Table of Contents

1.0   Purpose

This SOP describes the materials and methods needed to conduct an immunophenotyping analysis of a sample by flow cytometry using the Miltenyi Biotec 8-Color Immunophenotyping Kit.

2.0   Scope

This SOP applies to Biopharmaceutical Development Program (BDP) personnel performing an immunophenotyping analysis using the Miltenyi Biotec 8-Color Immunophenotyping Kit. The SOP applies only to the use of the Miltenyi Biotec 8-Color Immunophenotyping Kit for analysis and is not intended for the use of other immunophenotyping methods/kits. Similarly, this SOP is written for analysis performed on a Miltenyi Biotec MACSQuant Analyzer 10 flow cytometer and Miltenyi Biotec MACSQuantify software.

3.0   Authority and Responsibility

3.1   The Director, Process Analytics/Quality Control (PA/QC) has the authority to define this procedure.

3.2   PA/QC is responsible for training laboratory personnel and documenting this training to Biopharmaceutical Quality Assurance (BQA)

3.3   PA/QC personnel are responsible for the performance of this procedure.

3.4   PA/QC is responsible for reviewing the data and documentation of the results of this procedure.

3.5   BQA is responsible for quality oversight of this operation.

4.0 Equipment, Materials and Reagents

4.1   MACSQuant Analyzer 10 Flow Cytometer, Miltenyi Biotec

4.2   Biosafety cabinet

4.3   Refrigerator

4.4   Eppendorf Centrifuge 5417C, or BDP-approved equivalent

4.5   FACS Tubes with Cell Strainer (BDP PN 31168 or BDP-approved equivalent)

4.6   MACSQuant Running Buffer (BDP PN 31172) or BDP-approved equivalent

4.7   MACSQuant Calibration Beads (BDP PN 31171) or BDP-approved equivalent

4.8   MACS Comp Bead Kit, anti-REA (BDP PN 31176) or BDP-approved equivalent

4.9   8-Color Immunophenotyping Kit (BDP PN 31160) or BDP-approved equivalent

4.10   AutoMACS Rinsing Solution (BDP PN 31183) or BDP-approved equivalent

4.11   MACS BSA Stock Solution (BDP PN 31182 or BDP-approved equivalent)

4.12   Anti-CD8-VioGreen (BDP PN 31180 or 31261) or BDP-approved equivalent

4.13   Anti-CD8-VioBlue (BDP PN 31179) or BDP-approved equivalent

4.14   Anti-CD8-PE (BDP PN 31177) or BDP-approved equivalent

4.15   15 mL Centrifuge Tube (BDP PN 20006) or 50 mL Centrifuge Tube (BDP PN 20140) or BDP-approved equivalent

5.0 Procedure

5.1   Record reagents used on **Form 22206-01**.

5.2   Turn on the MACSQuant Analyzer 10 and allow the optical bench to warm up for at least 30 minutes, as described in *SOP 23131 - Operation of the MACSQuant Analyzer 10 Flow Cytometer*.

5.3   Perform a PMT calibration as described in *SOP 23131 - Operation of the MACSQuant Analyzer 10 Flow Cytometer* and document the calibration on Form 23131-01.

5.4   Adjust the FSC gain to approximately 250 on the X-axis of the FSC vs. SSC plot using a 50 µL aliquot of the sample to be analyzed as described in *SOP 23131 - Operation of the MACSQuant Analyzer 10 Flow Cytometer*.

5.5   Perform a spectral compensation as described in *SOP 23131 - Operation of the MACSQuant Analyzer 10 Flow Cytometer* using the MACS Comp Bead Kit, anti-REA and 4 µL (1:50 dilutions) of each antibody used in the compensation. Document the compensation incubation on Form 23131-01.

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NOTE: 0.1 mL aliquots of CD4-VioBright 667, CD8-APC-Vio 770, CD19-PE-Vio770 and CD56-VioBright 515 are provided with the 8-Color Immunophenotyping Kit for compensation. Due to lot-to-lot variations inherent in the production of these dyes, the provided aliquots (or an equivalent lot aliquot) should be used in the compensation whenever possible. Anti-CD8-VioGreen, anti-CD8-VioBlue and anti-CD8-PE should be purchased separately for compensation of the corresponding dyes (lot number used does not need to match what is present in the 8-Color Immunophenotyping Kit).

5.6   Prepare a Miltenyi Staining Solution in a 15 mL or 50 mL centrifuge tube by diluting MACS BSA Stock Solution 1:20 with autoMACS Rinsing Solution. Keep in the refrigerator (2-8°C) when not in use throughout the procedure.

NOTE: This gives a solution with 1X PBS, pH 7.2, 0.5% BSA and 2 mM EDTA. Record reagent volumes on **Form 22206-02**.

NOTE: The Miltenyi Staining Solution should be prepared fresh for each experiment.

5.7   Centrifuge up to 1E7 cells at 2,000 rpm (425 xg) for 5 minutes with a slow stop. Record the input volume of each sample on **Form 22206-02** as "Volume of Sample Aliquot Used for Staining (µL)".

5.8   Remove the supernatant and resuspend the pellet in 100 µL of Miltenyi Staining Solution.

5.9   Add 10 µL of 8-Color Immunophenotyping Cocktail and 1 µL of 7-AAD staining solution (part of 8-Color Immunophenotyping Kit) to each sample and pipet up and down to mix thoroughly.

5.10   Incubate the samples in the refrigerator (2-8°C) for 10 minutes. Record the incubation start/stop times on **Form 22206-02**.

5.11   Centrifuge each sample at 2,000 rpm (425 xg) for 5 minutes with a slow stop.

5.12   Resuspend each cell pellet in an appropriate volume of Miltenyi Staining Solution (typically 200 µL to 1000µL) depending upon the number of cells stained. Record the resuspension volume on **Form 22206-02** as "Post-Staining Sample Volume (µL)".

5.13   Analyze each sample directly following resuspension using a MACSQuant Analyzer 10 flow cytometer as described below (Section 6).

6.0   Sample Acquisition

6.1   Click on File and select New Workspace.

6.2   Click on File→ Open…and select Instrument setting on the left side of the Open box.

6.3   Browse through the Public, Private or External locations to find the compensation instrument setting prepared in step 5.5.

6.4   Select the correct instrument setting and click on Open.

6.5   Select the Channels Tab on the sidebar and click on Advanced.

6.6   Make sure that the Height box has been selected and press OK.

6.7   Click on File→ Open…and select Analysis on the left side of the Open box.

6.8   Browse through the Public, Private or External locations to find the proper analysis template.

    6.8.1   The Miltenyi Biotec 8-Color Immunophenotyping Analysis Template is depicted in Attachment 1.

    6.8.2   Prodigy Day 0, Day 7 and Day 9 Analysis Templates are depicted in Attachments 2, 3 and 4, respectively.

6.9   Select the correct Analysis template and click on Open.

6.10   A warning box will appear indicating that "All existing regions and windows will be removed! Proceed?". Click on OK.

6.11   Select the "Experiment" tab on the side toolbar.

6.12   Samples may be analyzed one tube at a time by selecting the "Single tube rack" or up to 24 at a time by selecting the "Chill 5 rack" in the Rack drop down menu.

6.13   Single tube rack

    6.13.1   Enter a Project name or QC Test Request number in the Project box.

    6.13.2   Enter sample information in the Sample ID and Description boxes.

    6.13.3   Select a flow rate and record the flow rate used on **Form 22206-02** as "Flow Rate Used".

        NOTE: Use a flow rate that is appropriate for the sample density (Low flow rate for samples that have a high cell density (>1E7), High flow rate for low cell densities (<1E6), Med flow rate for samples with an intermediate cell density).

    6.13.4   Select Mix gentle in the Mix sample drop-down menu.

    6.13.5   Select Extended in the Mode drop-down menu.

    6.13.6   Enter an appropriate volume for analysis in the Uptake volume box (Record volume on **Form 22206-02**) and enter the total sample volume (Post-Staining Sample Volume from **Form 22206-02**) in the Sample volume box.

    6.13.7   Select the Annotations tab and modify the annotations for the flow cytometer channels as follows: Channel V1 = CD14-VioBlue, Channel V2 = CD45-VioGreen, Channel B1 = CD16/CD56-VioBright 515, Channel B2 = CD3-PE, Channel B3 = 7-AAD-PerCP-Vio700, Channel B4 = CD19-PEVio770, Channel R1 = CD4-VioBright 667, Channel R2 = CD8-APC-Vio770

    6.13.8   Place the tube in the single tube rack and start acquisition by clicking on the Start

        Measurement button in the instrument status bar.

6.14   Chill 5 Rack

    6.14.1   Enter a Project name or QC Test Request number in the Project box.

    6.14.2   Select the wells to be used on the rack window.

6.14.3 Click on Group.

6.14.4 Select a flow rate and record the flow rate used on **Form 22206-02** as "Flow Rate Used".

6.14.5 Select Mix gentle in the Mix sample drop-down menu.

6.14.6 Select Extended in the Mode drop-down menu.

6.14.7 Select the Annotations tab and modify the annotations for the flow cytometer channels as follows: Channel V1 = CD14-VioBlue, Channel V2 = CD45-VioGreen, Channel B1 = CD16/CD56-VioBright 515, Channel B2 = CD3-PE, Channel B3 = 7-AAD-PerCP-Vio700, Channel B4 = CD19-PEVio770, Channel R1 = CD4-VioBright 667, Channel R2 = CD8-APC-Vio770

6.14.8 Select Ungroup.

6.14.9 For each tube, enter sample information in the Sample ID and Description boxes for each sample in the Chill 5 rack.

6.14.10 For each tube, enter an appropriate volume for analysis in the Uptake volume box (Record volume on **Form 22206-02**) and enter the total sample volume (Post-Staining Sample Volume from **Form 22206-02**) in the Sample volume box.

6.14.11 Place the tubes in the Chill 5 rack in the correct locations and start the sample acquisition by clicking on the Start Measurement button in the instrument status bar.

7.0   Data Analysis

7.1   The data may be analyzed on the MACSQuant Analyzer 10 instrument or may be copied to an external drive (Scientific Data network location or USB drive) and analyzed on an alternative computer (see *SOP 23131 - Operation of the MACSQuant Analyzer 10 Flow Cytometer* for details on how to copy files to an external USB drive).

NOTE: Cell count estimates are typically needed real time for Day 0 samples from a Prodigy run. In this case, a preliminary analysis of the samples is performed using the MACSQuantify software on the MACSQuant Analyzer 10 instrument and phenotyping data are provided to manufacturing personnel as needed. Final analysis is performed as described below and is recorded on **Forms 22206-03, 22206-04, 22206-05, 22206-06, 22206-07, 22206-08**. The results of the final analysis may differ slightly from the estimated cell counts provided in the BPR.

7.2   Click on File and select New Workspace.

7.3   Click on the Sample tab.

7.4   Right-click and select "Add…".

7.5   In the Select Data files to add window, browse through the folders to find the data file to be analyzed, select the file and click on Open.

NOTE: MACSQuant Analyzer 10 data files have a *.mqd extension.

7.6   The flow data may be visualized using the immunophenotyping analysis templates in either of two ways:

　　7.6.1   Since an immunophenotyping analysis template was selected prior to data acquisition (Step 6.9), that analysis template may be applied to the data by going to the Samples tab, right-clicking on the sample name and selecting Apply analysis template.

　　7.6.2   Alternatively, the proper immunophenotyping analysis template may be applied to the data file by clicking on File → Open. Select Analysis and browse through the folders to find the proper immunophenotyping analysis template and click on Open.

7.7   Double-click on each plot to enlarge it and check to ensure that the targeted population of cells is included in the gate.

　　7.7.1   Use the gate edit points to adjust the size and shape of the gate.

　　7.7.2   Click and drag a gate to move it to a different area of the plot.

7.8   For the Live Cells plot, the CD45-positive Leukocyte plot, the CD3-positive plot, the CD4-positive plot and the CD8-positive plot, click on Edit and select "Copy plot".

7.9   Paste the plots in the indicated locations on **Form 22206-03** (Live Cells), **Form 22206-04** (CD45-positive Leukocytes), **Form 22206-05** (CD3-positive cells), **Form 22206-06** (CD4-positive cells) and **Form 22206-07** (CD8-positive plot).

7.10   Select the Samples tab in the MACSQuantify software and right-click on the analyzed sample name.

7.11   Select "Export sample list…".

7.12   In the "Export sample list" window, "Options" tab, ensure that the Clipboard box is checked and uncheck/deselect the File box.

7.13   Click on the "Region functions" tab and in the Regions section select the parent, Live Cells, Leukocytes, CD3+ T Cells, CD4+ T cells and CD8+ T cells regions. In the Functions section, select Name, Path, %-T, %-# and Count/mL.

7.14   Click on OK.

7.15   Open the "Immunophenotyping Analysis" Excel File.

　　7.15.1 See **Attachment 5** for a representative example of a completed "Immunophenotyping Analysis" Excel file.

7.16   Select Cell B3 in the Immunophenotyping Analysis Excel spreadsheet and press Ctrl-v to paste the data into the spreadsheet.

7.17   Enter the "Sample Dilution Factor" from **Form 22206-02** into Cells G4, G5, G6 G7, G8 and G9.

7.18   The Dilution-adjusted concentration in Counts/mL will populate cells H4, H5, H6, H7, H8 and H9.

7.19   Enter the total volume of the production sample in cells I4, I5, I6, I7, I8 and I9.

7.20   The Total Cell Count will populate cells J4, J5, J6, J7, J8 and J9.

7.21   Save the Immunophenotyping Analysis file with the QC test request number and date to the following location:

\\at-s-is2-as.ncifcrf.gov\BdpVlan\PA\MACSQuant 10 Data\Immunophenotyping Analysis

7.22   Select the cells of the Excel spreadsheet and select Copy

7.23   Paste the results from the Excel analysis in the indicated area on **Form 22206-08**.

8.0   Documentation

Record all reagent part numbers, lot numbers and expiration dates on **Form 22206-01**. Record the calibration and compensation details on **Form 23131-01**. Record sample preparation details on **Form 22206-02**. Include the flow plots for Live Cells, CD45-positive Leukocytes, CD3-positive cells, CD4-positive cells and CD8-positive cells on **Form 22206-03, Form 22206-04, Form 22206-05, Form 22206-06** and **Form 22206-07**, respectively. Record the analyzed data from the flow analysis on **Form 22206-08**.

9.0   References and Related Documents

SOP 23131   *Operation of the MACSQuant Analyzer 10 Flow Cytometer*

Form 23131-01 *Calibration and Compensation Completion*

Form 22206-01 *Immunophenotyping Flow Cytometry Reagents*

Form 22206-02 *8-Color Immunophenotyping Sample Information*

Form 22206-03 *Immunophenotyping Flow Cytometry Results – Live Cells*

Form 22206-04 *Immunophenotyping Flow Cytometry Results – CD45-Positive Leukocytes*

Form 22206-05 *Immunophenotyping Flow Cytometry Results – CD3-Positive Cells*

Form 22206-06 *Immunophenotyping Flow Cytometry Results – CD4-Positive Cells*

Form 22206-07 *Immunophenotyping Flow Cytometry Results – CD8-Positive Cells*

Form 22206-08 *Immunophenotyping Data Analysis*
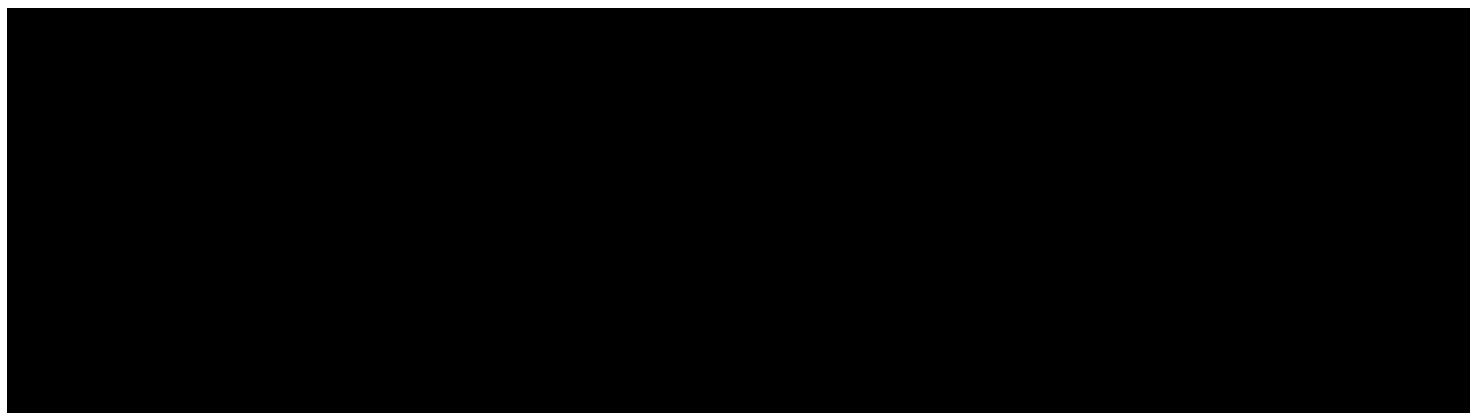
10.0   Attachments

Attachment 1   Miltenyi Biotec 8-Color Immunophenotyping Analysis Template

Attachment 2   Prodigy Day 0 Immunophenotyping Analysis Template

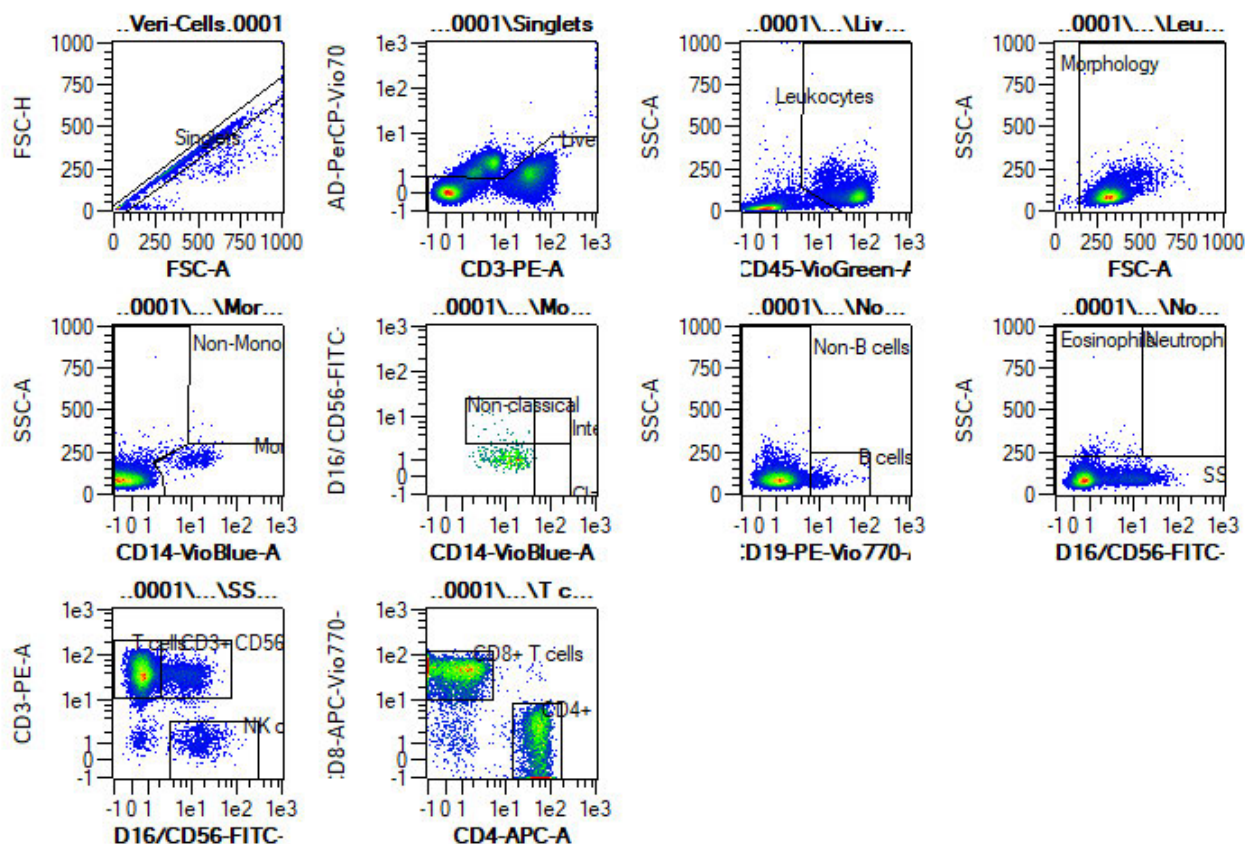**Attachment 3**   Prodigy Day 7 Immunophenotyping Analysis Template

Attachment 4   Prodigy Day 9 Immunophenotyping Analysis Template

Attachment 5   Representative Example of a Completed "Immunophenotyping Analysis" Excel Spreadsheet
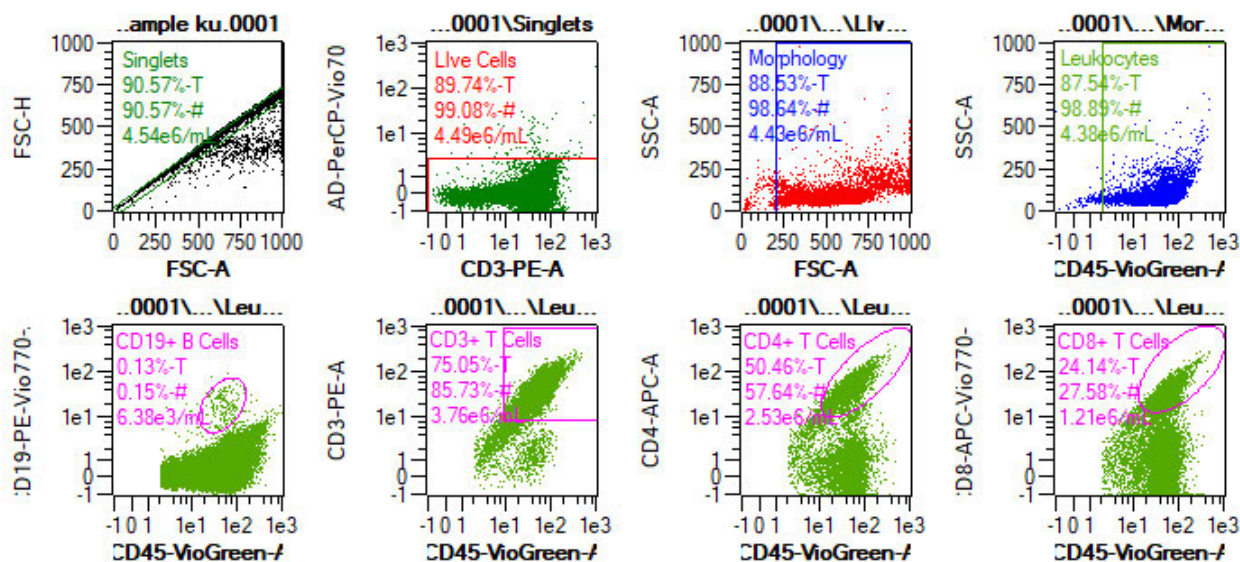
Attachment 1
## Miltenyi Biotec 8-Color Immunophenotyping Analysis Template

Attachment 2
Prodigy Day 0 Immunophenotyping Analysis Template

File	KU2018-12-12Day_0_selected sample ku.0001.mqd	2018-Dec-12 14:13	
SID	Day_0_selected sample ku	Descr.	Day_0_selected sample ku

Path	%-T	%-#	Count/mL
Singlets	90.57	90.57	4.54e6
Singlets\LIve Cells	89.74	99.08	4.49e6
Singlets\LIve Cells\Morphology	88.53	98.64	4.43e6
Singlets\LIve Cells\Morphology\Leukocytes	87.54	98.89	4.38e6
Singlets\LIve Cells\Morphology\Leukocytes\CD19+ B Cells	0.13	0.15	6.38e3
Singlets\LIve Cells\Morphology\Leukocytes\CD3+ T Cells	75.05	85.73	3.76e6
Singlets\LIve Cells\Morphology\Leukocytes\CD4+ T Cells	50.46	57.64	2.53e6
Singlets\LIve Cells\Morphology\Leukocytes\CD8+ T Cells	24.14	27.58	1.21e6

Attachment 3
## Prodigy Day 7 Immunophenotyping Analysis Template
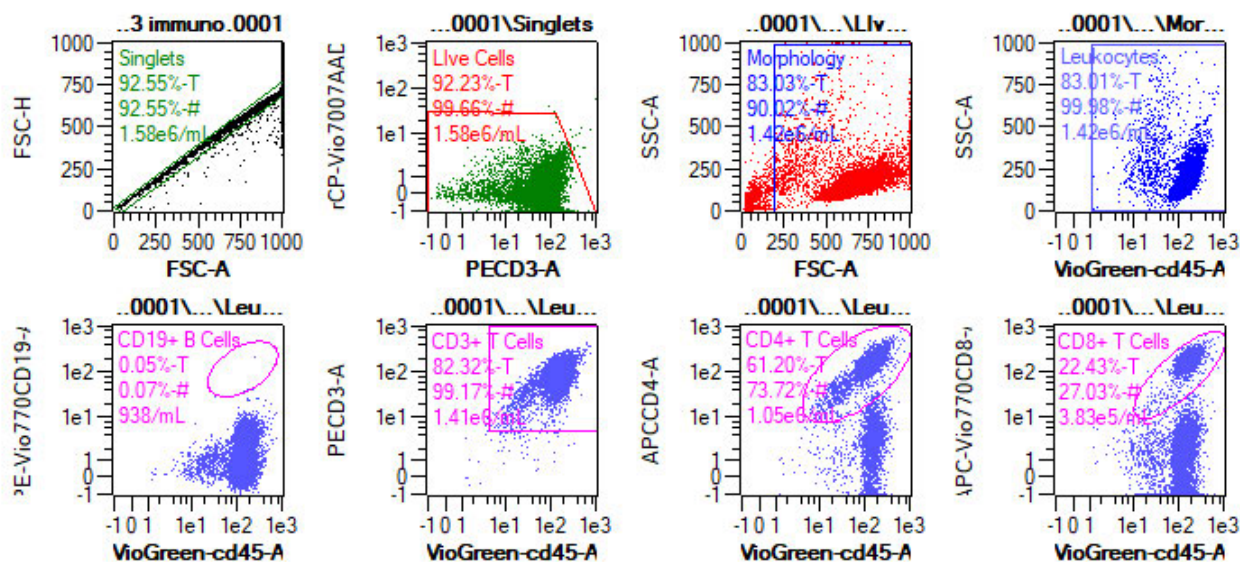
File	KU2018-12-19transduced CD33 immuno.0001.mqd	2018-Dec-19 05:07	
SID	Transduced CD33 QC 60235	Descr.	transduced CD33 immuno

Path	%-T	%-#	Count/mL
Singlets	92.55	92.55	1.58e6
Singlets\LIve Cells	92.23	99.66	1.58e6
Singlets\LIve Cells\Morphology	83.03	90.02	1.42e6
Singlets\LIve Cells\Morphology\Leukocytes	83.01	99.98	1.42e6
Singlets\LIve Cells\Morphology\Leukocytes\CD19+ B Cells	0.05	0.07	938
Singlets\LIve Cells\Morphology\Leukocytes\CD3+ T Cells	82.32	99.17	1.41e6
Singlets\LIve Cells\Morphology\Leukocytes\CD4+ T Cells	61.20	73.72	1.05e6
Singlets\LIve Cells\Morphology\Leukocytes\CD8+ T Cells	22.43	27.03	3.83e5

Attachment 4
Prodigy Day 9 Immunophenotyping Analysis Template
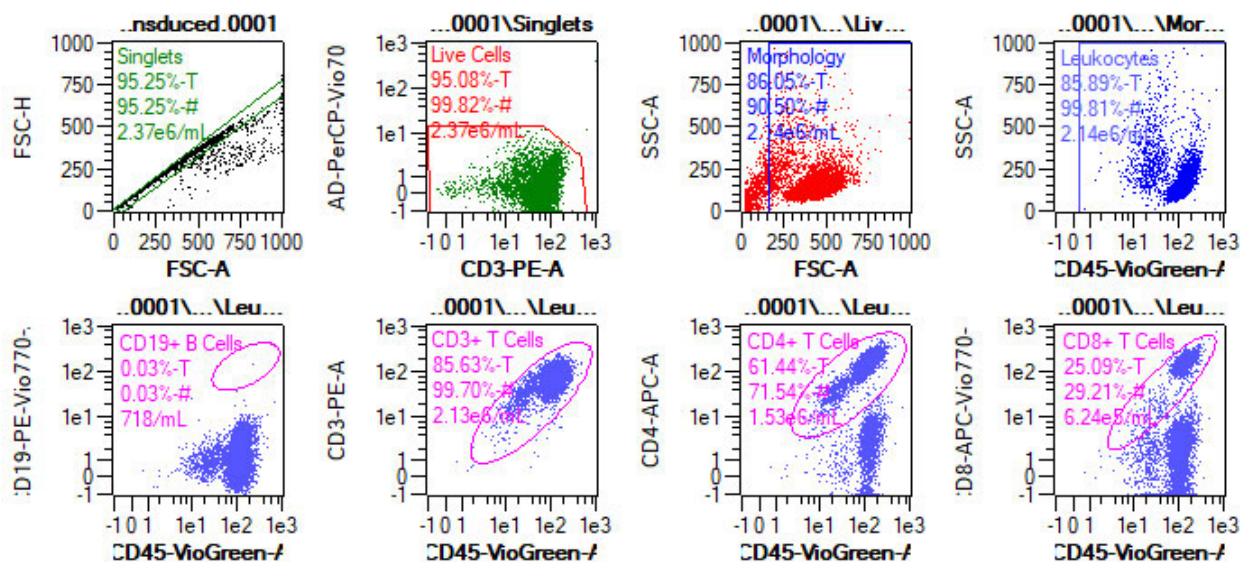
File	BSB2018-12-21Plasmalight transduced.0001.mqd	2018-Dec-21 02:27
SID	Plasmalight -transduced immuno ku	Descr. Plasmalight transduced

Path	%-T	%-#	Count/mL
Singlets	95.25	95.25	2.37e6
Singlets\Live Cells	95.08	99.82	2.37e6
Singlets\Live Cells\Morphology	86.05	90.50	2.14e6
Singlets\Live Cells\Morphology\Leukocytes	85.89	99.81	2.14e6
Singlets\Live Cells\Morphology\Leukocytes\CD19+ B Cells	0.03	0.03	718
Singlets\Live Cells\Morphology\Leukocytes\CD3+ T Cells	85.63	99.70	2.13e6
Singlets\Live Cells\Morphology\Leukocytes\CD4+ T Cells	61.44	71.54	1.53e6
Singlets\Live Cells\Morphology\Leukocytes\CD8+ T Cells	25.09	29.21	6.24e5

Attachment 5
Representative Example of a Completed "Immunophenotyping Analysis" Excel Spreadsheet

Raw data and column headings pasted from MACSQuantify Software

Value entered from Form 22206-02

Total production volume entered by analyst

Name	Path	%-T	%-#	Count/mL	Dilution Factor	Dilution-adjusted Concentration (Counts/mL)	Total Volume of Production Sample (mL)	Total Cell Count
KU2019-04-02Transduced Day 7 Phenotyping.0001	KU2019-04-02Transduced Day 7 Phenotyping.0001	100	---	2.26E+06	10	2.26E+07	100	2.26E+09
LIve Cells	Singlets\LIve Cells	94.96	98.71	2.15E+06	10	2.15E+07	100	2.15E+09
Leukocytes	Singlets\LIve Cells\Morphology\Leukocytes	93.01	99.85	2.11E+06	10	2.11E+07	100	2.11E+09
CD3+ T Cells	Singlets\LIve Cells\Morphology\Leukocytes\CD3+ T Cells	92.69	99.65	2.10E+06	10	2.10E+07	100	2.10E+09
CD4+ T Cells	Singlets\LIve Cells\Morphology\Leukocytes\CD4+ T Cells	55.14	59.28	1.25E+06	10	1.25E+07	100	1.25E+09
CD8+ T Cells	Singlets\LIve Cells\Morphology\Leukocytes\CD8+ T Cells	37.36	40.17	8.46E+05	10	8.46E+06	100	8.46E+08

Excel calculation
Count/mL * Dilution Factor

Excel calculation
Dilution-adjusted concentration * Total production volume