ACTG Consensus Method for 3-color Advanced Flow

1. PRINCIPLE

Specific molecules present on the cell surface can define characteristics of lymphocytes such as lineage-restriction, state of activation or functional capabilities. The phenotype of peripheral blood lymphocytes can be determined using fluorochome-conjugated monoclonal antibodies and flow cytometric techniques.

In HIV infection, changes in the size of specific phenotypically defined lymphocyte subsets relate to stage of disease, predict rate of disease progression or indicate response to treatment. The advanced panel immunophenotyping assay enumerates peripheral blood lymphocytes in certain phenotypically defined subsets by determining the expression of additional surface antigen on either $CD4^+$ or $CD8^+$ lymphocytes.

Separate guidelines currently exists for quantitating the major, lineage-restricted subsets of CD3/CD4 and CD3/CD8 lymphocytes (1-3) and the importance of using CD45 gating for these determinations. An electronic link to the current NIAID/DAIDS guideline is located on the AACTG web site at http://aactg.s-3.com/immlab.htm under the heading, "Flow Cytometry Methods."

This Version 3.0 Updated Consensus Protocol for Advanced Panel Immunophenotyping is for quantitating additional subcategories of $CD4^+$ or $CD8^+$ lymphocyte lineage-restricted subsets**. It is imperative that laboratories adhere strictly to the reagents and methodology contained in this protocol in order to minimize interlaboratory variability and to increase the precision of these determinations.**

2. SPECIMEN REQUIREMENTS

2.1 EDTA (K_2 or K_3) (4) is the recommended anticoagulant for immunophenotyping performed within 30 hours of blood draw.

2.2 Ideally, 2mL of blood for Adult protocols and 1ml of blood for Pediatric protocols is required.

2.3 Specimens must be kept at room temperature (18-22^{o}C) and stained and fixed within 30 hours of specimen draw. Stained and fixed specimens may be stored at 2-8^{0}C up to 24 hours before data acquisition using the flow cytometer.

2.4 Criteria for specimen rejection based on suboptimal specimen conditions (e.g.hemolysis, clotting) and are described in the NIAID guideline (1).

3. REAGENTS

3.1 Monoclonal antibody panel members are specified by the protocol.

3.1.1 Store reagents at 2–8 ^{o}C to assure proper performance. Avoid exposing

reagents to light. Discard reagents after expiration date.

3.1.2 Fluorochrome designations for monoclonal antibody combinations will be protocol-specified and must be followed.

3.1.3 Monoclonal antibody combinations will be obtained from a protocol-specified vendor. Maintain lot usage log for each antibody panel.

3.1.4 Sphero™ Rainbow Calibration Particles (8-peak) from Spherotech, Inc, 800-368-0822, http://www.spherotech.com, (catalog #RCP-30-5A) are a required reagent for flow cytometer calibration.

3.2 The cell washing buffer is Dulbecco's phosphate buffered saline (dPBS, e.g. Sigma, catalog #5652). Store unused wash buffer at 2 - 8 ^{o}C.

3.3 Approved commercial red cell lysing buffers include FACS™ Lysing Solution (BD Biosciences) and Q-Prep lysing reagent (Beckman Coulter).

3.4 The recommended fixative reagent is methanol-free 2% formaldehyde. A suggested source of this fixative is a 10% Formalin Neutral Buffered Solution from Sigma (catalog # HT50-1-1) that is diluted 1:2 with dPBS to obtain a working concentration of 2% formaldehyde. Another source is a 10% Formaldehyde Solution from PolySciences (#0418), which must be diluted 1:5 with dPBS to obtain a working concentration of 2% formaldehyde. Use of methanol-free 2% (w/v) Paraformaldehyde (Sigma, #P-6148) in a buffered salt solution, pH 7.4 – 7.6, is also acceptable. Store in an amber glass container or wrap container in aluminum foil to avoid exposure to light. Diluted 2% formaldehyde may be stored up to 1 week at 2 – 8 ^{o}C, while stock solutions of formaldehyde should be stored no longer than one year. All formaldehyde solutions are highly toxic and carcinogenic.

4. EQUIPMENT AND SUPPLIES

4.1 Flow cytometer capable of 3-color detection
4.2 Class 2 Biosafety cabinet
4.3 12 x 75 mm polystyrene staining tubes (Falcon #352008)
4.4 Micropipettes capable of dispensing 20 μL, 100 μL and 250 μL volumes
4.5 100 μL and 500 μL pipette tips
4.6 Bulk volume dispensers for cell washing buffer and red cell lysing buffer
4.7 Centrifuge with swinging bucket rotor, capable of centrifugation of 300 x g and equipped with aerosol containment canisters
4.8 Liquid waste container with bleach
4.9 Vortex
4.10 Disposable gloves
4.11 Disposable lab coat
4.12 Safety glasses

5. CALIBRATION PROTOCOLS AND SCHEDULES

5.1 Each laboratory will have a comprehensive quality assurance program that will assure proper instrument operation and calibration, reagent stability and personnel training.

5.2 Laboratories must participate in the NIAID DAIDS Quality Assessment Program for Advance Flow Cytometry administered by the Immunology Quality Assurance (IQA) Laboratory. Further contact information about the IQA can be obtained through an electronic link listed on the AACTG web site at: http://aactg.s-3.com/immlab.htm.

6. PROCEDURES

6.1 Universal Precaution Guidelines are based on the assumption that medical history and examination cannot reliably identify all patients infected with potentially infectious disease. Therefore, blood and body fluids precautions must consistently be used with ALL patient samples.

6.2 For some patient specimens, an artifactual staining occurs because of a plasma factor (5-6). Therefore, for Becton Dickinson (BD) flow cytometer users, an aliquot of blood must first be washed before it is stained with monoclonal antibodies. Place 2mL of anticoagulated blood in a 15mL conical centrifuge tube and add 10-12mL of cell washing buffer. (For pediatric samples, this may be a smaller volume). Mix by inversion. Centrifuge at 300 x g for 5 minutes and aspirate clear supernate. Resuspend cells in cell washing buffer to original 2mL volume.

6.3 However, for Beckman Coulter (BC) flow cytometer users, washed cells do not lyse well using the Coulter Q-Prep system unless they are resuspended in a solution which contains a protein level equivalent to that of normal serum. If a sample appears to need prewashing because of immunoglobulin artifact effects (see Appendix), the cells must be resuspended in normal goat serum, bovine serum, or some equivalent before lysing on the Q-Prep. Do not resuspend the cells in the patient's plasma as this will reintroduce the artifact problem.

6.4 Label 12 x 75 mm test tubes with donor and reagent profile tube number.

6.5 Add 20 μl of the appropriate panel antibody combination to 100 μL of whole blood (50 μL – 100 μL whole blood for pediatric samples) in each labeled 12 x 75 mm test tube.

6.6 Mix tube contents by low speed vortexing for less than 3 sec. Incubate for 30 minutes at room temperature (20 – 25^{o}C) in the Biosafety cabinet with the cabinet lamp turned off. Remix tubes at 15 min.

6.7 Add 2mL of room temperature red cell lysing buffer to each tube. Mix tubes by low speed vortexing for approximately 5 sec. Incubate tubes for 10 minutes with the cabinet lamp turned off. Then centrifuge tubes for 5 min at 300 x g.

6.8 Decant supernatant and add 2mL of cell washing buffer to each tube. Centrifuge for 5 min at 300 x g. Samples prepared using the Beckman Coulter Q-Prep system must receive an additional wash with cell washing buffer before the fixative is added. After decanting this additional wash supernatant, add 250 μL of fixative, and mix thoroughly. Let tubes stand for at least 15 min. Store fixed samples for up to 24 hrs at 2 – 8 ^{o}C in the dark and protect from evaporation.

6.9 Flow cytometric analysis will utilize linear forward light scatter (FLS), linear side light

scatter (SS) and log fluorescence parameters. Prior to daily use, verify that the cytometer is properly aligned and calibrated (7). Set PMT voltages using Sphero™ Rainbow Calibration Particles as follows:

Input the instrument settings normally used for whole blood specimens in your laboratory. **Turn off all compensation**.
Collect the following plots:
Forward Scatter-linear vs. Side Scatter-linear
FLl-log
FL2-log
FL3-log (BD instruments) or FL4-log (Coulter XL instruments)
FL1 -log vs FL2-log

Increase the FSC gain to place the 3 µm beads on scale and set a gate around the singlet
bead population. Gate all of the fluorescence histograms on the singlet beads.
You will see 6 to 8 bead peaks in each histogram, although you may only see the dimmer
peaks by looking at the FL1 vs. FL2 plot. Remember that all **compensation should be off**. Collect 10,000 events in the light scatter gate and adjust each PMT voltage so that the third most bright peak (from the right-hand axis) is in the following positions:

BD	Coulter
FL 1 228 (+/-4	22.8 (+/-2)
FL2 614 (+/-4)	61.4 (+/-2)
FL3 368 (+/-4)	36.8 (+/-2)

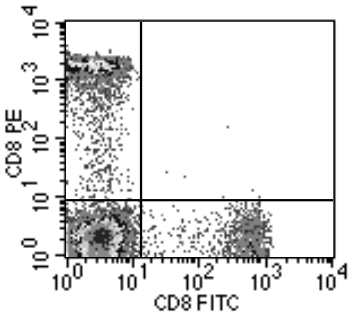
It is advised that each laboratory keep documentation of Sphero™ Rainbow Calibration Particles lot usage, as well as what voltages were used for FL1, FL2, and FL3 in the calibrations. A day to day comparison of these voltages may help alert the lab to instrument fluorescence intensity problems that may be arising.

6.10 Each laboratory should prepare CD8 compensation tubes for FITC, PE and PerCP (or CyChrome) fluorescence for each clinical specimen that will be stained and analyzed. To adjust compensation, readjust the light scatter gains to accommodate the (3) CD8-stained blood samples, but do not change the PMT voltages obtained using the Rainbow Particles. When adjusting the compensation values, do not alter your PMT voltages from those used to achieve the rainbow particle target channels. For best visual resolution during analysis, use probability plots with a setting of 10%. The compensation settings should be adjusted so that the cell distributions are orthogonal for FITC vs. PE and FITC vs. PerCP (or CyChrome) (not possible for BD cytometers) for the FITC-stained cells; FITC vs. PE and PE vs. PerCP (or CyChrome) for the PE-stained cells; FITC vs. PerCP (CyChrome) (not possible for BD cytometers) and PE vs. PerCP (or CyChrome) for the PerCP (or CyChrome)-stained cells. The mean channel of the negative and positive populations should be within 1.0 channel on a log scale for BD cytometers or within 0.10 using the Mnl statistic for Beckman Coulter cytometers. After all three tubes are analyzed in this manner, approximately 100ul from each of the three single fluorochrome tubes should be combined together in a fourth tube. This combined

3-color compensation tube should be analyzed to verify that the compensation settings have been correctly set. See Figure 6.10 below for an example of a combined fourth tube compensation analysis.

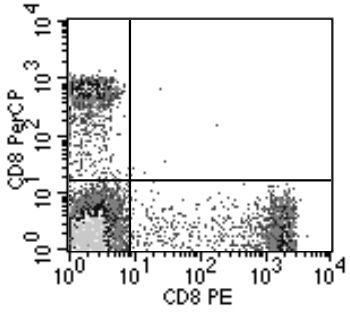
Fig. 6.10 Combined fluorochrome compensation analysis:

1. BD Immunocytometry Systems Cytometer



Gate: G1
Gated Events: 20072
Total Events: 59801
X Parameter: FL2-H CD8 PE (Log)
Y Parameter: FL3-H CD8 PerCP (Log
Quad Location: 9, 15

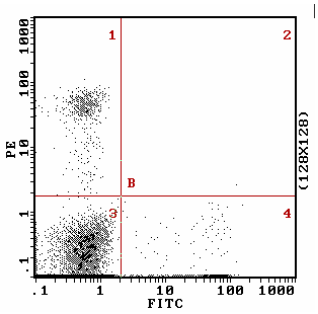
Quad	Events	% Gated	% Total	X Mean	Y Mean
UL	2779	13.85	4.65	2.06	574.38
UR	7	0.03	0.01	39.91	131.78
LL	13826	68.88	23.12	2.14	2.45
LR	3460	17.24	5.79	1517.16	2.08

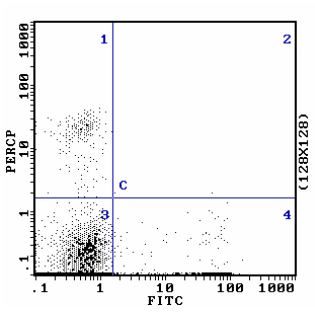


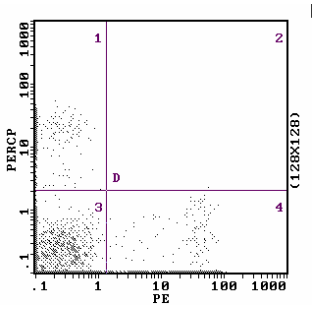
Gate: G1
Gated Events: 20072
Total Events: 59801
X Parameter: FL1-H CD8 FITC (Log
Y Parameter: FL2-H CD8 PE (Log)
Quad Location: 13, 9

Quad	Events	% Gated	% Total	X Mean	Y Mean
UL	3456	17.22	5.78	2.94	1518.91
UR	10	0.05	0.02	398.63	30.08
LL	14027	69.88	23.46	3.50	2.19
LR	2579	12.85	4.31	548.28	1.76

b) Beckman Coulter Cytometer







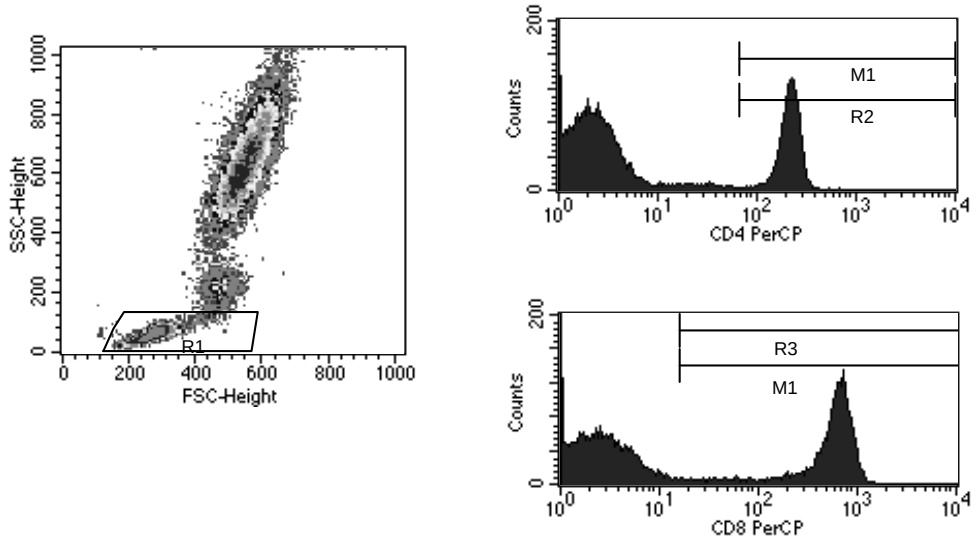
6.11 The lymphocyte population is gated using linear Forward Scatter versus linear Side Scatter (FS/SS) dot plot and a second anchor gate is set using a single parameter histogram of PerCP (or CyChrome) log fluorescence. When CD4 is the anchor marker, only $CD4^{bright}$ cells will be gated using the PerCP (or CyChrome) histogram. When CD8

is the anchor marker, both $CD8^{bright}$ and $CD8^{dim}$ will be gated to ensure consistency. This is done with the understanding that such a gate will include natural killer (NK) cells.

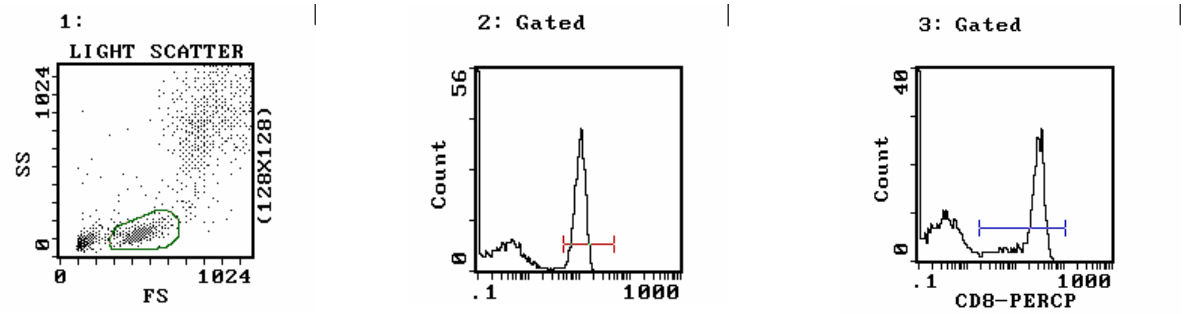
6.11.1 After gating on the lymphocyte population, an alternate method of anchor gating on a population of interest is to gate using an anchor marker log fluorescence versus linear SS dot plot. This method is allowed in some pediatric protocols.

Fig. 6.11 FS vs. SS lymphocyte gating combined with CD4 or CD8 anchor gating:

a) BD Immunocytometry Systems Cytometer



2. Beckman Coulter Cytometer



6.12 During sample acquisition, set the cytometer software to acquire approximately 20,000 events in the lymphocyte FLS/SS gate. This number of gated lymphocyte events helps

to assure that approximately 2,500 anchor marker-positive cells will be collected for each tube analyzed. Minimally, 500 anchor marker-positive cells will be permitted for those specimens where the CD4 subset is small (e.g, low CD4 absolute count). If the number of anchor marker-positive cells is below 500 events, an explanation citing insufficient events should be noted on the report form. Store data in list mode for at least 10 years.

7. DATA ANALYSIS AND CALCULATIONS

7.1 Initial cursor settings for data analysis will be set using the appropriate isotype control tubes, which should be set tightly around the negative-staining population (see Figure 1 and Figure 2 of Appendix 1: Consensus Protocol for Advanced Panel Immunopehnotyping, dated February 26, 2002). The lower left quadrant must contain between 98-99% of all events. Ideally, the isotype control quadrant settings should not be moved when analyzing the remaining tubes of the panel appropriate to that isotype control. However, isotype control cursor placements may divide a population of cells when analyzing subsequent tubes. Depending on the surface marker combination being analyzed, isotype control cursor placement may have to be adjusted on a patient sample basis. See Figure 4 and Figure 5 of Appendix 1: Consensus Protocol for Advanced Panel Immunopehnotyping, dated February 26, 2002

7.1.1 When analyzing a tube that requires FLS/SS lymphocyte gating, but not a subsequent anchor gate, use the appropriate isotype control to determine the appropriate placement of the quadrant statistical markers.

7.2 Percentages of events recorded in each quadrant will be reported as the percent of gated events. For anchor-gated cells, the events recorded will represent the percent of total $CD4^+$ cells or percent of total $CD8^+$ cells. For FLS/SSS lymphocyte-gated cells, the events recorded will represent the percent of total lymphocytes. Regardless of gating strategy, the sum of the quadrant percentage values for each 2-color histogram will equal 100.

8. QUALITY ASSURANCE ISSUES

8.1 Perform daily instrument quality control, 3-color compensation and set-up procedures. Laboratories will ensure quality of monoclonal antibody reagents by periodic testing on a blood specimen obtained from a normal donor or on stabilized, control cells.

8.2 The histogram appearances for the antibodies run for the A5001 Advanced Flow panel should be similar to those shown in Figure 2 and Figure 3 of Appendix 1: Consensus Protocol for Advanced Panel Immunopehnotyping, dated February 26, 2002. If not, a different lot of antibody reagent should be tested. Do not use suspect antibody reagents until the integrity of the reagent has been confirmed.

8.3 The Advanced Panel will be performed on all proficiency testing specimens received bimonthly from the NIAID Flow Cytometry Quality Assessment Program and results reported to Immunology Quality Assurance (IQA) Program in a timely fashion.

9. REFERENCES

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cytometric immunophenotyping: A report from the National Institute of Allergy and Infectious Diseases, Division of AIDS. Cytometry 14:702-715, 1993.

2. Nicholson, J., Kidd, P., Mandy, F., Livnat, D., and Kagan, J. Three-color supplement to the NIAID/DAIDS guideline for flow cytometric immunophenotyping. Cytometry (Communications in Clinical Cytometry) 26:227-230, 1996.

3. 1997 Revised Guidelines for Performing CD4+ T-cell Determinations in Persons Infected with Human Immunodeficiency Virus (HIV). CDC. MMWR 1997; 46 (No. RR-2), 1-29, 1997.

4. Shiba, A., Eastham, B. and Quist, M. Comparison of BD vacutainer plus K_2EDTA tubes and glass K_3 EDTA tubes for immunophenotyping with the BD FACS MultiSET system. Medical Affairs, BD Biosciences, San Jose, CA. Publication #1101 23-6492.

5. Ekong, T., Gompels, M. Clark, C. Parkin, J. and Pinching, A. Double-staining artifact observed in certain individuals during dual-colour immunophenotyping of lymphocytes by flow cytometry. Cytometry 14:679-684, 1993.

6. Nicholson, J.K., Rao, P.E., Calvelli, T., Stetler-Stevenson, M., Browning, S.W., Yeung, L., and Marti, G.E. Artifactual staining of monoclonal antibodies in two-color combinations is due to an immunoglobulin in the serum and plasma. Cytometry 18:140-146, 1994.

7. Schwartz, A. et al. Standardizing flow cytometry: Construction of a standardized fluorescence calibration plot using matching spectral calibrators. Cytometry (Communications in Clinical Cytometry) 26:22-31, 1996.

11. PROTOCOL VERSIONS EFFECTIVE DATES

Version 1.6 effective date: September 20, 1996
Version 2.0 effective date: June 29, 2000
Version 3.0 effective date: April 26, 2002

12. AUTHORS

Version 1.6 Keith A. Reimann, Alan Landay, Mary Ann Fletcher

Version 2.0 Laboratory Technologists Subcommittee of the
Data Management Committee of the AIDS Clinical Trials Group

NIAID DAIDS Advanced Flow Cytometry Quality Assurance Group

Version 3.0 NIAID DAIDS Advanced Flow Cytometry Quality Assurance Group

Procedure: ACTG Lab Man Consensus Method for Advanced Flow

Prepared by: ACTG Laboratory Technologist Committee

Preparation Date: 01 June 2004

Date Implemented into the Laboratory: _____

Updated on:

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Reviewed by: Date:

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Supersedes Archived Protocol: DAIDS Virology Manual for HIV Laboratories, Version January 1997