# Rapid, column-free two-step procedure for the enrichment of human Th17 cells from peripheral blood

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## Introduction

Th17 cells play a key role in anti-microbial immunity but have also been implicated in the development and progression of several autoimmune diseases. They are phenotypically identified as CXCR3 negative CD4$^+$ T-cells that express CCR6, CCR4, CD161, and IL-23R. Their cytokine expression profile can be heterogeneous; however, they typically produce IL-17 and can be functionally differentiated from Th1 cells by the lack of IFNγ production. Although not suitable for cell isolation, the lineage-specific transcription factor RORC2 has been identified as a definitive marker of human Th17 cells and is the master switch that drives their differentiation. Current magnetic isolation methods for Th17 cells require *in vitro* activation to induce IL-17 secretion and capture. To avoid *in vitro* activation, and in agreement with published flow cytometric sorting approaches, we have developed a two-step immunomagnetic, column-free method for the enrichment of human Th17 cells from fresh peripheral blood nucleated cells (PBNC). The isolation procedure involves the depletion of non-CD4$^+$ T-cells and CXCR3$^+$ cells using tetrameric antibody complexes directed against cell surface antigens that are crosslinked to dextran-coated magnetic particles. Targeted immunomagnetically labeled cells are depleted by placing the sample into a small hand held EasySep™ magnet. After a short incubation, enriched CD4$^+$CXCR3$^-$ T-cells are simply poured off into a new tube while the unwanted cells are retained in the magnet. To further enrich the Th17 subset, CCR6$^+$ cells present in the pre-enriched CD4$^+$CXCR3$^-$ sample are isolated using EasySep™ CCR6 positive selection. The frequency of human CD4$^+$CXCR3$^-$CCR6$^+$ T-cells in fresh PBNCs is 5 ± 2%. Following enrichment, purities of 94 ± 3% (n=10) can be obtained. Enriched CD4$^+$CXCR3$^-$CCR6$^+$ T-cells have increased levels of IL-17 production and minimal IFNγ production as assessed by ELISA and intracellular cytokine staining. Increased RORC2 mRNA expression is also found in the enriched CD4$^+$CXCR3$^-$CCR6$^+$ T-cells compared to total CD4$^+$ or CD4$^+$CXCR3$^+$ T-cells. The entire EasySep™ enrichment of human Th17 cells can be completed in 1 hour and 20 minutes and the can be automated using the RoboSep™ cell separator. Enrichment of unstimulated human Th17 cells enables the investigation of adaptive immune responses and regulation mechanisms required for the development of future therapies.

## Methods

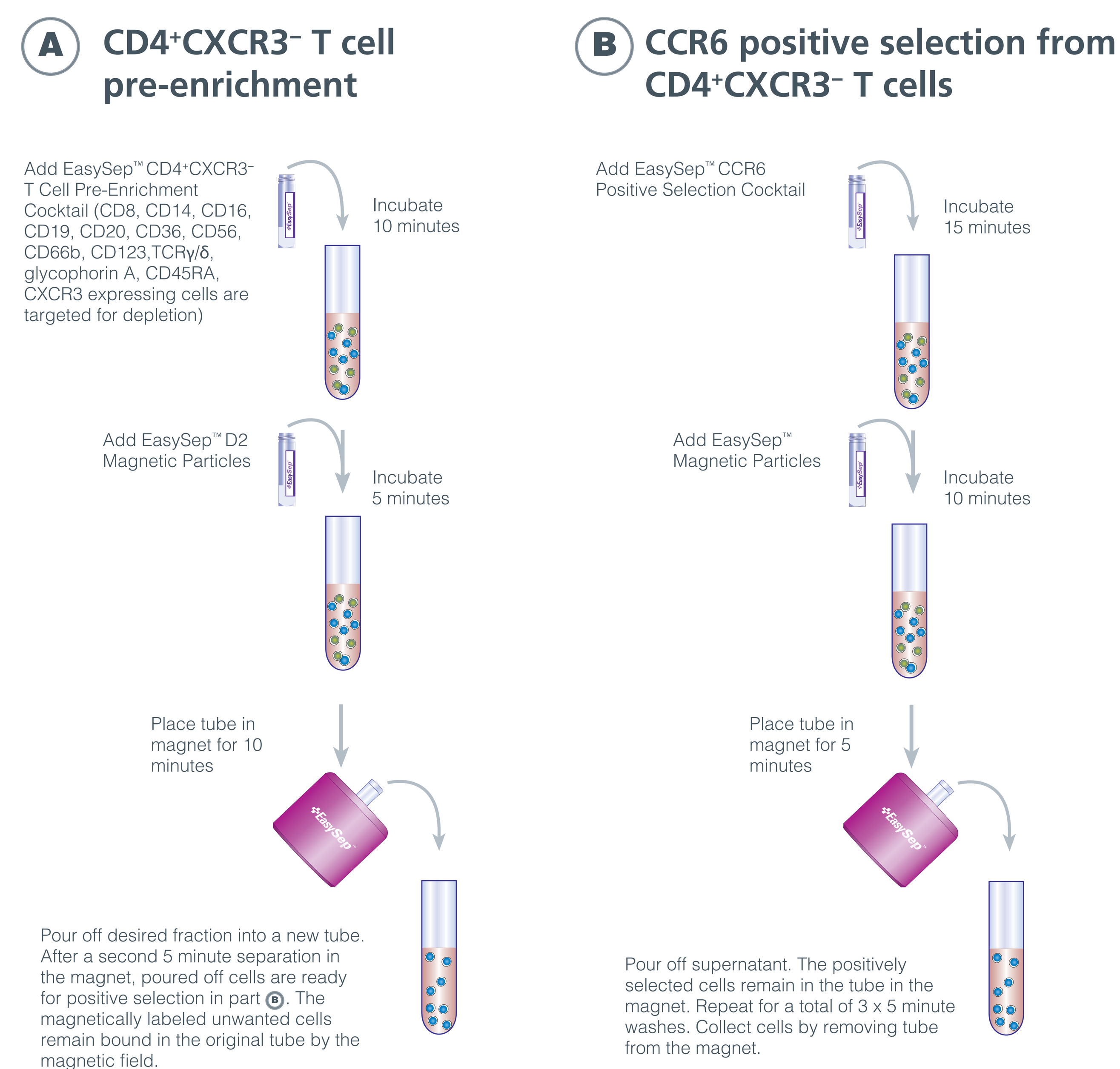
### Preparation of Starting Cell Suspension

A single cell suspension of mononuclear cells (PBMC) was prepared from either fresh whole blood or buffy coat suspensions of peripheral blood using Ficoll-paque PLUS. Alternatively, peripheral blood apheresis (Leucopak PBNC) cells were used following red blood cell lysis and one or more washes to remove platelets. Use of fresh (≤24 hours) cells is recommended. Typically start cells were resuspended at 5x10$^7$ cells/mL in PBS + 2% FBS and 1mM EDTA.

### Assessing Enriched Cells

The purity of Th17 (CD4$^+$CXCR3$^-$CCR6$^+$) T cells can be measured by flow cytometry after staining with fluorochrome-conjugated anti-CD4, anti-CXCR3, and anti-CCR6 antibodies. In addition, intracellular staining of IL-17 cytokine was assessed after stimulation of cells with PMA-Ionomycin. For ELISA, enriched cells were stimulated with anti-CD3/anti-CD28 beads for 24, 48, and 72 hours. Supernatants were collected and analysed for secreted cytokines.

**FIGURE 1: Two-step EasySep™ procedure for column-free enrichment of Th17 (CD4$^+$CXCR3$^-$CCR6$^+$) cells from human peripheral blood**




## Results

**TABLE 1: Purity and recovery of human Th17 (CD4$^+$CXCR3$^-$CCR6$^+$) cells enriched from peripheral blood by manual EasySep™ or RoboSep™**

| n | % start | % purity enriched | Typical recovery of CD4$^+$CXCR3$^-$CCR6$^+$ T cells from start |
|---|---|---|---|
| 16 | 4.7 ± 1.8 | 91.3 ± 3.7 | 1.5x10$^6$ CD4$^+$CXCR3$^-$CCR6$^+$ T cells from 1x10$^8$ peripheral blood nucleated cells |

Purities determined by flow cytometry. Values are expressed as means ±SD.

**FIGURE 2: Phenotypic assessment of human Th17 (CD4$^+$CXCR3$^-$CCR6$^+$) cells enriched using EasySep™**

Start: 5.4% CD4$^+$CXCR3$^-$CCR6$^+$ T Cells

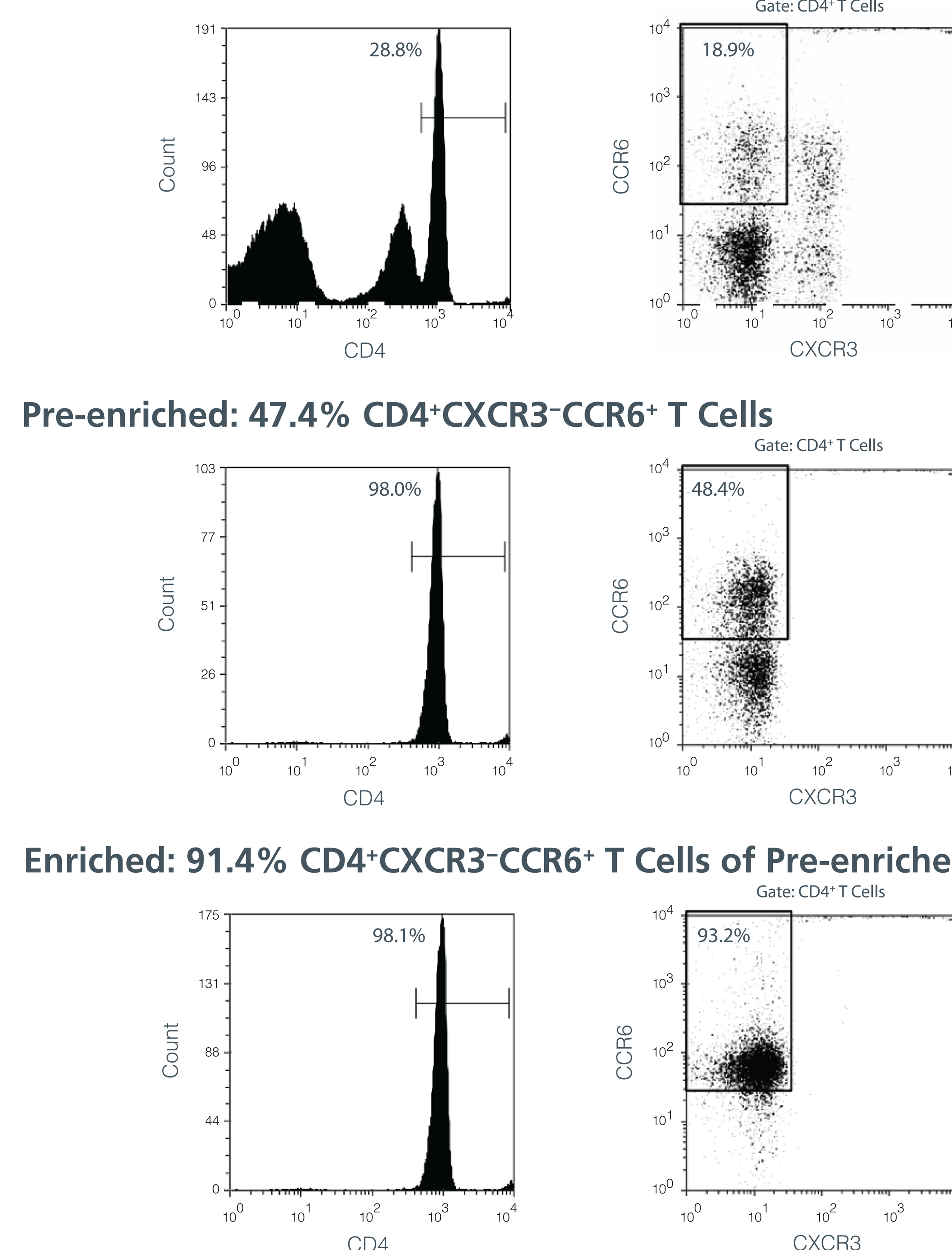
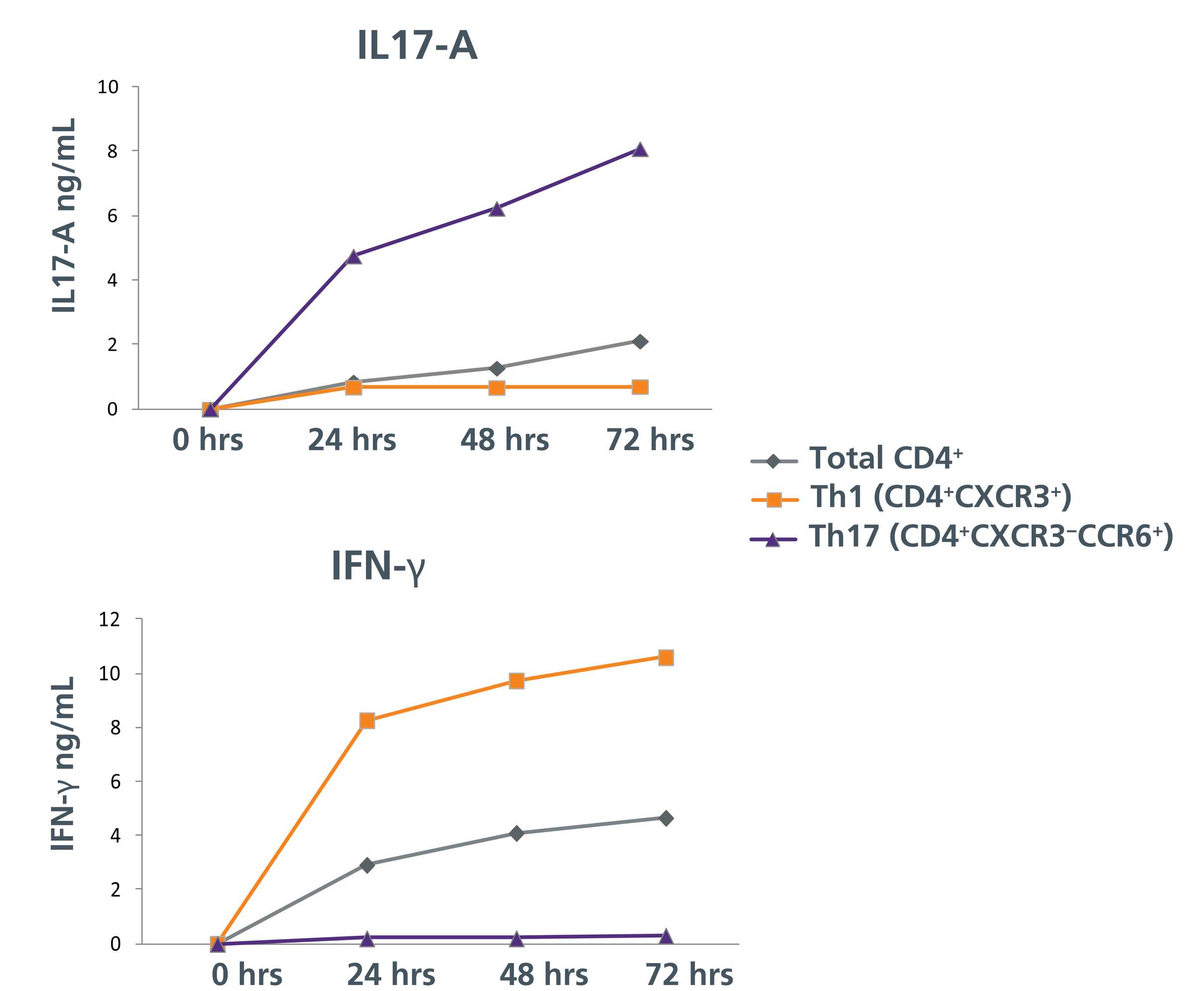
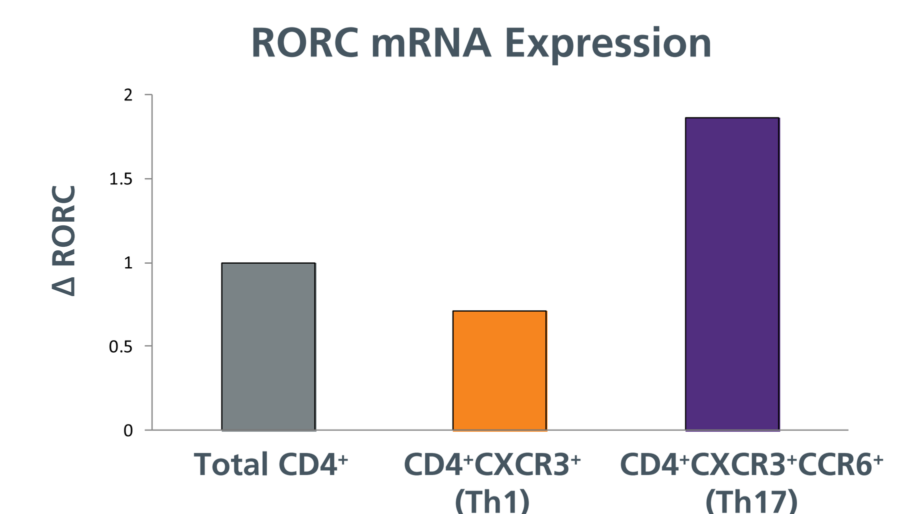



**FIGURE 3: Enriched CD4$^+$CXCR3$^-$CCR6$^+$ T cells show increased levels of IL17-A secretion by ELISA with minimal IFN-γ secretion compared to CD4$^+$CXCR3$^+$ T cells**




Freshly isolated CD4$^+$CXCR3$^-$CCR6$^+$ T cells were stimulated with anti-CD3/anti-CD28 coated beads at a ratio of 8:1 (cells: beads). Supernatants were collected at 24, 48, and 72 hours and cytokines were assessed by ELISA. Results for one of three comparable experiments are shown. A similar trend was observed with intracellular staining.

**FIGURE 4: Enriched CD4$^+$CXCR3$^-$CCR6$^+$ T cells show an increased level of RORC mRNA**




Freshly isolated CD4$^+$CXCR3$^-$CCR6$^+$ T cells were stimulated with anti-CD3/anti-CD28 beads at a ratio of 8:1 (cells: beads) for 48 hours. Expression of RORC relative to that of 18S was analysed by quantitative RT-PCR.

## Conclusions

- Highly enriched Th17 (CD4$^+$CXCR3$^-$CCR6$^+$) cells are obtained in 80 minutes; procedure can be automated using RoboSep™.
- Enriched Th17 (CD4$^+$CXCR3$^-$CCR6$^+$) cells demonstrate the following benefits:
  - can be obtained without prior *in vitro* stimulation
  - can be expanded by culturing with soluble anti-CD3 monoclonal antibody in the presence of irradiated APC for 14 days
  - increased levels of IL17 cytokine with minimal IFN-γ (compared to total CD4$^+$ or CD4$^+$CXCR3$^-$ T cells) as assessed by intracellular cytokine staining (data not shown) or analysis of supernatants by ELISA
  - increased level of RORC mRNA expression compared to total CD4$^+$ or CD4$^+$CXCR3$^+$ T cells