

Flow Cytometric quantitation of CD38 expression on CD8+ T lymphocytes

1. Principle

The purpose of this assay is to quantitate the surface expression of the CD38 molecule on CD8 positive T lymphocytes. Determination of this parameter has been shown to be a poor prognostic indicator in HIV-infected individuals. The use of a quantitative flow method will likely lead to reduced inter-laboratory variability as compared to the standard approach of reporting percent positive events as an estimate of CD38 expression.

Whole blood is stained with a combination of fluorochrome labeled monoclonal antibodies (CD8-FITC/CD38-PE/CD3-PerCP). The CD38 antibody is labeled with PE at a 1:1 fluorochrome to antibody ratio (ABC). This allows determination of the number of antibodies bound per cell by extrapolation of fluorescent intensities from a standard curve. The standard curve is generated by the use of PE labeled bead preparations with known number of PE molecules per bead.

2. Specimen Requirements

EDTA anticoagulated whole blood is the required specimen. After collection, keep specimen at room temperature. Staining and acquisition must be completed within 24 hours of blood draw.

3. Reagents

- 3.1 CD38-Q Reagent Kit. PharMigen Cat. # BP80051Z
Components: CD8-FITC / IgG1-PE / CD3-PERCP
CD8-FITC / CD38-PE* / CD3-PERCP
*1:1 fluorochrome to antibody ratio.
- 3.2 QuantiBRITE PE Fluorescent Quantitation kit. BDIS, Cat. #340495.
- 3.3 SPHERO Rainbow Calibration Particles. Spherotech, Inc. Cat. #RCP-30-5A.
- 3.4 FACSLyse. BDIS, Cat. #349202
- 3.5 1% Paraformaldehyde in PBS
- 3.6 PBSA [PBS (without Ca or Mg)/2% calf serum/0.1% NaAzide]

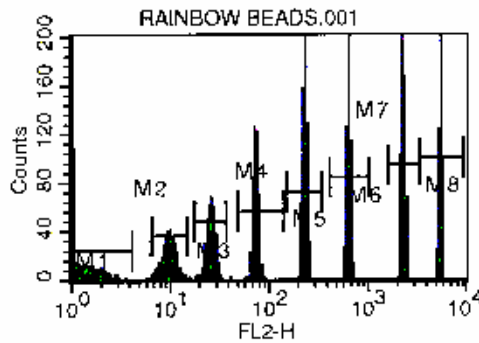
4. Instrument calibration

Flow cytometers should be calibrated according to each laboratory's standard operating procedure. Additionally, **calibration with Rainbow Beads is highly recommended for this protocol, although not required. If you calibrate with Rainbow Beads, turn compensation off until after completing instrument calibration with the Rainbow Beads.** This calibration is designed to evaluate logarithmic amplifier performance and should be performed before the laboratory analyzes the CD38 protocol specimens and after instrument service. It is not intended to be performed on a daily basis.

- 4.1 Procedure for calibration with Rainbow Beads.
 - 4.1.1 Add 1 – 2 drops of well-mixed Rainbow Beads to 0.5mL of PBSA (prepared fresh).
 - 4.1.2 Input instrument settings normally used for whole blood specimens and turn compensation off.

- 4.1.3 Increase the FSC gain to place the 3 um beads on scale and set a gate on the singlet bead population.
- 4.1.4 Place the mean of the 3rd brightest bead (M6) in the indicated target channel by setting cursors around the M6 peak and adjusting the PMT voltages (FL-2) until the target channel (± 2) is obtained. Collect 10,000 events and store in list mode.

Instrument	Target Channel (PE)
BDIS	614
Coulter	61.4



Marker	Left, Right	Events	% Gated	% Total	Mean	CV	Median	Peak	Peak Ch
All	1, 9910	10000	100.00	100.00	1026.82	164.51	214.80	623	1
M1	1, 4	1275	12.78	12.78	1.25	31.01	1.02	623	1
M2	6, 15	1194	11.94	11.94	9.62	12.65	9.56	41	10
M3	18, 35	1237	12.37	12.34	25.28	7.36	25.25	67	26
M4	49, 141	1234	12.34	12.34	71.77	3.91	71.69	124	71
M5	149, 355	1331	13.31	13.31	223.81	2.51	224.68	216	223
M6	418, 1037	1243	12.43	12.43	613.51	1.89	615.27	263	615
M7	1625, 3460	1185	11.85	11.85	2131.33	1.33	2128.75	341	2148
M8	3460, 9390	1294	12.94	12.94	5061.16	1.04	5048.07	468	5048

Note: A single parameter FL-2 histogram may not resolve these lower intensity bead preparations. You may have to display an FL-1 vs FL-2 dot plot and gate on individual populations of beads to resolve the lower intensity beads.

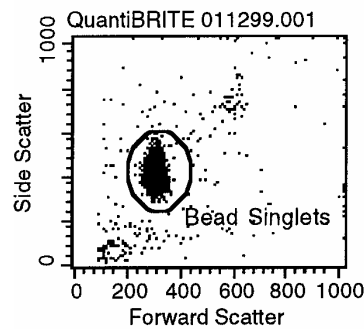
5. CD38 quantitation method.

5.1 Monoclonal Antibody Staining and Acquisition

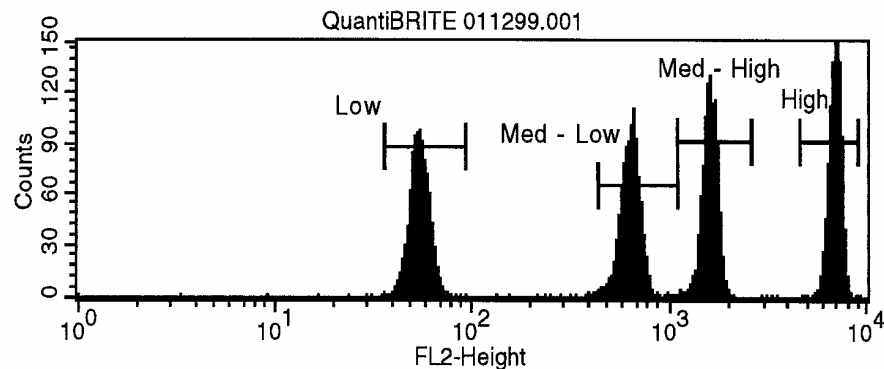
- 5.1.1 Pipette 100uL of whole blood into each of (2) 5mL tubes. Add 20uL of CD8/IgG1/CD3 reagent to tube 1 and 20uL of CD8/CD38/CD3 reagent to tube 2.
 - 5.1.2 Incubate for 30 minutes at room temperature.
 - 5.1.3 Add 2mL of FACSlyse, mix and incubate 10 minutes at room temperature.
 - 5.1.4 Centrifuge for 5 minutes at 300 x g and decant supernatant.
 - 5.1.5 Add 2mL of PBSA, mix and centrifuge at 300 x g for 5 minutes.
 - 5.1.6 Decant supernatant and add 0.5mL of 1% paraformaldehyde.
 - 5.1.7 Optimize compensation and acquire 5,000 lymphocytes from the isotype control tube. Next, collect a minimum of 2,000 CD8⁺ T cell from the CD38 stained tube.
- 5.2 QuantiBRITE Bead Acquisition and Analysis - CellQuest 3.1 or later version users.

Note: It is imperative that you do not change any instrument settings after you have acquired your patient(s) sample(s).

- 5.2.1 Reconstitute 1 tube of Quantibrite Beads with 0.5mL of PBSA and vortex.
- 5.2.2 Open the quantitation acquisition document in the Sample Files folder in the CellQuest folder. Modify to include the plots for the CD38 assay.
- 5.2.3 Run the Quantibrite bead tube thresholding on FSC or SSC and collect 10,000 events. Do not adjust PMT's or compensation after acquiring these events.
- 5.2.4 Display a FSC vs. SSC dot plot and adjust the gate around the singlet bead population.



- 5.2.5 Display an FL-2 histogram and adjust markers around the 4 bead peaks as shown below.



5.2.6 View histogram statistics (make sure geometric means are displayed).

Marker	Left, Right	Events	Geo Mean	CV
All	1, 9910	9721	737.07	119.11
Low	36, 92	2468	52.95	10.70
Med - Low	433, 1084	2439	610.96	10.76
Med - High	1094, 2595	2349	1535.97	7.82
High	4573, 9058	2420	6571.27	6.17

5.2.7 Select histogram statistics view and choose quantitative calibration from the Acquire menu.

5.2.8 Enter the lot-specific PE/bead values from the flyer in the kit.

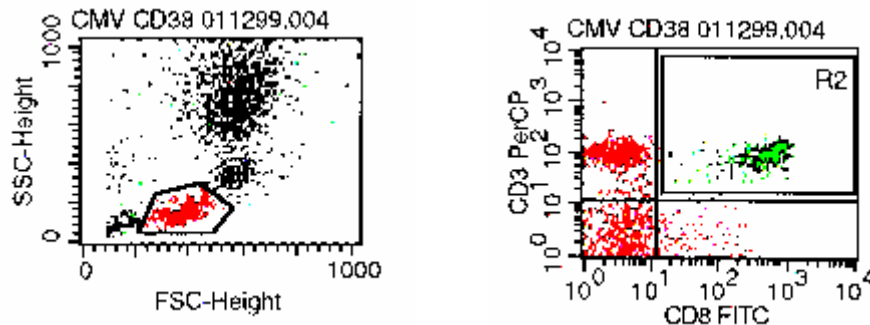
5.2.9 Click the copy means button to copy the geometric means of the 4 bead peaks from the histogram stats window.

5.2.10 Click calibrate for CellQuest to perform regression analysis and to display the slope, intercept and correlation coefficient.

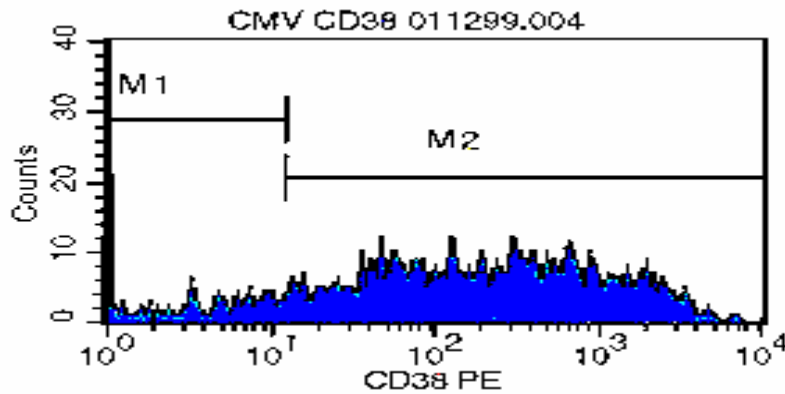
5.2.11 Save the document

5.3 Analysis of patient samples

5.3.1 Gate on lymphocytes and display an FL-1 vs FL-3 dot plot. Gate on lymphocytes (R1). Create a 2nd region around the CD3/CD8 positive cells (R2)



5.3.2 Display an FL-2 histogram of the CD3/CD8 positive cells (R1 + R2). The geometric mean of the **entire** CD3/CD8 population is then used to determine the CD38 ABC. This can be done automatically with the CellQuest Software.



6. Calculations

6.1 Manual Analysis:

- 6.1.1 On a statistics spreadsheet, enter the geometric means from the histogram statistics view for the 4 beads.
- 6.1.2 Enter the lot specific values for PE molecules per bead from the flyer in the kit.
- 6.1.3 Calculate the \log_{10} for each FL-2 geometric mean and for the PE molecules per bead.
- 6.1.4 Plot a liner regression of \log_{10} PE molecules per bead against \log_{10} fluorescence using the equation $y = mx + c$.
- 6.1.5 Determine the antibodies bound per cell (ABC) for the CD38 stained sample by substituting the log FL2 geometric mean of that population and solving for the log ABC (the antilog of this number is the ABC value).

7. Controls

Laboratories should ensure the quality of the monoclonal antibodies and reagents used in this assay by periodic testing of a normal donor. Testing of normal donors should be performed once per week every week for which a protocol subject sample is tested. If staining patterns on the normal donor do not appear suitable, the reagents should be retested. If on retest, the results do not appear satisfactory, the vendor's technical service department should be notified and a new kit should be obtained.

8. Expected values

Examples of histograms and dot plots are provided in this procedure. Normal ranges have not been established for this assay although normal donors are expected to give lower values.

9. Procedure Notes

Rainbow beads are recommended but not required. They are intended for use to validate the proper function of the logarithmic amplifiers. They should also be run after an instrument is serviced.

The CD38 PE monoclonal antibody supplied with the CD38 Q-reagent kit is at a 1:1 fluorochrome to antibody ratio. This allows determination of the ABC when Quantibrite beads are run under the same PMT and compensation settings as the whole blood sample.

10. Limitations

Time of analysis, method of fixation, lysing method and other factors may all influence the quantitative values obtained with this procedure. As such, this protocol must be precisely followed to assure comparability between laboratories.

11. References

CD38-Q Reagent Kit package insert. Pharmingen.

Quantibrite PE Quantitation kit package insert. BDIS.

SPHERO Rainbow Calibration Particles package insert. Spherotech, Inc.

Hultin, LE and JV Giorgi. Protocol for Estimating the Number of CD38 Molecules on the CD8+ T Lymphocytes of HIV-Infected Individuals. UCLA.

Please Note: The last reference is on the ACTG Immunology web site for your assistance: <http://aactg.s-3.com/pub/download/imm/imcd38.pdf>

Procedure: Flow Cytometric quantitation of CD38 expression on CD8+ T Lymphocytes

Prepared by: ACTG Laboratory Technologist Committee

Preparation Date: 01 June 2004

Date Implemented into the Laboratory: _____

Updated on:

Reviewed by:

Date:

Supersedes Archived Protocol: The Web Site Immunology Protocol, Version 4/1/99.