FlowCellect™ Human B Cell FAS Kit

100 Tests

Cat. No. FCCH100137

FOR RESEARCH USE ONLY
Not for use in diagnostic procedures.

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Application

The FlowCellect Human B Cell FAS kit allows for the detection and comparison of CD95/FAS antigen in B cell lymphocyte 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 (1). 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 and has also seen to be strongly upregulated on activated T cells, B cells, NK cells and thymocytes (4 – 5). FAS also widely expressed on cell lines of T, B, NK and myeloid lineage.

Fas/CD95 expression levels in immune cell subpopulations play a critical role in immune system development and disease (6). FAS expression on B cells and CD95-mediated death play an essential role in maintaining B cell tolerance (7, 8). CD95 -mediated apoptosis can contribute to the deletion of autoreactive B cells and/or B cells activated by "bystander" interactions with T cells in an antigen nonspecific fashion. Resting B cells constitutively express very low levels of CD95 and are not susceptible to CD95-mediated apoptosis induction. Ligation of CD40, by interaction with an activated T cell expressing CD154 (CD40L), causes up-regulation of CD95 expression on B cells and renders them susceptible to CD95-mediated apoptosis (9-11). FAS-induced apoptosis mediates the process of negative selection of B cells within the germinal center. Additionally, it has been suggested that FAS acts as a tumor suppressor gene (8). Multiple studies have demonstrated alterations in FAS expression levels in B cells in multiple disease states such as HIV, chronic leukemia and systemic lupus (12-14). B cell FAS levels have also been found to be altered in the treatment of cytotoxic chemotherapic drugs used in the treatment of leukemia. B cell FAS levels have also shown to be modulated on treatment with specific cytokines (15). The study of FAS expression levels in B cell subpopulations thus provides important insights into the mechanism of death.

The FlowCellect Human B Cell FAS kit allows for the detection and identification of CD45 lymphocytes and CD19 B lymphocytes and the FAS/CD95 expression levels in these populations in either whole blood or PBMC's using simplified no-wash assays and flow cytometry. The performance of the assays on the guava easyCyte 8HT platform along with the Incyte software allows for cell count information on CD45, CD19 cells and count and % of populations expressing CD95/FAS. The simplified identification of FAS expression levels can be of great utility in understanding B lymphocytes development, in research related to multiple diseases and understanding mechanism of autoimmunity and tolerance.

Test Principle

Millipore's FlowCellect™ Human B cell FAS Kit provides a simplified and rapid method to assess FAS/CD95 levels in B Lymphocytes and non-B lymphocytes. The kit includes all components needed to perform the assay on whole blood or peripheral blood mononuclear cells (1) CD19-FITC/CD45-PerCP Antibody Cocktail (2) Anti-human CD95 (FAS)-PE Antibody (3) Mouse IgG1 Isotype Control (4) 1X Assay Buffer BA and (5) 1X Lysing Solution to lyse erythrocytes.

The CD19-FITC/CD45-PerCP cocktail consists of two anti-human antibodies CD45-PerCP and CD19-FITC which allow for B cell lymphocyte detection and identification. The mouse anti-human CD19 antibody (clone HD37) reacts with CD19, a 120 kDa transmembrane glycoprotein, a critical signal transduction molecule that regulates B lymphocyte development, activation, and differentiation (4). CD19 is a broad lineage specific B cell marker whose expression is restricted to normal and neoplastic B cells, being absent from T cells, monocytes, and granulocytes (2). The CD19 antigen appears early during B cell maturation, persists during all stages of B cell maturation and is lost upon terminal differentiation to plasma cells. The mouse anti-human CD45 antibody (clone 2D1) recognizes CD45, a single chain type I transmembrane protein typically is expressed at high levels on nucleated cells of haematopoietic origin (1). CD45 is present on T and B lymphocytes, granulocytes, monocytes and macrophages. There are five different isoforms of CD45 whose MW ranges from 180,000-220,000 Da. All CD45 isoforms share the same intracellular segment which has been shown to have tyrosine phosphatase activity and which has a functional role in lymphocyte activation and differentiation. Antibodies that recognize all five isoforms are known as anti-CD45.

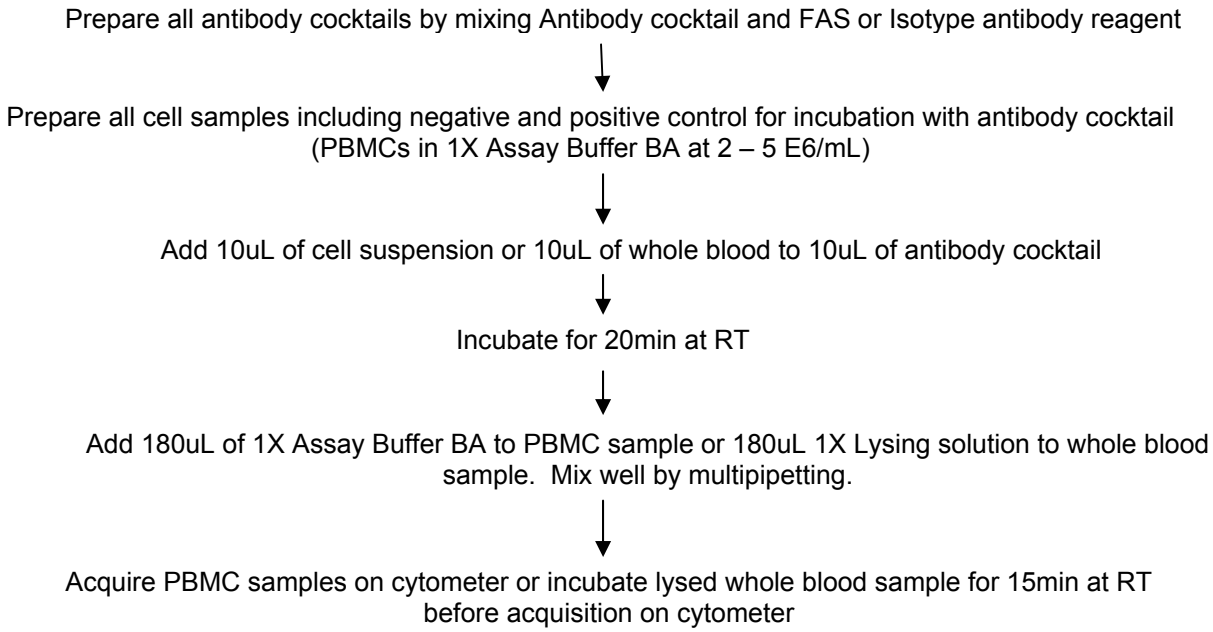
The mouse anti-human CD95 antibody (clone DX2) recognizes CD95 is a 45 kDa cell membrane receptor protein that is a member 6 of the tumour necrosis factor receptor superfamily and functions as a mediator of apoptosis (16). CD95 is expressed in a population of resting peripheral blood T cells, B cells and monocytes. A minority of resting NK cells and thymocytes express the antigen. Reactivity with granulocytes has been observed, although inconsistently. CD95 has been found to be strongly upregulated on activated B cells, T cells, NK cells and thymocytes

Total lymphocytes are identified by identifying populations based on scatter profile and high CD45 expression. The use of the CD19 antibody further delineates the B lymphocytes and the percentage of B lymphocytes in the total CD45 positive lymphocyte populations. Use of the CD95 antibody further allows for studying the level of expression of FAS/CD95 level in the B lymphocyte population as well as in the non-B lymphocyte population providing an understanding of FAS /CD95 levels in multiple sub-populations.

The FlowCellect™ Human B Cell FAS kit can distinguish multiple populations (1) CD45 Lymphocytes (2) B Lymphocytes (3) FAS/CD95 positive B lymphocytes (4) FAS/CD95 positive non- B lymphocytes. The kit thus provides a detailed picture of FAS/CD95 expression levels in B cell population, its response to treatment, development or diseases. 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 CD19-FITC/CD45-PerCP antibodies with a) isotype control and b) with FAS/CD95-PE antibody to clearly identify the expression of FAS/CD95 on B and non-B lymphocytes. The entire assay can be performed in 30-45 minutes in a simple no wash manner on PBMC's or whole blood.

Sufficient reagents are provided for 100 tests. The kit includes all optimized fluorescently labeled antibody conjugates, buffers and lysing solutions necessary for cell preparation and analysis.

Flow chart for performing the FlowCellect™B Cell FAS Kit.



Kit Components

- CD19 FITC/CD45-PerCP Cocktail (Part No.4700-1385) 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 CD19-FITC/CD45-PerCP 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 B cells in whole blood or PBMCs. For optimal throughput, final cell concentrations should be between 2×10^4 and 1×10^5 cells/well (or 2×10^6 to 1×10^7 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 blood volumes ranging from 10-30 µL.

Cells should be acquired shortly after the sample preparation had been completed. While some cell lines 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™ B 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 of culture with various reagents to induce activation.

Example Cell Staining Protocol

Procedure for Staining Whole Blood Samples Using the B 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 CD19-FITC/CD45-PerCP Antibody cocktail with Isotype control-PE.
      i. For each test, in a polypropylene microtube:
      ii. Add 5 μL of CD19-FITC/CD45-PerCP 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 CD19-FITC/CD45-PerCP Antibody cocktail with CD95-PE
      i. For each test, in a polypropylene microtube:
      ii. Add 5 μL of CD19-FITC/CD45-PerCP 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 CD19-FITC/CD45-PerCP 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 μL 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 B 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 CD19-FITC/CD45-PerCP Antibody cocktail with Isotype control-PE.

i. For each test, in a polypropylene microtube:
ii. Add 5 μL of CD19-FITC/CD45-PerCP 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 CD19-FITC/CD45-PerCP Antibody cocktail with CD95-PE
i. For each test, in a polypropylene microtube:
ii. Add 5 μL of CD19-FITC/CD45-PerCP 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 CD19-FITC/CD45-PerCP 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

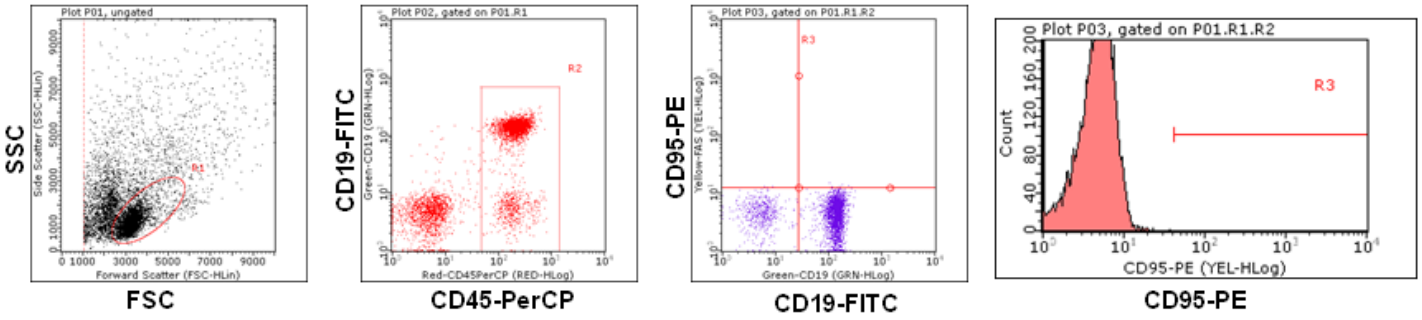


Figure 1 Display of Plots for Sample Acquistion: Set up of plots for data acquisition for samples treated with the B Cells FAS Kit. Plot 1 provides the plot of FSC vs. SSC which is typically used to set a lymphocyte gate. Plot 2 provides detection of CD19+ B cells (y-axis,Green channel) within CD45+ cells Red (x-axis, Red channel) , also gate R2 in this plot serves as the counting gate; typically 5000 CD45+ lymphocyte events are counted; Plot 3 provides comparison of CD19+ B 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 B cells. Use the uninduced sample stained with CD19-FITC/CD45-PerCP 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.

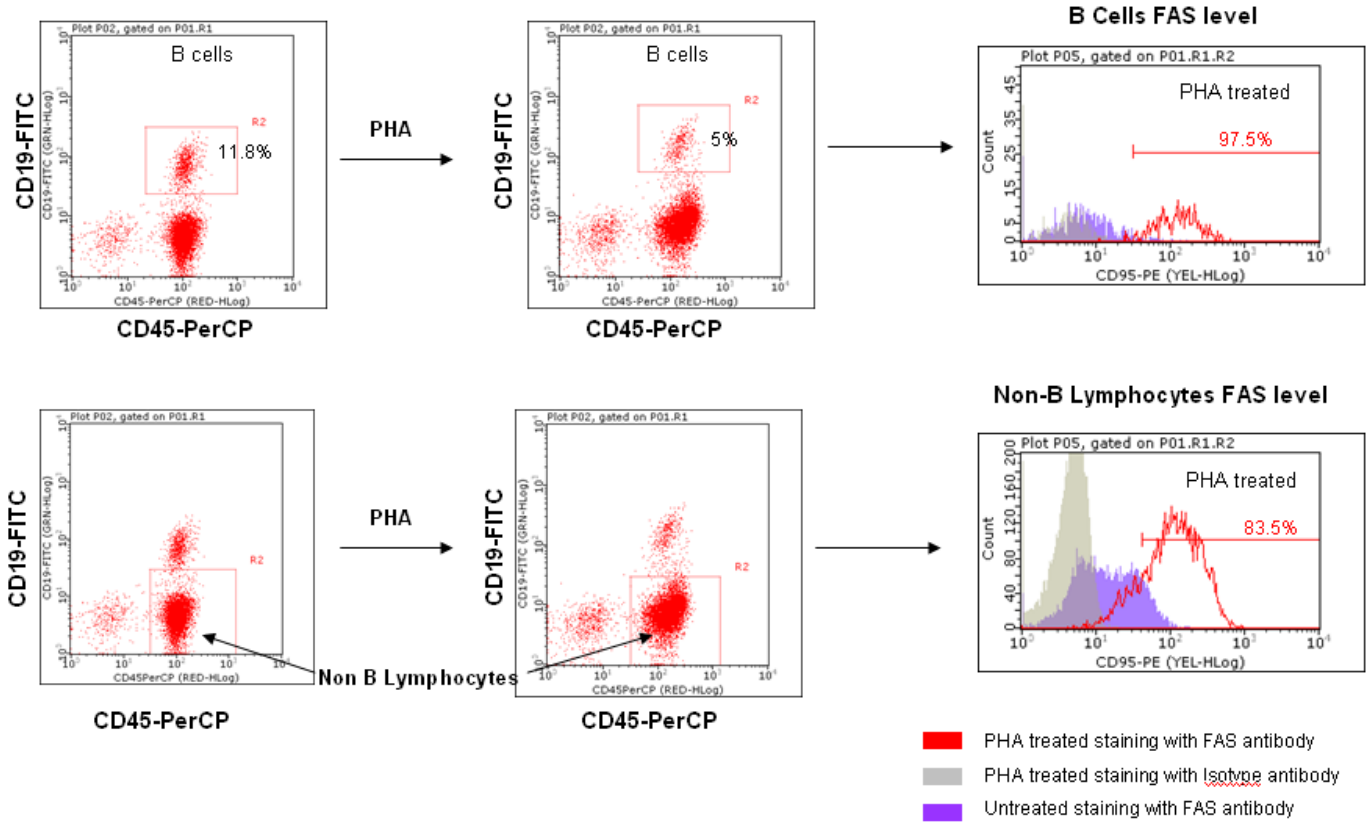


Figure 2. Analyzed Dual Parameter Data: Dot plots depicting PBMCs activated with 5ug/mL PHA (phytohemagglutinin) for 2 days and stained using FlowCellect™ B Cell FAS Kit. Dot Plots show the percentage of positive cells for 1) CD19+ B lymphocytes (upper panel) or Non-B Lymphocytes (lower panel) in CD45 (Red) and CD19 (Green) plot with and without PHA treatment- 2)Histogram Plots on right show FAS/CD95 levels on B cell lymphocyte (upper histogram) and non-B lymphocytes (lower histogram) B cells did not show any endogenous FAS expression, however treatement with PHA upregulated the expression of FAS in these populations. Non-B Lymphocytes show endogenous FAS/CD95 expression which is further elevated on PHA treatment. 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, CD19-FITC/ CD45-PerCP 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™ B 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 Instrument clogging 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. • 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 blood 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. • 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. • 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 number 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. • Although the assay procedure has been optimized to function utilizing both Lysed Whole Blood and PBMC's, donors may respond differently. A lack of signal may indicate low or absent level of FAS; in some cases, increasing may be of help in improving staining
Variability in day to day experiments	• If the FlowCellect™ B 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. • 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 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)
10. FlowCellect™ MitoStress Kit (Catalog No. FCCH100109)
11. FlowCellect™ Cytochrome *c* Kit (Catalog No. FCCH100110)

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