CONTROLLING LABORATORY VARIABLES TO IMPROVE PRECISION AND ACCURACY OF CD4+ T-CELL ENUMERATION ACROSS FLOW CYTOMETRY METHODS.

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DECLARATION

I, Mandy Wilja Mirembe declare that this dissertation is my own work. It is being submitted for the degree of Master of Science in Molecular Medicine and Haematology, in the Faculty of Health Sciences of the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.

Signature of candidate

October 19, 2009.

DEDICATION

To my father from whom all that is beautiful and excellent in my life is a result of.

ABSTRACT

This study assessed the effect that certain logistical and methodological factors in the laboratory could have on influencing precision and accuracy of enumeration of CD4+ cells. The efficacy of a new blood stabiliser to extend the window of CD4 testing, was also evaluated.

CD4+ counts were derived using the 2-colour Pan-*leuco*gating, 4-colour TetraONE and MultiTEST/TruCount protocols on the EPICS-XL, FC-500 or FACSCalibur flow cytometers. Statistical analyses included the paired-t-test, Spearman's correlation and Bland Altman comparisons.

The results showed that the reliability of CD4+ count results was heavily dependent on how blood samples were handled prior to and after receipt into the laboratory and on how samples were processed and analysed. The factors, motion, operator pipetting and analysis skills, storage temperature, use of different protocols, different gating strategies and the use of different flow cytometers, were found to influence accurate and precise enumeration of CD4+ counts.

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AFREQAS	African Regional External Quality Assessment Scheme for CD4+ Lymphocyte Enumeration
APC	Allophycocyanin
BC	Beckman Coulter
BDB	Becton Dickinson Biosciences
CAP	College of American Pathologists
DP	Dual Platform
ECD	Energy coupled dye viz. Phycoerythrin-Texas Red–x
FITC	Fluorescein Isothiocyanate
FP	Forward Pipetting
FSC	Forward Scatter
FT	Refrigerator Temperature
K_3EDTA	Tripotassium Ethylene Diamine Tetra-Acetic Acid
LOA	Limits of Agreement
MS/TC	MultiTEST /TruCount protocol
No	Number
PC5	Phycoerythrin-Cyanin 5
PE	Phycoerythrin
PerCP	Peridinin Chlorophyll Protein
QASI	Quality Assessment and Standardisation for Immunological Measures Relevant to HIV/AIDS
RD1	Phycoerythrin
RP	Reverse Pipetting
RT	Room Temperature
R^2	Correlation Coefficient for Regression Analysis
SANAS	South African National Accreditation System
SP	Single Platform
SSC	Side Scatter
STDEV	Standard Deviation
System	Manufacturer CD4 Testing Method (including the Manufacturer Sample Preparation Reagents i.e.Antibody and Red Cell Lysing Agents, and the Manufacturer Flow Cytometer)
UK NEQAS	United Kingdom National External Quality Assessment Scheme
WBC	White Blood Cell Count
%CV	Percentage Coefficient of Variation
95% CI	95% Confidence Interval

1.0 INTRODUCTION

1.1 Background

Twenty eight years after the first reported death from Acquired Immune Deficiency Syndrome (AIDS), the epidemic continues to be the leading cause of death globally. It is estimated that 33 million people were living with the Human Immunodeficiency Virus (HIV-1) and 2 million deaths occurred worldwide from infection with HIV in 2007(1). Approximately 5700 deaths and 6800 new infections were estimated to have occurred daily in 2007(2). The deaths and incident cases seen over the years were mainly attributed to a lack of access to HIV prevention and treatment services(2).

Concerted efforts to address these inadequacies at both national government level and globally, has resulted in a decline in the effects of the HIV epidemics worldwide. In 2007 alone, a 16% decline was seen in the number of people living with HIV than were published in 2006(2). Seventy percent (70%) of this reduction in number was attributed to changes in estimates in six countries, five of which were in Sub-Saharan Africa(2) the region with the largest (67%) adult population globally infected and living with the virus(1).

The drop in number of deaths and incident HIV infections has also been attributed to uptake of HIV prevention strategies and to increasing access to treatment and care programmes(1, 2). The number of people receiving antiretroviral treatment (ART) globally increased after the WHO/UNAIDS "3 by 5" initiative in 2003. In Africa, the increase was by 710,000 persons from 100,000 persons between the end of 2003 and 2005(3). Expanded access to ART inevitably increased the need for CD4+ counts to guide the initiation and monitoring of ART. With the G8 group of nations commitment to universal access to ART

by 2010, an even further need and availability of CD4+ monitoring services will be required(4).

1.1.1 The Role and Importance of CD4+ Counts

CD4+ T lymphocytes play a central role in the pathogenesis of HIV, and measuring them is one of the most important immunological parameters used in the staging and management of HIV infected patients(5-7). The number of CD4+ cells measured as cells/µl correlate with the clinical staging of HIV infection, progression to AIDS and mortality(8-11). CD4+ counts are of prognostic value as predictors of progression to AIDS(12), risk to development of certain opportunistic infections such as *Pneumocystis carinii* pneumonia, for guiding decisions on commencement and administration of ART(13, 14), prophylaxis of opportunistic infections(15-18) and as a surrogate marker for drug efficacy in clinical trials(19). CD4+ cell counts have also been shown to be essential in estimating the impact of HIV and ART administration on the epidemiological progression of tuberculosis and malaria in sub-Saharan Africa(20, 21). Accurate and reliable enumeration of CD4+ cell counts is therefore crucial if systematic evaluation of new therapeutic modalities and adequate monitoring of treatment to HIV infected persons is to be provided(3, 22).

For effective management and monitoring of ART treatment, the World Health Organization (WHO) and United States Public Health Service recommended that HIV patient CD4+ cell counts be monitored every 3 - 6 months(23, 24).

1.1.2 Overview of CD4+ Count Methods

There are two main categories of CD4+ count methods, the manual non-flow cytometric methods and non-manual flow cytometric methods.

Manual methods have a role in certain settings especially where small numbers (< 20) of samples are tested. Their use on a wider scale however, is impeded by a number of factors.

Manual methods are generally more error prone, less accurate and have poorer repeatability because their cell counts are based on smaller numbers counted, under a light microscope in a counting chamber (about 250 cells per analysis compared to 5000 – 10,000 with flow cytometry) (25-31). Many of the manual methods have a poor ability to accurately enumerate CD4 T-cells because monocyte cells which also express the CD4 cell marker, and other sources of CD4 protein, tend to be counted along with the CD4 T- cells resulting in the generation of higher CD4+ cell counts than flow cytometry methods(26, 27, 32). Many of the manual methods are also not able to provide percentage values critical in the management of paediatric patients, they generally lack effective external quality assurance assessment, are labour intensive(28, 33), time consuming, and subjective.

The increase in treatment availability worldwide increased the need for faster, easier and more reliable methods for CD4 monitoring(34, 35). Flow methods, and in particular, the single platform technologies (SP), meet these criteria and would therefore be the most suited for the need ahead. SP methods have been shown to have better precision than the dual platform technologies (DP) both, within-(36) and between-laboratories(37, 38). They have also been shown to have better precision than the manual methods(31).

1.2 Problem Statement

Much technological advancement has been made over the years to improve the quality of CD4+ cell enumeration using flow cytometry(35). However, accurate enumeration of CD4+ counts remains a challenge due to the wide variation of CD4 methods used across laboratories, invariably leading to bias differences noted between laboratories.

Many documented CD4+ flow techniques continue to be performed with poor precision (measured as the coefficient of variation [CV%]), with variability noted both within- and between-laboratories(30, 36-46). The main concern about variation of CD4+ counts is the

impact that the variation could have on influencing decisions related to treatment and management of HIV infected persons.

Variation of CD4+ counts is typically reported to occur in both normal and HIV infected persons(47, 48) and has been attributed to two main reasons; biological factors(49) and methodological factors(42). It has been suggested that diurnal variation, a factor reported to account for nearly 50% of the measured variance of CD4+ counts(50), could be minimised by drawing blood samples at a specific time of the day(49, 51). Other cohort studies in patients with early or advanced HIV infection suggest that diurnal variability may be significantly blunted with disease progression and could therefore be negligible(49). While biological factors and specifically diurnal variation play some role, most documented variation of CD4+ counts has however been described in relation to technical differences e.g. differences in sample handling procedures(38) or differences in methods and instruments used(42). The major concern therefore, is the variance of CD4+ counts resulting from the technical differences within and between laboratories.

Counts in the clinically relevant range (i.e around the 200cells/µl level) are more problematic. Variability of CD4+ counts and difficulties in attaining precision have been reported(6, 39, 40, 50), testing samples that have low CD4+ counts because these tended to show even higher levels of within-subject variation(6, 47). Studies that assessed within-laboratory variation reported higher variability (median CV% >10%) for samples with lower CD4+ counts (≤ 200cells/µl)(36, 43, 44). Much greater variability (CV% as high as 61.4%) mostly because of the variety of CD4+ count techniques used, has however been reported from external quality assessment (EQA) studies (trimmed data) and other studies, that looked at between-laboratory variation(36-38, 41, 43, 46, 52).

A study that investigated whether use of a standard protocol (in this instance the use of $CD45^{bright}$ gating versus other variations of lymphocyte gating) would reduce inter-laboratory variation, showed that about 77% of the variation in results of percentage

lymphocyte subsets could be attributed to laboratory, sample, background fluorescence, type of flow cytometer and flow cytometer setup(53).

Difference (bias) in CD4+ counts between methods caused by inherent differences between instrument systems is not unusual and has also been reported in a number of studies(25, 33, 40, 54-59) as shown in tables 1.1 and 1.2. Here, a range of differences described as "acceptable" between methods, some wide enough to alter decisions related to treatment and management of HIV patients, have been reported.

Table 1. 1: Bias (Mean Difference) between Flow Cytometry CD4+ Count Methods

Study/Methods compared	N	Bias	LOA	95% CI	MPD(%)	Median (ratio %)	10th %ile	90th %ile	R^2
Strauss et al, 1996(45)									
DP flow vs FACSCount	118	N/R	N/R	N/R	N/R	N/R	N/R	N/R	0.986
Lopez et al, 1999(60)									
In Salamanca: DP flow vs FACSCount	24	N/R	N/R	N/R	N/R	N/R	N/R	N/R	0.979
In Barcelona: DP flow vs FACSCount	25	N/R	N/R	N/R	N/R	N/R	N/R	N/R	0.939
Glencross et al, 2002(59)									
U.K. NEQAS evaluation									
Overall pool mean vs DP PLG	20	-10	110 to -129	(18, -38)	0 ± 9%	N/R	N/R	N/R	0.927
SP bead pool mean vs DP PLG	20	-21	85 to -127	(4, -46)	2 ± 7%	N/R	N/R	N/R	0.936
SP volumetric pool mean vs DP PLG	20	-2.5	143 to -148	(31, -36)	1 ± 7%	N/R	N/R	N/R	0.873
DP pool mean vs DP PLG	20	-14.5	109 to -138	(14, -43)	1 ± 9%	N/R	N/R	N/R	0.918
TransFix evaluation						N/R	N/R	N/R	
SP volumetric vs SP beads	112	15.4	187 to -157	(31.6, -0.7)	4 ± 31%	N/R	N/R	N/R	0.923
SP volumetric vs DP PLG	112	16.8	82 to -49	(23, -10.7)	6 ± 17%	N/R	N/R	N/R	0.990
SP volumetric vs DP Lymphocyte gated	112	46.2	368 to -275	(76.3, -16)	14 ± 51%	N/R	N/R	N/R	0.758
Pattanapanyasat et al, 2005(54)									
SP volumetric CyFlow vs SP TruCount	200	-69.1	87.5 to -225.7	N/R	N/R	N/R	N/R	N/R	0.960
SP volumetric CyFlow vs SP FACSCount	200	-40	85.1 to -165.1	N/R	N/R	N/R	N/R	N/R	0.970
Pattanapanyasat et al, 2005(55)									
3-colour DP vs DP PLG	611	18	-97.4 to 133.1	N/R	N/R	N/R	N/R	N/R	0.950
Dieye et al, 2005(33)									
SP volumetric CyFlow vs SP TruCount	121	-4	-121 to -114	(-14, -7)	N/R	N/R	N/R	N/R	0.970
SP volumetric CyFlow vs FACSCount	121	-4	-161 to -153	(-18, -10)	N/R	N/R	N/R	N/R	0.970
SP TruCOUNT vs FACSCount	121	-12	109 to -134	(-23, -1)	N/R	N/R	N/R	N/R	0.980
Pattanapanyasat et al, 2006(56)									
3-colour SP vs SP PLG	67	15.1	-26.2 to 56.4	N/R	N/R	N/R	N/R	N/R	0.995
Denny et al, 2008(40)									
Shipped samples									
SP/DP vs DP PLG	495	45	N/R	N/R	N/R	31 (0.89)	-26 (0.68)	134 (1.11)	N/R
Local samples									
SP/DP vs DP PLG	58		N/R	N/R	N/R	23 (0.89)	-23 (0.73)	112 (1.10)	N/R

Abbreviations: N/R **– data not reported.**

Table 1. 2: Bias between Different CD4+ Count Methods

Study/Methods compared	N	Bias	LOA	95% CI	Median	10th %ile	90th %ile	R^2
Landay et al, 1993(29)								
DP flow vs Cytosphere	382	N/R	N/R	N/R	N/R	N/R	N/R	0.832
Carella et al, 1995(25)								
DP flow vs Cytosphere	117	N/R	N/R	N/R	N/R	N/R	N/R	0.930
Denny et al, 1995(58)								
DP flow vs Zymmune CD4/CD8	166	N/R	N/R	N/R	N/R	N/R	N/R	0.940
Lyamuya et al, 1996(27)								
DP flow vs FACSCount	173	N/R	N/R	N/R	N/R	N/R	N/R	0.891
DP flow vs TRAx ELISA	189	N/R	N/R	N/R	N/R	N/R	N/R	0.398
DP flow vs Dynabeads	189	N/R	N/R	N/R	N/R	N/R	N/R	0.882
Schnizlein-Bick et al, 2000(43)								
3-colour SP TruCount vs 2-colour DP								
For Shipped samples	411	N/R	N/R	N/R	7	-67	79	N/R
On-site samples:								
6 hours old	560	N/R	N/R	N/R	10	-29	72	N/R
24 hours old	560	N/R	N/R	N/R	2	-62	65	N/R
Didier et al, 2001(30)								
3-colour SP TruCount vs FACSCount	45	N/R	N/R	N/R	N/R	N/R	N/R	0.971
3-colour SP TruCount vs Cytosphere	55	N/R	N/R	N/R	N/R	N/R	N/R	0.453
3-colour SP TruCount vs Dynabeads	46	N/R	N/R	N/R	N/R	N/R	N/R	0.913
3-colour SP TruCount vs OptiCim	27	N/R	N/R	N/R	N/R	N/R	N/R	0.717
3-colour SP TruCount vs Capcellia	49	N/R	N/R	N/R	N/R	N/R	N/R	0.423
Diabouga et al, 2003(31)								
3-colour SP TruCount vs Dynabeads	657	N/R	N/R	(-22, -8)	-16	N/R	N/R	0.890
Spacek et al, 2006(57)								
In Uganda: FACSCount vs EasyCD4	141	-4.6	187.6 to -196.8	N/R	N/R	N/R	N/R	0.920
In United States : DP vs EasyCD4	77	9	163.3 to -145.3	N/R	N/R	N/R	N/R	0.970
Karcher et al, 2006(26)								
DP flow vs Cytosphere	131	-1.4	369 to -372	(34.1, 31.3)	N/R	N/R	N/R	0.526
DP flow vs SP volumetric CyFlow	128	92	402 to -217	(65.1, 120.4)	N/R	N/R	N/R	0.863

Abbreviations: N/R **– data not reported**.

The need to minimise variables that cause variation of CD4+ counts is therefore especially critical where HIV patient CD4+ counts are to be determined using different instruments, both within- and/or between-laboratories, across treatment networks or where CD4+ counts are used to determine endpoints across study-groups. This is particularly important because variation of the CD4+ counts could make longitudinal comparisons of the counts unreliable and could obscure their prognostic value. In this context, Sax P.E. et al, 1995(61), showed

that inter-laboratory variability in CD4+ cell counts could influence patient management or diagnosis of AIDS. In this study, laboratories reported different CD4+ cell counts for same patient samples and the variation in the counts was wide enough to cause conflicting diagnostic and therapeutic patient classification or qualification for either *Mycobacterium avium* complex (MAC) or *Pneumocystis carinii* pneumonia (PCP) prophylaxis.

Variation between patient counts and variation of counts noted between laboratories needs urgent address. There is a need therefore to establish more standardised testing protocols and systems both within- and between-laboratories, to ensure generation of reliable CD4+ counts. Some guidelines are available for performing absolute CD4+ counts using flow cytometry(62-64). A first step towards addressing variability of CD4+ counts would be to ensure that these guidelines are uniformly applied by laboratories, at least within groups of laboratories servicing the same population of patients.

This study assessed how certain logistical and methodological factors influence precision and accuracy of CD4+ cell enumeration in a laboratory. The study was carried in South Africa and Uganda using three widely used single platform (bead) protocols: (i) the 2-colour PanLeucogate (2-colour FLOWCARE^{TM} PLG-CD4, BCI, Miami, FL)(59); and two 4-colour systems, (ii) the TetraONE (BCI, Miami, FL)(36) and (iii) the MultiTEST/TruCount (MS/TC, BDB, San Jose, CA)(43).

1.3 Hypothesis and Objectives

The study hypothesizes that bias between two individual single platform flow techniques, used as described by the manufacturer and performed with absolute precision, will be zero or at least, consistent, if samples are handled and stored similarly. The objectives of this research were therefore:

i) To assess the impact of day-to-day sample handling and storage procedures such as temperature and motion, on the accuracy and precision of CD4+ cell counting.

ii) To establish the possible 'bias' introduced with use of various methodologies and systems

iii) To assess the impact some aspects of sample preparation specificially, pipetting methodology, have on the accuracy and precision of CD4+ cell counting

iv) To assess the impact of delaying sample testing and to determine how long the testing period can be extended (time from collection to testing) to without undermining the quality (accuracy and precision) of the CD4+ results.

2.0 LITERATURE REVIEW

2.1 CD4 Based Classification for HIV Infection and ART Initiation

A CD4+ cell count of ≤ 200 cells/µl or CD4+ lymphocyte percentage of <14% is used by the U.S. Centers for Disease Control and Prevention (CDC) to define "AIDS" in HIV infected adults and adolescents(5). In HIV positive children, AIDS is defined as having an absolute CD4+ cell count of < 750 cells/µl for < 1 year olds; < 500 cells/µl for (1-5) year olds; < 200 cells/µl for (6-12) year olds; or a CD4+ T cell percentage of < 15% for all children irrespective of age(65).

The World Health Organization (WHO) recommends ART for all persons in WHO clinical stage IV, regardless of CD4+ cell count; for all adults with CD4+ cell counts of < 200 cells/µl, regardless of clinical stage, and for persons in stage III with CD4+ cell counts < 350 cells/µl(3). For children, WHO treatment guidelines recommend that ART is initiated at a CD4+ cell count of < 200 cells/µl, regardless of clinical stage(3), at a CD4+ cell count of < 1500 cells/µl for ≤ 11 months old; at < 750 cells/µl for 12 – 35 months old; and at < 350 cells/µl for 36 – 59 months old(66).

2.2 Trends in Use of CD4+ Tests

The number of CD4+ cell count tests performed annually in South Africa increased with the introduction of ART programmes and continues to grow with increased access to treatment(67). Subsequent to the implementation of the National ART Programme in South Africa, a more than double increase was noted with testing, increasing from 303,351 tests in 2004/5 to 704,131 tests in 2005/6(68). An even greater need for CD4 laboratory services was seen in 2007(39, 69) during which the number of CD4+ cell count tests performed in 2006/7 (1,566,276 tests), was more than twice that in previous years; (704,131) in 2005/6. These figures were based on the reported number of tests done by approximately 35 newly

appointed National Health Laboratory Service (NHLS) CD4 laboratories performing PanLeucogated CD4 lymphocyte testing. In 2007/8, these figures increased to 1.96 million CD4 tests performed across more than 56 SA-NHLS laboratories(70). Annual figures of CD4+ cell count tests done by the CDC laboratory in Uganda were 30,287, 22,235, 21,711 and 19,159 in 2005, 2006, 2007 and 2008 respectively (personal communication – Kusemererwa A., CDC-Entebbe, Uganda). These data clearly indicate that the demand and utilisation of CD4+ cell counts in sub-Saharan Africa is high and is on the increase.

2.3 CD4+ Enumeration Techniques

CD4+ count techniques can be divided into two main categories, the manual (non-flow cytometric) methods and flow cytometric methods.

Manual (Non-Flow Cytometric) technologies include:

i) **Microscopy bead assays** such as the Cytospheres assay (Coulter Corporation, Florida, USA) in which antibody coated with latex spheres binds CD4-expressing cells to form CD4 cell-sphere rosettes that are then identified and counted in a counting chamber using a light microscope(25, 26, 29). The magnetic bead Dynabead method (Dynal, Compiégne, France)(27, 28) and the Immunoalkaline Phosphatase assay(71-73), both of which also require a counting chamber for cell counting.

ii) **Enzyme Linked Immunosorbent assays (ELISA)** such as the Total Receptor Assay (TRAx CD4) (T Cell Diagnostics, Inc. [TCD], Cambridge, Mass.)(74, 75), Zymmune fluorescence assay (Zynaxis, Inc., Malvern, Pa.)(58, 76) and Whole Blood Capcellia CD4/CD8 (Sanofi diagnostics Pasteur, Marnes la Coquette, France)(77), (all no longer commercially distributed).

iii) **The dried whole blood spot assay** (Filter paper ELISA)(proposed as a potential alternative method for CD4 count measurement)(78).

Flow cytometric methods include:

i) **Dual platform (DP) methods**: These methods use two different instruments, a flow cytometer and a haematology analyser to obtain two separate values used in the derivation of absolute CD4+ cell counts. The flow cytometer is used to obtain a CD4+ lymphocyte percentage of total lymphocytes in the conventional method(79), or in the case of the DP PanLeucogated CD4 counting (DP PLG), to obtain a CD4+ lymphocyte percentage of total blood leukocytes(59). In the conventional DP CD4 method, a haematology analyser (HA) is used to obtain a Total Lymphocyte Count (TLC) (derived from a total white cell count (WBC) and lymphocyte differential) which is then used as the reference population for calculating absolute CD4+ counts and the CD4% amongst total lymphocytes(63, 79). In DP PLG the WBC and not the TLC, is used as the reference population for calculating absolute CD4+ counts(59). Because DP PLG avoids use of TLC the main variable in conventional DP testing, it has been shown to improve within- and between-laboratory precision to a level similar to that of SP methods(40, 59).

ii) **Single platform (SP) flow cytometry methods**: These methods use one instrument (a flow cytometer) to directly provide absolute cell counts. They are of two kinds:

- Flow cytometers that support bead-based methods: These methods use a known volume and concentration of beads that are added to the sample during sample preparation and act as the reference population from which the cell concentration per unit of blood is calculated. These include: The FACSCount (BDB, San Jose, CA) a modified, portable flow cytometer that uses a green laser, two-colour monoclonal antibody reagent, calibrated reference beads and control beads(45); the SP PanLeucogate (PLG-CD4)(39, 59) and TetraONE T-Cell (Beckman Coulter, Miami, FL)(36, 46) methods that use beads suspended

in liquid; and the MultiTest/Trucount (BDB, San Jose, CA)(43, 80, 81) method which uses lyophilised beads. However, any bead enumeration products can be used with any antibody combination to derive SP CD4+ counts.

- Volumetric methods: These do not require reference beads to calculate absolute cell counts but may use beads as internal quality control tools to ensure reliable volumetric CD4 measurements. Volumetric methods are available as:

  i) Methods that use a built-in syringe system to deliver a unit volume of blood for analysis such as; the 2-colour Guava Easy CD4(57, 82, 83) and Guava PCA(84) microcapillary flow cytometry systems that require small sample volumes (10µl) and do not use sheath fluid; PointCare and PointCare Now (PointCare Technologies)(85, 86), which are fully automated systems that operate with collidal gold-labeled reagents, and combine flow cytometry and hematology analysis. These compact, portable, instruments detect scattered light at four different angles all at once and use the light and not fluorescence signals, to identify and emunerate cells; Cytoron-Absolute (Ortho Diagnostics) although it is obsolete (87, 88); hybrid multitasking flow cytometers such as the Luminex 100 (Luminex Corporation, Austin, Tx.)(89, 90) and the FACSArray (BDB, San Jose, CA).

  ii) Methods that use an electrode volumetric option such as; the Cyflow SL blue multi-parameter cytometer equipped with a 488 nm blue laser (33) and the single or dual-parameter CyFLOW^{green} cytometer equipped with a 532 nm green laser, from Partec GmbH, Münster, Germany(54, 91). Cell counts on these cytometers are based on using a fixed volume of sample. This process is achieved by using two electrodes that automatically detect the sample fluid level and initiate and stop the sample acquisition process.

iii) Methods that use the dual syringe and electrode volumetric options e.g. the single-parameter Partec CyFlow Counter that is equipped with a 532 nm green laser (Partec GmbH, Münster, Germany)(28, 92, 93).

2.4 Comparison of Flow Methods

Several studies(40, 45, 55, 59, 60) have looked at the performance and reliability of CD4 testing using the two types of flow methods (DP and SP) mentioned in section 2.3. Studies that specifically compared conventional DP and SP flow methods showed the conventional DP methods to have much higher variability(36, 37, 94). The high variability and bias seen with these conventional DP methods has been attributed to the use of different machines (flow cytometers and haematology analysers) and to the use of the total lymphocyte count (TLC) to derive absolute CD4+ counts(95, 96). Haematology analysers have several other disadvantages. In particular, they have a narrow window of testing (6 hours) within which reliable white blood cell differentials can be generated(95). The instruments also generally lack internal quality control and external quality assessment for the WCC differentials(87, 95), and are calibrated using blood cell controls that have wide ranges(50). These factors coupled with the fact that the instruments differ in operation i.e. how they differentiate and measure lymphocytes(96) with some using just the cell volume (size) as opposed to a combination of both volume and light scatter features (the Volume Conductivity and Scatter/Absorbance Cytochemistry and Volume principle), may compromise the precision of derived absolute lymphocyte counts.

Because SP technologies provided more reliable results and overcome the limitations of the conventional DP systems, they have become the and gold standard for CD4+ cell enumeration in several laboratories(45, 46, 87, 94, 97).

Given the rate ART implementation programmes are envisaged to expand, the availability and timely delivery of CD4 monitoring services to support the expansion of these

programmes, will be necessary. For this to be accomplished, quality CD4+ count methodologies with a capacity for high workloads and fast turn-around time will be required. SP flow cytometry methods have demonstrated these capabilities and appear to be the most suitable platform for effective implementation of ART programmes(39).

2.5 Factors that cause Variation in CD4+Cell Counts

Variability of CD4+ cell counts can be caused by biological factors and methodological factors.

The biological factors include:

i) **Age:** Absolute WBC counts tend to decrease with age. Higher CD4+ counts are seen in children than in adults(98-100), in younger persons than in the elderly (>50 years)(101), and in neonates and infants (≤12 months), who have higher counts than older children(99, 102).

ii) **Gender and Hormonal effects:** Females have been shown to have higher CD4+ cell counts than males(48, 98, 103, 104). Among ovulating females, pre-ovulation and menstruation have been associated with higher CD4+ percents than post-ovulation(103). Estrogen has been suggested as a possible explanation for the difference in CD4+ counts seen between the genders(103, 105).

iii) **Diurnal and Circannual variations:** Diurnal and circannual variation of CD4+ cell counts in HIV positive and HIV negative populations has been documented in several studies(47, 49, 51, 106, 107). The lowest CD4+ counts (nadir) and highest counts in healthy HIV-negative adults with normal sleep/wake cycles, are reported to be seen in the morning hours (0800-1100 hours) and evenings (2000-2200 hrs), respectively(49, 51, 108). Studies have also shown lymphocyte cell counts to vary with the seasons of the year (winter, spring, summer and autumn)(48, 108, 109). Possible explanations for the contrast in counts have included, trafficking and compartmentalisation of the

lymphocytic cells in and out of blood and other body organs (bone marrow, lymph nodes, spleen)(106), or as a result of diurnal and circannual patterns of cortisol, exposure to light, or emotions(51).

iv) **Recent/Concomitant Infections:** Viral infections such as cytomegalovirus infection, bacterial infections such as tuberculosis(105), sexually transmitted diseases(110), parasitic (helminthic) infections, and other disease conditions like malnutrition, marasmus(111), Hepatitis B, P. *carinii* pneumonia, iron deficiency(112), autoimmune thyroiditis(113), bronchial asthma(114), have been associated with causing alterations in lymphocyte cell homeostasis.

v) **Splenectomy:** Is shown to cause elevation of CD4+ cell counts probably as a result of lymphocytosis(115, 116).

vi) **Physical Stress:** Has been suggested to cause an increase in lymphocyte subsets(117).

vii) **Exercise and Rest:** While acute strenuous exercise has been associated with lymphocytosis followed by relative lymphopenia in the immediate recovery period, brief periods of rest (30 and 60-minutes) from normal activity have been associated with decline in CD4+ cell counts(118).

viii) **Ethnic Origin and Geographical Location:** Variation in CD4+ counts is said to occur between regions and within and among populations(98, 119-122). Altitude, exposure to different pathogens and infections, genetic differences, environmental and nutritional factors, have been offered as plausible explanations for the variation in lymphocyte subsets within and among populations.

ix) **Smoking:** Was found to be associated with an increase in absolute CD4+ cell counts, leucocytosis, lymphocytosis and increase in CD4 percentages(103, 123).

x) **Drugs:** ART enhances T-cell reconstitution through restoration of thympoiesis(124, 125), however, other medicines e.g. cephalosporins, corticosteroids and glucocorticoids(126, 127) have been associated with a trend towards lower absolute

CD4+ cell counts. Alcohol use has also been associated with lowering of CD4+ cell counts(128).

Methodological influence on CD4+ counts is shown to arise from procedures related to how whole blood samples are handled and processed after draw, and from methods employed in analysis and reporting of results. Factors that influence the accuracy and precision of CD4+ lymphocytes counts include:

i) **Anticoagulant.** The type of anticoagulant used(129) and incorrect blood to anticoagulant ratio which can arise from incorrect filling of blood collection tubes or use of liquid anticoagulant (acid citrate dextrose or heparin), can affect accurate enumeration of absolute WBC and lymphocyte subset counts(64, 130). Improper mixing of blood with the anticoagulant could also lead to clotting and erroneous results if clotted specimens are analysed. Better reproducibility of cell counts was observed when adequate draws of blood were made i.e. 3-4ml of blood in 5ml EDTA vacutainer tubes than was when inadequate volumes were drawn i.e. draws of 1-2ml or 5ml of blood in 5ml collection tubes(130).

ii) **Temperature:** Varying degrees of sample deterioration can occur depending on how and at what temperature samples are stored and handled from the time of draw to when they are processed in the laboratory. It is generally recommended that whole blood samples are stored at room temperature (18 – 22ºC) as increases in temperature can result in large variation of CD4+ counts from baseline at room temperature(63, 64). Storage at 4ºC has also been suggested since no significant loss of lymphocyte cells occurred from storage of samples at refrigerator temperature(131). However, extreme temperatures hot or cold, could affect the accuracy of flow cytometry measurements. Temperatures ≥ 37ºC have been shown to cause cell death(131).

iii) **Interval from draw of blood to analysis (Sample Age):** Major changes in the light scatter patterns of leucocytes and the respective cell differential counts have been shown to occur when whole blood is kept for periods longer than 12-24 hours before analysis. These changes, combined with the inherent variability of different instruments, could result in considerable variation in reported absolute CD4+ counts(95, 96, 130).

iv) **Antibody Staining:** Different staining protocols have been shown to give different results(132, 133). When using commercial reagents, deviation from the manufacturer protocol in terms of the volume of antibody used, the time and temperature of incubation with antibody reagent and the blood volume, could cause changes of staining intensity. Fluorochrome associated variation was observed with higher measurements with phycoerythrin (PE) than with fluorescein isothiocyanate (FITC)(97, 134, 135). Change of reagent lots could also result in differences in T-cell subset counts.

v) **Nature of Lysing Reagents:** The acidic nature of erythrocyte (RBCs) lysing reagents reduces sample pH which could affect the integrity of cell membranes or alter flow cytometric light scatter patterns and surface staining characteristics of WBCs. Changes in sample pH may also cause dissociation of some antibody/antigen complexes and cause variation in cell counts(136-139).

vi) **Sample Preparation Methods (Cell Lyse and Fixation Procedures):** There are three techniques commonly used to prepare WBCs for flow cytometric analyses; the Lyse-and-Wash (LW) technique(140, 141); Lyse-No-Wash (LNW)(140) and the No-Lyse-No-Wash technique(NLNW)(33). The LW technique involves a RBC lyse step and subsequent wash and centrifugation steps to remove the lysed RBCs before a final WBC fix step. In the more commonly used LNW technique which excludes any cell wash steps, lysis of RBCs and fixation of the WBCs occur in a single step. With the

NLNW technique, whole blood is neither lysed nor washed. It has been reported that fixatives can change the light scattering profile and fluorescence intensity of cells(142) and that wash procedures could cause significant cell loss(139, 140). Vortexing and centrifugation procedures have also been reported to damage malignant and/or activated cells that are fragile or differ in buoyant density from normal cells(133, 143). Additionally, cell loss may occur because of adherence to containers or failure to form complete pellets after wash and centrifugation procedures. Lower variation and tighter CVs for absolute CD4+ cell counts were observed when LNW or NLNW techniques were used suggesting that greater cell loss occurred during wash procedures in the LW technique(46, 140). Variability of lymphocyte cell counts may also result from inadequate mixing of blood and the reagents (antibody, lyse/fix solution) or from the interference of light scatter patterns of unlysed nucleated erythroid cells with lymphocyte scatter patterns(38, 53).

vii) **Pipetting of Blood and/or Beads:** There are two types of beads commonly used in single platform (SP) flow methods; the liquid form such as FlowCount beads, or the preloaded lyophilised form such as TruCount beads. Accuracy of bead-based counts is dependent on the precision of the pipetting steps i.e. of the blood and/or beads. However, several other factors could also affect the pipetting steps and thus the accuracy of absolute bead-based counts. These factors include, inadequate resuspension of bead solutions after storage which can cause changes in the proportion of beads aspirated and the use of inappropriately calibrated/faulty pipettes(144). The latter is especially relevant for protocols that use preloaded lyophilised beads as these protocols have a single pipetting step i.e., that of blood. The single pipetting step increases the probability of introducing errors due to pipette variability, errors that would otherwise be minimised if the same pipette is used to pipette both blood and beads. Use of wrong

pipette tips, the pipetting technique employed e.g. reverse pipetting described in Appendix B, is the recommended technique(64, 144) for methods that use lyophilised bead products, and poor pipetting skill which results in incorrect dispensing of either blood or beads or both, could also affect the pipetting steps. Errors may also arise as a result of reduction of bead concentration in suspension (vanishing bead phenomenon) caused when protein-free or protein-poor samples are vortexed(145), or due to use of incorrect bead counting gating strategies i.e., CD4 techniques that use TruCount beads require the bead counting strategy to include all singlet and doublelet bead aggregates while techniques that use FlowCount beads require inclusive of only the singlet beads(144).

viii) **Instrument Characteristics:** Lack of uniformity in the method and instrument used for CD4 testing(134, 135), differences in instrument set-up (compensation settings, type and level of threshold [also called "discriminator"] settings)(46), differences in instrument sensitivity to different fluorochromes(134) as well as protocol setups including choice of 'live" gates and order in which they are used, could influence accurate enumeration of CD4+ T cells.

ix) **Data Analysis:** Choice of gating strategy and the quality of the lymphocyte gate (i.e. exclusion of non lymphocyte cells from the lymphocyte gate) can have a significant effect on results obtained(46, 97). Nonlymphoid cells such as monocytes, unlysed nucleated erythroid cells, basophils, immature cells and artifacts such as cell aggregates and platelet clusters could interfere with lymphocyte purity and not be easily identified or excluded if light scatter (forward scatter [FSC] and side scatter [SSC]) gating is used alone(144). Methods that employed CD45 and SSC-based gating of lymphocytes have been shown to have lower variation and greater lymphocyte purity than methods that used light scatter-based gating or CD45 and CD14 backgating strategies(46, 146).

2.6 Measures to Reduce Variation of CD4+ Counts

In addition to promoting the use of robust SP technologies, a number of other measures have been suggested to improve the quality of CD4+ counts. These include amongst others, provision of adequate training to laboratory personnel(53) and use of stringent internal quality controls or other quality control tools such as the bead count rate (BCR) to monitor the sample preparation process, specifically quality controlling the pipetting of beads and/or blood(39, 147). Participation in external quality control assessment schemes (EQA) has also been shown to have an impact on the performance of laboratories(37, 41, 52). A study by the Canadian International Programme for Quality Assessment and Standardisation for Immunological Measures relevant to HIV/AIDS (QASI) showed a decrease in inter-laboratory variability of CD4+ counts from 14.2% to 8.8%, with increasing participation(52). In the African context, use of simplified technologies such as FACSCount and PLG (both as DP or SP), has been shown to significantly improve on reproducibility and precision between laboratories(39). Other measures have included standardising of CD4+ protocols since this demonstrated more reliable inter-laboratory performance(39, 45, 46, 60, 87, 97, 148), use of universal templates(149, 150) and adherence of laboratories to good clinical laboratory practice(151).

3.0 METHODOLOGY

This study was carried out at the SANAS accredited NHLS Flow Cytometry Laboratory at the Johannesburg Hospital in South Africa and at the CDC laboratory in Entebbe-Uganda. The reason the study was done in two countries was to provide a means of evaluating the impact that different conditions could have on local CD4 testing reproducibility, including different population groups, different geographical and climatic settings and different laboratory logistics.

Both laboratories participated in external quality control assessment programmes for leucocyte immunophenotyping. The Johannesburg laboratory participated in UK-NEQAS, the African Regional External Quality Assessment Scheme for CD4+ Lymphocyte Enumeration (AFREQAS)(41), Quality Assessment and Standardisation for Immunological Measures Relevant to HIV/AIDS scheme (QASI), as well as in the Beckman Coulter Inter-laboratory Quality Assessment Programme (IQAP). The CDC laboratory participated in the UK-NEQAS, College of American Pathologists (CAP) and QASI schemes. It needs to be noted however that the performance of the operator (the writer) could not be accomodated in any of these external quality control assessment schemes and was not assessed on these programmes. Within-operator precision of the operator was however assessed.

3.1 Ethics Clearance

This study was approved by the ethical committee of the University of the Witwatersrand, Faculty of Health Sciences Committee for Research on Human Subjects (Reference Protocol Number: M070231) and the Science and Ethics Committee of the Uganda Virus Research Institute (Reference Number: GC/127/04/07).

3.2 Blood Samples

A total of 286 K_3EDTA (BDB) adult (> 18 years) blood samples were used in this study. One hundred and ninety seven (197) of the 286 samples were selected from samples received for routine CD4+ testing at the laboratories on the basis that they were < 24 hours old. The remaining 89 of the 286 were samples specifically drawn in (1ml) blood stabilisation vacutainer tubes (BDB) to evaluate the ability of this new commercial product to extend the window of CD4 testing.

One hundred and thirty-eight of the 197 samples were received and analysed at the Johannesburg laboratory and 59 were samples received at the CDC laboratory in Uganda.

Twenty (20) of the 89 samples drawn in 2 ml blood stabilisation vacutainer tubes were collected in Johannesburg and were first processed within 4 hours from the time of draw (the samples were tested over several consecutive days). The remaining 69 blood stabilisation samples were collected in Uganda and were processed within 48 hours from the time of draw. These 69 samples were however > 24 hours old at the time of receipt at the CDC laboratory.

3.3 Enumeration of CD4+ Cells

CD4+ cell count enumeration was performed using three known, standard, single platform, bead based, Lyse No-Wash methods: 4-colour MultiTEST/TruCount™ (MS/TC, BDB)(43); TetraONE™ with FlowCount (BCI, Miami, FL)(36); and the 2-colour PanLeucogate™ CD4 with FlowCount (2-colour PLG-CD4, BCI, Miami, FL)(39). The methods were performed in accordance to stipulated manufacturer instructions. All three methods were used for this study at the Johannesburg laboratory: 2-colour PanLeucogate was the predicate CD4+ cell count protocol at this laboratory.

4-colour MultiTEST/TruCount was the predicate CD4+ cell count method at the CDC laboratory and used for all studies performed at this laboratory.

3.3.1 Preparation of Samples

i) Procedure followed for the 4-colour TetraONE CD4 Method.

Test tubes (BCI, Miami, FL) were setup (labeled) to correspond to the laboratory number of samples. TetraCHROME^{TM} monoclonal antibody reagent (CD45FITC/CD4RD1/CD8ECD/CD3PC5) (BCI, Miami, FL) (5µl) was added to each tube, followed by the addition of (100µl) of well mixed whole blood. The tubes were gently vortexed (5 seconds), placed within a carousel and loaded on a Multi-Q-Prep^{TM} work station (BCI, Miami, FL) to incubate for 10 minutes before the automated lyse procedure described in section 3.3.1 (iii) was commenced. After the latter sample preparation step and immediately prior to analysis, FlowCOUNT^{TM} beads (BCI, Miami, FL) (100µl) were added to each sample using the same pipette used to dispense the blood. The tubes were each vortexed once, re-placed back into the carousel and loaded onto the flow cytometer for acquisition. Listmode data was saved for retrospective re-analysis of outliers, if required.

Listmode data was also used to evaluate the bias potentially introduced between different gating strategies. In other words, the TetraONE 4-colour listmode data was re-analysed using a 2-colour PLG strategy. Such re-analysis of data allowed for true assessment of impact of gating on bias between CD4+ counts. Here, all aspects of biological sample variation as well as variation introduced through sample preparation and technical instrument variation during flow cytometric analysis are effectively removed.

ii) Procedure followed for the PLG-CD4 Method

Test tubes (BCI, Miami, FL) were setup (labeled) to correspond to the laboratory number of samples. FlowCARE antibody reagent (CD45 FITC/CD4 PE) (BCI, Miami, FL) (10µl) was added to each tube, followed by the addition of (100µl) of well mixed whole blood. The tubes were gently vortexed (5 seconds), placed within a carousel and loaded on a

Multi-Q-Prep™ work station (BCI, Miami, FL) to incubate for 10 minutes before the automated lyse procedure described in section 3.3.1 (iii) was commenced. After the latter sample preparation step and immediately prior to analysis, FlowCOUNT™ beads (BCI, Miami, FL) (100µl) were added to each sample using the same pipette used to dispense the blood. The tubes were each vortexed once, re-placed back into the carousel and loaded onto the flow cytometer for acquisition. Listmode data was saved for retrospective re-analysis of outliers, if required.

PLG prepared samples were also used to evaluate the bias potentially introduced between different models of flow cytometers from the same manufacturer. In other words, the PLG prepared samples were run on the Epics-XL and re-analysed on the FC-500. Such re-analysis of data, where all aspects of biological sample variation as well as variation introduced through sample preparation were effectively removed, allowed for true assessment of impact of instrument variation during flow cytometric analysis on bias between CD4+ counts.

iii) Description of the Sample Lysis Procedure using the Multi Q-Prep™ Work Station

Sample lysis using the Multi Q-Prep™ work station (BCI, Miami, FL) is a 35 second automated process in which Beckman Coulter Immunoprep reagents (A), (B) and (C), are each added to a sample.

Lysing solution (Reagent A: Formic Acid and Stabiliser) (600µl) is added to the sample to lyse erythrocytes and the tube is mixed and allowed to stand for 8 seconds. Stabilising solution (Reagent B: Sodium Carbonate/ SodiumChloride/ Sodium Sulphate/ Stabiliser) (265µl) is then added and the sample mixed and allowed to incubate for 10 seconds. Fixative (Reagent C: Paraformaldehyde and Buffers) (100µl) is finally added to the sample and the tube mixed and allowed to stand for 10 seconds. The prepared sample is rendered

non-infectious with the addition of paraformaldehyde and the sample is stable for analysis for up to one week post sample preparation, if stored at refrigerator temperature (4°C).

iv) Procedure used for the MultiTEST/TruCount CD4 Method

TruCount tubes (containing preloaded lyophilised beads) were setup (labeled) to correspond to the laboratory number of blood samples. MultiTEST^{TM} antibody reagent (CD3-FITC/CD8PE/CD45PerCP/CD4APC) (20µl) was added to each tube, followed by the addition of (50µl) of well mixed whole blood of the appropriate sample. The tubes were gently vortexed (5 seconds) to ensure thorough mixing then placed in the dark to incubate for 15 minutes at room temperature. FACS^{TM} lysing solution (BDB) (450µl) diluted in a 1:10 ratio with distilled water was added to each sample to stop the reaction and fix the cells. The tubes were gently vortexed and allowed to incubate for another 15 minutes at room temperature. A work-list of the prepared samples was created and the samples arranged onto a carousel in the order of the list and loaded onto the FACSCalibur for acquisition and analysis. The prepared sample is rendered non-infectious with the addition of formaldehyde and the sample is stable for analysis for up to 24 hours post sample preparation, if stored at room temperature (20-25°C).

Created listmode data files were saved for retrospective re-analysis of samples flagged for having questionable integrity. The data files were also used to evaluate the bias potentially introduced between different manufacturer systems as well as bias potentially introduced between different gating strategies. In the latter case, the 4-colour MultiTEST/TruCount data was re-analysed using a 2-colour PLG strategy to allow true assessment of impact of gating on bias between CD4+ counts using the Becton Dickinson system.

3.3.2 Flow Cytometry

3.3.2.1 Instrumentation used for the Laboratory Procedures

Samples were analysed using flow cytometers that allow for four–colour data acquisition. The cytometers used were:

i) The EPICS XL flow cytometer (BCI, Miami, FL) equipped with a blue 488 nm argon laser

ii) The Cytomics FC-500 flow cytometer (BCI, Miami, FL) equipped with a blue 488 nm argon laser and a 635 nm red diode laser (the latter mentioned was not used for the purposes of this study) and

iii) The FACSCalibur (BDB) equipped with a 488 nm argon-ion laser and a 635 nm red diode laser.

The Johannesburg laboratory had all three cytometers and the CDC laboratory had just one, the FACSCalibur (BDB).

For each assessment of variability carried out, blood (and for the Beckman Coulter methods, the fluorescence beads) was measured using a specific pipette. The three pipettes used (referred to as pipette A, B and C respectively from here on) were;

i) (100µl) fixed volume pipette (Gilson Inc., WI, USA), (pipette A) used in Johannesburg

ii) (200µl) pipette (Gilson Inc. WI, USA), (pipette B) used at the CDC laboratory

iii) (20-200µl) Eppendorf pipette (Brinkmann Instruments, Westbury, N.Y), (pipette C) also used at the Johannesburg laboratory.

Pipetting was performed using Diamond D200 (200ul) pipette tips (Gilson Inc. WI.USA). Other instruments used in general standard procedures such as calibration of pipettes and vortexing, are listed in Appendix A.

3.3.2.2 Acquisition and Analysis of Samples using the TetraONE Protocol

The TetraONE protocol, used at the Johannesburg laboratory, uses 4-colour analysis and the $CD45^{bright}$ lymphocyte gating strategy to derive absolute CD4+ cell counts. It is however possible to apply both the 4-colour $CD45^{bright}$ lymphocyte gating strategy and the 2-colour PLG gating strategy in a single acquisition and analysis to facilitate the simultaneous generation of both a $CD45^{bright}$/CD3 gated CD4+ lymphocyte count as well as a PLG CD4+ lymphocyte count. Such a strategy effectively uses the same set of listmode from a single sample acquisition to assess the contribution of gating to bias between CD4 methods.

In this 4-colour analysis (aspects identified within a red box in Figure 3.1), total lymphocytes were identified as bright $CD45^{++}$/low SSC events and defined in the CD45 fluorescence/SSC scatter plot [gate B in scatter plot 2 of Figure 3.1]. $CD3^{+}4^{+}$ and $CD3^{+}8^{+}$ T-cells within this $CD45^{++}$ lymphocyte gate were identified [region F and region G (populations shown by red dotted lines) in Scatter plot 6 and 7 respectively, of Figure 3.1] and using the FlowCount bead population (identified as a single, very bright, tight population in the light scatter scatter plot [FSC versus SSC]: Scatter plot 1 in Figure 3.1), the CD4 and CD8 absolute cell counts of the total $CD3^{+}$ lymphocytes derived using the formula:

(Number of $CD3^{+}CD4^{+}$events) / (Total number of beads acquired) x (Number of beads/µl). Note: $CD3^{+}CD8^{+}$events were used in the calculation of the absolute CD8 count.

The CD4% lymphocyte count was derived using the formula:

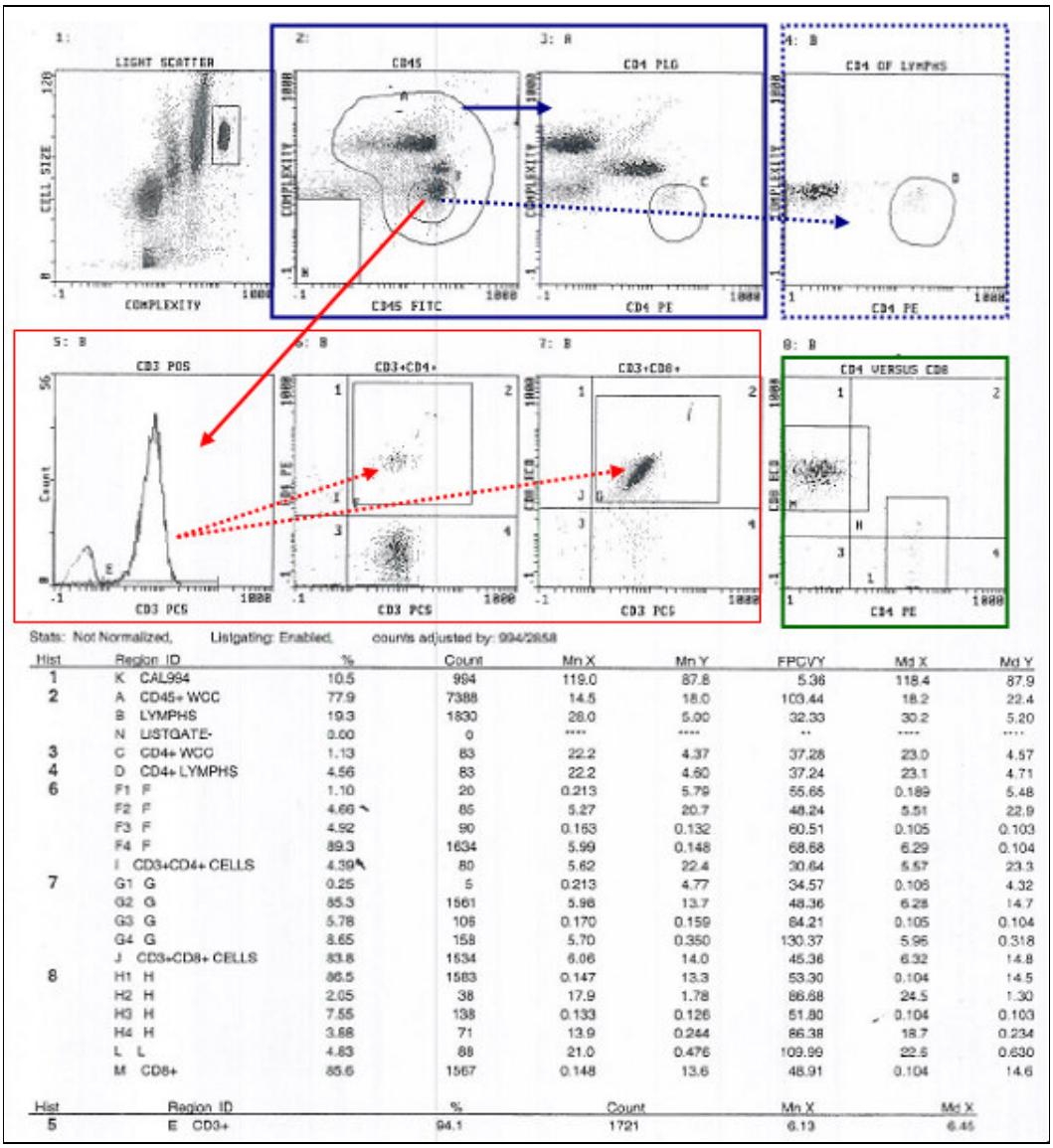
[Total $CD4^{+}$ lymphocyte events (events in gate D) / [Total lymphocyte events (events in gate B) x 100.

The CD8ECD vs. CD4PE scatter plot identified within a green box in Figure 3.1, was used to monitor color compensation.

An explanation of how absolute CD4+ counts were derived when the 4-colour TetraONE listmode data was re-analysed using the 2-colour PLG strategy [using only the data related to the CD45 and CD4 acquisition parameters of the 4-colour listmode data set – data in scatter plot 2 and 3 enclosed in a solid blue rectangle in Figure 3.1], is provided in section 3.3.2.3 that describes 2-colour PLG acquisition and analysis.

In the 2-colour PLG analysis, bright $CD4^{++}$ within the lymphocyte gate [region D in scatter plot 4, identified within a blue dotted blue box in Figure 3.1], were used to derive the CD4% count, in a similar fashion to the CD4% of lymphocytes generated within the $CD45^{bright}$ gated protocol.

An illustration of the TetraONE protocol performed incorporating both the 4-colour and 2-colour gating strategies in a single acquisition and analysis on the same sample, is shown in Figure 3.1.



Hist	Region ID	%	Count	Mn X	Mn Y	FPCVY	Md X	Md Y
1	K CAL994	10.5	994	119.0	87.8	5.36	118.4	87.9
2	A CD45+ WCC	77.9	7388	14.5	18.0	103.44	18.2	22.4
	B LYMPHS	19.3	1830	28.0	5.00	32.33	30.2	5.20
	N LISTGATE-	0.00	0	****	****	**	****	****
3	C CD4+ WCC	1.13	83	22.2	4.37	37.28	23.0	4.57
4	D CD4+ LYMPHS	4.56	83	22.2	4.60	37.24	23.1	4.71
6	F1 F	1.10	20	0.213	5.79	55.65	0.189	5.48
	F2 F	4.66	85	5.27	20.7	48.24	5.51	22.9
	F3 F	4.92	90	0.163	0.132	60.51	0.105	0.103
	F4 F	89.3	1634	5.99	0.148	68.68	6.29	0.104
	I CD3+CD4+ CELLS	4.39	80	5.62	22.4	30.64	5.57	23.3
7	G1 G	0.25	5	0.213	4.77	34.57	0.105	4.32
	G2 G	85.3	1561	5.96	13.7	48.36	6.28	14.7
	G3 G	5.78	105	0.170	0.159	84.21	0.105	0.104
	G4 G	8.65	158	5.70	0.350	130.37	5.96	0.318
	J CD3+CD8+ CELLS	83.8	1534	6.06	14.0	45.36	6.32	14.8
8	H1 H	86.5	1583	0.147	13.3	53.30	0.104	14.5
	H2 H	2.05	38	17.9	1.78	86.68	24.5	1.30
	H3 H	7.55	138	0.133	0.126	51.80	0.104	0.103
	H4 H	3.88	71	13.9	0.244	86.38	18.7	0.234
	L L	4.63	88	21.0	0.476	109.99	22.6	0.630
	M CD8+	85.6	1567	0.148	13.6	48.91	0.104	14.6

Hist	Region ID	%	Count	Mn X	Md X
5	E CD3+	94.1	1721	6.13	6.45

Figure 3. 1: Figure to illustrate the derivation of CD4+ counts and CD4% of Lymphocytes using the CD45^{bright} Lymphocyte Gating Strategy and the 2-colour PLG Gating Strategy in a Single Acquisition and Analysis using the Johannesburg TetraONE protocol on a Beckman Coulter Instrument.

3.3.2.3 Acquisition and Analysis of Samples using the 2-colour PLG-CD4 Protocol

The PLG-CD4 is a 2-colour based method that uses total leucocytes ($CD45^{+}$events) as a reference population for calculating absolute CD4+ cell counts.

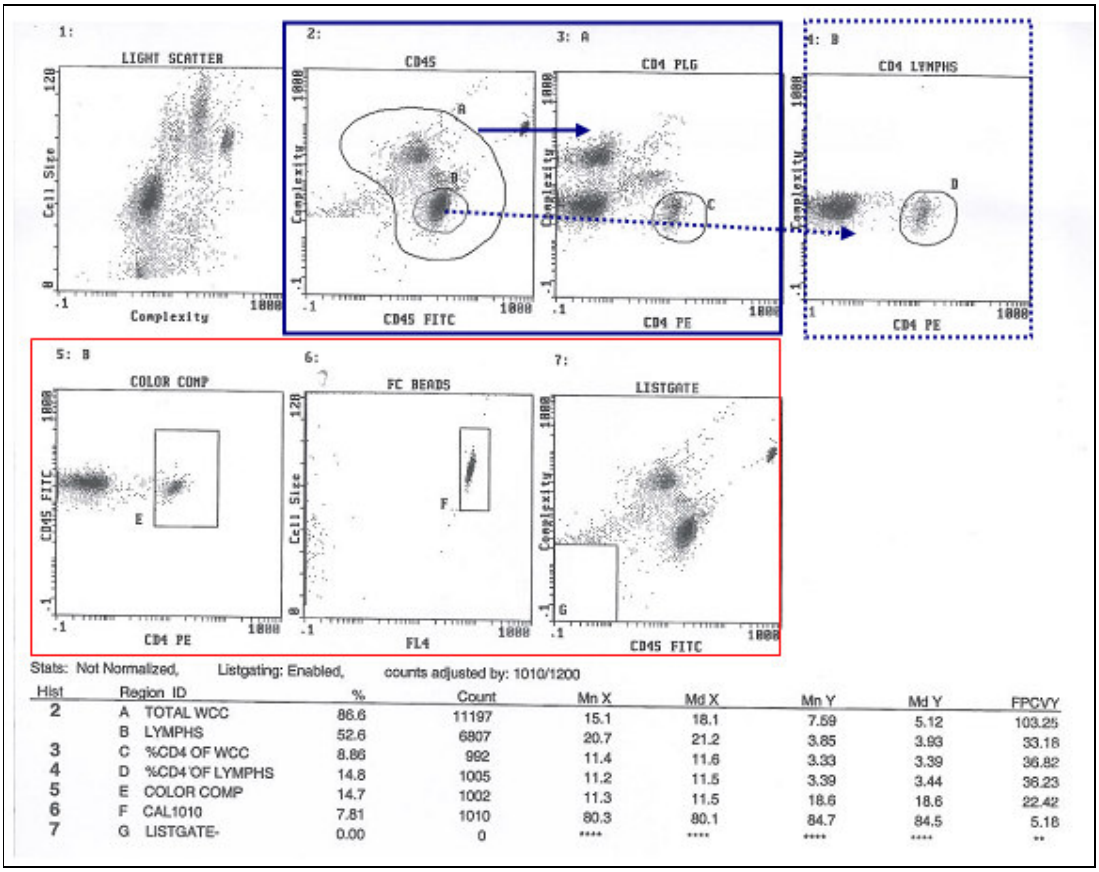
It is the predicate CD4+ cell count method used at the Johannesburg laboratory. Routine clinical samples, prepared following this 2-colour PLG-CD4, were analysed on the Epics-XL (BCI, Miami, FL) and on the FC-500 (BCI, Miami, FL) flow cytometers. This re-

acquisition of samples was performed to assess how using a different platform (instrument) of sample data acquisition impacts and contributes to bias between CD4 methods.

In the PLG analysis (scatter plots enclosed in a blue rectangle), a "Panleucogate" identifying all CD45+ events (total leucocytes) was first defined in the CD45 fluorescence/SSC scatter plot [gate A in Scatter plot 2 of Figure 3.2]. Bright $CD4^{++}$/low SSC cells within the Panleucogate were then identified [gate C in Scatter plot 3 of Figure 3.2] and using the FlowCOUNT^{TM} bead population (identified as a single, very bright, tight population in the light scatter plot: Plot 1 of Figure 3.2), the absolute CD4+ cell count derived using the formula:

(Total $CD45^{+}/CD4^{++}$ events) / (total number of beads acquired) x [number of beads/µl].

Figure 3.2 illustrates an ideal result report of a sample analysed using the PLG-CD4 method and protocol at the Johannesburg laboratory. The report includes relevant quality control steps (plots; 5-7 identified within a red box in Figure 3.2). Here, the light scatter plot is used to visualise the age and integrity of the sample. The CD45FITC vs. SSC scatter plot is used to distinguish all leucocytes and all $CD45^{bright}$ lymphocytes. The CD4PE vs. CD45FITC scatter plot is used to monitor colour compensation, the FL4 vs. Cell Size scatter plot is used to emunerate FlowCount beads and these used to monitor instrument performance and in the calculation of the absolute cells counts. The CD45FITC vs. Complexity scatter plot is used to identify debris (Gate G referred to as the Listgate) so that it is excluded from the listmode file.



Hist	Region	ID	%	Count	Mn X	Md X	Mn Y	Md Y	FPCVY
2	A	TOTAL WCC	86.6	11197	15.1	18.1	7.59	5.12	103.25
	B	LYMPHS	52.6	6807	20.7	21.2	3.85	3.93	33.18
3	C	%CD4 OF WCC	8.86	992	11.4	11.6	3.33	3.39	36.82
4	D	%CD4 OF LYMPHS	14.8	1005	11.2	11.5	3.39	3.44	36.23
5	E	COLOR COMP	14.7	1002	11.3	11.5	18.6	18.6	22.42
6	F	CAL1010	7.81	1010	80.3	80.1	84.7	84.5	5.18
7	G	LISTGATE-	0.00	0	****	****	****	****	**

Figure 3. 2: Figure to illustrate the derivation of CD4+ counts of Leucocytes and CD4% of Lymphocytes using the 2-colour PLG protocol on Beckman Coulter flow cytometers.

3.3.2.4 Acquisition and Analysis of Samples using the MultiTEST/TruCount CD4 Protocol

The MultiTEST/TruCount (BDB) is a 4-colour method that uses TruCount tubes that have encased at their bottom a lyophilised bead pellet. The pellet is of a known bead concentration and is provided per tube lot by the manufacturer (BDB). It dissolves during sample preparation, releasing a known number of fluorescence beads which are gated during analysis and used as the reference population to determine the absolute cell counts.

4-colour MS/TC (BDB) was the predicate CD4+ cell count method used at the CDC laboratory. The precision of MS/TC measurements is dependent on the single pipetting step of the sample. Reverse pipetting is reported to be the most reliable dispensing method for absolute cell count methodologies and is therefore the recommended technique for use in this sample pipetting step(64, 144). This dispensing technique was however not used by

the writer in performing the MS/TC method because the assessment of her pipetting skill showed that she had better pipetting precision using the forward pipetting technique.

Acquisition and analysis were performed using the automated MultiSET^{TM} software programme (BDB). A minimum of 5000 lymphocyte events (BDB defined threshold) were acquired for each sample and the un-gated data files for the samples saved.

In analysis, the TruCount bead population (defined as high SSC and very bright fluorescence signal events), CD4+ and CD8+ populations (defined as $CD3^{+}4^{+}$ and $CD3^{+}8^{+}$ in accordance to the lymphocyte gating strategy: Plot of CD4APC versus CD8PE in Figure 3.3), were automatically identified using in-built predefined Attractor^{TM} regions in the MultiSET^{TM} software. Absolute CD4+ cell counts were derived using the formula:

([Number of $CD3^{+}/CD4^{+}$ events] / [Number of acquired bead events]) x ([Number of beads/pellet] / [Volume of blood (50µl) used]).

Figure 3.3 shows a typical result report of a sample analysed using the 4-colour MS/TC method.

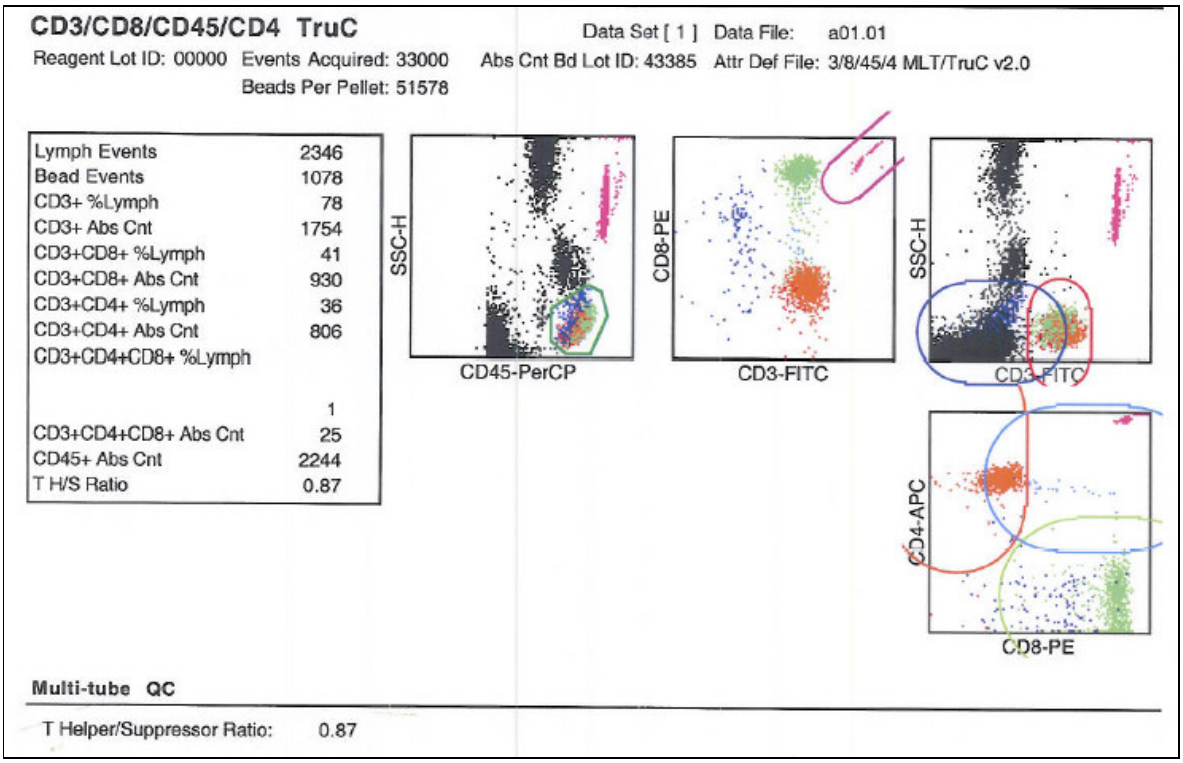


Figure 3. 3: Figure to illustrate the derivation of CD4+ counts and CD4% of Lymphocytes using the CD45*bright* Lymphocyte Gating Strategy in the 4-colour MultiTEST/TruCount protocol on a FACSCalibur flow cytometer.

3.3.3 Daily Quality Control (QC)

Daily internal quality control checks using CaliBRITE™ beads (BDB) for the FACSCalibur and Flow-CHECK™ beads (BCI, Miami, FL) for the Epics XL and FC-500 were performed, in accordance to manufacturer instructions to monitor the state of the respective flow cytometers. The beads have fluorescence properties and the ability to simulate unstained and stained cells and are used to assess laser linearity, check instrument sensitivity i.e. fluorescence settings, and to adjust fluorescence compensation, if required.

A daily check of the sample preparation process for Beckman Coulter methods (2-colour PLG-CD4 and TetraONE) was also performed using stabilised blood controls i.e. the "Low" and "Normal" value Immunotrol™ products (both, BCI, Miami, FL). The purpose of this check was to ensure the quality and state of the monoclonal antibody, and reagents used with the Multi Q-Prep™ system, for use with samples in the clinically relevant range (<200 cells/µl) and those with normal CD4+ counts. Verification of the sample preparation

process for the 4-colour MS/TC (BDB) methodology was not performed as such an equivalent product is not available from BDB.

Samples were only analysed on the flow cytometers after calibration and performance checks had been performed and passed by the technicians at the respective laboratories.

3.3.3.1 Procedure for Performing Daily QC for the FACSCalibur

The FACSCalibur linearity and performance check was performed using freshly prepared CaliBRITE™ beads (CaliBRITE™ 3 and CaliBRITE™ APC) (BDB). Levy-Jennings plots of the calibration results were automatically created and saved to facilitate day-to-day comparison of instrument performance and settings by the CDC laboratory technicians.

Two falcon tubes were set-up: To one, filtered sheath (1ml) and a drop of well mixed unlabeled beads (BDB) and APC beads (BDB) were added. To the second tube, filtered sheath (3ml) and a drop of unlabeled beads, APC, FITC, PE and PerCP labeled beads (BDB) were added. The tubes are gently vortexed (5 seconds), placed within a carousel, loaded onto the FACSCalibur and FACSComp software (BDB) launched to perform the automated instrument check.

If the calibration check passed, the new Lyse-No-Wash calibration settings were automatically set-up and used as the settings for sample acquisition.

3.3.3.2 Procedure for Performing Daily QC for the EPICS-XL and FC-500 Flow Cytometers

Although the Epics-XL and FC-500 Beckman Coulter flow cytometers are different models, the two were calibrated using a similar procedure and in accordance to the manufacturer's instructions. In this study, the calibrating material was prepared once and used for both instruments i.e., used to calibrate the Epics-XL then used for the FC-500.

A test tube with FlowCHECK™ fluorescent beads (BCI, Miami, FL) (15 drops) was acquired on the flow cytometer for 10 seconds to check the optical alignment of the laser and stability of the fluidic system. This was followed by performing the bead reproducibility check, to verify the stability of the flow count rate and assess precision of instrument fluidics(152). The latter was assessed by examining the consistency in the number of beads acquired when 11 replicate preparations of FlowCOUNT™ beads (BCI, Miami, FL) (100µl) in sheath fluid (BCI, Miami, FL) (900µl) were each acquired for 120 seconds. Lastly, a check to verify proper functioning of the sample preparation reagents and that of the Multi Q-Prep™ system was performed using stabilised blood controls (ImmunoTrols™, BCI, Miami, FL). The controls were samples of known CD4+ cell counts. One had a CD4+ cell count in the normal range and the other a low CD4+ cell count. These were prepared and analysed in a manner similar to that in which patient samples were processed when using the 4-colour TetraONE T-cell and 2-colour PLG-CD4 methods. The quality of the reagents and proper function of the Multi Q-Prep™ were confirmed if derived absolute and percentage results for the controls were within the acceptable ranges provided by the manufacturer (BCI, Miami, FL).

3.3.4 Gating Strategies

The method of data analysis (gating strategy and choice of flow cytometric threshold) has been shown to influence accurate enumeration of CD4+ counts and to have an impact on the duration the sample testing period can be extended.

Two standard gating strategies were used in the analysis of the flow data; the 2-colour PanLeucogating (PLG) strategy(39, 59) and the 4-colour Lymphocyte gating strategy ($CD45^{bright}$ Lymphocyte Gating) (62, 64). A comparison of CD4+ cell counts derived using both strategies was carried out to assess two aspects, (1) the impact of gating on bias

between different CD4 methods and systems and (2) which method was better able to extend the window period of testing.

3.3.4.1 The PanLeucogating Strategy (PLG)

The PLG is a strategy based on a combination of primary CD45 gating (i.e. CD45/Side Scatter [SSC]) and subsequent CD4 gating (i.e. CD4/SSC) [scatter plots enclosed in blue rectangle of Figure 3.4]. It typically uses Forward scatter [FSC] as a threshold (although CD45 fluorescence as a threshold is used in some instances with PLG, as in this study) and employs CD45/SSC to select (gate) all leucocytes [gate A in scatter plot 2 of Figure 3.4) as the primary reference population and subsequent CD4 expression and side scatter based on this total leucocyte gate, to define and enumerate absolute CD4+ cells [gate C in scatter plot 3 of Figure 3.4]. The use of CD4 expression and side scatter specifically excludes monocytes from the analysis and eliminates the need for CD3 to define lymphocytes(39, 40, 59). The conventional lymphocyte based ($CD45^{bright}$) gate is thus not applied for cell enumeration in PLG testing. However a CD4% of lymphocytes, as defined in the various international guidelines(62, 64, 94), can be obtained by incorporating $CD45^{bright}$ gating with PLG to define lymphocytes ($CD45^{bright}$ cells) [gate B of scatter plot 2 in Figure 3.4] and then, to assess the proportion of $CD45^{bright}$ lymphocytes that are CD4 bright/low SSC cells [gate D in scatter plot 4 of Figure 3.4].

The 2-colour PLG gating strategy, as well as the CD4% of lymphocytes are illustrated in Figure 3.4.

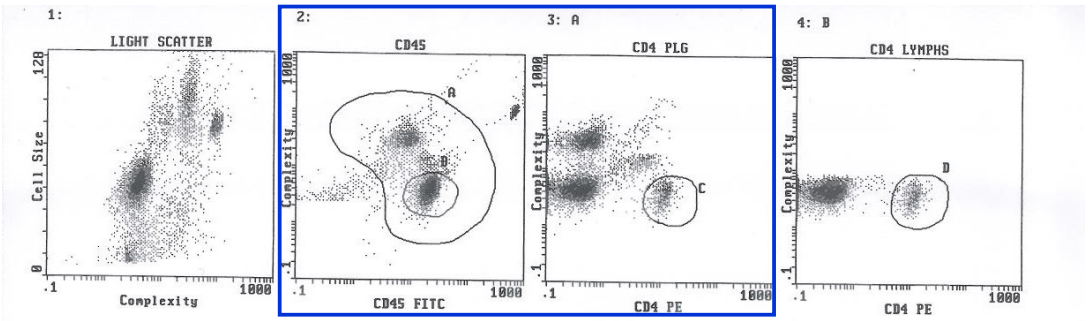


Figure 3. 4: Figure to illustrate the 2-colour PanLeucogating Strategy as performed on the Beckman Coulter flow Cytometers

Gate A (PLG Gate) showing total CD45 cells (leucocytes), Gate B is CD45^{bright} lymphocyte gate and Gate C are the CD4+ T-cells gated on Gate A.

Figure 3.5 following, shows how the writer re-analysed 4-colour MS/TC data files using the 2-colour PLG strategy on the FACSCalibur.

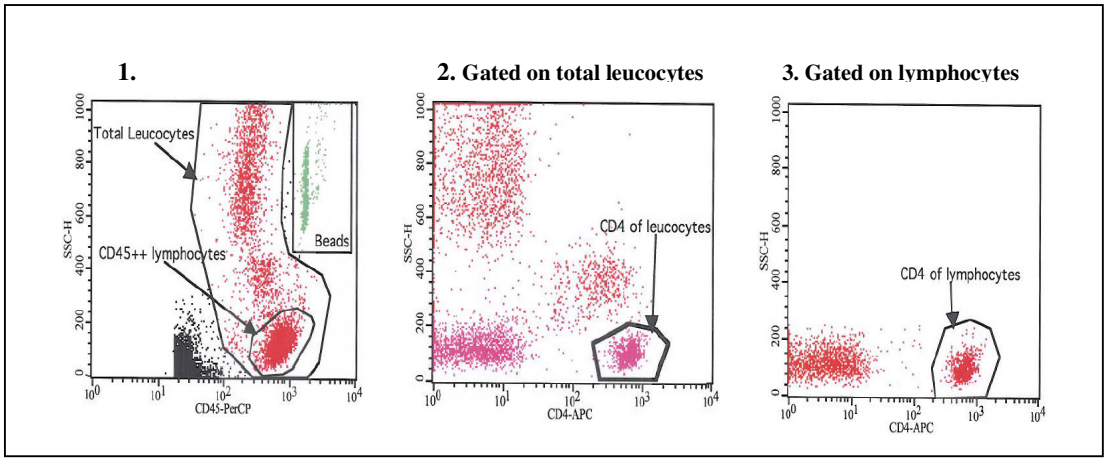


Figure 3. 5: Figure to illustrate the concept of the PanLeucogating strategy as performed on the FACSCalibur.

3.3.4.2 The Lymphocyte Gating Strategy (CD45^{bright} Lymphocyte Gating)

The lymphocyte gating strategy illustrated in Figure 3.6 differs from PLG in that it uses CD45 expression to define CD45^{bright} expressing cells lymphocytes, and CD3 to exclude monocytes from the analysis and to define CD4+ T cells.

This strategy typically identifies and uses the CD45^{bright} (CD45^{++}/low SSC) lymphocyte population from among leucocytes [gate A in scatter plot 1 of Figure 3.6] as the reference population. Then using a SSC/CD3 scatter plot gated on total leucocytes [gate B in scatter plot 2 of Figure 3.6], the T-lymphocyte cells (CD3^{+}) are defined. CD3^{+}4^{+} and CD3^{+}8^{+}

absolute cell counts and percentages are then identified from the $CD3^{+}$ population gated on $CD45^{bright}$ lymphocytes [Scatter plot 3 of Figure 3.6].

The lymphocyte gating strategy is used in the 4-colour MultiTEST/TruCount protocol (BD Biosciences) and in the 4-colour TetraONE/FlowCount (BC, Miami, FL) protocol.

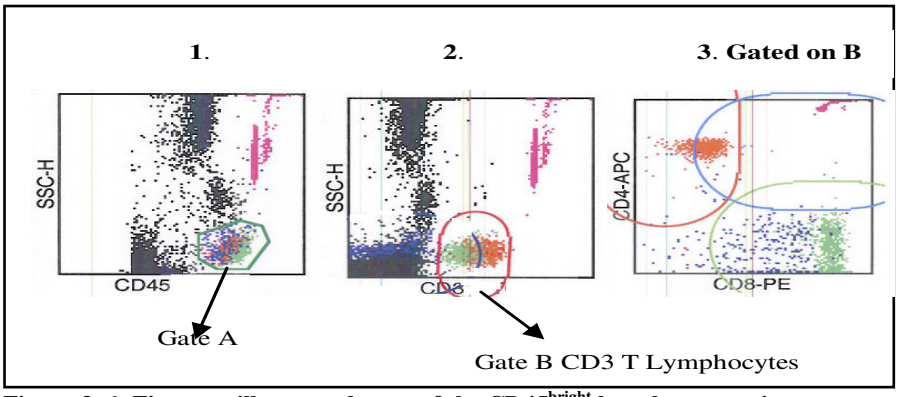


Figure 3. 6: Figure to illustrate the use of the $CD45^{bright}$ lymphocyte gating strategy as applied in the 4-colour MS/TC CD4 protocol.

3.4 Analyses performed to Assess Impact of CD4 Methodology including Specific Reagents, Flow Cytometry System, Protocol Setups and Gating on Bias of CD4 Reporting.

3.4.1 Assessment of Bias introduced through Poor Precision of Pipetting

The reliability of pipette measurements is dependant on proper calibration of pipettes and on an operator's pipetting skill(144). There are two known techniques of pipetting; (i). The forward displacement technique, also called forward pipetting (FP) and (ii) the reverse pipetting technique (RP). A description of both of these is provided in Appendix B.

Several pipettes were validated to enable the selection of properly functional pipettes for use in this study and to determine the precision of the operator (the writer) using the two pipetting techniques.

3.4.1.1 Validation of Pipettes used in this Study (Johannesburg and CDC laboratories)

In this process, the amount of distilled water repeatedly measured using a pre-set pipette was weighed, to ensure that the dispensed volume was precise and accurate (i.e. within the

range specified by the respective pipette manufacturer). The water pipetted, was weighed using weighing balances that had been calibrated and confirmed to be functionally suitable for use. Either 50µl or 100µl of water was pipetted depending on the pipette being validated and the volume of blood the pipette would be used to measure in the study assessments. This exercise was performed using the forward pipetting technique at the two laboratories.

An empty plastic test tube (BC, Miami, FL) was weighed and the balance tared (made to read 0.00g). Distilled water (50µl or 100µl) was then pipetted into the weighed test tube, the tube placed back onto the balance and the weight of the water determined. This process was repeated 20 times for each pipette. The water measurements (weights) taken were then grouped into tens to calculate the overall mean weight (corresponding to the average volume of water pipetted), standard deviation and coefficient of variation (%CV: [standard deviation/mean] x 100 – a measure of precision) for each pipette. The pipettes (A, B and C) that had the best precision in the hands of the writer (i.e. the lowest %CV readings) were selected for use in this study.

3.4.1.2 Operator Skill Assessment (Forward pipetting versus Reverse pipetting precision assessment of the operator [Intra-operator])

The purpose of validating pipettes and of evaluating the operator's (MM) pipetting skill prior to carrying out the other assessments of the study was to minimise the influence due to pipette error on other variables studied.

Using one of the validated pipettes [Pipette A, a fixed volume (100µl) pipette)], the two pipetting techniques, forward (FP) and reverse pipetting (RP) were assessed to determine the technique with which the operator (the writer), obtained better precision. Blood instead of distilled water was used in this exercise. The blood used was taken from routine CD4 samples after they had been tested at either of the two laboratories. The tube of blood was

thoroughly mixed to ensure homogeneity each time blood was pipetted off (from the same sample). Twenty-two samples (2 per day) were used over 11 days. Repeat volumes of blood (100µl) pipetted using RP or FP were weighed, the weights grouped into sets of 10s, averaged and the precision documented. A %CV was then determined in a manner similar to that done in the pipette validation exercise [section 3.4.1.1]. The technique (FP) with which the least variation was obtained (i.e. %CV<1) was chosen and used through out the study for all the methodologies including the 4-colour MultiTEST/TruCount method (BDB).

Upon establishing the pipetting technique (FP) to be used, the operator's (the writer) pipetting skill using the other two validated pipettes B and C, was also assessed.

3.4.1.3 Within-Laboratory Pipetting Skills Precision Assessment (Precision between different operators measured separately at two different geographically located sites [Inter-Operator])

i) Within-laboratory (within and between operator) precision

To assess the amount of variation that could result when different operators within the same laboratory processed the same patient sample, within-laboratory variability was determined. Ten laboratory technicians from each of the two laboratories were asked to participate in this assessment. Each technician's precision was first assessed and thereafter, the overall precision of the respective laboratory established using the results of all ten participating operators. Samples with CD4+ cell counts (274 cells/µl and 267 cells/µl) in the clinically significant HIV range (≤ 200 cells/µl to ≤ 350 cells/µl) were used.

At the Johannesburg laboratory, the blood sample was divided into 10 aliquots (1ml each) and each of the 10 operators given an aliquot to process ten times (stain with monoclonal antibody, fix and analyse) following the 4-colour TetraONE method. Each of the ten operators obtained 10 CD4+ cell count results and the mean, SD and %CV (precision of each operator) for each set was calculated. The laboratory %CV was then determined by

deriving the %CV of the hundred CD4+ counts obtained by the ten operators. This exercise was repeated by 10 technicians using the 4-colour MultiTEST/TruCount method on the FACSCalibur, at the CDC laboratory.

ii) Cell count related variation (within-operator variability across strata of clinical CD4+ counts)

The impact of an operator's pipetting skill on the precision of CD4+ counts in different strata, derived using the same method of sample preparation and model of flow cytometer, was assessed by the writer at the Johannesburg laboratory and by a different operator (MR) at the CDC laboratory. Three blood samples were used in this exercise; one with a low cell count (< 200 cells/µl), an intermediate count (300-500 cells/µl) and a high CD4+ count (> 500 cells/µl). This assessment was used to examine the precision of the two operators at the different CD4+ strata, especially at the clinically relevant range of < 200 cells/µl CD4+ counts.

Testing was performed using the 4-colour MultiTEST/TruCount method. Although it is not the required pipetting method for this system and because the measurable outcome was assessment of precision (and not accuracy), the forward pippetting technique was used in this precision assessment because both operators mentioned above demonstrated better pipetting precision using FP than when using the recommended RP technique(64, 144). Each sample from the different strata mentioned above was processed 10 times by the same operator (MR, or the writer) at the respective CDC and Johannesburg laboratories. The prepared samples were subsequently acquired and analysed on the FACSCalibur at each site. Each set of ten CD4+ count results for each sample, at each site, were averaged, the SD determined and the %CV derived. The %CV value was then used to assess precision of the two operators at the respective laboratory.

3.4.2 Assessment of the Bias introduced through use of Different Manufacturer Sample Preparation Systems and the Same Gating Strategy (CD45^{bright} Gating).

Forty one samples that were on average <16 hours old, were analysed on the Epics-XL, FC-500 and the FACSCalibur flow cytometers at the Johannesburg laboratory. Each sample was processed following both the Johannesburg's TetraONE method and the BDB 4-colour MultiTest/TruCount method. Samples prepared using the TetraONE method were analysed on the Epics-XL and the FC-500 instruments. Those samples prepared using the MultiTest/TruCount method were analysed on the FACSCalibur. Three CD4+ absolute count results were thus obtained for each sample for this part of the study. The mean absolute CD4+ counts from the respective flow cytometers were compared in order to assess the amount of bias introduced to CD4+ counts when samples were prepared using different manufacturer systems but applying the same gating strategy [i.e. 4-colour CD45^{bright} lymphocyte gating: (1). 4-colour MS/TC on the FACSCalibur (BDB) versus 4-colour TetraONE on the Epics XL (BC) and (2). 4-colour MS/TC on the FACSCalibur (BDB) versus 4-colour TetraONE on the FC-500 (BC)].

3.4.3 Assessment of the Bias introduced through use of Different Manufacturer Sample Preparation Systems including Application of Different Gating Strategies and Different Flow Cytometeric Threshold for Data Acquisition.

The purpose of this exercise was to mimic the reality of laboratory to laboratory testing and determine the potential bias introduced between CD4+ counts from different laboratories when different manufacturer sample preparation systems including using different gating strategies (2-colour PLG versus the 4-colour CD45^{bright} lymphocyte gating), are used in the determination of CD4+ cell counts.

Data of the 41 samples processed following the Johannesburg TetraONE protocol (in section 3.4.2) in which the 4-colour CD45^{bright} lymphocyte gating strategy and 2-colour PLG strategy were performed as a single analysis on the same samples, was used. CD4+ cell counts derived using the 2-colour PLG gating strategy and counts originally obtained using the 4-colour MultiTEST/Trucount protocol on the FACSCalibur in section 3.4.2 were compared [i.e. 4-colour MS/TC on the FACSCalibur (BDB) versus 2-colour PLG on the Epics XL (BC) and 4-colour MS/TC on the FACSCalibur (BDB) versus 2-colour PLG on the FC-500 (BC)].

3.4.4 Assessment of the Bias introduced when Samples are prepared using the Same Manufacturer System and Analysed on the Same Flow Cytometer but using a Different Gating Strategy.

In this analysis, CD4+ counts derived using the 4-colour CD45^{bright} lymphocyte gating strategy and counts of the same samples derived using the 2-colour PLG strategy on the same flow cytometer, were compared for each instrument [i.e. 4-colour CD45^{bright} lymphocyte gating on the Epics XL (BC) versus 2-colour PLG gating on the Epics XL (BC) and 4-colour CD45^{bright} lymphocyte gating on the FC-500 (BC) versus 2-colour PLG gating on the FC-500 (BC)]. Data of the 41 samples processed following the TetraONE protocol (in section 3.4.2) was used.

This assessment of the potential impact of bias introduced by use of different gating strategies in the same manufacturer method was also performed for the BDB system. Here, the 20 samples drawn in new blood stabilisation tubes at the Johannesburg laboratory were processed following the 4-colour MS/TC method (BDB) and analysed on the FACSCalibur (BDB). Data files of these 20 samples were retrospectively re-analysed using the 2-colour PLG gating approach to derive MS/TC-PLG CD4+ counts. The respective 2-colour PLG and 4-colour MS/TC CD4+ counts were then compared to assess bias introduced through gating alone on the BDB system.

3.4.5 Assessment of the Bias introduced when Samples prepared using the Same Manufacturer System were Analysed on Different models of Flow Cytometers from the Same Manufacturer.

In this analysis, an assessment was made of the bias that could potentially be introduced if different cytometers from the same manufacturer (Beckman Coulter) were used to derive a CD4+ cell count from the same prepared sample (the same prepared sample was re-run on a different model of flow cytometer from the same manufacturer). The cytometers are individually calibrated since there is typically no standardisation of instrument calibration.

Here, the writer used data of 61 samples that had been routinely processed by the technicians at the Johannesburg laboratory for CD4+ counts using the 2-colour PLG-CD4 protocol. Two thirds (45) of the 61 samples selected had CD4+ counts < 350 cells/µl so that bias in readings for this clinically significant range could be examined. The samples (N=61), were analysed on the Epics XL (XL-2) and immediately re-analysed on the FC-500 cytometer (by the laboratory scientist) and the absolute CD4+ cell counts from the two instruments compared [i.e. 2-colour PLG-CD4 (BC) on the Epics XL (BC) versus 2-colour PLG-CD4 (BC) on the FC-500 (BC)].

In addition, data of the 41 samples processed following the TetraONE protocol in Section 3.4.2, was also used. CD4+ cell counts derived using 4-colour $CD45^{bright}$ lymphocyte gating on the Epics XL (BC) were compared to counts derived using 4-colour $CD45^{bright}$ lymphocyte gating on the FC-500 (BC). Similarly, the CD4+ cell counts derived using 2-colour PLG gating on the Epics XL (BC) were compared to counts derived using 2-colour PLG gating on the FC-500 (BC).

3.5 Analyses performed to Assess Impact of Logistical Factors including Time from Venesection, handling during Transportation and Temperature on Bias of CD4+ Reporting.

The purpose of this exercise was to assess whether time, temperature and continuous motion affected the integrity of whole blood samples and thus, the precision of CD4+ cell counting.

Thirty two samples (average age between ≥12-24 hours old) were analysed using the 4-colour TetraONE protocol at the Johannesburg laboratory and 55 samples (average age < 6 hours old) were analysed using the 4-colour MS/TC protocol at the CDC laboratory for this exercise.

i) Samples were processed on the day they were received for baseline (day of draw) CD4+ cell counts and then each sample split into four (850µl) aliquots. One aliquot was stored at refrigerator temperature (FT), another at room temperature (RT) and the third at 37ºC in a water bath. The fourth aliquot was kept in motion on a mixer at RT. The choice to split samples into aliquots of 850mls (or 4 equal aliquots depending on the volume of the sample received) was based on the average 'fill' of 5ml EDTA samples received in the Johannesburg laboratory and the requirement of the study to test each aliquot up to 8 days post venesection. Each aliquot was processed and analysed for a CD4+ cell count every day, for a period of 8 days from the day of draw (unfortunately, there was only sufficient sample to analyse all 32 samples received at the Johannesburg laboratory up to Day 5). Refrigerator temperature was noted to be 4-6ºC in the Johannesburg laboratory and between 4.0-5.2ºC in the CDC laboratory. Room temperature was controlled and maintained by use of air-conditioners at both sites and ranged between 17-23ºC in Johannesburg and 20-23ºC at the CDC laboratory. Specimens kept at FT and water-bath temperatures were allowed to equilibrate to room temperature (30 minutes) before they were processed on days (1-8) of follow-up.

A comparison of mean CD4+ cell counts at baseline and mean CD4+ cell counts on each of days (1-8) for samples stored at the different temperatures (FT, RT and 37ºC) and those subjected to continuous motion, was done for each laboratory. Clinical significance of the CD4+ count results was assessed for two scenarios:

- When the similarity between the mean baseline count (Day 0) and mean day counts was > 90% or ≥ 95% and
- When the mean difference from baseline (BL) was defined as < 40 cells/µl or ≤ 20 cells/µl.

Two cutoffs were used in each scenario, one stricter than the other, so as to effectively determine the duration testing could be extended without compromising the quality of CD4 results.

The days of storage at which the similarity between day and baseline counts (mean difference) was more (less) than the above specified values, were accepted as the days to which samples could still be analysed for reliable results at each laboratory.

To further examine sample integrity, corresponding changes in CD4 percentages (CD4%) were also examined. For this, clinical significance was defined as the 95% confidence intervals outside the range of (>95 - <105).

ii) In order to assess if the type of gating strategy used influenced the precision of CD4+ counting and specifically, whether use of the simpler gating strategy (2-colour PLG) could extend the window of testing and analysis, CD4+ counts (N=32) derived using the two different gating strategies (4-colour CD45^{bright} lymphocyte gating strategy and 2-colour PLG strategy), were also compared. The mean change in CD4+ cell counts derived using the TetraONE protocol with 4-colour CD45^{bright} lymphocyte gating and the mean change of counts derived using 2-colour PLG stategy (i.e. 2-colour PLG TetraONE analysed data) were compared to determine which gating strategy was more reliable for analysis of aged samples.

3.6 Evaluation of the use of a Commercial Whole Blood Stabilising Agent to enable Extending the Window of CD4 Testing.

This aspect of the study was carried out to investigate the use of a stabiliser in maintaining the integrity of samples during transportation and the ability of the stabiliser to extend the window of CD4 testing beyond current expectations.

New (BDB) (2ml) CD4 blood collection tubes, specifically manufactured to address the challenges associated with transporting of whole blood specimens, were used. These collection tubes contained a stabiliser (Streck Laboratories, Omaha, NE) that was meant to preserve the integrity of leucocyte surface antigenic sites and thus improve reliability of CD4+ testing up to seven days if specimens were kept at room temperature (18-30°C) and up to three days if specimens are kept at 37°C.

In this study, the effect of extending the period of testing beyond that recommended by the manufacturer was also evaluated.

A total of 89 samples were tested following the 4-colour MS/TC method. This included 69 of the 89 samples analysed in Uganda and 20 samples analysed at the Johannesburg laboratory. In Johannesburg, all 20 samples were freshly bled and were on average < 2 hours old at the time of delivery to the laboratory. The 69 samples analysed at the CDC laboratory were all > 24 hours old at the time of delivery to the laboratory. These samples were drawn from patients in a CDC study cohort approximately 400 km away and were delivered to the laboratory in the afternoon of the following day.

Samples received at the Johannesburg laboratory were analysed for baseline (Day 0) CD4+ cell counts and the remaining blood stored at room temperature (17–23° C) for follow-up CD4+ cell counts on days 6, 7 and 8. The writer did not do the actual CD4+ testing for this assessment at the CDC laboratory as this had already been done by the laboratory (the 100 tubes provided by BDB for the evaluation had all been used by the time she returned to Uganda). Here, the samples were processed and analysed by three technicians depending

on the technicians on duty. Baseline CD4+ counts were measured a day after venesection (i.e. Day 1, considered as baseline) and subsequent CD4+ counts derived on days 4, 6 and eight from the day of venesection.

3.7 Statistics

All statistical analysis was performed with the help of Professor Piet Becker, a statistian from the Medical Research Council in South Africa. The CD4+ absolute count data were analysed using Microsoft Excel and Stata Statistical Software (StataCorp. 2003. Stata Statistical Software: Release 8.0. College Station, TX: Stata Corporation). Data from the two laboratories were analysed individually. Statistical methods used included Spearman's correlation, a paired-t-test and two-way analysis of variance. Box and Whisker plots of absolute CD4+ counts were also plotted to assess variation in CD4+ counts over time from the day of blood draw. Bland Altman comparisons were used to assess agreement between CD4+ cell counts at baseline to eight days after draw (days 1-8). The mean absolute difference (bias) between the day readings and the 95% limits of agreement (LOA = mean difference ± 2 standard deviations) as well as, day to baseline mean CD4+ count ratios and confidence intervals, were calculated. P values < 0.05 were considered significant.

4.0 RESULTS

4.1 Analyses performed to Assess Impact of CD4 Methodology Including Specific Reagents, Flow Cytometry System, Protocol Setups and Gating on Bias of CD4 Reporting.

4.1.1 Assessment of Bias introduced through Poor Precision Pipetting

4.1.1.1 Validation of pipettes used in this study (Johannesburg and CDC Laboratories)

Three pipettes (A, B and C) were validated for use in this study: Pipette (A) was a fixed volume pipette (100µl) that was used at the Johannesburg (WITS) laboratory to measure blood (100µl) and fluorescence beads (100µl) required for the 4-colour TetraONE method. Pipette (B) a (200µl) volume pipette was validated and used to measure blood (50µl) required for the 4-colour MS/TC method, used at the CDC laboratory. Pipette (C) a (20 - 200µl) volume pipette was validated and used to measure (50µl) and (100µl) required respectively for the 4-colour MS/TC and TetraONE CD4 testing methods performed at the Johannesburg laboratory. A summary of the mean volumes of distilled water measured and (%CV) obtained for each of these pipettes, selected for use in this study, is shown in Table (4.1) below.

Table 4. 1: Summary of the mean volumes of water and %CVs obtained for the pipettes selected for use in this study

Laboratory	Pipette	N	Set to Measure	Forward Pipetting		Forward Pipetting
				Mean Volume (µl)	Range (µl)	Mean %CV
Johannesburg	A	20	100µl	100.30	99.3-100.8	0.390
	C	20	50µl	49.95	48.8-50.5	0.705
	C	20	100µl	101.65	101.0-102.2	0.370
CDC	B	20	50µl	50.30	49.6-50.9	0.650

N: Represents the number of replicate measurements performed to assess the precision of each respective pipette.

4.1.1.2 Operator skill assessment (Forward Pipetting versus Reverse pipetting precision assessment of the operator [Intra-operator])

The pipetting skill of the operator/writer was assessed to determine the technique i.e. reverse pipetting (RP) or forward pipetting (FP), with which she obtained better precision. Figure 4.1 shows the development and improvement of the operator's (the writer) pipetting skill with time. Greater precision of blood measurements (measured as the coefficient of variation [%CV]) was obtained when the FP technique was used, with the %CV declining from 0.912% to 0.387% over 11 days. An average %CV of (0.513%) was demonstrated with FP compared to (1.214%) with the RP technique. FP was therefore chosen as the technique for use in all pipetting procedures used by the operator/writer in this study.

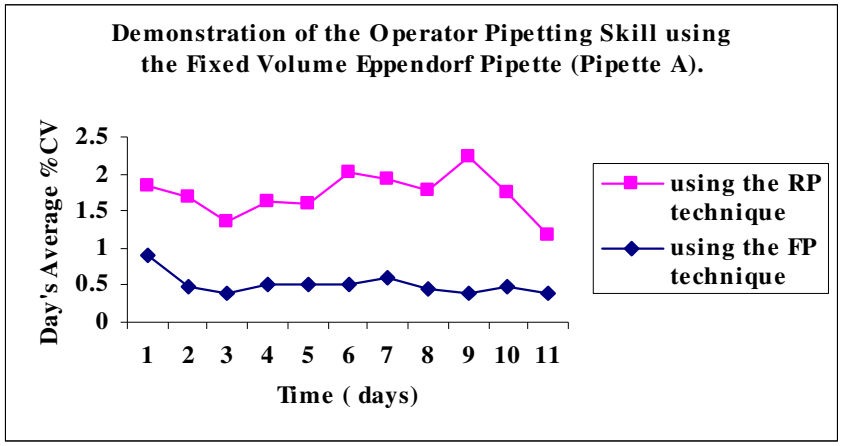


Figure 4. 1: A Figure Demonstrating the Improvement of an Operator's Pipetting Skill over Time.

Table (4.2) summarises the volumes measured and %CVs obtained by the operator using the FP technique with pipette A, B, and C.

Table 4. 2: Summary of the mean blood volumes and % CVs obtained for the pipettes used in the study

Laboratory	Pipette	N	Set to Measure	Forward Pipetting		Forward Pipetting	
				Range (µl)	Mean Volume (µl)	Mean %CV	Range (%CV)
Johannesburg	A	98	100µl	93.5-100.4	96.6	0.513	0.387-0.912
	C	30	50µl	47.7-50.3	48.7	0.804	0.647-0.985
	C	30	100µl	93.3-99.2	95.9	0.670	0.570-0.902
CDC	B	91	50µl	44.1-52.8	48.3	1.153	0.844-1.911

N: represents the number of replicate sets performed for each pipette.

4.1.1.3 Within-laboratory pipetting skills precision assessment (Precision between different operators measured separately at two different geographically located sites [Inter-operator])

i) Within-laboratory (within and between operator) precision.

A mean laboratory CD4+ count of 261cells/µl, range (225 – 293 cells/µl) was obtained when replicate CD4+ counts of the same patient sample were derived by ten different operators at the Johannesburg laboratory and a mean of 261cells/µl range (217 – 318 cells/µl) was obtained for the sample that was analysed by 10 technicians at the CDC laboratory.

Each operator's coefficient of variation was calculated using the 10 replicate CD4+ counts they each derived for the sample. Figure 4.2 shows the precision of the individual operators at the two laboratories. A mean laboratory %CV of (4.75%), with the individual %CV ranging from (2.51% – 6.20%) was obtained for the 10 operators at the Johannesburg laboratory, and a mean %CV of (7.5%), ranging from (3.61% - 9.28%) for the individual operators, obtained at the CDC laboratory. Overall, precision was better between the 10 participating technologists at the Johannesburg laboratory i.e. within-laboratory precision was better.

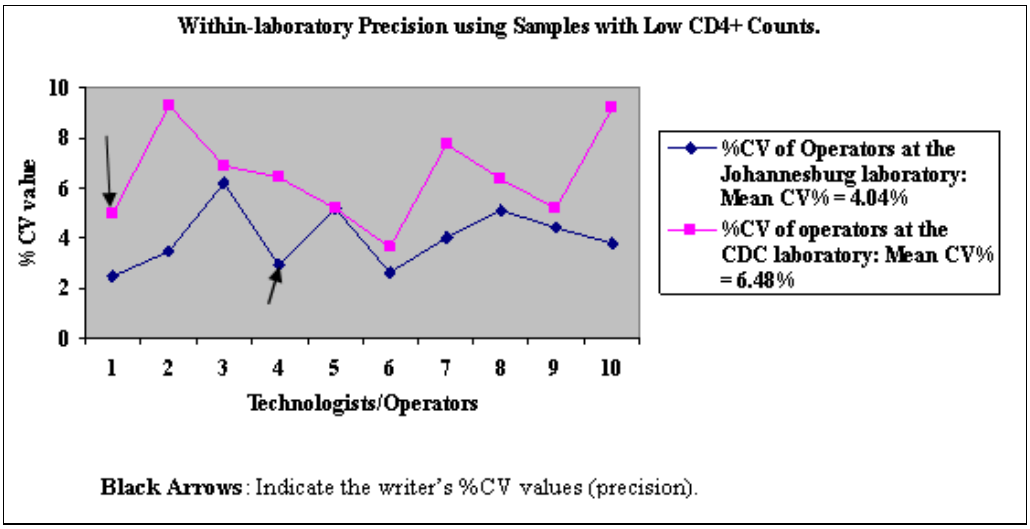


Figure 4. 2: Precision of different operators in two different laboratories using samples with low CD4+ Counts

ii) Cell count related variation (within-operator variability across strata of clinical CD4+ counts).

As expected, the assessment of within-operator variability (the writer's) at the Johannesburg laboratory showed that less precision (highest variation) in replicate CD4+ cell counts was noted in the sample that had a low CD4+ count; with a %CV of (8.46%) and (4.95%) after adjusting for an outlier reading caused by a pipetting error. Also as expected, better precision (%CV = 2.92%) was observed for CD4+ counts of the sample with a medium count (300-500cells/µl) and the least variation (%CV = 2.83%) was observed in CD4+ counts of the sample with a high CD4+ count (> 600cells/µl).

At the CDC laboratory where this assessment was carried out by a different operator, the highest variability was again observed in replicate CD4+ counts of the sample with a low CD4+ count [%CV = (9.19%), and (6.44%) after adjusting for an outlier reading most probably caused by a pipetting error too]. Less variation (5.41%) was observed in CD4+ counts of the sample with a high CD4+ count and the least variation (%CV = 5.20%) observed for the sample with the medium CD4+ count. Overall, greater precision of measurements was demonstrated by the operator/writer in Johannesburg using the FP technique.

4.1.2 Assessment of the Bias introduced when Samples prepared using the Same Manufacturer System were Analysed on the Different Models of Flow Cytometers from the Same Manufacturer.

The aim of this part of the study was to assess the contribution made by platform alone to bias in CD4 reporting (sample preparation, protocol set-up and gating strategy were identical and instruments were from the same manufacturer but different models of flow cytometers were used).

Two sets of data were also used in this analysis. In the first, CD4+ counts of 61 samples processed using the 2-colour PLG-CD4 method and analysed on the Epic-XL then immediately re-analysed using the same protocol on the FC-500, were compared.

The second data set used was that of the 41 samples analysed using the TetraONE protocol in which a 4-colour CD45^{bright} lymphocyte gated and 2-colour PLG gated CD4+ count were obtained simultaneously in a single analysis on the same sample. All samples were first analysed on the Epic-XL and thereafter, immediately re-analysed on the FC-500.

Significant differences were observed between the CD4+ counts of all three comparisons confirming that the two instruments/models from the same manufacturer (BC) showed intrinsic differences and could therefore not be used interchangeably for generating CD4+ counts . A summary of the results of this assessment are shown in Table 4.3.

A slightly higher correlation between CD4+ counts derived on the different models of instruments was obtained for the data set of the 41 samples which were processed by a single operator (the writer) [rho = 0.9909; mean difference = -15.24 cells/µl and rho = 0.9873; mean difference = -22.27 cells/µl] than for the CD4+ counts of the 61 samples that were processed by different operators [rho = 0.9597; mean difference = -39.20 cells/µl].

Table 4. 3: Assessment of variation between CD4+ Counts derived using the same system, protocol set-up and gating strategies but on different models of flow cytometers from the same manufacturer

Gating Strategies/Instruments	CD4 Range	N	Mean Diff (cells/µl)	95% CI	LOA	p-value	rho
2-colour PLG on Epics-XL vs. 2-colour PLG on FC-500	10 - 1009	61	-39.20[§]	(-52.13, -26.27)	[61.98, -139.98]	<0.0001	0.9597
CD45^{bright} gating on Epics-XL vs. CD45^{bright} gating on FC-500	32 - 906	41	-15.24*	(-21.8, -8.7)	[26.02, -56.50]	<0.0001	0.9909
2-colour PLG on Epics-XL vs. 2-colour PLG on FC-500	34 - 928	41	-22.27*	(-29.0, -15.5)	[20.45, -64.99]	<0.0001	0.9873

Abbreviations: **Mean Diff** - Mean difference in CD4+ count between instruments; **95% CI** - 95% Confidence Intervals for the mean difference; **LOA** - Limits of agreement; **p-value** - for the mean difference.
***** Single operator; [§] Several operators.

4.1.3 Assessment of the Bias introduced when Samples are prepared using the same Manufacturer System and Analysed on the same Flow Cytometer but using a Different Gating Strategy.

The aim of this part of the study was to assess the contribution made by gating alone to bias in CD4 reporting (all other known variables were identical including sample preparation, instrument settings and data acquisition). Two data sets were used in this analysis. In the first set, the CD4+ counts of 41 samples derived using the TetraONE protocol to generate both a 4-colour CD45^{bright} lymphocyte gated CD4+ count and a 2-colour PLG count in a single analysis on the Epics-XL (BC) and then on the FC-500 (BC), were compared for each instrument (i.e. 4-colour CD45^{bright} lymphocyte gated CD4+ counts vs. 2-colour PLG gated counts on the Epics-XL; 4-colour CD45^{bright} lymphocyte gated CD4+ counts vs. 2-colour PLG gated counts on the FC-500). The second data set comprised of the baseline CD4+ counts of the 20 samples drawn at the Johannesburg laboratory in the new special 2ml blood stabilisation tubes and processed following the 4-colour MS/TC protocol on the FACSCalibur. Listmode data files of these latter analyses were retrospectively re-analysed using a 2-colour PLG gating strategy. The results of these two sets of analyses are shown in Table 4.4.

Table 4. 4: Assessment of variation between CD4 counts derived using the same CD4 method but different gating strategies on the same instrument.

Instrument	Protocols	N	CD4 Range	Mean Diff (cells/µl)	95% CI	LOA	p-value	rho
Epics XL	4-colour CD45^{bright} – 2-colour PLG	41	35 - 860	-7.12	(-10.14, -4.11)	[11.98, -26.22]	<0.0001	0.9979
FC-500	4-colour CD45^{bright} – 2-colour PLG	41	32 - 928	-14.15	(-17.79, -10.50)	[8.96, -37.26]	<0.0001	0.9957
FACSCalibur	4-colour MS/TC – 2-colour PLG	20	54 - 774	-9.80	(-15.33, -4.27)	[13.84, -33.44]	0.0015	0.9925

Abbreviations: **Mean Diff** - Mean difference in CD4+ count between instruments; **95% CI** - 95% Confidence Intervals for the mean difference; **LOA** - Limits of agreement; **p-value** - for the mean difference.

Although the mean difference of CD4+ counts between these two gating strategies was statistically significant [i.e. (–7.12 cells/µl, p = <0.0001) on the Epics-XL; (–14.15 cells/µl, p = <0.0001) on the FC-500 and (-9.8 cells/µl, p = 0.0015) on the FACSCalibur], it would

not be clinically significant and can be interpreted to mean a constant but tight (small) difference between the two gating strategies.

Determination of the intra-class correlation coefficient indicated that the derived CD4+ counts were highly correlated; rho = 0.9979 between the two gating strategies on the Epics-XL; rho = 0.9957 on the FC-500 and rho = 0.9925 on the FACSCalibur.

4.1.4 Assessment of the Bias introduced through use of Different Manufacturer Sample Preparation Systems and Applying the Same Gating Strategy (CD45^{bright} Gating).

The aim of this part of the study was to mimic a real-life situation where two laboratories using completely different systems for CD4 preparation but similar gating strategies, can result in bias/differences of CD4 reporting. In this analysis, the mean CD4+ counts of 41 samples processed following the 4-colour TetraONE protocol on both the Epics–XL and FC-500 flow cytometers, and the 4-colour MS/TC protocol on the FACSCalibur, were compared (observations summarised in Table 4.5). The CD4+ count range was (35 – 843 cells/µl) on the Epics-XL, (32 – 906 cells/µl) on the FC-500 and (36 – 770 cells/µl) on the FACSCalibur. CD4+ counts derived on the FACSCalibur were closer to counts derived on the Epics-XL than to CD4+ counts derived using the FC-500.

Table 4. 5: Assessment of bias (Bland Altman analysis) in CD4+ counts derived using different protocols that use a similar gating strategy (CD45^{bright} lymphocyte gating) and different manufacturers' flow cytometers.

Instruments	CD4 Range	N	Mean Diff (cells/µl)	95% CI	LOA	p-value	rho
FACSCalibur - Epics XL	35 - 843	41	-2.59	(-13.3, 8.1)	[65.03, -70.21]	0.6271	0.9831
FACSCalibur - FC500	32 - 906	41	-17.83	(-28.3, -7.4)	[48.17, -83.83]	0.0013	0.9806

Abbreviations: **Mean Diff** - Mean difference in CD4 counts between instruments; **95% CI** - 95% Confidence Intervals for the mean difference; **LOA** - Limits of agreement; **p-value** - for the mean difference.

Bland-Altman analysis showed the mean difference in count and 95% confidence intervals between the FACSCalibur using 4-colour MS/TC and Epics-XL using the 4-colour

TetraONE protocol to be (–2.59[CI; -13.3, 8.1] and the limits of agreement (LOA) (65.03, -70.21). This difference was not significant ($p = 0.6271$).

However, the mean difference between the same patient CD4+ counts derived using the 4-colour MS/TC on the FACSCalibur and using the 4-colour TetraONE on the FC-500 (bias = -17.83 cells/µl) was significantly different from zero ($p = 0.0013$) implying that these two instruments could not be used interchangeably for reporting CD4 counts in the same laboratory.

As expected, because all instruments essentially measured the same parameter viz. CD4+ counts, determination of the intra-class correlation coefficient (rho), demonstrated a high correlation of the CD4+ counts between the different protocols performed on different flow cytometers; rho = 0.9831 between CD4+ counts derived using the 4-colour MS/TC on the FACSCalibur and the 4-colour TetraONE on the Epics-XL, and rho = 0.9806 between CD4+ counts derived using the 4-colour MS/TC on the FACSCalibur and the 4-colour TetraONE protocol on the FC-500.

4.1.5 Assessment of the Bias introduced through use of Different Manufacturer Sample Preparation Systems including Application of Different Gating Strategies and Different Flow Cytometeric Threshold for Data Acquisition.

The aim of this part of the study was to mimic a real-life situation where two laboratories using completely different systems for CD4 reporting but participating in the same network to support the same treatment programme, can result in differences in CD4 reporting and hence influence patient management according to CD4 method used. In this assessment, 41 CD4 results generated using the 4-colour MS/TC (BDB) were compared to CD4+ counts derived using the 2-colour PLG (BC) protocol on both the Epic-XL and FC-500 flow cytometers [see section 4.1.4]. A CD4+ count range of (35 – 860 cells/µl) was noted on the Epics-XL, a range of (34 – 928 cells/µl) noted on the FC-500 and a range of (36 – 770

cells/µl) noted on the FACSCalibur. The CD4+ counts derived on the FACSCalibur were again closer to counts derived on the Epics-XL than to CD4+ counts derived using the FC-500. Table 4.6 shows the results of this analysis.

Table 4. 6: Assessment of bias (Bland Altman analysis) of CD4+ counts derived using different manufacturers' flow cytometers, preparation systems and gating strategies: BC PLG versus BDB CD45^{bright} gated MS/TC.

Instruments	CD4 Range	N	Mean Diff (cells/µl)	95% CI	LOA	p-value	rho
FACSCalibur - Epics XL	35 - 860	41	-9.71	(-19.8, 0.41)	[54.41, -73.83]	0.0596	0.9838
FACSCalibur - FC500	34 - 928	41	-31.98	(-44.1, -19.9)	[44.60, -108.56]	<0.0001	0.9665

Abbreviations: **Mean Diff** - Mean difference in CD4+ count between instruments; **95% CI** - 95% Confidence Intervals for the mean difference; **LOA** - Limits of agreement; **p-value** - for the mean difference.

Bland-Altman analysis showed the mean difference in CD4+ count and 95% confidence intervals between the FACSCalibur using 4-colour MS/TC and Epics-XL using 2-colour PLG gating to be (–9.71[CI; -19.83, 0.41]) and the LOA (54.41, -73.83). The difference in CD4+ counts between these two machines showed borderline statistical significance (p = 0.0596). Use of these two different gating strategies appears to have slightly increased the bias between the two methods i.e., from a mean bias of -2.59 cells/µl or -17.83 cells/µl when both systems employed a CD45^{bright} gating strategy (Table 4.5), to a mean bias of -9.71 cells/µl or -31.98 cells/µl when different gating strategies (either CD45^{bright} or PLG) were used. However, this increase in bias could also be a reflection of differences in the flow cytometric acquisition and analysis.

CD4+ counts derived using 4-colour MS/TC on the FACSCalibur and counts derived using 2-colour PLG gating on the FC-500 were significantly different (p <0.0001) implying that these two different manufacturer sample preparation systems with different gating strategies, should not be used interchangeably for analysis of the same samples.

The correlation between 4-colour and 2-colour PLG CD4+ counts from the FACSCalibur and the FC-500 respectively was slightly lower (rho = 0.9665) than that observed when both systems employed a CD45^{bright} gating. However, once again, the correlation between

CD4+ counts derived using the 4-colour MS/TC on FACSCalibur and the 2-colour PLG on the Epics-XL was high (rho= 0.9838).

4.2 Analyses performed to Assess Impact of Logistical Factors including Time from Venesection, handling during Transportation and Temperature on Bias of CD4+ Reporting.

There are several factors that can affect the integrity of a sample prior to it getting to the laboratory for testing. These include the temperature at which the sample is stored or held, motion of the sample (typically during transportation of the sample to the laboratory) and time from venesection. The effect of these three factors was assessed using 32 samples processed following the 4–colour TetraONE method at the Johannesburg laboratory and using 55 samples processed following the 4–colour MS/TC method at the CDC laboratory. A single CD4 method was used at each site to enable meaningful assessment of impact of either motion, temperature or time from venesection on precision of CD4 counting.

4.2.1 Assessing the effect of Motion on Whole Blood Sample Integrity.

Samples were kept in motion on a blood mixer or rocker to mimic a situation were samples may be damaged (haemolysed) during transit in a vehicle to a laboratory.

Table (4.7) below summarises the CD4+ count results of the samples analysed using the 4-colour TetraONE protocol at the Johannesburg laboratory.

The mean absolute CD4+ count of the 32 samples analysed at 12-24 hours from venesection (baseline reading) was 306 cells/µl and the range (13 – 1237 cells/µl) but this dropped to a mean of 205 cells/µl, range (5 – 961 cells/µl) by Day 5.

Table 4. 7: Summary of the mean change in CD4+ counts of samples kept in motion and derived using the 4-colour TetraONE protocol at the Johannesburg laboratory

Day post venesection	Age of samples (hours)	No. of Samples	CD4 Count Range	Mean CD4 count	SD	LOA
Baseline (~1)	12-24	32	13 - 1237	306	223.56	
≥1-2	36-48	31	12 - 1133	284	209.44	(702.88, -134.88)
≥2-3	60-72	32	14 - 1152	273	211.92	(696.84, -150.84)
≥3-4	84-96	32	9 - 1000	249	192.38	(633.76, -135.76)
≥4-5	108-120	32	7 - 936	213	174.67	(562.34, -136.34)
≥5-6	132-144	32	5 - 961	205	174.25	(553.5, -143.50)
≥6-7	156-168	28*	6 - 952	194	181.34	(556.68, -168.68)
≥7-8	180-192	21*	4 - 786	169	171.01	(511.02, -173.02)
≥8-9	204-216	5*	3 - 592	164	243.83	(651.66, -323.66)

***** Lack of sufficient aliquot volumes for testing resulted in fewer numbers of samples available for testing after the Day 5 reading (> than 132 hours); the number of samples that could be statistically analysed reduced to 28, 21 and to 5 on testing days ≥6-7, ≥7-8 and ≥8-9 respectively (EDTA tubes are typically only filled with between 3ml and 5ml of blood). The aliquot volumes made were dependent on the original patient sample volumes venesected and sent to the laboratory. If a small sample volume had been sent, a small aliquot (< 850µl) was all that could be obtained which restricted the number of times for re-testing.

Table (4.8) below shows details of how long samples kept in motion on a rocker at room temperature (17 – 23ºC) could be kept before preparation and analysis (Johannesburg laboratory). When the >90% integrity limit was applied, samples could be used to derive precise and accurate CD4+ counts for up to 48 hours from venesection (only 1 day from receipt into the laboratory and up to 2 days from venesection, $p = 0.0015$). However, when the >95% limit ($p = 0.4557$) or the mean difference limit of <40 cells/µl ($p = 0.2453$), or the limit of <20 cells/µl ($p = 0.7642$) were applied, samples could not be used beyond 24 hours from venesection or the day of receipt in the laboratory. Beyond these specified days, CD4+ counts were significantly different from baseline counts (i.e. $p > 0.05$) suggesting that sample handling and motion could affect the sample integrity.

Table 4. 8: Change in mean CD4+ counts as a ratio of baseline and mean difference to baseline for samples kept in motion and derived using the 4-colour TetraONE protocol

Day post venesection	Ratio of Mean Day CD4 Count to Baseline as a (%)	SD	p value* at Cut-off		Bias	SD	95% CI	p value** at Cut-off	
			90%	95%				40cells/µl	20cells/µl
≥1-2	95.18	8.96	0.0015	0.4557	30.22	79.32	(1.6, 58.8)	0.2453	0.7642
≥2-3	89.04	9.35	0.717	0.9995	32.69	34.12	(20.4, 45.0)	0.1172	0.9782
≥3-4	80.85	16.67	0.998	1	56.97	65.27	(33.4, 80.5)	0.9242	0.9983
≥4-5	68.15	13.61	1	1	92.53	70.38	(67.2, 117.9)	0.9999	1
≥5-6	64.82	15.19	1	1	100.88	73.13	(74.5, 127.2)	1	1
≥6-7	64.97	45.49	0.9964	0.9992	131.27	229.41	(45.6, 216.9)	0.9812	0.9936
≥7-8	51.44	14.99	1	1	145.95	109.63	(96.1, 195.9)	0.9999	1
≥8-9	46.96	15.80	0.9982	0.9988	169.4	269.06	(-164.7, 503.5)	0.8286	0.8589

Clinical significance was defined as values > 0.05. When the p value is significant i.e. < 0.05, accept H_1 i.e. observed CD4 ratio exceeds 90% or 95 % and thus the associated day of storage is still acceptable;
SD - Standard Deviation; **CI** – 95% Confidence Interval.
* $\mathbf{H_0}$: CD4 Ratio = 90% (95%) versus $\mathbf{H_1}$: CD4 Ratio > 90% (95%)
** $\mathbf{H_0}$: the Mean difference in CD4+ counts = 40 cells/µl (20cells/µl) versus $\mathbf{H_1}$: Mean difference in CD4 + counts is < 40cells/µl (20cells/µl).

Corresponding CD4% values (data in Table 4.9) showed that samples could be reliably analysed up to 72 hours from venesection (or 2 days from receipt in the laboratory) [95% CI = (98.6, 104.8)].

Table 4. 9: Change in mean CD4% as a ratio of baseline over days for samples kept in motion and derived using the 4-colour TetraONE protocol

Day post venesection	Age of samples (hours)	Ratio of Mean CD4% to Baseline as a (%)	SD	95% CI	p value
≥1-2	36-48	100.08	5.99	(97.9, 102.3)	<0.0001
≥2-3	60-72	101.72	8.58	(98.6, 104.8)	0.0001
≥3-4	84-96	107.16	12.77	(102.6, 111.8)	<0.0001
≥4-5	108-120	123.39	26.62	(113.8, 133.0)	<0.0001
≥5-6	132-144	143.97	90.19	(111.5, 176.5)	0.0022
≥6-7	156-168	150.52	89.54	(115.8, 185.2)	0.0014
≥7-8	180-192	170.69	117.51	(115.7, 225.7)	0.0048
≥8-9	204-216	450.26	608.72	(-518.4, 1418.9)	0.1637

Further analysis was performed to assess whether use of the simplified 2-colour PLG gating strategy could extend the window of testing noted above (in samples that had been subjected to ongoing motion). No difference in time to testing was observed between the gating strategies except when the <40 cells/µl limit was applied (data shown in Appendix C). The 2-colour PLG gating strategy increased the window of testing to 72 hours from venesection (2 days from time of receipt of the samples into the laboratory or 3 days from

venesection) (p = 0.0443). This effect was however not observed when corresponding CD4% values were analysed. Merging of the $CD45^{bright}$ population with nearby monocytes resulted in the samples not being suitable for use beyond 1 day from receipt into the laboratory (or 48 hours from venesection) [95% CI = (99.9, 104.2)] (data shown in Appendix C).

The following figure (4.3) shows the deterioration in sample integrity over 8 days of follow-up of a sample kept in in motion at the Johannesburg laboratory.

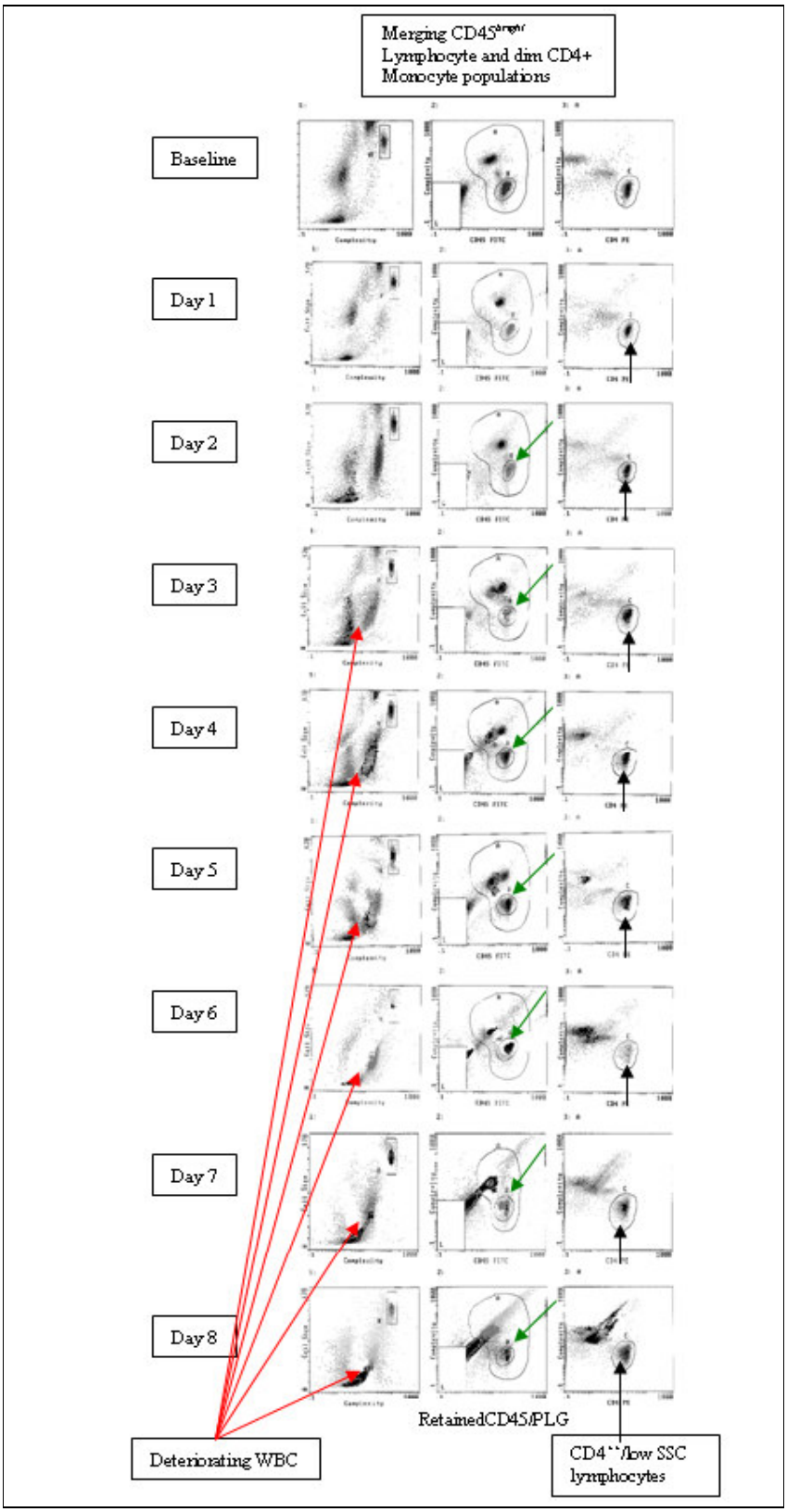


Figure 4. 3: A figure illustrating the deterioration in sample integrity over time for a sample kept in motion and derived using the 2-colour PLG protocol (Sample #31 was chosen as it included the least amount of fluorescent debris in the PLG gate).

Table (4.10) shows the changes in the CD4+ counts of the 55 samples that were kept in motion on a mixer and were analysed using the 4-colour MS/TC protocol (CDC laboratory). All 55 samples analysed at baseline had sufficient volumes for follow-up on all 8 days. A mean CD4+ count of 344 cells/µl, ranging from (17 – 814 cells/µl) was obtained at baseline and a mean of 205 cells/µl, range (11 – 634 cells/µl) obtained by Day 8.

Table 4. 10: Summary of the mean change in CD4+ counts of samples kept in motion and derived using the MS/TC protocol at the CDC laboratory

Day post venesection	Age of samples (hours)	No. of Samples	CD4 Count Range	Mean CD4 Count	SD	LOA
Baseline	<6	55	17 - 814	344	195.51	
1	24	55	13 - 854	337	198.83	(734.66, -60.66)
2	48	55	12 - 792	312	182.98	(677.96, -53.96)
3	72	55	13 - 697	302	167.99	(637.98, -33.98)
4	96	55	15 - 672	287	167.66	(622.32, -48.32)
5	120	55	16 - 673	273	160.55	(594.10, -48.10)
6	144	55	13 - 672	256	161.99	(579.98, -67.98)
7	168	55	9 - 724	235	150.37	(535.74, -65.74)
8	192	55	11 - 634	205	145.96	(496.92, -86.92)

Table 4.11 summarises the results obtained using the 4-colour MS/TC protocol. These were similar to those obtained at the Johannesburg laboratory. Samples could only be used to derive precise and accurate CD4+ counts for up to 24 hours (only 1 day from receipt into the laboratory) when the >90% integrity limit was used and only on the day of receipt into the laboratory if the >95% integrity limit was applied. Samples could however still be analysed by day 1 ($p < 0.0001$) when the <40cells/µl limit and the <20cells/µl limit were applied ($p = 0.0158$).

Table 4. 11: Change in mean CD4 counts as a ratio of baseline and mean difference to baseline for samples kept in motion, and derived using the 4-colour MS/TC protocol

Day post venesection	Ratio of Mean Day CD4 Count to Baseline as a (%)	SD	p value at Cut-off		Bias	SD	95% CI	p value at Cut-off	
			90%	95%				40cells/µl	20cells/µl
1	97.66	12.63	<0.0001	0.0623	6.25	46.20	(-6.2, 18.8)	<0.0001	0.0158
2	90.53	11.57	0.3688	0.9971	31.40	43.38	(19.7, 43.1)	0.0737	0.9717
3	89.65	13.55	0.5766	0.9975	41.18	50.83	(27.4, 54.9)	0.5681	0.9984
4	83.58	12.85	0.9997	1	56.80	53.75	(42.3, 71.3)	0.9879	1
5	80.02	14.32	1	1	70.38	64.75	(52.9, 87.9)	0.9995	1
6	74.26	17.16	1	1	87.60	79.16	(66.2, 109.0)	1	1
7	67.90	16.18	1	1	108.82	89.46	(84.6, 133.0)	1	1
8	58.26	16.93	1	1	138.20	97.87	(111.7, 164.7)	1	1

Corresponding CD4% values (Table 4.12) showed that sample integrity had deteriorated by day 1 and that samples could therefore not be analysed beyond the day of receipt into the laboratory.

Table 4. 12: Change in mean CD4% as a ratio of baseline over days for samples kept in motion and derived using the MS/TC protocol

Day of venesection	Age of samples (hours)	No of Samples	Ratio of Mean CD4% to Baseline as a (%)	SD	95% CI	p-value
1	24	55	102.71	9.12	(100.3, 105.2)	<0.0001
2	48	55	105.13	11.16	(102.1, 108.2)	<0.0001
3	72	55	107.58	12.07	(104.3, 110.8)	<0.0001
4	96	55	108.25	12.44	(104.9, 111.6)	<0.0001
5	120	55	112.66	14.24	(108.8, 116.5)	<0.0001
6	144	55	113.32	16.65	(108.8, 117.8)	<0.0001
7	168	55	116.76	18.86	(111.7, 121.9)	<0.0001
8	192	55	117.12	20.54	(111.6, 122.7)	<0.0001

Figure 4.4 shows the deterioration in sample integrity over 8 days of follow-up of a sample kept in in motion at the CDC laboratory.

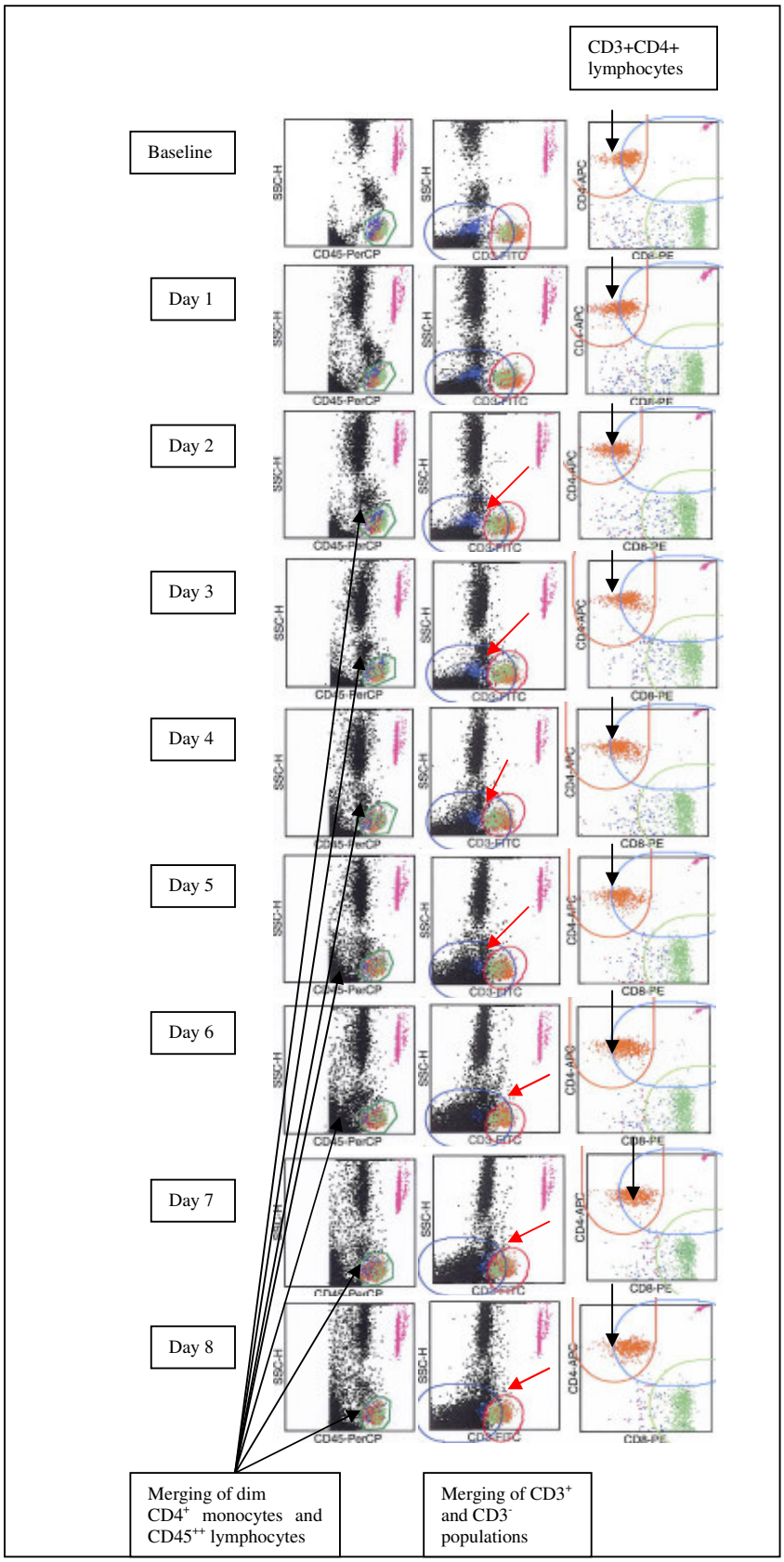


Figure 4. 4: A figure illustrating the deterioration in sample integrity over time for a sample kept in motion and analysed using the 4-colour MS/TC Protocol.

Figure 4.5 and 4.6 below, show the changes in CD4+ counts over time for blood samples kept in motion at the two laboratories.

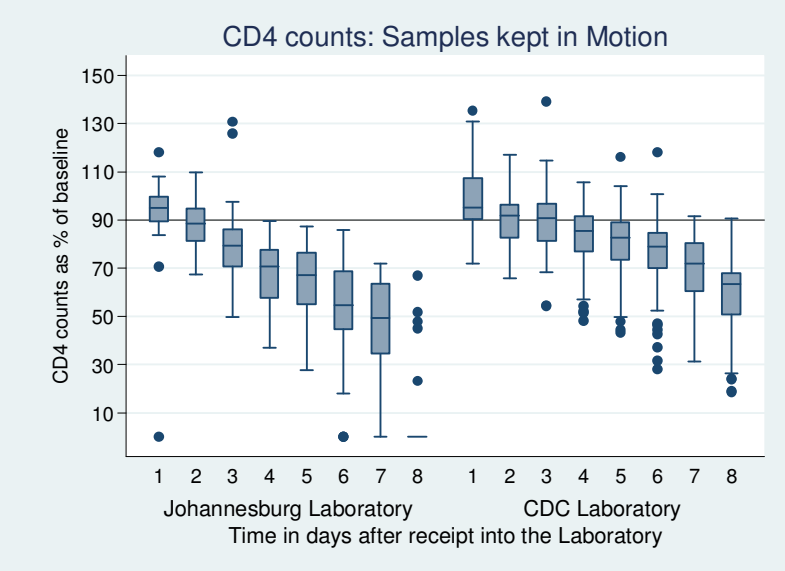


Figure 4. 5: Change in CD4+ Counts as a ratio of baseline over days for samples kept in motion and derived using the 4-colour TetraONE protocol at the Johannesburg Laboratory or using 4-colour MS/TC at the CDC laboratory
Legend: Horizontal line at 90 – shows the sample Integrity Limit set at 90% similarity of Day reading to Baseline.**Days labeled 1-8 for Johannesburg laboratory have actual estimated respective ages of** ≥1-2, ≥2-3, ≥3-4, ≥4-5, ≥5-6, ≥6-7, ≥7-8 and ≥8-9 days.

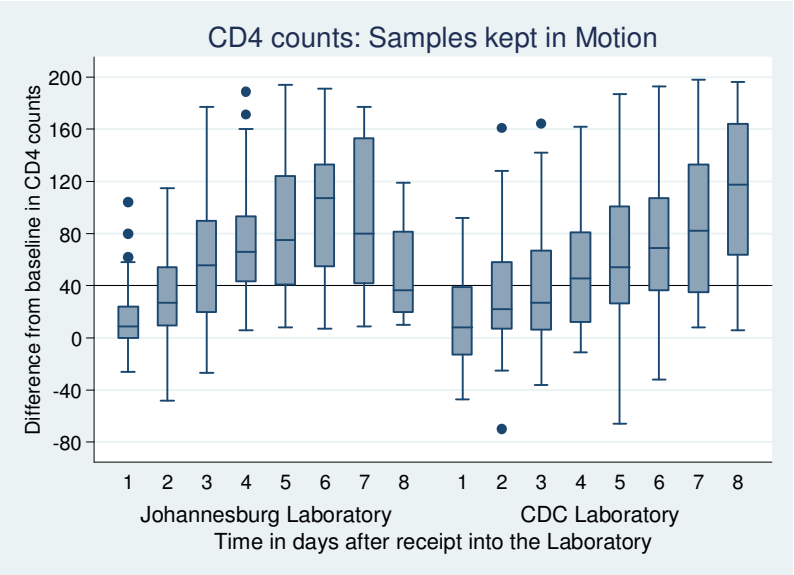


Figure 4. 6: Mean difference in CD4+ Counts to baseline over days for samples kept in motion and derived using the 4-colour TetraONE protocol at the Johannesburg Laboratory or using the 4-colour MS/TC at the CDC Laboratory
Legend: Horizontal line at 40 – shows the sample Integrity Limit set at a mean difference of 40cells/µl between day and baseline readings. **Days labeled 1-8 for Johannesburg laboratory have actual estimated respective ages of** ≥1-2, ≥2-3, ≥3-4, ≥4-5, ≥5-6, ≥6-7, ≥7-8 and ≥8-9 days.

A fast decline in mean CD4+ counts over the 8 days from baseline was observed of the samples analysed at both laboratories. The difference in the rate of sample disintegration at the two laboratories may have been caused by the difference in the age of the samples used and how the samples were kept in motion. Samples analysed at the Johannesburg laboratory were older at baseline (about 12-24 hours old versus 6 hours old samples used at the CDC laboratory) and possibly disintegrated faster since they had been kept in motion on a rocker which movement was more vigorous than if a mixer had been used.

4.2.2 Assessing the effect of Temperature on Whole Blood Sample Integrity.

4.2.2.1 Storage of Blood Samples at 37°C in a Water Bath

In order to assess the effect warmer temperatures could have on the integrity of blood, samples were kept at 37°C in a water bath to mimic ambient temperatures in some African countries.

Tables 4.13 and 4.14 provide an overview of CD4+ count results derived using the 4-colour TetraONE protocol at the Johannesburg laboratory. Flow cytometric analyses of samples kept in a water bath at 37ºC showed that sample integrity had deteriorated significantly a day after baseline. Blood samples were clearly hemolysed by day 2 and follow-up was discontinued on day 3 in order to prevent unnecessary blockages in the flow cytometer that could result from analysing markedly disintegrated samples.

Table 4. 13: Summary of the mean change in CD4+ counts of samples kept at 37°C in a water bath and derived using the 4-colour TetraONE protocol at the Johannesburg laboratory

Day post venesection	Age of samples (hours)	No. of Samples	CD4 Count Range	Mean CD4 count	SD	LOA
Baseline (~1)	12-24	32	13 - 1237	306	223.56	
≥1-2	36-48	31	18 - 1043	237	190.42	(617.84, -143.84)
≥2-3	60-72	30	5 - 590	214	126.46	(466.92, -38.92)
≥3-4	84-96	23	5 - 468	125	95.82	(316.64, -66.64)

Table 4. 14: Change in mean CD4+ counts as a ratio of baseline and mean difference to baseline for samples kept at 37°C in a water bath and derived using the 4-colour TetraONE protocol

Day post venesection	Ratio of Mean Day CD4Count to Baseline as a (%)	SD	p value at Cut-off		Bias	SD	95% CI	p value at Cut-off	
			90%	95%				40cells/µl	20cells/µl
≥1-2	79.36	16.80	0.9993	1	76.16	81.27	(46.9, 105.5)	0.9914	0.9998
≥2-3	73.53	17.40	1	1	100.77	117.76	(57.6, 144.0)	0.9963	0.9997
≥3-4	48	24.45	1	1	175.5	168.36	(107.5, 243.5)	0.9998	1

Significant differences from baseline CD4+ counts were observed as early as Day 1 (within 24 hours from venesection). Similar observations were made when CD4 % values were used (Table 4.15) and when the data was analysed using the 2-colour PLG gating strategy (data shown in Appendix D).

Table 4. 15: Change in mean CD4% as a ratio of baseline over days for samples kept at 37°C and derived using the 4-colour TetraONE protocol

Day post venesection	Age of samples (hours)	No of Samples	Ratio of mean CD4% to Baseline as a (%)	SD	95% CI	p value
≥1-2	36-48	31	103.54	12.31	(99.0, 108.1)	0.0003
≥2-3	60-72	30	93.10	18.43	(86.2, 100.0)	0.07119
≥3-4	84-96	23	130.23	42.24	(112.0, 148.5)	0.0003

Figure 4.7 shows the deterioration in sample integrity over 8 days of follow-up of a sample kept in a Water Bath at 37°C.

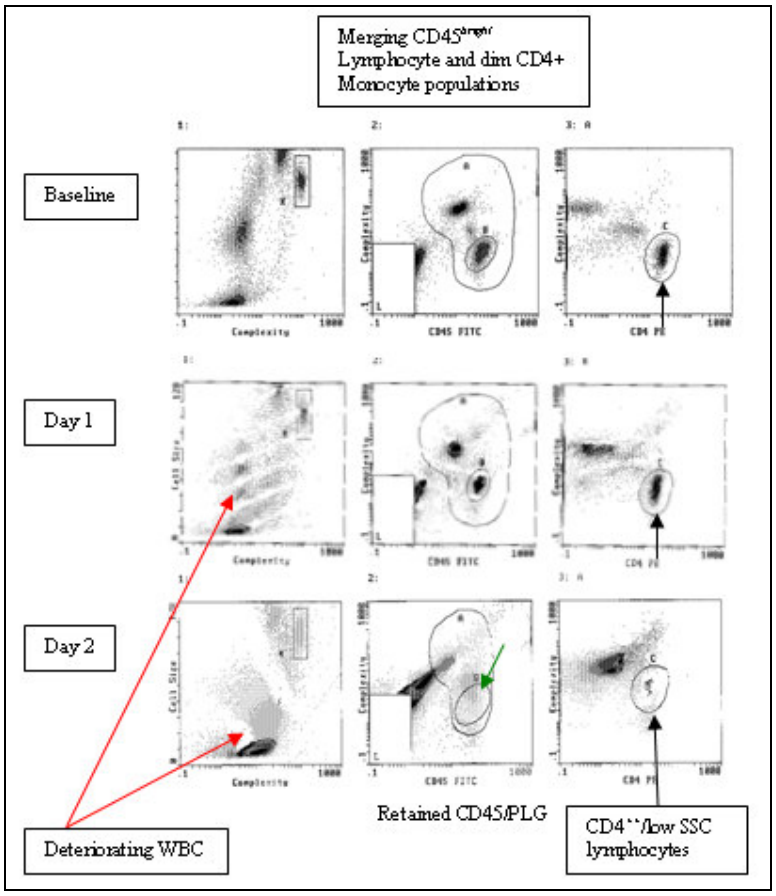


Figure 4. 7: A figure illustrating the deterioration of sample integrity over time for a sample kept in a water bath at 37°C and derived using the 2-colour PLG protocol (sample #31 was chosen as it included the least amount of fluorescent debris in the PLG gate).

Table 4.16 below gives an overview of the results obtained for the 55 samples kept at 37°C in a water bath at the CDC laboratory.

Similar to observations made at the Johannesburg laboratory, samples kept at this temperature were hemolysed by day 2 so follow-up was discontinued.

Table 4. 16: Change in mean CD4+ counts of samples kept in a water bath at 37°C and derived using the 4-colour MS/TC protocol at the CDC laboratory

Day post venesection	Age of samples (hours)	No. of Samples	CD4 Count Range	Mean CD4 count	SD	LOA
Baseline	<6	55	17 - 814	344	195.51	
1	24	55	11 - 704	298	179.40	(656.80, -60.80)
2	48	55	4 - 538	195	137.61	(470.22, -80.22)

Table 4.17 shows the CD4 count results for samples analysed following the 4-colour MS/TC protocol. These results were similar to those obtained using the 4-colour TetraONE

protocol. Samples kept at 37°C could not be used for accurate enumeration of CD4+ counts at all. On the contrary however, corresponding CD4% values (Table 4.18) showed that samples could still be analysed after being kept for a day at 37°C [95% CI = (98.0, 103.7)].

Table 4. 17: Change in mean CD4+ counts as a ratio of baseline and mean difference to baseline for samples kept at 37°C in a water bath and derived using the MS/TC protocol

Day post venesection	Ratio of Mean Day CD4 count to Baseline as a (%)	SD	p value at Cut-off		Bias	SD	95% CI	p value at Cut-off	
			90%	95%				40cells/µl	20cells/µl
1	84.29	13.26	0.9988	1.0000	45.38	42.96	(33.8, 57.0)	0.8215	1.0000
2	52.52	15.61	1.0000	1.0000	148.67	79.39	(127.2, 170.1)	1.0000	1.0000

Table 4. 18: Change in mean CD4% as a ratio of baseline over days for samples kept at 37°C in a water bath and derived using the MS/TC protocol

Day post venesection	Age of samples (hours)	No of Samples	Ratio of Mean CD4% to Baseline as a (%)	SD	95% CI	p-value
1	**24**	55	100.89	10.52	(98.0, 103.7)	0.0001
2	**48**	55	91.7	21.47	(85.9, 97.6)	0.8702

Figure 4.8 shows the deterioration in sample integrity over 8 days of follow-up of a sample kept in a Water Bath at 37°C.

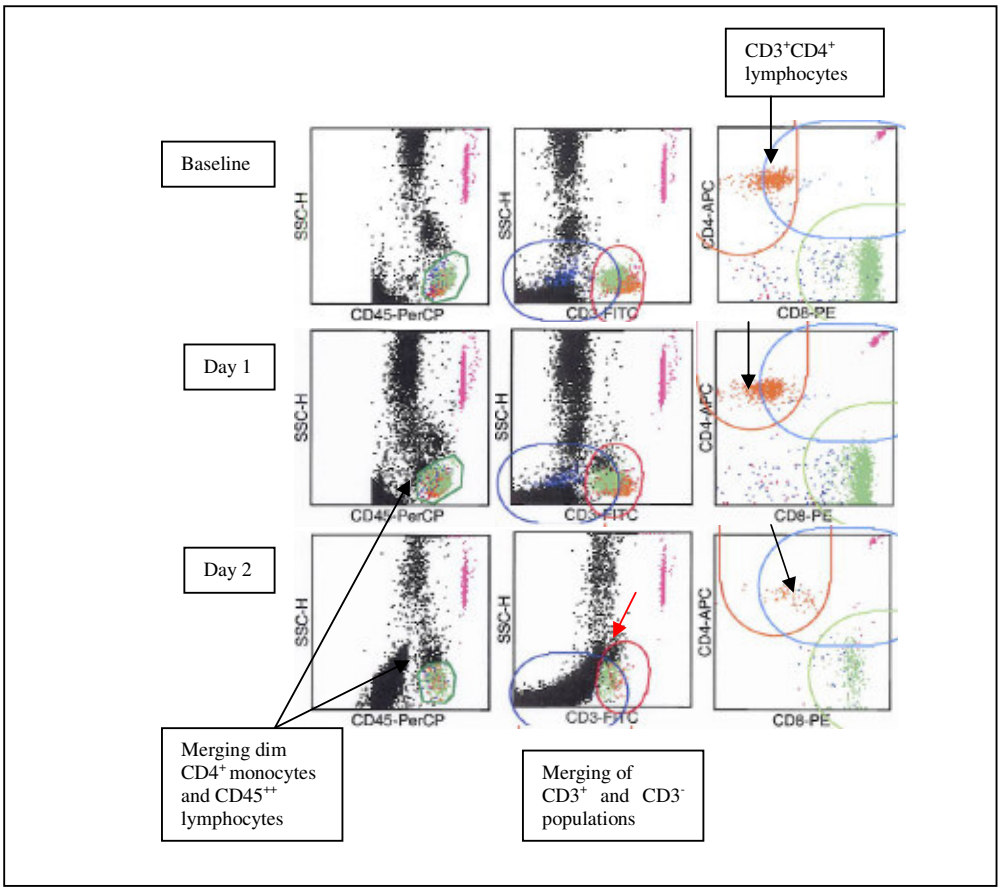


Figure 4. 8: A figure illustrating the deterioration in sample integrity over time for a sample kept in a water bath at 37°C and derived using the 4-colour MS/TC protocol

Figures 4.9 and 4.10 show the changes in CD4+ counts over time for blood samples kept at 37ºC at the two laboratories.

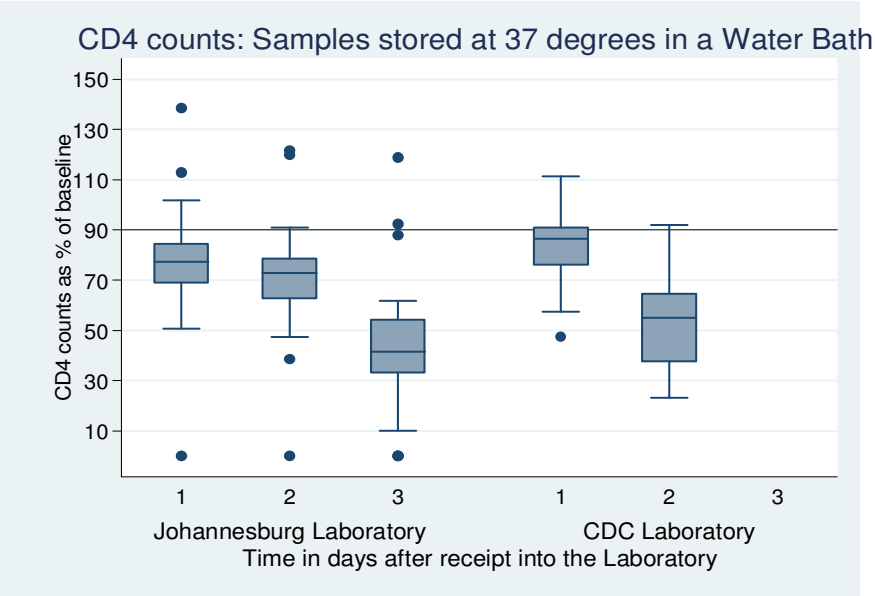


Figure 4. 9: Change in CD4+ counts as a ratio of baseline over days for samples stored at 37°C in a water bath and derived using the 4-colour TetraONE protocol at the Johannesburg Laboratory or using 4-colour MS/TC at the CDC laboratory

Legend: Horizontal line at 90 – shows the sample Integrity Limit set at 90% similarity of Day reading to Baseline.**Days 1-3 for Johannesburg laboratory have actual estimated respective ages of** ≥1-2, ≥2-3 and ≥3-4 days.

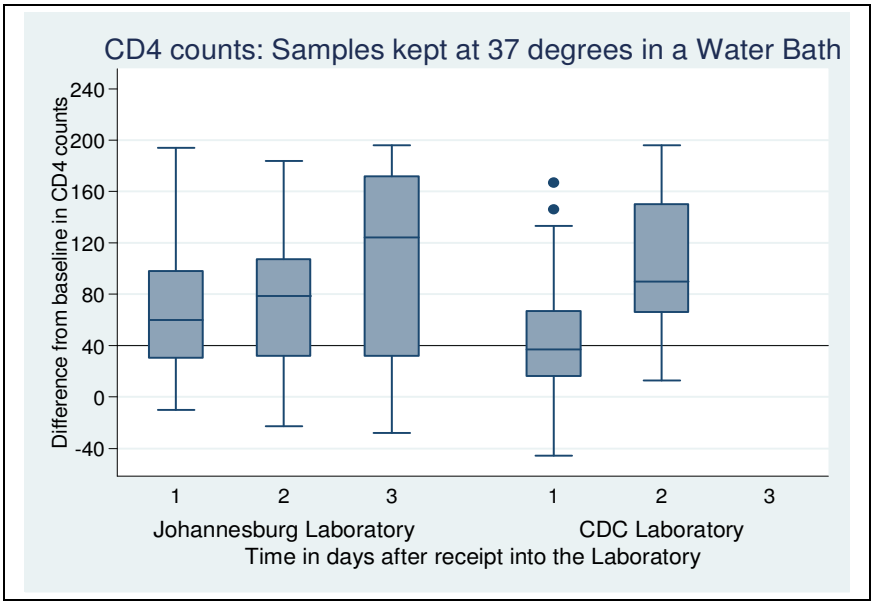


Figure 4. 10: Mean difference in CD4 Counts to Baseline over Days for samples stored at 37°C in a Water Bath and derived using the 4-colour TetraONE protocol at the Johannesburg Laboratory or using the 4-colour MS/TC at the CDC Laboratory

Legend: Horizontal line at 40 – shows the sample Integrity Limit set at a mean difference of 40 cells/µl between day and Baseline readings. **Days 1-3 for Johannesburg laboratory have actual estimated respective ages of** ≥1-2, ≥2-3 and ≥3-4 days.

4.2.2.2 Storage of Blood Samples at Room Temperature

Room temperature (RT) varies and is dependent on geographical location/region. To assess the effect different temperatures could have on sample integrity, samples were kept at RT and followed up for 8 days. RT was controlled and maintained within a specific range [between 17 - 23°C at the Johannesburg laboratory and between 20 - 23°C at the CDC laboratory] by use of air conditioners.

Tables (4.19) and (4.20) summarise the results obtained when CD4+ count data was analysed using the 4-colour TetraONE protocol at the Johannesburg laboratory. The mean absolute CD4+ count of the 32 samples analysed at baseline decreased at a slower rate than was observed when the samples were stored at a higher temperature (37°C): The mean CD4+ count was 306 cells/µl, range (13 – 1237 cells/µl) at baseline (average age of samples ≥12-24 hours old) and 266 cells/µl, range (10 – 1154 cells/µl) by Day 5 (average age of samples ≥132-144 hours old).

Table 4. 19: Summary of the mean change in CD4+ counts of samples kept at room temperature (17-23°C) and derived using the 4-colour TetraONE protocol at the Johannesburg laboratory

Day post venesection	Age of samples (hours)	No. of Samples	CD4 Count Range	Mean CD4 count	SD	LOA
Baseline (~1)	12-24	32	13 - 1237	306	223.56	
≥1-2	36-48	31	16 - 1249	293	235.16	(763.32, -177.32)
≥2-3	60-72	32	12 - 1195	285	216.56	(718.12, -148.12)
≥3-4	84-96	32	10 - 1162	272	212.16	(696.32, -152.32)
≥4-5	108-120	32	9 - 1024	259	195.02	(649.04, -131.04)
≥5-6	132-144	32	10 - 1154	266	207.92	(681.84, -149.84)
≥6-7	156-168	29*	10 - 846	243	177.71	(598.42, -112.42)
≥7-8	180-192	23*	9 - 1059	236	218.89	(673.78, -201.78)
≥8-9	204-216	5*	15 - 967	323	379.55	(1082.10, -436.1)

* Lack of sufficient aliquot volumes for testing resulted in fewer numbers of samples available for testing after the Day 5 reading; the number of samples that could be statistically analysed reduced to 29, 23 and to 5 on testing days ≥6-7, ≥7-8 and ≥8-9 respectively.

Table 4. 20: Change in mean CD4+ count as a ratio of baseline and mean difference to baseline over days for samples stored at room temperature (17-23°C) and derived using the 4-colour TetraONE protocol

Day post venesection	Ratio of Mean Day CD4 count to Baseline as a (%)	SD	p value at Cut-off		Bias	SD	95% CI	p value at Cut-off	
			90%	95%				40cells/µl	20cells/µl
≥1-2	97.44	12.26	0.001	0.1388	22	86.95	(-9.4, 53.4)	0.1253	0.5513
≥2-3	92.99	9.28	0.0395	0.8865	20.91	19.28	(13.9, 27.9)	<0.0000	0.6039
≥3-4	87.25	10.20	0.9313	0.9999	33.78	31.23	(22.5, 45.0)	0.1343	0.991
≥4-5	82.62	11.40	0.9995	1	46.53	40.94	(31.8, 61.3)	0.8131	0.9995
≥5-6	84.85	10.26	0.996	1	40.16	30.82	(29,0, 51.3)	0.5113	0.9996
≥6-7	79.97	11.25	1	1	75.9	91.67	(42.3, 109.5)	0.9814	0.999
≥7-8	77.11	15.10	1	1	70.17	75.51	(38.3, 102.1)	0.9687	1
≥8-9	82.66	20.18	0.7692	0.8784	92.20	103.60	(-36.4, 220.8)	0.8386	0.9029

Significant differences in CD4+ counts were observed by day 3 implying that samples could only be analysed up 72 hours (2 days from time of receipt into the laboratory) when the >90% limit was applied (p = 0.9313) and within 24 hours from venesection (on the day of receipt into the laboratory) when the >95% limit (p = 0.1388) and the two mean difference limits were each applied. Corresponding CD4% data shown in Table 4.21 below also showed that accurate results were obtainable only up to 72 hours (2 days from receipt into the laboratory and up to 3 days from the time of venesection).

Table 4. 21: Change in mean CD4% as a ratio of baseline over days for samples stored at room temperature (17-23°C) and derived using the 4-colour TetraONE protocol

Day post venesection	Age of samples (hours)	No of Samples	Ratio of Mean CD4% to Baseline as a (%)	SD	95% CI	p value
≥1-2	36-48	31	100.79	6.34	(98.5, 103.1)	<0.0001
≥2-3	60-72	32	102.06	5.58	(100.1, 104.1)	<0.0001
≥3-4	84-96	32	102.57	8.20	(99.6, 105.5)	<0.0001
≥4-5	108-120	32	106.09	13.36	(101.3, 110.9)	<0.0001
≥5-6	132-144	32	107.81	11.98	(103.5, 112.1)	<0.0001
≥6-7	156-168	30	109.66	15.22	(104.0, 115.4)	<0.0001
≥7-8	180-192	23	108.31	15.6	(101.6, 115.1)	0.0002
≥8-9	204-216	5	125.07	1978	(100.5, 149.6)	0.0136

No difference in the time to testing was observed when the data was analysed using the 2-colour PLG gating strategy if the >90% or >95% or the <20 cells/µl mean difference limit, were each applied. However, this simplified gating strategy was able to extend the window of testing to 96 hours (3 days from time of receipt of the samples in the laboratory or 4

days from venection), when the <40 cells/μl cutoff was applied (data shown in Appendix E). This effect though, was not observed with the corresponding CD4% data. Merging of the $CD45^{bright}$ population with nearby monocytes resulted in the samples not being suitable for use beyond 48 hours (1 day from receipt into the laboratory and up to 2 days from the time of venesection) (data shown in Appendix E).

Figure 4.11 shows the deterioration in sample integrity over 8 days of follow-up of a sample kept at RT.

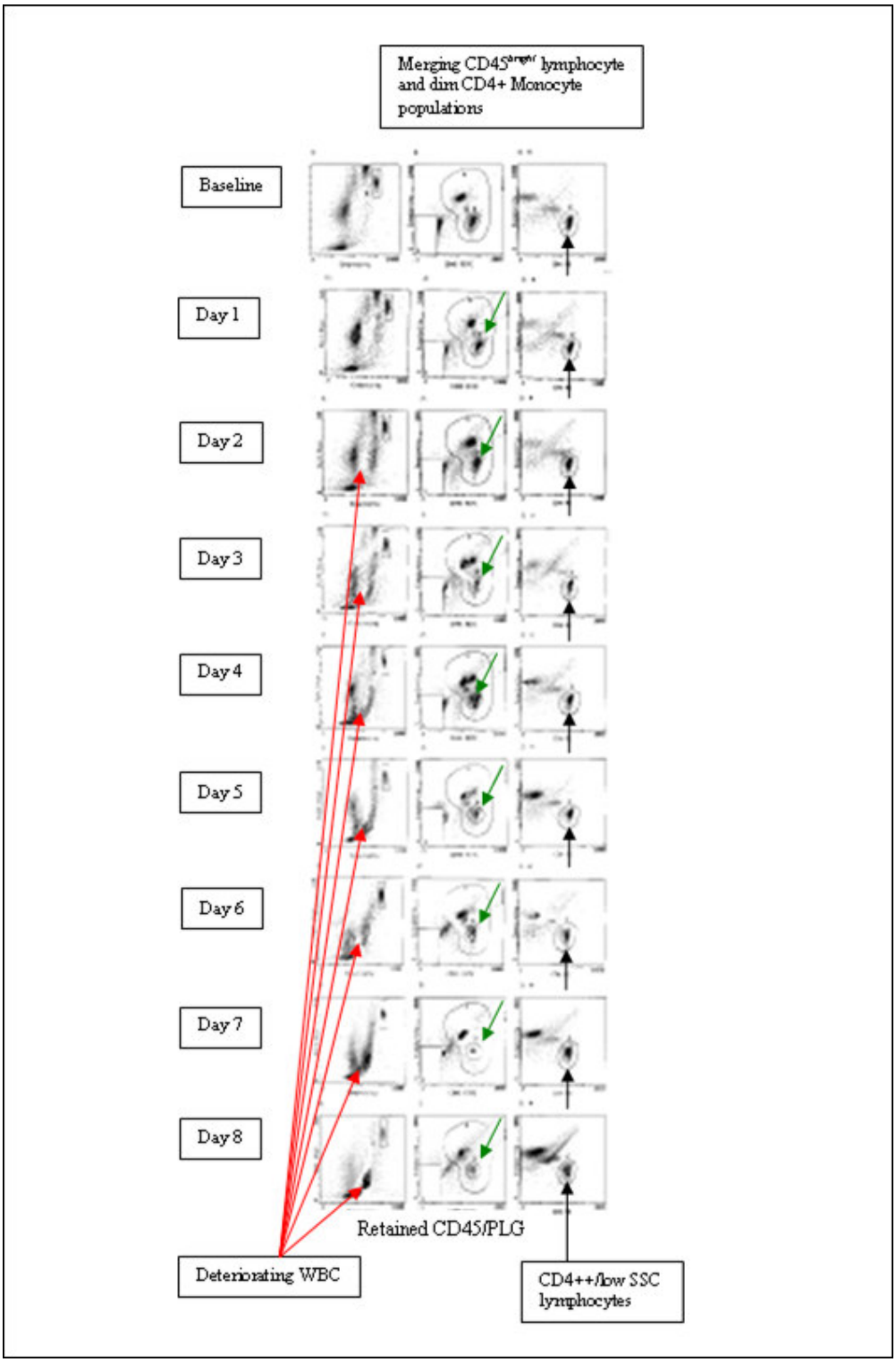


Figure 4. 11: A figure illustrating the deterioration of sample integrity over time for a sample kept at room temperature (17-23°C) and derived using the 2-colour PLG protocol (sample #31 was chosen as it included the least amount of fluorescent debris in the PLG gate).

Tables (4.22) and (4.23) summarise the results for the 55 samples analysed using the 4-colour MS/TC protocol at the CDC laboratory.

Table 4. 22: Summary of the mean change in CD4+ counts of samples kept at room temperature (20-23°C) and derived using the 4-colour MS/TC protocol at the CDC laboratory

Day post venesection	Age of samples (hours)	No. of Samples	CD4 count Range	Mean CD4 count	SD	LOA
Baseline	<6	55	17 - 814	344	195.51	
1	24	55	16 - 779	337	192.77	(722.54, -48.54)
2	48	55	13 - 774	325	188.98	(702.96, -52.96)
3	72	55	14 - 850	331	190.45	(711.90, -49.90)
4	96	55	14 - 776	300	171.54	(643.08, -43.08)
5	120	55	18 - 718	295	165.26	(625.52, -35.52)
6	144	55	11 - 679	279	169.16	(617.32, -59.32)
7	168	55	13 - 717	253	156.61	(566.22, -60.22)
8	192	55	15 - 571	224	149.77	(523.54, -75.54)

Table 4. 23: Change in mean CD4+ count as a ratio of baseline and mean difference to baseline for samples stored at room temperature (20-23°C) and derived using the 4-colour MS/TC protocol

Day post venesection	Ratio of Mean CD4 count to Baseline as a (%)	SD	p value at Cut-off		Bias	SD	95% CI	p value at Cut-off	
			90%	95%				40cells/µl	20cells/µl
1	98.33	8.22	<0.0001	0.0020	6.09	28.44	(-1.6, 13.8)	<0.0001	0.0003
2	94.13	13.19	0.0119	0.6857	18.29	37.99	(8.0, 28.6)	<0.0001	0.3700
3	96.09	12.76	0.0004	0.2648	12.87	44.89	(0.7, 25.0)	<0.0001	0.1221
4	88.85	13.62	0.7326	1	43.07	54.47	(28.4, 57.8)	0.6613	0.9986
5	87.56	12.40	0.9252	1	48.47	56.3	(33.3, 63.7)	0.8654	0.9998
6	79.92	16.27	1	1	64.40	61.29	(47.8, 81.0)	0.9977	1
7	73.24	16.07	1	1	90.60	80.61	(68.8, 112.4)	1	1
8	63.70	16.84	1	1	119.16	89.78	(94.9, 143.4)	1.00	1.00

Samples could not be analysed beyond 72 hours (day 3 from venesection) when the >90% cutoff limit (p = 0.7326) or the mean difference limit of <40 cells/µl were each applied (p <0.6613). However when the more strict limits (>95% and the mean difference limit of <20 cells/µl) were each applied, significant differences in CD4+ counts were observed 48 hours from venesection (by day 2). Significant differences from baseline counts were also observed by day 2 [95% CI: (100.1, 105.20)] when corresponding CD4% values were used (data shown in Table 4.24).

Table 4. 24: Change in mean CD4% as a ratio of baseline over days for samples stored at room temperature (20-23°C) and derived using the MS/TC protocol

Day post venesection	Age of samples (hours)	No of Samples	Ratio of Mean CD4% to Baseline as a (%)	SD	95% CI	p-value
1	24	55	102.4	7.70	(100.3, 104.5)	<0.0001
2	48	55	102.7	9.45	(100.1,105.2)	<0.0001
3	72	55	103.3	9.21	(100.8, 105.8)	<0.0001
4	96	55	103.2	11.99	(99.9, 106.4)	<0.0001
5	120	55	107.0	11.21	(103.9, 110.0)	<0.0001
6	144	55	105.5	15.65	(101.3, 109.7)	<0.0001
7	168	55	104.1	15.15	(100.0, 108.2)	<0.0001
8	192	55	103.4	15.75	(99.2, 107.7)	0.0001

Figure 4.12 shows the deterioration in sample integrity over 8 days of follow-up of a sample kept at RT and derived using the 4-colour MS/TC protocol.

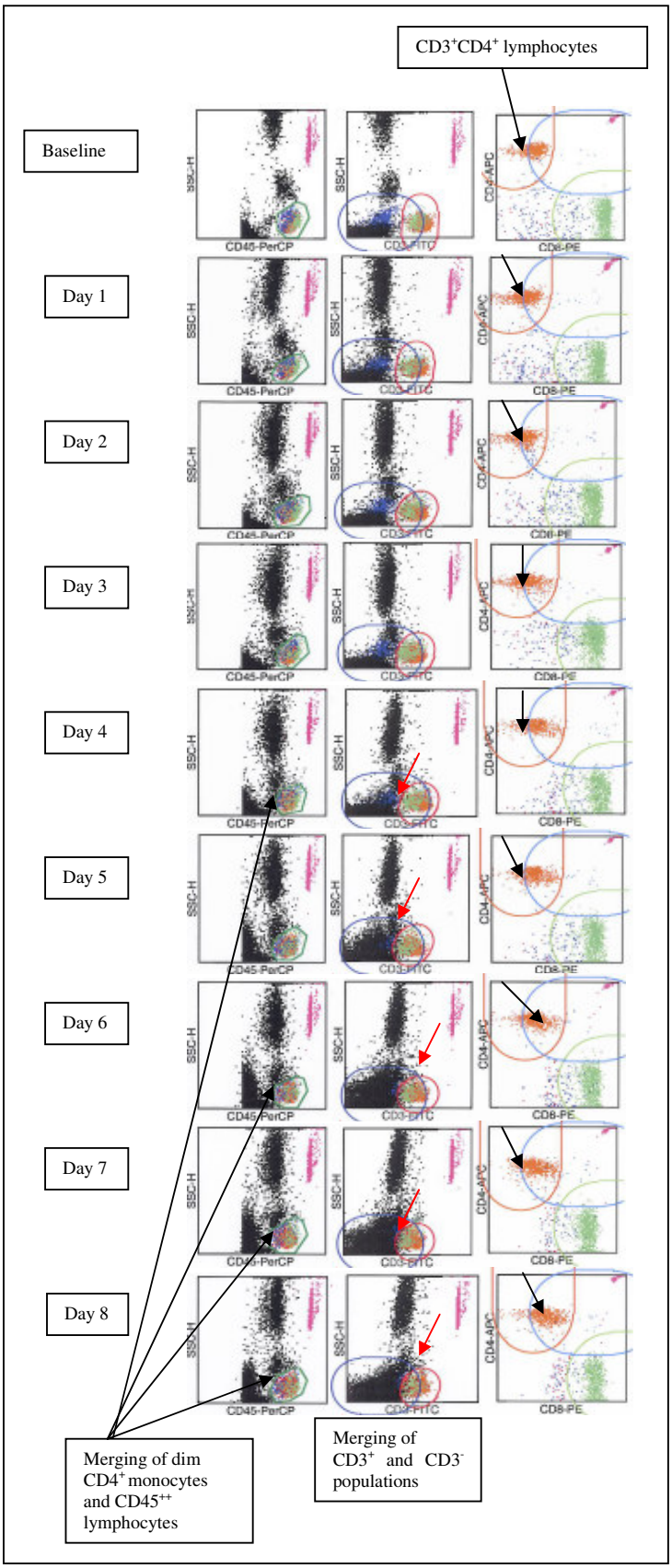


Figure 4. 12: A figure illustrating the deterioration in sample integrity over time for a sample kept at room temperature (20-23°C) and derived using the 4-colour MS/TC protocol

Changes over time in mean absolute CD4+ counts of samples stored at room temperature at the two laboratories are shown in Figures 4.13 and 4.14. A slightly faster decline in mean CD4+ counts over the eight days from baseline was again observed of the older samples (≥ 12-24 hours at baseline) analysed at the Johannesburg laboratory.

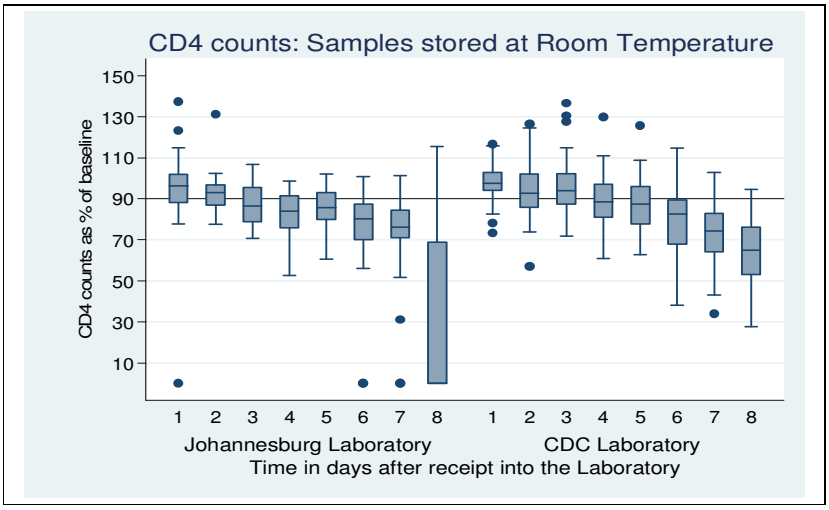


Figure 4. 13: Change in mean CD4+ counts as a ratio of baseline over days for samples stored at room temperature and analysed using the 4-colour TetraONE protocol at the Johannesburg Laboratory or using the 4-colour MS/TC at the CDC Laboratory.
Legend: Horizontal line at 90 – shows the sample Integrity Limit set at 90% similarity of Day reading to Baseline. **Days labeled 1-8 for Johannesburg laboratory have actual estimated respective ages of** ≥1-2, ≥2-3, ≥3-4, ≥4-5, ≥5-6, ≥6-7, ≥7-8 and ≥8-9 days.

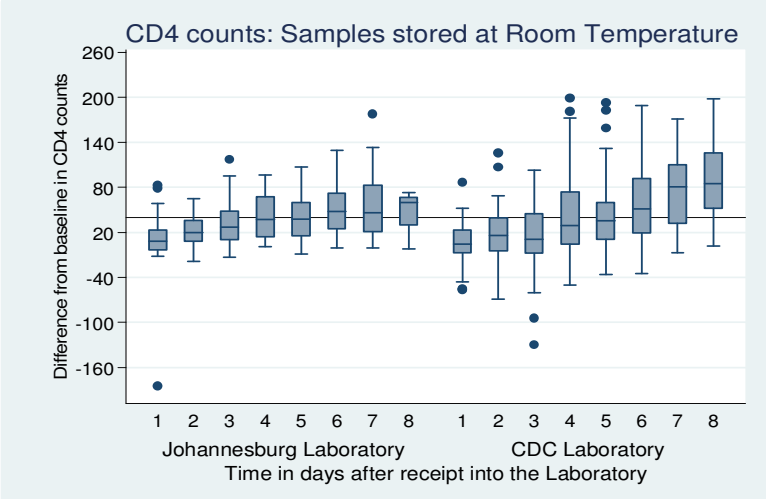


Figure 4. 14: Mean difference in CD4+ Counts to Baseline over Days for samples stored at Room Temperature and derived using the 4-colour TetraONE protocol at the Johannesburg Laboratory or using 4-colour MS/TC at the CDC Laboratory.
Legend: Horizontal line at 40 – shows the sample Integrity Limit set at a mean difference of 40cells/µl between day and baseline readings. **Days labeled 1-8 for Johannesburg laboratory have actual estimated respective ages of** ≥1-2, ≥2-3, ≥3-4, ≥4-5, ≥5-6, ≥6-7, ≥7-8 and ≥8-9 days.

4.2.2.3 Storage of Blood Samples at Refrigerator Temperature

Table (4.25) below summarises the results obtained when CD4+ count data for the 32 samples kept at 4-6°C at the Johannesburg laboratory were analysed using the 4-colour TetraONE protocol. The mean absolute CD4+ count of the 32 samples analysed at baseline was 306 cells/µl and the range (13 – 1237 cells/µl) but this dropped to a mean of 232 cells/µl, range (12 – 951 cells/µl) by Day 5. Only 29, 22 and 9 of the 32 samples had sufficient volumes for sample preparation and analysis on days 6, 7 and 8 respectively.

Table 4. 25: CD4+ count results of samples kept at refrigerator temperature (4–5°C) and derived using the 4-colour TetraONE protocol

Day post venesection	Age of samples (hours)	No. of samples	CD4 count range	Mean CD4 count	SD	LOA
Baseline (~1)	12-24	32	13 - 1237	306	223.56	
≥1-2	36-48	31	13 - 1199	287	216.72	(720.44, -146.44)
≥2-3	60-72	32	12 - 1141	279	209.03	(697.06, -139.06)
≥3-4	84-96	32	12 - 841	245	164.38	(573.76, -83.76)
≥4-5	108-120	32	13 - 905	238	180.97	(599.94, -123.94)
≥5-6	132-144	32	12 - 951	232	179.80	(591.60, -127.60)
≥6-7	156-168	29	9 - 1033	215	203.56	(622.12, -192.12)
≥7-8	180-192	22	9 - 497	176	140.97	(457.94, -105.94)
≥8-9	204-216	9	6 - 171	87	58.83	(204.66, -30.66)

Table (4.26) shows the details of how long samples stored at refrigerator temperature (4-6°C) could be kept before preparation and analysis (Johannesburg laboratory).

When the >90% integrity limit was applied, samples could be used to derive precise and accurate CD4+ counts for up to 48 hours (only 1 day from receipt into the laboratory and up to 2 days from the time of venesection ($p = 0.0002$). However, when the >95% limit ($p = 0.2447$), or the mean difference limit of <40 cells/µl ($p = 0.1887$) or that of <20 cells/µl ($p = 0.7050$) were applied, samples could not be used beyond 24 hours from venesection or the day of receipt in the laboratory.

Table 4. 26: Change in mean CD4+ counts as a ratio of baseline and mean difference to baseline for samples stored at refrigerator temperature and derived using the 4-colour TetraONE protocol

Day post venesection	Ratio of Mean CD4 count to to Baseline as a (%)	SD	p value at Cut-off		Bias	SD	95% CI	p value at Cut-off	
			90%	95%				40cells/µl	20cells/µl
≥1-2	96.06	8.39	0.0002	0.2447	27.56	78.57	(-0.8, 55.9)	0.1887	0.7050
≥2-3	91.01	9.44	0.2742	0.9884	26.94	30.25	(16.0, 37.9)	0.0102	0.8979
≥3-4	82.07	10.66	0.9999	1	60.25	72.90	(34.0, 86.5)	0.9369	0.9981
≥4-5	77.22	11.91	1	1	67.28	62.89	(44.6, 90.0)	0.99	0.9999
≥5-6	76.49	14.80	1	1	73.28	64.66	(50.0, 96.6)	0.9967	1
≥6-7	66.94	13.18	1	1	104.90	78.47	(76.1, 133.7)	1	1
≥7-8	59.94	17.79	1	1	129.82	151.27	(62.8, 196.9)	0.9945	0.9987
≥8-9	58.97	19.13	0.9994	0.9998	65.89	65.69	(15.4, 116.4)	0.8645	0.9653

Corresponding CD4% results (Table 4.27) showed that samples could be reliably analysed up to 72 hours from time of venesection (2 days from the day of receipt in the laboratory) [95% CI = (96.2, 101.7)].

Table 4. 27: Change in mean CD4% as a ratio of baseline over days for samples stored at refrigerator temperature and derived using the 4-colour TetraONE protocol

Day post venesection	Age of samples (hours)	No of Samples	Ratio of Mean CD4% to Baseline as a (%)	SD	95% CI	p value
≥1-2	36-48	31	97.78	5.56	(95.7, 99.8)	0.0046
≥2-3	60-72	32	98.94	7.54	(96.2, 101.7)	0.003
≥3-4	84-96	32	95.69	8.25	(92.7, 98.7)	0.319
≥4-5	108-120	32	92.46	13.42	(87.6, 97.3)	0.8541
≥5-6	132-144	32	99.62	25.48	(90.4, 108.8)	0.1564
≥6-7	156-168	29	96.55	10.94	(92.4, 100.7)	0.2263
≥7-8	180-192	22	100.07	14.18	(93.8, 106.4)	0.0541
≥8-9	204-216	9	93.99	9.64	(86.6, 101.4)	0.6194

When the TetraONE data was analysed using the 2-colour PLG gating strategy (data shown in Appendix F), this method of analysis was able to extend the window of testing to 72 hours (2 days from receipt into the laboratory and up to 3 days from the time of venesection ($p = 0.0277$) when the >90% integrity limit or the mean difference limit of <40 cells/µl, was applied. The 2-colour PLG strategy was also able to extend the window of testing to 48 hours (1 day from receipt into the laboratory and 2 days from the time of venesection) when the mean difference limit of <20 cells/µl was applied. It however had no impact when the >95% integrity limit was applied. Samples could not be used beyond

the day of draw. Corresponding PLG CD4% values showed that samples could be analysed for up to 96 hours (3 days from receipt into the laboratory or 4 days from the time of venesection) (data is shown in Appendix F).

Figure 4.15 shows the deterioration in sample integrity over 8 days of follow-up of a sample kept at FT at the Johannesburg laboratory.

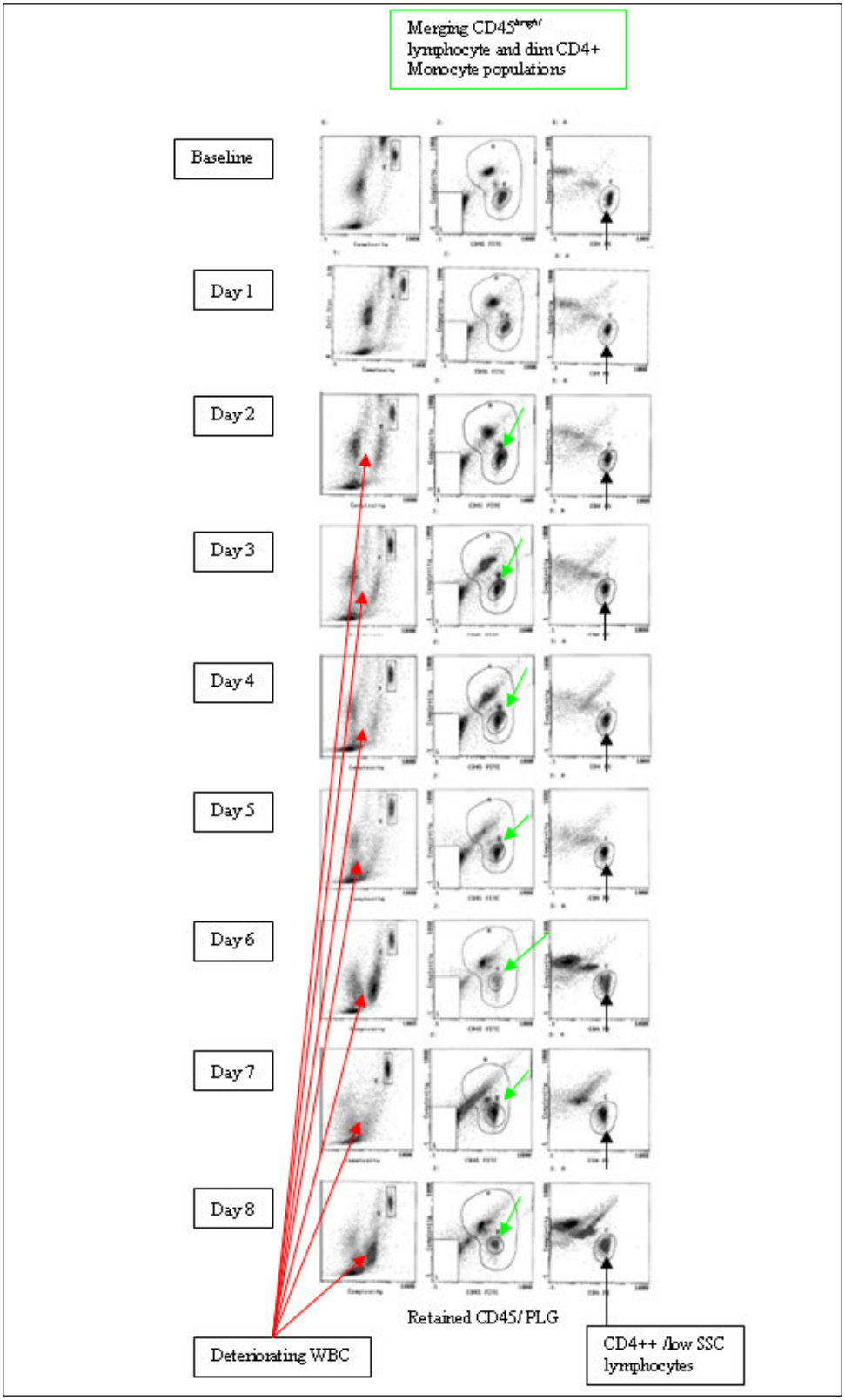


Figure 4. 15: A figure illustrating the deterioration of sample integrity over time for a sample kept at refrigerator temperature (4-6°C) and derived using the 2-colour PLG protocol (sample #31 was chosen as it included the least amount of fluorescent debris in the PLG gate).

Table (4.28) describes CD4+ count results for samples kept at 4.0–5.2°C and analysed using the 4-colour MS/TC protocol at the CDC laboratory. All 55 samples analysed at baseline were followed up for the 8 days. A mean CD4+ count of 344 cells/µl, ranging from (17 – 814 cells/µl) was obtained at baseline and a mean of 301 cells/µl, range (14 – 692 cells/µl) obtained by Day 8.

Table 4. 28: CD4+ count results of samples kept at refrigerator temperature (4.0–5.2°C) and derived using the 4-colour MS/TC Protocol at the CDC laboratory

Day post venesection	Age of samples (hours)	No. of Samples	CD4 Count Range	Mean CD4 count	SD	LOA
Baseline	<6	55	17 - 814	344	195.51	
1	24	55	15 - 811	329	188.07	(705.14, 47.14)
2	48	55	15 - 836	331	192.31	(715.62, -53.62)
3	72	55	15 - 843	327	187.04	(701.08, -47.08)
4	96	55	20 - 858	325	187.47	(699.94, -49.94)
5	120	55	14 - 825	316	185.87	(687.74, -55.74)
6	144	55	16 - 782	322	187.53	(697.06, -53.06)
7	168	55	14 - 769	313	180.16	(673.32, -47.32)
8	192	55	14 - 692	301	170.21	(641.42, -39.42)

Reliable CD4+ counts were obtained up to Day 6 (samples <144 hours old) (p = 0.0119) when the >90% integrity limit was applied and only on the day of receipt of the samples to the laboratory when the >95% limit was applied. A similar trend was observed when the <40 cells/µl (p = 0.0014) and <20 cells/µl (p = 0.1763) mean difference limits were each applied. These results are shown in the following table (4.29).

Table 4. 29: Change in mean CD4+ counts as a ratio of baseline and mean difference to baseline over days for samples stored at refrigerator temperature (4.0–5.2°C) at the CDC laboratory

Day post venesection	Ratio of Mean CD4 count to Baseline as a (%)	SD	p value at Cut-off		Bias	SD	95% CI	p value at Cut-off	
			90%	95%				40cells/µl	20cells/µl
1	96.27	11.01	<0.0001	0.1983	14.76	41.41	(3.6, 26.0)	<0.0001	0.1763
2	96.52	12.32	0.0001	0.1825	12.58	40.18	(1.7, 23.4)	<0.0001	0.0883
3	95.86	12.91	0.0007	0.3125	16.93	47.69	(4.0, 29.8)	0.0004	0.3174
4	95.43	9.64	0.0001	0.3714	18.46	35.33	(8.9, 28.0)	<0.0001	0.3734
5	92.11	9.61	0.0549	0.9852	27.09	32.47	(18.3, 35.9)	0.0024	0.9444
6	93.52	11.23	0.0119	0.8329	21.87	43.08	(10.2, 33.5)	0.0014	0.6258
7	91.08	12.42	0.2601	0.9885	30.94	47.68	(18.1, 43.8)	0.0824	0.9528
8	88.11	8.62	0.9449	1	42.6	42.53	(31.1, 54.1)	0.674	0.9999

Corresponding CD4% values (Table 4.30) showed that reliable results could still be obtained up to 8 days from the day of venesection, [95% CI = (97.3, 102.4)].

Table 4. 30: Change in mean CD4% as a ratio of baseline over days for samples stored at refrigerator temperature (4.0–5.2°C) at the CDC laboratory

Day post venesection	Age of samples (hours)	No of Samples	Ratio of Mean CD4% to Baseline as a (%)	SD	95% CI	p-value
1	24	55	100.38	10.11	(97.6, 103.1)	0.0001
2	48	55	100.84	9.20	(98.4, 103.3)	<0.0001
3	72	55	101.65	10.33	(98.9, 104.4)	<0.0001
4	96	55	101.31	9.80	(98.7, 104.0)	<0.0001
5	120	55	100.85	9.09	(98.4, 103.3)	<0.0001
6	144	55	101.05	11.07	(98.1, 104.0)	0.0001
7	168	55	99.79	11.76	(96.6, 103.0)	0.0019
8	192	55	99.85	9.31	(97.3, 102.4)	0.0002

Figure 4.16 shows the deterioration in sample integrity over 8 days of follow-up of a sample kept at refrigerator temperature and analysed using the 4-colour MS/TC protocol.

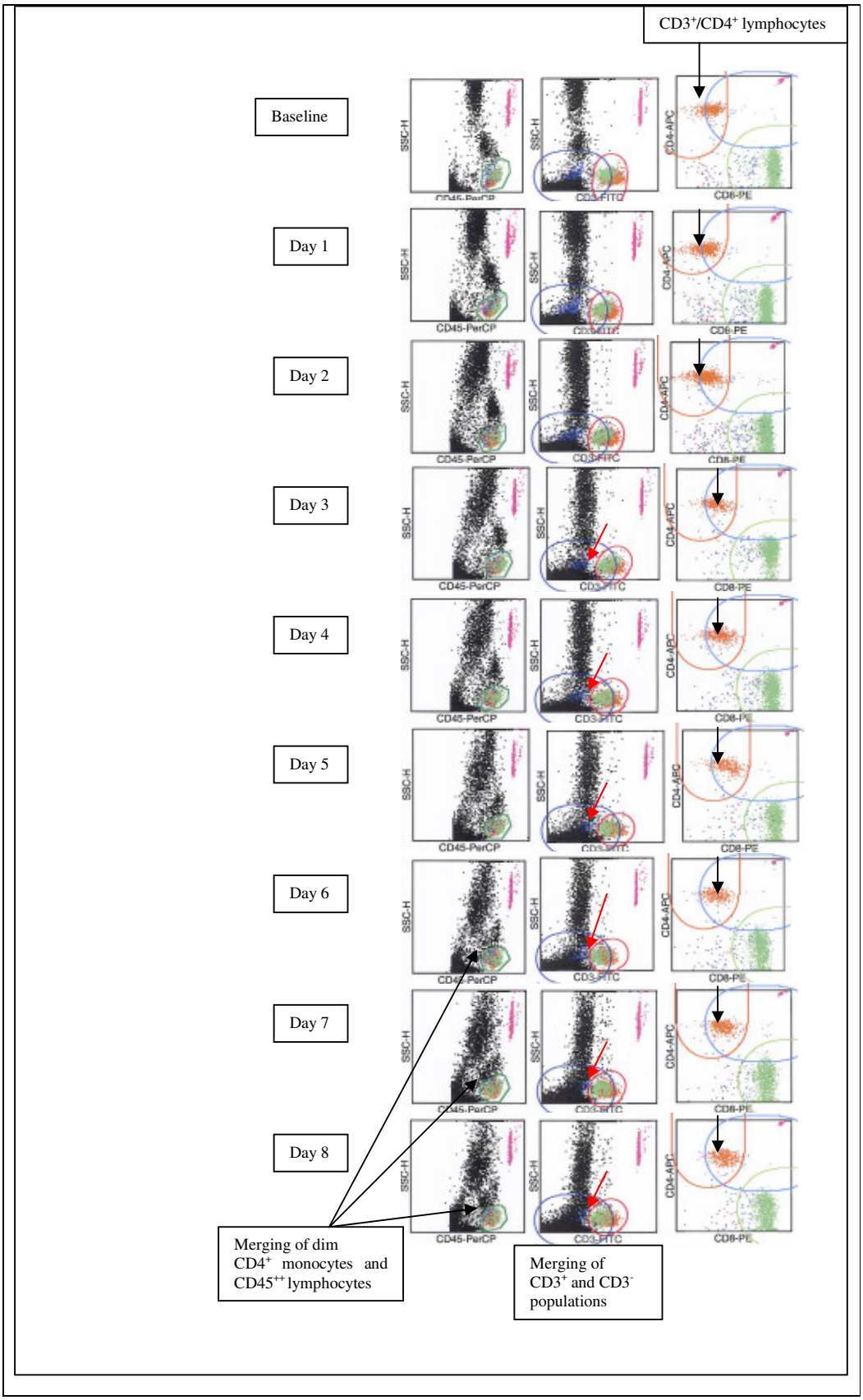


Figure 4. 16: A figure illustrating the deterioration of sample integrity over time for a sample kept at refrigerator temperature (4.0–5.2°C) and derived using the MS/TC protocol.

Changes in the CD4+ counts over days from baseline for samples stored at refrigerator temperature at the two laboratories are shown in Figures 4.17 and 4.18. On the whole, the deterioration in integrity and change from baseline CD4+ counts of samples analysed at the CDC laboratory occurred at a slower rate than was observed of samples analysed at the Johannesburg laboratory.

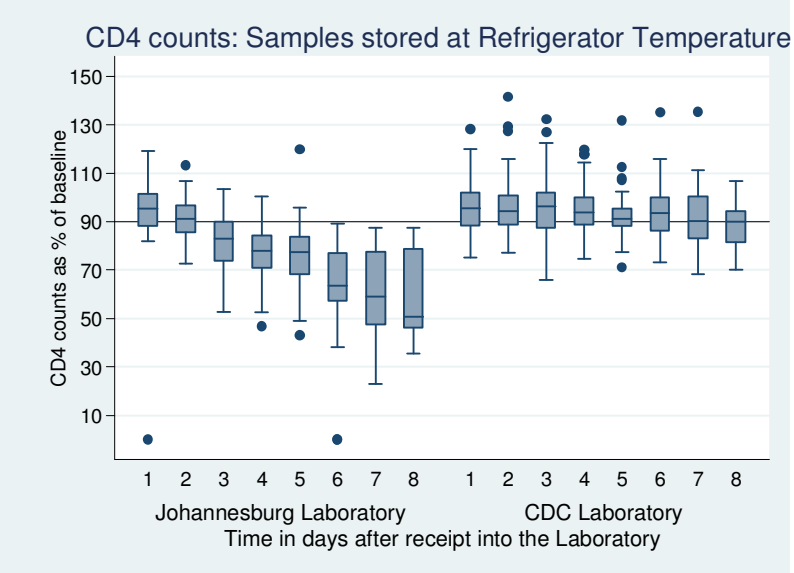


Figure 4. 17: Change in Mean CD4+ Counts as a Ratio of Baseline over Days for samples stored at Refrigerator Temperature and derived using the 4-colour TetraONE protocol at the Johannesburg Laboratory or using the 4-colour MS/TC at the CDC Laboratory.
Legend: Horizontal line at 90 – shows the sample Integrity Limit set at 90% similarity of Day reading to Baseline. **Days labeled 1-8 for Johannesburg laboratory have actual estimated respective ages of** ≥1-2, ≥2-3, ≥3-4, ≥4-5, ≥5-6, ≥6-7, ≥7-8 and ≥8-9 days.

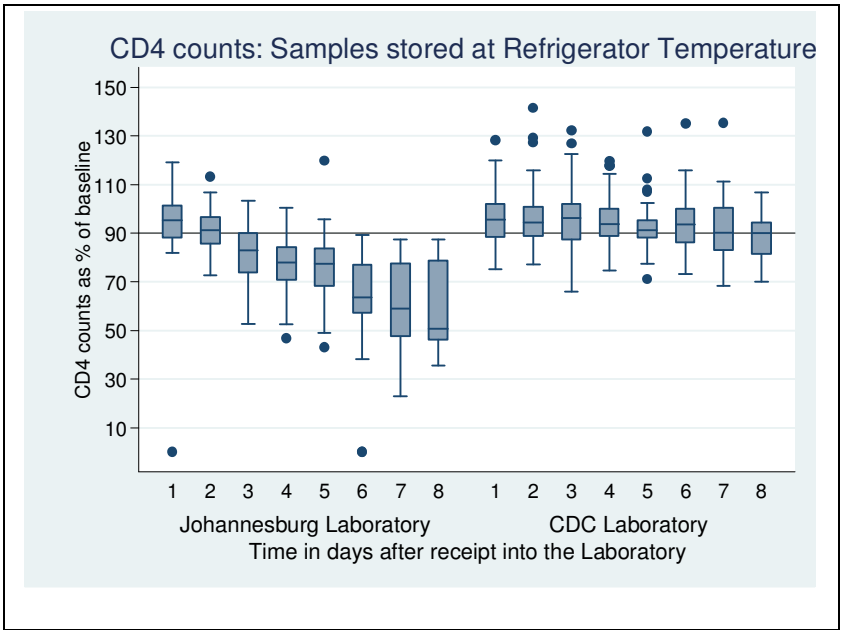


Figure 4. 18: Mean difference in CD4+ Counts to Baseline over Days for samples stored at Refrigerator Temperature and derived using the 4-colour TetraONE protocol at the Johannesburg Laboratory or using the 4-colour MS/TC at the CDC Laboratory.
Legend: Horizontal line at 40 – shows the sample Integrity Limit set at a mean difference of 40cells/µl between day and baseline readings. **Days labeled 1-8 for Johannesburg laboratory have actual estimated respective ages of ≥**1-2, ≥2-3, ≥3-4, ≥4-5, ≥5-6, ≥6-7, ≥7-8 and ≥8-9 days.

In summary, blood samples received at the Johannesburg hospital were on average ≥12-24 hours old at the time they were received for testing by the laboratory. Most had been bled in the late afternoon or early evening the day before they were delivered to Johannesburg hospital for testing when the laboratory opened.

In the hands of the writer, when the >90% integrity limit was used, samples processed using the international guideline protocol, 4-colour TetraONE, could only be used for up to 48 hours (only 1 day from receipt into the laboratory and up to 2 days from the time of venesection) if kept in motion or when stored at refrigerator temperature. However, if samples were stored at room temperature, they could be used to derive precise and accurate CD4+ counts for up to 72 hours (2 days from receipt into the laboratory and up to 3 days from the time of venesection. Reliable CD4+ counts could not be obtained beyond 24 hours from venesection (the day of receipt into the laboratory) for samples kept in motion

or in a refrigerator or at room temperature or at 37°C in a water bath, if the integrity limit of < 40 cells/µl was used.

Use of the 2-colour PLG gating strategy only extended the window of testing samples stored at refrigerator temperature when the >90% integrity limit was used. These samples could be kept and used for at least a day longer i.e. up to 72 hours (2 days from receipt into the laboratory and up to 3 days from the time of venesection). When the <40 cells/µl mean difference limit was used, use of the 2-colour PLG strategy extended the window of testing samples kept in motion and those stored at refrigerator temperature to 72 hours (2 days from receipt into the laboratory and up to 3 days from the time of venesection). It was also able to extend the window of testing samples stored at room temperature to 96 hours (3 days from receipt into the laboratory and up to 4 days from the time of venesection).

CD4 testing at the CDC laboratory was performed using the same international guideline but different manufacturer protocol, viz. the 4-colour MultiTEST/TruCount. All the samples tested at the CDC laboratory were on average about 5 hours old at the time of receipt into the laboratory and about 6 hours old when they were processed for baseline CD4+ counts.

When the >90% limit and the <40 cells/µl mean difference limit were used, samples kept in motion could only be used to derive precise and accurate CD4+ counts for a maximum of 24 hours (1 day from receipt into the laboratory). Samples stored at room temperature could be used for up to 72 hours (3 days from receipt into the laboratory) while those stored at refrigerator temperature could be kept up to 144 hours (6 days from receipt into the laboratory). Precise and accurate CD4+ counts could not be obtained at all when samples were kept at 37°C in a water bath.

4.3 Evaluation of the use of a Commercial Whole Blood Stabilising Agent to enable Extending the Window of CD4 Testing.

The change over time of CD4+ counts of samples drawn in new (2ml) commercial blood stabilisation tubes (BDB) was assessed in order to evaluate the efficacy of the stabiliser (Streck Laboratories, Omaha, NE) in the tubes to extend the window of CD4 testing.

Table (4.31) summarises the results obtained when CD4+ count data for the 20 samples kept at room temperature (17-23°C) were analysed using the 4-colour MS/TC protocol (BDB) at the Johannesburg laboratory. The mean CD4+ count of the 20 samples analysed at baseline was 303 cells/µl and the range (54 – 747 cells/µl) but this dropped to a mean of 281 cells/µl, range (50 – 850 cells/µl) by Day 7. Only 19 of the 20 samples had sufficient volumes for analysis on day 8 of follow-up.

Table 4. 31: Summary of mean change in CD4+ counts of samples drawn in the new BDB tubes and derived using the 4-colour MS/TC Protocol at the Johannesburg laboratory

Day post venesection	Age of samples (hours)	No. of Samples	CD4 count Range	Mean CD4 count	SD	LOA
Baseline	<4	20	54 - 747	303	161.11	
6	144	20	53 - 811	285	169.69	(624.38, -54.38)
7	168	20	50 - 850	281	181.98	(644.96, -82.96)
8	192	19	95 - 845	286	173.22	(632.44, -60.44)

Tables (4.32) below shows how long samples drawn in the new stabilisation tubes could be stored at room temperature before preparation and analysis. Samples could not be used to derive precise and accurate CD4+ counts by 144 hours (6 days from venesection) when the >90%, >95% limit and <20 cells/µl mean difference limit were each applied. Samples could however be kept and reliably analysed for up to 168 hours (7 days from venesection) when the <40 cells/µl mean difference cutoff was applied (p = 0.0447) confirming the manufacturer's (BDB) claim about the product.

Table 4. 32: Change in mean CD4+ counts as a ratio of baseline and mean difference to baseline for samples drawn in the new BDB Tubes and derived using the 4-colour MS/TC Protocol at the Johannesburg Laboratory

Day post venesection	Ratio of Mean Day CD4 count to Baseline as a (%)	SD	p value at Cut-off		Bias	SD	95% CI	p value at Cut-off	
			90%	95%				40cells/µl	20cells/µl
6	93.05	10.21	0.0986	0.7978	17.80	35.73	(1.1,34.5)	0.0060	0.3930
7	90.08	12.14	0.4882	0.9571	21.50	46.23	(-0.1, 43.1)	0.0447	0.5569
8	88.41	12.35	0.7090	0.9840	29.32	45.33	(7.5, 51.2)	0.1589	0.8089

Table (4.33) summarises the CD4+ count results of the 69 samples drawn in the new stabilisation tubes that were derived using the 4-colour MS/TC protocol (BDB) at the CDC laboratory. The mean CD4+ count of the 69 samples analysed on day 1 (considered as the baseline since the samples were > 24 hours old at time of receipt into the laboratory) was 472 cells/µl and the range (69 – 1217 cells/µl) but this dropped to a mean of 460 cells/µl, range (56 – 1452 cells/µl) by Day 6. Only 33 of the 69 samples had sufficient volumes for analysis on day 8 of follow-up.

Table 4. 33: Summary of mean change in CD4+ counts of samples drawn in the new BDB tubes and derived using the 4-colour MS/TC Protocol at the CDC Laboratory

Day post venesection	Age of samples (hours)	No. of Samples	Range of CD4 Counts	Mean CD4 Count	SD	LOA
Baseline	≥24-36	69	69-1217	472	234.05	
4	96	69	68 - 1187	449	236.25	(921.5, -23.5)
6	144	69	56 - 1452	460	246.10	(952.2, -32.2)
8	192	33	46 - 1041	430	215.30	(860.6, -0.6)

Samples at the CDC laboratory CD4+ counts could still be used to derive reliable CD4+ counts up to day 8 (192 hours from venesection) if the >90% similarity limit was applied but could not be used by day 4 (96 hours from venesection) if the other 3 limits were each applied (data is shown in Table 4.34).

Table 4. 34: Change in mean CD4+ counts as a ratio of baseline and mean difference to baseline for samples drawn in the new BDB tubes and derived using the 4-colour MS/TC Protocol at the CDC laboratory

Day post venesection	Ratio of Mean Day CD4 count to Day 1 as a (%)	SD	p value at Cut-off		Bias	SD	95% CI	p value at Cut-off	
			90%	95%				40cells/µl	20cells/µl
4	96.25	18.09	0.0028	0.2846	23.52	104.43	(-1.6, 48.6)	0.0972	0.6099
6	97.12	14.77	0.0001	0.1191	12.07	80.15	(-7.2, 31.3)	0.0026	0.2071
8	97.42	15.07	0.0040	0.1811	18.70	76.20	(-8.3, 45.7)	0.0590	0.4612

5.0 DISCUSSION

CD4+ T lymphocytes play a central role in the pathogenesis of HIV and are of prognostic value as predictors of progression to AIDS(8-10, 12). CD4+ counts are used in guiding decisions on the initiation and administration of ART(13, 14) and are used as a surrogate marker for drug efficacy in clinical trials(19). Measuring these T-cells is one of the most important immunological parameters used in the staging and management of HIV-1 infected persons(5-7). It is therefore critical that CD4+ counts are accurately and precisely enumerated to ensure adequate monitoring of treatment for HIV infected patients(3, 61).

Although CD4+ testing is now a routine test, variability between patient counts currently exists, especially in samples with low CD4+ counts (< 200 cells/µl)(6, 40, 47). Similar to reports from other studies(53, 60, 144), the results of this study showed that the reliability of reported CD4+ count results was heavily dependent upon the age of the sample at time of processing, procedures in which blood samples were handled, processed, and analysed in the laboratory, as well as the skills of the operator and methods used.

In this study, the effect that certain factors had on accurate and precise enumeration of CD4+ cell counts was assessed. Specific factors tested included operator's pipetting, preparative and analysis skills, gating (which specifically affect precision or reporting), storage temperature, motion, use of different protocols and manufacturer products, and the use of different flow cytometers. Furthermore, the efficacy of a new blood stabiliser was tested to evaluate the manufacturer's claim that it could extend the window of CD4 testing.

5.1 Analyses performed to Assess Impact of CD4 Methodology including Specific Reagents, Flow Cytometry System, Protocol Setup and Gating, on Bias of CD4 Reporting.

5.1.1 Assessment of Bias introduced through Poor Precision of Pipetting

Consistent with previous reports(37, 41, 52, 53), this study showed that a laboratory's competence to provide quality CD4+ count testing was dependent upon well trained operators and precise pipetting. Data on declining coefficients of variation (%CV) obtained in this study indicated that gradual improvement in precision was a direct consequence of time invested in the development of an operator's pipetting skill. The operator's (the writer's) %CV decreased from 0.912% to 0.387% over 11 days of practice, suggesting that the more skilled an operator became, the greater the probability of obtaining consistent and more reliable CD4+ counts. This observation is in agreement with the lower within-laboratory variation (%CV= 4.75%; range 2.51-6.20%) obtained at the Johannesburg laboratory. Technicians in Johannesburg received pipette training before participating in routine testing of patient samples and regularly had their pipetting skills assessed to ensure continued quality of CD4 results. In contrast, a higher within-laboratory variation (%CV= 7.5%; range 3.61-9.28%) was observed at the CDC laboratory where the technicians had not received ongoing pipette training.

5.1.1.1 Operator skill assessment (Forward pipetting versus Reverse pipetting precision assessment of the operator [Intra-operator])

In agreement with previous findings(153), this study also showed that establishing the pipetting technique upfront, i.e., either reverse pipetting or forward pipetting, showed improved operator consistency, with enhanced accuracy and precision of pipette measurements. The results showed that it was necessary to evaluate an individual's pipetting skill so that the individual consistently used the technique with which they

obtained better precision (lower %CVs)(153). In this study, the writer obtained better pipetting precision using the forward pipetting technique (mean %CV = 0.513%) than using the reverse technique (mean %CV = 1.214%). The forward pipetting technique was therefore used in all the pipetting procedures.

5.1.1.2 Within-laboratory pipetting skills precision assessment (precision between different operators at two different geographically located sites [Inter-operator])

i) Within-laboratory (within and between-operator) precision

Lower within-laboratory variation (%CV = 4.75% compared to %CV = 7.5%) resulted in lower variation in a patient's CD4+ count when a sample was processed and analysed by ten different technicians at the Johannesburg laboratory (mean CD4+ count for the 10 operators = 261 cells/µl; median = 260 cells/µl; range: 225-293 cells/µl; STDEV = 12.4). The greater variation observed in a patient's CD4+ count (mean CD4+ count for the 10 operators = 261 cells/µl; median = 262 cells/µl; range: 217-318 cells/µl; STDEV = 19.6) at the laboratory (CDC) with a higher within-laboratory %CV could impact the accuracy of CD4+ counts (see Table 5.1) by as much as 60 cells/µl between the best and worst operators; a 60 cells/µl difference would not have been an acceptable difference based on the criteria defined prior to the onset of this study. In contrast however, wider within-laboratory variation has been reported in a recent U.S. multisite study, where it was shown that the median within laboratory predicate CD4 method %CV ranged from 4.0-8.7%(40).

ii) Cell count related variation (within-operator variability) across strata of clinical CD4+ counts.

The operator (the writer) at the Johannesburg laboratory who received pipette training exhibited greater pipetting precision than the operator at the CDC laboratory when they each analysed samples in different CD4+ strata. Better within-operator precision (%CVs of

4.95%, 2.92% and 2.83% compared to 6.44%, 5.20% and 5.41%) for samples with low, intermediate, and high CD4+ counts, respectively, was obtained by the operator at the Johannesburg laboratory, demonstrating that pipette training enhanced pipetting precision and reduced variation in patient CD4+ counts. However, as noted in previous studies(6, 47), the precision of the two operators decreased with lower cell counts specifically, for CD4+ counts in the clinically relevant range. Here, variability was most likely to impact on treatment decisions and indicates the utmost importance of maintaining precision in this lower range of CD4+ counts.

In Table 5.1 below, the impact that laboratory imprecision could have on patient management is discussed using the example of a hypothetical sample with a CD4+ count of 200 cells/µl (the current CD4+ count level at which the WHO and U.S. Public Health Service recommend that adult and adolescent ART and other prophylaxis are initiated(3, 24).

Table 5. 1: A table illustrating hypothetical imprecision and estimated laboratory error around a CD4+ count in the clinically relevant range of 200 cells/µl at different levels of precision.

	Hypothetical scenario				
	CD4+ count	**Precision (%CV)**	**SD**	**2SD**	**200 cells/ul± 2SD range** Estimated expected range of error
Hypothetical	200	3 (ideal)	6	12	(188 – 212)
	200	5	10	20	(180 - 220)
	200	6	12	24	(176 - 224)
	200	7	14	28	(172 - 228)
	200	8	16	32	(138 - 232)
	200	9	18	36	(164 - 236)
	200	10	20	40	(160 - 240)
	200	12	24	48	(152 - 248)
	200	15	30	60	(140 - 260)
	200	18	36	72	(128 - 272)
	200	20	40	80	(120 - 280)
	200	25	50	100	(100 - 300)
	Summary of impact of within-laboratory pipetting precision on bias from this study				
CDC	261	7.5*	19.6	39.2	(161 - 239)
	Best precision	3.61	9.5	19.0	(181 - 219)
	Worst precision	9.28	23.9	47.8	(152 - 248)
Johannesburg	261	4.75*	12.4	24.8	(175 - 225)
	Best precision	2.51	6.5	13.0	(187 - 213)
	Worst precision	6.20	16.6	33.2	(167 - 233)
	Summary of impact of within-operator precision on bias from this study				
CDC	174	6.44	11.2	22.4	(152 – 196)
	500	5.20	26.0	52.0	(448 – 552)
	1327	5.41	71.8	143.6	(1183 – 1471)
Johannesburg	184	4.95	9.1	18.2	(166 - 202)
	449	2.92	13.1	26.2	(423 – 475)
	650	2.83	18.4	36.8	(613 - 687)

*** Mean laboratory %CV across 10 operators.**

The impact of poor precision on accuracy of CD4+ counts can be seen in Table 5.1. The table also reflects the findings of this study, which demonstrate that, with less precision, there is greater variance from the true value. This increases the probability that a patient would be falsely excluded from receiving treatment in time to prevent early death or in time to prevent the development of opportunistic infections and increased costs of treating these infections. Similarly, reporting inaccurately low CD4+ counts would also unnecessarily qualify patients for prophylaxis such as *Mycobacterium avium* complex

(MAC) therapy that is initiated at a CD4+ count of < 100 cells/µl(154). Further, for patients already on ART and where response to ART has been shown to be slow(155), reporting of small increases in CD4+ counts can be masked by wide variation of reporting from the servicing laboratory as illustrated in Table 5.1. Although viral load testing is typically used to decide response to therapy, in a scenario where CD4+ counts are the sole determinant for implementation and monitoring of ART, wide variation of CD4 reporting due to poor precision may give the false impression that a patient was showing poor immune and CD4 recovery (the CD4 recovery is masked by imprecision of reporting). The impact of the differences in precision noted across the laboratories evaluated in this study reveals that even relatively small differences in %CV/precision of reporting can result in differences of up to 40 cells/µl.

While variation in intermediate and high CD4+ counts may not put patients at risk of immediate death, it would increase the cost of treatment and overburden health care systems which, in many cases, already have limited financial resources(40). Treatment of individuals erroneously deemed eligible could also result in the exclusion of patients from treatment who, with the treatment, would otherwise regain their health and continue to contribute socially and economically to their communities.

5.1.2 Assessment of Bias introduced through use of Different Manufacturer Sample Preparation Systems, Different/Similar Gating Strategies, Different Flow Cytometers and Different Flow Cytometric Threshold.

Similar to reports from other studies(38, 40, 41, 46), this study also noted differences between CD4+ counts when same samples were analysed using different protocols, different flow cytometers, different gating strategies, and when different flow cytometric threshold were used for data acquisition. Differences between CD4+ counts were also

observed between protocols and flow cytometers from the same manufacturer even when the same gating strategy was used.

One instrument, the FC-500 cytometer (BCI, Miami, FL), consistently yielded results that were higher than those obtained from the other two instruments used viz.; the Epics-XL (BCI, Miami, FL) (see Table 4.3) and the FACSCalibur (BDB) (see Tables 4.5 and 4.6) .

There is an observed bias between CD4 results for a given sample analysed on different instruments. This occurred even when the same preparation methodology and the same gating analysis were used, which brings to light another critical factor; manufacturers do not typically calibrate their instruments with sufficient precision so that their instruments can be used interchangeably. Presently, the same model instrument cannot be used reliably for CD4 reporting, even within the same laboratory (personal communication – Lawrie D., Flow cytometry Laboratory, Johannesburg, South Africa)(152). Bias is frequently noted between instruments and between laboratories, even those using the same instruments. In some instances, differences have been noted even between laboratories using the same systems. An example is the differences in accuracy and precision observed in the BDB MS/TC system when two different gating methods are applied. One gating method which uses only primary CD3 gating (with CD4 and CD8) and the other which incorporates use of $CD45^{bright}$ gating (with CD3/CD4 and CD8), showed very different performances between laboratories(41) in terms of bias as well as precision between laboratories.

These bias differences need to be addressed by insisting that instruments are calibrated with precision during installation and during maintenance servicing (personal communication – Lawrie D., Flow cytometry Laboratory, Johannesburg, South Africa).

These results highlight the differences that are observed between instruments/methods/protocols used for CD4 lymphocyte enumeration. They highlight the need for laboratories that use more than one instrument or type of instrument, as well as groups of laboratories reporting CD4+ counts to the same network (e.g. across regional

study groups e.g. IAVI, AACTG, IMPAACT(156-158)) or across National country programmes, to validate CD4 instruments and additionally, preferably standardise the methods/protocols used. Usually, based on moderate workloads, laboratories only use a single instrument (flow cytometer) for CD4 testing. However, when multiple instruments are used to accommodate high workloads or when a group of laboratories supports the same service or study network, the impact of bias introduced across the numerous instruments can be marked. Further, the more variables there are in the sample preparation and analytical steps (viz. different methods, protocols, threshold, gating strategies), the greater the probability of introducing even more variation and hence more bias, between methods/instruments. Although the differences observed between instruments/methods/protocols were not clinically relevant in some parts of this study (clinical significance in this study was defined at two levels; a bias of 20 cells/µl and bias ≥ 40 cells/µl), these results demonstrate the relevance and importance of standardising or alternatively, minimising on the variables between testing procedures both within- and between laboratories, to ensure comparability of CD4+ count results. Better reproducibility and precision in laboratory performance has been reported in studies where standardised systems were used(39, 41, 60).

5.2 Analyses performed to assess the Impact of Logistical Factors and Time from Venesection, including Transportation and Temperature on Bias of CD4 Reporting.

5.2.1 Assessing the Effect of Motion on Whole Blood Sample Integrity

A large number of the samples received for testing at many of the central laboratories in Africa, are from remote areas. These samples are often transported to the testing laboratory by road for varying lengths of time and distances. However, most road networks in much of Africa are not well developed and so samples end-up being damaged in a vehicle during

transit. To assess the effect of sample handling on sample integrity, samples were kept in constant motion on a rocker or mixer for several days in an attempt to emulate a "bumpy drive" during transportation.

Significant levels of sample deterioration and higher variation in CD4+ counts from baseline CD4+ counts were observed for the samples kept in motion when compared to samples left lying still in a refrigerator or on a laboratory bench at room temperature. This finding strongly reflected the impact that poor handling can have on sample integrity. The samples kept motion could only be used to accurately derive CD4+ counts within one day after receipt into the laboratory if 4-colour $CD45^{bright}$ lymphocyte based gating protocols were used and extended up to 2 days after receipt into the laboratory (3 days after venesection), if the 2-colour PLG protocol was used. In this instance, the use of the $CD45^{bright}$ lymphocyte based gating obscured the definition of the $CD45^{bright}$ lymphocyte population due to its merging with the dimmer CD4+ monocyte population as samples deteriorated, making the enumeration of CD4+ lymphocytes more difficult. CD4+ counts by the PLG methodology were less affected in this context as the total white blood cell population is the reference population from which the CD4+ cells are counted (instead of the $CD45^{bright}$ gating). However, the CD4% lymphocyte values generated in the PLG protocol were similarly affected by the merging of $CD45^{bright}$ lymphocytes and dimmer CD4+ monocyte populations.

5.2.2 Assessing the effect of Temperature on Whole Blood Sample Integrity

The temperature at which samples are stored during handling and just before analysis in the laboratory can ultimately have an effect on the integrity of samples and on precise enumeration of CD4+ counts. In agreement with current recommendations for testing(64), results from this study showed that samples could be kept at temperatures of (17–23°C) and analysed for reliable CD4+ counts for up to 3 days from venesection if 4-colour

CD45^{bright} lymphocyte based gating protocols were used and longer (4 days), if the 2-colour PLG protocol was used. However, as previously reported(64), temperatures over 30°C caused the greatest variation from baseline CD4+ counts. This study showed that significant deterioration in sample integrity occurred soon after venesection when samples were kept at 37°C, causing samples to be unsuitable for reliable CD4+ counting.

Contrary to reports by Weiblen B.J et al, 1983(159) and Dzik W.H, et al, 1983(160), the study data were in agreement with findings by Paxton H and Bendele T, 1993(131) which showed that whole blood samples could be safely stored at refrigerator temperature (4-6°C) and reliably used for CD4+ count enumeration. Although conclusions on the duration samples could be kept before testing differed between the two laboratories, results from both laboratories still supported storage of samples at refrigerator temperature.

In an attempt to explain the difference in the window of testing, fluctuation in refrigerator temperature beyond (4-6°C) was ruled out since the refrigerators had been connected to stable power supplies (the CDC refrigerator in particular, was connected to an inventor supported power supply). As noted in this study and by others, possible explanations for the difference in results were attributed to the age of the sample at the time of refrigeration, the use of different CD4 testing protocols(38), differences in the populations that were studied(119) and to differences in the number of samples analysed at the two laboratories(161). In comparison with the samples received for testing in Uganda, the integrity of the samples received at the Johannesburg laboratory was compromised at the time of receipt and had deteriorated prior to processing (as evidenced by the deterioration of the granulocytes in the light scatter analysis). Samples received at the CDC laboratory were analysed for baseline CD4+ counts much sooner after venesection (6 hours) than samples at the Johannesburg laboratory (samples were estimated to be at least 12-24 hours old at the time of receipt in the laboratory). In addition, the samples processed at the CDC laboratory had been collected from sites in close proximity (5 – 38 km) to the laboratory

and may therefore have been less exposed to fluctuations in temperature and/or damage during transit. Fluctuations in temperature and movement have been reported to cause sample deterioration(162) and could perhaps explain why the results on the duration of testing samples at the Johannesburg laboratory differed from those previously shown(163). Scott, et al, showed that CD4 testing performed using the 2-colour PLG method could be extended up to over 5 days. However, samples used in their study had been drawn and delivered directly to the laboratory where they were stored at a constant temperature, left lying on the laboratory bench, and minimally handled for the entire period of testing. Further, although care was taken to ensure that the standardised Johannesburg TetraONE protocol including both a $CD45^{bright}$ and a PLG gate, was applied in this study, differences were noted between these results and those of Scott et al, or data from Beckman Coulter(162). Although a similar Forward Scatter (FSC) threshold was applied, higher FSC voltage settings than those used in the standardised protocol were applied resulting in non-leucocyte events (i.e., platelets and debris) being included in the PanLeucogate (Gate A) and negatively affecting the final PLG CD4+ count (see Figure 5.1 in Appendix G). This may also have been the main contributing factor in the significant bias noted between gating methods when all other variables were equal (i.e., $CD45^{bright}$ vs. PLG on the same listmode data).

Overall, these results strongly emphasise the need for attention to the manner in which samples are handled, stored, processed, and analysed. It has been reported that samples should be maintained at cooler temperatures (17-23°C) immediately after venesection, through transit, during any handling, and finally, in the laboratories, to avert deleterious effects that increased temperatures could have on sample integrity(64). The results here further suggest the need for laboratories to independently determine the most suitable method of handling samples. Relevant factors for consideration include transportation and storage temperatures and conditions prior to sample testing. Storage of samples at room

temperature would probably be the easiest and most practical option but it needs to be emphasised that temperatures should be maintained within a specified range, (preferably between 17-23°C).

Sample storage at refrigerator temperature is a good option for regions that experience temperatures that exceed 30ºC. However, it is important to stress that storage of samples is evaluated by laboratories to assess what works and best fits their local scenario. Local logistic analysis studies similar to these performed in this study would be of value in this context to determine best sample stability. The results of this study suggest that sample integrity is better maintained if samples are refrigerated as soon as possible after venesection.

5.3 Evaluation of the use of a Commercial Whole Blood Stabilising Agent to enable Extending the Window of CD4 Testing.

Stabilisers are added to blood to maintain the integrity of cells surface markers so that, if samples cannot be processed immediately after venesection, processing can be done at a later time and reliable results still obtained. A good stabilising agent should extend the window of testing without compromising the integrity of the samples. The advantage of using such a stabiliser is that it would improve accessibility to testing especially for remote sites that very often, in resource poor settings, lack laboratory facilities and have to send samples to centralised sites for testing. A good sample stabilising agent would also eliminate costs that accompany decentralised laboratory services and would therefore facilitate the centralisation of facilities and skills. This in turn, would simplify monitoring and implementation of quality control programmes, service delivery and logistics of maintaining the laboratories(164).

In this study, it was not possible to conclude whether the stabiliser actually ensured reliability of CD4 testing for the period claimed by the manufacturer. These results were in contrast to previously published work that confirmed extended window of testing using the new 2ml BDB tubes(165). In our study, data for 20 fresh samples (< 4 hours old at baseline) analysed at one of the laboratories showed that samples could not be used by day six from venesection. However, data from 69 samples analysed at the second laboratory showed that testing could be extended up to 8 days from venesection. This difference in results may have been due to differences related to the patient groups studied, differences in the batches of vacutainer tubes, differences in the TruCount kits used and possibly due to differences in operator skill at the two sites. The disparity of these results however, necessitates further evaluation of the efficacy of this stabiliser. If this is done, it should be noted that the 2ml tubes were taken off the market and re-launched as 5ml tubes after it was noticed that haemolysis of the samples occurred in high altitude regions if a full blood draw (2ml) was not made. Some haemolysis was noted in samples drawn in these special vacutainer tubes in the Johannesburg group (personal communication – Lawrie D.), which may also have contributed to the poorer results and failure to reach the supplier's claim for the product at this center.

Several other studies that evaluated the efficacy of different blood stabilising agents have shown that the window of CD4 testing could be extended for varying lengths of time (days) depending on the type of stabiliser, the concentration at which the stabiliser was used and depending on the temperature at which the stabilised/fixed samples were stored prior to analysis (i.e. 4°C, 25°C, 37°C or 42°C)(166-169).

6.0 CONCLUSIONS

Variation in patient CD4+ counts and variation of counts noted between laboratories has been reported to influence clinical management of HIV patients(61). Its impact, especially in patients with low CD4+ counts or those with full blown AIDS and therefore critically in need of ART and other prophylaxes, raises two main concerns. Firstly, there is a need to educate all concerned parties of the role and importance of ensuring quality diagnostic services for CD4 testing in the decision making process and clinical management of patients. Secondly, there is a need to train laboratory personnel and equip laboratories with tools to provide these high quality services. Some recommendations for optimal testing conditions therefore include:

Encouraging laboratories to use "Good clinical laboratory practice" (GCLP) including the use of daily internal quality control (IQC) tools/materials and participation in external quality assessment schemes (EQA). IQC enables a laboratory to monitor the day-to-day quality of sample processing, the performance of personnel, test procedures, reagents and instruments to ensure accuracy, reliability and reproducibility of results. EQA, on the other hand is the evaluation of the performance of the laboratory in question versus the performance of a number of laboratories using specially supplied samples. It is performed by an external agency and its main objective is to continually monitor the standard of a laboratory's performance and accuracy of reporting in relation to peer-laboratories. EQA is helpful for identifying systematic errors in a laboratory that IQC may fail to detect(41) and for assessing longitudinal performance over time. It is a valuable platform for assessing training needs, implementing on-going teaching and evaluation of how CD4 methodologies are used(41, 52). It is therefore a useful tool for observing inter-laboratory and inter-machine reliability and can serve as a reliable resource for policy makers who wish to

review performance of laboratories using various CD4 systems(41). Both IQC and EQA participation have been reported to significantly improve quality of testing(41, 46).

Where possible, CD4 methods, systems and protocols used could be to improve reliability and comparability of CD4 testing within and between-laboratories(39-41, 43-45, 55, 60, 87, 94, 148, 150). Standardisation would not only be more cost-effective and ensure a tighter between-laboratory precision for CD4 reporting, but would simplify procurement of supplies, service delivery, training, and management of quality assurance programmes(164). The same protocol/method/instrument must consistently be used and, if any changes need to be made, the new protocol/method/instrument must be validated before being put into use.

Laboratories may also have to take on the responsibility of ensuring that samples submitted for testing meet set criteria based on international guidelines(64). Samples must be drawn in the correct collection tubes (EDTA) and these filled to a correct volume (i.e. correct EDTA to blood ratio) to ensure the accuracy and precision of results(130). Samples must also be kept and transported at appropriate temperatures. Where available, ice packs or ice cubes wrapped in disposable plastic bags and newpaper to insulate and prevent direct contact with the samples, could be fitted around the samples in the transportation containers (cooler boxes) to cool temperatures during transit. Temperature-monitoring devices similar to the kind developed by WHO/UNICEF for monitoring vaccine deliveries(170) could also be placed in the transportation containers to monitor temperature during transit. Temperature readings could then be noted on delivery of samples to the laboratory and this information used to assess if samples were still suitable for testing.

In order to minimise damage of the samples during transit, samples could be placed into vertical tube racks and the racks placed into the transportation containers and fitted around with soft paper (old news paper, cotton wool or bubble wrap) to act as shock absorbers and

as a cushion to keep the samples in fixed positions. Transportation containers must not be roughly handled and should be kept from being tossed about or toppled over during the drive to the laboratory.

Field workers (phlebotomists, drivers) and laboratory staff should be educated, trained and provided with written, easy to follow standard operating procedures (SOPs)/guidelines on sample quality, sample collection, sample handling and transportation. However, as important as the education and training are, these would be pointless if the knowledge is not implemented. The onus would therefore be on the laboratory management teams to see to it that SOPs and good clinical laboratory practice (GCLP) are implemented and maintained.

Samples received for testing must be clearly labeled with a date and time of collection or submitted with this information to ensure that the samples are both suitable for testing and are processed within the acceptable time frame. On receipt, samples must be stored within the temperature range established as most suitable by the laboratory. During hot seasons in laboratories without air conditioners, samples could be stored in well ventilated areas and away from direct sunlight to minimise temperature increases. Alternatively, the samples could be left in the transportation containers/cooler boxes with ice packs/ice blocks until testing. In case the latter are unavailable, samples could be placed into vertical tube racks and the racks placed in open containers containing cold water (between 17–25°C) to immerse at least half the height of the sample tubes.

In preparation of samples for processing, prolonged mixing of the samples should be avoided and only proper blood rockers should be used (circular mixers tend to cause sedimentation and are only useful for chemistry analyses).

Test reagents and kits must validated before being put into use and used before the expiry date and in accordance with manufacturer guidelines.

Laboratories should ensure that pipettes are calibrated and validated before being put to use. The pipetting skills of operators should be assessed and regularly monitored as part of in-house quality control measures to minimise errors. Whole blood (not distilled water) must be used in these pipetting exercises to avoid volume errors that may otherwise occur due to the difference in viscosity of blood and water(64).

Several studies have also shown that the effect of diurnal variation is minimised if patient samples are repeatedly drawn at a specified time of day or time interval (e.g., between 8am – 12noon)(49, 51). Laboratories could be encouraged to do this, especially if they provide routine follow-up CD4 testing.

Lastly, laboratories (the users) and instrument manufacturers should be encouraged to partner in the effort to improve quality of CD4 testing. Closer interaction between the two groups could be used as a training/learning tool and to address loopholes in the systems/policies in use; the requirement to use specific pipettes/pipetting technique and bias between methods and instruments are cases in point.

This study provides information about causes of variation in CD4+ counts enumerated using different flow cytometry methods and provides some insight into how the quality and consistency of CD4+ testing can be improved both within and between laboratories.

7.0 LIMITATIONS

Repeatability of the study was not assessed between laboratories in the same country or using the same batch of samples. Such reproducibility and extended window studies would probably have provided a clearer conclusion of what temperature was most conducive for sample storage. Using the same number and batch of blood samples could have ruled out the differences in results obtained at the two laboratories and probably provided better data for comparison.

The 'actual' variability in CD4+ counts caused by the flow cytometers (instrument precision) was not assessed using a singly prepared sample. Performing this evaluation would have required that a prepared sample be acquired repeatedly (at least 10 times) for meaningful interpretation of a calculated %CV of the replicates. However, this could not be done because the prepared sample volume were too small to obtain 10 replicate CD4+ counts i.e. the (500µl) sample volume used in the MultiTEST/TruCount protocol or the (1ml) volume used in the PLG-CD4 and TetraONE protocols. Instrument precision was however tested daily (and optimally ensured) at the Johannesburg laboratory using bead count rates where precision of instrument fluidics was monitored daily(152).

An assessment of variation between same model cytometers i.e. Epics-XL versus Epics-XL or FACSCalibur versus FACSCalibur, to provide the basis for a comparison between different models from different manufacturers i.e. Epics-XL versus FACSCalibur, was not performed. It is recommended that such studies are followed up especially with the view to establishing standardisation of calibration between instruments and minimising bias in CD4+ counts within laboratories using more than one flow cytometer (as is the case in the Johanessburg laboratory where five Epics-XL instruments are used). With respect to the

BDB system, unfortunately, neither laboratory had more than one FACSCalibur flow cytometer to compare the MS/TC protocol between instruments in the same laboratory.

Further, as there already exists a good supporting body of literature which shows the existance of bias between CD4 methods(25-27, 29-31, 33, 40, 43, 45, 54-60), it was not considered within the scope of this study to include an assessment of bias between different platforms (DP versus SP) or bias introduced through use of other smaller systems (e.g., FACSCount, Guava and PointCARE systems).

In accordance with standardised protocols of the Johannesburg flow laboratory and published work by Scott et al, Glencross et al and data contained in the PLG package insert (BCI, Miami, FL), the extended window study, especially with respect to the 2-colour PLG protocol used, was not properly optimised by the writer for this study and very likely affected the data showing extended window to just 3 days. It is recommended that the unit repeat this aspect of the study with special attention to forward scatter threshold setup and size discrimination settings to properly ensure that all platelet and other particulate non-cellular fluorescenting debris (see Figure 5.1 in Appendix G) is excluded from the PLG gate. A specifically controlled study to assess the impact of transportation of samples (and temperature related issues during transportation) may further reveal interesting and relevant insights in how samples are potentially damaged during transit as well.

Although a comparison in performance of the new 2ml BDB tubes against the standard EDTA tubes has previously been done(165), it was not done in this study. It would have been important to assess the effect of the stabilising agent by comparing CD4+ counts of samples drawn in the new tubes to those drawn in standard EDTA tubes, at baseline.

APPENDICES

APPENDIX A: List of Other Instruments used in the Study.

Other instruments used in the study included:

i) Weighing balances

- AAA 250/L Adams Balance
- ALS Series Analytical Balance (Jencons – PLS, West Sussex, UK)

ii) Vortex-Genie2 mixer (Scientific Industries, INC. Bohemia, N.Y. USA)

iii) Roller Mixer (Thermo Denley SPIRAMIX)

APPENDIX B: Description of Pipetting Techniques

Procedure using the Forward Pipetting Technique.

In forward pipetting, the pipette is set to a desired volume and a correct tip fitted to its tip cone. Thumb pressure is gently applied to the plunger until the first stop and the tip placed beneath the surface of the blood sample to be pipetted. The plunger is then smoothly released to draw up blood, the tip carefully withdrawn from the blood, its exterior wiped to remove excess blood and the aspirated blood expelled into a test tube by gentle application of pressure to the plunger until the second stop.

Procedure using the Reverse Pipetting Technique.

In reverse pipetting, the pipette plunger is pressed all the way to the second stop to allow for a slight excess of sample to be aspirated. Excess blood on the exterior of the tip is carefully wiped and the blood expelled into a test tube by gentle application of pressure to the plunger until the first stop. The excess blood left in the tip is discharged.

APPENDIX C: Samples kept in motion and analysed using the TetraONE protocol with 2-colour PLG gating.

Table 4. 35: Change in Mean CD4+ counts as a Ratio of Baseline and Mean Difference to Baseline for samples kept in Motion and derived using TetraONE Protocol with 2-colour PLG Gating

Day post venesection	Ratio of Mean Day CD4 Count to Baseline as a (%)	SD	p value at Cut-off		Bias	SD	95% CI	p value at Cut-off	
			90%	95%				40cells/µl	20cells/µl
≥1-2	96.24	9.82	0.0007	0.244	16.19	31.17	(4.8, 27.6)	0.0001	0.2509
≥2-3	89.38	9.31	0.6431	0.9989	29.42	33.46	(17.1, 41.7)	0.0443	0.9362
≥3-4	83.78	17.11	0.974	0.9995	51	61.03	(28.6, 73.4)	0.8382	0.9959
≥4-5	71.34	14.46	1	1	89.94	68.22	(64.9, 115.0)	0.9998	1
≥5-6	66.97	14.89	1	1	102.74	71.56	(76.5, 129.0)	1	1
≥6-7	59.09	17.21	1	1	120.25	243.85	(25.7, 214.8)	0.9535	0.9807
≥7-8	53.54	15.12	1	1	152.15	106.73	(102.2, 202.1)	0.9999	1
≥8-9	54.28	12.64	0.9945	0.9962	199.25	263.52	(-220.1, 618.6)	0.8433	0.8666

Table 4. 36: Change in Mean CD4% as a Ratio of Baseline over Days for samples kept in Motion and derived using TetraONE Protocol with 2-colour PLG Gating

Day post venesection	Age of samples (hours)	Ratio of mean CD4% to Baseline as a (%)	SD	95% CI	p-value
≥1-2	36-48	102.08	5.85	(99.9, 104.2)	<0.0001
≥2-3	60-72	104.17	9.91	(100.5, 107.8)	<0.0001
≥3-4	84-96	110.55	11.4	(106.4, 114.7)	<0.0001
≥4-5	108-120	122.43	20.26	(115.0, 130.0)	<0.0001
≥5-6	132-144	129.74	27.52	(119.6, 139.8)	<0.0001
≥6-7	156-168	134.98	34.34	(121.7, 148.3)	<0.0001
≥7-8	180-192	145.25	21.53	(134.9, 155.6)	<0.0001
≥8-9	204-216	151.4	18.93	(121.3, 181.5)	0.0047

Merging of the CD45^{bright} population with nearby monocytes resulted in the samples not being suitable for use beyond 48 hours.

APPENDIX D: Samples stored at 37°C and analysed using the TetraONE protocol with 2-colour PLG gating.

Table 4. 37: Change in Mean CD4+ Counts as a Ratio of Baseline and Mean Difference to Baseline for samples kept at 37°C in a Water Bath and derived using the TetraONE Protocol with 2-colour PLG Gating

Day post venesection	Ratio of Mean CD4 count to Baseline as a (%)	SD	p value at Cut-off		Bias	SD	95% CI	p value at Cut-off	
			90%	95%				40cells/µl	20cells/µl
≥1-2	83.55	13.31	0.9943	1	44.55	31.44	(33.0, 56.1)	0.7865	0.9999
≥2-3	75.30	20.50	0.9997	1	74.17	57.83	(52.2, 96.2)	0.9982	1
≥3-4	41.39	23.09	1	1	174.77	147.61	(109.3, 240.20	0.9998	1

Table 4. 38: Change in Mean CD4% as a Ratio of Baseline over Days for samples kept at 37°C in a Water Bath and derived using the TetraONE Protocol with 2-colour PLG Gating

Day post venesection	Age of samples (hours)	No of Samples	Ratio of mean CD4% to Baseline as a (%)	SD	95% CI	p-value
≥1-2	36-48	31	103.59	10.61	(99.7, 107.5)	<0.0001
≥2-3	60-72	29	93.98	20.14	(86.32, 101.65)	0.606

APPENDIX E: Samples stored at 17-23°C and analysed using the TetraONE protocol with 2-colour PLG Gating.

Table 4. 39: Change in Mean CD4+ Counts as a Ratio of Baseline and Mean Difference to Baseline for samples stored at Room Temperature (17-23°C) and derived using the TetraONE Protocol with 2-colour PLG Gating

Day post venesection	Ratio of Mean CD4 count to Baseline as a (%)	SD	p value at Cut-off		Bias	SD	95% CI	p value at Cut-off	
			90%	95%				40cells/µl	20cells/µl
≥1-2	98.57	14.4	0.0012	0.0890	5.71	57.04	(-15.2, 26.6)	0.0011	0.0866
≥2-3	94.69	9.86	0.0064	0.5696	12.58	28.54	(2.1, 23.1)	<0.0001	0.0791
≥3-4	90.78	11.48	0.3544	0.9753	24.84	29.33	(14.1, 35.6)	0.0037	0.8172
≥4-5	86.83	9.75	0.9598	1	39.94	38.44	(25.8, 54.0)	0.4963	0.9964
≥5-6	86.73	10.16	0.9583	1	36.10	29.85	(25.1, 47.0)	0.2361	0.9973
≥6-7	82.24	10.72	0.9997	1	54.57	60.85	(31.0, 78.2)	0.8920	0.9972
≥7-8	80.50	10.62	1.00	1	55.83	38.52	(39.2, 72.5)	0.9692	1
≥8-9	75.95	11.61	0.9530	0.9768	103.00	73.43	(-13.9, 219.9)	0.9076	0.9456

Table 4. 40: Change in Mean CD4% as a Ratio of Baseline over days for samples stored at Room Temperature (17-23°C) and derived using the TetraONE Protocol with 2-colour PLG Gating

Day post venesection	Age of samples (hours)	No of Samples	Ratio of Mean CD4% to Baseline as a (%)	SD	95% CI	p-value
≥1-2	36-48	31	102.22	6.10	(100.0, 104.5)	<0.0001
≥2-3	60-72	31	106.51	6.85	(104.0, 109.0)	<0.0001
≥3-4	84-96	31	109.37	12.2	(104.9, 113.9)	<0.0001
≥4-5	108-120	31	109.1	12.28	(104.6, 113.6)	<0.0001
≥5-6	132-144	31	109.24	11.23	(105.1, 113.4)	<0.0001
≥6-7	156-168	28	111.11	12.27	(106.4, 115.9)	<0.0001
≥7-8	180-192	23	111.37	12.55	(106.0, 116.8)	<0.0001
≥8-9	204-216	4	117.26	4.55	(110.0, 124.5)	0.0011

Merging of the CD45^{bright} population with nearby monocytes resulted in the samples not being suitable for use beyond 48 hours.

APPENDIX F: Samples stored at 4-6°C and analysed using the TetraONE protocol with 2-colour PLG Gating.

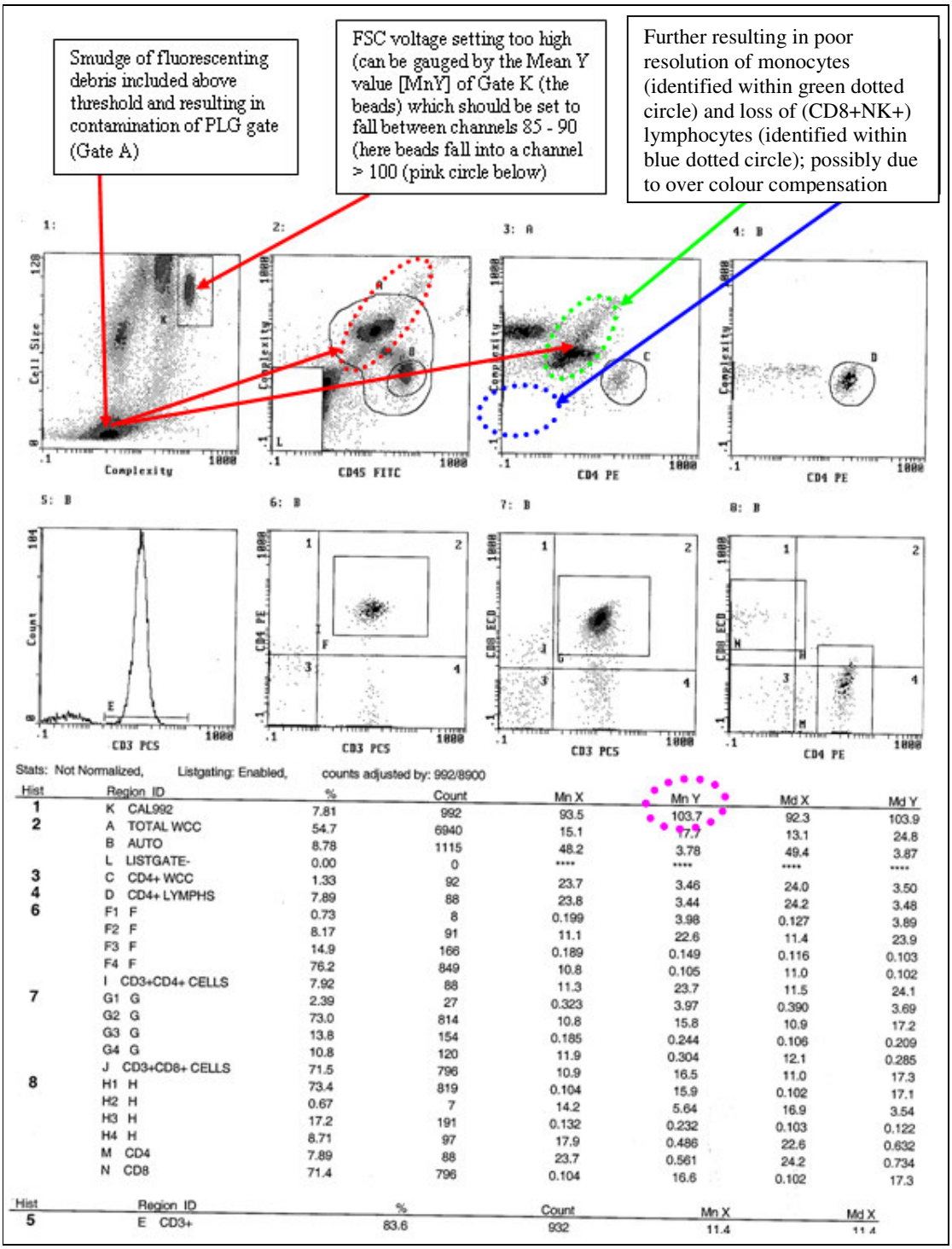
Table 4. 41: Change in Mean CD4+ counts as a Ratio of Baseline and Mean Difference to Baseline for Samples stored at Refrigerator Temperature (4-6°C) and derived using the TetraONE Protocol with 2-colour PLG Gating

Day post venesection	Ratio of Mean CD4 count to Baseline as a (%)	SD	p value at Cut-off		Bias	SD	95% CI	p value at Cut-off	
			90%	95%				40cells/µl	20cells/µl
≥1-2	97.26	8.75	<0.0001	0.0806	11.39	25.49	(2.0, 20.7)	<0.0001	0.0348
≥2-3	93.37	9.42	0.0277	0.8278	21.26	29.75	(10.3, 32.2)	0.0007	0.5923
≥3-4	85.16	10.3	0.9931	1	51.23	71.68	(24.9,77.5)	0.8049	0.9892
≥4-5	78.95	12.11	1	1	64.97	69.12	(39.6, 90.3)	0.9733	0.9995
≥5-6	78.73	14.88	0.9999	1	70.29	64.06	(46.8, 93.8)	0.9934	0.9999
≥6-7	69.27	14.20	1	1	84.82	47.13	(66.5, 103.1)	1	1
≥7-8	60.28	17.86	1	1	138.65	159.36	(64.1, 213.2)	0.9939	0.9982
≥8-9	62.19	17.18	0.9987	0.9995	73.25	62.34	(21.1, 125.4)	0.9124	0.9768

Table 4. 42: Change in Mean CD4% as a Ratio of Baseline over Days, for Samples stored at Refrigerator Temperature (4-6°C) and analysed using the TetraONE Protocol with 2-colour PLG Gating

Day post venesection	Age of samples (hours)	No of Samples	Ratio of Mean CD4% to Baseline as a (%)	SD	95% CI	p-value
≥1-2	36-48	31	98.93	5.66	(96.9, 101.0)	<0.0001
≥2-3	60-72	31	100.62	7.40	(97.9, 103.3)	0.0001
≥3-4	84-96	31	98.34	5.93	(96.2, 100,5)	0.0019
≥4-5	108-120	31	94.08	13.52	(89.1, 99.0)	0.6471
≥5-6	132-144	0	0			
≥6-7	156-168	28	97.62	11.16	(93.3, 102.0)	0.1121
≥7-8	180-192	20	99.90	13.87	(93.4, 106.3)	0.0653
≥8-9	204-216	8	96.79	8.70	(89.5, 104.1)	0.2890

APPENDIX G:



Hist	Region	ID	%	Count	Mn X	Mn Y	Md X	Md Y
1	K	CAL992	7.81	992	93.5	103.7	92.3	103.9
2	A	TOTAL WCC	54.7	6940	15.1	17.7	13.1	24.8
	B	AUTO	8.78	1115	48.2	3.78	49.4	3.87
	L	LISTGATE-	0.00	0	****	****	****	****
3	C	CD4+ WCC	1.33	92	23.7	3.46	24.0	3.50
4	D	CD4+ LYMPHS	7.89	88	23.8	3.44	24.2	3.48
6	F1	F	0.73	8	0.199	3.98	0.127	3.89
	F2	F	8.17	91	11.1	22.6	11.4	23.9
	F3	F	14.9	166	0.189	0.149	0.116	0.103
	F4	F	76.2	849	10.8	0.105	11.0	0.102
	I	CD3+CD4+ CELLS	7.92	88	11.3	23.7	11.5	24.1
7	G1	G	2.39	27	0.323	3.97	0.390	3.69
	G2	G	73.0	814	10.8	15.8	10.9	17.2
	G3	G	13.8	154	0.185	0.244	0.106	0.209
	G4	G	10.8	120	11.9	0.304	12.1	0.285
	J	CD3+CD8+ CELLS	71.5	796	10.9	16.5	11.0	17.3
8	H1	H	73.4	819	0.104	15.9	0.102	17.1
	H2	H	0.67	7	14.2	5.64	16.9	3.54
	H3	H	17.2	191	0.132	0.232	0.103	0.122
	H4	H	8.71	97	17.9	0.486	22.6	0.632
	M	CD4	7.89	88	23.7	0.561	24.2	0.734
	N	CD8	71.4	796	0.104	16.6	0.102	17.3

Hist	Region	ID	%	Count	Mn X	Md X
5	E	CD3+	83.6	932	11.4	11.4

Figure 5. 1: Figure illustrating the effect of poor threshold setting on accurate enumeration of CD4+ counts using the 4-colour TetraONE protocol.

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