

Deep Immune Profiling with the Maxpar Direct Immune Profiling System

Introduction

Immune profiling is the practice of identifying and quantifying immune populations according to their phenotypic and functional features. Performing immune profiling longitudinally, a process referred to as immune monitoring, is an especially valuable technique to study conditions where the immune system plays a key role in the pathology, progression, or resolution of disease. Immune monitoring can be performed using either whole blood or PBMC¹⁻⁴ samples and is commonly applied in the study of cancer, autoimmune diseases, and inflammation to provide phenotypic understanding of immune states prior to and following treatment⁵⁻¹¹. The wide diversity of immune cell populations demands a high-parameter technique to more fully and efficiently quantify these changes.

Flow cytometry has conventionally been used for immune profiling and monitoring. However, flow cytometry generally requires multiple tubes to comprehensively classify the diverse populations of immune cells in blood^{2, 12-16}. As a result, researchers are often limited to a small set of markers for defining each cell type.

Mass cytometry, which utilizes CyTOF® technology, is a single-cell analysis platform that uses metal-tagged antibodies to resolve over 50 markers in a single sample tube without the need for compensation^{12, 17}. It is an ideal solution for routine enumeration of immune cell populations. However, development of a robust, highly multiplexed assay requires panel optimization as well as standardization of instrument setup and an easy-to-use yet reliable data analysis solution.

The process of optimizing, validating, and preparing an in-house cell staining panel is laborious, time-consuming, and costly. In addition, workflows using multiple antibodies and multiple tubes increase the likelihood for user error. Deep immune profiling and monitoring call for a standardized solution that efficiently delivers trusted results.

The Maxpar® Direct™ Immune Profiling Assay™ provides a convenient and reliable solution for deep immune profiling of human peripheral whole blood and PBMC with the Helios™ mass cytometer.

The assay includes a dry-format 30-marker antibody panel with a viability stain in a standard 5 mL polypropylene tube (12 x 75 mm). The panel enables identification and enumeration of 37 immune cell populations when using the automated data analysis solution, Maxpar

Pathsetter™. PBMC or whole blood is directly added to a single tube for antibody staining and cell processing.

The assay panel was developed based on the recommendations of the Human ImmunoPhenotyping Consortium (HIPC)¹. Incorporating feedback from leading expert immunologists, eight markers were added to identify additional T cell subsets, NK cell subsets, neutrophils, basophils, and eosinophils (see Figure 1)^{20–25}. Metal-isotope-labelled antibody clones were selected to optimize the panel with minimal signal spillover. The detailed panel configuration is shown in Table 1.

Maxpar Pathsetter software is a data analysis and reporting software that uses a statistical method called probability state modeling^{18,19}. Pathsetter was developed specifically for mass cytometry and comes preloaded with statistical models for data cleanup (removal of doublets, aggregates, non-cell events, and dead cells) and automated analysis of FCS files generated by the Maxpar Direct Immune Profiling Assay. A customized statistical model can also be created in Maxpar Pathsetter for antibodies that are added to open channels in the panel to measure expression markers on existing classified populations or for further identification of additional immune cell subsets. For more information on adding antibodies to the Maxpar Direct Immune Profiling Assay, refer to the tech note Adding New Markers to the Maxpar Direct Immune Profiling Assay and Customizing Its Model and Report in Maxpar Pathsetter (PN 400239).

The Maxpar Direct Immune Profiling System, comprised of the Maxpar Direct Immune Profiling Assay, validated protocols, a Helios mass cytometer, and Maxpar Pathsetter software, has undergone rigorous analytical validation using both PBMC and whole blood. Here we show data supporting the analytical performance of the assay for standardized immune profiling by measuring intra-assay repeatability, intermediate precision, and accuracy of the dry panel format. This white paper also includes inter-site reproducibility data using peripheral human whole blood and PBMC. The results of the multi-site study demonstrate that the Maxpar Immune Profiling Assay is a robust solution for immune profiling.

With the complete Maxpar Direct Immune Profiling System, deep immune profiling information can be performed in a single tube consistently and conveniently.

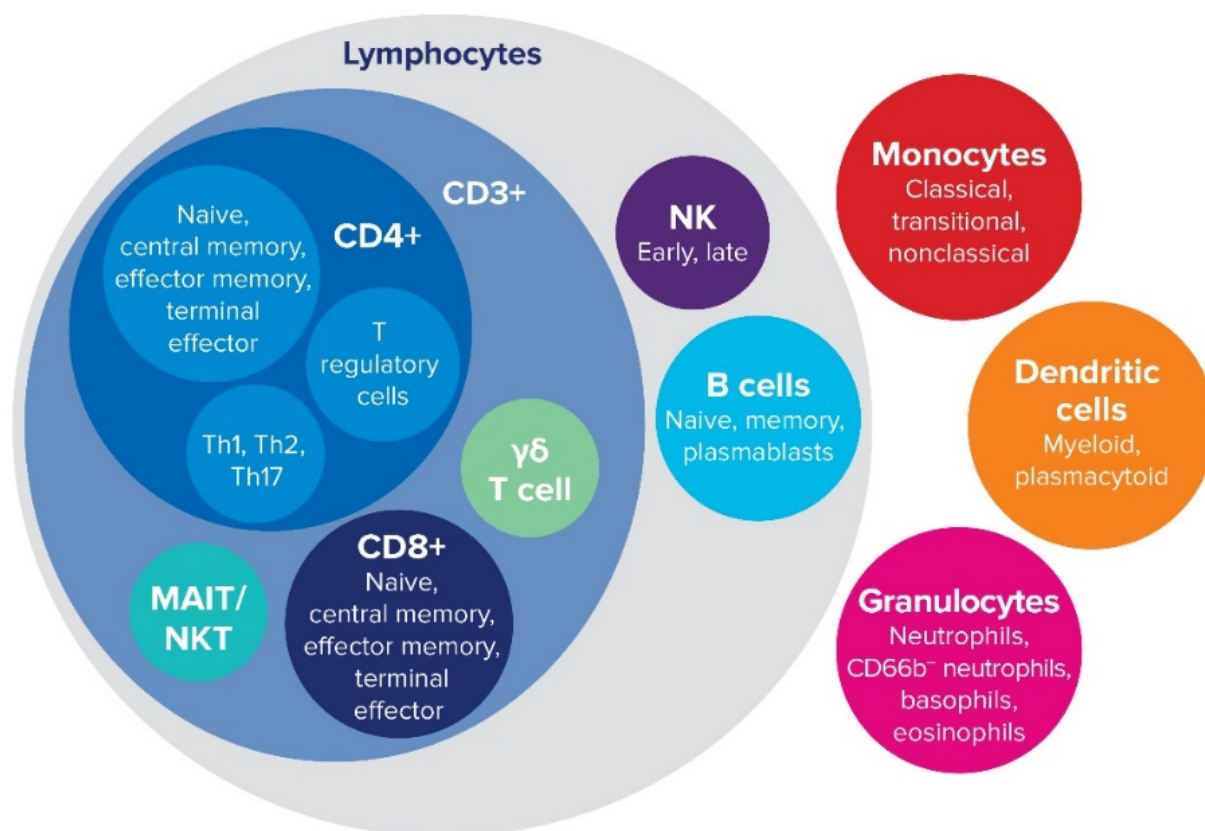


Figure 1. Populations automatically identified using the Maxpar Direct Immune Profiling Assay and Maxpar Pathsetter software

Table 1 Isotope-tagged antibodies included in the Maxpar Direct Immune Profiling Assay antibodies that is provided in a dry single-tube format

Antibody (clone)	Isotope	Antibody (clone)	Isotope
CD45 (HI30)	⁸⁹ Y	CD185/CXCR5 (J252D4)	¹⁵⁸ Gd
CD196 /CCR6 (G034E3)	¹⁴¹ Pr	CD28 (CD28.2)	¹⁶⁰ Gd
CD123 (6H6)	¹⁴³ Nd	CD38 (HB-7)	¹⁶¹ Dy
CD19 (HIB19)	¹⁴⁴ Nd	CD56/NCAM (NCAM16.2)	¹⁶³ Dy
CD4 (RPA-T4)	¹⁴⁵ Nd	TCRgd (B1)	¹⁶⁴ Dy
CD8a (RPA-T8)	¹⁴⁶ Nd	CD294 (BM16)	¹⁶⁶ Er
CD11c (Bu15)	¹⁴⁷ Sm	CD197/CCR7 (G043H7)	¹⁶⁷ Er
CD16 (3G8)	¹⁴⁸ Nd	CD14 (63D3)	¹⁶⁸ Er
CD45RO (UCHL1)	¹⁴⁹ Sm	CD3 (UCHT1)	¹⁷⁰ Er
CD45RA (HI100)	¹⁵⁰ Nd	CD20 (2H7)	¹⁷¹ Yb
CD161 (HP-3G10)	¹⁵¹ Eu	CD66b (G10F5)	¹⁷² Yb
CD194/CCR4 (L291H4)	¹⁵² Sm	HLA-DR (LN3)	¹⁷³ Yb
CD25 (BC96)	¹⁵³ Eu	IgD (IA6-2)	¹⁷⁴ Yb
CD27 (O323)	¹⁵⁴ Sm	CD127 (A019D5)	¹⁷⁶ Yb
CD57 (HCD57)	¹⁵⁵ Gd	Cell-ID Intercalator-103Rh	¹⁰³ Rh
CD183/CXCR3 (G025H7)	¹⁵⁶ Gd		

Relevant Fluidigm Documents

- Maxpar Direct Immune Profiling Assay Cell Staining and Data Acquisition User Guide (PN 400286)
- Maxpar Direct Immune Profiling Assay in Whole Blood Quick Reference (PN 400287)
- Maxpar Direct Immune Profiling Assay in PBMC Quick Reference (PN 400288)
- Customizing the Maxpar Direct Immune Profiling Assay Technical Note (PN 400239)

Materials and Methods

Whole Blood

Human peripheral whole blood from healthy donors (Canadian Blood Services, Vancouver, BC, Canada; STEMCELL™ Technologies, Vancouver, BC, Canada; or Discovery Life Sciences, Powell, OH, USA) was collected in BD Vacutainer® blood collection tubes (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) containing heparin as an anti-coagulant and shipped overnight. Blood was stained and processed no more than 24 hr following collection.

PBMC

Ficoll-isolated frozen PBMC samples were purchased from Discovery Life Sciences. Canadian Blood Services also isolated PBMC samples using BD Vacutainer CPT™ Mononuclear Cell Preparation Tubes (Becton, Dickinson and Company). Blood was collected in CPT tubes, centrifuged to separate the PBMC, and then shipped overnight on gel packs. When received the next day, the contents in the CPT tubes were washed in PBS and frozen in media containing 80% Gibco™ Advanced RPMI 1640 (Thermo Fisher Scientific, Waltham, MA, USA), 10% HyClone™ Cosmic Calf™ Serum (GE Healthcare, Chicago, IL, USA), and 10% DMSO (Sigma Aldrich, St. Louis, MO, USA). PBMC vials were thawed at 37 °C using CTL Anti-Aggregate Wash™ Solution (Immunospot®, Cleveland, OH, USA) according to the manufacturer's instructions. Cells were counted using a TC20™ cell counter (Bio-Rad, Hercules, CA, USA) and trypan blue (Bio-Rad) and determined to have ≥80% viability.

Cell Staining

Cell staining was performed according to the Maxpar Direct Immune Profiling Assay Cell Staining and Data Acquisition User Guide (PN 400286). For whole blood, an additional heparin blocking step was performed (Sigma Aldrich; 100 KU/mL) for 20 min at room temperature to reduce nonspecific antibody binding by granulocytes²⁶. For PBMC samples, cells were Fc-blocked with Human TruStain FcX Fc Receptor Blocking Solution (BioLegend®, San Diego, CA, USA) for 10 min at room temperature (5 µL Fc block per 3 x 10⁶ cells). For staining, 270 µL of heparin blocked human whole blood or PBMC (3 x 10⁶ cells resuspended in 270 µL of Maxpar Cell Staining Buffer + Fc Block) was added directly

to the dry-formulated antibody tube for antibody staining. Cells were washed and fixed after staining. For whole blood samples, an additional RBC lysis procedure using Cal-Lyse™ Lysing Solution (Thermo Fisher) was performed immediately after staining. The stained cells were stored overnight at 2–8 °C in Maxpar Fix & Perm Buffer containing 125 nM of Cell-ID™ Intercalator-Ir. The following day, samples were washed with Maxpar Cell Staining Buffer and Maxpar Cell Acquisition Solution. The cells were counted and spun down, and the supernatant was removed. Pelleted samples were left at 2–8 °C until sample acquisition.

Sample Acquisition

Samples were acquired according to the Maxpar Direct Immune Profiling Assay User Guide (PN 400286) using CyTOF Software v6.7.1016. All instruments were equipped with a WB Injector (PN 107950) and samples were acquired in Cell Acquisition Solution (CAS; PN 201239). Following instrument tuning and bead sensitivity test, the system was preconditioned with CAS for 15 min. Cells were resuspended at a concentration of 1×10^6 cells/mL in CAS solution containing 0.1X EQ™ Four Element Calibration Beads (PN 201078). Samples were acquired using the Maxpar Direct Immune Profiling Assay acquisition template. A target of 400,000 events for whole blood and 300,000 events for PBMC was set per file. Cells were acquired at an acquisition rate between 250 and 500 events/second.

Data Processing and Analysis

Generated FCS files were normalized in CyTOF Software v6.7.1016 and analyzed using Maxpar Pathsetter software (Fluidigm Corporation) and in Cytobank (Santa Clara, CA, USA).

Assay Performance Studies

Precision and accuracy of the Maxpar Direct Immune Profiling Assay in whole blood and PBMC samples were assessed: intra-assay repeatability, intermediate precision, accuracy (method comparison), and inter-site reproducibility. For all performance studies, 35 cell populations were evaluated (see Table 2). For technical reasons, total DC (mDC + pDC) and CD66b– neutrophils were not evaluated.

Accuracy-Liquid Panel vs. Dry Panel

Antibodies used in mass cytometry are formulated in liquid buffer with CANDOR antibody stabilizer (CANDOR Bioscience, Wangen, Germany). Cell-ID Intercalator-103Rh is formulated in ultrapure water. In the Maxpar Direct Immune Profiling Assay, antibodies and Cell-ID Intercalator-103Rh are delivered in a dry format. In order to test the accuracy²⁷ of the dry format reagents, staining was performed in parallel using tubes from the Maxpar Direct Immune Profiling Assay (dry format) and a panel containing the same antibodies in liquid form (Figure 2). A single whole blood or PBMC donor sample was stained by three technicians using both panel formats. Staining and cell processing were independently performed by each technician, and all samples were acquired on a single Helios instrument. Normalized FCS files were analyzed in Cytobank.

Definition. The accuracy of the analytical measurements involves the trueness and the precision of the assay.

Manual gating was performed in Cytobank to determine the population frequencies (% of live single cells) for whole blood and PBMC samples stained with liquid and dry format antibodies. The gating technician was blinded to results from the alternative formulation study. Frequencies for 35 cell populations were manually assessed for each panel format. The average frequency (among the three technicians) was calculated for each population and plotted (Figure 3). The coefficient of determination (R^2) for these datapoints was >0.99 for each sample type, demonstrating very good agreement between the two panel formats.

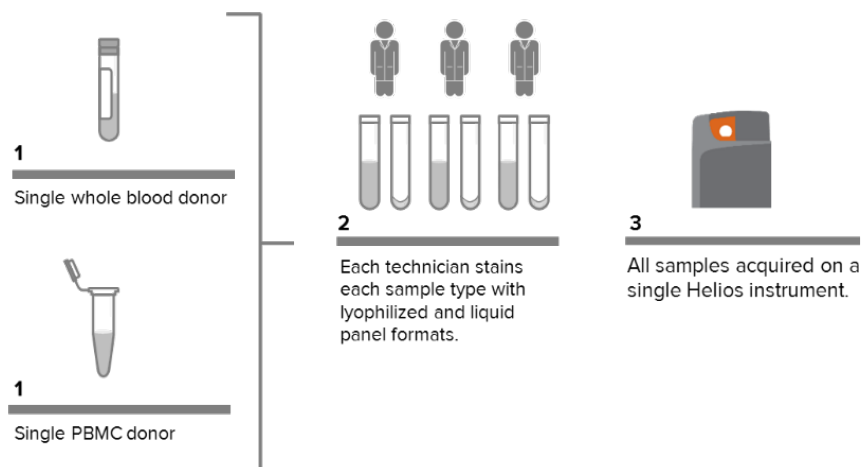


Figure 2. Workflow for assessing accuracy of the liquid vs. the dry antibody panel using the Maxpar Direct Immune Profiling Assay, with samples taken from a single whole blood donor and a single PBMC donor and acquired on a single Helios instrument.

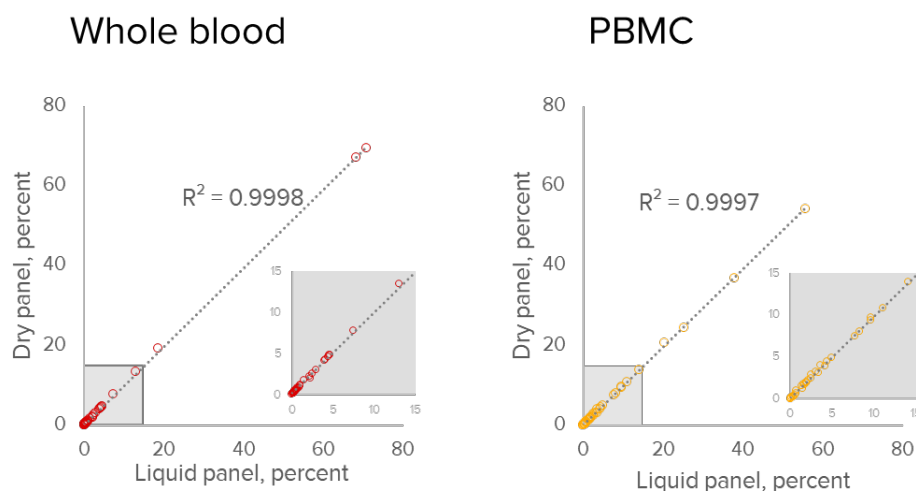


Figure 3. Accuracy of dry antibody format using the Maxpar Direct Immune Profiling Assay from a single whole blood donor and a single PBMC donor. The coefficient of determination (R^2) is shown for each sample type. Gray insert: enlarged figure for populations $\leq 15\%$.

Intra-Assay Repeatability

Repeatability is an important performance metric because it describes the fundamental reliability of the complete system. Here we define the Maxpar Direct Immune Profiling System as including protocols, reagents for cell staining, analytical instrument (Helios), and analysis software (Maxpar Pathsetter). In order to test the intra-assay repeatability²⁷ within the assay, Maxpar Direct Immune Profiling Assay tubes were stained in replicates and acquired on a single Helios instrument (Figure 4). A single whole blood donor sample was stained by a single technician on the same day using eight different Maxpar Direct Immune Profiling Assay tubes. For PBMC samples, three technicians stained the same sample in triplicate on the same day. The frequencies of 35 quantified populations generated by Maxpar Pathsetter were plotted for whole blood and PBMC replicates (Figure 5). The mean, standard deviation, coefficient of variation, and 95% confidence interval of the mean for 35 quantified populations were calculated (Table 2). For all populations with a frequency of $\geq 5\%$, the %CV of the mean for whole blood was $<12\%$ and for PBMC $<9\%$. These results demonstrate that the Maxpar Direct Immune Profiling System has a high degree of intra-assay repeatability.

Definition. Intra-assay repeatability refers to the precision of the assay within the run, typically in the same day, with the same technician and the same instrument.

Whole blood

Percent

Gran Neut Lymph CD3+ T cells CD4+ T cells CD8+ T cells

Percent

CD4 CM CD4 EM CD4 TE CD8 EM CD8 TE y8 T cells Th1-like B cells B naive Monocytes Class mono Eos NK cells Early NK Late NK

Percent

CD4 naive CD8 naive CD8 CM Treg MALT/NKT Th2-like Th17-like B mem Plasmablasts Trans mono NC mono Baso mDC pDC

PBMC

Percent

Lymph CD3+ T cells CD4+ T cells CD4 naive CD4 CM CD8 T cells B cells B naive Monocytes Class mono NK cells Late NK

Percent

Early NK CD8 naive CD8 EM CD4 TE Th1-like Th2-like Th17-like MALT/NKT B mem Gran Trans mono NC mono

Percent

Treg CD8 CM mDC pDC y8 T cells Plasmablasts Neut Baso Eos

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Table 2. Intra-assay repeatability cell frequencies obtained using Maxpar Pathsetter from whole blood and PBMC samples

Population	Whole Blood				PBMC			
	Mean	SD ^a	%CV ^b	95% CI ^c of Mean	Mean	SD ^a	%CV ^b	95% CI ^c of Mean
Lymphocytes	34.2	1.4	4.2	33.8–34.5	66.6	2.7	4.1	64.8–68.4
CD3 T cells	24.7	0.8	3.2	24.6–24.9	40.3	2.2	5.5	38.8–41.7
CD8 T cells	10.6	0.5	4.8	10.5–10.7	10.1	0.4	3.9	9.9–10.4
CD8 naïve	0.4	0.0	11.1	0.3–0.4	0.7	0.1	11.2	0.7–0.8
CD8 central memory (CM)	0.2	0.0	14.4	0.2–0.2	0.9	0.1	6.5	0.9–1.0
CD8 effector memory (EM)	2.0	0.2	9.9	1.9–2	2.7	0.2	8.7	2.6–2.9
CD8 terminal effector (TE)	8.1	0.3	3.9	8–8.2	2.8	0.1	3.5	2.8–2.9
CD4 T cells	11.9	0.4	3.7	11.8–12	27.0	1.5	5.7	26.0–28.0
CD4 naïve	0.9	0.1	14.6	0.8–0.9	8.5	0.5	6.2	8.2–8.9
CD4 central memory (CM)	3.1	0.3	9.4	3–3.1	8.3	0.6	7.7	7.9–8.8
CD4 effector memory (EM)	2.3	0.2	10.5	2.2–2.3	3.8	0.1	2.8	3.8–3.9
CD4 terminal effector (TE)	5.7	0.1	2.1	5.7–5.7	6.5	0.5	7.5	6.2–6.8
Treg	0.2	0.0	14.2	0.2–0.2	1.2	0.1	8.8	1.1–1.3
Th1-like	3.6	0.3	7.7	3.5–3.6	3.7	0.2	6.1	3.6–3.9
Th2-like	0.7	0.1	11.6	0.7–0.7	1.5	0.2	12.6	1.4–1.7
Th17-like	0.6	0.1	8.7	0.6–0.6	4.2	0.2	3.8	4.1–4.3
γδ T cells	1.5	0.1	4.2	1.5–1.6	0.7	0.0	5.4	0.7–0.7
MAIT/NKT	0.7	0.2	33.5	0.6–0.7	2.3	0.2	6.5	2.3–2.5
B cells (total)	6.0	0.7	11.6	5.8–6.2	10.8	0.3	2.7	10.6–11.0
B cells (naïve)	5.8	0.7	11.5	5.6–6	9.1	0.2	2.5	8.9–9.2
B cells (memory)	0.2	0.0	18.0	0.2–0.2	2.4	0.6	23.8	2.1–2.8
Plasmablasts	0.02	0.0	0.0	0.02–0.02	0.2	0.0	7.3	0.2–0.2
NK cells (total)	3.4	0.2	6.1	3.4–3.5	15.5	0.8	5.4	15.0–16.1
NK cells (early)	2.4	0.2	6.8	2.3–2.4	5.9	0.5	8.6	5.6–6.2
NK cells (late)	1.1	0.1	4.8	1–1.1	9.6	0.3	3.6	9.4–9.9
Monocytes (total)	3.3	0.6	17.6	3.2–3.5	20.7	1.1	5.4	20.0–21.5
Monocytes (classical)	2.7	0.5	18.0	2.5–2.8	17.1	1.1	6.4	16.4–17.8
Monocytes (nonclassical)	0.4	0.1	13.9	0.4–0.4	2.4	0.1	5.0	2.3–2.5
Monocytes (transitional)	0.2	0.1	23.9	0.2–0.3	2.8	0.5	16.7	2.5–3.1
pDC	0.1	0.0	13.9	0.1–0.1	0.7	0.0	4.3	0.7–0.7
mDC	0.3	0.0	11.0	0.3–0.3	0.9	0.2	22.9	0.8–1.1
Granulocytes	56.0	1.1	2.0	55.7–56.3	1.6	0.1	5.9	1.5–1.6
Neutrophils	53.7	1.0	1.9	53.4–53.9	0.8	0.3	38.5	0.6–1.0
Basophils	0.3	0.0	8.3	0.3–0.3	0.7	0.1	10.5	0.6–0.7
Eosinophils	1.5	0.1	4.6	1.5–1.5	0.1	0.0	26.6	0.1–0.1

- a. Standard deviation
- b. Coefficient of variation
- c. Confidence interval

Intermediate Precision

Routine use of an immune profiling assay in high-capacity labs requires multiple technicians and multiple instruments. The intermediate precision studies presented here describe the performance of the Maxpar Direct Immune Profiling System in these settings. The variability between different instruments and handling technicians was assessed to test intermediate precision²⁷. Three technicians stained and processed the same whole blood or PBMC donor sample using three Maxpar Direct Immune Profiling Assay tubes, and all nine sample tubes were acquired on two to three different Helios instruments (Figure 6) on the same day. All FCS files were analyzed using Maxpar Pathsetter. The frequencies of 35 quantified populations generated by Maxpar Pathsetter were plotted for whole blood and PBMC replicates (Figure 7). The mean, SD, %CV, and 95% CI of the mean for 35 quantified populations were calculated (Table 3). For all populations $\geq 5\%$ in frequency, the %CV of the mean was $<4\%$ for whole blood and $<10\%$ for PBMC. These results demonstrate that the Maxpar Direct Immune Profiling Assay shows a high degree of intermediate precision.

Definition. Intermediate precision assesses variabilities resulting from different technicians, instruments, or day-to-day operations within a single laboratory.

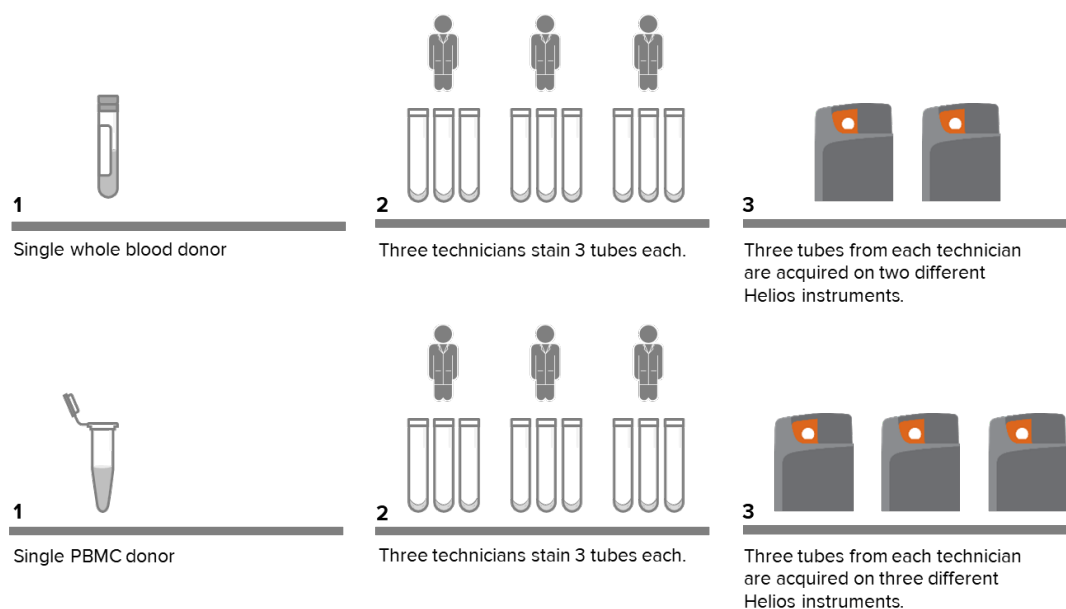


Figure 6. Workflow for assessing intermediate precision of the Maxpar Direct Immune Profiling Assay by testing results obtained by different technicians and instruments.

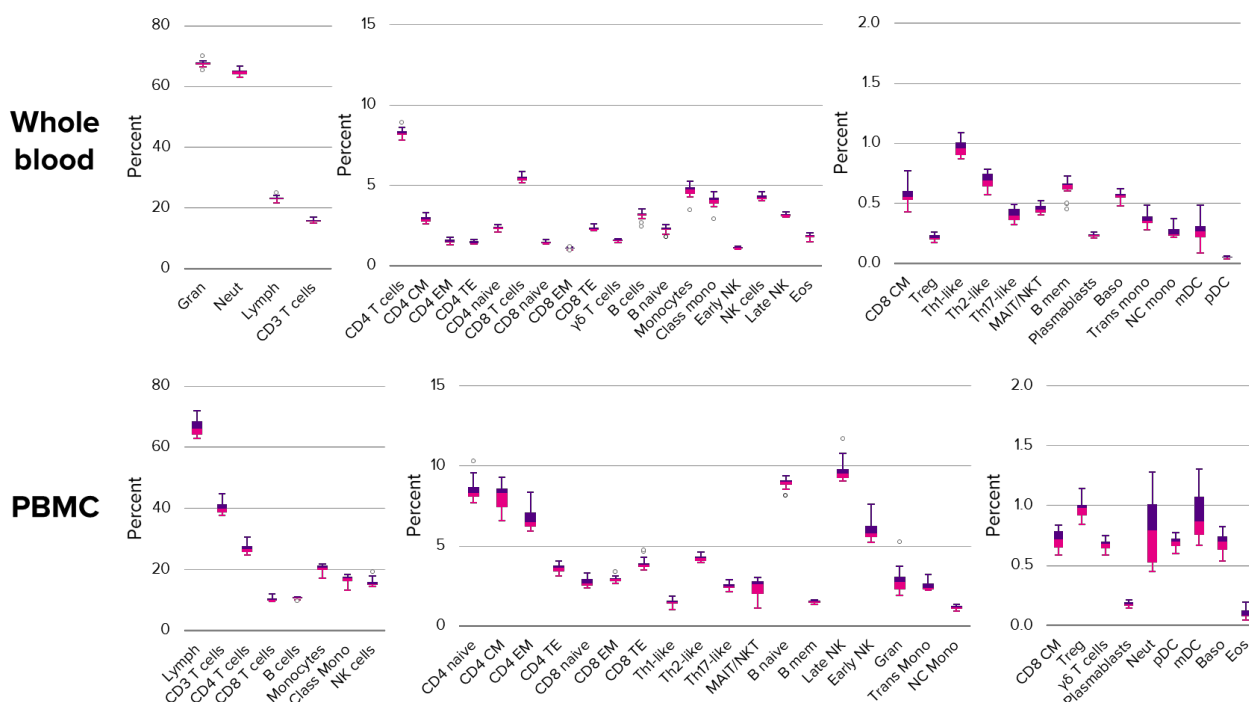


Figure 7. Replicate measurements of the same whole blood or PBMC samples were assessed for intermediate precision by measuring variability from different technicians and instruments for the Maxpar Direct Immune Profiling System at a single laboratory. Left panel: populations with average cell frequencies of > 10%. Middle panel: populations with average cell frequencies of 1–10%. Right panel: populations with average cell frequencies of < 1%. For the box and whisker plots: box, first quartile (pink) to third quartile (purple); color change, median; error bars, minimum/maximum values; open circles, outliers. The Y-axis is the measured % of total single live cells. The data shown is a representative of three independent experiments. The whole blood and PBMC samples in this experiment were obtained from different donors.

Table 3. Intermediate-precision results obtained using Maxpar Pathsetter measuring cell frequencies from whole blood and PBMC samples

Population	Whole Blood				PBMC			
	Mean	SD ^a	%CV ^b	95% CI ^c of Mean	Mean	SD ^a	%CV ^b	95% CI ^c of Mean
Lymphocytes	23.2	0.7	3.1	22.8–23.5	66.5	2.6	4.0	65.5–67.5
CD3 T cells	15.7	0.5	2.9	15.5–16	40.2	2.0	5.1	39.4–41
CD8 T cells	5.4	0.2	3.2	5.4–5.5	10.2	0.6	5.7	10–10.5
CD8 naïve	1.5	0.1	4.4	1.4–1.5	2.7	0.2	8.9	2.6–2.8
CD8 central memory (CM)	0.6	0.1	14.3	0.5–0.6	0.7	0.1	10.7	0.7–0.7
CD8 effector memory (EM)	1.1	0.1	7.4	1–1.1	2.9	0.2	5.8	2.9–3
CD8 terminal effector (TE)	2.3	0.1	5.0	2.3–2.4	3.9	0.3	7.6	3.7–4
CD4 T cells	8.3	0.2	2.9	8.2–8.4	26.9	1.5	5.6	26.3–27.4
CD4 naïve	2.4	0.1	5.6	2.3–2.4	8.5	0.6	7.2	8.3–8.7
CD4 central memory (CM)	2.9	0.2	6.7	2.8–3	8.1	0.7	9.2	7.8–8.3
CD4 effector memory (EM)	1.5	0.1	8.7	1.5–1.6	6.7	0.6	9.5	6.5–6.9
CD4 terminal effector (TE)	1.5	0.1	5.3	1.4–1.5	3.6	0.2	6.5	3.5–3.7
Treg	0.2	0.0	11.8	0.2–0.2	1.0	0.1	7.1	0.9–1
Th1-like	1.0	0.1	7.3	0.9–1	1.5	0.2	13.0	1.4–1.6
Th2-like	0.7	0.1	10.0	0.7–0.7	4.3	0.2	4.1	4.2–4.3
Th17-like	0.4	0.1	14.3	0.4–0.4	2.5	0.2	7.3	2.4–2.6
γδ T cells	1.6	0.1	3.5	1.6–1.6	0.7	0.0	6.1	0.7–0.7
MAIT/NKT	0.5	0.0	7.2	0.4–0.5	2.4	0.6	23.1	2.2–2.6
B cells (total)	3.1	0.2	7.7	3–3.3	10.7	0.3	3.0	10.6–10.8
B cells (naïve)	2.3	0.2	7.7	2.2–2.4	9.0	0.3	2.9	8.9–9.1
B cells (memory)	0.6	0.1	11.6	0.6–0.7	1.5	0.1	6.1	1.5–1.6
Plasmablasts	0.2	0.0	7.1	0.2–0.2	0.2	0.0	11.1	0.2–0.2
NK cells (total)	4.3	0.2	3.5	4.2–4.3	15.6	1.1	7.3	15.2–16.1
NK cells (early)	1.1	0.1	4.5	1.1–1.1	6.0	0.6	9.9	5.8–6.2
NK cells (late)	3.2	0.1	3.5	3.1–3.2	9.6	0.6	6.0	9.4–9.9
Monocytes (total)	4.7	0.4	8.5	4.5–4.8	20.4	1.2	5.7	19.9–20.8
Monocytes (classical)	4.0	0.4	9.1	3.9–4.2	16.7	1.2	6.9	16.3–17.2
Monocytes (nonclassical)	0.4	0.1	14.4	0.3–0.4	2.5	0.3	10.3	2.4–2.6
Monocytes (transitional)	0.3	0.0	16.8	0.2–0.3	1.2	0.1	10.7	1.1–1.2
pDC	0.0	0.0	14.6	0–0.1	0.7	0.0	6.2	0.7–0.7
mDC	0.3	0.1	40.1	0.2–0.3	0.9	0.2	21.7	0.8–1
Granulocytes	67.5	0.9	1.4	67.1–68	2.8	0.7	24.9	2.6–3.1
Neutrophils	64.6	0.9	1.4	64.2–65	0.8	0.3	34.6	0.7–0.9
Basophils	0.6	0.0	6.1	0.5–0.6	0.7	0.1	10.5	0.7–0.7
Eosinophils	1.8	0.1	6.8	1.8–1.9	0.1	0.0	39.1	0.1–0.1

- a. Standard deviation
b. Coefficient of variation
c. Confidence interval

Inter-Site Reproducibility

Multi-center studies and collaborative studies require use of multiple instruments and technicians. Longitudinal studies may also involve multiple technicians over time. Large-scale immune monitoring studies are increasingly being conducted in such settings. The inter-site reproducibility study presented here captures all sources of variability found in multi-site studies that use the Maxpar Direct Immune Profiling System. Inter-site reproducibility²⁷ of the Maxpar Direct Immune Profiling System was tested on whole blood and PBMC. Reproducibility was tested using external test sites, where each site had different instruments and technicians (Figure 8). A total of six test sites were provided with whole blood specimens from a single donor. Experiments were conducted from three sites during a two-day period (Week 1) and from the three other sites during a second two-day period (Week 2). Fresh whole blood was collected from the same donor for staining and processing. For PBMC sample staining and processing, a total of five test sites stained PMBC specimens from the same donor and provided frozen. Each site had a single technician stain four replicates of each of the whole blood and PBMC specimens, and samples were acquired on each site's Helios instrument. All FCS files were analyzed using Maxpar Pathsetter. The frequencies of 35 quantified populations generated by Maxpar Pathsetter were plotted for whole blood (Figure 9) and PBMC replicates (Figure 10). The mean, SD, %CV, and 95% confidence interval of the mean for 35 quantified populations were calculated for each week of blood collection (Table 4) and for PBMC (Table 5). For all populations $\geq 5\%$ in frequency, the %CV of the mean was $<10\%$ for whole blood and $<20\%$ for PBMC. These results demonstrate that the Maxpar Direct Immune Profiling System shows a high degree of inter-site reproducibility.

Definition. Inter-site reproducibility refers to the laboratory-to-laboratory variability and instrument-to-instrument variability. Inter-site reproducibility demonstrates precision of the analytical method.

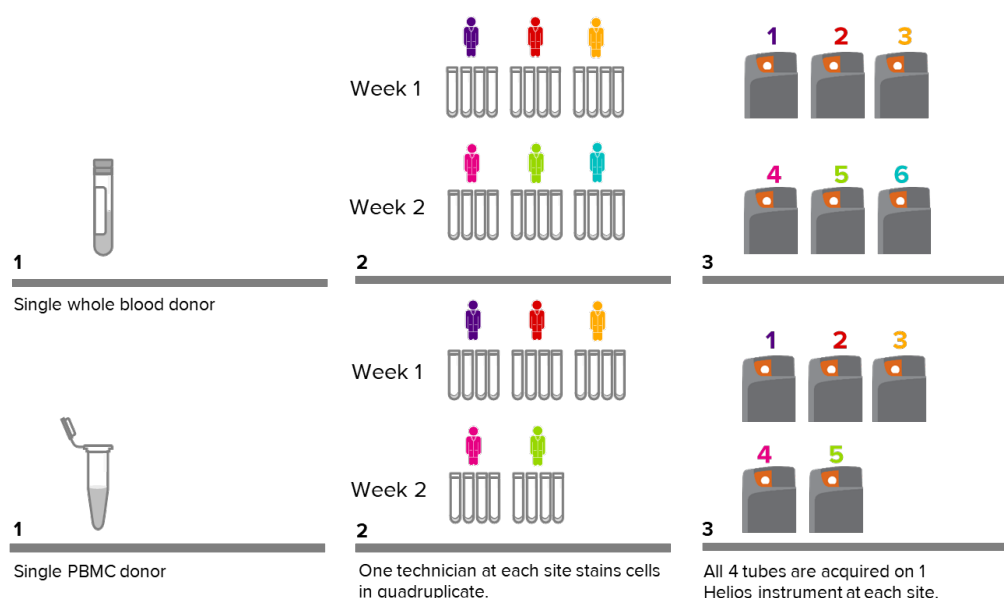


Figure 8. Workflow for assessing inter-site reproducibility of the Maxpar Direct Immune Profiling System for five external sites with a single PBMC donor or six external sites with a single whole blood donor in four¹ replicate samples acquired on the site's Helios system.

¹ One whole blood tube from one site during Week 1 was excluded from analysis due to technical reasons.

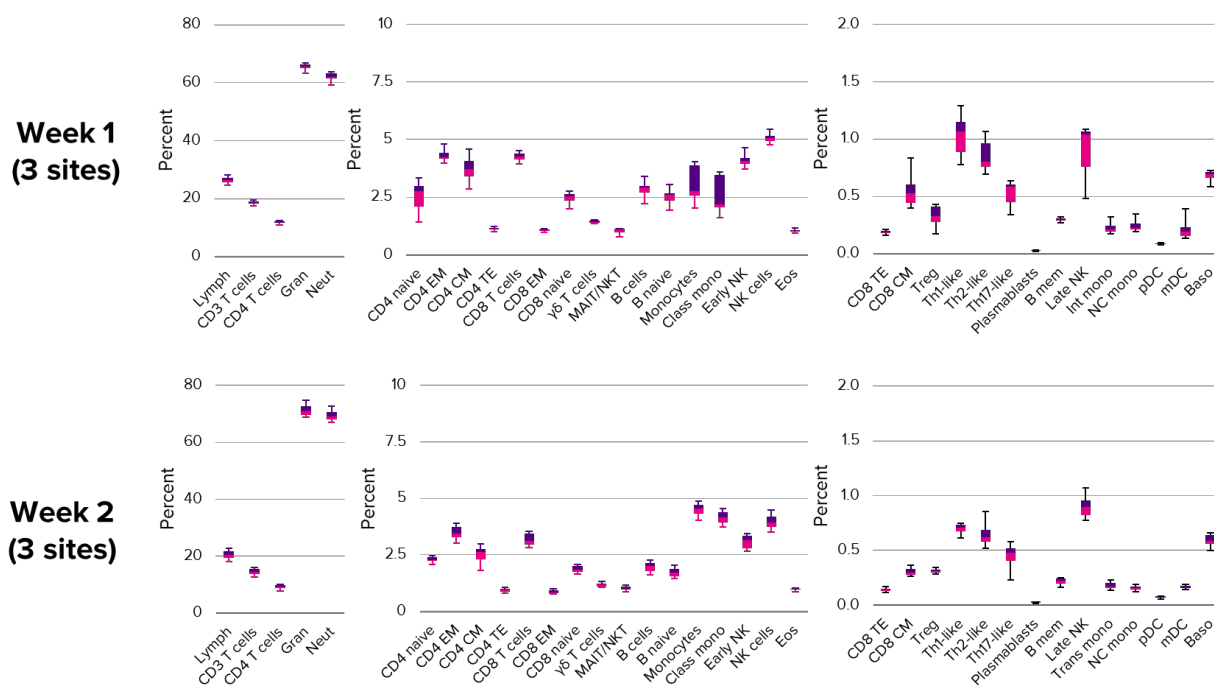


Figure 9. Replicate measurements of the same whole blood donor assessed for inter-site reproducibility. Variability from different technicians and instruments for the Maxpar Direct Immune Profiling System was measured at multiple sites. At two time points one week apart, blood from the same donor was drawn and analyzed at three different external sites. Left panel: populations with average cell frequencies of >10%. Middle panel: populations with average cell frequencies of 1–10%. Right panel: populations with average cell frequencies of <1%. For the box and whisker plots: box, first quartile (pink) to third quartile (purple); color change, median; error bars, minimum/maximum values. The Y-axis is the measured % of total single live cells.

Table 4. Inter-site reproducibility results obtained using Maxpar Pathsetter measuring cell frequencies from a single whole blood donor at two different time points

Population	Whole Blood—Week 1				Whole Blood—Week 2			
	Mean	SD ^a	%CV ^b	95% CI ^c of Mean	Mean	SD ^a	%CV ^b	95% CI ^c of Mean
Lymphocytes	26.3	1.1	4.1	25.7–27	20.6	1.6	7.8	19.6–21.5
CD3 T cells	18.5	0.6	3.4	18.2–18.9	14.6	1.2	8.0	13.9–15.3
CD8 T cells	4.3	0.2	3.9	4.2–4.4	3.2	0.3	8.5	3–3.3
CD8 naive	2.5	0.2	9.7	2.3–2.6	1.9	0.2	8.2	1.8–2
CD8 central memory (CM)	0.5	0.1	24.0	0.5–0.6	0.3	0.0	11.3	0.3–0.3
CD8 effector memory (EM)	1.1	0.0	4.6	1–1.1	0.9	0.1	9.5	0.8–0.9
CD8 terminal effector (TE)	0.2	0.0	7.3	0.2–0.2	0.1	0.0	10.5	0.1–0.1
CD4 T cells	11.8	0.5	4.0	11.5–12.1	9.2	0.8	8.4	8.8–9.7
CD4 naive	2.6	0.6	24.2	2.2–2.9	2.3	0.1	5.0	2.2–2.4
CD4 central memory (CM)	3.7	0.5	13.4	3.5–4	2.5	0.4	15.2	2.3–2.7
CD4 effector memory (EM)	4.4	0.4	8.1	4.2–4.6	3.5	0.3	8.4	3.3–3.6
CD4 terminal effector (TE)	1.1	0.1	5.6	1.1–1.2	0.9	0.1	9.2	0.9–1
Treg	0.3	0.1	28.4	0.3–0.4	0.3	0.0	6.5	0.3–0.3
Th1-like	1.0	0.2	17.6	0.9–1.1	0.7	0.2	20.6	0.7–0.8

Population	Whole Blood—Week 1				Whole Blood—Week 2			
	Mean	SD ^a	%CV ^b	95% CI ^c of Mean	Mean	SD ^a	%CV ^b	95% CI ^c of Mean
Th2-like	0.9	0.1	15.1	0.8–0.9	0.6	0.1	15.0	0.6–0.7
Th17-like	0.5	0.1	20.4	0.5–0.6	0.5	0.1	22.5	0.4–0.5
γδ T cells	1.4	0.1	3.5	1.4–1.5	1.2	0.1	7.3	1.1–1.2
MAIT/NKT	1.0	0.1	11.7	1–1.1	1.0	0.1	9.4	1–1.1
B cells (total)	2.8	0.5	17.4	2.5–3.1	2.0	0.2	11.8	1.8–2.1
B cells (naïve)	2.4	0.5	18.8	2.2–2.7	1.7	0.2	12.2	1.6–1.9
B cells (memory)	0.3	0.0	9.2	0.3–0.3	0.2	0.0	13.6	0.2–0.2
Plasmablasts	0.03	0.0	17.5	0.03–0.03	0.03	0.0	14.0	0.03–0.03
NK cells (total)	5.0	0.3	5.0	4.9–5.2	4.0	0.3	7.7	3.8–4.2
NK cells (early)	4.1	0.3	6.9	4–4.3	3.1	0.3	9.6	2.9–3.3
NK cells (late)	0.9	0.2	23.4	0.8–1	0.9	0.1	11.1	0.8–1
Monocytes (total)	3.0	0.7	23.3	2.6–3.4	4.4	0.6	14.4	4–4.7
Monocytes (classical)	2.5	0.7	29.0	2.1–3	4.0	0.6	15.0	3.7–4.4
Monocytes (nonclassical)	0.2	0.1	32.4	0.2–0.3	0.2	0.0	19.1	0.2–0.2
Monocytes (transitional)	0.2	0.1	21.0	0.2–0.3	0.2	0.0	12.1	0.1–0.2
pDC	0.1	0.0	9.0	0.1–0.1	0.1	0.0	11.1	0.1–0.1
mDC	0.2	0.1	35.9	0.2–0.3	0.2	0.0	10.1	0.2–0.2
Granulocytes	65.5	1.1	1.7	64.8–66.1	71.3	2.1	3.0	70–72.5
Neutrophils	62.1	1.4	2.2	61.3–63	69.5	2.0	2.8	68.3–70.6
Basophils	0.7	0.1	7.5	0.6–0.7	0.6	0.1	8.5	0.6–0.6
Eosinophils	1.0	0.1	5.9	1–1.1	1.0	0.0	4.2	0.9–1

- a. Standard deviation
b. Coefficient of variation
c. Confidence interval

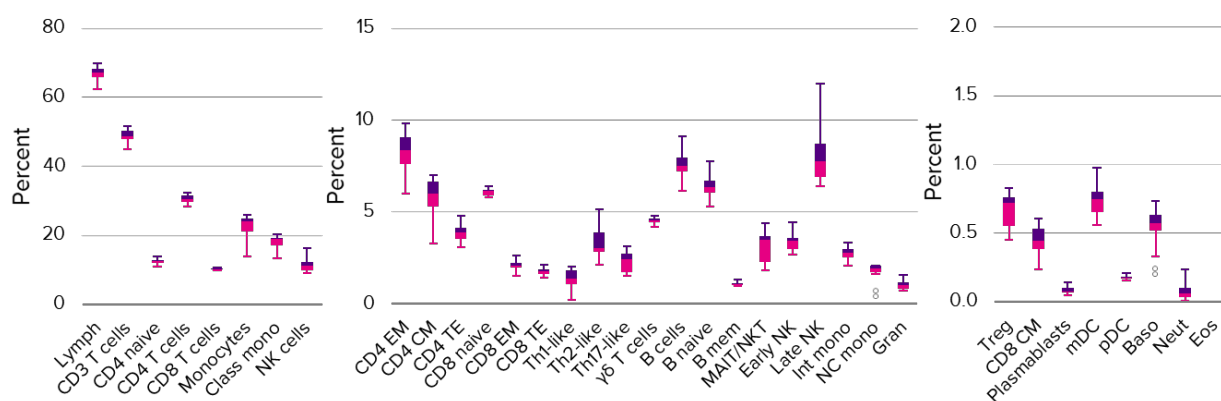


Figure 10. Replicate measurements from the same PBMC donor testing inter-site reproducibility by assessing variability from different technicians and instruments for the Maxpar Direct Immune Profiling Assay at five different sites. Left panel: populations with average cell frequencies of > 10%. Middle panel: populations with average cell frequencies of 1–10%. Right panel: populations with average cell frequencies of <1%. For the box and whisker plots: box, first quartile (pink) to third quartile (purple); color change, median; error bars, minimum/maximum values; open circles, outliers. The Y-axis is the measured % of total single live cells.

Table 5. Inter-site reproducibility results obtained using Maxpar Pathsetter measuring cell frequencies from a single PBMC donor

Population	PBMC			
	Mean	SD ^a	%CV ^b	95% CI ^c of Mean
Lymphocytes	67.5	2.9	4.3	66.2–68.8
CD3 T cells	48.4	2.6	5.4	47.3–49.6
CD8 T cells	10.4	0.4	3.7	10.2–10.5
CD8 naïve	6.1	0.3	4.1	6–6.2
CD8 central memory (CM)	0.4	0.1	23.0	0.4–0.5
CD8 effector memory (EM)	2.1	0.3	13.6	2–2.2
CD8 terminal effector (TE)	1.8	0.2	10.6	1.7–1.8
CD4 T cells	30.3	1.9	6.3	29.5–31.1
CD4 naïve	12.3	0.9	7.3	12–12.7
CD4 central memory (CM)	5.8	1.0	16.8	5.4–6.2
CD4 effector memory (EM)	8.2	1.2	14.0	7.7–8.7
CD4 terminal effector (TE)	3.9	0.4	11.4	3.7–4.1
Treg	0.7	0.1	19.4	0.6–0.7
Th1-like	1.3	0.6	47.0	1–1.5
Th2-like	3.3	1.0	28.7	2.9–3.7
Th17-like	2.3	0.6	24.4	2–2.5
γδ T cells	4.6	0.4	8.6	4.4–4.8
MAIT/NKT	3.2	0.8	26.9	2.8–3.5
B cells (total)	7.6	0.7	9.8	7.2–7.9
B cells (naïve)	6.4	0.7	10.4	6.1–6.7
B cells (memory)	1.1	0.1	12.8	1–1.1
Plasmablasts	0.1	0.0	28.3	0.1–0.1
NK cells (total)	11.5	2.1	18.0	10.6–12.5
NK cells (early)	3.4	0.5	14.9	3.2–3.6
NK cells (late)	8.2	1.6	19.9	7.5–8.9
Monocytes (total)	22.5	3.6	15.9	20.9–24
Monocytes (classical)	17.7	2.8	16.0	16.4–18.9
Monocytes (nonclassical)	2.8	0.3	12.1	2.6–2.9
Monocytes (transitional)	2.0	0.8	38.1	1.7–2.4
pDC	0.2	0.0	25.4	0.2–0.2
mDC	0.7	0.2	29.8	0.6–0.8
Granulocytes	1.1	0.4	36.6	0.9–1.3
Neutrophils	0.1	0.1	91.0	0.1–0.1
Basophils	0.5	0.1	27.0	0.5–0.6
Eosinophils	0.002	0.0	118.9	0.001–0.004

- a. Standard deviation
- b. Coefficient of variation
- c. Confidence interval

Conclusion

The analytical validation studies presented here demonstrate that the Maxpar Direct Immune Profiling System, which includes protocols, reagents for cell staining, use of a Helios mass cytometer, and Maxpar Pathsetter reporting and analysis software, provide repeatable and precise quantitation of a broad range of immune cell populations using whole blood and PBMC. In addition, we demonstrate the accuracy in performance of the dry panel compared to the equivalent liquid panel.

Combining Maxpar Pathsetter software with the Maxpar Direct Immune Profiling Assay reduces variability in sample preparation and subjectivity in data analysis, allowing researchers to have a streamlined solution for broad immune profiling using mass cytometry in individual labs as well as multi-center and collaborative studies.

References

- 1 Maecker, H.T., McCoy, J.P., Nussenblatt, R. Standardizing immunophenotyping for the Human Immunology Project. *Nature Reviews Immunology* (2012): 191–200.
- 2 Streitz, M. et al. Standardization of whole blood immune phenotype monitoring for clinical trials: panels and methods from the ONE study. *Transplantation Research* (2013): 17.
- 3 Olin, A. et al. Stereotypic immune system development in newborn children. *Cell* (2018): 1,277–92.
- 4 McCoy, J.P., Overton, W.R. Quality control in flow cytometry for diagnostic pathology: II. a conspectus of reference ranges for lymphocyte immunophenotyping. *Cytometry* (1994): 129–39.
- 5 Chew, V. et al. Immune activation underlies a sustained clinical response to yttrium-90 radioembolisation in hepatocellular carcinoma. *Gut* (2019): 335–46.
- 6 Roy Chowdhury, R. et al. A multi-cohort study of the immune factors associated with M. tuberculosis infection outcomes. *Nature* (2018): 644–8.
- 7 Coindre, S. et al. Mass cytometry analysis reveals the landscape and dynamics of CD32a+ CD4+ T cells from early HIV infection to effective cART. *Frontiers in Immunology* (2018): 1,217.
- 8 Krieg, C. et al. High-dimensional single-cell analysis predicts response to anti-PD-1 immunotherapy. *Nature Medicine* (2018): 144–53.
- 9 Subrahmanyam, P.B. et al. Distinct predictive biomarker candidates for response to anti-CTLA-4 and anti-PD-1 immunotherapy in melanoma patients. *Journal for ImmunoTherapy of Cancer* (2018): 18.

- 10 Lingblom, C.M.D., Kowli, S., Swaminathan, N., Maecker, H.T., Lambert, S.L. Baseline immune profile by CyTOF® can predict response to an investigational adjuvanted vaccine in elderly adults. *Journal of Translational Medicine* (2018): 153.
- 11 Smets, T., Stevenaert, F., Adams, H., Vanhoof, G. Deep profiling of the immune system of multiple myeloma patients using cytometry by time-of-flight (CyTOF). *In Methods in Molecular Biology* (2018): 47–54.
- 12 Cossarizza, A. et al. Guidelines for the use of flow cytometry and cell sorting in immunological studies. *European Journal of Immunology* (2017): 1,584–797.
- 13 Biancotto, A., Fuchs, J. C., Williams, A., Dagur, P.K., McCoy, J.P. High dimensional flow cytometry for comprehensive leukocyte immunophenotyping (CLIP) in translational research. *Journal Immunological Methods* (2011): 245–61.
- 14 Finak, G. et al. Standardizing flow cytometry immunophenotyping analysis from the Human ImmunoPhenotyping Consortium. *Scientific Reports* (2016): 20,686.
- 15 Kverneland, A.H. et al. Age and gender leucocytes variances and references values generated using the standardized ONE-Study protocol. *Cytometry Part A* (2016): doi:10.1002/cyto.a.22855.
- 16 Jamin, C. et al. Multi-center harmonization of flow cytometers in the context of the European “PRECISESADS” project. *Autoimmunity Reviews* (2016): 1038–45.
- 17 Simoni, Y. et al. Bystander CD8 + T cells are abundant and phenotypically distinct in human tumour infiltrates. *Nature* (2018): 575–9.
- 18 Bagwell, C.B. et al. Probability state modeling theory. *Cytometry Part A* (2015): doi:10.1002/cyto.a.22687.
- 19 Bruce Bagwell, C. High-dimensional modeling for cytometry: Building rock solid models using GemStone™ and Verity Cen-se’™ high-definition t-SNE mapping. *Methods in Molecular Biology* (2018): 11–36.
- 20 Borst, J. Distinct molecular forms of human T cell receptor gamma/delta detected on viable T cells by a monoclonal antibody. *Journal of Experimental Medicine* (2004): 1,625–44.
- 21 Dias, J., Leeansyah, E., Sandberg, J.K. Multiple layers of heterogeneity and subset diversity in human MAIT cell responses to distinct microorganisms and to innate cytokines. *Proceedings of the National Academy of Sciences of the United States of America* (2017): 5,434–43.
- 22 Björkström, N.K. et al. Expression patterns of NKG2A, KIR, and CD57 define a process of CD56 dim NK-cell differentiation uncoupled from NK-cell education. *Blood* (2010): 3,853–64.
- 23 Yoon, J., Terada, A., Kita, H. CD66b regulates adhesion and activation of human eosinophils. *The Journal of Immunology* (2007): 8,454–62.
- 24 Schaerli, P. et al. CXC chemokine receptor 5 expression defines follicular homing T cells with B cell helper function. *Journal of Experimental Medicine* (2000): 1,553–62.

- 25 Hirai, H. et al. Prostaglandin D2 selectively induces chemotaxis in T helper type 2 cells, eosinophils, and basophils via seven-transmembrane receptor CRTH2. *Journal of Experimental Medicine* (2001): 255-61.
- 26 Rahman, A.H., Tordesillas, L., Berin, M.C. Heparin reduces nonspecific eosinophil staining artifacts in mass cytometry experiments. *Cytometry Part A* (2016): 601–7.
- 27 *Evaluation of Precision of Quantitative Measurement Procedures; Approved Guideline—Third Edition*. CLSI (2014).

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