

ISOLATION OF DISTINCT HUMAN REGULATORY T CELL SUBSETS USING COMBINATIONS OF ANTIBODY-MEDIATED BUOYANT DENSITY CENTRIFUGATION AND/OR COLUMN-FREE IMMUNOMAGNETIC CELL SEPARATION

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ABSTRACT

Regulatory T cells (Tregs) have the ability to suppress T cell responses and play a critical role in peripheral tolerance and immune regulation. In recent years, interest in Tregs has exploded due to the great potential they hold in the treatment of autoimmune disorders and transplant rejection. Unlike their mouse counterparts, human Tregs appear to display a heterogeneous cell surface phenotype and regulatory capacity. To complicate matters further, human peripheral blood Tregs comprise only a small fraction of total CD4⁺ T cells and therefore must be highly enriched for their suppressive function to be detectable in vitro. Since Tregs lack a unique cell surface marker and often share phenotypic similarities with activated T cells, it is difficult to isolate highly purified Tregs in order to evaluate their suppressive function and therapeutic potential. Therefore, most strategies to purify Tregs rely on methods combining both negative and positive selection. Using STEMCELL Technologies' RosetteSep[®] and EasySep[®], distinct Treg populations are easily and rapidly isolated from virtually any normal peripheral blood sample. RosetteSep[®] is an antibody-mediated buoyant density centrifugation method used to isolate unlabelled cells specifically from whole blood or buffy coat samples. EasySep[®] is a column-free immunomagnetic cell separation method used to isolate cells from fresh or previously frozen peripheral blood mononuclear cells. For Treg pre-enrichment, a cocktail of bispecific tetrameric antibody complexes (TAC) selectively cross-links unwanted cells to either red blood cells (RosetteSep[®]) or magnetic particles (EasySep[®]), allowing their separation by Ficoll centrifugation or magnetic separation, respectively. RosetteSep[®] or EasySep[®] pre-enriched Treg populations consist of CD4⁺, CD4⁺CD127^{low} or CD4⁺CD49d⁺CD127^{low} T cells. Pre-enriched Tregs can be further purified using EasySep[®] positive selection to isolate Treg subsets expressing high levels of cell surface CD25. Depending on the Treg population, purities of 80% ± 10% CD4⁺CD25⁺FOXP3⁺ human Tregs are achievable.

TABLE 1: STEMCELL RosetteSep[®]/EasySep[®] Human Regulatory T Cell Isolation Kits

Starting Sample	Phenotype of Cells	STEMCELL Cell Isolation Kit	STEMCELL Catalog Number
Human			
Whole Blood or Buffy Coat	CD4 ⁺ CD25 ^{high} T cells	Complete Kit for Human CD4 ⁺ CD25 ^{high} T cells	15862
	CD4 ⁺ CD127 ^{low} CD25 ^{high} T cells	Complete Kits for Human CD4 ⁺ CD127 ^{low} CD25 ^{high} Regulatory T cells	15861
	CD4 ⁺ CD49d ⁺ CD127 ^{low} CD25 ^{high} T cells	Complete Kit for Human CD4 ⁺ CD49d ⁺ CD127 ^{low} CD25 ^{high} Regulatory T cells	15864
PMBC	CD4 ⁺ CD25 ^{high} T cells	EasySep [®] RoboSep [®] CD4 ⁺ CD25 ^{high} T cell Isolation Kit	18062

STARTING WITH WHOLE BLOOD OR BUFFY COAT

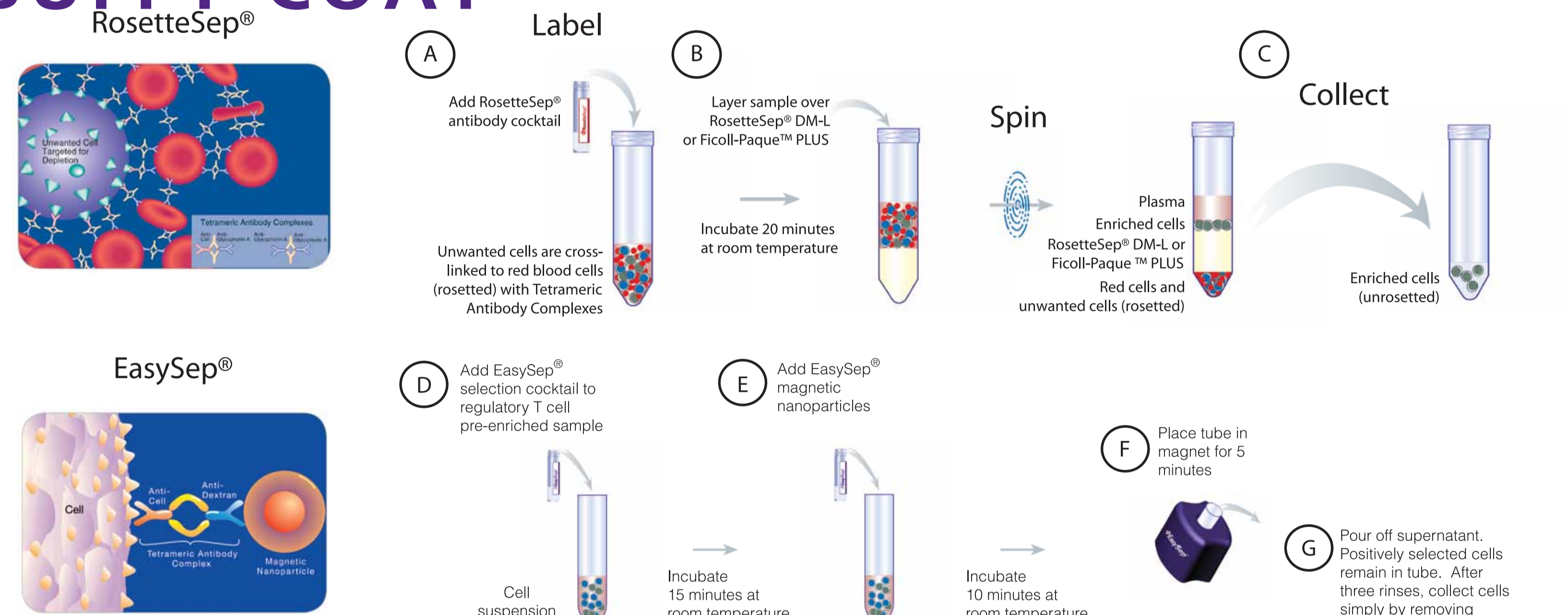


FIGURE 1: Two-Step Procedure for Isolating Human Regulatory T Cells from Whole Blood or Buffy Coat

Starting with whole blood or buffy coat samples, human Tregs are isolated in a two-step procedure. A-C) The RosetteSep[®] antibody cocktail cross-links unwanted cells in human whole blood to multiple red blood cells (RBCs), forming immunorosettes. Unwanted cells pellet along with the free RBCs when centrifuged over a buoyant density medium such as RosetteSep[®] DM-L or Ficoll-Paque[™] PLUS. Pre-enriched Tregs are collected from the interface between the plasma and the buoyant density medium and further purified using EasySep[®] CD25^{high} positive selection. D-G) Target cells are specifically labeled with dextran-coated magnetic particles using TACs that recognize both dextran and CD25. Magnetically-labeled Tregs are then separated from unlabeled cells using the EasySep[®] procedure.

STARTING WITH PBMC

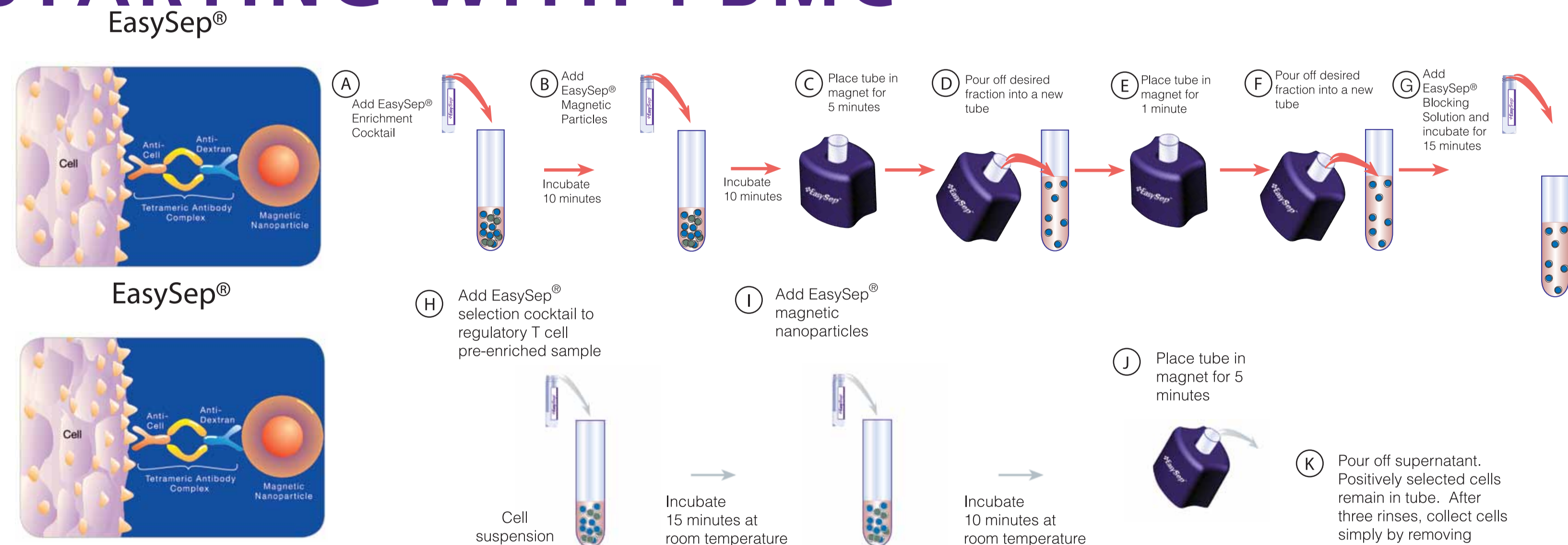


FIGURE 2: Isolation of Human CD4⁺CD25^{high} T Cells from PBMCs using RosetteSep[®] or EasySep[®]

Starting with PBMCs, human Tregs can be isolated in a two-step procedure using manual EasySep[®] or RoboSep[®]. A-G) PBMCs are incubated with the EasySep[®] CD4⁺ T cell enrichment cocktail followed by incubation with EasySep[®] magnetic D-particles. The tube containing the labeled PBMCs is then placed in an EasySep[®] magnet for 5 minutes. The EasySep[®]-enriched CD4⁺ T cells are simply poured off into a new tube while the magnetically-labeled non-CD4⁺ T cells are held in the original tube by the EasySep[®] magnet. Following an additional 1 minute magnetic separation, the enriched CD4⁺ T cells are incubated with the EasySep[®] blocking solution and washed once prior to EasySep[®] CD25^{high} positive selection (H-K). Both EasySep[®] cell separation steps can be performed on RoboSep[®], the fully automated cell separator.

RESULTS

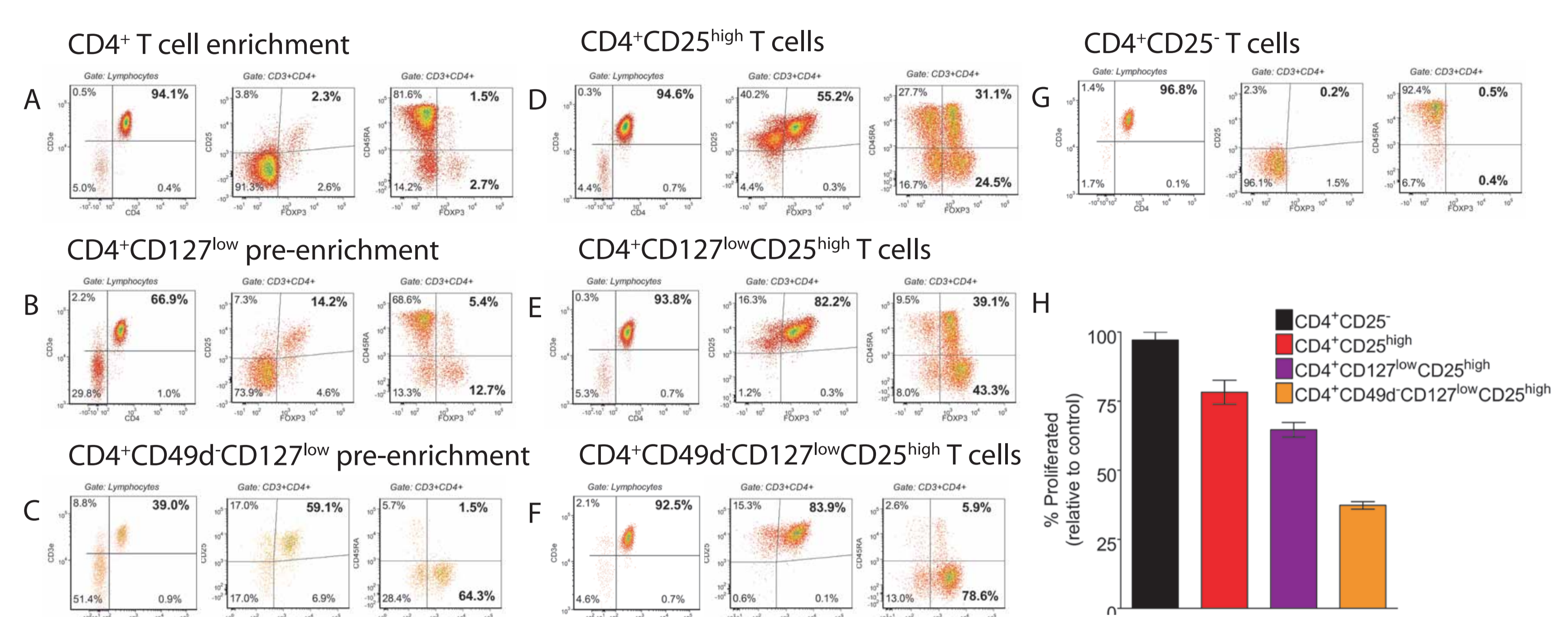


FIGURE 3: Purity, Phenotype and Functional Assessment of Human Regulatory T Cells Isolated using RosetteSep[®] and EasySep[®]

Human Tregs were isolated from whole blood using RosetteSep[®] followed by EasySep[®] CD25^{high} positive selection. Purity and phenotype of Tregs based on CD3, CD4, CD25, CD45RA and FOXP3 is shown following RosetteSep[®] A) CD4⁺, B) CD4⁺CD127^{low} and C) CD4⁺CD49d⁺CD127^{low} pre-enrichment followed by D-F) EasySep[®] CD25^{high} positive selection or G) EasySep[®] CD25 depletion. H) The ability of isolated Tregs to suppress anti-CD3/CD28 bead induced proliferation of CD4⁺CD25⁺ responder T cells at a 1:3 Treg to responder cell ratio was assessed using a CFSE based in vitro suppression assay.

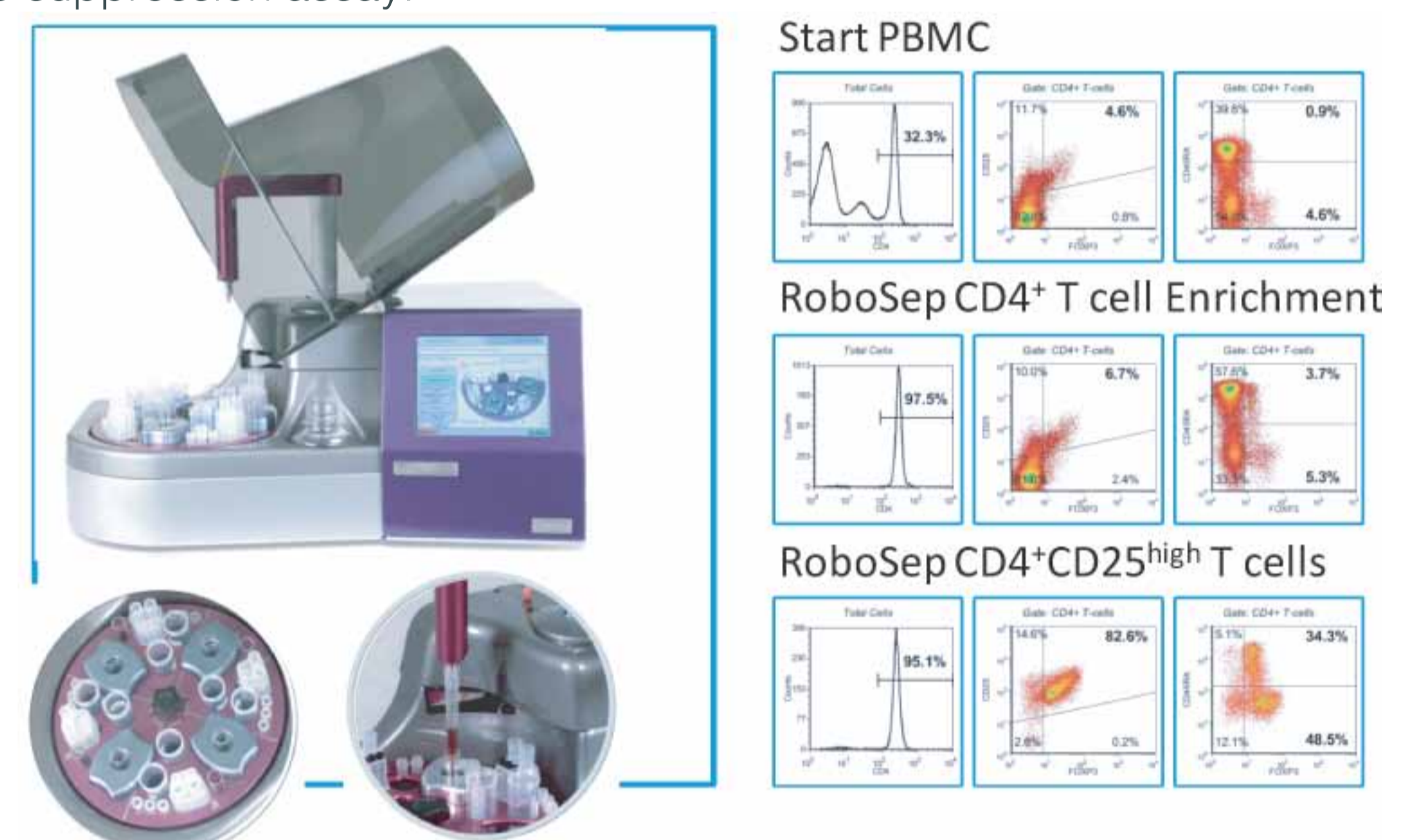


FIGURE 4: Highly Enriched Human CD4⁺CD25^{high} T Cells can be Isolated using RoboSep[®]

Human CD4⁺CD25^{high} T cells were isolated using RoboSep[®], the fully automated cell separator. Representative FACS plots: A) PBMC start sample; B) enriched CD4⁺ T cells and; C) enriched CD4⁺CD25^{high} T cells stained with CD4, CD25, CD45RA, and FOXP3.

TABLE 2: Protocol Comparisons of Human Treg Isolation Methods Starting with Whole Blood

Time	STEMCELL		COMPETITOR
	RosetteSep [®] /EasySep [®] Human Regulatory T Cell Isolation Kits	EasySep [®] /EasySep [®] Human CD4 ⁺ CD25 ^{high} T Cell Isolation Kit	Column-Based Human Regulatory T Cell Isolation Kit
0:00	20 min RosetteSep [®] cocktail incubation @ RT		
0:15		30 min Ficoll [™] spin	30 min Ficoll [™] spin
0:30	30 min Ficoll [™] spin		
0:45		2 - 10 min washes	2 - 10 min washes
1:00	2 - 10 min washes		
1:15			
1:30	resuspend in 500uL EasySep [®] buffer	15 min cocktail incubation; 10 min D-particle incubation; 5 min & 1 min magnetic separations; 15 min blocking solution incubation @ RT	10 min biotin cocktail incubation; 15 min microbead incubation; 10 min centrifuge spin; load negative selection column @ 2-8°C; 2 - 1mL column washes
1:45	15 min cocktail incubation @ RT		
2:00	10 min nanoparticle incubation @ RT	10 min centrifuge spin, wash, 10 min centrifuge spin, resuspend in 500uL EasySep [®] buffer	
2:15	4 - 5 min magnetic separations		
2:30		15 min cocktail incubation @ RT	
2:45	10 min centrifuge spin	10 min nanoparticle incubation @ RT	10 min centrifuge spin @ 2-8°C
3:00	resuspend Tregs in assay media	4 - 5 min magnetic separations	15 min microbead incubation @ 2-8°C
3:15			10 min centrifuge spin
3:30		10 min centrifuge spin	resuspend sample and load column
3:45		resuspend Tregs in assay media	3 - 500uL column washes
4:00			plunger column into second column
4:15			3 - 500uL column washes
4:30			10 min centrifuge spin
4:45			resuspend Tregs in assay media
	3 hr 00 min	4 hr 00 min	4 hr 45 min
	No columns	No columns	3 columns
	4 centrifugation steps	6 centrifugation steps	7 centrifugation steps

SUMMARY

- TREGS WITH HIGH PURITY CAN BE ISOLATED FROM WHOLE BLOOD USING A COMBINATION OF ROSETTESEP[®] AND EASYSEP[®] IN UNDER 3 HOURS.
- HIGHLY ENRICHED HUMAN CD4⁺CD25^{HIGH} T CELLS CAN BE ISOLATED FROM PBMCs USING EASYSEP[®] OR ROBOSEP[®] THE FULLY AUTOMATED CELL SEPARATOR.
- TREGS ISOLATED USING ROSETTESEP[®] AND EASYSEP[®] CAN SUPPRESS T CELL PROLIFERATION INDUCED BY ANTI-CD3/CD28 BEAD STIMULATION.