FlowCellect™ Mouse Viable Treg Characterization Kit

25 Tests

Cat. No. FCIM025168

FOR RESEARCH USE ONLY
Not for use in diagnostic procedures.

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Application

Regulatory T cells (Tregs) are a subpopulation of CD4+ T cells which constitutively express CD25 (IL-2 receptor alpha chain) and the transcription factor Foxp3. Characterization of these cells can be accomplished by performing multi-parameter flow cytometry to identify key combinations of the aforementioned surface and intracellular markers.

Tregs play a vital role in regulating the responses of the immune system. They suppress the activation of the immune system following infections, which helps maintain immune homeostasis (1, 2). It has also been shown that some cancer patients have increased numbers of Tregs which may allow some malignant cells to escape the activation of the immune system due to the suppressive nature of the Tregs. Researchers are interested in the mechanism by which cancers are evading anti-tumor immune responses as these could aid in the design of alternate treatment methods (3, 4).

A second and equally important function of Treg is promoting tolerance to self-antigens. Tregs act to ensure that self-antigens presented to the immune system do not illicit an immune response (5, 6). This is yet another way that Tregs help to maintain immune homeostasis by actively suppressing the activation and expansion of self-reactive T cells.

Many researchers are interested in the area of self-tolerance as a way to identify what can be tolerated and what signals a response to antigens. In particular, it would be of great benefit to understand the immune response that is common in organ, tissue, and cellular transplantation from donors to recipients. Knowing how to block/suppress the response to foreign antigens would allow the transplanted organ/tissue/cells to pass as self and escape rejection by the immune system (7).

All FlowCellect kits are optimized on guava® bench top flow cytometers. FlowCellect kits can be used on any flow cytometer following the same protocol providing researchers a reliable and fully validated solution to study mouse Tregs in the comfort of their own lab. All antibodies provided in the kit are carefully titrated and optimized together to ensure maximal performance when run in multiplex, alleviating the need for additional optimization.

In addition, Millipore has added a fixable viability dye that discriminates live and dead cells to eliminate false positive resulting from cytokine staining on dead cells that can occur during splenocyte preparations or in long term cell cultures. This viability dye can be used to irreversibly label dead cells prior to fixation and permeabilization procedures.

This kit also contains optimized fixation, permeabilization, wash, and assay buffers to provide researchers with a complete solution for characterizing mouse Foxp3+Tregs.

Test Principle

Millipore's FlowCellect™ Mouse Viable Treg Characterization kit includes three directly conjugated antibodies, Anti-CD4-PerCP/Cy5.5, Anti-CD25-PE, and Anti-Foxp3-Alexa Fluor® 488. A viability dye is also included to allow for the discrimination of only viable cells during sample analysis. This four color kit is designed to characterize viable Tregs within mixed cell populations.

Eliminating false results due to non-specific binding of antibodies to dead cells allows researchers to more accurately identify regulatory T-cells from different sample collections regardless of changes in

viability between samples. By doing so, Tregs can now be analyzed across multiple samples with a normalized and accurate measurement of their expression levels of each of the antibodies in the kit.

The antibodies provided have been carefully titrated to ensure the ability to accurately measure the expression of all markers simultaneously. Sufficient reagents are provided to perform 25 four-color tests. Detailed assay instructions are included to assist in analysis and to ensure the correct cell concentration is obtained during acquisition of sample data.

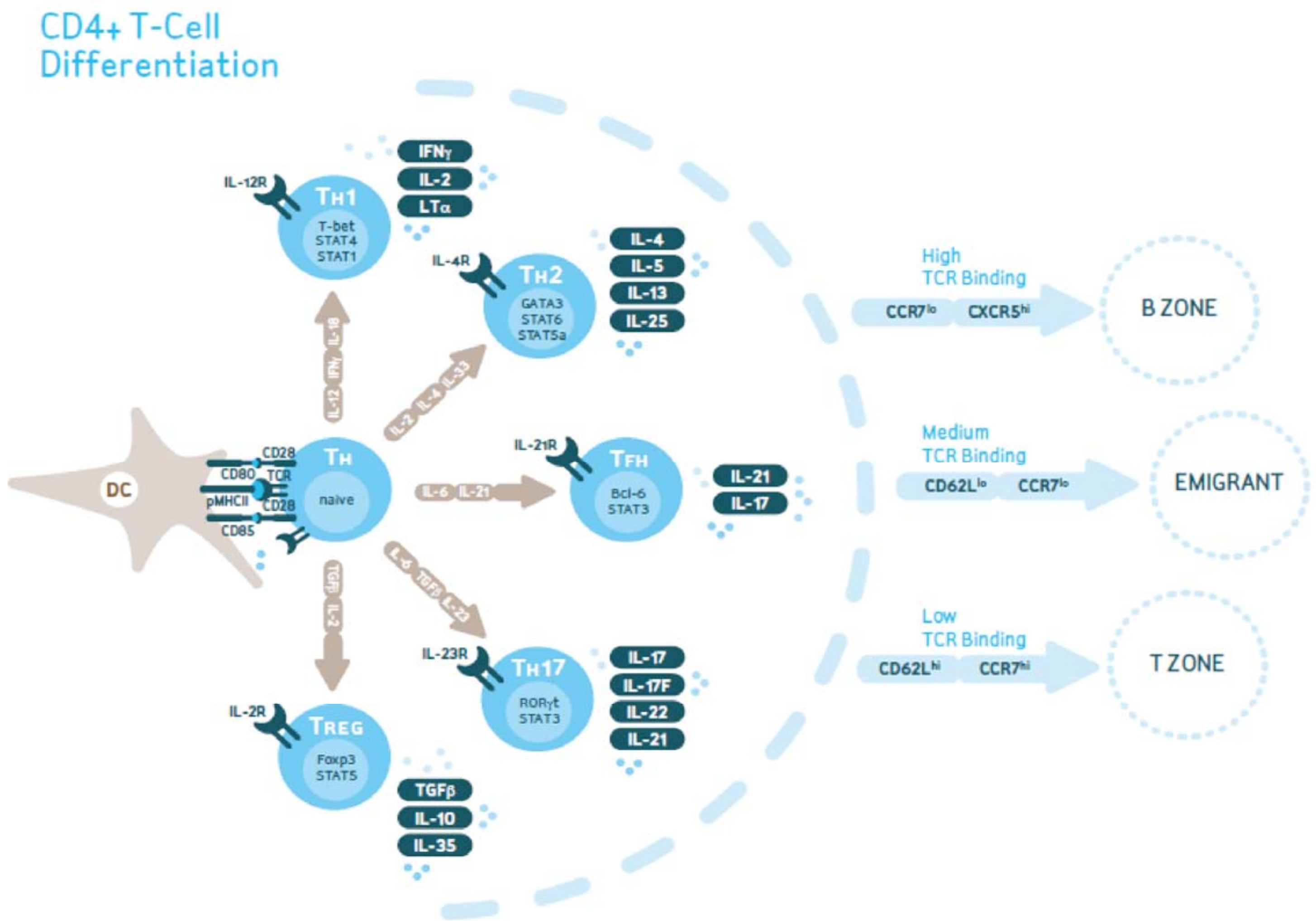


Fig. 1 Mouse CD4+ T cell Lineage Commitment
Naïve CD4+ T cells can polarize into lineages with distinct effector functions upon encountering foreign antigens resented by antigen-presenting cells such as dendritic cells (DC) and upregulated cytokine environment. Tregs possess the master regulator transcription factor Foxp3 and express CD25 (IL-2 receptor) on their surface. They play a crucial role in negative control of immune responses and the maintenance of immunological tolerance.

Kit Components

FCIM025168-1 (2-8 ºC)

- 20X Anti-CD4-PerCP/Cy5.5: (Part No. CS206550) One vial containing 150 µL.
- 20X Anti-CD25-PE: (Part No. CS206540) One vial containing 150 µL.
- 20X Anti-Foxp3-Alexa Fluor® 488: (Part No. CS206539) One vial containing 150 µL.
- 10X Wash Buffer: (Part No. CS202123) One bottle containing 13 mL.
- 4X Fixation/Permeabilization Buffer: (Part No. CS206472) One bottle containing 3.2 mL buffer.
- Fixation/Permeabilization Diluent: (Part No. CS206473) One bottle containing 9.5 mL buffer.
- 5X Assay Buffer: (Part No. CS202124) One bottle containing 55 mL.
- 10X Permeabilization Buffer: (Part No. CS206474) One bottle containing 6.5 mL buffer.

CF200098 (-80 ºC)

- 1000X Fixable Viability Dye eFluor®660: (Part No.CF200098) One vial containing 50 µL of 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, yellow, red1, and red2 fluorescence
2. 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\Tissue culture reagents, i.e. HBSS, PBS w/o Ca^{2+} or Mg^{2+}, cell dislodging buffers, etc.
3. Pipettors with corresponding tips capable of accurately measuring 0.1 – 1000 µL
4. Tabletop centrifuge capable of achieving 600 x g
5. Deionized water
6. Mouse splenocytes or cells of interest
7. Isotype controls (User Preference); Rat IgG2b for CD4 Conjugate, Rat IgG1 for CD25 conjugate, Mouse IgG1 for Foxp3 Conjugate.
8. ViaCount Reagent (Part No. 4700-0040) or Scepter (Part No. PHCC00000) for counting cells prior to staining.

Precautions

- 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.
- Wear proper laboratory attire (lab coat, gloves, safety glasses) when handling or using this product.
- Some assay components included in the kit may be harmful. Kit contains a fixation solution containing paraformaldehyde. Please refer to the MSDS sheet for specific information on hazardous materials (MSDS forms can be found by contacting Millipore technical services).
- 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.
- 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

- This kit must be stored at 2 - 8ºC except the fixable viability dye which must be stored at -80ºC.
- The 10X Wash Buffer can be stored at either 2 - 8ºC or at room temperature upon receipt.
- **Caution:** The fluorochrome conjugated antibody should always be stored at 2 - 8ºC and stored in the dark.

All kit components are stable up to six (6) months from date of receipt if stored and handled correctly. **Please avoid repeated changes in temperature as this will affect the integrity of the product.**

Preparation of Reagents

1. **Wash Buffer** is supplied at 10X concentration and should be diluted to 1X with deionized water prior to use. Prepared 1X Wash Buffer is stable up to one year. Store at 2 - 8ºC.
2. **Assay Buffer** is supplied at 5X concentration and should be diluted to 1X with deionized water prior to use. Prepared 1X Assay Buffer is stable up to one year. Store at 2 - 8ºC.

3. **Fix/Perm Buffer** (For 10 samples: scale up or down as necessary) Make 5 mL of 1X Fixation/Permeabilization Buffer. Mix 1.25 mL of 4X Fixation/Permeabilization Buffer with 3.75 mL of Fixation/Permeabilization Diluent. Place on ice. Mixed buffer is effective only for one day.
4. **Permeabilization Buffer** (For 10 samples: scale up or down as necessary) Make 25 mL of 1X Permeabilization Buffer. Mix 2.5 mL of 10X Permeabilization Buffer with 22.5 mL of deionized water. Place on ice.
5. **Fixable Viability Dye** If staining multiple samples on different days the Viability Dye must be aliquoted into individual vials to avoid repeated freeze and thaw cycles it should then be stored at -80ºC. One vial contains 50uL of dye; You use 1 µL of the dye for 2 million cells / mL. Please plan accordingly.

Assay Instructions

Note: This assay protocol has been optimized for both natural Tregs in mouse splenocytes and adaptiveTregs differentiated for several days in culture.

Staining Protocol for Flow Cytometry Analysis

Note: During all steps in the assay procedure, keep all reagents on ice. After preparation of the sprenocytes or cultured cells, follow the guidelines listed to ensure proper cell staining for optimal analysis.

Note: Step 1 to step 5 can be performed as a bulk staining (2 million cells in 1 mL of 1X Wash Buffer) with fixable viability dye-660 in a 1.5 mL tube or individual staining (200,000 cells in 100 µL of 1X Wash Buffer) in a 96-well plate.

1. Aliquot 2 million cells and wash with 1 mL of 1X Wash Buffer twice.
2. Resuspend cells with 1 mL of cold 1X Wash Buffer to bring the cell concentration to 2 million cells/ mL and place on ice.
3. Thaw an aliquot of 1000x Fixable Viability Dye just before use and keep it in the dark at all times.
4. Add 1 µL of 1000X Fixable Viability Dye to 1 mL of cell suspension (or 0.1 µL of 1000X Fixable viability Dye to 100 µL of cell suspension).
5. Incubate cells on ice in the dark for 15 minutes.
6. Spin down cells at 600 x g for 3 minutes and discard buffer.
7. Resuspend cells in 1 mL of 1X Wash Buffer and spin down cells at 600 x g for 3 minutes and discard buffer.
8. Resuspend cells at 2 million cells/ mL in 1x Assay Buffer.

9. Transfer 100 µL of cells (= 200,000 cells) in a well on a 96-well plate each test. This is the amount assumed though out the rest of this process.

10. Add 5 µL of 20X CD4-PerCP/Cy5.5 and 5 µL of 20X CD25-PE and incubate for 30 minutes on ice.

11. Prepare the 1X Fix/Perm Buffer during this incubation. Must mix that day to be effective.

12. Spin down cells at 600 x g for 5 minutes in 4°C centrifuge and discard buffer.

13. Add 200 µL of 1X Assay Buffer into each of the wells and spin at 600 x g for 5 minutes at 4°C. Discard buffer.

14. Resuspend each pellet in 100 µL of 1X Fix/Perm Buffer and incubate for 20 minutes at RT in the dark.

15. Spin down at 600 x g for 5 minutes in 4°C centrifuge and discard buffer.

16. Resuspend each pellet in 100 µL of 1X Perm Buffer.

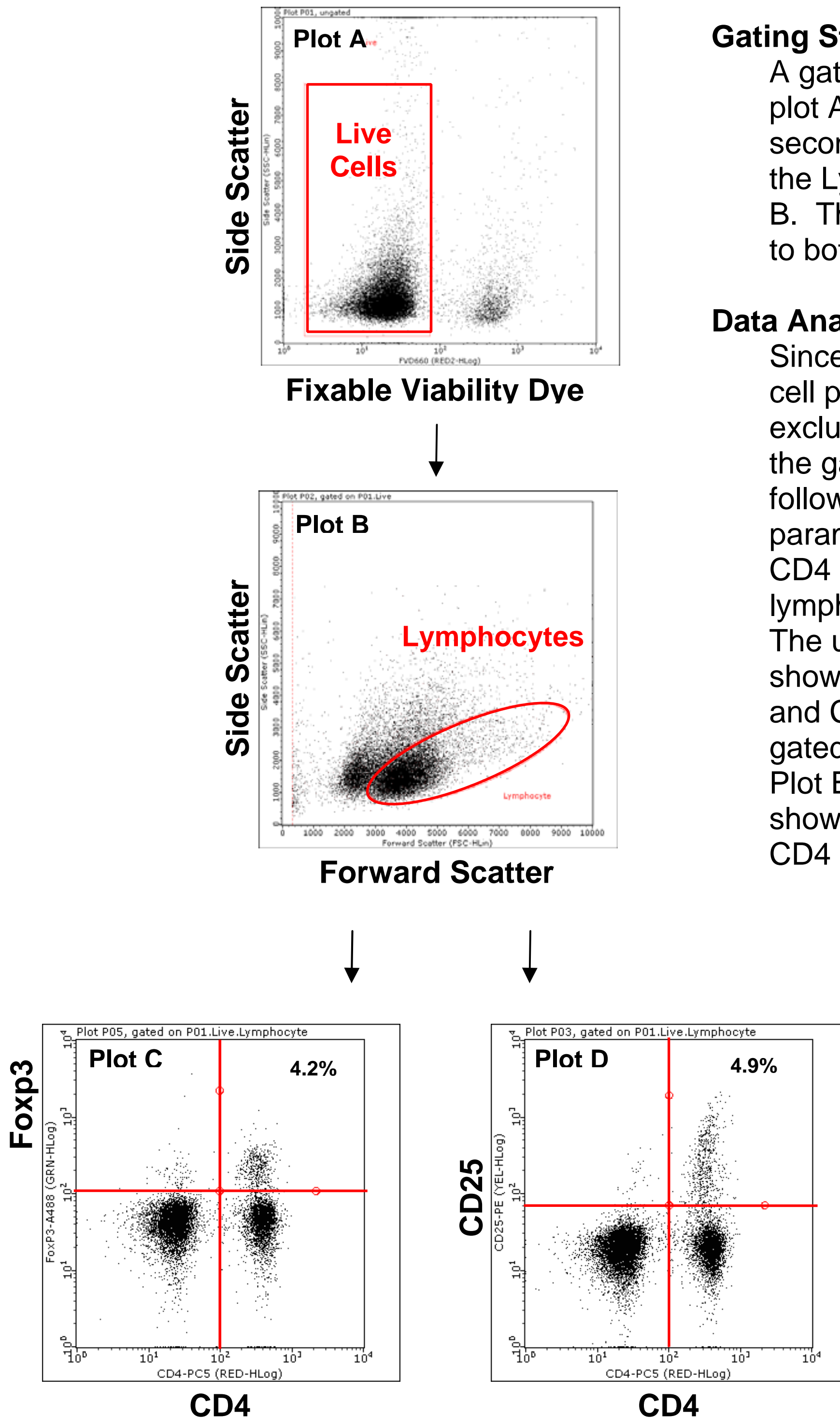
17. Add 5 µL of 20X Foxp3-Alexa Fluor 488 and incubate at RT for 30 minutes in the dark.

18. Centrifuge at 600 x g for 5 minutes and discard the supernatant.

19. Resuspend pellet with 200 µL of 1X Assay Buffer and spin down at 600 x g for 5 minutes. Discard buffer.

20. Resuspend pellet in 200 µL of 1X Assay Buffer and perform flow cytometry analysis
    Note: It is recommended to acquire more than 20,000 events per sample.

Sample Data



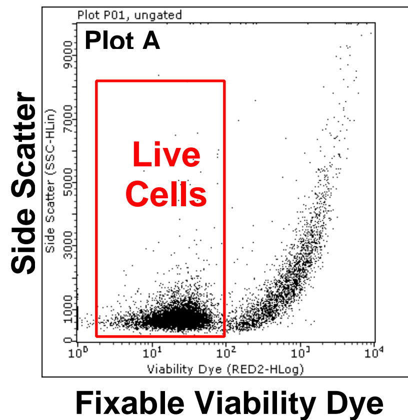
Gating Strategy:

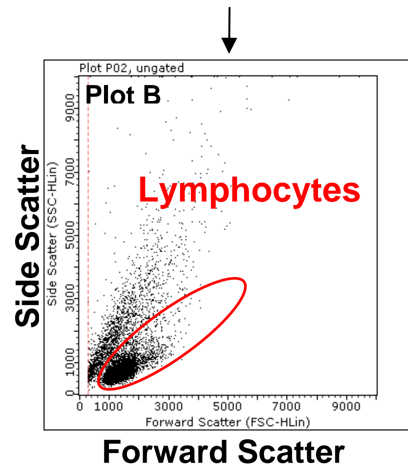
A gate is created for the live cells in plot A and applied to plot B. A second gate is created to surround the Lymphocyte population in plot B. The Lymphocyte gate is applied to both plots C and D.

Data Analysis:

Since plot A only includes the live cell population dead cells are excluded from the analysis when the gating strategy outlined above is followed. Plot C shows dual parameter analysis of Foxp3 and CD4 expression levels from the lymphocyte gate shown in plot B. The upper right quadrant of plot C shows 4.2% of the cells are Foxp3 and CD4 positive. Plot D is also gated from the Lymphocyte gate in Plot B and its upper right quadrant shows 4.9% of the cells are both CD4 and CD25 positive.

Figure 2: Representative flow cytometric data of mouse naïve splenocytes stained with Fixable Viability Dye (Plot A), gated out of lymphocyte region (Plot B) to dual stained anti-Foxp3/CD4 (Plot C) or CD25/CD4 (Plot D).





Gating Strategy:

A gate is created for the live cells in plot A and applied to plot B. A second gate is created to surround the Lymphocyte population in plot B. The Lymphocyte gate is applied to both plots C and D.

Data Analysis:

Following the same gating strategy as in Figure 2, only live lymphocytes are included in Plots B,C, and D.

The upper right quadrant of plot C shows 71.6% of the cells are Foxp3 and CD4 positive. Plot D is also gated from the Lymphocyte gate in Plot B and its upper right quadrant shows 67.4% of the cells are both CD4 and CD25 positive.

The majority of the cells have been directed to the Treg lineage as indicated by expression of Foxp3 as well as CD25 in plots C and D.

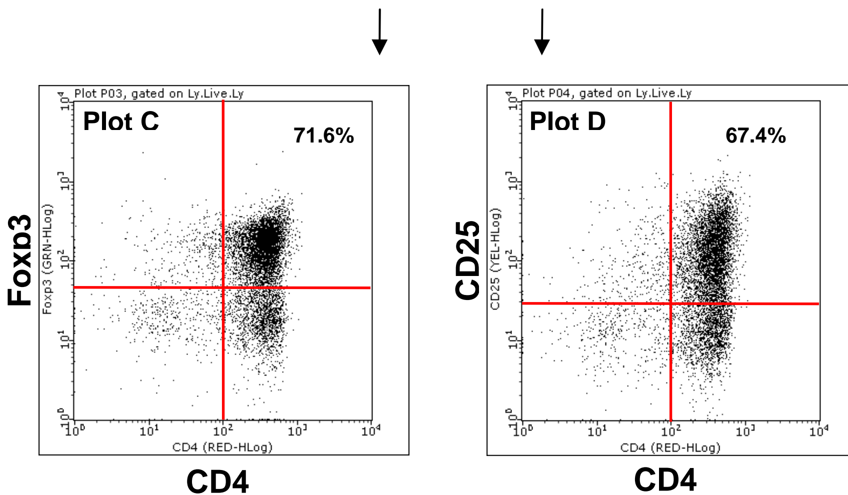


Figure 3: Representative flow cytometric data of mouse splenocytes following a 7 day directed differentiation to the iTreg lineage with optimized growth factor and antibody cocktails. The samples were stained with Fixable Viability Dye (Plot A), gated out of lymphocyte region (Plot B) to dual stained anti-Foxp3/CD4 (Plot C) or CD25/CD4 (Plot D).

Technical Hints

- If minor precipitate is detected in the 10X Wash Buffer place the bottle in a warm water bath for 30 minutes, followed by mixing the contents on a mechanical vortex.
- For cellular staining and analysis to be most effective, make sure that test cells have good viability prior to use.
- For certain cell cultures cell pellets may become hazy or transparent following the fixation step, making it difficult to see. If sampling a small collection of cells for flow analysis, it is recommended that all steps be performed in a smaller collection tube (e.g. centrifuge tube)
- Do not mix or interchange reagents from various kit lots.

Troubleshooting

Assay Step	Potential Problem	Experimental Suggestions
Reagent Preparation	Precipitation found in 10X Wash buffer	• If storing at -20ºC, precipitate can form in the 10X wash buffer. Prior to use, place bottle in a 37ºC water bath, swirling the contents occasionally. If this does not remove the precipitate completely, allow 10X Wash Buffer to sit at room temperature overnight.
Acquisition	Acquisition rate decreases dramatically	This usually indicates that the fluid pathway on the instrument may be blocked. This can be alleviated by the following: • Decreasing number of cells for analysis. Guava flow cytometers have the capacity of analyzing a steady stream of 300 – 500 cells per microliter. Any cell densities in excess can essentially block the normal flow, causing disruption during the assay. Decrease the number of cells being analyzed by diluting the sample to approximately 0.5 million cells per milliliter. • Adherent or sticky cells can result in cellular clumping. Use a more aggressive enzyme for dissociation such as trypsin during cell harvesting should help keep cells in single suspension. Alternatively, using a cell strainer can help disrupt cell clumping if needed (Catalog No. SCNY00060; 60 μM) • After many uses, it is possible that the fluid system on any standard flow cytometer will require cleaning. Run standard cleaning procedures to clean the fluid system during or after an assay. This will prevent any material from forming where the steady flow steam takes place.
Cellular Analysis	A loss or lack of signal	• Cell numbers may need to increase. Cell loss is common during washing steps in the assay procedure. A substantial decrease in cell numbers can lead to a loss of signal. Make sure that cell density remains at approximately 0.5 million cells per milliliter during analysis. • Although the assay procedure has been optimized to function utilizing many different cell types, further antibody titrations may be necessary for some cell types to capture the ideal staining concentration. A lack of signal may indicate that excess antibody will need to be used during the staining procedure.

Cellular Analysis	Background and/or non-specific staining of cells	• Although the assay procedure has been optimized to function utilizing many different cell types, further antibody titrations may be necessary for some cell types 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.
Cellular Analysis	Variability in day to day experiments	• When using the guava easyCyte™ Plus instrument for flow analysis, make sure that a quality check on the instrument (e.g. calibration) is performed on a daily basis prior to use. (**See Analytical Sensitivity and Detection Limits Section for Guava Check standards*)

For further support, please contact Millipore's Technical services at +1(800) 437-7500

References

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5. Sakaguchi S. Naturally arising Foxp3-expressing CD25+CD4+ regulatory T cells in immunological tolerance to self and non-self. *Nat Immunol.* 2005;6:345-52.
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7. Sakaguchi S. Regulatory T cells; Key controllers of immunologic self-tolerance. *Cell.* 2000; 101:455-458.

Related Products

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