PROCEDURE

EasySep™

POSITIVE SELECTION

STEMCELL™ TECHNOLOGIES

HUMAN TH17 CELL ENRICHMENT KIT

CATALOG #18162

PRODUCT DESCRIPTION

The EasySep™ Human Th17 Cell Enrichment Kit is a two-step method designed to enrich human Th17 ($CD4^+CXCR3^-CCR6^+$) T cells from fresh peripheral blood mononuclear cells and leukapheresis samples. *Please note that this kit is not recommended for use with frozen PBMC.* $CD4^+CXCR3^-$ T cells are first pre-enriched by negative selection using the EasySep™ Human $CD4^+CXCR3^-$ T Cell Pre-Enrichment Cocktail (Catalog #19152C.1). Next, CCR6 positive $CD4^+CXCR3^-$ T cells are selected using the EasySep™ Human CCR6 Positive Selection Cocktail (Catalog #18262C).

This kit is compatible for use with RoboSep™ **(Section A, page 1)**, the Purple EasySep™ Magnet **(Section B, page 2)**, and "The Big Easy" Silver EasySep™ Magnet **(Section C, page 3)**.

PLEASE NOTE: Two different magnetic particles are provided in the kit– EasySep™ D2 Magnetic Particles (Catalog #19650) and EasySep™ Magnetic Nanoparticles (Catalog #18150H) **(brown ●).** It is important to follow the procedures outlined in this Product Information Sheet carefully and use the EasySep™ D2 Magnetic Particles only for the Human $CD4^+CXCR3^-$ T Cell Pre-Enrichment protocol and the EasySep™ Magnetic Nanoparticles **(brown ●)** only for the Human CCR6 Positive Selection protocol. Please contact STEMCELL Technologies' Technical Support at techsupport@stemcell.com for more information.

SECTION A:

FULLY AUTOMATED PROTOCOL USING ROBOSEP™ (CATALOG #20000).

This procedure is used for processing **500 µL - 8 mL** of sample (up to 4×10^8 cells).

I. RoboSep™ Human $CD4^+CXCR3^-$ T Cell Pre-Enrichment

1. Prepare a cell suspension at a concentration of 5×10^7 cells/mL in RoboSep™ Buffer (Catalog #20104) (see Notes and Tips, page 3). Cells must be placed in a 14 mL (17 x 100 mm) polystyrene tube to properly fit into the RoboSep™ carousel.
   Falcon™ 14 mL Polystyrene Round-Bottom Tubes (BD Biosciences, Catalog #352057) are recommended.
2. Select the appropriate RoboSep™ protocol:
   - Human $CD4^+CXCR3^-$ T cell Pre-Enrichment 19152

   If a modified RoboSep™ protocol is required, please contact STEMCELL Technologies' Technical Support at techsupport@stemcell.com.
3. Load the RoboSep™ carousel as directed by the on-screen prompts. **Vortex EasySep™ D2 Magnetic Particles for 30 seconds before loading. Ensure that the particles are in a uniform suspension with no visible aggregates.** When all desired quadrants are loaded, press the green "Run" button. All cell labeling and separation steps will be performed by RoboSep™.
4. When cell separation is complete, remove the tube containing the pre-enriched cells from the RoboSep™ carousel. Collect the pre-enriched cells in the 14 mL tube located in the Q2 quadrant.
5. Centrifuge the pre-enriched cells at 200 x *g* for **10 minutes**, room temperature (15 - 25°C). Carefully aspirate or decant supernatant. Resuspend sample in **250 µL** (for 0.25 - 1.25 x 10^8 start cells), **500 µL** (for >1.25 - 2.0 x 10^8 start cells), or **1 mL** (for >2.0 - 4.0 x 10^8 start cells) of RoboSep™ Buffer (see Table 1, page 3). The sample is now ready for the CCR6 Positive Selection using RoboSep™ **(Section A II)**.

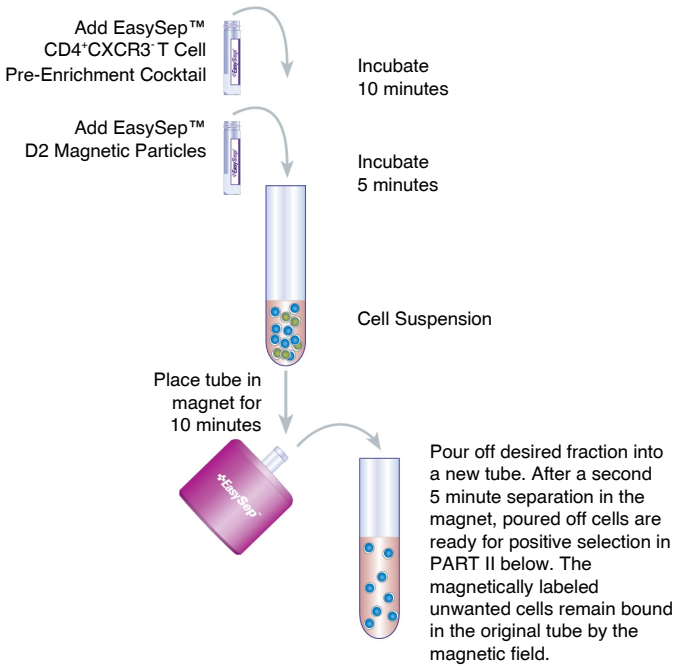
II. RoboSep™ Human CCR6 Positive Selection

1. Select the appropriate RoboSep™ protocol:
   - Human CCR6 Positive Selection 18262

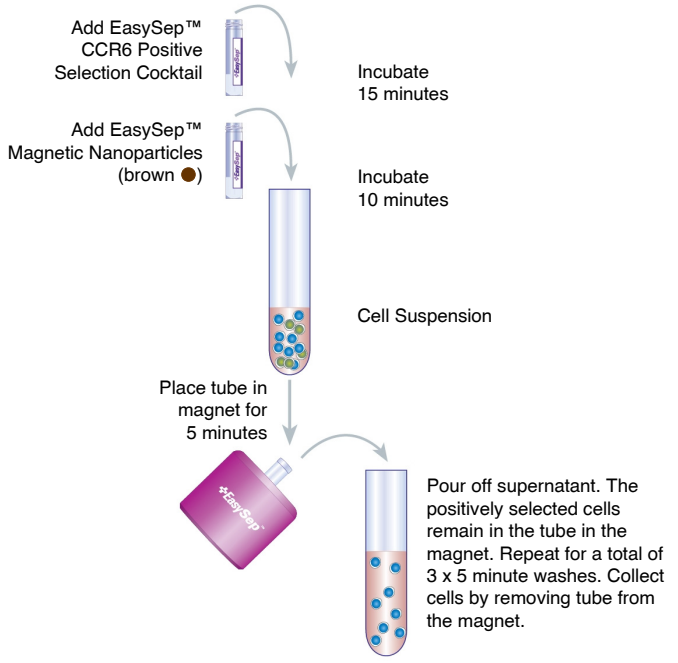
   If a modified RoboSep™ protocol is required, please contact STEMCELL Technologies' Technical Support at techsupport@stemcell.com.
2. Load the RoboSep™ carousel as directed by the on-screen prompts. Mix EasySep™ Magnetic Nanoparticles **(brown ●)** before loading to ensure that they are in a uniform suspension by vigorously pipetting up and down more than 5 times. *Vortexing is not recommended.* When all desired quadrants are loaded, press the green "Run" button. All cell labeling and separation steps will be performed by RoboSep™.
3. When cell separation is complete, remove the tube containing the isolated cells from the magnet. Resuspend in an appropriate amount of medium. The positively selected cells are now ready for use.

MANUAL EASYSEP™ PROTOCOL DIAGRAM

PART I: $CD4^+CXCR3^-$ T CELL PRE-ENRICHMENT



PART II: CCR6 POSITIVE SELECTION



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VERSION 1.2.1

DOCUMENT #29669

SECTION B:

MANUAL EASYSEP™ PROTOCOL USING THE PURPLE EASYSEP™ MAGNET (CATALOG #18000).

I. EasySep™ Human CD4$^+$CXCR3$^-$ T Cell Pre-Enrichment

This procedure is used for processing **500 µL - 2 mL** of sample (up to 1 x 10^8 cells).

1. Prepare a cell suspension at a concentration of 5 x 10^7 cells/mL in recommended medium (see Notes and Tips, page 3). Cells must be placed in a 5 mL (12 x 75 mm) polystyrene tube to properly fit into the Purple EasySep™ Magnet.

   Falcon™ 5 mL Polystyrene Round-Bottom Tubes (BD Biosciences, Catalog #352058) are recommended.
2. Add the EasySep™ Human CD4$^+$CXCR3$^-$ T Cell Pre-Enrichment Cocktail at **50 µL/mL cells** (e.g. for 2 mL of cells, add 100 µL of cocktail). Mix well and incubate at room temperature (15 - 25°C) for **10 minutes**.
3. Vortex the EasySep™ D2 Magnetic Particles for 30 seconds. Ensure that the particles are in a uniform suspension with no visible aggregates.
4. Add the D2 Magnetic Particles at **100 µL/mL cells** (e.g. for 2 mL of cells, add 200 µL of particles). Mix well and incubate at room temperature (15 - 25°C) for **5 minutes.**
5. Bring the cell suspension up to a **total volume** of **2.5 mL** by adding recommended medium. Mix the cells in the tube by gently pipetting up and down 2 - 3 times. Place the tube (without cap) into the magnet and incubate at room temperature (15 - 25°C) for **10 minutes**.
6. Pick up the EasySep™ Magnet, and in one continuous motion invert the magnet and tube, pouring off the desired fraction into a new 5 mL polystyrene tube. The magnetically labeled unwanted cells will remain bound inside the original tube, held by the magnetic field of the EasySep™ Magnet. Leave the magnet and tube in inverted position for 2 - 3 seconds, then return to upright position. *Do not shake or blot off any drops that may remain hanging from the mouth of the tube.*
7. Remove the original tube from the EasySep™ Magnet and place the new tube containing the desired cells into the magnet and set aside for **5 minutes**. Repeat Step 6 for a total of 2 separations (**1 x 10 minutes**, **1 x 5 minutes**) in the magnet.
8. Centrifuge pre-enriched cells in the new tube at 200 x *g* for **10 minutes**, room temperature (15 - 25°C). Carefully aspirate or decant supernatant. Resuspend sample in **250 µL** of the recommended medium (see Table 1, page 3) and continue with the EasySep™ Human CCR6 Positive Selection protocol **(Section B II below)**.

II. EasySep™ Human CCR6 Positive Selection

1. Add the EasySep™ Human CCR6 Positive Selection Cocktail at **50 µL/mL cells**. Mix well and incubate at room temperature (15 - 25°C) for **15 minutes**.
2. Mix the EasySep™ Magnetic Nanoparticles **(brown ●)** to ensure that they are in a uniform suspension by pipetting up and down vigorously more than 5 times. *Vortexing is not recommended.* Add the nanoparticles at **100 µL/mL cells** . Mix well and incubate at room temperature (15 - 25°C) for **10 minutes**.
3. Bring the cell suspension to a **total volume** of **2.5 mL** by adding recommended medium. Mix the cells in the tube by gently pipetting up and down 2 - 3 times. Place the tube (without cap) into the magnet. Set aside for **5 minutes**.
4. Pick up the EasySep™ Magnet, and in one continuous motion invert the magnet and tube, pouring off the supernatant fraction into a new 5 mL polystyrene tube. The magnetically labeled CCR6$^+$ cells will remain inside the tube, held by the magnetic field of the EasySep™ Magnet. Leave the magnet and tube in inverted position for 2 - 3 seconds, then return to upright position. *Do not shake or blot off any drops that may remain hanging from the mouth of the tube.*
5. Remove the tube from the magnet and add **2.5 mL** of the recommended medium. Mix the cell suspension by gently pipetting up and down 2 - 3 times. Place the tube back in the magnet and set aside for **5 minutes.**
6. Repeat Steps 4 and 5, then Step 4 once more, for a total of **3 x 5 minute** separations in the magnet. Remove the tube from the magnet and resuspend cells in an appropriate amount of desired medium. The positively selected cells are now ready for use.

SECTION C:

MANUAL EASYSEP™ PROTOCOL USING "THE BIG EASY" SILVER EASYSEP™ MAGNET (CATALOG #18001).

I. EasySep™ Human CD4$^+$CXCR3$^-$ T Cell Pre-Enrichment

This procedure is used for processing **500 µL - 8 mL** of sample (up to 4 x 10^8 cells).

1. Prepare a cell suspension at a concentration of 5 x 10^7 cells/mL in recommended medium (see Notes and Tips, page 3). Cells must be placed in a 14 mL (12 x 75 mm) polystyrene tube to properly fit into the Silver EasySep™ Magnet.

   Falcon™ 14 mL Polystyrene Round-Bottom Tubes (BD Biosciences, Catalog #352057) are recommended.
2. Add the EasySep™ Human CD4$^+$CXCR3$^-$ T Cell Pre-Enrichment Cocktail at **50 µL/mL cells** (e.g. for 2 mL of cells, add 100 µL of cocktail). Mix well and incubate at room temperature (15 - 25°C) for **10 minutes**.
3. Vortex the EasySep™ D2 Magnetic Particles for 30 seconds. Ensure that the particles are in a uniform suspension with no visible aggregates.
4. Add the D2 Magnetic Particles at **100 µL/mL cells** (e.g. for 2 mL of cells, add 200 µL of particles). Mix well and incubate at room temperature (15 - 25°C) for **5 minutes**.
5. Bring the cell suspension up to a **total volume** of **5 mL** (for ≤2 x 10^8 cells) or to **10 mL** (for >2.0 - 4.0 x 10^8 cells) by adding recommended medium. Mix the cells in the tube by gently pipetting up and down 2 - 3 times. Place the tube (without cap) into the magnet and incubate at room temperature (15 - 25°C) for **10 minutes**.
6. Pick up the EasySep™ Magnet, and in one continuous motion invert the magnet and tube, pouring off the desired fraction into a new 14 mL polystyrene tube. The magnetically labeled unwanted cells will remain bound inside the original tube, held by the magnetic field of the EasySep™ Magnet. Leave the magnet and tube in inverted position for 2 - 3 seconds, then return to upright position. *Do not shake or blot off any drops that may remain hanging from the mouth of the tube.*
7. Remove the original tube from the EasySep™ Magnet and place the new tube containing the desired cells into the magnet and set aside for **5 minutes**. Repeat Step 6 for a total of 2 separations (**1 x 10 minutes**, **1 x 5 minutes**) in the magnet.
8. Centrifuge pre-enriched cells in the new tube at 200 x *g* for **10 minutes**, at room temperature (15 - 25°C). Carefully aspirate or decant supernatant. Resuspend sample in **250 µL** (for 0.25 - 1.25 x 10^8 start cells), **500 µL** (for >1.25 - 2.0 x 10^8 start cells) or **1 mL** (for >2.0 - 4.0 x 10^8 start cells) of the recommended medium (see Table 1, page 3) and continue with the EasySep™ Human CCR6 Positive Selection protocol **(Section C II below)**.

II. EasySep™ Human CCR6 Positive Selection

1. Add the EasySep™ Human CCR6 Positive Selection Cocktail at **50 µL/mL cells**. Mix well and incubate at room temperature (15 - 25°C) for **15 minutes**.
2. Mix the EasySep™ Magnetic Nanoparticles **(brown ●)** to ensure that they are in a uniform suspension by pipetting up and down vigorously more than 5 times. *Vortexing is not recommended.* Add the nanoparticles at **100 µL/mL cells** . Mix well and incubate at room temperature (15 - 25°C) for **10 minutes**.
3. Bring the cell suspension to a **total volume** of **2.5 mL** by adding recommended medium Mix the cells in the tube by gently pipetting up and down 2 - 3 times. Place the tube (without cap) into the magnet. Set aside for **5 minutes**.
4. Pick up the EasySep™ Magnet, and in one continuous motion invert the magnet and tube, pouring off the supernatant fraction into a new 14 mL polystyrene tube. The magnetically labeled CCR6$^+$ cells will remain inside the tube, held by the magnetic field of the EasySep™ Magnet. Leave the magnet and tube in inverted position for 2 - 3 seconds, then return to upright position. *Do not shake or blot off any drops that may remain hanging from the mouth of the tube.*
5. Remove the tube from the magnet and add **2.5 mL** of the recommended medium. Mix the cell suspension by gently pipetting up and down 2 - 3 times. Place the tube back in the magnet and set aside for **5 minutes**.
6. Repeat Steps 4 and 5, then Step 4 once more, for a total of **3 x 5 minute** separations in the magnet. Remove the tube from the magnet and resuspend cells in an appropriate amount of desired medium. The positively selected cells are now ready for use.

CATALOG #18162

Components:	For labeling 10^9 total cells
• EasySep™ Human $CD4^+CXCR3^-$ T Cell Pre-Enrichment Cocktail	1.0 mL
• EasySep™ D2 Magnetic Particles	2 x 1.0 mL
• EasySep™ Human CCR6 Positive Selection Cocktail	0.5 mL
• EasySep™ Magnetic Nanoparticles Positive Selection (brown ●)	1.0 mL

EasySep™ POSITIVE SELECTION

REQUIRED EQUIPMENT:

EasySep™ Magnet (Catalog #18000), or "The Big Easy" EasySep™ Magnet (Catalog #18001), or RoboSep™ (Catalog #20000).

PRODUCT DESCRIPTION AND APPLICATIONS:

EasySep™ Human Th17 Cell Enrichment Kit (Catalog #18162) is a two-step isolation kit designed to enrich Th17 ($CD4^+CXCR3^-CCR6^+$) T cells from fresh peripheral blood mononuclear cells or ammonium chloride-lysed leukapheresis samples.

EASYSEP™ LABELING OF HUMAN CELLS:

Target cells are specifically labeled with dextran-coated magnetic particles using bispecific Tetrameric Antibody Complexes (TAC). These complexes recognize both dextran and the target cell surface antigen (Figure 1). The small size of the magnetic dextran iron particles allows for efficient binding to the TAC-labeled cells, and does not interfere with subsequent FACS analysis. Magnetically labeled cells are then separated from unlabeled cells using the EasySep™ procedure.

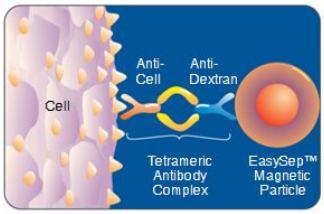


Figure 1.
Schematic Drawing of EasySep™ TAC Magnetic Labeling of Human Cells.

NOTES AND TIPS:

PREPARING THE CELL SUSPENSION

FROM WHOLE PERIPHERAL BLOOD

Prepare a mononuclear cell suspension from fresh whole peripheral blood by density gradient centrifugation.

FROM PERIPHERAL BLOOD APHERESIS (LEUKOPAK)

If working with large volumes (>150 mL), concentrate Leukopak cells first by centrifuging at 500 x *g* for 10 minutes. Remove the supernatant and resuspend the cells in 1/10th of the original Leukopak volume with recommended medium (e.g. for 300 mL of cells, resuspend in 30 mL of recommended medium). For small volumes (150 mL or less), add the Ammonium Chloride Solution (Catalog #07800/07850) directly to the cell suspension.

1. Add an equal volume of Ammonium Chloride Solution to the Leukopak suspension (e.g. for 5 mL of Leukopak suspension, add 5 mL Ammonium Chloride Solution).
2. Incubate 15 minutes on ice.
3. Centrifuge at 500 x *g* for 10 minutes at room temperature (15 - 25°C). Remove the supernatant.
4. Wash the cells by topping up the tube with recommended medium. Centrifuge the cells at 150 x *g* for 10 minutes at room temperature (15 - 25°C) with the brake off. Carefully remove the supernatant.
5. Repeat the wash step one or more times until most of the platelets have been removed (indicated by a clear supernatant).
6. Resuspend cells at recommended cell concentration, in the recommended medium.

TABLE 1. RECOMMENDED RESUSPENSION VOLUMES FOR CCR6 POSITIVE SELECTION PROTOCOL

MAGNET	$CD4^+CXCR3^-$ T CELL PRE-ENRICHMENT		$CCR6^+$ SELECTION
	STARTING CELL NUMBER	VOLUME	RESUSPENSION VOLUME
Purple	≤1 x 10^8	0.5 - 2.0 mL	250 µL
Silver/ RoboSep™	≤1.25 x 10^8	0.5 - 2.5 mL	250 µL
	>1.25 x 10^8 - 2 x 10^8	>2.5 - 4.0 mL	500 µL
	>2 x 10^8 - 4 x 10^8	>4.0 - 8.0 mL	1.0 mL

RECOMMENDED MEDIUM The recommended medium is RoboSep™ Buffer (Catalog #20104), or phosphate buffered saline (PBS) + 2% fetal bovine serum (FBS) (Catalog #07905) with 1 mM EDTA. Medium should be Ca^{++} and Mg^{++} free.

ASSESSING PURITY 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.

CCR6 antibody clone: G034E3 is recommended at a concentration of 0.25 µg/mL. CXCR3 antibody clone: G025H7 is recommended at a concentration of 4.0 µg/mL.

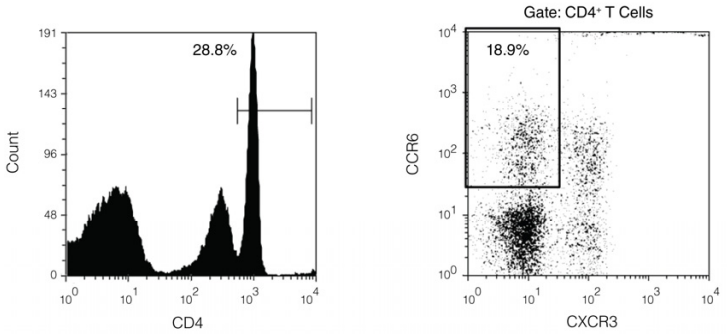
In addition, intracellular staining of IL-17 cytokine may be assessed after stimulation of cells with PMA-Ionomycin.

REFERENCES

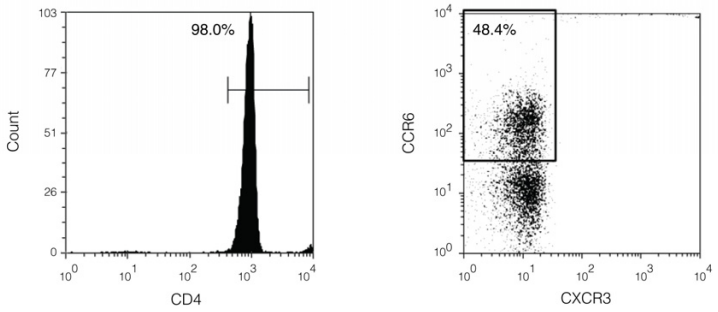
1. Crome SQ, et al. Inflammatory effects of ex vivo human Th17 cells are suppressed by regulatory T cells. J Immunol; 185 (6): 3199 - 208, 2010.
2. Rossi RL, et al. Distinct microRNA signatures in human lymphocyte subsets and enforcement of the naïve state in $CD4^+$ T cells by the microRNA miR-125b. Nat Immunology; 12(8): 796 - 803, 2011.

TYPICAL EASYSEP™ HUMAN TH17 CELL ENRICHMENT PROFILE:

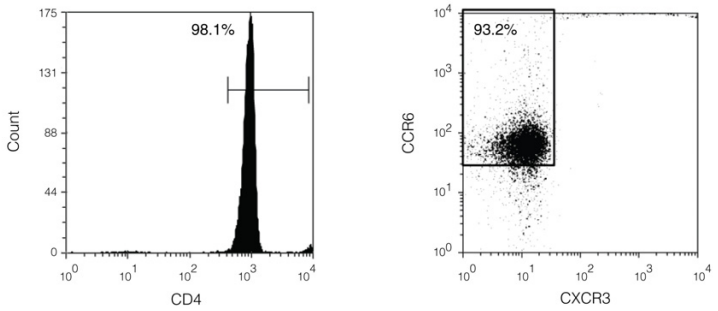
Start: 5.4% $CD4^+CXCR3^-CCR6^+$ T Cells



Pre-enriched: 47.4% $CD4^+CXCR3^-CCR6^+$ T Cells



Enriched: 91.4% $CD4^+CXCR3^-CCR6^+$ T Cells of Pre-enriched Fraction



Starting with fresh peripheral blood nucleated cells, the $CD4^+CXCR3^-CCR6^+$ T cell content of the enriched fraction typically ranges from 85 - 94%. Intracellular staining for IL-17 producing cells typically ranges from 5 - 20% $IL\text{-}17^+$ cells. These values vary widely amongst donors. IFN-γ producing cells are typically <5% of the enriched fraction.

COMPONENT DESCRIPTIONS:

EASYSEP™ HUMAN CD4$^+$CXCR3$^-$ T CELL PRE-ENRICHMENT COCKTAIL **CODE #19152C.1**

This cocktail contains a combination of monoclonal antibodies bound in bispecific Tetrameric Antibody Complexes (TAC) which are directed against cell surface antigens on human blood cells (CD8, CD14, CD16, CD19, CD20, CD36, CD56, CD66b, CD123, TCRγ/δ, glycophorin A, CD45RA, CXCR3) and dextran. The mouse monoclonal antibody subclass is IgG_1. It should be noted that this product is a biological reagent, and as such cannot be completely characterized or quantified. Some variability is unavoidable.

EASYSEP™ D2 MAGNETIC PARTICLES **CODE #19650**

A suspension of magnetic dextran iron particles in TRIS buffer.

EASYSEP™ HUMAN CCR6 POSITIVE SELECTION COCKTAIL **CODE #18262C**

This cocktail contains a combination of monoclonal antibodies bound in bispecific Tetrameric Antibody Complexes (TAC) which are directed against CCR6 and dextran. The mouse monoclonal antibody subclass is IgG_1. This cocktail is supplied in PBS and contains an antibody against human Fc receptor. It should be noted that this product is a biological reagent, and as such cannot be completely characterized or quantified. Some variability is unavoidable.

EASYSEP™ MAGNETIC NANOPARTICLES POSITIVE SELECTION (brown ●)

CODE #18150H

A suspension of magnetic dextran iron nanoparticles in water.

STABILITY AND STORAGE:

EASYSEP™ HUMAN CD4$^+$CXCR3$^-$ T CELL PRE-ENRICHMENT COCKTAIL
EASYSEP™ D2 MAGNETIC PARTICLES
EASYSEP™ HUMAN CCR6 POSITIVE SELECTION COCKTAIL
EASYSEP™ MAGNETIC NANOPARTICLES POSITIVE SELECTION

Product stable at 2 - 8°C until expiry date as indicated on label. Contents have been sterility tested. Do not freeze this product. This product may be shipped at room temperature (15 - 25°C), and should be refrigerated upon receipt.