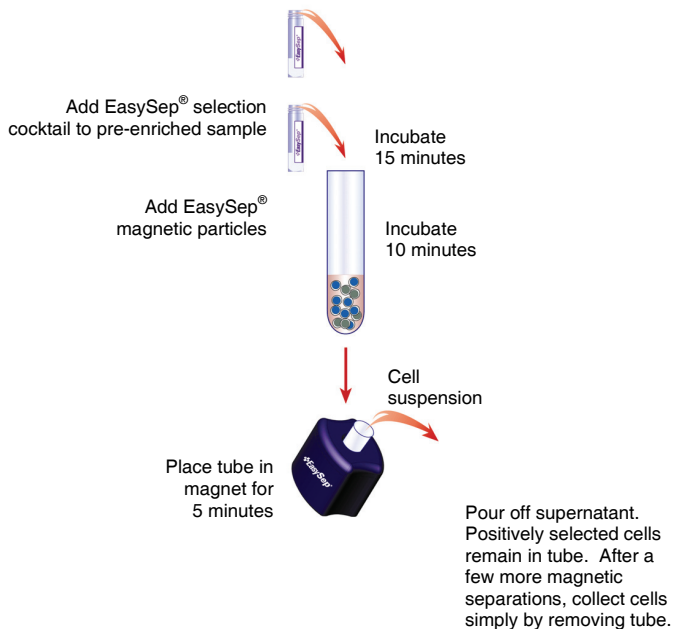


This procedure is used for processing samples that have been pre-enriched using STEMCELL's Complete Kit for Human Regulatory T Cell Isolation Kits (Catalog #15862, 15861 and 15864). The following procedure can also be found in the Product Information Sheet included in the Complete Kit for Human Regulatory T Cell Isolations for Catalog #15861 and 15864.

Fully Automated Protocol Using RoboSep® (Catalog #20000)

- Transfer 500µL 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.
- Select the appropriate RoboSep® protocol:
 - “Human CD25high Positive Selection 18231 – High Purity”
 - “Human CD25high Positive Selection 18231 – High Recovery”
 If a modified RoboSep® protocol is required, please contact STEMCELL Technologies' Technical Support at techsupport@stemcell.com.
- 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®.
- 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)

- Transfer 500 µL of pre-enriched sample into a 5 mL (12 x 75 mm) polystyrene tube to properly fit into the EasySep® Magnet.
- 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**.
- 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.
- 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**.
- 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.*
- 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**.
- 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)

- Transfer 500 µL of pre-enriched sample into a 14 mL (17 x 100 mm) polystyrene tube to properly fit into the “The Big Easy” EasySep® Silver Magnet.
- 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**.
- 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.
- 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**.
- 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.*
- 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**.
- 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.

Components:

• EasySep [®] Human CD25 Positive Selection Cocktail	1 mL
• EasySep [®] Magnetic Nanoparticles	1 mL



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 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 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).

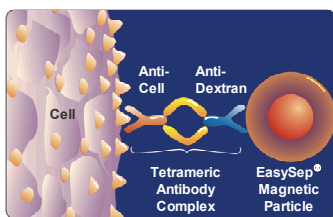


Figure 1.
Schematic Drawing of EasySep[®] TAC Magnetic Labeling of Human Cells.

NOTES AND TIPS:

Recommended Media. The recommended medium is RoboSep[®] Buffer (Catalog #20104) or Phosphate Buffered Saline (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.

CD25 Depletion. Following EasySep[®] CD25 Positive Selection, the negative fraction can be further depleted of CD25⁺ cells to obtain CD4⁺CD25⁻ cells following the Complete Kit for Human CD4⁺CD25⁺ Regulatory T Cell Isolation (Catalog #15862). Depending on the donor, purity of CD4⁺ T cells in the CD25 depleted fraction may vary. Please follow the optional CD25⁺ cell depletion procedure using the purple EasySep[®] Magnet (Catalog #18000):

1. If desired, CD4⁺CD25⁺ cells can be depleted from the supernatant fraction obtained in Step 5. Centrifuge the supernatant fraction at 200 x g for 10 minutes, then resuspend cells in 500 µL of recommended buffer.
2. Add EasySep[®] Positive Selection Cocktail at **100 µL/mL** cells (e.g. for 500 µL of cells, add 50 µL of cocktail). Mix well and incubate at room temperature (15 - 25°C) for **15** minutes.
3. Mix EasySep[®] Magnetic Nanoparticles by pipetting up and down vigorously more than 5 times to ensure that they are in a uniform suspension. Vortexing is not

recommended. Add the particles at **100 µL/mL** cells (e.g. for 500 µL of cells, add 50 µL of Nanoparticles). Mix well and incubate at room temperature (15 - 25°C) for **10** minutes.

4. Bring the cell suspension to a total 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 **10** minutes.
5. Pick up the EasySep[®] Magnet, and in one continuous motion invert the magnet and tube, pouring off the supernatant fraction into a new 14 x 100 mm polystyrene tube.
6. Leave the magnet and tube in inverted position for 2 - 3 seconds, then return to upright position. *Do no shake or blot off any drops that may remain hanging from the mouth of the tube.*
7. Remove the empty tube from the EasySep[®] Magnet and place the new tube containing the supernatant fraction into the magnet. Set aside for **10** minutes.
8. Repeat Step 5 and 6. The depleted cell suspension in the new tube is now ready for use.

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 IgG1. 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.