PN 400248 B1
TECHNICAL NOTE

Approach to Bivariate Analysis of Data Acquired Using the Maxpar Direct Immune Profiling Assay

Introduction

The Fluidigm Maxpar® Direct™ Immune Profiling Assay™ (Cat. No. 201325) was validated with Maxpar Pathsetter™ software (Cat. No. 401018). The software provides fast, reliable, and flexible automated analysis of FCS files from human PBMC and whole blood cells stained with the Maxpar Direct Immune Profiling Assay and acquired on a Helios™ mass cytometer. 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, bivariate gating can be used to define and enumerate these immune populations. The Maxpar Direct Immune Profiling Assay gating example, provided here, was developed using Cytobank, specifically for use with the Maxpar Direct Immune Profiling Assay. This gating strategy is based on the flow cytometry results data templates created by the Human Immunology Project Consortium (HIPC) [1], and on the Maxpar Direct Immune Profiling Assay probability state model used by Maxpar Pathsetter software. The gating example provides recommendations for gating immune populations in whole blood using markers available in the kit. The gating example can also be used for PBMC and is available as Fluidigm_Maxpar Direct Immune Profiling Assay_201325_Gating Example_v1.0.

Symbols and Abbreviations in This Document

Symbols

Purple squares denote navigation options within Cytobank.

Pink squares denote example populations in plots.

Terms and Abbreviations

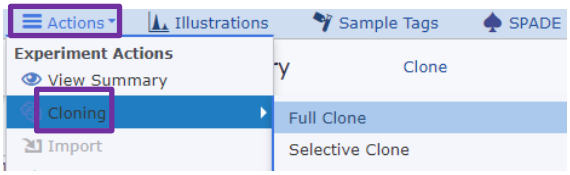
Beads: EQ™ Four Element Calibration Beads (Cat. No. 201078)
DC: dendritic cell
MAIT cell: mucosal-associated invariant T cell

mDC: myeloid dendritic cell
NK cell: natural killer cell
NKT cell: natural killer T cell
pDC: plasmacytoid dendritic cell
Treg: regulatory T cell
Th: T helper cell

Accessing the Public Experiment

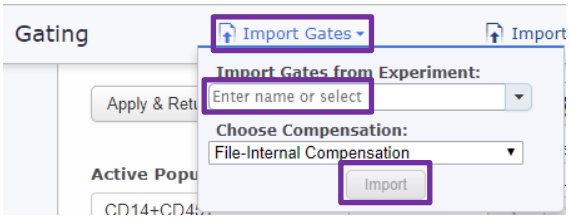
An example analysis for the panel kit, Fluidigm_Maxpar Direct Immune Profiling Assay_201325_Gating Example_v1.0, is available for reference at premium.cytobank.org.

Create a Clone of the Public Experiment



The Public Experiment is read-only. To access the Gating tab, create a personal copy by cloning the experiment (available under the Actions tab).

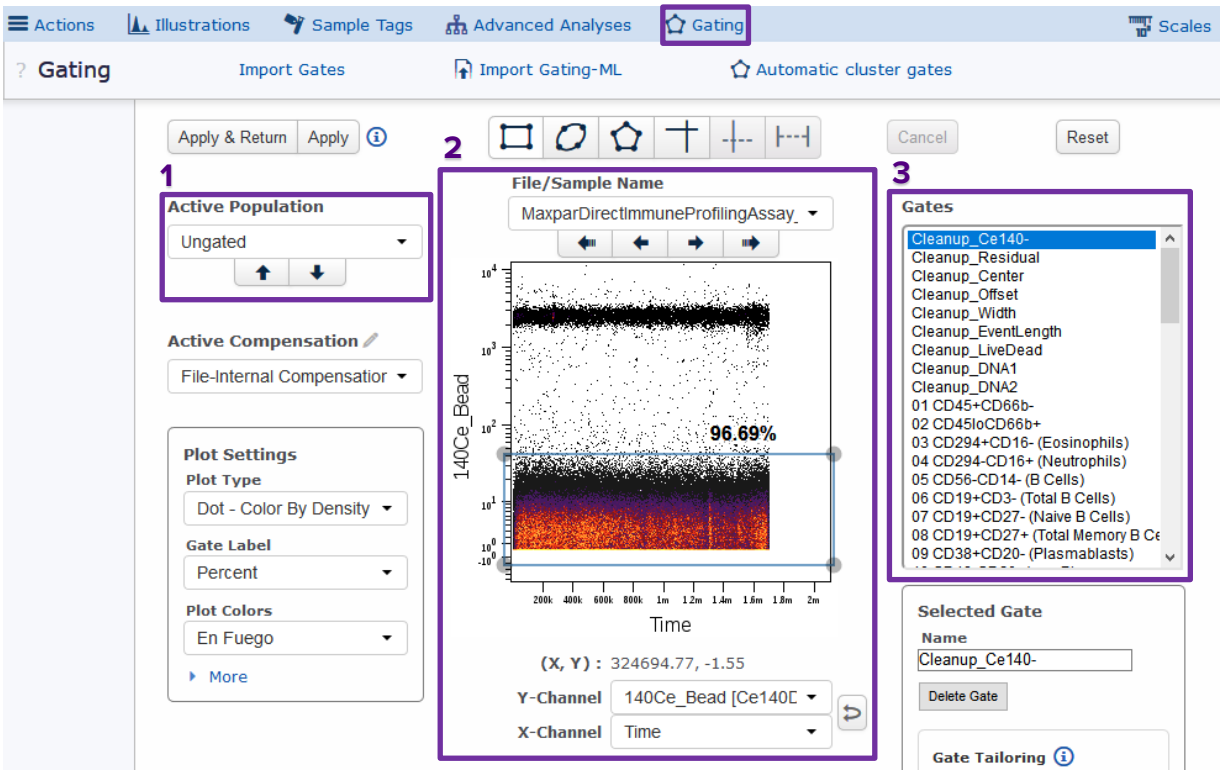
Apply Gating Strategy to New Data



The gating strategy can be applied to new experiments by using the Import Gates function in the Gates tab. Enter the Cytobank experiment number **221569** in the dialog box and click **Import**. This applies the gating strategy of the selected Cytobank experiment to the files in the new experiment.

Overview of the Cytobank Gating Tab

The Gating tab within Cytobank is used to create and adjust the gating strategies.



Key features:

1 **Active Population:** Choose this population to view and adjust the gate.

2 **File/Sample Name plot:** View the gate parameters for the active population.

3 **Gates:** Choose a gate name to view and edit.

Defining Gates and Populations

In Cytobank, the terms gate and population are not interchangeable.

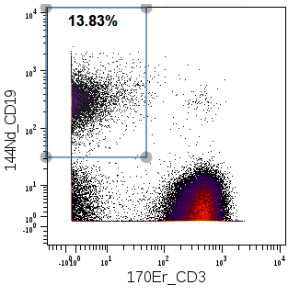
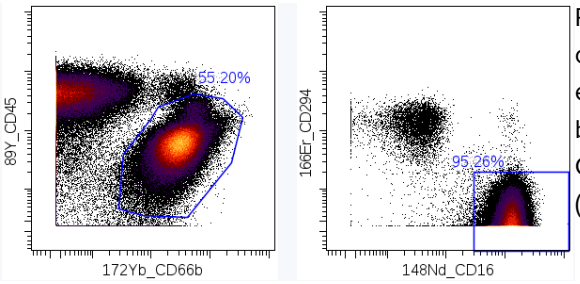


Figure 1. CD19+CD3– gate.

A gate is a selected region in the plot. Gates are defined on single parameters in histograms or two parameters in bivariate plots, for example, the CD19+CD3- gate (Figure 1). Gates can be rectangular, elliptical, or polygonal in shape.

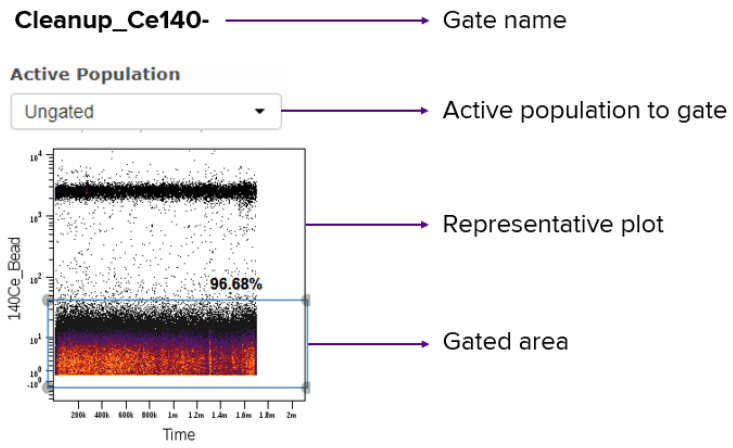


Populations are defined by the combination of gates used to identify each group. For example, neutrophils can be defined with two gates: CD45loCD66b+ and CD294-CD16+ (Figure 2).

Figure 2. CD45loCD66b+ and CD294-CD16+ gates used to define the neutrophil population

Using the Maxpar Direct Immune Profiling Assay Cell Gating Strategy

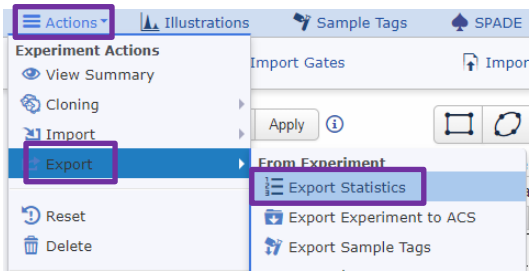
The steps for the cleanup and cell gating are outlined below with the gate name, Active Population, and a representative plot.



To adjust gates:

1. Select the gate name in the Gates box.
2. Select the **Active Population** from the drop-down menu.
3. Review the gate and modify to select the appropriate region if required.

Exporting Statistics from a Cytobank Experiment



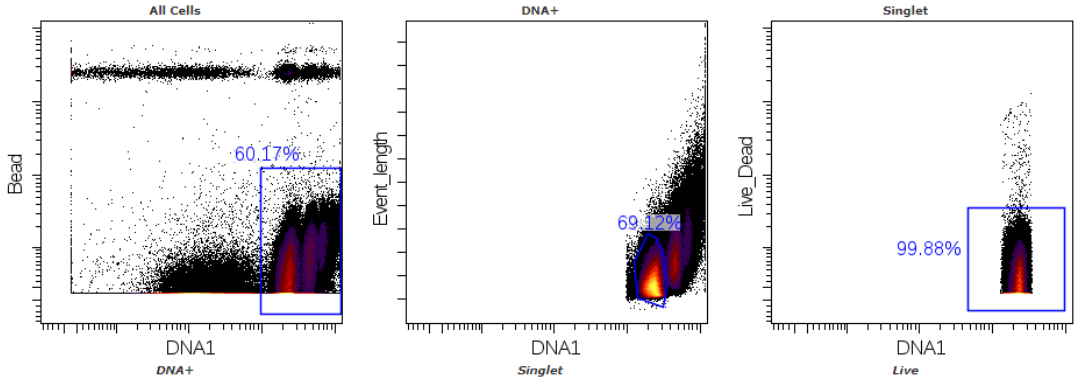
In addition to the gating strategy, statistics such as event counts and marker intensities can be exported using the Export Statistics tool.

A template for exporting event counts for each population is provided within the Public

Experiment (Fluidigm_Maxpar Direct Immune Profiling Assay_201325_Gating Example_v1.0). For more information on Cytobank features visit support.cytobank.org.

Data Cleanup

Prior to cell gating using antibody targets (markers), a cleanup strategy is used to remove debris, normalization beads, doublets, and dead cells [2]. A common cleanup method used in mass cytometry is depicted below. DNA+ events are gated on DNA+Bead- (191Ir+140Ce-), then singlet events are gated using Event Length vs. DNA. The most abundant DNA+ Event_Length (low/int) population is selected, followed by viable cell gating using a viability marker such as Cell-ID™ Intercalator-Rh (191Ir+103Rh-).

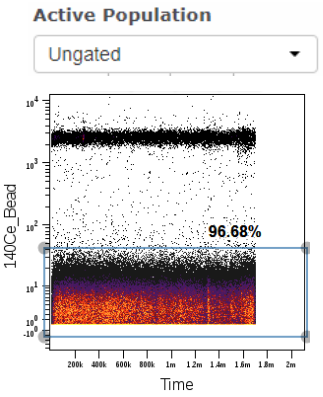
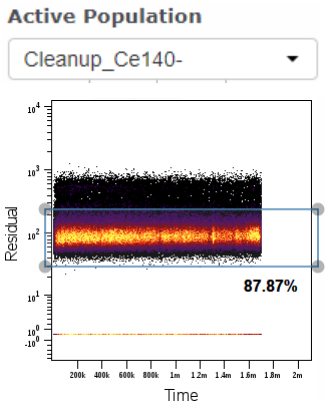
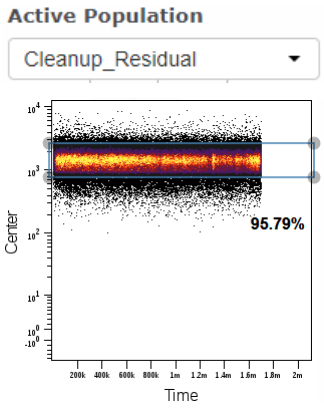


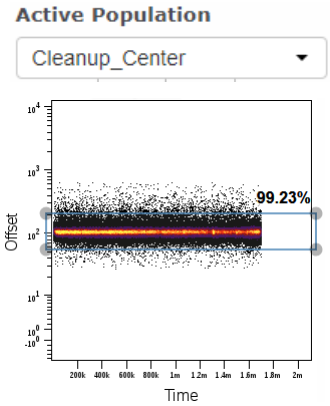
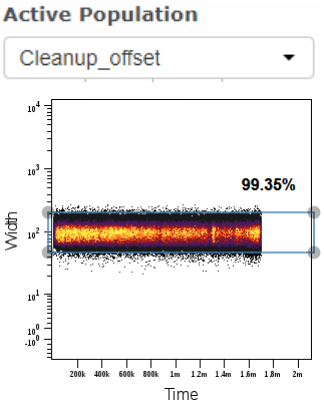
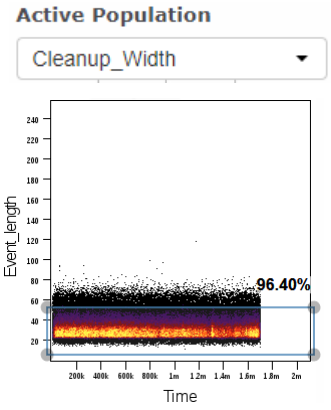
A new robust cleanup strategy was developed by Verity Software House and Fluidigm. This method has shown better aggregate and doublet removal than commonly applied gating strategies. In addition to the common parameters used (DNA, Bead, Event Length, Viability), this cleanup method uses Gaussian parameters for each event. The Gaussian discrimination (GD) channels (Center, Width, Offset, Residual) are recorded for each FCS file generated by Helios.

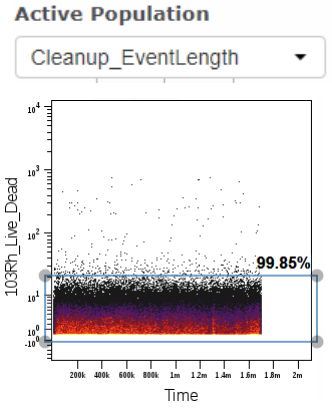
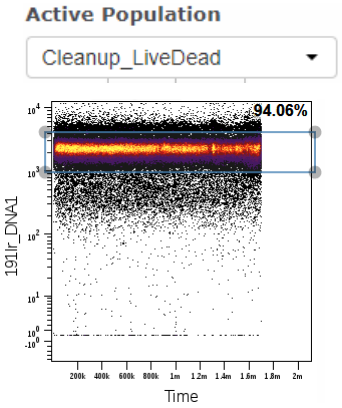
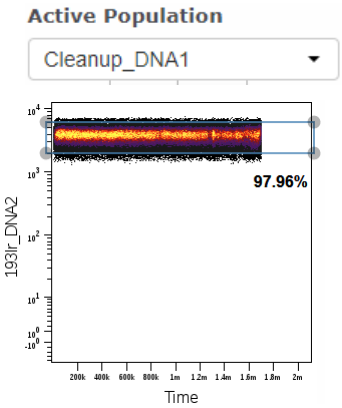
Cleanup Strategy

Performing the automated cleanup routine using Maxpar Pathsetter software (Cat. No. 401018) is recommended. Below is an example of the manual cleanup strategy, which can be applied to an FCS file normalized in CyTOF® Software version 6.7.1016 (or higher). Each cleanup parameter is plotted against time. The gates are adjusted to remove aggregates, debris, beads, doublets, and dead cells.

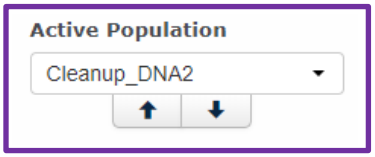
Cleanup Gates

Bivariate Plot	Active Population and Gating	Description
1 Bead vs. Time		**Beads removal** Adjust the Cleanup_Ce140- gate to select the low intensity events.
2 Residual vs. Time		**Gaussian discrimination, Residual** Adjust the Cleanup_Residual gate to select the largest band of events (mid-range intensity).
3 Center vs. Time		**Gaussian discrimination, Center** Adjust the Cleanup_Center gate to select the largest band of events (mid-to-high range intensity).

Bivariate Plot	Active Population and Gating	Description
4 **Offset vs. Time**		**Gaussian discrimination, Offset** Adjust the Cleanup_Offset gate to select the largest band of events (mid-range intensity).
5 **Width vs. Time**		**Gaussian discrimination, Width** Adjust the Cleanup_Width gate to select the largest band of events (mid-range intensity).
6 **Event Length vs. Time**		**Event Length** Adjust the Cleanup_EventLength gate to select the largest band of events (low range intensity).

Bivariate Plot	Active Population and Gating	Description
7 **Live Dead vs. Time**		**Cell viability** Adjust the Cleanup_LiveDead gate to select the largest band of events (low range intensity).
8 **DNA1 vs. Time**		**Nucleated (DNA-containing) cells, 191Ir** Adjust the Cleanup_DNA1 (191Ir) gate to select the largest band of events (mid-to-high range intensity).
9 **DNA2 vs. Time**		**Nucleated (DNA-containing) cells, 193Ir** Adjust the Cleanup_DNA2 (193Ir) gate to select the largest band of events (mid-to-high range intensity).

Global Parent Population

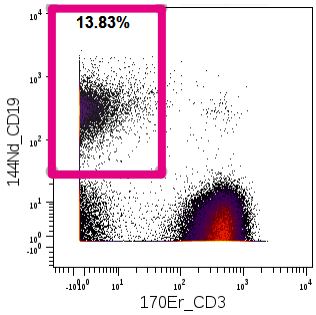


After each of the cleanup gates (Cleanup_Ce140- to Cleanup_DNA2) is applied, the Cleanup_DNA2 population is considered to be the live singlet population.

This is the global parent population that will be used for subsequent immune cell gating.

Immune Cell Gating

The ability to gate is dependent on the staining intensity of each marker and the resolution between positive and negative populations. Before analysis of critical samples, a preliminary pilot experiment should be performed on noncritical samples. Review pilot data for antibody staining quality. Evaluate pilot experiment marker intensities that are lower or higher than expected, which may affect the ability to identify populations.

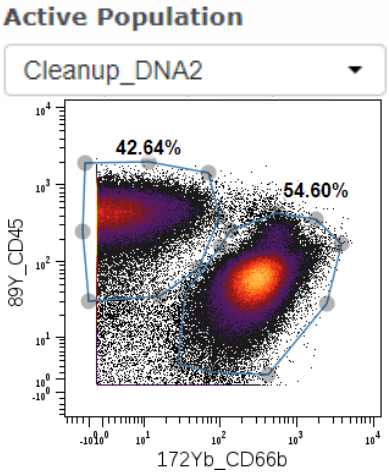


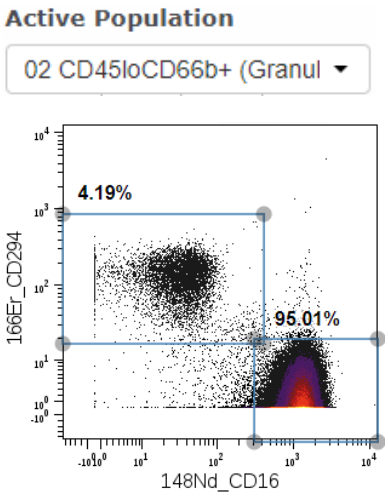
The Maxpar Direct Immune Profiling Assay gating strategy is a manual method that uses bivariate plots to gate on positive and negative regions to identify different immune populations. Gates are adjusted based on the marker expression. For instance, B lymphocytes are gated as CD3-CD19+. The CD3 population is used as a negative population for CD19+ events. For some markers, a negative population may not be available in PBMC samples. View each gate in each file to ensure correct gating.

Many gates are used to identify multiple populations. The gates used to define each population, including intermediate populations, are listed in Appendix: Population Gating Tables. In the gating descriptions below, populations starting with a checkbox sign (☑) are considered end populations.

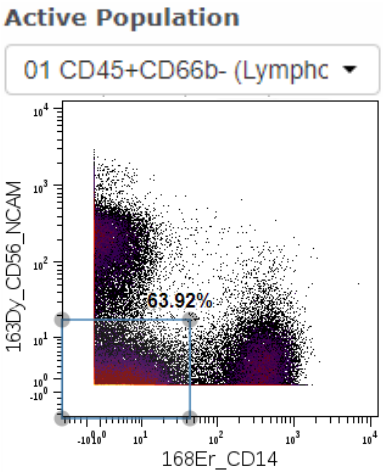
NOTE For more information, contact your local Fluidigm field application specialist.

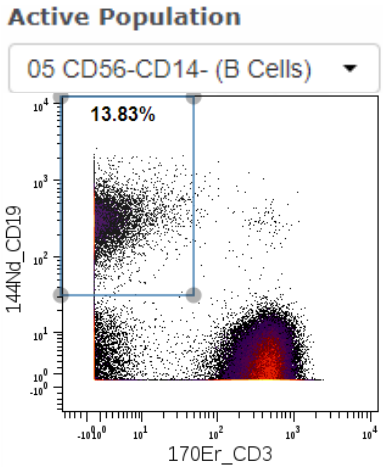
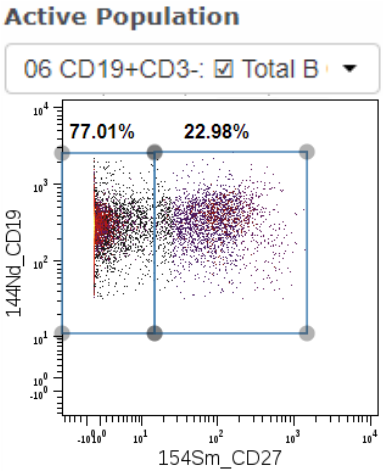
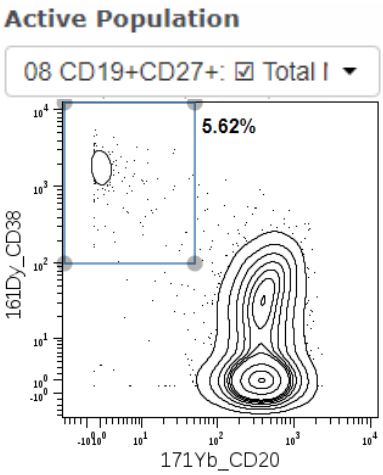
Neutrophils and Eosinophils

Bivariate Plot	Active Population and Gating	Description
1 **CD66b vs. CD45**		**Granulocyte exclusion** The CD45 vs. CD66b plot is used to gate on granulocytes (CD45loCD66b+) and excludes granulocytes from subsequent CD45+ populations: • *01 CD45+CD66b- (Lymphocytes, DCs, Monocytes)* • *02 CD45loCD66b+ (Granulocytes)* **NOTE** CD66b expression and granulocyte frequency are sensitive to sample preparation and sample type. PBMC preparations may have few granulocytes.

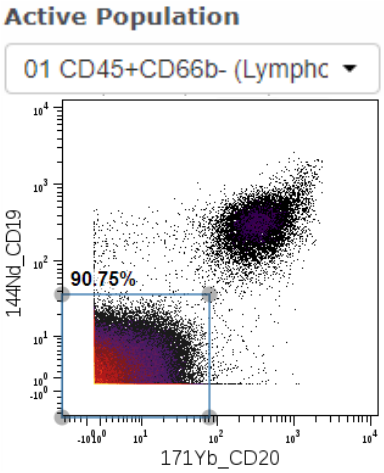
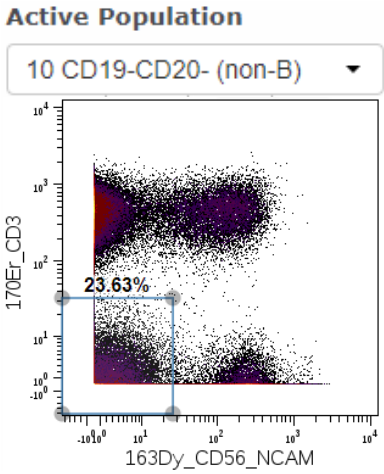
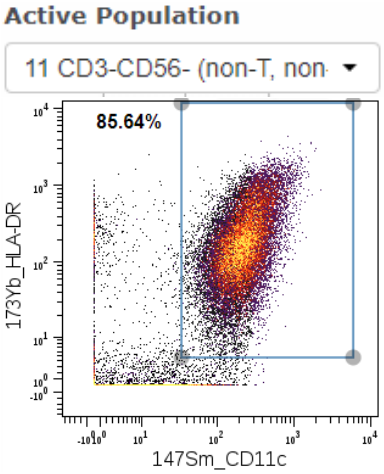
Bivariate Plot	Active Population and Gating	Description
2 CD16 vs. CD294		**☑ Eosinophils and neutrophils** From active population *#02 CD45loCD66b+ (Granulocytes)*, the CD16 vs. CD294 plot is used to identify the two following granulocyte populations: • *03 CD294+CD16-: Eosinophils* • *04 CD294-CD16+: Neutrophils* **NOTE** CD16 expression on granulocytes is sensitive to sample preparation and sample type. PBMC preparations may have reduced CD16 expression on granulocytes. **NOTE** Neutrophils and eosinophils have been observed to nonspecifically bind to certain antibodies. Adding heparin to whole blood samples prior to staining (as followed in the Maxpar Direct Immune Profiling Assay Cell Staining and Data Acquisition User Guide, PN 400286) reduces nonspecific binding [3].

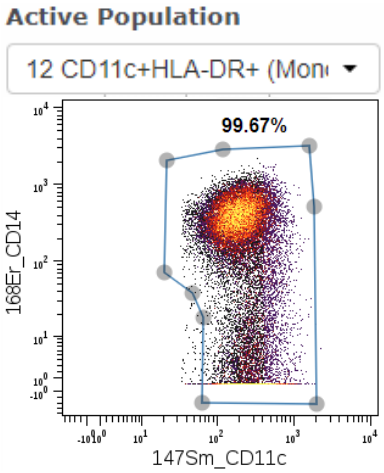
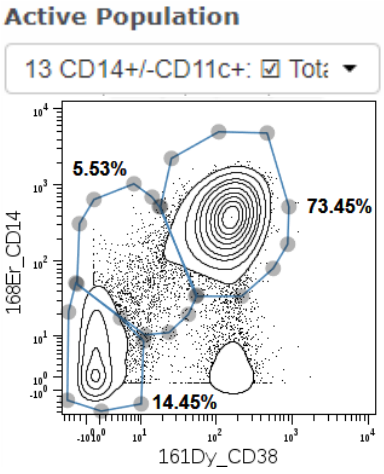
B Cell Subsets

Bivariate Plot	Active Population and Gating	Description
1 CD14 vs. CD56		**NK cell and monocyte exclusion** Starting from active population *#01 CD45+CD66b- (Lymphocytes, DCs, Monocytes)*, the CD56-CD14- gate is used to exclude natural killer cells and monocytes. • *05 CD56-CD14- (B Cells)*

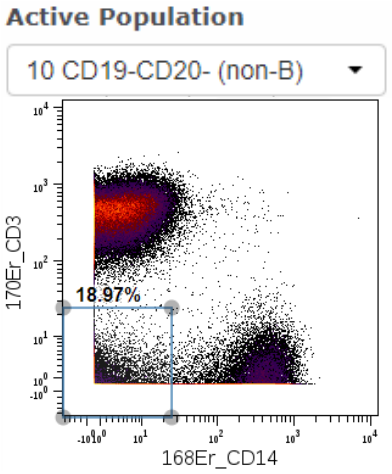
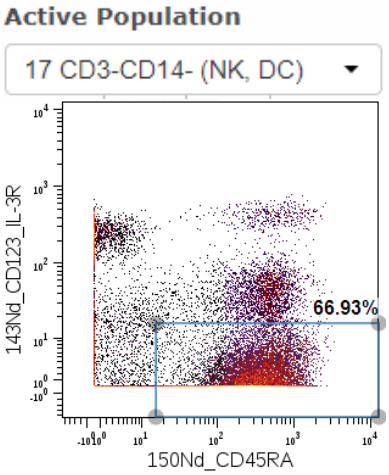
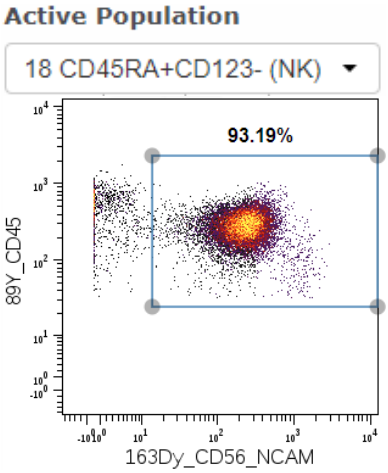
Bivariate Plot	Active Population and Gating	Description
2 CD3 vs. CD19		**☑ Total B cells** From active population *#05 CD56-CD14- (B Cells)*, the CD19+CD3- gate excludes T cells and gates on total B cells. • *06 CD19+CD3-: Total B Cells*
3 CD27 vs. CD19		**☑ Naive and total memory B cells** From active population *#06 CD19+CD3-: Total B Cells*, the CD27 vs. CD19 plot includes two gates to identify naive B cells and total memory B cells: • *07 CD19+CD27-: Naive B Cells* • *08 CD19+CD27+: Total Memory B Cells*
4 CD20 vs. CD38		**☑ Plasmablasts** From active population *#08 CD19+CD27+: Total Memory B Cells*, the CD38+CD20- gate identifies the plasmablast population: • *09 CD38+CD20-: Plasmablasts* **NOTE** For this gate it is recommended to use a contour plot to better identify rare plasmablast events.

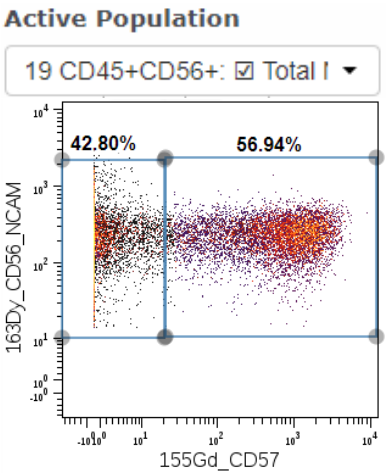
Monocytes

Bivariate Plot	Active Population and Gating	Description
1 **CD20 vs. CD19**		**B cell exclusion** Starting from active population *#01 CD45+CD66b- (Lymphocytes, DCs, Monocytes)*, the CD19-CD20- gate is used to exclude B cells from the subsequent populations: • *10 CD19-CD20- (non-B)*
2 **CD56 vs. CD3**		**T cells and NK cells exclusion** From active population *#10 CD19-CD20- (non-B)*, the CD3-CD56- gate is used to exclude T cells and NK cells from the subsequent populations: • *11 CD3-CD56- (non-T, non-NK)*
3 **CD11c vs. HLA-DR**		**Total monocytes, step 1 of 2** From active population *#11 CD3-CD56- (non-T, non-NK)*, the CD11c+HLA-DR+ gate is used to identify total monocytes: • *12 CD11c+HLA-DR+ (Mono)*

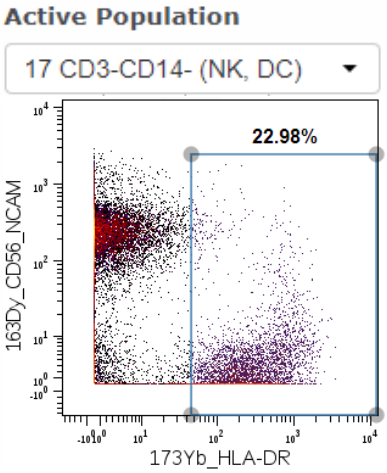
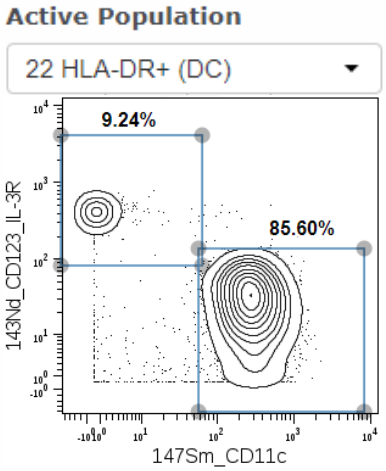
Bivariate Plot	Active Population and Gating	Description
4 CD11c vs. CD14		**☑ Total monocytes step 2 of 2** From active population *#12 CD11c+HLA-DR+ (Mono)*, the CD14+/-CD11c+ gate is used to confirm the presence of CD14 on total monocytes. CD14lo/-CD11clo/- events are excluded using this gate: • *13 CD14+/-CD11c+: Total Monocytes*
5 CD14 vs. CD38		**☑ Monocytes, classical, transitional, and nonclassical** From active population *#13 CD14+/-CD11c+: Total Monocytes*, the CD14 vs. CD38 plot is used to distinguish between classical, transitional, and nonclassical monocytes: • *14 CD14+CD38+: Classical Monocytes* • *15 CD38lo/-CD14int: Transitional Monocytes* • *16 CD38-CD14-: Nonclassical Monocytes* **NOTE** For this gate, using a contour plot is recommended to better identify transitional monocytes events.

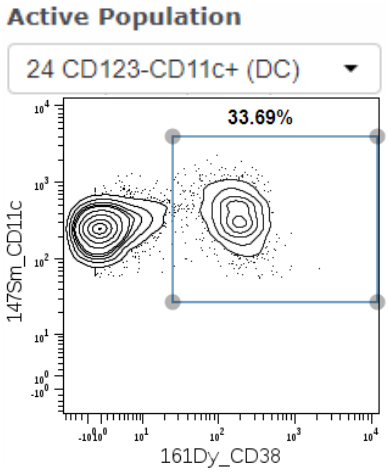
Natural Killer (NK) Cells

Bivariate Plot	Active Population and Gating	Description
1 CD14 vs. CD3		**T cell and monocyte exclusion** Starting from active population *#10 CD19-CD20- (non-B)*, the CD3-CD14- gate is used to exclude T cells and monocytes from the subsequent populations: • *17 CD3-CD14- (NK, DC)*
2 CD45RA vs. CD123		**Total NK cells, step 1 of 2** From active population *#17 CD3-CD14- (NK, DC)*, the CD45RA+CD123- gate excludes CD123+ events from the NK Cell population: • *18 CD45RA+CD123- (NK)*
3 CD56 vs. CD45		**☑ Total NK cells, step 2 of 2** From active population *#18 CD45RA+CD123- (NK)*, the CD45+CD56+ gate selects for the total NK cell population: • *19 CD45+CD56+: Total NK*

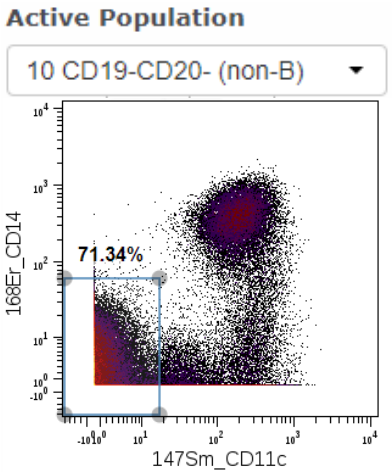
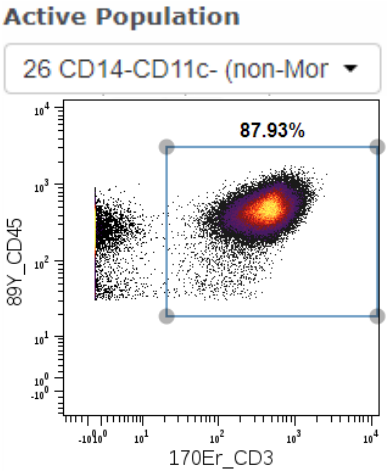
Bivariate Plot	Active Population and Gating	Description
4 CD57 vs. CD56		**☑ NK cells, early and late** From active population *#19 CD45+CD56+ (Total NK)*, CD57 is used to distinguish between early (CD57-) and late NK (CD57+) cells: • *20 CD56+CD57-: Early NKs* • *21 CD56+CD57+: Late NKs*

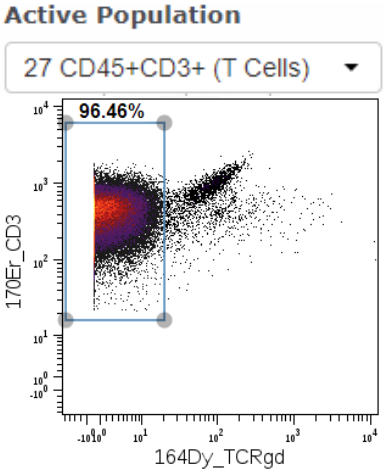
Dendritic Cells (DCs)

Bivariate Plot	Active Population and Gating	Description
1 HLA-DR vs. CD56		**HLA-DR positive cells** Starting from active population *#17 CD3-CD14- (NK, DC)*, HLA-DR+ cells are gated from the HLA-DR vs. CD56 plot: • *22 HLA-DR+ (DC)*
2 CD11c vs. CD123		**DC and ☑ plasmacytoid DC** From active population *#22 HLA-DR+ (DC)*, the CD11c vs. CD123 plot is used to distinguish between the plasmacytoid dendritic cells (CD123+CD11c-), and the CD123-CD11c+ DCs: • *23 CD123+CD11c-: pDC* • *24 CD123-CD11c+ (DC)*

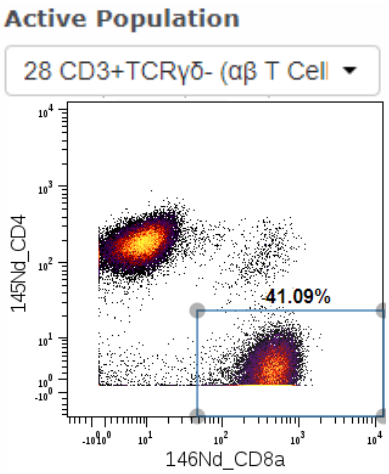
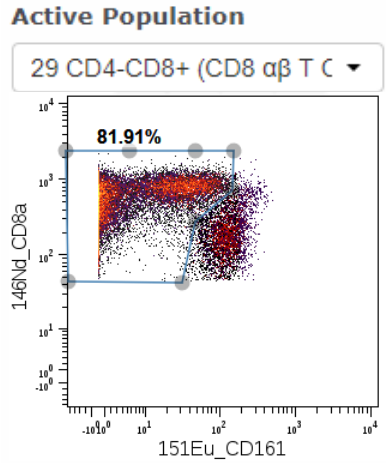
Bivariate Plot	Active Population and Gating	Description
3 CD11c vs. CD38		**☑ Myeloid DC** From active population *#24 CD123-CD11c+ (DC)*, the CD11c+CD38+ gate is used to identify myeloid dendritic cells (mDCs): • *25 CD11c+CD38+: mDC*

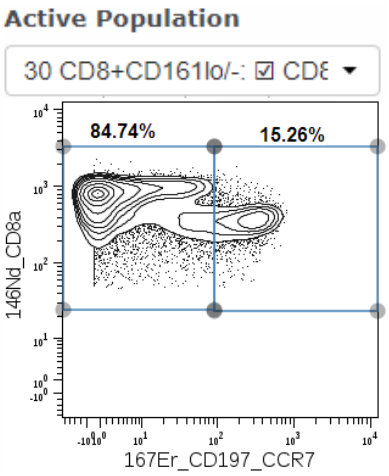
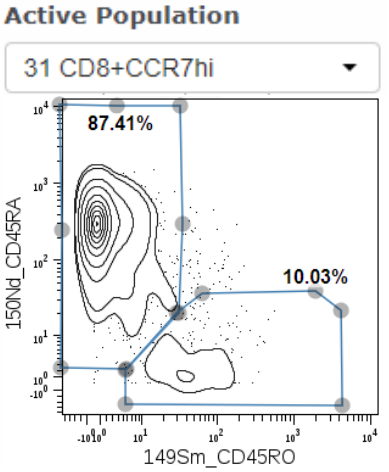
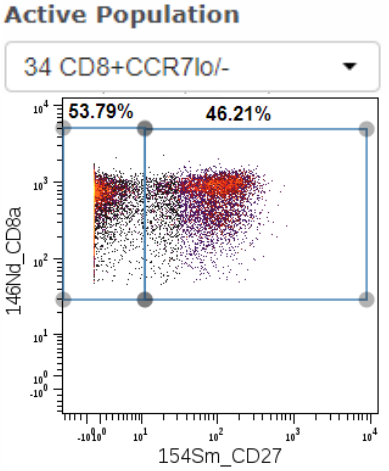
T Cell Subsets

Bivariate Plot	Active Population and Gating	Description
1 CD11c vs. CD14		**Monocyte exclusion** Starting from active population *#10 CD19-CD20- (non-B)*, the CD14-CD11c- gate is used to exclude monocytes: • *26 CD14-CD11c- (non-Mono)*
2 CD3 vs. CD45		**T cells** From active population *#26 CD14-CD11c- (non-Mono)*, the CD45+CD3+ gate identifies all CD3+ events: • *27 CD45+CD3+ (T Cells)*

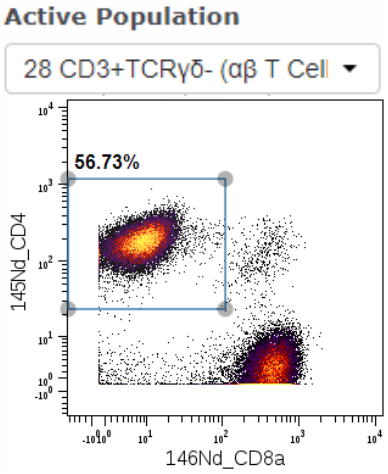
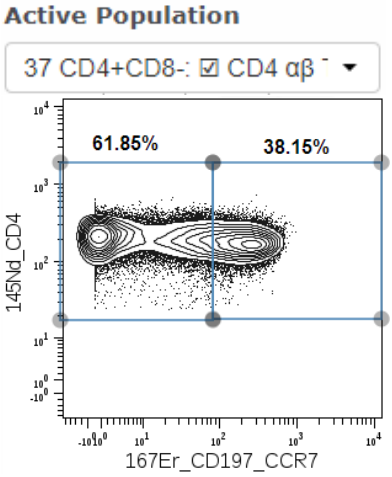
Bivariate Plot	Active Population and Gating	Description
3 TCRγδ vs. CD3		**αβ T cells** From active population *#27 CD45+CD3+ (T Cells)*, the CD3+TCRγδ- gate excludes all gamma delta (γδ) T cells from subsequent analyses: • *28 CD3+TCRγδ- (αβ T Cells)*

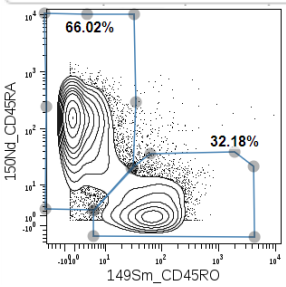
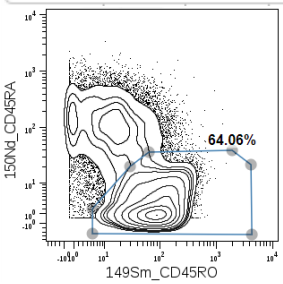
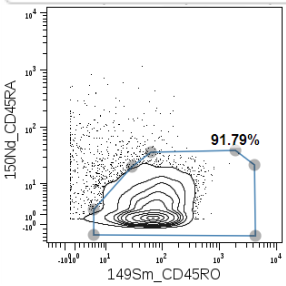
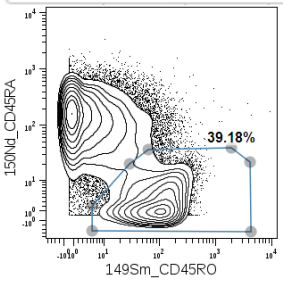
CD8 αβ T Cell Subsets

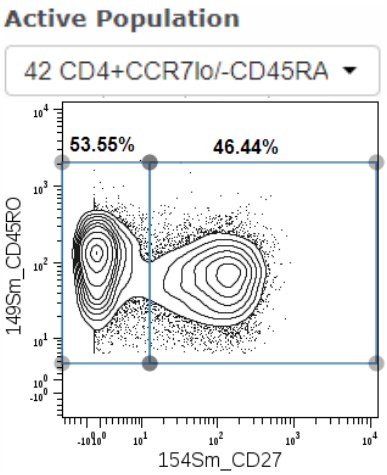
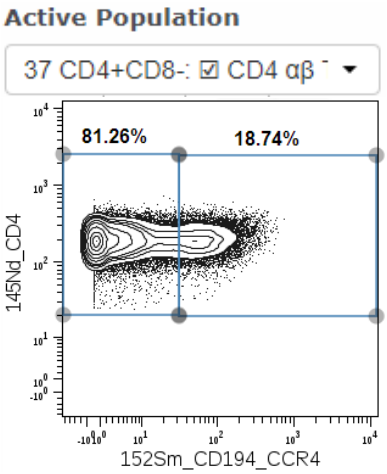
Bivariate Plot	Active Population and Gating	Description
1 CD8a vs. CD4		**CD8 αβ T cells, step 1 of 2** From active population *#28 CD3+TCRγδ- (αβ T Cells)*, the CD4-CD8+ gate identifies the CD8 T cell population: • *29 CD4-CD8+ (CD8 αβ T Cells)*
2 CD161 vs. CD8a		**☑ CD8 αβ T cells, step 2 of 2** From active population *#29 CD4-CD8+ (CD8 αβ T Cells)*, the CD161 vs. CD8a plot is used to gate out the CD8a+CD161hi cells from the subsequent CD8 populations: • *30 CD8+CD161lo/-: CD8 αβ T Cells*

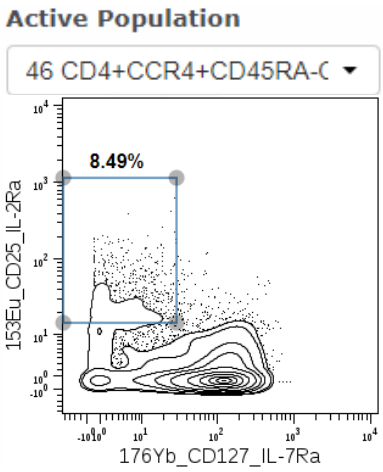
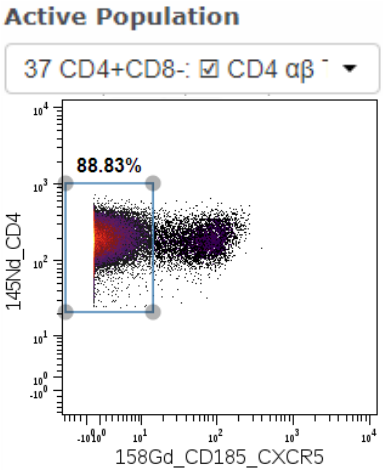
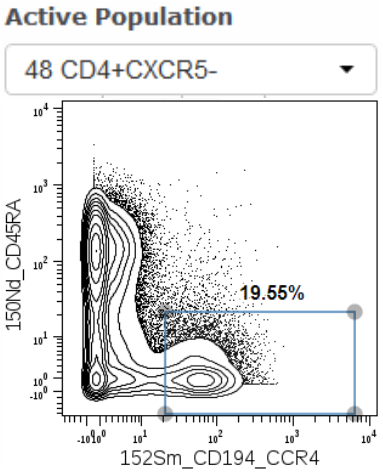
Bivariate Plot	Active Population and Gating	Description
3 CCR7 vs. CD8a		**CD8 αβ T cell stages, initial gate** From active population *#30 CD8+CD161lo/-: CD8 αβ T Cells*, the CCR7 vs. CD8a plot is used to distinguish between the CCR7+ and CCR7- populations. These are intermediate subsets: • *31 CD8+CCR7hi* • *34 CD8+CCR7lo/-* **NOTE** For this gate it is recommended to use a contour plot to better identify the transition between CCR7hi and CCR7lo/-, which typically occurs around 10^2.
4 CD45RO vs. CD45RA		**☑ CD8 αβ T cells, naive and central memory** From active population *#31 CD8+CCR7hi*, the CD45RO vs. CD45RA plot is used to identify naive (CD45RA+CD45RO-) and central memory (CD45RA-CD45RO+) CD8 T cells: • *32 CD45RA+CD45RO-: CD8 Naive* • *33 CD45RA-CD45RO+: CD8 Central Memory* **NOTE CD45RA+CD45RO- gate is used again in active population #39, and CD45RA-CD45RO+ gate is used again in active populations #40, #42, #46, and #50.**
5 CD27 vs. CD8a		**☑ CD8 αβ T cells, effector memory and terminal effector** From active population *#34 CD8+CCR7lo/-*, the CD27 vs. CD8a plot is used to gate on CD8 effector memory (CD27+) and CD8 terminal effector (CD27-) cells: • *35 CD8+CD27+: CD8 Effector Memory* • *36 CD8+CD27-: CD8 Terminal Effector*

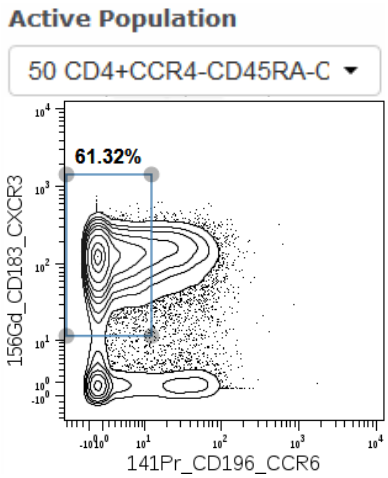
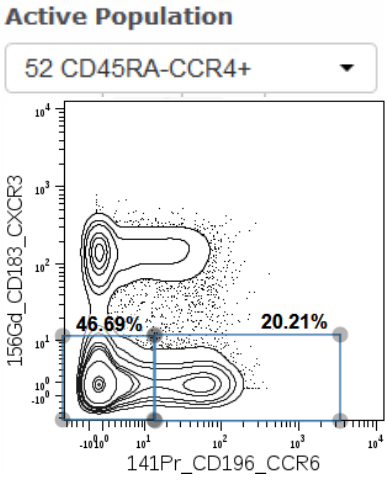
CD4 αβ T Cell Subsets

Bivariate Plot	Active Population and Gating	Description
1 CD8a vs. CD4		**☑ CD4 αβ T cells** From active population ***#28 CD3+TCRγδ- (αβ T Cells)***, the CD4+CD8- gate is used to identify CD4 T cells: • *37 CD4+CD8-: CD4 αβ T Cells*
2 CCR7 vs. CD4		**CD4 αβ T cell stages, initial gate** The CCR7 vs. CD4 plot is used to distinguish between the CCR7hi and CCR7lo/- populations. These are intermediate subsets: • *38 CD4+CCR7hi* • *41 CD4+CCR7lo/-* **NOTE The transition between CCR7hi and CCR7lo/- typically occurs around 10^2 and is observed more easily as a contour plot.**

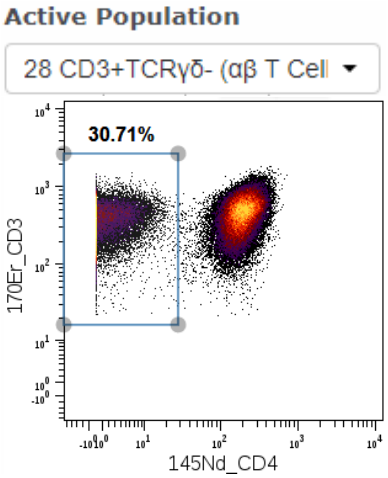
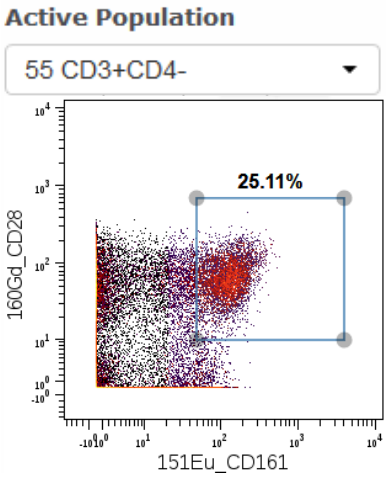
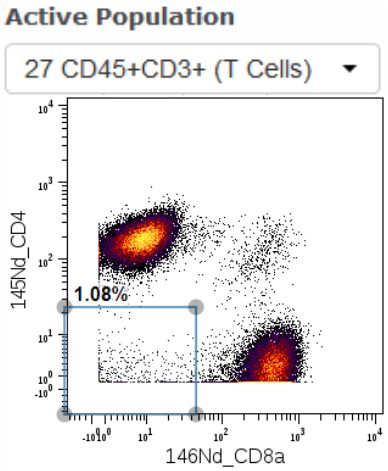
Bivariate Plot	Active Population and Gating	Description
3 CD45RO vs. CD45RA **NOTE** The CD45RA+CD45RO- gate was previously used for CD8 T cell subsets. The following are the same gates used in CD4 T cell populations.	Active Population 38 CD4+CCR7hi  Active Population 41 CD4+CCR7lo/-  Active Population 45 CD4+CCR4+  Active Population 49 CD4+CCR4- 	**☑ CD4 αβ T cells, naive and central memory** From active population *#38 CD4+CCR7hi*, the CD45RO vs. CD45RA plot is used to identify naive (CD45RA+CD45RO-) and central memory (CD45RA-CD45RO+) CD4 T cells: • *39 CD45RA+CD45RO-: CD4 Naive* • *40 CD45RA-CD45RO+: CD4 Central Memory* **CD4 αβ T cells, effector memory and terminal effector** From active population *#41 CD4+CCR7lo/-, CD45RA-CD45RO+* is used as an intermediate population to identify CD4 effector memory and terminal effector cells: • *42 CD4+CCR7lo/-CD45RA-CD45RO+* **CD45RA-CD45RO+ selection for intermediate population of Treg cells** From active population *#45 CD4+CCR4+*, CD45RA-CD45RO+ is used as an intermediate population to identify T regulatory cells: • *46 CD4+CCR4+CD45RA-CD45RO+* **CD45RA-CD45RO+ selection for intermediate population of Th1-like cells** From active population *#49 CD4+CCR4-*, CD45RA-CD45RO+ is used as an intermediate population to identify Th-1 like cells: • *50 CD4+CCR4-CD45RA-CD45RO+*

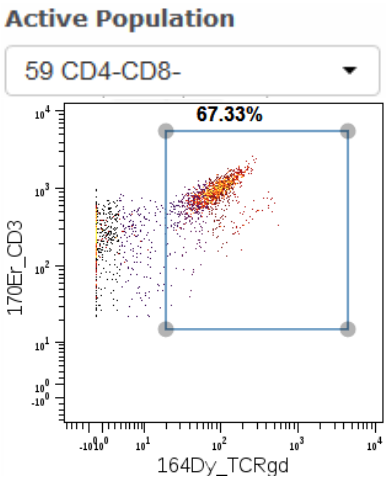
Bivariate Plot	Active Population and Gating	Description
4 CD27 vs. CD45RO		**☑ CD4 αβ T cells, effector memory and terminal effector** From active population *#42 CD4+CCR7lo/-CD45RA-CD45RO+*, the CD27 vs. CD45RO plot it used to identify CD4 effector memory and terminal effector cells: • *43 CD45RO+CD27+: CD4 Effector Memory* • *44 CD45RO+CD27-: CD4 Terminal Effector*
5 CCR4 vs. CD4		**CCR4 positive and negative selection for Treg and Th1-like intermediate populations** From active population *#37 CD4+CD8-: CD4 αβ T Cells*, the CCR4 vs. CD4 plot is used to identify the intermediate population T regulatory cells (CD4+CCR4+). From active population *#48 CD4+CXCR5-*, the CCR4 vs. CD4 plot is used to identify the intermediate population Th1-like cells (CD4+CCR4-): • *45 CD4+CCR4+* • *49 CD4+CCR4-* **NOTE** Active population #13 Total Monocytes can be used to identify the positive and negative threshold of CCR4.

Bivariate Plot	Active Population and Gating	Description
6 CD127 vs. CD25		**☑ Treg (T regulatory cells)** From active population *#46 CD4+CCR4+CD45RA-CD45RO+*, Treg cells are identified by gating on CD25hiCD127lo/- cells: • *47 CD25hiCD127lo/-: Treg*
7 CXCR5 vs. CD4		**CD4+CXCR5- selection for Th1-like, Th2-like, and Th-17 like intermediate populations** From active population *#37 CD4+CD8-: CD4 αβ T cells*, CXCR5- cells are gated as an intermediate population to identify Th-1-like, Th2-like, and Th17-like cells: • *48 CD4+CXCR5-* **NOTE** Chemokine receptor expression is sensitive to sample type, preparation, and treatment. For PBMC samples, the intensities of CXCR5 should be evaluated on noncritical samples.
8 CCR4 vs. CD45RA		**CD45RA-CCR4+ selection for Th2-like and Th17-like intermediate populations** From active population *#48 CD4+CXCR5-*, CD45RA-CCR4+ cells are gated as an intermediate population to identify Th2-like, and Th17-like cells: • *52 CD45RA-CCR4+*

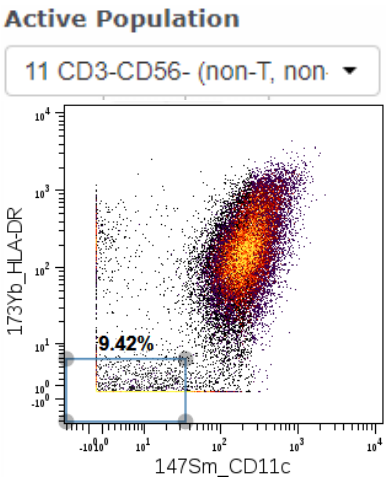
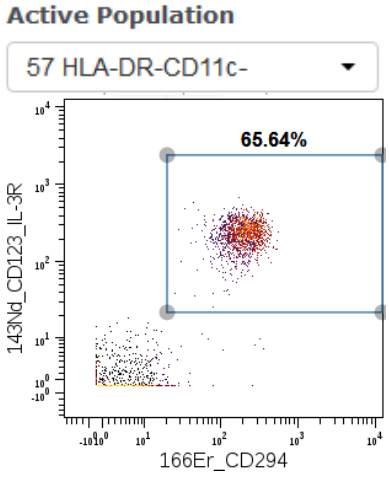
Bivariate Plot	Active Population and Gating	Description
		**☑ Th1-like** From active population *#50 CD4+CCR4-CD45RA-CD45RO+*, Th1-like cells are gated as CXCR3+CCR6-: • *51 CXCR3+CCR6-: Th1-like*
9 CCR6 vs. CXCR3		**☑ Th2-like and Th17-like** From active population *#52 CD45RA-CCR4+*, the CXCR3 vs. CCR6 plot is used to identify Th2-like and Th17-like cells: • *53 CXCR3-CCR6-: Th2-like* • *54 CXCR3-CCR6+: Th17-like* **NOTE** Chemokine receptor expression is sensitive to sample type, preparation, and treatment. For PBMC samples, the intensities of CXCR3 and CCR6 should be evaluated on noncritical samples to determine the ability to gate CD4 Th-like subsets.

CD4- MAIT/NKT Cells and CD4-CD8- γδ T Cells

Bivariate Plot	Active Population and Gating	Description
1 **CD4 vs. CD3**		**CD4 exclusion** From active population *#28 CD3+TCRγδ- (αβ T Cells)*, CD3+CD4- cells are gated as an intermediate population to identify CD4- MAIT/NKT cells: • *55 CD3+CD4-*
2 **CD161 vs. CD28**		**☑ MAIT/NKT cells** From active population *#55 CD3+CD4-*, CD28+CD161hi cells are gated to identify CD4- mucosal-associated invariant T (MAIT)/natural killer T (NKT) cells: • *56 CD28+CD161hi: CD4- MAIT/NKT* **NOTE** MAIT and NKT cells can be resolved with anti-TCR Vα7.2antibody.
3 **CD8a vs. CD4**		**γδ T cells, step 1 of 2** From active population *#27 CD45+CD3+ (T Cells)*, the CD4-CD8- cells are gated as an intermediate population to identify CD4-CD8- γδ T cells: • *59 CD4-CD8-*

Bivariate Plot	Active Population and Gating	Description
4 TCRγδ vs. CD3		**☑ γδ T cells, step 2 of 2** From active population *#59 CD4-CD8-*, CD3+TCRγδ+ cells are gated to identify γδ T cells: • *60 CD3+TCRγδ+: CD4-CD8- γδ T Cells* **NOTE** The majority of γδ T cells are CD4-CD8-. There are also CD4+ γδ T cells and CD8+ γδ T cells, which are not included in this gating strategy.

Basophils

Bivariate Plot	Active Population and Gating	Description
1 CD11c vs. HLA-DR		**Basophils, step 1 of 2** From active population *#11 CD3-CD56- (non-T, non-NK)*, the CD11c-HLA-DR- gates on an intermediate population used to identify basophils: • *57 HLA-DR-CD11c-*
2 CD294 vs. CD123		**☑ Basophils, step 2 of 2** From active population *#57 HLA-DR-CD11c-*, basophils are gated as CD123+CD294+: • *58 CD123+CD294+: Basophils* **NOTE** Basophils are a granulocyte population that can be found in PBMC preparations.

Additional Gating Comments

Monocyte Gating Strategy

Classical, transitional, and nonclassical monocytes are more commonly gated using CD14 and CD16 (Figure 3) [4–6]. The CD38 marker is also expressed by monocytes [7–11]. The Maxpar Pathsetter software probability state immunophenotyping model produces more robust results using CD38 instead of CD16. Manual gating of total monocytes and monocyte subsets—classical, transitional, and nonclassical—using CD38 vs. CD14 were comparable to Pathsetter immunophenotyping model. Results from back-gating each monocyte subset from CD14 vs. CD38 to CD14 vs. CD16 appear similar to the common strategy (Figure 4). This document shows a CD14 and CD38 gating strategy. However, each user can determine a preferred gating strategy.

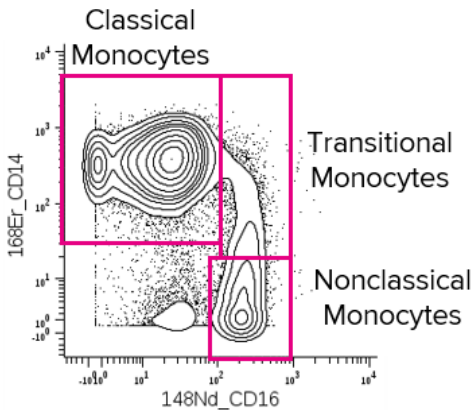


Figure 3. Common gating strategy for monocyte subsets using CD14 and CD16

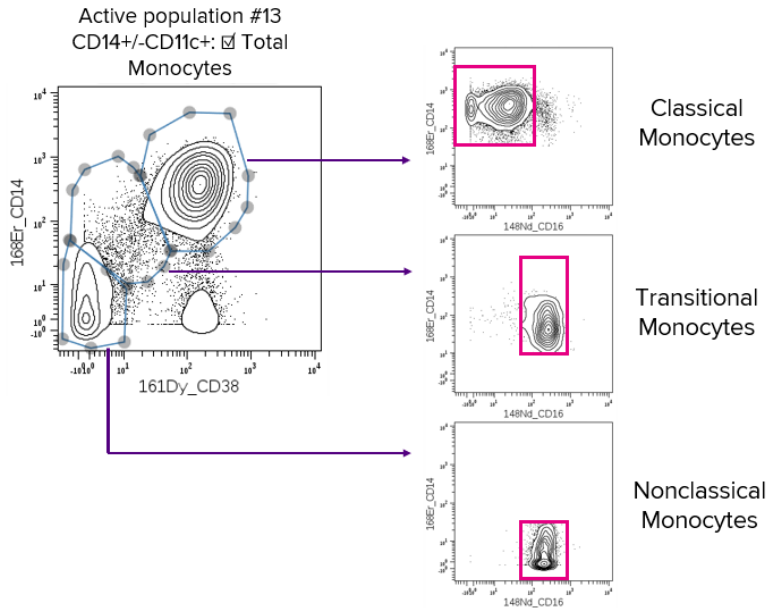


Figure 4. Back-gating the monocyte subsets from CD14 vs. CD38 to CD14 vs. CD16.

Further Gating Memory B Cells

Memory B cells can be further divided into IgD+ Memory B cells and IgD- Memory B cells. This can be visualized by gating IgD vs. CD27 on total B cells (active population #06).

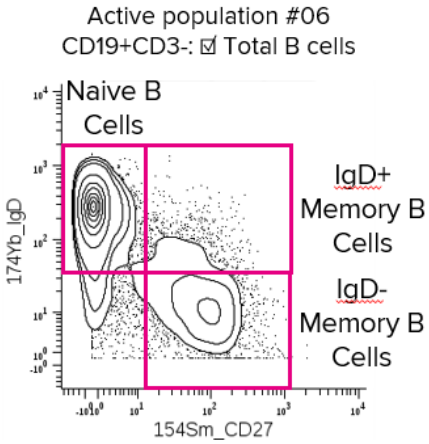


Figure 5. Gating strategy for identifying IgD+ and IgD- memory B cells.

CD66b- Neutrophils

The Maxpar Pathsetter probability state immunophenotyping model identifies a population that has been labeled CD66b- Neutrophils. This population is identified as lineage negative (CD3-CD19-CD56-HLA-DR-CD123-CD45-CD66b-) phenotype with sample-dependent CD16lo/+ expression observed. Neutrophils have been shown to express different isoforms of CD66, such as CD66a, CD66b, CD66c, and CD66d [12,13], which may not be captured by CD66b in the panel. When comparing the spatial localization of CD66b- Neutrophils to CD66b+ Neutrophils in the Pathsetter Cen-se′™ plot*, these two populations cluster close together, suggesting that they may be related. With the addition of more markers to the panel, the CD66b- Neutrophil population may be more accurately defined.

* Cen-se′ (Cauchy-Enhanced Nearest-neighbor Stochastic Embedding) is an unsupervised nonlinear dimensionality reduction algorithm developed by Verity Software House, based on the original t-SNE (t-distributed stochastic neighbor embedding) algorithms commonly used for visualizing high-dimensional mass cytometry data [14].

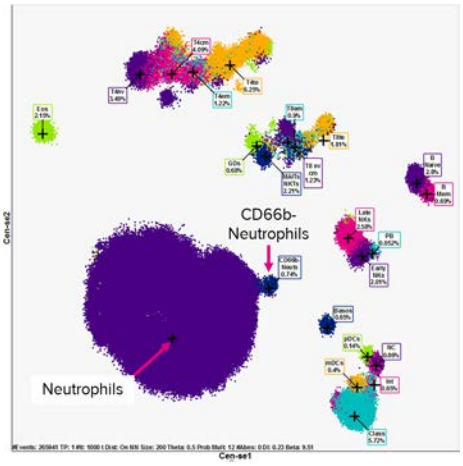


Figure 6. Cen-se′ plot in Pathsetter showing the nearby clustering of the Neutrophils and CD66b- Neutrophils in a whole blood sample. The Neutrophil populations are not displayed in the Cen-se′ plot of the default Pathsetter report.

Additional considerations

- Marker intensities may vary across different samples. To adjust the gate for each specific file and/or population, use the Gate Tailoring box below the Gates box. For instance, the CD45RA vs. CD45RO gate can be adjusted for CD4 and CD8 T cells.
- Gates can be added and removed for each population in the Population Manager.
- Always check all gates for each file.
- For more information on Cytobank features go to support.cytobank.org.

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Appendix: Population Gating Tables

Table 1. Gates used to distinguish cell populations stained by the Maxpar Direct Immune Profiling Assay (Cat. No. 201325). All listed cell populations should also include the cleanup gates described in the cleanup strategy.

		Population	Gate #	Gates	Population	Gate #	Gates
	Granulocytes	Neutrophils	02	CD45loCD66b+	Eosinophils	02	CD45loCD66b+
			04	CD294-CD16+		03	CD294+CD16-
		Basophils	01	CD45+CD66b-			
			10	CD19-CD20-			
			11	CD3-CD56-			
			57	HLA-DR-CD11c-			
			58	CD123+CD294+			
Lymphocytes	CD8 T Cells	CD8 αβ T cells (Total; CD161lo/-)	01	CD45+CD66b-	CD8 αβ T cells, Naïve	01	CD45+CD66b-
			10	CD19-CD20-		10	CD19-CD20-
			26	CD14-CD11c-		26	CD14-CD11c-
			27	CD45+CD3+		27	CD45+CD3+
			28	CD3+TCRγδ-		28	CD3+TCRγδ-
			29	CD4-CD8+		29	CD4-CD8+
			30	CD8+CD161lo/-		30	CD8+CD161lo/-
						31	CD8+CCR7hi
						32	CD45RA+CD45RO-
		CD8 αβ T cells, Central Memory	01	CD45+CD66b-	CD8 αβ T cells, Effector Memory	01	CD45+CD66b-
			10	CD19-CD20-		10	CD19-CD20-
			26	CD14-CD11c-		26	CD14-CD11c-
			27	CD45+CD3+		27	CD45+CD3+
			28	CD3+TCRγδ-		28	CD3+TCRγδ-
			29	CD4-CD8+		29	CD4-CD8+
			30	CD8+CD161lo/-		30	CD8+CD161lo/-
			31	CD8+CCR7hi		34	CD8+CCR7lo/-
			33	CD45RA-CD45RO+		35	CD8+CD27+
		CD8 αβ T cells, Terminal Effector	01	CD45+CD66b-			
			10	CD19-CD20-			
			26	CD14-CD11c-			
			27	CD45+CD3+			
			28	CD3+TCRγδ-			
			29	CD4-CD8+			
			30	CD8+CD161lo/-			
			34	CD8+CCR7lo/-			
			36	CD8+CD27-			

		Population	Gate #	Gates	Population	Gate #	Gates
Lymphocytes	CD4 T Cells	CD4 αβ T cells (Total)	01	CD45+CD66b-	CD4 αβ T cells, Naïve	01	CD45+CD66b-
			10	CD19-CD20-		10	CD19-CD20-
			26	CD14-CD11c-		26	CD14-CD11c-
			27	CD45+CD3+		27	CD45+CD3+
			28	CD3+TCRγδ-		28	CD3+TCRγδ-
			37	CD4+CD8-		37	CD4+CD8-
						38	CD4+CCR7hi
						39	CD45RA+CD45RO-
		CD4 αβ T cells, Central Memory	01	CD45+CD66b-	CD4 αβ T cells, Effector Memory	01	CD45+CD66b-
			10	CD19-CD20-		10	CD19-CD20-
			26	CD14-CD11c-		26	CD14-CD11c-
			27	CD45+CD3+		27	CD45+CD3+
			28	CD3+TCRγδ-		28	CD3+TCRγδ-
			37	CD4+CD8-		37	CD4+CD8-
			38	CD4+CCR7hi		41	CD4+CCR7lo/-
			40	CD45RA-CD45RO+		40	CD45RA-CD45RO+
						43	CD45RO+CD27+
		CD4 αβ T cells, Terminal Effector	01	CD45+CD66b-	Treg	01	CD45+CD66b-
			10	CD19-CD20-		10	CD19-CD20-
			26	CD14-CD11c-		26	CD14-CD11c-
			27	CD45+CD3+		27	CD45+CD3+
			28	CD3+TCRγδ-		28	CD3+TCRγδ-
			37	CD4+CD8-		37	CD4+CD8-
			41	CD4+CCR7lo/-		45	CD4+CCR4+
			40	CD45RA-CD45RO+		46	CD45RA-CD45RO+
			44	CD45RO+CD27-		47	CD25hiCD127lo/-
		Th1-like	01	CD45+CD66b-	Th2-like	01	CD45+CD66b-
			10	CD19-CD20-		10	CD19-CD20-
			26	CD14-CD11c-		26	CD14-CD11c-
			27	CD45+CD3+		27	CD45+CD3+
			28	CD3+TCRγδ-		28	CD3+TCRγδ-
			37	CD4+CD8-		37	CD4+CD8-
			48	CD4+CXCR5-		45	CD4+CXCR5-
			49	CD4+CCR4-		52	CD45RA-CCR4+
			50	CD45RA-CD45RO+		53	CXCR3-CCR6-
			51	CXCR3+CCR6-			
		Th17-like	01	CD45+CD66b-			
			10	CD19-CD20-			
			26	CD14-CD11c-			
			27	CD45+CD3+			
			28	CD3+TCRγδ-			
			37	CD4+CD8-			
			45	CD4+CXCR5-			
			52	CD45RA-CCR4+			
			54	CXCR3-CCR6+			

		Population	Gate #	Gates	Population	Gate #	Gates
Lymphocytes	Other T cells	Gamma-delta T cells, CD4-CD8-	01	CD45+CD66b-	MAIT/NKT CD4- cells	01	CD45+CD66b-
			10	CD19-CD20-		10	CD19-CD20-
			26	CD14-CD11c-		26	CD14-CD11c-
			27	CD45+CD3+		27	CD45+CD3+
			59	CD4-CD8-		55	CD3+CD4-
			60	CD3+TCRγδ+		56	CD28+CD161hi
	B cells	Total B cells	01	CD45+CD66b-	Naive B cells	01	CD45+CD66b-
			05	CD56-CD14-		05	CD56-CD14-
			06	CD19+CD3-		06	CD19+CD3-
						07	CD19+CD27+
		Total Memory B cells	01	CD45+CD66b-	Plasmablasts	01	CD45+CD66b-
			05	CD56-CD14-		05	CD56-CD14-
			06	CD19+CD3-		06	CD19+CD3-
			08	CD19+CD27+		08	CD19+CD27+
						09	CD38+CD20-
	NK cells	Total NK cells	01	CD45+CD66b-	Early NK cells	01	CD45+CD66b-
			10	CD19-CD20-		10	CD19-CD20-
			17	CD3-CD14-		17	CD3-CD14-
			18	CD45RA+CD123-		18	CD45RA+CD123-
			19	CD45+CD56+		19	CD45+CD56+
						20	CD56+CD57-
		Late NK cells	01	CD45+CD66b-			
			10	CD19-CD20-			
			17	CD3-CD14-			
			18	CD45RA+CD123-			
			19	CD45+CD56+			
			21	CD56+CD57+			
	Monocytes	Total Monocytes	01	CD45+CD66b-	Classical Monocytes	01	CD45+CD66b-
			10	CD19-CD20-		10	CD19-CD20-
			11	CD3-CD56-		11	CD3-CD56-
			12	CD11c+HLA-DR+		12	CD11c+HLA-DR+
			13	CD14+/-CD11c+		13	CD14+/-CD11c+
						14	CD38+CD14hi
		Transitional Monocytes	01	CD45+CD66b-	Nonclassical Monocytes	01	CD45+CD66b-
			10	CD19-CD20-		10	CD19-CD20-
			11	CD3-CD56-		11	CD3-CD56-
			12	CD11c+HLA-DR+		12	CD11c+HLA-DR+
			13	CD14+/-CD11c+		13	CD14+/-CD11c+
			15	CD38lo/-CD14int		16	CD38-CD14-
	Dendritic cells	Plasmacytoid Dendritic cells	01	CD45+CD66b-	Myeloid Dendritic cells	01	CD45+CD66b-
			10	CD19-CD20-		10	CD19-CD20-
			17	CD3-CD14-		17	CD3-CD14-
			22	HLA-DR+		22	HLA-DR+
			23	CD123+CD11c-		24	CD123-CD11c+
						25	CD11c+CD38+

Table 2. Active cell populations identified in Cytobank for manual gating of the Maxpar Direct Immune Profiling Assay (Cat. No. 201325). Active populations that contain parentheses are descriptors of intermediate populations for which those gates are used. Populations ending with a descriptor following a colon and starting with a checkbox sign (☑) are end populations.

01 CD45+CD66b- (Lymphocytes, DCs, Monocytes)	**31 CD8+CCR7hi**
02 CD45loCD66b+ (Granulocytes)	**32 CD45RA+CD45RO-: ☑ CD8 Naive**
03 CD294+CD16-: ☑ Eosinophils	**33 CD45RA-CD45RO+: ☑ CD8 Central Memory**
04 CD294-CD16+: ☑ Neutrophils	**34 CD8+CCR7lo/-**
05 CD56-CD14- (B cells)	**35 CD8+CD27+: ☑ CD8 Effector Memory**
06 CD19+CD3-: ☑ Total B cells	**36 CD8+CD27-: ☑ CD8 Terminal Effector**
07 CD19+CD27-: ☑ Naive B cells	**37 CD4+CD8-: ☑ CD4 αβ T cells**
08 CD19+CD27+: ☑ Total Memory B cells	**38 CD4+CCR7hi**
09 CD38+CD20-: ☑ Plasmablasts	**39 CD45RA+CD45RO-: ☑ CD4 Naive**
10 CD19-CD20- (non-B)	**40 CD45RA-CD45RO+: ☑ CD4 Central Memory**
11 CD3-CD56- (non-T, non-NK)	**41 CD4+CCR7lo/-**
12 CD11c+HLA-DR+ (Mono)	**42 CD4+CCR7lo/-CD45RA-CD45RO+**
13 CD14+/-CD11c+: ☑ Total Monocytes	**43 CD45RO+CD27+: ☑ CD4 Effector Memory**
14 CD14+CD38+: ☑ Classical Monocytes	**44 CD45RO+CD27-: ☑ CD4 Terminal Effector**
15 CD38lo/-CD14int: ☑ Transitional Monocytes	**45 CD4+CCR4+**
16 CD38-CD14-: ☑ Nonclassical Monocytes	**46 CD4+CCR4+CD45RA-CD45RO+**
17 CD3-CD14- (NK, DC)	**47 CD25hiCD127lo/-: ☑ Treg**
18 CD45RA+CD123- (NK)	**48 CD4+CXCR5-**
19 CD45+CD56+: ☑ Total NK	**49 CD4+CCR4-**
20 CD56+CD57-: ☑ Early NKs	**50 CD4+CCR4-CD45RA-CD45RO+**
21 CD56+CD57+: ☑ Late NKs	**51 CXCR3+CCR6-: ☑ Th1-like**
22 HLA-DR+ (DC)	**52 CD45RA-CCR4+**
23 CD123+CD11c-: ☑ pDC	**53 CXCR3-CCR6-: ☑ Th2-like**
24 CD123-CD11c+ (DC)	**54 CXCR3-CCR6+: ☑ Th17-like**
25 CD11c+CD38+: ☑ mDC	**55 CD3+CD4-**
26 CD14-CD11c- (non-Mono)	**56 CD28+CD161hi: ☑ CD4- MAIT/NKT**
27 CD45+CD3+ (T cells)	**57 HLA-DR-CD11c-**
28 CD3+TCRγδ- (αβ T cells)	**58 CD123+CD294+: ☑ Basophils**
29 CD4-CD8+ (CD8 αβ T cells)	**59 CD4-CD8-**
30 CD8+CD161lo/-: ☑ CD8 αβ T cells	**60 CD3+TCRγδ+: ☑ CD4-CD8- γδ T cells**

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