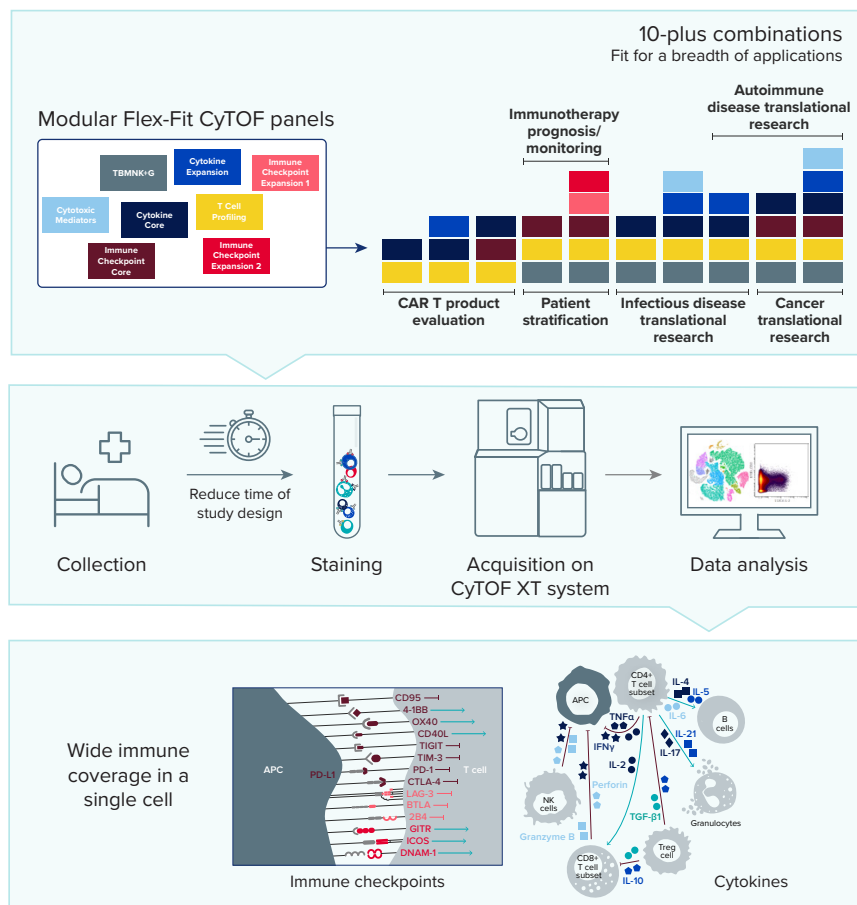


Accelerating Immuno-Oncology Research by Profiling Cell Function with Flex-Fit CyTOF Panels

Graphical Abstract



Objectives

- **Reveal functional diversity** of immune checkpoint markers and cytokines in immuno-oncology applications using Flex-Fit CyTOF™ Panels
- **Resolve rare cell populations** with exceptional resolution to uncover true functional biology
- Demonstrate **flexibility, modularity and customizability** of Flex-Fit CyTOF Panels to reduce time of study design while maximizing functional target coverage

Introduction

Accurate phenotyping of immune cells from cancer patients is critical for understanding mechanism of action, disease prognoses and monitoring clinical efficacy of immunotherapies. Characterization of immune checkpoint markers and cytokines produced in single cells provides the functional readout necessary to decipher mechanisms of immune evasion and antitumor responses in patients. Therefore, building comprehensive high-parameter flow panels is essential for deep immune profiling to accelerate immunotherapy research.

Here, we introduce a series of pre-optimized and predefined panels called Flex-Fit CyTOF Panels (Figure 1). These modular panels are designed to identify various immune cell populations, characterize key immune checkpoints regulating immune cell response and evaluate immune cell function, such as cytokine expression. With low signal spillover, no autofluorescence and no need for reference controls, Flex-Fit CyTOF Panels can be easily mixed and matched to form high-parameter 40-plus-marker bundles that enable deep immunophenotyping analysis. The modular nature allows Flex-Fit panels within high-parameter bundles to be added, removed or swapped to interrogate different biological questions without the need to redesign and optimize a new high-parameter panel, which overcomes common challenges of fluorescence-based flow cytometry panel design.

Detection of rare cytokine-secreting cells via fluorescence cytometry has been historically difficult due to signal spillover and spread, which can affect the accurate resolution of cytokines and result in the potential loss of cell populations. Enabled by CyTOF technology, 3 Flex-Fit cytokine panels have been designed to capture both rare and abundant cytokine-producing immune cells at exceptional single-cell resolution, providing functional insights into immune cell biology.

In this application note, we demonstrate the power of Flex-Fit panels by employing 2 high-parameter bundles, built using different combinations of Flex-Fit panels, to evaluate the phenotypic and functional characteristics of PBMC collected from a cancer patient.

Study design










Two high-parameter bundles were created using Flex-Fit panels (Figure 2 and Appendix B and C).

The Human Broad Immune Checkpoint CyTOF Bundle, 34 Antibodies (hereafter referred to as the Checkpoint Bundle) was built to characterize a variety of immune checkpoint markers expressed on T cells, providing cellular insights into the potential mechanism of action for disorders such as cancer, autoimmunity and infectious diseases.

To further evaluate the functionality of cancer patient T cells, the 2 immune checkpoint Flex-Fit panels from the Checkpoint Bundle were swapped with 3 cytokine panels, and a “drop-in” antibody (TGF- β 1) was added to build the Human T Cell Immune Checkpoint and Cytokine CyTOF Bundle, 40 Antibodies (hereafter referred to as the Cytokine Bundle). This bundle enables evaluation of T cell function by assessing the cytokine profile of T cell responses and the simultaneous characterization of key immune checkpoints.

All antibodies were titrated for optimal signal detection. Ordering information, antibody clones and corresponding metal conjugates for each Flex-Fit panel and high-parameter bundle can be found in Appendix A, B and C.

Frozen PBMC from 1 melanoma patient and 1 healthy donor were thawed and rested with or without stimulation before staining (see the Methods section for details). Briefly, corresponding antibodies were pooled to form an antibody cocktail and frozen at $-20\text{ }^{\circ}\text{C}$ as single-use aliquots to be used for all staining. PBMC were stained with surface antibodies, barcoded using the Cell-ID™ 20-Plex Pd Barcoding Kit (201060) and combined in a single tube. Checkpoint Bundle samples proceeded with Cell-ID Intercalator-Ir staining overnight at $4\text{ }^{\circ}\text{C}$ while Cytokine Bundle samples were first permeabilized and stained with intracellular antibodies before Cell-ID Intercalator-Ir staining overnight. Both samples were frozen at $-80\text{ }^{\circ}\text{C}$ and thawed before acquisition on the CyTOF XT system. This optional cryopreservation is a unique feature of CyTOF technology that enhances workflow flexibility. Samples can be conveniently acquired with no compromise to data quality.

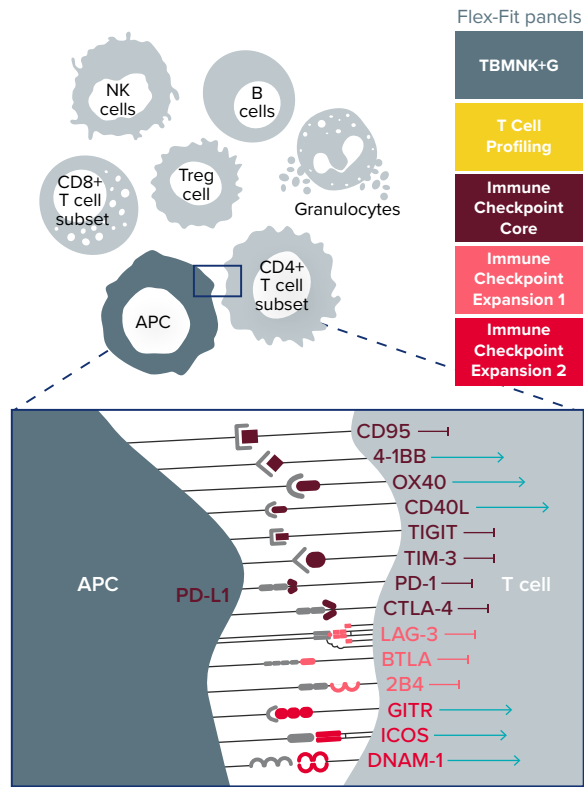
| Flex-Fit Panel | Markers | Cell Populations | Number of Cell Subsets |
|----------------|-------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------|
| TBMNK+G | CD3 CD4 CD8 CD14 CD16 CD19 CD45 CD56 CD66b |  CD4+ T cell  CD8+ T cell  Double-negative T cell (CD4-CD8-)  Monocyte Classical (CD14+CD16-) Transitional (CD14+CD16+)  Natural killer cell Mature (CD16+CD56+) Immature (CD16-CD56hi)  B cell (CD3-CD19+)  Granulocyte (CD45loCD66b+) | 15 |
| | CCR7 CD25 CD27 CD28 CD38 CD45RA CD45RO CD127 CD161 TCRαβ | Naive (CD45RA+CCR7+) Central memory (CD45RA-CCR7+) Naive (CD45RA+CCR7+) Central memory (CD45RA-CCR7+) Terminal effector (CD45RA+CCR7-) Terminal effector (CD45RA-CCR7-) Effector memory (CD45RA-CCR7-) Effector memory (CD45RA-CCR7-) Activated (CD38+CD25+) Activated (CD38+CD25+)  Treg-like cell (CD4+CD45RO+CD127lo/-CD25hi)  CD4- MAIT/NKT cell (CD4-CD28+CD161+) | 16 |

| Flex-Fit Panel | Markers | Functional Insights |
|-------------------------------|-------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Immune Checkpoint Core | PD-1, PD-L1, CTLA-4, CD40L, CD95, TIM-3, TIGIT, OX40, 4-1BB | These crucial immune checkpoint markers play essential roles in the regulation of T cell responses. |
| Immune Checkpoint Expansion 1 | LAG-3, 2B4, BTLA | These immune checkpoint molecules exert inhibitory effects on immune responses. |
| Immune Checkpoint Expansion 2 | DNAM-1, GITR, ICOS | These immune checkpoint molecules exert stimulatory effects on immune responses. |
| Cytokine Core | IL-2, IL-4, TNFα, IFNγ, IL-17 | These Th1/Th2/Th17 cytokines have important implications in immunopathology including autoimmune diseases and cancer. |
| Cytokine Expansion | IL-5, IL-10, IL-21 | These cytokines are produced by Th2 T cells, Tregs, and T follicular helper cells (Tfh), respectively. |
| Cytotoxic Mediators | IL-6, perforin, granzyme B | Granzyme B and perforin are commonly released by cytotoxic CD8 T cells and NK cells, mediating apoptosis in target cells. IL-6 is a pleiotropic cytokine with both pro- and anti-inflammatory functions. |

Figure 1. Flex-Fit CyTOF Panels enable phenotypic characterization of cell populations with functional insights. A) When combined, 2 Flex-Fit panels identify over 30 cell populations. Key markers used to define immune cell types are shown in parentheses. Refer to Supplementary Data 2 for the cell phenotypes of all identified cell populations. B) Six Flex-Fit panels (3 immune checkpoint panels and 3 cytokine panels) provide functional insights into immune potency and responses.

To view the expansive compatibility of Flex-Fit panels, go to standardbio.com/resources/panel-design to access Maxpar™ Panel Designer and a panel compatibility guide.

**Human Broad Immune Checkpoint
CyTOF Bundle, 34 Antibodies
(PN 201347)**
17 open channels



**Human T Cell Immune Checkpoint and
Cytokine CyTOF Bundle, 40 Antibodies
(PN 201348)**
11 open channels

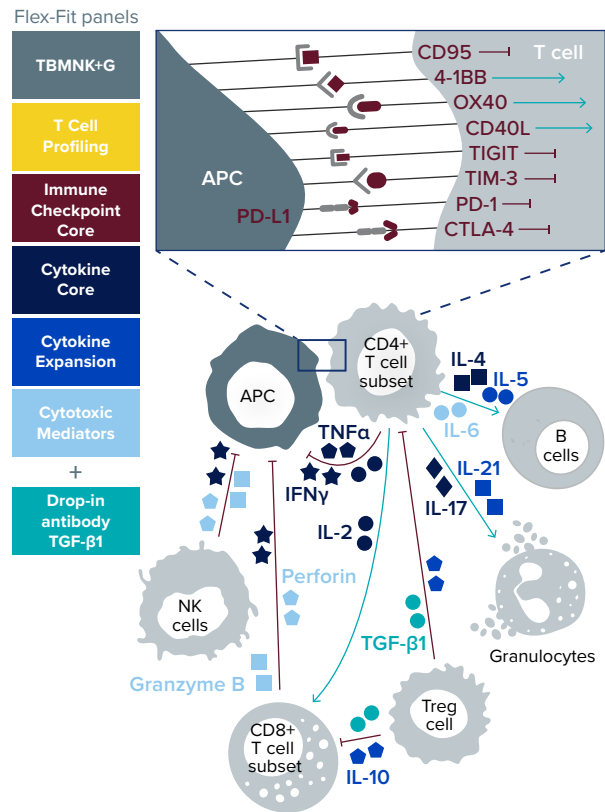


Figure 2. Two high-parameter bundles built from Flex-Fit CyTOF Panels allow for comprehensive characterization of cell populations and functional dynamics. Two high-parameter bundles were created using Flex-Fit panels listed in Figure 1. Left: Human Broad Immune Checkpoint CyTOF Bundle, 34 Antibodies consists of 5 Flex-Fit panels. Right: Human T Cell Immune Checkpoint and Cytokine CyTOF Bundle, 40 Antibodies consists of 6 Flex-Fit panels and the drop-in antibody TGF-β1. This second high-parameter bundle was created by swapping 2 Flex-Fit panels for 3 different Flex-Fit panels and adding 1 more catalog antibody. These 2 bundles can be further expanded or customized by adding antibodies in the available open channels to address a variety of research questions.

Results

Flex-Fit panels enable high-dimensional and deep immunophenotyping of over 30 cell populations

The combination of the Human TBMNK+G CyTOF Panel, 9 Antibodies TBMNK+G and the Human T Cell Profiling CyTOF Panel, 10 Antibodies T Cell Profiling Flex-Fit panels enables identification of over 30 immune cell types in peripheral blood (Figure 1A). Refer to Supplementary data Data 1 and 2 for information on gating strategy and identified cell populations. Using dimensionality reduction (opt-SNE) and FlowSOM clustering analysis, each distinct cell population is visualized as a uniquely colored cluster (Figure 3A).

The Checkpoint Bundle provides comprehensive functional characterization of immune checkpoints within T cell subsets

Immune checkpoint markers are expressed in activated T cells

The Checkpoint Bundle is able to evaluate 15 immune checkpoint markers in healthy and melanoma T cells. Overall, the majority of immune checkpoint markers have low expression on unstimulated T cells. Upon stimulation, all 15 immune checkpoint molecules are detectable on both healthy and melanoma T cells (Appendix E).

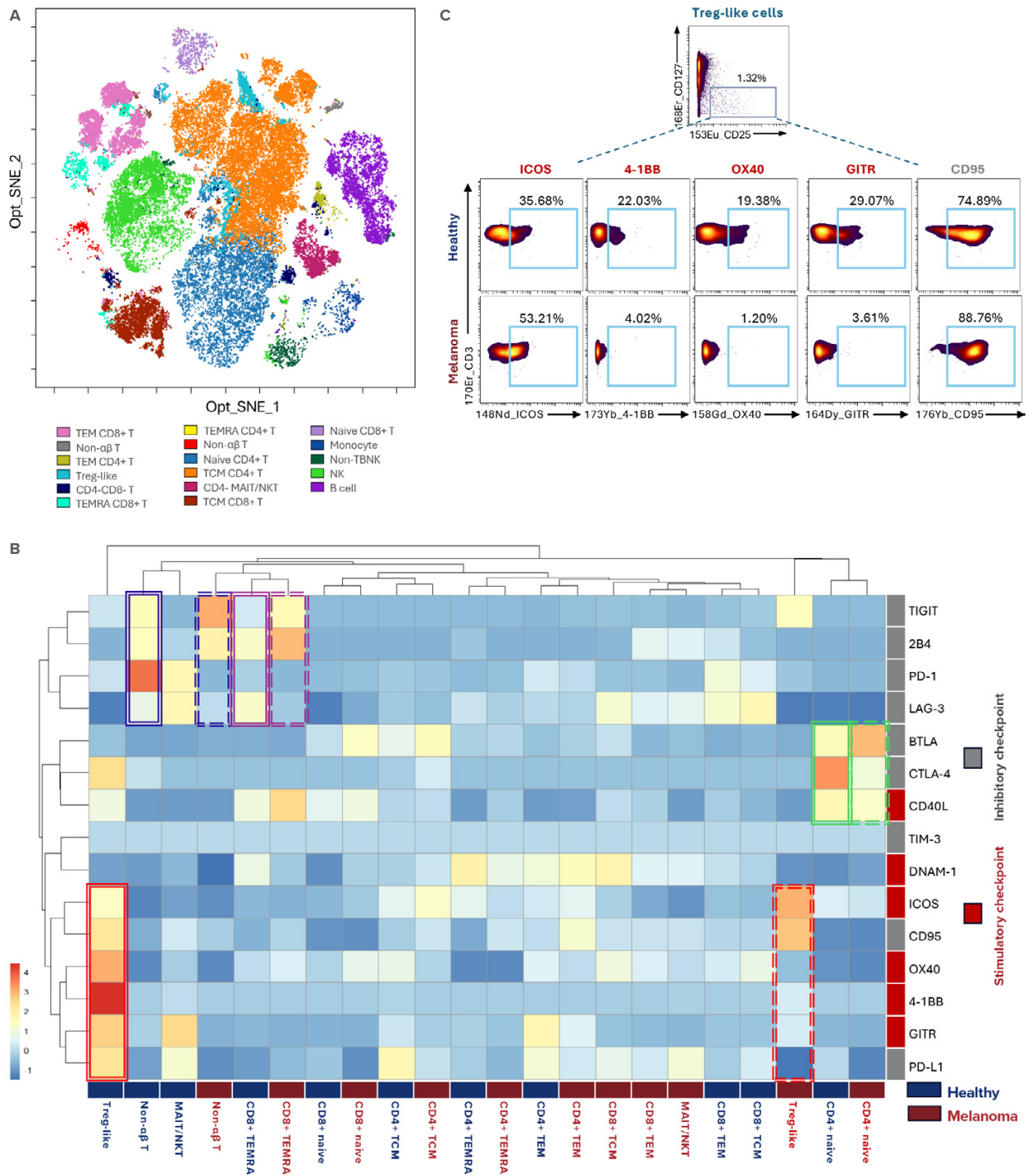


Figure 3. The Checkpoint Bundle enables comprehensive functional characterization of 15 immune checkpoint markers across 30-plus cell populations in PBMC. A) Visualization of various cell populations using opt-SNE and FlowSOM analysis. Fifty thousand events from the viable singlet PBMC population were proportionately sampled. The representative plot shows events within different cell populations from melanoma patient PBMC. The legend illustrates the different cell populations identified by FlowSOM clustering analysis. B) The median signal intensity of 15 immune checkpoint markers within each cell subset of unstimulated T cells was scaled, centered and hierarchically clustered in a heat map. The X-axis denotes melanoma T cells in red and healthy T cells in blue. The Y-axis denotes inhibitory checkpoint markers in grey and stimulatory checkpoint markers in red. Within the same cell subsets, the expression of selected clustered immune checkpoint markers in healthy (solid line) and melanoma (dashed line) T cells are shown in colored rectangles [red: Treg-like cell; green: CD4+ naive; purple: CD8+ TEMRA (terminal effector memory T cell); blue: non- $\alpha\beta$ T cell]. C) The biaxial plots of CD3 versus representative immune checkpoint markers (ICOS, CD95, OX40, GITR, 4-1BB) are displayed within the Treg-like cell subset of healthy and melanoma PBMC. Gate labels display percent of Treg-like cells positive for the immune checkpoint marker.

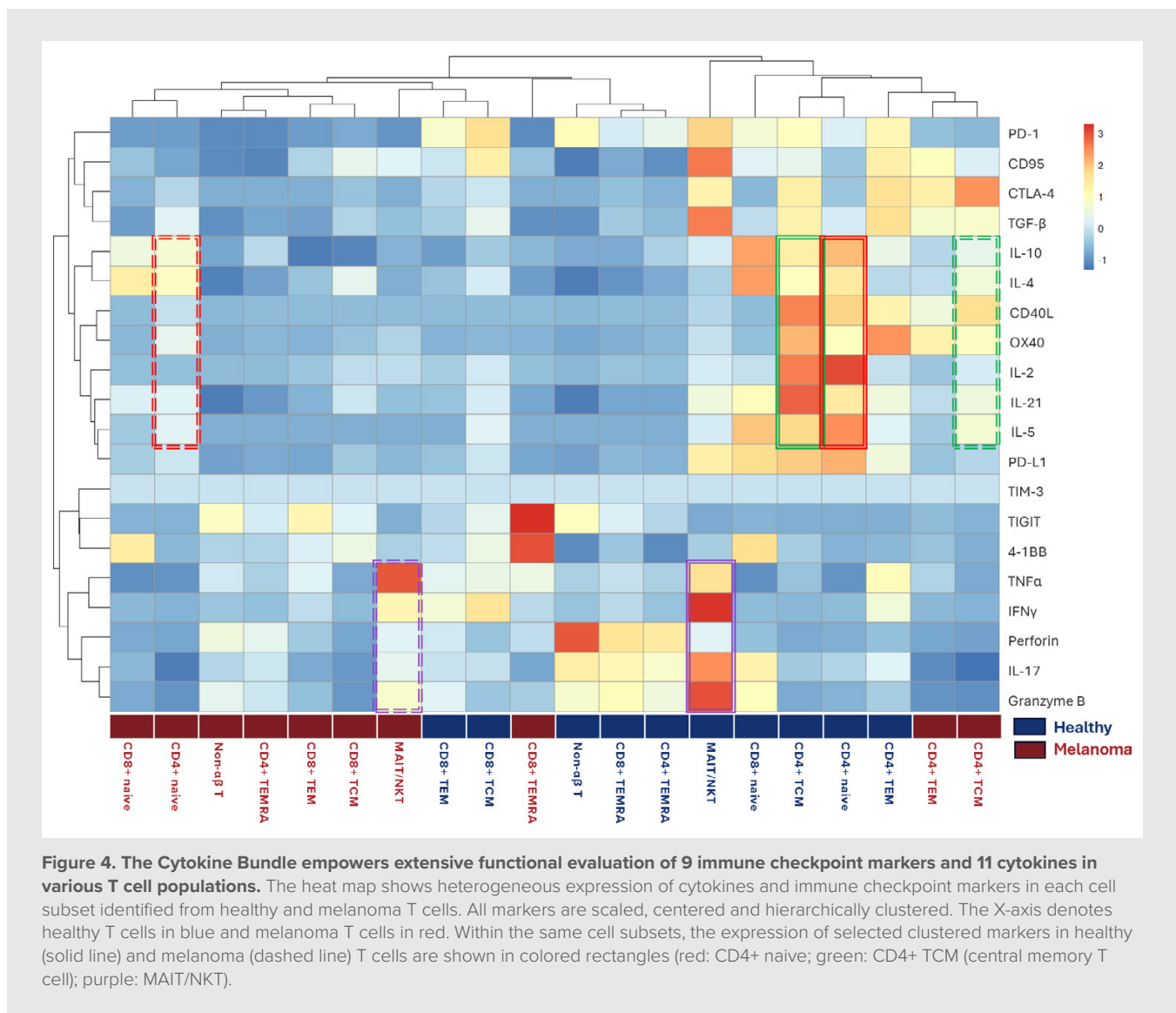
Immune checkpoint markers are differentially expressed in melanoma T cell subsets

The signal intensity of immune checkpoint markers from each subset of unstimulated T cells identified in Figure 3A is plotted in a hierarchical clustering heat map (Figure 3B). Interestingly, despite low basal-level expression of immune checkpoint markers, a comparison between healthy and melanoma T cells shows visible differences in several cell subsets (highlighted with colored rectangles in Figure 3B), with the most prominent difference seen in Treg (regulatory T cell)-like cells. A deeper exploration into this rare cell population (approximately 1% of CD4+ T cells) shows elevated expression of ICOS and CD95 and lower expression of OX40, 4-1BB and GITR in the melanoma patient sample (Figure 3C), further

exemplifying the findings of the clustering heat map. Overall, the upregulation of inhibitory checkpoints and downregulation of stimulatory checkpoints suggest a suppressive immune system in this melanoma patient¹.

The Cytokine Bundle simultaneously evaluates key immune checkpoints and cytokines in T cells

Cytokines play a pivotal role in cancer biology, acting as messengers to orchestrate cellular interactions and communications of the immune system. To further assess in-depth functionality of T cells from this melanoma patient, the Cytokine Bundle was used to evaluate cytokines and key immune checkpoint markers.



Immune checkpoint markers and cytokines are differentially expressed in melanoma T cell subsets

As expected, the Cytokine Bundle detects upregulation of all 9 immune checkpoint markers and 11 cytokines across diverse T cell subsets upon stimulation (Appendix F).

The signal intensity of all markers within each population of T cells is plotted in a hierarchical clustering heat map to evaluate differences between marker expression (Figure 4). Reduced cytokine production is seen within each subset of melanoma T cells compared with healthy T cells (visible differences are highlighted with colored rectangles in Figure 4), suggesting compromised immune competency of melanoma T cells².

Functional signatures of cytokines are identified in T cells

opt-SNE cluster maps demonstrate the distinct expression and distribution of 11 cytokines (Figure 5A). Functional signatures of low-expressing cytokines (for example, IL-4, IL-10, TGF- β) showcase the exceptionally high resolution of CyTOF technology.

Correlations between select cytokines and immune checkpoint markers are observed in melanoma T cells

Subsequent analysis of cytokine signaling pathways reveals intriguing correlations between cytokines and immune checkpoint markers, namely IL-2 and CD40L, and TGF- β and CTLA-4, which co-localize on the opt-SNE cluster map (Figure 5B). It is well known that the CD40 pathway is a key regulator of IL-2 production and antitumor immune response. Higher expression of IL-2 generated by the interaction of CD40L and CD40 between T helper cells and macrophages predicts better clinical outcomes in colorectal cancer³. On the other hand, the TGF- β autocrine loop drives FoxP3-mediated expression of CTLA-4 in Tregs, which in turn restrains co-stimulation of T cells⁴. Upregulation of TGF- β /CTLA-4 and downregulation of IL-2/CD40L in localized clusters suggest dysregulated cytokine signaling pathways, implying the presence of activated Tregs and compromised T cell function in this melanoma patient.

In summary, these 2 high-parameter bundles enable deep immune profiling of PBMC by comprehensively evaluating immune checkpoints and cytokines, thereby capturing functional insights into the immune competency of this melanoma patient.

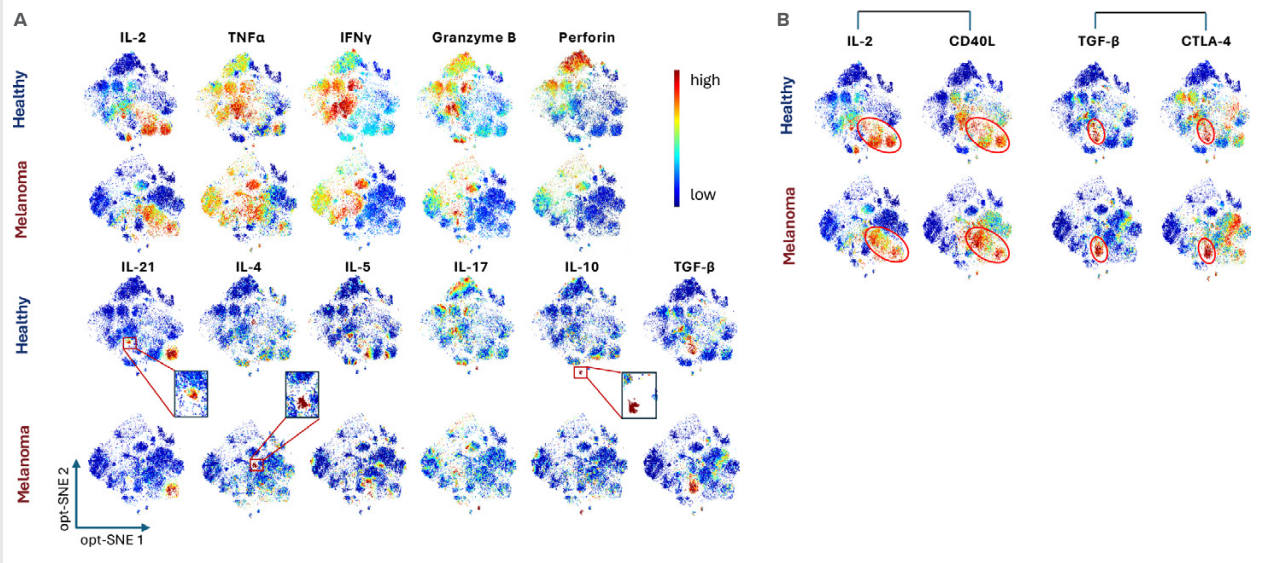


Figure 5. The Cytokine Bundle captures functional signatures of cytokines and immune checkpoints in T cells. A) opt-SNE cluster map of cytokine expression. opt-SNE analysis was performed on T cells to generate maps that represent the expression of each cytokine on the opt-SNE cell clusters of healthy and melanoma T cells. Top 2 rows: cytokines that express on multiple clusters; bottom 2 rows: cytokines whose expression is limited to a single cluster or a few small island clusters. Insets indicate rare populations detected by the Cytokine Bundle. B) Co-localization of select cytokines and immune checkpoint markers (outlined in red) was detected by the Cytokine Bundle. These correlations suggest the presence of a dysregulated cytokine signaling pathway, implying that T cell functionality is compromised in this melanoma patient.

Discussion

Explicit detection of intracellular cytokines uncovers the true functional biology of immune cells

As messengers between various immune cell types, cytokines play a crucial role in immune responses by controlling activation, differentiation and exhaustion of immune cells. Therefore, unveiling the complex crosstalk of cytokines and their signaling pathways that are masked by surface markers provides insights into the true biology of immune evasion and antitumor responses.

The Cytokine Bundle provides functional readouts of 12 cytokines and cytotoxic mediators at the single-cell level, enabling the evaluation of polyfunctionality of the diverse T cell subsets identified by this bundle. The remarkable resolution of CyTOF technology clearly resolves even rare cytokine-secreting cell populations (Figure 5A). Furthermore, this bundle facilitates the understanding of cytokine signaling pathways in cancer patients by tying in corresponding immune checkpoint markers (Figure 5B).

Simplifying high-parameter cytometry with Flex-Fit panels

Modularity

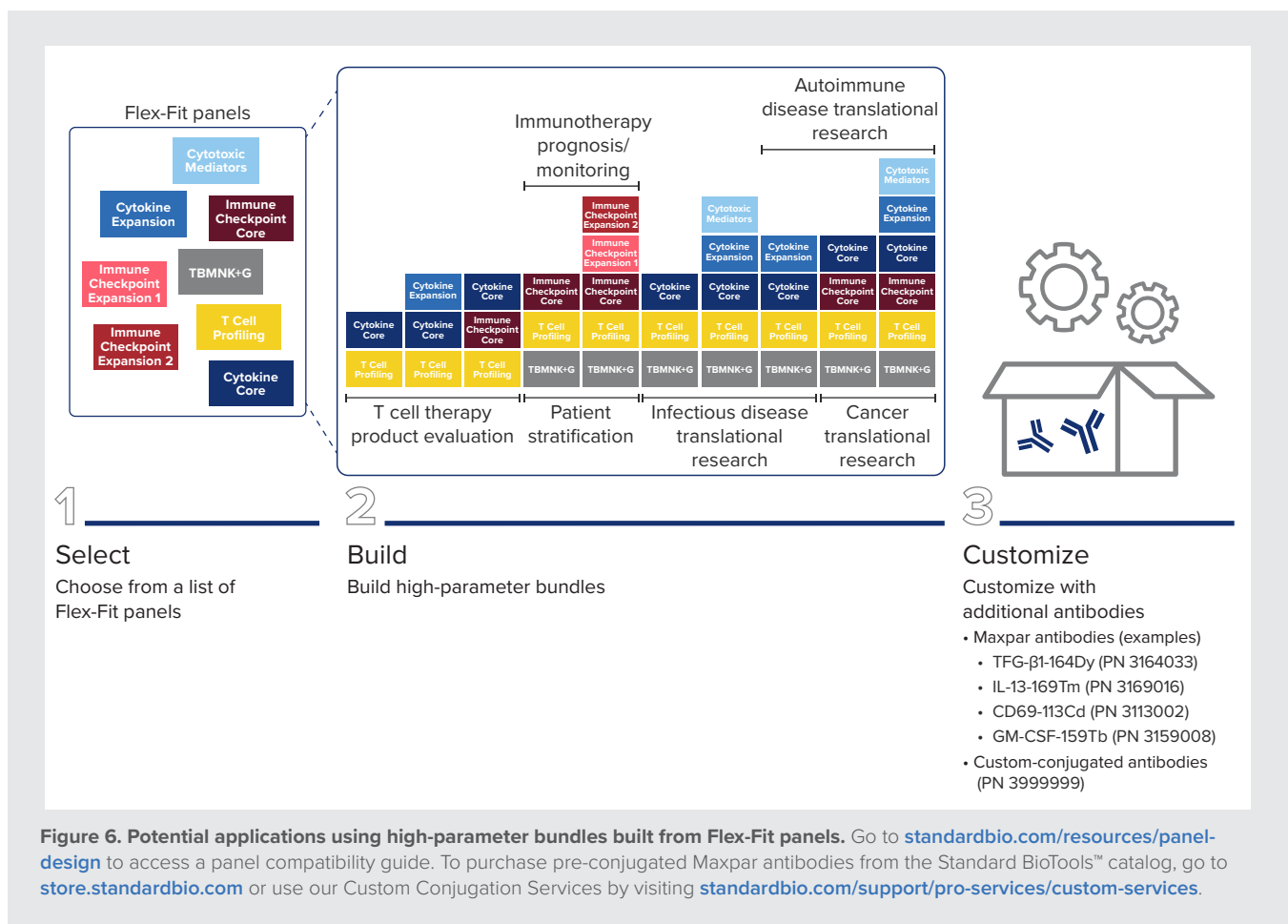
In this application note, we present 2 examples of high-parameter bundles built from Flex-Fit panels, the Checkpoint Bundle and the Cytokine Bundle. The modular nature of Flex-Fit panels allows us to interrogate the cytokine profile of immune cells by simply swapping out 2 panels from the Checkpoint Bundle, thereby avoiding lengthy and laborious panel redesign and validation.

Flexibility

Beyond these examples, different combinations of Flex-Fit panels can be created to address various research questions, such as translational research for autoimmune disease and infectious disease (Figure 6).

Customizability

Flex-Fit panels may be further customized by adding additional targets of interest into the open and available channels. For instance, to assess T cell activation, CD69 (available in the Maxpar antibody catalog in a compatible channel) can be added to the panel (Figure 6).



Note: The Human Broad Immune Profiling CyTOF Panel, 20 Antibodies (201339) is also compatible with the Human Immune Checkpoint Expansion 1 CyTOF Panel, 3 Antibodies (201342), the Human Immune Checkpoint Expansion 2 CyTOF Panel, 3 Antibodies (201343), the Human Cytokine Core CyTOF Panel, 5 Antibodies (201344) and the Human Cytotoxic Mediators CyTOF Panel, 3 Antibodies (201345).

Conclusions

- Two high-parameter bundles built from Flex-Fit panels revealed a cellular signature of compromised immune competency in melanoma PBMC by:
 - Enabling deep immune profiling on 15 immune checkpoint markers and 12 cytokines on a single-cell level across 30-plus immune cell populations
 - Identifying rare cell populations with exceptional resolution
- Flex-Fit panels can be mixed and matched to build application-specific bundles for investigating applications such as autoimmunity, infectious disease, CAR T cell therapies and cancer translational research

CyTOF features

This application note demonstrates a variety of unique CyTOF features, including:

- **Frozen antibody cocktail**

Metal-tagged antibodies used in CyTOF technology can be pooled as an antibody cocktail and frozen at -20°C , as demonstrated in this application note. This unique feature of CyTOF platforms can significantly improve data consistency in studies with repetitive staining and acquisition cycles, either spread over time or across different sites for sample collection and analysis⁵.

- **Sample multiplexing**

In this study, palladium (Pd) fixed-cell barcoding was performed on surface-stained samples prior to intracellular staining using the Cell-ID 20-Plex Pd Barcoding Kit. The use of cell barcoding maximizes the efficiency of CyTOF systems by enabling sample multiplexing for high-parameter single-cell analysis⁶. Barcoding is especially advantageous in large studies by simplifying workflows, increasing sample throughput, improving data consistency and minimizing variability in sample staining.

- **Flexible whole blood workflows**

In this application note, 2 high-parameter bundles were used to evaluate PBMC only. However, each antibody in the bundles is compatible with fresh and stabilized whole blood. Compatibility of Flex-Fit panels with stabilization reagents, such as Proteomic Stabilizer PROT1 (Smart Tube Inc.) is summarized in the product technical datasheet, available to download from store.standardbio.com.

- **Streamlined data analysis**

CyTOF Software accelerates data analysis as no data manipulation is required post-acquisition. For seamless analysis, pre-designed gating strategies and templates are available for use with each high-parameter bundle. Data generated with these bundles can be analyzed in third-party flow cytometry software such as FlowJo and Cytobank Analysis software, further minimizing the time required to transition from data collection to biological insights.

Tips for success

- Calculate the amount of antibody needed based on the study design and prepare 10% excess cocktail for multiple samples. Freeze the cocktail in single-use aliquots.
- Expression of markers may vary depending on disease state and stimulation conditions. We recommend titration of the panel antibodies in the specific context of the application.
- When using healthy donor samples, the majority of immune checkpoint markers and cytokines require stimulation to be detected. For the best results, we recommend phytohemagglutinin (PHA) stimulation for all immune checkpoint markers and PMA/I stimulation for all cytokines except TGF- β 1.
- Despite being classified as a cytokine, TGF- β 1/LAP can only be detected when surface staining is performed. Also, apart from other cytokines, TGF- β 1 is only visible under specific stimulation (for example, PHA) when healthy donor samples are utilized.
- Although they are surface markers, CD40L and CTLA-4 were best detected when stained using an intracellular staining workflow
- We recommend staining cells in excess to ensure the required number of events are acquired
- For maximum cell recovery, rinse each individual sample tube after combining the barcoded samples
- Scale up buffers and antibody volumes according to cell number as described in the Cell-ID 20-Plex Pd Barcoding Kit User Guide (PRD023)

Methods

Frozen antibody cocktail preparation

Surface antibodies (including Cell-ID Intercalator-103Rh – 500 μ M) and intracellular antibodies from each bundle were pooled to make separate antibody cocktails, respectively, and frozen at -20°C as single-use aliquots the day before the experiment. The frozen antibody cocktails were thawed at room temperature (RT) and immediately used for staining cells. All staining in this study was performed using frozen antibody cocktails.

PBMC handling, stimulation and staining

PBMC from a healthy donor and a melanoma patient were obtained from STEMCELL Technologies and Discovery Life Sciences, respectively. A multistep staining procedure was carried out (Appendix D). Cells were thawed in complete RPMI 1640 (cRPMI) containing Anti-Aggregate Wash supplement (Cellular Technology) following manufacturer instructions. After resting for 4 hr, 1×10^6 cells were stimulated with 10 μ g/mL PHA and 1 μ g/mL lipopolysaccharide (LPS) overnight at 37°C . The next day, cells designated for Cytokine Bundle staining were further stimulated with 25 ng/mL phorbol 12-myristate 13-acetate and 500 ng/mL ionomycin (PMA/I) for 6 hr at RT in the presence of 5 μ g/mL brefeldin A (BFA) and 2 μ M monensin.

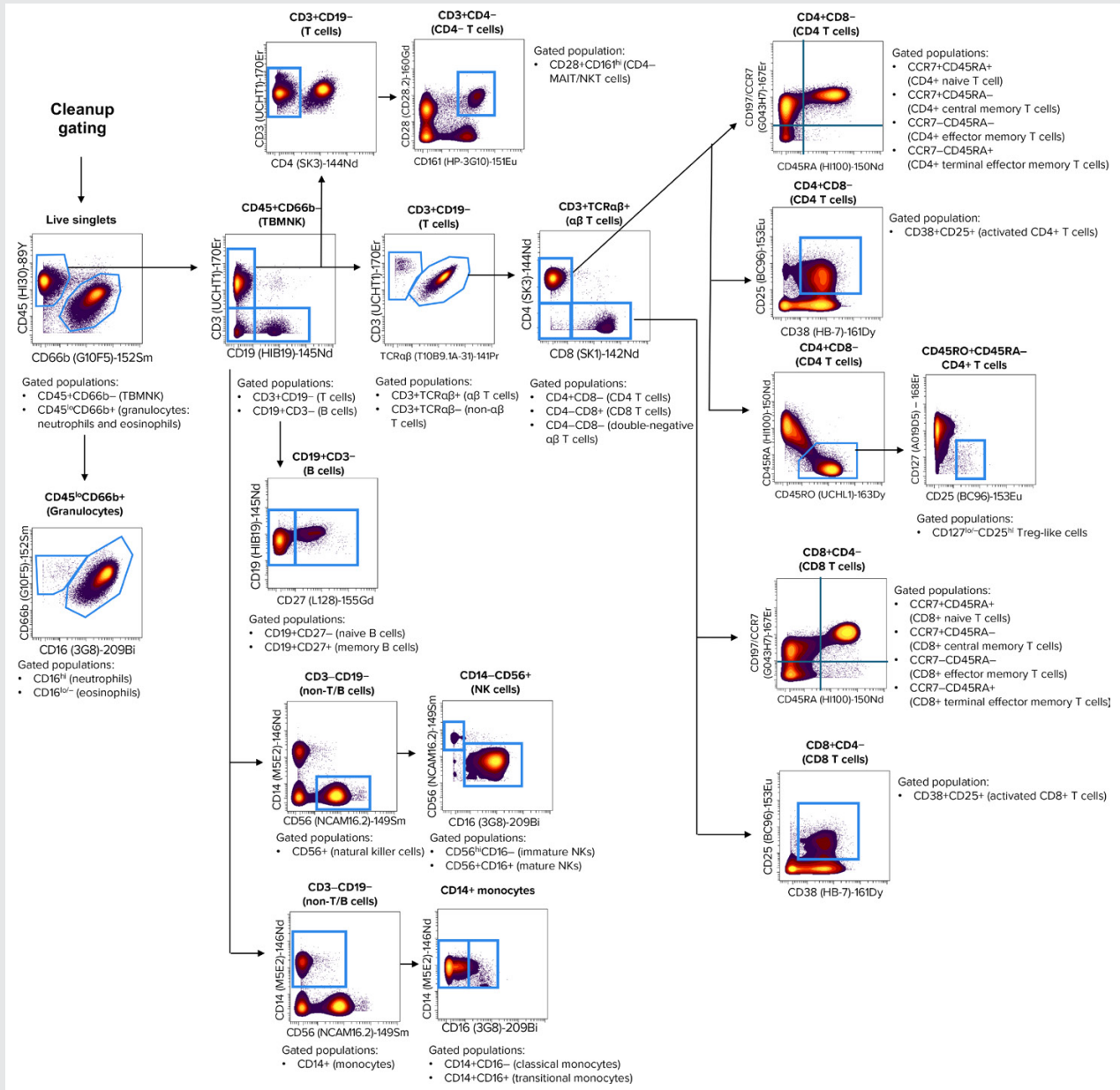
After 6 hr, cells were harvested, washed with Maxpar Cell Staining Buffer (CSB) and blocked with Human TruStain FcX (BioLegend) before surface staining at RT for 30 min using frozen surface antibody cocktails⁷. Cells were then barcoded using the Cell-ID 20-Plex Pd Barcoding Kit according to the user guide and pooled together in a single tube. The Checkpoint Bundle sample was fixed and incubated with 25 nM of Cell-ID Intercalator-Ir overnight at 4°C . The Cytokine Bundle sample was permeabilized and stained with the frozen intracellular antibody cocktail before intercalator staining overnight at 4°C ⁷. Both samples were frozen and thawed before acquisition on the CyTOF XT system.

Acquisition and analysis

Briefly, antibody-stained samples were washed with CSB and Maxpar Cell Acquisition Solution Plus, filtered through a cell strainer and counted according to the sample preparation for acquisition instructions in the Maxpar Cell Staining with Fresh Fix User Guide (FLDM-01319). All samples in this study were acquired on a CyTOF XT system.

Original FCS files were normalized using CyTOF Software v9.0.2 and subsequently debarcoded to obtain individual FCS files. The FCS files were analyzed using Cytobank Analysis software.

Supplemental data 1: Gating strategy*



* Plots are derived from a combination of whole blood and PBMC samples.

Supplemental data 2: Population gating table for 31 immune cell populations

| No. | Name | Phenotype |
|-----|--------------------------------|-----------------------------------------------------------------------|
| 1 | Granulocytes | CD45loCD66b+ |
| 2 | Neutrophils | CD45loCD66b+CD16hi |
| 3 | Eosinophils | CD45loCD66b+CD16lo/- |
| 4 | CD45+ (TBMNK) | CD45+CD66b- |
| 5 | Monocytes | CD45+CD66b-CD3-CD19-CD14+ |
| 6 | Classical monocytes | CD45+CD66b-CD3-CD19-CD14+CD16- |
| 7 | Transitional monocytes | CD45+CD66b-CD3-CD19-CD14+CD16+ |
| 8 | Natural killer cells | CD45+CD66b-CD3-CD19-CD14-CD56+ |
| 9 | Immature natural killer cells | CD45+CD66b-CD3-CD19-CD14-CD56hiCD16- |
| 10 | Mature natural killer cells | CD45+CD66b-CD3-CD19-CD14-CD56+CD16+ |
| 11 | Total B cells | CD45+CD66b-CD3-CD19+ |
| 12 | Naive B cells | CD45+CD66b-CD3-CD19+CD27- |
| 13 | Memory B cells | CD45+CD66b-CD3-CD19+CD27+ |
| 14 | Total T cells | CD45+CD66b-CD19-CD3+ |
| 15 | CD4- MAIT/NKT cells | CD45+CD66b-CD19-CD3+CD4-CD28+CD161hi |
| 16 | $\alpha\beta$ T cells | CD45+CD66b-CD19-CD3+TCR $\alpha\beta$ + |
| 17 | Non- $\alpha\beta$ T cells | CD45+CD66b-CD19-CD3+TCR $\alpha\beta$ - |
| 18 | CD4+ T cells | CD45+CD66b-CD19-CD3+TCR $\alpha\beta$ +CD8-CD4+ |
| 19 | CD4+ naive T cells | CD45+CD66b-CD19-CD3+TCR $\alpha\beta$ +CD8-CD4+CCR7+CD45RA+ |
| 20 | CD4+ central memory T cells | CD45+CD66b-CD19-CD3+TCR $\alpha\beta$ +CD8-CD4+CCR7+CD45RA- |
| 21 | CD4+ effector memory T cells | CD45+CD66b-CD19-CD3+TCR $\alpha\beta$ +CD8-CD4+CCR7-CD45RA- |
| 22 | CD4+ terminal effector T cells | CD45+CD66b-CD19-CD3+TCR $\alpha\beta$ +CD8-CD4+CCR7-CD45RA+ |
| 23 | Activated CD4+ T cells | CD45+CD66b-CD19-CD3+TCR $\alpha\beta$ +CD8-CD4+CD38+CD25+ |
| 24 | Treg-like cells | CD45+CD66b-CD19-CD3+TCR $\alpha\beta$ +CD8-CD4+CD45RO+CD127lo/-CD25hi |
| 25 | CD8+ T cells | CD45+CD66b-CD19-CD3+TCR $\alpha\beta$ +CD4-CD8+ |
| 26 | CD8+ naive T cells | CD45+CD66b-CD19-CD3+TCR $\alpha\beta$ +CD4-CD8+CCR7+CD45RA+ |
| 27 | CD8+ central memory T cells | CD45+CD66b-CD19-CD3+TCR $\alpha\beta$ +CD4-CD8+CCR7+CD45RA- |
| 28 | CD8+ effector memory T cells | CD45+CD66b-CD19-CD3+TCR $\alpha\beta$ +CD4-CD8+CCR7-CD45RA- |
| 29 | CD8+ terminal effector T cells | CD45+CD66b-CD19-CD3+TCR $\alpha\beta$ +CD4-CD8+CCR7-CD45RA+ |
| 30 | Activated CD8+ T cells | CD45+CD66b-CD19-CD3+TCR $\alpha\beta$ +CD4-CD8+CD38+CD25+ |
| 31 | Double-negative T cells | CD45+CD66b-CD19-CD3+TCR $\alpha\beta$ +CD8-CD4- |

Appendix A: Ordering information

| Standard BioTools Reagents | Part Number |
|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------|
| Human Broad Immune Checkpoint CyTOF Bundle, 34 Antibodies* | 201347 |
| Human T Cell Immune Checkpoint and Cytokine CyTOF Bundle, 40 Antibodies* | 201348 |
| Maxpar Cell Surface Staining Kit, containing: <ul style="list-style-type: none"> • Cell-ID Intercalator-Ir, 12.5 μM – 50 μL • Maxpar Cell Acquisition Solution Plus – 200 mL • Maxpar Cell Staining Buffer – 500 mL • Maxpar PBS – 100 mL • Maxpar Fix and Perm Buffer – 25 mL | 201601 |
| Maxpar Cytoplasmic/Secreted Antigen Staining Kit, containing: <ul style="list-style-type: none"> • Cell-ID Intercalator-Ir, 12.5 μM – 50 μL • Maxpar Cell Acquisition Solution Plus – 200 mL • Maxpar Cell Staining Buffer – 500 mL • Maxpar Fix I Buffer (5X) – 15 mL • Maxpar Fix and Perm Buffer – 25 mL • Maxpar PBS – 100 mL • Maxpar Perm-S Buffer – 250 mL | 201602 |
| Maxpar Cell Acquisition Solution Plus for CyTOF XT – 1,000 mL | 201244 |
| Cell-ID Intercalator-Rh – 500 μ M | 201103A |
| Cell-ID 20-Plex Pd Barcoding Kit | 201060 |
| Maxpar Water – 500 mL | 201069 |

* Refer to Appendix B and C for ordering information for individual antibodies in the bundle.

Appendix B: Antibody composition in the Checkpoint Bundle

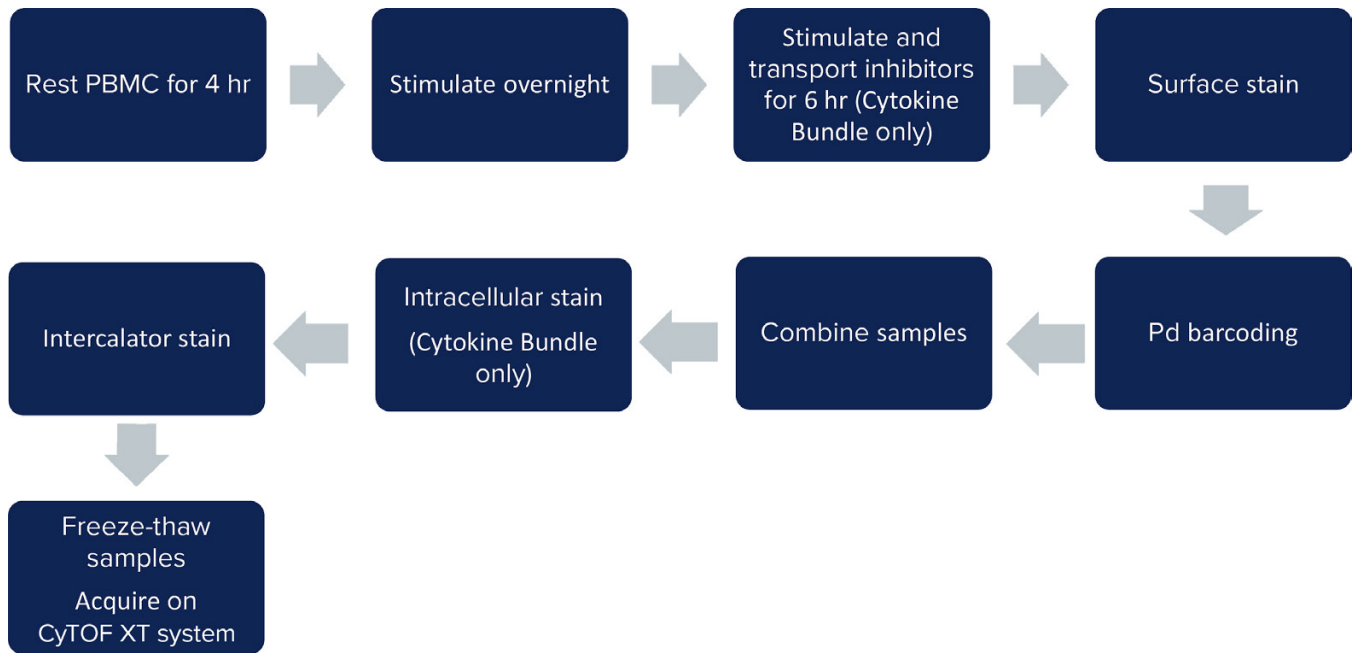
| Flex-Fit Panel | Target | Clone | Metal | Part No. | |
|--------------------------------------------------------------------|------------------------------------------------------------------------|--------------|-------------|----------|---------|
| Human Broad Immune Checkpoint CyTOF Bundle, 34 Antibodies (201347) | CD45 | HI30 | 89Y | 3089003 | |
| | CD8a | SK1 | 142Nd | 3142021 | |
| | CD4 | SK3 | 144Nd | 3144029 | |
| | CD19 | HIB19 | 145Nd | 3145020 | |
| | CD14 | M5E2 | 146Nd | 3146022 | |
| | CD56 | NCAM16.2 | 149Sm | 3149021 | |
| | CD66b | G10F5 | 152Sm | 3152019 | |
| | CD3 | UCHT1 | 170Er | 3170001 | |
| | CD16 | 3G8 | 209Bi | 3209002 | |
| | Human T Cell Profiling CyTOF Panel, 10 Antibodies (201340) | TCRαβ | T10B9.1A-31 | 141Pr | 3141022 |
| | | CD45RA | HI100 | 150Nd | 3150034 |
| | | CD161 | HP-3G10 | 151Eu | 3151029 |
| | | CD25 | BC96 | 153Eu | 3153032 |
| | | CD27 | L128 | 155Gd | 3155001 |
| | | CD28 | CD28.2 | 160Gd | 3160003 |
| | | CD38 | HB-7 | 161Dy | 3161034 |
| | | CD45RO | UCHL1 | 163Dy | 3163032 |
| | | CD197/CCR7 | G043H7 | 167Er | 3167009 |
| | | CD127/IL-7Rα | A019D5 | 168Er | 3168017 |
| | Human Immune Checkpoint Core CyTOF Panel, 9 Antibodies (201341) | CD154/CD40L | 24-31 | 143Nd | 3143033 |
| | | CD366/TIM-3 | F38-2E2 | 154Sm | 3154010 |
| | | CD279/PD-1 | EH12.2H7 | 156Gd | 3156038 |
| | | CD134/OX40 | ACT35 | 158Gd | 3158012 |
| | | CD152/CTLA-4 | 14D3 | 162Dy | 3162039 |
| | | TIGIT | MBSA43 | 166Er | 3166033 |
| | | CD137/4-1BB | 4B4-1 | 173Yb | 3173015 |
| | | CD274/PD-L1 | MIH1 | 174Yb | 3174027 |
| | | CD95/Fas | DX2 | 176Yb | 3176029 |
| | Human Immune Checkpoint Expansion 1 CyTOF Panel, 3 Antibodies (201342) | CD244/2B4 | PP35 | 165Ho | 3165046 |
| | | CD223/LAG-3 | 11C3C65 | 172Yb | 3172035 |
| | | CD272/BTLA | MIH26 | 175Lu | 3175042 |
| | Human Immune Checkpoint Expansion 2 CyTOF Panel, 3 Antibodies (201343) | CD278/ICOS | C398.4A | 148Nd | 3148019 |
| | | CD357/GITR | 108-17 | 164Dy | 3164034 |
| | | CD226/DNAM-1 | DX11 | 171Yb | 3171013 |

Appendix C: Antibody composition in the Cytokine Bundle

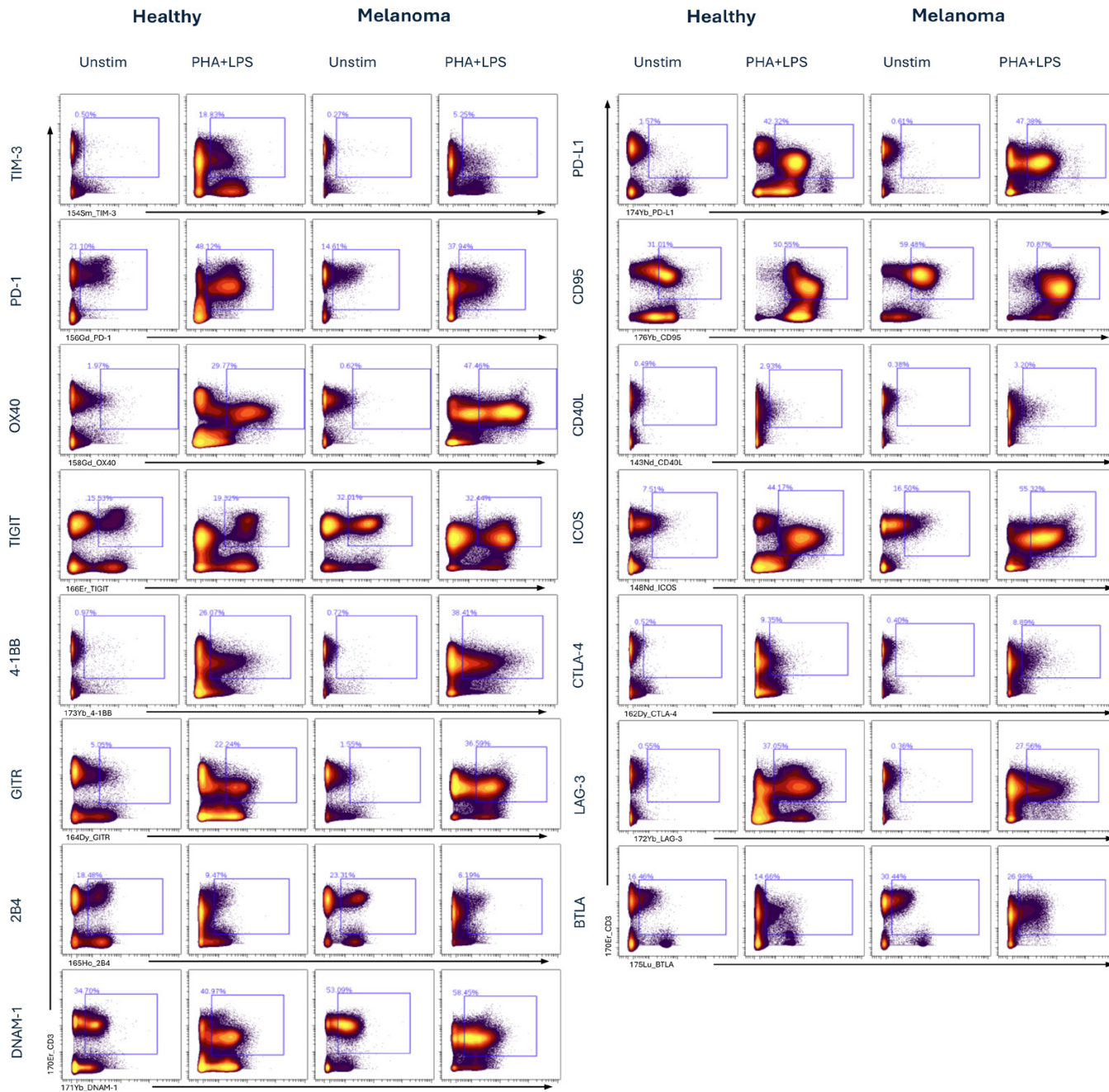
| Flex-Fit Panel | Target | Clone | Metal | Part No. | |
|----------------------------------------------------------------------------------|-----------------------------------------------------------------|--------------|-------------|----------|---------|
| Human T Cell Immune Checkpoint and Cytokine CyTOF Bundle, 40 Antibodies (201348) | CD45 | HI30 | 89Y | 3089003 | |
| | CD8a | SK1 | 142Nd | 3142021 | |
| | CD4 | SK3 | 144Nd | 3144029 | |
| | CD19 | HIB19 | 145Nd | 3145020 | |
| | CD14 | M5E2 | 146Nd | 3146022 | |
| | CD56 | NCAM16.2 | 149Sm | 3149021 | |
| | CD66b | G10F5 | 152Sm | 3152019 | |
| | CD3 | UCHT1 | 170Er | 3170001 | |
| | CD16 | 3G8 | 209Bi | 3209002 | |
| | Human T Cell Profiling CyTOF Panel, 10 Antibodies (201340) | TCRαβ | T10B9.1A-31 | 141Pr | 3141022 |
| | | CD45RA | HI100 | 150Nd | 3150034 |
| | | CD161 | HP-3G10 | 151Eu | 3151029 |
| | | CD25 | BC96 | 153Eu | 3153032 |
| | | CD27 | L128 | 155Gd | 3155001 |
| | | CD28 | CD28.2 | 160Gd | 3160003 |
| | | CD38 | HB-7 | 161Dy | 3161034 |
| | | CD45RO | UCHL1 | 163Dy | 3163032 |
| | | CD197/CCR7 | G043H7 | 167Er | 3167009 |
| | | CD127/IL-7Rα | A019D5 | 168Er | 3168017 |
| | Human Immune Checkpoint Core CyTOF Panel, 9 Antibodies (201341) | CD154/CD40L | 24-31 | 143Nd | 3143033 |
| | | CD366/TIM-3 | F38-2E2 | 154Sm | 3154010 |
| | | CD279/PD-1 | EH12.2H7 | 156Gd | 3156038 |
| | | CD134/OX40 | ACT35 | 158Gd | 3158012 |
| | | CD152/CTLA-4 | 14D3 | 162Dy | 3162039 |
| | | TIGIT | MBSA43 | 166Er | 3166033 |
| | | CD137/4-1BB | 4B4-1 | 173Yb | 3173015 |
| | | CD274/PD-L1 | MIH1 | 174Yb | 3174027 |
| | CD95/Fas | DX2 | 176Yb | 3176029 | |
| | Human Cytokine Core CyTOF Panel, 5 Antibodies (201344) | IL-2 | MQ1-17H12 | 112Cd | 3112002 |
| | | TNFα | MAb11 | 114Cd | 3114002 |
| | | IFNγ | B27 | 116Cd | 3116002 |
| | | IL-4 | MP4-25D2 | 171Yb | 3171028 |
| | | IL-17A | BL168 | 195Pt | 3195002 |
| | Human Cytotoxic Mediators CyTOF Panel, 3 Antibodies (201345) | IL-6* | MQ2-13A5 | 106Cd | 3106003 |
| | | Perforin | B-D48 | 196Pt | 3196002 |
| | | Granzyme B | GB11 | 198Pt | 3198002 |
| | Human Cytokine Expansion CyTOF Panel, 3 Antibodies (201346) | IL-5 | TRFK5 | 147Sm | 3147023 |
| | | IL-10 | JES3-9D7 | 148Nd | 3148025 |
| | | IL-21 | 3A3-N2 | 172Yb | 3172011 |
| | Individual Maxpar antibody | TGF-β1/LAP | S20006A | 164Dy | 3164033 |

* IL-6 was not used in this application note due to incompatibility with the Cell-ID 20-Plex Pd Barcoding Kit.

Appendix D: Workflow overview of staining procedure



Appendix E: Expression of immune checkpoint markers on unstimulated and PHA+LPS-stimulated T cells using the Checkpoint Bundle



Appendix F: Expression of immune checkpoint markers and cytokines in unstimulated and PHA+LPS+PMA/I-stimulated T cells using the Cytokine Bundle



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