DATA EXAMPLES

Differentiation of naïve CD4$^+$ T cells into Treg cells is confirmed by intracellular staining for FoxP3 (Figure 1).

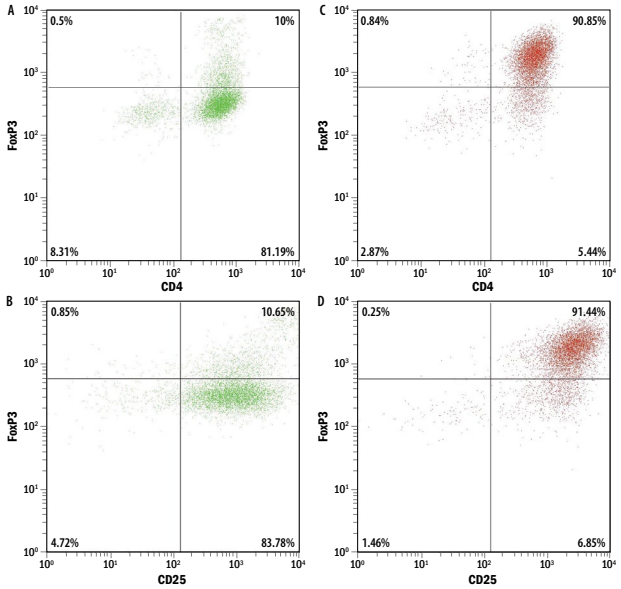

Figure 1: Intracellular Staining of Differentiated Treg Cells. Flow cytometry data showing human peripheral blood naïve CD4$^+$ T cells without (A, B) and with (C, D) a 5 day differentiation using reagents included in the Human Treg Cell Differentiation Kit. On day 5 of differentiation, the cells were fixed, permeabilized, and stained using the FlowX Human Regulatory T Cell Multi-Color Flow Kit (R&D Systems, Catalog # FMC021). Quadrants were set based on isotope-stained samples.

REFERENCES

1. Feuerer, M. *et al.* (2009) Nat. Immunol. **10**:689.
2. Liston, A. and D.H. Gray (2014) Nat. Rev. Immunol. **14**:154.
3. Campbell, D.J. and M.A. Koch (2011) Nat. Rev. Immunol. **11**:119.

NOTES

CellXVivo™

Human Treg Cell Differentiation Kit

Catalog Number: CDK006

BACKGROUND

CD4$^+$ T cells differentiate into T helper cells under the influence of various cytokines and cellular interactions that induce expression of specific transcription factors. Naïve CD4$^+$ T cells can be induced to Forkhead Box P3 (FoxP3)$^+$ regulatory T (Treg) cells by activation in the presence of IL-2 and TGF-β *in vitro* (1). Treg cells are a suppressive subset of CD4$^+$ T cells that function to antagonize immune responses. Treg cells have the capacity to prevent potentially damaging autoimmune and protective immune responses, so the number of Treg cells is a crucial determinant of the regulatory burden on the immune system (2). Treg cells prevent autoimmune disease, maintain immune homeostasis, and modulate immune responses during infection (3). The Human Treg Cell Differentiation Kit contains specially formulated reagents and growth factors to differentiate human naïve CD4$^+$ T cells into FoxP3$^+$CD25$^+$ Treg cells.

This package insert must be read in its entirety before using this product.
For research use only. Not for use in diagnostic procedures.

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MATERIALS PROVIDED & STORAGE CONDITIONS

Store the unopened kit at -20 °C in a manual defrost freezer.
Do not use past kit expiration date.

COMPONENTS	PART #	# VIALS	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
Mouse Anti-Human CD3	967817	1 vial	May be stored at 2-8 °C under sterile conditions for up to 30 days or at -20 °C to -70 °C in a manual defrost freezer for up to 3 months.*
Human Treg Reagent 1	967819	1 vial	
Human Treg Reagent 2	967820	1 vial	
Human Treg Reagent 4	967822	1 vial	
Human Treg Reagent 3	967821	1 vial	May be stored at -20 °C to -70 °C under sterile conditions in a manual defrost freezer for up to 3 months.*
Reconstitution Buffer 1	967552	1 vial	May be stored under sterile conditions for up to 3 months at 2-8 °C.*
Reconstitution Buffer 2	967553	1 vial	
20X Wash Buffer	967557	3 vials	

* Provided this is within the expiration date of the kit.

OTHER MATERIALS & SUPPLIES REQUIRED

- Ficoll-Hypaque™
- MagCellect™ Human Naïve CD4⁺ T Cell Isolation Kit (R&D Systems, Catalog # MAGH115, or equivalent)
- X-VIVO™15 Chemically Defined, Serum-free Hematopoietic Cell Medium (Lonza, or equivalent)
- Sterile deionized water
- Tissue culture flasks and/or plates
- Microscope
- Hemocytometer
- 37 °C, 5% CO_2 incubator
- Centrifuge
- Pipettes and pipette tips

REAGENT PREPARATION

Human Treg Differentiation Media

1. Reconstitute Human Treg Reagent 1 and Human Treg Reagent 2 each with 125 µL of Reconstitution Buffer 1; these are 400X stocks.
2. Reconstitute Human Treg Reagent 4 with 125 µL of Reconstitution Buffer 2; this is a 400X stock.
3. Add 62.5 µL each of Human Treg Reagents 1, 2, and 4, and 25 µL of Human Treg Reagent 3 (1000X) to 24.8 mL of X-VIVO 15 serum-free medium.

Human CD3 Antibody

1. Reconstitute the Mouse Anti-Human CD3 antibody with 125 µL of Reconstitution Buffer 2; this is a 100X stock.
2. Add 10 mL of 20X Wash Buffer to 190 mL of sterile deionized water to prepare 200 mL of 1X Wash Buffer.
3. Just before coating, dilute the 100X antibody stock 100-fold with 1X Wash Buffer.

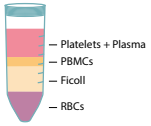
PROTOCOL FOR Treg DIFFERENTIATION

1. Coat a plate with Mouse Anti-Human CD3 antibody.
   a. For a 24-well plate, add 250 µL/well of diluted CD3 antibody. For a 6-well plate, add 1 mL/well of diluted CD3 antibody.
   b. Incubate at 2-8 °C overnight.
   c. Wash the plate with 1X Wash Buffer once before use.
2. Isolate human peripheral blood mononuclear cells (PBMCs) from human blood using Ficoll-Hypaque density gradient centrifugation.
3. Isolate human naïve CD4⁺ T cells from human PBMCs using the MagCellect Human Naïve CD4⁺ T Cell Isolation Kit.
4. Suspend human naïve CD4⁺ T cells at 0.5 -1.0 x 10^6 cells/mL in Human Treg Differentiation Media.
5. Add the cells to a human CD3 antibody-coated plate. For a 24-well plate, add 1 mL/well. For a 6-well plate, add 4 mL/well.
6. Incubate the cells in a 37 °C, 5% CO_2 humidified incubator for 5 days.
7. After 5 days of differentiation, the differentiated Treg cells are ready for downstream applications.
8. **Optional:** To verify Treg cell differentiation via flow cytometry, collect the cells and wash with PBS once. Process, stain, and analyze the cells using the FlowXTM Human Regulatory T Cell Multi-Color Flow Kit (R&D Systems, Catalog # FMC021). Analyze marker expression via flow cytometry as shown in the Data Examples.

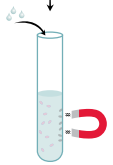
PROTOCOL OUTLINE

Coat wells of a 24-well plate with Anti-Human CD3 Antibody.

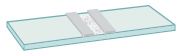
Isolate PBMCs from human blood.



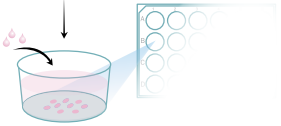
Isolate human naïve CD4⁺ T cells from PBMCs (e.g., using magnetic cell selection).



Perform a cell count.



Suspend 0.5-1.0 x 10^6 naïve CD4⁺ T cells/mL in Human Treg Differentiation Media. **Culture** the cells on plates pre-coated with CD3 antibody for 5 days.



Verify Treg cell differentiation by analyzing marker expression via flow cytometry **(optional)**.