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### 1.0 Purpose

This SOP describes the materials and methods needed for the release of CliniMACS CD4 Reagent and CliniMACS CD8 Reagent. CliniMACS CD4 reagent is composed of murine anti-CD4 monoclonal antibodies conjugated to superparamagnetic iron dextran particles while CliniMACS CD8 reagent is composed of murine anti-CD8 antibodies conjugated to superparamagnetic iron dextran particles. These reagents are used in the selection of CD4-positive and CD8-positive cells for downstream expansion and/or manipulation.

### 2.0 Scope

This SOP applies to BDP personnel analyzing CliniMACS CD4 Reagent and/or CliniMACS CD8 Reagent for raw material release. 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 CliniMACS CD4 Reagent (BDP PN 10694) and/or CliniMACS CD8 Reagent (BDP PN 10695) – Material to be tested

4.2 Veri-Cells PBMC, (BDP PN 31201)

4.3 LS Columns, (BDP PN 31229)

4.4 MACS MultiStand, Miltenyi Biotec, Order no: 130-042-303

4.5 QuadroMACS Separator, Miltenyi Biotec, Order no: 130-090-976 or MidiMACS Separator, Miltenyi Biotec, Order no: 130-042-302

4.6 Low Binding Microcentrifuge Tube (1.7 mL) (BDP PN 31129 or BDP-approved equivalent)

4.7 MACSQuant Analyzer 10 Flow Cytometer, Miltenyi Biotec

4.8 Biosafety Cabinet

4.9 Eppendorf Centrifuge 5417C, or equivalent

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

4.11 MACSQuant Running Buffer (BDP PN 31172)

4.12 MACSQuant Calibration Beads (BDP PN 31171)

4.13 MACS Comp Bead Kit, anti-REA (BDP PN 31176)

4.14 8-Color Immunophenotyping Kit (BDP PN 31160)

4.15 autoMACS Rinsing Solution (BDP PN 31183)

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

4.17 Anti-CD8-VioGreen (BDP PN 31180-1)

4.18 Anti-CD8-VioBlue (BDP PN 31179-1)

4.19 Anti-CD8-PE (BDP PN 31177-1)



## 5.0 Procedure

- 5.1 Remove a new vial of lyophilized Veri-Cells PBMC from the refrigerator and allow it to warm to room temperature. Record Veri-Cells PBMC reagent information on **Form 22968-01**.
- 5.2 Add 325  $\mu$ L of Buffer A (included with Veri-Cells PBMC) to the lyophilized Veri-Cells, replace the rubber stopper in the vial and invert the vial for 5 seconds to facilitate complete mixing.
- 5.3 Incubate the vial at room temperature for 5 minutes  $\pm$  1 minute to allow for complete rehydration of the lyophilized cells (record rehydration incubation start/end times on **Form 22968-01**).
- 5.4 Record the date that the vial was rehydrated and the rehydration expiration date as 5 days from the date of rehydration on **Form 22968-01**.
- 5.5 Record the rehydration date and rehydration expiration date on the Veri-Cells packaging along with analyst initials.
- 5.6 Prepare Miltenyi Staining Solution by diluting MACS BSA Stock Solution 1:20 with autoMACS Rinsing Solution. Record the reagent details and volumes used on **Form 22968-01**.
- 5.7 Aliquot 50  $\mu$ L of reconstituted Veri-Cells into 1 microcentrifuge tube for each selection reagent to be assayed plus one extra microcentrifuge tube for a No Selection Bead negative control.
- 5.8 Spin each aliquot of Veri-Cells at 2,000 rpm for 5 minutes and remove the supernatant.
- 5.9 Resuspend each Veri-Cells pellet in 80  $\mu$ L of Miltenyi Staining Solution.
- 5.10 Add 20  $\mu$ L of CliniMACS CD4 Reagent, 20  $\mu$ L of CliniMACS CD8 Reagent or 20  $\mu$ L of Miltenyi Staining Solution (No Selection Bead Negative Control) to a resuspended Veri-Cells pellet.
- 5.11 Vortex gently to ensure complete mixing and place each tube on a rotator in the refrigerator (2-8°C) for 15 minutes  $\pm$  1 minute. Record the incubation start/end times on **Form 22968-02**.
- 5.12 Add 500  $\mu$ L of Miltenyi Staining Solution to each tube and spin at 2,000 rpm for 5 minutes.
- 5.13 Remove the supernatant and resuspend the cells in 500  $\mu$ L of Miltenyi Staining Solution.
- 5.14 Label an LS Column with the sample identity for each sample or control to be tested (1 LS Column per sample).
- 5.15 Prepare LS Columns for separation as follows (may be performed during the 15-minute sample incubation and spin (Steps 5.11-5.12)).



- 5.15.1 For each CliniMACS Reagent sample and the No Selection Bead negative control, place an LS column into the magnetic field of the QuadroMACS Separator or MidiMACS separator, with the column wings facing the analyst.
- 5.15.2 Rinse each column by adding 3 mL of Miltenyi Staining Solution. Allow the Miltenyi Staining Solution to pass through the column and collect in a 15 mL conical tube placed below the bottom of the column.
- 5.16 Apply each cell suspension to a column and allow the liquid to pass through the column and into a 15 mL conical tube placed below the bottom of the column.
- 5.17 Wash each column 3 times by adding 3 mL of Miltenyi Staining Solution and allowing the buffer to completely pass through the column into the 15 mL conical below the column.
- 5.18 Remove the column from the separator and place it in a 1.7 mL microcentrifuge tube.
- 5.19 Add 1.0 mL of Miltenyi Staining Solution to the column and push the contents through the column into the microcentrifuge tube using the column plunger.
- 5.20 Spin each microcentrifuge tube at 2,000 rpm for 5 minutes and remove the supernatant.
- 5.21 Resuspend each cell pellet in 100  $\mu$ L of Miltenyi Staining Solution.
- 5.22 Separately, spin a 50  $\mu$ L aliquot of reconstituted Veri-Cells at 2,000 rpm for 5 minutes and remove the supernatant. Resuspend in 100  $\mu$ L of Miltenyi Staining Solution. This serves as an Immunophenotyping Positive Control.
- 5.23 Label the resuspended cells using the Miltenyi Biotec 8-Color Immunophenotyping kit as described in **SOP 22206 - Cell Phenotyping by Flow Cytometry Using the Miltenyi Biotec 8-Color Immunophenotyping Kit**, with the following parameters:
  - 5.23.1 Following labeling, resuspend each cell pellet in 250  $\mu$ L of Miltenyi Staining Solution.
  - 5.23.2 Use a medium flow rate for analysis on the flow cytometer.
  - 5.23.3 Set the uptake volume to 50  $\mu$ L.
  - 5.23.4 Use the CD4 Selection Beads analysis template (see **Attachment 1**) for CliniMACS CD4 Reagent samples. Use the CD8 Selection Beads analysis template (see **Attachment 2**) for CliniMACS CD8 Reagent samples. Use the CD4 Selection Beads analysis template for the No Selection Bead Negative Control and the Immunophenotyping Positive Control.

## 6.0 Data Analysis

- 6.1 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).
- 6.2 Click on "File" and select "New Workspace."



- 6.3 Click on the “Sample” tab.
- 6.4 Right-click and select “Add...”
- 6.5 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.”
- 6.6 **NOTE:** MACSQuant Analyzer 10 data files have a \*.mqd extension.
- 6.7 The flow data may be visualized using the selection beads analysis templates in either of two ways:
  - 6.7.1 Since either the CD4 Selection Beads analysis template or CD8 Selection Beads analysis template was selected prior to data acquisition (Step 5.23.4), 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.
  - 6.7.2 Alternatively, either the CD4 Selection Beads analysis template or the CD8 Selection Beads 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 selection beads analysis template and click on Open.  
**NOTE:** The No Selection Bead Negative Control and the Immunophenotyping Positive Control should be analyzed using both selection beads templates if a CliniMACS CD4 Reagent and a CliniMACS CD8 Reagent were tested.
- 6.8 Double-click on each plot to enlarge it and check to ensure that the targeted population of cells is included in the gate.
  - 6.8.1 Use the gate edit points to adjust the size and shape of the gate.
  - 6.8.2 Click and drag a gate to move it to a different area of the plot.
- 6.9 For CliniMACS CD4 Reagent release
  - 6.9.1 Double-click on the “No Selection Bead Negative Control” CD4 plot, click on “Edit” and select “Copy plot”.
  - 6.9.2 Paste the “No Selection Bead Negative Control” CD4 plot in the indicated location of **Form 22968-03**.
  - 6.9.3 Double-click on the “Immunophenotyping Positive Control” CD4 plot, click on “Edit” and select “Copy plot”.
  - 6.9.4 Paste the “Immunophenotyping Positive Control” CD4 plot in the indicated location of **Form 22968-03**.
  - 6.9.5 Double-click on the CliniMACS CD4 Reagent CD4 plot, click on “Edit” and select “Copy plot”.



- 6.9.6 Paste the CliniMACS CD4 Reagent CD4 plot in the indicated location of **Form 22968-03**.
- 6.9.7 Indicate “Yes” on Form 22968-03 “Positive for CD4 Binding (Yes/No)” if the %-T in the CD4 gate for the CliniMACS CD4 Reagent is higher than the %-T in the CD4 gate for the “No Selection Bead Negative Control”.
- 6.9.8 If the %-T in the CD4 gate for the CliniMACS CD4 Reagent is not higher than the %-T in the CD4 gate for the “No Selection Bead Negative Control” and the “Immunophenotyping Positive Control” is also negative, indicate “Inconclusive” for the “Positive for CD4 Binding (Yes/No)” entry and repeat the assay.
- 6.9.9 The assay may be repeated with supervisor approval. Include an explanation in the Comments section of **Form 22968-03**.
- 6.10 For CliniMACS CD8 Reagent release
  - 6.10.1 Double-click on the “No Selection Bead Negative Control” CD8 plot, click on “Edit” and select “Copy plot”.
  - 6.10.2 Paste the “No Selection Bead Negative Control” CD8 plot in the indicated location of **Form 22968-04**.
  - 6.10.3 Double-click on the “Immunophenotyping Positive Control” CD8 plot, click on “Edit” and select “Copy plot”.
  - 6.10.4 Paste the “Immunophenotyping Positive Control” CD8 plot in the indicated location of **Form 22968-04**.
  - 6.10.5 Double-click on the CliniMACS CD8 Reagent CD8 plot, click on “Edit” and select “Copy plot”.
  - 6.10.6 Paste the CliniMACS CD8 Reagent CD8 plot in the indicated location of **Form 22968-04**.
  - 6.10.7 Indicate “Yes” on Form 22968-04 “Positive for CD8 Binding (Yes/No)” if the %-T in the CD8 gate for the CliniMACS CD8 Reagent is higher than the %-T in the CD8 gate for the “No Selection Bead Negative Control”.
  - 6.10.8 If the %-T in the CD8 gate for the CliniMACS CD8 Reagent is not higher than the %-T in the CD8 gate for the “No Selection Bead Negative Control” and the “Immunophenotyping Positive Control” is also negative, indicate “Inconclusive” for the “Positive for CD8 Binding (Yes/No)” entry and repeat the assay.
  - 6.10.9 The assay may be repeated with supervisor approval. Include an explanation in the Comments section of **Form 22968-04**.



## 7.0 Documentation

Record flow cytometry reagent part numbers, lots, and expiration dates on Form 22206-01. Record the calibration and compensation details on Form 23131-01. Record the preparation of Veri-Cells PBMC and Miltenyi Staining Solution on **Form 22968-01**. Record sample preparation details on **Form 22968-02**. Record CliniMACS CD4 Reagent flow cytometry results on **Form 22968-03**. Record CliniMACS CD8 Reagent flow cytometry results on **Form 22968-04**.

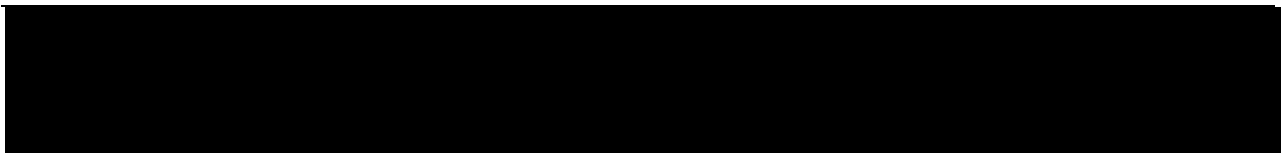
## 8.0 References and Related Documents

<b>SOP 22206</b>	Cell Phenotyping by Flow Cytometry Using the Miltenyi Biotec 8-Color Immunophenotyping Kit
<b>SOP 23131</b>	Operation of the MACSQuant Analyzer 10 Flow Cytometer
<b>Form 22968-01</b>	Veri-Cells PBMC and Miltenyi Staining Solution Preparation
<b>Form 22968-02</b>	CD4/CD8 Selection Sample Preparation
<b>Form 22968-03</b>	CD4 Flow Cytometry Results
<b>Form 22968-04</b>	CD8 Flow Cytometry Results

## 9.0 Attachments

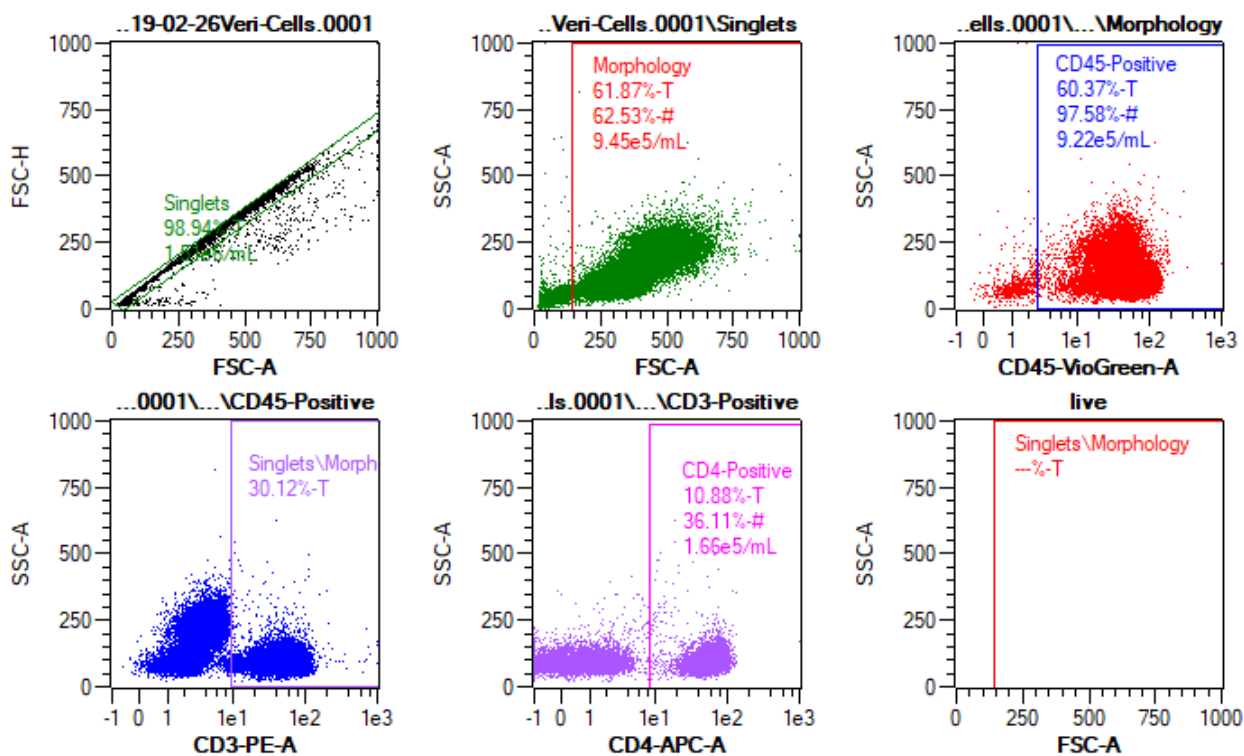
- 9.1 **Attachment 1** CD4 Selection Bead Analysis Template
- 9.2 **Attachment 2** CD8 Selection Bead Analysis Template

## 10.0 Change Summary





Attachment 1 – CD4 Selection Bead Analysis Template

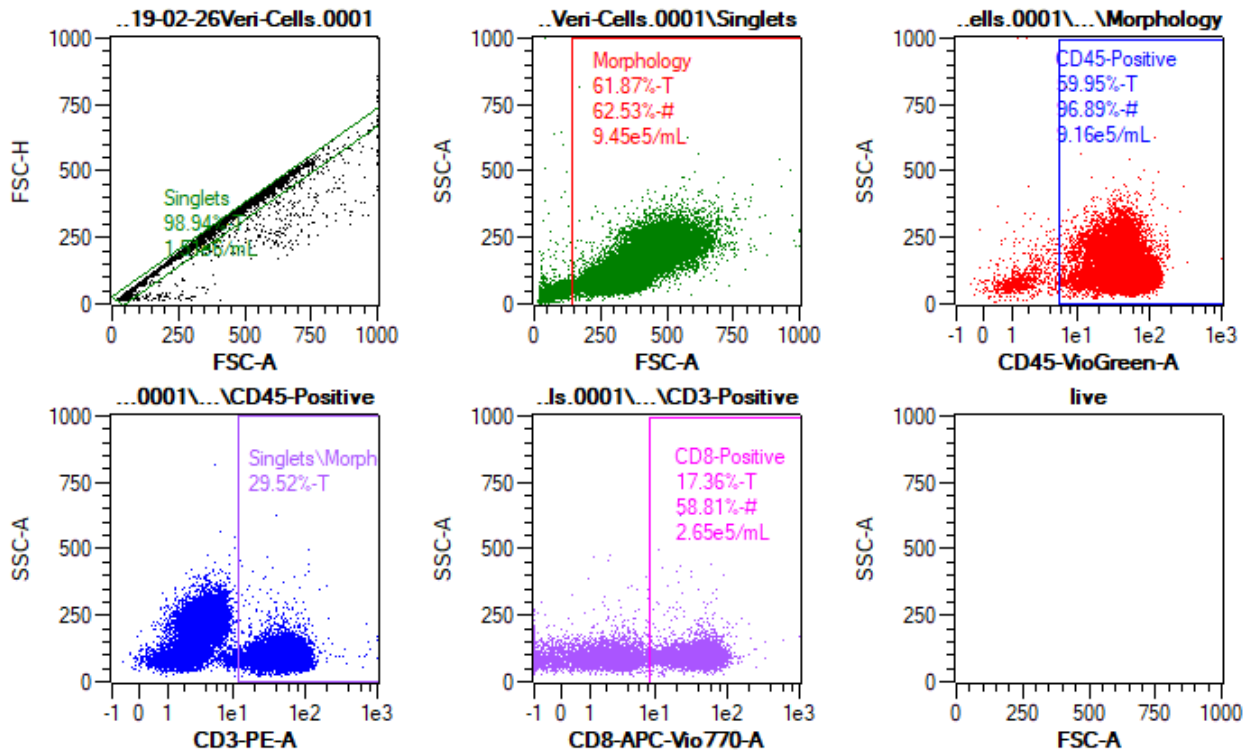


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Attachment 2 – CD8 Selection Bead Analysis Template



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