FlowCellect™ Mouse Breg Identification Kit

25 Tests

Cat. No. FCIM025154

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Application

Regulatory B cells are a unique subset of small white blood cells that are recognized as a critical component in the immune system. Also known as “Bregs”, the primary function of regulatory B cells are to prevent or dampen overzealous immune responses. This regulatory function appears to be directly mediated by the production of IL-10 (interleukin-10), a potent cytokine which allows the ability of B cells to interact with pathogenic T cells to inhibit harmful immune responses [1]. Bregs have been identified in numerous experimental models such as inflammation, autoimmunity, and cancer, indicating that Breg-mediated suppression is important for maintaining peripheral tolerance [1,2]. IL-10 production by B cells may suppress the B cell and T cell-mediated immune responses and inflammation that are necessary to efficiently clear pathogens [2]. Understanding Bregs relevance to autoimmune diseases and cancer can assist in the development of novel therapeutic strategies [1].

Recent studies have identified a potent regulatory B cell subset (B10 cells) within the rare $CD1d^{hi}CD5^{+}$ B cell subset of the spleen that regulates acute inflammation and autoimmunity through the production of IL-10 [3]. IL-10 production is a hallmark of B10 cells, and although B10 cells normally represent only 1-3% of spleen B cells they dramatically inhibit the induction of Ag-specific inflammatory reactions and autoimmunity [4]. IL-10 competent spleen B cells were found to predominantly localize within a $CD1d^{hi}CD5^{+}CD19^{hi}$ subset, so having the ability to monitor CD1d, CD5, and CD19 expression levels simultaneously in a mixed cell population allows for the identification of Bregs which occur at low frequencies. B cell subsets are identified by the expression of CD19 and CD5, whereas regulatory B cells with the ability to secrete IL-10 were immunophenotypically identified by CD1d. Categorization of B10 cells as $CD1d^{hi}CD5^{+}$ is useful for defining the novel character of these B cells and for analyzing their distributions.

Regulatory B cells play major roles during immune responses to pathogens where B10 cells function to protect host tissues from immunopathology during infections. IL-10 release from B10 cells exerts anti-inflammatory and suppressive effects on most hematopoietic cells. This cytokine was initially associated with T helper (Th)2 cells and was described to inhibit Th1 cytokine production [2]. IL-10 also suppresses pro-inflammatory cytokine production by monocytes and macrophages and the proliferation of Ag-specific CD4+ T cells by inhibiting the Ag-presenting capacity and decreasing co-stimulatory molecule expression by professional APCs, including dendritic cells, macrophages, Langerhan’s cells, and B cells [2].

Understanding the relationship between regulatory B cells and their presence in certain diseases can offer new opportunities to develop novel therapeutic strategies. For example, as noted in Watanabe, et al. (2010), B10 cells and other regulatory B cell subsets have been indicated to negatively regulate inflammation and autoimmune disease in mice, including hypersensitivity, inflammatory bowel diseases, and arthritis [3]. This has also been confirmed by Mizoguchi, et al. (2006), where activated murine B10 cells produced large amounts of IL-10, which was detected in vivo under a variety of experimental inflammatory conditions such as IBD, EAE, arthritis, lupus, and UV radiation. Moreover, the presence of regulatory B cells has also been identified in some Crohn’s disease-like disease models [5]. The identification of regulatory B cells in disease models should provide important clues to how the immune system regulates itself in response to vaccines as well as infectious agents [6]. Moreover, this information should enable researchers design ways to help the immune system control infections more effectively, which could be useful knowledge as researchers refine approaches to preventing HIV infection.

Test Principle

We have developed a multi-parameter flow cytometry assay for identification of mouse regulatory B cells from mouse splenocytes. Millipore's FlowCellect™ Mouse Breg Identification Kit includes three directly conjugated antibodies: Anti-Mouse CD5-APC, Anti-Mouse CD19-FITC, and Anti-Mouse CD1d-PE, along with optimized assay buffers and a blocking antibody to provide researchers the ability to phenotypically distinguish cell types.

Antibodies against CD5 and CD19 are provided in the kit to phenotype cells by their surface marker expression. CD5 is used to identify B-cell subsets, while CD19 is a mature B-cell marker. CD1d is used to phenotypically identify mouse regulatory B cells, which can serve useful utility in identifying the presence of this B cell subset during certain disease models. Purified Anti-CD16/32 is included for blocking non-specific binding of immunoglobulin to the Fc receptors.

Sufficient reagents are provided for 25 three-color tests. Detailed assay instructions are included to assist in analysis and to ensure the correct cell populations are analyzed during acquisition of sample data. The kit includes all optimized fluorescent labeled antibodies, buffers, and a blocking antibody necessary for proper cell preparation and analysis.

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 and identify regulatory B cells right in the comfort of their own lab. All three antibodies provided in the kit are carefully titrated and optimized together to ensure maximal performance when run in multiplex, alleviating the need for any additional optimization.

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Kit Components

- 20X Anti-Mouse CD5-APC, clone 53-7.3: (Part No. CS206436) One vial containing 150 µL.
- 20X Anti-Mouse CD19-FITC, clone 1D3: (Part No. CS206434) One vial containing 150 µL.
- 20X Anti-Mouse CD1d-PE, clone 1B1: (Part No. CS206435) One vial containing 150 µL.
- 20X Anti-Mouse CD16/CD32 Purified, clone 93: (Part No. CS206424) One vial containing 150 µL.
- Fixation Buffer: (Part No. CS202122) One bottle containing 13 mL.
- 10X Wash Buffer: (Part No. CS202123) One bottle containing 13 mL.
- 5X Assay Buffer: (Part No. CS202124) One bottle containing 55 mL.

Materials Not Supplied

1. Test tubes for sample preparation and storage
2. 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 10 – 1000 µL
4. Tabletop centrifuge capable of achieving 300 x g
5. Mechanical vortex
6. Flow Cytometer
7. Deionized water
8. Red Blood Cell Lysing Buffer (Sigma: Part No. R7757)
9. ViaCount Reagent (Part No. 4700-0040)
10. Isotype controls: rat IgG2a, kappa FITC and APC; rat IgG2b, kappa PE

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.
- Some assay components included in the kit may be harmful. Kit contains a fixation solution which may be hazardous if improperly handled. Please refer to the MSDS sheet for specific information on hazardous materials (MSDS forms can be found by contacting Millipore technical services).
- The conjugated antibodies are light sensitive and must be stored in the dark at 4°C.
- During storage and shipment, the product supplied in vials may condense within the vial. For maximum recovery of the product, centrifuge original vial prior to removing cap.
- Do not use reagents beyond the expiration date of the kit.

Storage

This kit must be stored at 2 - 8ºC. The 10X Wash Buffer (Part # CS202123) and Fixation Buffer (Part # CS202122) can be stored at either 2 - 8ºC or at room temperature upon receipt. **Caution:** The fluorochrome conjugated antibodies 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**

   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**

   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.

Assay Instructions

Note: This assay protocol has been optimized for mouse splenocytes. However, this kit is suitable for measuring mouse regulatory B cells from other tissues. Wash and Fixation Buffers are included in the kit but are not required for proper cell preparation.

Flow Kit Staining Protocol

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

I. Splenocyte Isolation

- Remove mouse spleen and place the spleen directly into a petri dish containing RPMI media supplemented with 10% fetal bovine serum (FBS). Keep mouse spleen hydrated.
- Homogenize the spleen to obtain a single cell suspension. Techniques include disrupting the spleen between the frosted ends of two glass microscopy slides, rubbing the slides together to break up the tissue.
- Pass the homogenized spleen through a 60 µM cell strainer (or nylon mesh) mounted on a 50 mL conical to collect single cell suspension. Wash through with RPMI media supplemented with 10% fetal bovine serum (FBS).
- Spin cells at 300 x g for 5 minutes.

- Discard supernatant and resuspend pellet in 1 mL Red Blood Cell Lysing Buffer. Incubate at room temperature for 5-10 minutes. Add 9 mL RPMI media supplemented with 10% fetal bovine serum (FBS).
- Spin cells at 300 x g for 5 minutes (washing step).
- Discard supernatant and resuspend pellet in 10mL RPMI media supplemented with 10% fetal bovine serum (FBS).
- Determine cell numbers by using ViaCount or a hemacytometer and note cell viability.
- Rususpend cells in 1X Assay Buffer (80 µL buffer for every $1x10^6$ cells).

II. Fc Blocking

- For every $1x10^6$ cells (in 80 µL 1X Assay Buffer) , Add 5 µL of 20X Anti-Mouse CD16/32 purified antibody. Incubate cells for 30 minutes on ice.

III. Antibody Staining

- Add 5 µL of 20X Anti-Mouse CD19-FITC, 5 µL of 20X Anti-Mouse CD1d-PE, and 5 µL of 20X stock Anti-Mouse CD5-APC to 85 µL of cell suspension ($1x10^6$ cells in 85 µL containing 1X Assay Buffer and 1X Fc blocking antibody) for staining. Allow to incubate on ice for 30 minutes in the dark.
- Wash cells once by adding 200 µL 1X Assay Buffer for every $1x10^6$ cells. Again, carefully disrupt cell pellet to homogeneity, and remove assay buffer by centrifugation at 300 x g for 5 minutes. Aspirate and discard supernatant, only saving the cell pellet.

IV. Analysis

- If performing cell analysis on a guava easyCyte 8HT instrument, cells can be aliquoted either in a 96-well plate (200 µL volumes) or single tube (500 µL volumes).
- If using an alternative flow cytometer, dilute cells according to instrument manufacturer's recommendations.
- Begin cellular analysis and sample acquisition.

Doublet Discrimination (Area vs. Width)

Doublet discrimination can be achieved by implementing gating strategies plotting area versus width. By doing such, it can be easily determined which cells are truly in single cell suspension versus cells which are clumped together based on the increased width of a given cell population.

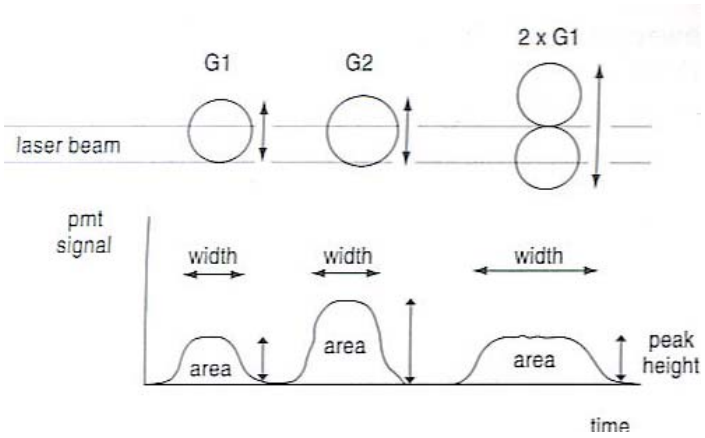

Figure 1. Time of cell flight distinguishes doublets from single cells in suspension As cells pass through the interrogation point, the laser beam width and area increases as the width of the cell increases. Hence, a larger cell (e.g. aggregates) will cause an increase in cell width.

A. Ungated sample **B. Gated sample**

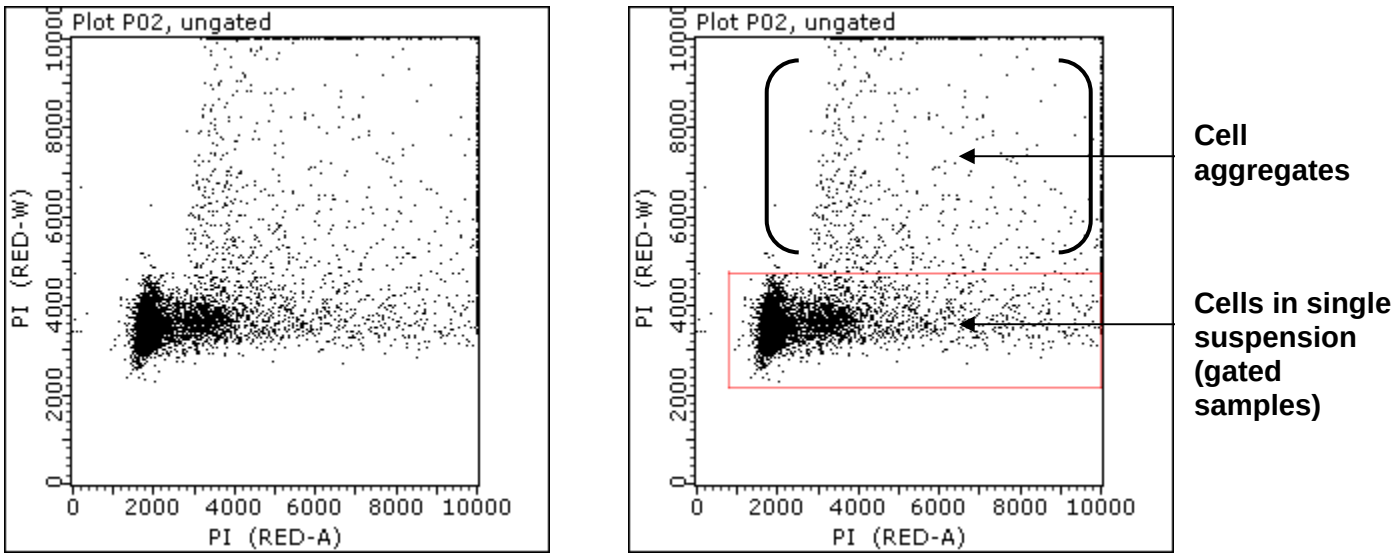

Figure 2. Doublet discrimination: Plotting Area vs. Width Aggregate cells can mistakenly be interpreted as single cells, leading to inaccurate results. Eliminate cell doublets by gating only accounting for cells giving a constant width. Plot A shows an ungated sample containing excessive cell scattering which will account for all the cell aggregates or coincidence events. In B, only cells of interest are used for analysis purposes.

Sample Data

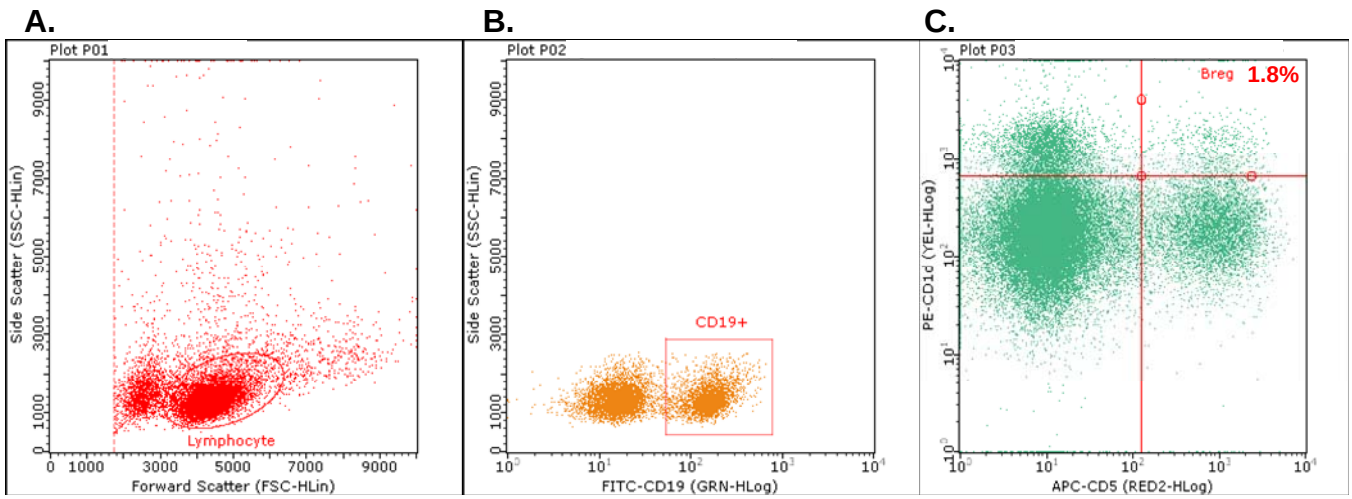

Figure 3. Isolation and Identification of Mouse Regulatory B cells (Bregs) from mouse splenocytes Mouse splenocytes are isolated from mouse spleens and regulatory B cells are phenotypically identified by using specific mouse CD markers: Mouse CD5, CD19, and CD1d. A rare subset of IL-10 competent spleen Breg cells (also known as B10 cells) was found to predominantly localize within a $CD1d^{hi}CD5^{+}CD19^{hi}$ subset. In (**A**), lymphocyte populations are gated, and B cells are further isolated by using a mouse $CD19^{+}$ antibody (**B**). From here, Breg subset cells (B10 cells) are identified by double positive staining of mouse $CD1d^{hi}CD5^{+}$ (**C**). This rare Breg cell subset from mouse spleens (represent only 1-3%) has been indicated to regulate acute inflammation and autoimmunity through the production of IL-10.

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	• 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. • 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) 548-7853

References

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2. DiLillo, D.J., *et al.* (2010). B10 cells and regulatory B cells balance immune responses during inflammation, autoimmunity, and cancer. *Ann N Y Acad Sci.*;1183:38-57.
3. Watanabe, R., *et al.* (2010). Regulatory B cells (B10 cells) have a suppressive role in murine lupus: CD19 and B10 cell deficiency exacerbates systemic autoimmunity. *J Immunol.*; 184(9):4801-9.
4. Carter, R.H., *et al.* (2004). Signaling by the CD19/CD21 complex on B cells. *Curr Dir Autoimmun.*;7:4-32.
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