# FlowCellect™ Human CD8 T Cell FAS Kit

100 Tests

## Cat. No. FCCH100140

**FOR RESEARCH USE ONLY**
**Not for use in diagnostic procedures.**

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## Application

The FlowCellect™ CD8 T Cell FAS kit allows for the detection and comparison of CD95/FAS antigen in CD8 T cell populations. FAS (Apo-1 or CD95) belongs to the subgroup of the tumor necrosis factor receptor (TNF-R) family that contains an intracellular death domain and triggers apoptosis. The interaction and ligation between CD95 and CD95L is recognized as a major pathway for the induction of apoptosis in cells and tissues (2-3). Fas / CD95 antigen is expressed on a substantial proportion of peripheral CD4+ cells, CD8+ cells and B cells but on a minor proportion of NK cells. It is also variably expressed on granulocytes and monocytes. Fas / CD95 has been seen to be strongly upregulated on activated T cells, B cells, NK cells and thymocytes (4 – 5). It is also widely expressed on cell lines of T, B, NK and myeloid lineage

FAS expression and apoptosis has been shown to play a significant role in T-cell selection in the thymus for the acquisition of self-tolerance (6), T-cell mediated cytotoxicity and the downregulation of the immune response. It has also a role in the clonal deletion of activated T-cells in order to maintain T cell homeostasis, down-regulation of inflammatory T cells and the regulation of activated B cells (7). The presence or absence of FAS and/or FasL on cells of the immune system and their interaction with FAS and FasL on cells of other tissues plays a major factor in immune system homeostasis. It has been proposed that activation induced cell death (AICD) serves a role in limiting the expansion of an immune response by eliminating lymphocytes that are no longer necessary at the end of an infection. Recent studies have also indicated that in addition to a critical role in receptor mediated apoptosis, FAS has a growth-promoting role during tumorigenesis. The study of FAS expression levels plays a critical role in immune system development and disease. Changes in FAS expression levels are important in the process of AICD. FAS expression levels in CD8 T cell subsets have been found to be altered in HIV disease progression, gastric cancer, breast cancer as well as rejection status in liver transplants (8-13). FAS levels have also been shown to be upregulated with the treatment of specific cytokines and studies performed on its correlation with apoptosis (14). FAS has also been shown to be upregulated in tumor cells The study of FAS expression levels in immune cell subpopulations thus provides important insights into the mechanism of death.

The FlowCellect CD8 T Cell FAS kit allows for the identification and counts of CD3 T Cells and CD8 cytotoxicity T cells and the level of FAS expression levels by allowing the detection and quantitation of CD95 (Apo1, FAS) marker on either whole blood or PBMC using simplified no-wash assays. The kit can be utilized on any flow cytometry system equipped with blue laser. The performance of these assays on the guava easyCyte 8HT platform along with the Incyte software allows for cell count information on CD8, CD3 T cells and count and % of populations expressing CD95/FAS. Furthermore, easy identification of activated T cells can be performed using heat map features or measurement of the kinetics of activation using the dose response features. The simplified identification of activated samples can be of great utility in drug development, kinetic studies and understanding mechanism of compound action and disease.

## Test Principle

Millipore's FlowCellect™ CD8 T Cell FAS Kit includes (1) Antibody Cocktail containing CD8-FITC and CD3-PECy5 antibodies, (2) Anti-human CD95 (FAS)-PE Antibody, (3) Isotype Control Mouse IgG1-PE, (4) 1X Lysing solution to lyse erythrocytes and (5) 1X Assay Buffer BA solution.

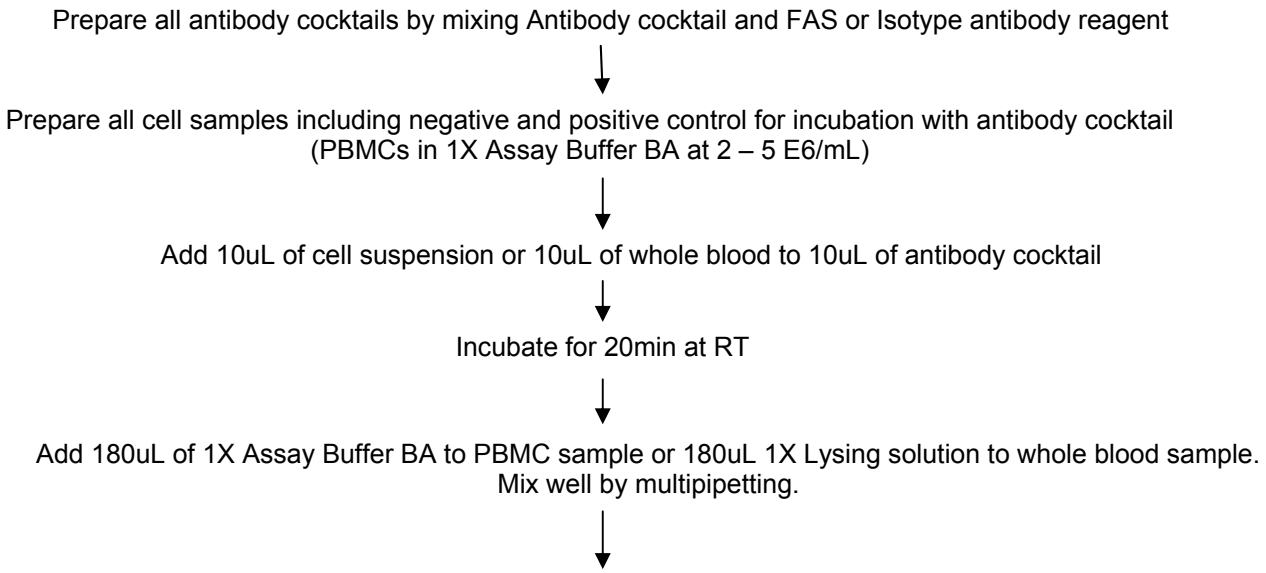
The CD8-FITC/CD3-PECy5 cocktail consists of two anti-human antibodies, CD3-PECy5 and CD8-FITC antibodies, which allow for cytotoxicity T cell detection and identification
The CD3 antibody (clone UCHT1) reacts with the ε-chain of the CD3 part of the TCR/CD3 complex. CD3 is a pan-T marker expressed by normal and neoplastic T cells and uniquely allows the identification of all T cell lymphocytes. The CD8 antibody (clone DK25) recognizes a 68 kDa disulphide-linked, transmembrane glycoprotein. Receptor CD8 is expressed by the great majority of cortical thymocytes and approximately 30% of medullary thymocytes, and by class I major histocompatibility complex restricted, mature suppressor/cytotoxic T cells. In addition, a proportion of T cells and NK cells express CD8. Thus, CD8 is expressed by about 25-35% of peripheral T cells, and about 30% of NK cells express low levels of CD8. The use of the CD3 antibody along with CD8 antibody allows the identification of CD8 cytotoxic T cells when using the kit.

The mouse anti-human CD95 antibody (clone DX2) recognizes CD95 which is a 45 kDa cell membrane receptor protein that is a member 6 of the tumour necrosis factor receptor superfamily and functions as a mediator of apoptosis (15). CD95 functions as a mediator of apoptosis and is expressed by a substantial minority of resting T and B cells, and by about 5% of resting NK cells. Among T cells, CD95 is preferentially expressed by CD45RA low CD45R0 high memory T cells. CD95 has been found to be strongly upregulated on activated B cells, T cells, NK cells and thymocytes.

The kit can thus distinguish multiple populations (1) CD3 T cells and their CD95 level, (2) CD8 cytotoxcicity T cells with no CD95 expression and (3) CD8 cytotoxcicity T cells with CD95 expression. The kit thus provides a complete picture of cytotoxicity T cell, their FAS expression level and its response for inducer treatment conditions or diseases. The entire assay can be performed in 30-45 minutes, simple no wash manner without loss of precious activated cells. The kit contains reagents to perform the assay on whole blood in a no wash procedure or on PBMCs. An isotype control conjugated to PE is also included in the kit to allow for clear identification of CD95 positive populations. The samples are thus recommended to be run with CD8-FITC/CD3-PECy5 antibodies with a) isotype control and b) with FAS/CD95-PE antibody to clearly identify the expression of FAS/CD95 on CD8 T-cells.

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

**Flow chart for performing the FlowCellect™CD8 T Cell FAS Kit**.




## Kit Components

- CD8-FITC/ CD3-PECY5 Cocktail (Part No.4700-1405) One vial containing 500 µL of Antibody Cocktail .
- Anti-Human CD95(FAS)-PE Antibody (Part No. 4700-1395) One vial containing 500 µL of labeled antibody .
- Isotype control mouse IgG1-PE (Part No. 4700-1390) One vial containing 500 µL of labeled antibody .
- 1X Assay Buffer BA ((Part No.4700-1360) One bottle containing 50 mL of 1x Assay Buffer
- Guava 1X Lysing Solution (Part No.4700-0082) One bottle containing 40 mL of 1X Lysing Solution

## 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 and red fluorescence
2. ViaCount™ reagent (Catalog No. 4000-0041) or ViaCount Flex reagent (Catalog No. 4700-0060)
3. Whole blood or PBMCs samples
4. Tissue culture instruments and supplies (including 37°C incubator, growth media, plates, detachment buffer, etc.)
5. Polypropylene tubes and or bottles for sample and buffer preparation and storage.
6. Pipettors with corresponding tips capable of accurately measuring 1 – 1000 μL
7. Tabletop centrifuge capable of exceeding x300G.
8. Vortex mixer
9. Reagent reservoirs, optional
10. Guava® Instrument Cleaning Fluid (ICF) (Cat. No. 4200-0140), optional
11. guava easyCheck Kit (Cat. No. 4500-0025), optional
12. Milli-Q™ Distilled Water or DI water.
13. 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 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, store the CD8-FITC/CD3-PECY5 and 1X Assay Buffer BA at 2-8°C.

Upon receipt, store the Guava 1X Lysing Solution at room temperature.

***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.*

## Before You Begin

### Specimen Collection and Preparation

The blood used for the procedure should be collected by venipuncture into a sterile K3 EDTA (lavender top) or Sodium heparin (green top) blood collection tube.

**WARNING:** Blood samples that are hemolyzed, clotted, lipemic, discolored or containing interfering substances should be discarded.

**NOTE:** Blood should be stained within 30 hours of collection for optimal results. Unstained anticoagulated blood should be maintained at 18-25°C prior to sample processing.

**NOTE:** Leave the capped tubes of blood standing upright or lying on their sides if it is stored overnight. Do not rock or agitate blood in any way during extended storage.

This protocol was developed to allow direct determination of the percent of activated T cells in whole blood or PBMCs. For optimal throughput, final cell concentrations should be between 2 x $10^4$ and 1 x $10^5$ cells/well (or 2 x $10^6$ to 1 x $10^7$ cells/mL). EMD-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. The kit may also be used for 10-30 µL of blood.

Cells should be acquired shortly after the sample preparation had been completed. While some cells have been shown to yield stable results for up to 3 hours, others might vary depending on the donors. Hence, you should determine the stability of results for your own cells.

**Time considerations**: The process of staining cells with the FlowCellect™CD4 T Cell FAS 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 2 to 72 hours (or longer) of culture with various reagents to induce activation.

## Example Cell Staining Protocol

### Procedure for Staining Whole Blood Samples Using the CD8 T Cell FAS Kit

1. Prepare blood samples including positive and negative controls to cause activation of the cells.
2. Prepare the antibody cocktail for staining:
   a. Determine number of control samples that will need CD8-FITC/CD3-PECy5 Antibody cocktail with Isotype control-PE.
      i. For each test, in a polypropylene microtube:
      ii. Add 5 μL of CD8-FITC/CD3-PECy5 Antibody Cocktail (per test) and 5 μL of Isotype control (per test).
      iii. Mix by vortexing at medium speed for 2 seconds. This cocktail will be used for all samples that serve as control.
   b. Determine number of test samples that will need CD8-FITC/CD3-PECy5 Antibody cocktail with CD95-PE
      i. For each test, in a polypropylene microtube:
      ii. Add 5 μL of CD8-FITC/CD3-PECy5 Antibody Cocktail (per test) and 5 μL of CD95-PE antibody (per test).
      iii. Mix by vortexing at medium speed for 2 seconds.
3. Pipet 10μL of Control antibody cocktail or CD8-FITC/CD3-PECy5/CD95-PE cocktail from step 2 into each well or tube depending on if it is a control or test sample.
4. Pipet 10 μL of blood to each well or tube.
   **NOTE**: Blood in the tubes should be thoroughly resuspended by gentle agitation for a few minutes before removing an aliquot for sample preparation.
5. Mix the sample thoroughly by multipipetting the wells or cap the tubes and then vortex each sample immediately at medium intensity for 3 – 5 seconds.
   **CAUTION**: Avoid leaving blood to dry on the side of the well or tube. This may cause erroneous results.
6. Incubate the samples for 20 minutes at room temperature (18 to 25ºC) in the dark.
7. Pipet 180 mL of 1X Lysing Solution directly into one of the wells or tubes to bring total sample volume to 200 μL.
   **NOTE:** If using a flow cytometer other than the easyCyte HT System, add 380uL of 1X Lysing Solution.
8. Immediately mix the sample thoroughly by multipipetting the wells or cap the tubes and then vortex each tube on medium intensity for 3-5 seconds.
9. Incubate for 15 minutes at room temperature (18 to 25ºC) in the dark.
10. Samples are ready for acquisition and analysis on the easyCyte HT System or other flow cytometer.
    **NOTE:** Batch your preparations to avoid over-incubation of samples. Samples must be acquired within 3 hours after preparation.

### Procedure for Staining PBMC Using the CD8 T Cell FAS Kit

1. Prepare PBMC samples including positive and negative controls to cause activation of the cells.
2. Centrifuge and resuspend cells at 5 x $10^6$ cells/mL in 1x Assay Buffer BA.
3. Prepare the antibody cocktail for staining:
   a. Determine number of control samples that will need CD8-FITC/CD3-PECy5 Antibody cocktail with Isotype control-PE.
      i. For each test, in a polypropylene microtube:

ii. Add 5 μL of CD8-FITC/CD3-PECy5 Antibody Cocktail (per test) and 5 μL of Isotype control (per test).
iii. Mix by vortexing at medium speed for 2 seconds. This cocktail will be used for all samples that serve as control.

b. Determine number of test samples that will need CD8-FITC/CD3-PECy5 Antibody cocktail with CD95-PE
   i. For each test, in a polypropylene microtube:
   ii. Add 5 μL of CD8-FITC/CD3-PECy5 Antibody Cocktail (per test) and 5 μL of CD95-PE antibody (per test).
   iii. Mix by vortexing at medium speed for 2 seconds.

4. Pipet 10μL of Control antibody cocktail or CD8-FITC/CD3-PECy5/CD95-PE cocktail from step 3 into each well or tube depending on if it is a control or test sample.
5. Pipet 10 μL of PBMC to each well or tube.
6. Mix the samples thoroughly by pipetting up and down or cap the tubes and then vortex each tube on medium intensity for 3-5 seconds.
   **CAUTION**: Avoid leaving cells to dry on the side of the well or tube. This may cause erroneous results.
7. Incubate the samples for 20 minutes at room temperature (18 to 25ºC) in the dark.
8. Pipet 180 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 380uL of 1X Assay Buffer BA.
9. Immediately mix the sample thoroughly by pipetting up and down or cap the tubes and then vortex each tube on medium intensity for 3-5 seconds.
10. 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

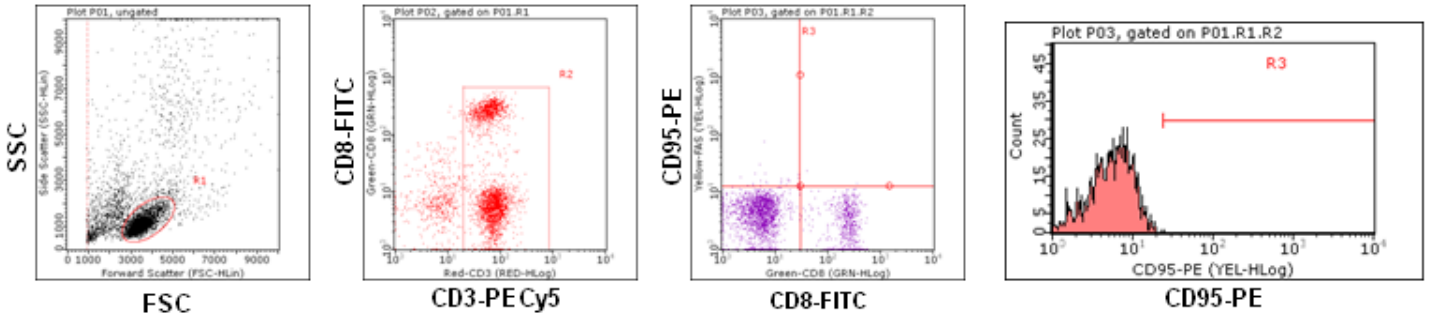



**Figure 1 Display of Plots for Sample Acquistion:** Set up of plots for data acquisition for samples treated with the CD8 T Cells FAS Kit. Plot 1 provides the plot of FSC vs. SSC which is typically used to set a lymphocyte gate. Plot 2 provides detection of CD8+ T cells (y-axis,Green channel) within CD3+ T cells Red (x-axis, Red channel) , also gate R2 in this plot serves as the counting gate; typically 3000 CD3+ events are counted . Plot 3 provides comparison of CD8+ T cells (x-axis, Green channel) vs. Isotype control or CD95-PE (y-axis, Yellow channel). Plot 4 shows a histogram depicting the level of CD95 (FAS)-PE/ Isotype control-PE expression on CD8 T cells. Use the uninduced sample stained with CD8-FITC/CD3-PECy5 cocktail and isotype control mouse IgG1-PE to adjust settings for green, yellow and red channels. Adjust settings for the all three channels so as to place the double negative population within the first decade of the dotplot.

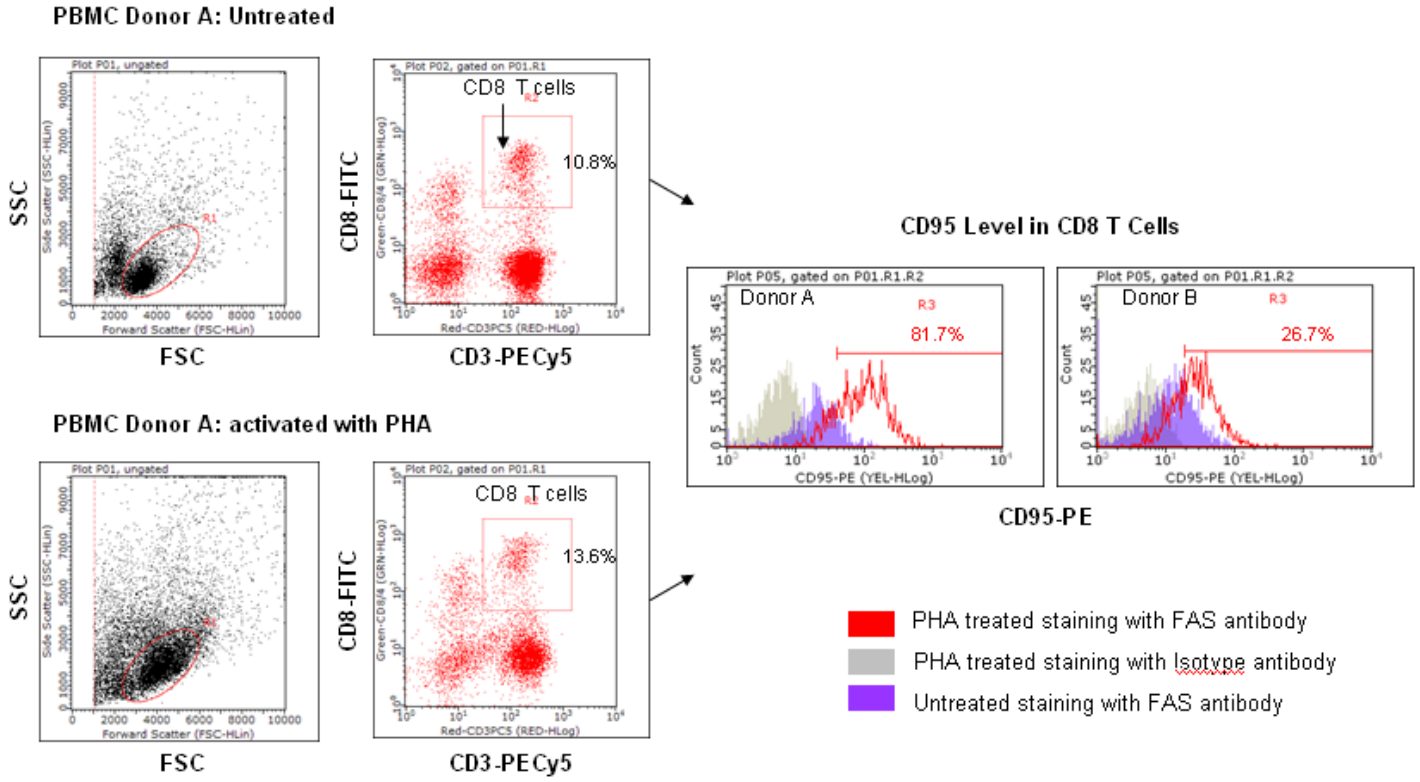



**Figure 2 Analyzed Dual Parameter Data**: Dot plots depicting PBMCs activated with 5ug/mL PHA (phytohemagglutinin) for 2 days and stained using FlowCellect™ CD8 T Cell FAS Kit. Dot plots show the percentage of positive cells for CD8 T cells as a % of CD3 T cells as shown in the plots on the left with and without PHA treatment (upper and lower plots). The histograms on the right depict the level of FAS/CD95 expression on CD8 T cells. PHA treatment upregulates the expression of FAS/CD95 on the CD8 T cells. The gating was set up on an unstimulated control sample stained with isotype control and applied to the stimulated samples.

## Technical Hints

- All kit reagents, CD8-FITC/ CD3-PECY5 Cocktail, 1X Lysing Solution 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™ CD8 T Cell Kit yield optimal results when the stained cell sample used for acquisition is between 1 x $10^6$ to 5 x $10^6$ 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<br>Instrument clogging<br>Too many cells | • Cell concentration too high - Decrease the number of cells per microliter by diluting sample to 300 – 500 cells/µL. The Guava EasyCyte™ Plus or guava easyCyte HT systems gives the most accurate data when the flow rate is less 500 cells per microliter.<br>• 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 | • Repeat experiment with increased number of cells. 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. However, cell numbers in donors vary. 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 both PBMC's and Lysed Whole Blood, further antibody titrations may be necessary for some donor cells to capture the ideal staining concentration. Non-specific staining and background may indicate that less antibody will need to be used during the staining procedure.<br>• 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 using the Post-acquisition Compensation feature of Incyte software 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. Low level may also be obtained if temperatures are sub-optimal and fall below 18 ºC. |
| Dim Staining | • Dim or false negative staining obtained with the CD4/CD3 antibody cocktail or CD95 antibody may indicate reagent degradation. Verify that the reagent is not past its expiration dates before using.<br>• Dim staining may also be a sign that the cell concentration was too high (>500 cells/ µL) and thus the concentration of reagents was insufficient to properly stain the cells. Repeat the experiment, using a lower numbe of cells per well. |
| Samples appear to be activated when low level of activation is expected | • Some cell cultures may have high level of constitutive CD95 expression. Negative controls should be a sample from your cell culture, not treated and stained with isotype control mouse IgG1 |
| Low level of staining of | • Cells may have low endogenous expression of FAS. If treatment was |

| | |
|---|---|
| CD95 | performed, cells may not have undergone activation to express FAS. To determine optimal activation, conduct a time-course study in order to achieve the best results for CD95 detection. Positive control samples are recommended for each experiment. Positive controls should be appropriate for comparison with the test procedure or test cell population. Use a reagent previously characterized as cell activator. Treatments to induce activation of T cells include, but are not limited to a) PHA, b) activation of cell surface receptors such as Fas, TNFR1, or TCR, c) PMA ± Ionomycin, and d) treatment with various interleukins.<br>• 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 low or absent level of FAS; in some cases, increasing staining time may be of help in improving staining |
| Variability in day to day experiments | • If the FlowCellect™CD8 T Cell 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.<br>• 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.<br>• 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 MitoDamage Kit (Catalog No. FCCH100139)
3. FlowCellect™ Human T Cell Activation Kit (Catalog No. FCCH100141)
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5. FlowCellect™ Human CD4 T Cell Fas Kit (Catalog No. FCCH100154)
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)
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11. FlowCellect™ Cytochrome *c* Kit (Catalog No. FCCH100110)

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