Technical Data Sheet

Mouse Th17/Treg Phenotyping Kit

Product Information

Material Number:	**560767**
Size:	50 Tests
Storage Buffer:	Aqueous buffered solution containing ≤0.09% sodium azide.

Description

Components:

51-9006647	Mouse Th17/Treg Phenotyping Cocktail	1.0 ml
	Containing the following: Mouse CD4 PerCP-Cy5.5 (clone: RM4-5) Mouse IL-17A PE (clone: TC11-18H10.1) Mouse Foxp3 Alexa Fluor® 647 (clone: MF23)	
51-9006124	Mouse Foxp3 Fixation Concentrate (20x)	10 ml
51-9006125	Mouse Foxp3 Permeabilization Concentrate (5x)	80 ml
51-2092KZ	BD GolgiStop™ Protein Transport Inhibitor (containing monensin)	0.7 ml

The immune system protects individuals from a broad range of pathogenic microorganisms while avoiding inappropriate or extreme immune responses, such as autoimmune responses that could be harmful. The peripheral CD4+ T cell pool includes multiple functionally-distinct T cell subsets that arise through thymic differentiation or as a consequence of antigen-driven expansion and differentiation of peripheral naïve T cells. The early response of naïve CD4+ T cells to antigenic stimulation may be characterized by high level proliferation and a limited cytokine repertoire. Further differentiation yields cells with a more diverse potential for cytokine expression. Depending upon the balance of local cytokines, costimulatory molecules, antigen levels, and genetic factors, Th17 effector/memory cells or inducible CD4+ T regulatory cells (iTregs) can be generated from the naïve CD4+ T cell pool. In addition, the peripheral T cell pool contains natural CD4+ T regulatory cells (nTregs) that are generated in the thymus as a functionally mature subpopulation of T cells.

Functionally-polarized CD4+ T cell subsets have been identified based on their distinctive patterns of cytokine secretion, transcription factor expression and function. As a signature cytokine, Th17 express high levels of interleukin-17A (IL-17A) whereas Treg are characterized by the expression of the FoxP3 transcription factor. Through the secretion of IL-17A and other factors, Th17 cells recruit and activate neutrophils and mediate immune responses against extracellular bacteria and fungi. Th17 cells are also implicated in mediating autoimmune responses. Natural and inducible Tregs can suppress the function of other T cells or cell types involved in the immune response through a variety of mechanisms including cytokines. In this way, Treg safeguard against the immune system's responsiveness to self antigens and restrain excessive responsiveness to foreign antigens that could be harmful to the host.

The Th17/Treg paradigm provides a useful model system for investigating the cellular and molecular mechanisms that mediate protective as well as harmful immune responses including autoimmune diseases. The BD Mouse Th17/Treg Phenotyping Kit provides an easy-to-use three-color cocktail of fluorescent antibodies-specific for mouse CD4, IL-17A (for Th17) and Foxp3 (for Treg)-that will enable researchers to identify and characterize the nature of the CD4+ T cell types present in their system by multicolor flow cytometric analysis. The kit can be used to successfully analyze ex vivo lymphoid cell samples (eg, for the types of in vivo-generated mouse CD4+ T cell subsets) or to monitor CD4+ T cell differentiation of cells cultured within various experimental model systems.

Investigators should note that the appearance of BD GolgiStop™ Protein Transport Inhibitor may range in color from clear (colorless) to light yellow.

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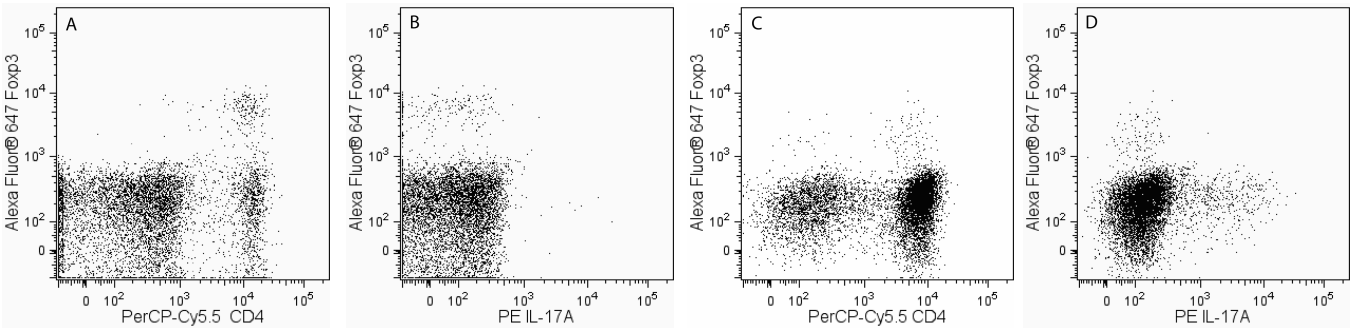

Flow cytometric analysis of IL-17A and FoxP3 expression by mouse CD4+ Th17 cells. *Mouse splenocytes were either unstimulated (panels A and B) or polarized and stimulated in culture for IL-17 production (panels C and D). The cells were then fixed, permeabilized and stained with a cocktail of fluorescent antibodies (PE anti-mouse IL-17A, Alexa Fluor® 647 anti-mouse Foxp3, and PerCP-Cy™5.5 anti-mouse CD4) as described in the protocol. Two-color flow cytometric dot plots showing correlated expression patterns of Foxp3 and CD4 (panels A and C) and IL-17A and Foxp3 (panels B and D) were derived from gated events with the forward and side light-scatter characteristics of viable lymphocytes. Flow cytometry was performed using a BD™ LSRII System.*

Preparation and Storage

Store undiluted at 4°C and protected from prolonged exposure to light. Do not freeze.
The monoclonal antibody was purified from tissue culture supernatant or ascites by affinity chromatography.
The antibody was conjugated with R-PE under optimum conditions, and unconjugated antibody and free PE were removed.
The antibody was conjugated with PerCP-Cy5.5 under optimum conditions, and unconjugated antibody and free PerCP-Cy5.5 were removed. Storage of PerCP-Cy5.5 conjugates in unoptimized diluent is not recommended and may result in loss of signal intensity.
The antibody was conjugated to Alexa Fluor® 647 under optimum conditions, and unreacted Alexa Fluor® 647 was removed.

Application Notes

Application

Flow cytometry	Tested During Development

Recommended Assay Procedure:
A. Stimulation of the Cells

Various methods have been reported for the polarization and stimulation of T helper cell subsets to produce IL-17 in vitro. Short term activation with phorbol myristate acetate (PMA; protein kinase C activator) plus ionomycin (Ca2+ ionophore) has been useful for quickly inducing and characterizing polyclonal cytokine-producing cells. However, *ex vivo* stimulation of freshly explanted lymphoid cells from mice with PMA and ionomycin in culture for several hours typically results in the detection of only a small percentage of IL-17A-producing cells. For this kit, we recommend a 5 day polarization of enriched CD4+ T-cells cultured in the presence of TGFβ followed by a 4 hour restimulation with PMA + ionomycin in the presence of BD GolgiStop™ Protein Transport Inhibitor.

Note: Kinetic studies need to be performed to determine the optimal incubation time for each experimental system.

Procedure for generation of mouse IL-17A producing cells

1. Prepare a single cell suspension of splenocytes from a BALB/c mouse.
2. Lyse the red blood cells (1× BD PharmLyse™ Lysing Buffer, Cat. No. 555899).
3. Isolate CD4+ T cells by either a negative or positive cell selection method.
4. Culture cells for four days in the presence of plate-bound anti-CD3 antibody (Cat. No. 553057; 10 µg/ml; coat tissue culture plate wells overnight at 4°C; wash wells three times with Dulbecco's PBS), soluble anti-CD28 antibody (Cat. No. 553294; 2 µg/ml final concentration), and recombinant mouse IL-1β (Cat. No. 554577; 50 ng/ml), mouse IL-6 (Cat. No. 554582; 25 ng/ml), and TGFβ(Cat. No. 356039; 5 ng/ml) proteins in complete RPMI-1640 tissue culture medium. Add fresh media at day 3 or 4 if necessary.
5. On day 5 harvest cells and wash once with complete RPMI-1640 tissue culture medium.
6. Restimulate cells with 50 ng/ml of PMA (Sigma Cat. No. P-8139) and 1 µg/ml of ionomycin (Sigma Cat. No. I-0634) in the presence of Monensin (BD Golgistop™ Protein Transport Inhibitor (provided) Cat. No. 554724) in complete RPMI-1640. Incubate cells for four to five hours.
7. Harvest cells.
8. Wash cells once in BD Pharmingen™ Stain Buffer (FBS)* (Cat. No. 554656).
9. Fix cells according to the Foxp3 staining procedure described below.

Optional (Cells may be frozen and stored for later use after fixation)

1. Fix cells with 1× BD Pharmingen™ Mouse Foxp3 Fixation Buffer (provided, Cat. No. 560409) at a concentration of 10 million cells/ml for 30 minutes on ice.
2. Wash cells once in BD Pharmingen™ Stain Buffer (FBS)* (5ml wash buffer per 10 million cells).

3. Count cells and suspend the cells at 10 million cells/ml in 10% DMSO/90% FCS.
4. Freeze cells at -80°C in a freezer vial.

B. Staining of the Cells

Collect cells from stimulatory cell cultures by centrifugation (300x*g*) and suspend them in BD Pharmingen Stain Buffer (FBS)*. Count the cells and adjust their concentration to 10 million cells/ml in BD Pharmingen Stain Buffer (FBS)*.

For frozen cells, remove DMSO by washing thawed cells with 5 ml/frozen vial with BD Pharmingen Stain Buffer (FBS)*, and centrifuge 300x*g* for 5 minutes at RT. Suspend pellet at 10 million cells/ml in BD Pharmingen Stain Buffer (FBS)* and aliquot 1 million cells per well of a 96 well plate. Centrifuge, remove buffer and continue procedure at step 7.

Staining Procedure
Note: This procedure is for staining cells in 96-well U bottom plates. If staining cells in tubes is preferred, then adjust wash volumes to 1 ml.

1. Remove clumps of cells and/or debris by passing the cell suspension through a BD Falcon™ 70-µm nylon cell strainer (Cat. No. 352235).
2. Dilute the cells to 10 million cells/ml.
3. Aliquot 1 million cells per well, centrifuge at 300x*g* for 5 minutes, and remove buffer.
4. To fix the cells, gently suspend the cell pellet in the residual volume of staining buffer and then add 200 µl of freshly prepared cold 1× BD Pharmingen™ Mouse Foxp3 Fixation Buffer (provided, Cat. No. 560409). Mix well. Incubate for 30 minutes at 4°C in the dark.
5. Centrifuge 300x*g* for 5 minutes, and remove fixative.
6. To wash cells, suspend each pellet in 200 µl of freshly prepared pre-warmed (37°C) 1× BD Pharmingen™ Mouse Foxp3 Permeabilization Buffer (provided, Cat. No. 560409), and centrifuge 300x*g* for 5 minutes. Remove permeabilization buffer.
7. To permeabilize the cells, gently suspend the cell pellet in another 200 µl of freshly prepared pre-warmed (37°C) 1× BD Pharmingen™ Mouse Foxp3 Permeabilization Buffer. Incubate for 30 minutes at 37°C in the dark.
8. Centrifuge the cells at 300x*g* for 5 minutes, and remove buffer.
9. To wash cells, add 200 µl of BD Pharmingen™ Stain Buffer (FBS) to each tube, centrifuge 300x*g* for 5 minutes. Remove buffer. Repeat.
10. Add 20 µl/test of the mouse Th17/Treg phenotyping cocktail or appropriate negative staining control. Incubate at RT for 30 minutes in the dark. Cells should be protected from light throughout the staining and storage.
11. Repeat wash step 9 above two times.
12. Suspend cell pellet in 200 µl stain buffer and proceed with flow cytometric analysis.

C. Flow Cytometric Analysis

Set PMT voltage and compensation using unstained cells and appropriate cell surface markers

Note: It has been reported that CD4 expression on T cells is decreased after cell activation.

* BD Pharmingen Stain Buffer (FBS) (Cat No. 554656) is recommended for initial surface staining and all wash steps and covering tubes during incubation steps with caps or parafilm.

Warnings & Precautions

Danger: BD GolgiStop™ Protein Transport Inhibitor (component 51-2092KZ) contains 99.61% ethanol (w/w) and 0.26% monensin, mononatriumsalz (w/w).

Hazard statements:
Highly flammable liquid and vapor.
Causes serious eye irritation.

Precautionary statements:
Keep away from heat/sparks/open flames/hot surfaces. No smoking.
Wear protective gloves / eye protection.
Wear protective clothing.
IF ON SKIN (or hair): Remove / Take off immediately all contaminated clothing. Rinse skin with water / shower.
IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.
Dispose of contents / container in accordance with local / regional / national / international regulations.

Danger: Mouse Foxp3 Fixation Concentrate (20x) (component 51-9006124) contains 4.2% formaldehyde (w/w).

Hazard statements
Harmful if inhaled.
Causes skin irritation.
Causes serious eye damage.
May cause an allergic skin reaction.
Suspected of causing genetic defects.
May cause cancer. Route of exposure: Inhalative.
May cause respiratory irritation.

Precautionary statements
Wear protective clothing / eye protection.
Wear protective gloves.
Do not breathe mist/vapours/spray.
IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.
If skin irritation or rash occurs: Get medical advice/attention.

Suggested Companion Products

Catalog Number	Name	Size	Clone
553925	PE Rat IgG1, κ Isotype Control	0.1 mg	R3-34
557691	Alexa Fluor® 647 Rat IgG2b, κ Isotype Control	0.1 mg	A95-1
554656	Stain Buffer (FBS)	500 mL	(none)

Product Notices

1. Please observe the following precautions: Absorption of visible light can significantly alter the energy transfer occurring in any tandem fluorochrome conjugate; therefore, we recommend that special precautions be taken (such as wrapping vials, tubes, or racks in aluminum foil) to prevent exposure of conjugated reagents, including cells stained with those reagents, to room illumination.
2. Alexa Fluor® 647 fluorochrome emission is collected at the same instrument settings as for allophycocyanin (APC).
3. PerCP-Cy5.5–labelled antibodies can be used with FITC- and R-PE–labelled reagents in single-laser flow cytometers with no significant spectral overlap of PerCP-Cy5.5, FITC, and R-PE fluorescence.
4. PerCP-Cy5.5 is optimized for use with a single argon ion laser emitting 488-nm light. Because of the broad absorption spectrum of the tandem fluorochrome, extra care must be taken when using dual-laser cytometers, which may directly excite both PerCP and Cy5.5™. We recommend the use of cross-beam compensation during data acquisition or software compensation during data analysis.
5. Alexa Fluor® is a registered trademark of Molecular Probes, Inc., Eugene, OR.
6. The Alexa Fluor®, Pacific Blue™, and Cascade Blue® dye antibody conjugates in this product are sold under license from Molecular Probes, Inc. for research use only, excluding use in combination with microarrays, or as analyte specific reagents. The Alexa Fluor® dyes (except for Alexa Fluor® 430), Pacific Blue™ dye, and Cascade Blue® dye are covered by pending and issued patents.
7. Cy is a trademark of Amersham Biosciences Limited. This conjugated product is sold under license to the following patents: US Patent Nos. 5,486,616; 5,569,587; 5,569,766; 5,627,027.
8. Source of all serum proteins is from USDA inspected abattoirs located in the United States.
9. Caution: Sodium azide yields highly toxic hydrazoic acid under acidic conditions. Dilute azide compounds in running water before discarding to avoid accumulation of potentially explosive deposits in plumbing.
10. This product is subject to proprietary rights of Amersham Biosciences Corp. and Carnegie Mellon University and made and sold under license from Amersham Biosciences Corp. This product is licensed for sale only for research. It is not licensed for any other use. If you require a commercial license to use this product and do not have one return this material, unopened to BD Biosciences, 10975 Torreyana Rd, San Diego, CA 92121 and any money paid for the material will be refunded.
11. For fluorochrome spectra and suitable instrument settings, please refer to our Multicolor Flow Cytometry web page at www.bdbiosciences.com/colors.
12. Please refer to www.bdbiosciences.com/pharmingen/protocols for technical protocols.

References

Carlson MJ, West ML, Coghill JM, et al. In vitro-differentiated TH17 cells mediate lethal acute graft-versus-host disease with severe cutaneous and pulmonary pathologic manifestations. *Blood.* 2009; 113:1365-1374. (Biology)
Dong C. Th17 cells: Current understanding of their generation and regulation. *Eur J Immunol.* 2009; 39(3):640-644. (Biology)
Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. *Science.* 2003; 299(5609):1057-1061. (Biology)
Liang SC, Tan XY, Luxenberg DP, et al. Interleukin (IL)-22 and IL-17 are coexpressed by Th17 cells and cooperatively enhance expression of antimicrobial peptides. *J Exp Med.* 2006; 203:2271-2279. (Biology)
Ono M, Yaguchi H, Ohkura N, et al. Foxp3 controls regulatory T-cell function by interacting with AML1/Runx1. *Nature.* 2007; 446(7136):685-689. (Biology)
Shevach EM. Mechanisms of Foxp3+ T regulatory cell-mediated suppression. *Immunity.* 2009; 30:636-645. (Biology)
Zheng Y, Danilenko DM, Valdez P, et al. Interleukin-22, a T(H)17 cytokine, mediates IL-23-induced dermal inflammation and acanthosis. *Nature.* 2007; 445:648-651. (Biology)
Ziegler, SF. FOXP3: of mice and men. *Annu Rev Immunol.* 2006; :209-226. (Biology)

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