FlowCellect™ Mouse Treg Differentiation Tool Kit

12 Tests

Cat. No. FCIM025166

FOR RESEARCH USE ONLY
Not for use in diagnostic procedures.

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Application

$CD4^+$ 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).

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. 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 (5-8).

In vitro generated mouse induced Treg cells have been utilized for intracellular cytokine analysis using flow cytometry, secreted cytokine analysis by ELISA or Luminex and *in vivo* transplantations for adoptive immunotherapy (9, 10).

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

Assay Principle

The FlowCellect Mouse™ Treg Differentiation Tool Kit includes a CD3 Coated Activation Plate, complete Differentiation Media and Activators. Detailed assay instructions are included to assist in two culture steps: Activation and Expansion to complete Treg cell generation in 7 days.

Day 1=Activation: Culture CD4+ cells with growth factors on an anti-CD3 coated 96-well plate for 3 days.
Day 4=Expansion: Expand activated/developing Treg cells in a larger tissue culture plate.
Day 7=Harvest: Differentiated Treg cells are harvested for Foxp3, CD25 and CD4 expression analysis.

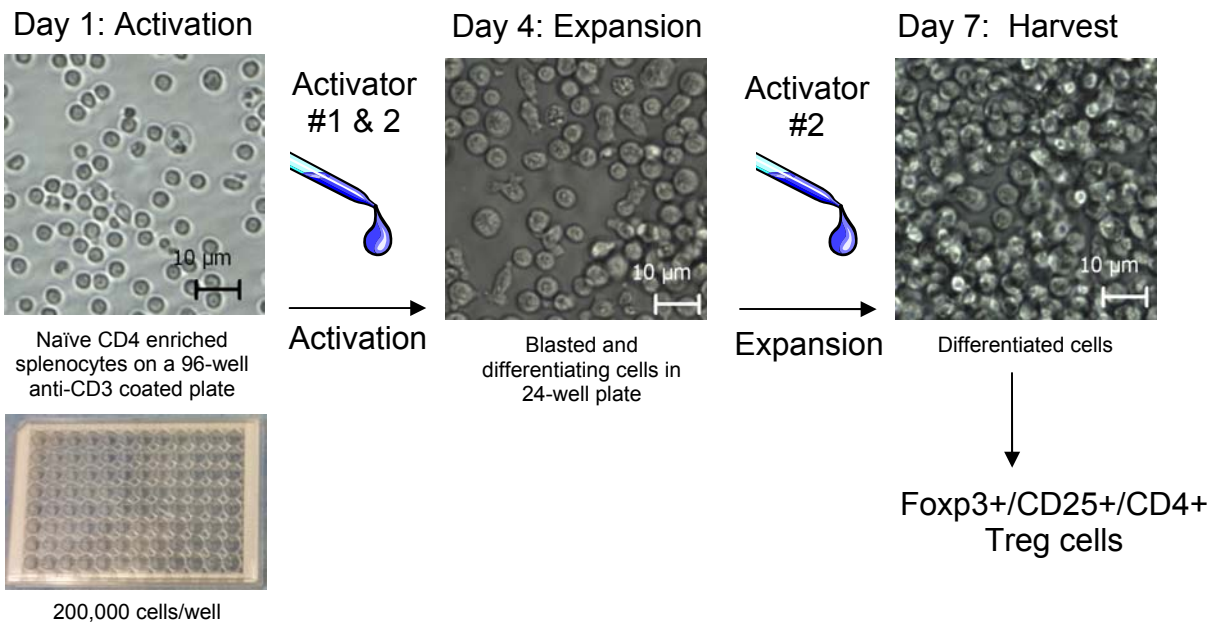


Figure 1. Overview of the differentiation process.
CD4+ Splenocytes are differentiated to the induced Treg lineage with single addition of an activator mixture on day 1 and day 4, then harvested on day 7 for Foxp3, CD25 and CD4 analysis.

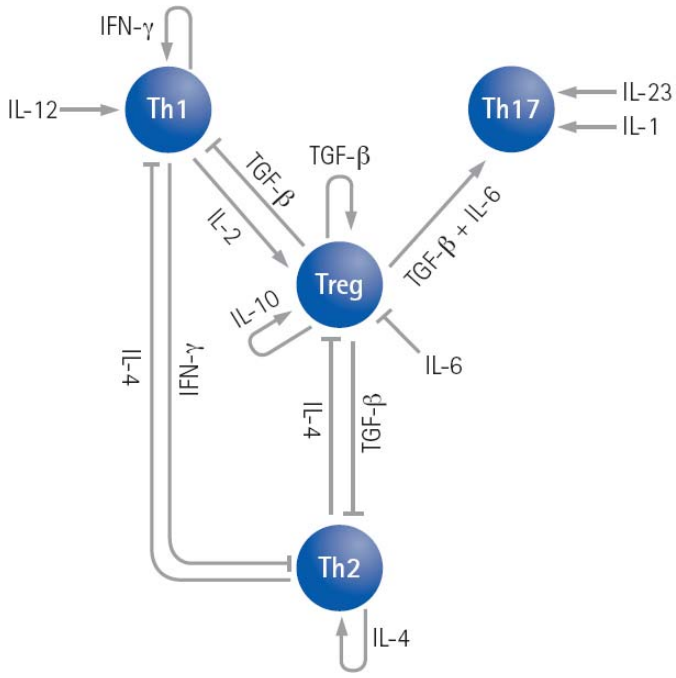


Fig. 2 Cytokine Balance in Effector T Cell Differentiation
Each type of effector T cell counter balances each other to achieve immune homeostasis. For example, IL-6 induces Th17 differentiation, however it inhibits the Treg lineage. *In vitro* Treg differentiation assay, Treg activator TGFbeta is added in the culture along with neutralizing antibody anti-IL-6 to enhance and maintain the lineage commitment. Thus, cytokines play crucial roles as both agonists and antagonists for the lineage.

Kit Components

FCIM025166-1 (2-8 ºC)

- Mouse CD3 Coated Activation Plate: (Part No. CS206556) One 96-well plate with strips
- Treg Activator #1: (Part No. CS206530) One vial containing 12 µL of neutralizing antibodies
- Differentiation Media: (Part No. CS206524) One vial containing 200 mL of complete Treg differentiation culture media

FCIM025166-2 (-20 ºC)

- Treg Activator #2: (Part No.CS206531) One vial containing lyophilized growth factors

Materials 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 600 x g
5. Sterile Deionized water
6. FlowCellect™ Mouse Viable Treg Characterization Kit (Viability Dye, Conjugated antibodies for detection of CD4, CD25, and Foxp3). **Cat. No. FCIM025168**
7. Scepter Cell counter. **Cat. No. PHCC20040**

Precautions

1 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.

2 Wear proper laboratory attire (lab coat, gloves, safety glasses) when handling or using this product.

3 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).

4 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.

5 Avoid microbial contamination of all solutions, which may cause erroneous results.

6 Do not use reagents beyond their expiration date.

Storage/Handling

- Anti-CD3 Coated 96-well Activation Plate, Differentiation Media and Treg Activator #1 must be stored at 2 – 8 ºC. Unopened vial of lyophilized Treg Activator #2 must be stored at -20 ºC.

- After combined Treg Activator #1 and #2, the mixture must be stored at 2 – 8 ºC.

All kit components are stable up to six (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 Treg Activator #2;** After a brief spin, reconstitute in 140 µL 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. **Mixture of Treg Activator #1** and **#2;** Transfer 30 µL of reconstituted Treg Activator #2 into Treg Activator #1 vial. Once combined, the mixture must be stored at 2 – 8 ºC. See Figure 3.

Note: After making Treg Activator #1 and #2 mixture, keep unused portion of Activator #2 at 2 – 8 ºC for the Day 4 expansion step.

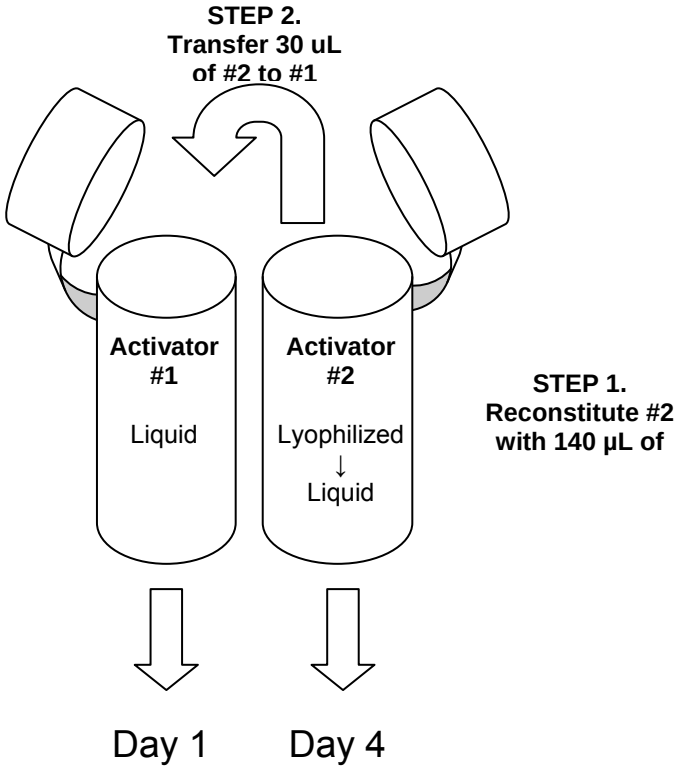
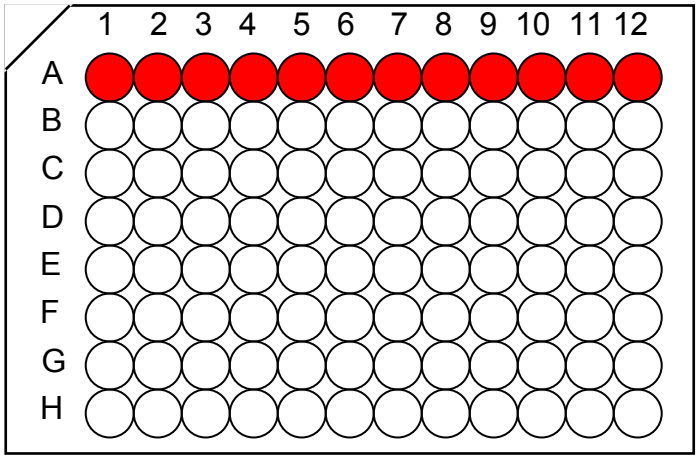


Figure 3. Reconstitution and Mixing of Activation Reagent.

3. **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.



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

5. **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 Treg 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. 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 Treg Activator #1 and #2 (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 3 µL of mixture of Treg Activator #1and #2 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 Treg Cells

Materials for Day 4

- Mixture of Treg Activator #2
- Differentiation Media
- 24-well plate not included

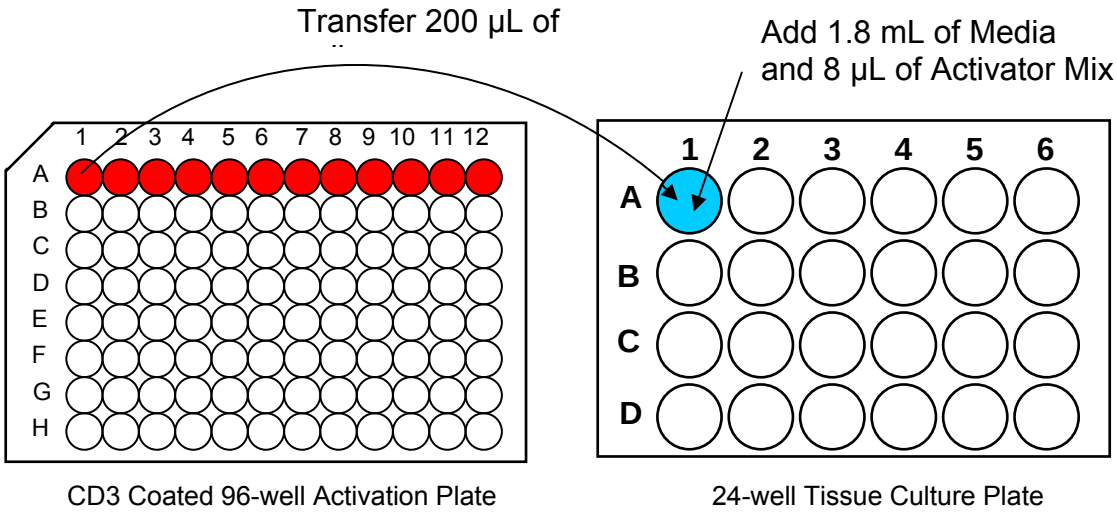
Expand activated cells at 1:10 in Differentiation Media. We recommend using single well of a 24-well plate.

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

24-well plate:

6. Suspend Treg activated cells in the CD3 Coated Activation 96-well Plate vigorously by pipeting up side down and scraping out from bottom of the well with pippet tip.

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



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

9. Apply 8 µL of Treg Activator #2 to each well.

10. Incubate at 37 ºC in a CO_2 humidified incubator for 3 days.

Day 7: Harvest

On day 7, differentiated Treg cells are harvested for Foxp3, CD25 and CD4 analysis. Total cell numbers should be approximately 1-5 $X10^6$ per well by day 7.

Sample Data

Intracellular Cytokine Analysis by Guava Flow Cytometry using FlowCellect™ Mouse Viable Treg Characterization Kit.

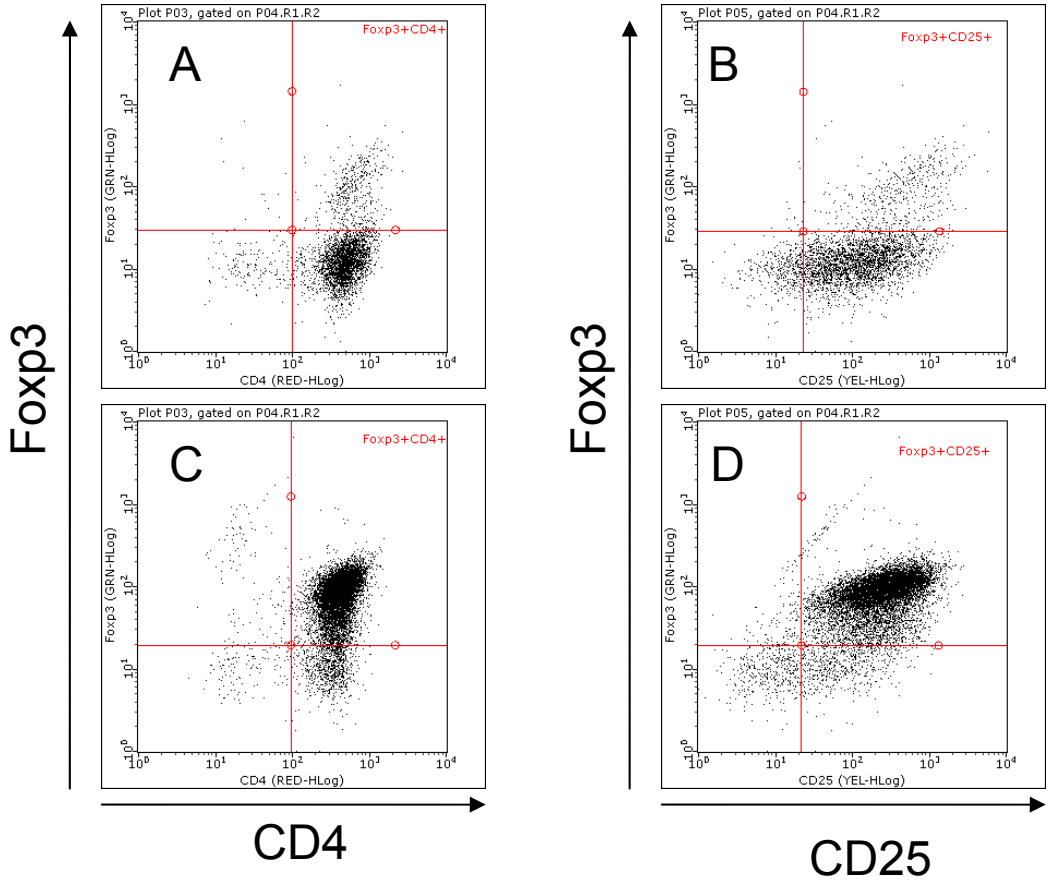


Figure 4: Representative flow cytometric data of mouse naïve splenocytes (top panels A and B) and following a 7 day directed differentiation to the Treg lineage with optimized growth factor and antibody cocktails (bottom panels C and D) . The samples were stained with Fixable Viability Dye to gate out dead cells and only live cells were analyzed for anti-Foxp3/CD4 (left column A and C) or anti-Foxp3/CD25 (right column B and D).

Troubleshooting

To obtain reliable and producible results the operator should carefully read this manual and fully understand all aspects of each assay step before beginning the culture.

Assay Step	Potential Problem	Experimental Suggestions
Reagent Preparation		• If storing at -20 ºC 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 µL of Activator #2 ONLY on day 2 (not the mixture of #1 and #2) and incubate another 3 days.
Culture	Many 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.
Culture	contamination	• Check all material used was sterile including cell preparation step and water added for reconstitution of Activator(s). All materials in this kit are provided as sterile.

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

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.
- Use a Scepter for performing cell counts to reduce the amount of sample loss during determination of cell concentrations.

References

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