Sorting and Downstream Functional Assessment of Regulatory T Cells

Isolating live cells with the BD FACSMelody™ cell sorter

Features

- Sort regulatory T cells (Tregs) and responder T cells (Tresps)
- Assess Treg-mediated suppression of CD69 and CD154 activation
- Assess intracellular cytokine production by freshly sorted and stimulated cells
- Evaluate in vitro expansion of sorted Tregs and assess viability
- Perform immunophenotyping of expanded Tregs
- Determine suppression of peripheral blood mononuclear cell (PBMC) proliferation by expanded Tregs

Tregs play a critical role in maintaining immune homeostasis and self-tolerance. Altered frequencies or impaired functions of Tregs have been implicated in conditions such as graft-versus-host disease and various autoimmune diseases including type I diabetes, systemic lupus erythematosus, multiple sclerosis and rheumatoid arthritis. These cells also play a role in immune suppression during tumor progression and chronic infections. Tregs display a high level of phenotypic complexity, and understanding the biological implication(s) of this phenotypic diversity is critical for harnessing the therapeutic utility of Tregs. Therefore, there is great interest in isolating this cell population with high purity, yield and viability.

In this data sheet, we demonstrate the use of the BD FACSMelody™ cell sorter to sort Tregs with high purity and viability. The system can also be used to study the expansion and immunophenotyping of these cells, which retain functionality as demonstrated in suppression and cytokine production assays. The BD FACSChorus™ software guides researchers throughout the entire sorting process. This advanced automation technology simplifies the workflow, thereby minimizing the requirement for cell sorting technical expertise to make sorting available for more researchers.

Sorting regulatory T cells and responder T cells

The CD4$^+$ T-cell compartment is comprised of conventional T helper cells and Tregs. Treg-driven immunosuppression assays typically require co-culturing of Tregs with responder cells (Tresps) that comprise conventional T cells. Staining human cells for surface CD3, CD4, CD25 and CD127 molecules provides a robust method for identifying and sorting viable Tregs and Tresps. Tregs can be identified as CD25$^{high/+}$CD127$^{low/-}$ cells within the CD4$^+$ T cell population, as well as from positive staining of the transcription factor FoxP3, which controls Treg development and suppressive function. However, staining for FoxP3 to identify Tregs requires fixing and permeabilizing cells prior to intracellular staining and is incompatible with live-cell sorting. Tresps can be identified as CD25$^{low/-}$CD127$^{high/+}$ cells within the CD4$^+$ T cell population.

To isolate Tregs and Tresps, PBMCs were isolated from the whole blood of a normal healthy donor. BD IMag™ Human CD4 T Lymphocyte Enrichment Set-DM was used to enrich CD4$^+$ T cells prior to staining, thus minimizing sort time. The enriched CD4$^+$ T cell population was stained using the multicolor panel outlined in Table 1 to identify dead cells, lineage-positive cells, Tregs and Tresps. Cells were then re-suspended in BD FACS™ Pre-Sort Buffer to minimize cell clumping and preserve viability prior to sort. The BD FACSMelody cell sorter was prepared for cell sorting utilizing the automated setup. Fluorescence compensation was performed using single-color compensation controls and the temperature control setup in the BD FACSChorus™ software maintained the cells at 4°C before and during the sort to preserve cellular viability and function.

The staining panel enabled exclusion of dead (7-AAD$^+$) and other lineage-positive cells (ie, those expressing CD8, CD14 or CD19) to ensure viability and enrichment of CD4$^+$T cells. Tregs and Tresps (Figure 1A) were sorted into sterile polypropylene collection tubes coated with heat-inactivated fetal bovine serum (FBS). The purity of sorted Tregs and Tresps was assessed immediately after the sort by analyzing the sorted population on the BD FACSMelody system. Post-sort purity of both populations was determined to be over 98% pure within the parent population (Figure 1B and 1C). For functional studies, cells were immediately transferred to complete RPMI 1640 media supplemented with 10% heat-inactivated FBS for culture.

Table 1. Flow cytometry panel for sorting Treg and Tresp populations

Specificity	Fluorochrome
CD3	APC-H7
CD4	BV786
CD25	APC
CD127	PE
CD8/CD14/CD19	PerCP-Cy™5.5
Dead Cells	7-AAD

Figure 1

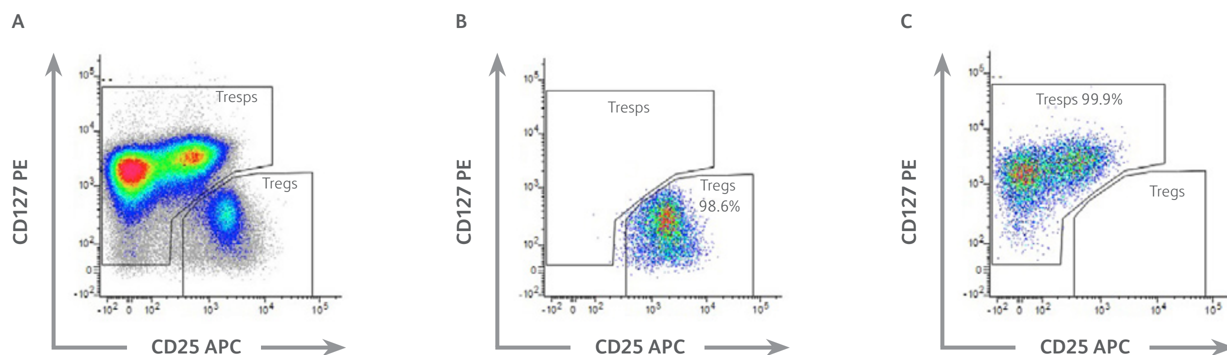


Figure 1. Sorting Treg and Tresp populations

CD4$^+$ T cells were enriched using the BD IMag Human CD4 T Lymphocyte Enrichment Set-DM and stained using the panel outlined in Table 1. Samples were acquired and sorted using the BD FACSMelody cell sorter. Single cells were gated based on forward and side light scatter (not shown). Dead (7-AAD$^+$) and other lineage-positive cells (ie, those expressing CD8, CD14 or CD19) were excluded, and CD4$^+$ T cells were identified as CD3$^+$CD4$^+$ (not shown). **1A**. Representative final sorting gates of Tregs identified as CD25$^{high/+}$CD127$^{low/-}$ and Tresps as CD25$^{low/-}$CD127$^{high/+}$ are shown within CD4$^+$ T cells. The purity of sorted Treg and Tresp populations was assessed by re-acquiring the cells using the BD FACSMelody system. Sorted Tregs (**1B**) and Tresps (**1C**) had greater than 98% purity within the CD4$^+$ T cell gate. Population percentage statistics are shown.

Treg-mediated suppression of CD69 and CD154 expression by activated Tresps

The BD FastImmune™ Regulatory T-Cell Function Kit enables flow-cytometric assessment of Treg function within 7 hours by analyzing the expression of well-established activation markers CD69 and CD154 on responder T cells. Using a similar strategy, we assessed the ability of sorted Tregs to suppress Tresps. Sorted Tresps were stimulated in the presence or absence of sorted Tregs. Upregulated CD69 and CD154 expression on Tresps was assessed by staining the cells using the multicolor panel listed in Table 2. Purified Tregs suppressed the expression of CD69 and CD154 on freshly-sorted and activated Tresps by approximately 40%, when co-cultured at a ratio of 0.5 Treg per Tresp, and approximately 25% at a 0.25:1 Treg:Tresp ratio (Figure 2A and 2B), showing that the purified Tregs retained their suppressive function after sorting with the BD FACSMelody.

Table 2. Panel for assessing Treg suppression of CD69 and CD154 activation on Tresps

Specificity	Fluorochrome
CD3	APC-H7
CD4	BV786
CD25	APC
CD127	PE
CD69	BV421
CD154	BB700

Figure 2A

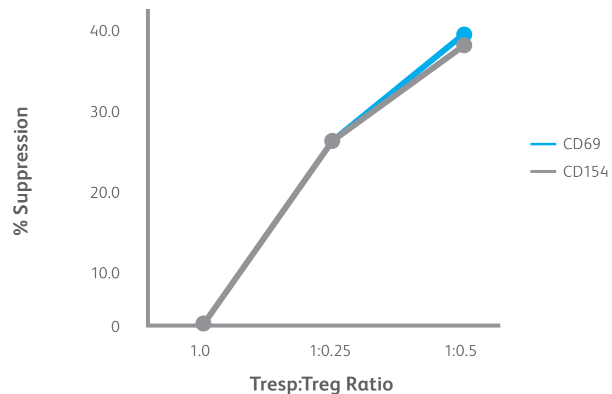


Figure 2B

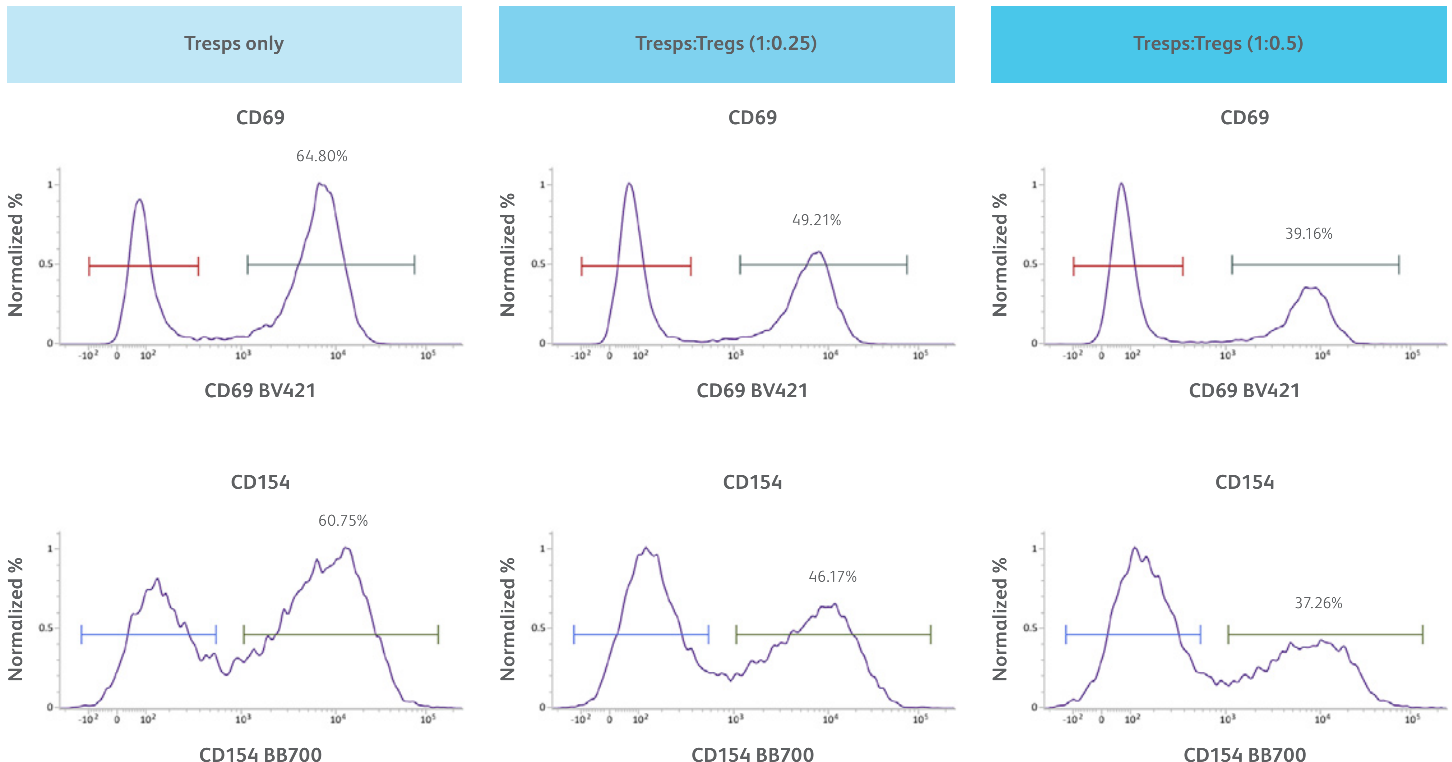


Figure 2. Treg mediated suppression of CD69 and CD154 activation on Tresps

Sorted Tresps were stimulated using anti-CD3/CD28 magnetic beads (Gibco™ Dynabeads™ Human T-Activator CD3/CD28) in the presence or absence of sorted BD Horizon™ CFSE-labeled Tregs (BD Horizon CFSE-labeled) at two different Tresp:Treg ratios (1:0.25 and 1:0.5). Upregulated CD69 and CD154 expression on Tresps was assessed by staining the cells with the fluorescent antibodies listed in Table 2 prior to acquisition on a BD FACSLyric™ flow cytometer. Tresps were identified as CFSE$^-$cells (not shown). **2A**. Plotted percent suppression of Tresp activation, as measured by expression of CD69 and CD154 is shown (% suppression in marker frequency was calculated for CD69 and CD154 separately as: {100 – [(% positive in the presence of Tregs / % positive in the absence of Tregs) × 100]}. **2B**. Histograms of CD69 and CD154 expression on Tresps are shown for each ratio tested (1:0.25 and 1:0.5), and % positive statistics are presented on the corresponding histograms.

Intracellular cytokine production by freshly sorted and stimulated cells

Analogous to conventional CD4$^+$ T helper cell lineages, Tregs can be categorized into Th1-, Th2-, Th17- and Th22-like subsets based on the expression patterns of chemokine receptors, transcription factors and the cytokines they produce. The diverse cytokine milieu that drives conventional T helper cell specialization during inflammation also drives the polarization of Th-like Tregs that can suppress the specific inflammatory conditions in which they arise.

Similar to T helper cells, Th1-like Tregs have been shown to produce IFN-γ, Th2-like Tregs produce IL-4, IL-5 and IL-13 and Th17-like Tregs produce IL-17A. To assess post-sort cytokine production capabilities, intracellular cytokine profiles of Tresps and Tregs from autologous fresh PBMCs and freshly sorted Tresps and Tregs after stimulation were assessed (Figure 3) using the fluorescent antibody panel listed in Table 3. Both sorted and fresh PBMC Tresps had comparable intracellular cytokine responses. Similarly, sorted and fresh PBMC Tregs showed comparable cytokine responses, thereby confirming that the sorted cells were functionally similar to their freshly-isolated counterparts.

Table 3. Intracellular cytokine assessment panel

Specificity	Fluorochrome
CD3	APC-H7
CD4	BV786
CD25	APC
CD127	PE
IFN-γ	BV605
IL-17A	APC-R700

Figure 3

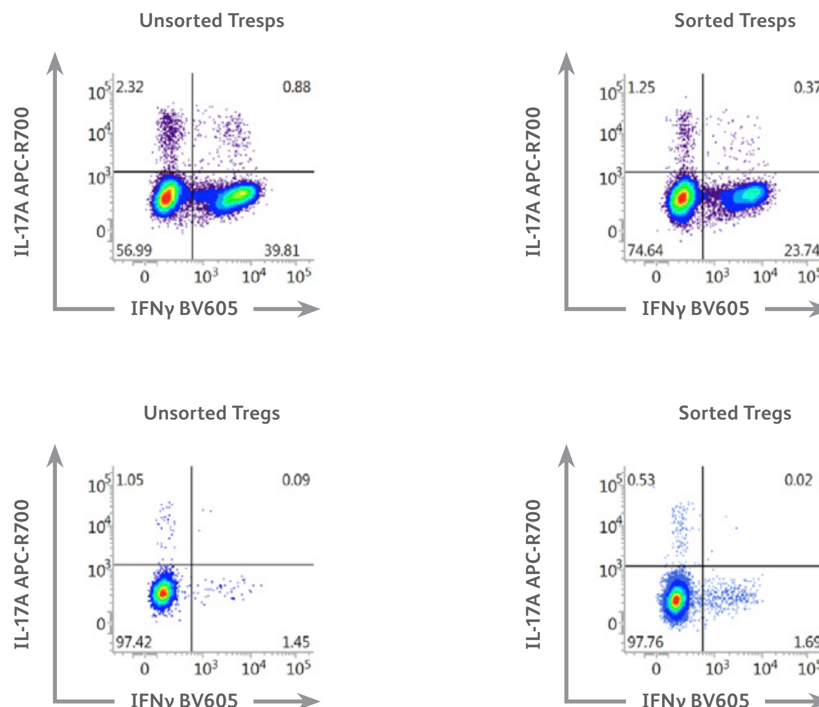


Figure 3. Assessment of intracellular cytokines in freshly isolated PBMCs and sorted cells upon PMA/ionomycin stimulation

Freshly isolated/unsorted total PBMC Tresps (top left) and Tregs (bottom left), sorted Tresps (top right) and sorted Tregs (bottom right) from the same healthy donor were used to assess intracellular cytokine production (IFN-γ and IL-17A). 100,000 total PBMCs, sorted Tresps or sorted Tregs were seeded at a density of 0.5 × 10⁶ cells/mL and treated with 0.05 µg/mL phorbol 12-myristate 13-acetate (PMA) and 0.5 µg/mL ionomycin (Stemcell Technologies) for 8 hours in the presence of BD FastImmune™ Brefeldin A and BD GolgiStop™ Protein Transport Inhibitor containing Monensin. Following stimulation, cells were stained with fluorescently labeled antibodies listed in Table 3 in combination with the BD Cytofix/Cytoperm™ Fixation/Permeabilization Solution Kit, per the manufacturer's instructions. Samples were acquired using the BD FACSLyric flow cytometer. Freshly isolated total PBMCs (unsorted Tresps, unsorted Tregs), sorted Tresps and sorted Tregs were gated as previously described (Figure 1) and intracellular cytokine profiles were analyzed. The percentage of cells positive for each marker is indicated in each quadrant. Sorted Tresps and Tregs had comparable cytokine production to freshly isolated counterparts from the same donor.

In vitro expansion of sorted Tregs and assessment of viability

Tregs are relatively rare, comprising approximately 5-10% of the total $CD4^+$ T cell population in healthy donors. We tested the expansion ability of sorted Tregs over the course of 2 weeks. Viability and expansion measurements were performed after expansion for 7 and 14 days. Over the course of 14 days as shown in Figure 4, the sorted Tregs expanded approximately 55-fold in culture while maintaining >98% viability.

Phenotypic characterization of expanded Tregs

We next performed phenotypic characterization of the expanded Treg population to ensure that the expanded cells maintained their Treg phenotype prior to functional assessment of these cells. Tregs are known to express high levels of CD25 in the normal $CD4^+$ T cell compartment with low-to-no expression of CD127. The most reliable marker for Tregs is FoxP3, the key transcription factor controlling Treg development and function.

To evaluate the expanded Tregs phenotype, freshly isolated PBMCs and Tregs expanded post-sort from the same healthy donor were phenotypically characterized with the multicolor panel listed in Table 4. The expanded sorted Treg population showed a high level of purity (data not shown), as well as a $FoxP3^+$ ratio similar to that of fresh PBMCs from the same donor indicating that cells sorted with the BD FACSMelody system maintained their viability and high purity after in vitro expansion (Figure 5).

Table 4. Panel for Treg phenotypic characterization

Specificity	Fluorochrome
CD3	APC-H7
CD4	FITC
CD25	BV421
CD127	BB700
FoxP3	Alexa Fluor® 647

Figure 4

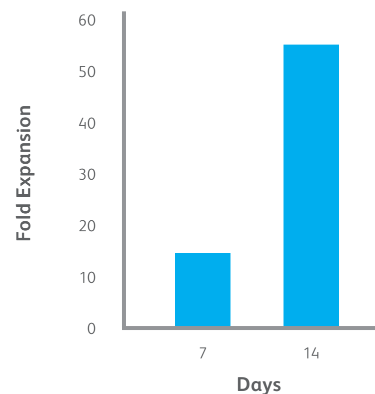


Figure 4. Expansion of Tregs in vitro and assessment of viability

Sort-purified Tregs were cultured at a density of 0.5 × 10^6 cells/mL in the presence of anti-CD3/CD28 coated magnetic beads at a ratio of 1 bead per every 2 cells in complete RPMI medium supplemented with 10% heat-inactivated human serum AB male (Sigma-Aldrich) and 300 Units/mL of recombinant human IL-2 protein (Roche). Viability and expansion measurements were performed using Trypan blue-exclusion staining and subsequent cell counting after expansion on days 7 and 14. The graph displays fold expansion of sorted Tregs which maintained >98% viability.

Figure 5

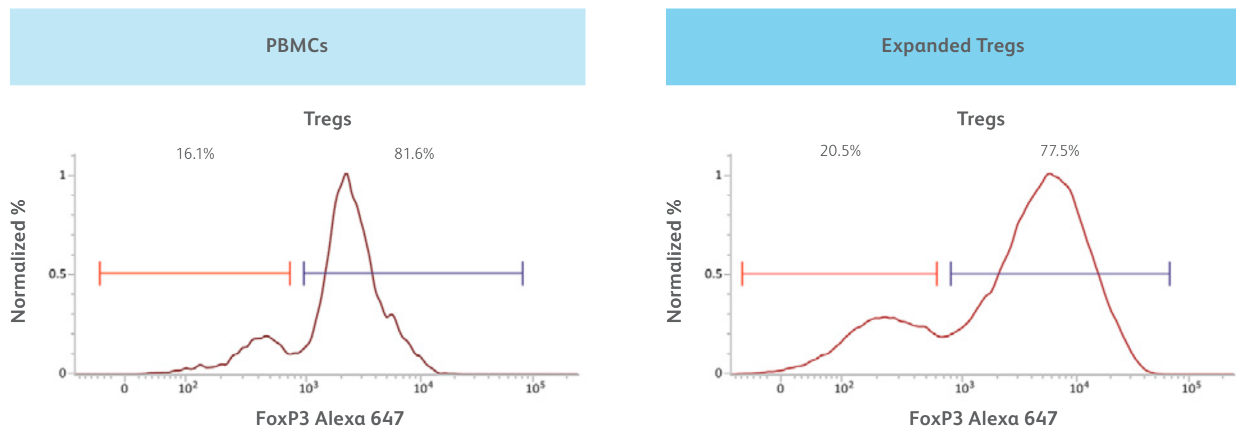


Figure 5. Assessment of Treg phenotype in freshly isolated PBMCs and expanded Tregs

Freshly prepared PBMCs (left) and Tregs expanded post-sort (right) were stained with the multicolor panel outlined in Table 4 using the BD Pharmingen™ Transcription Factor Buffer Set according to the manufacturer's recommendations. Lymphocytes were gated based on forward and side light scatter (data not shown). Tregs were identified within the $CD3^+CD4^+$ cell gate as $CD25^{high/+}$ $CD127^{low/-}$ cells as described previously and the percentage of cells positive for FoxP3 was determined (histograms). Numbers displayed represent the percentages of $FoxP3^-$ and $FoxP3^+$ cells.

Secreted cytokine production by expanded Tregs

While Tregs are known to produce signature inflammatory cytokines similar to their conventional T helper cell counterparts, Tregs also secrete anti-inflammatory cytokines such as IL-10, TGF-β and IL-35 that are capable of suppressing pro-inflammatory responses of T cells, NK cells, B cells, dendritic cells and macrophages. To evaluate the functionality of sorted and expanded Tregs, we evaluated the cytokine production of these cells during a three-week in vitro expansion time course. Sorted Tregs maintained their ability to produce IL-17A, IFN-γ, IL-4 and IL-10 throughout the expansion process, indicating that the proliferating Tregs retained cytokine production capabilities (Figure 6).

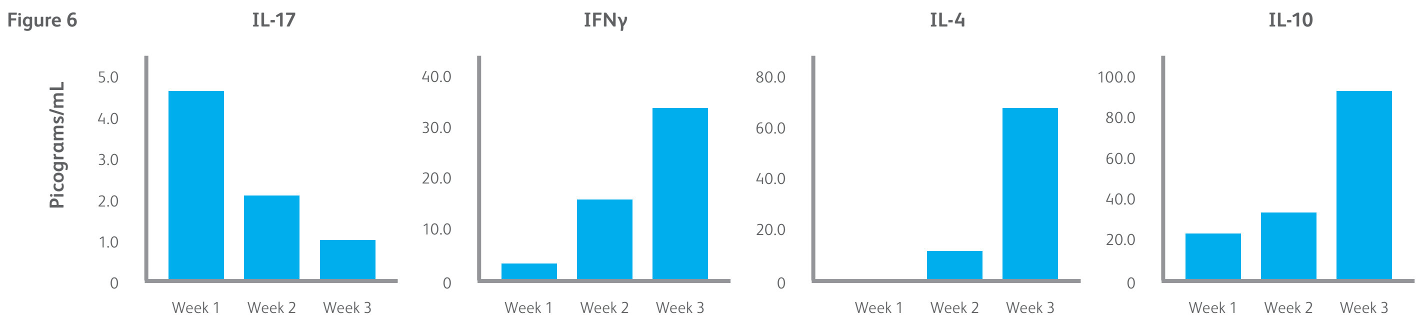
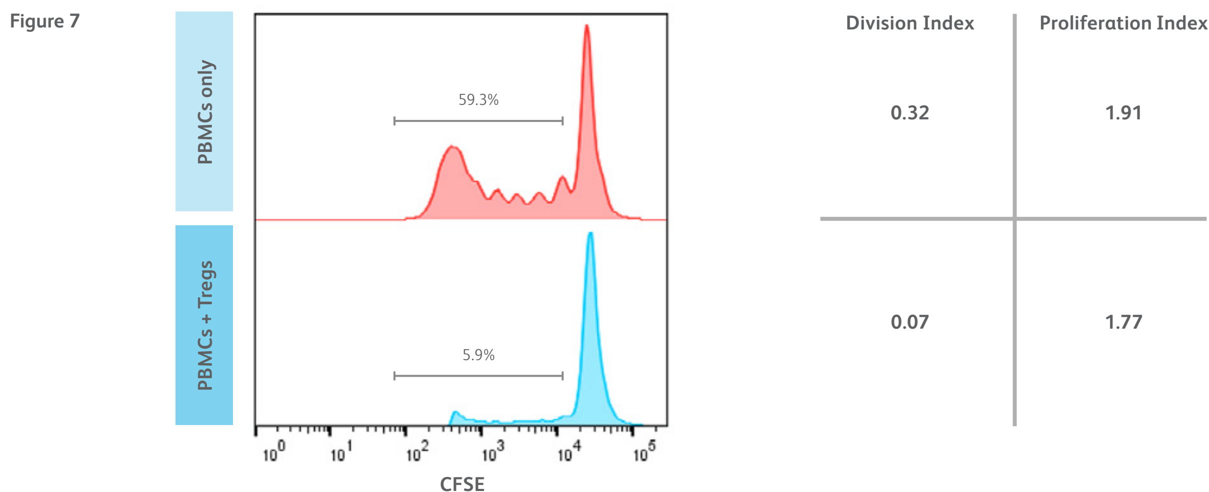


Figure 6. Cytokine production by expanding Tregs

Tissue culture media was collected at multiple time points (1, 2 and 3 weeks) during Treg expansion and cytokine levels were measured using the BD™ Cytometric Bead Array (CBA) Enhanced Sensitivity Flex Set for IL-17A, IFN-γ, IL-4 and IL-10 according to manufacturer's instructions. Samples were acquired using the BD FACSLyric flow cytometer and analysis was performed using FCAP Array™ 4 software.

Suppression of PBMC proliferation by expanded Tregs

To assess the functional capabilities of expanded sorted Tregs, we assessed their ability to suppress TCR-stimulated PBMC proliferation as measured by CFSE-dilution of target responder cells. Following target cell activation, approximately 60% of the PBMC population underwent proliferation as measured by CFSE-dilution using FlowJo™ software (Figure 7). This proliferative response was almost completely suppressed following the addition of expanded Tregs (Figure 7), indicating the maintenance of suppressive capabilities after sorting with the BD FACSMelody.



Division Index	Proliferation Index
0.32	1.91
0.07	1.77

Figure 7. Suppression of proliferation by in vitro expanded Tregs

Freshly isolated and BD Horizon CFSE-labeled autologous PBMCs were cultured in complete RPMI media supplemented with 10% heat-inactivated human serum in the presence of 100 Units/mL of recombinant human IL-2 and anti-CD3/CD28 coated magnetic beads at a ratio of 1 bead per 30 cells. Tregs were added at a 1:0 or 1:1 ratio. The cells were cultured for 5 days and acquired using the BD FACSLyric flow cytometer. CFSE dilution in PBMCs for both conditions is shown as PBMCs only (top histogram) or PBMCs + Tregs (bottom histogram). The percentage of cells that have undergone division is indicated on the histograms. Proliferation analysis was performed using FlowJo™ software. Division and proliferation indexes are shown (Division index: average number of cell divisions that a cell has undergone. Proliferation index: the total number of divisions divided by the number of cells that went into division).

It is often necessary to identify and purify distinct viable cell subpopulations of interest from heterogeneous cell mixtures, such as human peripheral blood. This capacity allows researchers to study the biology and function of the specific subpopulations of interest at the single cell level, thus enabling deep scientific insights. As presented in this data sheet, cellular subpopulations purified by the BD FACSMelody cell sorter are phenotypically and functionally similar to their freshly isolated or cultured counterparts, thereby making them suitable for functional studies designed to characterize the nature of the isolated cell types.

Ordering information

Systems and software
Description
BD FACSMelody™ 9-Color Cell Sorter Blue, Red, and Violet Laser Configuration, with Plate Sorting
BD FACSChorus™ software v1.1 (or later)

Reagents		
Description	Clone	Cat. No.
APC-H7 Mouse Anti-Human CD3	SK7	641397
FITC Mouse Anti-Human CD4	SK3	340133
BD Horizon™ BV786 Mouse Anti-Human CD4	SK3	563877
BD Pharmingen™ PerCP-Cy™5.5 Mouse Anti-Human CD8	SK1	565310
BD Pharmingen™ PerCP-Cy™5.5 Mouse Anti-Human CD14	MψP9	562692
BD Pharmingen™ PerCP-Cy™5.5 Mouse Anti-Human CD19	HIB19	561295
APC Mouse Anti-Human CD25	2A3	340939
BD Horizon™ BV421 Mouse Anti-Human CD25	2A3	564033
BD Horizon™ BV421 Mouse Anti-Human CD69	FN50	562884
BD Pharmingen™ PE Mouse Anti-Human CD127	HIL-7R-M21	557938
BD Horizon™ BB700 Mouse Anti-Human CD127	HIL-7R-M21	566398
BD OptiBuild™ BB700 Mouse Anti-Human CD154	TRAP1	745814
BD Pharmingen™ Alexa Fluor® 647 Mouse anti-Human FoxP3	259D/C7	560045
BD Horizon™ BV605 Mouse Anti-Human IFN-γ	B27	562974
BD Horizon™ APC-R700 Mouse Anti-Human IL-17A	N49-653	565163
BD Horizon™ CFSE		565082
BD Pharmingen™ 7-AAD		559925
BD IMag™ Human CD4 T Lymphocyte Enrichment Set-DM		557939
BD FastImmune™ Brefeldin A Solution		347688
BD GolgiStop™ Protein Transport Inhibitor (Containing Monensin)		554724
BD Cytofix/Cytoperm™ Fixation/Permeabilization Solution Kit		554714
BD Pharmingen™ Transcription Factor Buffer Set		562725
BD™ Cytometric Bead Array (CBA) Human IL-4 Enhanced Sensitivity Flex Set		561510
BD™ Cytometric Bead Array (CBA) Human IL-10 Enhanced Sensitivity Flex Set		561514
BD™ Cytometric Bead Array (CBA) Human IFN-γ Enhanced Sensitivity Flex Set		561515
BD™ Cytometric Bead Array (CBA) Human IL-17A Enhanced Sensitivity Flex Set		562143
BD FACS™ Pre-Sort Buffer		563503

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