A Simple and Versatile Method for the Isolation of Human Regulatory T Cells from Peripheral Blood Samples Ranging from 5 to 500 mL

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Summary

The isolation of $CD4^+CD25^+FOXP3^+$ regulatory T cells (Tregs) from human peripheral blood is difficult and requires multiple steps, primarily because these cells are extremely rare and lack a unique marker that distinguishes them from activated $CD4^+$ T cells. We have recently developed a two-step method for the isolation of Tregs directly from whole blood. The first step combines density centrifugation and $CD4^+$ T cell enrichment by negative selection using RosetteSep® technology, which cross-links red blood cells to unwanted cells in the sample and causes them to pellet when centrifuged over density medium. The second step is column-free immunomagnetic selection of $CD25^{+bright}$ cells. The combination of these two steps significantly reduces the time required to obtain highly purified Tregs from whole blood. In this study we have automated the positive selection step using RoboSep®, a pipetting robot with true walk-away capability. We have also optimized the procedure for samples as small as 5 mL or as large as 500 mL. $CD4^+$ T cells were isolated directly from small samples of whole blood or large buffy coat suspensions using RosetteSep®. $CD25^{+}$bright T cells were then positively selected using the RoboSep® cell separator. The purified cell suspensions were 94±2% $CD4^+CD25^+$ (avg. ± 1SD; n=14, 7 donors), 84±8% $CD25^{+bright}$, and typically around 90% $FOXP3^+$ based on flow cytometry analysis. While the first step of $CD4^+$ T cell isolation using RosetteSep® density centrifugation makes this method scalable to large samples, the highly selective second step of $CD25^{+bright}$ positive selection ensures high purity. We report here new advancements to a method that simplifies the challenge of isolating rare regulatory T cells from human peripheral blood.

Methods: Step 1 Isolation of $CD4^+$ T cells directly from whole blood using RosetteSep®

Figure 1. Rosette of unwanted cell and red blood cells formed by RosetteSep® tetrameric antibody complexes (TAC)

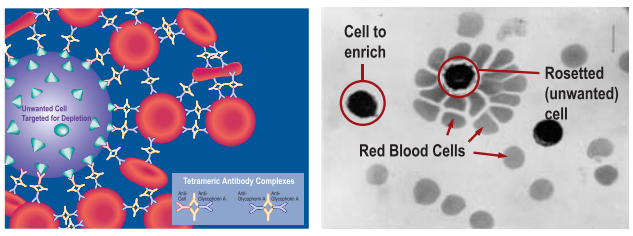


Figure 2. RosetteSep® procedure

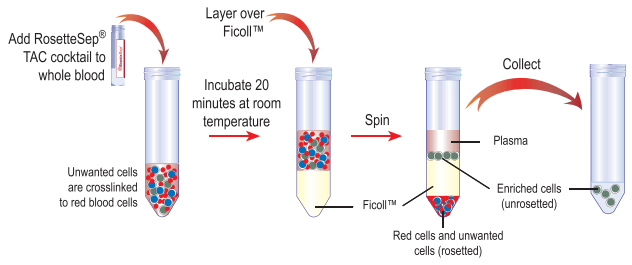
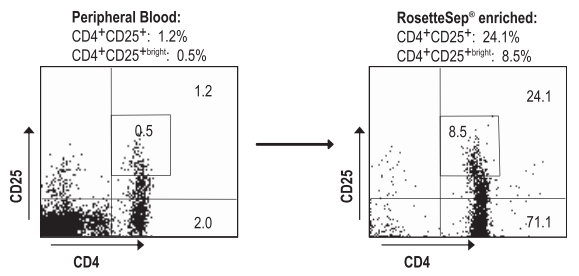
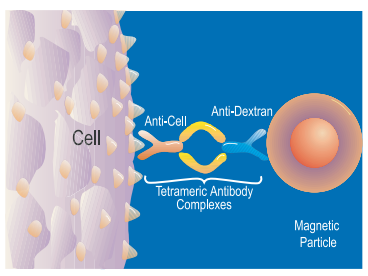


Figure 3. A typical FACS profile of $CD4^+$T cells isolated directly from whole blood using RosetteSep® technology



Methods: Step 2 Fully automated isolation of $CD25^{+bright}$ cells using the RoboSep® Cell Separator.

Figure 4. RoboSep® uses TAC based, column free magnetic cell selection technology.



TAC are comprised of two mouse IgG_1 monoclonal antibodies held in tetrameric array by two rat anti-mouse IgG_1 monoclonal antibody molecules. One mouse antibody recognizes the specific cell surface antigen while the other recognizes dextran on the EasySep® magnetic particle.

Figure 5. RoboSep® is a fully automated cell separator.

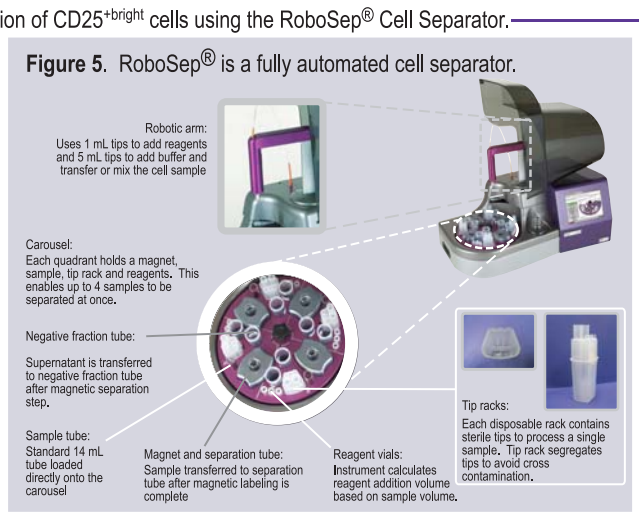


Table 1. RoboSep® protocols have been optimized to select $CD25^{+bright}$ cells from $CD4^+$ T cell fractions obtained from blood samples ranging from 5 to 500mL.

Start Sample	Application	RoboSep® Protocol Code	Recommended $CD4^+$ T Cell Concentration	Sample Volume Range	Total Cells Labeled per Kit
RosetteSep®-enriched $CD4^+$ T cells from **small** whole blood samples (~5-25 mL)	$CD25^{+bright}$ positive selection	Human CD25 Bright Positive Selection (WB) 18231 - small volume	5 x 10^6 cells/mL	0.50 - 4.0 mL	2 x 10^8 cells
RosetteSep®-enriched $CD4^+$ T cells from **large** whole blood samples (~25-150 mL)	$CD25^{+bright}$ positive selection	Human CD25 Bright Positive Selection (WB) 18231 - large volume	5 x 10^7 cells/mL	0.25 - 2.0 mL	1 x 10^9 cells
RosetteSep®-enriched $CD4^+$ T cells from **buffy coat** samples (for buffy coats performed on whole blood samples >50 mL)	$CD25^{+bright}$ positive selection	Human CD25 Bright Positive Selection 18231 - buffy coat	5 x 10^7 cells/mL	0.50 - 4.0 mL	2 x 10^9 cells
Negative fraction obtained after $CD25^{+bright}$ positive selection	CD25 depletion	Human CD25 Depletion 18231 - high purity	5 x 10^7 cells/mL	0.25 - 8.0 mL	5 x 10^8 cells

Results

Figure 6. FACS profile of cell populations isolated by combining RosetteSep® $CD4^+$T cell enrichment and RoboSep® $CD25^{+bright}$ positive selection

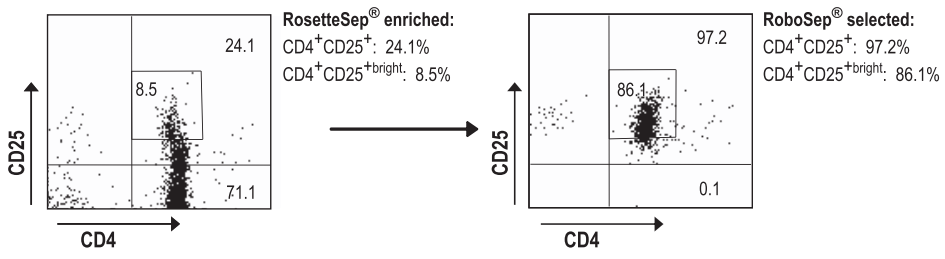
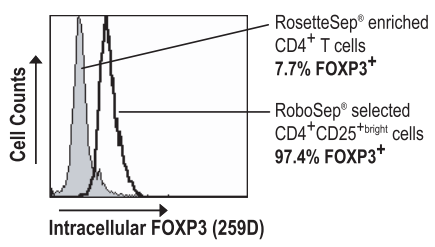


Table 2. Summary of purities obtained by combining RosetteSep® $CD4^+$T cell enrichment and RoboSep® $CD25^{+bright}$ positive selection

n=14, 7 donors	%$CD4^+CD25^+$	%$CD25^{+bright}$
Average*	94.0	83.7
STDEV	2.4	7.6

*Results were obtained using fresh whole blood samples

Figure 7. Intracellular FOXP3 measurements of isolated $CD4^+$ T cell populations.



Conclusions

- $CD4^+CD25^{+bright}FOXP3^+$ regulatory T cells can be rapidly isolated directly from whole blood with minimal effort.
- This two-step method is highly versatile and enables the processing of a wide range of sample sizes.