TECHNICAL NOTE

Assay Validation Processes to Ensure Accuracy and Precision in Immune Profiling – Advancing Single-Cell Proteomic Applications

Summary:

- The Maxpar™ Direct Immune Profiling Assay maintains high sensitivity and specificity across all markers in the panel
- Cross-validation of Maxpar Direct Immune Profiling Assay liquid vs. lyophilized panel demonstrates no difference in results
- Validation testing on the Maxpar Direct Immune Profiling Assay panel confirms high reproducibility across multiple sites, high repeatability across sample types, including whole blood and PBMC, and robust intermediate precision when used by different technicians

Introduction

Immune profiling assists in identifying and quantifying immune populations according to their phenotypic and functional features. Performing immune profiling longitudinally, known as immune monitoring, is a valuable technique to study conditions in which 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 PBMC[1–4] 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 treatment[5–11]. The wide diversity of immune cell populations demands a high-parameter technique to quantify these changes more fully and efficiently.

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], which hinders high-parameter single cell phenotyping and limits discovery of more complex or rare phenotypes. 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. Even so, 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.

With the Maxpar Direct Immune Profiling System, deep immune profiling information can be performed in a single tube consistently and conveniently. Here we show data supporting the analytical performance of the Maxpar Direct Immune Profiling Assay for standardized immune profiling by verifying assay sensitivity and specificity, intra-assay repeatability, intermediate precision and accuracy of the dry panel format, as well as inter-site reproducibility and biological robustness of data using peripheral human whole blood and PBMC.

31-plex Maxpar Direct Immune Profiling Assay panel

Antibody (clone)	Isotope	Antibody (clone)	Isotope	Antibody (clone)	Isotope
CD45 (HI30)	89Y	CD11c (Bu15)	147Sm	CD25 (BC96)	153Eu
CD185/CXCR5 (J252D4)	158Gd	CD197/CCR7 (G043H7)	167Er	IgD (IA6-2)	174Yb
CD196 /CCR6 (G034E3)	141Pr	CD16 (3G8)	148Nd	CD27 (0323)	154Sm
CD28 (CD28.2)	160Gd	CD14 (63D3)	168Er	CD127 (A019D5)	176Yb
CD123 (6H6)	143Nd	CD45RO (UCHL1)	149Sm	CD57 (HNK-1)	155Gd
CD38 (HB-7)	161Dy	CD3 (UCHT1)	170Er	Cell-ID Intercalator-103Rh	103Rh
CD19 (HIB19)	144Nd	CD45RA (HI100)	150Nd	CD183/CXCR3 (G025H7)	156Gd
CD56/NCAM (NCAM16.2)	163Dy	CD20 (2H7)	171Yb		
CD4 (RPA-T4)	145Nd	CD161 (HP-3G10)	151Eu		
TCRγδ (B1)	164Dy	CD66b (G10F5)	172Yb		
CD8a (RPA-T8)	146Nd	CD194/CCR4 (L291H4)	152Sm		
CD294 (BM16)	166Er	HLA-DR (LN3)	173Yb		

Figure 1. Isotope-tagged antibodies included in the Maxpar Direct Immune Profiling Assay provided in a dry single-tube format

The Maxpar Direct Immune Profiling Assay is a pre-configured and validated solution for deep immune profiling of human peripheral whole blood and PBMC. The assay includes a dry-format 30-marker antibody panel (Figure 1) with a viability stain in a standard 5 mL polypropylene tube. The panel enables automated 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. To profile expression markers, change cell type definitions or identify new immune cell types, the assay panel can be expanded by adding at least 18 additional antibodies.

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.

The Maxpar Direct Immune Profiling Assay has undergone rigorous analytical validation using both PBMC and whole blood.

Assay design and testing process

1 Determination of required antibody targets based on recommendation by the Human ImmunoPhenotyping Consortium (HIPC) and the Human Immunology Project and recommendations from key opinion leaders.

2 Testing of clones on both whole blood and PBMC to determine optimal concentrations.

- Specificity determined from assessing staining of clones on different cell subsets
- Sensitivity determined from assessing dynamic range across different donors

3 Lyophilization of antibody panel and testing on whole blood and PBMC

4 Repeatability and reproducibility verification

- Repeatability tested by single technician staining replicate samples
- Intermediate precision tested by multiple technicians staining replicate samples on 2–3 instruments at a single site
- Reproducibility tested with samples stained at different sites by different technicians, and acquired on different instruments

Assay validation process and biological robustness testing on whole blood and PBMC from multiple donors

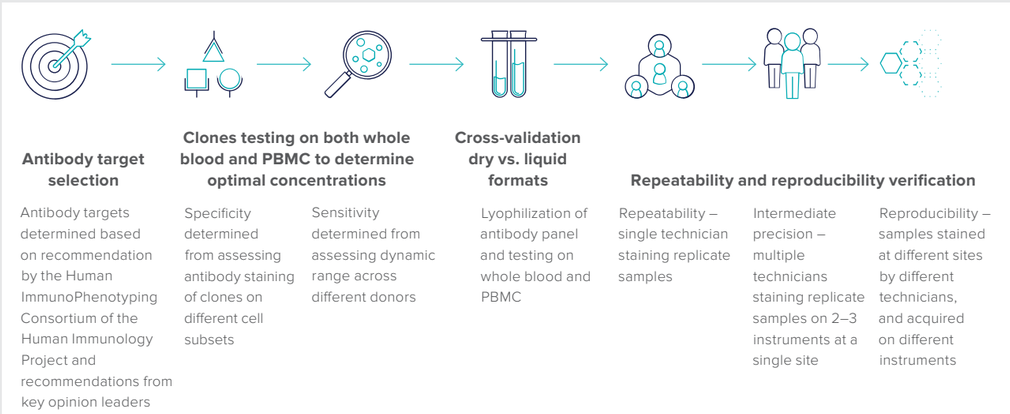


Figure 2. Standard BioTools™ process for designing and validating panels and assays for use with CyTOF systems

Validating assay performance to ensure standardized results

Performance validations confirm and ensure that accurate and precise results can be consistently collected from a standardized assay. At Standard BioTools, quality and validation testing make up a significant step in product development. Assay validation for the Maxpar Direct Immune Profiling System provides proof of specificity, sensitivity, repeatability, reproducibility and precision (Figure 2).

The assay panel was developed based on the recommendations of the HIPC[1]. Incorporating feedback from leading expert immunologists, 8 markers were added to identify additional T cell subsets, NK cell subsets, neutrophils, basophils and eosinophils[20–25]. Metal-isotope-labeled antibody clones were selected to optimize the panel with minimal signal spillover.

Validation includes the use of Maxpar Pathsetter software for fast, reliable and flexible automated analysis of FCS files from human PBMC and whole blood cells stained with the Maxpar Direct Immune Profiling Assay. Maxpar Pathsetter automatically reports on 37 immune populations and is the recommended tool for reporting and analysis of results from the Maxpar Direct Immune Profiling Assay. Alternatively, manual bivariate gating can be used to define and enumerate these immune populations.

Specificity verification

Specificity is the ability of the assay to detect and measure only the target protein, differentiating from other components without interference or cross-reactivity. This is essential to gain an unbiased understanding of sample composition and translation to actionable insights.

Each of the 30 antibody targets was determined based on recommendations by the HIPC and from key opinion leaders. Clones are tested on both whole blood and PBMC to determine optimal concentrations. Specificity is then determined from assessing staining of clones on different cell subsets (Figure 3).

Detecting 37 cell subsets simultaneously with the Maxpar Direct Immune Profiling Assay in a single tube

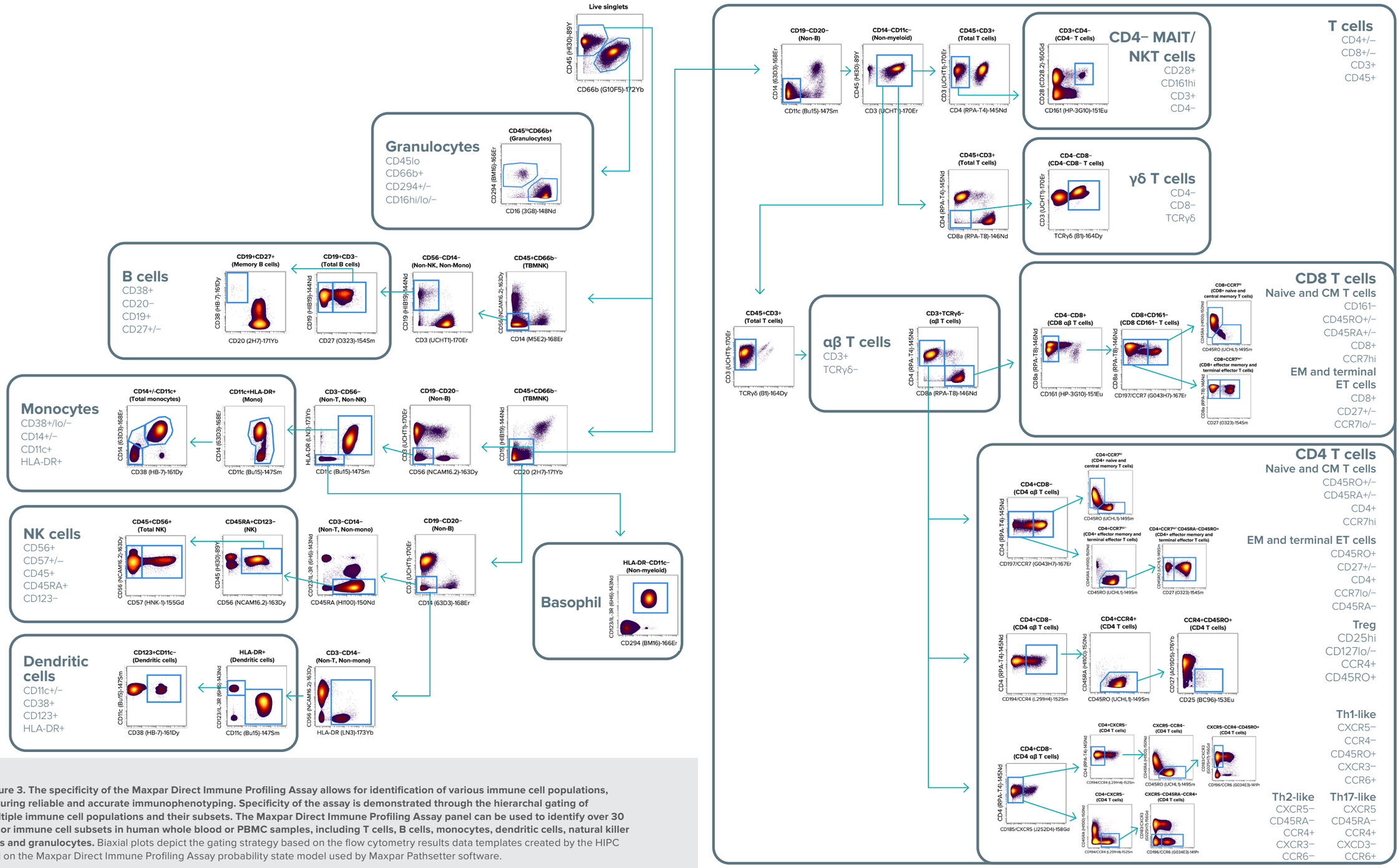


Figure 3. The specificity of the Maxpar Direct Immune Profiling Assay allows for identification of various immune cell populations, ensuring reliable and accurate immunophenotyping. Specificity of the assay is demonstrated through the hierarchal gating of multiple immune cell populations and their subsets. The Maxpar Direct Immune Profiling Assay panel can be used to identify over 30 major immune cell subsets in human whole blood or PBMC samples, including T cells, B cells, monocytes, dendritic cells, natural killer cells and granulocytes. Biaxial plots depict the gating strategy based on the flow cytometry results data templates created by the HIPC and on the Maxpar Direct Immune Profiling Assay probability state model used by Maxpar Pathsetter software.

Sensitivity of the Maxpar Direct Immune Profiling Assay allows for accurate identification of immune cell populations across different donors

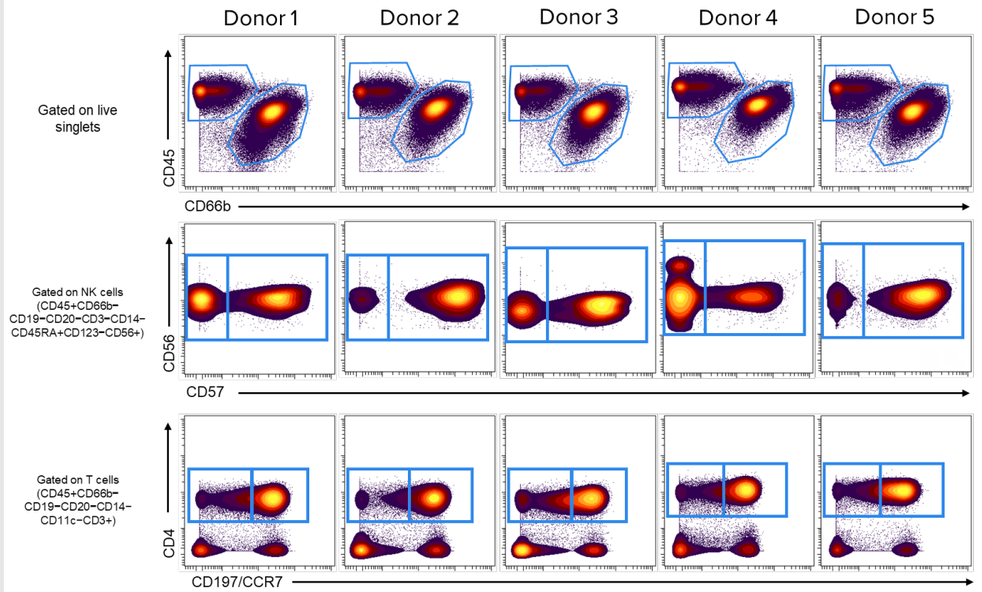


Figure 4. The sensitivity of the Maxpar Direct Immune Profiling Assay is demonstrated in the bivariate plots of 5 whole blood donors stained within a single day using a single lot of the assay. The bivariate plots illustrate the distinct separation and sensitivity in (top row) CD45 vs. CD66b used for identifying lymphocytes and granulocytes, (middle row) CD56 vs. CD57 used for identifying early and late NK cells, and (bottom row) CD4 vs. CD197/CCR7 used for identifying CD4 naive/central memory/effector memory/ terminal effector memory T cells.

Sensitivity verification

Verification of sensitivity confirms whether the antibody can detect the target protein at any concentration, testing at what low concentration detection is successful. This is important because it demonstrates the assay can reliably detect various levels of even a low-expressing target among different samples and sample types.

Clones are tested on both whole blood and PBMC to determine optimal concentrations. Sensitivity is then determined by assessing dynamic range across different donors.

The Maxpar Direct Immune Profiling Assay standardizes panel processing with dry format

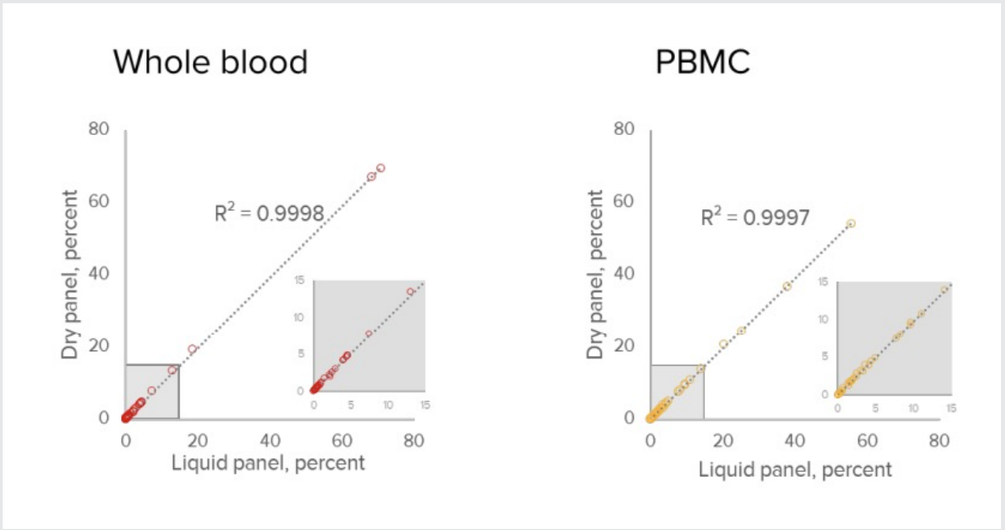


Figure 5. Accuracy assessment of the liquid vs. the dry antibody panel using the Maxpar Direct Immune Profiling Assay, with samples taken from a single whole blood donor and a single PBMC donor and acquired on a single mass cytometer. Normalized FCS files were analyzed in Cytobank. Correlaton of marker expression between dry and liquid panel formats in whole blood and PBMC demonstrate comparable performance. The coefficient of determination (R2) is shown for each sample type. Gray insert: enlarged figure for populations ≤15%

Cross-validation of liquid vs. lyophilized panel

Pre-configured dry antibody panels can significantly reduce processing time, technical variation and errors that can result from working with multiple individual antibodies, offering an advantage over liquid panels that is amplified when developing larger panels. Antibodies used in mass cytometry are formulated in liquid buffer with Antibody Stabilizer (CANDOR Bioscience). 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. To test the accuracy[26] 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 5). A single whole blood or PBMC donor sample was stained by 3 technicians using both panel formats.

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 3 technicians) was calculated for each population and plotted (Figure 5). The coefficient of determination (R2) for these datapoints was >0.99 for each sample type, demonstrating very good agreement between the 2 panel formats. Thus, there is no difference in results when comparing a liquid version of the Maxpar Direct Immune Profiling Assay antibody panel and the lyophilized Maxpar Direct Immune Profiling Assay panel.

High assay repeatability across sample types

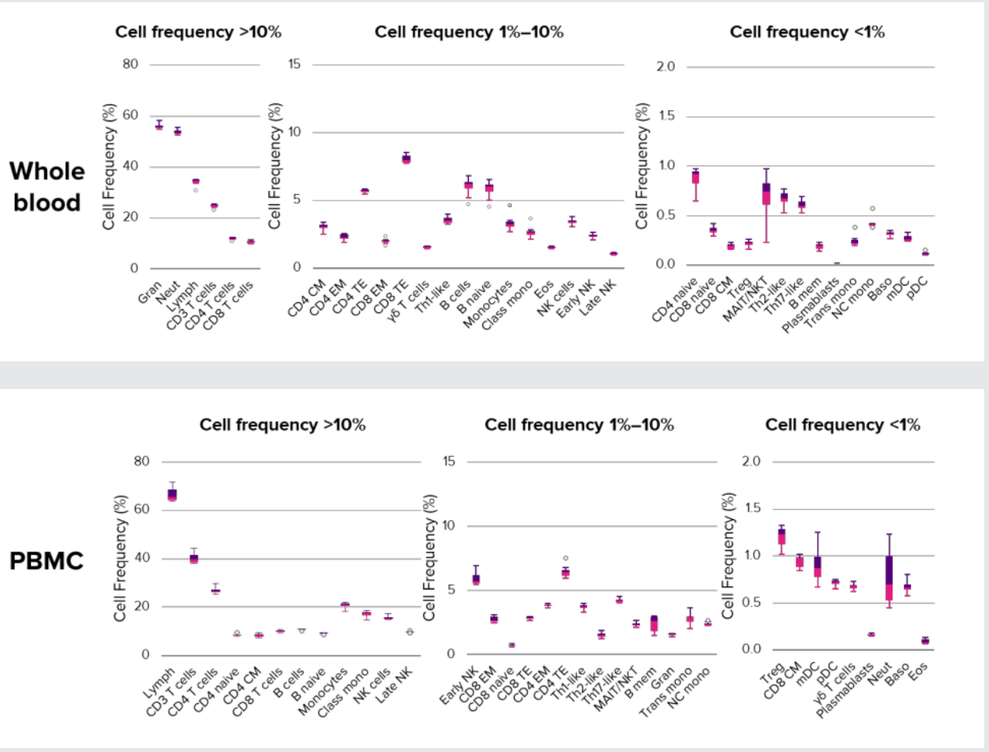


Figure 6. Assessment of the intra-assay repeatability of the Maxpar Direct Immune Profiling Assay using whole blood and PBMC. Replicate measurements of the same whole blood or PBMC sample were assessed for intra-assay repeatability of the Maxpar Direct Immune Profiling Assay. Left panel: populations 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 (lower half of dataset; pink) to third quartile (upper half of dataset; 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 4 independent experiments. The whole blood and PBMC samples in this experiment were obtained from different donors.

Repeatability verification

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, an analytical instrument and analysis software (Maxpar Pathsetter). To test the intra-assay repeatability[26] within the assay, Maxpar Direct Immune Profiling Assay tubes were stained in replicates and acquired on a single mass cytometer.

The frequencies of 35 quantified populations generated by Maxpar Pathsetter were plotted for whole blood and PBMC replicates (Figure 6). For all populations with a frequency of ≥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. The populations with cell frequencies less than 1% displayed higher variability, as expected. The Maxpar Direct Immune Profiling Assay is highly repeatable for both human whole blood and PBMC samples as demonstrated by narrow error bars of plotted results.

The Maxpar Direct Immune Profiling Assay ensures high assay reproducibility across sites

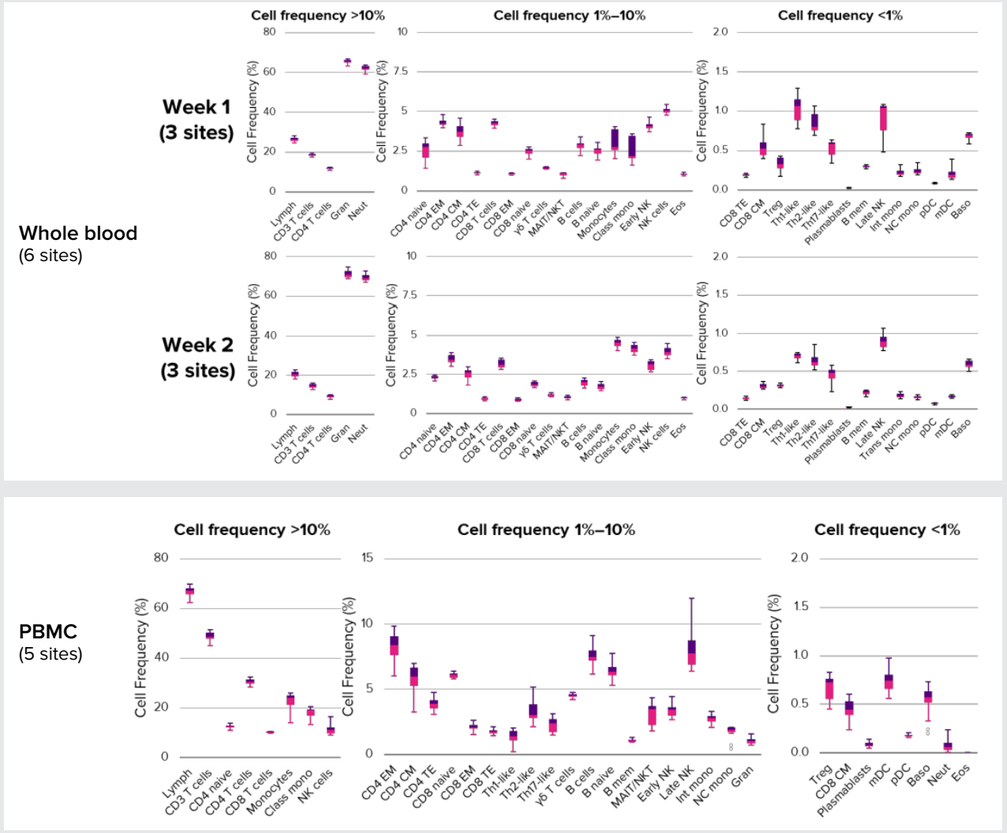


Figure 7. Assessment of the inter-site reproducibility of the Maxpar Direct Immune Profiling Assay for 5 external sites with a single PBMC donor or 6 external sites with a single whole blood donor in 4 replicate samples. Replicate measurements of the same whole blood donor assessed for inter-site reproducibility. Variability from different technicians and instruments for the Maxpar Direct Immune Profiling Assay was measured at multiple sites. At 2 time points 1 week apart, blood from the same donor was drawn and analyzed at 3 different external sites. Left: populations with average cell frequencies of >10%. Middle: populations with average cell frequencies of 1–10%. Right: populations with average cell frequencies of <1%. For the box and whisker plots: box, first quartile (lower half of dataset; pink) to third quartile (upper half of dataset; purple); color change, median; error bars, minimum/maximum values. The Y-axis is the measured % of total single live cells.

Reproducibility verification

Multi-center 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 reproducibility[26] 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 7). A total of 6 test sites were provided with whole blood specimens from a single donor.

All FCS files were analyzed using Maxpar Pathsetter. The frequencies of 35 quantified populations generated by Maxpar Pathsetter were plotted for whole blood (Figure 7 top) and PBMC replicates (Figure 7 bottom). For all populations ≥5% in frequency, the %CV of the mean was <10% for whole blood and <20% for PBMC. The Maxpar Direct Immune Profiling Assay is highly reproducible across multiple sites and can be reproducibly used across multiple sites as an immune monitoring application.

Maxpar Direct Immune Profiing Assay precision across multiple technicians

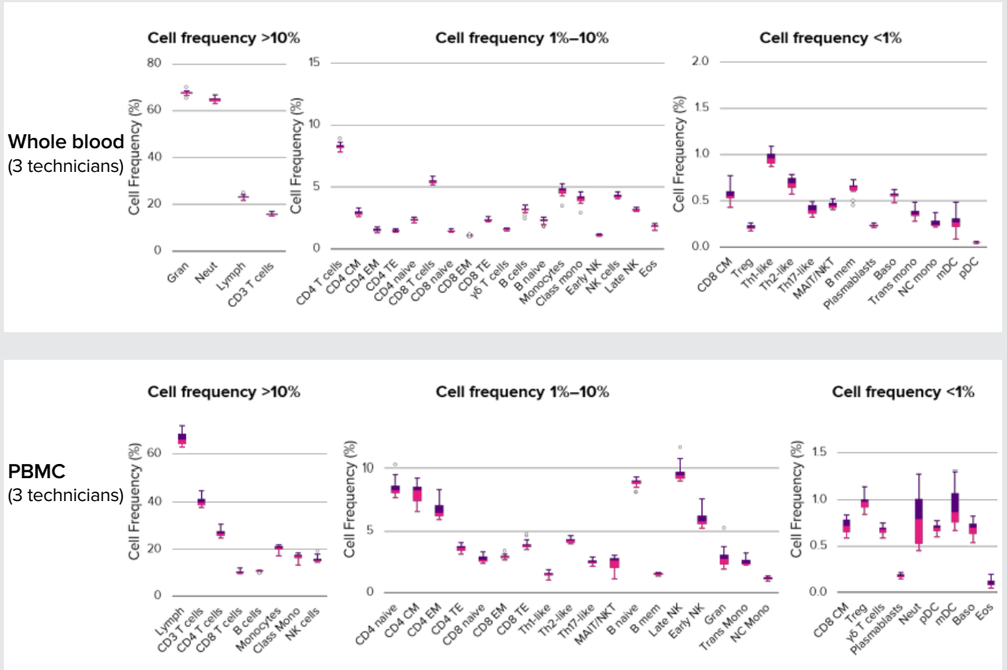


Figure 8. Intermediate precision assessment of the Maxpar Direct Immune Profiling Assay by testing results obtained by 3 technicians using 3 Maxpar Direct Immune Profiling Assay tubes acquired on 2–3 different instruments on the same day. Replicate measurements of the same whole blood or PBMC samples 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%. The data shown is a representative of 3 independent experiments. The whole blood and PBMC samples in this experiment were obtained from different donors.

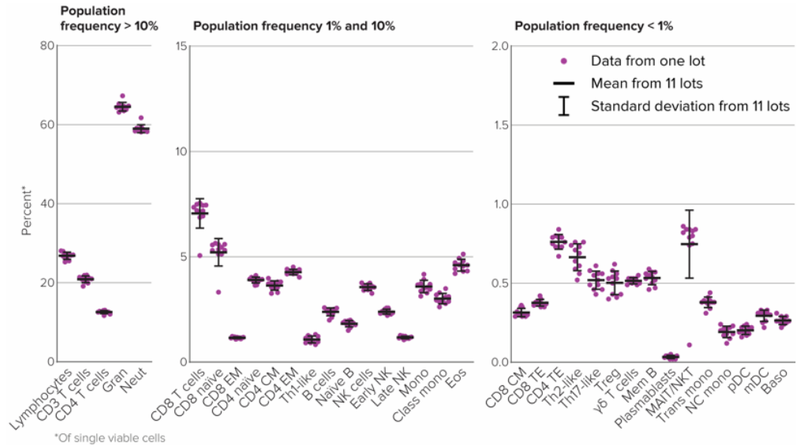
Intermediate precision validation

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, assessing variabilities resulting from different technicians, instruments or day-to-day operations within a single laboratory. 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 (Figures 8 and 9).

For the results obtained by multiple technicians (Figure 8), all populations ≥5% in frequency, the %CV of the mean was <4% for whole blood and <10% for PBMC. Figure 9 demonstrates that population frequencies and median signal intensities were highly consistent lot to lot. These results demonstrate that the Maxpar Direct Immune Profiling Assay shows a high degree of intermediate precision.

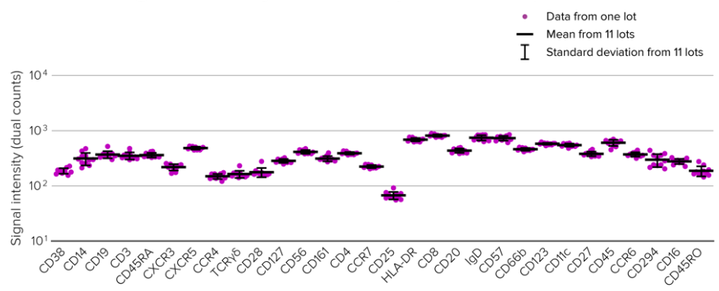
Low lot-to-lot variability shown with the Maxpar Direct Immune Profiling Assay for standardized longitudinal study results

Population frequency results: Consistency across multiple lots



For all whole blood populations ≥5% in frequency, the %CV of the inter-lot mean was 12.5%.

Signal intensity: Consistency across multiple lots



For all 30 markers in the antibody panel, the mean %CV of signal intensity was 11.4%.

Figure 9. The Maxpar Direct Immune Profiling Assay has low lot-to-lot variability as demonstrated by narrow error bars of plotted results. Lot-to-lot variability assessment of the Maxpar Direct Immune Profiling Assay by testing results obtained by 1 technician using 11 different lots. Consistency across multiple lots is observed. Left panel: For all whole blood populations ≥5% in frequency, the %CV of the inter-lot mean was 12.5%. Right panel: For all 30 markers in the antibody panel, the mean %CV of signal intensity was 11.4%.

Biological robustness testing

Validated for multiple donors, sites and sample types

Validation testing for the Maxpar Direct Immune Profiling Assay thoroughly tested whole blood and PBMC from multiple donors. From feasibility through validation testing, the Maxpar Direct Immune Profiling Assay was tested on 131 different whole blood samples and 48 different PBMC samples. Thus, the Maxpar Direct Immune Profiling Assay has been robustly tested on unique whole blood and PBMC samples.

Conclusions

The analytical validation studies presented here demonstrate that the Maxpar Direct Immune Profiling System, which includes protocols, reagents for cell staining, use of a mass cytometer and Maxpar Pathsetter reporting and analysis software, provides 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 with 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. et al. "Standardizing immunophenotyping for the Human Immunology Project." *Nature Reviews Immunology* 12 (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* 2 (2013): 17.
3. Olin, A. et al. "Stereotypic immune system development in newborn children." *Cell* 174 (2018): 1,277–1,292.e14.
4. McCoy, J.P. and Overton, W.R. "Quality control in flow cytometry for diagnostic pathology: II. A conspectus of reference ranges for lymphocyte immunophenotyping." *Cytometry Part A* 18 (1994): 129–139.
5. Chew, V. et al. "Immune activation underlies a sustained clinical response to yttrium-90 radioembolisation in hepatocellular carcinoma." *Gut* 68 (2019): 335–346.
6. Chowdhury, R.R. et al. "A multi-cohort study of the immune factors associated with *M. tuberculosis* infection outcomes." *Nature* 560 (2018): 644–648.
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* 9 (2018): 1217.
8. Krieg, C. et al. "High-dimensional single-cell analysis predicts response to anti-PD-1 immunotherapy." *Nature Medicine* 24 (2018): 144–153.
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* 6 (2018): 18.
10. Lingblom, C.M.D. et al. "Baseline immune profile by CyTOF can predict response to an investigational adjuvanted vaccine in elderly adults." *Journal of Translational Medicine* 16 (2018): 153.
11. Smets, T. et al. "Deep profiling of the immune system of multiple myeloma patients using cytometry by time-of-flight (CyTOF)." *Methods in Molecular Biology* 1,792 (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* 47 (2017): 1,584–1,797.
13. Biancotto, A. et al. "High dimensional flow cytometry for comprehensive leukocyte immunophenotyping (CLIP) in translational research." *Journal of Immunological Methods* 363 (2011): 245–261.
14. Finak, G. et al. "Standardizing flow cytometry immunophenotyping analysis from the Human ImmunoPhenotyping Consortium." *Scientific Reports* 6 (2016): 20686.
15. Kverneland, A.H. et al. "Age and gender leucocytes variances and references values generated using the standardized ONE-Study protocol." *Cytometry Part A* 89 (2016): 543–564.
16. Jamin, C. et al. "Multi-center harmonization of flow cytometers in the context of the European "PRECISESADS" project." *Autoimmunity Reviews* 15 (2016): 1,038–1,045.
17. Simoni, Y. et al. "Bystander CD8+ T cells are abundant and phenotypically distinct in human tumour infiltrates." *Nature* 557 (2018): 575–579.
18. Bagwell, C.B. et al. "Probability state modeling theory." *Cytometry Part A* 87 (2015): 646–660.
19. Bagwell, C.B. "High-dimensional modeling for cytometry: Building rock solid models using GemStone and Verity Cen-se′ high-definition t-SNE mapping." *Methods in Molecular Biology* 1,678 (2018): 11–36.
20. Borst, J. et al. "Distinct molecular forms of human T cell receptor gamma/delta detected on viable T cells by a monoclonal antibody." *Journal of Experimental Medicine* 167 (1988): 1,625–1,644.
21. Dias, J. et al. "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* 114 (2017): e5,434–e5,443.
22. Björkström, N.K. et al. "Expression patterns of NKG2A, KIR, and CD57 define a process of CD56dim NK-cell differentiation uncoupled from NK-cell education." *Blood* 116 (2010): 3,853–3,864.
23. Yoon, J. et al. "CD66b regulates adhesion and activation of human eosinophils." *The Journal of Immunology* 179 (2007): 8,454–8,462.
24. Schaerli, P. et al. "CXC chemokine receptor 5 expression defines follicular homing T cells with B cell helper function." *Journal of Experimental Medicine* 192 (2000): 1,553–1,562.
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* 193 (2001): 255–261.
26. *Evaluation of Precision of Quantitative Measurement Procedures; Approved Guideline—Third Edition*. CLSI (2014).

Interested in the Maxpar Direct Immune Profiling Assay? Learn more at **standardbio.com**

LAB-00027 Rev 01 012025

Maxpar Direct Immune Profiling Assay Validation Application Note

For Research Use Only. Not for use in diagnostic procedures.