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THE MANAGEMENT OF HIV POSITIVE PATIENTS USING A CD8/38 FLOW CYTOMETRY ASSAY AS AN ALTERNATIVE TO VIRAL LOAD TESTING

THE MANAGEMENT OF HIV POSITIVE PATIENTS USING A CD8/38 FLOW CYTOMETRY ASSAY AS AN ALTERNATIVE TO VIRAL LOAD TESTING

Keshendree Moodley

Dissertation submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Master of Science in Medicine.

Johannesburg, 2011

DECLARATION:

I, Keshendree Moodley declare that that this dissertation is my own work. It is being submitted for the degree of Master of Science in Medicine in the branch of Molecular Medicine and Haematology to the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.

……

Keshendree Moodley

……day of……., 2011.

DEDICATION:

To my loving parents whose unconditional love and support have allowed me to achieve my dreams.

To my darling husband, thanks for your patience, love and understanding.

PUBLICATIONS AND PRESENTATIONS ARISING FROM THIS STUDY:

- Poster presentation entitled “From research tool to routine test: CD38 Monitoring in HIV patients”- WITS Open day September 2008.
- Oral and poster presentation entitled “Extended window period for CD38 activation marker testing to 48 hours post venesection”. Oral presentation at PATHTECH Durban 2009 and poster presentation at 4th International Workshop on HIV treatment, Pathogenesis and Prevention Research in Resource Poor Settings in Mozambique 2010 together with implementation of the CD38 assay at a secondary site.

ABSTRACT:

BACKGROUND: Human Immunodeficiency Virus (HIV) is a global epidemic with growing numbers of people on highly active anti-retroviral therapy (HAART) programmes. Effectiveness of treatment needs to be monitored to ensure the uncompromised well being of patients. This is currently done using both Viral Load (VL) and CD4 cell counts for HAART initiation and follow-up. Although VL is the best predictor of disease progression it is often too expensive for monitoring patients in resource-limited settings. There is thus a need for a cheaper, more accessible alternative to monitor long term patient response to therapy.

METHODS: This study evaluated the use of a recently described flow cytometric assay of CD38 expression (previously developed at the Johannesburg Flow Cytometry Reference Laboratory) in a cohort of HIV+ patients failing 1st line therapy, who were subsequently enrolled onto 2nd line HAART. CD38 and CD8 were "piggy -backed" onto the PLG/CD4 protocol and mean fluorescence intensity (MFI) of the CD8/38 expression was monitored longitudinally. Patterns of CD38 expression were compared to 1st line treatment observations to establish equivalence in the predictive power of CD38 expression of fluctuation in viral load on 2nd line treatment patients. In addition, the effect of sample age on assay accuracy was tested before implementation of the CD38 assay at a secondary testing site.

RESULTS: The patterns observed in the cohort of 2nd line therapy patients mirrored patterns previously seen in 1st line therapy with 55% of patients showing a continuous decline in CD38 MFI that mimicked changes in VL. The remaining 33% of patients had non-specific increases in CD38 MFI without concurrent increases in VL and one patient showed irregular VL and CD38 MFI (non-responder). The CD38 assay showed acceptable accuracy and reproducibility up to 48 hours after venesection (%CV<5%). Implementation at the secondary testing site was successful with 98% similarity (%CV<5%) compared to the reference laboratory.

CONCLUSION: CD38 monitoring of 2nd line therapy patients showed comparable patterns to observations in 1st line therapy patients. The assay proved stable over time and easy to implement at another PLG/CD4 testing facility. As such, the CD38 assay offers a cost-effective, reliable real time supplementary test to long-term VL monitoring of HIV infected patients on the national ART programme.

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ABBREVIATIONS USED IN THE TEXT:

AIDS	Acquired Immune Deficiency Syndrome
ART	Anti-Retroviral Treatment
BC XL	Beckman Coulter XL Flow Cytometer
BD	Becton Dickinson
BCR	Bead Count Rate
CMJAH	Charlotte Maxeke Johannesburg Academic Hospital
CD38 MFI	CD38 Mean Fluorescent Intensity
CIPRA	Comprehensive International Program of Research on AIDS
EDTA	Ethylenediaminetetraacetic
ELISA	Enzyme-linked Immunosorbent Assay
FL4	Fluorescent Channel 4
MFI	Mean Fluorescence Intensity
HAART	Highly Active Anti-Retroviral Treatment
HIV	Human Immunodeficiency Virus
HJH	Helen Joseph Hospital
LMD	Listmode Data
NHLS	National Health Laboratory Services
PLG	Pan-Leuko Gating
QC	Quality Control
SOP	Standard Operating Procedures
TB	Tuberculosis
T Cells	T Lymphocyte cells
VL	Viral Load
WHO	World Health Organisation
NSI	Non-specific increases

1.0 INTRODUCTION

Human Immunodeficiency Virus (HIV) infection has emerged as the cause of one of the major infectious pandemics of the 21st century. At the end of 2008 there were around 33.4 million people infected worldwide according to the UNAIDS report on the Global AIDS Epidemic (1). South Africa is the main contributor to this figure with a population of 5.7 million infected with the disease (2). Huge efforts are being made by governments and private sector alike to try to curb the spread of the disease through education campaigns and the implementation of treatment programmes (issuing of anti-retroviral treatment (ART) to HIV infected people). Currently there are over 950 000 HIV infected people receiving treatment in the private and public sectors (3). This wide scale treatment implementation in South Africa requires monitoring of ARV treatment effectiveness and ensuring uncompromised safety of patients. To achieve this, different laboratory tests and analyses are required as per recommendations by WHO (4).

CD4 counts and viral load (VL) testing are just two of the many safety tests employed for HIV surveillance by all laboratories of the South African National Health Laboratory Service (NHLS-SA). CD4 lymphoid cells are the primary target of the HIV virus and the decrease in the CD4 absolute count is an indicator of the immune status of HIV+ patients. Therefore measuring the number of CD4+ T cells in blood reflects the activity of the virus on the immune system (5). The HIV virus replicates daily producing billions of new HIV copies (6). The best way to quantify this replication is to conduct a HIV viral

load test which allows one to establish the level of HIV infection and help to monitor treatment efficacy in patients that have already been diagnosed as HIV positive (7).

1.1 Current Laboratory Tests for Monitoring HIV

1.1.1 CD4 testing

In untreated HIV there is a massive depletion of CD4+ T cells. This is mainly due to HIV-1 causing an increase in immune activation resulting in an increased production of T cells. This overproduction leads to exhaustion and decreased renewal capability of CD4+ T cells and thus a decrease in the total CD4+ lymphocyte population over time (8-9), commonly referred to as the "tap and drain" hypothesis. In addition, an increase in abnormal apoptosis of uninfected CD4 Tcells also contributes to a decrease in circulating CD4+ T cells (10-13). There has also been new research focusing on the gastrointestinal tract (GIT) as the reason for this huge depletion, as the majority of CD4 T cells are found at this site (14-15). It was shown by Brenchley et al that the CCR5+CD4+ T cells are specifically depleted which could be due to CCR5 being the receptor for the HIV virus (14) while Shacklett et al postulate that once infection occurs in the GIT the CD4 cells are killed directly by NK cells or cytotoxic T lymphocytes (CTL) or through bystander apoptosis thereby causing the decrease in CD4 cells (15).

WHO guidelines recommend that a CD4 count be carried out to either establish HIV status and monitor treatment efficacy (4).

There are numerous CD4 technologies available in the market today, of which flow cytometry is still the most popular. These typically include single platform assays using

either bead based or alternatively true volumetric measurement capabilities, or the less frequently used dual platform methods that require cell counting on a haematology analyser to produce lymphoid percentages.

Several manufacturers provide products and instruments (flow cytometers) for single platform CD4 enumeration methods, including Becton Dickinson (BD), Beckman Coulter (BC), Guava and Partec. BD flow cytometers include the semi-automated FACSCount™. This technology is a 2-tube; 2-colour immunoflourescence assay that has pre-dispensed beads and bead calibrators within the tube that requires minimal technical input. It however requires accurate manual pipetting of blood, with no lysis of red blood cells. Sample analyses take around 60 minutes/sample. As such these instruments are ideally suited for low volume CD4 testing facilities. Long term reliability in the field has been proven (16-19).

TruCount™ is another CD4 technology from BD used on the FACSCalibur™ instrument. It is a pre-dispensed bead based, SP method that requires accurate reverse manual pipetting of blood, with option for fully automated, algorithm-driven sample analyses (TruCountTM Multiset™). It is a high throughput system that can analyse approximately 150 samples per day (20). Equivalent systems have been described by other manufacturers that use beads dispensed with blood i.e. Beckman Coulter Epics flow cytometers (21). Here, either reverse or forward pipetting can be used (22) for accurate cell enumeration as long as the same pipette is used to dispense both (23). Most of the new generation flow cytometers have automated or semi-automated

preparation systems to minimize pipetting error (i.e. Immunoprep™, PrepPlus2™ and CellMek™ by Beckman Coulter).

Lesser used flow systems like Guava Technologies (which include the Easy CD4™ and PCA™ systems) require small volumes of blood and minimal volumes of reagents. These methods however require accurate manual reverse pipetting and skilled computer literate laboratory personnel to operate the systems. As these systems are not fully automated they are mainly used in low throughput laboratories (24).

Instruments made in Germany which are very similar to the Guava instruments are the Partec CyFlow and CyFlow SL_Blue and Green instruments. They are also SP volumetric flow cytometers which require manual reverse pipetting with manual loading of samples and are primarily used in low volume laboratories. These instruments do not require beads or a lyse-prep system; however, they do require technologists with a good understanding of flow cytometry for data interpretation (25).

Several alternative low-end CD4 technologies have been described for use in resource poor settings. In 1997, O'Gorman and colleagues (26) validated a volumetric capillary cytometric (VCC) assay against the dual platform assay in different laboratories and found that interlaboratory variability was similar although the VCC instrument gave lower CD4 and CD8 absolute results. This study concluded that the VCC method could be used as a cheaper alternative to the standard methods providing single platform (SP) absolute count reports. A manual CD4+ count kit assay using cytospheres has also been described for use. Several studies have validated this method against flow

cytometry (27-32), although outcomes have been poor and this system is not generally advocated for use.

There are also point-of-care (POC) CD4 technologies that are now being developed for use in resource poor countries. These diagnostic instruments are portable, fast and easy to use, providing immediate CD4 results thus eliminating patient lost to follow-up due to delays in laboratory tested CD4 results. The PIMA POC CD4 test system was recently tested in Zimbabwe at a VCT centre (33). Results revealed that there were no significant differences between the conventional CD4 testing (BD FACSCalibur) and the PIMA CD4 analyser. Although the PIMA instrument is advocated for use in both rural and centralised clinics and hospitals and could alleviate the high burden of CD4 testing at centralised sites, more studies need to be conducted on the reproducibility and accuracy of the instrument.

In South Africa, a cost effective simplified flow cytometric CD4 assay (PLG/CD4)(34) was developed and rolled out to National Health Laboratories in 2002 (35). This assay differs from previous flow cytometry CD4 methods in that it references absolute CD4 count to the total leukocyte count and not the total lymphocyte count as with previous methods (34, 36). This assay employs only two monoclonal antibodies, namely CD45 FITC and CD4 PE and therefore allows two additional fluorescence channels to be used for additional markers. Numerous quality control procedures have been implemented to ensure the accuracy, reproducibility and stability of the assay (22, 37-40). The PLG/CD4 test is performed and analysed within a turnaround time (TAT) of ~24-48

hours and can be used on any make/model of flow cytometer equipped with one or more lasers.

Currently this technology is used in 69 laboratories in SA (37), has been validated internationally (41) and used in other countries such as Mozambique (42), the Caribbean (43) and the Far East (44-45). PLG is also described using generic antibodies against the standard 6-tube-2-colour and 3-tube-3-colour panels (44) with good correlations and no bias in CD4 values, confirming the findings of other studies (34, 37, 41-42, 46).

1.1.2 HIV Viral Load (VL) Testing

Viral Load is reported to be the best tool to monitor the progression rate of HIV disease to AIDS as well as a patient's response to treatment (47-49). There are numerous VL tests available in the market today but only two are routinely used in South African laboratories. These include the AMPLICOR® HIV-1 test from (50) Roche and the NucliSENS HIV-1 QT Assay . Some of the collective advantages of these assays are that they can be used for other diseases besides HIV and are useful for high throughput laboratories (50-53). However, the disadvantages are that they are typically time consuming, labour intensive, require good laboratory infrastructure with dedicated equipment and space and good technical support as well as skilled staff to follow stringent protocols. These tests also require a specific volume of plasma (1.0 ml), sample separation facilities at remote laboratories to separate plasma prior to testing at a centralised site and need to be performed within 24 hours of blood collection. Fully

automated, walk away options are available but reagents and testing systems for VL monitoring remain typically expensive (50-54).

For this reason, certainly in a resource-poor setting, current WHO guidelines advocate VL testing as non-essential for monitoring HIV patients on treatment (55). Thus vital laboratory information of treatment failure and/or development of drug resistance are not accessible to clinicians. A decision to change therapy is typically made on declining CD4 counts and/or clinical profiles, leaving fewer options available for 2nd and 3rd line treatment (56). Viral load testing is however still essential for monitoring patients on ART to manage response to treatment, drug resistance and overall well being of patients (57-58).

1.2 Alternative monitoring to conventional VL testing

There is thus a real need for more accessible and affordable alternative approaches to VL testing that facilitate assessment of HIV virologic rebound and/or treatment failure. Many studies have looked into different methods and alternative assays. These include the Perkin-Elmer heat denatured, signal boosted p24 antigen test as well as the Cavidi Exavir load quantitative HIV-RT kit (51, 59-60). The benefit of the p24 test is that equipment can be shared with ELISA, it can be used for monitoring paediatrics, training is easily available and it has < 1-day turnaround time with high throughput, requiring only small volumes (<0.1ml) of plasma. However, the drawback is that it needs a dry heat block and the lysing reagent used to improve its sensitivity is not commercially available (51-52, 59-60). The Cavidi test is easier to perform and can be used for all

sub-types of HIV and is relatively inexpensive. This test takes more than 3 days to perform and has a low throughput, requires a dedicated incubator and vacuum pump and needs further evaluation before it can be applied to the clinical management of patients (50-54, 56, 59-60). Whole dried blood spots taken from children have been suggested as an alternative solution to conventional VL testing by Patton et al (61). They evaluated the improved ultra- sensitive p24 antigen ELISA and found that regardless of how blood is collected and stored; the test had a sensitivity of 97-100% as long as the assay was performed within 6 weeks of collection (59). This can be applied in third world countries where paediatric HIV diagnosis is restricted till 12 months of age due to the extreme cost of molecular VL tests. The use of these dry blood spots (DBS) can also be used for cost effective monitoring of VL by allowing centralization of monitoring thereby minimizing the need to set up such instrumentation in the periphery. The latest trend in viral load testing is Point-of-Care testing where a viral load can be performed at clinic level/bedside. These include the Liat HIV Quant Assay (62) and the SAMBA HIV-1 test (63). These simplified tests are quick to perform and can be done by non-laboratory trained personnel, however they are not sensitive enough compared to the molecular based tests. Therefore in spite of described alternatives, a truly accessible VL alternative is still not routinely available to monitor virological failure in HIV infected individuals.

1.3 Flow cytometry based alternatives to VL testing

HIV promotes increased immune activation, specifically T cell activation (64-66). Activation markers such as HLA-DR, CD28, CD69, CD40L, CD95 (Fas) and CD71 have been widely studied as possible alternatives for monitoring HIV+ patients receiving ART. Programmed Death Receptor (PD-1) has also been found to be upregulated on CD8+ T cells in HIV untreated patients (67-68). Day et al (69) found PD-1 to correlate positively with viral load and disease progression but negatively with CD4 counts. Holm et al (70) compared PD-1 to CD38 expression and HIV RNA in predicting loss of CD4 cells, and found it to be a better predictor than either of the above.

TIM-3 which is a negative immune regulator is also up-regulated on HIV-1-specific CD8+ T cells. It was found to correlate positively with HIV-1 viral load and negatively with CD4 counts in a population of dysfunctional T cells in a study conducted by Jones et al (71).

Another T-cell activation marker, CD127 (interleukin 7 receptor) ,was found to be a good monitoring tool in patients receiving ART as it showed a positive correlation with CD4 count and a negative correlation with viral load (72).

However the above markers have proven to be suboptimal compared to CD38 expression which has been shown to be the best characterised activation marker on CD8+ T cells (73-76)

1.4 CD38 expression as an alternative to VL testing

CD38 is a type two transmembrane glycoprotein that can act independently as an enzyme and receptor (77). In humans it is expressed according to age with 90% lymphocytes showing significant CD38 expression in newborns. In adults, CD38 expression is found on natural killer cells, T and B cells, monocytes, macrophages and sometimes on platelets and erythrocytes. CD38 is also expressed in the pancreas, prostate epithelial cells and Purkinje cells of the cerebellum (77-79). CD38 has a multitude of associations in the diagnosis of different human diseases for example as an activation molecule in HIV infected T cells and a differentiation antigen in chronic B lymphocytic leukaemia and myeloma (77, 79-83). Strong CD38 expression is largely associated with T cell activation and as a result CD38 is over-expressed on activated CD8-positive T cells in HIV and other infectious conditions (HIV/TB) (84) It is thus not exclusive to HIV infection, but rather a consequence of any type of infection that results in cell activation. HIV infection is characterized by activation of both B and T cells (especially in CD4 and CD8 cells) and consequently an increase in the surface expression of CD38 is seen in HIV+ individuals compared to negligible expression in HIV negative adults (78-79, 85). The CD38 antigen is described as a potential prognostic marker in pre-ART HIV+ populations, as expression patterns parallel downward trends in VL after initiation of highly active anti-retroviral therapy (HAART) (76-77, 85-88).

1.5 Literature review of CD38 Expression in HIV:

CD38 expression as an activation marker in HIV disease was first described by Giorgi and Detels in 1989 (89) when they observed an increase in CD38 expression on CD8+ T lymphocytes that characterised HIV disease progression in patients with low CD4 counts. Following up these initial observations with several published studies (76, 86-87, 90-92) and using different instruments, techniques and reporting methods for CD38 (i.e. CD38%, CD38ABC/ul and CD38 RFI), Giorgi and others concluded that CD38 expression on CD8+ T cells was the best predictor of HIV progression and the development of AIDS (93-95).

Further studies conducted on this activation marker, including one in 1996 by Bouscarat et al (96), looked at the correlation between CD8 lymphocyte activation and viral load, and concluded that CD38 had predictive value and could be used as a monitoring tool in patients on highly active anti-retroviral therapy (HAART). In 1999, a follow up study employing more patients monitored for a longer period of time was carried out by the same group (97). In this study it was found that CD38 activation mirrored viral load results but that CD4 count was dependent on both VL and CD38 (97). Tilling et al, in 2002 (78) found that CD8+/CD38++ expressing T cell counts mimicked viral load, however it was stated that this pattern only occurred in patients receiving HAART with undetectable VL. In 2004, Benito et al (98) also concluded that CD38 expression on CD8 lymphocytes can be employed as an indicator of residual viral replication in HIV treated patients.

All of the above mentioned studies were conducted in first world countries in well resourced centres where patients had access to good quality medication and state of the art laboratory technologies for monitoring response to HAART. Similar studies on CD38 expression in 3rd world resource poor settings where VL and CD4 testing are still considered luxuries were done in Cote d'Ivoire, Africa by Ondoa et al (99). In this study, the percentage of CD38+CD8+ was found to be sensitive in predicting HIV outcome in different types of HIV treatment responders but not specific enough for early detection of virological drug failure. This study was followed up in 2006 with another study assessing the mean fluorescent intensity (MFI) of CD38 on memory (CD45RO+) CD8+ T cells, instead of CD38 percentage. Here a better correlation with VL than previously reported for CD38% was noted (100). In a study conducted in Thailand, use of CD38 expression to monitor HAART has been advocated. However, this study showed that the level of CD38 expression depended on the monoclonal antibody used and it was more reliable when combined with HLA-DR expression (101). Further confirmation of the usefulness of CD38 monitoring was noted in a recent study done on HIV positive patients participating in a scheduled treatment interruption trial where it was found that in 94% of patients, CD38 MFI and VL concomitantly increased during treatment interruption and resumed to pre-interruption levels once treatment was started again (102).

CD38 has thus been the focus of many studies over the past years and proved to be a good prognostic marker in HIV disease. In 2008 data relating to the first year follow-up of patients on ART from the SA-CIPRA study was published (103). These results based

on an in-house developed protocol, using global CD38 MFI on CD8++ gated cells, were very promising and followed the trends of most of the previously published data by other groups. This CD8 ECD and CD38 PC5 assay, expanding on the pre-existing protocol by piggy-backing the CD38 MFI assay onto the well established CD4 monitoring (PLG/CD4) test, has been customized specifically for use on Beckman Coulter XL cytometers (currently in use in ~69 SA-NHLS CD4 laboratories). Long term follow-up of CD38 expression on patients on ART is not well described in the published literature and as such the study by Glencross et al (103) provided valuable information on long-term CD38 response of patients on 1st line ART in the South African setting.

In this study, (103), using the CD38 marker expressed on CD8 T cells (CD8/38) on a cohort of patients on ART followed longitudinally it was found that at baseline, before ART initiation, both the VL and the mean fluorescent intensity (MFI) of CD8/38 was elevated in all patients. However, after the initiation of ART it was noticed that the CD38 MFI and VL both decreased while the CD4 count gradually increased. A continued decrease of expression of CD38 MFI was seen in 60% of patients with undetectable HIV VL levels responding to ART. In the remaining 15-20% of patients in the Glencross study, fluctuations of CD38 were matched to VL increases and in the remaining 20-25% of patients, fluctuations of CD38 expression appeared to reflect lymphocyte activation due to secondary infections. Further investigation into the distribution and patterns of CD38 expression in HIV+ patients failing therapy or patients with underlying causes of persistent lymphocyte CD38 activation, including treatment non-compliance, concomitant viral or other underlying infection including tuberculosis

is thus needed to improve the sensitivity and specificity of CD38 MFI monitoring of HIV+ patients on ART.

2.0 AIM:

It is postulated that the need for regular VL testing can be reduced in at least 60% of patients monitored longitudinally, thus substantially reducing the number of VL tests required across a national ART programme and subsequently reducing the global cost of VL testing (103).

The aim of this study was to confirm the use of a flow cytometric CD38 expression-based assay as a useful alternative and/or supplementary assay to monitor patients on second line ART and establish if similar patterns of CD38 expression as previously reported for patients on 1st line treatment exist in patients who have failed 1st line therapy. This study also investigated the stability of the assay in samples older than 24 hours and further development of standardisation and quality control measures for the CD38 assay in order to decentralize testing to national laboratories already performing flow cytometric PLG/CD4 testing.

3.0 MATERIALS AND METHODS

3.1 Study type and site:

This study was conducted at the NHLS Flow Cytometry Research and Development laboratory at the Charlotte Maxeke Johannesburg Academic Hospital (CMJAH) of the National Health Laboratory Service (NHLS), South Africa. It was a prospective analysis of

data collected on 2nd line patients who were followed longitudinally from 2005 on the "Safeguard the Household" (Grant U19/ AI53217, PI Prof J McIntyre) CIPRA Cohort (Comprehensive International Programme of Research on AIDS) after initially failing first line treatment. The patients were seen at the Thembelethu Clinic at the Helen Joseph Hospital, which is located 15 km from the reference laboratory site. Blood samples were transported to the reference laboratory within 12 hours of venepuncture as part of the coordinated CIPRA effort. The principle investigator at the site was Dr Ian Sanne whose clinic manages 6000 patients on ART, as part of the national ART rollout programme, as well as being responsible for coordinating patient recruitment for the CIPRA network study.

3.2 Study size and subjects:

Patients were enrolled onto the CIPRA cohort during 2005 (103). Specifically, patients were identified on the basis that they had CD4 and VL data available from baseline (pre- antiretroviral treatment) through first line therapy, with longitudinal follow-up of CD4 and viral load with subsequent virological failure and enrolment onto second line ART. Treatment duration on the different regimens differed between patients. On 1st line treatment, duration ranged from 4 to 96 weeks while on 2nd line treatment, it ranged from 36 to 168 weeks. Treatment failure was defined as an increase of VL to greater than 1000 copies in patients already on 1st line therapy. Nine of the original 120 patients enrolled, failed first line treatment and went on to second line ART. These patients (n=9) were monitored longitudinally for CD4, VL and CD38 expression for at

least 1 year after starting on 2nd line treatment. Some patients continued follow-up on the national roll-out programme (Right to Care) after the CIPRA study concluded in December 2009 and data on these patients are reported to June 2010.

In the selected patient cohort, changes in CD38 expression on CD8 T cells were measured as the mean fluorescence intensity (MFI) and monitored in relation to VL changes, with special reference to each patient's baseline values (CD38/8 MFI) and previous visit (percentage of baseline or previous visit).

Patients were classified into three groups, namely low, medium and high responders, based on their baseline CD38 MFI according to the method of classification described for the CIPRA cohort at baseline before commencement of treatment (103). The patient's own baseline CD38 MFI was used as a longitudinal within-patient control for all subsequent follow-up visits because of the relatively wide range of baseline CD38 MFI expression noted between patients, except in those few patients where no 1st line baseline level was available. In order to standardize the rate of decrease of CD38 MFI between patients over time, all individual CD38 MFI values at each subsequent visit were expressed as a percentage of the baseline reading according to methods previously described (104-105).

Although some adverse events were reported for the overall CIPRA study cohort, detailed information on ART failure, development of other concurrent infections and clinical information relating to poor compliance or drug toxicity was unfortunately not available at the time of this write-up on the described patient cohort followed for CD38 MFI expression.

3.3 Anti-retroviral Treatment regimen:

The CIPRA programme used the two South African Comprehensive Care Management and Treatment (CCMT) standard regimens for all patients. This 1st line regimen consisted of the following drugs: Lamivudine (3TC), Stavudine (d4T), Efavirenz (EFV)/ Nevirapine (NVP). The 2nd line regimen contained: Didanosine (ddl), Zidovudine (AZT) and Kaletra (Lopinavir).

3.4 Laboratory analysis:

Samples comprised of 5 ml of whole EDTA blood drawn from patients at the clinic and subsequently transported to the reference laboratory. The CD38/8 analysis was conducted within 24 hours of venepuncture on the Beckman Coulter (BC) Epics XL flow cytometer instrument and prepared and analysed as described below. All samples were tested anonymously with only study identifiers used to link follow-up visits. Ethical clearance to perform testing was obtained (M080432).

3.5 Sample preparation:

Samples were kept at room temperature and mixed well, before being prepared for flow cytometry analysis. For each blood sample, two tubes per patient per visit were labelled with the corresponding laboratory ID's. The first tube was labelled with 10µl of the 4-colour reagent, CYTOSTAT®/tetraCHROME™ (CD45FITC /CD4RD1/ CD8ECD/ CD3PC5) as per the manufacturers' instructions. In the second tube, the CD3 was replaced with 10 µl of CD38 PC5, 10µl PLG/CD4 (FlowCare™) (CD45 FITC and CD4 PE)

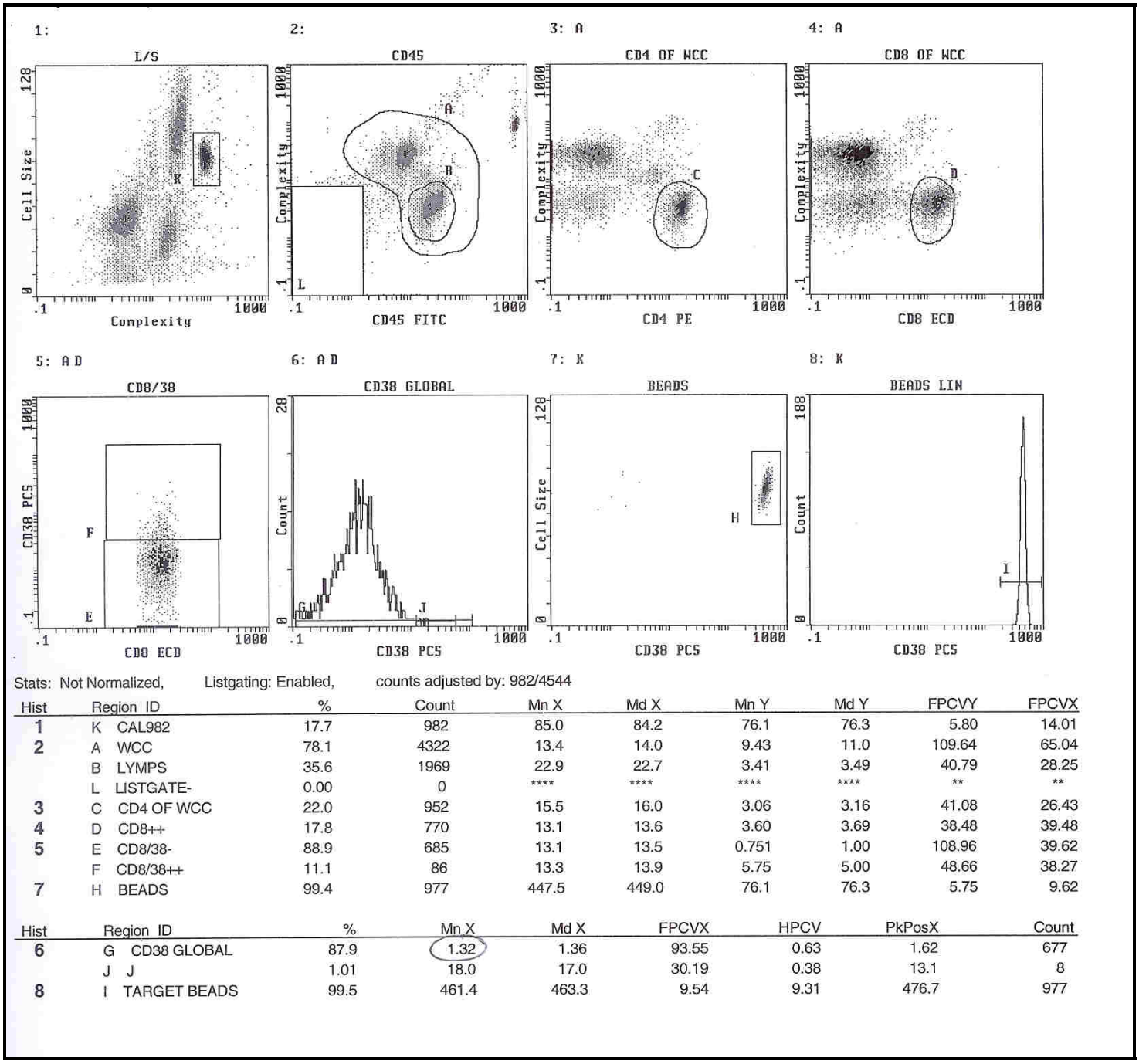
and 10 µl of CD8 ECD which was added to the bottom of each tube (all reagents obtained from Beckman Coulter, Miami, Florida). The CD45FITC/CD4RD1 antibody clones from the tetraCHROME^{TM} reagent used are the exact clones used in the PLG/CD4 kit. Thereafter 100 µl of whole blood was accurately pippetted into the bottom of each tube. The samples were gently vortexed and left to incubate for 10 minutes in the dark followed by sample lysis using the automated Immunoprep™ station (Beckman Coulter, Miami, FL). All samples were analysed within 2 hours of sample preparation. Just prior to analysis by flow cytometry, 100 µl of FlowCount^{TM} flourospheres (beads) (BC, Miami, FL) was added to each tube using the same pipetting technique and pipette used for adding the blood to ensure accurate 1:1 ratio of blood to beads for absolute cell enumeration. Samples were analysed on both the standardised NHLS T-cell 4-color protocol and the CD45/4/38/8 study protocol on the same Beckman Coulter XL flow cytometer. All data was stored as listmode files on the XL instrument for retrospective analysis and backup of data was regularly made to compact disk.

3.6 Protocol setup and gating strategies:

The reference tetraCHROME^{TM} T-cell 4-color protocol was used as a base protocol for establishing the CD38/8 standardised protocol, by changing the CD3 PC5 for a CD38 PC5 antibody, while keeping the CD45FITC/CD4RD1/CD8ECD intact.

In short, the CD38/8 protocol (Figure 1) is based on the PLG/CD4 concept (CD4 vs. Side Scatter) and incorporates identical Side Scatter (SS) gating strategies to identify small

lymphocytes and thus enumerate bright CD4 expressing T-cell lymphocytes (histograms 3 and 4). The same SS gating identification strategy was used to identify CD8 bright lymphocytes (CD8 vs. SS). The gated CD8 bright lymphocytes were then displayed in a dual histogram (histogram 5) to distinguish CD8+/CD38^{bright} (Region F) from CD8+/CD38^{negative} (Region E). The global CD38 expression on the gated CD8 bright T-cells were reported as the Mean Channel value (MnX of Region G, histogram 6). As an internal quality control measure of standard fluorescence expression, and for the purpose of single platform analyses, FlowCount^{TM} beads were monitored in histogram 7 (region H) and the MFI value recorded (Region K, histogram 8).



Stats: Not Normalized, Listgating: Enabled, counts adjusted by: 982/4544

Hist	Region ID	%	Count	Mn X	Md X	Mn Y	Md Y	FPCVY	FPCVX
1	K CAL982	17.7	982	85.0	84.2	76.1	76.3	5.80	14.01
2	A WCC	78.1	4322	13.4	14.0	9.43	11.0	109.64	65.04
	B LYMPS	35.6	1969	22.9	22.7	3.41	3.49	40.79	28.25
	L LISTGATE-	0.00	0	****	****	****	****	**	**
3	C CD4 OF WCC	22.0	952	15.5	16.0	3.06	3.16	41.08	26.43
4	D CD8++	17.8	770	13.1	13.6	3.60	3.69	38.48	39.48
5	E CD8/38-	88.9	685	13.1	13.5	0.751	1.00	108.96	39.62
	F CD8/38++	11.1	86	13.3	13.9	5.75	5.00	48.66	38.27
7	H BEADS	99.4	977	447.5	449.0	76.1	76.3	5.75	9.62

Hist	Region ID	%	Mn X	Md X	FPCVX	HPCV	PkPosX	Count
6	G CD38 GLOBAL	87.9	1.32	1.36	93.55	0.63	1.62	677
	J J	1.01	18.0	17.0	30.19	0.38	13.1	8
8	I TARGET BEADS	99.5	461.4	463.3	9.54	9.31	476.7	977

Figure 1: An example of the standardised CD38/8 protocol, indicating the gating strategies used to identify CD8 bright T-cells that express CD38, reported as the global MFI (e.g. 1.32). In addition an absolute CD38 value (86 cells/µl), a total white cell count (WCC) ($4.32x10^9$/l), total lymphocyte count (Lymph) ($1.96x10^9$/l) and absolute CD4 (952 cells/µl) and CD8 counts (770cells/µl) were derived from this protocol. The MFI value of the beads (Region I; 461.4) were recorded as an internal quality control measure.

3.7 Quality Control procedures:

Quality control (QC) procedures were carried out routinely on the flow cytometer used for CD38 analyses to include both daily as well as weekly QC measures as described below.

Daily quality control was performed on the instrument according to the manufacturer's guidelines and the laboratory standard operating procedures (SOP's) in order to monitor laser performance, optics, and fluidic stability and ensure optimal sample preparation and analysis. FlowCheck™ (laser alignment), 2 levels of ImmunoTrol™ (Normal and Low), carry-over and background counts were analysed and a bead reproducibility test was done to ensure instrument stability and reproducibility over time (Appendix A).

A weekly quality control protocol was also implemented to monitor instrument stability and protocol integrity (Appendix B). This was achieved by the automated panel setup-function on the instruments, as previously described (106). This procedure use 6 tubes, the first being FlowSet™ (BC, Miami, FL), used to standardise light scatter and set fluorescence intensities (channel values) to pre-defined values for optimal cell separation. The remaining tubes contained ImmunoTrol™ blood product, dual stained with FITC/RD1, RD1/ECD, RD1/PC5 and ECD/PC5 (Cytocomp Kit, BC, Miami, FL) that allow compensation for the "spill over" between fluorescence channels. As a confirmation, ImmunoTrol™ samples labelled for both the tetraCHROME™ and CD38/8 assay were acquired to ensure that the cytosettings (voltages and colour compensation values) obtained from the auto-setup procedure were automatically saved to these

protocols for acquisition of patient samples and to establish a cut-off between $CD38^{positive}$ and $CD38^{negative}$ $CD8^{bright}$ T-cells (Region F of Figure 1). All ImmunoTrol™ samples were prepared in the same fashion as patient samples described above. In addition, confirmed HIV positive and HIV negative patient samples were tested weekly as extra controls to confirm the established cut-off limits for CD38 positivity.

Random reproducibility studies were conducted over the course of the study, using an ImmunoTrol™ sample and a confirmed HIV positive sample, prepared as 10 replicate tubes for CD38 analyses as described. The MFI of the global CD38 expression on CD8+ T-lymphocytes for each sample was entered on an XL spreadsheet to calculate a %CV (co-efficient of variation) to measure the reproducibility of both the instrument and accuracy of the protocol setup.

In addition to daily and weekly QC procedures, every sample tested was monitored for bead count rate (BCR; calculated as the number of bead events/acquisition time) to ensure accurate pipetting (37, 39) and fluidic stability of the flow cytometer within runs (22, 107). The MFI of the bead population was also recorded as part of the CD38 protocol standardization and continued quality control from sample-to-sample and between batches (days) for this assay. Since samples were tested in parallel using the tetraCHROME™ 4-color reference method (as per instructions for the CIPRA study), absolute CD4 and CD8 counts were compared with results obtained with the CD38 protocol as an additional internal quality check.

The tetraCHROME™ results from the flow cytometer used for CD38 analyses, participated on the South-African NHLS AFREQAS programme and a z-score value was reported for both absolute CD4 and absolute CD8 values.

3.8 Testing the extended window period for analyzing samples for CD38 activation:

As a secondary objective, this study investigated the reproducibility of CD38 MFI reporting in samples up to 48 hours after venesection. Random EDTA blood samples from the routine PLG/CD4 laboratory were stained with a combination of PLG/CD4 antibody (CD45 FITC/CD4 PE), CD8 ECD and CD38 PC5 and analysed on the BC XL using the same preparation method described above. Samples were prepared on day of arrival (0hrs) and re-prepared and analysed 24 and 48 hours after receipt. In addition, lysates prepared at time 0 were re-analysed after 24 hours. The MFI of global CD38 and FlowCount™ beads were compared at each time point.

3.9 Implementation of CD38 monitoring at a Secondary Site:

As a final aim, this study set out to assess the implementation of the CD38 assay at a secondary laboratory site. Helen Joseph Hospital was chosen as it has a well established PLG/CD4 laboratory that uses Beckman Coulter XL flow cytometers and was close to the reference laboratory for transport of samples. The standardised CD38 protocol and related QC protocols were loaded onto a single XL flow cytometer at the secondary site where a member of staff was trained on preparation and analysis of samples using the CD38 assay (method described above) and the relevant weekly CD38

testing QC procedures. Additional training included trouble shooting and result reporting. Once the staff member was deemed competent, actual testing at the site began. Random samples earmarked for routine CD4 testing at HJH were selected for the pilot CD38 implementation study. Samples were prepared at HJH and analysed before they were sent to CMAJH for verification. Samples were re-prepared and analysed at the reference site and LMD files from HJH were also replayed for analyses on the standardised protocol. QC was performed daily as per PLG/CD4 SOP and once a week (for CD38) according to the QC guidelines from the reference laboratory.

3.10 Additional laboratory tests: (Viral load testing):

HIV Viral load tests were done on all patient samples as part of the CIPRA programme. These were performed on the COBAS AmpliPrep/Amplicor system RT-PCR version 1.5 and the ultra-sensitive version of the kit was used to detect lower viral load limits (below 50 copies/ml). Results were reported on the NHLS DISA patient database and were retrieved from the system to compare to CD38 MFI and CD4 absolute count at correlating time points.

3.11 Statistical Analysis:

All statistical analyses were performed using GraphPad Prism Version 5.03 Software.

1. Overall trends: individual patient data and group mean values for CD38 MFI were plotted longitudinally to identify trends. In addition, the percentage change in CD38 MFI from each visit to the previous and to baseline was

calculated and plotted longitudinally for individual patients and mean values for the group.

2. Comparison of different test methodologies (tetraCHROME™ vs. PLG/CD38) was done using non parametric tests (t-test with Mann-Whitney test, Spearman correlation) for different parameters (absolute CD4 and CD8).
3. Quality Control: (FlowCheck™, ImmunoTrol™, Colour Compensation, HIV+ and HIV-): Means and Standard deviations were calculated as well as the percentage coefficient of variation (%CV) for longitudinal monitoring of reproducibility on replicate assays.
4. Testing the 48 hour window period for CD38 expression: Bland Altman graphs (108) (plotted as differences on the vertical axis vs. the average of readings on the horizontal axis) were plotted to compare overall agreement in CD38 MFI results at different time points. Non parametric One-Way ANOVA analyses with a 95% confidence interval were calculated to compare grouped results of the different parameters at four different time points. Percentage similarity analyses (109) were also carried out, where time 24, 36 and 48 were compared to time zero results.
5. Implementation of the assay at the secondary site: The percentage similarity model was used to compare results to the reference site (109).
6. All bar graphs (scatter dot plots) include lines that represent the mean ± 1SD range unless otherwise specified.

4.0 RESULTS

4.1 PATIENT RESULTS

All patients were followed longitudinally for CD4 and VL from the start of 1st line therapy. Of the original cohort of 120 patients with 1st line longitudinal CD38 MFI follow-up, only 9 patients who failed 1st line therapy were followed longitudinally for CD38 MFI assessment. Patients were followed on through initiation of second line treatment up until the end of the study in December 2009. Patients were classified into three groups based on their baseline CD38 MFI values according to Glencross et al (103). Four patients had a baseline MFI of 0.1-1.99, making them low responders, while 3 were classed as medium responders (MFI 2.0-3.9) and 2 as high responders (MFI>4.0). Longitudinal response classification (true negative, true positive, false negative and false positive responders) according to Glencross et al (105) was not used due to the small sample size (n=9) of the current study.

4.1.1 Group Results:

4.1.1.1 CD4 absolute count, CD38 MFI and Viral Load results

The results for the whole group (n=9) are shown below (Table 1). At initiation of ART (baseline) the VL and CD38 MFI was elevated and CD4 counts <200cells/µl. On failure of 1st line therapy (discontinuation 1st line) CD4 counts were above 200cells/µl although the VL and CD38 MFI were close to pre-treatment values. At the end of the study in December 2009 (study conclusion), patients were doing well on 2nd line therapy as reflected in a lowered VL, CD38 MFI and elevated CD4 counts of >400cells/µl.

Table1: Results of the cohort of 9 patients at baseline, end of 1st line therapy (Discontinuation 1st line) and end of the study (Study Conclusion), expressed as mean values with 95% confidence interval (CI) results in brackets.

	VIRAL LOAD (Log)	CD38 MFI	CD4 absolute count (cells/µl)
BASELINE	4.995 (4.3-5.7)	3.721 (1.6-5.9)	166.0 (125.4-206.6)
DISCONTINUATION 1ST LINE	3.520 (2.7-4.3)	3.301 (1.5-5.1)	287.5 (230.1-344.9)
STUDY CONCLUSION	2.279 (1.4-3.2)	1.213 (0.97-1.5)	439.3 (309.8-568.8)

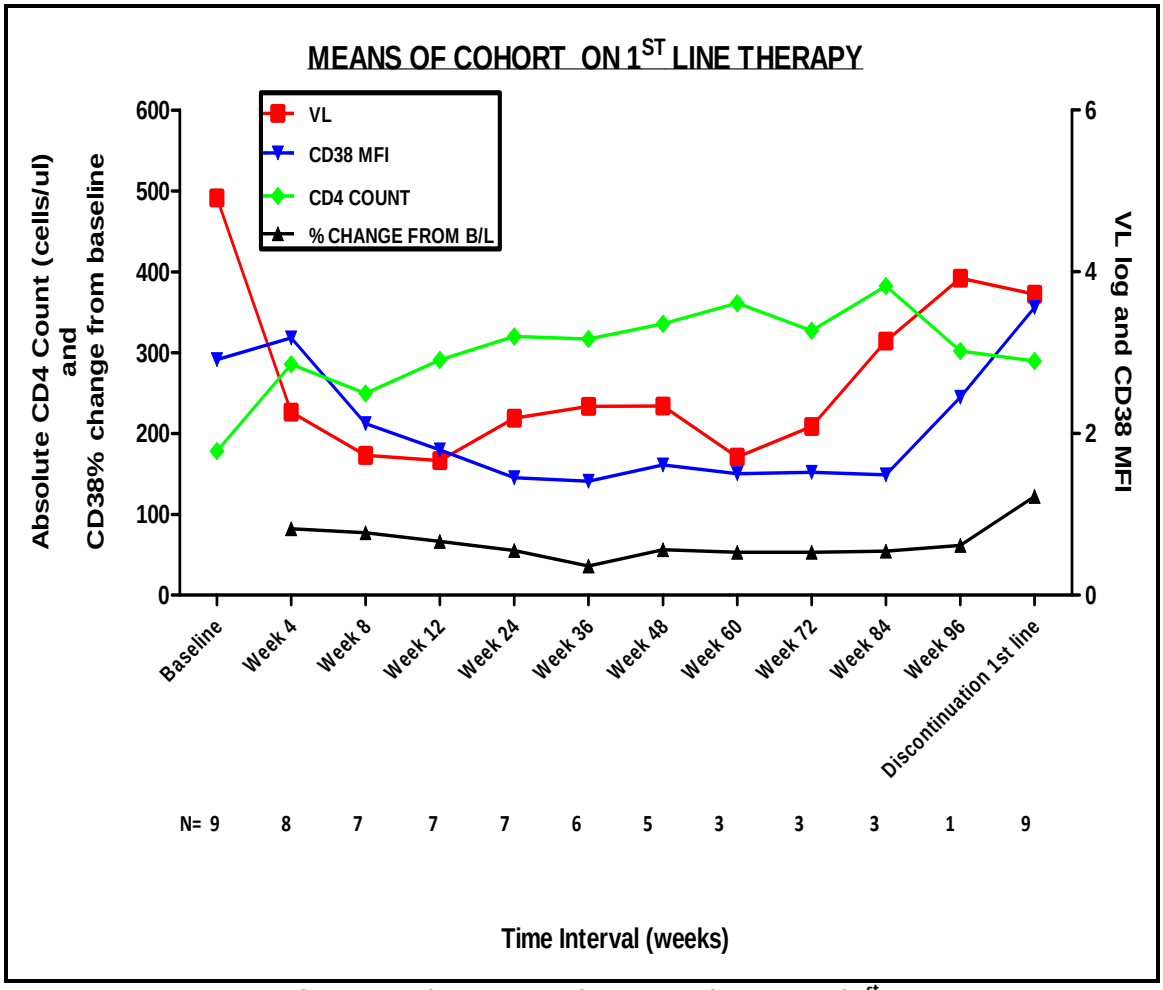


Figure 2 : The longitudinal follow-up of the cohort of 9 patients from start of 1st line therapy to discontinuation of 1st line therapy, reflecting mean values of parameters followed on 1st line treatment: absolute CD4 count (green), Viral Load (red), CD38 MFI (blue) and CD38 % change from Baseline (black). N is the number of patients for that particular visit week

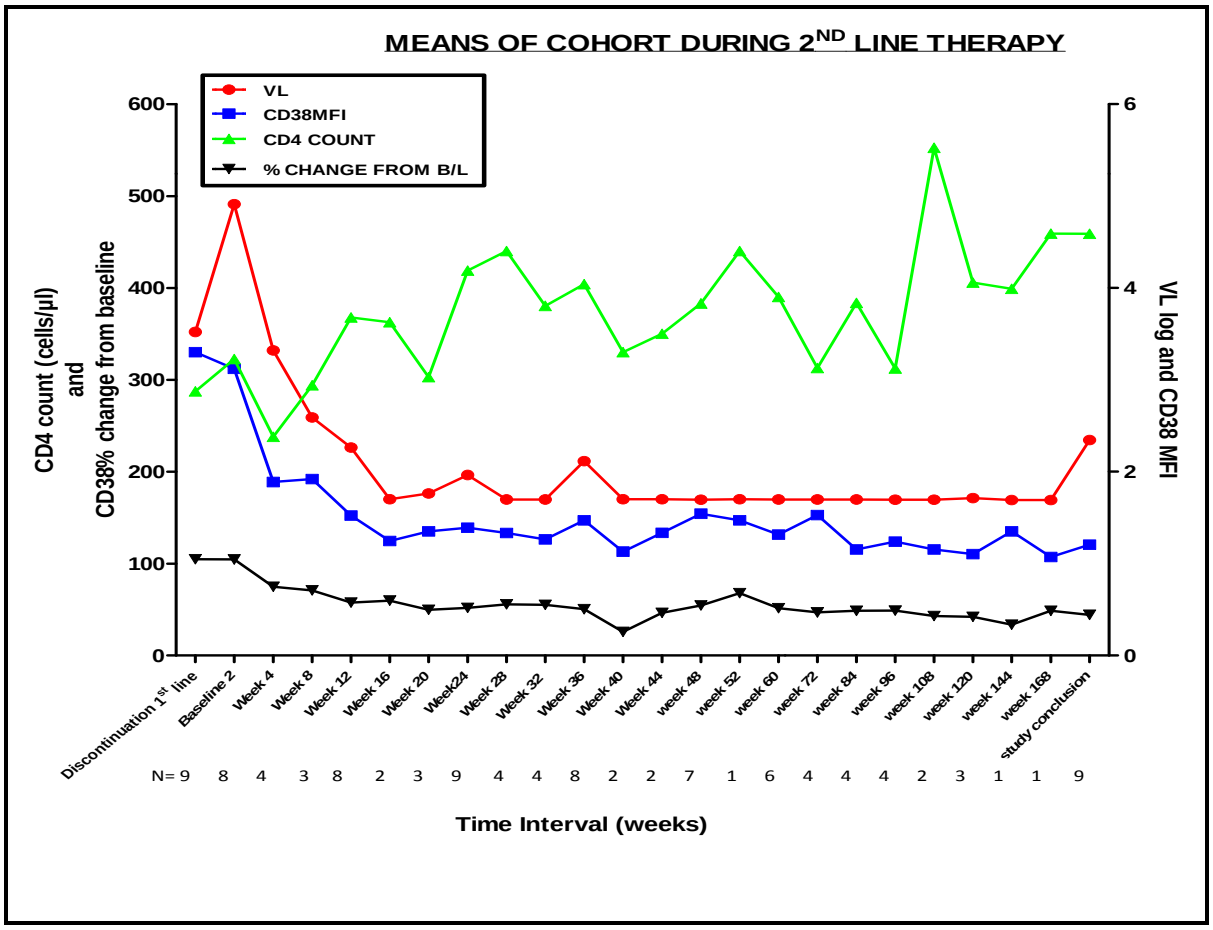


Figure 3: The longitudinal follow-up of the cohort of 9 patients from start of 2nd line therapy to conclusion of the study, showing the mean absolute CD4 count (green), Viral Load (red), CD38 MFI (blue) and CD38 % change from Baseline (black). N is the number of patients for that particular visit week

The mean values per visit for the cohort were expressed for their reaction to 1st line therapy (Figure 2) and 2nd line therapy (Figure 3). Patients were on 1st and 2nd line therapy for variable time periods. Therefore not all time intervals shown in Figures 2 and 3 represent the mean of the 9 patients but rather represent the number of patients per visit. Both graphs show a general trend of an initial rapid decrease in VL from start of therapy to about week 24, which corresponded with a steady decline in CD38 MFI and a slow increase in absolute CD4 counts.

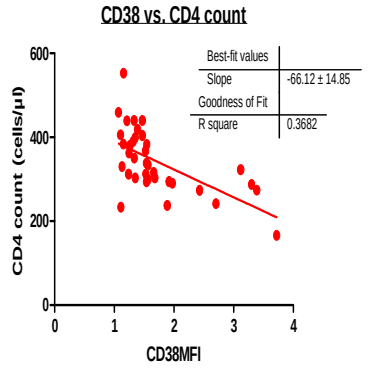
At failure of 1st line therapy (discontinuation 1st line, Figure 2) the mean CD4 count was 287.5 cells/µl, the HIV VL was 3.52 log and CD38 MFI was 3.3. However three patients in this cohort did not have CD38 follow up on 1st line treatment. During the first 12 weeks on 2nd line therapy there was a dramatic decrease in VL to undetectable levels and a corresponding steady decline in CD38 MFI. Patients continued to have undetectable VL and stable low levels of CD38 MFI through the course of 2nd line therapy, while the absolute CD4 counts increased steadily up to conclusion of the study. In the graphs above, both CD38 MFI and CD38 MFI as a percentage change from baseline were calculated. The CD38 % of baseline mimicked the pattern CD38 MFI over time. As a group, the CD38% of baseline results exceeded 80% of baseline at discontinuation of 1st line therapy only, confirming significant elevations in CD38 MFI values at that time point.

4.1.2 CORRELATIONS BETWEEN IMMUNOLOGIC AND VIROLOGIC PARAMETERS

Spearman Rank Correlation and linear regression analyses were conducted to evaluate any significant relationships between CD4 Absolute count, VL and CD38 MFI for the cohort in this study.

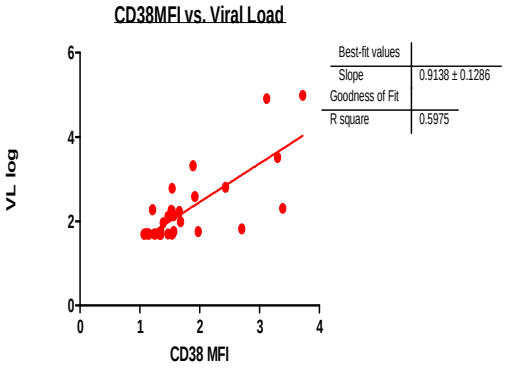
CD38 MFI showed good correlation with both CD4 count and VL (Figure 4). There was a confirmed positive correlation with viral load (r=0.72) and a negative correlation with CD4 count (r=-0.65). A strong negative correlation was found between CD4 count and viral load (R=-0.56). Correlation for VL versus CD38 and CD4 count was limited by the cut-off for VL detection of 1.69 log.

(i)



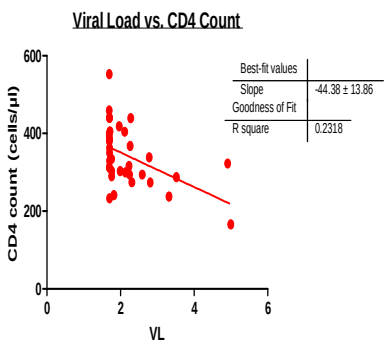
Number of XY Pairs	36
Spearman r	-0.65
95% confidence interval	-0.81 to -0.40
P value (two-tailed)	< 0.0001
P value summary	***
Exact or approximate P value?	Gaussian Approximation
Is the correlation significant? (alpha=0.05)	Yes

(ii)



Number of XY Pairs	36
Spearman r	0.72
95% confidence interval	0.50 to 0.85
P value (two-tailed)	< 0.0001
P value summary	***
Exact or approximate P value?	Gaussian Approximation
Is the correlation significant? (alpha=0.05)	Yes

(iii)



Number of XY Pairs	36
Spearman r	-0.56
95% confidence interval	-0.76 to -0.28
P value (two-tailed)	0.0004
P value summary	***
Exact or approximate P value?	Gaussian Approximation
Is the correlation significant? (alpha=0.05)	Yes

Figure 4: Spearman Rank correlation coefficients and linear regression results showing the relationship between CD38 MFI and both immunological (CD4) (i) and virological (VL) parameters (ii) for the study cohort (n=9) from the start to conclusion of the study. The relationship between VL and CD4 counts is depicted in figure (iii).

4.1.3 Individual Patient results

4.1.3.1 Longitudinal follow-up of individual patients

As a group, there was a defined pattern of response to treatment as reflected in the VL, CD38 MFI and absolute CD4 counts. However, individual patients showed variations of established patterns relative to the general trend. Individual patient results are discussed in more detail to reflect nuances of the differences in CD38 MFI response over time.

1. PATIENT BMR

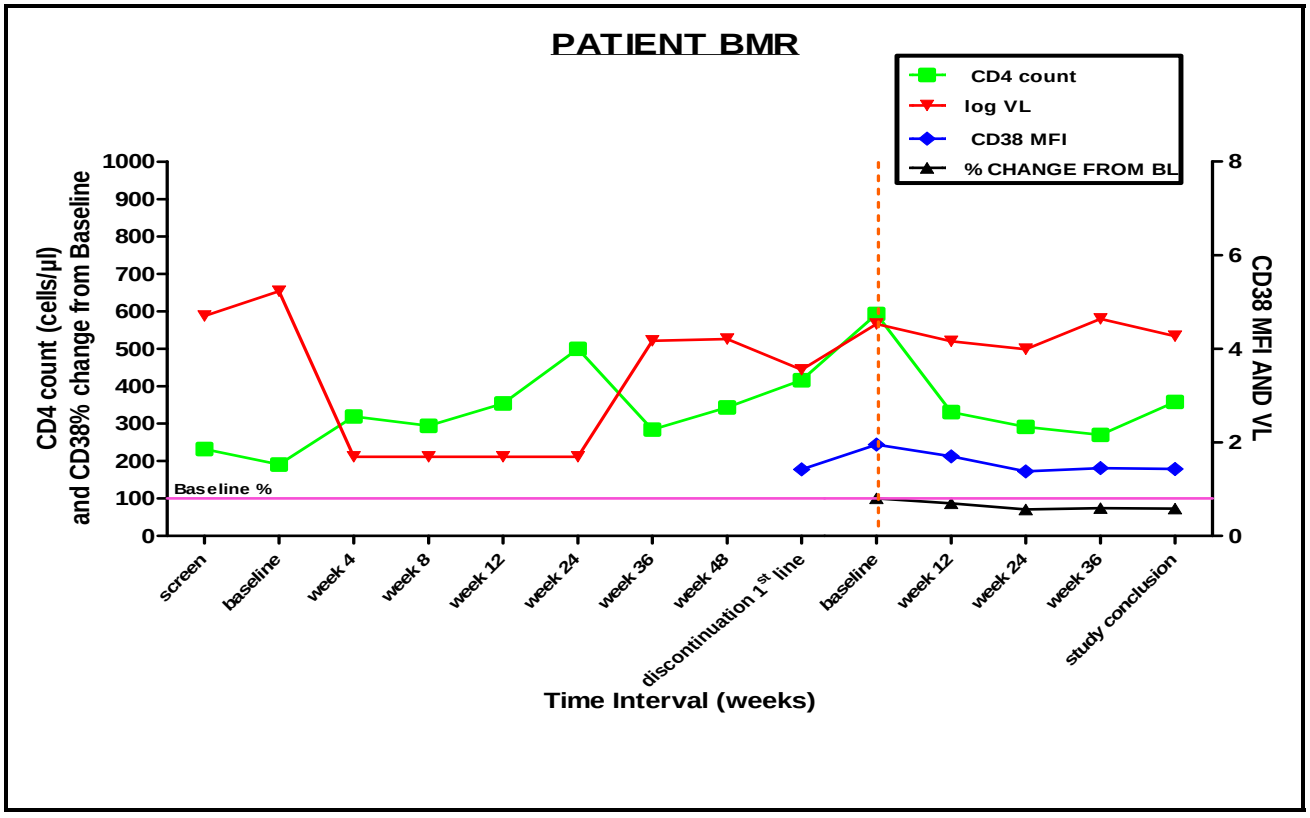


Figure 5: Longitudinal results of patient BMR showing absolute CD4 counts (green), Viral load (red), CD38 MFI (blue) and CD38 % change from baseline (black). The solid pink line represents CD38 MFI % change at baseline (100%). The orange dotted vertical line indicates start of 2nd line treatment.

Patient BMR was followed for CD38 expression for only 6 consecutive visits before the study concluded. Although this patient showed a quick response in decline of VL after initiation of 1st line therapy, the VL values spiked to >1000 copies at week 36 and stayed high through discontinuation of 1st line therapy and start of 2nd line therapy. Unfortunately, this patient was not followed for CD38 expression on 1st line therapy, but only from discontinuation onward. The MFI value at baseline of 2nd line treatment was 1.4 and stayed relatively stable at values between 1.9 to 1.43. Second line therapy baseline was used as no CD38 MFI results were available for baseline 1st line to confirm the patient's classification as a possible low responder. The fact that the CD38 MFI values stayed below 100% of 2nd line baseline, supports this classification of a low CD38 response status (based on 2nd line baseline). There were no significant changes in CD38 MFI from one visit to the next.

2. PATIENT B-S

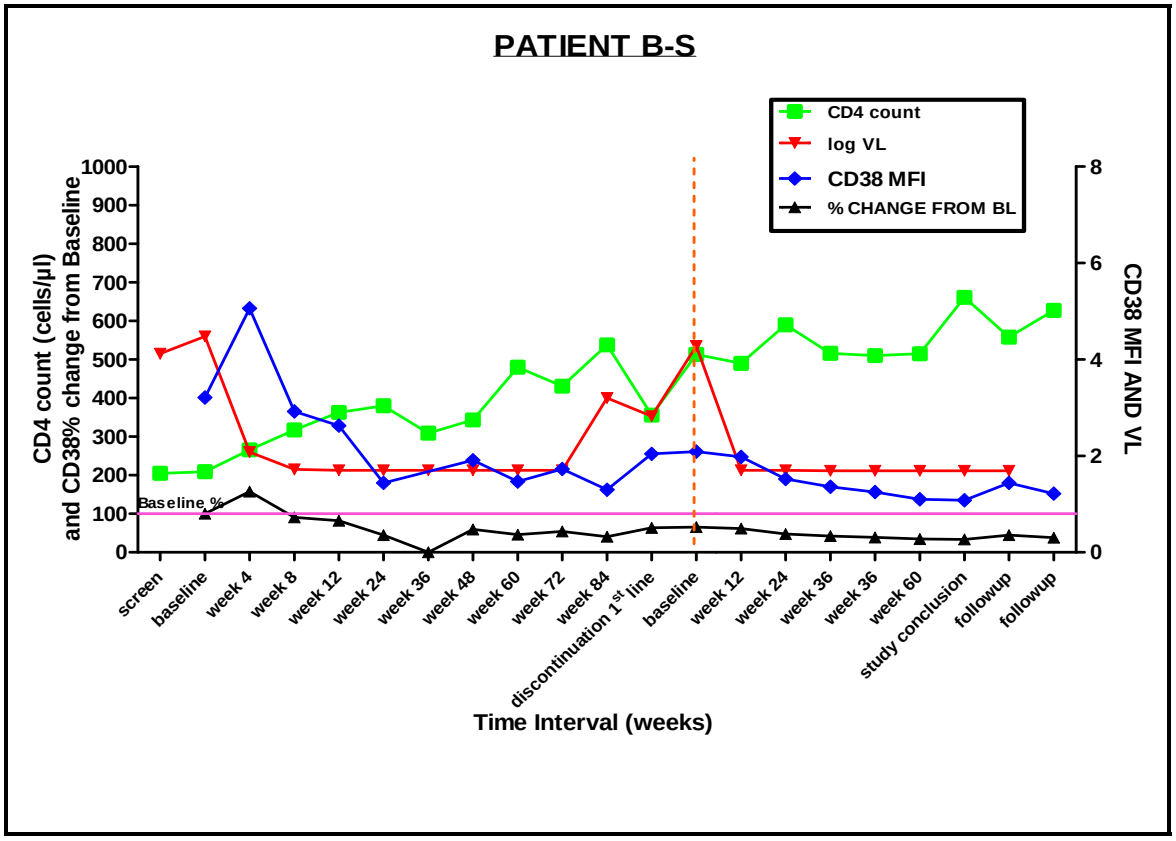


Figure 6: Longitudinal results of patient B-S showing absolute CD4 counts (green), Viral load (red), CD38 MFI (blue) and CD38 % change from baseline (black). The solid pink line represents CD38 MFI % change at baseline (100%). The orange dotted vertical line indicates start of 2nd line treatment.

Patient B-S was classified as a medium responder as at initiation of 1st line treatment a CD38 MFI of 3.21 was noted. By week 8 of treatment, VL had become undetectable illustrating effectiveness of treatment. At week 84, a spike of 3.2 logs was observed in VL with a corresponding increase in CD38 MFI to >2.0 and as a result, failure of 1st line ART was declared and 1st line therapy ended. At baseline of initiation of second line treatment this patient had a CD38 MFI of 2.09 and a CD4 count of 537 cells/µl. By week 12 of second line treatment, this patient once again had undetectable levels of VL indicating response to ART with a corresponding gradual rise of CD4 count until the end

of the study. This patient showed reduced levels of immune activation / lowered expression of CD38 sustained until study conclusion. This patient returned for two follow-up visits on the Right-to-Care programme, showing no changes in VL and no clinically significant changes in CD38 MFI, confirming continued good response to 2nd line ART.

3. PATIENT PMM

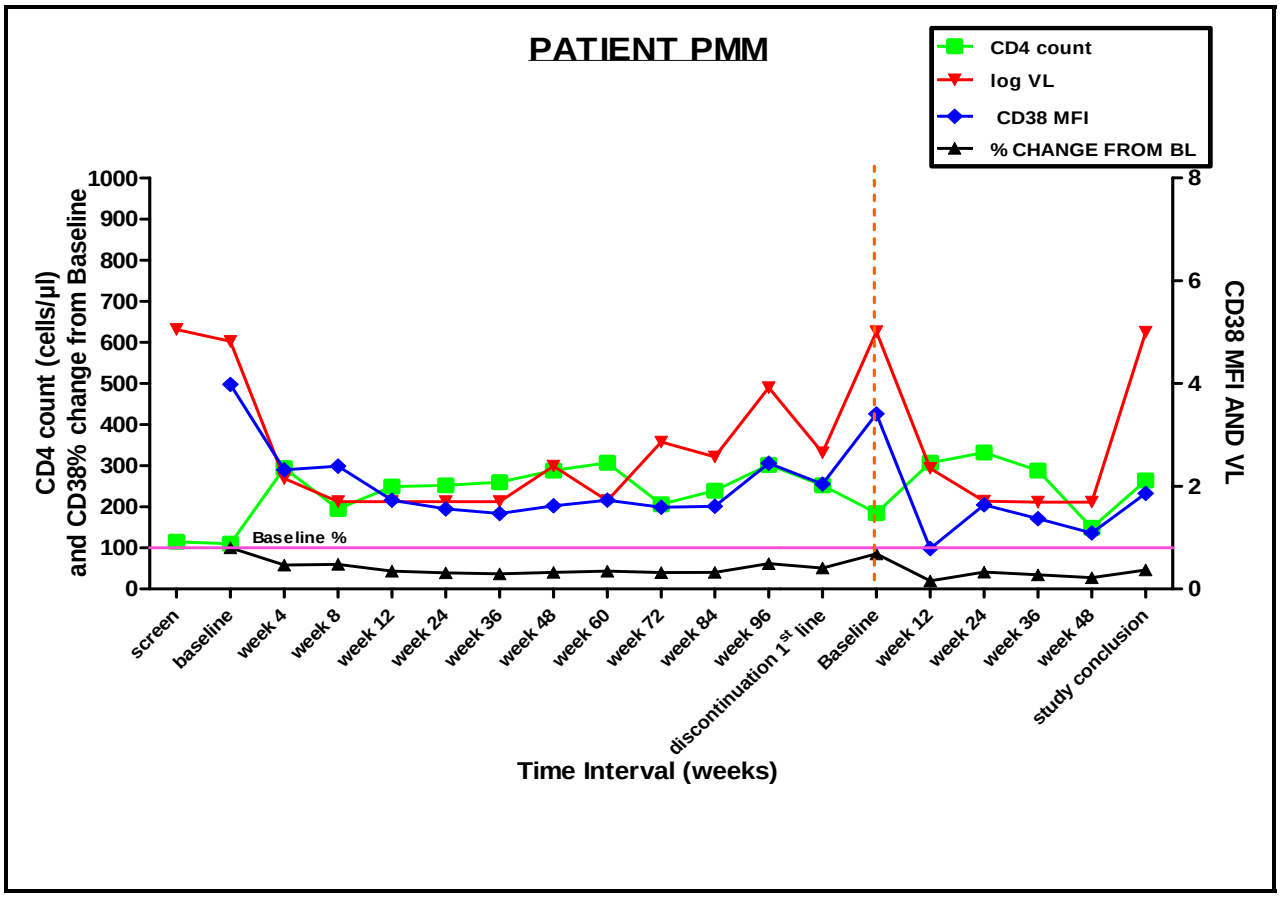


Figure 7: Longitudinal results of patient PMM showing absolute CD4 counts (green), Viral load (red), CD38 MFI (blue) and CD38 % change from baseline (black). The solid pink line represents CD38 MFI % change at baseline (100%). The orange dotted vertical line indicates start of 2nd line treatment

Patient PMM was classified as a medium responder (baseline CD38 MFI of 3.98) and reacted well to first line treatment as seen with undetectable VL by week 8 and stable CD38 values of around 1.5. From week 72 onwards this patient showed increases in VL, corresponding with higher CD38 MFI. This patient was discontinued from 1st line ART when the HIV VL reached a log of 2.65. A corresponding increase of CD38 MFI of 2.04 and drop of CD4 count to 256 cells/µl was also noted at the time. Although the patient was initiated immediately on 2nd line ART, the VL only decreased at week 24 of treatment. A sudden increase to >4 log VL was noted at the end of the study suggesting failure to 2nd line ART as well. Although CD38 declined significantly after initiation of 2nd line therapy, the CD38 increased at the last visit at termination of this study (corresponding with high VL). At the end of this study this patient's VL increased again to 5 log copies and CD4 count dropped significantly from 438 to 243 cells/µl. CD38 MFI also increased to >150% from previous visit confirming 2nd line ART failure. This patient did not return on the Right-to-Care program and as such failure of treatment could not be confirmed.

In the remaining 6 patients (FBD, A-M, APD, BUM, RSZ and CM), similar trends in CD38 expression was noted. Overall, both HIV VL log and CD38 values suggested adequate response to ART. In these patients there were fluctuations in CD38 expression that did not relate to HIV VL i.e. there was no corresponding VL elevation. None of these individual CD38 fluctuations corresponded to the main list of adverse events published on the CIPRA cohort.

4. PATIENT FBD

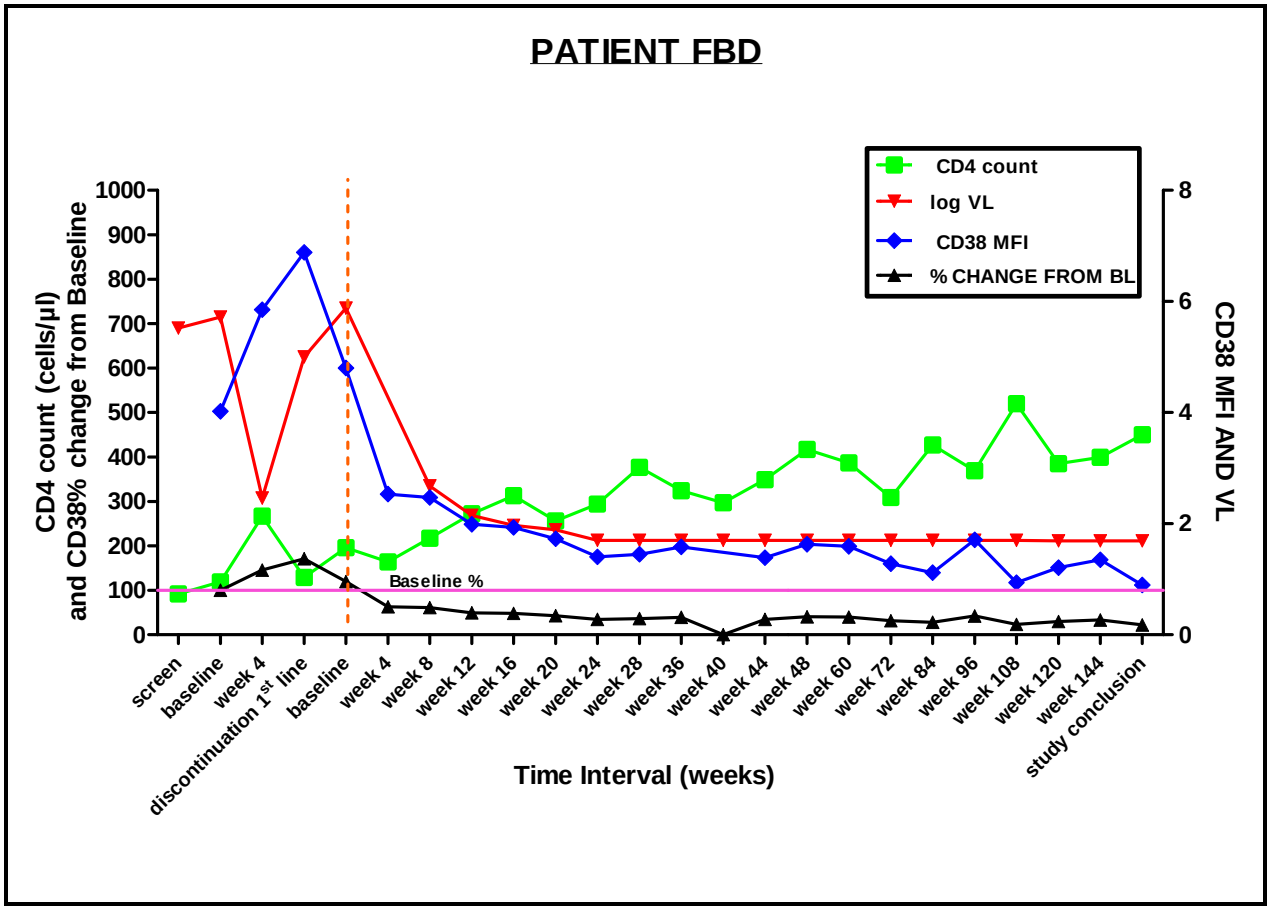


Figure 8: Longitudinal results of patient FBD showing absolute CD4 counts (green), Viral load (red), CD38 MFI (blue) and CD38 % change from baseline (black). The solid pink line represents CD38 MFI % change at baseline (100%). The orange dotted vertical line indicates start of 2nd line treatment

Patient FBD did not respond well to first line therapy as indicated by high VL and corresponding high CD38 MFI levels (>4.0). At discontinuation of 1st line therapy, this patient had a VL of 5 log copies and a CD38 MFI of 6.88 (making the patient a high responder), with a CD4 count of 131 cells/µl. After initiation of 2nd line therapy this patient did well, with VL becoming undetectable after week 24 and correspondingly CD38 stabilized at 1.4. At week 96 there was an increase in CD38 MFI to 1.7. Although it was not significantly above baseline (<100%), it was however 153% higher than the

previous visit. At this time point, no changes were noted in the HIV VL while a small decline in CD4 count occurred. At the next visit (week 108), CD38 MFI dropped to pre-week 96 levels (±1.0) while CD4 count increased by 95 cells/µl to 520. This random increase in CD38 MFI could not be accounted for by changes in protocol setup (gating or cytosettings), sample preparation (FCR within acceptable limits) or instrument instability (daily QC result within preset limits). Concomitant other viral infection or failure to adhere to treatment should be considered as possible causes of the transient rise in CD38 expression.

5. PATIENT A-M

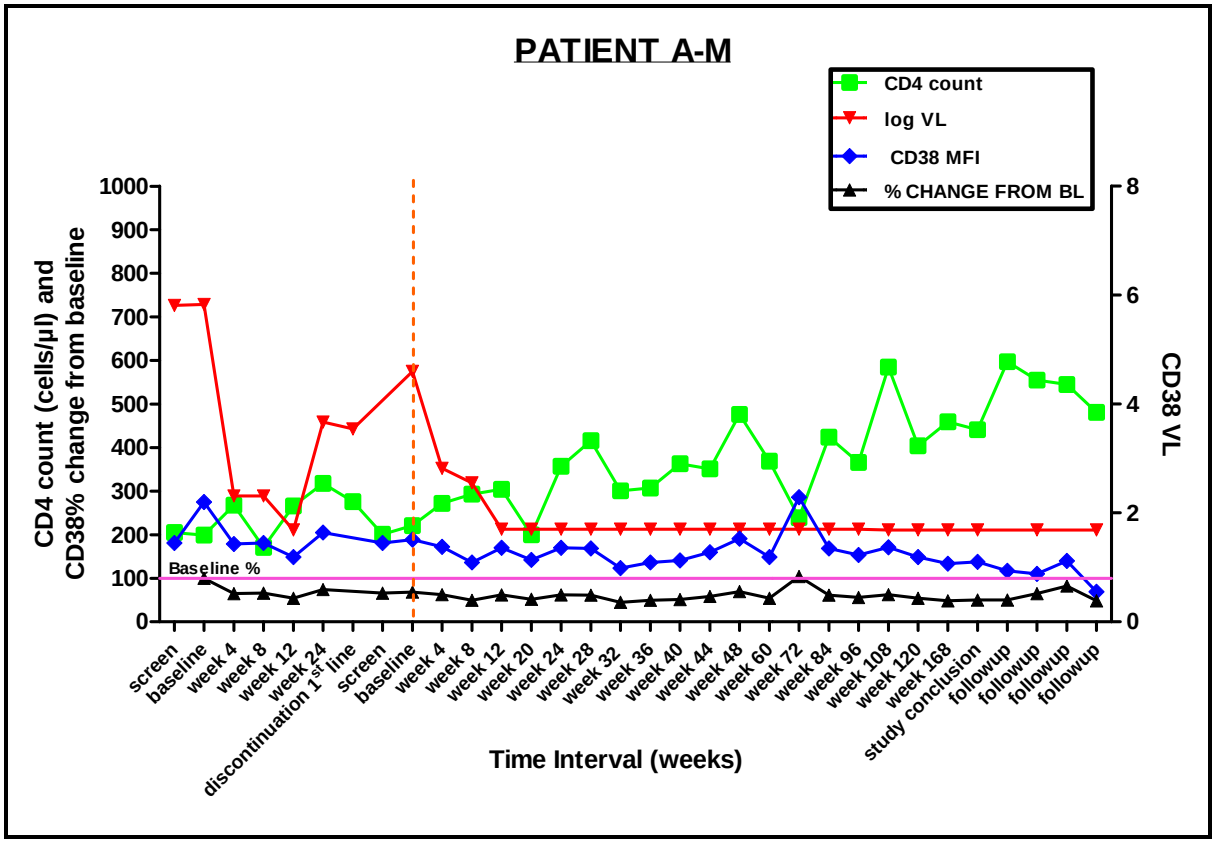


Figure 9: Longitudinal results of patient A-M showing absolute CD4 counts (green), Viral load (red), CD38 MFI (blue) and CD38 % change from baseline (black). The solid pink line represents CD38 MFI % change at baseline (100%). The orange dotted vertical line indicates start of 2nd line treatment.

Patient A-M did not respond very well to first line treatment and was put onto second line ART after only 24 weeks when the HIV VL peaked to 3.54 logs. This patient was classified as a low responder as at initiation of 1st line therapy the CD38 MFI was 1.45. By week 12 on second line therapy, the VL dropped to undetectable levels, the CD4 count gradually increased and CD38 MFI declined. However at week 72 there was a sudden increase of CD38 MFI to above 150% from the previous visit and above 100% of baseline value to 2.29. There was however no change in VL, but CD4 count saw a significant drop of >200 cells/µl. At the next visit (week 96) this patient's CD38 recovered to pre week 72 values (MFI of 1.25) and CD4 count started to increase once again. During this time there was no change in VL until the end of the study. This patient was followed up for an additional 4 visits (to June 2010) on the National Right-to-Care programme and results confirm good compliance and reaction to current therapy regime (undetectable VL, low CD38 and stable CD4 counts).

6. PATIENT APD

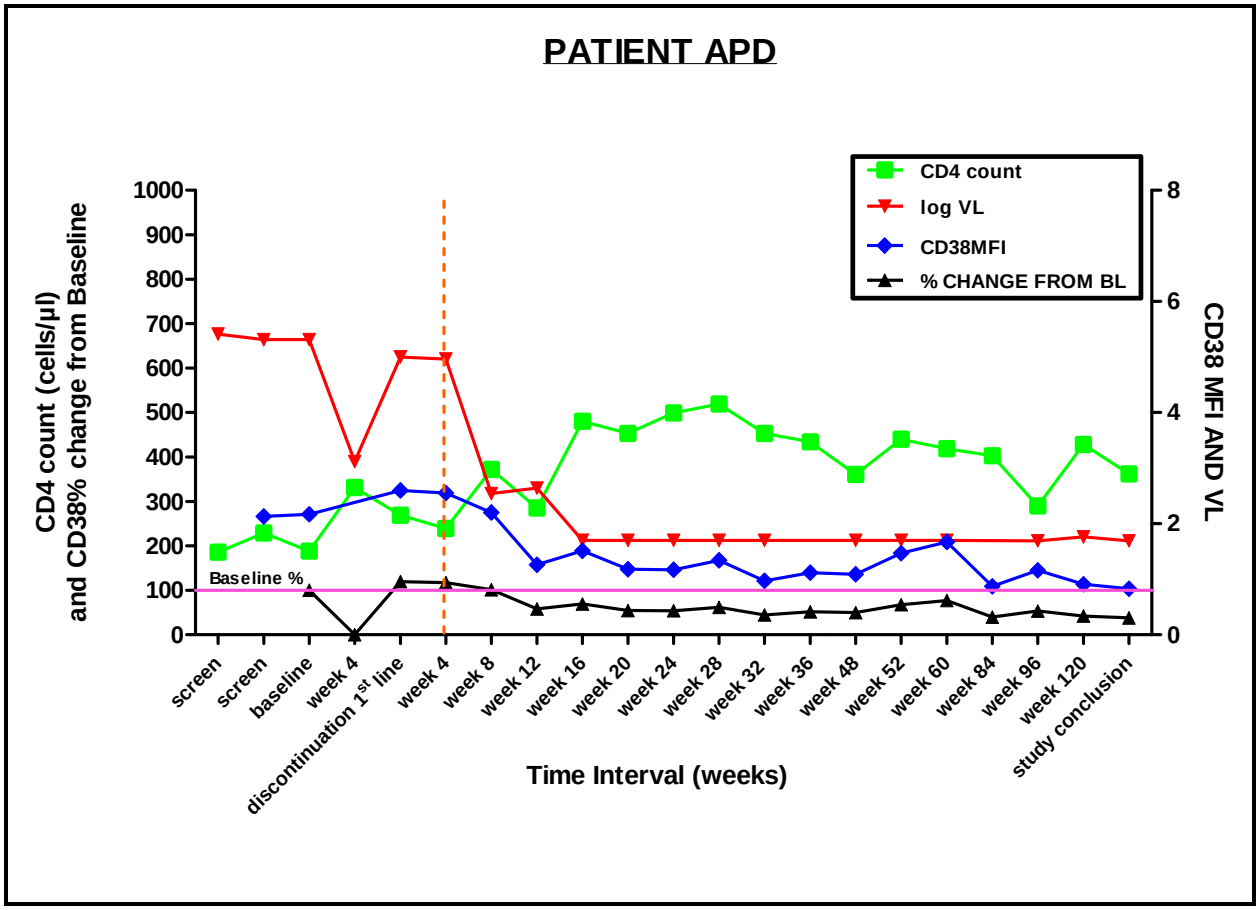


Figure 10: Longitudinal results of patient APD showing absolute CD4 counts (green), Viral load (red), CD38 MFI (blue) and CD38 % change from baseline (black). The solid pink line represents CD38 MFI % change at baseline (100%). The orange dotted vertical line indicates start of 2nd line treatment

Patient APD also followed a similar trend noted in the previous patient and was discontinued on first line therapy after only week 4. By week 16 this patient responded well to second line treatment with VL being undetectable and CD4 count showing a gradual increase. CD38 also decreased from >2 at discontinuation of 1st line therapy to <1.5 after week 16. Between weeks 48-60 a gradual increase (upward trend) was noted in CD38 MFI but with no changes noted in VL. At week 60, a significant increase in CD38 MFI (114%) from previous visit was seen. CD4 count also dropped slightly but

not significantly. After week 72 the CD38 stabilized at levels < 1.0 with no further CD38 MFI rises noted. The CD4 count however did not make a quick recovery and stabilized at around 350 cells/µl.

7. PATIENT BUM

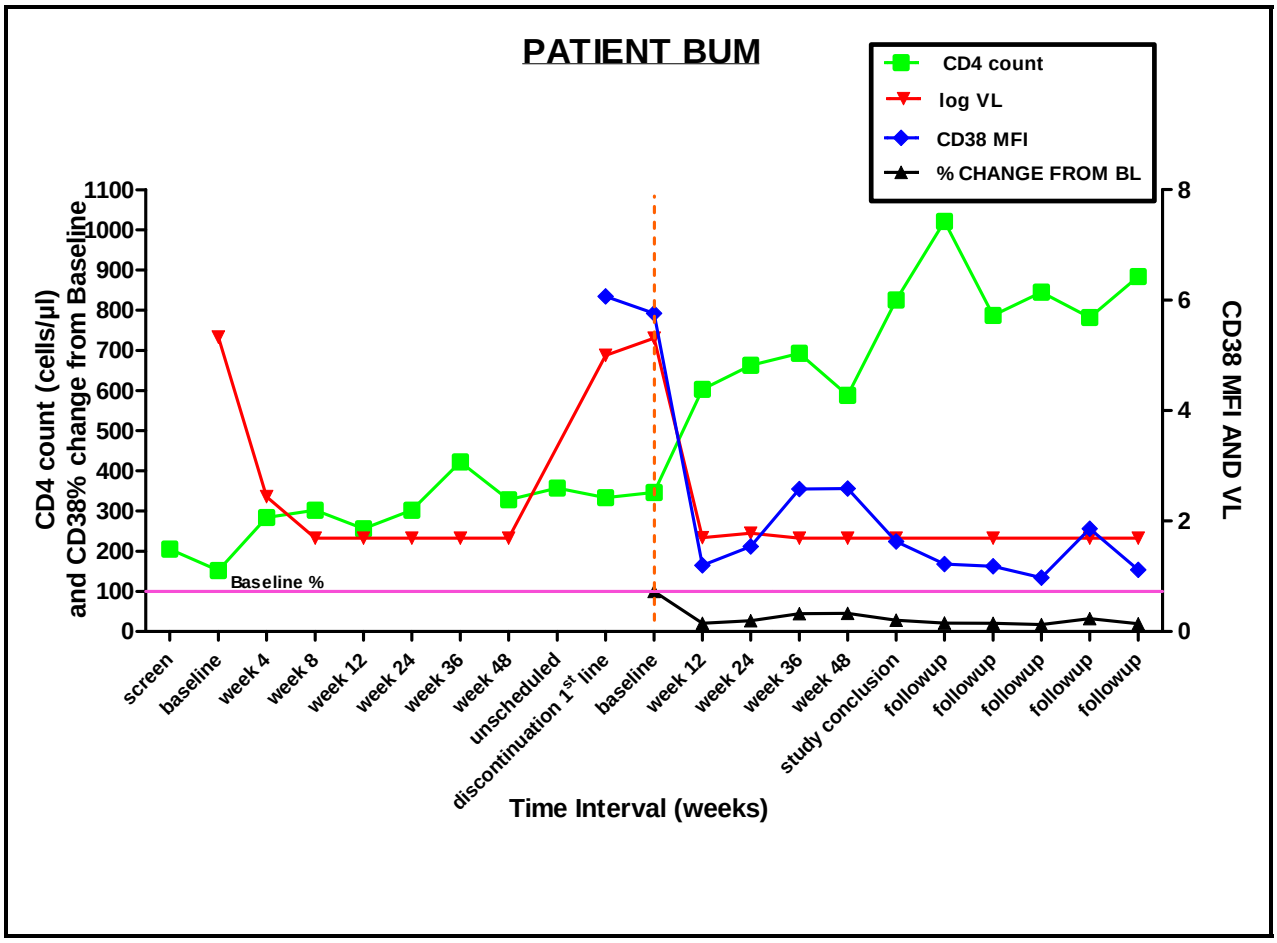


Figure 11: Longitudinal results of patient BUM showing absolute CD4 counts (green), Viral load (red), CD38 MFI (blue) and CD38 % change from baseline (black). The solid pink line represents CD38 MFI % change at baseline (100%). The orange dotted vertical line indicates start of 2nd line treatment

Patient BUM did not have CD38 MFI follow-up through first line therapy but was initiated onto 2nd line therapy once 1st line treatment failed at a VL of 5 log copies with

a corresponding high CD38 MFI of 6.07 (high responder). Between weeks 24-48 there were increases in CD38 MFI to >100% above previous visit but not more than 50% of baseline. During this period however VL remained stable and undetectable. After week 48, CD4 count spiked to 645 cells/µl and continued to increase steadily until the end of the study. VL continued to remain undetectable and CD38 MFI remained low. This patient attended 5 follow-up visits on the Right-to-Care programme during which time VL remained undetectable and CD38 stable with one blip at the 4th follow-up visit (MFI 1.86) that returned to lower levels (MFI 0.96) at the next visit. No changes in VL were noted and CD4 count stabilised at around 800cells/µl.

8. PATIENT RSZ

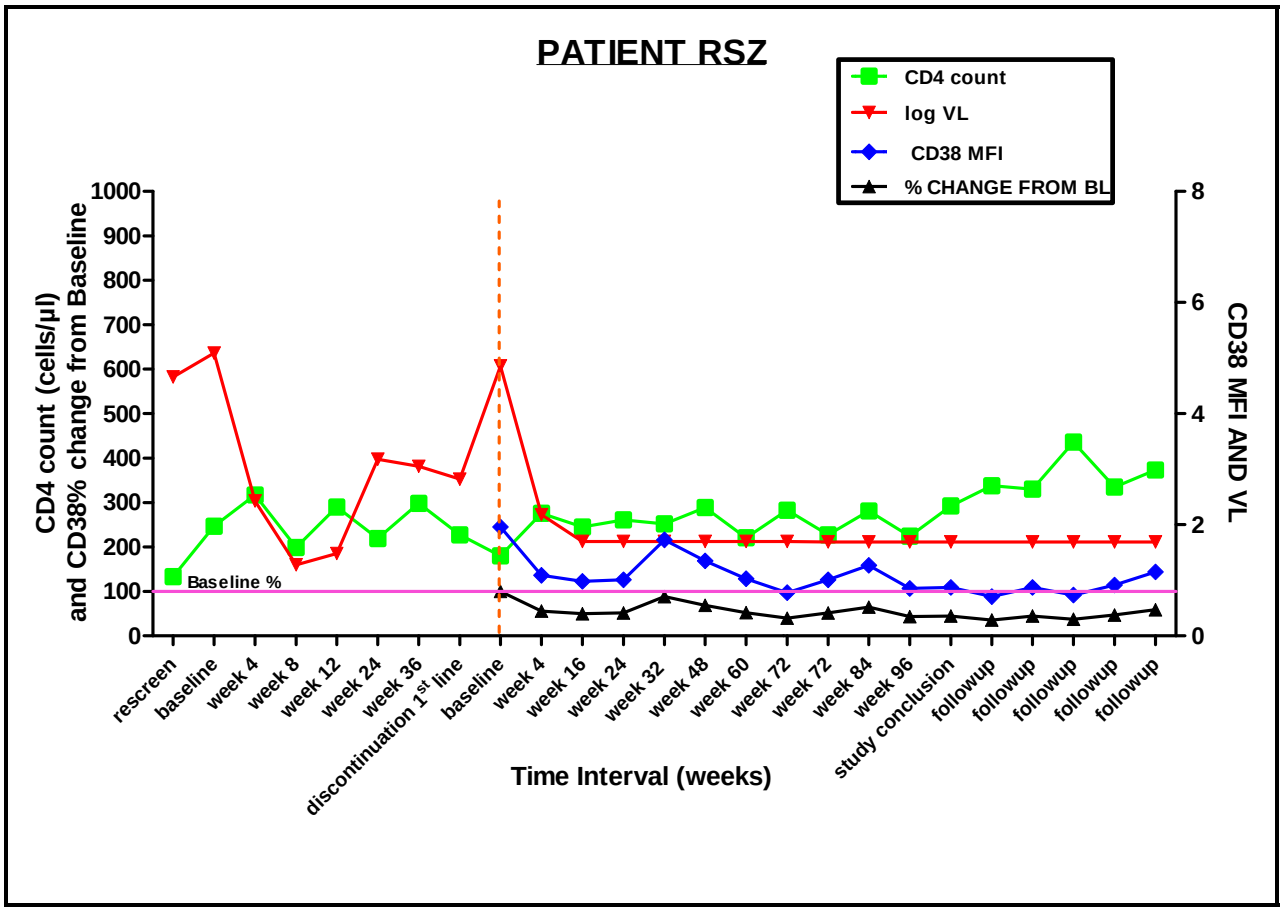


Figure 12: Longitudinal results of patient RSZ showing absolute CD4 counts (green), Viral load (red), CD38 MFI (blue) and CD38 % change from baseline (black). The solid pink line represents CD38 MFI % change at baseline (100%). The orange dotted vertical line indicates start of 2nd line treatment

Patient RSZ was not followed for CD38 from the start of the study. This patient however showed a quick response after initiation of 2nd line therapy with a significant initial decline of VL to week 16. VL values remained undetectable for the remainder of the study. MFI values at baseline was 1.9 and stayed relatively stable at values between 0.7 to 1.96, indicating that this patient was a low CD38 responder (MFI values at baseline <2.0). However this patient also had two rises in CD38 MFI at week 32 and 84.

These were 80% above baseline and >50% from previous visit with no corresponding increase or change in the VL. CD4 counts at both these time points were >250 cells/µl. At the subsequent visit (week 96) CD38 MFI dropped to <1.0 and CD4 counts gradually increased. This patient attended the Right-to-Care programme and had 5 subsequent visits, which indicate good compliance and response to current treatment.

9. PATIENT C-M

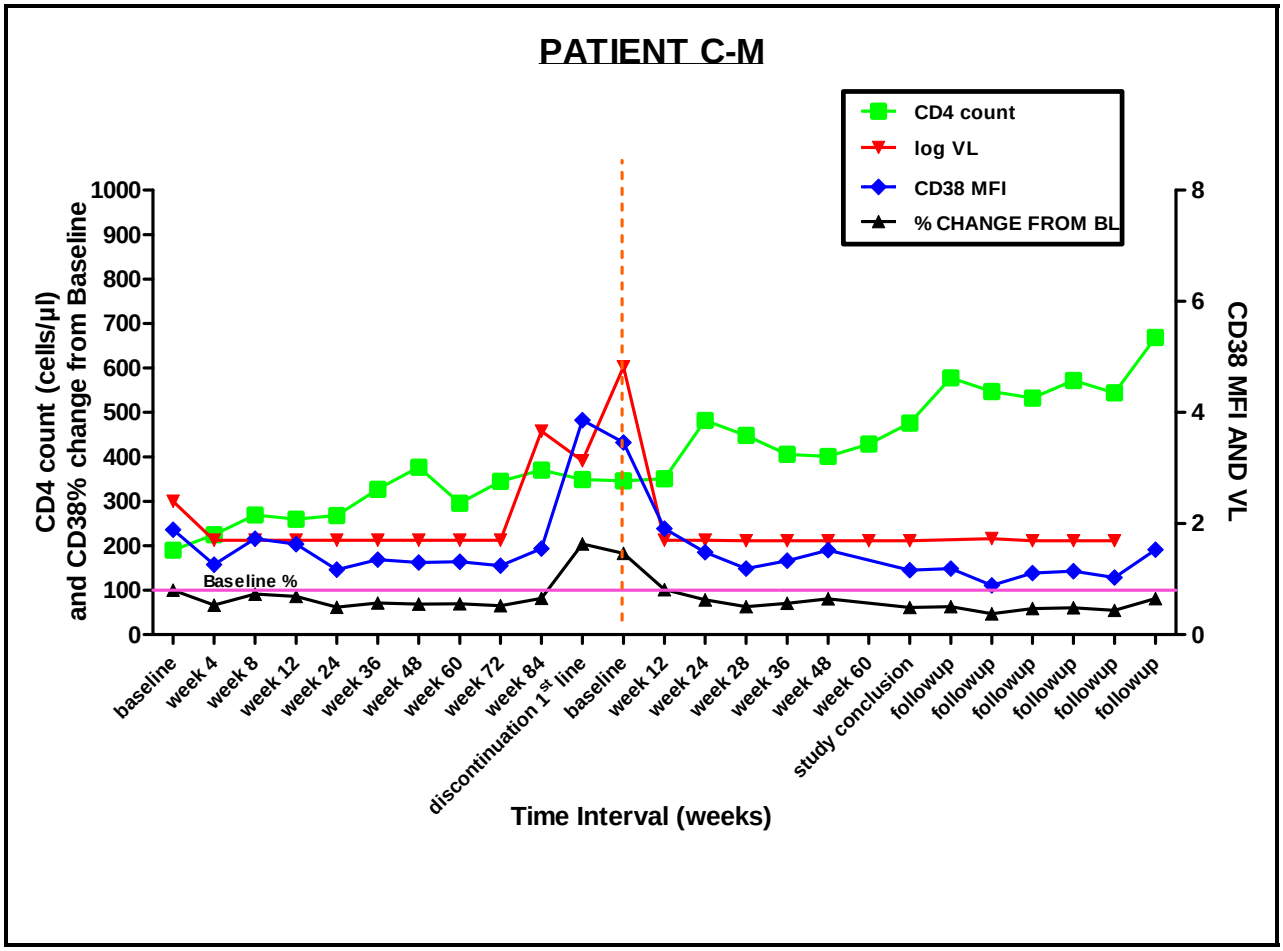


Figure 13: Longitudinal results of patient C-M showing absolute CD4 counts (green), Viral load (red), CD38 MFI (blue) and CD38 % change from baseline (black). The solid pink line represents CD38 MFI % change at baseline (100%). The orange dotted vertical line indicates start of 2nd line treatment.

Patient C-M (medium responder CD38 MFI of 1.89) responded well to 1st line treatment and by week 4, VL became undetectable and CD4 count started increasing steadily. CD38 MFI levels were stable at around 1.2 at this time. At week 84 a spike in VL of 3.66 logs with a corresponding increase in CD38 MFI of 1.55 to 3.86 was seen. At this time point, the CD4 count was not significantly affected. At discontinuation of 1st line therapy this patient had a CD38 MFI of 3.86, more than 200% above 1st line baseline with a corresponding increase in VL (4.82 logs). There was excellent response to 2nd line ART. By week 12 of 2nd line treatment this patient's VL was undetectable and CD38 MFI stabilised to <2. At week 48 a significant change from baseline was noted in CD38 MFI (1.52) without a concomitant increase in VL. At the next visit (Week 60) CD38 MFI returned to pre week 48 values (1.16) suggesting the presence of other causes of immune activation. Additional follow-up visits on the Right-to-Care programme demonstrated continued undetectable VL and stable CD38 with increasing CD4.

All data points were checked for accurate transcription of data and raw data scrutinized to eliminate gating errors or sample preparation errors (pipetting) that could impact CD38 MFI values.

4.2 PROTOCOL VERIFICATION AND QUALITY CONTROL

The gating strategy employed was sensitive enough to distinguish all levels of CD38 activation, i.e. normal/low levels as seen with normal HIV-controls to elevated levels as seen in ARV naïve HIV infected patients (Figure 14).

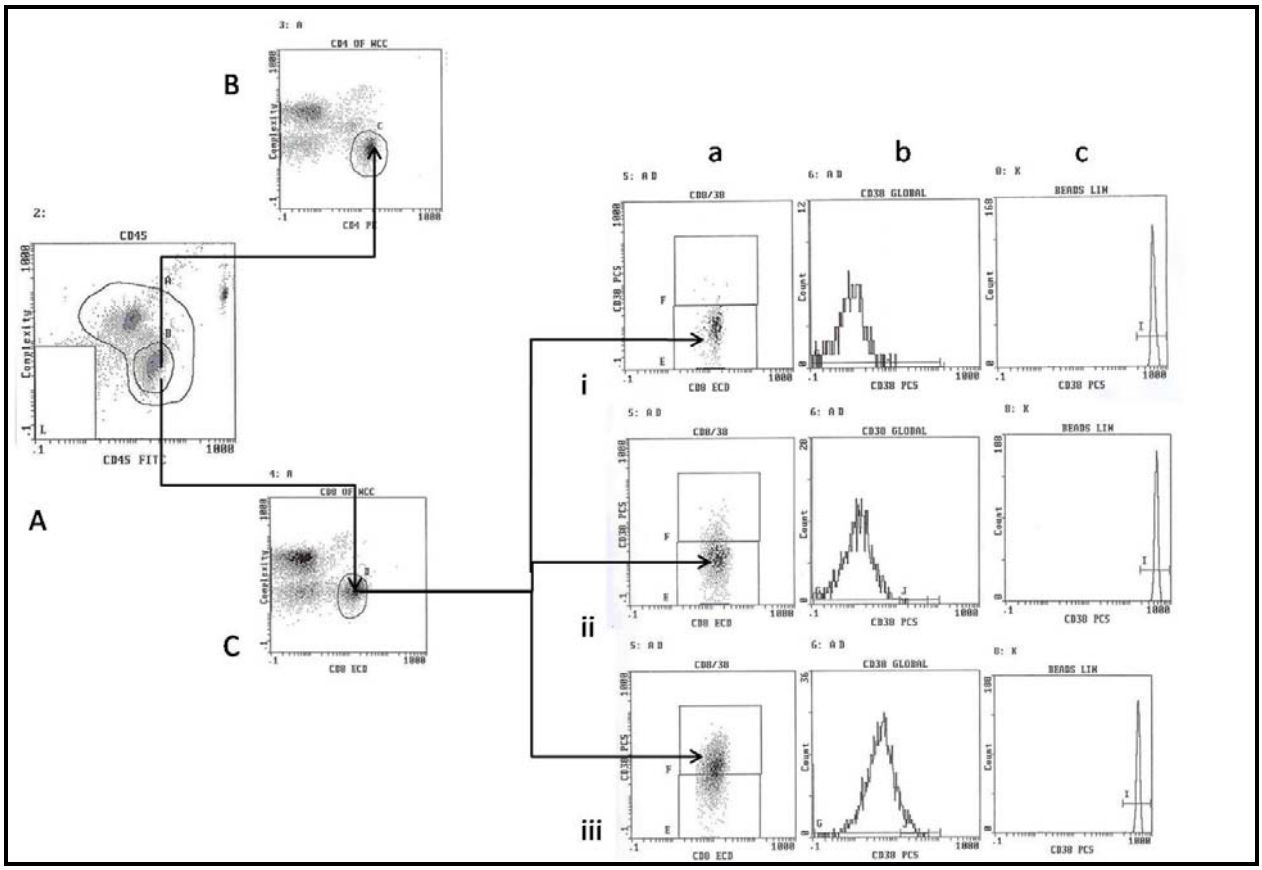


Figure 14: An extract from the CD38 protocol to re-iterate the gating strategy used for CD38 activation monitoring. Here (A) represents a histogram of CD45 FITC vs. Side scatter, where the total WCC population is gated. From this, (B) CD4+ Tcells are gated using CD4FITC against SSC and (C) CD8+ Tcells using CD8ECD and SSC. After identification of (C) the CD8+ cells, CD8ECD is gated against CD38PC5 (Figure a i-iii). In figure (i, a-c) a truly CD38 negative sample using a normal biological control (Immunotrol^{TM}), with a MFI of 0.91 is shown. Figure (ii, a-c) shows an example of a HIV-negative control with low level CD38 expression and a corresponding CD38 MFI of 1.32 compared to (iii, a-c) a HIV+ patient (pre-ARV), indicating high levels of CD38 activation with a corresponding CD38 MFI of 4.17.

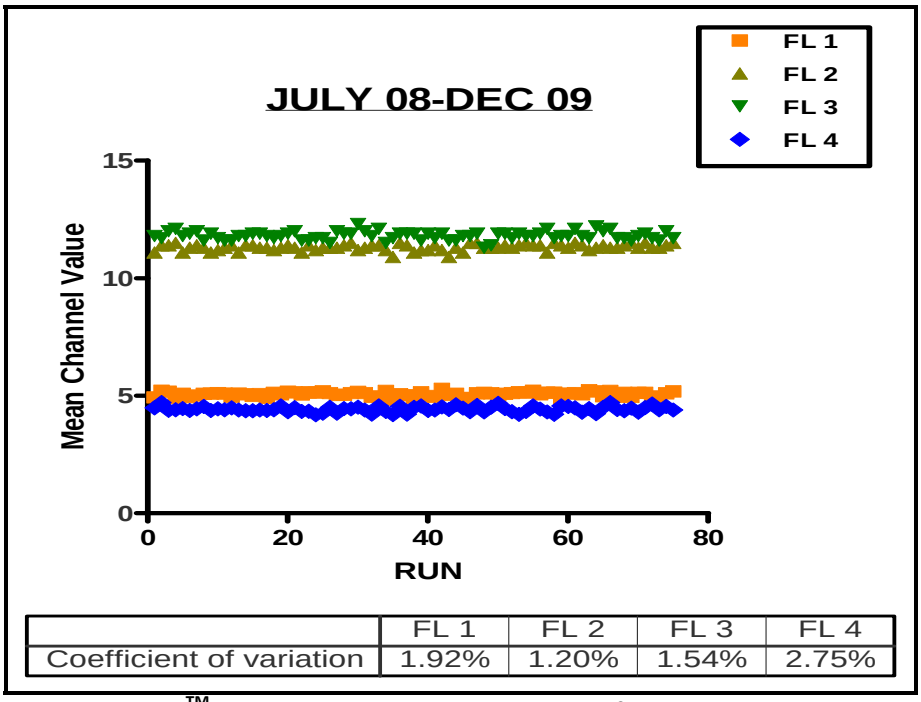
Figures b (i-iii) represents the global expression of CD38 MFI while c (i-iii) shows the internal quality control of the added FlowCount beads. This protocol was used to accurately place gates E and F in the correct positions so that true shifts in CD38 expression could be identified.

Daily as well as weekly quality control material was analysed on the flow cytometer (Examples are given in Appendix A and B). Daily QC measures were within the preset limits of acceptance with longitudinal CV's of <5% for FlowCheck™ and ImmunoTrol™.

Weekly QC included the use of FlowSet™ for laser stability and adjustment of fluorescence channel to predefined values; ImmunoTrol™ for verification of protocol and CytoComp™ for compensation of fluorescence spill over between channels. In addition, both HIV negative and HIV positive patient samples were analysed to confirm (i) optimal placement of gates to ensure differentiation of $CD38^{dim}$ vs. $CD38^{bright}$ cells and (ii) stability of instrument settings and protocol setup over time. To test the reproducibility (precision) of CD8 T cell enumeration, every sample set up for CD38 analyses was run in duplicate as a tetraCHROME™ assay to confirm accuracy of the absolute CD8 counts used for assessing CD38 expression.

4.2.1 FLOW SET

FlowSet™ was used to set fluorescence channel numbers to predefined values for optimal cell positioning and discrimination.



	FL 1	FL 2	FL 3	FL 4
Coefficient of variation	1.92%	1.20%	1.54%	2.75%

Figure 15: Weekly FlowSet™ results in a Levy Jennings style plot for the period July 2008 to December 2009. Mean channel values for the different fluorescent channels (FL1, FL2, FL3 and FL4) are shown.

The above figure (Figure 15) illustrates the stability of fluorescence channels of the XL flow cytometer over time. Over the one and a half year period (79 weekly runs) no fluctuations were noted, indicating excellent reproducibility of the instrument. The overall %CV was <3% for all fluorescence channels for the period of the study.

4.2.2 COMMERCIAL BIOLOGICAL CONTROL (IMMUNOTROL™)

ImmunoTrol™ normal was used to verify gating strategies on the protocol by comparing both CD4 and CD8 absolute counts to the reference value (per package insert Appendix B). It was also used to set the negative gate for CD38 activation of patient samples, as ImmunoTrol™ is a true CD38 negative preparation (stabilized blood product). For purpose of instrument and protocol stability, the MFI values of FlowCount™ beads in these QC samples were also monitored longitudinally.

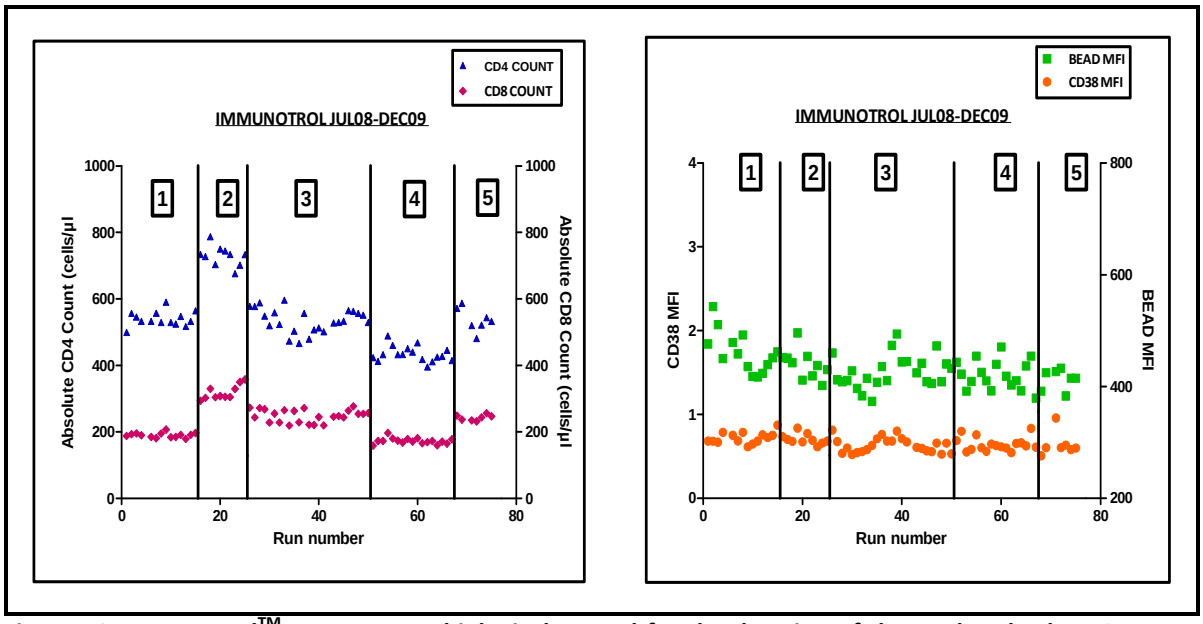


Figure 16: Immunotrol™ was run as a biological control for the duration of the study. Absolute CD4 (blue) and CD8 (pink) counts as well as CD38 MFI (orange) and Bead MFI (green) were plotted over time. Vertical lines represent different lots/ batch number of Immunotrol™ used.

In batch 1-5 as indicated above for both graphs the %CV for CD4 absolute count were 4.17% 4.38%, 6.68%, 5.37% and 6.54% respectively and for CD8 absolute count the %CV were 3.86%, 7.12%, 7.6%, 5.17% and 3.54% respectively. The 95% confidence interval ranges for CD38 and Bead MFI were 0.51-0.96 (mean of 0.67) and 374-543 (mean of 432) for all batches respectively.

4.2.3 COLOUR COMPENSATION

During 4 colour analyses, it is important to eliminate spill over of fluorescence between channels, especially in the channels of interest for CD38 analyses (i.e. FL3 for CD8 and FL4 for CD38) to ensure accurate distinction between true positive and true negative cells.

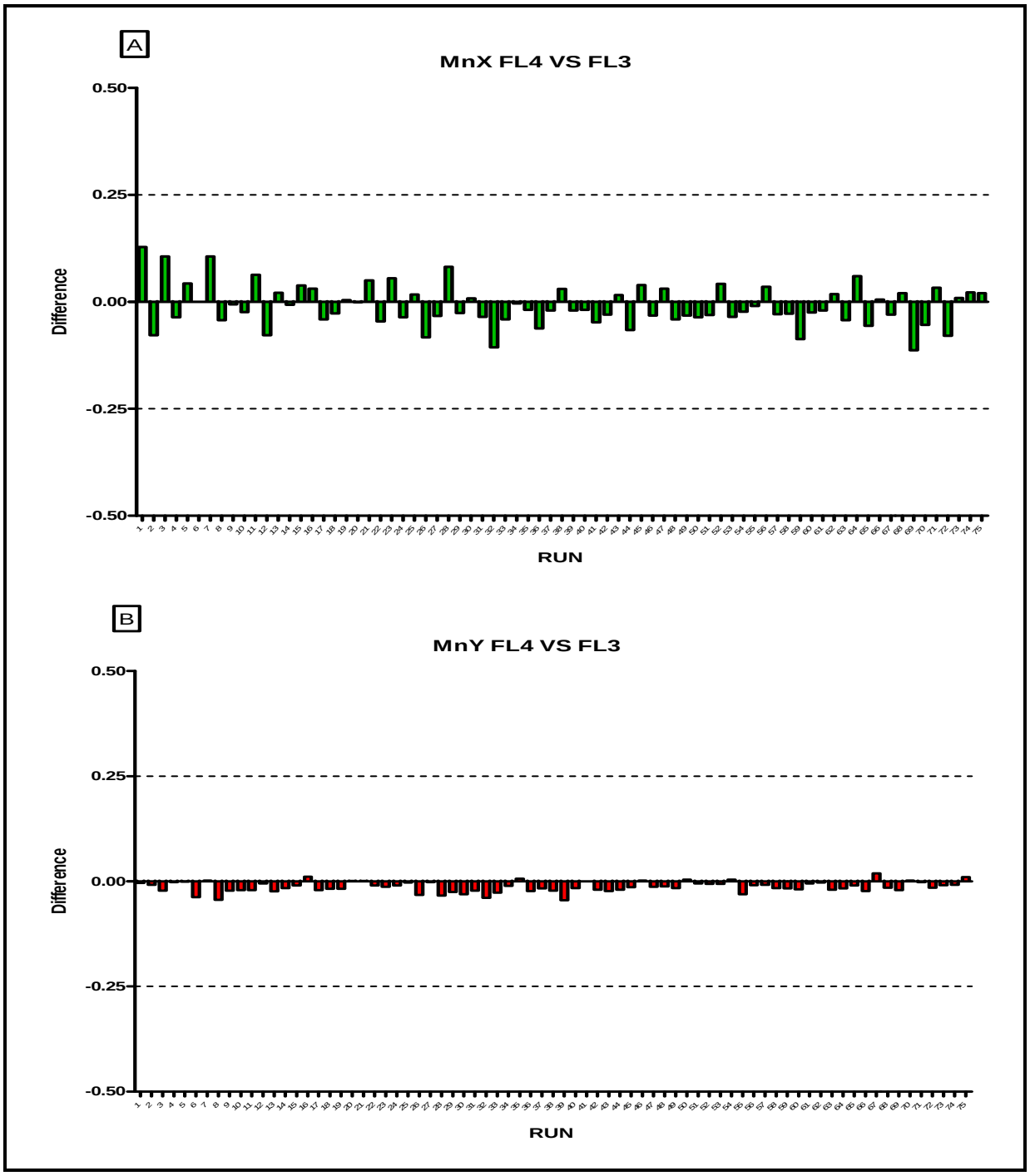


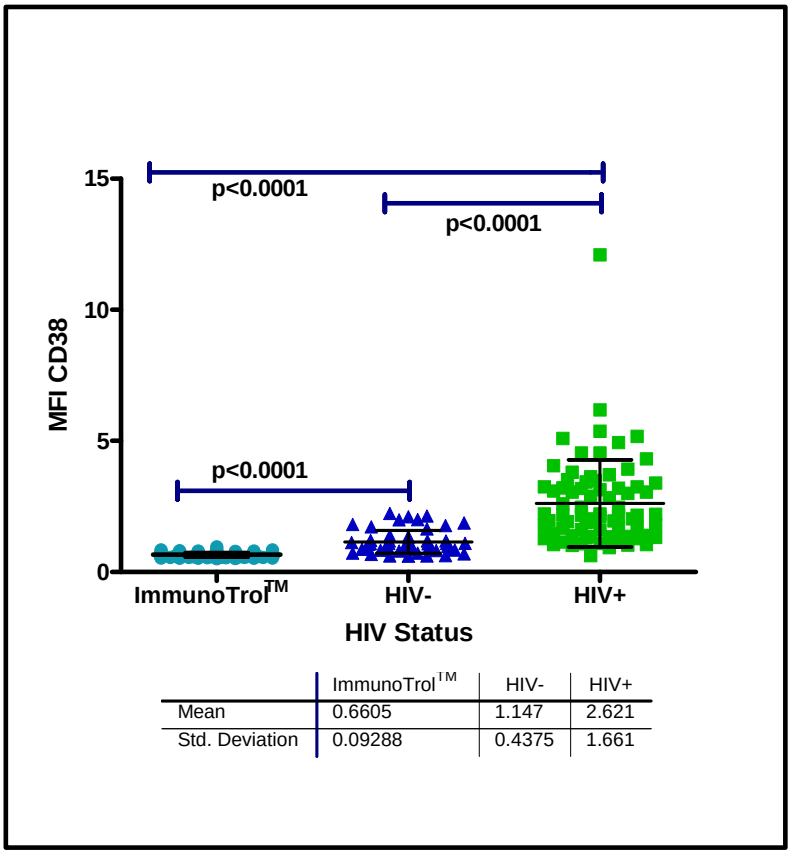
Figure 17: The difference in MnX (A) and MnY (B) values between FL4 and FL3 channels indicating adequate colour compensation, allowing accurate gating of CD8 and CD38 cells

Results show that the differences calculated between the mean X channel values (-0.01) and mean Y channel values (-0.13) for FL3 and FL4 were less than the acceptable

limit of <0.2, confirming optimal color compensation and thus optimal cell separation between fluorescence channels of interest.

4.2.4 HIV POSITIVE AND NEGATIVE CONTROLS

HIV negative and positive patient samples were analysed to ensure good separation of CD38+ versus CD38- CD8 T-cells.



	ImmunoTrol™	HIV-	HIV+
Mean	0.6605	1.147	2.621
Std. Deviation	0.09288	0.4375	1.661

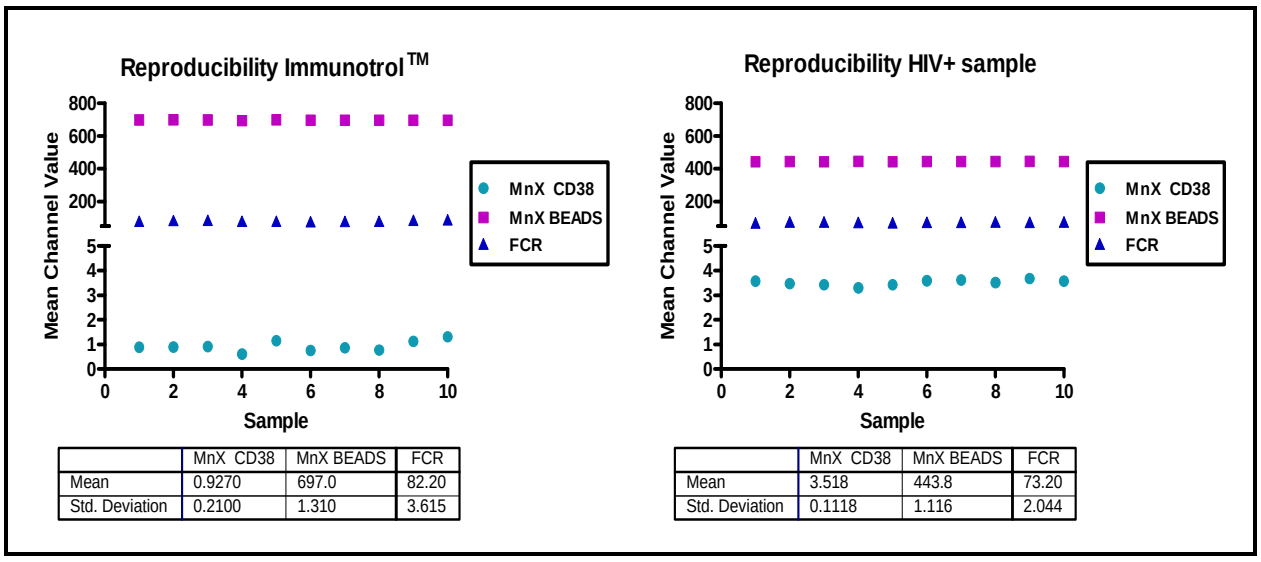
Figure 18: Comparison of CD38 MFI of ImmunoTrol™ (turquoise; n=75), HIV NEGATIVE (blue; n=51) and HIV POSITIVE (green; n=75) patient samples collected over the period of this study. Statistical significance (differences) between the various groups is indicated by the respective p values.

As is evident in Figure 18 above, CD38 was expressed at an MFI range of 0.384 to 1.58 (mean of 0.66) on the biological control, versus an MFI range of 0.644 to 1.98 (mean of 1.147) on HIV negative samples. A significant difference was noted between these two control groups (p<0.001). This indicates that HIV-negative patients show some

evidence of underlying low level CD38 activation. HIV+ patients (treatment naïve) showed an MFI range of 0.807 to 12.3 with a corresponding mean of 3.106 that was significantly higher than either the HIV-group or the true negative ImmunoTrol™ group ($p<0.001$).

4.2.5 REPRODUCBILITY (Precision of testing)

Reproducibility was established on both commercial control material (ImmunoTrol™) and actual HIV+ patient samples. Results indicate excellent reproducibility of MFI values for both CD38 expression and beads in patient samples and ImmunoTrol™. In addition sample flow count rates (FCR) also indicated stability of the instrument with respect to fluidic stability of the instrument for the duration of the analyses of 10 consecutive samples (%CV<5%, Figure 19).



	MnX CD38	MnX BEADS	FCR
Mean	0.9270	697.0	82.20
Std. Deviation	0.2100	1.310	3.615

	MnX CD38	MnX BEADS	FCR
Mean	3.518	443.8	73.20
Std. Deviation	0.1118	1.116	2.044

Figure 19: Reproducibility testing with ImmunoTrol™ (left) and an HIV+ patient sample (right), prepared as 10 replicates and analysed on the flow cytometer. FCR was also calculated as an internal quality control measure to establish instrument fluidic stability.

4.3 CORRELATION BETWEEN THE DIFFERENT TEST METHODOLOGIES

As per CIPRA Project V study protocol (DK Glencross, personal communication) patient samples were run in duplicate using the well established tetraCHROME™ 4-color method concurrently with the 4-color PLG CD4 with CD8/CD38 activation assay as CD45/CD4/ CD8/ CD38. This apparent duplication of testing was primarily to establish reliability of PLG CD4 enumeration against the established 4-colour tetraCHROME™ method, and to further ascertain reliability of PLG without use of CD3 and in combination with CD38.

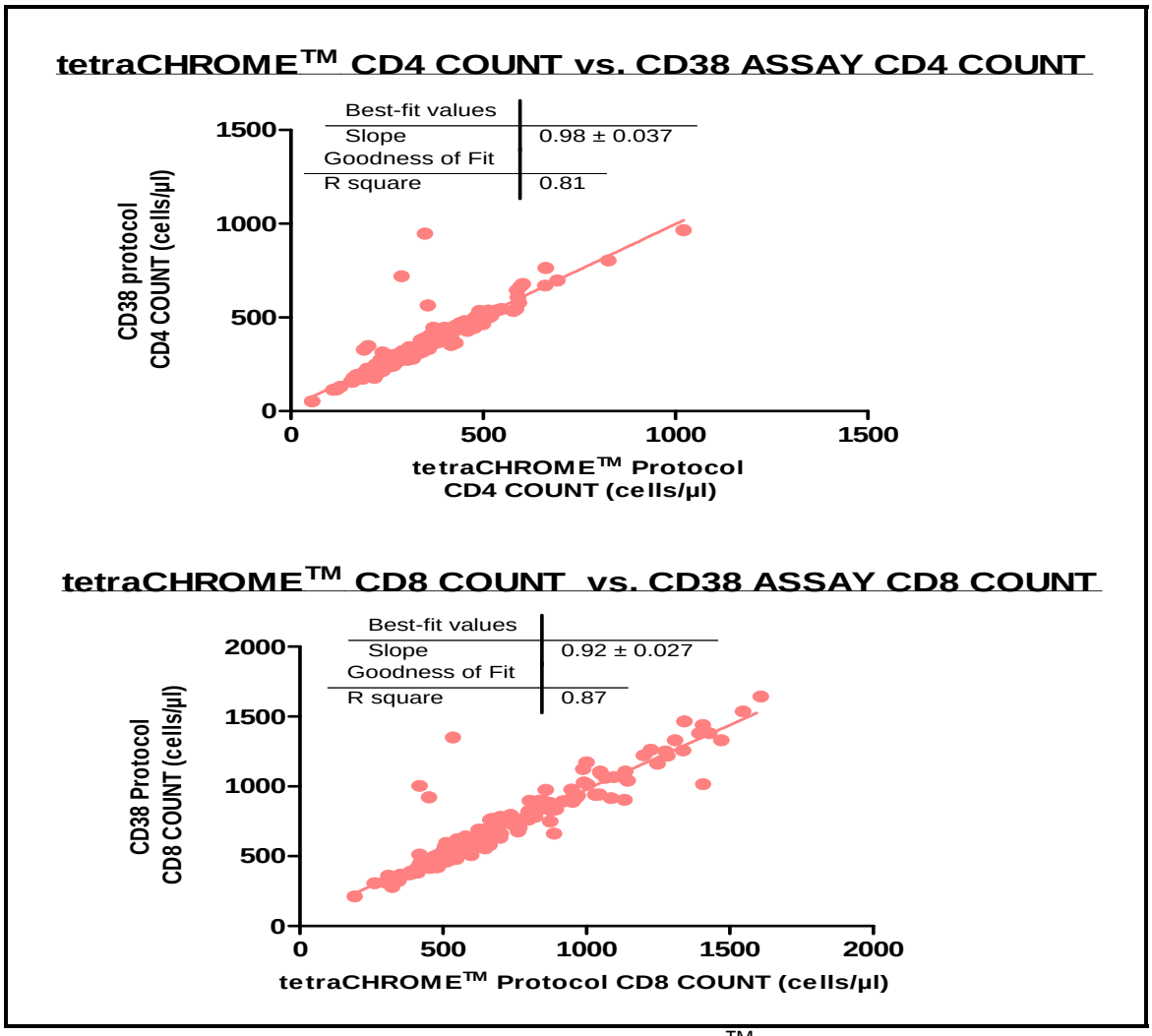
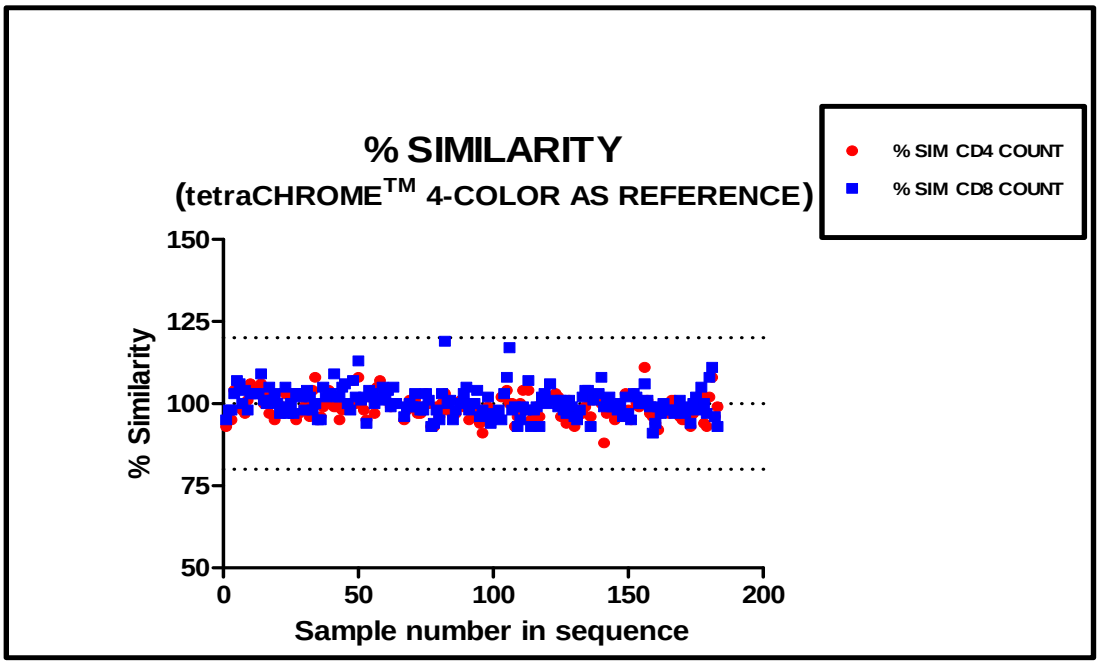


Figure 20: Correlation between the established tetraCHROME™ method against the CD8/CD38 activation assay for CD4 and CD8 absolute counts.

The above figures showed excellent agreement between the two different test methodologies with respect to generating accurate absolute CD4 and CD8 counts in patients on 2nd line therapy, indicated by the linear regression results and Spearman r correlation analyses for CD4 of 0.942 (95% CI of 0.921 to 0.957) and CD8 of 0.931 (95% CI of 0.907 to 0.949).

The overall % similarity was 99±3.6% for CD4 and 100.4±4.2% for CD8 with corresponding %CV values (precision to standard) of <5% (see Figure 21).



	% SIM CD4 COUNT	% SIM CD8 COUNT
Number of values	177	177
Minimum	88.00	91.00
25% Percentile	97.00	98.00
Median	99.00	100.0
75% Percentile	101.0	103.0
Maximum	111.0	119.0
Mean	99.04	100.4
Std. Deviation	3.614	4.272
Std. Error	0.2716	0.3211
Lower 95% CI of mean	98.50	99.75
Upper 95% CI of mean	99.58	101.0
Coefficient of variation	3.65%	4.26%
Sum	17530	17768

Figure 21: Percentage Similarity between CD4 (red) and CD8 (blue) absolute counts, where CD38 assay CD4 and CD8 counts were compared to tetraCHROME™ CD4 and CD8 assay counts.

4.4 PARTICIPATION IN THE SOUTH AFRICAN NHLS/ AFREQAS PROGRAMME

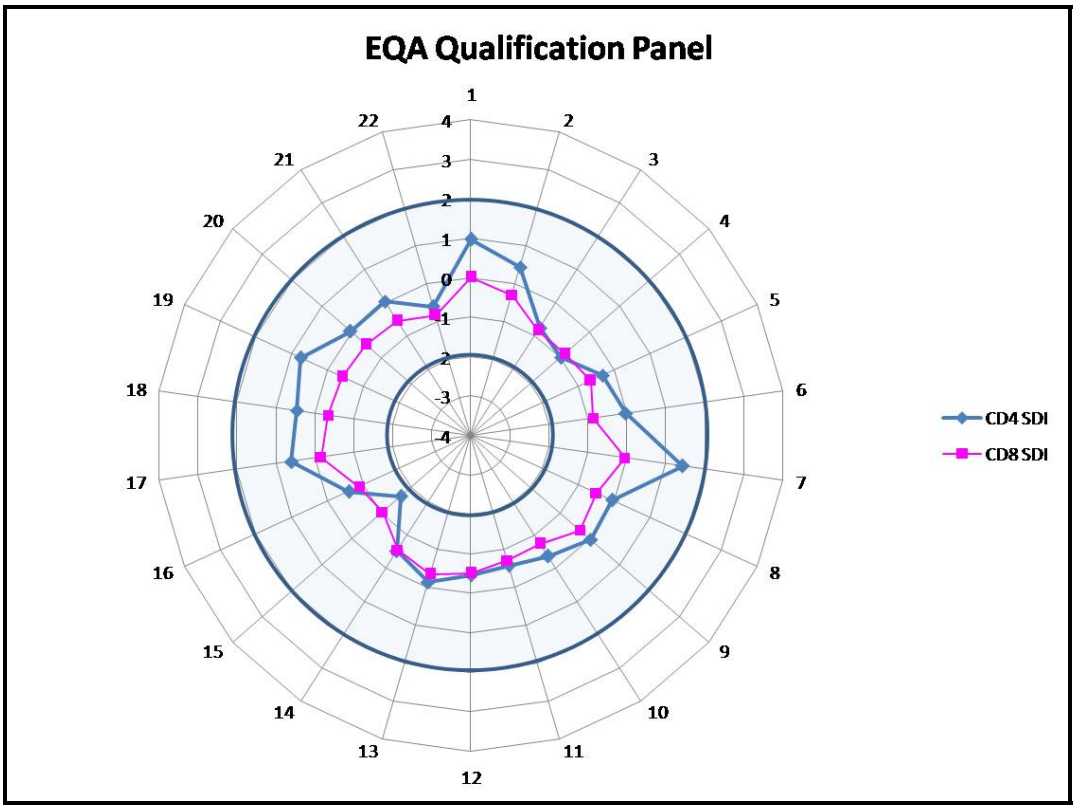
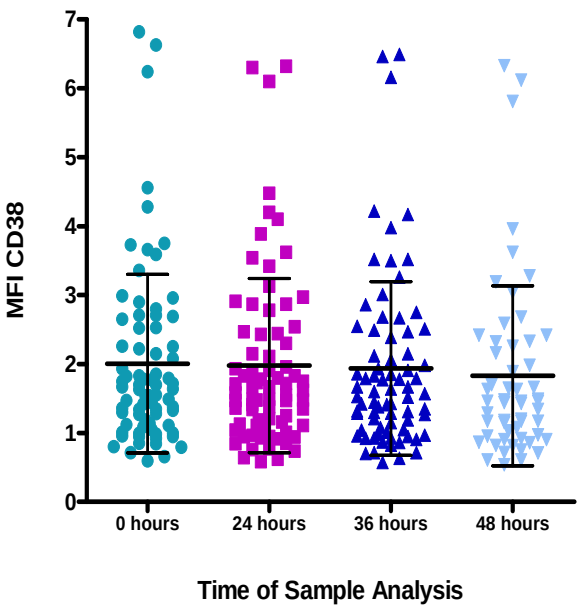


Figure 22: Radar graph showing accuracy and precision of CD4 and CD8 absolute count test results submitted on the South African NHLS AFREQAS programme. Dark blue lines and shaded area represent the ideal range and 2SD limits respectively. Light blue line represents the CD4 SDI and pink line represents the CD8 SDI. Results are from the period March 2008 to November 2009 (data used with permission, Laboratory Head, Prof DK GLENCROSS).

The results from the EQA scheme for the period March 2008 to November 2009 showed that the instrument used for CD38 analyses produced reliable results (within the 2SDI limits of acceptance) for both absolute CD4 and CD8 counts using the tetraCHROME^{TM} 4-color method. As the protocol setup and cytosettings of the tetraCHROME^{TM} CD45/4/8/3 protocol was identical to the CD38 4-color (CD45/4/8/38) assay protocol, (CD3 substituted for CD38) one can deduct that the CD38 protocol setup will produce reliable reproducible CD4 and CD8 absolute count results.

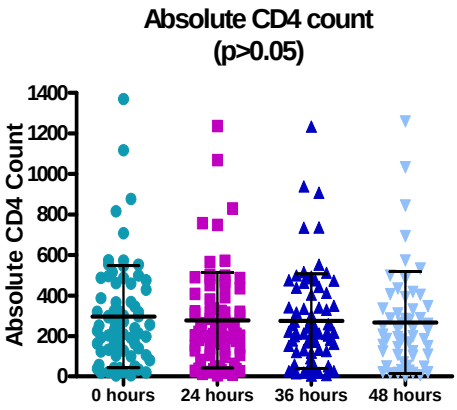
4.5 EXTENDED WINDOW PERIOD FOR TESTING CD38 EXPRESSION

In addition to the longitudinal follow up of patients on 2nd line HAART, the CD38 assay was evaluated for stability up to 48 hours after sampling to ensure that no *in vitro* activation occurred between sampling and sample analyses. This is necessary for the decentralisation of the CD38 assay to other PLG/CD4 testing sites where sample transport to a testing laboratory for analysis could take longer than 24 hours. In total, 75 randomly chosen fresh EDTA HIV+ blood samples were analysed for CD38 expression on day of receipt (within 6 hours of venesection; named T0) and re-analysed after 24 and 48 hours. At each time interval, one sample was prepared 10 times to test reproducibility.

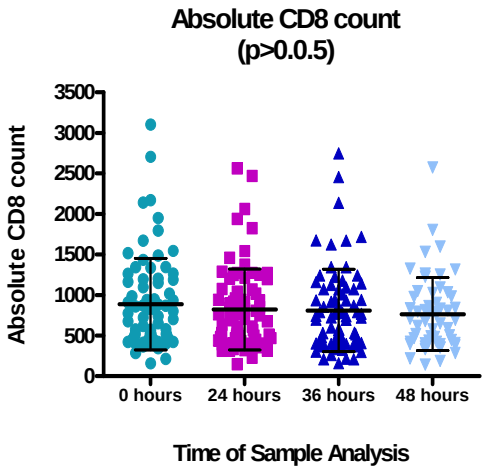


	0 hours	24 hours	36 hours	48 hours
Number of values	75	75	75	55
Minimum	0.5960	0.5860	0.5720	0.5400
25% Percentile	1.120	1.120	1.050	0.9530
Median	1.640	1.620	1.570	1.460
75% Percentile	2.530	2.440	2.470	2.320
Maximum	6.820	6.320	6.490	6.330
Mean	2.005	1.978	1.934	1.829
Std. Deviation	1.295	1.264	1.260	1.307

Figure 23: Comparison of CD38 MFI at time intervals, T0, T24, T36 and T48. No significant differences were noted for CD38 MFI at any time interval (p>0.05, One-way ANOVA).Due to instrument breakdown, only 55 samples could be analysed at T48.



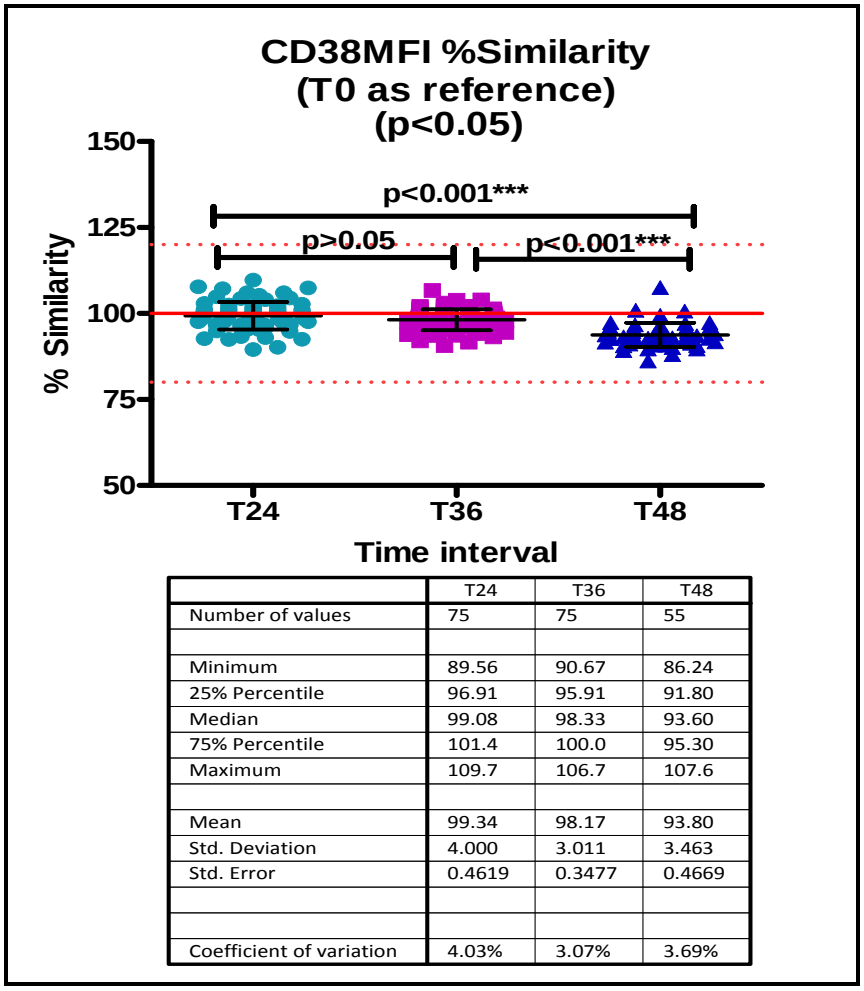
	0 hours	24 hours	36 hours	48 hours
Number of values	75	75	75	55
Minimum	5.000	6.000	5.000	2.000
25% Percentile	128.0	129.0	121.0	103.0
Median	238.0	221.0	216.0	207.0
75% Percentile	429.0	381.0	404.0	347.0
Maximum	1370	1237	1234	1259
Mean	295.3	277.4	274.5	266.1



	0 hours	24 hours	36 hours	48 hours
Number of values	75	75	75	55
Minimum	156.0	146.0	160.0	140.0
25% Percentile	475.0	447.0	416.0	423.0
Median	738.0	708.0	736.0	693.0
75% Percentile	1188	1068	1073	982.0
Maximum	3101	2561	2746	2575
Mean	887.7	821.1	808.6	763.5

Figure 24: Comparison of absolute CD4 and CD8 counts generated using the CD38 protocol assay at T0, T24, T36 and T48. No significant differences were noted for any of the parameters tested (p>0.05, One-way ANOVA).

T0 was used as the "reference" for comparing CD38 MFI (Figure 23) and absolute CD4 and CD8 counts (Figure 24) at T24, T36 and T48. There were no significant differences noted at any time interval for any parameter tested.



	T24	T36	T48
Number of values	75	75	55
Minimum	89.56	90.67	86.24
25% Percentile	96.91	95.91	91.80
Median	99.08	98.33	93.60
75% Percentile	101.4	100.0	95.30
Maximum	109.7	106.7	107.6
Mean	99.34	98.17	93.80
Std. Deviation	4.000	3.011	3.463
Std. Error	0.4619	0.3477	0.4669
Coefficient of variation	4.03%	3.07%	3.69%

Figure 25: CD38 MFI % Similarity at the different time points compared to T0 as the reference value indicated significant differences between T24 vs. T48 and T36 vs. T48 (P<0.001; one-way ANOVA).

Results indicated (Figure 25) good overall agreement, between 98-100%, with corresponding CV's of <5% and a bias of <0.2MFI across time intervals. There was no significant difference between T24 and T36 (p>0.05). At T48 the agreement was slightly less at 93.8% (CV<4%), but the differences noted were significant (p<0.05) and was most probably due to sample disintegration over time.

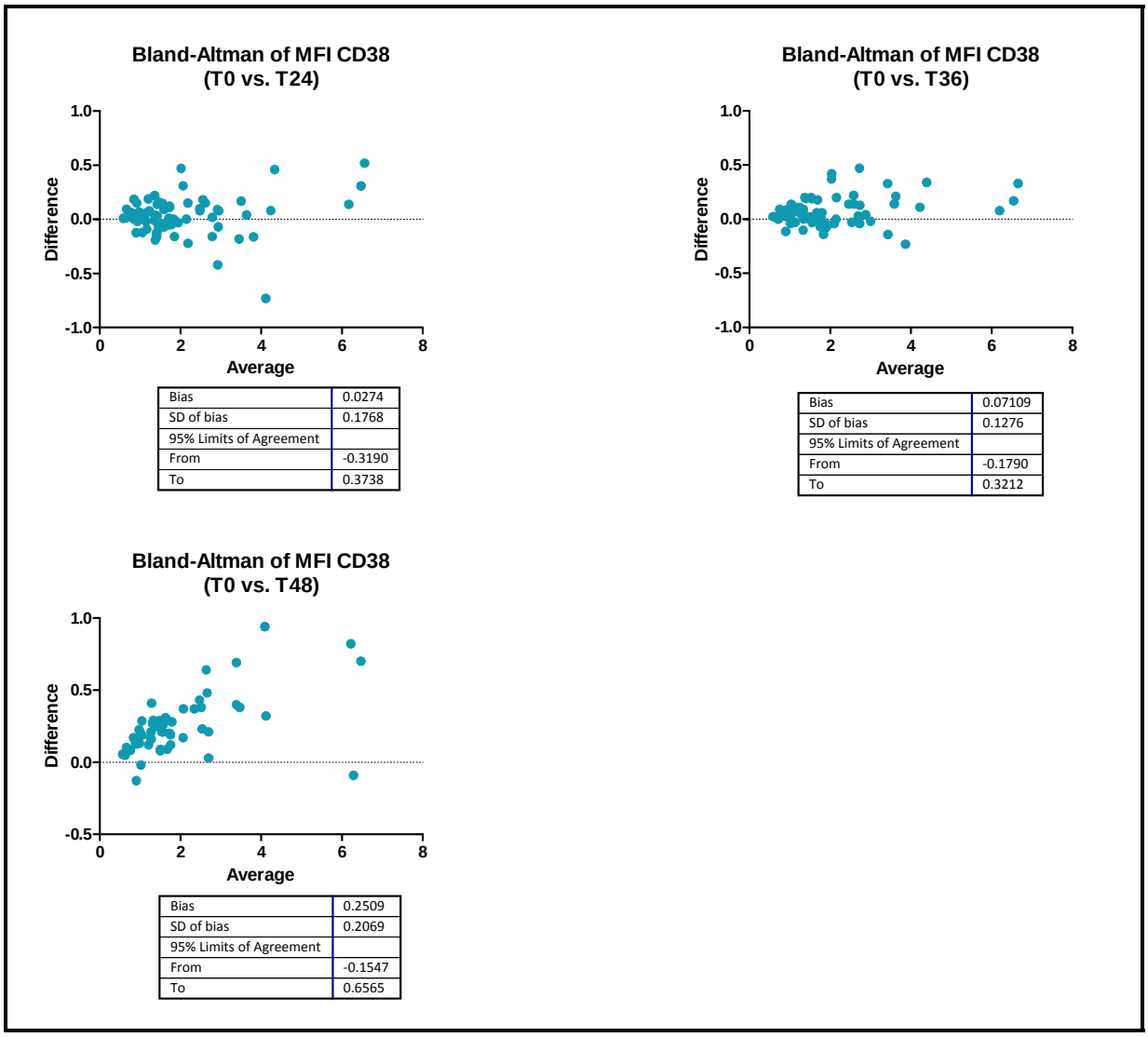
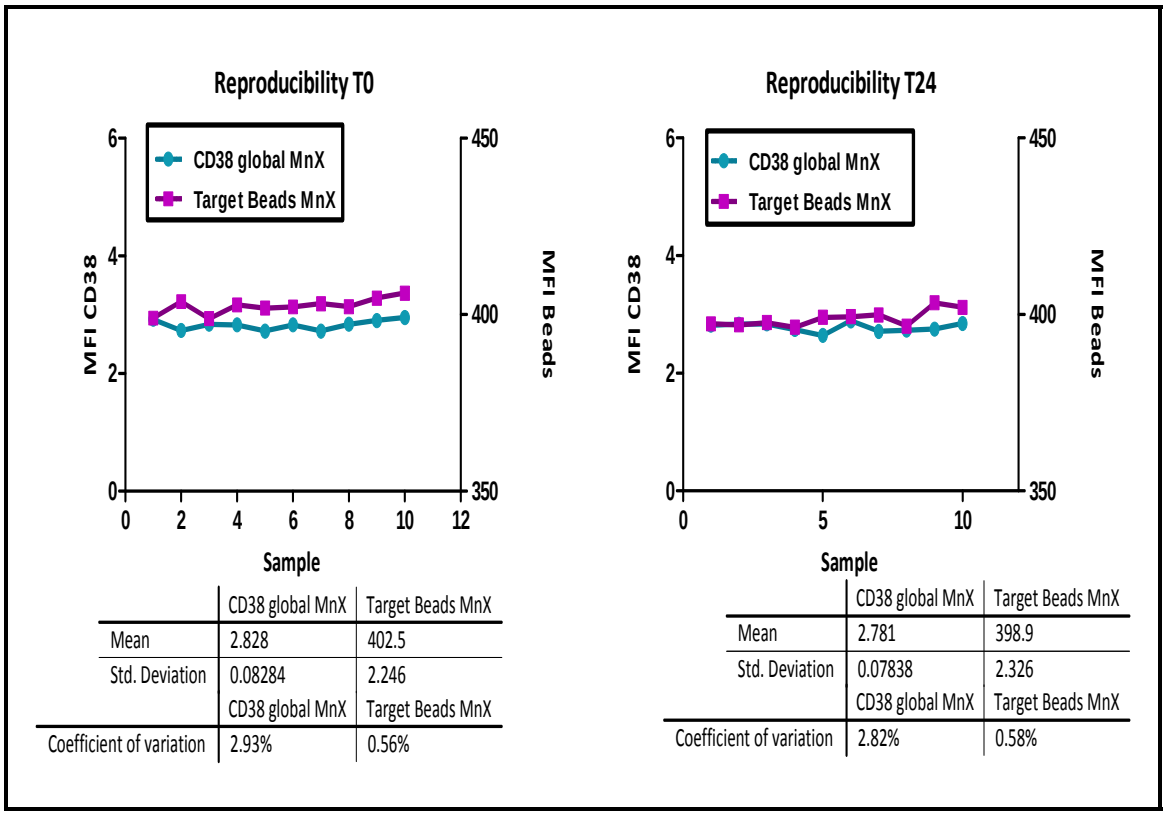


Figure 26: Bland-Altman analyses of T0 vs. T24, T36 and T48, indicating insignificant differences/bias of <1 (T0 used as reference minus T24/36/48).

The percentage similarity (Figure 25) and Bland Altman analyses (Figure 26) of CD38 MFI confirmed that insignificant differences were noted up to 36 hours, however at T48, sample disintegration may have already impacted on accurate CD38 MFI reporting.



	CD38 global MnX	Target Beads MnX
Mean	2.828	402.5
Std. Deviation	0.08284	2.246
	CD38 global MnX	Target Beads MnX
Coefficient of variation	2.93%	0.56%

	CD38 global MnX	Target Beads MnX
Mean	2.781	398.9
Std. Deviation	0.07838	2.326
	CD38 global MnX	Target Beads MnX
Coefficient of variation	2.82%	0.58%

Figure 27: Reproducibility with 10 replicates at each time interval of one selected sample is shown for T0 and T24.

The reproducibility exercise done at T0 and T24, showed excellent reproducibility with CV values <3% for both CD38 MFI and Target Bead MFI values.

4.6 IMPLEMENTATION AT A SECONDARY SITE

Results suggest that CD38 MFI is not affected by *in vitro* activation due to sample aging therefore the CD38 assay was implemented at a secondary NHLS CD4 testing laboratory. After uploading the CD38 assay on an XL instrument, 40 HIV+ EDTA whole blood samples were randomly chosen in the secondary laboratory for CD38 analysis. For comparison of results between sites, samples were sent to the reference laboratory (CMJAH) for preparation and re-analysis using the standardized CIPRA CD38 protocol. Differences between the instruments at the different sites were noted between the sensitivity of fluorescence channel 4 (FL4), even after intervention by service engineers tried to resolve the problem. In addition listmode data collected at the secondary site was re-analysed in the Johannesburg laboratory to establish whether any differences in protocol setup and sensitivity existed between the reference and the testing (implemented) sites.

CD4 and CD8 absolute counts and CD38 MFI were recorded and compared between the laboratories.

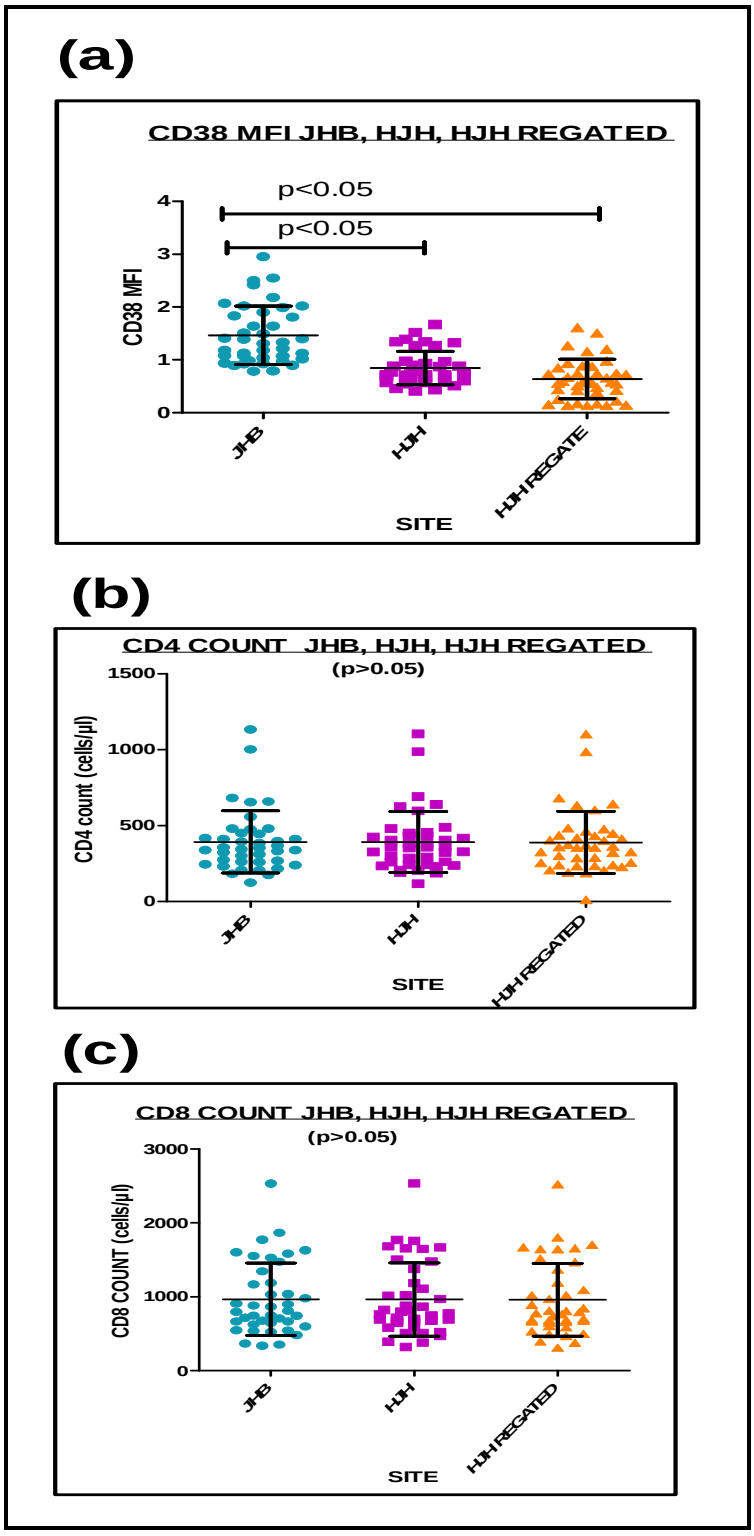


Figure 28: Correlations of (a) CD38 MFI, (b) CD4 and (c) CD8 absolute counts between the two laboratories. (Johannesburg in blue, Helen Joseph in purple and Helen Joseph listmode data re-analysed in the Johannesburg laboratory in orange). Error bars represent mean±1SD limit.

It is evident that the comparison for CD4 and CD8 absolute counts between the laboratories were excellent. The CD38 MFI results however showed statistically significant differences ($p<0.05$). This was due to the differences noted in FL4 channel sensitivity between the two instruments. This is a common problem as engineers are not able to calibrate instruments with precision but rather try to get to values prescribed by manufacturer or to previous settings. To accommodate possible differences of sensitivity of FL4 results (CD38 expression), results were calculated as a ratio of CD38 MFI to Bead MFI (as bead reproducibility had tight %CV<5%) and compared these results between sites.

4.6.1 RATIO OF CD38 MFI IN JOHANNESBURG VS HELEN JOSEPH HOSPITAL

	JHB	HJH
Number of values	40	40
Minimum	1.790	1.640
25% Percentile	2.345	2.345
Median	3.020	2.895
75% Percentile	4.270	3.738
Maximum	6.680	6.070
Mean	3.342	3.214
Std. Deviation	1.257	1.173
Std. Error	0.1987	0.1855
Lower 95% CI of mean	2.940	2.839
Upper 95% CI of mean	3.743	3.589

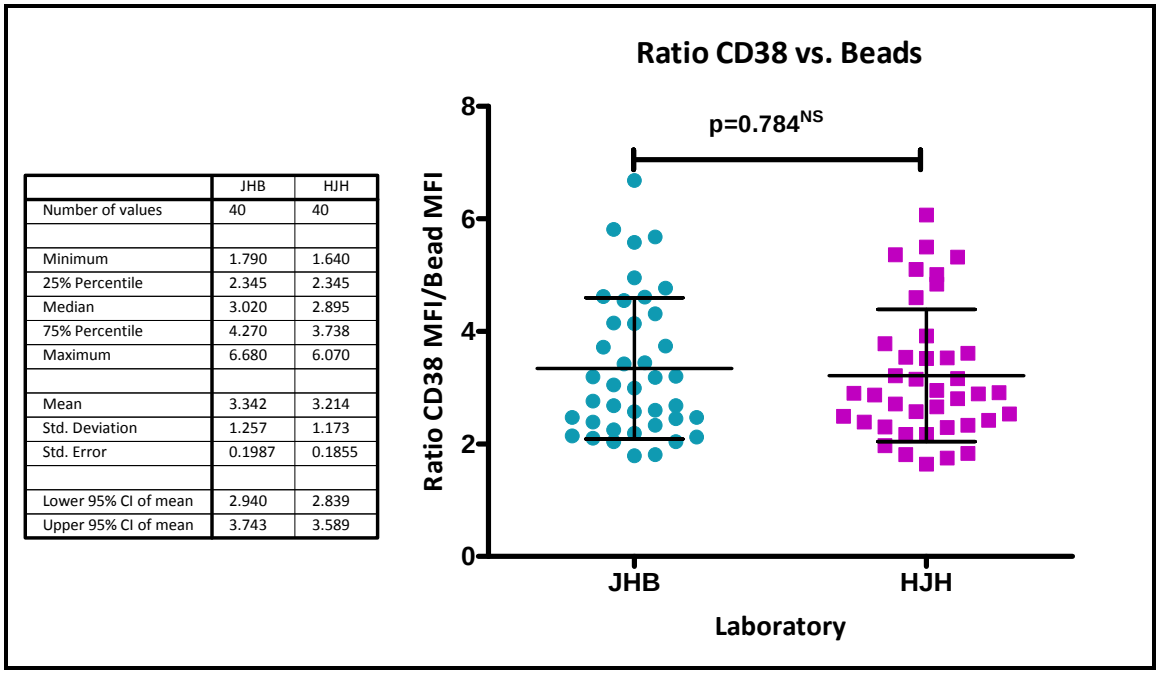


Figure 29: CD38 MFI/Bead MFI was used as a ratio to compare results between the two laboratories, (JHB in blue and HJH in purple). No significant differences were noted (p>0.05, t-test).

No significant difference were noted between laboratories for the ratio of CD38 MFI vs. Bead MFI (p=0.78), confirming that the differences noted in global CD38 MFI was not clinically significant. This was confirmed by the %similarity analyses (figure 30 below)

	%Sim ratio
Number of values	40
Minimum	84.67
25% Percentile	94.02
Median	100.5
75% Percentile	103.1
Maximum	109.6
Mean	98.65
Std. Deviation	5.881
Std. Error	0.9299
Lower 95% CI of mean	96.76
Upper 95% CI of mean	100.5
Coefficient of variation	5.96%

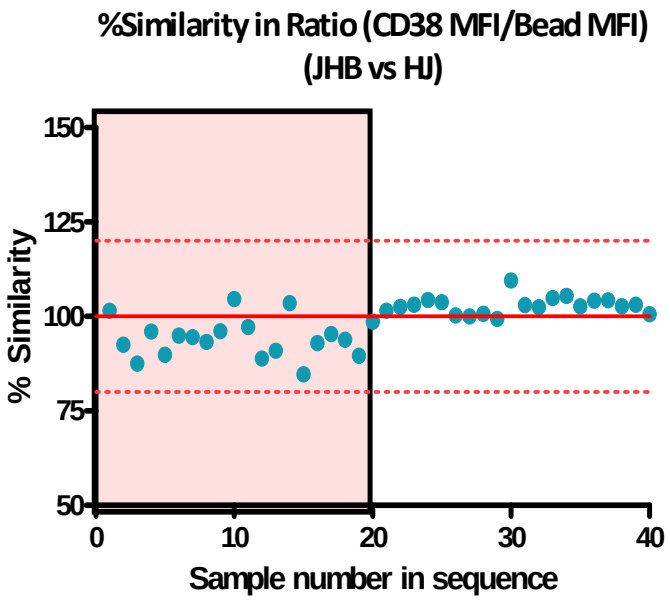


Figure 30: % Similarity for CD38/Bead MFI ratio between Johannesburg versus Helen Joseph results.

From the sequence plot, it is clear that during the initial phase on this implementation some variability occurred probably due to the staff not being familiar with the protocol and analyses (%Similarity 94.3%, CV 5.5%). Accuracy however improved after this initial set of 20 samples analysed (%similarity 103%, CV 2.2%), indicating that initial setup issues were overcome successfully and staff were able to prepare and analyse samples effectively (overall agreement 98.6% with CV of 5.9%) without further intervention.

5.0 DISCUSSION

The current study reported in this dissertation is a follow-up of a sub group from the initial 2005 cohort who subsequently failed 1st line therapy and were continued onto 2nd line therapy. This subset of patients was monitored longitudinally for CD38 expression until the conclusion of the study at the end of 2009. Overall, this study showed that patients on 2nd line therapy, who continue to respond well to treatment, show similar and equivalent CD38 response patterns observed in patients being followed on 1st line therapy. Furthermore, this study confirmed that the CD38 assay described works well in combination with the routinely used PLG CD4 assay.

Mean CD38 MFI for the study group decreased significantly over time from 3.271 (start of 1st line therapy) to 1.213 (study conclusion after 2nd line therapy) with corresponding mean VL decreases from 4.995 to 2.279 log copies indicating overall response to ART. Second line therapy patients (n=9) as a group showed similar results to previously published data on good responders to 1st line therapy up to weeks 48 and 96 (104) in that CD38 MFI followed VL downward until the termination of the current study.

The overall results of the SA-CIPRA group for the four year follow up period (104, 110) has showed that CD4 counts increased steadily through first line therapy. The current study confirmed that absolute CD4 counts continued to increase through switch of therapy (to 2nd line ART) in the cohort of 9 patients, before it plateau at 459± 181cells/µl. Benito et al (111) observed a similar plateau in absolute CD4 counts of

between 270-646 cells/µl and contributed it to the fact that CD4+ T cells recover independently from activated CD8+ T cells and therefore although CD38 expression might decrease rapidly after initiation of therapy, it could take much longer for CD4 counts to return to "normal" levels. An alternative explanation could be that the lack of CD4 gains due to increased levels of CD8+ T cell activation and decreased levels of naïve CD4 T cells in combination with patient age result in the plateaus seen in patients (112). It is further possible that the initial low baseline CD4 count seen specifically in the SA-CIPRA cohort contributed to the relative slow gain in CD4 count over time, as most patients presented with CD4 counts <200cells/µl (103).

Of note however is that the absolute CD4 counts at conclusion of the study were still lower than local normal reference ranges (500-2010 cells/µl) (113), although the percentage increase (272%± 126%) from baseline were significant for individual patients.

In accordance with previously published studies (17, 78, 86, 111), CD4 absolute counts had a good (expected) negative correlation with CD38 MFI and VL results in the current patient cohort. Equally, and also as expected, there was a significant positive correlation between CD38 MFI and VL for this study.

5.1 Individual patient results

Patients fell into two distinct groups. The first group of patients failed 1st line therapy but responded well to 2nd line therapy as reflected by undetectable VL levels and concomitant decreases in CD38 MFI. The second group of patients showed some non-specific increase (NSI) in CD38 MFI while on 2nd line therapy, without a corresponding increase in VL. No recorded adverse events were noted in these patients at the time of the observed CD38 MFI NSI. It is speculated that these non-specific rises may possibly be attributed to short-term non-adherence to therapy (<4 weeks) or the presence of a secondary viral/bacterial infection since CD38 MFI values returned to preceding visit values at subsequent visits. Although CD38 values correspond well with VL, CD8 T-cell activation is not exclusive to HIV infection. In the context of actual clinical follow-up of patients, it is thus important to correlate CD38 MFI results with clinical evidence of secondary infections and/or interruption/non-adherence to treatment, especially in cases where non-specific increases of CD38 MFI are noted.

A major finding of this study, of second line patients, was that there were no patients who showed an increase in VL without a corresponding increase in CD38 MFI, in keeping with the initial published outcomes to 48 weeks on first line ART (104-105, 110). This suggests that the CD38 assay was sensitive enough to reflect all observed changes in VL. In the current cohort, 5/9 patients (55%) showed CD38 MFI following changes in VL, while 3/9 (33%) patients showed transient increases in CD38 MFI at one time point with no corresponding increase in VL, while 1 patient had irregular CD38

FMI and VL values throughout 2nd line therapy, indicating either non-adherence or possible resistance to 2nd line therapy. The patterns observed in this study correlated with the published and reported data on 1st line response for the total SA-CIPRA cohort (104-105, 110). Similar patterns of a NSI in CD38 matched with a corresponding increase in VL were also reported in 1st line ART patients monitored for one year, in a paper published by Tauillon et al (114). They concluded that CD38 bright cells on CD8+ T cells mimics the results of VL and that CD38 can be a good monitoring tool in patients on ART. However, they suggested the use of different cut-off points for CD38 depending on the patients CD4 count at initiation of therapy, whereas the current study recommends that a baseline CD38 MFI be used as the reference point to monitor individual patients longitudinally on ART. Although the current study only included 9 patients followed for 2nd line therapy, the data suggests that CD38 MFI monitoring is as useful for 2nd line follow-up as it is in 1st line and that the CD38 assay is sensitive enough to detect subtle changes in the immune system due to increased viral burden, secondary infection or non-adherence to therapy.

In order to decentralise implementation of the described CD38 assay, stringent quality control procedures needed to be established. Accurate reporting of CD38 depends on several variables, as highlighted by Lenkei et al (115) and include selection of antibody (clone and fluorochrome), instrument standardization, sample preparation and the availability of suitable calibrators/reference material. The current study reports CD38 using mean fluorescent intensity as per Glencross et al (103) unlike previous studies

that looked at CD38 expression either as a percentage or Antibody Binding Capacity (ABC) (76, 87, 90, 96, 99-101). Recommendations by Lenkei et al (115) did not include MFI standardisation as a method for reporting CD38 activation.

Several methods have been described to standardise CD38 expression in different patient cohorts. CD4 expression on CD4+ T lymphocytes was used as a biological calibrator for converting CD38 PE fluorescent intensity into units of CD38 antibodies bound per CD8+ T cell (116). This study by Hultin et al used the relative fluorescent intensity (RFI) of PE (the RFI multiplier) to estimate the number of CD38 antibodies bound per CD8 cell. This method of cell enumeration was specifically developed for use on Beckton Dickinson flow cytometers (FACSCalibur™/FACSScan™) and had the disadvantage in that the test had to be validated periodically using 20 normal donors, resulting in increased reagent usage, time and operating costs. This method was compared to a commercially available PE labelled bead preparation (QuantiBRITE™) by Schmitz et al (117). QuantiBRITE™ did not need periodic validation like the biological calibrator, but significant differences in results were noted between different laboratories while yielding similar results if compared within laboratory. (117). Thus it was recommended that a laboratory should choose one method for CD38 enumeration/reporting that suits them and use it consistently. Losses of fluorescence on the QuantiBRITE™ beads over time may however lead to over estimation of MFI if the beads are used over an extended period of time which could also lead to variation between reporting sites (118).

Another approach to define true CD38 positivity was reported by Tilling et al (78), who used cord blood as a positive control to establish a cut-off as the latter has very high expression of CD38. Cord blood is however difficult to obtain and may present ethical issues and as such this method is not suitable for routine CD38 quality control testing.

An in-house quality control procedure was developed and implemented to set limits for fluorescence expression and monitor protocol stability over time. The data presented in the current study is a continuation of data collection post publication of Coetzee et al (106). The procedure included the use of FlowSet™ beads to optimize fluorescence channels to preset values, while colour compensation was done to ensure minimal spectral overlap. Optimal cytosettings were confirmed using the CD38 assay on ImmunoTrol™ stabilized blood, while monitoring FlowCount™ bead MFI as an additional internal control for both ImmunoTrol™ and patient samples. One of the disadvantages of CD38 flow cytometric assays in the past was defining the cut-off/cut-on value for true positive CD38 expression as it is known to be diffuse (78, 98). The "in-house" developed quality control procedures allowed for the clear definition of CD8+/CD38- cells using non-activated ImmunoTrol™. In addition, both HIV- and HIV+ (treatment naive) patient samples were analysed as part of the weekly QC to confirm the pre-set differentiation between CD38+ and CD38- cells. The data confirmed a statistically significant difference between HIV- and ImmunoTrol™ CD38 levels, re-iterating that ImmunoTrol™ is a better negative control for use to optimize gating of true CD38 positive cells. As the immune system is a dynamic system, low levels of cell activation is always present even in HIV- patient samples. As ImmunoTrol™ is

commercially available and already used in PLG/CD4 laboratories as part of their daily quality control procedures it does not impact on cost for the implementation and maintenance of the CD38 assay.

Monitoring of bead MFI in each sample tested, not only confirmed stability of the instrument over time (CV<5%) but ensured that changes seen in CD38 MFI expression reflected true activation and not instrument/operator induced shifts in fluorescence.
In addition to the daily and weekly QC measures, all samples tested were done in duplicate, using the CD38 4-color protocol ("piggy-backed" onto PLG/CD4) and the tetraCHROME^{TM} 4-color protocol (equivalent CD45/CD4 reagent clones to PLG/CD4). Results confirmed that the CD38 assay delivered equivalent results to the "gold standard" for both absolute CD4 and CD8 counts, indicating that the addition of CD8/CD38 to the PLG/CD4 protocol did not adversely affect absolute cell enumeration. This is a positive finding as the potential roll-out of a combined CD4/ CD38 assay will not compromise absolute CD4 reporting for patients already on therapy or new patients qualifying for therapy.

For consideration of implementation of the CD38 MFI assay into routine practice, it was imperative to establish the robustness of the assay and eliminate possible *in vitro* activation of cells (artefactual increases) due to sample handling and aging. Results showed that the comparison of CD38 MFI, Bead MFI, CD4 and CD8 absolute counts at the different time points of sample preparation did not show any significant differences

(p> 0.05, One-way ANOVA). Percentage similarity analysis at T24 and T48 versus T0 showed a similarity of 98-99.3% across all parameters, with excellent precision to predicate methodology (CV<5%). This confirmed the good overall agreement of results if samples are analysed within 24-48 hours of receipt in the laboratory. The Bland-Altman analysis revealed some loss of CD38 MFI at T48 but this was not significant (p>0.05). An insignificant bias of <0.2 MFI was observed at all time points versus T0 and overall reproducibility showed excellent results. The CD38 assay was deemed stable for up to 24 hours with some loss of MFI at 48 hours post venesection, irrespective of the level of in vitro lymphocyte activation. CD38 expression on CD8 cells are thus not significantly affected by sample aging and can be adequately tested 24 hours after venesection. However it must be noted that this experiment was conducted on the bench, at room temperature, with minimal sample handling. A more detailed study may however be required to assess the impact of sample handling on CD38 activation. Previous work conducted by Mandy et al (119) showed that even samples left on a rocker for 24 hours, under strict controlled temperatures in a laboratory setting, could not be processed due to deterioration of sample integrity. Therefore special attention needs to be considered with regards to sample packaging and transport from outlying laboratories to PLG/CD4 testing sites (for proposed roll out of CD38 assay across the SA-NHLS network).

A secondary aim of this study was to assess the feasibility of implementing the described CD38 assay in an off-site 'pilot' laboratory before considering national

implementation as a routine test across a network of CD4 laboratories. Helen Joseph CD4 laboratory was selected as a pilot site as it is a well established NHLS PLG/CD4 testing laboratory using the same equipment and protocols as the reference laboratory according to National NHLS standardisation protocols. A few problems were experienced on initial implementation, due to the differences in fluorescence sensitivity in channel 4 between the two instruments. However despite the lack of instrument calibration standardisation, comparative results between laboratories were not compromised. Standardisation between laboratories was facilitated through use of a calculation of a ratio of CD38 MFI to Bead MFI at each site, used to compare results between the two instruments. The calculated ratios showed no statistically significant differences, with corresponding % similarity of 98.6% and precision to predicate (CV) noted of 5.9%. This data emphasises the importance of a within-assay standardised quality system, like the addition of FlowCount™ beads to compare results between sites. These beads are however, not specific to the CD38 assay but are the same beads currently used in the PLG method for CD4 enumeration onto which the CD38 assay is "piggy-backed". In this context any uniform bead products would be comparable and of use, although it was not in the scope of the current study to evaluate the performance of different commercial bead products with the CD38 assay.

This study thus confirms the ease of implementation in the laboratory as evidenced by the smooth operation of the implementation and the good overall acceptable agreement between the two sites. After an initial learning curve period (Figure 30,

sample number 20 onwards), differences between matched site results were minimised and showed excellent correlation to results obtained in the Johannesburg laboratory reference site. Staff at the site commented that the assay was easy to use with very little additional work required. This study is the first to our knowledge to document the implementation of a standardised CD38 assay into a secondary laboratory for possible implementation across a network of national CD4 testing laboratories. Previous studies mostly looked at CD38 expression in isolation as a once off study at one site under strict clinical settings. This in-house developed CD38 assay is thus novel in the sense that it is tailor-made for current South African needs, making it user friendly and easy to implement, permitting staff with no prior experience in CD38 monitoring to apply this assay. As PLG/CD4 operators are skilled in the use of flow cytometers, training is modular and based on skills learned through use of PLG CD4 testing and additional training would concentrate more on quality control implementation and data interpretation of CD38 MFI monitoring. This study opens the door for CD38 monitoring to become an integral part of the National Rollout as a supplementary monitoring tool for HIV positive patients on ART. This study in second line treatment patients confirms previous reports, that CD38 MFI is a reliable and cheaper alternative in up to 60% of patients on 1st line ART, who are responding to treatment. Such an approach could potentially reduce the need for VL testing as per current national guidelines (3) if specific algorithms are developed which facilitate "reflex testing' whereby VL PCR testing is only performed when a rise of CD38 MFI is noted in two or more consecutive visits. By having the CD38 assay piggy backed onto

the PLG assay it will give additional information on the status of the patient's continued response to treatment and allow clinicians to better manage HIV patients in real time.

5.2 LIMITATIONS OF THE STUDY

A CIPRA document of adverse and opportunistic events was made available. However due to the small number of patients in the current study which only consisted of a subgroup of 9 patients from the initial well controlled CIPRA cohort (n= 472) and CD38 sub-study of 120 patients , detail in this document was insufficient to address all the possible causes of the non-specific increases in CD38 MFI noted in the study patients. Patient compliance to ART as well as minor infections causing NSI of CD38 was unfortunately not recorded on this database. It is most probable though that the latter are the most likely events for transient CD38 increases noted. It is unlikely that infections such as TB and EBV were the cause of these spikes in CD38 as one would assume that these would not be resolved within a 4 week period and in keeping with this one would expect elevated CD38 levels for a longer period (these infections would also have been listed in the document related to opportunistic infections and adverse events).

The current cohort did not include children and youth. However in a recent study conducted by Rosso et al (95) in youths aged between 7-18 years, similar patterns in CD38 expression were noted in responder and non-responder groups. Although this group looked at CD38 antibody binding capacity and CD38%, their assay had 75%

sensitivity and 93.8% specificity for both measurements and illustrates that it could be used successfully in children. In a previously published paper by Sherman et al (85), in HIV-infected paediatric patients, it was also found that increased CD38 expression on CD8+ T cells had the same prognostic power as that seen in HIV-infected adults.

Due to the unexpected good compliance of patients on the greater CIPRA cohort, the number of patients failing 1st line therapy was much lower than anticipated. However, similar trends were noted for patients continuing on 1st line therapy vs. 2nd line therapy regarding the CD38 expression which followed VL trends. Another limitation would be that roll-out of this test into the routine setting cannot be recommended without more extensive testing in a less controlled cohort.

5.3 COST OF CD38 ASSAY

Due to limited funding and South Africa having the highest burden of HIV in the world (1) , the impact of reduced testing for VL could positively impact both financially and logistically on our National CCMT programme. This would reduce overall programme costs thereby optimising VL testing to when absolutely needed for patient management. Details of potential breakdown of savings are outlined in Table 2 below.

Table 2: Cost assessment using 2007/2008 data as per NHLS Financial report.

Time of Testing	Current Strategy 100 Patients	Alternative strategy 60 % of Patients	Savings
Baseline	VL + PLG (R360)	VL+PLG+CD38 (R400)	-
6 months	VL + PLG (R360)	PLG + CD38 (R100)	
12 months	VL + PLG (R360)	PLG+ CD38 (R100)	
SUBTOTAL	R108 000 (R1080/p/y)	R60 000 (R600/p/y)	*R48 000* *(R480/p/y)*
Year 2 (x2)	VL + PLG (R720) R72 000	CD38 (R200) R20 000	*R52 000* *(R520/p/y)*
Year 3 (x2)	VL + PLG (R720) R72 000	CD38 (R200) R20 000	*R52 000* *(R520/p/y)*
TOTAL COST 3 years/100 patients	R252 000 (R2 520/p)	R100 000 (R1 000/p)	R152 000 (R1520/p)
400 000 Patients need to start ARV (3year estimate)	1.008 billion (336 million/year)	400 million (133 million/year)	608 million/3 years (203 million/y)
CURRENT EXPENDITURE 07/08 (Assume 350 000 patients on ARV)	286.8 million/year 860.4 million/3years		153.8 million/year 461.4 million/3 years

Although it was not the aim of this study to evaluate the potential cost savings of implementing the CD38 assay as a supplementary test to VL testing, a preliminary cost estimation was done using the current SA-NHLS cost of a CD4 count (R60-00) and VL test (R300-00). The assumed cost of a combined CD4/CD38 test at R100-00 was used as

the maximum price as combining antibodies into a single vial cocktail will probably only effectively put the current CD4 price up by R20-00. It is estimated that the cost savings per annum using the CD38 assay will be significant (around 150 million per annum). In view of the recent announcement by the Department of Health (120) to increase the number of people tested for HIV and put more people on therapy, these savings could imply that more people qualifying for initiation of ART (CD4 count <350cells/µl) will receive therapy, improving the quality of life and life expectancy of thousands of infected South Africans.

5.4 FUTURE RECOMMENDATIONS

To make the interpretation of CD38 MFI results more user friendly, an algorithm is in development that would indicate to laboratory personnel (and hence clinicians) when to request VL testing and how to interpret longitudinal CD38 MFI monitoring. Such systems relate to use of standardised MFI reporting as a % of MFI change from baseline or from previous visit as an indicator to reflex to VL testing (104-105, 110, 121) (personal communication DK Glencross).

The CD38 assay can be further streamlined by asking the manufacturers to produce a 4-color product (CD45/4/8/38) cocktail that will cost considerably less than adding two separate antibodies to the current PLG/CD4 reagent kit and will greatly improve the ease of use as only one pipetting step will be needed for antibody dispensing into tubes. This may also prove valuable on new systems like the Beckman Coulter MPL and CellMek automated preparation system, where it would be easy to replace one bottle

of antibody (PLG/CD4) with another. It is foreseen that should the CD38 assay be rolled out to all SA-NHLS CD4 testing laboratories, that it can be used on the current and replacement systems as the protocol has also been set up successfully on the FC500 (equivalent to the new MPL).

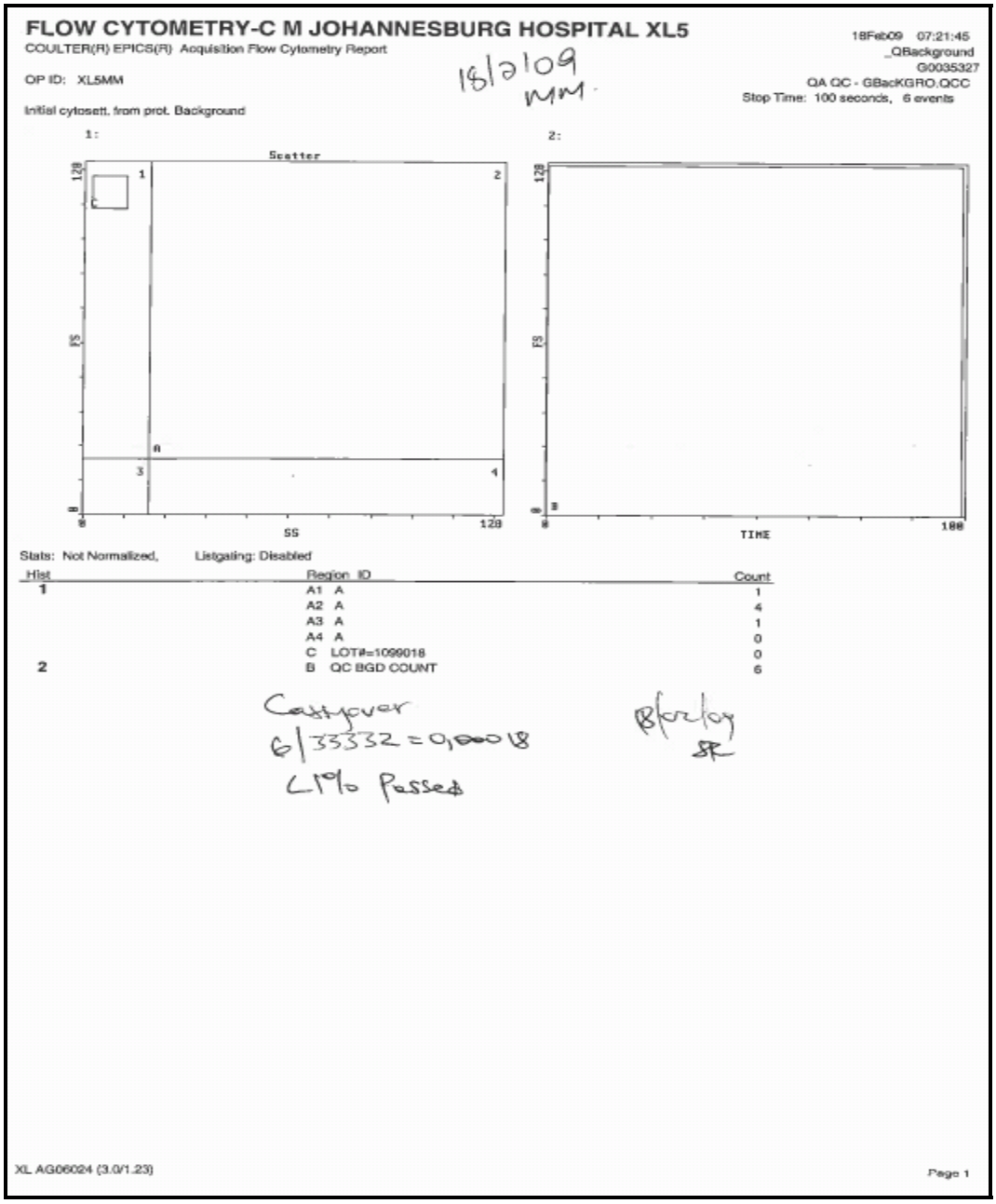
The current study was conducted in a strict controlled environment with an exceptionally reliable cohort (re compliancy). The next step would be to test the impact of this assay in a longitudinally followed cohort from a typical routine HIV clinic where compliancy is not so strictly controlled and patients have co-infection with TB.

5.5 CONCLUSION

CD38 expression on CD8+ T cells is a prospective laboratory monitoring tool for longitudinal follow-up of patients responding to 1st or 2nd line ART. In practical terms, the data revealed similar patterns in 2nd line patients as seen with 1st line therapy. In more than 60-80% of patients CD38 MFI in relation to baseline measurements identified patients responding to/or failing therapy (1st and/or 2nd line). The development of algorithms mentioned above to perform CD38 testing coupled with clear indications of when to reflex to HIV VL testing can potentially save millions across the South African National HIV/AIDS treatment programme. It can also be considered as a measurement of wellness and further be used to detect patients not adhering to therapy. This combined PLG CD4/ CD8/38 assay provides both absolute CD4 count as well as the CD38 MFI, using the same sample, preparation, analyses platform (flow

cytometry instruments) and labour already deployed at 69 NHLS laboratories. If national roll-out is adopted, the test cost will be further cut as consumable antibody cost of a single vial 4-color reagent will be less than the current sum of the PLG/CD4 reagent and the two additional purchased antibodies. This data does not advocate abandoning viral load testing, but suggests promoting the CD38 assay as a supplementary test that could reduce the number of VL tests required per patient dramatically, whilst reserving VL testing for patients that show significant aberrant CD38 increased expression from previous and/or baseline visits. The simplicity of both the assay and its quality control measures make it easy for large scale implementation across a network already geared for CD4 testing and the procedures and protocols developed for the XL flow cytometers can easily be implemented on other makes and models of flow cytometers and used on automated preparation systems.

APPENDIX A: Daily Quality Control



FLOW CYTOMETRY-C M JOHANNESBURG HOSPITAL XL5

COULTER(R) EPICS(R) Acquisition Flow Cytometry Report

18Feb09 07:21:45
_QBackground
G0035327
QA QC - GBackGRO,QCC
Stop Time: 100 seconds, 6 events

OP ID: XL5MM

18/2/09 MM

Initial cytoset, from prot. Background

1: Scatter

2:

Stats: Not Normalized, Listgating: Disabled

Hist	Region	ID	Count
1	A1	A	1
	A2	A	4
	A3	A	1
	A4	A	0
	C	LOT#=1099018	0
2	B	QC BGD COUNT	6

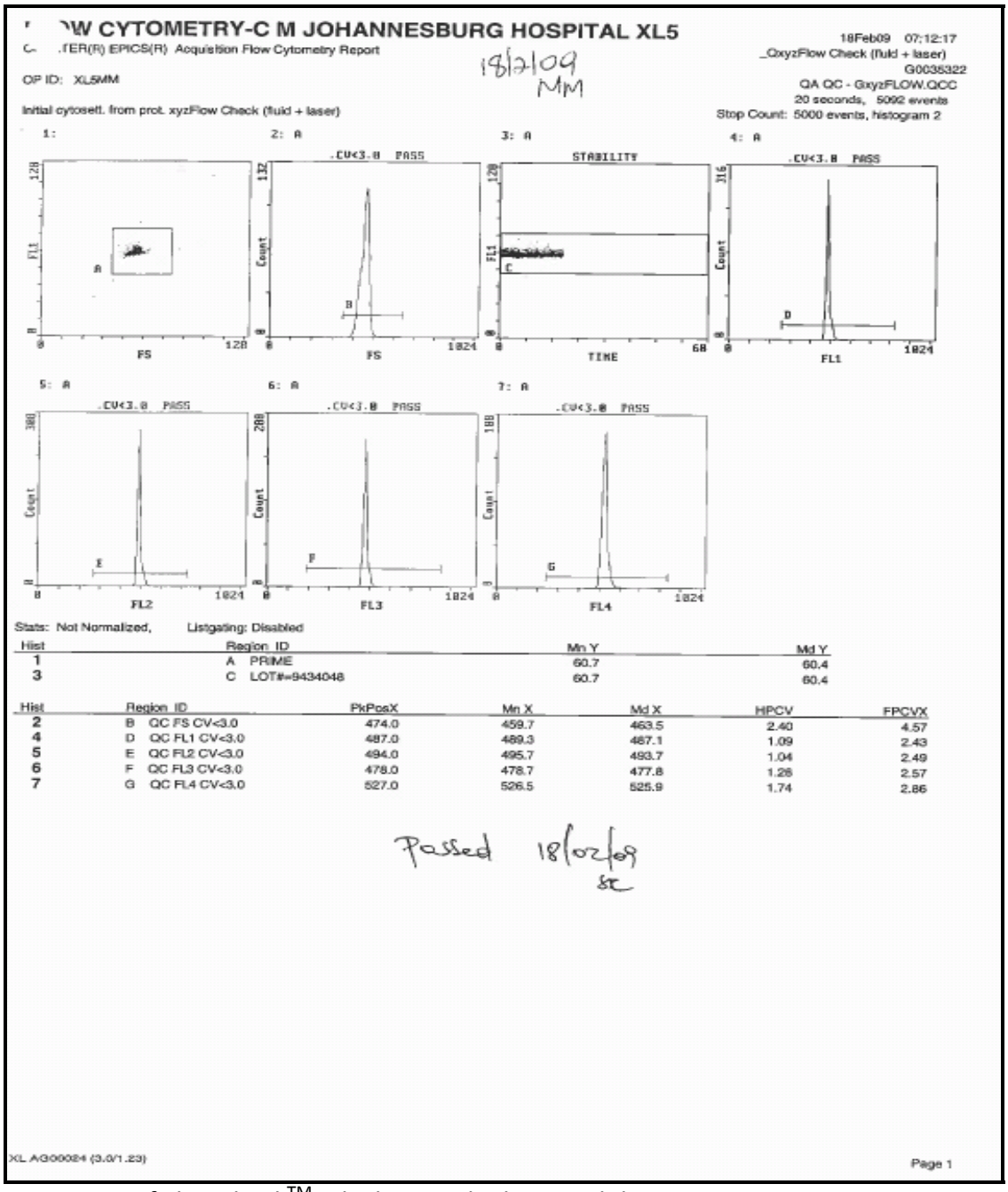
Carryover
6/33332 = 0,00018
<1% Passed

Before
SR

XL AG06024 (3.0/1.23)

Page 1

1. Printout of Carryover test using sheath fluid



W CYTOMETRY-C M JOHANNESBURG HOSPITAL XL5

.TER(R) EPICS(R) Acquistion Flow Cytometry Report

18/2/09 MM

18Feb09 07:12:17
_OxyzFlow Check (fluid + laser)
G0035322
QA QC - GxyzFLOW.QCC
20 seconds, 5092 events
Stop Count: 5000 events, histogram 2

OP ID: XL5MM

Initial cytosetf. from prot. xyzFlow Check (fluid + laser)

1: 2: A .CV<3.0 PASS 3: A STABILITY 4: A .CV<3.0 PASS

5: A .CV<3.0 PASS 6: A .CV<3.0 PASS 7: A .CV<3.0 PASS

Stats: Not Normalized, Listgating: Disabled

Hist	Region	ID	Mn Y	Md Y
1	A	PRIME	60.7	60.4
3	C	LOT#=9434048	60.7	60.4

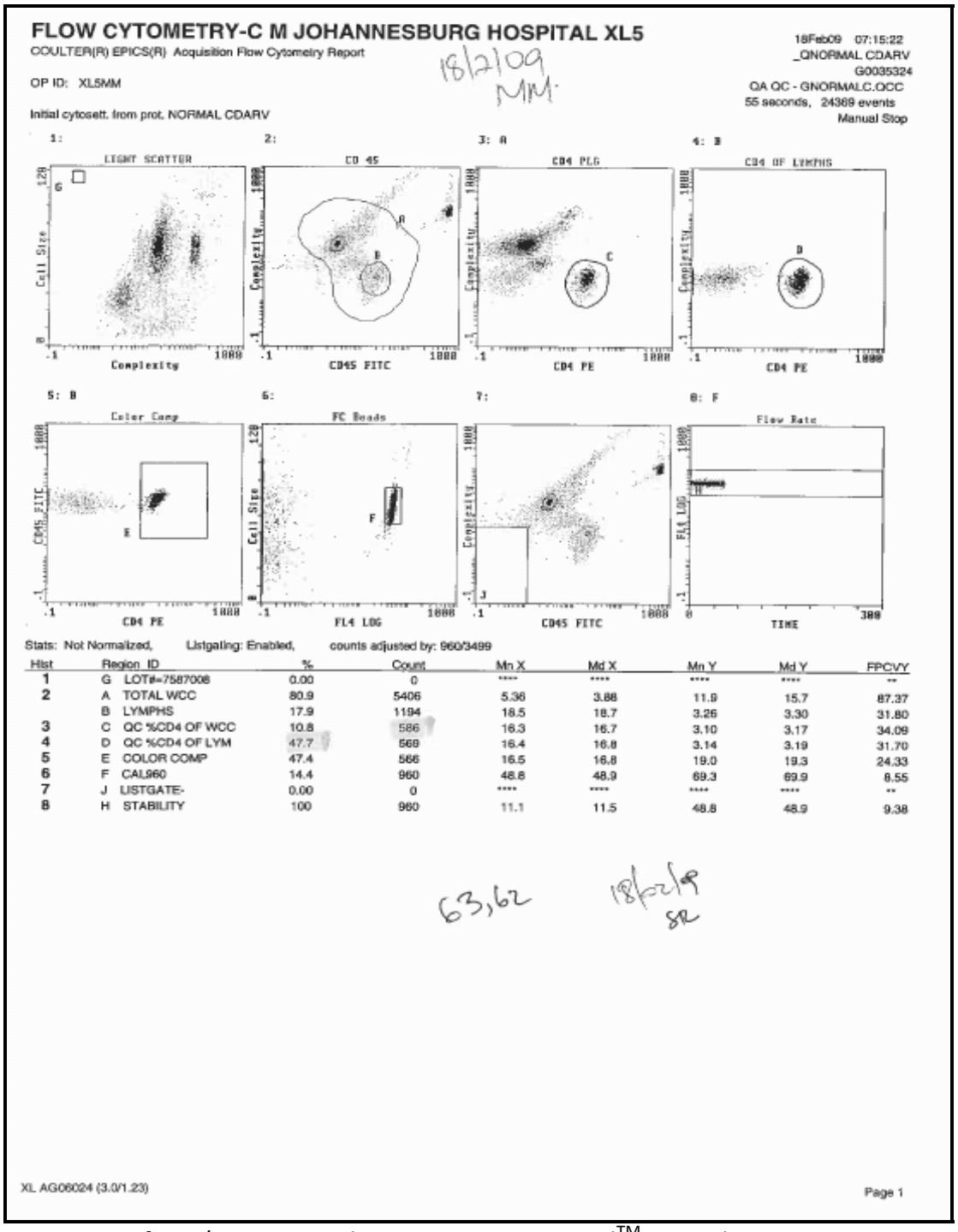
Hist	Region	ID	PkPosX	Mn X	Md X	HPCV	FPCVX
2	B	QC FS CV<3.0	474.0	459.7	463.5	2.40	4.57
4	D	QC FL1 CV<3.0	487.0	489.3	487.1	1.09	2.43
5	E	QC FL2 CV<3.0	494.0	495.7	493.7	1.04	2.49
6	F	QC FL3 CV<3.0	478.0	478.7	477.8	1.28	2.57
7	G	QC FL4 CV<3.0	527.0	526.5	525.9	1.74	2.86

Passed 18/02/09 SE

XL AG00024 (3.0/1.23)

Page 1

2. Printout of FlowCheck™ which tests the laser stability



FLOW CYTOMETRY-C M JOHANNESBURG HOSPITAL XL5

COULTER(R) EPICS(R) Acquisition Flow Cytometry Report

18/2/09 MM.

18Feb09 07:15:22
_QNORMAL CDARV
G0035324
QA QC - GNORMALC.OCC
55 seconds, 24369 events
Manual Stop

OP ID: XL5MM

Initial cytoset, from prot. NORMAL CDARV

Stats: Not Normalized, Listgating: Enabled, counts adjusted by: 960/3499

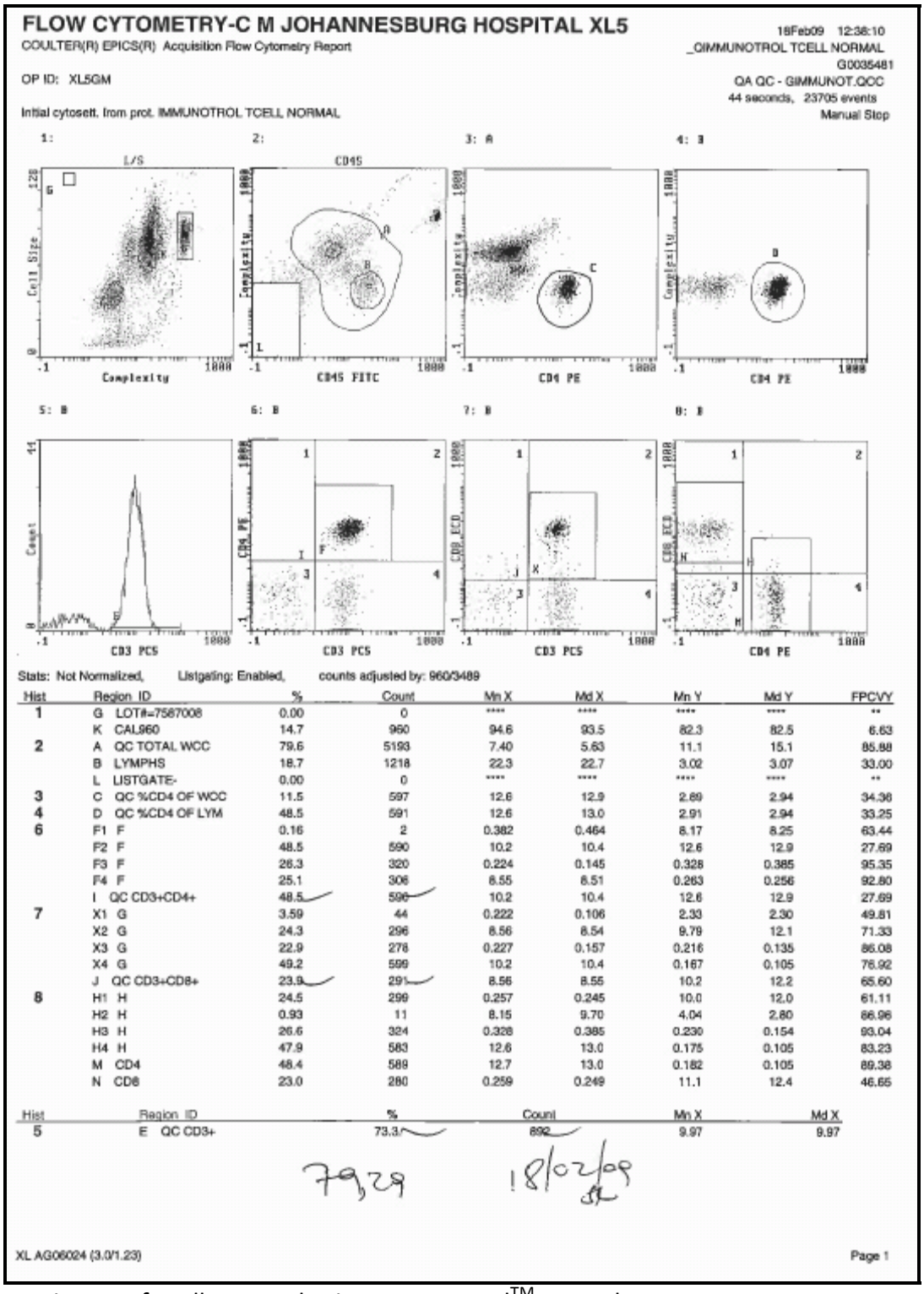
Hist	Region	ID	%	Count	Mn X	Md X	Mn Y	Md Y	FPCVY
1	G	LOT#=7587008	0.00	0	****	****	****	****	**
2	A	TOTAL WCC	80.9	5406	5.36	3.88	11.9	15.7	87.37
	B	LYMPHS	17.9	1194	18.5	18.7	3.26	3.30	31.80
3	C	QC %CD4 OF WCC	10.8	586	16.3	16.7	3.10	3.17	34.09
4	D	QC %CD4 OF LYM	47.7	569	16.4	16.8	3.14	3.19	31.70
5	E	COLOR COMP	47.4	566	16.5	16.8	19.0	19.3	24.33
6	F	CAL960	14.4	960	48.8	48.9	69.3	69.9	8.55
7	J	LISTGATE-	0.00	0	****	****	****	****	**
8	H	STABILITY	100	960	11.1	11.5	48.8	48.9	9.38

63,62 18/02/09 82

XL AG06024 (3.0/1.23)

Page 1

3. Printout of PLG/CD4 protocol run, using ImmunoTrol™ Normal



FLOW CYTOMETRY-C M JOHANNESBURG HOSPITAL XL5

COULTER(R) EPICS(R) Acquisition Flow Cytometry Report

18Feb09 12:38:10
_GIMMUNOTROL TCELL NORMAL
G0035481
QA QC - GIMMUNOT.QOC
44 seconds, 23705 events
Manual Stop

OP ID: XL5GM

Initial cytosett. from prot. IMMUNOTROL TCELL NORMAL

Stats: Not Normalized, Listgating: Enabled, counts adjusted by: 960/3489

Hist	Region ID	%	Count	Mn X	Md X	Mn Y	Md Y	FPCVY
1	G LOT#=7587008	0.00	0	****	****	****	****	**
	K CAL960	14.7	960	94.6	93.5	82.3	82.5	6.63
2	A QC TOTAL WCC	79.6	5193	7.40	5.63	11.1	15.1	85.88
	B LYMPHS	18.7	1218	22.3	22.7	3.02	3.07	33.00
	L LISTGATE-	0.00	0	****	****	****	****	**
3	C QC %CD4 OF WCC	11.5	597	12.6	12.9	2.89	2.94	34.36
4	D QC %CD4 OF LYM	48.5	591	12.6	13.0	2.91	2.94	33.25
6	F1 F	0.16	2	0.382	0.464	8.17	8.25	63.44
	F2 F	48.5	590	10.2	10.4	12.6	12.9	27.69
	F3 F	26.3	320	0.224	0.145	0.328	0.385	95.35
	F4 F	25.1	306	8.55	8.51	0.263	0.256	92.80
	I QC CD3+CD4+	48.5	590	10.2	10.4	12.6	12.9	27.69
7	X1 G	3.59	44	0.222	0.106	2.33	2.30	49.81
	X2 G	24.3	296	8.56	8.54	9.79	12.1	71.33
	X3 G	22.9	278	0.227	0.157	0.216	0.135	86.08
	X4 G	49.2	599	10.2	10.4	0.167	0.105	76.92
	J QC CD3+CD8+	23.9	291	8.56	8.55	10.2	12.2	65.60
8	H1 H	24.5	299	0.257	0.245	10.0	12.0	61.11
	H2 H	0.93	11	8.15	9.70	4.04	2.80	86.96
	H3 H	26.6	324	0.328	0.385	0.230	0.154	93.04
	H4 H	47.9	583	12.6	13.0	0.175	0.105	83.23
	M CD4	48.4	589	12.7	13.0	0.182	0.105	89.38
	N CD8	23.0	280	0.259	0.249	11.1	12.4	46.65

Hist	Region ID	%	Count	Mn X	Md X
5	E QC CD3+	73.3	892	9.97	9.97

79,29 18/02/09

XL AG06024 (3.0/1.23)

Page 1

4. Printout of Tcell protocol using ImmunoTrol™ Normal

FLOW CYTOMETRY-C M JOHANNESBURG HOSPITAL XL5

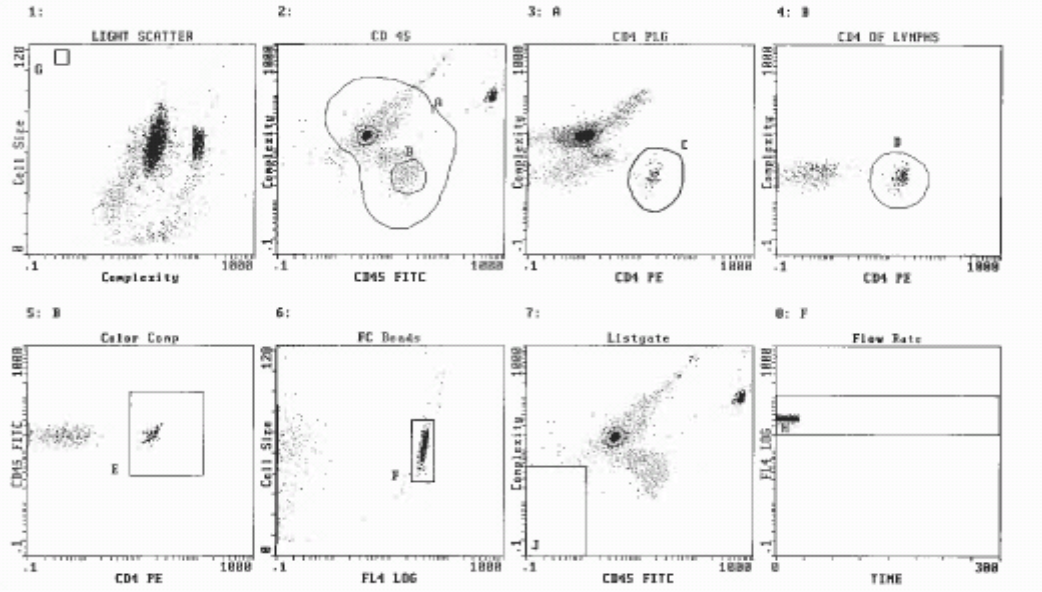
COULTER(R) EPICS(R) Acquisition Flow Cytometry Report

18Feb09 07:13:54
_QCDARV LOW IMMUNOTROL
G0035323
QA QC - GCDARVLO.QCC
32 seconds, 13790 events
Manual Stop

18/2/09 MM.

OP ID: XL5MM

Initial cytosett. from prot. CDARV LOW IMMUNOTROL



Stats: Not Normalized, Listgating: Enabled, counts adjusted by: 960/2003

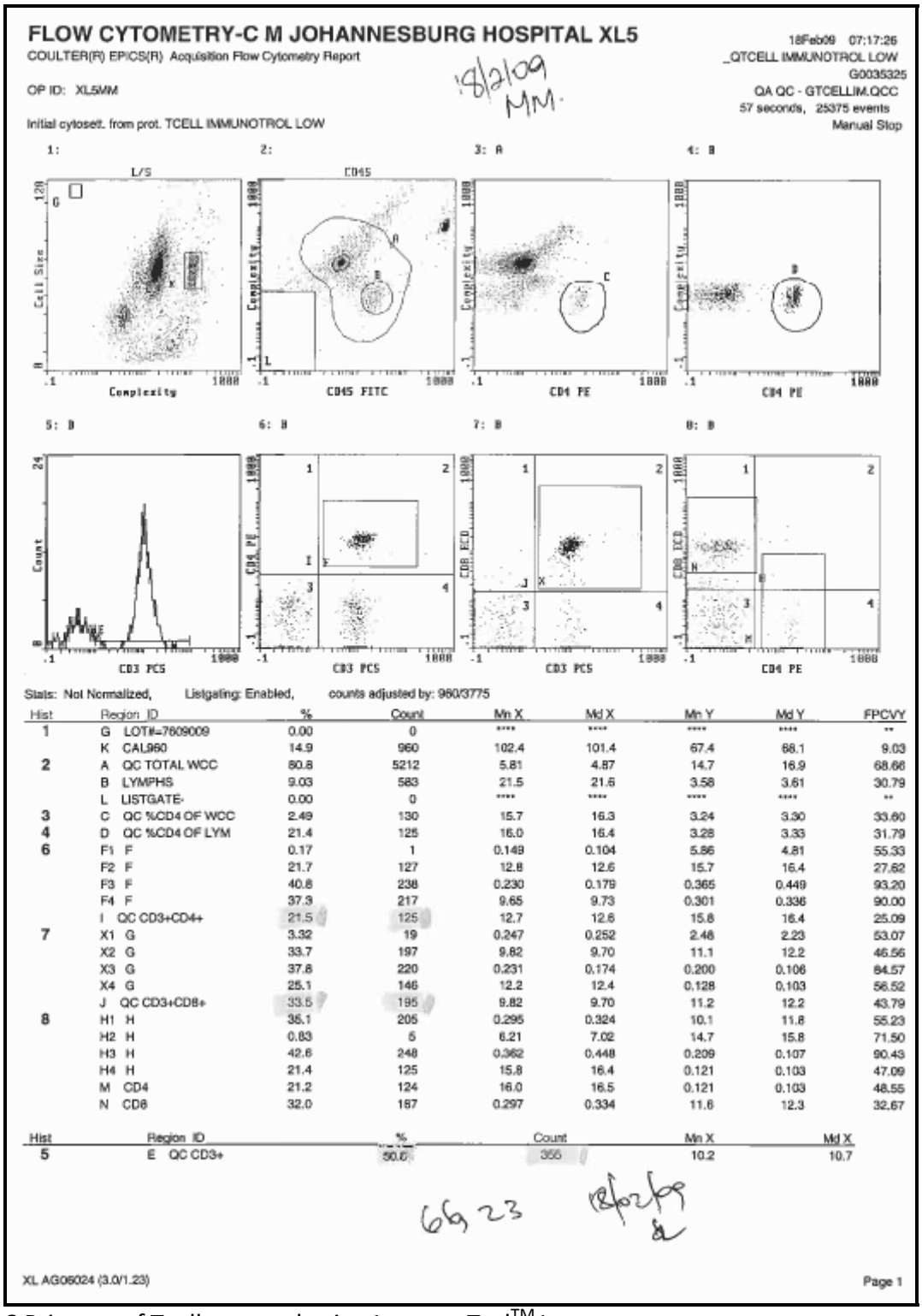
Hist	Region	ID	%	Count	Mn X	Md X	Mn Y	Md Y	FPCVY
1	G	LOT#=7609009	0.00	0	****	****	****	****	**
2	A	TOTAL WCC	82.2	5434	5.22	4.27	14.6	16.5	72.71
	B	LYMPHS	8.94	591	19.4	19.4	3.36	3.40	30.64
3	C	QC %CD4 OF WCC	2.41	131	16.0	16.5	3.01	3.05	35.24
4	D	QC %CD4 OF LYM	21.1	125	16.2	16.6	3.03	3.07	32.11
5	E	COLOR COMP	21.1	125	16.2	16.6	19.1	19.1	21.43
6	F	CAL960	14.5	960	39.2	39.3	65.8	66.7	9.63
7	J	LISTGATE-	0.00	0	****	****	****	****	**
8	H	FLOW RATE	100	960	6.2	6.2	39.2	39.3	10.00

62,59 18/02/09 SR

XL AG06024 (3.0/1.23)

Page 1

5. Printout of PLG/CD4 protocol using ImmunoTrol™ Low



FLOW CYTOMETRY-C M JOHANNESBURG HOSPITAL XL5

COULTER(R) EPICS(R) Acquisition Flow Cytometry Report

18Feb09 07:17:26
_QTCELL IMMUNOTROL LOW
G0035325
QA QC - GTCELLIM.QCC
57 seconds, 25375 events
Manual Stop

18/2/09 MM.

OP ID: XL5MM

Initial cytoset. from prot. TCELL IMMUNOTROL LOW

1: L/S — Cell Size vs Complexity
2: CD45 — Complexity vs CD45 FITC
3: A — Complexity vs CD4 PE
4: B — Complexity vs CD4 PE
5: B — Count vs CD3 PC5
6: B — CD4 PE vs CD3 PC5
7: B — CD8 ECD vs CD3 PC5
8: B — CD8 ECD vs CD4 PE

Stats: Not Normalized, Listgating: Enabled, counts adjusted by: 960/3775

Hist	Region	ID	%	Count	Mn X	Md X	Mn Y	Md Y	FPCVY
1	G	LOT#=7609009	0.00	0	****	****	****	****	**
	K	CAL960	14.9	960	102.4	101.4	67.4	68.1	9.03
2	A	QC TOTAL WCC	80.8	5212	5.81	4.87	14.7	16.9	68.66
	B	LYMPHS	9.03	583	21.5	21.6	3.58	3.61	30.79
	L	LISTGATE-	0.00	0	****	****	****	****	**
3	C	QC %CD4 OF WCC	2.49	130	15.7	16.3	3.24	3.30	33.60
4	D	QC %CD4 OF LYM	21.4	125	16.0	16.4	3.28	3.33	31.79
6	F1	F	0.17	1	0.149	0.104	5.86	4.81	55.33
	F2	F	21.7	127	12.8	12.6	15.7	16.4	27.62
	F3	F	40.8	238	0.230	0.179	0.365	0.449	93.20
	F4	F	37.3	217	9.65	9.73	0.301	0.336	90.00
	I	QC CD3+CD4+	21.5	125	12.7	12.6	15.8	16.4	25.09
7	X1	G	3.32	19	0.247	0.252	2.48	2.23	53.07
	X2	G	33.7	197	9.82	9.70	11.1	12.2	46.56
	X3	G	37.8	220	0.231	0.174	0.200	0.106	84.57
	X4	G	25.1	146	12.2	12.4	0.128	0.103	56.52
	J	QC CD3+CD8+	33.5	195	9.82	9.70	11.2	12.2	43.79
8	H1	H	35.1	205	0.295	0.324	10.1	11.8	55.23
	H2	H	0.83	5	6.21	7.02	14.7	15.8	71.50
	H3	H	42.6	248	0.362	0.448	0.209	0.107	90.43
	H4	H	21.4	125	15.8	16.4	0.121	0.103	47.09
	M	CD4	21.2	124	16.0	16.5	0.121	0.103	48.55
	N	CD8	32.0	187	0.297	0.334	11.6	12.3	32.67

Hist	Region	ID	%	Count	Mn X	Md X
5	E	QC CD3+	90.0	355	10.2	10.7

66 23 18/02/09

XL AG06024 (3.0/1.23)

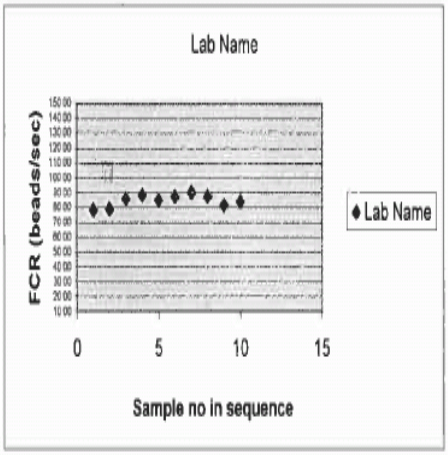
Page 1

6.Printout of Tcell protocol using ImmunoTrol™ Low

Department of Molecular Medicine and Haematology

STARTUP BEAD REPRODUCIBILITY

No in seq	Seconds	Bead count	Beads/sec
1	60.00	4699.00	78.32
2	60.00	4736.00	78.93
3	60.00	5148.00	85.80
4	60.00	5306.00	88.43
5	60.00	5104.00	85.07
6	60.00	5236.00	87.27
7	60.00	5415.00	90.25
8	60.00	5248.00	87.47
9	60.00	4892.00	81.53
10	60.00	5034.00	83.90
Average			**84.70**
STDEV			**4.01**
%CV			**4.74**
XL 5	18-Feb	2009	



Optimal %CV <3.0% but Acceptable <5.0%

Signed SR

Date 18-02-09

7. Spreadsheet of the Start-up Bead Reproducibility used to test stability of flow count rate on the instrument

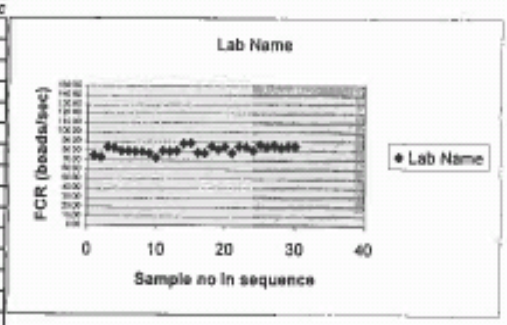
Department of Molecular Medicine and Haematology

Verified and signed SL

Date 18-02-2009

FIRST CAROUSEL AFTER OPENING UP XL'S

No in seq	Seconds	Bead count	Beads/sec
1	54.00	4038.00	74.78
2	76.00	5549.00	73.01
3	36.00	3018.00	83.83
4	51.00	4228.00	82.90
5	20.00	1598.00	79.90
6	29.00	2316.00	79.86
7	59.00	4678.00	79.29
8	52.00	4106.00	78.96
9	33.00	2548.00	77.21
10	29.00	2105.00	72.59
11	300.00	23962.00	79.87
12	23.00	1823.00	79.26
13	39.00	3107.00	79.67
14	61.00	5339.00	87.52
15	26.00	2295.00	88.27
16	198.00	15452.00	78.04
17	32.00	2463.00	77.09
18	90.00	7625.00	84.72
19	42.00	3418.00	81.38
20	65.00	5464.00	84.06
21	28.00	2174.00	77.64
22	110.00	9280.00	84.36
23	42.00	3516.00	83.71
24	19.00	1528.00	80.42
25	45.00	3893.00	86.51
26	32.00	2680.00	83.75
27	34.00	2919.00	85.85
28	29.00	2408.00	83.03
29	32.00	2702.00	84.44
30	55.00	4656.00	84.65
31			
32			
AVERAGE			81.46
STDEV			4.04
CV%			4.96
+2STDEV			89.54
-2STDEV			73.38
2	32.00	2123.00	66.34



xl5

Acceptable results

%CV <5.0

18-Feb 2009

Comment.....

Passed

Signature.....

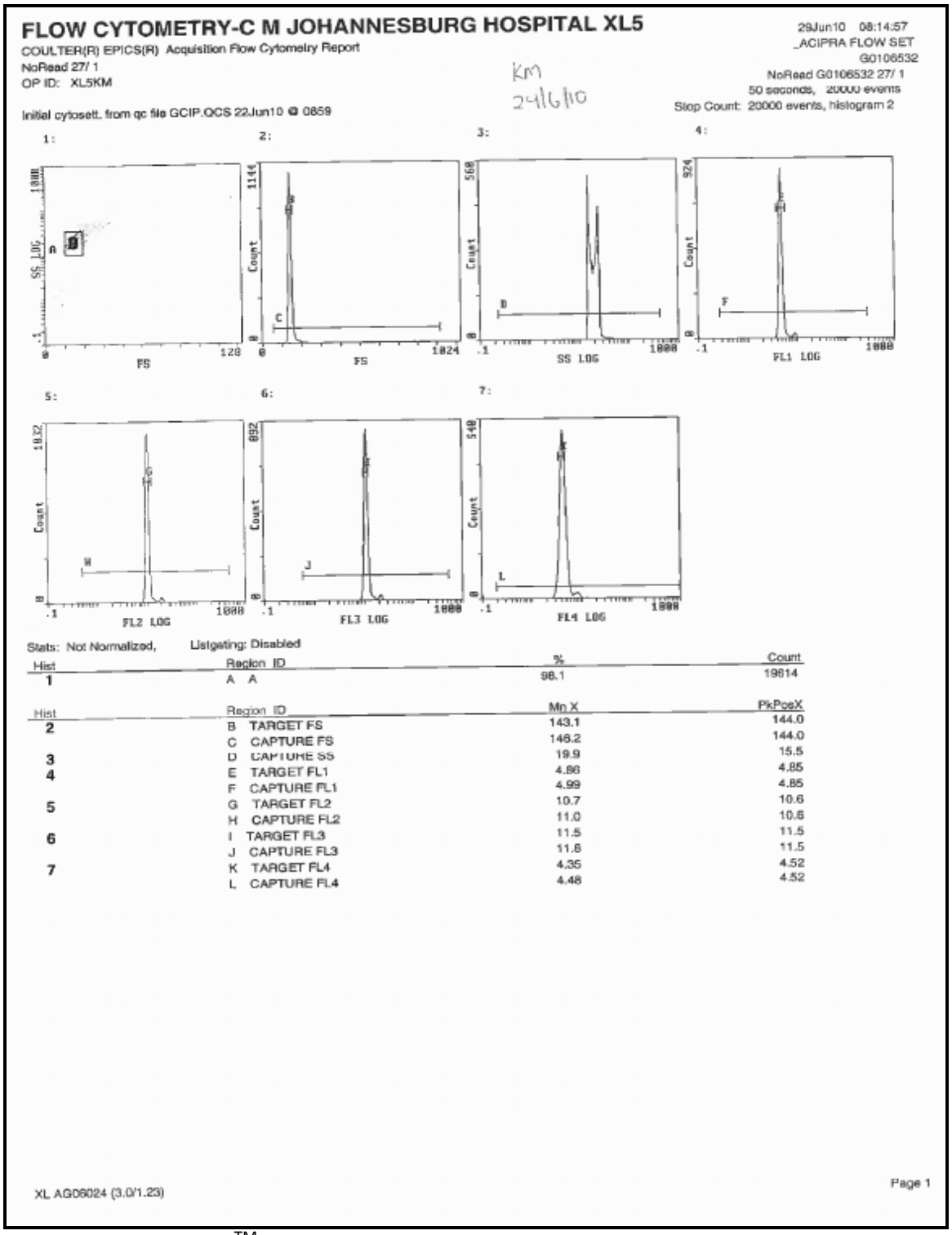
Date.....

FOR WCC

FOR WCC

8.Flow count rate for the first carousel run on the flow cytometer after daily QC was done

APPENDIX B: Weekly Quality Control



FLOW CYTOMETRY-C M JOHANNESBURG HOSPITAL XL5

COULTER(R) EPICS(R) Acquisition Flow Cytometry Report
NoRead 27/ 1
OP ID: XL5KM

29Jun10 08:14:57
_ACIPRA FLOW SET
G0106532
NoRead G0106532 27/ 1
50 seconds, 20000 events
Stop Count: 20000 events, histogram 2

KM
24/6/10

Initial cytoset. from qc file GCIP.QCS 22Jun10 @ 0859

1: 2: 3: 4:

5: 6: 7:

Stats: Not Normalized, Listgating: Disabled

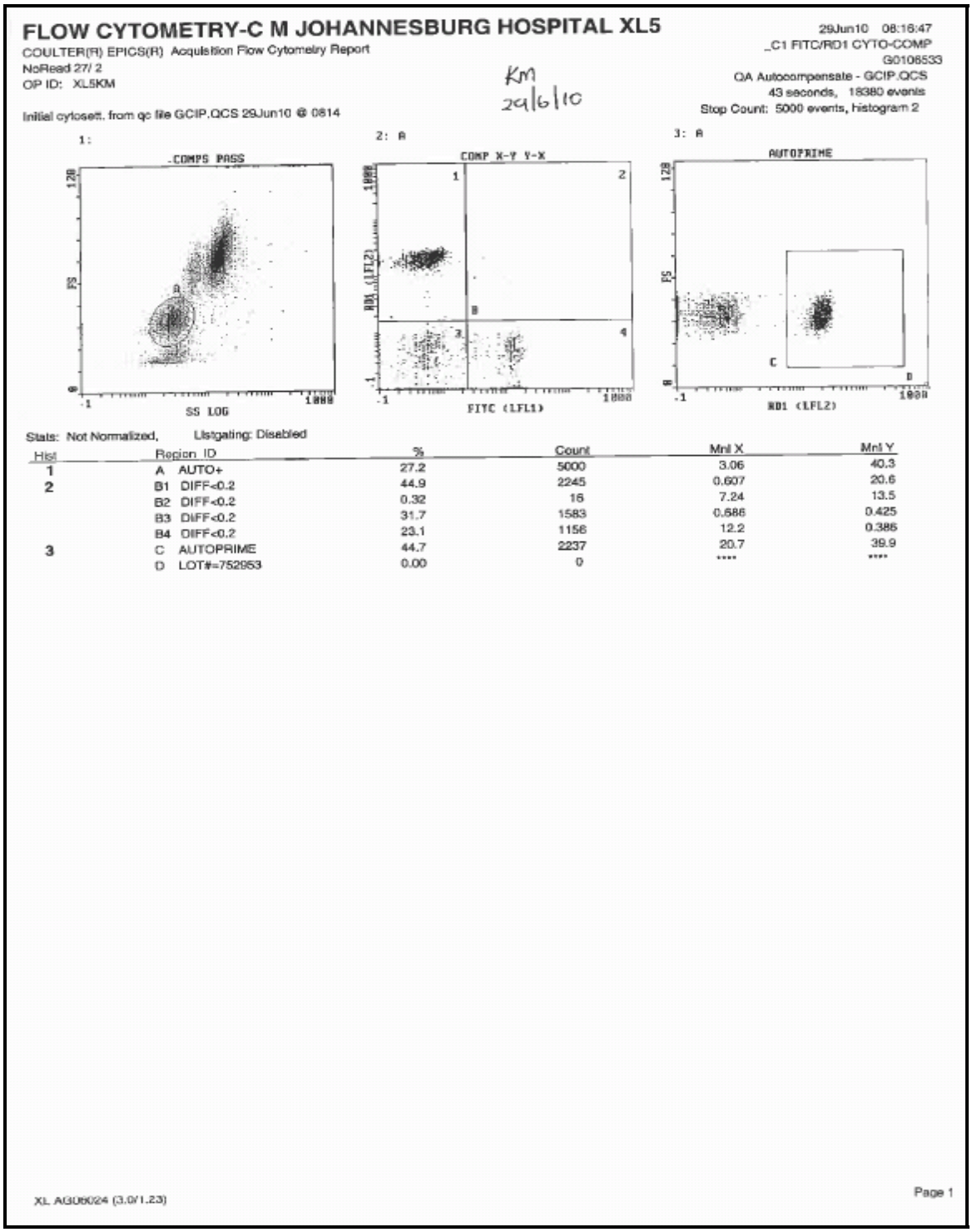
Hist	Region ID	%	Count
1	A A	98.1	19614

Hist	Region ID	Mn X	PkPosX
2	B TARGET FS	143.1	144.0
	C CAPTURE FS	146.2	144.0
3	D CAPTURE SS	19.9	15.5
4	E TARGET FL1	4.86	4.85
	F CAPTURE FL1	4.99	4.85
5	G TARGET FL2	10.7	10.6
	H CAPTURE FL2	11.0	10.6
6	I TARGET FL3	11.5	11.5
	J CAPTURE FL3	11.8	11.5
7	K TARGET FL4	4.35	4.52
	L CAPTURE FL4	4.48	4.52

XL AG06024 (3.0/1.23)

Page 1

1. Printout of FlowSet™ results



FLOW CYTOMETRY-C M JOHANNESBURG HOSPITAL XL5

COULTER(R) EPICS(R) Acquisition Flow Cytometry Report
NoRead 27/ 2
OP ID: XL5KM

29Jun10 08:16:47
_C1 FITC/RD1 CYTO-COMP
G0106533
QA Autocompensate - GCIP.QCS
43 seconds, 18380 events
Stop Count: 5000 events, histogram 2

KM
29/6/10

Initial cytoset. from qc file GCIP.QCS 29Jun10 @ 0814

1: .COMPS PASS — FS vs SS LOG
2: A COMP X-Y Y-X — RD1 (LFL2) vs FITC (LFL1)
3: A AUTOPRIME — FS vs RD1 (LFL2)

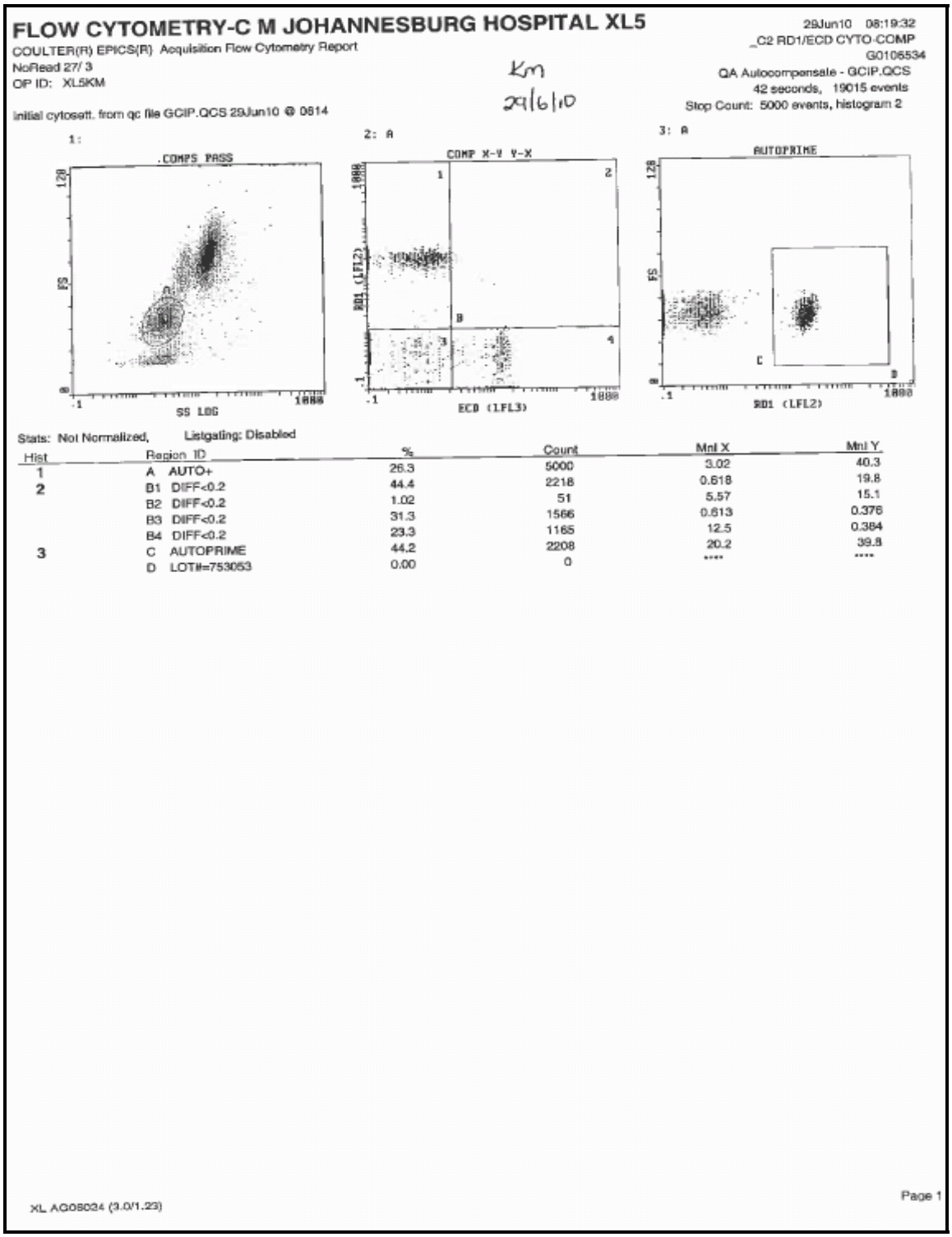
Stats: Not Normalized, Ustgating: Disabled

Hist	Region	ID	%	Count	Mnl X	Mnl Y
1	A	AUTO+	27.2	5000	3.06	40.3
2	B1	DIFF<0.2	44.9	2245	0.607	20.6
	B2	DIFF<0.2	0.32	16	7.24	13.5
	B3	DIFF<0.2	31.7	1583	0.686	0.425
	B4	DIFF<0.2	23.1	1156	12.2	0.386
3	C	AUTOPRIME	44.7	2237	20.7	39.9
	D	LOT#=752953	0.00	0	****	****

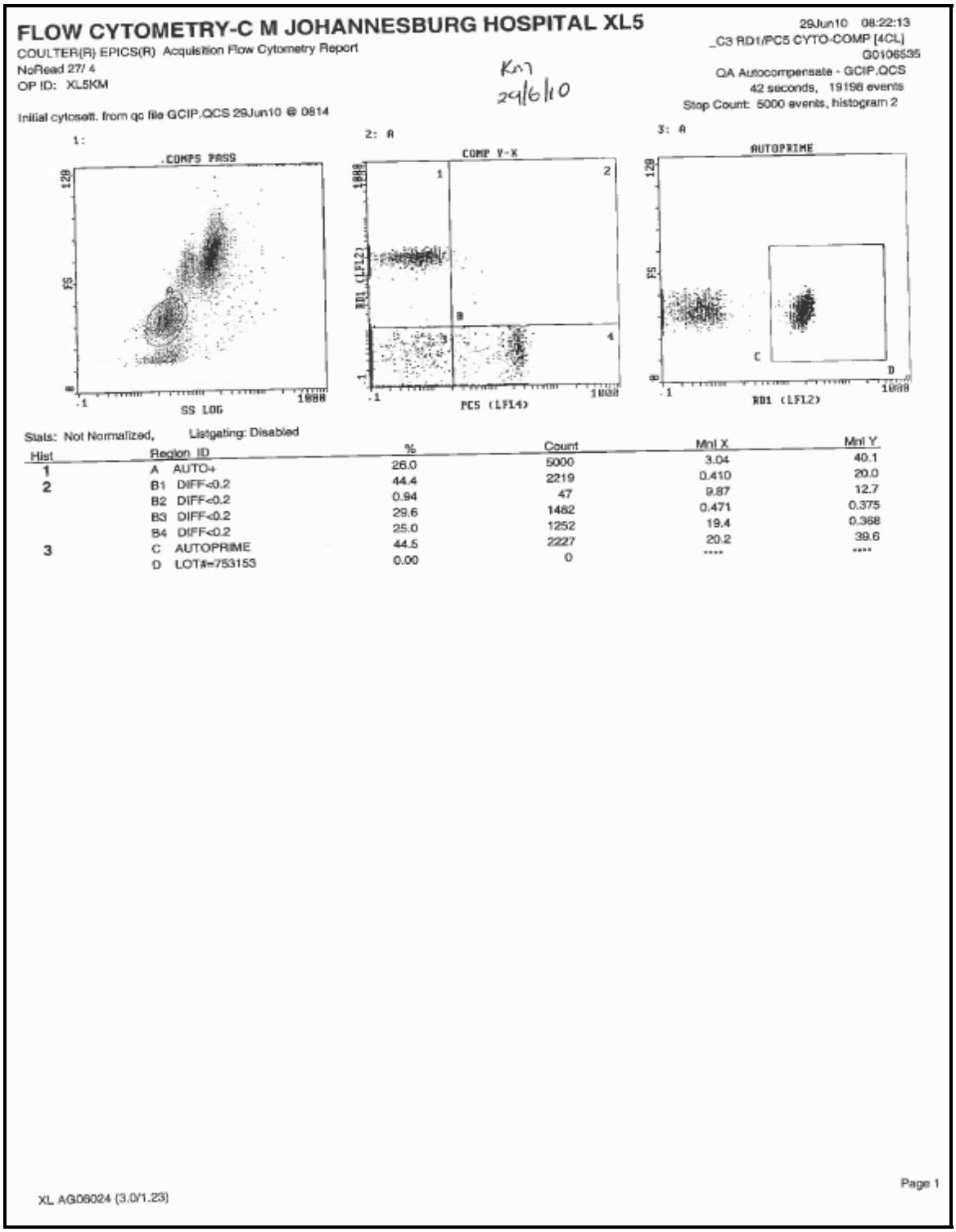
XL AG06024 (3.0/1.23)

Page 1

2. Colour compensation 1 between Abs FITC and RD1(PE)



3. Colour compensation 2 between Abs RD1 and EC D



FLOW CYTOMETRY-C M JOHANNESBURG HOSPITAL XL5

COULTER(R) EPICS(R) Acquisition Flow Cytometry Report
NoRead 27/ 4
OP ID: XL5KM

29Jun10 08:22:13
_C3 RD1/PC5 CYTO-COMP [4CL]
G0106535
QA Autocompensate - GCIP.QCS
42 seconds, 19198 events
Stop Count: 5000 events, histogram 2

Km
29/6/10

Initial cytosett. from qc file GCIP.QCS 28Jun10 @ 0814

1: .COMPS PASS — FS vs SS LOG
2: A — COMP Y-X — RD1 (LFL2) vs PC5 (LFL4)
3: A — AUTOPRIME — FS vs RD1 (LFL2)

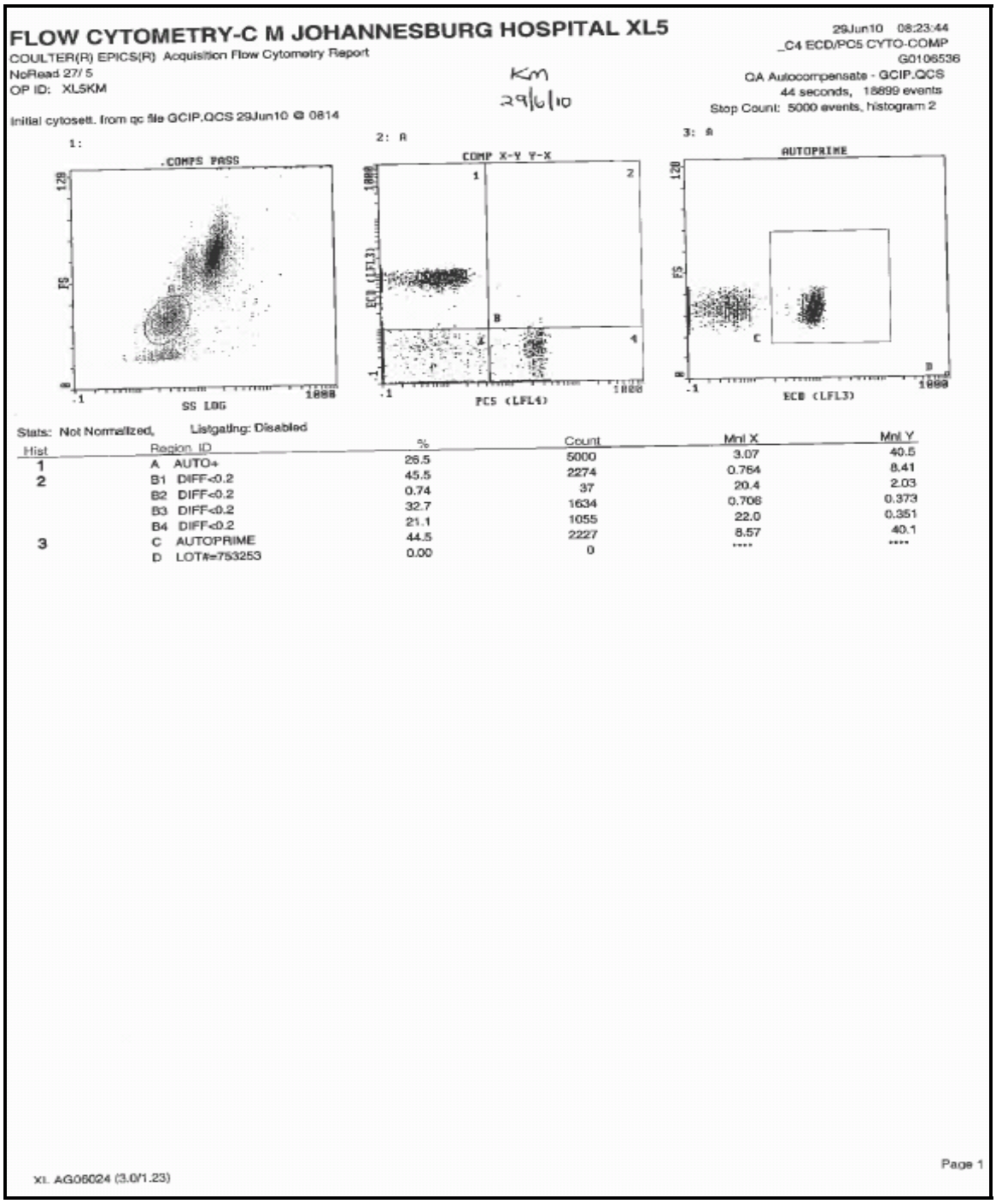
Stats: Not Normalized, Listgating: Disabled

Hist	Region	ID	%	Count	Mnl X	Mnl Y
1	A	AUTO+	26.0	5000	3.04	40.1
2	B1	DIFF<0.2	44.4	2219	0.410	20.0
	B2	DIFF<0.2	0.94	47	9.87	12.7
	B3	DIFF<0.2	29.6	1482	0.471	0.375
	B4	DIFF<0.2	25.0	1252	19.4	0.368
3	C	AUTOPRIME	44.5	2227	20.2	39.6
	D	LOT#=753153	0.00	0	****	****

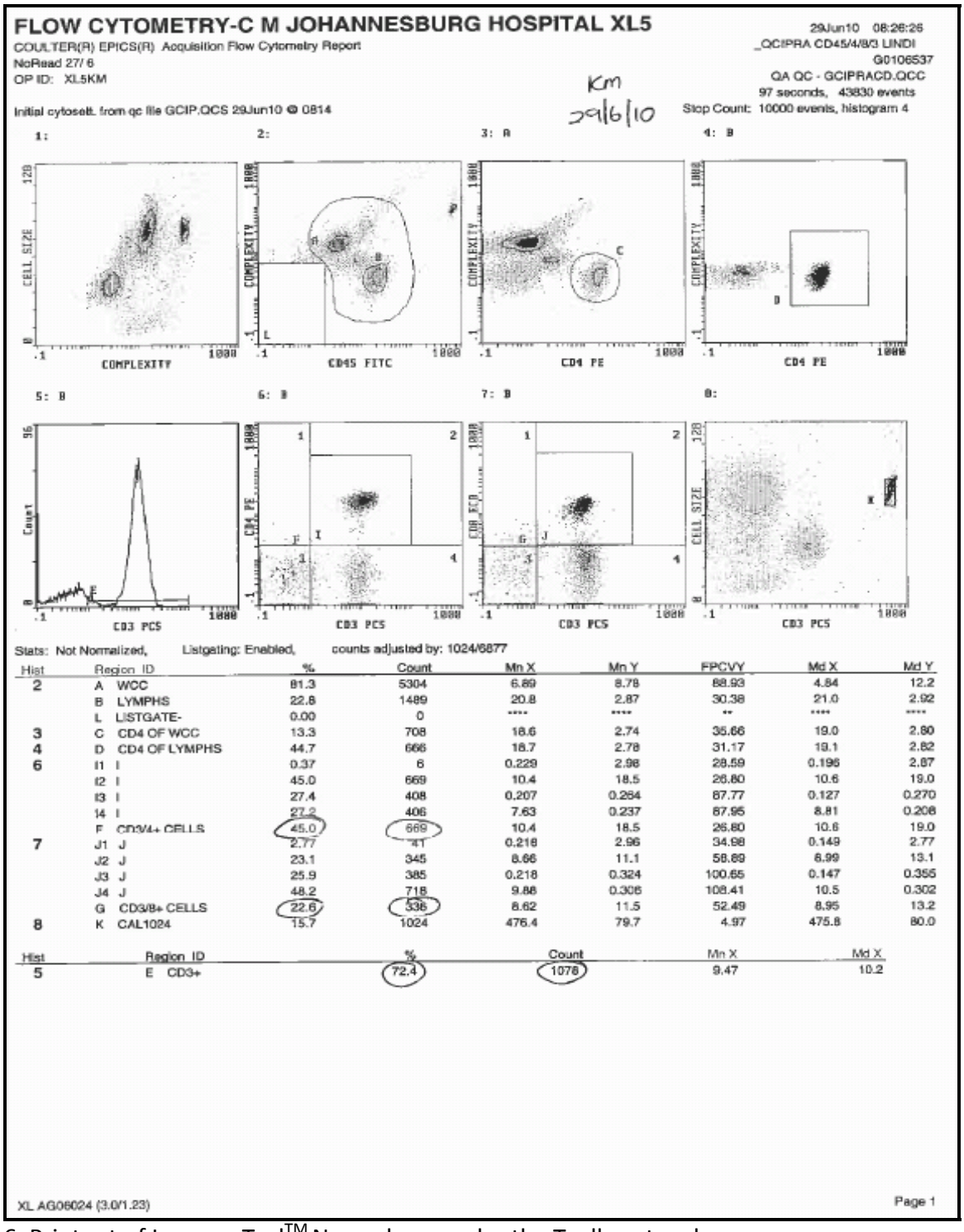
XL AG08024 (3.0/1.23)

Page 1

4. Colour compensation 3 between Abs RD1 and PC5



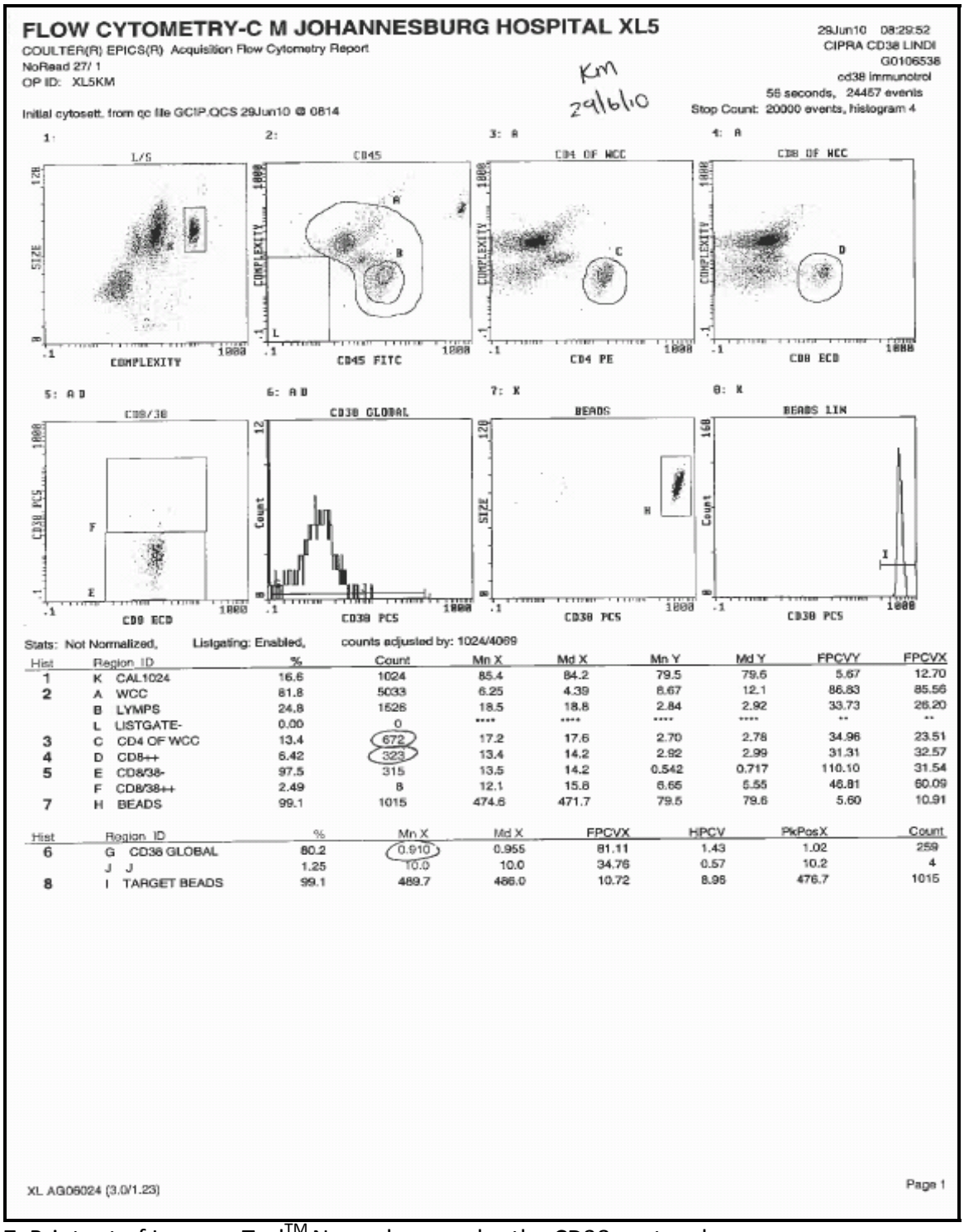
5. Colour compensation 4 between Abs ECD and PC5



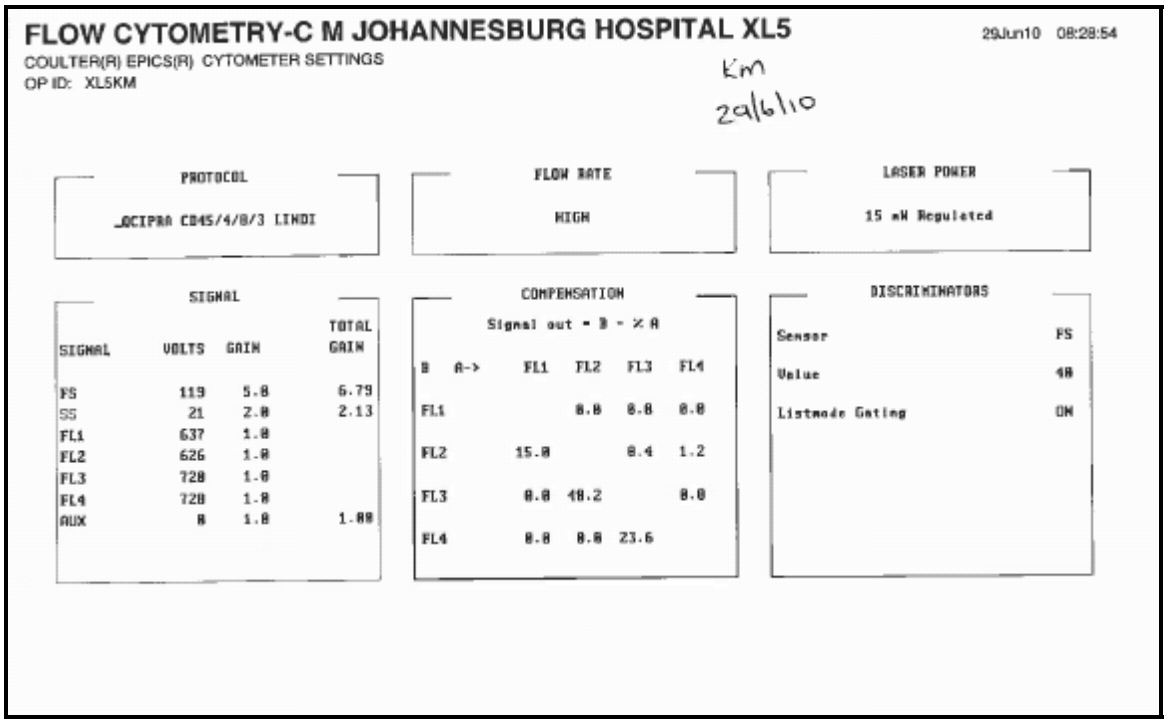
Hist	Region ID		%	Count	Mn X	Mn Y	FPCVY	Md X	Md Y
2	A	WCC	81.3	5304	6.89	8.78	88.93	4.84	12.2
	B	LYMPHS	22.8	1489	20.8	2.87	30.38	21.0	2.92
	L	LISTGATE-	0.00	0	****	****	**	****	****
3	C	CD4 OF WCC	13.3	708	18.6	2.74	35.66	19.0	2.80
4	D	CD4 OF LYMPHS	44.7	666	18.7	2.78	31.17	19.1	2.82
6	I1	I	0.37	6	0.229	2.98	28.59	0.196	2.87
	I2	I	45.0	669	10.4	18.5	26.80	10.6	19.0
	I3	I	27.4	408	0.207	0.264	87.77	0.127	0.270
	I4	I	27.2	406	7.63	0.237	87.95	8.81	0.206
	F	CD3/4+ CELLS	45.0	669	10.4	18.5	26.80	10.6	19.0
7	J1	J	2.77	41	0.218	2.96	34.98	0.149	2.77
	J2	J	23.1	345	8.66	11.1	58.89	8.99	13.1
	J3	J	25.9	385	0.218	0.324	100.65	0.147	0.355
	J4	J	48.2	718	9.88	0.306	108.41	10.5	0.302
	G	CD3/8+ CELLS	22.6	336	8.62	11.5	52.49	8.95	13.2
8	K	CAL1024	15.7	1024	476.4	79.7	4.97	475.8	80.0

Hist	Region ID		%	Count	Mn X	Md X
5	E	CD3+	72.4	1078	9.47	10.2

6. Printout of ImmunoTrol™ Normal run under the Tcell protocol



7. Printout of ImmunoTrol™ Normal run under the CD38 protocol



FLOW CYTOMETRY-C M JOHANNESBURG HOSPITAL XL5

29Jun10 08:28:54

COULTER(R) EPICS(R) CYTOMETER SETTINGS

OP ID: XL5KM

Km 29/6/10

PROTOCOL

_OCIPBA CD45/4/8/3 LINDI

FLOW RATE

HIGH

LASER POWER

15 mW Regulated

SIGNAL

SIGNAL	VOLTS	GAIN	TOTAL GAIN
FS	119	5.8	6.79
SS	21	2.8	2.13
FL1	637	1.8	
FL2	626	1.8	
FL3	728	1.8	
FL4	728	1.8	
AUX	8	1.8	1.88

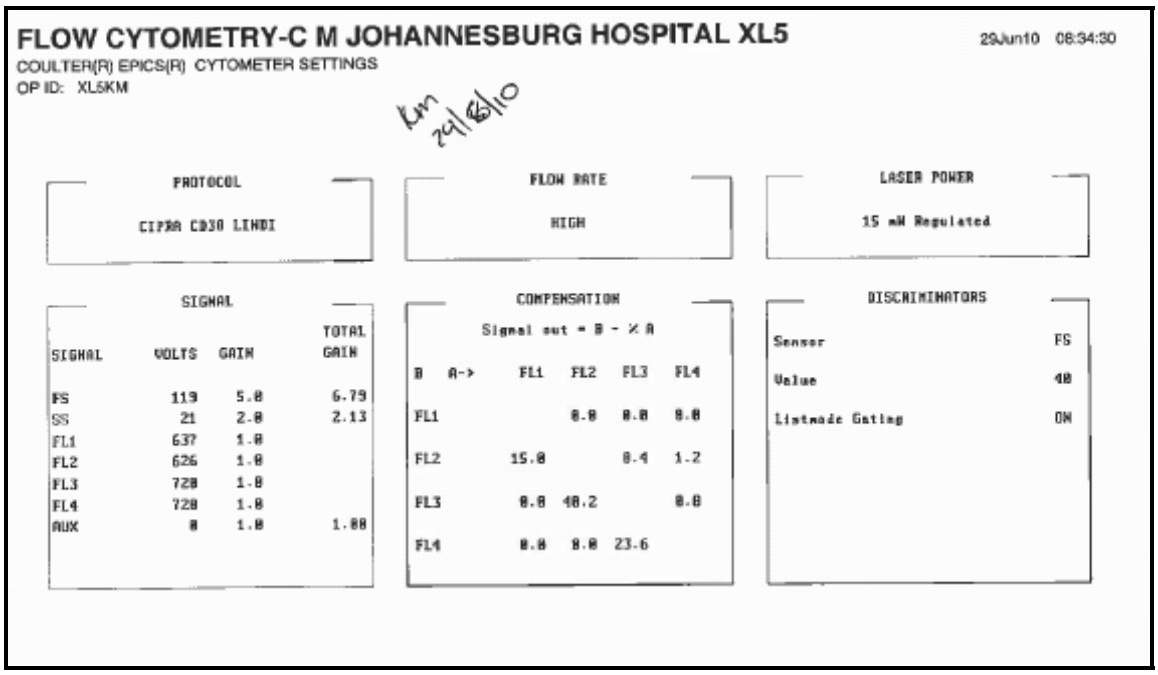
COMPENSATION

Signal out = B - % A

B A->	FL1	FL2	FL3	FL4
FL1		8.8	8.8	8.8
FL2	15.8		8.4	1.2
FL3	8.8	48.2		8.8
FL4	8.8	8.8	23.6	

DISCRIMINATORS

Sensor	FS
Value	48
Listmode Gating	ON



FLOW CYTOMETRY-C M JOHANNESBURG HOSPITAL XL5

29Jun10 08:34:30

COULTER(R) EPICS(R) CYTOMETER SETTINGS

OP ID: XL5KM

Km 29/6/10

PROTOCOL

CIPBA CD38 LINDI

FLOW RATE

HIGH

LASER POWER

15 mW Regulated

SIGNAL

SIGNAL	VOLTS	GAIN	TOTAL GAIN
FS	119	5.8	6.79
SS	21	2.8	2.13
FL1	637	1.8	
FL2	626	1.8	
FL3	728	1.8	
FL4	728	1.8	
AUX	8	1.8	1.88

COMPENSATION

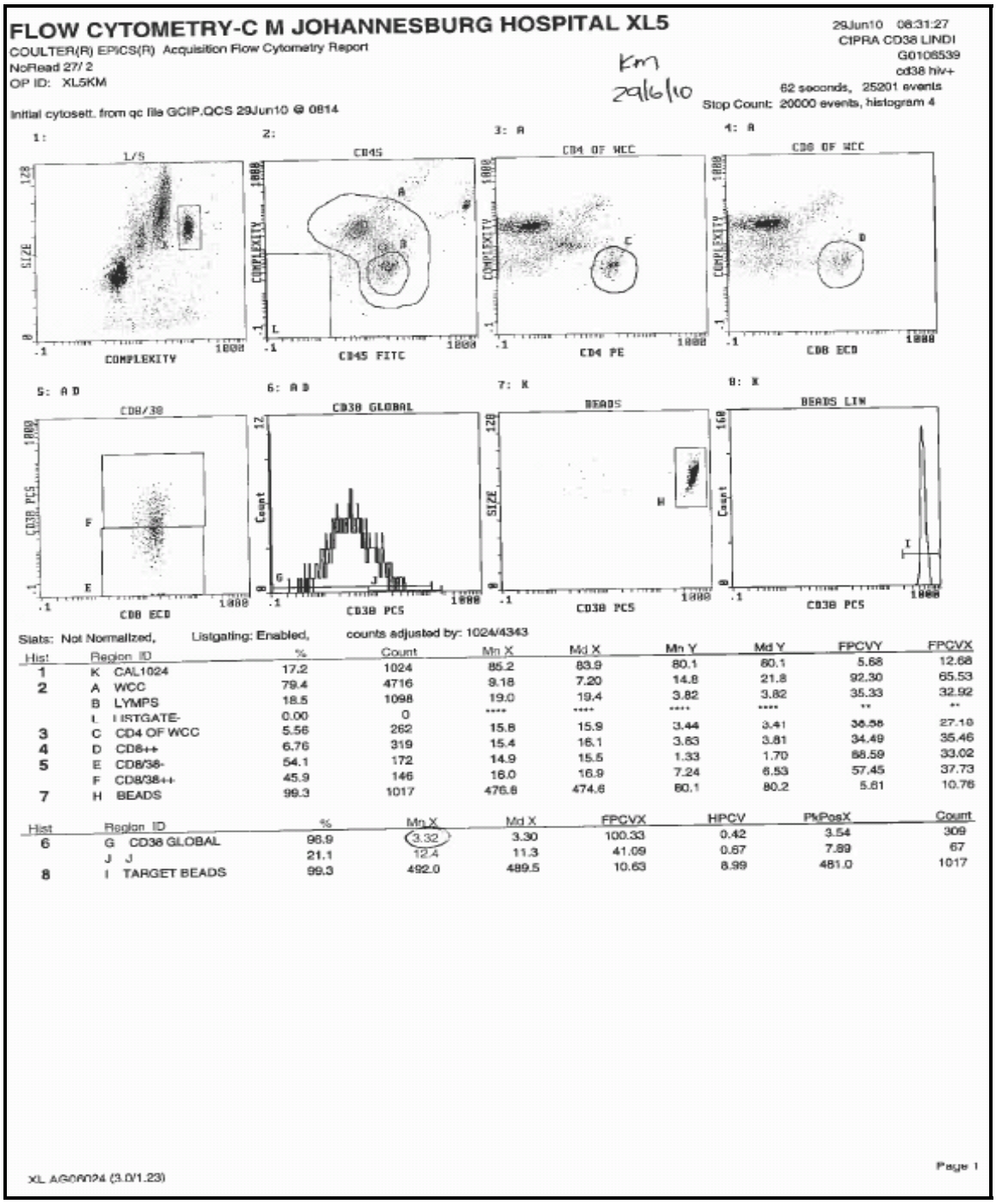
Signal out = B - % A

B A->	FL1	FL2	FL3	FL4
FL1		8.8	8.8	8.8
FL2	15.8		8.4	1.2
FL3	8.8	48.2		8.8
FL4	8.8	8.8	23.6	

DISCRIMINATORS

Sensor	FS
Value	48
Listmode Gating	ON

8. Cytosettings from the Tcell protocol (above) and from the CD38 protocol (below) (same settings are used for both protocols).



FLOW CYTOMETRY-C M JOHANNESBURG HOSPITAL XL5

29Jun10 08:31:27
CIPRA CD38 LINDI
G0108539
cd38 hiv+
62 seconds, 25201 events
Stop Count: 20000 events, histogram 4

COULTER(R) EPICS(R) Acquisition Flow Cytometry Report
NoRead 27/ 2
OP ID: XL5KM

Km
29/6/10

Initial cytosett. from qc file GCIP.QCS 29Jun10 @ 0814

1: L/S — COMPLEXITY / SIZE
2: CD45 — CD45 FITC / COMPLEXITY
3: A — CD4 PE / COMPLEXITY — CD4 OF WCC
4: A — CD8 ECD / COMPLEXITY — CD8 OF WCC
5: A D — CD8 ECD / CD38 PC5 — CD8/38
6: A D — CD38 PC5 / Count — CD38 GLOBAL
7: K — CD38 PC5 / SIZE — BEADS
8: K — CD38 PC5 / Count — BEADS LIN

Stats: Not Normalized, Listgating: Enabled, counts adjusted by: 1024/4343

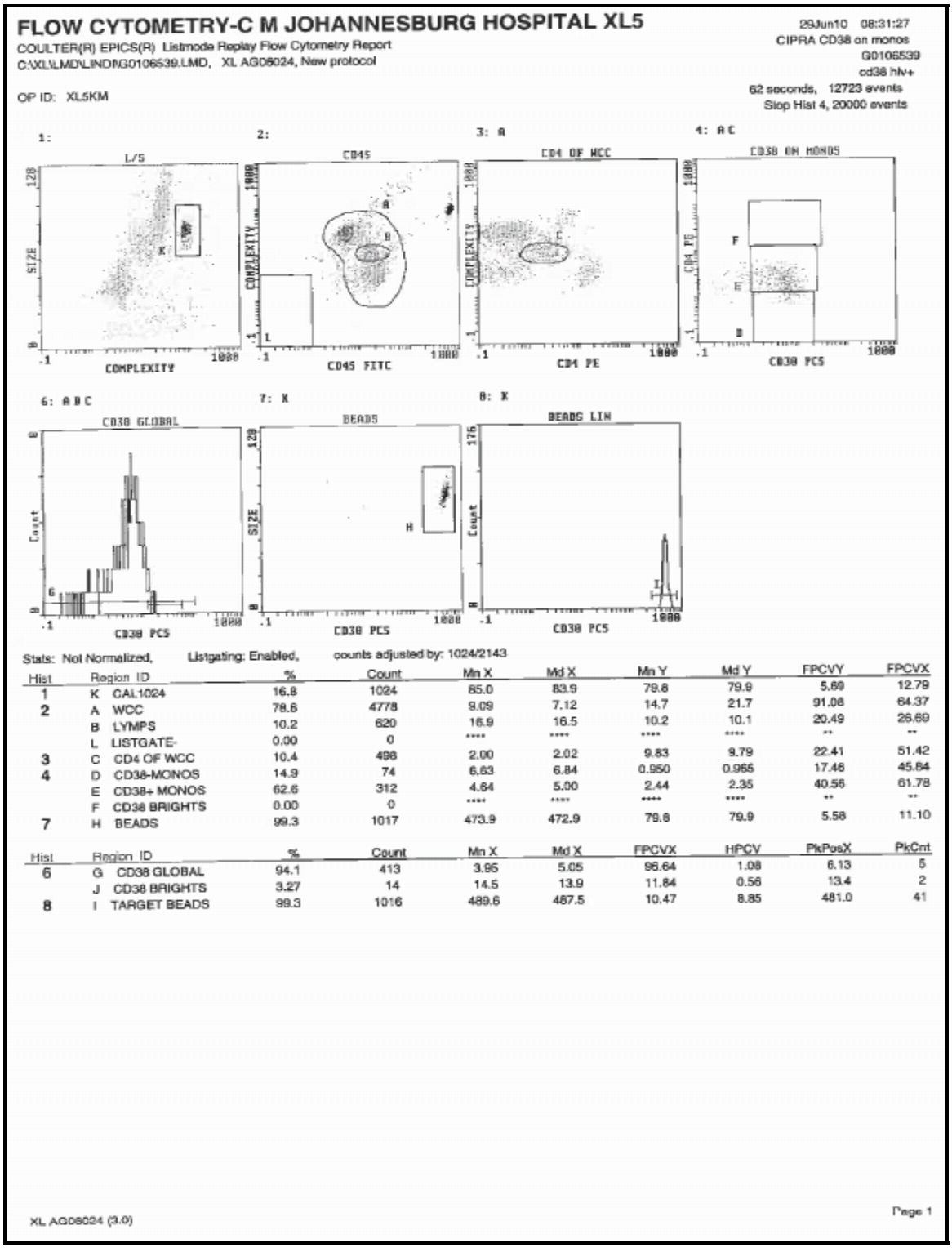
Hist	Region	ID	%	Count	Mn X	Md X	Mn Y	Md Y	FPCVY	FPCVX
1	K	CAL1024	17.2	1024	85.2	83.9	80.1	80.1	5.68	12.68
2	A	WCC	79.4	4716	9.18	7.20	14.8	21.8	92.30	65.53
	B	LYMPS	18.5	1098	19.0	19.4	3.82	3.82	35.33	32.92
	L	LISTGATE-	0.00	0	****	****	****	****	**	**
3	C	CD4 OF WCC	5.56	262	15.8	15.9	3.44	3.41	36.58	27.18
4	D	CD8++	6.76	319	15.4	16.1	3.83	3.81	34.49	35.46
5	E	CD8/38-	54.1	172	14.9	15.5	1.33	1.70	68.59	33.02
	F	CD8/38++	45.9	146	16.0	16.9	7.24	8.53	57.45	37.73
7	H	BEADS	99.3	1017	476.8	474.6	80.1	80.2	5.81	10.76

Hist	Region	ID	%	Mn X	Md X	FPCVX	HPCV	PkPosX	Count
6	G	CD38 GLOBAL	98.9	3.32	3.30	100.33	0.42	3.54	309
	J	J	21.1	12.4	11.3	41.09	0.67	7.89	67
8	I	TARGET BEADS	99.3	492.0	489.5	10.63	8.99	481.0	1017

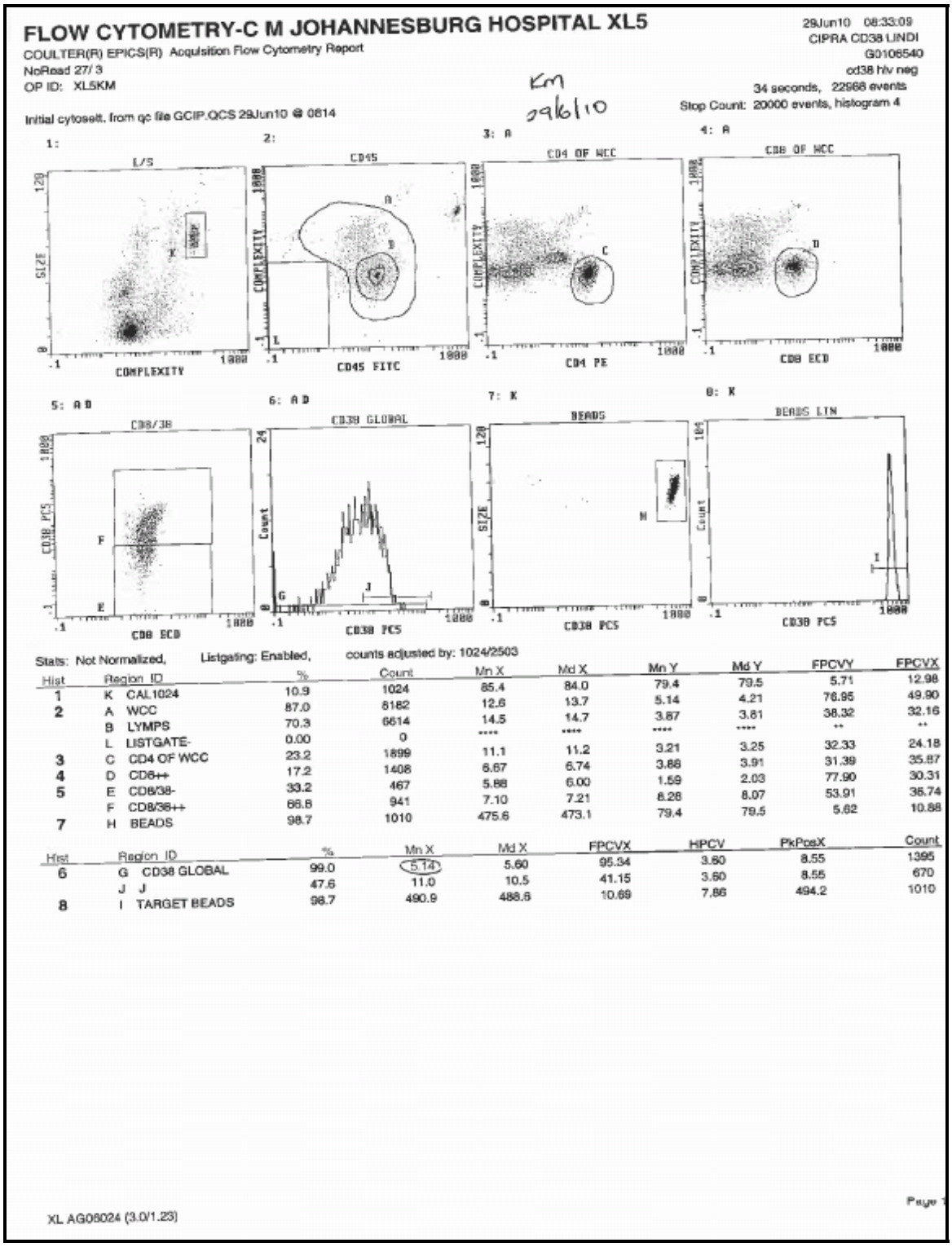
XL AG0024 (3.0/1.23)

Page 1

9. Printout of an HIV positive patient, used as a control for the CD38 protocol



10. The CD38 check for the HIV positive control re-gated on monocytes



FLOW CYTOMETRY-C M JOHANNESBURG HOSPITAL XL5

29Jun10 08:33:09
CIPRA CD38 LINDI
G0106540
od38 hiv neg
34 seconds, 22968 events
Stop Count: 20000 events, histogram 4

COULTER(R) EPICS(R) Acquisition Flow Cytometry Report
NoRead 27/ 3
OP ID: XL5KM

KM 29/6/10

Initial cytoset. from qc file GCIP.QCS 29Jun10 @ 0814

1: L/S — SIZE vs COMPLEXITY
2: CD45 — COMPLEXITY vs CD45 FITC
3: A CD4 OF WCC — COMPLEXITY vs CD4 PE
4: A CD8 OF WCC — COMPLEXITY vs CD8 ECD
5: A D CD8/38 — CD38 PC5 vs CD8 ECD
6: A D CD38 GLOBAL — Count vs CD38 PC5
7: K BEADS — SIZE vs CD38 PC5
8: K BEADS LIN — Count vs CD38 PC5

Stats: Not Normalized, Listgating: Enabled, counts adjusted by: 1024/2503

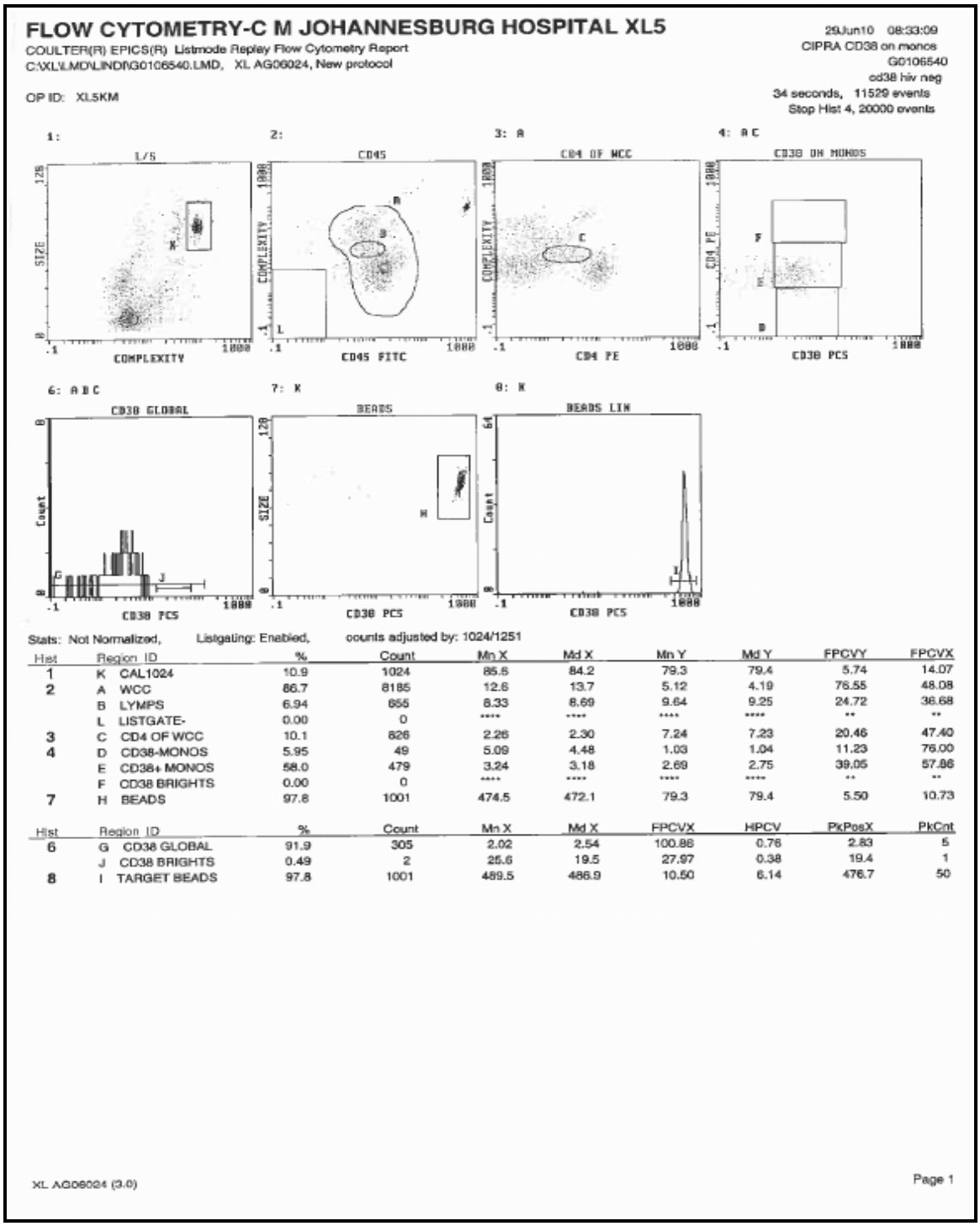
Hist	Region ID	%	Count	Mn X	Md X	Mn Y	Md Y	FPCVY	FPCVX
1	K CAL1024	10.9	1024	85.4	84.0	79.4	79.5	5.71	12.98
2	A WCC	87.0	8182	12.6	13.7	5.14	4.21	76.95	49.90
	B LYMPS	70.3	6614	14.5	14.7	3.87	3.81	38.32	32.16
	L LISTGATE-	0.00	0	****	****	****	****	**	**
3	C CD4 OF WCC	23.2	1899	11.1	11.2	3.21	3.25	32.33	24.18
4	D CD8++	17.2	1408	6.67	6.74	3.88	3.91	31.39	35.87
5	E CD8/38-	33.2	467	5.88	6.00	1.59	2.03	77.90	30.31
	F CD8/38++	66.8	941	7.10	7.21	8.28	8.07	53.91	36.74
7	H BEADS	98.7	1010	475.6	473.1	79.4	79.5	5.62	10.88

Hist	Region ID	%	Mn X	Md X	FPCVX	HPCV	PkPosX	Count
6	G CD38 GLOBAL	99.0	5.14	5.60	95.34	3.60	8.55	1395
	J J	47.6	11.0	10.5	41.15	3.60	8.55	670
8	I TARGET BEADS	98.7	490.9	488.6	10.69	7.86	494.2	1010

XL AG06024 (3.0/1.23)

Page 1

11. Printout of an HIV negative patient, used as a control for the CD38 protocol



Hist	Region	ID	%	Count	Mn X	Md X	Mn Y	Md Y	FPCVY	FPCVX
1	K	CAL1024	10.9	1024	85.6	84.2	79.3	79.4	5.74	14.07
2	A	WCC	86.7	8185	12.6	13.7	5.12	4.19	76.55	48.08
	B	LYMPS	6.94	655	8.33	8.69	9.64	9.25	24.72	36.68
	L	LISTGATE-	0.00	0	****	****	****	****	**	**
3	C	CD4 OF WCC	10.1	826	2.26	2.30	7.24	7.23	20.46	47.40
4	D	CD38-MONOS	5.95	49	5.09	4.48	1.03	1.04	11.23	76.00
	E	CD38+ MONOS	58.0	479	3.24	3.18	2.69	2.75	39.05	57.86
	F	CD38 BRIGHTS	0.00	0	****	****	****	****	**	**
7	H	BEADS	97.8	1001	474.5	472.1	79.3	79.4	5.50	10.73

Hist	Region	ID	%	Count	Mn X	Md X	FPCVX	HPCV	PkPosX	PkCnt
6	G	CD38 GLOBAL	91.9	305	2.02	2.54	100.86	0.76	2.83	5
	J	CD38 BRIGHTS	0.49	2	25.6	19.5	27.97	0.38	19.4	1
8	I	TARGET BEADS	97.8	1001	489.5	486.9	10.50	6.14	476.7	50

12. The CD38 check for the HIV negative control re-gated on monocytes

APPENDIX C: ImmunoTrol™ Reagent Data Sheet

CE

IVD

PN 4238091-LA

IMMUNO-TROL™ Cells

REF 6607077

Table of Expected Results

7587020	LOT	Unit					Unit			
		US		SI 2			US		SI 2	
2010-06-08		%+	±	%+	±		cells/µL	±	x10⁹/L	±
CD2+	LY %	83	8	0.83	0.08	LY #	1195	313	1.195	0.313
CD3+	LY %	73	9	0.73	0.09	LY #	1095	260	1.095	0.260
CD4+	LY %	49	5	0.49	0.05	LY #	728	180	0.728	0.180
CD3+/CD4+	LY %	48	9	0.48	0.09	LY #	709	165	0.709	0.165
CD5+	LY %	73	12	0.73	0.12	LY #				
CD8+	LY %	26	7	0.26	0.07	LY #	378	137	0.378	0.137
CD3+/CD8+	LY %	22	6	0.22	0.06	LY #	330	118	0.330	0.118
CD14+	MO %	77	20	0.77	0.20	MO #				
CD19+	LY %	15	5	0.15	0.05	LY #	220	67	0.220	0.067
CD45+	LY %					LY #				
CD3-/CD56+	LY %	9	4	0.09	0.04	LY #	142	56	0.142	0.056

The CD populations listed below are not cleared by the United States Food and Drug Administration For In Vitro Diagnostic Use.		Unit			
		US		SI 2	
		%+	±	%+	±
CD7+	LY %	74	18	0.74	0.18
CD10+	GR %				
CD13+	MO %	95	15	0.95	0.15
CD13+	GR %	98	13	0.98	0.13
CD19+/kappa+	CD19+LY %	61	12	0.61	0.12
CD19+/lambda+	CD19+LY %	41	9	0.41	0.09
CD22+	LY %	20	11	0.20	0.11
CD33+	MO %	83	17	0.83	0.17
HLA-DR	LY %	22	6	0.22	0.06

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BECKMAN COULTER

APPENDIX D: **Means, medians and ranges of the individual patient results**

	CD4 Abs count	VL	CD38 MFI
PATIENT A-M			
Mean	351	2.3	1.3
Median	335	1.7	1.3
Range	(171-597)	(1.4-5.8)	(0.55-2.3)
PATIENT APD			
Mean	369	2.7	1.5
Median	388	1.7	1.1
Range	(186-519)	(1.7-5.4)	(0.83-2.6)
PATIENT BMR			
Mean	341	3.6	1.6
Median	325	4.3	1.4
Range	(191-593)	(1.7-5.2)	(1.4-2.0)
PATIENT B-S			
Mean	437	2.2	1.9
Median	480	1.7	1.5
Range	(205-661)	(1.4-4.5)	(1.1-5.1)
PATIENT BUM			
Mean	523	2.3	2.3
Median	422	1.7	1.6
Range	(152-1021)	(1.4-5.3)	(0.98-6.1)
PATIENT C-M			
Mean	402	2.0	1.6
Median	377	1.7	1.3
Range	(190-669)	(1.4-4.8)	(0.89-3.9)
PATIENT FBD			
Mean	305	2.5	2.3
Median	311	1.7	1.6
Range	(92-520)	(1.7-5.9)	(0.90-6.9)
PATIENT PMM			
Mean	242	2.8	1.9
Median	252	2.4	1.7
Range	(110-332)	(1.7-5.1)	(0.79-4.0)
PATIENT RSZ			
Mean	271	2.2	1.1
Median	276	1.2	1
Range	(133-436)	(1.3-5.1)	(0.71-2.0)

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