

## PRODUCT DESCRIPTION

This kit contains five conjugated antibodies and buffers that can be used for staining of human Th17 cells (1-12).

## MATERIALS PROVIDED & STORAGE

Store the unopened kit at 2-8 °C **in the dark**. Refer to the kit label for date of expiration.

PART	PART #	DESCRIPTION
Positive Markers	968421	125 µL of CD4 Alexa Fluor® 594 Mouse IgG <sub>2A</sub> (Clone 11830)
	968422	125 µL of IL-17A Alexa Fluor® 700 Mouse IgG <sub>2B</sub> (Clone 41809)
	968423	250 µL of RORα-PE Mouse IgG <sub>2B</sub> (Clone 784652)
	968424	125 µL of RORγt Alexa Fluor® 488 Rabbit IgG (Clone 1181A)
	968425	250 µL of CXCR6 APC Mouse IgG <sub>2B</sub> (Clone 56811)
FoxP3/Transcription Factor Fixation Concentrate (4X)	894065	8 mL of a formaldehyde solution.
FoxP3/Transcription Factor Fixation Diluent	894068	25 mL of a buffered detergent.
FoxP3/Transcription Factor Permeabilization & Wash Buffer (10X)	894356	25 mL of a buffered protein base with detergent and preservatives. <i>May contain a precipitate but will not affect product performance.</i>
Staining Buffer (1X)	895068	50 mL of a 1X staining buffer.

## INTENDED USE

This product is designed for the flow cytometric analysis of Th17 cells using five fluorochrome-conjugated antibodies.

## PRECAUTIONS

Some components in this kit contain formaldehyde which is a suspected carcinogen. Avoid contact with skin, eyes, and mucous membranes, and avoid inhaling fumes. In case of contact, wash immediately with water and seek medical advice.

Some components in this kit contain sodium azide which may react with lead and copper plumbing to form explosive metallic azides. Flush with large volumes of water during disposal.

## REFERENCES

1. Wilson, N.J. *et al.* (2007) *Nature Immunol.* **8**:950.
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3. Harrington, L.E. *et al.* (2005) *Nature Immunol.* **6**:1123.
4. Stockinger, B. and M. Veldhoen (2007) *Curr. Opin. Immunol.* **19**:281.
5. Palm, N.W. and R. Medzhitov (2007) *Nature Immunol.* **8**:549.
6. Weaver, C.T. *et al.* (2007) *Ann. Rev. Immunol.* **25**:821.
7. Korn, T. *et al.* (2007) *Nature* **448**:484.
8. Nurieva, R. *et al.* (2007) *Nature* **448**:480.
9. Liang, S.C. *et al.* (2006) *J. Exp. Med.* **203**:2271.
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11. Amadi-Obi, A. *et al.* (2007) *Nature Med.* **13**:711.
12. Bagwell, B. and E.G. Adams (1993) *Ann. N.Y. Acad. Sci.* **677**:167.

## GENERATION OF HUMAN Th17 DIFFERENTIATED CD4<sup>+</sup> T CELLS

The following protocol may be used to differentiate human PBMCs to Th17 cells. Alternatively, the CellXVivo™ Human Th17 Cell Differentiation Kit (R&D Systems®, Catalog # CDK003C), or other protocols may be used to generate Th17 cells.

1. Prepare cell culture flasks or plates containing immobilized anti-human CD3 and CD28 antibodies: Add 5.0 ug/mL of anti-human CD3 $\epsilon$  antibody (R&D Systems®, Catalog # MAB100) and 5.0 ug/mL of anti-human CD28 antibody (R&D Systems®, Catalog # AF-342-PB) in PBS to the cell culture flask or plate and incubate at 2-8 °C overnight. The next day, wash two times with PBS.
2. Purify CD4<sup>+</sup> T cells from total human PBMCs using a cell selection protocol, such as MagCollect™ Human CD4<sup>+</sup> T cell Isolation Kit (R&D Systems®, Catalog # MAGH102).
3. Resuspend cells in complete RPMI media containing 20 ng/mL of recombinant human IL-2 (R&D Systems®, Catalog # 202-IL), 10 ng/mL of human TGF- $\beta$ 1 (R&D Systems®, Catalog # 100-B), 20 ng/mL of recombinant human IL-23 (R&D Systems®, Catalog # 1290-IL), 40 ng/mL of recombinant human IL-6 (R&D Systems®, Catalog # 206-IL), 10 ng/mL of recombinant human IL-1 $\beta$  (R&D Systems®, Catalog # 201-LB) and 10  $\mu$ g/mL of anti-human IFN- $\gamma$  antibody (R&D Systems®, Catalog # AF-285-NA) and incubate at 37 °C for 7 days. Media and soluble factors should be replenished every 2-3 days.
4. On day 7, stimulate the cells with 50 ng/mL PMA and 200 ng/mL Calcium Ionomycin for 1 hour, then add a secretion inhibitor such as Monensin (3.0  $\mu$ M) for an additional 3 hours.

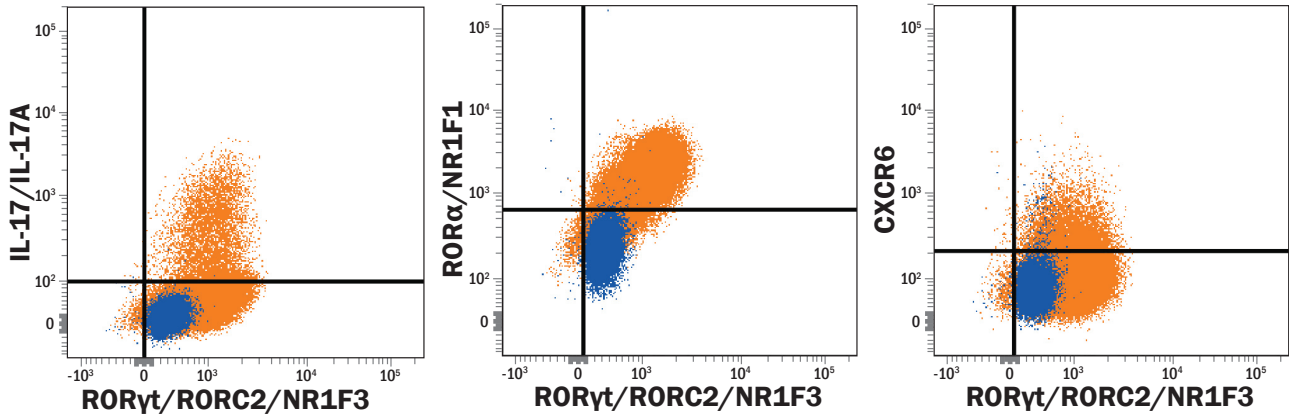
## INTRACELLULAR STAINING PROTOCOL

1. Harvest cells and wash with 2.0 mL of Flow Cytometry Staining Buffer (R&D Systems®, Catalog # FC001) or other BSA-containing buffer, by spinning at 300 x g for 5 minutes, using 5.0 mL flow cytometry tubes.
2. Fc-block cells with blocking IgG (1.0  $\mu$ g IgG/10<sup>6</sup> cells) for 10 minutes at 2-8 °C, if desired.
3. Surface stain the cells by adding 5.0  $\mu$ L of CD4-Alexa Fluor® 594 and 10  $\mu$ L of CXCR6-APC.
4. Incubate for 30-45 minutes at 2-8 °C **in the dark**.
5. Wash the cells two times with cold 1X PBS. During the washes, make up fresh 1X FoxP3/Transcription Factor Fixation Buffer by diluting FoxP3/Transcription Factor Fixation Concentrate (4X) with FoxP3/Transcription Factor Fixation Diluent (*i.e.* 100  $\mu$ L FoxP3/Transcription Factor Fixation Concentrate (4X) + 300  $\mu$ L FoxP3/Transcription Factor Fixation Diluent).
6. Resuspend the cells in fresh 1X FoxP3/Transcription Factor Fixation Buffer using 0.5 mL/tube. Incubate at 2-8 °C for 30 minutes. During this incubation, make up 1X FoxP3/Transcription Factor Permeabilization and Wash Buffer by diluting FoxP3/Transcription Factor Permeabilization and Wash Buffer (10X) with distilled water (*i.e.* 100  $\mu$ L FoxP3/Transcription Factor Permeabilization and Wash Buffer (10X) + 900  $\mu$ L diH<sub>2</sub>O) and keep at 2-8 °C.
7. Wash two times with fresh, cold, 1X FoxP3/Transcription Factor Permeabilization and Wash Buffer.
8. Add 5.0  $\mu$ L of IL-17A-Alexa Fluor® 700, 10  $\mu$ L of ROR $\alpha$ -PE, and 5.0  $\mu$ L of ROR $\gamma$ t Alexa Fluor® 488 to the cells and incubate for 30 minutes at 2-8 °C.
9. Wash the cells one time with cold 1X FoxP3/Transcription Factor Permeabilization and Wash Buffer.
10. Resuspend the cells in Flow Cytometry Staining Buffer and run on a flow cytometer.

## TECHNICAL HINTS

- Isotype controls may be used to set quadrant markers if desired: Mouse IgG<sub>2A</sub>-Alexa Fluor® 594 (R&D Systems®, Catalog # IC003T), Mouse IgG<sub>2B</sub>-Alexa Fluor® 700 (R&D Systems®, Catalog # IC0041N), Mouse IgG<sub>2B</sub>-PE (R&D Systems®, Catalog # IC0041P), Mouse IgG<sub>2B</sub>-APC (R&D Systems®, Catalog # IC0041A), and Rabbit IgG-Alexa Fluor® 488 (R&D Systems®, Catalog # IC1051G).
- A live/dead fixable viability dye may be used to exclude dead cells from analysis.
- Doublet exclusion gating is recommended to gate specifically on single cells.

## DATA EXAMPLES



**Figure 1:** Th17 cells were generated using the protocol above. Cells were harvested and stained with the indicated antibodies following the procedure. Live, single, CD4<sup>+</sup> cells are shown in the dot plots (determined using a fixable viability dye, doublet exclusion, and staining with anti-human CD4-Alexa Fluor® 594). Dot plots show relative RORγt<sup>+</sup>, IL-17A<sup>+</sup>, RORα<sup>+</sup>, and CXCR6<sup>+</sup> cells in CD4<sup>+</sup> resting (blue dots, lower left) and Th17-differentiated (orange dots, upper right quadrants). Quadrant markers were set based on isotype controls.