eBioscience™ Essential Human Th1/Th17 Phenotyping Kit

Catalog Number A42927

Pub. No. MAN0018285 **Rev.** A.0

WARNING! Read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves. Safety Data Sheets (SDSs) are available from **thermofisher.com/support**.

Product description

The Invitrogen™ eBioscience™ Essential Human Th1/Th17 Phenotyping Kit is comprised of markers to identify Th-1 and Th-17 cells.

- **CD4**: The RPA-T4 monoclonal antibody reacts with human CD4, a 59 kDa cell surface receptor expressed by a majority of thymocytes, subpopulation of mature T cells (T-helper cells) and in low levels by monocytes.
- **IFN-γ**: The 4S.B3 monoclonal antibody reacts with interferon-gamma (IFN-γ), a 17 kDa factor produced by activated T and NK cells. Th1 cells are CD4+ and generate high levels of IFN-γ
- **IL-17A**: The eBio64DEC17 monoclonal antibody reacts with human Interleukin-17A (IL-17A), a CD4+ T cell-derived cytokine, that promotes inflammatory responses. Th17 cells are CD4+ and generate high levels of IL-17A.

Contents and storage

Table 1 eBioscience™ Essential Human Th1/Th17 Phenotyping Kit, (Cat. No. A42927)

Components	Cat. No.	Storage
eBioscience™ Essential Human Th1/Th17 Phenotyping Panel with Staining & Stimulation Reagents	**A42926**	**2°C to 8°C**
eBioscience™ Essential Human Th1/Th17 Phenotyping Panel • CD4 (RPA-T4)-FITC- Mouse IgG1, kappa • IFN-γ (4S.B3)-PE- Mouse IgG1, kappa • IL-17A (eBio64DEC17)-APC- Mouse IgG1, kappa	A42810	2°C to 8°C
• eBioscience™ Cell Stimulation Cocktail (plus protein transport inhibitors) (500X) • CONTROL –eBioscience™ Protein Transport Inhibitor Cocktail		–5 to –30°C
eBioscience™ Intracellular Fixation and Permeabilization Buffer Set • IC Fixation Buffer • Permeabilization Buffer (10X)	88-8824-00	2°C to 8°C
eBioscience™ Essential Human Th1_Th17 Phenotyping Isotype Control	**A42811**	**2°C to 8°C**
• Mouse IgG1, kappa Isotype Control- PE • Mouse IgG1, kappa Isotype Control- APC		

Note: Cat. No. A42926 contains A42810 and 88-8824-00.

Required materials not supplied

Unless otherwise indicated, all materials are available through **thermofisher.com**. MLS: Fisher Scientific (**fisherscientific.com**) or other major laboratory supplier.

Item	Source
Reagents	
eBioscience™ Flow Cytometry Staining Buffer	00-4222-26
eBioscience™ Fixable Viability Dye eFluor™ 506	65-0866-14
UltraComp eBeads™ Compensation Beads	01-2222-42
Instruments and equipment	
12 × 75 mm round-bottom test tubes	MLS
Flow cytometer equipped with at least three lasers (488 nm, 405 nm and 633 nm), with optics capable of detecting fluorophores in the kit.	MLS
Centrifuge (Compatible with 75 mm round bottom test tubes)	MLS
Vortex mixer	MLS
Pipettes	MLS
Refrigerator or ice bucket	MLS
65°C heat block or water bath	MLS
Countess™ II Automated Cell Counter or equivalent counting device	AMQAX1000

Suggested experimental setup

	Laser	488 Laser		633 Laser	405 Laser
	Fluorophore	**FITC**	**PE**	**APC**	**eFluor™ 506**
	Antibody	**CD4**	**IFN-γ**	**IL17A**	**Viability**
Calibration	Calibration Beads	—	—	—	—
Compensation	Ultracomp-CD4	CD4	—	—	—
	Ultracomp-IFN-γ	—	IFN-γ[1]	—	—
	Ultracomp-IL17A	—	—	IL17A[1]	—
Controls	Cells unstained	—	—	—	—
	Cells 2 isotypes	CD4	mIgG1-PE[1]	mIgG1-APC[1]	Viability eFluor™ 506
FMO controls	Cells CD4 FMO	—	IFN-γ[1]	IL17A[1]	Viability eFluor™ 506
	Cells IFN-γ FMO	CD4	—	IL17A[1]	Viability eFluor™ 506
	Cells IL17A FMO	CD4	IFN-γ[1]	—	Viability eFluor™ 506
Viability	Cells viability	—	—	—	Viability eFluor™ 506
Test samples	Cells multiplex	CD4	IFN-γ[1]	IL17A[1]	Viability eFluor™ 506

[1] Do not add intracellular marker during surface staining.

IMPORTANT! Cells must be stimulated prior to staining and fixation/permeabilization.

Stimulate cells

Stimulation will result in increased levels of IFN-γ and IL-17A. Without stimulation these markers will not be detectable by antibody staining and Flow Cytometry. Stimulation and subsequent staining can be carried out with either cells in culture or frozen cells directly from thaw. If using frozen cells thaw according to recommended protocol for the cell type being used.

1. Resuspend the cells at a density of 0.5×10^6 cells/mL in cell culture media.
2. Evenly divide cell suspension between two T25 flasks and label one "Stimulated" and the other "Unstimulated".
3. Add Protein Transport Inhibitor to the "Unstimulated" flask at a ratio of 2 µL per mL of media.
4. Add Cell Stimulation Cocktail to the "Stimulated" flask at a ratio of 2 µL per mL of media.

   Note: Cell Stimulation Cocktail also includes Protein Transport Inhibitor.
5. Gently mix the flasks by tilting them back and forth several times.
6. Put both flasks in a cell culture incubator at 37°C and 5% CO_2 for 4–6 hours.

Perform surface marker staining

1. Count cells using a hemocytometer or automated cell counter, such as the Countess™ II Automated Cell Counter.

   Record the cell counts.
2. Collect the cell suspension from the culture vessel, and add to a sterile 15 mL conical tube.
3. Spin for 5 minutes at 200 × *g* at room temperature and discard the supernatant.

   If the cell type being used has a different recommended centrifugation speed use that setting throughout this protocol.
4. Resuspend cells in Flow Cytometry Staining Buffer so that the concentration is 2×10^5–1×10^6 cells per 100 µL.

   (2×10^6–1×10^7 cells/mL)
5. Aliquot 100 µL of the cells from step 4 into as many tubes (12 × 75 mm tubes) as are needed for experimentation.

   For the Th1/Th17 panel (see "Contents and storage" on page 1), the recommended experimental setup uses 7 samples.
6. Positive control for viability (*optional*): Remove 50 µL of the "Viability" sample and place in a 65°C heat block for 15–20 minutes. Afterward, transfer the heat-treated cells back into the same tube with the untreated cells.

   Note: This step is recommended if the percentage of dead cells is expected to be less than 5%. This step allows for visualization of the distinct population of dead cells in order to enable effective gating between live and dead cells.
7. Add 5 µL of each antibody to the cell suspensions prepared in step 5 and step 6 for the appropriate tubes according to the experimental setup shown in "Suggested experimental setup" on page 2.

   Do not add IL17A, IFN-(γ), and isotype controls prior to fixation/permeabilization.
8. Add 1uL of Viability Dye eFluor™ 506 to the samples designated in the eFluor™ 506 Column, of "Suggested experimental setup" on page 2.
9. Briefly vortex all sample tubes. Incubate at 4°C for 30 minutes in the dark.
10. Add 2 mL of Flow Cytometry Staining Buffer quickly vortex to resuspend the cell pellet, and centrifuge at 200 × *g* for 5 minutes at room temperature. Discard supernatant.
11. Repeat step 10.
12. Proceed to intracellular marker staining.

Perform intracellular marker staining (cytoplasmic)

1. Prepare a 1X working solution of Permeabilization Buffer by mixing 1 part 10X concentrate with 9 parts distilled water.

   Each sample will require 8.5 mL of 1X Permeabilization Buffer.
2. Discard the supernatant from the last spin in "Perform surface marker staining" on page 3 and pulse vortex the sample to completely dissociate the pellet.

   Approximately 100 µL residual volume remains.
3. Fix the cells by adding 100 µL of IC Fixation Buffer and pulse vortex to mix.
4. Incubate 30 minutes at room temperature. Protect from light.
5. Add 2 mL of 1X Permeabilization Buffer, quickly vortex to resuspend the cell pellet, and centrifuge at 400–600 × *g* for 5 minutes at room temperature. Discard the supernatant.
6. Repeat step 5.
7. Resuspend the cell pellet in 100 µL 1X Permeabilization Buffer.
8. Add 5 µL of each antibody or 0.125 µL IFN-γ Isotype Control or 0.3 µL IL-17A Isotype Control to the prepared cell suspensions for the appropriate tubes according to the experimental setup shown in "Suggested experimental setup" on page 2.

   Antibodies and isotype controls to be added during this step are denoted with a "[1]" in "Suggested experimental setup" on page 2.
9. Incubate for 30 minutes at room temperature. Protect from light.

10. Add 2 mL 1X Permeabilization Buffer, quickly vortex to resuspend the cell pellet, and centrifuge at 400–600 × *g* for 5 minutes at room temperature. Discard supernatant.

11. Repeat step 10.

12. Resuspend stained cells in 0.5 mL Flow Cytometry Staining Buffer.

13. Analyze samples by flow cytometry.

    Vortex each sample before acquisition. It is recommended to collect a minimum of 30,000 events for each sample.

    Note: Fixed cells will have a different forward/side scatter profile as compared to live cells, check and adjust voltages accordingly prior to collecting samples.

Data acquisition and analysis

Performance Tracking Beads: Startup your Attune™ NxT instrument and software and follow the software prompt to run the performance tracking beads. See *Attune™ Performance Tracking Beads User Guide* (Pub. No. MAN0002636).

Compensation Bead Staining: Follow instructions as per manufacturer's instructions. See ***UltraComp eBeads™ Compensation Beads Technical Data Sheet***.

Typical gating results

Note: All example plots generated using FlowJo.

Th1/Th17 Panel initial gates

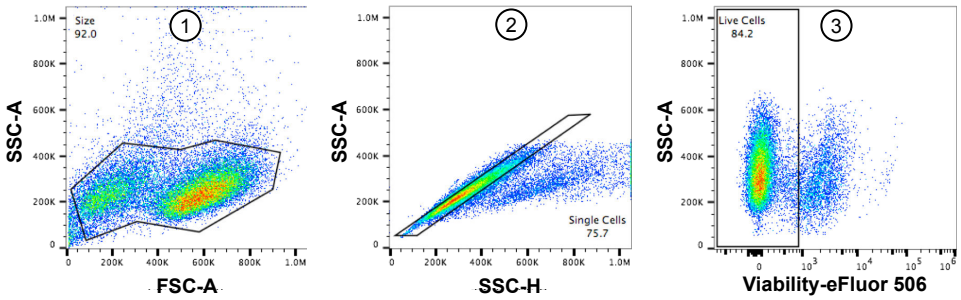

Figure 1 Setting gates to exclude unwanted cells

① **Exclude debris**. Parent Gate: Ungated

② **Exclude doublets**. Parent Gate: Size/Debris

③ **Exclude dead cells**. Parent Gate: Single Cells

Th1/Th17 Panel Controls

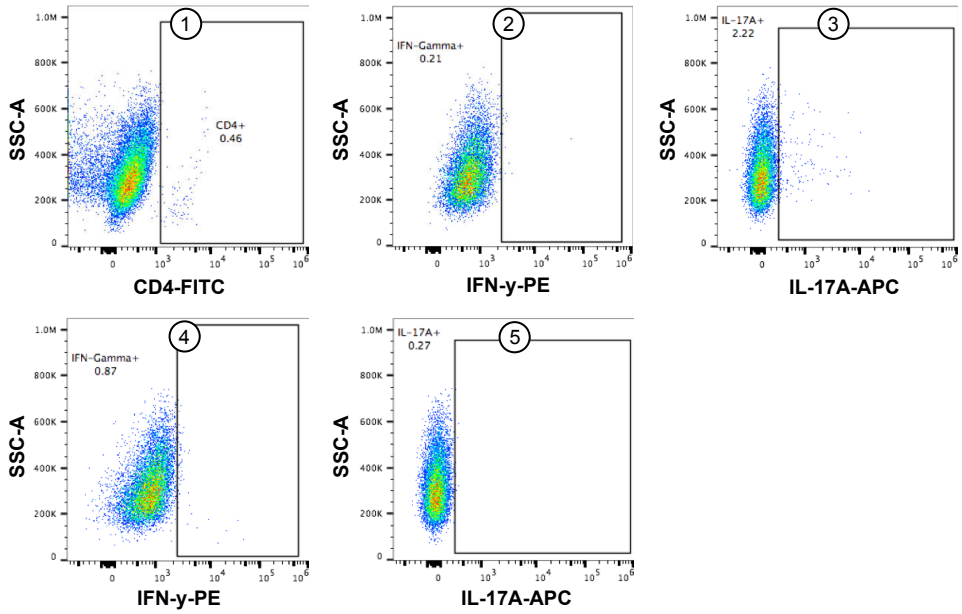

Figure 2 Setting gates with FMO and Isotype Control

① **CD4 FMO**. Parent Gate: Live Cells
② **IFN-γ FMO**. Parent Gate: CD4+ Cells
③ **IL-17A FMO**. Parent Gate: CD4+ Cells
④ **IFN-γ Isotype Control**. Parent Gate: CD4+ Cells
⑤ **IL-17A Isotype Control**. Parent Gate: CD4+ Cells

Th1/Th17 analysis

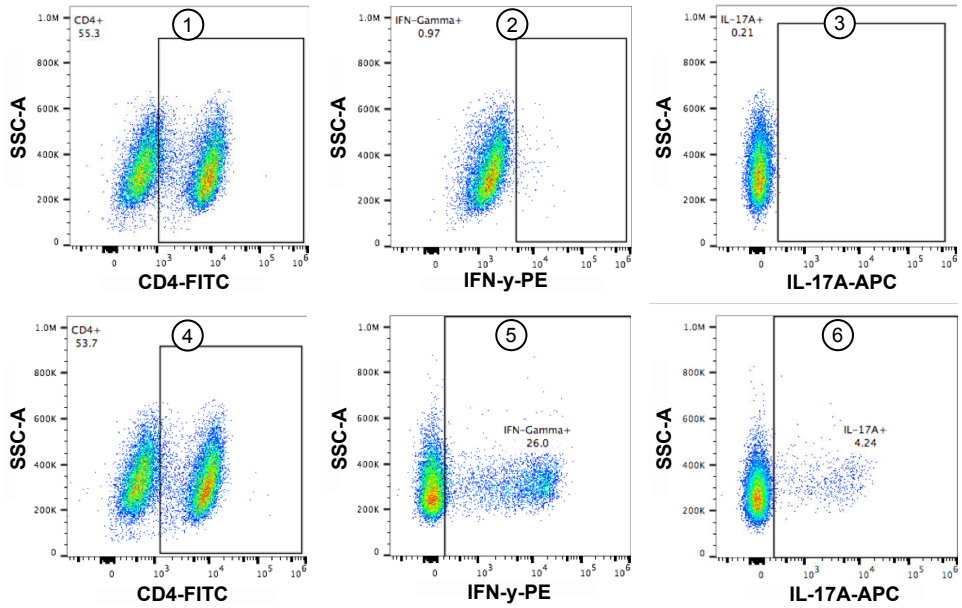

Figure 3 Analysis of cell samples

① **CD4 Gating–Unstimulated** . Parent Gate: Live Cells
② **IFN-γ Gating– Unstimulated**. Parent Gate: CD4+ Cells
③ **IL-17A Gating– Unstimulated**. Parent Gate: CD4+ Cells
④ **CD4 Gating– Stimulated**. Parent Gate: Live Cells
⑤ **IFN-γ Gating– Stimulated**. Parent Gate: CD4+ Cells
⑥ **IL-17A Gating– Stimulated**. Parent Gate: CD4+ Cells

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Limited product warranty

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