FLUIDIGM® | Application Note

Impact of Cryopreservation on Performance of the Maxpar Direct Immune Profiling System

Introduction

The Maxpar® Direct™ Immune Profiling System has set a new standard for comprehensive immunophenotyping of human PBMC and whole blood by enabling simultaneous identification and enumeration of 37 immune subsets (Figure 1). Combining a 30-marker antibody panel in a single tube, with acquisition on the Helios™ mass cytometer and analysis by Maxpar Pathsetter™ software, the Maxpar Direct Immune Profiling System provides a convenient and reliable product. The Maxpar Pathsetter data analysis software is fully automated and generates a detailed phenotypic report of results within 5 minutes per sample. A 6-site study demonstrated a high degree of reproducibility, with all population frequencies >5% having a coefficient of variation (%CV) of less than 10% in whole blood[1]. A detailed analysis is described in the white paper Deep Immune Profiling with the Maxpar Direct Immune Profiling System (FLDM-400247).

While fluorophores can be sensitive to fixation, freezing and long-term storage, samples stained with a metal-tagged antibody cocktail that had been frozen for up to 4 weeks showed comparable results compared to samples stained with the freshly made cocktail[2]. Researchers have also shown that data from samples stained for mass cytometry analysis and stored at either 4 °C or –80 °C does not show significant differences when assessing signal intensity of metal-labeled antibodies targeting surface proteins, iridium and palladium barcodes[3].

This application note describes a proof-of-concept study where human whole blood samples stained with the Maxpar® Direct™ Immune Profiling Assay™ (Cat. No. 201325) were cryopreserved for up to 120 days then thawed, data was acquired on a Helios mass cytometer and analyzed with Maxpar Pathsetter software (Figure 2). This intra-assay study reports the same degree of reproducibility, with all population frequencies >5% having a coefficient of variation (CV) of less than 10% after the cryopreservation process.

Summary

- Whole blood samples stained with the Maxpar Direct Immune Profiling Assay are stable when frozen at –80 °C for short-term or long-term storage.
- Freezing of samples processed over time allows samples to be acquired in a single batch if desired.
- Stability of frozen stained samples enables shipment of samples on dry ice to external sites for acquisition when necessary.

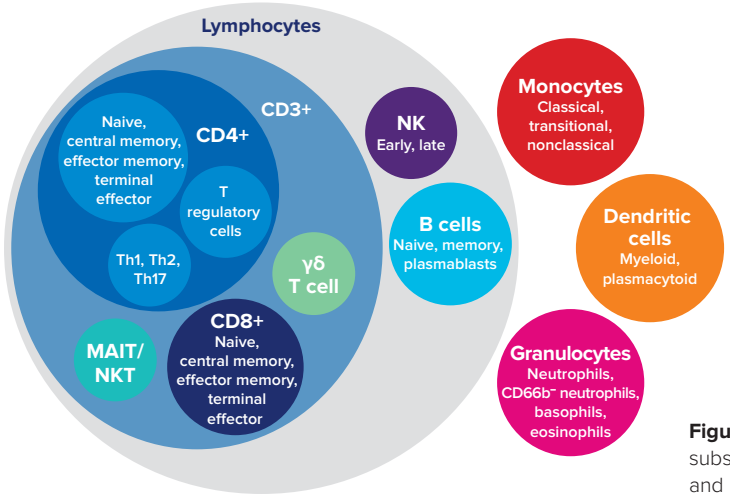


Figure 1. A comprehensive immune profile. The 37 immune cell subsets identified using the Maxpar Direct Immune Profiling Assay and Maxpar Pathsetter software.

Study Design

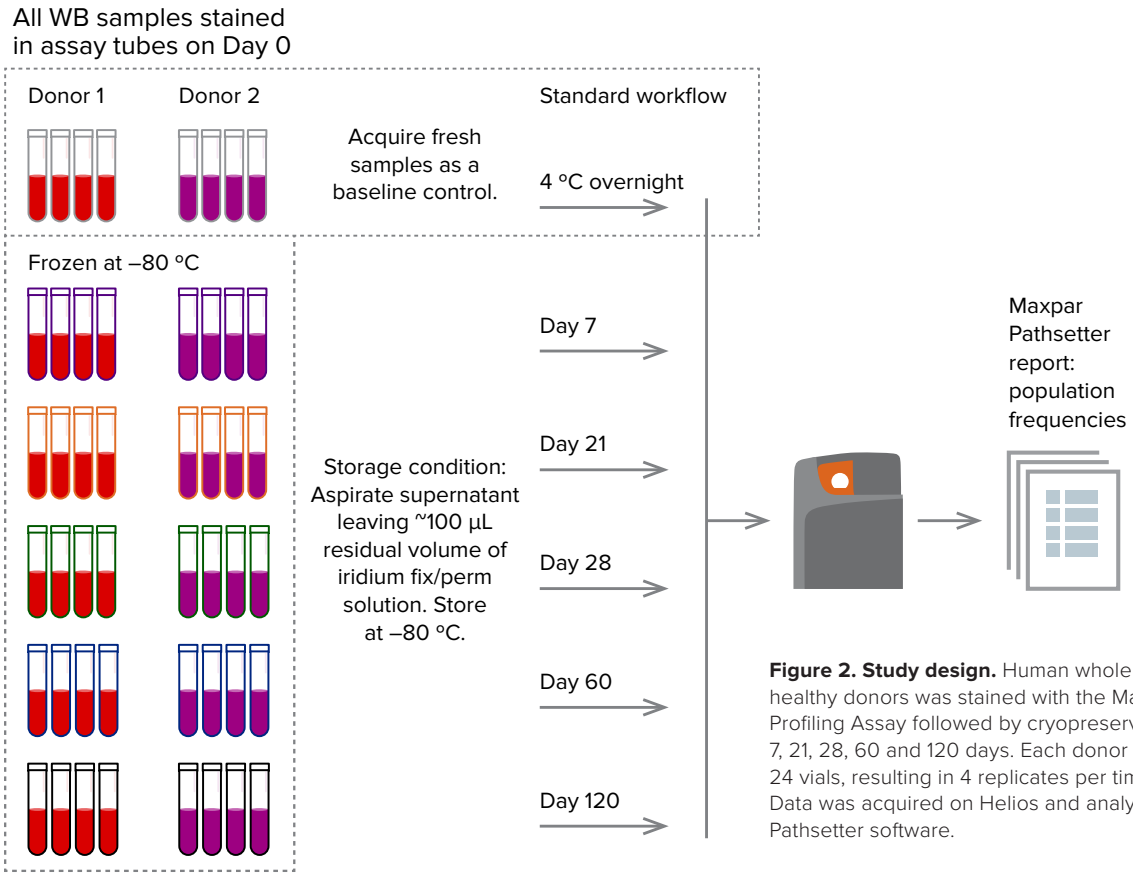


Figure 2. Study design. Human whole blood (WB) from 2 healthy donors was stained with the Maxpar Direct Immune Profiling Assay followed by cryopreservation at –80 °C for 7, 21, 28, 60 and 120 days. Each donor sample was split into 24 vials, resulting in 4 replicates per time point per donor. Data was acquired on Helios and analyzed with Maxpar Pathsetter software.

Cryopreservation Workflow

1

STOP
at Step 3 of the Stain Cells with Cell-ID™ Intercalator-Ir section in the Maxpar Direct Assay User Guide (PN 400286).

2

1. Centrifuge tube at 800 x *g* for 5 min.
2. Aspirate supernatant leaving ~100 µL residual volume of iridium fix/perm solution.
3. Resuspend the cell pellet in residual volume and transfer into a labeled 1.5 mL microcentrifuge tube.
4. Place 1.5 mL tubes in a plastic container for storage at –80 °C.

   Note: Frozen samples can be shipped overnight on dry ice for acquisition at an external site if needed.
5. On the day of acquisition, thaw 1.5 mL tubes by holding in hand for 1 min.
6. Add 1 mL of Cell Staining Buffer (CSB) to the 1.5 mL tube and then transfer contents into a labeled 5 mL round-bottom tube.
7. Rinse 1.5 mL tube with an additional 1 mL of CSB and transfer to the same 5 mL tube.

3

CONTINUE
with Step 1 of the Wash and Count Cells section in the Maxpar Direct Assay User Guide (PN 400286).

Figure 3. Cryopreservation workflow. The cryopreservation and thawing protocol for short- or long-term storage of stained cells from whole blood. After incubation with iridium overnight at 2–8 °C, the cells are transferred from 5 mL tubes to 1.5 mL tubes for cryopreservation at –80 °C until ready to be acquired on a Helios mass cytometer. Alternatively, for shipping to an external site, frozen samples can be placed on dry ice for acquisition on a Helios. Once the samples have been thawed, continue at Step 1 of the Wash and Count Cells section in the Maxpar Direct Immune Profiling Assay Cell Staining and Data Acquisition User Guide (PN 400286).

Results

Human whole blood samples were obtained and stained on Day 0 and were frozen the next day after an overnight incubation with iridium. Data acquisition and analysis were performed for the freshly stained samples and after freezing for 7, 21, 28, 60, and 120 days (Figure 2). Mean frequencies of 35 immune cell subsets were reported by Maxpar Pathsetter. These frequencies for each time point separated by donor are listed in Table 1. The mean signal intensities for all 30 markers across the 6 different time points separated by donor are shown in Table 2.

Population Frequencies

As demonstrated in Table 1, the majority of immune subsets (30 of 35) showed highly consistent results across all time points for both donors. For the 5 populations that had a variation greater than 10%, the maximal changes in population frequency are on the order of 0.1%. Significant differences seen in some subsets for one donor were not replicated in the other donor. Graphs of the individual datasets can be found in the appendix (Supplementary Figure 1).

Rare populations such as myeloid DCs and plasmablasts were reproducibly detected in each time point, though they comprised less than 0.5% of the total live cell count. Other subsets that constitute only 2% of the total live cell count, such as NKT, Tregs and subsets of monocytes, were also detected throughout the time course without significant differences due to cryopreservation.

As expected, because of biological variation, there was a significant difference in population frequencies between Donor 1 and Donor 2. Donor 1 had a 1.5 times higher mean frequency of lymphocytes and related subsets compared to Donor 2, whereas Donor 2 had more granulocytes, neutrophils and CD8 effector memory cells.

Signal Intensities

Results summarized in Table 2 show median signal intensities, SD, %CV and 95% confidence interval (CI) of the mean for all 30 markers for 4 technical replicates. Antibody signal intensity (reported as dual ion counts) did not differ significantly between freshly acquired samples and cryopreserved samples acquired at Day 120. Furthermore, the CV for the majority of markers was ≤15% on Day 0 as well as with cryopreservation for up to 120 days. The only exceptions were CD28, CD127 and CD27, which had a CV of ≤20% across all time points.

Maxpar Pathsetter also provides a stain index report that evaluates the separation of the negative and positive populations for each marker. This report showed that the stain index for all markers was consistent across all 6 time points, indicating preservation of staining quality after freezing (data not shown).

These results demonstrate that the data acquired from samples stained with the Maxpar Direct Immune Profiling Assay show a high degree of reproducibility across multiple cryopreservation time points up to 120 days vs. fresh.

Table 1. Reproducibility of cell frequencies after cryopreservation. A comparison is shown of the 35 immune populations from fresh (Day 0) and frozen stained whole blood samples (7, 21, 28, 60 and 120 days). The mean population frequencies of each subset per time point and the %CV of each population are displayed. An asterisk (*) indicates significant differences in the cell population over time.

Cell Populations	Donor 1							Donor 2						
	Day 0	Day 7	Day 21	Day 28	Day 60	Day 120	%CV	Day 0	Day 7	Day 21	Day 28	Day 60	Day 120	%CV
Lymphocytes	31.0	32.9	32.5	32.3	32.7	32.2	4.1	20.8	22.9	22.4	22.7	22.4	22.9	6.2
CD3 T cells	21.5	22.9	22.6	22.5	22.9	22.4	4.1	16.5	18.0	17.6	17.8	17.6	18	6.1
CD8 T cells	3.0	3.1	3.1	3.0	3.1	3.1	3.4	3.5	3.8	3.8	3.8	3.6	3.7	5.6
CD8 naive	1.5	1.6	1.6	1.6	1.6	1.6	4.0	1.0	1.0	1.1	1.1	1	1	6.5
CD8 central memory (CM)	0.2	0.2	0.2	0.2	0.2	0.2	8.2	0.4	0.5	0.5	0.6	0.5	0.5	11.4
CD8 effector memory (EM)	0.6	0.7	0.7	0.6	0.6	0.7	5.7	1.8	1.9	1.8	1.8	1.8	1.9	5.6
CD8 terminal effector (TE)	0.6	0.7	0.6	0.6	0.6	0.7	8.5	0.3	0.3	0.4	0.4	0.3	0.3	12.4
CD4 T cells	18.3	19.5	19.3	19.3	19.5	19.1	4.3	11.6	12.7	12.4	12.6	12.5	12.8	6.7
CD4 naive	6.9	7.4	7.3	7.3	7.3	7.0	5.8	2.8	2.9	2.8	2.8	2.9	2.8	7.4
CD4 central memory (CM)*	6.2	6.8	6.8	7.0	7.3	6.8	6.7	4.5	5.1	5.0	5.4	5.2	5.3	9.6
CD4 effector memory (EM)	4.2	4.4	4.1	4.0	3.9	4.3	7.3	3.6	3.8	3.7	3.6	3.6	3.8	8.5
CD4 terminal effector (TE)	1.0	1.0	1.0	1.0	0.9	1.1	8.8	0.8	0.8	0.9	0.9	0.8	0.8	9.1
Treg	0.5	0.6	0.6	0.6	0.6	0.6	4.2	0.3	0.4	0.3	0.3	0.4	0.3	7
Th1-like	1.7	1.9	1.8	2.0	1.8	1.7	8.1	1.3	1.4	1.4	1.5	1.4	1.5	9.7
Th2-like	1.2	1.3	1.2	1.3	1.2	1.3	6.4	0.9	1.0	1.0	1	1	1	6.2
Th17-like*	0.6	0.6	0.6	0.6	0.6	0.7	10.2	0.8	0.9	0.9	0.9	0.9	0.9	6.8
γδ T cells	0.1	0.1	0.1	0.1	0.1	0.1	6.0	0.3	0.4	0.4	0.4	0.4	0.4	5.8
MAIT/NKT	0.2	0.2	0.2	0.1	0.2	0.2	12.5	1.1	1.2	1.1	1.1	1.1	1.1	6.3
B cells (total)	3.9	4.3	4.4	4.6	4.7	4.3	9.4	2.2	2.4	2.4	2.6	2.6	2.8	12
B cells (naive)*	3.2	3.5	3.7	3.8	3.8	3.6	9.7	1.6	1.7	1.8	2	1.9	2.1	13.5
B cells (memory)	0.7	0.8	0.7	0.8	0.8	0.7	13.2	0.6	0.7	0.6	0.6	0.7	0.7	12.4
Plasmablasts	0.05	0.06	0.1	0.1	0.1	0.1	12.7	0.02	0.03	0.0	0.03	0.03	0.03	13.4
NK cells (total)	5.5	5.7	5.4	5.2	5.2	5.4	5.6	2.2	2.4	2.3	2.2	2.2	2.2	5.5
NK cells (early)	3.0	3.1	2.9	2.8	2.8	2.9	5.1	1.4	1.5	1.5	1.4	1.4	1.4	5.8
NK cells (late)	2.5	2.6	2.5	2.4	2.4	2.5	6.4	0.8	0.9	0.8	0.8	0.8	0.8	6.4
Monocytes (total)	5.5	6.2	5.9	6.0	6.2	5.8	8.3	3.7	4.2	3.9	4.1	4.5	4.3	11
Monocytes (classical)	4.6	5.2	4.9	5.0	5.2	4.8	9.1	3.1	3.5	3.3	3.4	3.7	3.6	11.6
Monocytes (nonclassical)	0.3	0.4	0.3	0.4	0.4	0.3	13.0	0.3	0.4	0.4	0.4	0.4	0.4	10.2
Monocytes (transitional)	0.6	0.7	0.6	0.6	0.6	0.6	6.0	0.3	0.3	0.3	0.3	0.3	0.3	11
pDC*	0.04	0.06	0.1	0.1	0.1	0.0	23.4	0.06	0.08	0.1	0.08	0.08	0.08	17.2
mDC	0.3	0.3	0.3	0.2	0.3	0.3	15.2	0.2	0.2	0.2	0.2	0.2	0.2	10.5
Granulocytes	60.3	57.6	58.1	58.3	57.7	58.2	2.6	71.4	68.9	69.4	69.4	69.1	68.7	2.5
Neutrophils	57.5	54.4	55.4	55.3	54.6	55.5	2.9	69.5	66.7	67.5	67	66.5	66.4	2.9
Basophils	0.7	0.7	0.7	0.7	0.8	0.7	8.5	0.4	0.4	0.4	0.4	0.5	0.5	9.5
Eosinophils*	1.9	2.1	1.7	1.7	1.8	1.8	9.6	1.2	1.4	1.0	1.2	1.4	1.3	14

Table 2. Reproducibility of signal intensities after cryopreservation. Median signal intensities, SD, %CV and 95% confidence interval (CI) of the mean of 4 replicates for fresh and 120-day time points for all 30 markers are shown. Signal intensity is expressed as dual ion count per metal channel for the associated cell surface marker.

Channel	Marker	Donor 1 (Days 0–120)				Donor 2 (Days 0–120)			
		Mean	SD[a]	%CV[b]	95% CI[c] of Mean	Mean	SD[a]	%CV[b]	95% CI[c] of Mean
89Y	CD45 (HI30)	506	44	9	488–525	448	27	6	422–474
141Pr	CD196/CCR6 (G034E3)	231	10	5	226–235	142	14	10	129–155
143Nd	CD123 (6H6)	568	44	8	549–587	350	43	12	308–392
144Nd	CD19 (HIB19)	381	25	7	371–392	360	38	11	322–398
145Nd	CD4 (RPA-T4)	325	15	5	318–331	294	18	6	276–312
146Nd	CD8a (RPA-T8)	575	31	5	562–587	601	54	9	548–654
147Sm	CD11c (Bu15)	408	32	8	394–421	213	12	6	201–225
148Nd	CD16 (3G8)	208	17	8	201–216	184	11	6	173–195
149Sm	CD45RO (UCHL1)	131	15	12	124–137	68	6	9	62–74
150Nd	CD45RA (HI100)	146	19	13	138–154	85	9	11	76–94
151Eu	CD161 (HP-3G10)	259	22	8	250–268	202	15	8	187–217
152Sm	CD194/CCR4 (L291H4)	115	15	13	109–121	130	17	13	113–147
153Eu	CD25 (BC96)	59	4	6	57–61	54	2	4	52–56
154Sm	CD27 (O323)	345	42	12	327–362	213	35	16	179–247
155Gd	CD57 (HCD57)	701	21	3	692–709	692	26	4	666–718
156Gd	CD183/CXCR3 (G025H7)	218	17	8	211–225	171	21	13	150–192
158Gd	CD185/CXCR5 (J252D4)	399	29	7	386–411	310	30	10	280–340
160Gd	CD28 (CD28.2)	128	26	20	117–139	135	12	9	123–147
161Dy	CD38 (HB-7)	155	10	7	150–159	197	16	8	182–212
163Dy	CD56/NCAM (NCAM16.2)	304	16	5	298–311	226	24	11	202–250
164Dy	TCRgd (B1)	140	13	9	135–146	186	16	8	171–201
166Er	CD294 (BM16)	400	24	6	390–410	356	12	3	344–368
167Er	CD197/CCR7 (G043H7)	403	22	5	394–413	147	8	6	139–155
168Er	CD14 (63D3)	375	32	8	362–388	313	37	12	276–350
170Er	CD3 (UCHT1)	445	30	7	432–457	401	30	8	372–431
171Yb	CD20 (2H7)	484	34	7	469–498	231	26	11	205–257
172Yb	CD66b (G10F5)	351	23	6	342–361	252	9	3	243–261
173Yb	HLA-DR (LN3)	454	39	9	438–471	408	44	11	365–451
174Yb	IgD (IA6-2)	498	26	5	488–509	311	28	9	284–338
176Yb	CD127 (A019D5)	248	48	19	228–268	183	18	10	166–200

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

Conclusion

The Maxpar Direct Immune Profiling System is designed to simplify deep immune profiling, providing a quick and easy method for PBMC and whole blood analysis. The results in this application note indicate that whole blood samples can be stained with the Maxpar Direct Immune Profiling Assay, cryopreserved for either short-term (days) or long-term storage (up to 4 months) and, if necessary, shipped to an external site for acquisition on a Helios mass cytometer without compromising data quality.

This is not a full validation study, but a recommendation that is intended to assist with sample acquisition and storage.

Materials and Methods

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)

Blood Handling, Sample Preparation and Storage

Human peripheral whole blood from healthy donors (Canadian Blood Services, Vancouver, BC, Canada) was collected in BD Vacutainer® blood collection tubes (Becton, Dickinson, Franklin Lakes, NJ, USA) containing heparin as an anticoagulant. Blood was processed and stained no more than 14 hr following collection.

Blood was stained in accordance with the Day 1: Cell Staining section of the Maxpar Direct Immune Profiling Assay Cell Staining and Data Acquisition User Guide (PN 400286). In brief, blood cells were counted on a TC20™ cell counter using trypan blue exclusion to assess viability (Bio-Rad). Next, 10 mL of 10 KU/mL sodium heparin (Sigma-Aldrich®) was added per 1 mL of whole blood and incubated for 20 min at room temperature (RT). Then 270 µL of heparin-blocked whole blood was added to a 5 mL tube containing the antibody pellet and incubated for 30 min at RT. Immediately after staining, red blood cells were lysed using Cal-Lyse™ Lysing Solution (Thermo Fisher Scientific™). Cells were incubated in 1 mL Cell-ID Intercalator-Ir (Cat. No. 201192A) diluted in Maxpar Fix and Perm Buffer (Cat. No. 201067) (final concentration 125 nM) and incubated overnight at 2–8 °C. Cells were spun down in 5 mL tubes, resuspended in 100 µL residual volume, transferred to 1.5 mL microcentrifuge tubes and stored at –80 °C for up to 120 days.

Thawing of Samples and Final Processing

Prior to acquisition on Helios, samples were placed in a box of dry ice for up to 19 hr to simulate overnight shipment to an external site. After this time, a 1.5 mL microcentrifuge tubes were taken out of the freezer and thawed by hand, holding for 1 min in hand and then transferred back into 5 mL polystyrene round bottom tubes. At this time, the final washes and cell counting were completed according to the protocol in the Wash and Count section of the Maxpar Direct Immune Profiling Assay User Guide (PN 400286).

Sample Acquisition and Processing

All samples were acquired on a Helios mass cytometer using CyTOF® Software v7.0.5189. The Helios was tuned using a WB Injector (PN 107950) and Tuning Solution (Cat. No. 201072). A full protocol was run. After instrument tuning and the bead sensitivity test were completed, the system was preconditioned with Cell Acquisition Solution (CAS, Cat. No. 201239) for 15 min. Cells were resuspended at a concentration of 1×10^6 cells/mL in CAS containing 0.1X EQ™ Four Element Calibration Beads (Cat. No. 201078). Samples were acquired using the Maxpar Direct Immune Profiling Assay acquisition template. A total of 400,000 events was collected for each sample. Cells were acquired at an acquisition rate between 250 and 500 events/second.

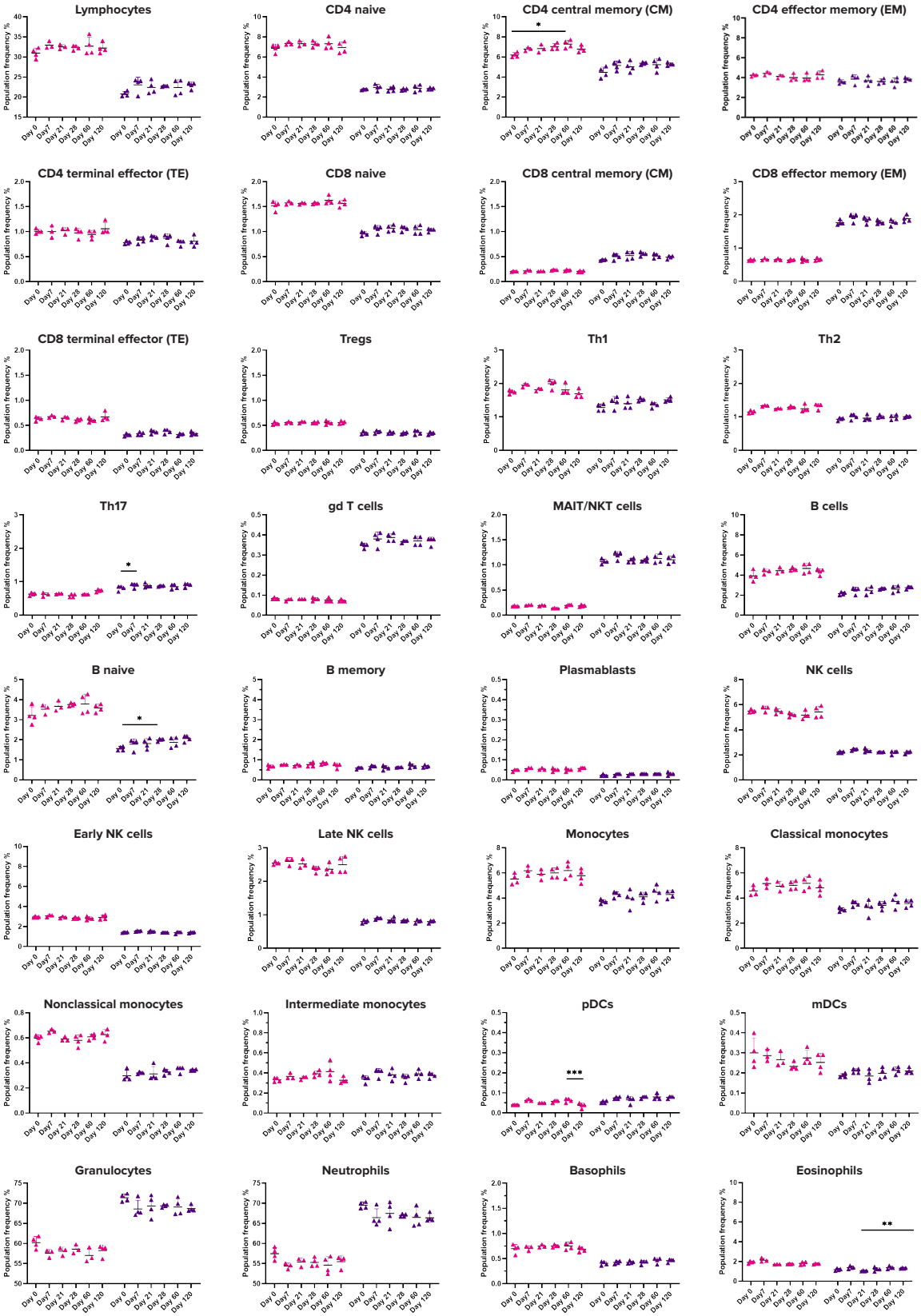
Data Analysis with Maxpar Pathsetter

FCS files were normalized using CyTOF Software v7.0.5189 and then analyzed using Maxpar Pathsetter, a data analysis and reporting software that uses a statistical method called probability state modeling[4,5]. 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).

The reports generated were used to analyze data collected from each time point. Statistical significance was reported for each time point using the two-way ANOVA with Sidak's multiple comparison test. Significance was defined as $^{*}p \leq 0.05$, $^{**}p \leq 0.01$ and $^{***}p \leq 0.001$ are indicated in Supplementary Figure 1.

Appendix

Supplementary Figure 1. Reproducibility of population frequencies of immune subsets after cryopreservation. Population frequencies of 32 immune cell subsets for Donors 1 (magenta) and 2 (purple) at Days 0, 7, 21, 28, 60 and 120 are shown. Each datapoint represents 1 of 4 replicates acquired at each time point with the mean frequency of each subset denoted by the horizontal bar. Error bars denote mean and SD of replicates. Significance is denoted by the bar where *p ≤0.05, **p ≤0.01 and ***p ≤0.001.



Ordering Information

Product Name	Catalog Number
Maxpar® Direct™ Immune Profiling Assay™, 30 Marker—25 Tests	201325
Maxpar Pathsetter™	401018

References

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FLDM-00089 Rev 01