

# **Canadian Guidelines for Flow Cytometric Immunophenotyping**

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**2001 Edition**

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National Laboratory for HIV Immunology  
Bureau of HIV/AIDS, STD and TB  
Centre for Infectious Disease Prevention and Control  
Population and Public Health Branch  
Health Canada

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# PREFACE

This section has been developed by the National Laboratory for HIV Immunology (NLHI) within the Bureau of HIV/AIDS, STD & TB. It was approved by the Canadian Immunology Advisory Committee and the Technologist Sub-committee which provide guidance and advice to NLHI. This document was designed to help laboratory personnel across Canada who are performing lymphocyte immunophenotyping in human immunodeficiency virus (HIV) -infected persons.

Although scientific advancements in the field of biotechnology have been rapid, implementation and validation of some of these advancements have been slow. The decision making dilemma arises from the need to remain current and to implement the latest technical innovations, counter-balanced with the need to maintain the “status quo”, continuing to use diagnostic methods for the purposes of seamless disease monitoring during longitudinal clinical trials. The incorporation of new technologies must be introduced with caution, and with significant discretion. Clearly, the methodology for immunophenotyping T-cell subsets has been improving over time, and the importance of being able to reliably monitor patient response to experimental medication in multi-site clinical trials cannot be over-emphasized (1-6). In order to ultimately benefit all Canadians living with HIV, there must be a judicious balance between the frequency of adjustments needed to improve the precision and accuracy of a test, and the advantage of continuity in monitoring drug efficacy both in clinical practice and clinical trial. The technological advancements made in the area of immunophenotyping have reached the point where they can no longer be ignored. The recommendations presented here are comprehensive. They cover laboratory safety, specimen collection, transport, maintenance of integrity, and sample processing. They also address the general issue of quality control in a flow cytometry laboratory as well as issues related to data analysis.

# INTRODUCTION

Nearly twenty years ago, several reports were published in the United States regarding severe acquired immunodeficiency in gay men. By 1983, the CD4+ T-cell assay was recognized as the hallmark clinical surrogate marker for staging disease progression for this new syndrome (2,4,5,7). There was a direct association between decreased percentages and numbers of CD4+ T-cells in peripheral blood, and the severity of acquired immunodeficiency (1,8-13). Within a year, the causative agent was determined to be a newly discovered retrovirus. This human retrovirus was subsequently named as the human immunodeficiency virus (HIV). The underlying characteristic of HIV infection is the breakdown of the immune defense systems, primarily cellular immunity, thus allowing opportunistic infections and some malignancies to establish a foothold in the host. Hence, the descriptive name, acquired immunodeficiency syndrome (AIDS) was associated with the disease.

T-cell subset testing has been around for almost two decades. Until the mid 90's, T-cell subsets had been the sole laboratory marker physicians followed for the routine monitoring of immune status in HIV infected individuals (14-16). CDC had revised AIDS surveillance case definition to include all HIV-infected persons who had less than 200 CD4+ T-cells (7). More recently, the quantitative viral load assay joined the limited number of valuable surrogate markers of disease progression that are available. The Canadian federal endeavor to monitor and standardize CD4+ T-cell counting began in 1989 (17). It was established to provide a national quality assessment programme for physicians conducting clinical trials for managing patients with HIV disease. The National Laboratory for HIV Immunology, NLHI (then called the National Laboratory for Analytical Cytology) with the cooperation of the Centers for Disease Control (CDC) in the U.S., published a bilingual guideline for CD4+ T-cell enumeration in 1994 (18). During the past six years, improvements have emerged, both in the field of immunophenotyping, and especially in the domain of drug discoveries.

Given that today most individuals living with HIV are asymptomatic and their disease status is largely dependent on surrogate markers, progress in diagnostic

immunology dictates that the whole approach to effective monitoring and management of treatment of individuals living with HIV is reviewed. Two examples of this progress are worth mentioning. Historically, the measurement of absolute CD4+ T-cell levels in whole blood was the product of three laboratory technologies (3,19). The white blood cell (WBC) count, the percentage of WBCs that were lymphocytes (differential), and the percentage of lymphocytes that are CD4+ T-cells were all required to generate an absolute count. This complex procedure has been replaced by a single-platform technology (SPT) where all required measurements are obtained simultaneously from a clinical flow cytometer (20). Although the merits of SPT were first reported in 1982 (21), today, in Canada, the implementation of this improved technique is still far from being complete . The slow implementation of the heterogeneous gating strategies is another example of the lag between technological advancement and implementation in the laboratory. The benefits of a heterogeneous gating strategy, where a lineage specific marker is combined with light scatter for CD3 or CD45 gating, were first published in 1992, and again in 1993 and 2000 (22-30). Yet, the integration of these more effective gating strategies for clinical trials in Canada and the USA is only now, in 2001, commencing.

# LABORATORY BIOSAFETY

According to Schmid et. al. , in *Biosafety considerations for flow cytometric analysis of human immunodeficiency virus-infected samples* (31) “HIV may be transmitted directly either by percutaneous (e.g., through a needle stick) or mucous membrane exposure by splashing or spattering of infectious fluids. Another route of infection is the exposure of non-intact skin with infectious fluids where the virus may inoculate the worker through minor cuts, scratches, abrasions, and skin lesions. Aerosol transmission of HIV has been hypothesized, but up to date has not been documented. Thus, it is important that flow cytometer operators be protected from exposure to contaminated surfaces, accidental splashes, droplets, and aerosols.”

## **SPECIMEN**

### **HANDLING**

HIV is identified as a risk group III agent (32). All manipulations should be performed in a containment level 2 laboratory (BSL2) using a class I or class II biosafety cabinet. These manipulations include opening of collection tubes, aliquoting, adding reagents, vortexing, aspirating, etc. To minimize splashes and spraying of blood, always remove the rubber stopper of blood collection tubes using a gauze pad or available commercial safety devices to avoid spattering of infectious fluids. Centrifugation must be carried out using closed containers (bucket) which should be opened only in a biological safety cabinet. Proper protective clothing, such as laboratory coats and gloves, should be worn when processing specimens, and during analysis on the flow cytometer (32-39). Appendix A provides the basic requirements for laboratories using infectious or toxic agents (32).

## **TRANSPORT**

Specimens should be maintained at room temperature (18-25°C) and should be transported as quickly as possible. Overnight carriers should be used to ensure arrival the following day, at the latest (40-43).

**INTERNAL** To move collection blood tubes within a laboratory site, use a container labeled with the biohazard symbol which is able to contain the specimen in case of breakage of the blood tubes; e.g. a plastic bag with leak proof seal or a plastic carrier with a secure lid (31).

**EXTERNAL** The shipping of specimens containing infectious agents is regulated by the Transportation of Dangerous Goods Act and the International Air Transport Association (IATA) Dangerous Goods Regulations (44). HIV is classified as an infectious class 6.2 substance under the United Nations (UN) number 2814. Along with proper labeling instructions, the packaging must meet UN class 6.2 specifications. Proper packaging requires a three layer system; (i) a watertight inner container, (ii) a watertight secondary inner container which contains absorbent material and (iii) a strong outer package.

## **FIXATION**

After staining and lysing, fix all samples with buffered (pH 7.0-7.4) 1%-2% formaldehyde (final concentration). The time required to inactivate cell-associated HIV and other viruses after fixation with formaldehyde is not well established. Therefore, all samples should remain in fixative during analysis (45-49).

## **SPILL & DISPOSAL**

Disinfect spills with a freshly made 1/10 (V/V) dilution of sodium hypochlorite (domestic bleach ~5%) or an appropriate dilution of

mycobactericidal disinfectant. Contain the area of spill by setting absorbent paper around it and pouring disinfectant on the paper and toward the spill. Let it sit for 30 minutes before wiping up. All waste must be disposed of according to institutional regulations for biohazardous waste. Autoclaving of formaldehyde fixed specimens is prohibited, as it may release toxic formaldehyde gas (31). Fixed specimens could be aspirated to a container with an appropriate amount of sodium hypochlorite (50).

## **FLOW CYTOMETER**

### **LASER**

Most flow cytometers contain low-power lasers which are fully contained. Stream-in-air flow cytometers, mostly cell sorters, may contain high-power lasers, not fully enclosed. Safety goggles should be worn whenever the laser beams are exposed (31).

### **AEROSOL**

Stream-in-air flow cytometers pose a risk to operators through potential exposure to droplets and aerosols generated during sample acquisition. A partially clogged nozzle tip, or air in the fluidic system, may substantially increase aerosol production. It is important to test the efficiency of aerosol control measures on these flow cytometers. A detailed protocol for testing efficiency of flow cytometer aerosol containment has been described (50).

### **WASTE COLLECTION**

Waste fluid from the flow cytometer must be disinfected. Disinfect flow cytometer wastes by adding a volume of undiluted sodium hypochlorite to the waste container so that the final concentration of sodium hypochlorite is 0.5% when the container is full (31).

## **FLUID LINES**

Disinfect the flow cytometer as recommended by the manufacturer. A generally recommended method is to disinfect the fluid pathways of your instrument regularly by flushing the fluidic lines with diluted 1/10 (V/V) sodium hypochlorite solution for at least 10 minutes. Because the disinfectant solution is corrosive it is important to rinse well with distilled water for at least 10 minutes (31,50).

## **TRAINING**

Training personnel is essential to the safety of the workers and co-workers in the same environment. The actions of those who work in the laboratory determine their own safety and that of their colleagues. Laboratory equipment and design contribute to safety only if they are properly used. The Canadian Labour Code requires that each employer provide safe working conditions and that employees be properly trained regarding all hazards they will face in the course of their duties. Strict adherence to safety protocols should be emphasized and monitored regularly (32).

## **BLOOD**

### **ANTICOAGULANT**

The most commonly used anticoagulant for immunophenotyping is K<sub>3</sub>EDTA. The primary reason for using K<sub>3</sub>EDTA was to eliminate the need to draw a separate tube for hematology (information specific to hematology is in Appendix B). As a result, the commercial lysing preparations were optimized for K<sub>3</sub>EDTA (51). K<sub>3</sub>EDTA should NOT be used for specimens held for >30 hours before testing because the proportion of some lymphocyte populations may change after this period.

## **STABILITY**

Maintain specimens at room temperature (18-25° C). Avoid extremes in temperature so that specimens do not become too cold or too hot. Extreme temperatures will affect the flow cytometric measurements (40-43).

## **INTEGRITY**

Inspect the tube and its contents immediately upon arrival. If the specimen is hot or cold to the touch, but not obviously hemolyzed or frozen, process it, but note the temperature condition on the worksheet and report form. Abnormalities in light-scattering patterns will reveal a compromised specimen. If blood is hemolyzed or frozen, reject the specimen and request a fresh specimen. If clots are visible, reject the specimen and request another. Reject a specimen that cannot be processed within 30 hours and request another. This time may be extended if CD45 gating is used (24). The use of the CD45 as a gating tool will allow the accurate relative and absolute (by single-platform method) enumeration of T-cell subset over a period of time exceeding 48 hours (24,30,51).

# **INSTRUMENT SETUP**

## **ALIGNMENT**

Most of the current clinical flow cytometers have a fixed optical system, meaning that the relative position of the flow cell with respect to the optical elements is fixed. The alignment can only be verified. Alignment is checked with alignment standards such as wide spectrum fluorescent microbeads with measurable light scatter characteristics. Physical characteristics of various types of standards for instrument setup and calibration have been classified according to their applications (see Appendix C) (52). Alignment is most critically assessed, and most easily optimized, using particles with very uniform scatter and fluorescence (< 2% C.V.). The alignment standard material is used to ensure that light scatter and fluorescence signals are as bright and narrow as possible. Alignment standards

should be used daily to verify, monitor and detect significant variations in power output and mean channel number (52,53). It is important to note that some research flow cytometers require regular alignment adjustment. In all cases, manufacturer's instructions should be followed.

## **PMT VOLTAGES**

Unstained and lysed fresh whole blood is suitable for adjusting the PMT voltages. The autofluorescence from the unstained lymphocytes should be completely visible, and should fall within the lower left quadrant of the dot plot for every PMT detector in use. To ensure a reproducible analysis range, reference standards with spectral properties similar to cells could also be used to set up PMT voltages (see Window of Analysis, in Quality Control section). Light scatter parameters should be adjusted so that all of the leukocyte populations are clearly visible (52-54).

## **COLOUR COMPENSATION**

Compensation describes the process of correcting for spectral overlap of one fluorochrome into the fluorescence spectrum of another. This correction is done by adjusting the electronic compensation circuits on the flow cytometer to place each population in their respective fluorescence quadrants with no overlap into the double-positive quadrant. At the same time, it is important to avoid overcompensation (55).

Compensation should be set using cell populations that have the brightest signal. If a dimmer-than-expected signal is used to set compensation, undercompensation for the brightest signal can result. It is important to reset compensation when photomultiplier tube voltages or optical filters are changed. Some manufacturers have developed software and compensation controls to assist the operator in the process of colour compensation.

### **Summary**

1. Choose compensation controls that include cells with both a positive and a negative population. Prepare a compensation control for each fluorescence parameter. Select the compensation control, so it will match the brightest specimen signal.
2. Gate lymphocytes
3. Adjust compensation settings until the positive and negative clusters appear to be level with each other (visual) or match their median values (x or y).
4. Run a HIV-negative multi-colour stained specimen to verify that the distribution of each population is appropriate.

When spectral compensation of a particular specimen appears to be inappropriate, repeat the sample preparation. Remove plasma by prewashing the specimen with phosphate-buffered saline (PBS), pH 7.2, before the monoclonal antibodies are added. About 5% of the HIV infected individuals have a factor in their plasma and serum that may give the impression of a dual expression for a number of markers commonly used for phenotyping (56,57).

## **PHENOTYPING T-CELL SUBSET**

### **RELATIVE AND ABSOLUTE VALUES**

Until recently, the conventional method for reporting absolute lymphocyte subset values was based on a double-platform method involving both a hematology analyzer and a flow cytometer. Since 1996, single-platform technology has been available. The basic principle consists of adding beads of known concentration to the sample preparation. By collecting both the number of events in the bead region and in the cell region, we can calculate the number of cells per volume of blood, an absolute measure (20,28,58,59).

### **STAINING**

Manufacturers have developed reagents to phenotype whole blood specimens. The classic protocol involves a short incubation of whole blood and conjugated monoclonal antibodies. The mixture is then lysed and fixed. The additional fixation step should be performed even if the commercial lysing reagent already contains a fixative. The characteristics of formaldehyde may vary from lot to lot. Formaldehyde may also lose its effectiveness over time. Therefore, prepared fixatives should be made fresh weekly, filtered and pH monitored.

For single-platform absolute count determination, the pipetting technique is crucial to the precision and accuracy of the determinations. There are commercially available bead preparations for absolute enumeration. Flow-Count™ (Beckman-Coulter) and FCSC Count Standard™ (Flow Cytometry Standards Corporation) beads are contained in suspension where as TruCount™ (Becton Dickinson) beads are lyophilized and pre-dispensed in test tubes. Absolute count beads in suspension should be added to the stained preparation just prior to acquisition. Table 1. “Staining”, summarizes the principle steps in sample preparation.

**TABLE 1**

**STAINING**

<p><b>RELATIVE VALUE</b> %</p>	<p><b>ABSOLUTE VALUE</b> cells/ul</p>
<p>Use a direct three-colour or four-colour immunofluorescence whole blood lysis method.</p>	
<p>Use the "stain, lyse " commercial procedure and follow manufacturer's instructions.</p>	<p>Use the "stain, lyse, no wash " commercial procedure and follow manufacturer's instruction. Samples prepared for single-platform technology must not be washed.</p>
<p>Use the commercial premixed monoclonal antibody. The panels are listed by CD nomenclature in TABLE 2. If single colour reagents are combined, each must be titered in combination with each other to determine optimal concentration for use (60).</p>	
<p>Pipetting is not critical for relative measurements.</p>	<p>The pipetting of the blood and of the beads is critical for absolute count measurements. See "pipetting tips", Appendix D. See "pipetting technique", page 30</p>
<p>Fix all sample tubes after staining and lysing. Store all stained samples in the dark and at 4-10°C until analysis.</p>	
	<p>Add beads to the stained samples unless TruCount absolute count tubes containing beads are used.</p>
<p>Vortex samples immediately before analysis to optimally disperse cells.</p>	

## **MONOCLONAL ANTIBODY COMBINATION**

A two-colour monoclonal antibody panel is described in Appendix E (61-63). This panel is no longer recommended if your instrument has the capability for three or four-colour analysis. Laboratories may perform T-cell subset determinations by choosing a three or four-colour monoclonal antibody panel (see Table 2), depending on the ability of the flow cytometer to do three or four-colour analysis. The three-colour monoclonal antibody combination allows T-cell subset determinations in two tubes, whereas the four-colour monoclonal antibody panel allows T-cell subset determination in a single tube.

The use of CD45 gating, in combination with side scatter, defines with certainty the location of the lymphocyte population and effectively distinguishes lymphocytes from debris (23,24). Lymphocytes have bright CD45 positive expression and low side scattering properties. Since gating is based on a cell lineage specific marker, all events meet the criteria of being lymphocytes (24,26).

The three-colour combination without CD45 (CD3/CD4/CD8) is not recommended as there is no way to validate the lymphocyte gate in this tube (purity and recovery)(63). A recent study has shown that non-CD45 gating laboratories are more likely to report unacceptable results, sacrificing both accuracy and reproducibility (64).

The proportion of all lymphocytes (T+B+NK) present in the specimen and enclosed in the lymphocyte gate cannot be determined using the short panel A . The expanded panel B, used for pediatric studies, can be useful in accounting for additional cell populations for quality control purposes (23,25,26).

### **TABLE 2**

### THREE AND FOUR-COLOUR MONOCLONAL ANTIBODY PANEL

PANEL	3 COLOUR	4 COLOUR	PANEL
A	CD45/CD3/CD4	CD45/CD3/CD4/CD8	B
	CD45/CD3/CD8		
	CD45/CD3/CD19	CD45/CD3/CD19/CD16 &/or 56	
	CD45/CD3/CD16 &/or 56		

#### Panel A

- Isotype controls are not needed since CD3, CD4 and CD8 positive clusters are easily identified and distinctly separated (24,25). Unlabeled cells may serve as an isotype control.
- CD45 is used for gating lymphocytes
- CD3 and CD4 are used for enumeration of CD4+T-cells
- CD3 and CD8 are used for enumeration of CD8+T-cells

#### Panel B

- Pediatric studies should include B cells and NK cells(25). The complete panel including B and NK markers may be used to generate lymphosum. (26)
- Useful in defining lymphocyte recovery

### ANALYSIS

Each manufacturer has developed protocols to optimize the analysis on their respective instruments. NLHI developed a universal protocol for single-platform absolute counting that can be used with any clinical instrument, lysing protocol, monoclonal antibody and absolute count beads (17,28).

#### Universal template

Below is a detailed description of the universal template shown in Figures 1 & 2. To address the differences in terminology between Beckman Coulter and Becton Dickinson users, we included terms typically used by each in square brackets, separated by a semi-colon: [ Becton Dickinson term ; Beckman Coulter term].

## **STEP A**

### **DOUBLE ANCHOR GATE**

#### DOT PLOT #1, CD45 X SS

Set [threshold; discriminator] on CD45 detector, setting it as near as possible to the leukocyte populations. Adjust SS to visualize all leukocyte populations. Draw a region [R1; A] around the bright CD45 positive cells with low side scatter (lymphocytes); you may include some contaminants.

#### DOT PLOT #2, CD3 X SS: GATED ON [R1; A]

Set a region [R2; B] around the T cell cluster; being sure to include all the bright CD3 positive events. A minimum of 2000-2500 T-cells should be collected.

## **STEP B**

### **T-CELL ABSOLUTE COUNT**

#### DOT PLOT #3 AND #4: GATED ON [R1 \* R2; AB]

Determine the number of events for CD3+4+ and CD3+8+ in the quadrant [UR; C2,D2] on dot plot #3 and #4 respectively. Both dot plots display only T-cells. The number of CD3 positive cells may be collected from the region [R2; B] on dot plot #2.

#### DOT PLOT #5 OR HISTOGRAM #5

Determine the number of beads using an ungated dot plot or histogram. The fluorescence signal emitted by the calibrator beads will appear in several fluorescence detectors (PMT). Please select the PMT that gives you the best fluorescence resolution between beads and cells. Different commercial bead preparations have different levels of fluorescence intensity.

A minimum of 1 $\mu$ l of blood should be analyzed which can be estimated by the bead concentration. If the concentration of beads is 1050 per  $\mu$ l then you should not stop the acquisition before this number has been reached.

### **CALCULATION of ABSOLUTE CD4 (example)**

$$\frac{\# \text{ events in cell subset region [UR;C2]}}{\# \text{ events in bead region [M1;E]}} \times \frac{\text{total number of beads}}{\text{volume of blood}} = \# \text{ cells}/\mu\text{l}$$

Example: FACSCALIBUR , FIG.1 (TruCount 50225 beads)

$$\frac{1510}{1094} \times \frac{50225}{50} = 1386 \text{ CD4 cells}/\mu\text{l}$$

Example: EPICS-XL, FIG. 2 (Flow-Count 1050 beads /  $\mu\text{l}$ )

$$\frac{1335}{1213} \times \frac{1050 \times 100}{100} = 1156 \text{ CD4 cells}/\mu\text{l}$$

Both Beckman Coulter and Becton Dickinson manufacturers offer softwares which assist the user with calculations of absolute counts.

## STEP C

### T-CELL PERCENTAGES

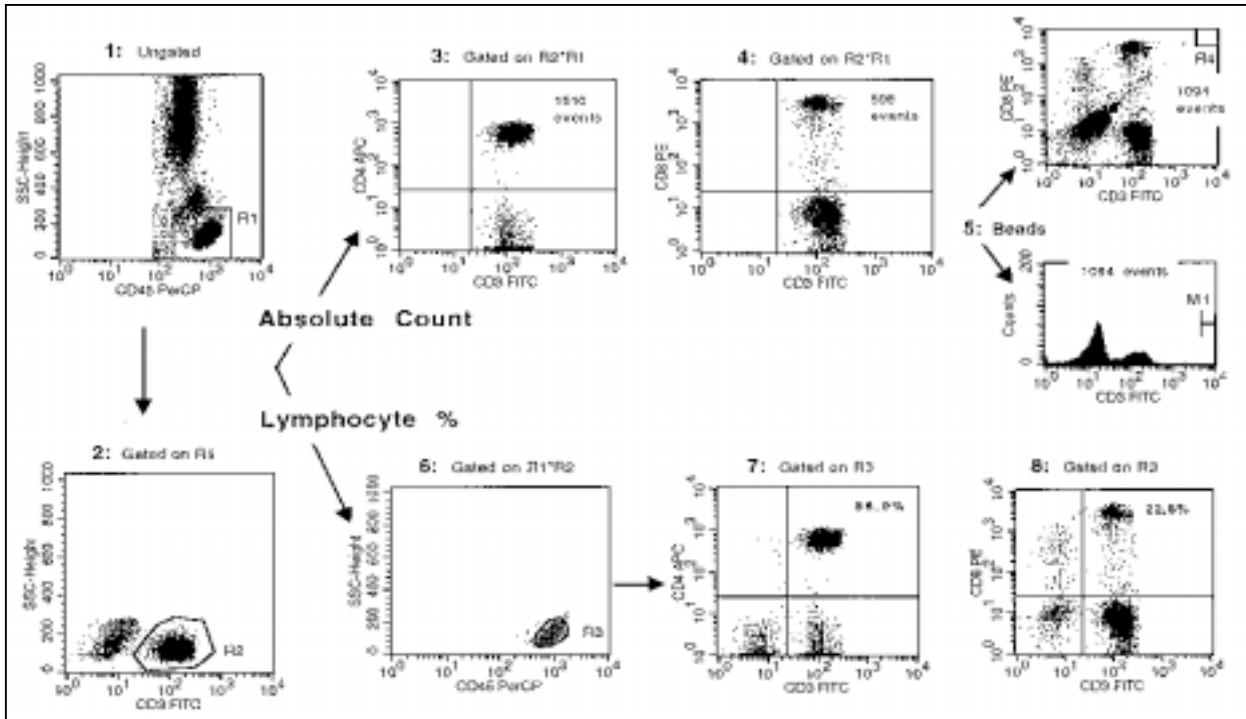
#### DOT PLOT #6, CD45 X SS: GATED ON [R1\*R2; AB]

Dot plot #6 displays only T cells as it is gated on CD45 [R1;A] and CD3[R2;B] positive cells. Be careful drawing the gate [ R3; G] around the cluster. Do not use “autogating”; you may exclude B and NK cells, generating false high percentages. Gate [R3;G] should be drawn as close as possible to the cell cluster. A gate too large would result in serious contaminations by monocytes. The contamination can be monitored by the CD3<sup>-</sup> 4<sup>+</sup> events on dot plot #7. The gate [R3; G] should include at least 98% of the T-cells.

#### DOT PLOT #7 AND #8: GATED ON [R3; G]

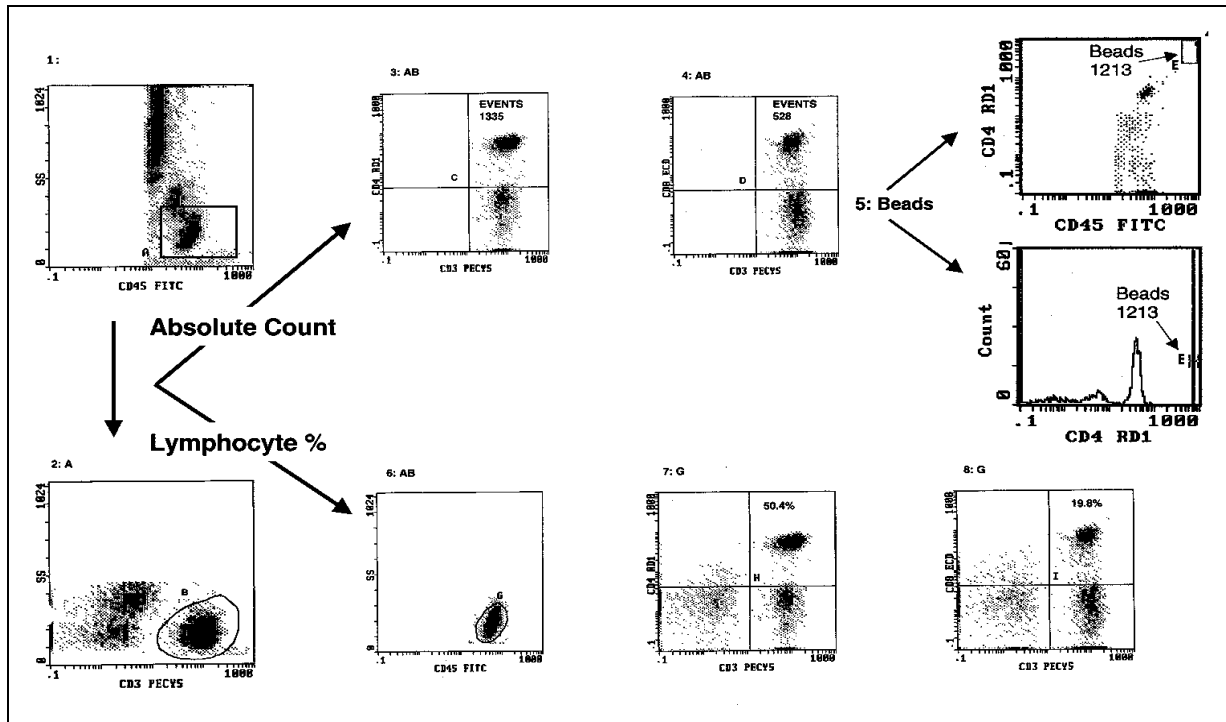
Both CD3+4+ and CD3+8+ percentages are collected from the quadrant [UR; H2, I2] on dot plot #7 or #8 respectively. The CD3 values are determined by adding the upper and lower right quadrant [UR + LR; H2+H4 or I2 + I4] from either dot plot #7 or #8. Values should be identical for both dot plots. The monocyte contamination contained in gate [R3; G] can be monitored on dot plot #7, in the upper left quadrant [UL; H1]. Monocyte contamination should not exceed 2%.

FIGURE 1. UNIVERSAL TEMPLATE (FACSCalibur)



Analysis of whole blood sample. Data was acquired on a Becton Dickinson, FACSCalibur flow cytometer. The blood was stained with CD45PERCP/CD3FITC/CD4APC/CD8PE. The preparation was analyzed using the “universal template” to generate both relative and absolute T-cell values. This template also applies to the two tubes, three-colour combination CD45PERCP/CD3FITC/CD4PE and CD45PERCP/CD3FITC/CD8PE.

FIGURE 2. UNIVERSAL TEMPLATE (EPICS-XL)



Analysis of whole blood sample. Data was acquired on a Beckman Coulter, EPICS-XL flow cytometer. The blood was stained with CD45FITC/CD3PC5/CD4RD1/CD8ECD. The preparation was analyzed using the “universal template” to generate both relative and absolute T-cell values. This template also applies to the two-tubes, three-colour combination CD45FITC/CD3PC5/CD4RD1 and CD45FITC/CD3PC5/CD8RD1.

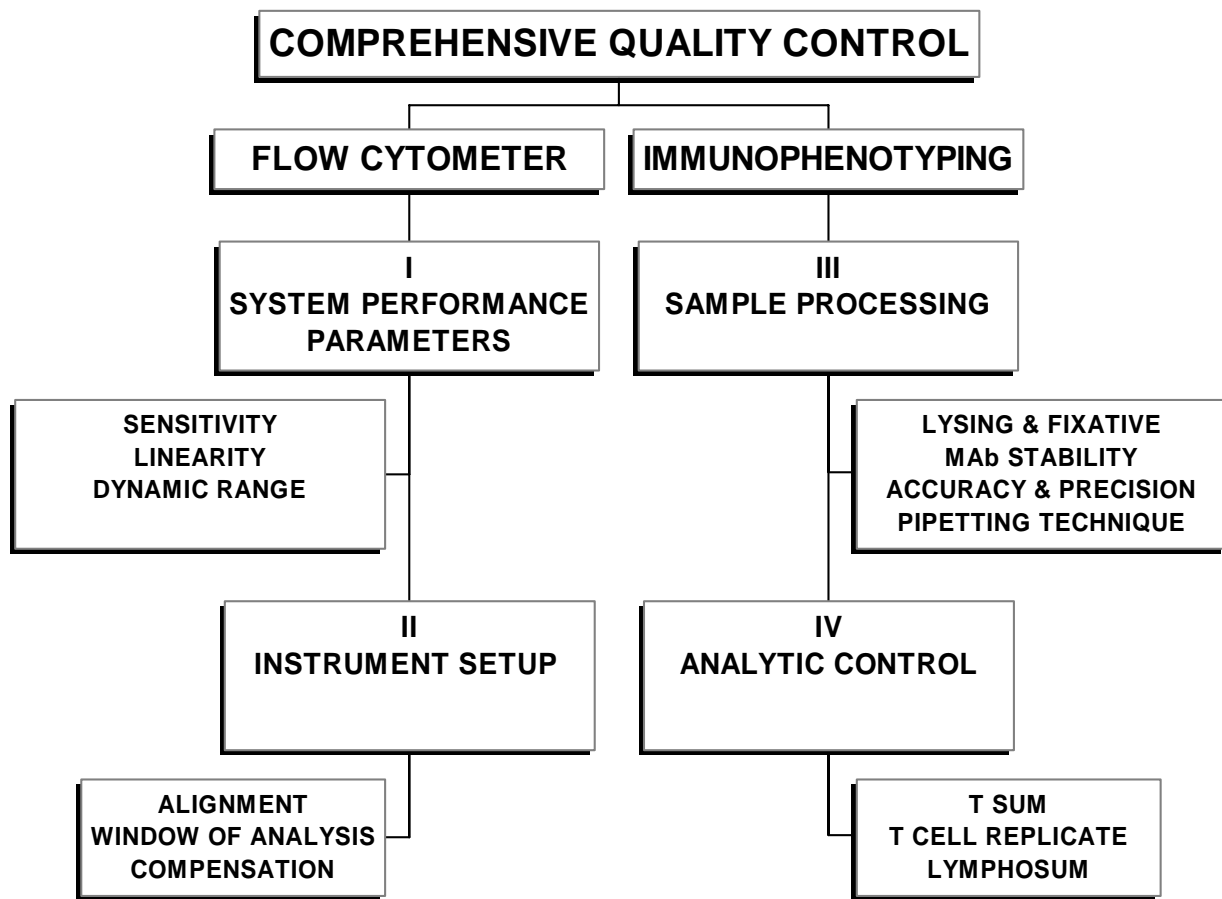
## QUALITY CONTROL

CD4+ T-cells have prognostic value in the development of AIDS in HIV-infected individuals, as well as evaluating response to therapy. The degree of variability in CD4 measurements is directly linked to the uncertainty in determining which patients who will or will not benefit, from therapy. Furthermore there are often discordant results from some patients and inconsistency between HIV-1, plasma RNA and absolute CD4+ T-cell counts. The use of quality control procedures reduces this variability. Quality control is associated with the assessment of measurement error. It is defined as the systematic procedures implemented in one laboratory to evaluate and monitor the accuracy and precision of any analytical process. The main objective is to evaluate the results of testing by means of assessing their quality. Quality control involves the

monitoring of intra-laboratory variation by ensuring the day to day consistency of measurements. A comprehensive quality control for immunophenotyping by flow cytometry implies monitoring of instrumentation as well as of sample processing (see Org. chart 1 and Table 3 ) (17,54).

**ORGANIZATIONAL CHART 1.**

**TABLE 3. COMPREHENSIVE QUALITY CONTROL**



	QC FUNCTION	MATERIAL USED	MONITORING
--	-------------	---------------	------------

<b>I – SYSTEM PERFORMANCE PARAMETERS</b>	CALIBRATION	CALIBRATION STANDARDS	<ul style="list-style-type: none"> <li>• LINEARITY</li> <li>• SENSITIVITY</li> <li>• DYNAMIC RANGE</li> </ul>
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	<b>QC FUNCTION</b>	<b>MATERIAL USED</b>	<b>MONITORING</b>
<b>II - INSTRUMENT SETUP</b>	ALIGNMENT	ALIGNMENT STANDARD	<ul style="list-style-type: none"> <li>• HPCV</li> <li>• PEAK CHANNELS</li> <li>• PMT VOLTAGES</li> </ul>
	WINDOW OF ANALYSIS	UNSTAINED FRESH WHOLE BLOOD AND REFERENCE STANDARD	<ul style="list-style-type: none"> <li>• PMT VOLTAGES</li> <li>• PEAK CHANNEL</li> </ul>
	COLOUR COMPENSATION	MULTICOLOUR STAINED FRESH WHOLE BLOOD	<ul style="list-style-type: none"> <li>• COMPENSATION SUBTRACTION</li> <li>• MATRIX</li> </ul>
<b>III – SAMPLE PROCESSING</b>	LYSING & FIXATIVE	MULTICOLOUR STAINED FRESH WHOLE BLOOD	<ul style="list-style-type: none"> <li>• OBSERVATION</li> </ul>
	MAb STABILITY		<ul style="list-style-type: none"> <li>• MEAN FLUORESCENCE INTENSITY</li> </ul>
	ACCURACY PRECISION	STABILIZED CELL MATERIAL	<ul style="list-style-type: none"> <li>• PERCENTAGES</li> <li>• ABSOLUTE COUNT</li> </ul>
	PIPETTING TECHNIQUE	WHOLE BLOOD AND DISTILLED WATER	<ul style="list-style-type: none"> <li>• MEAN, S.D., % C.V.</li> </ul>
<b>IV – ANALYTIC CONTROL</b>	PHENOTYPIC RELIABILITY	PATIENTS SPECIMENS CONTROL SPECIMENS	<ul style="list-style-type: none"> <li>• T SUM</li> <li>• T CELL REPLICATES</li> <li>• LYMPHOSUM</li> </ul>

### **I - SYSTEM PERFORMANCE PARAMETERS**

Proper instrument calibration is a key factor when performing quantitative flow cytometry determination of antibody binding as well as providing information to assess instrument performance. Primary performance parameters such as

instrument linearity, sensitivity and dynamic range can be determined from a standardization calibration plot and can be used to monitor instrument performance. An intra-instrument evaluation refers to the consistency of data obtained from a single instrument over time (52,54,65,66).

## CALIBRATION

A standardized calibration plot can provide comprehensive quantitative quality control against acceptable ranges, which can be used to monitor instrument performance. The use of calibration material permits the measurement of instrument response, and the quantification of sample fluorescence signals (54).

WHAT TO USE: Calibration standards: beads of multiple fluorescence intensities or beads with different antibody binding capacities.

HOW TO USE : The material can be used daily, once a month, once a week and every time maintenance or repair has been done on the instrument. Always check the alignment before proceeding. Calibration can be used to assess the overall performance by monitoring sensitivity, linearity and dynamic range of the instrument on Levey-Jennings plots (65).

## **II - INSTRUMENT SETUP**

### ALIGNMENT

Many modern flow cytometers do not require daily optical alignment because they have fixed optical systems. However, it is strongly recommended that laboratories use alignment standards to verify instrument alignment and the fluidics system, making sure that samples do not appear to be shifted, or to

appear broader than expected. It is recommended that alignment standards be used daily to verify and monitor optimal resolution and to detect significant variations in power output and mean channel number (52).

WHAT TO USE: Alignment standards: beads with fluorescence intensities exceeding the range normally used in applications related to cell surface marker analysis. Proper alignment should give C.V. less than 2 %

HOW TO USE: Establish stability of the instrument by daily monitoring of the PMT voltages, peak channel and C.V. for each detector, and recording the data points on Levey-Jennings plots.

### WINDOW OF ANALYSIS

To achieve reproducibility of the analysis range and standardization of the position of cell clusters, a window of analysis may be established. This is accomplished by placing reference standards in specified target channel, which will normalize fluorescence intensity from day to day (52).

WHAT TO USE: Reference standards: beads with distributions of 5 to 10% C.V., and without the high uniformity required to obtain or monitor reliable optical alignment of an instrument.

HOW TO USE: Set voltages based on the autofluorescence of your specimen. Adjust voltages to observe your signal in the first log decade of each PMT detector fluorescence scale. Run your reference standards and record your "initial target channel". To conserve this window of analysis for subsequent runs, readjust your PMT voltages each day to maintain the target channels for each detector. Monitor daily voltages of each detector over time on Levey -Jennings plots.

## COLOUR COMPENSATION

To assure consistency related to colour compensation setup, you may establish a window of analysis with compensation circuitry turned on. To accomplish this setup, the window of analysis is established as described in the previous section. Compensation controls are then run and compensation adjusted accordingly. To verify the compensation matrix run an HIV-negative control stained with the appropriate antibody combination and verify that each cluster falls into proper position. To achieve reproducibility of the analysis range, run the reference standards under these settings and record the specific target channels.

WHAT TO USE: Reference standards: beads with distributions of 5 to 10% C.V. and without the high uniformity required to obtain or monitor reliable optical alignment of an instrument.

HOW TO USE: Following alignment, positioning of the window of analysis and compensation, run the reference standards and record the specific bead target channels of each fluorescence detector. Daily, readjust your PMT voltages to keep the fluorescence signal of the reference beads in their respective specific target channel. If your “verifier” compensation controls show abnormal distribution, compensation settings must be reset with adequate control cells. Monitor daily PMT voltages of each detector overtime on Levey-Jennings plots.

**INTER-INSTRUMENT NORMALIZATION.** If a laboratory has two or more flow cytometers and wants to be able to directly compare histograms, all instruments can be normalized to the same analysis range by positioning the same reference material in the same equivalent target channel. To achieve inter-instrument normalization, it is essential to use reference standards that are spectrally matched (52,63,65).

### **III – SAMPLE PROCESSING**

#### **LYSING AND FIXATIVE**

Lysing and fixing effectiveness can be evaluated qualitatively. The inclusion of CD45 for gating purposes permits good analysis even with poorly lysed specimens. It eliminates the inclusion of debris, RBC and platelets (23). Completeness of the lysing procedure can be assessed by verifying good resolution between leukocyte populations and debris on a dual light scatter dot plot (FS x SS).

An HIV-negative specimen, properly fixed, should give a similar light scatter profile from day to day. Use a fresh whole blood specimen to detect any abnormal fixative effect. Mark date of fixative preparation, monitor pH, and identify the solution with an expiry date.

#### **MONOCLONAL ANTIBODY STABILITY**

Each lot of monoclonal antibody can be quality controlled by staining fresh whole blood specimen and monitoring the mean fluorescence intensity of each antibody over time using a Levey-Jennings plot.

#### **ACCURACY & PRECISION**

A number of stabilized cell preparations are commercially available (67). These control materials allow the evaluation of the accuracy in lymphocyte percentages and absolute counts by comparing values obtained with the range of expected values supplied by the manufacturer. A preparation processed in triplicate will permit the assessment of precision (reproducibility) on a given day.

Levey-Jennings plots should be used to monitor cell count measurements, both percent and absolute, over time.

PIPETTING TECHNIQUE

The single-platform methodology for absolute count requires accurate and precise measurement. Reverse pipetting technique is recommended. (see pipetting notes in Appendix D)

TESTING PIPETTING PRECISION

- Using the reverse pipetting technique, pipet 10 replicates of blood and record the weight.
- Using the reverse pipetting technique, pipet 10 replicates of bead suspension and record the weight.
- The C.V. should be less than 2% (Table 4 )

TESTING PIPETTING ACCURACY

Following the reverse pipetting technique, pipet 10 replicates of distilled water and record the weight. 100 ul of water should weight 0.1000 grams. C.V. must be less than 2% (range 0.098-0.102)

**TABLE 4.**

**EVALUATION OF PRECISION AND ACCURACY**

TO EVALUATE:	ACCURACY	PRECISION	PRECISION
Replicate #	100 µl of water (grams)	100 µl of blood (grams)	100 µl of microbeads (grams)
1	0,1036	0,1072	0,1056
2	0,1018	0,1071	0,1056
3	0,1020	0,1067	0,1055
4	0,1026	0,1069	0,1056

5	0,1008	0,1067	0,1052
6	0,1002	0,1060	0,1055
7	0,0989	0,1072	0,1056
8	0,1019	0,1090	0,1047
9	0,1009	0,1070	0,1050
10	0,1027	0,1066	0,1050
<b>MEAN</b>	<b>0,1015</b>	<b>0,1070</b>	<b>0,1053</b>
<b>S.D.</b>	<b>0,0014</b>	<b>0,0008</b>	<b>0,0003</b>
<b>%C.V.</b>	<b>1,35</b>	<b>0,73</b>	<b>0,31</b>

## **IV – ANALYTIC CONTROL**

### **PHENOTYPIC RELIABILITY**

**T SUM:** Optimally the sum of the percentage of CD3+4+ and CD3+8+ cells should equal the total percentage of CD3+ cells within  $\pm 5\%$ . In specimens containing a considerable amount of T  $\gamma\delta$  cells, this reliability check may exceed the maximum variability (14,63,68,69).

**T CELL REPLICATES:** A three-colour monoclonal antibody panel consists of at least two tubes with the CD3 marker. Differences between replicate CD3 results should be less than  $\leq 2\%$  (23).

**LYMPHOSUM:** The lymphosum (T+B+NK=  $100\% \pm 10\%$ ) is used to serve as a check on the overall accuracy of the immunophenotyping result (63).



## DISCUSSION

Current antiretroviral therapy effectively suppresses HIV replication in a significant number of patients and has radically extended the length of time for which patients remain relatively healthy with minimal or no symptoms. A growing body of clinical evidence suggests that immune functions improve in most patients after suppression of plasma viral load. Until better measures are available, the T-cell subset profile in conjunction with plasma viral RNA measurement remains the best approach for assessing the immune status of such individuals. In HIV infected individuals immunophenotyping remains the hallmark test for the evaluation of their prognosis, immune deficiency status, response to therapy, and diagnosis of clinical AIDS.

In 1992, the Centers for Disease Control (CDC) developed and published guidelines for performing CD4+ T-cell enumeration. Two years later the Laboratory Centre for Disease Control (LCDC) in Canada, with permission, reissued the same guidelines as a bilingual document.

A decade has passed since the initiation of the Canadian programme for assessing the immunophenotyping performance of the Canadian HIV Trials Network members. Over this time the quality of immunophenotyping has improved. The factors responsible are numerous. While much of the improvement can be attributed to the diligence of the clinical laboratory professionals across Canada, it is also true that flow cytometers, reagents, software and algorithms used to analyze leukocytes have also improved. The overall impact of the frequency of participation on immunophenotyping performance in Canada has been published (17).

As we enter a new decade the capacity to generate reliable absolute lymphocyte counts across Canada has, for the first time, become feasible. Until now, this clinically useful parameter was not available for multi-center studies. Absolute counts were performed using a multi-platform technology, and monitoring the performances of laboratories across Canada with that technology was not possible. Relying on results that were in part generated by a hematology laboratory made the centralized quality assessment of such integrated systems impractical. In a study conducted by NIAID in 1999, it was observed that the between-laboratory coefficient of variation (%C.V.) for

CD4+ T-cell absolute counts was 1.5 times higher than the percent C.V. for CD4+ T-cell percentages. The increase in variability due to hematology procedures suggested that single-platform technology would be beneficial. In May 2000, two publications dealing with this specific issue appeared. Both were comparisons of single platform technology versus traditional double-platform absolute counting. These two papers convincingly demonstrated a significant advantage of the single-platform technology for both within laboratory and between laboratory variations for absolute counting. In addition, the two studies also indicated that the use of CD45 gating as a heterogeneous gating method was preferable. When CD45 fluorescence and side scatter are correlated, the precision of the lymphocyte subset improved significantly over the traditional homogeneous gating method which requires correlation of forward and side light scatter. The latter approach ignores the benefits of cell identification based on lineage specific markers.

With the recent availability of single-platform technology and the capacity to utilize heterogeneous gating strategies, it is possible to provide reliable CD4 and CD8 T-cell absolute values for multi-center studies. The clinical flow cytometry laboratory will be able to provide absolute count immunophenotyping values with the reliability, precision and accuracy to a degree previously obtainable only with lymphocyte percentages. The implementation and validation of the improvements described in this guideline will enhance multi-center studies in HIV.

## **APPENDIX A**

### **UNIVERSAL SAFETY PRACTICES**

1. All laboratory personnel and others whose work requires them to enter the laboratory must understand the biological and other hazards with which they will come in contact through their normal work in the laboratory, and be trained in appropriate safety precautions and procedures. A laboratory safety manual must be prepared and adopted. And it is the responsibility of the laboratory director/principal investigator to ensure it identifies known and potential biohazards and specifies practices and procedures to eliminate or minimize such risks. The manual must also contain an emergency response plan. Personnel must be required to know, understand, and follow standard practices and procedures. Training in laboratory safety must be provided, and competence in safe technique demonstrated before work is allowed with hazardous agents or toxins.
2. Laboratories should have a biological safety officer (BSO) and/or a biological safety committee whose responsibilities include ensuring that all work is carried out in accordance with safety practices established at the institution. The duties of the BSO should include providing technical advice on safety procedures and equipment, developing emergency plans, conducting safety inspections, providing biosafety training, conducting or supervising testing of containment systems, and providing guidance and information related to compliance with pertinent regulations.
3. The laboratory must be kept neat, orderly and clean, and storage of materials not pertinent to the work should be minimized.
4. Protective laboratory clothing (uniforms, coats, gowns) must be available and be worn properly fastened by all personnel, including visitors, trainees and others entering or working in the laboratory. Protective laboratory clothing must not be worn in non-laboratory areas. Suitable footwear with closed toes and heels and preferably with non-slip soles must be worn in all laboratory areas.
5. Gloves must be worn for all procedures that might involve direct skin contact with toxins, blood, infectious materials, or infected animals. Rings or hand jewelry which would interfere with glove functioning should be removed before gloving. Gloves should be removed carefully and decontaminated with other laboratory wastes before disposal. Reusable gloves (e.g. insulated, chemical resistant, etc.) may be used only where necessary and must be appropriately decontaminated.
6. Protective face and eyewear (e.g. glasses, goggles, face shields, or other protective devices) must be worn when necessary to protect the face and eyes from splashes, impacting objects, harmful substances, UV light, or other rays.
7. Eating, drinking, smoking, storing food, personal belongings or utensils, applying cosmetics, and inserting or removing contact lenses is not permitted in any laboratory work area. Contact lenses should be worn only when other forms of

corrective eyewear are not suitable. The wearing of jewelry should be discouraged in the laboratory.

8. Oral pipetting of any substance is prohibited in any laboratory.
9. Long hair must be tied back or restrained.
10. Hands must be washed after gloves are removed, before leaving the laboratory, and at any time after handling materials known or suspected to be contaminated.
11. Work surfaces must be cleaned and decontaminated with a suitable disinfectant at the end of the day and after any spill of potentially dangerous material. Loose or cracked work surfaces must be replaced or repaired.
12. All technical procedures must be performed in a manner that minimizes the creation of aerosols.
13. All contaminated or infectious liquid or solid materials must be decontaminated before disposal or reuse. Contaminated materials that are to be autoclaved or incinerated at a site away from the laboratory must first have the outside of the container disinfected chemically, or be double-bagged.
14. Access to the laboratories must be strictly limited (containment Levels 3 and 4). Decisions on entry into containment Level 1 and 2 laboratories should be at the discretion of the laboratory director/ principal investigator (e.g. only persons who have been advised of the potential hazards and meet any specific entry requirements such as immunization should be allowed to enter the laboratory area). Children under the age of 16 should not be permitted in the laboratory or support areas. Pregnant women or immunocompromised people who work in or enter the laboratory should be advised of the associated risks.
15. Hazard warning signs, indicating the risk level of the agents being used, must be posted outside each laboratory. Where infectious agent(s) used in the laboratory require special provisions for entry, the relevant information must be included in the sign. The agent must be identified, and the name of the laboratory supervisor and other responsible person(s) as well as any special conditions for staff entry must be listed.
16. The use of needles, syringes and other sharp objects should be strictly limited. Needles and syringes should be used only for parenteral injection and aspiration of fluids from laboratory animals and diaphragm bottles. Extreme caution should be used when handling needles and syringes to avoid autoinoculation and the generation of aerosols during use and disposal. Procedures should be performed in a biological safety cabinet. Needles should not be bent or sheared. They should not be replaced in the sheath or guard. They should be promptly placed in a puncture-

proof container and decontaminated, preferably by incineration or autoclaving, before disposal.

17. All spills, accidents, and overt or potential exposures must be reported in writing to the laboratory supervisor or acting alternate as soon as circumstances permit; this person should file this report with management and the appropriate biosafety officer or committee. Appropriate medical evaluation, surveillance, and treatment should be sought and provided as required. Actions taken to prevent future occurrences should be documented.
18. Baseline serum for laboratory and other at-risk personnel (eg. laboratory support and maintenance staff) should be collected and stored. Additional serum specimens may be collected periodically, depending on the agents handled or the function of the facility.
19. Laboratory workers should be protected by appropriate immunization where possible. Levels of antibody considered to be protective should be documented. Particular attention must be given to individuals who are or may become immunocompromised, as vaccine administration may be different than for immunologically competent adults.

## **APPENDIX B**

### **BLOOD COLLECTION FOR HEMATOLOGY**

#### **ANTICOAGULANT**

Use tripotassium ethylenediamine tetra-acetate ( $K_3EDTA$ ,  $1.5 \pm 0.15$  mg/ml blood) and perform the test within the time frame allowed by the manufacturer of your hematology analyzer (9). Laboratories using such instruments are justified in extending the period

between specimen collection and hematologic testing only after they have validated their instrument. The validation requires that the maximum time that specimens from both HIV-negative and HIV-infected persons show no significant variation in count from time 0.

## **BLOOD COLLECTION**

Collect blood specimens by venipuncture into evacuated tubes containing an appropriate anticoagulant, completely expending the vacuum in the tubes. Draw pediatric specimens in pediatric tubes to avoid underdrawing. Mix the blood well with the anticoagulant to prevent clotting. Draw the appropriate number of tubes. When hematology and flow cytometric immunophenotyping will be performed in the same laboratory on the same specimen, use one tube containing K<sub>3</sub>EDTA. In all other circumstances, draw two separate tubes (K<sub>3</sub>EDTA for hematologic determinations and K<sub>3</sub>EDTA, ACD, or heparin for flow cytometric immunophenotyping).

# APPENDIX C

## FLUORESCENCE STANDARDS CLASSIFICATION (52)

CLASSIFICATION SYSTEM OF FLUORESCENCE STANDARDS

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Table 1  
*Physical Characteristics of Various Types of Standards for Instrument Setup and Calibration*

Characteristics	Type 0— certified blank	Type I— alignment		Type II— reference			Type III— calibration		
		Ia	Ib	IIA	IIB	IIC	IIIA	IIIB	IIIC
Size relative to lymphocytes	Equivalent	Smaller	Equivalent	Equivalent	Equivalent	Equivalent	Equivalent	Equivalent	Equivalent
Size uniformity (i.e., CV < 2%)	No	Yes	Yes	No	No	No	No	No	No
Fluorescence intensity level	Very low	Very bright	Very bright	Bright	Bright	Bright <sup>a</sup>	Dim to bright	Dim to bright	Dim to bright <sup>a</sup>
Number of fluorescence intensity levels	Single	Single	Single	Single	Single	Single <sup>a</sup>	Multiple	Multiple	Multiple <sup>a</sup>
Excitation and emission spectra matching with sample	NA <sup>b</sup>	No	No	No	Yes	Yes <sup>a</sup>	No	Yes	Yes <sup>a</sup>
Environmentally responsive	No	No	No	No	Yes	Yes <sup>a</sup>	No	Yes	Yes <sup>a</sup>
Antibody binding capacity	No	No	No	No	No	Yes	No	No	Yes

<sup>a</sup>After binding of fluorescently labeled antibodies.

<sup>b</sup>NA, not applicable.

CLASSIFICATION SYSTEM OF FLUORESCENCE STANDARDS

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Table 2  
*Applicability of Various Types of Standards for Instrument Set-Up and Calibration\**

Function	Types of standards (see Table 1 for explanation)								
	0	Ia	Ib	IIA	IIB	IIC	IIIA	IIIB	IIIC
Instrument set-up									
Alignment	—	+	+	±	—	—	±	—	—
Positioning of FL window of analysis	±	—	—	+ <sup>a</sup>	+	±	+ <sup>a</sup>	+	±
Color compensation	—	—	—	—	±	+	—	±	+
Instrument calibration									
Linearity	—	—	—	—	—	—	+	+	+
Noise level	+ <sup>b</sup>	—	—	—	—	—	—	—	—
Dynamic range	—	—	—	—	—	—	+	+	+
Zero channel value	—	—	—	—	—	—	± <sup>a</sup>	+	+
Quantitative fluorescence measurements									
Arbitrary units (RFI)	—	—	—	—	—	—	+ <sup>a</sup>	—	—
MESF units per fluorochrome, antibodies bound per cell	—	—	—	—	—	—	—	+	—
Antibody binding capacity	—	—	—	—	—	—	—	—	+

\*Suitability for the specified function: + good; ± poor; — none.

<sup>a</sup>Due to the lack of spectral matching between standards and sample, Type IIA or IIIA standards only yield consistent results for a single instrument, but not between multiple instruments (for explanation, see text).

<sup>b</sup>When used in combination with type IIIB and IIIC calibration standards.

# APPENDIX D

## PIPETTING NOTES

This is a brief consolidation of operational instruction tips from the Instruction Manual of several pipettes i.e. Eppendorf® series 2000, Pipetman® P, Eppendorf® Repeater™ 4780, Eppendorf® Response 4850 electronic pipette—for complete information and more detailed instructions, refer to your pipette Instruction Manual. An Eppendorf Standard Operating Procedure for pipettes is available on their site: [www.eppendorf.com/pipet/SOP](http://www.eppendorf.com/pipet/SOP). (70,71)

- Select desired volume (with manual pipettes, it is recommended that higher volumes be set first---if going from a lower volume to a higher volume, first surpass the desired volume and then slowly decrease the volume until the required setting is reached)
- Select desired mode (if applicable) i.e. **reverse pipette** (this is recommended for best precision and reproducibility)
- Reverse pipetting can be done with a manual pipette by pressing the control button slightly past the first stop when aspirating, taking up more liquid than will be dispensed, then pressing the control button only to the first stop when dispensing---there will be a small volume remaining in the tip after dispensing
- Select appropriate tip (usually colour matches colour of control button)

### Filling:

- For optimal precision and accuracy,
  - Volumes > 10 µL - pre-rinse 2-3 times for each new tip  
(this involves aspirating and dispensing liquid several times)
  - Volumes < 10 µL - DO NOT pre-rinse  
(rinse tip AFTER dispensing to ensure whole volume dispensed)

### Reasons for pre-rinsing

- ✓ System pressure compensation
- ✓ Compensation for *slight* differences in temperature between pipette and liquid
- ✓ Compensation for properties of the liquid (liquids form a thin film on the inside of pipette---without pre-rinsing, the first volume would be too small due to retention of a thin film on the inside wall of the tip---the “thickness” and nature of this film, and therefore the potential source of error, will vary depending on the nature of the liquid being pipetted)
- ✓ For the smaller volumes, pre-rinsing is not recommended as the dispensed volume would be too great

- Make sure tip is securely attached
- Hold pipette upright
- When aspirating, try to keep the tip at a constant depth below the surface of the liquid
- Glide control button *slowly* and *smoothly* (electronic pipettes perform this step for you)
- When pipetting viscous liquids (i.e. serum, blood), leave tip in the liquid for 1-2 seconds after aspirating before withdrawing it
- Once there is liquid in the tip, NEVER lay pipettes on their sides

Dispensing:

- Hold tip at an angle, against the inside wall of the vessel/tube if possible
- Glide control button slowly and smoothly (electronic pipettes perform this step for you)

Other Recommendations:

- To ensure optimal performance, the temperature of the pipetted solution and the pipette and tips should be the same (volume errors may occur due to changes in air displacement and viscosity of the liquid) --- do not pipette liquids with temperatures greater than 70°C
- Volume errors may also occur with liquids that have a high vapor pressure or a density/viscosity which differs greatly from water (water is generally used to calibrate the pipette and check inaccuracy and imprecision) --- it may be possible to recalibrate the pipette for liquids with densities that vary greatly from that of water
- Pipettes should be checked regularly for precision and accuracy
- Regular maintenance (cleaning etc.) should be performed either by the user or a service technician according to manufacturer's instruction
- READ PIPET INSTRUCTION MANUAL!

## APPENDIX E

### TWO-COLOUR PANEL

The results for the panel provide data useful for defining the T-cell population and subpopulations; determining the proportion and purity of the lymphocytes in the gate; setting cursors for positivity; accounting for all lymphocytes in the sample; monitoring tube-to-tube variability; and monitoring T-cell, B-cell, and natural killer (NK)-cell levels in sequential patient specimens. The following internal controls are included in the panel (61).

Tube	FITC*	PE**	Reason for using
1	CD45	CD14	To draw gates; lymphocytes are brightly positive for CD45 and negative for CD14 (62)
2	Isotype	Isotype	To set cursors, or discriminators, for positivity in the samples to follow
3	CD3	CD4	To measure CD4+ T-Cells; only cells positive for both CD4 and CD3 should be considered CD4+ T-cells.
4	CD3	CD8	To measure CD8+ T-cells; only cells that are positive for both CD8 and CD3 should be considered CD8+ T-cells. The remainder of the CD8 cells (CD3-) are natural killer (NK)-cells.
5	CD3	CD19#	To measure B-cells for quality assurance and to account for all lymphocytes.
6	CD3	CD16 56#	To measure NK-cells (negative for CD3, and/or positive for CD16 and/or CD56) and for quality assurance to help account for all lymphocytes.

\* Fluorescein IsoThioCyanate

\*\* Phycoerythrin or RD-1 (Beckman Coulter trademark)

# A minimal acceptable panel omits these two monoclonal antibody combinations.

CD3 monoclonal antibody in tubes 3-6 serves as a control for tube-to-tube variability and is also used to determine T-cell populations. All CD3 values should be within 3% of each other. If the CD3 value of a tube is >3% of any of the others, that tube should be repeated (new aliquot of blood labeled, lysed, and fixed). Monoclonal antibodies that label T-cells, B-cells and NK-cells are used to account for all lymphocytes in the sample.

## APPENDIX F

### Definitions

**Alignment standard:** A single population of fluorospheres of high uniformity and high fluorescence intensity.

**Antibody binding capacity ABC:** Number of antibody molecules bound by a particle when specific binding sites are saturated.

**Calibration standards:** Particles with multiple fluorescence intensity populations used to calibrate the response of the instrument, assess the system performance parameters and to quantify the fluorescence signals of samples.

**Certified blank standard:** Particles that do not have any added fluorescent dyes and have been certified to have fluorescence intensity lower than the autofluorescence of unlabeled cells of a healthy individual.

**Double anchor gate:** The sequential use of two lineage specific markers to characterize a target population. The combination gate defined by this method is referred to as double anchor gate.

**Dynamic range:** Range of fluorescence intensity measurable by the instrument.

**Linearity:** Ability (within a given range) to provide results that are directly proportional to the concentration of the analyte in the test sample.

**Quality assessment:** External quality assessment refers to a system of retrospectively and objectively comparing results from different laboratories by means of surveys organized by an external agency. The main objective is to establish between-laboratory and between-instrument comparability. External quality assessment schemes may be regional, national or international.

**Quality assurance :** All steps taken by the director of a laboratory to ensure reliability of laboratory results and to increase accuracy, reproducibility and between lab comparability. This should include laboratory accreditation, proficiency surveillance and the constant use of an internal quality assessment scheme. It also includes participation in additional advanced training courses, biotechnology transfer workshops, collaborative studies of instruments and laboratory methods. In addition, defining standards for personnel, methods, equipment maintenance, and report generation intended for the improvement of laboratory performance are a necessary component of a quality assurance program.

**Reference standards:** Particles with fluorescence intensity comparable to cells used to set up the analysis range (see window of analysis).

**Sensitivity:** Fluorescence limit of detection of the instrument.

**Spectral matching:** Excitation and emission properties of the standard have to match those of the samples across the spectrum.

**Target channels:** A set reference point (mean peak channel) within the window of analysis.

**Window of analysis:** Range of fluorescence intensity (analysis range) measured by the flow cytometer at a particular instrument setting.

## REFERENCES

- (1) Graham NM, Piantadosi S, Park LP, Phair JP, Rinaldo CR, Fahey JL. CD4+ lymphocyte response to zidovudine as a predictor of AIDS- free time and survival time. *J Acquir Immune Defic Syndr* 1993; 6(11):1258-1266.
- (2) Turner BJ, Hecht FM, Ismail RB. CD4+ T-lymphocyte measures in the treatment of individuals infected with human immunodeficiency virus type 1. A review for clinical practitioners. *Arch Intern Med* 1994; 154(14):1561-1573.
- (3) Fei DT, Paxton H, Chen AB. Difficulties in precise quantitation of CD4+ T lymphocytes for clinical trials: a review. *Biologicals* 1993; 21(3):221-231.

- (4) Hoover DR, Graham NM, Chen B, Taylor JM, Phair J, Zhou SY, Munoz A. Effect of CD4+ cell count measurement variability on staging HIV-1 infection. *J Acquir Immune Defic Syndr* 1992; 5(8):794-802.
- (5) De G, V, Gelman R, Lagakos S. Uses of CD4-lymphocyte count in AIDS treatment decisions. *Infect Agents Dis* 1993; 2(5):304-313.
- (6) Rachlis AR, Zarowny DP. Guidelines for antiretroviral therapy for HIV infection. Canadian HIV Trials Network Antiretroviral Working Group. *CMAJ* 1998; 158(4):496-505.
- (7) CDC. 1993 revised classification system for HIV infection and expanded surveillance case definition for AIDS among adolescents and adults. *MMWR Morb Mortal Wkly Rep* 1992; 41(RR-17):1-19.
- (8) Stein DS, Korvick JA, Vermund SH. CD4+ lymphocyte cell enumeration for prediction of clinical course of human immunodeficiency virus disease: a review. *J Infect Dis* 1992; 165(2):352-363.
- (9) De Wolf F, Roos M, Lange JM, Houweling JT, Coutinho RA, van der Noordaa J, Schellekens PT, Goudsmit J. Decline in CD4+ cell numbers reflects increase in HIV-1 replication. *AIDS Res Hum Retroviruses* 1988; 4(6):433-440.
- (10) Fahey JL, Taylor JM, Detels R, Hofmann B, Melmed R, Nishanian P, Giorgi JV. The prognostic value of cellular and serologic markers in infection with human immunodeficiency virus type 1. *N Engl J Med* 1990; 322(3):166-172.
- (11) Lang W, Perkins H, Anderson RE, Royce R, Jewell N, Winkelstein WJ. Patterns of T lymphocyte changes with human immunodeficiency virus infection: from seroconversion to the development of AIDS. *J Acquir Immune Defic Syndr* 1989; 2(1):63-69.
- (12) Masur H, Ognibene FP, Yarchoan R, Shelhamer JH, Baird BF, Travis W, Suffredini AF, Deyton L, Kovacs JA, Falloon J. CD4 counts as predictors of opportunistic pneumonias in human immunodeficiency virus (HIV) infection. *Ann Intern Med* 1989; 111(3):223-231.
- (13) Hanson DL, Chu SY, Farizo KM, Ward JW. Distribution of CD4+ T lymphocytes at diagnosis of acquired immunodeficiency syndrome-defining and other human immunodeficiency virus-related illnesses. The Adult and Adolescent Spectrum of HIV Disease Project Group. *Arch Intern Med* 1995; 155(14):1537-1542.
- (14) Landay A, Ohlsson-Wilhelm B, Giorgi JV. Application of flow cytometry to the study of HIV infection. *AIDS* 1990; 4(6):479-497.
- (15) Loken MR, Stall AM. Flow cytometry as an analytical and preparative tool in immunology. *J Immunol Methods* 1982; 50(3):R85-112.

- (16) Nicholson J, Landay AL. Use of Flow Cytometry to Enumerate Lymphocyte Populations in HIV Disease. AIDS Testing: A Comprehensive guide, 2 edition. Springer-Verlag; 1994. p 170-195.
- (17) Bergeron M, Faucher S, Minkus T, Lacroix F, Ding T, Phaneuf S, Somorjai R, Summers R, Mandy F. Impact of unified procedures as implemented in the Canadian Quality Assurance Program for T lymphocyte subset enumeration. Participating Flow Cytometry Laboratories of the Canadian Clinical Trials Network for HIV/AIDS Therapies. Cytometry 1998; 33(2):146-155.
- (18) CDC. Guidelines for the performance of CD4+ T-cell determinations in persons with human immunodeficiency virus infection. MMWR Morb Mortal Wkly Rep 1992; 41(RR-8):1-17.
- (19) Koepke JA, Landay AL. Precision and accuracy of absolute lymphocyte counts. Clin Immunol Immunopathol 1989; 52(1):19-27.
- (20) Barnett D, Granger V, Whitby L, Storie I, Reilly JT. Absolute CD4+ T-lymphocyte and CD34+ stem cell counts by single-platform flow cytometry: the way forward. Br J Haematol 1999; 106(4):1059-1062.
- (21) Stewart CC, Steinkamp JA. Quantitation of cell concentration using the flow cytometer. Cytometry 1982; 2(4):238-243.
- (22) Mandy FF, Bergeron M, Recktenwald D, Izaguirre CA. A simultaneous three-color T cell subsets analysis with single laser flow cytometers using T cell gating protocol. Comparison with conventional two-color immunophenotyping method. J Immunol Methods 1992; 156(2):151-162.
- (23) Nicholson JK, Hubbard M, Jones BM. Use of CD45 fluorescence and side-scatter characteristics for gating lymphocytes when using the whole blood lysis procedure and flow cytometry. Cytometry 1996; 26(1):16-21.
- (24) Nicholson JK, Jones BM, Hubbard M. CD4 T-lymphocyte determinations on whole blood specimens using a single-tube three-color assay. Cytometry 1993; 14(6):685-689.
- (25) Nicholson J, Kidd P, Mandy F, Livnat D, Kagan J. Three-color supplement to the NIAID DAIDS guideline for flow cytometric immunophenotyping. Cytometry 1996; 26(3):227-230.
- (26) Mercolino TJ, Connelly MC, Meyer EJ, Knight MD, Parker JW, Stelzer GT, DeChirico G. Immunologic differentiation of absolute lymphocyte count with an integrated flow cytometric system: a new concept for absolute T cell subset determinations. Cytometry 1995; 22(1):48-59.

- (27) Brando B, Barnett D, Janossy G, Mandy F, Autran B, Rothe G, Scarpati B, D'Avanzo G, D'hautcourt JL, Lenkei R, Schmitz G, Kunkl A, Chianese R, Papa S, Gratama JW. Cytofluorometric methods for assessing absolute numbers of cell subsets in blood. *Cytometry* 2000; 42(6):327-346.
- (28) Mandy F, Brando B. Enumeration of absolute cell counts using immunophenotypic techniques. In: Robinson JP, Darzynkiewicz Z, Dean PN, Dressler LG, Rabinovitch PS, Stewart CC, Tanke HJ, Wheelless LL, editors. *Current Protocols in Cytometry, Supplement 13* edition. New York: John Wiley & Sons, Inc.; 2000. p 6.8.1-6.8.26.
- (29) Nicholson JKA, Mandy FF. Immunophenotyping in HIV infection. In: Stewart C, Nicholson JKA, editors. *Immunophenotyping, 1st* edition. New York: Wiley-Liss; 2000. p 261-289.
- (30) Bergeron M, Nicholson JKA, Phaneuf S, Ding T, Soucy N, Badley A, Hawley-Foss NC, Mandy F. The selection of lymphocyte gating protocol has impact on the level of reliability of T-cell subsets in aging specimens. How to obtain robust values with 4-color flow cytometer. [IN PREPARATION] 2001.
- (31) Schmid I, Kunkl A, Nicholson JK. Biosafety considerations for flow cytometric analysis of human immunodeficiency virus-infected samples. *Cytometry* 1999; 38(5):195-200.
- (32) Office of Biosafety, Health Canada. *LABORATORY BIOSAFETY GUIDELINES*. 2nd edition., 1996.
- (33) CDC. Update: universal precautions for prevention of transmission of human immunodeficiency virus, hepatitis B virus, and other bloodborne pathogens in health-care settings. *MMWR Morb Mortal Wkly Rep* 1988; 37(24):377-378.
- (34) CDC. Agent summary statement for human immunodeficiency viruses (HIVs) including HTLV-III, LAV, HIV-1, and HIV-2. *MMWR Morb Mortal Wkly Rep* 1988; 37 Suppl 4:1-17.
- (35) CDC. Recommendations for prevention of HIV transmission in health-care settings. *MMWR Morb Mortal Wkly Rep* 1987; 36 Suppl 2:1S-18S.
- (36) CDC. Acquired immune deficiency syndrome (AIDS): precautions for clinical and laboratory staffs. *MMWR Morb Mortal Wkly Rep* 1982; 31(43):577-580.
- (37) CDC. Acquired immunodeficiency syndrome (AIDS): precautions for health-care workers and allied professionals. *MMWR Morb Mortal Wkly Rep* 1983; 32(34):450-451.

- (38) CDC. Recommendations for preventing transmission of infection with human T-lymphotropic virus type III/lymphadenopathy-associated virus in the workplace. *MMWR Morb Mortal Wkly Rep* 1985; 34(45):681-685.
- (39) Westgard JO, de Vos DJ, Hunt MR, Quam EF, Carey RN, Garber CC. Concepts and practices in the evaluation of clinical chemistry methods. I. Background and approach. *Am J Med Technol* 1978; 44(4):290-300.
- (40) McCoy JP, Jr., Carey JL, Krause JR. Quality control in flow cytometry for diagnostic pathology. I. Cell surface phenotyping and general laboratory procedures. *Am J Clin Pathol* 1990; 93(4 Suppl 1):S27-37.
- (41) Ekong T, Kupek E, Hill A, Clark C, Davies A, Pinching A. Technical influences on immunophenotyping by flow cytometry. The effect of time and temperature of storage on the viability of lymphocyte subsets [published erratum appears in *J Immunol Methods* 1993 Dec 3;166(2):301]. *J Immunol Methods* 1993; 164(2):263-273.
- (42) Shield CF, Marlett P, Smith A, Gunter L, Goldstein G. Stability of human lymphocyte differentiation antigens when stored at room temperature. *J Immunol Methods* 1983; 62(3):347-352.
- (43) Paxton H, Bendele T. Effect of time, temperature, and anticoagulant on flow cytometry and hematological values. *Ann N Y Acad Sci* 1993; 677:440-443.
- (44) International Air Transport Association (IATA). *Dangerous Goods Regulations*. 41th edition. IATA, 2000.
- (45) Cory JM, Rapp F, Ohlsson-Wilhelm BM. Effects of cellular fixatives on human immunodeficiency virus production. *Cytometry* 1990; 11(5):647-651.
- (46) Aloisio CH, Nicholson JK. Recovery of infectious human immunodeficiency virus from cells treated with 1% paraformaldehyde. *J Immunol Methods* 1990; 128(2):281-285.
- (47) Lifson JD, Sasaki DT, Engleman EG. Utility of formaldehyde fixation for flow cytometry and inactivation of the AIDS associated retrovirus. *J Immunol Methods* 1986; 86(1):143-149.
- (48) Martin LS, Loskoski SL, McDougal JS. Inactivation of human T-lymphotropic virus type III/lymphadenopathy-associated virus by formaldehyde-based reagents. *Appl Environ Microbiol* 1987; 53(4):708-709.
- (49) Nicholson JK, Browning SW, Orloff SL, McDougal JS. Inactivation of HIV-infected H9 cells in whole blood preparations by lysing/fixing reagents used in flow cytometry. *J Immunol Methods* 1993; 160(2):215-218.

- (50) Schmid I, Nicholson JK, Giorgi JV, Janossy G, Kunkl A, Lopez PA, Perfetto S, Seamer LC, Dean PN. Biosafety guidelines for sorting of unfixed cells. *Cytometry* 1997; 28(2):99-117.
- (51) Nicholson JK, Green TA. Selection of anticoagulants for lymphocyte immunophenotyping. Effect of specimen age on results. *J Immunol Methods* 1993; 165(1):31-35.
- (52) Schwartz A, Marti GE, Poon R, Gratama JW, Fernandez-Repollet E. Standardizing flow cytometry: a classification system of fluorescence standards used for flow cytometry. *Cytometry* 1998; 33(2):106-114.
- (53) Schwartz A, Fernandez-Repollet E. Development of clinical standards for flow cytometry. *Ann N Y Acad Sci* 1993; 677:28-39.
- (54) Schwartz A, Fernandez-Repollet E. Standardization for flow cytometry. *Methods Cell Biol* 1994; 42 Pt B:605-626.
- (55) Roederer M. Compensation in Flow Cytometry. In: Robinson JP, Darzynkiewicz Z, Dean PN, Orfao A, Rabinovitch PS, Stewart CC, Tanke HJ, Wheelless LL, editors. *Current Protocol in Cytometry*, New York: John Wiley & Sons, Inc.; 2000. p 1-19.
- (56) Ekong T, Gompels M, Clark C, Parkin J, Pinching A. Double-staining artefact observed in certain individuals during dual-colour immunophenotyping of lymphocytes by flow cytometry. *Cytometry* 1993; 14(6):679-684.
- (57) Nicholson JK, Rao PE, Calvelli T, Stetler-Stevenson M, Browning SW, Yeung L, Marti GE. Artifactual staining of monoclonal antibodies in two-color combinations is due to an immunoglobulin in the serum and plasma. *Cytometry* 1994; 18(3):140-146.
- (58) Schnizlein-Bick CT, Spritzler J, Wilkening CL, Nicholson JK, O'Gorman MR. Evaluation of TruCount absolute-count tubes for determining CD4 and CD8 cell numbers in human immunodeficiency virus-positive adults. Site Investigators and The NIAID DAIDS New Technologies Evaluation Group. *Clin Diagn Lab Immunol* 2000; 7(3):336-343.
- (59) Reimann KA, O'Gorman MR, Spritzler J, Wilkening CL, Sabath DE, Helm K, Campbell DE. Multisite comparison of CD4 and CD8 T-lymphocyte counting by single- versus multiple-platform methodologies: evaluation of Beckman Coulter flow-count fluorospheres and the tetraONE system. The NIAID DAIDS New Technologie Evaluation Group. *Clin Diagn Lab Immunol* 2000; 7(3):344-351.
- (60) Stewart CC, Stewart SJ. Cell preparation for the identification of leukocytes. *Methods in Cell Biology* vol. 41, New York: Academic Press, Inc.; 1994. p 39-60.

- (61) Schenker EL, Hultin LE, Bauer KD, Ferbas J, Margolick JB, Giorgi JV. Evaluation of a dual-color flow cytometry immunophenotyping panel in a multicenter quality assurance program. *Cytometry* 1993; 14(3):307-317.
- (62) Loken MR, Brosnan JM, Bach BA, Ault KA. Establishing optimal lymphocyte gates for immunophenotyping by flow cytometry. *Cytometry* 1990; 11(4):453-459.
- (63) CDC. 1997 Revised guidelines for performing CD4+ T-cell determinations in persons infected with human immunodeficiency virus (HIV). 46(No. RR-2), 1997. Atlanta GA.
- (64) Gelman R, Wilkening C. Analyses of quality assessment studies using CD45 for gating lymphocytes for CD3(+)4(+)% . *Cytometry* 2000; 42(1):1-4.
- (65) Schwartz A, Fernandez RE, Vogt R, Gratama JW. Standardizing flow cytometry: construction of a standardized fluorescence calibration plot using matching spectral calibrators. *Cytometry* 1996; 26(1):22-31.
- (66) Henderson LO, Marti GE, Gaigalas A, Hannon WH, Vogt RFJ. Terminology and nomenclature for standardization in quantitative fluorescence cytometry. *Cytometry* 1998; 33(2):97-105.
- (67) Nicholson JK, Hubbard M, Dawson CD. Evaluation of stabilized whole blood control materials for lymphocyte immunophenotyping. *Cytometry* 1999; 38(6):268-273.
- (68) De Paoli P, Gennari D, Martelli P, Basaglia G, Crovatto M, Battistin S, Santini G. A subset of gamma delta lymphocytes is increased during HIV-1 infection. *Clin Exp Immunol* 1991; 83(2):187-191.
- (69) Margolick JB, Scott ER, Odaka N, Saah AJ. Flow cytometric analysis of gamma delta T cells and natural killer cells in HIV-1 infection. *Clin Immunol Immunopathol* 1991; 58(1):126-138.
- (70) Curtis R.H. Performance verification of manual action pipets, Part I. *American Clinical Laboratory* 12[7], 8-9. 1994.
- (71) Curtis R.H. Performance verification of manual action pipets, Part II. *American Clinical Laboratory* 12[9]. 1994.