Multiparametric Immunophenotyping of Human Hematopoietic Stem Cells and Progenitor Cells by Flow Cytometry

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Application Note

Contents

Abstract

Umbilical cord blood, peripheral blood, and bone marrow are the major sources of hematopoietic stem cells (HSCs) and progenitor cells. The characterization and enumeration of HSCs and progenitor cells from these samples can provide valuable information for clinical research. Multiparametric flow cytometry is a well established method for immunophenotyping of HSCs and various subpopulations of the progenitor cells. Using 8-color panels, a lyse/no-wash assay was developed to analyze cell surface phenotypic markers of HSCs and the various progenitor cell populations such as multipotent progenitor cells (MPPs), common myeloid progenitor cells (CMPs), common lymphoid progenitor cells (CLPs), megakaryocyte erythroid progenitor cells (MEPs), and granulocyte macrophage progenitor cells (GMPs). In addition, regulatory T cells (Tregs) and mesenchymal stem cells (MSCs) were also measured. During the development of this research assay, cord blood and bone marrow samples were tested for identification and enumeration of cells in each of the subpopulations. Additionally, enumeration of $CD34^+$ cells was also compared with the 3-color $CD34^+$ BD™ Stem Cell Enumeration (SCE) assay using a limited sample set. The percent $CD34^+$ data from the samples tested showed less than 10% difference between the 8-color and 3-color assays. Cell count data revealed variations in the HSC and progenitor subpopulations from sample to sample, which might have a significant impact on the recovery rate of neutrophils, platelets, and the overall immune system in stem cell recipients after transplantation. Overall, these 8-color panels could be used as a valuable research tool for characterization and enumeration of HSCs and progenitor cells in research transplantation units.

Introduction

Hematopoiesis is a complex and highly orchestrated process by which pluripotent HSCs differentiate into functional blood cells. In this hierarchical proliferation and differentiation process, self-renewing HSCs first differentiate into MPPs. The MPPs further differentiate into lineage-committed lymphoid or myeloid progenitor cells. The lineage-committed progenitors finally differentiate into terminal functional lymphoid cells, myeloid cells, or erythrocytes (Figure 1).

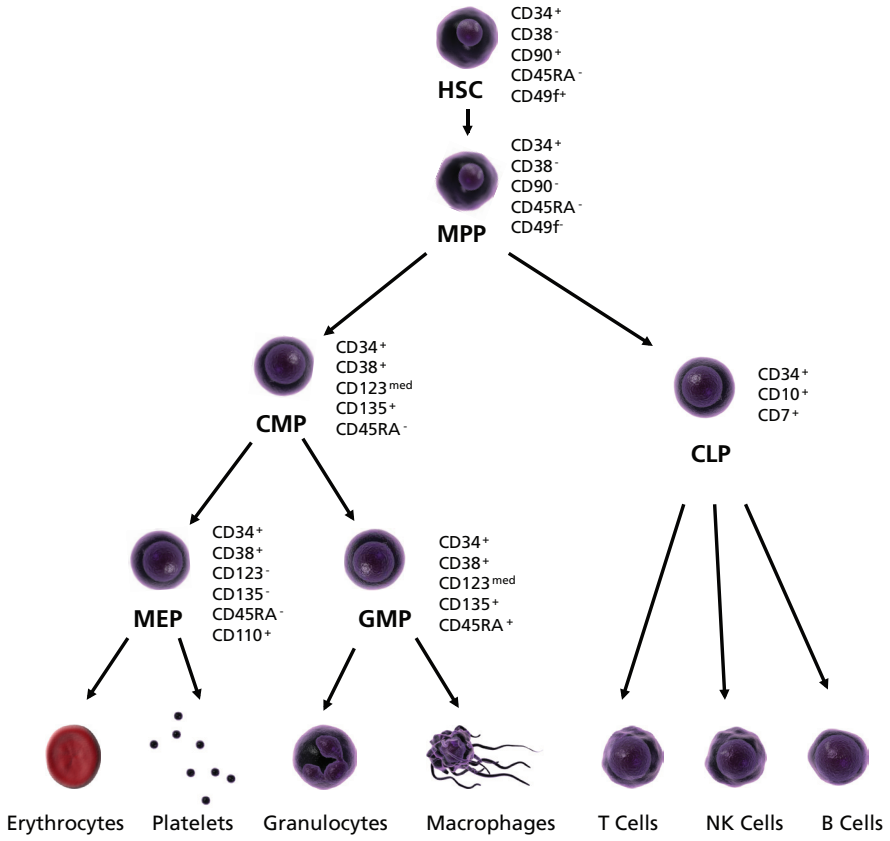


Figure 1. Overview of hematopoietic stem cell differentiation

Umbilical cord blood, peripheral blood, and bone marrow are commonly used as the transplant units for hematopoietic stem cell transplantation (HSCT). HSCs and progenitor cells present in a transplant unit are known to be key cell populations responsible for successful transplantation.[1] After infusion of a transplant unit, the functional cells normally die within a few days. However, the HSCs can survive in the recipient long-term, where they differentiate into terminal functional lymphoid or myeloid cells leading to a successful engraftment. MPPs and lineage-committed progenitor cells are also important. Although these cells do not survive long-term, they provide functional cells short-term and ensure an early engraftment.

HSCs and progenitor cells express unique surface markers that make them distinguishable from other cell types. These markers have been well characterized and various combinations of multiple markers characteristic for each cell subpopulation have been reported. Figure 1 and Table 1 show some of the commonly expressed markers reported in literature for various subpopulations

of HSCs and progenitor cells. For example, CD34 is an adhesion molecule that is expressed on all HSC and progenitor cells. It plays a central role in HSC and progenitor cell recognition. CD90 is another important cell surface marker expressed on early stage hemotopoietic cells. On the other hand, the absence of CD38 is normally associated with an earlier stage of hematopoiesis. CD10 and CD7 are important markers for early lymphoid lineage development. CD123, an interleukin-3 receptor, and CD135 (which is also called Flt3) have been shown to be important for myeloid lineage development. CD110, a thrombopoietin receptor, is important for platelet development.

Table 1. Characteristic marker combinations of different cell subpopulations.

Cell Type	Marker Definition	Reference
HSC	$CD34^+CD38^-CD90^+CD45RA^-CD49f^+$	2, 3
MPP	$CD34^+CD38^-CD90^-CD45RA^-CD49f^-$	2, 3
CLP	$CD34^+CD10^+CD7^+$	4
CMP	$CD34^+CD38^+CD123^{med}CD135^+CD45RA^-$	5, 6
GMP	$CD34^+CD38^+CD123^{med}CD135^+CD45RA^+$	5, 6
MEP	$CD34^+CD38^+CD123^-CD135^-CD45RA^-CD110^+$	5, 6
Treg	$CD4^+CD25^+CD127^{low}CD45RA^{+/-}$	7
MSC	$CD45^-CD34^-CD73^+CD105^+CD90^+$	8, 9

Flow cytometry is a powerful tool for detecting cell surface markers and, by using polychromatic flow cytometry, multiple markers on a cell can be characterized. With the availability of a variety of fluorochrome-labeled antibodies against surface markers and state-of-the-art instruments, flow cytometry is now a well established method for studying HSCs and other cell subpopulations in blood.

An accurate characterization and enumeration of cells in these HSC and progenitor cell subpopulations, as well as Treg and MSC subpopulations, that are present in a blood unit, might be an important quality indicator and might help in understanding the likelihood of a successful engraftment. We have designed an 8-color flow cytometry assay to characterize and enumerate the cells within each subpopulation in a sample (Table 2). The assay detects HSCs, MPPs, CLPs, CMPs, GMPs, and MEPs, as well as Tregs and MSCs. The characteristic marker combinations for each subpopulation are shown in Table 2.

Table 2. 8-color assay panel.

Tube	FITC	PE	PerCP-Cy™5.5	PE-Cy™7	APC	APC-H7	BD Horizon™ V450	BD Horizon™ V500	Subpopulation Identified
1	CD10	CD135	7-AAD	CD34	CD90	CD45RA	CD38	CD45	HSC, MPP, CLP, CMP, GMP, MEP
2	CD45RA	CD127	7-AAD	CD25	–	–	CD4	CD45	Treg
3	CD71	CD105	7-AAD	CD34	CD90	CD44	CD73	CD45	MSC

Objective

The objective of this application note is to demonstrate the use of an 8-color flow cytometry assay panel for the characterization of HSC, MPP, CLP, CMP, MEP, GMP, Treg, and MSC subpopulations found in cord blood and bone marrow samples using a BD FACSCanto™ II flow cytometer.

Methods

Antibodies

Antibody Specificity	Clone	Fluorochrome	Isotype	Vendor	Cat. No.
CD4	RPA-T4	BD Horizon V450	Ms IgG_1, κ	BD Biosciences	560345
CD10	HI10a	FITC	Ms IgG_1, κ	BD Biosciences	340925
CD25	2A3	PE-Cy7	Ms IgG_1, κ	BD Biosciences	335789
CD34	8G12	PE-Cy7	Ms IgG_1, κ	BD Biosciences	348791
CD38	HB7	V450	Ms IgG_1, κ	BD Biosciences	646851
CD44	G44-26	APC-H7	Ms IgG_{2b}, κ	BD Biosciences	560532
CD45	2D1	BD Horizon V500-C	Ms IgG_1, κ	BD Biosciences	647449
CD45RA	L48	FITC	Ms IgG_1, κ	BD Biosciences	347513
	HI100	APC-H7	Ms IgG_{2b}, κ	BD Biosciences	560674
CD71	L01.1	FITC	Ms IgG_{2a}, κ	BD Biosciences	340717
CD73	AD2	BD Horizon V450	Ms IgG_1, κ	BD Biosciences	561255
CD90	5E10	APC	Ms IgG_1, κ	BD Biosciences	559869
CD105	266	PE	Ms IgG_1, κ	BD Biosciences	560839
CD127	hIL-7R-M21	PE	Ms IgG_1, κ	BD Biosciences	557938
CD135	4G8	PE	Ms IgG_1, κ	BD Biosciences	558996

Other Reagents and Materials

Product Description	Vendor	Catalog Number
BD™ CompBead Anti-Mouse Ig, κ	BD Biosciences	552843
BD Pharmingen™ 7-AAD Staining Solution	BD Biosciences	559925
BD Pharm Lyse™ Lysing Buffer (10X concentrate)	BD Biosciences	555899
BD Pharmingen™ Stain Buffer (BSA)	BD Biosciences	554657
BD™ Cytometer Setup and Tracking (CS&T) Bead Kit	BD Biosciences	641319
BD Falcon™ Round-Bottom Tubes, 5 mL	BD Biosciences	352052
BD Stem Cell Enumeration Kit	BD Biosciences	344563
BD Stem Cell Control Kit (Bi-Level Control)	BD Biosciences	340991

Instrument Configuration

Laser			Detector	Dichroic Mirror (LP)	Bandpass Filter (nm)	Fluorochrome
Wavelength (nm)	Power (mW)	Type				
405	30	Point Source™ iFLEX2000™-P-1-405-0.65-30-NP	B	N/A	450/50	BD Horizon V450
			A	502	510/50	BD Horizon V500
488	20	Coherent® Sapphire™ Solid State	E	502	530/30	FITC
			D	556	585/42	PE
			B	655	670 LP	PerCP-Cy5.5
			A	735	780/60	PE-Cy7
633	17	JDS Uniphase™ HeNe Air Cooled	C	N/A	660/20	APC
			A	735	780/60	APC-H7

Specimens

Fresh (0 to 48 hours post-collection) cord blood and bone marrow samples were purchased from AllCells Inc.

Instruments and Software

Flow cytometry data was acquired on a BD FACSCanto II analyzer equipped with three lasers. The instrument was set up using BD Cytometer Setup and Tracking (CS&T) beads. BD FACSDiva™ software (v6.1.3) was used for data acquisition and analysis. Application settings were established to optimize the cytometer's photomultiplier tube (PMT) voltages. For details about application settings, see the Tips and Tricks section.

Methods

Sample Staining

Antibody reagent cocktails were made in 5-mL round-bottom tubes, immediately prior to use, as specified in Table 3.

One hundred microliters of sample was added to each tube. Tubes were incubated for 20 minutes in the dark at room temperature. Red blood cells (RBCs) were lysed by adding 1 mL of 1X BD Pharm Lyse lysing buffer to each tube followed by 10 minutes of incubation in the dark at room temperature. Samples were then placed on ice and were analyzed within 1 hour of lysis.

Table 3. Antibody cocktails.

Tube	Marker	Volume Used (µL)
Tube 1	CD10 FITC	20
	CD135 PE	10
	7-AAD	2
	CD34 PE-Cy7	2
	CD90 APC	2
	CD45RA APC-H7	5
	CD38 BD Horizon V450	2.5
	CD45 BD Horizon V500-C	5
Tube 2	CD45RA FITC	5
	CD127 PE	1
	7-AAD	2
	CD25 PE-Cy7	5
	CD4 BD Horizon V450	5
	CD45 BD Horizon V500-C	5
Tube 3	CD71 FITC	10
	CD105 PE	5
	7-AAD	2
	CD34 PE-Cy7	2
	CD90 APC	2
	CD44 APC-H7	3
	CD73 BD Horizon V450	2
	CD45 BD Horizon V500-C	5

Compensation Setup

Compensation was determined using BD CompBead particles and the compensation setup tool in BD FACSDiva software. Nine compensation controls were prepared in 5-mL round-bottom tubes according to Table 4. All tubes were stained for 20 minutes at room temperature in the dark. After staining, 2 mL of bovine serum albumin (BSA) staining buffer was added to tubes 1–7. These tubes were then centrifuged for 10 minutes at 1,400*g* at room temperature. The supernatants were aspirated and pellets resuspended in 0.5 mL of BSA staining buffer. The tubes were kept on ice in the dark.

The RBCs present in tubes 8 and 9 were lysed by incubating with 2 mL of 1X BD Pharm Lyse lysing buffer for 10 minutes in the dark at room temperature. After lysing, these two tubes were centrifuged for 5 minutes at 200*g* at room temperature. The supernatants were aspirated, and the pellets were resuspended in 300 µL of BSA staining buffer. The contents of tubes 8 and 9 were then combined into a single tube that was placed on ice and protected from light.

Samples were acquired on the cytometer using the BD FACSDiva software compensation setup tool.

Table 4. Reagents for compensation control tubes.

Tube	Reagent	Sample/Control and Buffer
1	5 µL CD38 BD Horizon V450	100 µL of BSA staining buffer + 1 drop of positive BD CompBead Control + 1 drop of negative BD CompBead Control
2	5 µL CD45 BD Horizon V500-C	
3	20 µL CD10 FITC	
4	20 µL CD135 PE	
5	5 µL CD34 PE-Cy7	
6	2 µL CD90 APC	
7	5 µL CD45 APC-H7	
8	5 µL 7-AAD	100 µL of BD Stem Cell Control (High or Low)
9	5 µL 7-AAD	100 µL of fresh blood sample

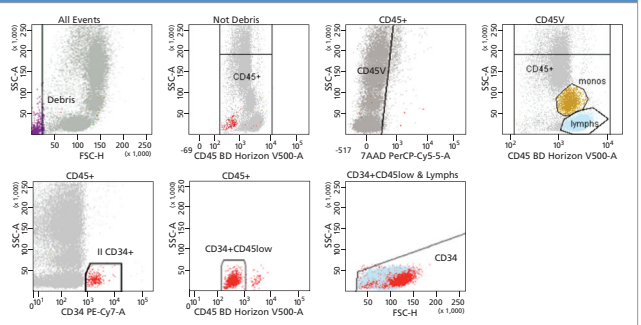
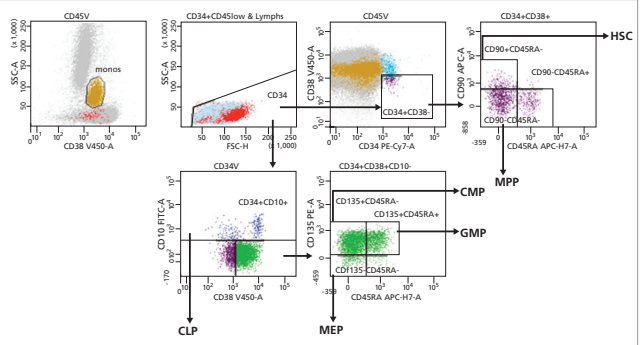
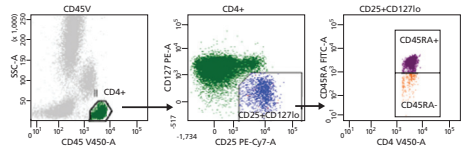
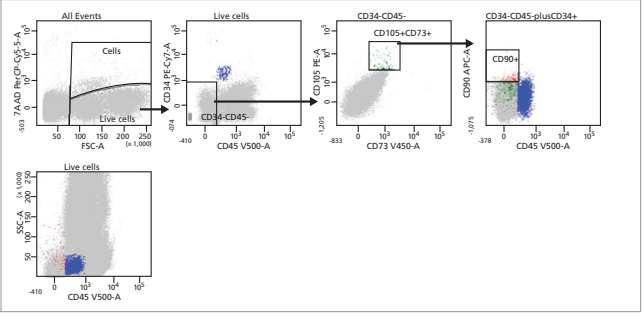
Data Acquisition

The entire sample volume in each tube was acquired using the high flow rate setting. In order to exclude debris, tubes 1 and 2 (Table 2) were acquired with the acquisition threshold set on the channel containing CD45 (BD Horizon V500-C); Tube 3 (MSC) was acquired with the threshold set on forward scatter (FSC) (3,000) and side scatter (SSC) (6,000) to preserve the MSC-containing CD45$^-$ population.

3-Color CD34$^+$ Cell Enumeration Using the BD Stem Cell Enumeration Kit

CD34$^+$ counts were also determined using a 3-color BD™ Stem Cell Enumeration Kit (CD45 FITC, CD34 PE, and 7-AAD) following the machine setup and protocol described in the product technical data sheet (TDS).

Gating Strategy

Subpopulation	Description	Gating Hierarchy	Gating Strategy
CD34$^+$	CD34$^+$ cells were gated combining CD34, CD45, SSC, and FSC parameters in a sequential Boolean gating strategy following the Clinical and Laboratory Standards Institute (CLSI) H42-A2 approved guideline.[10] The dead cells were excluded by 7-AAD–positive staining. The CD34$^+$, CD45^{low}, low-to-intermediate SSC, and low-to-high FSC cells were the true CD34$^+$ cells.	All Events Debris Not Debris CD45+ CD34+ CD34+CD45low CD34 CD45V lymphs monos CD34V	
HSC, MPP, CLP, CMP, GMP, and MEP (Tube 1)	Viable CD34$^+$ cells (CD34V) were further gated into CD34$^+$CD38$^-$ and CD34$^+$CD38$^+$ populations. The CD34$^+$CD38$^-$ events were then subdivided into HSCs (characterized as CD34$^+$CD38$^-$ CD90$^+$CD45RA$^-$) and MPPs (characterized as CD34$^+$CD38$^-$CD90$^-$CD45RA$^-$). CD34$^+$CD10$^+$ events were CLPs. CD34$^+$CD38$^+$CD10$^-$ events were subdivided into CMP (CD34$^+$CD38$^+$CD135$^+$CD45RA$^-$), GMP (CD34$^+$CD38$^+$CD135$^+$CD45RA$^+$), and MEP (CD34$^+$CD38$^+$CD135$^-$CD45RA$^-$) populations.	All Events Debris Not Debris CD45+ CD34+ CD34+CD45low CD34 CD45V lymphs monos CD34V CD34+C38- CD90+CD45RA- CD90-CD45RA+ CD34+CD38+ CD34+CD10+ CD34+CD10- CD34+CD38+CD10- CD135+CD45RA- CD135+CD45RA+ CD135-CD45RA-	
Treg (Tube 2)	Tregs were identified based on their cell surface markers. Viable CD45$^+$ cells were gated on CD4$^+$ lymphocyte events. These were then gated on CD25$^+$CD127^{low} (Tregs). The Tregs were further divided into the naive (CD45RA$^+$) and the memory (CD45RA$^-$) Treg subpopulations.	All Events Debris NOT(Debris) CD45+ CD45V CD4+ CD25+CD127lo CD45RA+ CD45RA-	
MSC (Tube 3)	The live cell population was defined by 7-AAD staining and FSC gating. The viable cells were gated on CD45$^-$CD34$^-$ events, then further gated on CD105$^+$, CD73$^+$, and CD90$^+$ events. These CD45$^-$CD34$^-$CD105$^+$CD73$^+$CD90$^+$ cells were MSCs. CD34$^+$ cells are also shown in the plots (in blue).	All Events Cells Live cells CD34-CD45- CD105+CD73+ CD90+	

Results

Assay Verification

A proof-of-principle experiment was performed to check the accuracy of the data collection on a limited number of samples to compare the percentages of viable CD34⁺ cells within the viable CD45⁺ cell population obtained from the 8-color assay and the 3-color BD Stem Cell Enumeration assay. As shown in Table 5 and Figure 2, a comparison of the %CD34⁺ population in six cord blood samples and three bone marrow samples using the 8-color assay was within 10% of the CD34 Stem Cell Enumeration Kit. The regression plot of this small data set suggests that there might be a correlation between these two assays as evident from the near unity R^2 value.

Table 5. Comparison of CD34⁺ enumeration using the 8-color assay and the CD34⁺ BD Stem Cell Enumeration Kit.

Sample	%CD34V in CD45V		Difference
	8-Color Assay	Stem Cell Enumeration Kit	
Cord Blood #1	0.19	0.19	0.00%
Cord Blood #2	0.24	0.26	-9.62%
Cord Blood #3	0.44	0.45	-3.33%
Cord Blood #4	0.58	0.64	-9.38%
Cord Blood #5	0.30	0.30	0.00%
Cord Blood #6	0.21	0.22	-4.55%
Bone Marrow #1	0.88	0.88	0.00%
Bone Marrow #2	1.26	1.34	-6.34%
Bone Marrow #3	1.16	1.15	0.43%

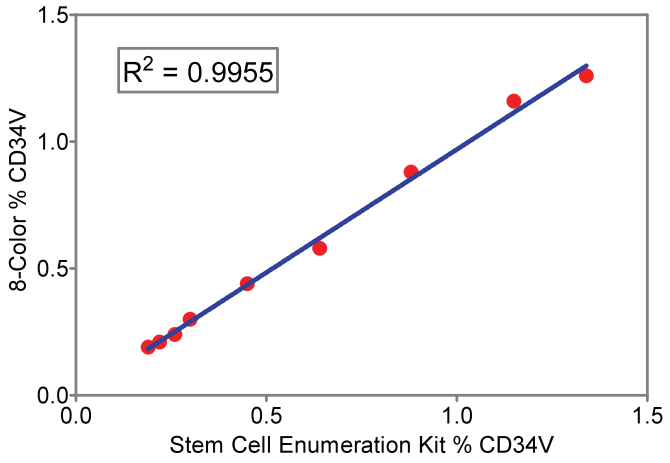


Figure 2. Comparison of CD34⁺ enumeration using the 8-color assay and the CD34⁺ BD Stem Cell Enumeration Kit.

The percentages of viable CD34⁺ cells within the viable CD45⁺ cell population obtained from six cord blood and three bone marrow samples are plotted against each other in graphic form with a linear regression line (R^2 = 0.9955).

Enumeration of Cells in Different Subpopulations

Six cord blood samples and three bone marrow samples were tested using the 8-color assay. Within each sample type, the data showed wide variations in the cell counts for each cell subpopulation (Table 6 and Figure 3). Further, cell counts from different sample types also showed differences. The cell counts were similar between bone marrow and cord blood samples in the HSC, MPP, CMP, GMP, or MEP subpopulations. CLP counts were higher in bone marrow samples. Treg cell counts were higher in cord blood than in bone marrow. MSC cell counts were much higher in the bone marrow samples than those from cord blood samples.

Table 6. The median and range of number of cells in different subpopulations in cord blood and bone marrow samples using 8-color panels.

			Number of Cells		
Subpopulation	Markers	Sample Type	Min	Max	Median
HSC	$CD34^+CD38^-CD90^+CD45RA^-$	Bone Marrow	297	890	553
		Cord Blood	33	250	168
MPP	$CD34^+CD38^-CD90^-CD45RA^-$	Bone Marrow	447	1,914	641
		Cord Blood	372	1,473	606
CLP	$CD34^+CD10^+$	Bone Marrow	2,374	8,123	4,280
		Cord Blood	61	276	169
CMP	$CD34^+CD38^+CD10^-CD135^+CD45RA^-$	Bone Marrow	860	2,055	1,938
		Cord Blood	147	759	318
GMP	$CD34^+CD38^+CD10^-CD135^+CD45RA^+$	Bone Marrow	1,532	2,822	2,555
		Cord Blood	662	2,804	968
MEP	$CD34^+CD38^+CD10^-CD135^-CD45RA^-$	Bone Marrow	214	575	435
		Cord Blood	156	563	253
Treg	$CD4^+CD25^+CD127^{low}$	Bone Marrow	1,987	3,736	2,454
		Cord Blood	8,031	25,155	10,246
Treg $CD45RA^+$	$CD4^+CD25^+CD127^{low}CD45RA^+$	Bone Marrow	329	1392	734
		Cord Blood	4,835	18,113	6,109
Treg $CD45RA^-$	$CD4^+CD25^+CD127^{low}CD45RA^-$	Bone Marrow	1,658	2,345	1,720
		Cord Blood	1,067	7,041	3,441
MSC	$CD45^-CD34^-CD73^+CD105^+CD90^+$	Bone Marrow	264	815	747
		Cord Blood	33	117	63

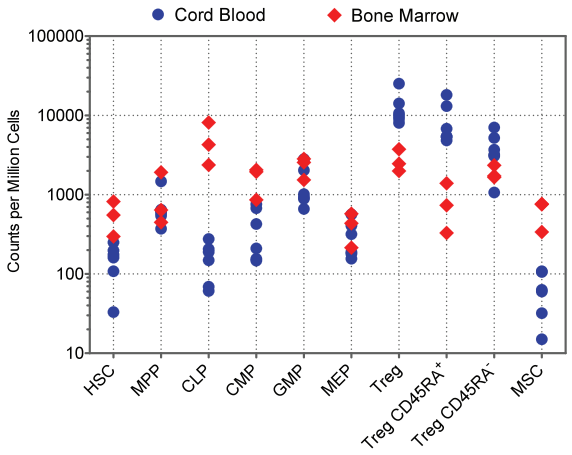


Figure 3. Enumeration of cells in different subpopulations in cord blood and bone marrow samples using 8-color panels.

The blue markers represent the six cord blood samples and the red markers represent the three bone marrow samples. The number of cells in the HSC, MPP, CLP, CMP, GMP, and MEP subpopulations were from Tube 1. The cell numbers in the Treg and $CD45RA^+$ or $CD45RA^-$ Treg subpopulations were obtained from Tube 2. The number of MSCs was obtained from Tube 3.

Discussion

In recent years, there has been a significant increase in the number of HSCTs using apheresis and cord blood units compared to the more conventional source, bone marrow. With its low requirement for HLA matching, cord blood has become a very important alternative source for HSCT and, as a result, cord blood banking has been growing rapidly. Cord blood transplantation has shown reduced acute and chronic graft-versus-host disease with disease-free survival similar to bone marrow and peripheral blood transplantation. However, it has also shown delayed granulocyte and platelet engraftment associated with the low cell dose obtainable from a single cord blood unit.[1] Regardless of the source of HSCTs, the recovery rate of neutrophils, platelets, and other immune cells in the recipient (after transplantation) is hard to predict. Here we provide a flow cytometry–based tool that researchers might use to analyze the HSC and progenitor cell populations in the pre-transplant samples in a research setting. This immunophenotyping-based analysis might give researchers a better understanding of the potential for engraftment success for research samples to be transplanted.

Using an 8-color assay on a BD FACSCanto II flow cytometer, this application note demonstrates the identification and enumeration of various subpopulations of HSC and progenitor cells from cord blood and bone marrow samples. The assay design was based on the existing literature[2-6] on unique marker combinations for HSC, MPP, CLP, CMP, GMP and MEP cells (Table 1). After testing three samples of bone marrow and six samples of cord blood using this assay, wide variations in subpopulations were observed from sample to sample and among different sample types. Since these HSCs ultimately differentiate into the functional blood cell populations, the variations observed in the number of HSCs might have an impact on the recovery rates of blood cell populations in recipients after transplantation. Overall, this study clearly shows the characterization and enumeration of functional cell populations that might aid in the selection of suitable transplant units and might further aid in the understanding of successful engraftment.

Conclusions

The 8-color assay presented in this application note can be used in research settings to identify and enumerate HSCs, a variety of progenitor cells including multipotent, common lymphoid, common myeloid, megakaryocyte erythroid, and granulocyte macrophage progenitor cells, and also regulatory T cells and mesenchymal stem cells in cord blood and bone marrow samples. The analysis of a limited number of samples using this assay showed that the cell counts in each subpopulation among different blood units were variable. We hypothesize that this variation might contribute to the difference in recovery rates of the cell populations in the recipient after transplantation, and therefore, accurate cell counts in these different cell populations might aid in the understanding of the potential engraftment capability of a unit.

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11. Standardizing Application Setup Across Multiple Flow Cytometers Using BD FACSDiva™ Version 6 Software. BD Biosciences Technical Note. 2012. 23-13661-00.

Tips and Tricks

Establishing Application Settings

In order to get consistent flow cytometry data, we recommend standardizing and establishing assay-specific application settings on an instrument. Establishing application settings involves optimization of the PMT voltages by determining the best dynamic range for the specific reagent being used in each detection channel, and ensuring that the negative events are above the electronic noise level of the instrument. After creating the application settings for the 8-color assay, these settings can be saved and applied to future experiments to obtain reproducible results. Details on how to perform this procedure can be found in the technical note on standardizing application setup across multiple flow cytometers using BD FACSDiva Version 6 software.[11] A brief overview of the technique is as follows.

To create application settings in BD FACSDiva software, a set of nine tubes containing unstained cells and CD8-stained cells was prepared as described in Table 7. CD8 is an abundant surface molecule on lymphocytes. Anti-CD8, therefore a very bright reagent for staining this population, was used as the reagent of choice to maximize the dynamic range of the PMT voltages for this specific application. A set of compensation control tubes was also prepared as described in Table 4 in the Methods section. Cord blood or bone marrow samples (20 µL) were stained for 20 minutes at room temperature in the dark. RBCs present in the samples were lysed by incubating with 1 mL of 1X BD Pharm Lyse lysing buffer for 10 minutes at room temperature in the dark. Tubes were placed on ice and protected from light until acquired.

Table 7. Reagents for application settings tubes.

Tube	Antibody Reagent	Sample and Buffer
1	10 µL CD8 FITC	80 µL of BSA staining buffer + 20 µL of sample (cord blood or bone marrow)
2	10 µL CD8 PE	
3	10 µL CD8 PerCP-Cy5.5	
4	3 µL CD8 PE-Cy7	
5	3 µL CD8 APC	
6	3 µL CD8 APC-H7	
7	3 µL CD8 V450	
8	3 µL CD8 V500	
9	NA	

To optimize the PMT voltages for this application, a series of verification steps was performed:

- To verify that the negative cell populations were above background noise, 5,000 events from the unstained cells tube were acquired. For each fluorescence parameter, the robust standard deviation (rSD) (found in the statistics view in BD FACSDiva software) of the lymphocyte population was examined. The PMT voltage of each parameter was adjusted as needed to ensure that the actual rSD was between the calculated 2.5x to 3x electronic noise robust standard deviation (ENrSD). The unstained tube was re-acquired after changing the PMT voltages to verify that the rSDs for each parameter were within range, and further adjustments made as needed.
- To verify that the positive cell populations were on scale, 5,000 events from each CD8-stained tube from Table 7 were acquired. PMT voltages were adjusted so that the mean fluorescence intensity (MFI) of the positive cells for each parameter was less than 120,000 or the Linearity Max Channel value.
- To verify that the compensation controls were on scale, 5,000 events from each compensation control tube (Table 4) were acquired. Singlet beads were gated, and PMTs were lowered as necessary to lower the mean of the positive beads below the Linearity Max Channel values.

Once all tubes were acquired and all PMTs were optimized, the application settings were saved and used in subsequent experiments.

Table 8 is provided as a template for recording cytometer PMT voltages. ENrSDs and Linearity Max Channel values can be obtained from the instrument's baseline report after running CS&T beads, and then the 2.5x and 3x ENrSDs can be calculated manually.

Table 8. 8-color application settings parameters.

Fluorescence Parameter	ENrSD	2.5x ENrSD	3x ENrSD	Unstained (Neg) Cells rSD	Lin. Max Channel	Positive Cells Mean	Compensation Control Mean
FITC							
PE							
PerCP-Cy5.5							
PE-Cy7							
APC							
APC-H7							
BD Horizon V450							
BD Horizon V500							

6-Color Panels for Immunophenotyping HSCs and Progenitor Cells

The 8-color panel described in this application note is ideal for 3-laser instruments such as the BD FACSCanto II. For 2-laser instruments which lack the ability to detect violet dyes such as BD Horizon V450 and BD Horizon V500, an alternative option of using a 6-color panel using five tubes was also optimized for the characterization of HSC, MPP, CLP, CMP, MEP, GMP, Treg, and MSC subpopulations from cord blood and bone marrow samples (Table 9). Although the 6-color panel provides similar information to the 8-color panel, it loses some dimensions for marker co-expression. In addition, tube 2 in the 6-color assay can provide more information on $CD34^+CD7^+$ CLPs.

Antibodies for 6-Color Panels

Antibody Specificity	Clone	Fluorochrome	Isotype	Vendor	Cat. No.
CD4	SK3	APC	Ms IgG_1, κ	BD Biosciences	340443
CD7	M-T701	FITC	Ms IgG_1, κ	BD Biosciences	340737
CD10	HI10a	PE	Ms IgG_1, κ	BD Biosciences	340921
CD25	2A3	PE-Cy7	Ms IgG_1, κ	BD Biosciences	335789
CD34	8G12	PE-Cy7	Ms IgG_1, κ	BD Biosciences	348791
CD38	HB7	APC	Ms IgG_1, κ	BD Biosciences	340439
		PE	Ms IgG_1, κ	BD Biosciences	347687
CD45	2D1	APC-H7	Ms IgG_1, κ	BD Biosciences	641399
CD45RA	L48	FITC	Ms IgG_1, κ	BD Biosciences	347723
CD73	AD2	FITC	Ms IgG_1, κ	BD Biosciences	561254
CD90	5E10	APC	Ms IgG_1, κ	BD Biosciences	559869
		PE	Ms IgG_1, κ	BD Biosciences	555596
CD105	266	PE	Ms IgG_1, κ	BD Biosciences	560839
CD110	1.6.1	APC	Ms IgG_{2b}, κ	BD Biosciences	562199
CD123	9F5	PE	Ms IgG_1, κ	BD Biosciences	340545
CD127	hIL-7R-M21	PE	Ms IgG_1, κ	BD Biosciences	557938

Panel Design

Table 9. 6-color panel.

Tube	FITC	PE	PerCP-Cy5.5	PE-Cy7	APC	APC-H7	Cell Population Tested
1	CD45RA	CD90	7-AAD	CD34	CD38	CD45	HSC, MPP
2	CD7	CD10	7-AAD	CD34	CD38	CD45	CLP
3	CD45RA	CD123	7-AAD	CD34	CD38	CD45	CMP, GMP, MEP
4	CD45RA	CD127	7-AAD	CD25	CD4	CD45	Treg
5	CD73	CD105	7-AAD	CD34	CD90	CD45	MSC

Table 10. Antibody cocktails for the 6-color assay.

Tube	Marker	Volume Used (µL)
Tube 1	CD45RA FITC	5
	CD90 PE	2
	7-AAD	2
	CD34 PE-Cy7	2
	CD38 APC	3
	CD45 APC-H7	3
Tube 2	CD7 FITC	10
	CD10 PE	20
	7-AAD	2
	CD34 PE-Cy7	2
	CD38 APC	3
	CD45APC-H7	3
Tube 3	CD45RA FITC	5
	CD123 PE	10
	7-AAD	2
	CD34 PE-Cy7	2
	CD38 APC	3
	CD45 APC-H7	3
Tube 4	CD45RA FITC	5
	CD127 PE	1
	7-AAD	2
	CD25 PE-Cy7	5
	CD4 APC	5
	CD45 APC-H7	3
Tube 5	CD73 FITC	20
	CD105 PE	5
	7-AAD	2
	CD34 PE-Cy7	2
	CD90 APC	2
	CD45 APC-H7	3

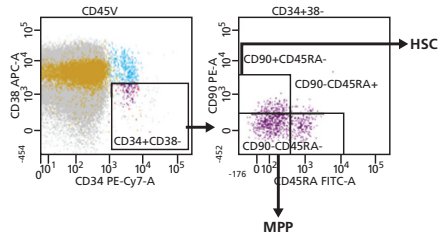
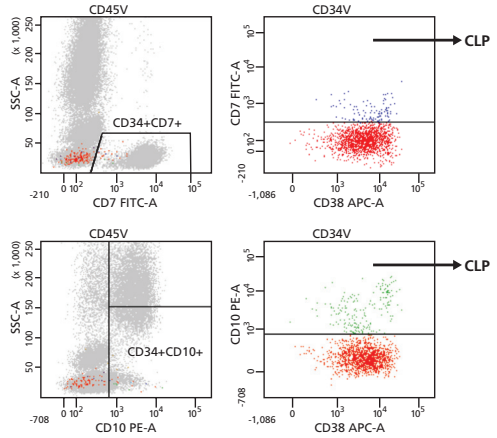
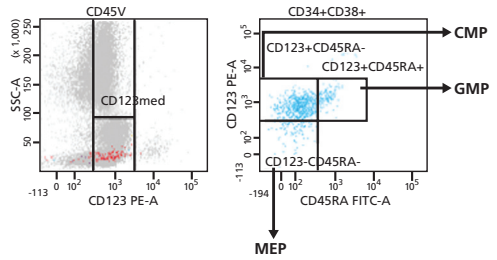
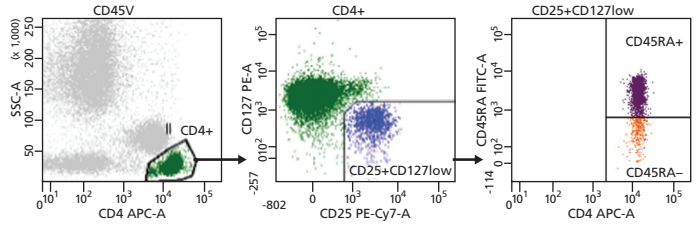
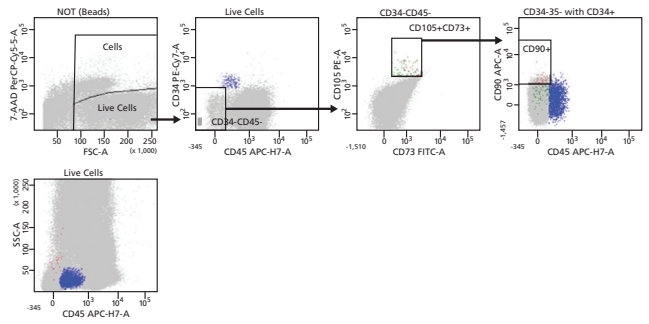
Sample Staining and Compensation Setup

Antibody reagent cocktails were made fresh in 5-mL round-bottom tubes as specified in Table 10.

Sample staining and compensation were performed as previously described for the 8-color assay, except that a subset of the tubes excluding tubes containing BD Horizon V450 and BD Horizon V500 dyes was used for compensation.

Gating Strategy for the 6-Color Assay

Viable CD34⁺ cells were gated as described for the 8-color panels.

Subpopulation	Description	Gating Hierarchy	Gating Strategy
HSC and MPP (Tube 1)	CD34⁺ events were gated into CD34⁺CD38⁻ and CD34⁺CD38⁺ populations. The CD34⁺CD38⁻ population was then gated into CD90⁺CD45RA⁻ events (HSCs). The MPPs are the CD34⁺CD38⁻ CD90⁻CD45RA⁻ events.	All Events Debris Not Debris CD45+ CD34+ CD34+45low CD34 CD45V lymphs monos CD34V CD34+38- CD90-CD45RA+ CD90-CD45RA- CD90-CD45RA- CD34+CD38+	
CLP (Tube 2)	CD34⁺ events were divided into CD34⁺CD10⁺ or CD34⁺CD7⁺ populations. These are the CLP cells that give rise to lymphoid progeny as reported in in vitro and in vivo studies.[3,4]	All Events Debris Not Debris CD45+ CD34+ CD34+45low CD34 CD45V lymphs monos CD34V CD34+38- CD34+CD38+ CD34+CD7+ CD34+CD10+	
CMP, GMP, and MEP (Tube 3)	CD34⁺CD38⁺ events were gated as CMPs (CD34⁺CD38⁺CD123^{med}CD45RA⁻ population), GMPs (CD34⁺CD38⁺CD123^{med}CD45RA⁺ popluation), and MEPs (CD34⁺CD38⁺CD123⁻CD45RA⁻ population).	All Events Debris Not Debris CD45+ CD34+ CD34+45low CD34 CD45V lymphs monos CD34V CD34+38- CD34+CD38+ CD123+CD45RA- CD123+CD45RA+ CD123-CD45RA- CD123med	
Treg (Tube 4)	Viable CD45⁺ events were gated on CD4⁺. This CD4⁺ poplation was gated on CD25⁺ CD127^{low} events (Tregs). The Tregs were further gated into the naive (CD45RA⁺) and the memory (CD45RA⁻) Treg subpopulations.	All Events Debris NOT(Debris) CD45+ CD45V CD4+ CD25+CD127low CD45RA+ CD45RA-	
MSC (Tube 5)	The viable cell population was defined by 7-AAD staining and FSC gating, then gated on CD45⁻CD34⁻ events. This CD45⁻CD34⁻ population was further gated on CD105⁺CD73⁺CD90⁺ events (MSCs). CD34⁺ cells are also shown in the plots (in blue).	All Events Cells Live cells CD34-CD45- CD105+CD73+ CD90+	

Single-Tube Assay for Immunophenotyping HSCs and MPPs (CD49f+ HSC Panel)

CD49f is an HSC marker which has been recently reported for isolation of human HSCs capable of long-term multilineage engraftment regardless of CD90 expression.[3] An 8-color panel was designed to characterize $CD34^+CD38^-CD90^-CD49f^+$ HSCs (Table 11). This tube can also be used as a replacement for tube 1 in the 8-color panel (described in this application note) to provide more information on HSCs. However, it will not generate information for CLPs, as CD49f is used instead of CD10.

Additional Antibody for the CD49f HSC Panel

Antibody Specificity	Clone	Fluorochrome	Isotype	Vendor	Cat. No.
CD49f	GoH3	FITC	Rat IgG_{2a}, κ	BD Biosciences	555735

Panel Design

Table 11. 8-color panel for immunophenotyping of $CD34^+CD38^-CD90^-CD45RA^-CD49f^+$ HSCs.

Fluorochrome	FITC	PE	PerCP-Cy5.5	PE-Cy7	APC	APC-H7	BD Horizon V450	BD Horizon V500	Cell Populations Tested
Reagent	CD49f	CD123	7-AAD	CD34	CD90	CD45RA	CD38	CD45	HSC,MPP, CMP, GMP, MEP

Sample Staining and Compensation Setup

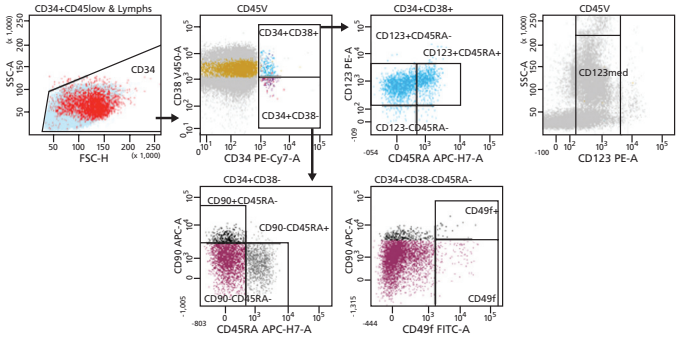
Antibody reagent cocktails were made fresh in 5-mL round-bottom tubes as specified in Table 12.

Sample staining and compensation were performed as previously described for the 8-color assay.

Table 12. Antibody cocktail for the $CD49f^+$ HSC panel.

Marker	Volume Used (µL)
CD49f FITC	5
CD123 PE	10
7-AAD	2
CD34 PE-Cy7	2
CD90 APC	2
CD45RA APC-H7	2.5
CD38 BD Horizon V450	2.5
CD45 BD Horizon V500-C	5

Gating Strategy

Subpopulation	Description	Gating Hierarchy	Gating Strategy
HSC, MPP, CMP, MEP, GMP	$CD34^+$ events were gated into $CD34^+CD38^-$ and $CD34^+CD38^+$ populations. The $CD34^+CD38^-$ population was then subdivided into $CD90^+CD45RA^-$ (HSC) and $CD90^-CD45RA^-$ (MPP) populations. The $CD34^+CD38^-CD45RA^-$ events were further gated on $CD49f^+$ HSCs. The $CD34^+CD38^+$ population was subdivided into $CD34^+CD38^+CD123^{med}CD45RA^-$ (CMP), $CD34^+CD38^+CD123^{med}CD45RA^+$ (GMP), and $CD34^+CD38^+CD123^-CD45RA^-$ (MEP) populations.	CD34V CD34+CD38- CD90+CD45RA- CD49f+ CD90-CD45RA- CD49f+ CD90-CD45RA+ CD34+CD38+ CD123+CD45RA- CD123+CD45RA+ CD123-CD45RA- CD123med	

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