



FlowCollect™ Mouse T_H1 Intracellular Cytokine Kit
25 Tests

Cat. No. FCIM025123

FOR RESEARCH USE ONLY
Not for use in diagnostic procedures.

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Background

There are five main subsets of differentiated CD4+ T-cells; T_{H1} , T_{H2} , T_{H17} , T_{Reg} and T_{FH} cells. Each subtype expresses a signature cytokine or transcription factor that directs the immune response and acts to regulate differentiation. T_{H1} CD4+ T-cells are important for protection against intracellular bacteria, fungi, protozoa and viruses and are involved in some autoimmune responses (1). They mediate immune responses to intracellular pathogens and respond by producing interferon gamma (IFN- γ). Through secretion of IFN- γ , T_{H1} CD4+ T-cells activate macrophages, natural killer (NK) cells and CD8+ T-cells. IFN- γ also increases the production of interleukin-12 by dendritic cells and macrophages, and via positive feedback, stimulates the production of IFN- γ , thereby promoting the T_{H1} profile. IFN- γ also inhibits the production of interleukin-4, an important cytokine associated with the T_{H2} subtype, and thus it also acts to preserve its own pathway of differentiation.

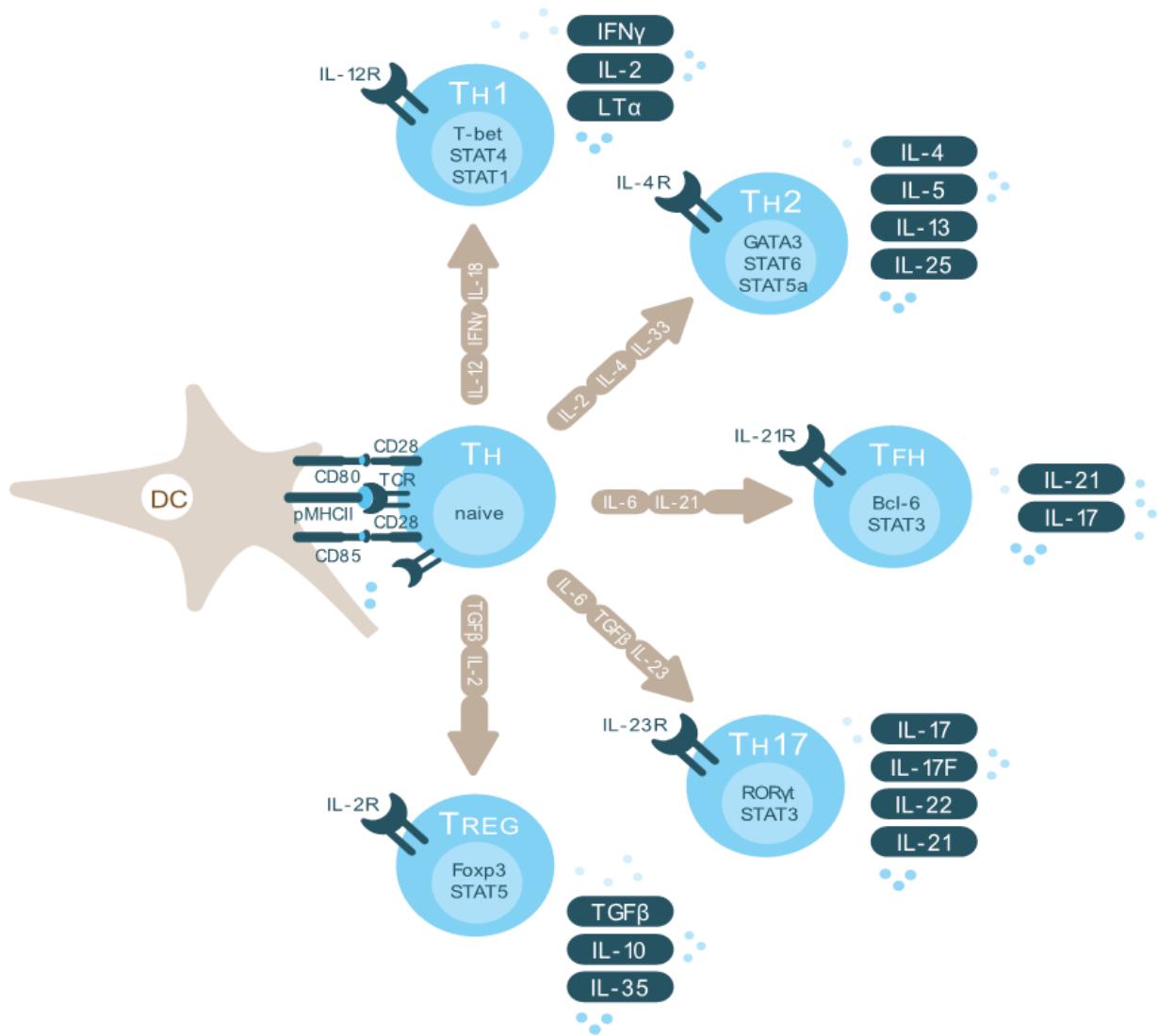


Figure 1: Schematic diagram of CD4+ T-cell differentiation. Five different subtypes of CD4+ T-cells can develop from a common naïve precursor depending on the cytokine environment and interaction with dendritic cells (DC).

Application

Millipore's FlowCollect™ Mouse T_H1 Identification Kit is designed to enable a researcher a quick and easy way to detect IFN- γ expression in mouse T_H1 CD4+ T-cells.

Millipore's FlowCollect™ Mouse T_H1 Identification Kit contains a PE conjugated antibody to IFN γ and a PerCP conjugated antibody to CD4 as well as optimized cytokine blocking reagents and buffers to aid in identifying T_H1 CD4+ T-cell subsets in ex vivo lymphocyte populations or to monitor T_H1 CD4+ T-cell differentiation in culture. In addition, Millipore has added a fixable viability dye that eliminates false positive data resulting from cytokine staining on dead cells that can occur in long term cultures. The Millipore's FlowCollect™ Mouse T_H1 Identification Kit contains sufficient reagents for 25 3-color tests. Detailed assay instructions are included to assist in analysis and to ensure that the correct cell concentration is obtained during acquisition of sample data. This kit is not designed to be used in the analysis of whole spleen cells from SJL mice.

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Kit Components

FCIM025123-1, FlowCollect™ Mouse T_H1 Intracellular Cytokine Kit, store at 2-8°C

1. 20X Anti-CD4-PerCP clone GK1.5: (Part No. CS205790) 1 vial containing 150 μ L
2. 20X Anti-IFN γ -PE, clone XMG1.2: (Part No. CS206353) 1 vial containing 150 μ L
3. Fixation Buffer: (Part No. CS202122) One bottle containing 13 mL buffer
4. 10X Wash Buffer: (Part No. CS202123) One bottle containing 13 mL buffer
5. 5X Assay Buffer: (Part No. CS202124) One bottle containing 55 mL buffer
6. Permeabilization Buffer Concentrate: (Part No. CS206346) One bottle containing 15 mL buffer
7. 1000X Brefeldin A Buffer: (Part No. CS206347) One vial containing 50 μ L

CF200098, store at -80°C

8. 1000X Fixable Viability Dye eFluor®660: One vial containing 50 μ L*

***Note: Refer to the Preparation of Buffers and Storage sections of the manual for detailed instructions on Fixable Viability Dye eFluor®660 storage and preparation.**

Materials Not Supplied

1. Flow Cytometer with a 488nm and a 633nm laser
2. Mouse splenocytes
3. Pipettors with corresponding tips capable of accurately measuring 1 – 1000 μ L
4. Tabletop centrifuge capable of exceeding 400Xg.
5. Tubes capable of holding 1 mL and 2 μ L of sample
6. Red blood cell lysis buffer (Millipore catalog #4700-0088)
7. Activators and stimulants to differentiate T_H1 cells

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. The kit includes a fixation solution containing formaldehyde. Please refer to the MSDS sheet which can be found at www.millipore.com 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.
- Do not use reagents beyond 4 months from date of receipt.

Storage

Caution: The fixable viability dye should be protected from light and moisture and stored at -80°C with desiccant. Upon receipt, all antibodies and buffers should be stored at 2-8°C. Fluorochrome conjugated antibodies should always be stored at 2-8°C. Do not freeze fluorescent antibodies. Any deviation in temperature for long periods of time may compromise the performance of the antibodies.

Preparation of Buffers

The 1000X fixable viability dye is sensitive to light, moisture and temperature changes and therefore should be thawed in the dark and stored at -80°C into single use aliquots with a desiccant pack.

- Thaw vial of 1000X Fixable Viability Dye eFluor®660 in the dark. Spin down vial briefly and aliquot 2 μ L in 25 single use tubes. Use 1 μ L per test.

Prepared 1X Assay Buffer, 1X Wash Buffer and Permeabilization Buffer are stable up to four months if stored at 2-8°C, and can be prepared when you receive the kit or when you start the first assay.

- Make 100 mL of 1X Assay Buffer: Mix 20 mL of 5X Assay Buffer with 80 mL of deionized water.
- Make 30 mL of 1X Wash Buffer: Mix 3 mL of 10X Wash Buffer with 27 mL of deionized water.
- Make 30 mL of Permeabilization(Perm) Buffer: Mix 6 mL of 5X Assay Buffer with 0.3 mL of Permeabilization Buffer Concentrate and 24 mL of deionized water.

Prepared Permeabilization/Fixation Buffer must be made fresh each time the assay is performed.

- Make 1 mL of Fixation/Permeabilization(Fix/Perm) Buffer/test: Mix 0.5 mL of Fixation Buffer with 0.05 mL of Permeabilization Buffer Concentrate and 0.45 mL of 1X Assay Buffer.

Cell Staining Protocol

Note: Before starting protocol prepare all buffers and place on ice or store at 2-8°C.

1. Add 1000X Brefeldin A Buffer to cells of interest 5 hours before staining. **Note:** Brefeldin A can be added to cells during stimulation.
2. Count cells and place 5×10^6 cells into a sample tube.
3. Spin down cells at 2500 rpm (600Xg) for 3 minutes and discard buffer.
4. Resuspend the cells in 1 mL of 1X Wash Buffer by gently pipetting up and down.
5. Spin down cells at 2500 rpm (600Xg) for 3 minutes and discard buffer.
6. Resuspend the cells in 1 mL of 1X Wash Buffer by gently pipetting up and down. Place cells on ice.
7. Thaw an aliquot of 1000X Fixable Viability Dye eFluor®660 just before use and keep it in the dark at all times. Add 1 μ L of 1000X Fixable Viability Dye for every 5×10^6 cells. Immediately mix cells and dye gently by pipetting up and down. **Note:** Once cells are stained with dye keep them away from light as much as possible.
8. Incubate cells on ice for 15 minutes in the dark.
9. Spin down cells at 2500 rpm (600Xg) for 3 minutes and discard buffer.
10. Resuspend the cells in 1mL of 1X Assay Buffer/ 5×10^6 cells by gently pipetting up and down.
11. Repeat steps 9 and 10 once and then go to step 12.
12. Resuspend cells in 0.5 mL Permeabilization/Fixation Buffer/ 5×10^6 cells by gently pipetting up and down.
13. Incubate cells at room temperature for 20 minutes in the dark.
14. Add 1 mL of 1X Perm Buffer/ 5×10^6 cells.
15. Spin down cells at 2500 rpm (600Xg) for 3 minutes and discard buffer.
16. Repeat steps 14 and 15 once and then go to step 17..
17. Resuspend cells in 1 mL of Permeabilization Buffer/ 5×10^6 cells by gently pipetting up and down. **Note:** At this point the cells can be stored overnight at 4 degrees in the dark.
18. Mix cells thoroughly and place 0.1 mL of cells in a 1.5 mL tube or 96-well plate.
19. Add 5 μ L of 20X Anti-CD4-PerCP and 5 μ L of 20X Anti-IFN γ -PE to each tube or well.
20. Incubate cells in the dark and on ice for 1 hour.

21. Add 0.1 mL of Permeabilization Buffer to cells.
22. Spin down cells at 2500 rpm (600Xg) for 3 minutes and discard buffer.
23. Resuspend cells in 0.2 mL of Permeabilization Buffer by gently pipetting up and down.
24. Spin down cells at 2500 rpm (600Xg) for 3 minutes and discard buffer.
25. Resuspend cells in 0.2 mL of 1X Assay Buffer by gently pipetting up and down.
26. Dilute cells in 1X Assay Buffer to an appropriate concentration for flow cytometer to be used and analyze.

Sample Data

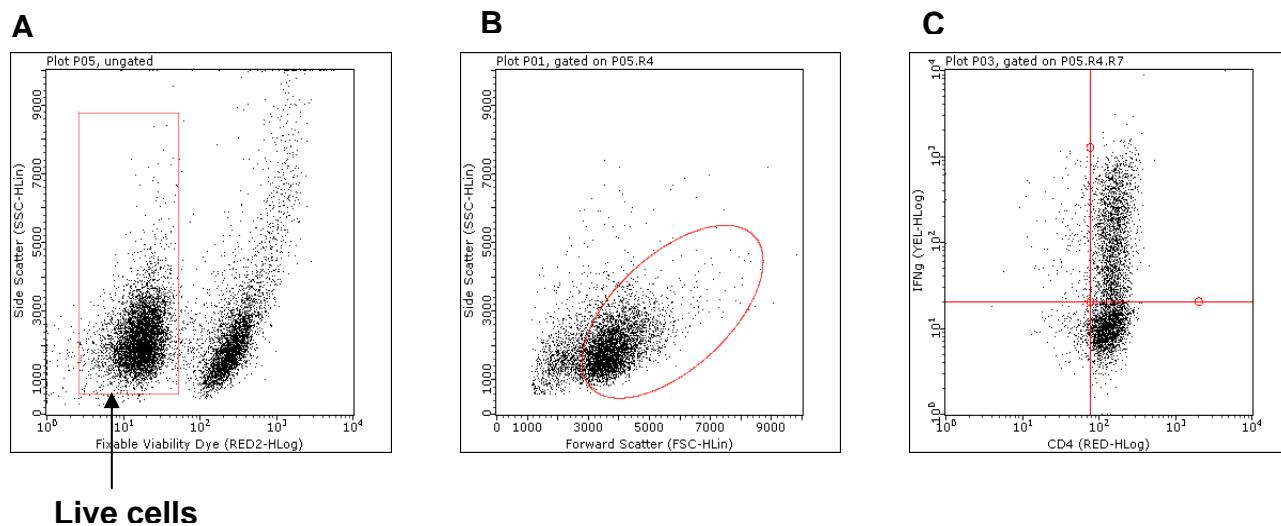


Figure 2: Representative flow cytometric data of T_{H1} differentiated CD4+ mouse T-cells stained with Fixable Viability Dye eFluor®660, anti-CD4-PerCP and anti-IFN γ -PE. Dot plot of ungated cells stained with fixable viability dye (A). Forward and side scatter dot plot of live gated cells from plot A (B). Differentiated T_{H1} CD4+ T-cells gated on lymphocyte population in plot B and stained with anti-CD4-PerCP and anti-IFN γ -PE(C).

Technical Hints

- The Permeabilization Buffer concentrate may have crystallization or precipitation when it is stored at 2-8°C, however, it's normal and does not affect the buffer performance. *Do not heat, vortex or filter.* If crystals form, avoid them when taking out an aliquot.
- 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.
- Certain cell cultures cell pellets may become hazy or transparent following the fixation step, making it difficult to see. After the washing steps they will become easier to visualize.
- 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

Potential Problem	Experimental Suggestions
Acquisition rate decreases dramatically Instrument clogging Too many cells	<ul style="list-style-type: none">• Cell concentration too high - Decrease the number of cells per microliter by diluting sample to 300 – 500 cells per microliter. Guava cytometers give the most accurate data when the flow rate is less 500 cells per microliter.• Run three Quick Cleans (for Guava cytometers) to rinse out the flow cell. 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	<ul style="list-style-type: none">• Spin down cells and resuspend in a smaller volume. The assay instructions are optimized to give you a range of cells between 100-500 cells/µL in the final sample volume. However, cell loss is common during washing steps in the assay procedure. A substantial decrease in cell numbers can lead to difficulty in adjusting settings. Make sure to leave the cell pellet intact when discarding buffer. If the cells are not generating a compact pellet after centrifugation, increase the time to 5 minutes and/or increase the speed by 500 rpm until a compact and visible cell pellet forms.
Background staining and/or non-specific staining of cells	<ul style="list-style-type: none">• This assay was optimized using mouse spleen cells. Therefore, further antibody titrations may be necessary for other cell types and conditions 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.
Variability in day to day experiments	<ul style="list-style-type: none">• Monitor cell viability and cell numbers being analyzed are consistent. Any changes in culture conditions or viability can influence experimental results.• Make sure that a quality check on the flow cytometer to be used (e.g. calibration) is performed on a daily basis prior to use.

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

References

1. Wan YY. Multitasking of helper T cells. *Immunology* 2010 Jun;130 (2):166-71.

Related Products

1. FlowCelect™ Mouse FoxP3 Treg Identification Kit (Catalog No. FCIM025126)
2. FlowCelect™ Human Lymphocyte ZAP-70 Characterization Kit (Catalog No. FCIM025122)

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