



## STEM CELL LABORATORY (STCL)



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**DOCUMENT TITLE:**

Flow Cytometric Analysis of CD45RA+ Cell Depletion Test Samples

**DOCUMENT NOTES:**

### Document Information

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## **FLOW-GEN-043**

### **FLOW CYTOMETRIC ANALYSIS OF CD45RA+ CELL DEPLETION TEST SAMPLES**

#### **1 PURPOSE**

- 1.1 This protocol describes the determination of CD45RA+ T-cells before and after an automated separation process from an HPC-A product using the CliniMACS Cell Separator.

#### **2 INTRODUCTION**

- 2.1 Analysis of CD45A+ T-cells is performed by fluorochrome-conjugated monoclonal antibody labeling of the target and non-target cells followed by determination using a flow cytometer.

#### **3 SCOPE AND RESPONSIBILITIES**

- 3.1 The Laboratory Director, Laboratory Manager, Flow Cytometry supervisor, and designated flow cytometry staff are responsible for ensuring that the requirements of this procedure are successfully met.

#### **4 DEFINITIONS/ACRONYMS**

- 4.1 CD45RA+ T-cells – Naïve T-cell
- 4.2 Original Fraction – Leukapheresis product after labeling and washing, before depletion
- 4.3 Target cell fraction – Depleted target cells after CliniMACS separation (CD3+CD45RA-CD45RO+)
- 4.4 Non-target cells – CD3+CD45RA+CD45RO-
- 4.5 DI water – Deionized water from spigot 1 (filtered)
- 4.6 7-AAD – 7 amino-actinomycin-D used to determine cellular viability
- 4.7 HPC-A - Hematopoietic Progenitor Cell – Apheresis
- 4.8 BD – Becton Dickinson
- 4.9 PPE – Personal Protective Equipment
- 4.10 WBC - white blood cell

#### **5 MATERIALS**

- 5.1 CD45RA APC, Miltenyi Biotec (130-092-249)
- 5.2 CD45RO FITC, Miltenyi Biotec (130-095-462)
- 5.3 CD3 PE, Miltenyi Biotec (130-091-374)
- 5.4 MsIgG2a FITC, Miltenyi Biotec (130-091-837)
- 5.5 MsIgG2b APC, Miltenyi Biotec (130-092-217)
- 5.6 PBS with 1%BSA, Gibco (Duke Cell Culture Facility)

- 5.7 BD Pharmlyse (10x), BD Biosciences Cat# 555899
  - 5.7.1 Dilute using 1 part BD Pharmlyse to 9 parts DI water.
- 5.8 7-AAD, BD Pharmingen Cat# 51-68981 E
- 5.9 Status Flow Process Control cells, R&D Systems

## 6 EQUIPMENT

- 6.1 BD FACSCalibur Dual-laser flow cytometer equipped with two lasers 488 nm and 635 nm to allow simultaneous analysis of FITC, PE and APC and appropriate software
- 6.2 Table top centrifuge – Sorvall RT 7
- 6.3 Pipettes and tips- 10, 100, 2000

## 7 SAFETY

- 7.1 Wear appropriate personal protective equipment (PPE) at all times when handling potentially infectious blood and body fluids to include, but not limited to, gloves, lab coats, etc.

## 8 STAINING PROCEDURE

- 8.1 Prepare the required amount of 1x Pharmlyse red blood cell lysing solution prior to beginning:
- 8.2 Add 2 mls of 10X Pharmlyse to 18 mls of DI water and mix well. Store solution at room temp and discard remaining solution at completion of use for the day.
- 8.3 Label tubes (2) for each fraction (A, B, or C) as they arrive for staining.



Isotype control  
(CD3 PE/MsIgG2a Fitc/  
MsIgG2b APC/7AAD)



Test tube  
(CD3 PE/CD45RO FITC  
CD45RA APC/7AAD)

- 8.4 Transfer  $3 \times 10^6$  WBCs to each tube or 50 microliters of control cells.
- 8.5 Calculate the correct volume to transfer by using the formula:
 
$$\# \text{ Cells needed} / \text{WBC concentration} = \text{volume of sample}$$

$$3 \times 10^6 / 43.0 \times 10^6 = 0.0698 \text{ or } 70 \text{ microliters (round to whole number)}$$
- 8.6 Add 10 times the volume of 1X Pharmlyse solution to lyse red blood cells. Using the example above this would be 700 microliter. Use 500 microliters for controls.
- 8.7 Incubate for 10 minutes in the dark at room temperature. (Do not extend beyond this time.)
- 8.8 Vortex gently and centrifuge at 1200 rpm (300 g) 5 minutes (refrigerated).

- 8.9 Decant the supernatant and touch off the last drop to a paper towel.
- 8.10 Wash the cells by adding 1 ml PBS with 1% BSA buffer.
- 8.11 Repeat centrifugation and decant steps.
- 8.12 Resuspend the cells in ~70-100  $\mu$ l of PBS with 1% BSA buffer and add antibodies as follows to the tubes:
- 8.13 20 $\mu$ l - MsIgG2a FITC (tube 1) or CD45RO FITC (tube 2)
- 8.14 10 $\mu$ l - MsIgG2b APC (tube1) or CD45RA APC (tube 2)
- 8.15 10 $\mu$ l - CD3 PE (both tubes)
- 8.16 7-AAD is added later (both tubes). **Do not add to control cells.**
- 8.17 Mix well and incubate the tubes for 10 minutes in the dark in the refrigerator (2-8 $^{\circ}$  C)
- 8.18 Wash the cells by adding 1 ml of PBS with 1% BSA buffer and centrifuging at 1200 rpm for 5 minutes (refrigerated).
- 8.19 Decant the supernatant and touch off the last drop to a paper towel.
- 8.20 Resuspend the cells in an adequate (~300 $\mu$ l) PBS with 1% BSA buffer.
- 8.21 Add 10 $\mu$ l of 7-AAD to each tube and vortex tube prior to acquisition. **Do not add to control cells.**

## 9 FLOW CYTOMETER ACQ/ ANALYSIS PROCEDURE

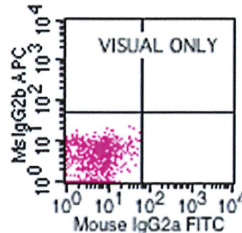
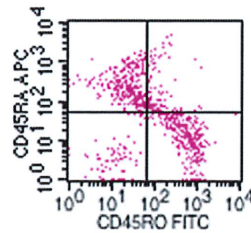
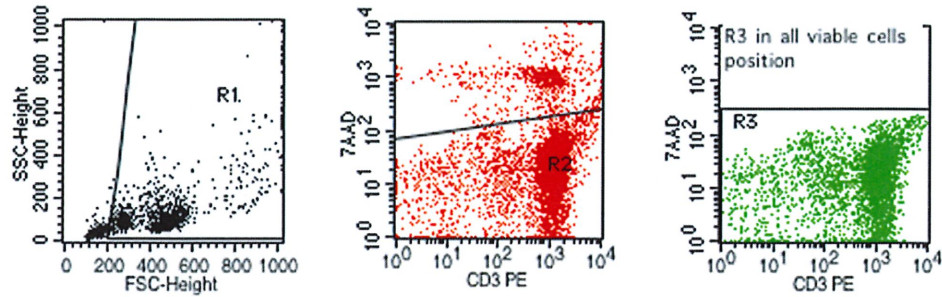
- 9.1 Wash the compensation tubes, prepared for immune reconstitution optimization at the beginning of the day, when the test samples receive the final wash step (8.18-20 above). Optimize the Lyse/Wash settings by following guidelines found in procedure FLOW-GEN-032 Fluorescence Overlap Compensation Optimization for the BD FACSCalibur Flow Cytometer.
- 9.2 The BD Cell Quest Pro Acquisition/Analysis template CD45RA selection should be used to acquire and analyze the test samples and control cells. For test samples enter the WBC concentration and product volume into the appropriate function boxes prior to starting the acquisition of each sample. This will allow the proper stats to be obtained according to the Region 3 placement explained later. *This step is not required for control cells.*
- 9.3 Place tube 1 on the instrument and while in Setup mode, place the R1 region around the cellular events on the ungated FSC/SSC plot to eliminate debris. Place the R2 region around the 7AAD negative population on the debris free (red) gated plot.
- 9.4 For the A and B sample, set acquisition stop at 50,000 viable events.
- 9.5 For the C sample, set acquisition stop at 100,000 viable CD3+ events.
- 9.6 Tube 1 (isotype control) from each sample may be stopped after enough events to establish background are acquired.
- 9.7 In order to obtain the % of CD45RA cells in the product Region 3 must be inclusive of all viable events as illustrated in Example 1 below. When in this

position, the displayed CD3 value is invalid and should be marked out in GMP fashion with the date and initial of the person making the correction. R3 must then be moved as in Example 2 to obtain the CD3 total. The % CD45RA is then invalid and should be marked out as explained previously. **It is important to recognize that the results are linked to the placement of region 3 when reporting results from this template.**

- 9.8 The viability, total CD3+, and % CD45 RA are calculated automatically within the template expression editors from statistics generated through region and quadrant placements.

**EXAMPLES BEGIN ON NEXT PAGE**

**Example 1 showing A or B sample with R3 positioned to obtain CD45RA%. R3 needs to be adjusted as in Example 2 below to obtain CD3 results.**



Gated Events: 103883  
Total Events: 168486  
X Parameter: CD3 PE (Log)  
Y Parameter: 7AAD (Log)

Gate	Events	% Gated
WBCs	103883	100.00
VIABLE WBC	103883	100.00
VIABLE CD3+	103883	100.00

Gated Events: 103883  
Total Events: 168486  
X Parameter: CD45RO FITC (Log)  
Y Parameter: CD45RA APC (Log)  
Quad Location: 64, 50

Quad	Events	% Gated
UL	40079	38.58
UR	15250	14.68
LL	7815	7.52
LR	40739	39.22

WBC =  $150.00 \times 10^6/\text{ml}$

WBC Viability: 83.84 %

Sample volume 100.00 mls

Report %CD45RA of viable events only  
when R3 is placed around All viable events

%CD45RA OF VIABLE EVENTS IN SEL A AND B  
(CONTAMINANT IN FINAL PRODUCT): 53.26 %

Gated Events: 123907  
Total Events: 168486  
X Parameter: CD3 PE (Log)  
Y Parameter: 7AAD (Log)

Gate	Events	% Gated
WBCs	123907	100.00
VIABLE WBC	103883	83.84
VIABLE CD3+	103883	83.84

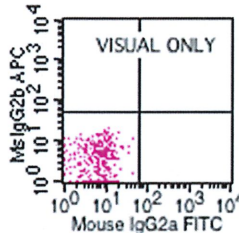
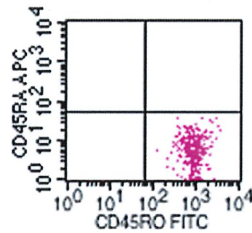
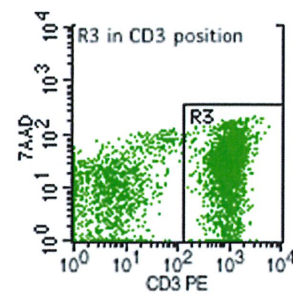
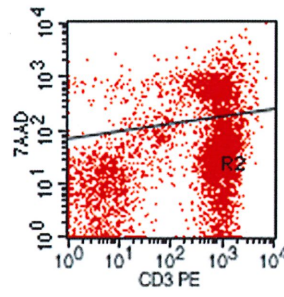
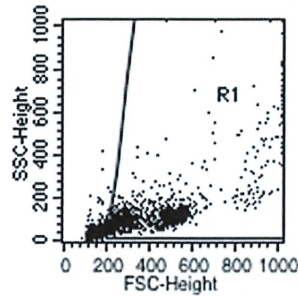
TOTAL VIABLE CD3 IS USED ONLY WHEN REGION 3  
(R3) IS PLACED AROUND CD3+ POPULATION

Total viable CD3+:

Invalid number when R3 is  
positioned around all events.  
Mark through result, sign,  
and date.

Report

Example 2 showing sample C post selection with R3 positioned to obtain CD3 total. R3 must be adjusted to position in Example 1 to obtain CD45RA%.



Gated Events: 103786		
Total Events: 280186		
X Parameter: CD3 PE (Log)		
Y Parameter: 7AAD (Log)		
Gate	Events	% Gated
WBCs	103786	100.00
VIABLE WBC	103786	100.00
VIABLE CD3+	70968	68.38

Gated Events: 70968  
Total Events: 280186  
X Parameter: CD45RO FITC (Log)  
Y Parameter: CD45RA APC (Log)  
Quad Location: 64, 50

Quad	Events	% Gated
UL	0	0.00
UR	197	0.28
LL	8	0.01
LR	70763	99.71

WBC = 56.00 x 10e6/ml

WBC Viability: 75.19 %

Sample volume 100.00 mls

Report %CD45RA of viable events only  
when R3 is placed around All viable events

%CD45RA OF VIABLE  
(CONTAMINANT IN

Result is invalid when R3 is positioned around CD3+ events. Mark through results sign and date.

TOTAL VIABLE CD3 IS USED ONLY WHEN REGION 3 (R3) IS PLACED AROUND CD3+ POPULATION

Total viable CD3+: 3829.23 x 10e6 cells

Report

Gated Events: 138037		
Total Events: 280186		
X Parameter: CD3 PE (Log)		
Y Parameter: 7AAD (Log)		
Gate	Events	% Gated
WBCs	138037	100.00
VIABLE WBC	103786	75.19
VIABLE CD3+	70968	51.41

9.9 The following results of testing should be reported to the processing tech:

9.9.1 A and B sample: CD3+ total and % CD45RA+ of viable cells

9.9.2 C sample: Total CD3 and % CD45RA + contamination

9.10 Region 3 must be moved back and forth to obtain CD45RA% and CD3 totals for all 3 samples (A-C).

9.11 The control cell analysis should be reviewed for acceptability prior to reporting results and turned in to the flow supervisor to be filed.

## 10 RELATED DOCUMENTS/FORMS

FLOW-GEN-032 Fluorescence Overlap Compensation Optimization for the BD FACSCalibur Flow Cytometer

## 11 REFERENCES

CliniMACS Special protocol - Proposal for CD45RA+ Cell determination by flow cytometry after depletion of CD45RA+ cells.

## 12 REVISION HISTORY

Revision No.	Author	Description of Change(s)
03	M. Reese	<ul style="list-style-type: none"> <li>Changed resuspension volume of sample in 8.13</li> <li>Clarified acquisition and analysis steps</li> <li>Added better analysis examples</li> <li>Clarified what results to report</li> <li>Reformatted procedure steps to include Staining and Acq/Analysis</li> <li>Added Status Flow controls to the process</li> </ul>

**Signature Manifest****Document Number:** FLOW-GEN-043**Revision:** 03**Title:** Flow Cytometric Analysis of CD45RA+ Cell Depletion Test Samples

All dates and times are in Eastern Time.

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**Quality**

Name/Signature	Title	Date	Meaning/Reason
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**Document Release**

Name/Signature	Title	Date	Meaning/Reason
Sandy Mulligan (MULLI026)		14 Jul 2017, 06:59:09 PM	Approved