SUGGESTED REAGENTS FOR FLOW CYTOMETRY

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<th>CATALOG #</th>
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<td>IC317A, and IC0041A</td>
<td>Human IL-17 MAB (Clone 41809), Mouse IgG1, and Mouse IgG1, APC Isotype Control (Clone 133303)</td>
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<td>IC285P, and IC0041P</td>
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<td>Human CD4 PerCP MAB (Clone 11831), Mouse IgG1, and Mouse IgG1, PerCP Isotype Control (Clone 20102)</td>
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<td>FC004</td>
<td>Flow Cytometry Fixation Buffer (1X)</td>
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<td>FC005</td>
<td>Flow Cytometry Permneabilization/Wash Buffer (1X)</td>
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REFERENCES

**REAGENT PREPARATION**

**Human Th17 Differentiation Media**

1. Reconstitute Human Th17 Reagents 1, 2, and 3 each with 250 μL of Reconstitution Buffer 1, this is a 400X stock.
2. Reconstitute Human Th17 Reagents 4 and 5 each with 250 μL of Reconstitution Buffer 2, this is a 400X stock.
3. Add 62.5 μL each of Human Th17 Reagent 1, 2, 3, 4, and 5 to 24.6 mL of cell culture media (X-VIVO 15 medium, 100 units/mL Penicillin, and 100 μg/mL Streptomycin).

**Human CD3 and CD28 Antibodies**

1. Reconstitute the Mouse Anti-Human CD3 and CD28 antibodies each with 100 μL of Reconstitution Buffer 2, these are 40X stocks.
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 and CD28 antibodies.
   a. For a 24-well plate, add 125 μL/well of diluted CD3 antibody and 125 μL/well of diluted CD28 antibody.
   b. For a 96-well plate, add 25 μL/well of diluted CD3 antibody and 25 μL/well of diluted CD28 antibody.
   c. Incubate at 2-8 °C overnight.
   d. 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.
4. Suspend human naïve CD4+ T cells at 1-2 x 10⁵ cells/mL in Human Th17 Differentiation Media.
5. Add the cells to a human CD3 and CD28 antibody-coated plate.
6. Incubate the cells in a 37 °C, 5% CO₂ humidified incubator for 2-3 days.
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 the day before the next refresh.
8. After 10 days of differentiation, the differentiated Th17 cells are ready to be used for downstream applications.
9. To verify Th17 cell differentiation via ELISA, remove the supernatant on day 10 and analyze via ELISA.
10. To verify Th17 cell differentiation via flow cytometry, collect the cells and wash with X-VIVO medium once, resuspend the cells in 1 mL X-VIVO medium, 100 units/mL penicillin, 100 μg/mL streptomycin, 50 ng/mL PMA, and 1 μg/mL ionomycin. Incubate the cells in a 37 °C, 5% CO₂ humidified incubator for 1 hour. Then add monensin at 3 μM and incubate for an additional 6 hours. Analyze cytokine expression via flow cytometry.

**OTHER MATERIALS & SUPPLIES REQUIRED**

- MagCellect Human Naive CD4+ T Cell Isolation Kit (R&D Systems, Catalog # MAGH115, or equivalent).
- Monensin
- PMA
- Ficoll-Hypaque®
- X-VIVO® 15 Chemically Defined, Serum-free Hematopoietic Cell Medium (Lonza, or equivalent).
- Sterile deionized water
- Ionomycin
- Penicillin-Streptomycin
- Tissue culture flasks and/or plates
- Microscope
- Hemocytometer
- 37 °C, 5% CO₂ Incubator
- Centrifuge
- Pipettes and pipette tips

**PROTOCOL OUTLINE**

1. Coat wells of a 24-well plate with Anti-Human CD3 and CD28 Antibodies.
2. Isolate PBMCs from human blood.
3. Perform a cell count.
4. Suspending 1-2 x 10⁵ naive CD4+ T cells/mL in the Human Th17 Differentiation Media. Culture the cells on plates pre-coated with CD3 and CD28 antibodies for 10 days.
5. Refresh the Differentiation Media every 2-3 days.
6. Re-stimulate cells with mitogens.
7. Verify Th17 cell differentiation by analyzing cytokine expression via flow cytometry.

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