

# PROTOCOL FOR ESTIMATING THE NUMBER OF CD38 MOLECULES ON THE CD8<sup>+</sup> T LYMPHOCYTES OF HIV-INFECTED INDIVIDUALS

(UPDATES TO THIS PROTOCOL MAY BE AVAILABLE AT (<http://cyto.mednet.ucla.edu/>))

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## **1. MATERIALS**

- Rainbow beads 8 bead mix (Spherotech Inc., Libertyville, IL, CAT# RCP 30-5a).
- PE-CD4 (~100 tests) and PE-CD38 (sufficient for 6 months of patient tests) with F/P ratios of 1.0.
- PE-IgG1 isotype control, FITC-CD8 and PerCP-CD3.
- Ortho-mune ammonium chloride lysing reagent (Ortho Diagnostic Systems Inc., Raritan, NJ).
- PBSA 1X PBS without calcium or magnesium (Gibco BRL, Life Technologies, Grand Island, NY, CAT# 14190-144) supplemented with 2% heat normal calf serum and 0.1% sodium azide (pH 7.2).
- Protein-free 1X PBS with 1% paraformaldehyde; (pH 7.2). A commercially available 10% solution of methanol free formaldehyde made from paraformaldehyde, EM grade from Electron Microscopy Sciences, Fort Washington, PA, CAT#15712-S has been verified for CD38 RFI stability when used as directed in 5.2A and optionally in 5.2B
- Four Excel spreadsheets (Rainbow beads for FL-2 and FL-1, Healthy Control, and CD38 worksheets) in a single Excel file. If you need the spreadsheets, you can download them from the web site (<http://cyto.mednet.ucla.edu/>) or request a disk containing them by sending an email to Lance Hultin whose address is [hultinl@mednet.ucla.edu](mailto:hultinl@mednet.ucla.edu).
- EDTA collected blood specimens (stability of CD38 RFI on ACD and heparin has not been verified).
- Optional FACS lysing solution (Becton Dickinson, San Jose, CA, CAT# 349202) is an optional lysing reagent used only for CD38 measurement on patient samples. It has been verified for CD38 RFI stability when used as directed in section 5.2B.

## **2. RECOMMENDATION OF VENDOR FOR PE-CD38 AND PE-CD4**

PE-CD38 for quantitation on patient samples and PE-CD4 for instrument characterization must be at a consistent F/P ratios of 1.0. To date, Becton Dickinson Immunocytometry Systems (BDIS, San Jose, CA) is the sole vendor who has guaranteed the required 1.0 F/P ratio. mAb for other specificities may be purchased from any vendor.

## **3. VALIDATION OF LOGARITHMIC AMPLIFIER LINEARITY AND SENSITIVITY**

### **3.1. INTRODUCTION**

All intensity measurements in this document are given in terms of the Relative Fluorescence Intensity (RFI) on a scale of 1–10,000 with 4 decade logarithmic amplifiers as provided by BDIS flow cytometers, except as noted for Coulter instrumentation. All fluorescence intensity measurements must be converted to RFI.

### 3.2. PREPARE RAINBOW BEADS

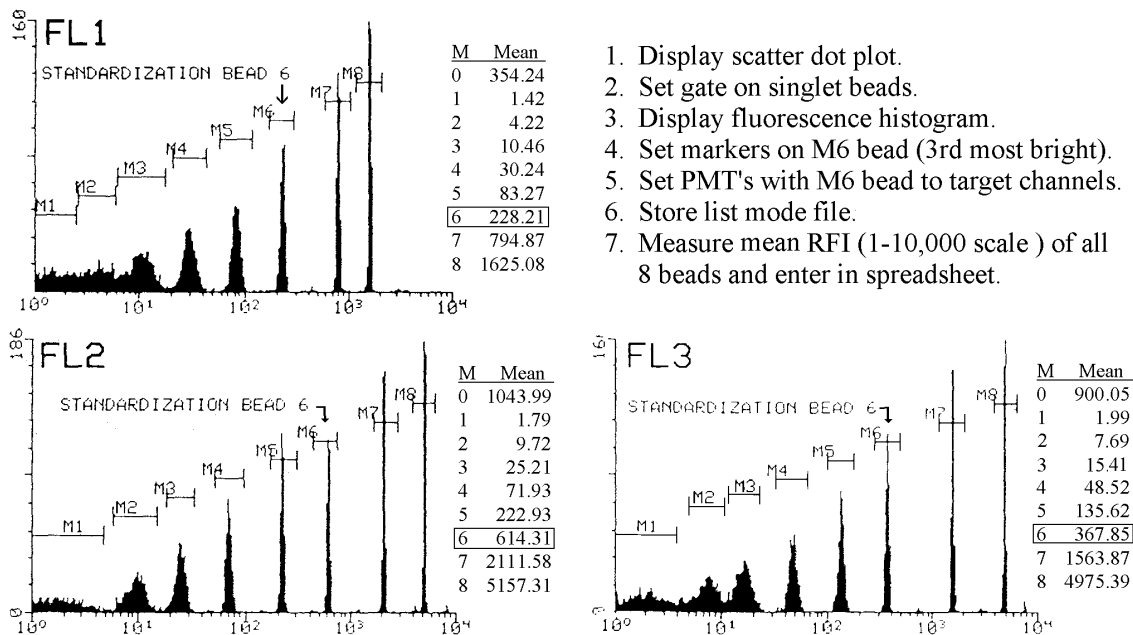
Shake bottle vigorously then add 1–2 drops of beads to 0.5 ml PBSA. Make this preparation fresh daily.

The Rainbow bead mixture will be used to set PMT voltages and assess logarithmic amplifiers/PMT performance. The PMTs will be set for FL1 (FITC), FL2 (PE) and the 3rd color detector. The 3rd color fluorescence detector may be designated as FL3 or FL4 depending on the cytometer manufacturer and the 3<sup>rd</sup> color fluorochrome. BDIS instrument users will use FL3 and Coulter XL instrument users will use FL4 for the 3rd color. The PMT voltage for FL2 was selected to place the PE-CD4 distribution in the first portion of the fourth decade of the logarithmic scale because logarithmic amplifiers show the greatest non-linearity at the ends of the scale. The third most bright bead, marked M6 on Figure 1, will be used to set PMTs. The mean of the M6 peak will be placed at the target channels specified in the target channel tables below. This bead mixture does not use true FITC or PE fluorochrome and cannot be compensated. To set instrument PMT voltages using channel number (0–1023) rather than the relative fluorescence intensity (RFI 1–10,000), the target channels are included.

**Table 1. Target Channels for BDIS and Coulter Instruments**

BDIS	1024 linear channels	4 decade RFI (1–10,000)	Coulter	4 decade RFI (.1 – 1000)
FL1	604	228	FL1	22.8
FL2	714	614	FL2	61.4
FL3	657	368	FL3/FL4	36.8

**Figure 1. Validation of Logarithmic Linearity and Daily Setup Using Rainbow Beads.**



### 3.3. SET PMT VOLTAGES

Input instrument settings normally used for whole blood specimens in the laboratory. Turn compensation OFF. The FSC gain will need to be increased to place the 3 μm beads on scale. Set a gate on the singlet bead population. The PMTs for FL1, FL2, and either FL3 (for FACScan users) or FL4 (for XL users) need to be adjusted using the Rainbow beads. Place the mean of the third most bright bead (labeled M6 bead in Figure 1) of the diluted beads into the target channels noted in Table 1, depending on the instrument. Briefly, cursors are set around the M6 bead and PMT voltages are adjusted until the mean for this bead matches the mean listed in Table 1.

*It is important that the Rainbow beads are placed into the appropriate target channels. Regardless of which scale is used, try to keep the setting to within 1 or 2 of that indicated in the table.*

Collect 10,000 events and store in list mode. Collect this file before compensation is set.

### 3.4. ANALYSIS OF INSTRUMENT PERFORMANCE

*The analysis of instrument performance is done at the beginning of the study and following instrument service. It is not done on a daily basis as is setting PMT voltages.*

After the Rainbow bead file is collected, determine the mean fluorescence of all 8 beads. It has been our experience that the three dimmest beads are not always clearly resolved on a single parameter histogram but they are usually resolvable on a two parameter dot plot. For this reason it may be necessary to create a display of FL1 versus FL2 and draw a region around the 8 individual bead populations and use region statistics to determine the mean RFI of each bead for the FL1 and FL2 detectors.

### 3.5. RAINBOW BEAD SPREADSHEET (DISPLAY OF FL2 DATA)

The Rainbow Bead spreadsheet, provided on a 3½ floppy disk or by downloading from this web site, is used to evaluate instrument performance. (Enter the mean RFI values for the 8 beads into the spreadsheet. The spreadsheet will already have data entered, so just click on the appropriate cell and replace this with the new data. The following tables illustrate a portion of the Rainbow Bead spreadsheet. Numbers in bold in Table 2A are the FL2 mean RFI values. Table 2B represents calculated values used to assess instrument performance.

Bead	MEPE	Mean RFI	Channel #	Peak to Peak(2 point)		
				chan/dec	# Decades	Residual %
Blank 1	70	<b>1.79</b>	64.3	---	---	---
Bead 2	400	<b>9.72</b>	252.84	222.08	4.6	8.22
Bead 3	1200	<b>25.21</b>	358.80	232.85	4.4	1.81
Bead 4	3800	<b>71.93</b>	475.37	251.83	4.1	6.70
Bead 5	12000	<b>222.93</b>	601.13	249.16	4.1	3.04
Bead 6	34000	<b>614.31</b>	713.83	244.28	4.2	0.73
Bead 7	124000	<b>2111.58</b>	851.10	258.75	4.0	0.39
Bead 8	300000	<b>5157.31</b>	950.38			5.21

**Table 2B.**

<b>Bead</b>	<b>MEPE</b>
slope	243.65
intercept	-389.52
R-Sqrd	<b>0.999</b>
AvgRes%	<b>3.73</b>
Logarithmic Decades	<b>4.20</b>
Coeff of Response	243.65
Blank Threshold	<b>73.17</b>

Information in bold in Table 2B are calculated from a regression equation included in the spreadsheet and is based on the mean values obtained for each bead and the fluorescence units assigned by the manufacturer (MEPE, molecules of equivalent PE, in example above). The R-SQUARED is a measure of how good the fit of the regression line is and should be >0.95. The AVERAGE RESIDUAL PERCENT is a composite measure of how close the measured beads are to the regression line. If the points are

not very linear, the average residual percent will be >5% and could indicate a logarithmic amplifier that is non-linear. The LOG DECADES indicates the overall dynamic range of the logarithmic amplifier and should be between 3.8 and 4.2. The AVERAGE RESIDUAL PERCENT and LOG DECADES measurements are averages over the entire scale encompassed by the beads. Individual areas of a logarithmic amplifier could vary and these data are also provided on the spreadsheet under the columns labeled as “Peak to Peak” in Table 2A. This bead method is a quick method to reveal gross problems in logarithmic amplifier performance. The BLANK THRESHOLD measures where on this regression line the blank bead lies. This is a measure of sensitivity and should be <200 MEPE. If all 8 beads can be identified in this particular lot of beads on a FL1 versus FL2 display, the instrument’s sensitivity is satisfactory. This spreadsheet does not include manufacturer values for the FL3 detector. If the instrument does not meet one or more of the criteria noted for R-SQUARED, AVERAGE RESIDUAL PERCENT, LOG DECADES, or BLANK THRESHOLD, contact the instrument service person.

**4. CONSERVATION OF THE LEVEL OF CD4 ANTIGEN EXPRESSION ON CD4<sup>+</sup> LYMPHOCYTES AND ITS USE AS A BIOLOGIC STANDARD FOR FLOW CYTOMETER INSTRUMENT CHARACTERIZATION**

To characterize the flow cytometer, we have chosen to utilize a biological standard, i.e., CD4 antigen among healthy donors. CD4 antigen is expressed with a C.V. (coefficient of variation) among healthy controls of <5% (Hultin, Cytometry. Suppl. 8:90, 1996.). *HIV infection decreases the RFI of CD4 lymphocytes in some individuals, so only uninfected donors should be used to perform instrument characterization.* For purposes of this protocol, the level of CD4 staining on CD4<sup>+</sup> T lymphocytes is taken to be 50,000 molecules per cell (Poncelet, Methods of immunological analysis, Masseyeff, Albert, Staines, eds., 388-417).

The median relative fluorescence intensity (RFI) of PE-CD4 staining is determined on the lymphocytes of 20 healthy control donors. The mean PE-CD4 RFI of the 20 healthy donors is calculated. This mean is divided into 50,000 to calculate the number of PE molecules detected per RFI unit. For example, if the mean RFI = 1,000, then 50,000 ÷ 1,000 = 50. This value (50 in the example) is referred to as the “RFI multiplier”. Each instrument may have a different “RFI multiplier” associated with it and this should remain relatively stable over a period of months to years.

## 5. SAMPLE PREPARATION METHODS 1 AND 2

*Sample Preparation Method 1, which does not have a fixation step, is utilized for the PE-CD4 measurement on the uninfected healthy control donor specimens that are used to characterize the flow cytometer. A fixation step is avoided because there can be significant decreases in PE-CD4 fluorescence after fixation. Note that characterization of the flow cytometer is completed as a separate task prior to making CD38 measurement on patient samples and is only repeated if instrument servicing that would effect fluorescence detection is performed or to check stability of the detection. Sample Preparation Method 2 which uses fixation of cells in paraformaldehyde is used for CD38 antigen quantitation on HIV-infected samples. CD38 RFI is effected minimally or not at all by fixation depending on the fixative. An optional method using FACS lyse and 1% fixation has been recently verified and added as an optional method for patient samples.*

Prepare ammonium chloride lysing solution by diluting one vial to a total volume of 100 ml with double distilled H<sub>2</sub>O. Mix well. Hold at room temperature. Make fresh daily.

### 5.1. METHOD 1: HEALTHY CONTROLS (NOT FIXED) FOR INSTRUMENT CHARACTERIZATION

Whole blood sample preparation using Ammonium Chloride lysing solution

1. Add 50 µl of staining buffer (PBSA) to 12 x 75mm round bottom tubes.
2. Add 20 µl each of appropriate antibodies. Each three-color tube will require 3 separate aliquots of 20 µl each.
3. Add 100 µl of well-mixed whole blood.
4. Gently vortex tubes individually in order to observe that all blood is mixed with antibodies.
5. Incubate in the dark at room temperature for 30 min.
6. Lyse with 1 ml of 1X NH<sub>4</sub>Cl lysing solution. Vortex tubes then add 1ml lysing solution and vortex tubes again. Place in the dark at room temperature for **5 min**. The NH<sub>4</sub>Cl lysing solution must be at room temperature to properly lyse the erythrocytes.
7. Spin cells at 4°C for five min at 300 X g, (the 4°C temperature is used to inhibit lysis in the centrifuge).
8. Aspirate supernatant and vortex tubes vigorously to break up pellet.
9. Repeat steps 6, 7, and 8 EXCEPT lyse samples for only 3 min. This lysis is at room temperature also.
10. After careful aspiration of the supernatant vortex tubes vigorously and add 500 µl of PBSA.
11. Run Method 1 samples immediately to prevent loss of fluorescence of over time. Perform daily instrument setup just prior to running samples to minimize the effect of instrument drift over time.
12. Each sample should be vortexed individually immediately before being analyzed.

**5.2. METHOD 2: PATIENT SAMPLES (FIXED IN 1% PARAFORMALDEHYDE) FOR CD38 QUANTITATION (CHOOSE EITHER METHOD 5.2A OR 5.2B)**

**5.2A AMMONIUM CHLORIDE LYSING OPTION**

1. Perform steps 1 through 9 of Sample preparation Method 1.
2. Aspirate the supernatant and vortex tubes.
3. Add 1 ml PBSA wash and spin cells at 4°C for 5 min at 300 X g.
4. Aspirate the PBSA wash and resuspend the cells in 500 µl of protein free 1X PBS with 1% paraformaldehyde.
5. Allow samples to stabilize at 4°C in the dark for 1 hr prior to running.
6. Perform daily instrument setup just prior to running samples to minimize the effect of instrument drift over time.
7. Each sample should be vortexed individually immediately before being analyzed.

**5.2B FACS LYSE OPTION**

1. Perform steps 1 through 5 of Sample preparation Method 1.
2. Lyse with 2 ml of 1X FACS lysing solution. Vortex tubes then add 2ml lysing solution and vortex tubes again. Place in the dark at room temperature for **10 min**. The FACS lysing solution must be at room temperature to properly lyse the erythrocytes.
3. Spin cells at 4°C for five min at 300 X g,
4. After careful aspiration of the supernatant vortex tubes.
5. Add 1 ml PBSA wash and spin cells at 4°C for 5 min at 300 X g.
6. Aspirate the PBSA wash and resuspend the cells in 500 µl of protein free 1X PBS with 1% paraformaldehyde.
7. Allow samples to stabilize at 4°C in the dark for 1 hr prior to running. Samples have been verified to remain stable for 24 hours with this method.
8. Perform daily instrument setup just prior to running samples to minimize the effect of instrument drift over time.
9. Each sample should be vortexed individually immediately before being analyzed.

**5.3. METHOD 1 OR 2: COMPENSATION TUBES**

See Section 6.3. for details. Use unfixed single stained samples to set compensation for the unfixed healthy controls and fixed single stained samples to set compensation for the fixed HIV-infected samples.

**5.4. OTHER TUBES FOR IMMUNOPHENOTYPIC ANALYSIS CAN BE STAINED AND RUN USING PROTOCOLS ALREADY IN PLACE IN THE LABORATORY.**

## 6. DAILY INSTRUMENT SETUP (PMT VOLTAGES AND COMPENSATION)

### 6.1. INTRODUCTION

Set the PMT voltages and compensation just prior to each set of patient samples. The PMTs are set to place a control bead in a predetermined channel, thereby standardizing fluorescence intensity readings over time. After the PMT voltages are set, set compensation. This PMT setup procedure is the same as that described in Section 3.3. See Table 1 for target channels and set up the instrument accordingly.

### 6.2. SET PMT VOLTAGES TO PLACE CONTROL BEAD (M6) IN THE TARGET CHANNEL

**6.2.1.** Prepare the Rainbow beads by shaking vigorously and adding 1–2 drops of beads to 0.5 ml PBSA. Make this preparation fresh daily.

**6.2.2.** Input instrument settings normally used for whole blood specimens in the laboratory. Turn compensation OFF. The FSC gain will need to be increased to place the 3 µm beads on scale. Set a gate on the singlet bead population as shown in Figure 1. The PMTs for FL1, FL2, and the 3rd color fluorescence detector need to be adjusted using the Rainbow beads. Place the mean of the third most bright bead (labeled M6 bead in Figure 1) of the diluted beads into the target channels noted below, depending on the instrument. Briefly, cursors are set around the M6 bead and PMT voltages are adjusted until the mean for the M6 bead matches the mean listed in Table 1.

*It is important that the Rainbow beads are placed into the appropriate target channels. Regardless of which scale is used try to keep the setting to within 1 or 2 of that indicated in the table.*

**6.2.3.** Collect 10,000 events and store in list mode. Collect this file before compensation is set.

### 6.3. SET INSTRUMENT COMPENSATION

Compensation tubes (one set of compensation tubes per run on the cytometer)

Tube 1 FITC-CD8

Tube 2 PE-CD4

Tube 3 PerCP-CD3 (optional if needed)

1. Stain and run a single stained FITC-CD8. Set a lymphocyte gate using Forward and Side Scatter. Display FL1 versus FL2 gated on the lymphocytes. Next, set FL1 versus FL2 compensation.
2. Stain and run a single stained PE-CD4 to set FL1 versus FL2 and FL2 versus FL3 compensation.
3. Stain and run PerCP-CD3 to set appropriate compensations if the instrumentation requires it.



- 6.4. Caution:** *The PE-CD4 compensation tube should not be rerun for RFI measurements because of the possibility that a decreased RFI can result presumably from back drips of the sheath fluid into the sample. Always aliquot samples into separate tubes if a sample will be run more than once.*
- 6.5. Repeat before each run:** PMT voltages and Compensation must be set carefully prior to each new experimental or patient run due to the slight instrumentation drift observed during a day's operation. Use unfixed single stained samples to set compensation for the unfixed healthy controls and use fixed single stained samples to set compensation for the fixed HIV-infected samples.

## 7. CHARACTERIZATION OF THE NUMBER OF MOLECULES OF PE PER RFI CHANNEL

### 7.1. OVERVIEW

The object is to characterize the flow cytometer to determine how many PE molecules are detected per linear fluorescence channel. Stain whole blood from 20 healthy adult control donors using the “not fixed” method 1 described in Section 5.1. and the mAb panel below. Only 4 or 5 donors should be run each day to smooth day to day variability that might be expected in instrument performance. The “not fixed” method is used because the fluorescence from CD4-stained cells is decreased significantly and variably on paraformaldehyde fixed samples. The twenty controls should be collected over a one week time span. This procedure can be repeated on 20 donors every 3 months thereafter to verify the stability or to stimulate re-characterization of the instrument. The median RFI for PE-CD4 is determined for each control donor as described in Data Analysis (Section 7.5. below) and the data are entered into the Healthy Control spreadsheet (Section 7.6. below). This spreadsheet contains mAb lot information and calculates the mean PE-CD4 RFI for the samples. The spreadsheet also calculates the number of PE molecules per RFI channel. This value, termed the “RFI multiplier” is calculated as:

$50,000 \text{ CD4 molecules} \div \text{mean of median PE-CD4 RFI of the 20 control donors} = \text{RFI multiplier}$

### 7.2. PANEL FOR PE-CD4 MEDIAN RFI

Tube 1: PE-IgG1 isotype control

Tube 2: PE-CD4

*PE-CD4 should be run as a single stained sample. Combining other markers with this measurement can variably alter the PE-CD4 RFI measurement.*

### 7.3. SAMPLE PREPARATION

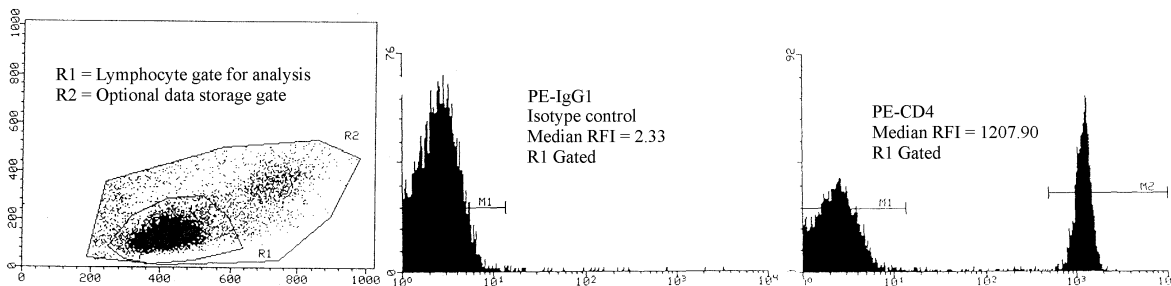
Method 1 Sample Preparation: (Section 5.1.)

### 7.4. SAMPLE ACQUISITION

After the daily instrument setup has been performed, collect data from the Method 1 prepared samples (PE-CD4 and PE-IgG1 control) samples. It is recommended that data

from all cells be stored in list mode but if data storage space is of concern a large gate set around the lymphocytes and monocytes can be used as shown in Figure 2. This large mononuclear cell gate can be used for data storage. Various software have different utilities for setting a stop-counter for data storage. Collect 5000 lymphocytes for tube 1 (the isotype control tube) and 5000 lymphocytes for measurement of the median RFI for PE-CD4 on each healthy control.

**Figure 2. Characterization of the Number of PE Molecules per RFI Channel by Measurement of Median RFI of PE-CD4 on Healthy Controls.**



**Tube 1 and 2:**

1. Display scatter dot plot.
2. Set gate (R1) on lymphocytes for tubes 1 and 2.

**Tube 1: (PE-IgG1)**

3. Display FL2 histogram gated on lymphocytes.
4. Set a statistical region (M1) to include ~98% of the lymphocytes. The lower cursor must be set at channel 1.
5. Measure the median RFI.

**Tube 2: (PE-CD4)**

6. Display FL2 histogram gated on lymphocytes.
7. Set a statistical region (M2) to include all of the CD4<sup>+</sup> lymphocytes and exclude any dim staining monocytes.
8. Measure the median RFI.
9. Record Median RFI data of tube 1 and 2 in the Healthy Control Spreadsheet.

**7.5. DATA ANALYSIS**

Please refer to Data Analysis Figure 2 above. A brief description is provided.

Median RFI measurements are used for all biological fluorescence intensity data. A lymphocyte gate is set using forward and side scatter. The lymphocyte gate is used for all biological data analysis.

1. For measurement of the background PE fluorescence on the PE-IgG1 isotype control tube, display an FL2 (PE-IgG1) histogram that is gated on the lymphocyte population defined by forward scatter and side scatter. Set a lower cursor at RFI channel 1 the lowest channel and set an upper cursor to include ~98 % of the lymphocytes. Next determine the median RFI of the lymphocytes and record and enter into the spreadsheet for the Healthy Controls. This background fluorescence is subtracted from the median RFI measurements for PE-CD4 to yield a corrected CD4 RFI that is used to calculate the number of PE molecules per RFI channel (RFI multiplier). In this way the autofluorescence is subsequently subtracted out and the median RFI measurements for PE-CD4 are due to antibody binding.

2. For measurement of the median RFI of PE-CD4, cursors are set around the positive peak of the CD4 stained cells using the FL2 histogram display so as to exclude any dim staining monocytes. The median RFI is determined, recorded and entered into the Healthy Control spreadsheet.

## **7.6. “HEALTHY CONTROL” SPREADSHEET**

The Healthy Control spreadsheet calculates the “RFI multiplier” (the number of PE molecules per RFI channel on a 1–10,000 RFI scale) that is needed for use in the CD38 spreadsheet to calculate the number of CD38 molecules. There are places for entering the median RFIs for the PE-CD4 tube and the PE-IgG1 isotype control tube run on the healthy controls using the Method 1 sample preparation procedure. This spreadsheet also permits mAb lot numbers to be entered for all mAb reagents. Assemble healthy control data and enter the name or laboratory identifier number of each patient (P.I.D. on the spreadsheet). Next, enter the median PE-IgG1 RFI data into the PE-IgG1 column and then enter the median PE-CD4 RFI into the PE-CD4 column. The spreadsheet subtracts the median RFI of the PE-isotype control tube from the median RFI of the PE-CD4 under the column labeled “corrected CD4 RFI”. The spreadsheet calculates the average corrected PE-CD4 RFI of the healthy control population as well as the standard deviation and the coefficient of variation. In our experience, the coefficient of variation (C.V.) for PE-CD4 of the healthy control population has been <5%. C.V.’s much greater than 5% should be investigated for proper sample preparation and instrument quality control. Larger C.V.’s have been observed on small sized samples (n = <20).

## **8. DETERMINATION OF THE NUMBER OF CD38 MOLECULES PER CD8<sup>+</sup> CELL**

This method is used for patient specimens to determine CD38 expression.

### **8.1. PANEL FOR MEDIAN PE-CD38 RFI**

Tube 1: PE-IgG1 isotype control

Tube 2: FITC-CD8/PE-CD38/PerCP-CD3

Tube 3–X: Other tubes in the panel

### **8.2. SAMPLE PREPARATION**

Tubes 1 and 2 must be prepared using the Method 2 Sample Preparation (Section 5.2.).

Tubes 3–X can be prepared using standard laboratory protocol.

### **8.3. SAMPLE ACQUISITION**

After the daily instrument setup has been performed, collect the samples tube 1 PE-IgG1 control and tube 2 FITC-CD8/PE-CD38/PerCP-CD3. It is recommended that data from all cells be stored list-mode but if data storage space is of concern, a large gate set around the lymphocytes and monocytes can be used. This large mononuclear cell gate can be used for data storage. Various software have different utilities for setting a stop-counter for data

storage. Collect 5000 lymphocytes on the control tube and attempt to collect at least 2000 CD8<sup>+</sup> T cells on the FITC-CD8/PE-CD38/PerCP-CD3 tube. Depending on the software, the stop counter may need to be increased to 10,000–20,000 mononuclear cells to achieve 2000 CD8<sup>+</sup> T-cells on subjects with lower CD8<sup>+</sup> T cell percentages. This is true especially if the median RFI for PE-CD38 are performed on the healthy control group.

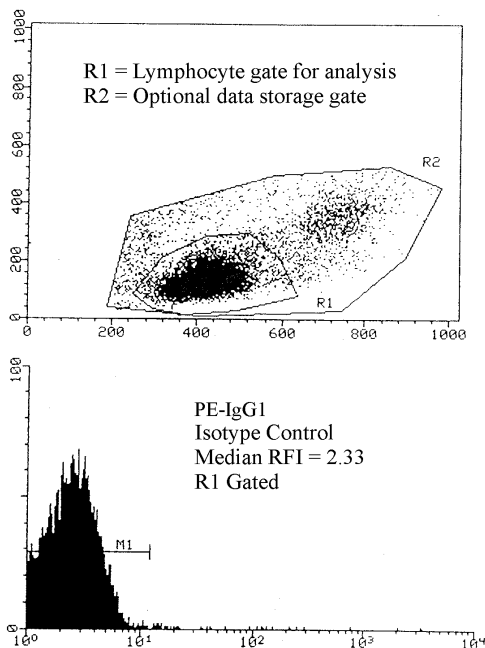
#### **8.4. DATA ANALYSIS**

Please refer to Data Analysis Figure 3. A brief description is provided below.

Median RFI measurements are used for all biological fluorescence intensity data. A lymphocyte gate is set using forward and side scatter. The lymphocyte gate is used for all biological data analysis. Measurement of CD38 RFI will include an additional CD8<sup>+</sup>CD3<sup>+</sup>cell gate.

1. For measurement of the background PE fluorescence on the PE-IgG1 isotype control tube, display an FL2 (PE-IgG1) histogram that is gated on the lymphocyte population defined by forward scatter and side scatter. Set a lower cursor at RFI channel 1 (the lowest channel) subsequently and set an upper cursor to include ~98% of the lymphocytes. Next determine the median RFI of the lymphocytes and record and enter into the CD38 spreadsheet. The background fluorescence is subtracted from the median RFI measurements for PE-CD38 to yield a corrected CD38 RFI.
2. For measurement of the median RFI of PE-CD38, display a dot plot histogram of FL3 (PerCP-CD3) versus FL1 (FITC-CD8) gated on lymphocytes. Set quadrant statistics to enumerate the lymphocyte populations. Set a gate around the CD8<sup>+</sup> T cells that matches quadrant 2 of the display. Display an FL2 (PE-CD38) histogram gated on the lymphocytes from the scatter display and on CD8<sup>+</sup> T cells from the FL3 versus FL1 display. The median RFI of PE-CD38 is measured on all the CD8<sup>+</sup> T lymphocytes. Some software may require a lower cursor setting at RFI channel 1 out of 10,000 and an upper cursor at RFI channel 10,000 out of 10,000 to get a median RFI of PE CD38 gated on all the CD8 T lymphocytes while some software does not require any cursors to be set. The percentage of cells in this display should read 100% of the gated events and should include only CD8<sup>+</sup> T cells. Enter the median RFI of PE-CD38 data in the PE-CD38 spreadsheet.

**Figure 3. Determination of the Number of CD38 Molecules per CD8<sup>+</sup> Cell by Measurement of Median RFI of PE-CD38 on Patient Samples**

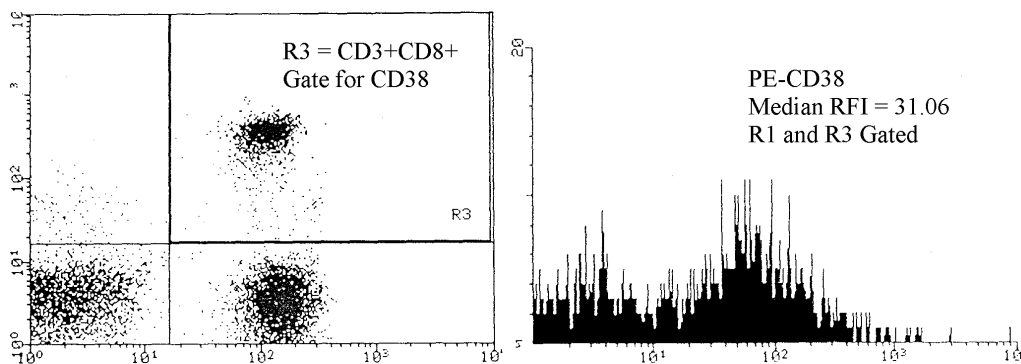


**Tube 1 and 2**

1. Display scatter dot plot.
2. Set gate (R1) on lymphocytes for tube 1 and 2.

**Tube 1 (PE-IgG1)**

3. Display FL2 histogram gated on lymphocytes.
4. Set a statistical region (M1) to include ~98% of the lymphocytes. The lower cursor must be set at channel 1.
5. Measure the median RFI.



**Tube 2 (FITC-CD8/PE-CD38/PerCP-CD3)**

6. Display FL3 (CD3) versus FL1 (CD8) dot plot gated on lymphocytes.
7. Set quadrant statistical region to enumerate the lymphocyte populations.
8. Set a gate (R3) on the CD3<sup>+</sup>CD8<sup>+</sup> cells that matches quadrant 2 of the dot plot.
9. Display FL2 (CD38) histogram gated on R1 and R3 which includes only CD3<sup>+</sup>CD8<sup>+</sup> lymphocytes.
10. Measure the median RFI of all the cells in the display.
11. Record median RFI data of tube 1 and 2 in the CD38 spreadsheet.

## 8.5. CD38 SPREADSHEET

The CD38 spreadsheet is used for calculation of the median number of CD38 molecules on the CD8<sup>+</sup> T cells of subjects stained using the Method 2 sample preparation procedure. Assemble the CD38 data and enter the name or patient identifier number in the P.I.D. column, the median RFI for the PE-IgG1 tube into the IgG1 column, and the median RFI for PE-CD38 into the CD38 column. Click on the appropriate cell and enter the data. The spreadsheet calculates a corrected CD38 median in the corrected PE-CD38 column. The corrected PE-CD38 RFI is then multiplied by the “RFI multiplier” from the Healthy Control Spreadsheet and determines the median number of CD38 molecules on the CD8<sup>+</sup> T cells of the individual samples. A serostatus column is provided for entering whether an individual is infected with HIV. This spreadsheet is linked to the healthy control spreadsheet.

## 9. CLINICAL USE OF THE NUMBER OF CD38 MOLECULES

The median number of CD38 molecules expressed per CD8<sup>+</sup> T lymphocyte is referred to as “CD38 on CD8.” A high CD38 on CD8 measurement is a marker of CD8<sup>+</sup> T lymphocyte activation and is a poor prognostic indicator in HIV-infected individuals. In order to facilitate CD38 on CD8 being applied clinically, data from a study conducted at UCLA can be used. Four categories of CD38 on CD8, namely low, medium, high and very high, are suggested. The suggested range of measurements to include in these categories of CD38 on CD8, measured in number of molecules per CD8<sup>+</sup> T lymphocyte, is <2350 molecules (low), 2350-4000 molecules (medium), 4001-7000 molecules (high) and >7000 molecules (very high). Over 3 years, 4%, 19%, 40%, and 73%, respectively, of those in the low, medium, high and very high categories of CD38 on CD8 expression developed AIDS. Taking time at which AIDS developed into consideration as well, individuals with medium, high, and very high CD38 on CD8 measurements had approximately 5, 12 and 40 times greater risk of developing AIDS as compared with individuals with CD38 expression <2350 molecules per CD8<sup>+</sup> T lymphocyte. Categories of CD38 on CD8 expression can be modified as additional clinical data are accumulated.

IF YOU NEED THE SPREADSHEETS TO FACILITATE ESTABLISHING THIS PROTOCOL, YOU CAN DOWNLOAD THEM FROM THE WEB SITE (<http://cyto.mednet.ucla.edu/>) OR REQUEST A DISK CONTAINING THEM BY SENDING AN EMAIL TO LANCE HULTIN WHOSE ADDRESS IS HULTINL@MEDNET.UCLA.EDU.