FlowCellect™ Mouse Th17 Differentiation Tool Kit

Catalog No. FCIM025163

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Not for use in diagnostic procedures.

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Introduction

CD4+ T cells play a central role in the function of the immune system: They help B cells produce antibodies, enhance and maintain responses of $CD8^+$ T cells, regulate macrophage function, orchestrate immune responses against a wide variety of pathogenic microorganisms, and regulate immune responses (1).

Effector T cells are derived from naïve $CD4^+$ progenitor T cells, after maturational complex interactions with antigen-presenting cells in a permissive milieu, including antigenic type and load, and cytokine signaling. Committed $CD4^+$ T cells can differentiate into four major lineages: Th1, Th2, Th17 and regulatory T cells with distinct cytokine products and biological functions (2-4).

Th17 cells are key mediators in host defense, inflammatory disorders and autoimmune diseases by producing abundant inflammatory cytokines such as IL-17, IL-21 and IL-22. The differentiation to the Th17 lineage is promoted through a series of complex signaling events such as TGFβ and IL-6. They act cooperatively to promote Th17 differentiation. IL-23 is also critical for survival and proliferation of differentiated Th17 cells (5, 6).

In vitro generated mouse Th17 cells have been utilized for intracellular cytokine analysis using flow cytometry, secreted cytokine analysis by ELISA or Milliplex and *in vivo* transplantations for adoptive immunotherapy (7).

EMD-Millipore's FlowCellect™ Mouse Th17 Differentiation Tool Kit is a complete primary culture system designed for the optimal differentiation of naive $CD4^+$ cells into Th17 polarized cells.

Product Overview

The FlowCellect™ Mouse Th17 Differentiation Tool Kit includes a CD3 Coated Activation Plate, complete Differentiation Media and Activator/Re-Stimulation Reagents. Detailed assay instructions are included to assist in three culture steps: Activation, Expansion and Re-Stimulation to complete Th17 cell generation in 6 days.

Day 1=**Activation**: Culture $CD4^+$ cells with growth factors and TCR co-stimulator on an anti-CD3 coated 96-well plate for 3 days.

Day 4=**Expansion**: Expand activated/developing Th17 cells in a larger tissue culture plate.

Day 6=**Re-stimulation**: Differentiated Th17 cells are re-stimulated with mitogens to induce Th17 signature cytokine production on day 6.

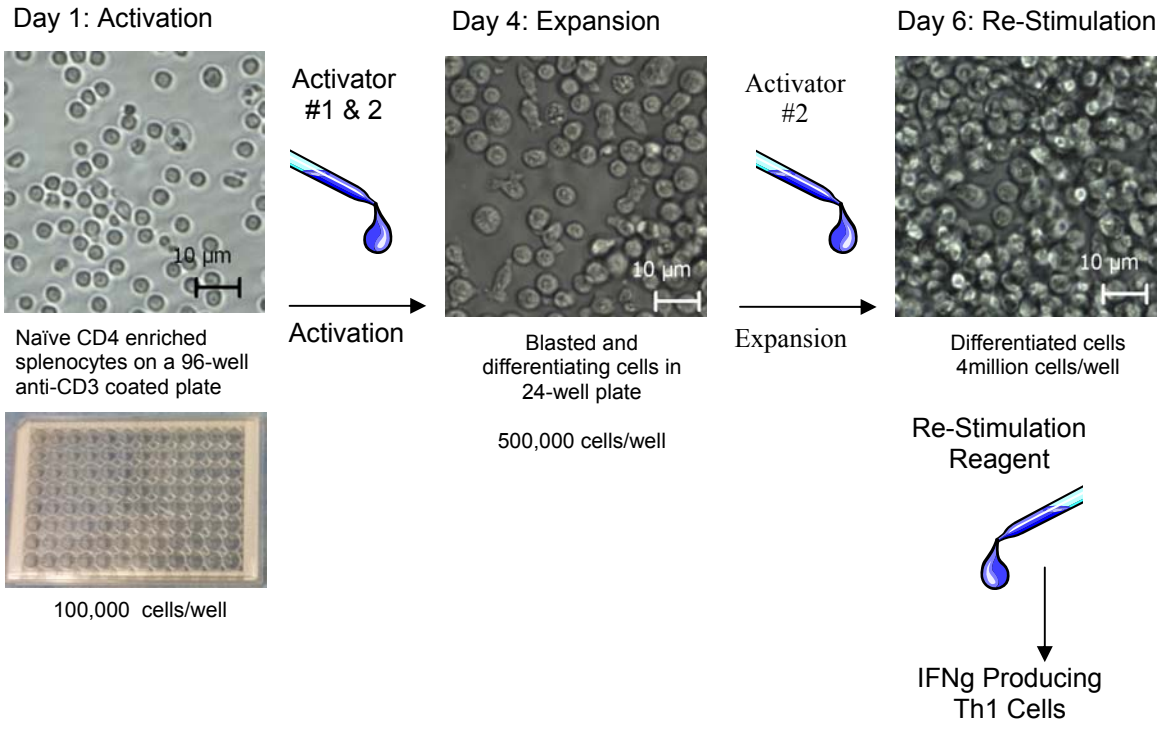


Figure 1. Overview of the differentiation process.

CD4+ Splenocytes are differentiated to the Th2 lineage with single addition of an activator mixture on day 1 and day 4, then re-stimulated on day 6 for cytokine analysis. This optimized process allows for consistent differentiation results.

Each type of effector T cell counter balances each other to achieve immune homeostasis. For example, IFNγ and IL-4 induce Th1 and Th2 differentiation respectively; however they inhibit the Th17 lineage. *In vitro* Th17 differentiation assay, Th17 activator TGFβ, IL-6 and IL-23 are added in the culture along with neutralizing antibodies of IFNγ and IL-4 to enhance and maintain the lineage commitment. Thus, cytokines play crucial roles as both agonists and antagonists for the lineage.

Materials Provided

FCIM025163-1, Store at 2-8°C

1. Mouse CD3 Coated Activation Plate: (Part No. CS206556) One 96-well plate with strips

2. Th17 Activator #1: (Part No. CS206518) One vial containing 63 µL of neutralizing antibodies,

3. Th17 Activator #2: (Part No. CS206519) One vial containing 510 µL of neutralizing antibody

4. Differentiation Media: (Part No. CS206521) One vial containing 200mL of complete Th17 differentiation culture media

FCIM025163-2, Store at -20 ºC

1. Th17 Activator #3: (Part No.CS206516) One vial containing lyophilized growth factors

2. Th17 Activator #4: (Part No.CS206517) One vial containing lyophilized growth factors

3. Re-Stimulation Reagent: (Part No.CS206520) One vial containing 48uL cocktail of PMA and ionomycin in DMSO

Materials Required But Not Supplied

1. Mouse $CD4^+$ enriched splenocytes
2. 24-well tissue culture plates
3. Pipettors with corresponding tips capable of accurately measuring 0.1 – 1000 µL
4. Tabletop centrifuge capable of achieving 400 x g
5. Sterile Deionized water
6. Scepter for performing cell counts
7. FlowCellect™ Mouse Th17 Intracellular Cytokine Kit (Viability dye, Brefeldin-A, Primary conjugated antibodies for signature cytokine detection).

Warnings and 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. Please refer to the MSDS sheet for specific information on hazardous materials (MSDS forms can be obtained by contacting Millipore technical services).
- 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 all solutions, which may cause erroneous results.
- Do not use reagents beyond their expiration date.
- Wear gloves when using this product. Avoid skin contact or ingestion of all reagents and chemicals used in this protocol.
- Protease Inhibitor Cocktail III contains DMSO, avoid contact with skin.

Storage and Stability

- Anti-CD3 Coated 96-well Activation Plate, Differentiation Media and Th17 Activator #1 and Th17 Activator #2 must be stored at 2 – 8 ºC.
- Unopened vial of lyophilized Th17 Activator #3, Th17 Activator #4 and Re-Stimulation Reagent must be stored at -20 ºC.
- After combined Th17 Activator #1, #2 and #3, or #2, #3 and #4, those mixtures must be stored at 2 – 8 ºC.

All kit components are stable for four (4) 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

Note: Perform all steps under a certified tissue culture hood using sterile materials and aseptic technique.

1. **Reconstitution of Th17 Activator #3;** after a brief spin, reconstitute in 70 uL of sterile water and mix by gentle pipetting then spin down again. Make sure that all lyophilized powder is dissolved in water. Avoid repeated freeze and thaw cycles.

2. **Reconstitution of Th17 Activator #4;** after a brief spin, reconstitute in 70 uL of sterile water and mix by gentle pipetting then spin down again. Make sure that all lyophilized powder is dissolved in water. Avoid repeated freeze and thaw cycles.

3. **Mixture of Th17 Activator #1**, **#3 and #4;** Transfer 8 µL of reconstituted Th17 Activator #3 and 8 uL of reconstituted Th17 Activator #4 into Th17 Activator #1 vial. Once combined, the mixture must be stored at 2 - 8ºC. See Figure 2.

Note: After making Th17 Activator #1, #3 and #4 mixtures, keep unused portion of Activator #3 and #4 at 2 - 8ºC for the Day 4 expansion step.

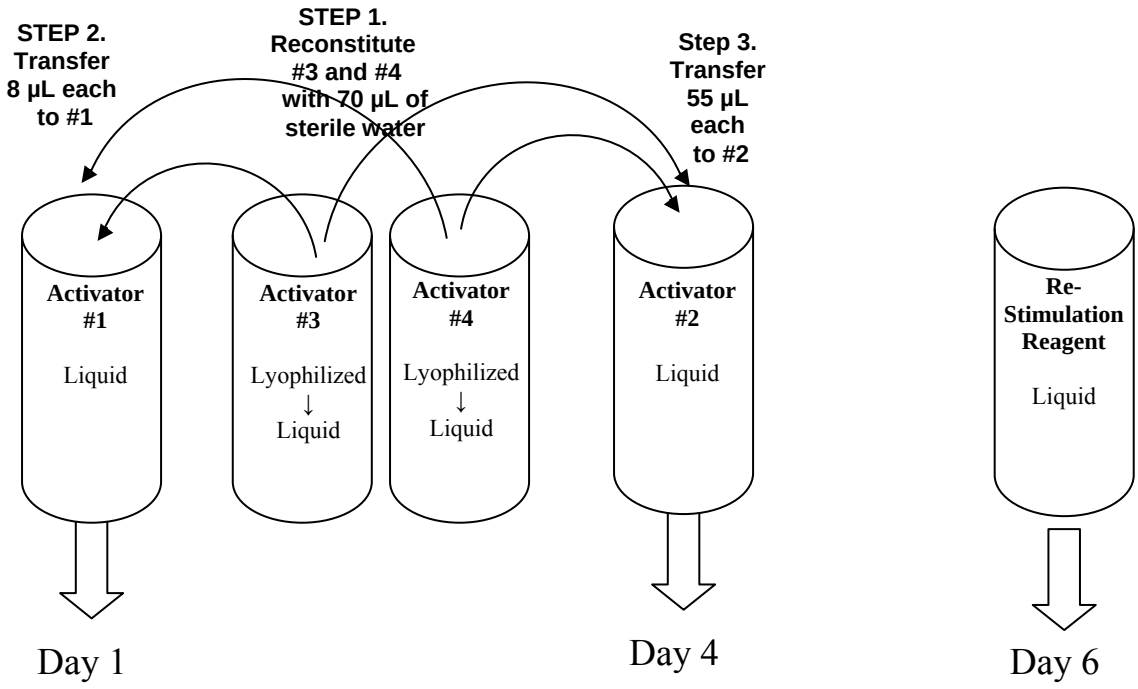
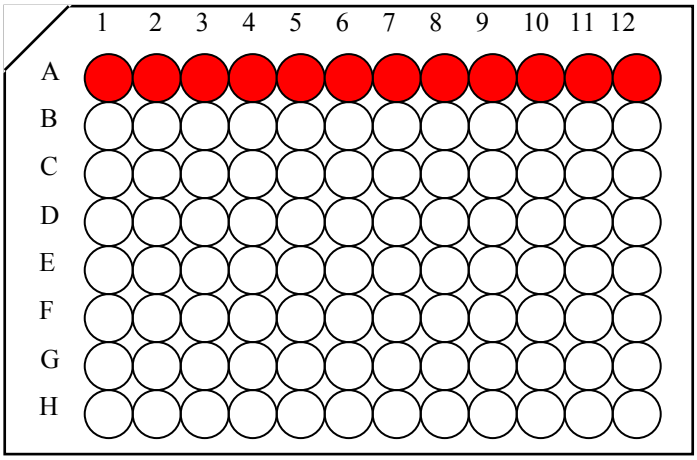


Figure 2. Reconstitution and Mixing of Activation Reagent.

4. **Mixture of Th17 Activator #2**, **#3 and #4;** Transfer 55 µL of reconstituted Th17 Activator #3 and 55 µL of reconstituted Th17 Activator #4 into Th17 Activator #2 vial. Once combined, the mixture must be stored at 2 – 8 ºC. **CD3 Coated Activation Plate:** Pre-warm strips needed at RT.

   Note: Anti-CD3 antibody is coated only on 12 wells of Row A (from well #A1 to A12). Unused strips should be kept in sterile condition in the foil pouch with desiccant provided and stored at 2-8 ºC. The strips only fit in one direction on the plate so Row A will always contain the coated wells.



5. **Differentiation Media:** Pre-warm media at RT.

6. **Re-Stimulation Reagent:** Thaw and aliquot if using on different days. One vial contains 48 µL; You use 2 µL of the reagent per 2 million expanded cells on Day 6. Store the aliquots at -20 ºC, single use only.

Preparation of Cells

Isolate CD4+ T cells from mouse splenocytes with your preferred sorting method. We recommend starting with CD4+ T cell enriched splenocytes with more than 90% CD4+ purity and 90% viability.

Assay Instructions

Note: This assay protocol has been optimized for naïve CD4+ enriched splenocytes derived from both naïve BALB/c and C57BL strains, 7-12 week-old mice. Other strains have not been tested, but, are expected to perform similarly.

Protocol for Th17 Differentiation

Note: Perform all steps including reagent preparations under a certified tissue culture hood using sterile materials and aseptic technique. Keep all activator vials on ice except Re-Stimulation Reagent which contains DMSO. Pre-warm the plate and media at RT.

Day 1: Start Activation of Naïve CD4+ T Cells

Materials for Day 1

- CD3 Coated Activation Plate
- Mixture of Th17 Activator #1, #3 and #4 (See "Preparation of Reagents" section for details)
- Differentiation Media
- CD4+ enriched splenocytes not included

1. Prepare CD4+ enriched splenocytes.
2. Wash cells with Differentiation Media once and resuspend in Differentiation Media at a concentration of $8X10^5$ cells / mL.
3. Plate $2X10^5$ cells (250 µL) into a CD3 coated well on Row A.
4. Apply 6 µL of mixture of Th17 Activator #1, #3 and #4 to each well. Mix well.
5. Place the 96-well plate at 37 ºC in a CO_2 humidified incubator for 3 days.

Day 4 : Expansion of Activated Th1 Cells

Materials for Day 4

- Mixture of Th17 Activator #2, #3 and #4 (See "Preparation of Reagents" section for details)
- Differentiation Media
- 24-well plate not included

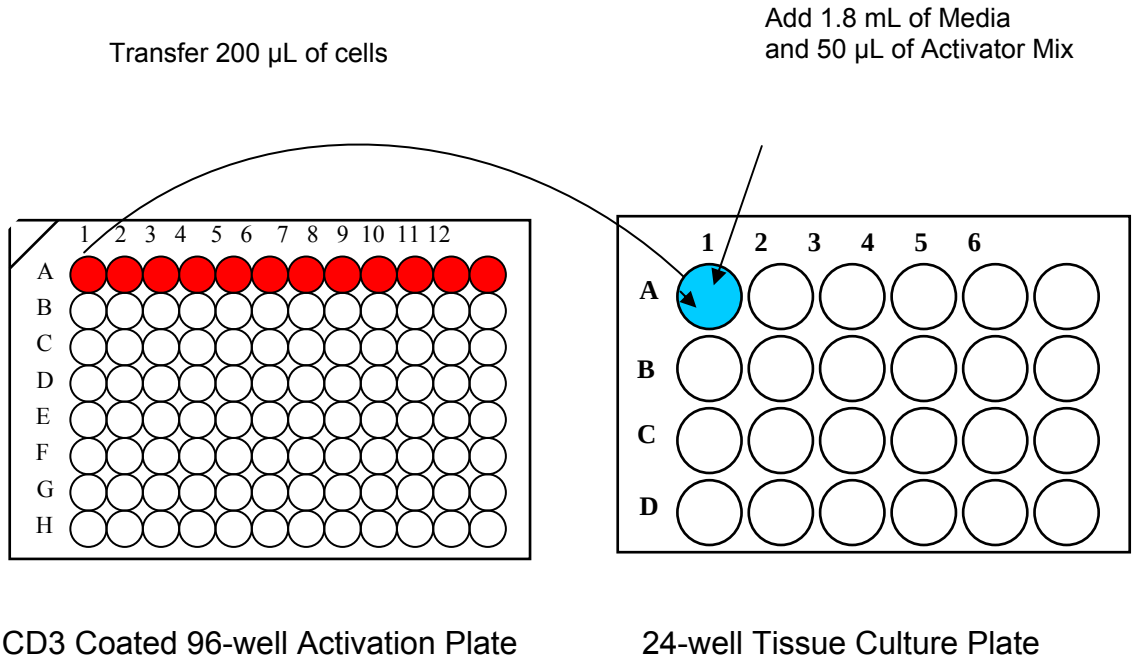
Expand activated cells at 1:10 in Differentiation Media. We recommend using single well of a 24-well plate.

Note; Cells should start blasting and forming small cell aggregates which can be seen under a microscope at this point in the culture.

<u>24-well plate;</u>

6. Suspend Th17 activated cells in the CD3 Coated Activation 96-well Plate vigorously by pipeting upside down and scraping out from bottom of the well with pipette tip.

7. Transfer 200 µL of the activated cell suspension from a 96-well plate to a single well of a 24-well plate.



CD3 Coated 96-well Activation Plate 24-well Tissue Culture Plate

8. Apply 1.8 mL of Differentiation Media to each well for a total media volume of 2.0 mL.

9. Apply 50 µL of Th1 Activator #2, #3 and #4 mixture to each well.

10. Incubate at 37 ºC in a CO_2 humidified incubator for 2 days.

Day 6: Re-Stimulation

Materials for Day 6

- Re-Stimulation Reagent
- Differentiation Media
- 24-well plate not included

On day 6, differentiated Th17 cells are re-stimulated with mitogens to induce Th17 cytokine production. We recommend using a 24-well plate for intracellular cytokines by flow analysis or a 96-well plate for secreted cytokine analysis by ELISA or Milliplex. Total cell numbers should be approximately 1-5 $X10^6$ per well by day 6.

24-well plate;

11. Count cells using Scepter or other method which uses a small sample size and aliquot 2 X 10^6 cells from each well into conical tubes.

12. Spin down the cells at 400 x g for 5 minutes in 4 ^oC centrifuge and discard supernatant.

13. Add 2 mL of Differentiation Media to the cell pellet (2 X 10^6) to bring the cell concentration to 1x 10^6 cells/mL. Resuspend well.

Note: For intracellular cytokine analysis, add a protein transporter inhibitor such as Brefeldin A at the same time of adding Re-Stimulation Reagent.

15. Add 2 µL of Re-Stimulation Reagent to each tube. Mix well.

16. Transfer 2 mL of the cell suspension to each well of a 24-well plate.

17. Incubate at 37ºC in a CO_2 humidified incubator for 3-5 hours.

18. Harvest cells for analysis.

This step can be scaled up or down using 6- or 96-well plates as follows;

96-well plate; Plate 4 X 10^5 cells / 200 µL into each well of a 96-well plate and add 0.2 µL of Re-Stimulation Reagent. Incubate 3-5 hours.

6-well plate; Plate 6 X 10^6 cells / 6 mL into each well of a 6-well plate and add 4 µL of Re-Stimulation Reagent. Incubate for 5 hours.

Sample Data

Intracellular Cytokine Analysis by Guava Flow Cytometry using combinations of FlowCellect™ Mouse Th1, Th2 and Th17 Intracellular Cytokine Kits.

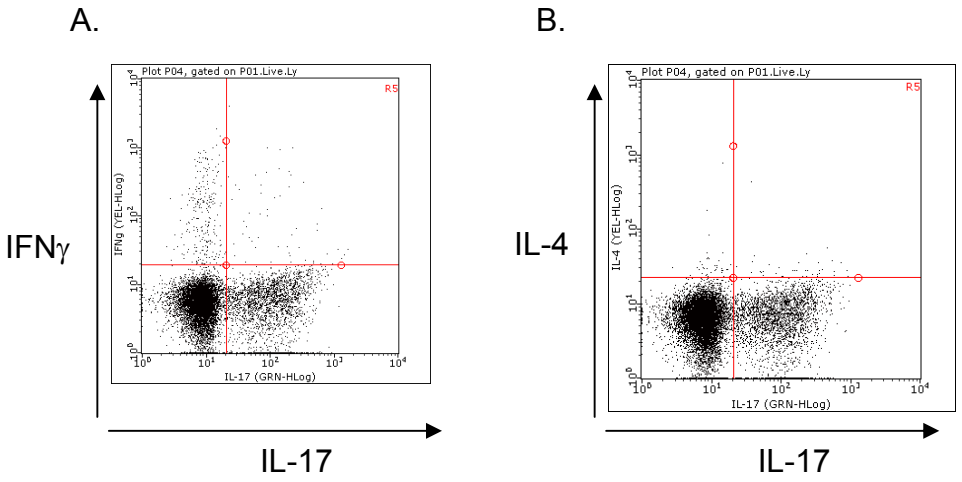


Figure 3: Representative flow cytometric data of mouse splenocytes following a 6 day directed differentiation to the Th17 lineage with optimized growth factor and antibody cocktails. The samples were stained with Fixable Viability Dye to gate out of live cells and CD4+ cells were dual stained with anti-IFNγ/IL-17 (Plot A), anti-IL-4/IL-17 (Plot B).

Secreated Cytokine Analysis by Milliplex®.

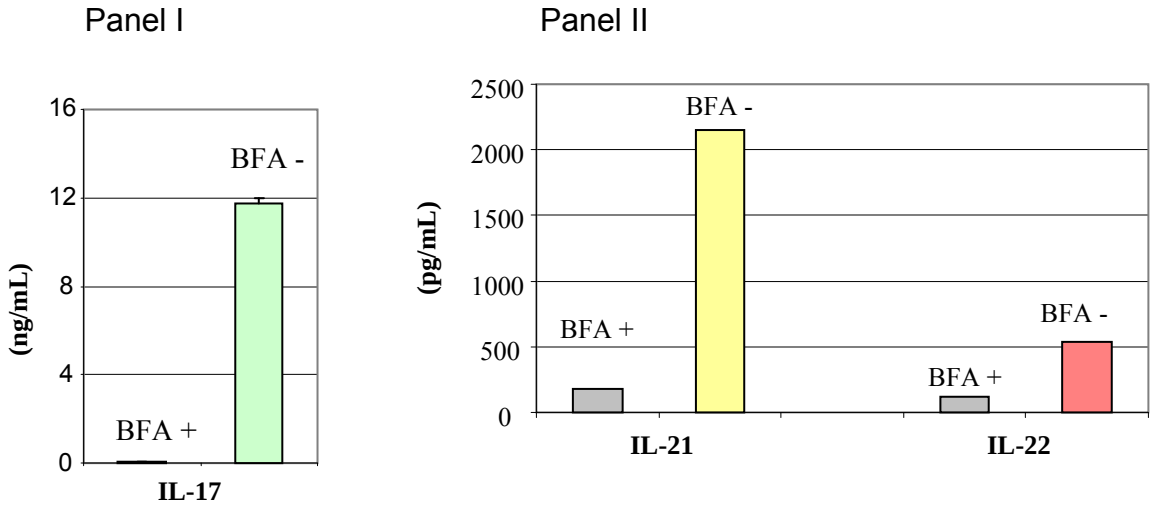


Figure 4: Representative Milliplex® data of Th17 differentiated cell culture supernatants. Milliplex® Mouse Cytokine/Chemokine Panel I and Panel II were used to run the culture supernatants. The data was then analyzed for Th17 signature cytokines IL-17, IL-21, and IL-22. BFA=Brefeldin A.

Technical Hints

- For activation and differentiation to be most effective, make sure that enriched $CD4^+$ cells have good viability and purity prior to culture.
- Perform all steps including reagent preparations under a certified culture food using sterile materials and aseptic technique.
- Expansion and re-stimulation steps can be scaled up and down for your needs. Make sure that you have enough reagents before alter the protocols.
- Do not mix or interchange reagents from various kit lots.
- Scepter can be used throughout the protocol to determine cell concentration without significant loss of sample.

Troubleshooting

Step	Potential Problems	Experimental Suggestions
Reagent Preparation		• If storing at -20ºC or for lyophilized agents, place tubes at room temperature and briefly spin, prior to use. • After reconstitution of Activator #2 with sterile water, set the vial at room temperature for 5 minutes to allow powder to dissolve completely and spin briefly.
Culture	Poor cell proliferation	• Cells should start blasting and proliferating by the end of 2nd day of the culture. Check cells under a microscope and see if you see any morphological changes such as increase in cell size or irregular cell shape as shown in pictures on page XX in this manual. • Check an orientation of cell culture strip on 96-well plate and make sure CD3-coated wells are used. The strips only fit one way so use only 12 wells of Row A. Wells of from row B to H are non-coated; using these wells gives no TCR activation which is required for the primary CD4+ cell proliferation. • Check Activator #1 for Day 1 culture and make sure Activator #2 was added correctly. If you did not add this mixture on day1, add 0.5 uL of Activator #2 ONLY on day 2 (not the mixture of #1 and #2) and incubate another 3 days.
Culture	Cell death	• A concentration of cells was too low or too high. Count cells on day 1 before plating cells as well as on day 6 before re-stimulation. Make sure you use right cell concentration which we suggested in this manual. • Check an orientation of cell culture strip on 96-well plate and make sure CD3-coated wells are used. The strips only fit one way so use only 12 wells of Row A. Wells of from row B to H are non-coated; using these wells gives no TCR activation which is required for the primary CD4+ cell proliferation.

Related Products

FCIM025123 - FlowCellect™ Mouse Th1 Intracellular Cytokine Kit

FCIM025124 - FlowCellect™ Mouse Th2 Intracellular Cytokine Kit

FCIM025125 - FlowCellect™ Mouse Th17 Intracellular Cytokine Kit

FCIM025137 - FlowCellect™ Mouse Th1/Th2 Intracellular Cytokine Kit

FCIM025138 - FlowCellect™ Mouse Th1/Th17 Intracellular Cytokine Kit

FCIM025154- FlowCellect™ Mouse Breg Characterization Kit

FCIM025168- FlowCellect™ Mouse Viable Treg Characterization Kit

FCIM025159- FlowCellect™ Human Memory B Cell Function Kit

FCIM025118 - FlowCellect™ Human FOXP3 Treg Characterization Kit

FCIM025161- FlowCellect™ Mouse Th1 Differentiation Tool Kit

FCIM025162- FlowCellect™ Mouse Th2 Differentiation Tool Kit

FCIM025166-FlowCellect™ Mouse Treg Differentiation Tool Kit

References

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