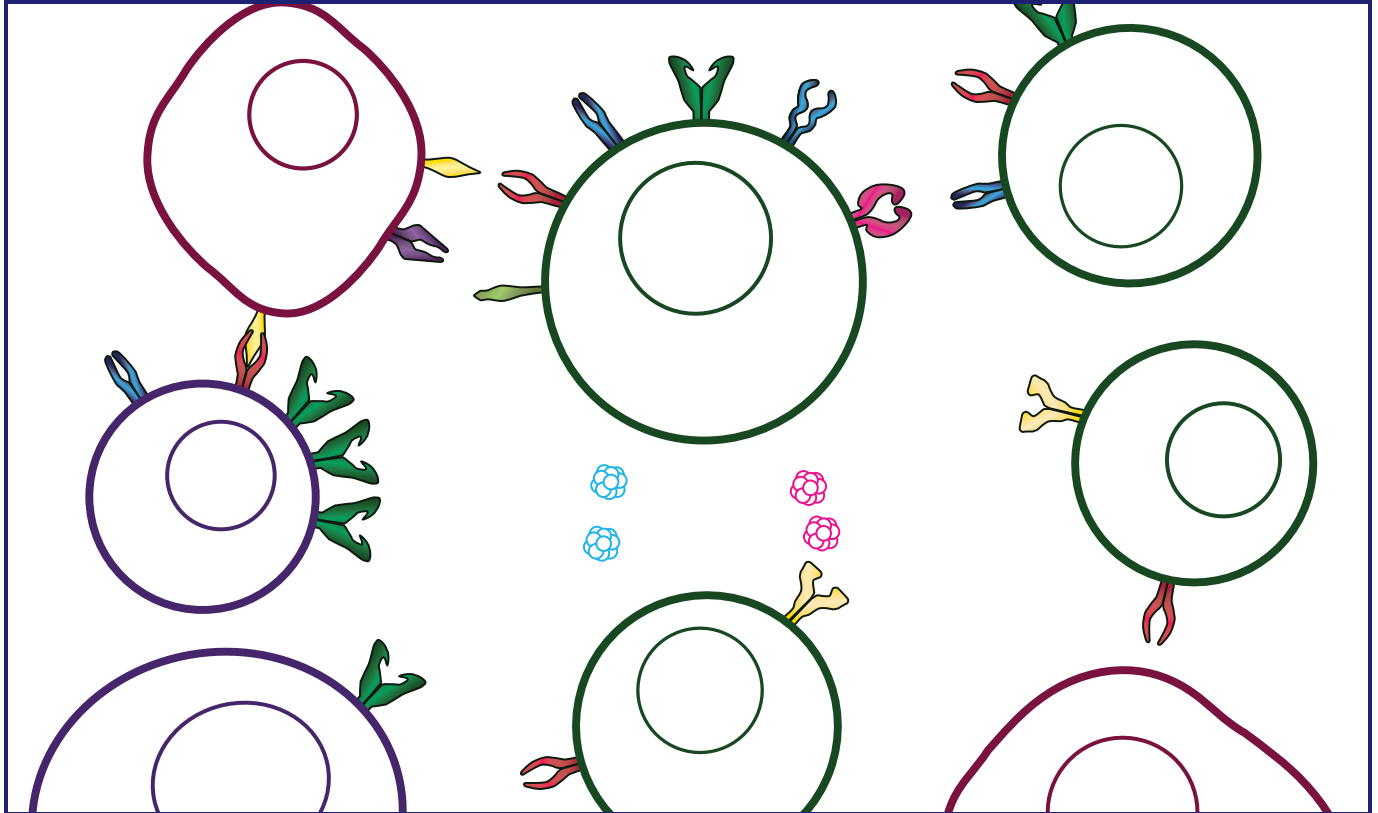


Regulatory T Cells

Isolation and Characterization of Viable Human Regulatory T Cells



The following application note describes a method of isolating Regulatory T (Treg) cells by using CD4-enriched cells and sorting on the CD4⁺ CD25^{int-hi} CD127^{lo} phenotype for increased efficiency and higher yields.

Introduction

Sorting a population of Regulatory T cells for use in assays to characterize these cells has been problematic. The traditional method, sorting cells via the established phenotype of CD4⁺ expressing lymphocytes that have the highest expression of CD25, yields relatively few cells. This application note describes a new method of obtaining a population of Treg cells based on the low expression of CD127 on Treg cells. This expression pattern closely follows the expression of the FoxP3 transcription factor, which is the most definitive marker for identification of Treg cells. This indicates that CD4 T lymphocytes with low expression of CD127 (CD127^{lo}) and intermediate-high (int-hi) expression of CD25 (CD25^{int-hi}) are Treg cells.¹

A significant advantage of using this new marker over that of intracellular expression of FoxP3 is that the cells do not have to be permeabilized. Instead, viable cells can be isolated through cell sorting and used in culture and in other *in vitro* assays. Another advantage of using this sorting strategy is that it allows for a two to four-fold increase in the recovery of the sorted Treg cells over previous methods.^{1,2}

Researchers have searched for mechanisms of immunologic tolerance for over 50 years. Intrinsic CD25⁺ CD4⁺ Treg

cells were originally identified in the mid 1990s, and different subsets are still being identified. The various types of Treg cells may not be mutually exclusive, and the interrelationships between them have not been completely defined. However, knowledge of the immunosuppressive mechanisms used by Treg cells is already providing a potential basis for new clinical protocols to attenuate autoimmune disease and transplant rejection³ and to promote resistance to tumors and parasites.

We can now isolate Treg cells in larger numbers using CD127 along with the previously defined CD4⁺ CD25⁺ phenotype. The overall time to isolate purified Treg cells can be reduced further by adding a CD4 enrichment step through magnetic cell separation prior to cell sorting.

The method described isolates a purified population of viable Treg cells, increases the number of the target population compared with traditional methods, and allows for performing further *in vitro* studies with the isolated cells.^{1,2}

MATERIAL	VENDOR	CAT. NO.
Ficoll-Paque™ PLUS density gradient medium	GE Healthcare	17-1440-02
*Alternative density gradient product: BD™ CPT cell preparation tube with sodium citrate, 8-mL draw capacity	BD Vacutainer	362761
BD IMag™ Human CD4 T Lymphocyte Enrichment Set - DM	BD Biosciences	557939
BD IMagnet cell separation magnet	BD Biosciences	552311
CD4 PerCP	BD Biosciences	347324
CD25 APC	BD Biosciences	340939
CD127 PE	BD Biosciences	557938
mIgG1 PE	BD Biosciences	550617
mIgG1 PerCP	BD Biosciences	550672
mIgG1 APC	BD Biosciences	554681
BD IMag Buffer	BD Biosciences	552362
BD Falcon™ 12 x 75-mm, 5-mL polystyrene round-bottom test tube	BD Biosciences	352008
PBS Wash Buffer (1X)	BD Biosciences	554781
Fetal Bovine Serum (FBS) Low IgG, US origin	Hyclone	SH30151.03
Sodium Azide	EMD Chemicals	SX0300-1
BD™ Cell Viability Kit	BD Biosciences	349480
FoxP3 Alexa Fluor® 488	BD Biosciences	560047

Method

Overview of the procedure for Treg cell isolation

1. Peripheral Blood Mononuclear Cell (PBMC) isolation with Ficoll-Paque PLUS density gradient medium
2. BD IMag CD4 enrichment of the PBMCs
3. Antibody staining for cell sorting
4. Treg isolation by cell sorting

PBMC isolation with Ficoll-Paque PLUS

The following procedure describes how to isolate the PBMCs using a Ficoll-Paque PLUS density gradient. Refer to the product insert for further details about this product and how to perform this procedure.

Note: The BD CPT cell preparation tube with sodium citrate can be used as an alternative density gradient product for the isolation of PBMCs for this application. Refer to the product insert for further details about this product and how to perform this procedure.

1. Label a 50-mL conical tube for every 10 mL of whole blood.
2. Add 25-mL 18°C – 22°C D-PBS to each labeled 50-mL conical tube.
3. Add 10-mL blood to each tube containing 25-mL of D-PBS and mix by inverting.
4. Underlay 10 mL of Ficoll-Paque PLUS in each 50-mL tube.
5. Centrifuge cells at 700g for 25 min at 18°C – 22°C with the brake turned off.
6. Remove the tubes and aspirate 20 mL to 25 mL of the top layer of fluid. Be careful not to disturb the cellular layer.
7. Remove the PBMC fraction at the interface with a 10-mL pipette and transfer this PBMC fraction into a new 50-mL conical tube.
8. Repeat step 7 for every gradient tube that has been run.
9. Fill the conical tubes with PBS wash buffer and centrifuge at 225g for 7 min.

Note: Wash buffer is PBS + 1% FBS + 0.09% sodium azide.

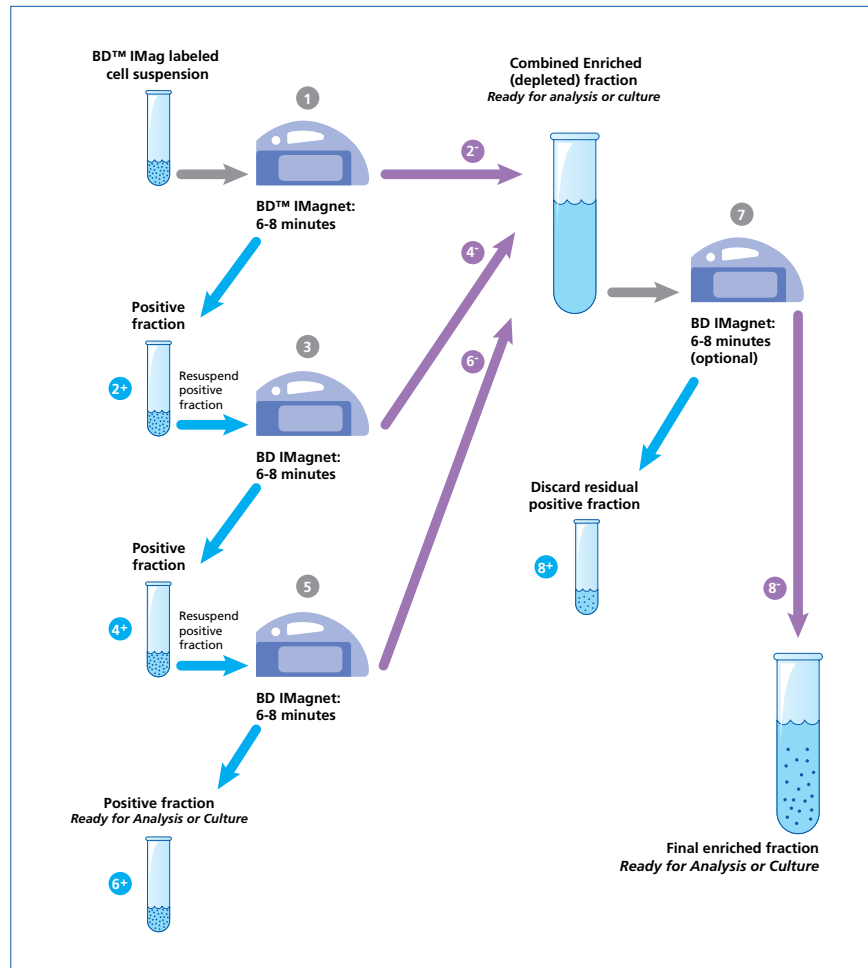
10. Aspirate the supernatant and resuspend the cells in 1 mL BD IMag Buffer for every 10 mL of whole blood that was processed.
11. Perform a viable cell count to determine the number of cells that have been isolated. For the procedure, see the BD Cell Viability Kit package insert.

BD IMag CD4 enrichment of the PBMCs

The following procedure describes how to negatively select the CD4 T lymphocytes from the PBMCs using the BD IMag Human CD4 T Lymphocyte Enrichment Set – DM. This enrichment procedure captures the erythrocytes, platelets, and peripheral leukocytes that are not CD4 T lymphocytes by magnetically removing them from the sample to create an enriched CD4 cell population. Refer to the *BD IMag Human CD4 T Lymphocyte Enrichment Set – DM* product insert for more information about this product.

1. Aliquot 5×10^6 PBMCs in 500 μ L into a 12 \times 75-mm round bottom test tube. Place the tube on ice. This tube will be used later for compensation control staining.
2. Centrifuge the cells at 225g for 7 min and resuspend the cell pellet at 10×10^6 cells/mL in BD IMag buffer.
3. Add the Biotinylated Human CD4 T Lymphocyte Enrichment Cocktail at 5 μ L per 1×10^6 cells and incubate at 18°C – 22°C for 15 min.
4. Wash the labeled cells with a 10 \times excess volume of 1 \times BD IMag buffer.
5. Centrifuge the sample at 225g for 7 min and carefully aspirate the supernatant.
6. Vortex the BD IMag streptavidin particles – DM thoroughly, add 5 μ L of particles for every 1×10^6 cells, and resuspend by gently pipetting up and down.
7. Incubate the sample at 18°C – 22°C for 30 min.
8. Resuspend the sample to a final cell concentration of 20×10^6 cells/mL - 80×10^6 cells/mL with 1 \times BD IMag buffer.
9. Transfer a maximum volume of 1 mL of the labeled cells to a 12 \times 75-mm round bottom test tube. If the volume exceeds 1 mL, use additional tubes.
10. Place the cell suspension on the BD IMagnet cell separation magnet for 8 min.
11. Carefully aspirate the supernatant with a Pasteur pipette and transfer the supernatant to a labeled tube. The tubes should be labeled negative fraction #1, negative fraction #2, etc, depending on the number of samples that are being processed
12. Remove the positive fraction from the BD IMagnet cell separation magnet and resuspend the positive fraction in 1 mL BD IMag buffer by gently pipetting the suspension up and down.
13. Repeat steps 10 through 12 for each tube.
14. Combine the negative fractions into a freshly labeled 17 \times 100-mm tube and place this negative fraction tube on the BD IMagnet cell separation magnet for 6 min.
 - Note:* If a 17 \times 100-mm tube is not available, add no more than 3 mL of the combined negative fraction per tube to multiple 12 \times 75-mm tubes.
15. Transfer the supernatant using a Pasteur pipette into a tube labeled “CD4 enriched fraction” and perform a viable cell count. For the procedure, see the *BD Cell Viability Kit* package insert.
16. Centrifuge the cells at 225g for 7 min and resuspend the cells at 10×10^6 cells/mL in PBS wash buffer.

BD IMag Enrichment Protocol Flow Chart



Antibody staining for cell sorting

The following procedure describes how to stain the controls and sort sample for cell sorting.

- Label five 12 × 75-mm tubes as follows:
 - Tube 1: Unstained PBMCs
 - Tube 2: PE Compensation
 - Tube 3: PerCP Compensation
 - Tube 4: APC Compensation
 - Tube 5: Isotype Control
- Aliquot the PBMCs (1×10^6 cells in 100 μ L per tube) that were set aside in step 1 of the BD IMag CD4 enrichment of the PBMCs section into tubes 1 through 5.
- Add the following antibodies to the appropriate control tube.
 - PE Compensation tube: 20 μ L of CD4 PE
 - PerCP Compensation tube: 20 μ L of CD4 PerCP
 - APC Compensation tube: 5 μ L of CD4 APC
 - Isotype Control tube: 0.5 μ g mIgG₁-PE, 0.0625 μ g mIgG₁-PerCP, and 0.0625 μ g mIgG₁-APC
- Incubate tubes 1-5 at 18°C – 22°C in the absence of light for 30 min.
- For the CD4 enriched population to be sorted, add the

appropriate amount of antibody needed for the sample size. For every 1×10^6 cells, add the following amount of antibody:

- For CD4 PerCP, add 20 μ L.
 - Example: for 40×10^6 cells, add 800 μ L of the CD4 PerCP antibody.
 - For CD25 APC, add 5 μ L.
 - For CD127 PE, add 0.5 μ g.
- Once the appropriate amounts of antibody are added to the sort sample, incubate the sample at 18°C – 22°C in the absence of light for 30 min.
 - Add 10× volume PBS wash buffer to the sort sample and 2 mL of PBS wash buffer to tubes 1-5 and centrifuge at 225g for 7 min.
 - Aspirate the supernatant and resuspend the sort sample in 50 mL of wash buffer and tubes 1-5 in 2 mL PBS wash buffer.
 - Centrifuge the sample at 225g for 7 min.
 - Aspirate the supernatant and resuspend the sort sample at a concentration of 5 - 10×10^6 cells/mL in PBS wash buffer. Resuspend tubes 1-5 in 500 μ L of PBS wash buffer.
 - Use the cell sorter to isolate the CD4⁺/CD25^{int-hi}/CD127^{lo} cells.

Treg isolation by cell sorting

The following section describes how to establish the sorting gates for isolating the Treg cell population in a cell sorter.

Treg sort gates

Sort gates were established on the populations shown on the following plots to isolate the Treg cells.

Post-sort analysis of sorted fractions

The Treg population that was isolated by cell sorting was reanalyzed for purity and whether it was positive for FoxP3, a traditional Treg marker.

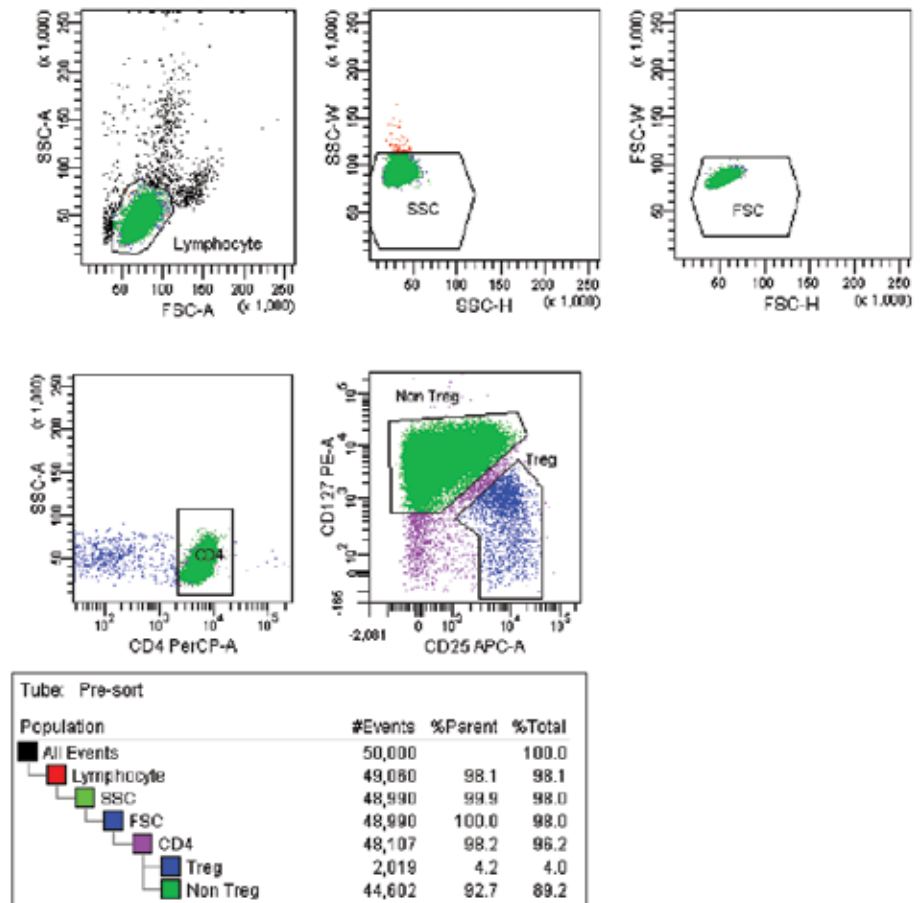


Figure 1a. Human PBMCs were enriched for CD4⁺ cells and then stained with a combination of CD4-PerCP/CD25-APC/CD127-PE. Based on their phenotype, the cells were sorted on the BD FACSAria™ cell sorter into CD4⁺CD25^{int-hi}CD127^{lo} (Treg cell) and CD4⁺CD25^{int-hi}CD127^{hi} (non-Treg cell) populations. The Treg cell population defined as CD4⁺CD25^{int-hi}CD127^{lo} is 4% of the CD4⁺ parent population.

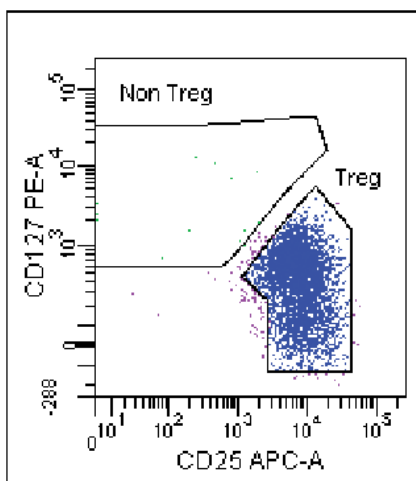


Figure 1b. The post-sort purity of the Treg isolated population was 87% of the total population.

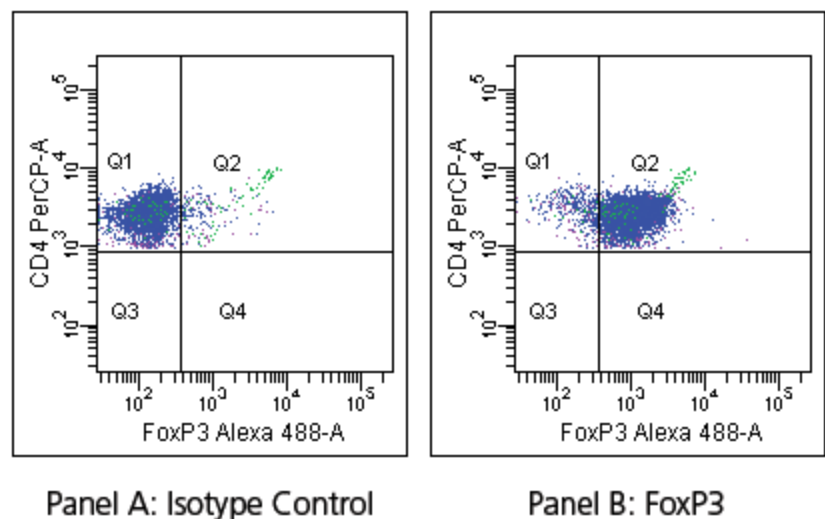


Figure 1c. To confirm that the CD4⁺CD25^{int-hi}CD127^{lo} isolated population consisted of Tregs, we stained the cells with FoxP3, an intracellular stain, after the sort. The sorted cells were stained with an isotype control (Panel A) or FoxP3 (Panel B). The Treg-isolated cells showed a high degree of FoxP3 expression over the isotype control (91.8% in Q2 for Panel B vs. 4.2% in Q2 for Panel A) when both were gated on CD4⁺ cells, thus indicating that they are actual Treg cells. The CD4⁺CD25^{int-hi}CD127^{hi} (non-Treg) population also was stained with FoxP3 and showed no increase in mean fluorescence intensity (MFI) when compared to the isotype control (data not shown).

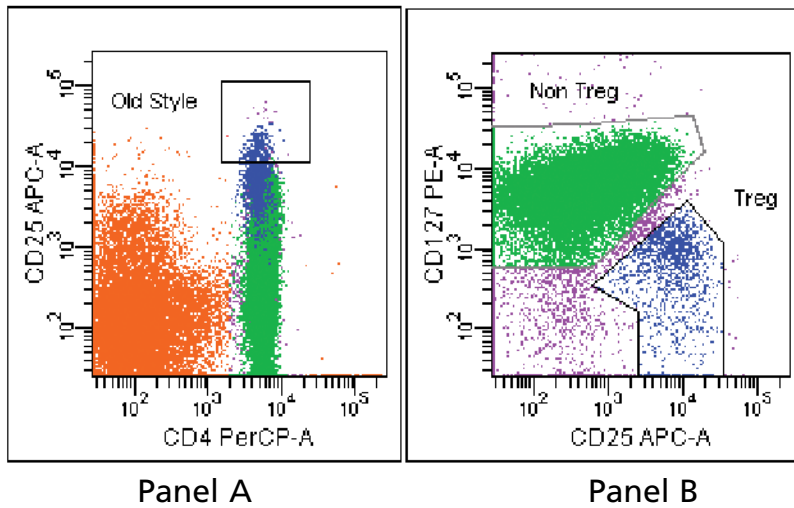


Figure 2. PBMCs gated with the traditional Treg sort gates: CD25⁺CD4⁺ (Panel A) and with the new Treg sort gates: CD4⁺CD25^{int-hi}CD127^{lo} (Panel B).

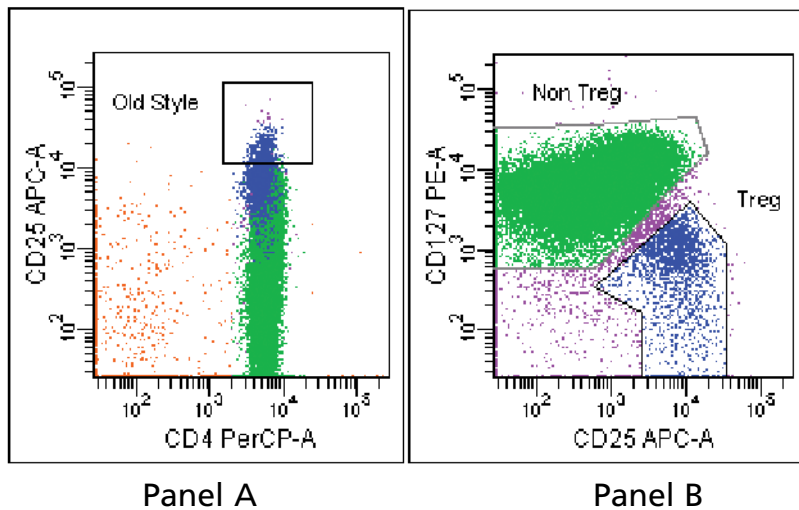


Figure 3. BD IMag CD4 enriched cells gated with the traditional Treg sort gates: CD25⁺CD4⁺ (Panel A) and with the new Treg sort gates: CD4⁺CD25^{int-hi}CD127^{lo} (Panel B).

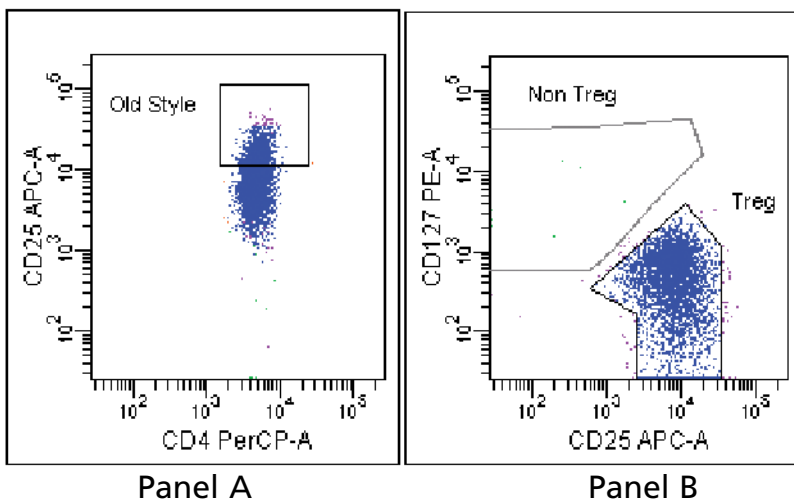


Figure 4. Post-sort analysis of the Treg sorted population gated with the traditional Treg sort gates: CD25⁺CD4⁺ (Panel A) and with the new sort Treg gates: CD4⁺CD25^{int-hi}CD127^{lo} (Panel B).

Results

Immunophenotyping data for PBMCs and the BD IMag CD4 enriched population demonstrates a significant reduction in the time to sort 10⁶ Treg cells. Pre-enrichment with the BD IMag human CD4 T lymphocyte enrichment method resulted in a three-fold decrease in the total time to sort 10⁶ Treg cells by significantly increasing the percentage of Treg-positive cells. Post-sort viability of the Treg cells averaged 85%. However, the mean will vary for each lab depending on its cell processing procedures and the sort conditions used.

	PBMCs	CD4 enriched	Improvement due to IMag enrichment
%CD25 ^{int-hi} CD127 ^{lo}	1.40%	4.00%	186% of target cells
Total time to sort 10 ⁶ Treg cells	6.9 hours	2.4 hours	65% faster

The new sort gates shown in this application note demonstrate that this method significantly enriches the Treg population (CD4⁺CD25^{int-hi}CD127^{lo}), thus allowing for a more rapid isolation of a purified population of Tregs by cell sorting. The following data (and chart) show a two to three-fold increase in the percentage of the Treg population using the new sort gates vs traditional Treg sort gates (CD25⁺CD4⁺).

The following chart summarizes the differences between each cell population using the traditional Treg sort gates and the new Treg sort gates.

Population	CD25 ⁺ CD4 ⁺ of total population (traditional sort gates for Tregs)	CD4 ⁺ CD25 ^{int-hi} CD127 ^{lo} of total population (new sort gates for Tregs)	Increase
Pre-enriched (PBMCs)	0.40%	1.40%	250%
BD IMag enriched	1.10%	4.10%	273%
Post sort	23.60%	85.00%	260%

Summary

Isolation of Treg cell populations has posed a challenge. Now there is a way to sort a purified Treg population in a shorter period of time, resulting in an increase of viable cells that can be used in subsequent functional assays. Magnetic enrichment of the CD4 positive cells using the BD IMag selection method selects for the cells of interest and reduces the overall sorting time compared to sorting unenriched PBMCs. The CD127 sort gate also increases the percentage of Treg cells sorted by two to four-fold compared to traditional gating of the CD4 expressing lymphocytes with the highest expression of CD25. The steps described in this application note produce a large population of purified, viable Treg cells.

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