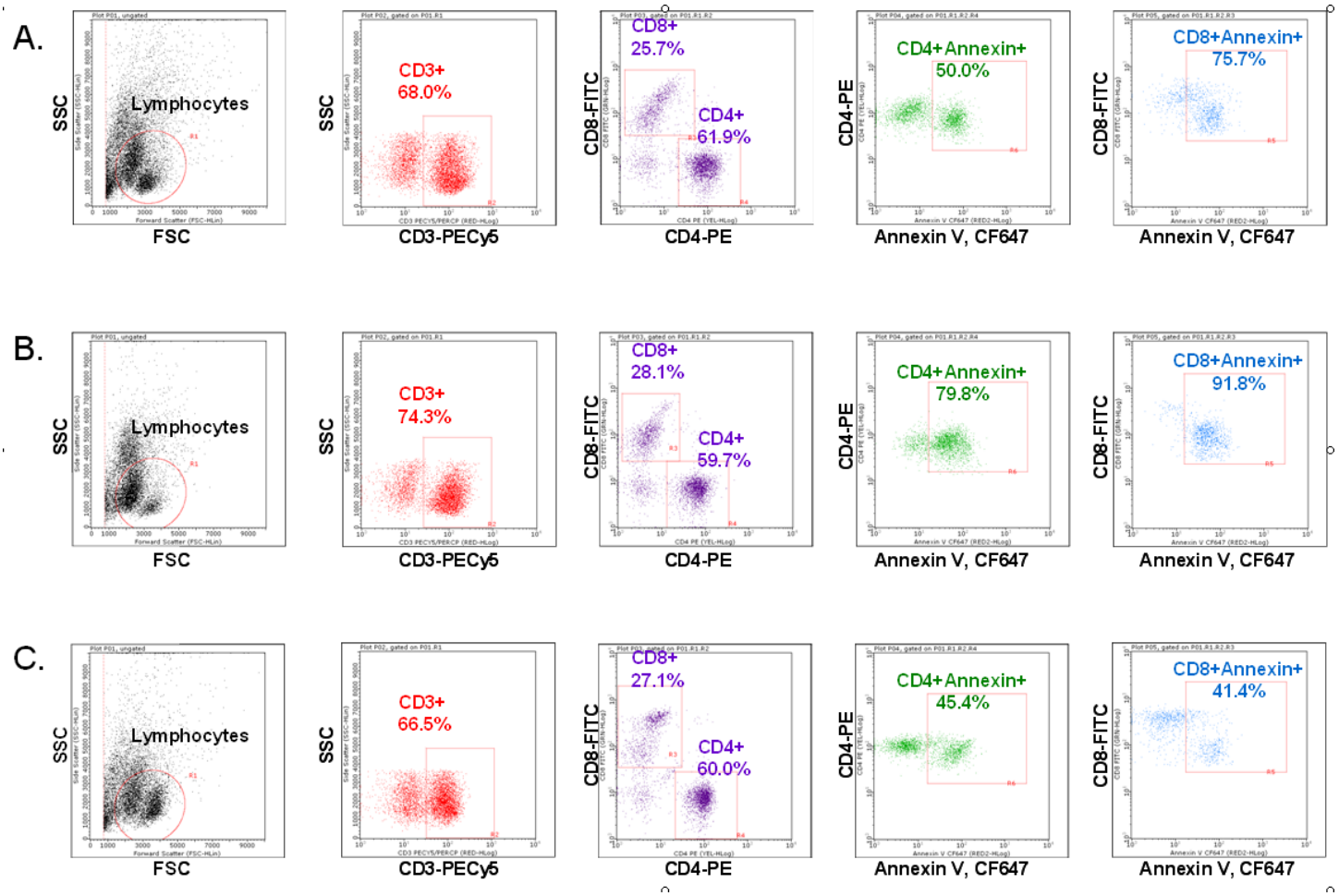

Figure 2 Analyzed Dual Parameter Data: Dot plots depicting PBMC treated overnight with A. 2uM Staurosporine, B. *3.13uM Gambogic Acid, or C.60uM Anisomycin and* stained with the T Cell Apoptosis Kit. Plots show the percentage of positive cells for 1) CD3 T cells, 2) CD4 T cells and CD8 T cells, 3) CD4 T cells and Annexin responsive cells, and 4) CD8 T cells and Annexin responsive cells. Treatment with different inducers causes populations with increased Red2 fluorescence to appear as shown in the CD4 vs. Annexin V CF647 (Red2) and CD8 vs. Annexin V CF647 plots. In all cases, the gating was set up on an uninduced control sample and applied to the induced samples.

Technical Hints

- All kit reagents, CD8-FITC/CD4-PE/CD3-PECy5, Annexin V, CF647, 1X Assay Buffer HSC and 1X Assay Buffer BA, should be brought to room temperature prior to staining and washing.
- 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™ T Cell Apoptosis 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.
Too high cell concentration during acquisition	• If the concentration of the stained cell sample for acquisition is high (>500cells/ μL), the accuracy of data will most likely be compromised. Dilute the sample further with 1X Assay Buffer HSC to have the cell concentration below 500cells/ μL. For best results, it is recommended that the cell concentration is in the range of 200 – 300 cells/ μL.
Background staining and/or non-specific staining of cells	• If cells have high background staining, the cells may be damaged as dead cells tend to aggregate and nonspecifically adsorb fluorescent reagent. Avoid damaging cells when handling them in culture. • 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 both Lysed Whole Blood and 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 or Low level of Annexin V positive staining	• Cells may not have induced or the Annexin V may have not been taken up correctly by the cells. To determine optimal apoptotic induction, conduct a time-course study in order to achieve the best results for Annexin V, CF647 staining. Positive control samples are recommended for each experiment. Positive controls should be appropriate for comparison with the test procedure or test cell population.
Poor resolution of stained populations.	• Poor resolution could indicate that the staining time was too short. Make sure that the cells were stained for the appropriate amount of time at room temperature. • Increase the staining time to 30 minutes for both the cocktail and annexin V incubations. • Wash the samples and resuspend in fresh Assay Buffer HSC to increase separation.
Variability in day to day experiments	• If the FlowCellect Cell Apoptosis 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 MitoDamage Kit (Catalog No. FCCH100139)
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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