Naïve CD4/Th1/Th2/Th17 Isolation from peripheral blood

Procedure for the Isolation of Naïve CD4+, Th1, Th2, and Th17 cells, and expansion of memory subsets.

Ordering Information:

Reagent	**Catalogue Number**	**Maunfacterer**
antiCCR6-PE	559562	BD
antiCCR4-PE-Cy7	557864	BD
antiCXCR3-PE-Cy5	558047	BD
antiCD4-Qdot 655	Q10007	Invitrogen
antiCD3-APC-AF750	27-0078-13	eBioscience
antiCD45RO-FITC	11-0477-73	eBioscience
antiCD25-APC	555434	BD
PBS	D8537	Sigma
Ficoll-Paque	17-1440-03	GE
BSA	A2153	Sigma
EDTA	EDS	Sigma
Trypan Blue	T6146	Sigma
MACS CD4 T cell Isoaltion Kit II	130-091-155	Miltenyi
MACS LS Columns	130-042-401	Miltenyi
AIM-V media	12055-083	Invitrogen
Human AB Serum	35-060-CI	Cellgro
Penicillin-Streptomycin Solution	15070-063	Invitgogen
IL-4	CRI104-20	Cell sciences
IL-12	CRI111B	Cell sciences
IL-1b	CRI133B	Cell sciences
IL-2	Proleukin	Prometheus
antiCD3/CD28 Dynabeads	111.3110	Invitrogen
PMA	P1585	Sigma
Ionomycin	I0634	Sigma
Brefeldin A	B7651	Sigma
Perm/Wash	51-2091KZ	BD
Cytofix/Cytoperm	51-2090KZ	BD
antiIL-4-FITC	53-7049-42	eBioscience
antiIL-13-APC	501907	BD
antiIL-17-PE (Cap17)	12-7178-42	eBioscience
antiIFNg-PerCP-Cy5.5	502526	Biolegend
antiIL-17-biotin (Dec17)	13-7179-85	eBioscience
antiCD45-biotin	304004	Biolegend
Streptavidin	SNN1001	Biosource

Materials List

500 mL Corning 0.2 µm Filter System (Cat# 430758)
15 mL Corning Polypropylene Conical Centrifuge Tubes (Cat# 430766)
50 mL Corning Polypropylene Conical Centrifuge Tubes (Cat# 430828)

Graduated pipets (5, 10, 25, 50 mL)
Hemocytometer
Micropipet with P20 tips
Micropipet with P200 tips
Micropipet with P1000 tips
.7μm filter
Microscope (preferably phase contrast)
Eppendorf Refrigerated Centrifuge 5810R
MACs seperator
FACS AriaII Cell Sorter
FACS LSRII Flow Cytometer

Reagent Preparation

MACs buffer
.5% BSA and 2mM EDTA in PBS, sterile filtered, degassed

FACs buffer
1% BSA in PBS, sterile filtered

Growth Media
AIM V media supplemented with 2% AB serum and 1% Penicillin/Streptomycin, sterile filtered

Isolation of PBMCs from peripheral blood

1) Collect 100-400 mL of whole blood from donor.

2) Aliquot blood into 50 mL conical tubes.

3) Centrifuge at 1300 rpm/10 min (Break OFF).

4) Remove white middle band (buffy coat) along with small portion of plasma and RBC portions.

5) Dilute each collected buffy coat (from 50 mL of whole blood) to 50 mL in sterile PBS.

6) Aliquot 20 mL of Ficoll in 50mL conical tubes. One conical tube of Ficoll will be needed for each 30 mL of whole blood processed. Overlay 30 mL of PBS-diluted buffy coat over each of the Ficoll aliquots.

7) Centrifuge at 1800 rpm/20 min (Break OFF).

8) Collect 15-20 mL of PBMC layer and dilute to 50 mL in sterile PBS.

9) Centrifuge at 1300 rpm/10 min.

10) Resuspend cell pellets in 5mL MACs Buffer and filter through 70 μM filter, collecting all PBMCs in 50 mL conical. Rinse filter with 5-10 mL MACs buffer.

11) Take a 10 μL volume and dilute to 100 μL in 1:10 Trypan Blue:PBS. Count cells with hemocytometer.

MACs enrichment of CD4+ T cells

1) Centrifuge at 1300 rpm/10 min.

2) Resuspend cell pellet in 40 μL MACs buffer/10^7 cells. Add 10 μL of Biotin-Antibody Cocktail (Tcell Isolation Kit II) per 10^7 cells. Incubate 10 min at 4^0 C.

3) Add 30 μL MACs buffer/10^7 cells. Add 20 μL of anti-Biotin-Microbeads (Tcell Isolation Kit II) per 10^7 cells. Incubate 15 min at 4^0 C

4) Wash cells by adding MACs buffer to 50 mL and centrifuge at 1300 rpm for 10 min. Resuspend pellet in 500 μL MACs buffer/10^8 cells

5) Place LS columns (1 per 10^8 cells) in magnetic field of MACs Seperator

6) Prepare column with 3 mL MACs buffer

7) Discard effluent and change collection tube (15 mL conical)

8) Apply 500 μL of PBMCs in MACs buffer to column

9) Wash column with 3 x 3 mL MACs buffer. Collect effluent (contains CD4+ Tcells)

10) Combine collected CD4+ T cells and count on hemocytometer as before

FACS separation of T cell subsets

1) Aliquot 1 x 10^6 cells into each of 8 wells in 96 well plate and use cells as single stain compensation controls

2) Add FACs buffer to CD4+ T cells to 50 mL and centrifuge 1300 rpm/10 min

3) Resuspend cells in 200 mL of FACs buffer. Add:
   50 μL antiCCR6-PE
   100 μL antiCCR4-PE-Cy7
   100 μL antiCXCR3-PE-Cy5
   5 μL antiCD4-Qdot 655

100 μL antiCD3-APC-Cy7
100 μL antiCD45RO-FITC
100 μL antiCD25-APC

4) Stain 30 min on ice

5) Wash cells with 50 mL of FACs buffer and centrifuge 1300 rpm/10 min (2x)

6) Resuspend cells at approximately 20 x 10^6 cells/ml in growth media and filter through 70 μm filter.

7) Sort cells on FACS Aria II (4 way sort) into 6mL tubes containing 1mL of growth media. Isolate 4 T cell populations. CD3+CD4+CD25-:

    1) naive: CD45RO-CCR6-CCR4-CXCR3-
    2) Th1: CD45RO+CCR6-CCR4-CXCR3+
    3) Th17: CD45RO+CCR6+
    4) Th2: CD45RO+CCR6-CCR4+CXCR3-

Process Naïve CD4+ cells directly for either DHS treatment and/or RNA isolation

See relevant protocols

Stimulation and expansion of Th1, Th2, Th17 cells

1) Wash cells with growth media, centrifuge 1300 rpm/10 min, and resuspend at 10^5 cells/mL

2) Add media, IL-2, antiCD3/28 microbeads, and relevant cytokine (IL-12 for Th1, IL-4 for Th2, IL-1b for Th17) to final concentrations of 5x10^5 cells/mL, 50 U/mL IL-2, 10 μL/mL antiCD3/CD28 microbeads (vortex beads before pipetting), and 10 μg/mIL-12, IL-4, or IL-1b.

3) Place cells in culture container of appropriate size and incubate at 37^0C, 5% CO_2 for 72 hours.

4) After 72 hours add equal volume of culture media and fresh cytokines to final concentrations of 50 U/mL IL-2 and 10 μg/mL IL-4, IL-12, or IL-1b.

5) Repeat splitting of culture every 48-72 hours while adding fresh cytokines.

Confirmation of cytokine profile by intracellular cytokine staining

1) On day 7, take 1x10^5 cells from each effector cell type and restimulate with .7 μM Ionomycin and 10 ng/mL PMA in presence of BFA (10 μg/mL) in growth media. Add 2 μL/mL anti-CD4-Qdot 655.

2) Stained cells with BD cytofix/cytoperm kit and protocol with:
    a. antiIL17-PE

b. antiIFNg-PerCP-cy5.5
c. antiIL4-FITC
d. antiIL13-APC

3) Analyze culture and verify purity of effector populations. >99% CD4+. For Th1, >50% cells producing IFNg; <2% producing IL-17 or IL-4. For Th2, >50% of cells producing IL-4 or IL-13; <2% producing IL-17 or IFNg.

On Day 8 process Th1 and Th2 cells for either DHS treatment and/or RNA isolation

See relevant protocols

Cytokine Capture for enrichment of IL-17 producing cells

1) On day 9, count Th17 cells as described.

2) Adjust Th17 cells to 10^6 cells/mL in growth media. Add 5 ng/mL PMA and 0.5 μM Ionomycin.

3) Incubate 1 1/2 hours at 37C, 5% CO_2.

4) Transfer cells to 50mL conical tube and wash cells with FACs buffer, and centrifuge 1300 rpm/10 min.

5) Stain cells sequentially in FACs buffer at 10^8 cells/mL on ice with:

   a. 20 μL/mL aCD45-biotin (.5mg/mL) for 30 min
   b. 5 μL/mL streptavidin (1mg/mL) for 15 min
   c. 40 μL/mL anti IL-17-biotin for 15 min

   Wash with 50 mL FACs buffer between each stain. *Note: no wash step after final stain with anti-IL-17.

6) Dilute to $1x10^6$ cell/mL in growth media and incubate on rotator at 37 C for 1 hour.

7) Spin down at 1300 rpm/10 min, wash once with FACs buffer (50 mL), and stain at 10^8 cells/mL with 200 μL/mL aIL-17-PE and 200 μL/mL aCD4-APC in FACs buffer for 30 min on ice.

8) Wash with 50 mL FACs buffer, spin down, and resuspend at $2x10^7$ cells/mL in AIM-V with 2% AB serum and filter through 70 μm filter.

9) Sort PE^+APC^+ cells on FACs Aria II (PE^-APC^+ as controls).

Expansion of IL-17 producing cells

1) Resuspend sorted cells at $1x10^6$ cells/mL in growth media + IL-2 (50 U/mL) and incubate for 48 hours.

2) After 48 hours, stimulate cells by adding media, IL-2, antiCD3/28 microbeads, IL-1b to final concentrations of $5x10^5$ cells/mL, 50 U/mL IL-2, 10 μL/mL antiCD3/CD28 microbeads (vortex beads before pipetting), and 10 μg/m IL-1b.

3) After 72 hours add equal volume of culture media and fresh cytokines to final concentrations of 50 U/mL IL-2 and 10 μg/mL IL-1b.

4) Repeat splitting of culture every 48-72 hours while adding fresh cytokines.

7 days post stimulation, verify IL-17 production of cells by intracellular cytokine staining as before

Verify purity of Th17 as >99% CD4+, >50% cells producing IL-17; <2% producing IFNg or IL-4.

Process Th1 and Th2 cells for either DHS treatment and/or RNA isolation