eBioscience™ Essential Human Treg Phenotyping Kit

Catalog Number A42925

Pub. No. MAN0018284 **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 Treg Phenotyping Kit is comprised of both positive and negative markers to identify Regulatory T-Cells (Tregs).

- **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.
- **CD25**: The CD25-4E3 monoclonal antibody reacts to human CD25, which is also known as the low-affinity interleukin (IL)-2 receptor alpha. CD25 is expressed on activated T cells (including a subset of regulatory T cells), B cells, and macrophages. Co-expression of CD4 and CD25 has become widely used as an indicator of Tregs.
- **CD127**: The eBioRDR5 monoclonal antibody reacts with human CD127 (Interleukin-7 Receptor alpha.) It has been demonstrated that CD127 expression is down-regulated on $CD4^+CD25^+$ Tregs.
- **FoxP3**: The PCH101 monoclonal antibody reacts with the amino terminus of human foxp3 protein. Constitutive high expression of Foxp3 mRNA has been shown in $CD4^+CD25^+$ Tregs.

Contents and storage

Table 1 eBioscience™ Essential Human Treg Phenotyping Kit, (Cat. No. A42925)

Components	Cat. No.	Storage
eBioscience™ Essential Human Treg Phenotyping Panel & Staining Buffers	**A42924**	**2°C to 8°C**
eBioscience™ Essential Human Treg Phenotyping Panel • CD4 (RPA-T4)-FITC- Mouse IgG1, kappa • CD25 (CD25-4E)- PerCP-eFluor710- Mouse IgG1, kappa • CD127 (eBioRDR5) - PE- Mouse IgG1, kappa • FoxP3 (PCH101) – eFluor450- Rat IgG2a, kappa	A42808	2°C to 8°C
FoxP3/TF Staining Buffer Set • Fixation/Permeabilization Concentrate • Fixation/Permeabilziation Diluent • Permeabilization Buffer (10X)	00-5523-00	2°C to 8°C
eBioscience™ Essential Human Treg Phenotyping Isotype Control	**A42809**	**2°C to 8°C**
• rat IgG2a, kappa Isotype Control- eFluor450		

Note: Cat. No. A42924 contains A42808 and 00-5523-00.

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**			**405 Laser**	
	Fluorophore	**FITC**	**PE**	**PerCP-eFluor™ 710**	**eFluor™ 450 Pac Blue**	**eFluor™ 506**
	Antibody	**CD4**	**CD127**	**CD25**	**FoxP3**	**Viability**
Calibration	Calibration beads	—	—	—	—	—
Compensation	Ultracomp-CD4	CD4	—	—	—	—
	Ultracomp-CD127	—	CD127	—	—	—
	Ultracomp-CD25	—	—	CD25	—	—
	Ultracomp-FoxP3	—	—	—	FoxP3[1]	—
Controls	Cells unstained	—	—	—	—	—
	Cells 1 isotype	CD4	CD127	CD25	rIgG2a-eF450[1]	Viability eFluor™ 506
FMO controls	Cells CD4 FMO	—	CD127	CD25	FoxP3[1]	Viability eFluor™ 506
	Cells CD127 FMO	CD4	—	CD25	FoxP3[1]	Viability eFluor™ 506
	Cells CD25 FMO	CD4	CD127	—	FoxP3[1]	Viability eFluor™ 506
	Cells FoxP3 FMO	CD4	CD127	CD25	—	Viability eFluor™ 506
Viability	Cells viability	—	—	—	—	Viability eFluor™ 506
Test samples	Cells multiplex	CD4	CD127	CD25	FoxP3[1]	Viability eFluor™ 506

[1] Indicates intracellular marker not to be added during surface staining.

Perform surface marker staining

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

   Record the cell counts.

2. Collect an appropriate amount of 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 Treg panel (see "Contents and storage" on page 1), the recommended experimental setup uses 8 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, see "Suggested experimental setup" on page 2.

   Do not add Foxp3 or Foxp3 isotype control prior to fixation/permeabilization.

8. Add 1uL of Viability Dye eFluor™ 506 to the samples designated in the eFluor™ 506 Column in "Suggested experimental setup".

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 (nuclear)

1. Prepare 1X Permeabilization Buffer by mixing 1 part 10X Permeabilization Buffer with 9 parts distilled water.

   Each sample will require 8.5 mL of 1X Permeabilization Buffer.

2. Prepare 1X Foxp3 Fixation/Permeabilization Solution by mixing 1 part of Foxp3 Fixation/Permeabilization Concentrate with 3 parts Foxp3 Fixation/Permeabilization Diluent.

   Each sample will require 1 mL of 1X Foxp3 Fixation/Permeabilization Solution.

3. Discard the supernatant from the last spin in the Surface Marker Staining section and pulse vortex the sample to completely resuspend the pellet.

   Approximately 100 µL residual volume remains.

4. Add 1 mL of 1X Foxp3 Fixation/Permeabilization Solution to each tube and pulse vortex.

5. Incubate for 30 minutes at room temperature.

   Protect from light.

6. Add 2 mL of 1X Permeabilization Buffer to each tube and centrifuge samples at 400–600 × *g* for 5 minutes at room temperature. Discard the supernatant.

7. Repeat step 6.

8. Resuspend pellet in residual volume of 1X Permeabilization Buffer.

   This is typically ~100 µL.

9. Add 5 µL of each antibody or 2.5 µL Foxp3 isotype control to the prepared cell suspensions for the appropriate tubes according to the experimental setup, see "Suggested experimental setup".

   Note: Antibodies and isotype controls to be added during this step are denoted with a "[1]" in "Suggested experimental setup" on page 2

10. Incubate for 30 minutes at room temperature.

    Protect from light.

11. 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.

12. Repeat step 11.

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

14. Analyze samples by flow cytometer.

    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.

Treg panel initial gates

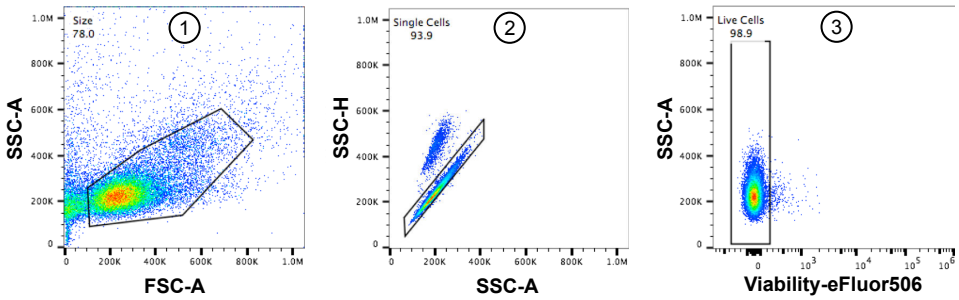

Figure 1 Setting gates to exclude unwanted cells

① **Exclude debris**. Parent Gate: Ungated
② **Exclude doublets**. Parent Gate: Size/Debris
③ **Exclude dead cells**. Parent Gate: Single Cells

Treg panel controls

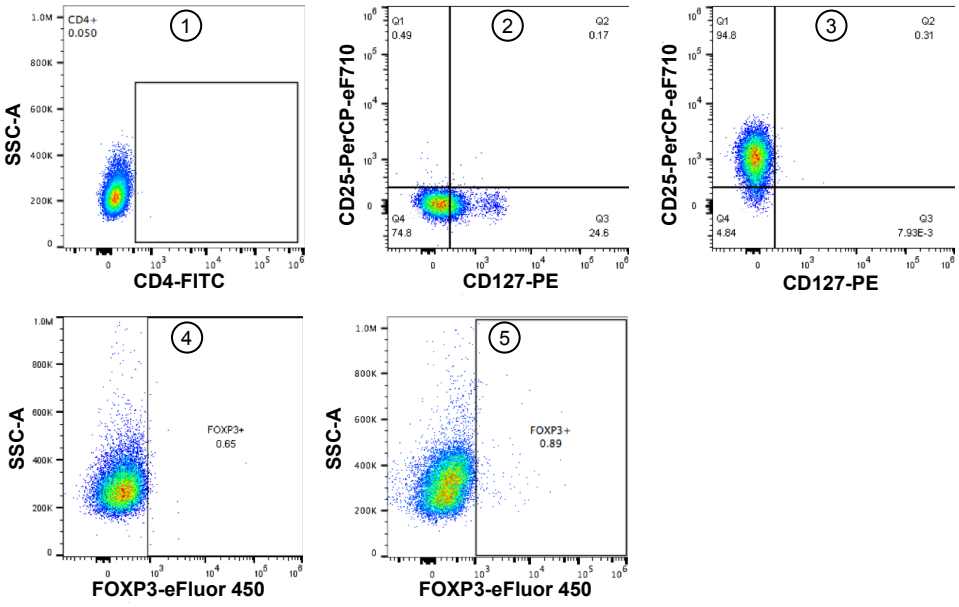

Figure 2 Setting gates with FMO and Isotype Control

① **CD4 FMO**. Parent Gate: Live Cells
② **CD25 FMO**. Parent Gate: CD4+ Cells
③ **CD127 FMO**. Parent Gate: CD4+ Cells
④ **Foxp3 FMO** . Parent Gate: CD25+ CD127- Cells
⑤ **Foxp3 Isotype Control**. Parent Gate: CD25+ CD127- Cells

Treg analysis

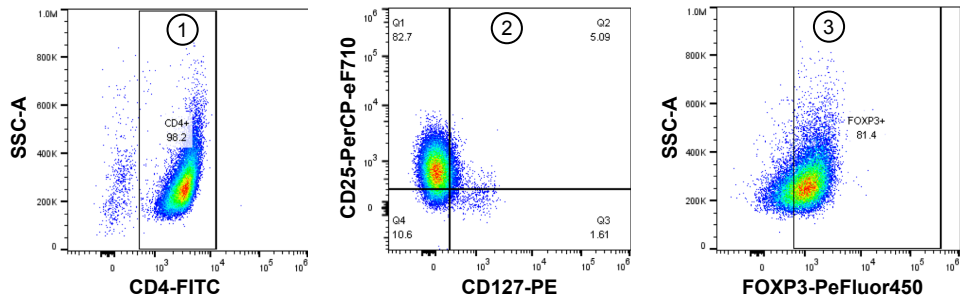

Figure 3 Analysis of cell samples

① **CD4 gating**. Parent Gate: Live Cells
② **CD25 and CD127 gating**. Parent Gate: CD4+ Cells
③ **FOXP3 gating**. CD25+ CD127- Cells

Limited product warranty

Life Technologies Corporation and/or its affiliate(s) warrant their products as set forth in the Life Technologies' General Terms and Conditions of Sale at **www.thermofisher.com/us/en/home/global/terms-and-conditions.html**. If you have any questions, please contact Life Technologies at **www.thermofisher.com/support**.

Life Technologies Corporation | 5781 Van Allen Way | Carlsbad, CA 92008
For descriptions of symbols on product labels or product documents, go to **thermofisher.com/symbols-definition**.