DATA EXAMPLES

Differentiation of naïve CD4$^+$ T cells into Th17 cells is confirmed by intracellular staining for IL-17 (Figure 1) and secretion of IL-17 (Figure 2). The corresponding tests for IFN-γ (Th1 cell marker) and IL-4 (Th2 cell marker) are low/negative.

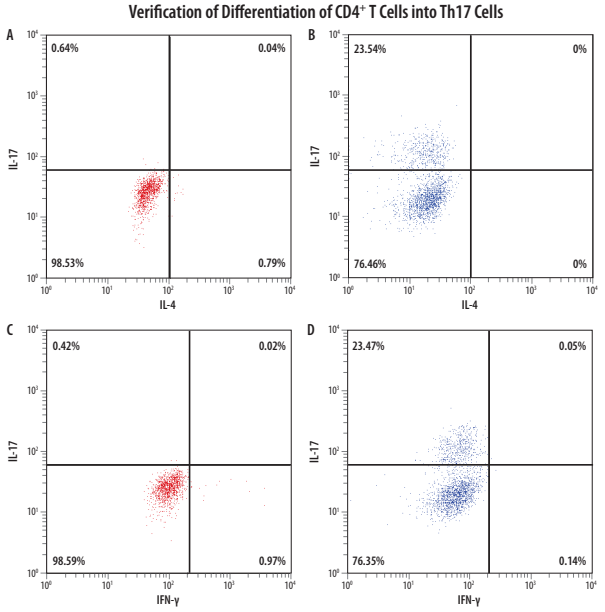

Figure 1: Intracellular Cytokine Staining of Differentiated Human Th17 Cells. Flow cytometry data showing human peripheral blood naïve CD4$^+$ T cells without (A, C) and with (B, D) a 10 day differentiation using reagents included in the Human Th17 Cell Differentiation Kit. On day 10 of differentiation, the cells were stimulated with Cell Activation Cocktail (Tocris®, Catalog # 5476) and stained with Human IL-17, Human IFN-γ, and Human IL-4 Monoclonal Antibodies. Quadrants were set based on isotype-stained samples. All R&D Systems® antibodies and corresponding catalog numbers used in this figure are shown below.

SUGGESTED REAGENTS FOR FLOW CYTOMETRY

CATALOG #	DESCRIPTION
IC317A , and IC0041A	Human IL-17 APC MAb (Clone 41809), Mouse IgG_{2B}, and Mouse IgG_{2B} APC Isotype Control (Clone 133303)
IC285P, and IC0041P	Human IFN-γ PE (Clone 25723), Mouse IgG_{2B}, and Mouse IgG_{2B} PE Isotype Control (Clone 133303)
IC204F, and IC002F	Human IL-4 Fluorescein MAb (Clone 3007), Mouse IgG_1, and Mouse IgG_1 Fluorescein Isotype Control (Clone 11711)
FAB3791C, and IC003C	Human CD4 PerCP MAb (Clone 11830), Mouse IgG_{2A}, and Mouse IgG_{2A} PerCP Isotype Control (Clone 20102)
FC004	Flow Cytometry Fixation Buffer (1X)
FC005	Flow Cytometry Permeabilization/Wash Buffer (1X)

DATA EXAMPLES *CONTINUED*

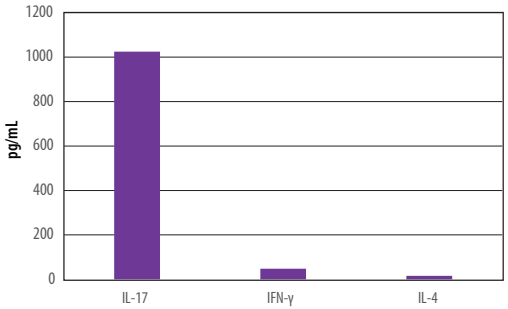

Figure 2: Th17-differentiated Human CD4$^+$ Cells Secrete IL-17. Human peripheral blood naïve CD4$^+$ T cells were differentiated for 10 days using the reagents included in the Human Th17 Cell Differentiation Kit. On day 10, cell culture supernatant was collected and cytokine secretion was determined using the Human IL-17 Quantikine® ELISA Kit, the Human IFN-γ Quantikine® ELISA Kit, and the Human IL-4 Quantikine® ELISA Kit. All relevant R&D Systems Quantikine® ELISA kits and corresponding catalog numbers are listed below.

SUGGESTED REAGENTS FOR ELISA

CATALOG #	DESCRIPTION
D1700, or DY317	Human IL-17 Quantikine® ELISA Kit, or Human IL-17 DuoSet® ELISA
DIF50, or DY285	Human IFN-γ Quantikine® ELISA Kit, or Human IFN-γ DuoSet® ELISA
D4050, or DY204	Human IL-4 Quantikine® ELISA Kit, or Human IL-4 DuoSet® ELISA

REFERENCES

1. Sundrud, M.S. and C. Trivigno (2013) Semin. Immunol. **25**:263.
2. Luckheeram, R.V. *et al.* (2012) Clin. Dev. Immunol. **2012**:925135.
3. Hirahara, K. *et al.* (2011) Immunology **134**:235.

CellXVivo™

Human Th17 Cell Differentiation Kit

Catalog Number: CDK003C

BACKGROUND

T helper type 17 (Th17) cells are a lineage of CD4$^+$ effector T cells that protect against extracellular bacteria and fungi. They function as pro-inflammatory agents by recruiting other inflammatory immune cells and opposing some regulatory T cell (Treg) functions. Th17 cells also mediate autoimmune and inflammatory disease pathogenesis (1). Differentiation of CD4$^+$ effector T cells into the Th17 lineage is promoted by cytokines such as TGF-β and IL-6, while their survival and expansion are dependent on IL-21 and IL-23 (2, 3). Th17 cells secrete TNF-α, IL-6, IL-9, IL-17A, IL-17F, IL-21, IL-22, and (human) IL-26/AK155. The CellXVivo™ Human Th17 Cell Differentiation Kit contains anti-human CD3 antibody, anti-human CD28 antibody, and optimized Th17 differentiation reagents. The quantity of the components in the kit is sufficient to differentiate one 24-well plate, or approximately 2.5-5x10^6 naïve CD4$^+$ T cells, and generate a 20-fold increase of Th17 polarized cells within your starting CD4$^+$ T cell population.

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. Do not use past kit expiration date.

COMPONENTS	PART #	# VIALS	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
Mouse Anti-Human CD3	968087	1 vial	May be stored at 2-8 °C under sterile conditions for up to 1 month or at -20 °C to -70 °C in a manual defrost freezer for up to 3 months.*
Mouse Anti-Human CD28	968088	1 vial	
Human Th17 Reagent 1	967565	1 vial	
Human Th17 Reagent 2	967566	1 vial	
Human Th17 Reagent 3	967567	1 vial	
Human Th17 Reagent 4	967568	1 vial	
Human Th17 Reagent 5	967592	1 vial	
Reconstitution Buffer 1	967552	2 vials	May be stored under sterile conditions for up to 3 months at 2-8 °C.*
Reconstitution Buffer 2	967553	2 vials	
Reconstitution Buffer 3	968014	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 Naive 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)
- Penicillin/Streptomycin (optional)
- Sterile deionized water
- Cell Activation Cocktail 500X (Tocris, Catalog #5476)
- Tissue culture flasks and/or plates
- Pipettes and pipette tips
- Inverted microscope
- Hemocytometer
- 37 °C, 5% CO2 incubator
- Centrifuge

REAGENT PREPARATION

Human Th17 Reagent 1 (400X) - Add 250 µL of Reconstitution Buffer 1 to Human Th17 Reagent 1 to produce Human Th17 Reagent 1 (400X).

Human Th17 Reagent 2 (400X) - Add 250 µL of Reconstitution Buffer 3 to Human Th17 Reagent 2 to produce Human Th17 Reagent 2 (400X).

Human Th17 Reagent 3 (400X) - Add 250 µL of Reconstitution Buffer 1 to Human Th17 Reagent 3 to produce Human Th17 Reagent 3 (400X).

Human Th17 Reagent 4 (400X) - Add 250 µL of Reconstitution Buffer 2 to Human Th17 Reagent 4 to produce Human Th17 Reagent 4 (400X).

Human Th17 Reagent 5 (400X) - Add 250 µL of Reconstitution Buffer 2 to Human Th17 Reagent 5 to produce Human Th17 Reagent 5 (400X).

Mouse Anti-Human CD28 (400X) - Add 250 µL of Reconstitution Buffer 2 to Mouse Anti-Human CD28 to produce Mouse Anti-Human CD28 (400X).

Human Th17 Differentiation Media - Add Human Th17 Reagent 1 (400X), Human Th17 Reagent 2 (400X), Human Th17 Reagent 3 (400X), Human Th17 Reagent 4 (400X), Human Th17 Reagent 5 (400X), and Mouse Anti-Human CD28 (400X) to desired amount of X-VIVO™ 15 Medium. Addition of Penicillin (100 units/mL) and Streptomycin (100 µg/mL) is recommended.

REAGENT PREPARATION *CONTINUED*

Mouse Anti-Human CD3 Antibody

1. Reconstitute Mouse Anti-Human CD3 with 200 µL of Reconstitution Buffer 2, this is a 40X 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 each 40X antibody stock 1:40 with 1X Wash Buffer.

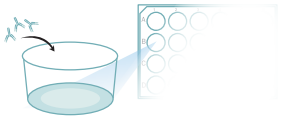
PROTOCOL FOR Th17 DIFFERENTIATION

1. Coat a plate with Mouse Anti-Human CD3.
   a. For a 24-well plate, add 250 µL/well of diluted CD3 antibody. For a 96-well plate, add 50 µL/well of diluted CD3 antibody.
   b. Incubate at 2-8 °C overnight.
   c. Wash the plate with 1X Wash Buffer twice 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. Perform a cell count.
4. Suspend human naïve CD4+ T cells at 1-2 x 10^5 cells/mL in Human Th17 Differentation Media.
5. Add the cells to a human CD3 antibody-coated plate. For a 24-well plate, add 1 mL/well. For a 96-well plate, add 0.2 mL/well.
6. Incubate the cells in a 37 °C, 5% CO_2 humidified incubator for 10 days. Refresh the Human Th17 Differentiation Media every 2-3 days according to step 7.
7. Refresh the Human Th17 Differentiation Media by removing 900 µL of the media from each well of a 24-well plate or 180 µL of the media from each well of a 96-well plate and replenishing with the same volume of fresh Human Th17 Differentiation Media every 2-3 days.

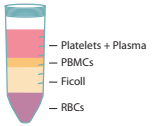
   Note: *If the culture medium is turning yellow, the cell density has likely expanded above 1.5 x 10^6 cells/mL and the cells must be split. Split the cells 1:2 using Human Th17 Differentiation Media.*
8. After 10 days of differentiation, the differentiated Th17 cells are ready to be used for downstream applications, such as flow cytometry or ELISA.
9. To verify Th17 cell differentiation via flow cytometry, collect the cells and wash with X-VIVO™ 15 Medium once, resuspend the cells in 1 mL X-VIVO™ 15 Medium and Cell Activation Cocktail (1X). Incubate the cells in a 37 °C, 5% CO_2, humidified incubator for 6-7 hours. Analyze cytokine expression via flow cytometry. Addition of Penicillin (100 units/mL) and Streptomycin (100 µg/mL) is recommended.

PROTOCOL OUTLINE

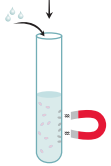
Coat with Mouse Anti-Human CD3 antibody.



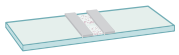
Isolate PBMCs from human blood.



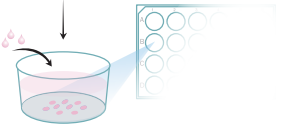
Isolate human naïve CD4+ T cells from PBMCs (e.g., using magnetic cell selection).



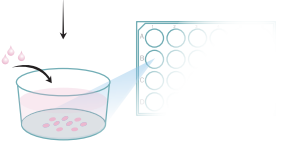
Perform a cell count.



Suspend 1-2 x 10^5 naïve CD4+ T cells/mL in Human Th17 Differentiation Media. **Culture** the cells on plates pre-coated with CD3 antibody.



Refesh the Differentiation Media every 2-3 days for a total of 10 days.



Verify Th17 cell differentiation by analyzing cytokine expression via flow cytometry or ELISA (optional).