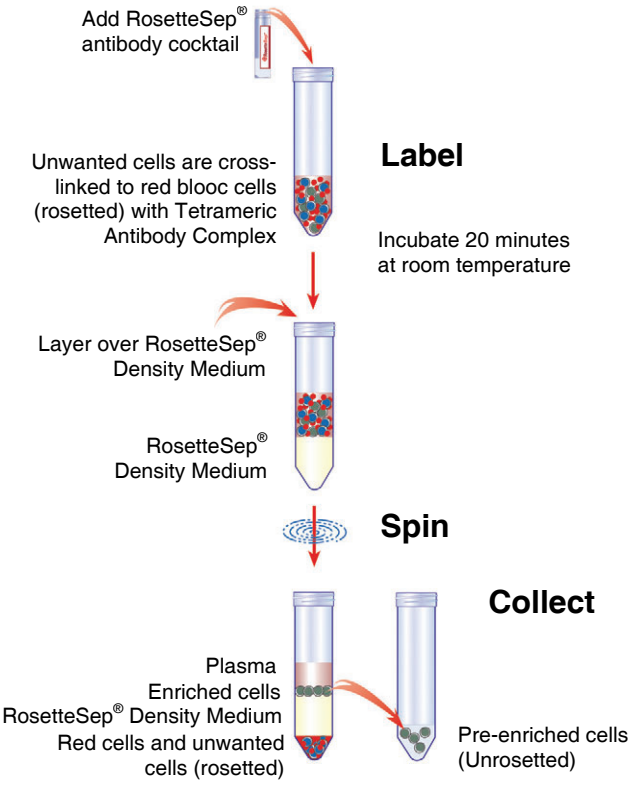
PRODUCT DESCRIPTION

The Complete Kit for Human $CD4^+CD127^{low}CD49d^-CD25^+$ Regulatory T Cells (Catalog # 15861) is a two-step cell isolation kit. $CD4^+CD127^{low}$ $CD49d^-$ T cells are first pre-enriched from whole blood or buffy coat using the RosetteSep® Human $CD4^+CD127^{low}$ $CD49d^-$ Pre-Enrichment Cocktail (Catalog # 15364) by negative selection (Section A, page 1). Following pre-enrichment, cells expressing high levels of CD25 are positively selected using EasySep® Human CD25 Positive Selection Kit (Catalog #18231) (Section B, page 3).

ROSETTESEP® PROTOCOL DIAGRAM



SECTION A:

ROSETTESEP® HUMAN $CD4^+CD127^{LOW}CD49d^-$ T CELL PRE- ENRICHMENT COCKTAIL PROTOCOL

Ensure that blood sample, recommended medium (see Note and Tips on page 2), density medium (RosetteSep® DM-L (Catalog #15705) or Ficoll-Paque™ PLUS (Catalog #07957)) and centrifuge are all at room temperature (15 – 25°C).

1. Add RosetteSep® Human $CD4^+CD127^{low}CD49d^-$ Regulatory T Cell Pre-Enrichment Cocktail at **50 µL/mL** of whole blood (e.g. for 2 mL of whole blood, add 100 µL of cocktail).
2. Mix well and incubate **20 minutes** at room temperature (15 – 25°C).
3. Dilute sample with an equal volume of PBS + 2% FBS and mix gently.
4. Layer the diluted sample on top of the density medium OR layer the density medium underneath the diluted sample. Be careful to minimize mixing of the density medium and sample (see Table 1 for volume recommendations). With 50 mL centrifuge tubes, we suggest using 15 mL density medium to easily remove the enriched layer.

Table 1: Recommended Volumes and Tube Sizes

Whole Blood (mL)	PBS + 2% FBS (mL)	Density Medium (mL)	Tube Size (mL)
1	1	1.5	5
2	2	3	14
3	3	3	14
4	4	4	14
5	5	15	50
10	10	15	50
15	15	15	50

5. Centrifuge for **30 minutes** at 400 *x g* (see page 2 for conversion of *g* to RPM) at room temperature (15 – 25°C), with the brake off.
6. Remove the enriched cells from the density medium:plasma interface.

   NOTE: Due to the low frequency of $CD4^+CD127^{low}CD49d^-$ regulatory T cells in whole blood samples, no cells will be visible at the interface. It is advisable to remove most of the density medium along with the enriched cells, while leaving the red blood cell pellet undisturbed in order to ensure their complete recovery
7. Wash enriched cells once with PBS + 2% FBS and centrifuge cells for 10 minutes at 200 x *g* at room temperature. Aspirate or decant the supernatant. Repeat.
8. Resuspend the enriched cells in **500 µL** of the recommended media.
9. Transfer 500 µL of pre-enriched sample into a 5 mL (12 x 75 mm) polystyrene tube to properly fit into the EasySep® Magnet (Catalog #18000) or into a 14 mL (17 x 100 mm) polystyrene tube to properly fit into the "The Big Easy" EasySep® Silver Magnet (Catalog #18001) and continue with the EasySep® Human CD25 Positive Selection Procedure (see Section B, page 3).

   Falcon™ 5 mL Polystyrene Round-Bottom Tubes (BD, Catalog #352058) are recommended.

   Falcon™ 14 mL Polystyrene Round-Bottom Tubes (BD, Catalog #352057) are recommended.

CATALOG #15864 For labeling 200 mL of whole blood

Components:

- RosetteSep® Human $CD4^+CD127^{low}CD49d^-$ Pre-Enrichment Cocktail 5 x 2 mL
- EasySep® Human CD25 Positive Cocktail 1 mL
- EasySep® Magnetic Particle 1 mL

RosetteSep® EasySep®

PRODUCT INFORMATION SHEET

PRODUCT DESCRIPTION & APPLICATIONS:

The RosetteSep® Human $CD4^+CD127^{low}$ $CD49d^-$ T cell enrichment cocktail is designed to enrich $CD4^+CD127^{low}$ $CD49d^-$ T cells from whole blood or buffy coat.

ROSETTESEP® LABELING OF HUMAN CELLS:

The RosetteSep® antibody cocktail crosslinks unwanted cells in human whole blood to multiple red blood cells (RBCs), forming immunorosettes (Figure 1). This increases the density of the unwanted (rosetted) cells, such that they pellet along with the free RBCs when centrifuged over a buoyant density medium such as RosetteSep® DM-L or Ficoll-Paque™ PLUS. Desired cells are never labeled with antibody and are easily collected as a highly enriched population at the interface between the plasma and the buoyant density medium.

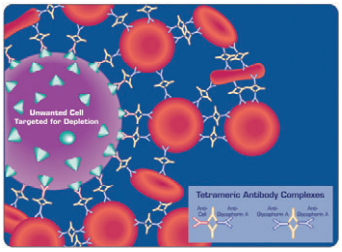


Figure 1.

Rosette of Unwanted Cells and RBCs Formed by RosetteSep® Tetrameric Antibody Complexes (TAC)

NOTES AND TIPS:

Sample Preparation. Ensure that the fresh whole blood sample is at room temperature (15 - 25°C) prior to using RosetteSep®. Do not store fresh whole blood at 2 - 4°C. Samples should be processed immediately within 12 – 24 hours. We do not recommend using samples that are more than 24 hours old as it will give sub-optimal cell isolation results due to cell death.

Samples other than whole blood. Although RosetteSep® has been optimized for use with whole blood, cells can be enriched from other sources such as buffy coat. For buffy coat, we recommend using RosetteSep® provided that the concentration of nucleated cells does not exceed 5 x 10^7 cells/mL and red blood cells (RBCs) are present at a ratio of at least 50 RBCs per nucleated cell in the sample.

Recommended Medium. The recommended medium is RoboSep® Buffer (Catalog #20104) or Phosphate Buffered Saled (PBS) with 2% FBS (Catalog #07905) + 1 mM EDTA. Medium should be Ca^{++} and Mg^{++} free.

Density Medium. Density medium refers to RosetteSep® DM-L (Catalog #15705) or Ficoll-Paque™ PLUS (Catalog #07957). Recovery of lymphocytes may be improved with the use of STEMCELL's unique density medium, RosetteSep® DM-L.

Sample dilution prior to Ficoll-Paque™ PLUS. Diluting the blood sample with a 3 -4 fold volume of PBS + 2% FBS may result in increased recovery and lower red blood cell contamination.

Conversion of *g* to RPM. To convert *g* to rpm, use the following formula:

$$RPM = \sqrt{\frac{RCF}{(1.118 \times 10^{-5}) \times (Radius)}}$$

Where: RCF = relative centrifugal force (*g*)
RPM = centrifuge speed in revolutions per minute
Radius = radius of rotor in cm

ASSESSING PURITY:

Purity of $CD4^+$ T cells can be measured by flow cytometry after staining with a fluorochrome-conjugated anti-CD4 antibody (e.g. FITC anti-CD4, Catalog #10403), and anti-CD127 antibodies. For CD127 staining, we recommend the clone h-IL-7R-M21 since it is not blocked by the anti-CD127 TAC present in the RosetteSep® cocktail (BD, Catalog #557938 (PE) or #558598 (Alexa 647)). For $CD49d^-$ staining, we recommend staining with anti-CD49d (9F10) PE-Cy5 (BioLegend, Catalog # 304305).

COMPONENT DESCRIPTION:

ROSETTESEP® HUMAN $CD4^+CD127^{low}CD49d^-$ REGULATORY T CELL PRE-ENRICHMENT COCKTAIL **CODE #15324C**

This cocktail contains a combination of monoclonal antibodies purified from mouse ascites fluid or hybridoma culture supernatant, by affinity chromatography using Protein A or Protein G Sepharose. These antibodies are bound in bispecific TAC which are directed against cell surface antigens on human hematopoietic cells and glycophorin A on red blood cells. The mouse monoclonal antibody subclass is IgG_1. It should be kept in mind that this product is a biological reagent, and as such can not be completely characterized or quantified. Some variability is unavoidable.

STABILITY AND STORAGE:

ROSETTESEP® HUMAN $CD4^+CD127^{low}CD49d^-$ REGULATORY T CELL PRE-ENRICHMENT COCKTAIL

Product stable at 2 - 8°C until expiry date as indicated on label. Contents have been sterility tested. Do not use cocktail if vial contents have leaked. Unused cocktail may be disposed of according to standard laboratory procedures for non-hazardous liquids. Do not freeze this product. This product may be shipped at room temperature (15 – 25°C), and should be refrigerated upon receipt.

SECTION B:

The EasySep® Human CD25 Positive Selection Kit is designed to select $CD25^+$ cells from samples that have been enriched using the RosetteSep® Human $CD4^+CD127^{low}CD49d^-$ Regulatory T Cell Pre-Enrichment Cocktail (Catalog #15364). This procedure is compatible with the purple EasySep® Magnet (Catalog #18000), "The Big Easy" EasySep® Magnet (Catalog #18001), or RoboSep® (Catalog #20000).

Note: Although this procedure is compatible with "The Big Easy" Silver EasySep® Magnet, we recommend using the purple EasySep® Magnet for better recovery.

FULLY AUTOMATED PROTOCOL USING ROBOSEP® (CATALOG #20000)

1. Transfer 500 µL of pre-enriched sample into a 14 mL (17 x 100 mm) polystyrene tube to properly fit into the RoboSep® carousel.

   Falcon™ 14 mL Polystyrene Round-Bottom Tubes (BD, Catalog #352057) are recommended.

2. Select the recommended RoboSep® protocol:

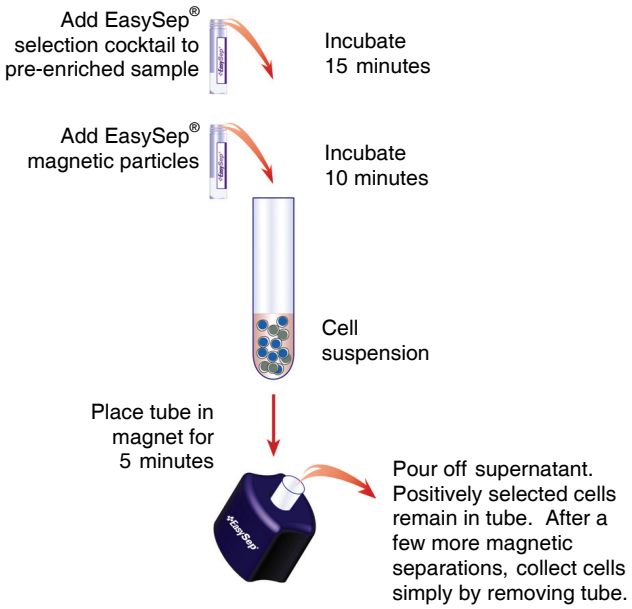
   - "Human CD25high Positive Selection 18231 – High Recovery"

   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. Mix EasySep® Magnetic Nanoparticles 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®.

4. When cell separation is complete, remove the tube containing the isolated cells from the magnet. The positively selected cells are now ready for use.

MANUAL EASYSEP® PROTOCOL DIAGRAM



MANUAL PROTOCOL USING EASYSEP® MAGNET (CATALOG #18000)

1. Transfer 500 µL of pre-enriched sample into a 5 mL (12 x 75 mm) polystyrene tube to properly fit into the EasySep® Magnet.
2. Add EasySep® Positive Selection Cocktail at **50 µL/mL** cells (i.e. for 500 µL of cells, add 25 µL of cocktail). Mix well and incubate at room temperature (15 –25°C) for **15** minutes.
3. Mix EasySep® Magnetic Nanoparticles to ensure that they are in a uniform suspension by vigorously pipetting up and down at least 5 times. Vortexing is not recommended. Add the Nanoparticles at **50 µL/mL** cells (e.g. for 500 µL of cells, add 25 µL of nanoparticles). Mix well and incubate at room temperature (15 – 25°C) for **10** minutes.

   Note: EasySep® Positive Selection Cocktail and Nanoparticles may be provided in excess.

4. 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.
5. Pick up the magnet, and in one continuous motion invert the magnet and tube, pouring off the supernatant fraction. The magnetically labeled cells will remain inside the tube, held by the magnetic field of the EasySep® Magnet. Leave the magnet and tube inverted 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.*
6. Remove the tube from the magnet and add 2.5 mL recommended medium. Mix the cell suspension by gently pipetting up and down 2 - 3 times. Place the tube back in the magnet and let sit for another **5** minutes.
7. Repeat steps 5 and 6 twice, and then step 5 once more, for a total of four 5-minute separations in the magnet. Remove tube from magnet and resuspend cells in an appropriate volume of recommended medium. The positively selected cells are now ready for use.

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

1. Transfer 500 µL of pre-enriched sample in to a 14 mL (17 x 100 mm) polystyrene tube to properly fit into the "The Big Easy" EasySep® Silver Magnet.
2. Add EasySep® Positive Selection Cocktail at **50 µL/mL** cells (i.e. for 500 µL of cells, add 25 µL of cocktail). Mix well and incubate at room temperature (15 – 25°C) for **15** minutes.
3. Mix EasySep® Magnetic Nanoparticles to ensure that they are in a uniform suspension by vigorously pipetting up and down at least 5 times. Vortexing is not recommended. Add the Nanoparticles at **50 µL/mL** cells (e.g. for 500 µL of cells, add 25 µL of nanoparticles). Mix well and incubate at room temperature (15 – 25°C) for **10** minutes.

   Note: EasySep® Positive Selection Cocktail and Nanoparticles may be provided in excess.

4. Bring the cell suspension to a total volume of 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.
5. Pick up the magnet, and in one continuous motion invert the magnet and tube, pouring off the supernatant fraction. The magnetically labeled cells will remain inside the tube, held by the magnetic field of the EasySep® Magnet. Leave the magnet and tube inverted 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.
6. Remove the tube from the magnet and add 5 mL recommended medium. Mix the cell suspension by gently pipetting up and down 2 -3 times. Place the tube back in the magnet and let sit for another **5** minutes.
7. Repeat Steps 5 and 6 twice, and then step 4 once more, for a total for four 5-minute separations in the magnet. Remove tube from magnet and resuspend cells in an appropriate volume of recommended medium. The positively selected cells are now ready for use.

REQUIRED EQUIPMENT:

EasySep® Magnet (Catalog #18000) or RoboSep® (Catalog #20000).

PRODUCT DESCRIPTION AND APPLICATIONS:

EasySep® Human CD25 Positive Selection Cocktail and EasySep® Magnetic Particles are designed to positively select $CD25^+$ cells corresponding to $CD4^+CD25^+FoxP3^+$ regulatory T cells from RosetteSep®-enriched $CD4^+CD127^{low}CD49d^-$ T cell fractions.

EASYSEP® LABELING OF HUMAN CELLS:

Target cells are specifically labeled with dextran-coated magnetic nanoparticles using bispecific Tetrameric Antibody Complexes (TAC). These complexes recognize both dextran and the 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 target cells using the EasySep® procedure (reverse side).

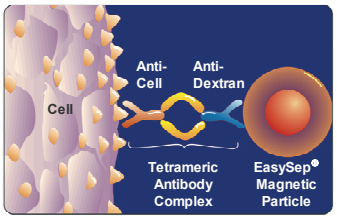


Figure 1.
Schematic Drawing of EasySep® TAC Magnetic Labeling of Human Cells.

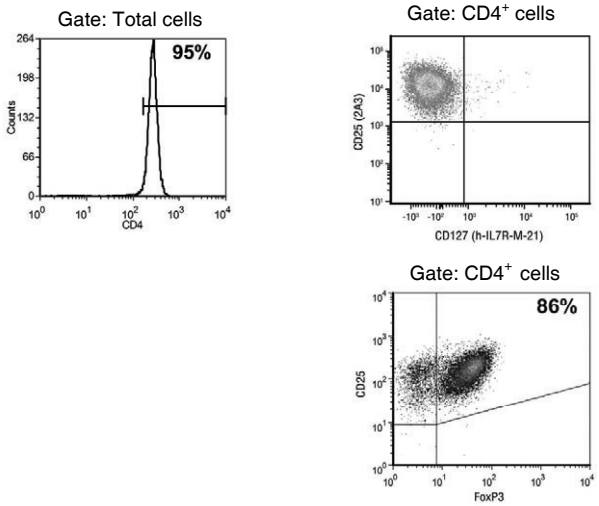
NOTES AND TIPS:

Recommended Media. The recommended medium is RoboSep® Buffer (Catalog #20104) or Phosphate Buffered Saled (PBS) with 2% FBS (Catalog #07905) + 1 mM EDTA. Medium should be Ca^{++} and Mg^{++} free.

Assessing Purity. The CD25 positive selection cocktail uses the anti-CD25 antibody clone M-A251, which recognizes epitope B of the CD25 antigen and may block some anti-CD25 antibody clones used to assess purity by flow cytometry. We recommend using the clone 2A3 (Catalog #10512), which recognizes epitope A of the CD25 antigen, to assess purity by flow cytometry. One of the following methods can be used to assess purity:

1. Add PE-labeled antibodies at the same time as the cocktail: Add the fluorochrome conjugated anti-CD25 antibody at a concentration of **0.4 µg/mL** immediately after adding the cocktail to provide a strong detection signal without affecting separation performance. This method labels the CD25 positive cells in the entire sample.
2. Use a secondary fluorochrome-conjugated antibody, such as FITC labeled sheep anti-mouse IgG.

TYPICAL FACS ANALYSIS OF HUMAN $CD4^+CD127^{low}CD49d^-CD25^+$ REGULATORY T CELL ISOLATION KIT:



Starting with fresh whole blood, the $CD4^+$ cell fraction is typically 78.8 - 98.9% and 67.4 - 92.9% $CD25^+FoxP3^+$.

COMPONENT DESCRIPTION

EASYSEP® HUMAN CD25 POSITIVE SELECTION COCKTAIL **CODE #18231C.2**

This cocktail contains a combination of monoclonal antibodies purified from hybridoma culture supernatant by affinity chromatography using Protein A or G Sepharose. These antibodies are bound in bispecific TAC which are directed against CD25 and dextran. The mouse monoclonal antibody subclass is IgG_1. This cocktail is supplied in PBS + 0.1% Bovine serum albumin and contains an antibody against human Fc receptor. It should be noted that this product is a biological reagent, and as such can not be completely characterized or quantified. Some variability is unavoidable.

EASYSEP® MAGNETIC NANOPARTICLES **CODE #18150**

A suspension of magnetic dextran iron particles in water.

STABILITY AND STORAGE

EASYSEP® HUMAN CD25 POSITIVE SELECTION COCKTAIL AND EASYSEP® MAGNETIC NANOPARTICLES

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.