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External Quality Assessment on $CD4^+$ T-Cell count using In-house Proficiency Testing Panels for CD4 count Laboratories in Addis Ababa, Ethiopia.

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A Thesis Submitted to the School of Graduate Studies, Addis Ababa University, in Partial Fulfillment of the Requirements for the Degree of Masters in Clinical Laboratory Sciences (Diagnostic and Public Health Microbiology Speciality Track)

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ADDIS ABABA UNIVERSITY
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Abstract

Back ground: A CD4+ T-cell count External Quality Assessment (EQA) program is important for the evaluation of performance of CD4 count laboratories.

Objective: The purpose of the present study is to assess the quality of CD4 count laboratory performance using in-house Proficiency Testing (PT) panels that perform routine CD4 counts in Addis Ababa, Ethiopia.

Methods: Voluntary sampling technique was employed and 20, 23, and 25 laboratories were participated in trials 1, 2, 3 respectively. In-house prepared fresh whole blood samples both with 68 "normal" and 68 "low" CD4 count materials were sent according to WHO guidelines to participating laboratories. The percentage and absolute counts of CD4+ T-lymphocytes were done using their routine procedures. The data were analyzed using Microsoft Excel™, sigma plot version 12.3, and Graph Pad® Prism version 5 for each trial, for each participant which included; the trimmed mean, standard deviation (SD), the percent coefficient of variation (%CV), the residual, and the standard deviation index (SDI) values for both the absolute counts and percentages of CD4+ lymphocytes (%CD4). Feedback was provided to each respective participant within one month of result submission in each trial.

Results: Most participating laboratories produced results that were within 2SD of the mean (Mean ± 2SD). The average inter-laboratory precision (trimmed %CV) was 10.87% and 5.14% for CD4 absolute counts and CD4% of Lymphocytes respectively. For the normal level CD4 EQA material, the trimmed mean %CV (between-laboratory precision) was 9.59% and 3.23% for CD4 absolute counts and %CD4 respectively. For the low level CD4 EQA material, the trimmed mean %CV (between-laboratory precision) was 12.15% and 7.05% for CD4 absolute counts and %CD4 respectively. The percentage outlier rate of absolute CD4+ counts and % CD4 for all trials were 4.3% and 4.1% respectively.

Conclusion: CD4 EQA program using In-house PT panels was found to help facilities in early identifying their gaps with regard to their CD4 count performance. In-house prepared PT panels was helpful in avoiding the challenges encountered during participation in external EQA providers like the high cost, transportation problem, feedback delay, CD4 laboratory coverage.

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List of Abbreviations

AAU	Addis Ababa University
AFREQAS	African Regional External Quality Assessment Scheme
AIDS	Acquired Immune Deficiency Syndrome
ART	Anti-retroviral Therapy
CD	Cluster of Differentiation
EHNRI	Ethiopian Health and Nutrition Research Institute
CV	Coefficient of Variation
EQA	External Quality Assessment
EQAS	External Quality Assessment Scheme
HIV	Human Immunodeficiency Virus
IQC	Internal Quality Control
MBL	Medical Biotech Laboratory
PT	Proficiency Testing
QA	Quality Assurance
QASI	Quality Assessment Scheme International
QC	Quality Control
SD	Standard Deviation
SDI	Standard Deviation Index
SOP	Standard Operating Procedure
WHO	World Health Organization

Operational definition

Accuracy: It expresses the closeness of agreement between the consensus mean and the results obtained by participant laboratories.

Coefficient of variation or Relative standard deviation: Measure used to compare the dispersion or variation in **groups** of CD4 count participant laboratories. CV = $\frac{SD}{Mean} x$ 100%

Consensus value ("trimmed mean"): is the mean of all CD4 count results submitted by participants after removal of outliers (Grubbs' test of outlier applied) that will distort the distribution and hence affect the mean

External Quality Assessment (EQA): A program that allows laboratories or testing sites to assess the quality of their performance by comparison of their results with other laboratories, through analyzing proficiency panels.

External quality assessment scheme (EQAS): A recognized scheme for organizing EQA. This can be a local scheme or may be organized at national, regional or international level.

Internal quality control (IQC): It is a set of procedures undertaken by laboratory staff using BD FACSCount control kit for the continuous monitoring of CD4 count in order to decide whether results are reliable enough to be released to the requesting clinicians.

Outlier: It is defined as a submitted participant laboratory result falling outside of ±2SD of the trimmed pool mean.

Precision: Closeness of agreement between participant laboratory results (= %CV).

Proficiency panel: Set of CD4 stabilization tube that holds whole blood sample with known count of normal and low CD4 T-cells that is packed and distributed to participant laboratories to ascertain the proficiency of the laboratories in performing CD4 testing.

Participant: A laboratory that receives proficiency test items and submits results for review by the proficiency testing scheme provider.

Pooled standard deviation: The standard deviation of all CD4 count results submitted by participant laboratories after elimination of obvious blunders and outliers.

Proficiency testing (PT): It is a periodic assessment of the performance of individual CD4 laboratories and groups of laboratories that is achieved by the distribution of in-house prepared proficiency panels for unsupervised analysis by the participants.

Proficiency testing scheme (PTS): Organized system for proficiency testing.

Standard deviation: Statistic that shows the spread or dispersion of results in a distribution of laboratory results.

$$SD = \sqrt{\frac{\Sigma(X - \bar{X})^2}{n - 1}}$$

Standard deviation index (SDI): This relates individual deviation from the mean or median to the SD, so that it is a measure of bias and calculated as follows:

$$SDI = \frac{Individual\ result\ (R) - Trimmed\ mean}{Trimmed\ SD\ (S')}$$

1. INTRODUCTION

1.1. Back ground

An EQA scheme is designed to provide an independent check of laboratory results. It allows comparison of different methods and instruments for the same test and therefore is an assessment of reliability between laboratories. EQA is an evaluation of the performance of a number of laboratories by an outside organization on specially supplied PT panels. The EQA scheme monitors the performance of each laboratory over time and identifies those laboratories that require training or corrective action to improve their performance. One of the best ways for a laboratory to monitor its performance, against both its own requirements and the performance of other laboratories, is to participate regularly in an EQA scheme (1).

Analysis of performance of a laboratory is, however, a retrospective exercise and cannot override good internal quality control (IQC) procedures. The EQA provides the laboratory with a snapshot of what would have occurred on a "normal" routine day in a laboratory. The objective of an EQA scheme is to promote between-laboratory and between-method comparability, whereas, it is the prerogative/privilege of a given laboratory to attain good within laboratory precision. The EQA scheme enables participating laboratories to relate their performance to their peers, either nationally or internationally (1).

Good Clinical Laboratory Practice (GCLP) (2) enables maintaining quality and encompasses quality control comprising (i) IQC: the laboratory uses procedures and reagents recommended by the manufacturers to ensure accuracy and reproducibility of the testing system, test by test, day by day and (ii) EQA. Quality control (QC) is supported by the procedures performed with a set of operating procedures to ensure standardization of testing.

EQA schemes in flow cytometry play an important role of laboratory testing of immune monitoring and testing. With the outbreak of the acquired immune deficiency syndrome (AIDS) epidemic and the need for reliable CD4 testing, scientists and pathologists were given a stimulus to develop accurate and reliable methods for enumeration of CD4 cells (3). This led to an increased demand for EQA in flow cytometry and initiated a series of multinational collaborative studies (4).

Laboratories can benefit from participation in an EQA scheme. It confirms the competency of the laboratory, identifies problems with test methods, monitors participant improvement of performance over time, educates staff, generates confidence, monitors competency of staff, determines accuracy and precision of test methods and can satisfy local auditing and accreditation organizations, depending on the type of scheme used by a laboratory. EQA programmes operate through a combination of three approaches that include participation in external PT programmes, supervisory site visits by external experts, and re-testing a subset of specimens in another competent laboratory or site at a higher level (5, 6).

PT refers to an EQA scheme in which sanctions are linked to inadequate performance; for example, a laboratory may be required to repeat testing if poor performance is noted on a single trial. These laboratories can however have their accreditation status rescinded in the event of ongoing poor performance and they would only be re-instated once adequate performance is re-established (6-9).

Participation in an EQA/PT scheme improves between-laboratory performance and can act as a tool to identify problems experienced by the participating laboratory (10, 11). There are several well recognized international immune monitoring EQA schemes available for CD4 testing including the United Kingdom National External Quality Assessment Scheme (UK NEQAS) for Leukocyte Immunophenotyping (www.ukneqas.org.uk), Health Canada Quality Assessment Scheme International (QASI) (www.qasi-lymphosite.ca) with the purpose to improve the accuracy and precision of cell identification and testing of lymphocyte subsets and leukaemia/lymphoma immunophenotyping. In comparison with their international counterparts, relatively few laboratories in Africa (including Sub-Saharan Africa) participate in international EQA schemes.

In Africa, the proportion of countries with a national QA programme for HIV has increased from 51% in 2003 to 61% in 2007, but participation was limited mainly to reference laboratories. In 2007, a large majority of countries (91%) participate in an HIV serology EQA programme, but only 47% of the countries do so for CD4+ T-cell enumeration (1, 12). African regional external quality assessment scheme (AFREQAS) (13) was introduced in 2002 in support of this need and initially underwritten by WHO (14) and later supported through the South African National Health Laboratory Service (SA-NHLS) (15). In 2004, the programme was extended beyond

Southern Africa to include regional centers across the continent with shipments reaching Ethiopia, and other African countries (13).

1.2. Statement of the Problem

The human immunodeficiency virus (HIV) global epidemic has necessitated the routine enumeration of T-lymphocyte subsets, which has created a need for EQA (11). The first case of HIV in Ethiopia was reported in 1984. Since then, HIV/AIDS has become a major public health concern, leading the Government of Ethiopia to declare a public health emergency in 2002. In 2011, adult HIV/AIDS prevalence in Ethiopia was estimated at 1.5 percent. Approximately 1.2 million Ethiopians were living with HIV/AIDS in 2010 (16). Since it is now well established that CD4+ T lymphocytes are the primary target for HIV, it is important to monitor $CD4^+$ T lymphocytes for the proper management of HIV infected patients (17).

In CD4+ T cell enumeration, accurate absolute CD4+ cell counts, as well as their percentage values, are two crucial assays used for the monitoring of HIV infection. They are required for the following: (1) to assess the degree of immune deterioration and rate of progression towards AIDS (defined as a CD4+ lymphocyte count of $< 0.2 \times 10^9$ /litre or < 14%), (2) to group HIV seropositive patients into cohorts according their baseline CD4+ counts before starting treatment, (3) to determine the appropriate time for prophylaxis of opportunistic infections, and (4) to monitor the efficacy of antiretroviral and/or interleukin 2 (IL-2) treatment and candidate vaccines. Therefore, there is a clinical need for the accurate and precise enumeration of CD4+ T cells (18).

Measurement of CD4+ T lymphocytes is usually performed by the use of immunophenotyping and flow cytometric analysis, whereby cells are counted after being labeled with a fluorescently conjugated monoclonal antibody that recognizes a specific surface antigen (e.g., CD3 and CD4) (19). This necessitates the evaluation of CD4 count which is fundamental for the consistent management of patients with HIV/AIDS.

The use or misuse of $CD4^+$ T-lymphocyte measurements has a crucial impact on the effective management of HIV/AIDS patients. It is essential that accurate daily IQC and PT or EQA programs are employed to ensure reliable results. Satisfactory performances in $CD4^+$ T-lymphocyte EQA are recommended for HIV research and clinical trial programs within many

parts of the world, including Ethiopia. The CD4 EQA program has two main objectives. Firstly, it must conduct a low-cost national EQA and standardization of flow cytometer $CD4^+$ T-lymphocyte determinations in HIV/AIDS patients. Secondly, the EQA provider should assist in the country's flow cytometer laboratory performance evaluations, and incorporate scientifically and educationally-based schemes in order to monitor their improvement (20).

International expansion of $CD4^+$ T lymphocyte testing is underway with many laboratories developing standard operating procedures (SOPs), programmes for training staff and validating testing platforms. However, there are a number of challenges specific to the introduction and continued monitoring of $CD4^+$ T lymphocytes, including: the remote locations of many laboratories, equipment and personnel validations, and the lack of reference or validation controls similar to those that are available for many routine analytic testing procedures (19).

Now that global laboratory scale up and operational efforts are well underway, additional efforts are required to monitor accuracy and precision of the laboratory parameters being tested. As highlighted, the development of comprehensive IQC and participation in EQA using In-house prepared PT panel is therefore extremely important (19, 21). However, EQA turnaround times from sample issue to data reporting can delay the implementation of changes that are required to improve performance. This delay can impact upon the need for additional staff training or rectification of problems related to equipment/reagent performance (10, 11, 22).

The international EQA partnership between the National Institute of Allergy and Infectious Disease—Division of Aids Immunology Quality Assessment Program (DAIDS— IQA) and the UK NEQAS for Leucocyte Immunophenotyping programmes [the latter based within the national health service of England and Wales (a nonprofit-making Government Health Care organization)], have developed, to address many of the issues discussed earlier, a novel training panel that provides instant verification of competence or performance, of either instrument or individual, using data reporting and analysis (23). Therefore, PT is the most widely used approach to monitor the quality of CD4 testing, and each testing facility should participate in an external PT program. Demonstration of proficiency in these panels provides information to health care providers on the quality of testing in each laboratory and builds confidence in the accuracy of results on patient specimens (13, 23).

However, participation in such EQA programme is difficult in terms of cost, transportation, coverage of all laboratories, on time feedback, and communication. As a result, the problem of quality is best addressed when In-house prepared PT panel is used in the EQA programme.

A study done on the Quality Assurance (QA) in the HIV/AIDS laboratory network of China showed that many laboratories performing CD4 count do not participate in PT programmes. According to this study, these challenges will need to be resolved to guarantee sound development of the Chinese HIV/AIDS testing laboratory network and its QA system (24).

According to Alemnji et al, in developing countries stringent QA practices for HIV diagnostic and follow up testing, which include appropriate training, development of SOPs, maintenance of operator proficiency, routine use of QC specimens, standardized data management, equipment calibration and maintenance, and biohazard safety with proper disinfection/disposal procedures are not routinely followed to ensure reliability of results and a safe work environment (25).

This study also showed that the introduction of point-of-care testing technologies involving the use of non-laboratorians in routine testing has further increased the complexity of QA. Therefore, special consideration is needed in implementing a step-wise approach towards quality improvement, strengthening of the supply chain management, human capacity development, infrastructure upgrade, and strong public private partnerships to ensure long term sustainability of these efforts. Development of a functional laboratory tiered network that facilitates communication, referral, training and problem solving could further enhance confidence in laboratory testing (25).

Considering the nature and complexity of EQA to include the need for follow up supervision to resolve non-conformances, it is recommended that countries should set up functional EQA programmes to be coordinated at the national level with the capacity to either produce or procure PT panels to distribute to the other laboratories (23, 25).

One of the key challenges in running PT programmes in developing countries has been that of the cost and distribution to include the issues of maintaining cold chain, packaging and shipment with maximum safety to laboratory staff and people involved in the shipment exercise (25). As a result, establishment of PT scheme for CD4 count using in-house made PT panel is fundamental to resolve the problem.

According to Brando et al, EQA programs for clinical cell analysis should not be doomed to remain monopolized by a few major providers worldwide even if challenges are there. According to this study, the history of the last 20 years of EQA programs in clinical cell analysis has shown that every project has germinated first in a soil made of clinical need and scientific enthusiasm, and then it was transplanted in a solid institutional background, where it could mature, get stronger, and disseminate its educational fruits (26).

According to WHO Regional Office for Africa an assessment done on HIV/AIDS laboratory capacity in Africa from 2005-2007 revealed that although some countries are on course towards the 2010 Universal Access target, the African Region as a whole needs to put in a lot more effort to achieve this goal. Those countries found to be lagging behind will have to quickly strengthen their National Reference Laboratories (NRLs); improve on QA insufficiencies; establish and improve on EQA for CD4+ T-cell enumeration; and recruit and train the required number of qualified laboratory scientists and technicians to do the work. Generally the assessment shows that low levels of inter-laboratory networking in the African Region; absence of a national quality assurance programme in nearly half of the respondent countries, insufficient EQA Scheme for CD4+ T-cell enumeration and HIV drug resistance monitoring, and absence of designated National Reference Laboratory (NRL) in some countries (12).

In Ethiopia specifically in Addis Ababa, there are extensive expansion of public and private ART sites including public health centers and hospitals as well as private clinics and hospitals for the treatment and monitoring of HIV infected patients. The two most commonly available and used flow cytometry machines in Ethiopia for ART initiation and monitoring are BD FACSCount, which is available almost in all ART laboratories, and BD FACSCalibur that is available at a referral level in the regional laboratories and some private laboratories only (27). Despite extensive expansion of care and treatment and progress in ART monitoring of HIV infected patients, participating in EQA/PT for the CD4 count remains severely limited.

Different ART CD4 count laboratories in Ethiopia participate in EQA programmes through QASI (that is coordinated by Ethiopian Health and Nutrition Research Institute (EHNRI)) and/or AFREQAS that send-out lyophilized materials. Since the imported lyophilized panels need reconstitution upon supplier's instructions, this process reduces the commutability of the materials and feedback takes long time (above six months) after result submission. Another

problems associated with participation in the international EQAS include, the high cost of EQA schemes, logistical problems with sample transport to testing sites and lack of infrastructure and computers to enter data onto websites for submission of results. In addition, international EQA schemes are frequently commercial investments and their focus may not necessarily be the development of quality laboratory capacity building in resource-poor settings (1, 11, 13).

Promotion of locally or nationally supported EQA schemes would therefore have more impact in Ethiopia if the scheme is run with an understanding of regional difficulties and circumstances, methods used and even local languages where applicable. Moreover, since several laboratories are involved, analysis based on SD variation obtained from such laboratories is very wide and undesirable performance results could fall within normal range (mean ±2SD).

This is specially a major gap that should be given high emphasis in our county Ethiopia. Because there is no established EQA scheme for CD4 count that can assess and monitor periodically the performance of the numerous CD4 count laboratories by giving feedback for amendment and consistency of the CD4 count testing. In addition to this, participating in the established international EQAS is difficult in terms of resources, time, and coverage of all CD4 count laboratories in Ethiopia. The increased application of CD4 count machines in public and private health sector is helpful for treatment and ART monitoring of HIV patients but it is essential to participate in CD4 EQA by establishing local EQA scheme using in-house PT panels for the sustainable improvement of their CD4 testing performance.

1.3. Significance of the Study

CD4 EQA using In-house prepared PT panels provides substantial value to the practice of laboratory medicine by assessing the performance of individual $CD4^+$ count laboratories and, when commutable samples are used, the status of standardization or harmonization among different measurement procedures as indicated in different researches conducted elsewhere. However, there is no data about the EQA using In-house PT panels for CD4+ T-cell count assessing the performance of laboratories in Ethiopia. Thus, it was essential to establish the CD4 EQA scheme using In-house PT panels to fill this gap and thereby improve performance of CD4 count Laboratories in Addis Ababa, Ethiopia.

Participation in commercialized EQA scheme is too difficult in resource poor country like Ethiopia. This is due mainly to high cost, coverage of all CD4 laboratories, feedback delay, and logistical problems with sample transport to testing sites and lack of infrastructure and computers to enter data onto websites for submission of results. In addition, international EQA schemes are frequently commercial investments and their focus may not necessarily be the development of quality laboratory capacity building in resource-poor settings.

Thus, CD4 EQA using In-house prepared PT panels was found very important in solving the above problems by increasing the CD4 laboratory coverage, by providing with easy transportation and on time feedback, by diverting all attentions towards the improvement of performance of CD4 count Laboratories since it is non profitable programme and aims in improving the quality of CD4 count services. As a result, findings obtained from CD4 EQA using In-house PT panels help to avoid being dependent externally.

The findings of this study are helpful to see the performance of the participating laboratories and to stimulate standardization of procedures to achieve more uniform results among participating laboratories in Addis Ababa. This intern have impact for monitoring of HIV infected patients because PT panel is intended to verify on a recurring basis that laboratory results conform to expectations for the quality required for patient care. Of all, this data generated from this study will be used as a baseline for future large scale proficiency testing schemes.

2. LITERATURE REVIEW

Whether a test is in the clinical research phase or widely performed on a daily basis, appropriate quality control (QC) is critical to assuring that accurate and clinically relevant comparisons can be made at multiple levels: cell to cell within a sample, sample to sample within the laboratory, day to day for a patient, patient to patient, and laboratory to laboratory (20, 23, 25).

Internal quality control (IQC) provides the means for evaluation of analytic test results at the time of testing in order to decide whether they are reliable enough to be released to the requesting clinicians. EQA, on the other hand, refers to a system of retrospective and objective comparison of results from different laboratories by means of PT organized by an external agency. The main purpose is to establish between-laboratory and between-method (including

between-instrument) comparability, and agreement with a reference standard where one exists. IQC and EQA complement each other and must never be considered as alternatives (28).

The generation of quality requirements must be a dynamic process by which requirements are not specified once and for all, but are revised at regular intervals taking into account the evolution of the analytical state of the art and the introduction of new and more sophisticated methodologies as well as the continuous improvement in the performance of laboratories (29).

According to Miller et al, Key factors for interpreting PT/EQA results are knowledge of the commutability of the PT panels used and the process used for target value assignment. A commutable PT panel demonstrates the same numeric relationship between different measurement procedures as that expected for patients' samples. Non-commutable PT panels frequently have a matrix-related bias of unknown magnitude that limits interpretation of results (30). This shows that preparations of commutable in-house PT panels are critical in the PT scheme.

PT/EQA results for commutable samples can be used to assess accuracy against a reference measurement procedure or a designated comparison method. PT/EQA results for non-commutable samples must be compared to a peer group mean/median of results from participants who use measurement procedures that are expected to have the same or very similar matrix-related bias. Peer group evaluation is used to assess whether a laboratory is using a measurement procedure in conformance to the manufacturer's specifications and/or in conformance to other laboratories using the same technology (30).

In an EQA, during laboratory report analysis consensus mean values and observed SDs of measurement/analytical results of laboratories participating in PT are insufficiently reliable for the assessment of a laboratory's performance in a PT with a limited number of participants (less than 20–30). For this reason, traceable assigned values of test items (portions of a certified reference material, of an in-house reference material or of a spike) and externally set performance criteria (usually expressed as a SD of PT results for a z-score) acceptable for all participants should be used wherever at all possible (31).

The Impact of WHO EQA program on the laboratory performance for CD4 enumeration for monitoring of ART in resource limited settings showed that CD4 counts play an important role

in the decision making process for initiating and monitoring ART. Thus, ensuring reliable results through investment in EQA program is a key for individual patients and for cost-effective public health expenditure (14).

According to Whitby et al, the UK NEQAS showed that the adoption of single-platform technology resulted in a reduction of the overall mean inter-laboratory CV for absolute CD4 T lymphocytes from 56% (prior to the widespread use of single-platform technology) to 9.7%. Individual laboratory deficiencies were also identified using a performance monitoring system and, through re-education by collaboration with the coordinating center, satisfactorily resolved. (10).

In conclusion, during the last 9 years, the UK NEQAS for Immune Monitoring program has made significant advances in the quality control and monitoring of national and international laboratories undertaking the measurement of lymphocyte subsets. The timely production and distribution of scientific and educational material by the program, coupled with a robust performance monitoring system, has ensured that participants can use EQA (complementary to good IQC procedures) as a tool to rapidly respond to technological advances. As a result, the quality of results generated by participating centers has improved significantly (10).

A study done on the Impact of the International Program for Quality Assessment and Standardization for Immunological Measures Relevant to HIV/AIDS (QASI) showed that laboratories new to participation in EQA programmes have a higher degree of variation from the aggregate mean, when compared to those that have participated in an EQA programme for some time. This was mainly due to lack of proper communication and early degradation of the stabilized material due to improper packaging. In this study laboratory results from five consecutive shipments were analyzed and the CV decreased from 7.2% to 4.7% and from 14.2% to 8.8% for percent and absolute CD4 T-cell counts, respectively (11).

According to this study, the most important factors contributing to the reduction of overall immunophenotyping error were the frequency of participation in an EQA program, carefully orchestrated pre-shipment communications with appropriate follow-up, and reduction of the time interval between results returned by participating laboratories and notification of the laboratory's performance. This study illustrates that a quality assessment program have improved the overall

performance of laboratories and reducing inter-laboratory variation enhanced significantly the effectiveness of multicenter HIV vaccine or drug trial evaluation (11).

Another research carried out on EQA Program on CD4+ T-Lymphocyte Counts for Persons with HIV/AIDS in Thailand revealed that most participating laboratories produced results that were within 2SD of the mean, while the average inter-laboratory %CV were less than 8% for CD4+ T-lymphocytes. The program was found to improve the reliability of CD4+ T-lymphocyte determinations and is becoming increasingly important as Thailand and other Southeast Asian countries scale up their national programs that provide access to antiretroviral therapy for persons living with HIV/AIDS. In this study, although there were no complications with the lysis and lymphocyte gating procedures, some flow cytometers encountered technical problems arising from inconsistent maintenance issues or because participants did not perform calibration tests prior to sample runs, leading to inaccuracies (20).

A study done on Flow Cytometric Lymphocyte Subset Enumeration: 10 Years of EQA in the Benelux Countries showed that the implementation of EQA scheme was effective in reducing test variation as a function of time. According to this study, the first send-out was exceptional with a high variability of all subset counts, but especially of the $CD3^+$, $CD4^+$, and $CD8^+$ T cells. Thereafter, test variation declined with occasional exceptions. Nevertheless, inspection of the variation of $CD3^+$, $CD4^+$, and $CD8^+$ T-cell counts as a function of time reveals a trend toward lower variation (32).

In this study result report were analyzed using robust multivariate regression and five variables were associated with significant positive or negative bias of absolute lymphocyte subset counts: (i) platform methodology (i.e., single platform assays yielded lower CD4+ and CD8+ T-cell counts than did dual-platform assays); (ii) sample preparation technique (i.e., assays based on mononuclear cells isolation yielded lower T-cell counts than those based on red cell lysis); (iii) gating strategies based on CD45 and sideward scatter gating of lymphocytes yielded higher CD4+ T-cell counts than those based on ‘‘back-gating’’ of lymphocytes guided by CD45 and CD14); (iv) stabilized samples were generally associated with higher lymphocyte subset counts than non-stabilized samples; and (v) significant bias and variability of results, independent of the variables tested for in this analysis, were also associated with individual laboratories (32).

According to Pandolfi et al, the Italian quality control study for evaluation of CD4 cells in centers involved in the treatment of HIV-1 patients revealed that unsatisfactory performance for percentage of CD4 cells were identified as a CD4 analysis with residual values ≥ ± 5% and with deviates ≥ ± 2 and that of absolute numbers of CD4 cells as CD4 counts with residual > ± 100 CD4 cells/mm^3 and with deviates ≥ ± 2. This research findings showed that most Italian laboratories provide reliable results in evaluating the numbers of CD4 cells in HIV-1 positive samples and unsatisfactory performance (Only 3.7% of the laboratories) were associated with delayed processing of the samples due to shipment problems like unavailability of rapid shipment of HIV-infected blood samples in the same day of blood drawing, limited inter-laboratory quality control program at national level, and lack of instrument calibration. It also showed that unsatisfactory performance was not related to the usage, in the individual laboratories, of different commercial anti-CD4 MAbs or cytometers (33).

Another study done by Prachongsai et al, showed that alternative methods for preparation of blood stabilization is necessary to avoid high cost of the existing preparation of QC materials for IQC and EQA of CD4 T-lymphocyte testing (34). This study show us preparation of in-house PT material is necessary to avoid being dependent on the commercialized PT materials and local capacity building which is crucial for long-term sustainability.

Another research done on QASI, an International Quality Management System (QMS) for CD4 T-Cell Enumeration Focused to Make a Global Difference showed that decreased inter-laboratory variation for both low and medium CD4 T-cell levels. After three cycles of consecutive participation, there is an average of 38 and 26% error reduction reported for the mid and low CD4 levels, respectively resulting from continuous participation in the QASI-QMS (35).

In the AFREQAS which was implemented from Johannesburg showed that the Overall AFREQAS between-laboratory reproducibility (trimmed %CV) was 10.5% and 9.1% for absolute CD4 and CD4%, respectively. For the respective CD4 absolute and CD4% of Lymphocyte values in the trials where ‘‘normal’’ material was shipped trimmed %CV of 10.9 and 7.3% were noted, and in ‘‘low’’ value shipments %CV of 13.8% and 12.4% were noted. Cumulative absolute CD4 SDI analysis revealed the best between-laboratory precision amongst BD FACSCount and PanLeucogating (PLG CD4) users (both SD of SDI = <1.2 and %CV of <<8%). Dual Platform or Single Platform algorithm-based systems and certain volumetric

methods (laboratories who used Partec CyFlow instruments) had higher numbers of outlying laboratories (>12–25%CV and SD (SDI) > 2.2 noted), indicating that additional technical training and/or manufacturer support was required (13).

In this AFREQAS, some of the CD4 methodologies such as the Multiset TM with TrucountTM (TC/MS; BDS), appears to be used incorrectly by some African users and this takes place more frequently than with the other methods and this was probably due to misconception of many users that the TC/MS system is ‘‘automatic,’’ ‘‘walk away’’ and ‘‘does not need to be interfered with.’’ In other words, the perception is that it can be used like the FACSCount TM system. A further potential misunderstanding was to assume that the blood-pipetting step in a single tube analysis does not require quality control when used in combination with pre-dispensed (e.g., Trucount) beads. According to this research result, participation in an AFREQAS with feedback and remedial action improved the quality of CD4 testing (13).

3. OBJECTIVE OF THE STUDY

3.1. General objective

The general objective of this study is to assess the quality of $CD4^+$ count laboratory performance using in-house PT panels and see its feasibility in Addis Ababa, Ethiopia.

3.2. Specific objectives

- To assess a pilot-feasibility of preparation of in-house PT panel for CD4 enumeration
- To assess the performance of individual $CD4^+$ count laboratories and establish the requirements of implementing EQA for $CD4^+$ T-cell count
- To establish methods for data analysis of all submitted results and further establishes a database for analysis of cumulative trial data.

4. MATERIALS AND METHODS

4.1. Study setting and period

This was a cross-sectional study where results were collected from laboratories in Addis Ababa participating in the Inter-laboratory Proficiency Testing Programme using in-house PT panel for $CD4^+$ T-Cell Count. The study was conducted from December 2012 to July 2013.

4.2. Study Participants

Public CD4 laboratories (governmental laboratories), Non-governmental organization laboratories, and private laboratories were included in the study. The CD4 EQA programme using in-house prepared PT panels was implemented at local level. Prior to participation, the CD4 EQA scheme coordinator met with each participating laboratory manager to discuss the aim and benefits of the programme.

During the initial site visit, to these and subsequent participants, laboratories were made aware of the guidelines and standards available on Good Laboratory Practice (GLP) to enable them to introduce GLP into their laboratories (2). Participants were further informed that results generated from their laboratories are in the interest of patient care, by generating reproducible results, to standardize techniques across centers and improve the CD4 laboratory diagnostic service. The aim of CD4 EQA scheme was to provide an assessment scheme (advisory) and not an assurance scheme (policing). The CD4 EQA scheme offered assistance to laboratories with regard to corrective actions but was not responsible for ensuring implementation of these advised corrective actions.

4.3. Eligibility criteria

4.3.1. Inclusion criteria

- Any laboratory that carry out CD4 count in Addis Ababa.

4.3.2. Exclusion criteria

- Those laboratories that did not do CD4 count due to several reasons during the scheme

4.4. Sampling procedures

Voluntary sampling technique was used to include participant laboratories according to inclusion criteria in Addis Ababa. These laboratories were chosen across the three trials as they voluntary to participate in the programme. Following Trial 1 pilot, Trial 2 and 3 were extended to the rest of laboratories that were not participated in the first and second trials respectively.

4.5. Documentation

Various documents were generated, both to ensure the success of the scheme and to obtain information and data required for this thesis. These include registration form (Annex E1 and E2),

safety advice and instructions (Annex E3), instruction sheet (Annex E4), data repot form and In-house prepared PT material condition (Annex C and Annex E5).

4.6. Preparation of EQA material for shipment

4.6.1. CD4 External Quality Assessment (EQA) material

Fresh whole blood obtained from one donor was prepared for distribution on the CD4 EQA scheme at Medical Biotech Laboratory (MBL). MBL participates in CD4 EQA scheme with digital PT Canada (organized through EHNRI) and in AFREQAS (organized through National Health Laboratory Service, Johannesburg, South Africa), quarterly and every two monthly respectively. EQA performance results of MBL are excellent. The advantages of fresh whole blood preparations for CD4 EQA include wide compatibility with most flow cytometers, ability to be used with various sample-processing procedures, such as lysing, fixing, washing, etc. but since it can be processed only within 48 hours, it cannot be used for sites that cannot be reached at same date of blood collection. Similar preparation of fresh whole blood for CD4 EQA scheme was reported by Kunkl et al, in 2002 (46).

Labels were printed and fixed for each vial of quality control material using local adhesives. Labels contained a sequential quality assessment number (trial number), storage conditions, as well as low and normal CD4 panel code, for example, sample-1A, sample-1B.

4.6.2. Preparation of CD4 EQA material

In-house PT material was prepared by drawing HBsAg, HCV, and HIV-1 and HIV-2 negative and with predetermined CD4 value fresh whole blood from one donor in to 4ml K3-EDTA BD vacutainer tubes (8-12 tubes). The collected blood was mixed thoroughly in automated hematology-mixer machine to insure homogeneity of the materials. Once mixed, the collected blood (from one donor but in 8-12 K3-EDTA BD vacutainer tubes) was pooled into a large sterile container with 50 ml volume capacity (Costar,Corning, Inc., Corning, NY) and gently mixed again to ensure uniformity of pooled samples. The above blood was separated into two samples (sample-A and -B) to prepare the normal (sample-A) and low (sample-B) EQA materials. Normal EQA material was prepared in such a way that coincides with Ethiopian CD4 T-cell counts (753±227 cells/µl for adult male) (37). Low EQA material was prepared by

continuous dilution of sample-B using phosphate buffer saline (PBS) till desirable low CD4 count was attained (100-300 cells/µl range) (Annex B).

The samples was visually inspected for haemolysis by checking the plasma to see if it had a clear straw colour appearance as this would indicate no hemolysis, haemolysed red cells would give a red tinge to the plasma. A dark brown colour could be indicative of bacterial contamination. The blood was not shown any discolouration, since it was carried out by strictly following SOPs. Strict safety procedures were in place for dispensing material in accordance with standard operating procedures of the flow cytometry laboratory. A calibrated pipette was used to dispense/aliquot 1ml of well-mixed CD4 EQA material into pre-labeled vials (2ml cryovials). After dispensing, the vials were capped, thoroughly checked for leakages, and packed according to WHO guidelines before dispatch time (42, 47, 50) (Annex E4).

4.6.3. Dispatch of CD4 EQA material

All shipments included In-house prepared proficiency material, a data return form with unique participant identifier (number), clear instructions of the date of submission deadline together with product instruction sheets and safety information. It was personally delivered by the Principal investigator (PI) to each participant throughout the three trials. To distribute to all participants at the same day of blood collection, the PI was used Motorcycle. In those dispatch date the CD4 EQA material was sent in plastic transport bag box to maintain optimal temperature by avoiding direct sunlight (at ambient temperature (18-22°).

Thermometer was placed inside the box to check the temperature fluctuation throughout the day. Monday and Tuesday were the preferred date for the distribution. This was to take corrective action if any problem is occurred within the next consecutive working days. All participants received their samples within 1-8 hours of EQA material dispense. All communications were mainly based on telephone and personal contact before and during all the dispatch dates (Annex H). Table 4.1 gives a summary of the CD4 EQA material across the three trials.

Table 4.1: Summary of CD4 EQA material dispatch

Trial Number	**Dates of sample dispatch**	**Type of proficiency testing panels (PTP)**	**Type of samples**	№ of participants
1	2012 December	In-house PTP	2	20
2	2013 March	In-house PTP	2	23
3	2013 July	In-house PTP	2	25

4.7. Quality assurance and Quality control

Specific SOPs were followed during blood collection, preparation of blood for CD4 count, registration of results that was already in place at MBL. FACSCount Controls run was carried out in each trial in addition to the normal schedule of the laboratory. Preparation of in-house PT panels as well as its transportation was done based on standard guide lines (23, 28, 41, 42).

It was essential to establish the stability of the in-house prepared PT material during transportation to the participating laboratories. The aim was to assess the stability of the CD4 EQA material at ambient temperature. This was performed as follows: one set of EQA material tested and recorded its result at the beginning of the dispatch and round throughout the day to all participants. When distribution finished, this material was returned to the MBL in which this material was prepared and tested. The returned CD4 EQA material was visually checked for haemolysis and bacterial contamination. This CD4 EQA material was analyzed by BD FACSCount^{TM} at MBL to ascertain that the T-cell subset enumeration was not adversely affected by sample transport. Finally, the two results (pre-and post-shipment) were checked if any difference is occurred due to the transportation process.

4.8. Statistical analysis of CD4 EQAS results

Participant laboratory results were collected by the PI by visiting to their respective sites. After statistical analysis and generation of individual laboratory reports, feedback also delivered to participants by the PI.

Statistics were performed on two levels: (i) individual laboratory statistics and (ii) pooled statistics of all participants to assess the success of the EQA scheme. Both sets of statistics are

reported to participating sites to allow bias estimation and comparison of individual performance against a pool of participants. Microsoft Word™, Microsoft Excel™, sigma plot version 12.3, and Graph Pad® Prism version 5, were used to capture data and to generate statistics, graphs, documents, letters and feedback to all participating laboratories.

4.8.1. Statistical analyses of all participants results

Each participant's data was analyzed according to international practice (5, 6, 10, 13, 51). On receipt of all participant data for each trial, CD4 absolute counts and CD4% of Lymphocyte values were entered into Microsoft Excel™. The mean, standard deviation ($SD = [\sqrt{\Sigma}(X1 - X)^2/(n -1)]$), and coefficient of variation (%CV = SD/Mean x100) was calculated automatically by the Microsoft Excel™ for the combined Graph Pad® Prism of all results, and a ±2SD limit determined for both CD4 parameters (untrimmed pooled data). Results that fell outside the ±2SD limit were identified and removed as outliers and pool data re-analyzed to calculate a pooled trimmed mean, pooled trimmed SD and pooled trimmed %CV.

4.8.2. Statistical analyses of individual participant results

Each participant's ''Residual'' expressed in CD4 cells/µl or CD4% (equal to participant's submitted CD4 absolute or CD4% result minus the trimmed pool mean result) and the standard deviation index (SDI) (equal to participant's ''Residual'' divided by SD of the trimmed mean, expressed as a ratio) was calculated and compared to the pooled trimmed results (as described above) to indicate bias. Laboratories identified as outliers (outside of ±2SD of the trimmed pool mean) were contacted to troubleshoot and perform corrective actions (45). These participants were not allowed to resubmit corrected results.

4.9. Laboratory performance report

The performance report was the main interface with all participants. For all trials the data were represented in tabular and graphic form.

4.9.1. Tables

As shown below (Table 4.2 A), the participant performance report included: the unique participant number, the trial/survey number as well as the date of report, the no lyse no wash protocol, and flow cytometer type.

The summary table of statistics was divided into two columns (Table 4.2 B). The first column reports the participant's results as absolute counts and CD4% of Lymphocytes. The 2nd column reports the results of all participants (pooled value). The individual participant's results reported for the individual participant included their reported results, the residual value, and SDI with no outlier. The pooled results reported the number of participants, the group trimmed mean value, SD of the trimmed pool mean and %CV.

A: An example of a laboratory's participation details

Inter-laboratory Proficiency Testing Programme

MBL **for CD4 Cell Count** **AAU, CHS**

Results of survey number 1, Sample-A of 2012/13	
Date of report	20/12/2012
Laboratory	Lab 1
Protocol	No lyse no wash
Flow-cytometry (Instrument)	BD FACSCount™
Monoclonal antibody panel	Two-color immunofluorescence method

B: An example of a participant's results

					All Methodologies				
Test	**Results**	**Residual**	**SDI**	**>±2.0**	**Method**	**№**	**mean**	**SD**	**CV**
CD4+ lymphocyte absolute count/µl	724	91.1	1.83	*	All	20	632.9	49.84	7.87
CD8+ lymphocyte absolute count/µl	606	72.7	1.53	*	All	10	533.3	47.6	8.93
CD3+ lymphocyte absolute count/µl	1,461.00	161	1.6		All	10	1,299.8	100.63	7.74
CD4 % of lymphocytes	NA				All	9	35.79	0.8	2.24

Indicates an outlying flagged result, if >±2SDI

Participant's results

Pooled trimmed results

Table 4.2: Examples of tables used as part of the Laboratory Performance Report to indicate (A) participant details and (B) participant results.

CD4 methodologies were not separated and as such a participant's results were compared to the consensus pooled trimmed results for all methodologies on the scheme. Data was analyzed and outliers identified and removed (trimmed data). The latter was analyzed for mean, SD, %CV and SDI for each lymphocyte subset parameter reported. Individual participant's results were compared directly to the trimmed SDI value, as recommended by international EQA programs (5, 13).

4.9.2. Graphs

In addition to a tabled summary of participant's performance, results were also represented graphically (Figure 4.1). This graph represents a summary of SDI values for all participants, to help laboratories interpret their own results versus that of the pool. The limits (±2SDI) were highlighted to visualize outliers easily.

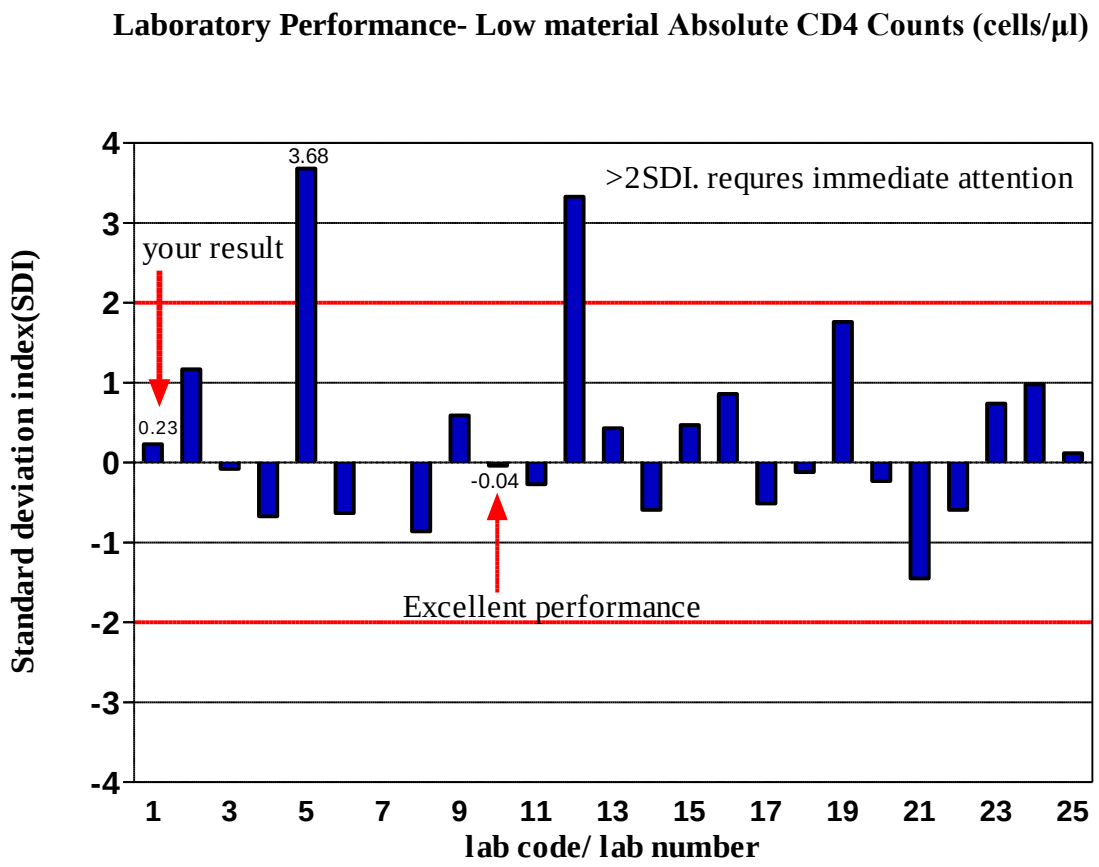


Figure 4.1: A bar chart depicting the participant laboratory number versus the calculated SDI value of individual participants. Acceptable SDI limits indicated by red lines (within ± 2SDI).

Further, included in the result sheet was an explanation of how to interpret the graphs for laboratories that were not familiar with quality assurance procedures. In this context, a SDI of less than 0.5 denotes excellent performance. A SDI between 0.5 and 1.0 is considered satisfactory. A SDI between 1.0 and 1.5 is considered acceptable, a SDI between 1.5 and 2.0 is considered borderline and lastly, a SDI of greater than 2.0 requires immediate attention and corrective action (47,51) (Annex G).

4.10. Feedback

On joining the CD4 EQA programme using In-house prepared PT panels, participants were made aware of the procedure of feedback and identifying problems to assist laboratories with trouble shooting. Those participants whose results were outside the ±2SD range were contacted immediately to effect corrective action timeously (Annex E5). These were based on advice on

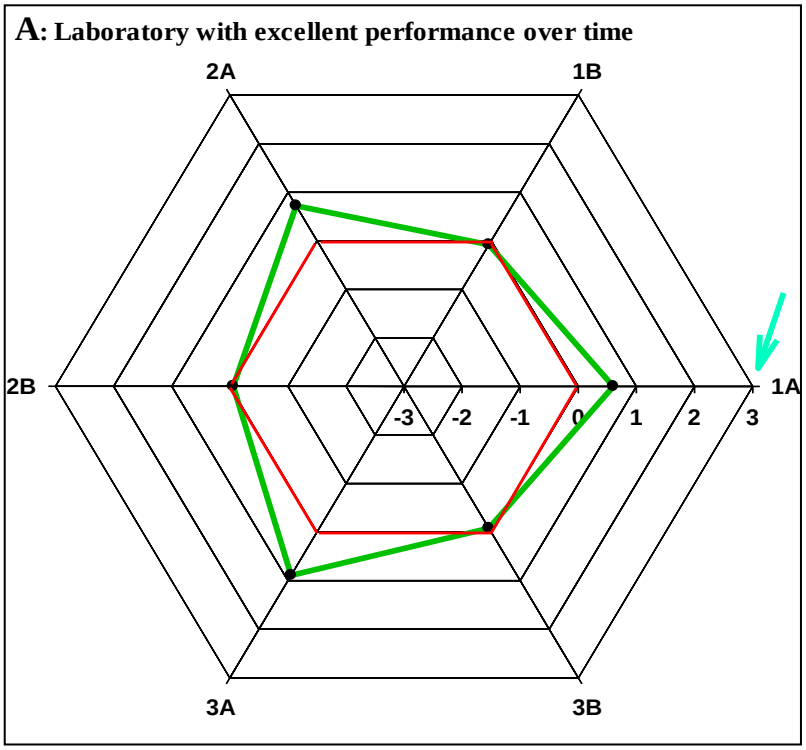
different technologies and guidelines for equipment maintenance and regular service of instruments. Participant laboratories were also advised to use a Levey-Jennings wall chart to plot and monitor their performance over time. This was based on the assumption that it is used at their discretion for teaching and training purposes.

4.11. Assessment of longitudinal performance of individual participants

For the purposes of this study, individual SDI performance of laboratories was monitored longitudinally by graphically representing the SDI values versus trial number on a radar graph (13, 21). These radar plots enabled visual representation of the accuracy and precision of CD4 absolute counts and CD4% of Lymphocytes for individual participants.

The radar graphs depict the longitudinal follow-up of SDI values (X-axis; range of -3 to +3) versus the trial number (Y-axis; range of 1-3) (Figure 4.2). Radar graphs allowed visual representation of performance during consecutive trials in one snapshot view showing point of enrolment, non-submission of results or switched to alternative technologies. In addition, this type of graph depicted improvement or problems in accuracy and precision over time by clearly indicating outliers (13, 20).

Examples of Laboratory with excellent and good performance over time.



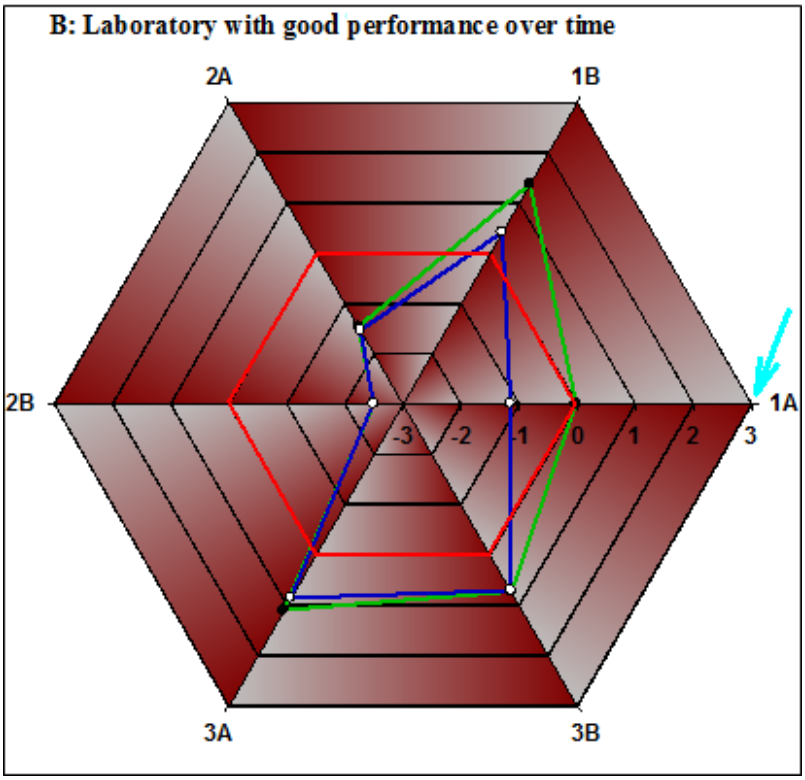


Figure 4.2: Examples of the use of radar graphs to evaluate participant performance over time.

The X-axis is from -3 to +3 and trial numbers are indicated from 1A to 3B on the Y-axis. SDI values are plotted for both the CD4 abs counts (Green line) and CD4% of Lymphocyte values (Blue line) for a particular laboratory (B) and only CD4 absolute counts for laboratory (A). The blue arrow at 1A indicates when the laboratory enrolled on the CD4 EQA scheme. The target SDI of 0 (red line) is indicated for comparison of data to the expected target.

4.11.1. Statistical analysis of overall precision of participant groups

Within each of the three trials, separate sub-analyses were performed, to assess between laboratory precision performances of laboratories using the same methodology. The most commonly used methodology was BDS FACSCount™ throughout the trials.

For each trial, submitted results for both absolute counts and CD4% of Lymphocytes for the BD FACSCount™ group was pooled and a mean, SD, and %CV calculated to assess accuracy and precision of the group of laboratory. The longitudinal precision of this BD FACSCount™ group was analyzed (%CV over the three trials) for both absolute counts and CD4% of Lymphocytes. These results were plotted on scatter plots using Graph Pad Software.

Table 4.3: Summary of outliers and non-submissions, for the four CD4 methodology used by participants across 3 trials of the CD4 EQA scheme using In-house PT panels.

	Absolute counts		**CD4% Lymphocytes**	
Methodology	**% Outliers**	**% Non Submissions**	**% Outliers**	**% Non Submissions**
BD FACSCount™	2.04	0.0	1.92	0.00
BD FACSCalibur™	6.25	0.0	6.25	0.00
Pima™ CD4	16.67	0.0	NA	NA
Partec® Cy Flow	0.00	0.0	NA	NA

(NA = not applicable as these methods could not generate a CD4% of lymphocytes at the time of the study).

4.11.2. Cumulative assessment of longitudinal precision of BD FACSCount™ users

To assess the longitudinal accuracy and precision of a group of laboratories using BD FACSCount™, the standardized individual laboratory SDI values were pooled across the first 3 trials. Thus a mean (SDI) was calculated per trial. These mean (SDI) values were used in two ways.

(i) Radar plots show the mean of the pooled SDI values of a BD FACSCount™) users, plotted trial by trial. (ii) The mean (SDI) values were plotted using Graph Pad Prism software in a Gaussian distribution plot. In this pooled SDI analysis, the calculated mean of the pooled SDI's, i.e., the mean (SDI) reflects longitudinal accuracy within BD FACSCount™ users. The SD (SDI) of the mean of the SDI namely, SD (Mean SDI), reflects longitudinal precision. In this analysis, ideal accuracy mean (SDI) values should be expected to be 0.0 with an expected range of -1 to +1. Ideal precision SD (Mean SDI) values should be expected to be 1.0 with an expected range of 0.0 to 1.0. Results were plotted as Gaussian distribution graphs for visual interpretation with acceptable ranges indicated and outliers identified as values outside these ranges.

4.12. Ethical Consideration

Ethical clearance was obtained from the School of Medical Laboratory Sciences, College of Health Sciences, Addis Ababa University and Addis Ababa City Administration Health Bureau, and a letter was submitted to each participating laboratories found in Addis Ababa to get

permission for participation. A unique participant code number was used throughout the trials to maintain their confidentiality (Annex E2).

5. RESULT

5.1. Study participants

The inter-laboratory CD4+ Count proficiency testing programme using In-house PT panel was expanded gradually from first to third trial. Since the inception of the prodramme in December 20, 2012, the number of participants in the scheme had increased from 20 to 27 participating laboratories (almost all laboratories in Addis Ababa) by Trial 3 in July 2013, representing an average monthly growth rate of the scheme of 11.9% (Figure 5.1). However, 2 laboratories were not participated by trial 3 due to lack of BD FACSCount reagents. As a result, 25 laboratories were participated in the programme by the end of the trial in Addis Ababa, Ethiopia.

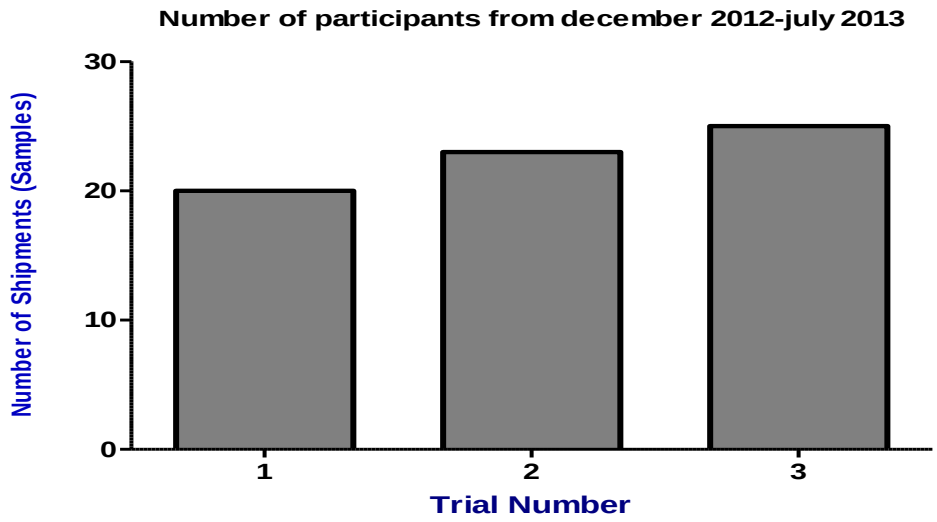


Figure 5.1: A graphical representation of the growth of the inter-laboratory CD4 Proficiency testing programme from December 2012 to July 2013 (Trial 1-3).

5.1.1. Participant Response Rate

The inter-laboratory CD4 proficiency testing programme had issued 3 trials by the end of 2013, equating to 136 In-house prepared PT panels of which 68 were “normal” CD4 value materials and 68 “low” CD4 value materials. The response rate, defined as the number of returned results

divided by the number of shipped samples multiplied by one hundred to obtain a percentage, with an average response rate of 100% was noted. A summary of the user's outlier and participation rates per trial is outlined in Table 5.1.

Table 5.1: Participant numbers, percentage increase, response rates and outlier rates over 3 trials

Phase	Level	Trial №	Shipped samples (n)	% increase since previous trial	Response rates (%)	Outlier rates (%)	
						CD4 Absolute Count	CD4 % of Lymphocyte
One	local	1A	20		100	0.00	0.00
		1B	20		100	5	0.00
Two	Local	2A	23	15	100	4.3	8.3
		2B	23	15	100	8.7	8.3
Three	Local	3A	25	8.7	100	0.00	7.7
		3B	25	8.7	100	8	0.00

5.2. Relevant information from participant Data Report Forms

5.2.1. Sample Condition and Result submission

There was positive feedback on the sample condition. No samples were reported hemolysed, contaminated, clotted or insufficient across the three trials. All returned results were received by or before the due date. Feedback was not forthcoming from laboratories citing confidentiality constraints.

5.2.2. Flow Cytometer and Antibody Panel Used

Eighty three point six percent (83.6%) of participants used Beckton Dickinson (BD). From these 11% and 72.6% of participants used FACSCalibur™ and FACSCount™ respectively. Four percent (4%) of participants used the volumetric Partec® based instruments, while the remaining 12.4% of participants used Pima^{TM} CD4. Throughout the Trial, all of the participants used single platform methodology. Eleven percent (11%) of participants used lyse no wash methods and 89% used a no wash no lyse method.

Seventy two point six percent (72.6%) of participants used 2-color immunofluorescence method (BD FACSCount), and 16.4% of participants used one color method: Partec cyflow and Pima™ CD4, and the other 11% of participants used 3 color method (BD FACSCalibur).

5.2.3. Bead Product Used

Eleven percent (11%) of participants used BD FACSCalibur™ with TruCOUNT™ beads. Seventy two point six percent (72.6%) of participants used bead product supplied in ready prepared kit. This prepared kit comprised of both single (53%) and two (47%) tube format. Four percent (4%) of participants used Partec GmbH, M¨ unster, Germany product. The other 12.4% of participants used PimaCD4 test Cartridge from Alere™ and Pima™.

5.2.4. IQC and Pipetting method

All BD FACSCount™ users undergo Control run with new reagent lot number. Most of the participants described that manufacturer user's guide and SOPs are well followed especially during quality control run but regular training is not established for the persons responsible for CD4 count. Some laboratories have experience in EQA which was coordinated by EHNRI. Almost all BD FACSCount™ users used manual pipettes. BD FACSCalibur™, Partec®, and pimaCD4 used manual pipetting methods. Twelve percent (12%) of laboratories calibrated their pipettes either once or twice a year or 88% do not calibrate at all.

5.2.5. Workload of laboratory

Almost all of the participants stated the number of CD4 samples tested per day, week, and month in their respective laboratories. Except some laboratories that used Pima CD4 which have low patient flow, the average number of CD4 samples tested in the participant laboratories per day were approximately 30. Pima™ CD4 users run from 28-168 samples per month, BD FACSCount™ users run from 56-1176 per month.

5.3. Assessment of CD4 EQA material

In the first trial, two different EQA materials were evaluated, by distributing to 20 participants to ascertain which product is most suitable for future trials. Stabilized whole blood that was prepared at MBL was distributed to 20 laboratories but most of the flowcytometry cannot read the stabilized materials as reported by the participants. As a result, we stopped it from any another steps. Instead, we prepared and dispatched a fresh whole blood obtained from one donor

to all participants and the results were more satisfactory. This coincides with works cited in another parts of the world like in Milan, Italy (46).

Table 5.2: In-house prepared PT panels in the pilot study

	ABSOLUTE CD4 COUNTS				**CD4 % OF LYMPHOCYTES**			
Whole blood								
Trial №	Pool mean	SD	%CV	N(Labs)	Pool mean	SD	%CV	N(Labs)
1A	632.9	49.8	7.9	20	35.8	0.8	2.2	9
1B	280.4	28.9	10.3	20	34.2	1.1	3.4	9
	Mean %CV		9.1		Mean %CV		2.8	

Tighter between-laboratory precision was demonstrated with in-house prepared Normal and Low PT panels (Table 5.2). Therefore, this in-house prepared materials showed acceptable overall precision between laboratories and were deemed to be the better product for distribution, in line with previously published works (46).

5.4. In-house prepared PT panel stability

As shown below (Table 5.3 and Figure 5.2), transport and temperature did not affect In-house PT panel material stability in this audit. Samples tested at ambient temperature were in transit for 1 day (from MBL to all participants and back to MBL at the end of the shipment day).

Table 5.3: A summary of trimmed SDI values for low and normal CD4 absolute counts stability of EQA material at ambient temperature

CD4 EQA pooled trimmed SDI Results					
		Normal abs CD4 Counts		**Low Abs CD4 count**	
Site	**Method**	**Pre-shipment**	**Post-shipment**	**Pre-shipment**	**Post-shipment**
MBL	BD FACSCount	-0.14	-0.18	-0.32	-0.32
MBL	BD FACSCount	-0.54	-0.58	-0.84	-0.91
MBL	BD FACSCount	0.01	0.02	0.86	0.74

Good accuracy and precision for absolute $CD4^+$ counts was reported in keeping with the first audit (Figure 5.2). The mean "normal" CD4 absolute counts was 644.33 and 642 for pre-shipment and post shipment respectively, and the mean "low" CD4 absolute counts was 233.33 and 231.67 for pre-shipment and post shipment respectively.

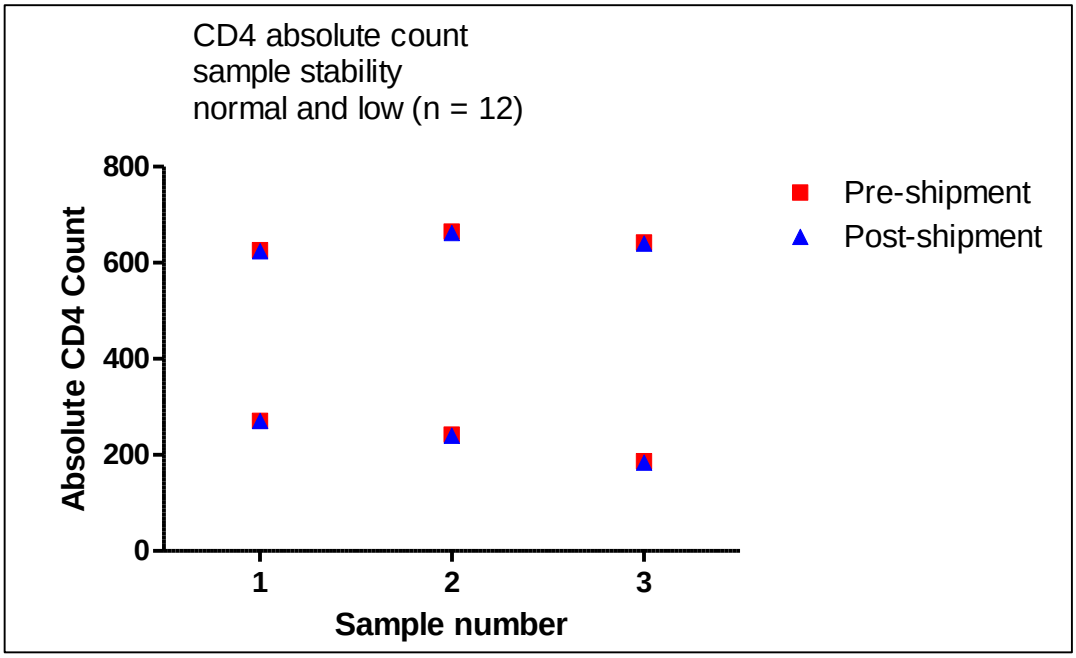


Figure 5.2: Comparison of low and normal CD4 absolute counts used for sample stability audit study at ambient temperature.

5.5. Performance of all participant Laboratories

Over the three trials, the laboratories participating on the EQA Scheme using In-house prepared PT panels showed an average between laboratory precision/reproducibility for absolute CD4 counting of 10.87% CV, ranging between 7.88% (Trial 1A) to 15.49% (Trial 3B). The trimmed mean between laboratory precision for CD4% of Lymphocytes was 5.14% CV ranging between 2.25% (Trial 1A) to 10.44% (Trial 2B).

For the normal level CD4 EQA material, the trimmed mean %CV was 9.59% and 3.23% for CD4 absolute counts and CD4% of Lymphocytes respectively. For the low level CD4 EQA material, the trimmed mean %CV for CD4 absolute counts and CD4% of Lymphocytes was

12.15% and 7.05% respectively. Overall the participating laboratories showed relatively poorer precision (%CV) with "low" material than with "normal" material for both CD4 absolute counts and CD4% of Lymphocytes.

Accuracy was also poorer with low material than with that of the normal material. In trials 1 and 2 only 2 results (from trial 2) were out of ± 2SD with the normal material but 4 results (three from trial 2 and one form trial 1) were out of ± 2SD from low material. In the same manner, one result from normal material and 2 results from low material were out of ± 2SD in trial 3 as well.

Table 5.4: Within trial performance of Laboratories and trimmed results of all methodologies showing precision between participants.

	ABSOLUTE COUNT				**CD4% OF LYMPHOCYTES**			
Trial №	**Mean**	**SD**	**%CV**	**Participant (n)**	**Mean**	**SD**	**%CV**	**Participant (n)**
1A	632.9	49.84	7.88	20	35.79	0.80	2.25	9
1B	280.35	28.89	10.31	20	34.25	1.15	3.35	9
2A	703.91	71.91	10.22	23	36.68	1.70	4.63	12
2B	265.82	28.35	10.66	23	33.63	3.51	10.44	12
3A	641.16	68.55	10.69	25	27.03	0.76	2.81	13
3B	165	25.56	15.49	25	25.14	1.85	7.36	13
Mean	448.19	45.52	10.87		32.09	1.63	5.14	

(*Shaded cells indicate trials with relatively higher trimmed %CV and **A** & **B** represents normal and low material respectively)

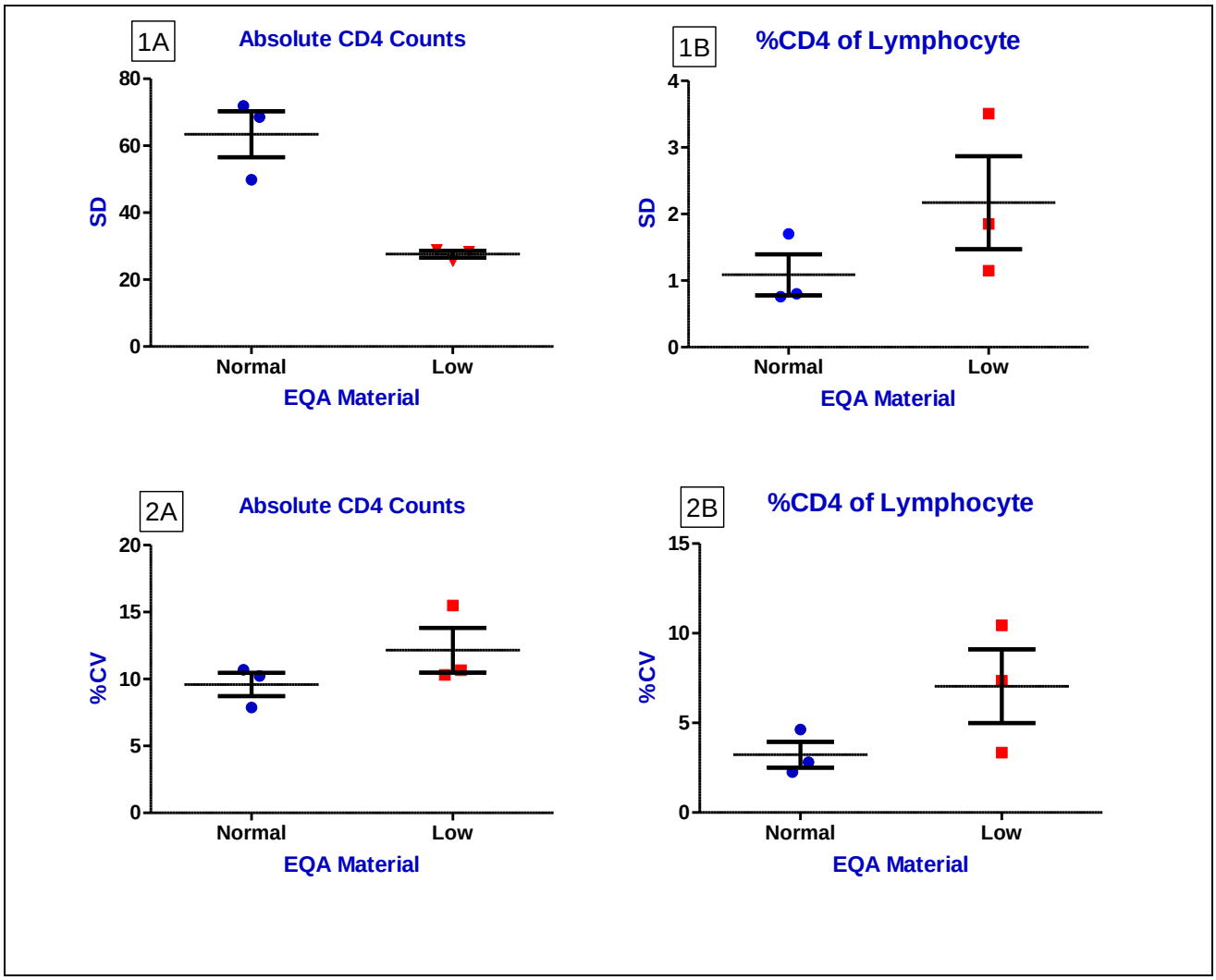


Figure 5.3: Comparison of SD (1A and 1B) and %CV (2A and 2B) values of the first 3 CD4 EQAS trials for both normal and low material. Each dot represents either trimmed SD or %CV from a single trial.

Participant performance was characterized as excellent and good based on the number of outliers and non-submissions. Excellent performers had neither outliers nor non-submissions during their time of participation. Good performers had 2 or less outliers/non-submissions for the duration of their participation on the CD4 EQAS using In-house prepared PT panels. Similar grouping criteria was used elsewhere (1, 13).

Table 5.5: Summary of overall performance of participant laboratories noted post input from CD4 EQA scheme

Performance of total participants across three trials			
	Outliers and non Submissions	**Absolute CD4 counts**	**CD4 % of Lymphocytes**
Excellent	None	81.48	76.92
Good	≤ 2	18.52	23.08

5.5.1. Performance of laboratories reporting CD4 absolute counts

Overall, 81.48% of participants show excellent serial continued performance across 3 trials. Here the methodologies used comprised of BD FACSCount™ users, Pima™ CD4, and BD FACSCalibur™. A single laboratory using Partec CyFlow counter test was also included in this group. A second group of users defined as good performers comprised a further 18.52% (Table 5.5). The methodologies here are mainly Pima™ CD4.

5.5.2. Performance of laboratories reporting CD4% of Lymphocyte

Overall, 76.92% of participants show excellent serial continued performance across three trials. Here the methodologies used comprised of BD FACSCount™ and BD FACSCalibur™ users.
A second group of users defined as good performers comprised a further 23.08% with the same methodologies.

To assess whether after in the groups defined as good performers, a further analysis of the group was undertaken. In the group with "good performance", some of them showed random as opposed to consecutive outliers, 40% participants, after the first trial outliers, showed improvement in the third trial in this group.

5.6. Assessment of individual participant results

5.6.1. Feedback reports

For each participant, the residual value was calculated. Here a negative value implies that the participant was reading lower than the pool mean, while a positive residual value indicated that the participant read higher than the pooled mean. In Figure 5.4 below, the participant showed a

positive residual for all CD4 absolute count parameters. CD8+ Lymphocyte and CD3+ Lymphocyte absolute cell counts were included in the feedback report for those who report it but these are omitted from further analysis since they were optional parameters.

The SDI was within the acceptable ±2SDI limit as shown below. A flagged result automatically raised a need for a corrective action for the concerned laboratory.

					All Methodologies				
Test	**Results**	**Residual**	**SDI**	**> ± 2**	**Method**	**№**	**Mean**	**SD**	**CV**
CD4+ Lymphocyte Absolute cell count/µl	724	91.1	1.83	*	All	20	632.9	49.8	7.9
CD8+ Lymphocyte Absolute cell count/µl	606	72.7	1.53	*	All	10	533.3	47.6	8.9
CD3+ Lymphocyte Absolute cell count/µl	1,461	161	1.6	*	All	10	1,299.8	100.6	7.7
CD4 Lymphocyte %	NA				All	9	35.8	0.8	2.2

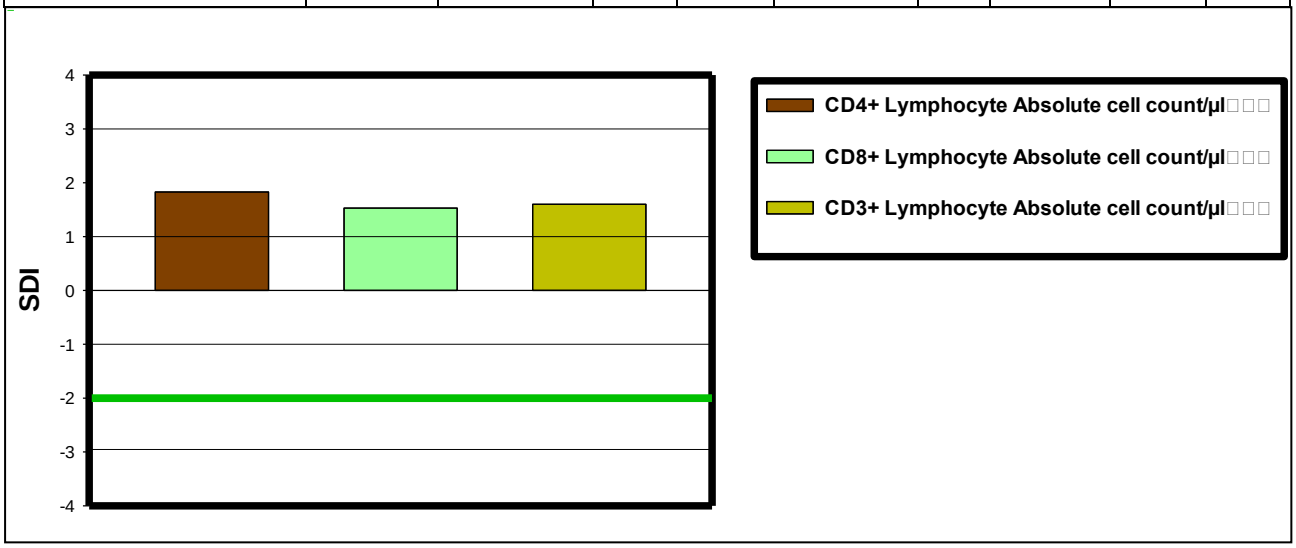


Figure 5.4: Feedback report

5.6.2. Longitudinal performance of individual participants

The results shown in Figure 4.2 are examples of excellent versus a good performing laboratory (irrespective of methodologies used) for the first 3 CD4 EQAS trials using In-house prepared PT panels. Radar graphs (Figure 4.2) were generated to view serial longitudinal performance for

each participating laboratory to identify outliers (>±2SDI) and non-submission of results. These graphs enabled graphic representation of laboratories showing excellent consistency and accuracy of CD4 absolute counts (Figure 4.2A). Due to confidentiality and objectivity and to ensure that all technologies were reflected fairly, no pre-selection of individual participant graphs are represented in this study. However, selected examples were used to illustrate problems (Annex F).

5.7. Precision of Laboratories using the same CD4 Methodology

During the three trials, BD FACSCount™, Pima™ CD4, BD FACSCalibur™, and Partec CyFlow counter was performed as a single platform test but except BDS FACSCount™, their numbers were insufficient for statistical analysis as a group. As a result, they were excluded from group comparison but their performance was assessed together with all participants. The numbers of BD FACSCalibur™ and PimaTM CD4 users were 4 for each methodology by the end of the third trial that is below 10 which are the minimum number of participants to be assessed. Partec® methodology was a single participant throughout the trials. In trial 2, 13.78% CV was recorded which was relatively poor and this was due to a single outlier where one participant from Pima™ users reported a CD4 absolute count of 386 cells/µl for low material. On removing this outlying result (Grubbs' test of outlier applied), the overall trimmed mean for all technology users was 265.82 with a %CV reduced by 3.12% CV.

5.7.1. BD FACSCount™ methodology

Across the three trials, the performance of the BD FACSCount™ participants (comprising 72.67% of participants) for CD4 absolute counts showed consistently good performance (Figure 5.6 and 5.8). The overall mean precision (%CV) was 9.13% and 5.15% for absolute CD4 counts and CD4% of lymphocytes respectively. In trials where normal CD4 value material was shipped, a trimmed mean %CV of 8.58% and 3.07% was recorded for absolute CD4 count and CD4% of lymphocytes respectively. For low CD4 value material, the trimmed mean %CV was 9.67% and 7.22% for absolute CD4 count and CD4% of lymphocytes respectively (Table 5.6).

Table 5.6: Within trial performance and trimmed results of Laboratories using BD FACSCount™ methodology showing precision between participants.

	ABSOLUTE COUNT				CD4% OF LYMPHOCYTES			
Trial №	**Mean**	**SD**	**%CV**	**Participant (n)**	**Mean**	**SD**	**%CV**	**Participant (n)**
1A	644	44.58	6.92	17	35.77	0.86	2.39	8
1B	281.54	26.55	9.43	17	34.03	1.01	2.95	8
2A	713.27	67.27	9.43	15	36.50	1.88	5.15	9
2B	262.93	26.52	10.08	15	32.62	3.42	10.48	9
3A	661.71	62.18	9.39	17	27.09	0.45	1.66	9
3B	164.94	15.68	9.51	17	24.65	2.03	8.24	9
Mean	454.73	40.46	9.13		31.78	1.61	5.15	

The precision of BD FACSCount users for absolute $CD4^+$ counts and percentage of CD4 lymphocytes is shown in Figure 5.5 and 5.7 below.

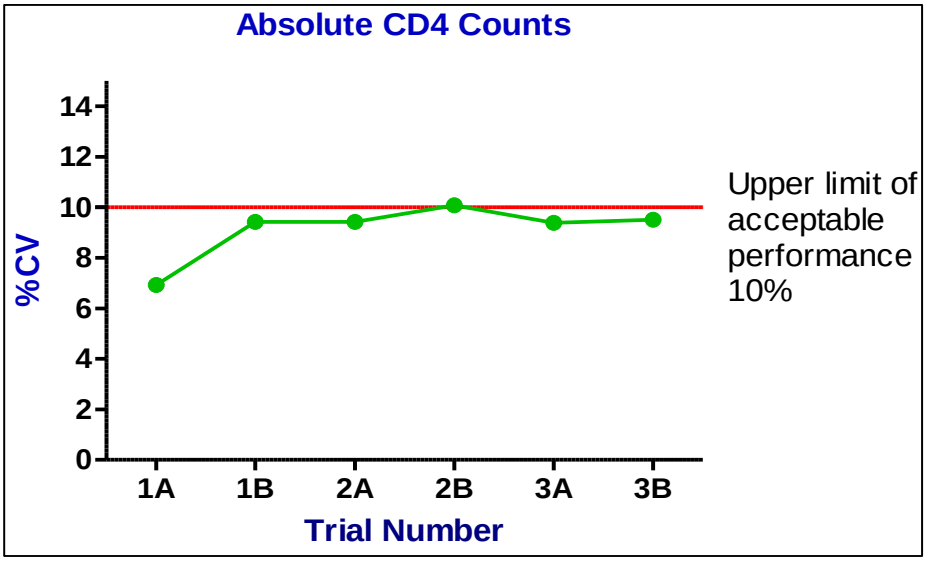


Figure 5.5: Precision between laboratories using BD FACSCount for CD4 absolute counts over 3 trials

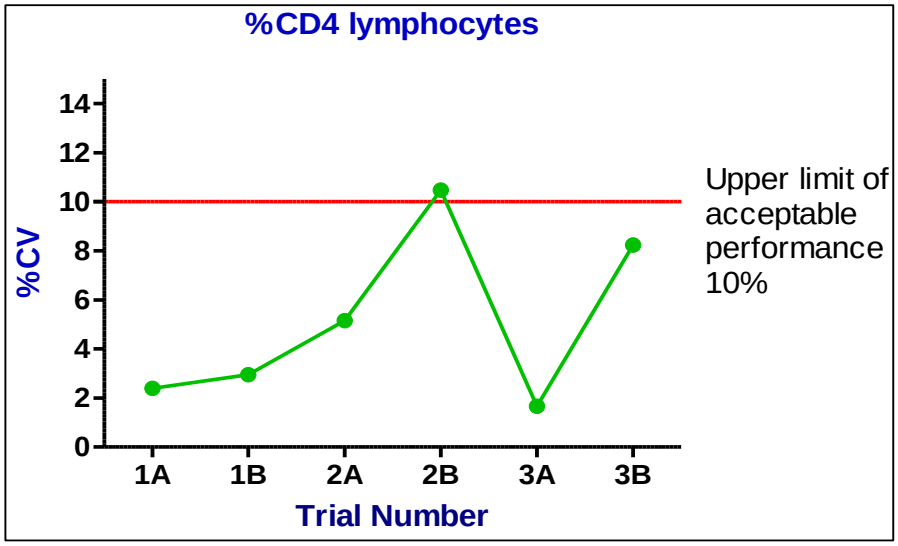


Figure 5.6: Precision between laboratories using BD FACSCount for CD4% of lymphocyte over 3 trials

The following Figure 5.7 shows the average precision of BD FACSCount users for both normal and low materials across the three trials

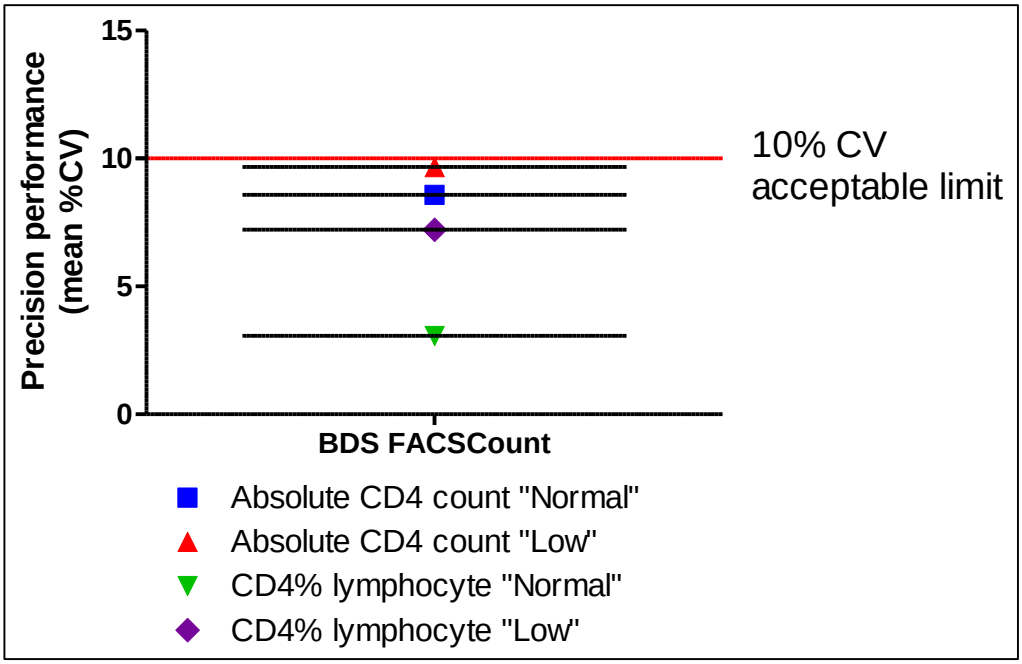


Figure 5.7: The average precision (mean %CV of 3 trials) of laboratories using BD FACSCount, split into normal and low levels of EQA material.

5.8. Longitudinal precision of laboratories using BD FACSCount™

To assess the longitudinal accuracy and precision of BD FACSCount™ users the standardized individual laboratory SDI values were pooled across 3 trials and a mean (SDI) was calculated per trial. Radar plots showed the mean of the pooled SDI values, plotted trial by trial. Separate analyses were performed overall and for normal and low EQA material data. All submitted results (including outliers) were used. The first analysis plots the Mean SDI, trial by trial, in a radar plot distribution (Figure 5.8). The second analysis plots these same Mean SDI values but instead in Gaussian distribution to view the spread of the data (Figure 5.9-5.10). Gaussian distribution of the Mean (SDI) values gives an indication of overall accuracy, whilst the spread of the data reflects precision i.e. the SD (of the Mean SDI).

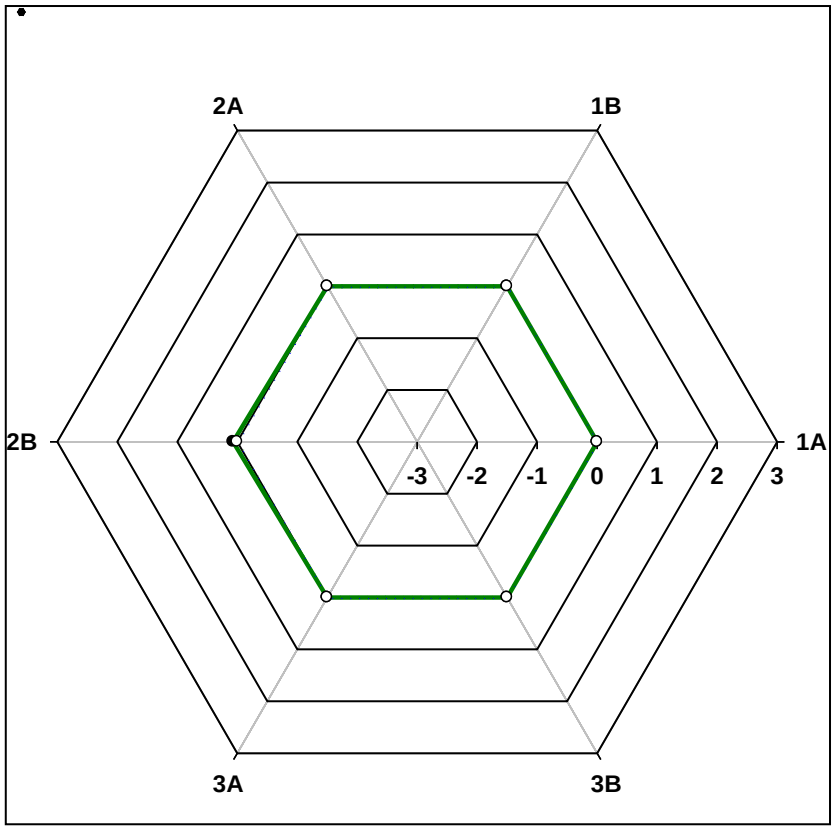


Figure 5.8: Radar plots illustrating the longitudinal performance (SDI) of BD FACSCount™ methodology across the three trials.

SDI values were plotted for both the absolute CD4 count (Green line) and CD4% of lymphocyte values (totally obscured by the target line because they have same value i.e. zero). As a result; it is hidden.

Pooled SDI data reflecting longitudinal accuracy and precision across three trials showed that laboratories using BD FACSCount™ could consistently generate accurate and precise absolute CD4 counts irrespective of whether normal or low value EQA material was used (Figure 5.9).

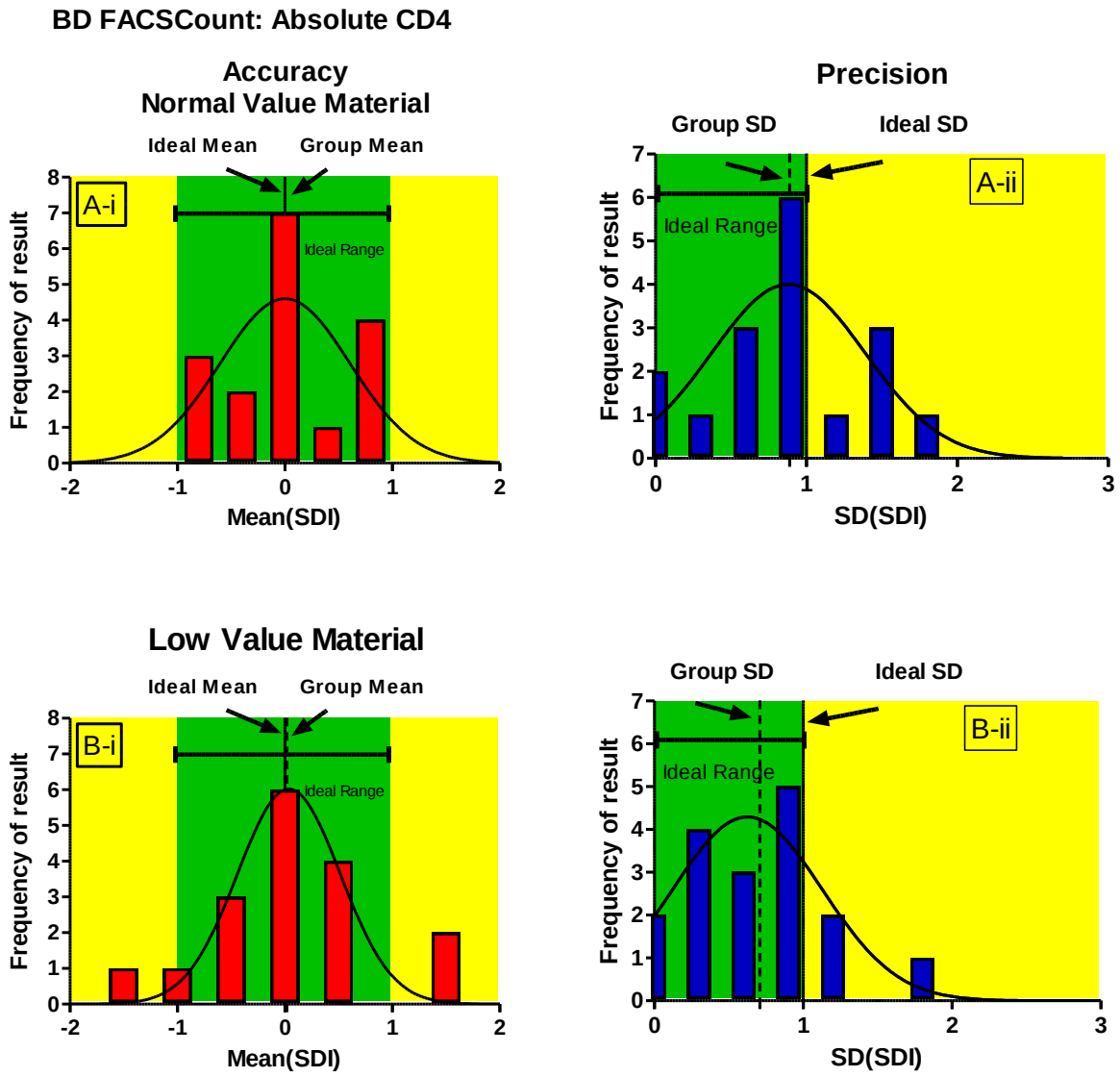


Figure 5.9: Gaussian distribution curves showing longitudinal accuracy (mean SDI, Ai and B i) and precision (SD of mean SDI), Aii and B ii) for BDS FACSCount™ users for CD4 abs counts

Green areas represent the acceptable e limits for both Mean SDI and SD of (mean SDI) values, while yellow areas indicate values outside the acceptable limits. Ideal values for both mean SDI (0) and SD of (mean SDI) (1) are indicated.

The distribution curves were tight and majority of results indicated good longitudinal accuracy and precision for absolute CD4 counts (in green area, Figure 5.9 above). On low EQA material, the longitudinal follow-up showed slightly poorer accuracy (mean SDI) and precision than with normal EQA material. This however did not impact on the overall good performance of this methodology on the CD4 EQA scheme.

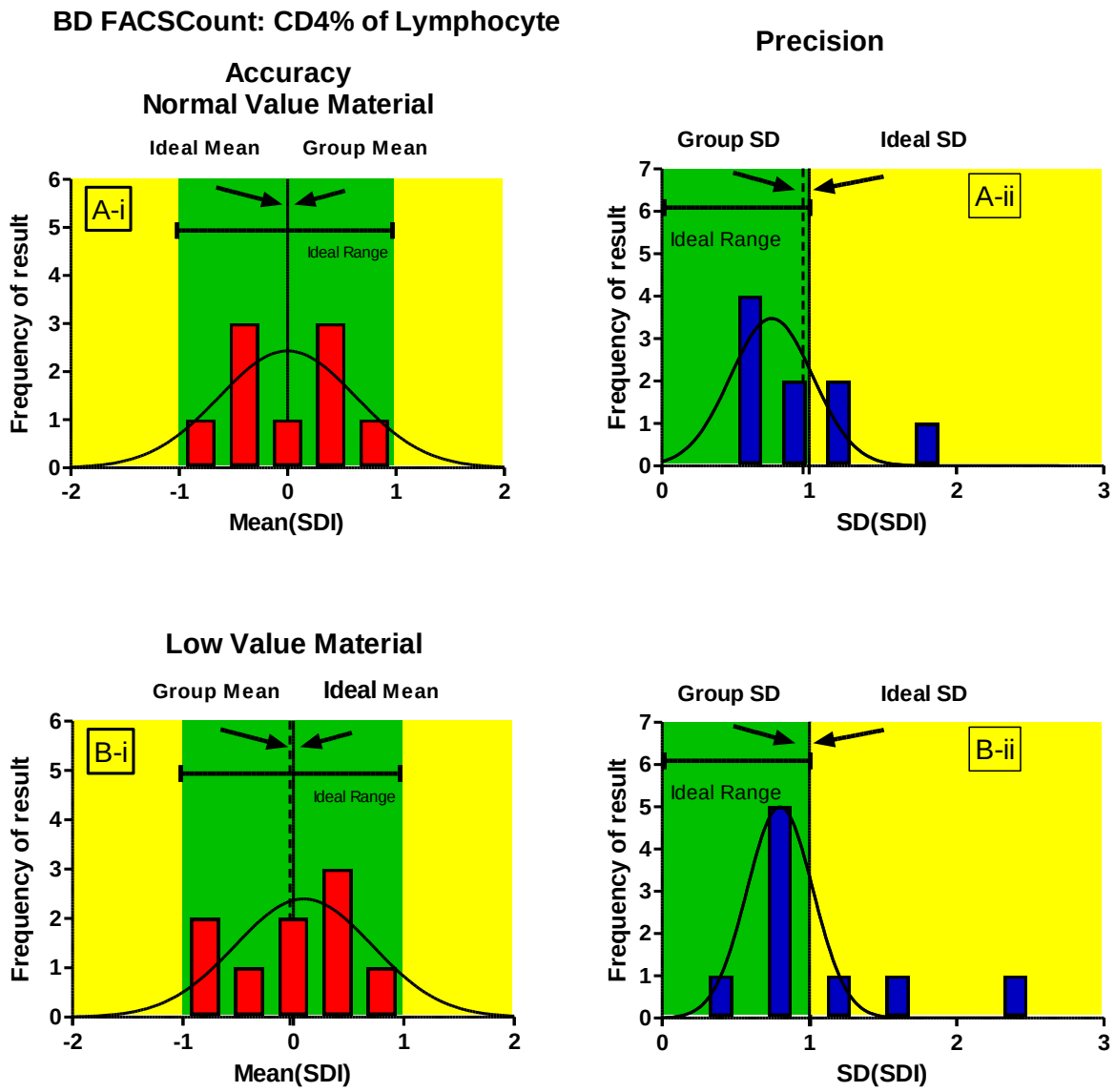


Figure 5.10: Gaussian distribution curves showing longitudinal accuracy (mean SDI, Ai and B i) and precision (SD of mean SDI), Aii and B ii) for BD FACSCount™ users for CD4% of lymphocyte.

Green areas represent the acceptable limits for both Mean SDI and SD of (mean SDI) values, while yellow areas indicate values outside the acceptable limits. Ideal values for both mean SDI (0) and SD of (mean SDI) (1) are indicated.

On low EQA material, the longitudinal follow-up showed slightly poorer accuracy (mean SDI) and precision than with normal EQA material. This however did not impact on the overall good performance of this methodology on the CD4 EQA scheme. Only one trial showed relatively poorer precision (Trial 2). This was due to one outlier where one participant reported CD4% lymphocyte for EQA sample (Trial 2B) of 24.92% (pool mean 32.62).

5.9. Investigation of laboratories with outlying results

An outlier was defined as a submitted result falling outside of ±2SD of the trimmed pool mean. The percentage outlier rate of absolute CD4 counts and CD4% of lymphocytes for all trials were 4.3% and 4.1% respectively (Table 5.1). The percentage outlier rate for normal CD4 EQA material was 1.4% and 5.3% for absolute counts and CD4% of Lymphocyte respectively. The percentage outlier rate for low CD4 EQA material was 7.2% and 2.8% for absolute counts and CD4% of Lymphocyte respectively. The percentage outlier rate and non-submission of the four methodologies is summarized in Table 4.2.

Overall BD FACSCount™ and BD FACSCalibur users showed the least outliers across 3 trials with an average of <5% and <7% respectively. The Pima™ CD4 user group showed higher outliers across the three trials with an average of <17%. The single Partec® user laboratory had no outliers and non-submission of results across the first 2 trials.

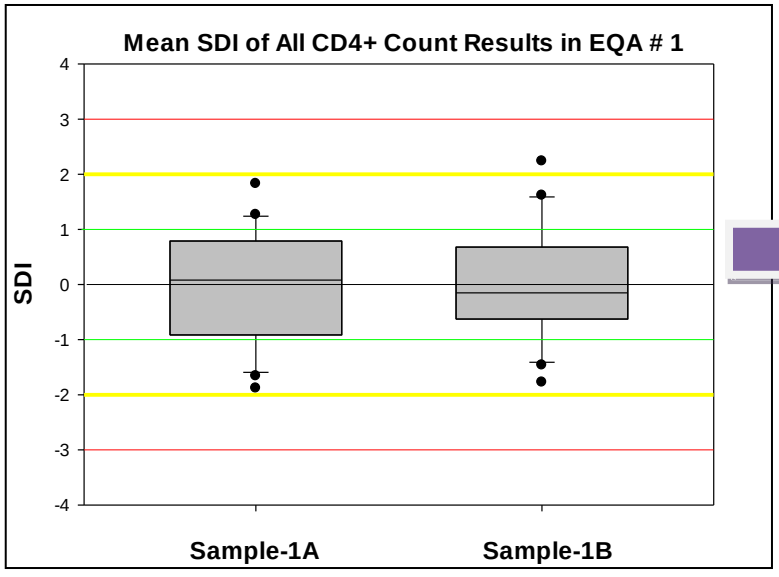


Figure 5.11. Box and whisker plot showing outlier of participant's SDI (>±2SDI) across three trials of the EQAS using In-house PT panels with Normal and Low values

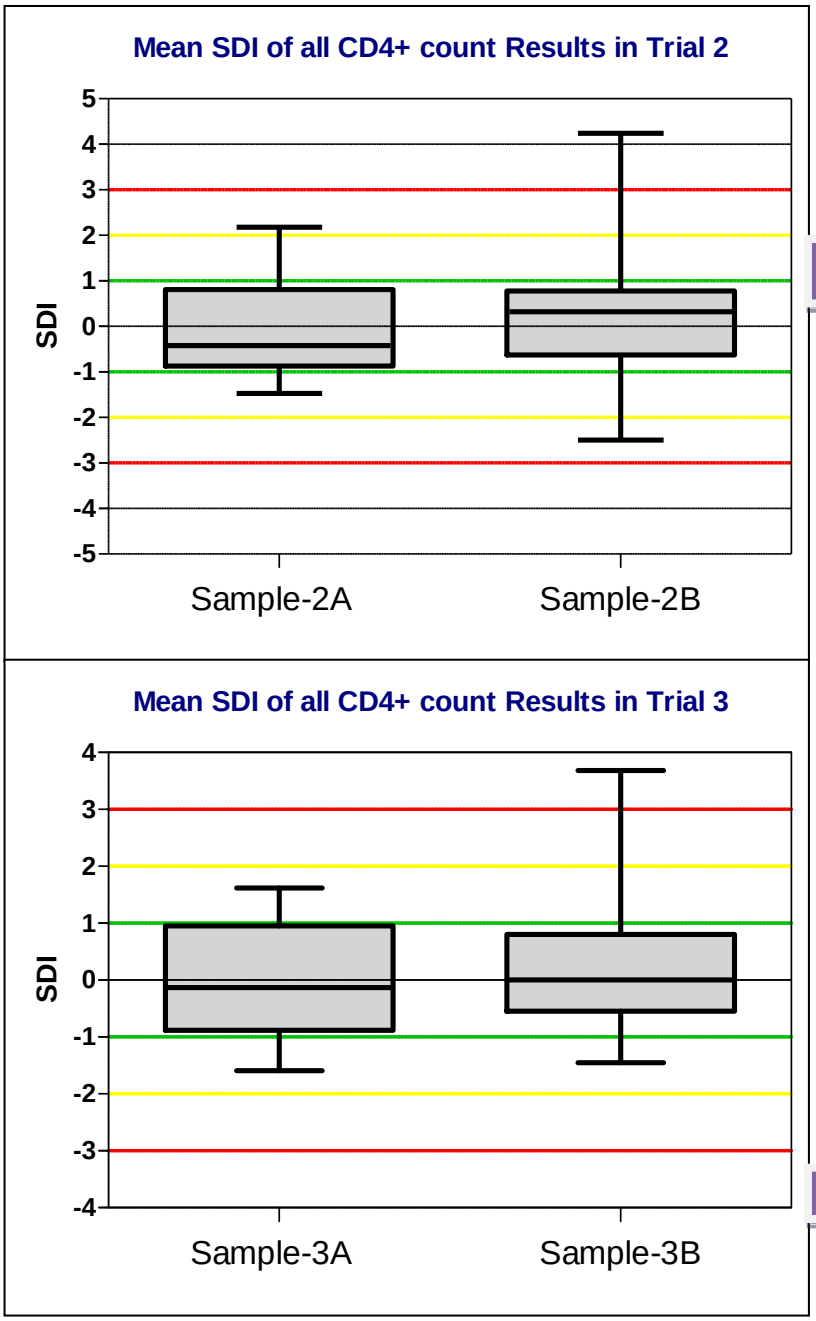


Figure 5.11. Box and whisker plot showing outlier of participant's SDI (>±2SDI) across three trials of the EQAS using In-house PT panels with Normal and Low values .

5.10. Effects of Feedback for Remedial Action

Feedback was individualized and remedial input given was specific to the problem of the CD4 method used by that site. These include appropriate feedback and advice on corrective action. An outlier on CD4 absolute counts was noted on Trial 1 which fell outside the acceptable ±2SDI limits from submitted participant laboratory results on the CD4 EQA scheme. The laboratory

was subsequently contacted to investigate the inaccuracy and imprecision of pipetting through the validation of pipettes based on the BDS FACSCout user's guide. .

With corrective action feedback to this laboratory, consistently reported with no outliers recorded for the last two subsequent trials.

6. DISCUSSION

With the arrival of HIV/AIDS, CD4 count laboratories and their professional personnel in Ethiopia have a huge cross to bear. This load can be lightened by establishing and consolidating quality laboratory management practices in laboratories that are performing CD4 counting in the country. This local capacity-building and ‘‘empowerment’’ is crucial to long-term sustainability (12, 13). The need for quality testing, recognition of the impact of EQA scheme (EQAS) participation and feedback on improved performance of laboratories participating on such programmes (13, 19, 20, 21, 46) and the absence of national CD4 EQA programme in Ethiopia was initiated to carry out CD4 EQA program using In-house prepared PT panels.

Consistent and accurate performance can be achieved where there is well-planned investment by governments or grant-funding agencies to organize technical training initiatives and implement quality control (1, 5, 13). This includes establishment of local CD4 EQAS supported by local and regional expertise with technical training programs (13, 15, 19, 21). The impact of constructive feedback to CD4 EQAS participating laboratories with a plan of remedial action has been documented in this report and elsewhere (3, 4, 10, 13, 15, 19-24).

During the three trials of CD4 EQAS over a period of 6 months, the overall participation rate was excellent with average 93.1%. The average response rate was 100%. Overall, reasons for 100% response rate were due to consistent information exchange between the CD4 EQAS coordinator and managers of laboratory participants. During each trial, EQA materials were shipped after the participants have made ready to carry out the test. In addition to this, results were collected from each participant sites by the EQAS coordinator throughout the trials.

The other 6.9% of potential laboratories were not participated. This was because participants don't want to use their reagents to participate in the programmme. Another reason for this problem were poorly developed flow-cytometry maintenance, lack of BD FACSCount Reagents,

lack of supplier and vendor availability for support and maintenance of equipment, unreliable power supplies, and poorly developed communication transport and custom clearance infrastructure.

The average precision of all participant laboratories across the three trials was 10.87 and 5.14 %CV for absolute CD4 counts and CD4% of Lymphocytes respectively. This performance is better than the overall precision (%CV) reported on the AFREQA scheme across 20 trials of 11.9 CV% for absolute CD4 counts and 10.7 CV% for CD4% of Lymphocytes (13). This can be due to different reasons. In this study, In-house PT panels were shipped within the same day of preparation to participants but in case of AFREQAS transportation of EQA materials to participants that are found in different countries take long time. As a result, the commutability of materials can be reduced through time and temperature fluctuation. In AFREQAS, the huge numbers of participants were used single and dual platform technology that may increase the %CV but in this study all participants were used only single platform technology.

Another study done on An Inter-laboratory Study of the FACSCount System using Peripheral Blood of HIV-Infected Patients has reported similar improved precision mean CV of less than 10% (7).

It is a little beat higher than the overall precision %CV reported on the EQA Program on CD4+ T-Lymphocyte Counts for Persons with HIV/AIDS in Thailand across 30 trials of less than 8% for CD4 T-lymphocytes (20). According to information obtained from participant's "data return form" in CD4 EQA programme using In-house prepared panels, most of the participants do not calibrate their pipettes and in a few laboratories mismatched reagent lot number (ID) and control run reagent lot number (ID) was found. This is probably the reason for the increased %CV.

Two levels of CD4 count In-house PT materials were shipped throughout the CD4 EQAS trials (Table 4.1). With the "normal" and "low" value CD4 EQA material, similar findings were reported in other studies (13, 18), with overall slightly poorer precision with "low" count material than "normal" count material amongst participants. For "normal" count material, the trimmed %CV was below 10% CV for both CD4 absolute counts and CD4% of Lymphocytes. For "low" count material, the trimmed %CV was greater than 10% CV (12.15%) and 7.05% for CD4 absolute counts and CD4% of Lymphocytes respectively (Table 5.4).

BD FACSCount™ group showed very good between-laboratory precision with a mean %CV of less than 10% CV for CD4 absolute counts and CD4% of lymphocytes over three trials. In each of the trials, the % CV was below 10% CV. In only one trial (Trial 2B) was greater than 10% CV seen, with a 10.08 and 10.48%CV for absolute and CD4% of lymphocytes respectively (Table 5.6). This is similar to findings reported elsewhere (7, 13).

Overall accuracy and precision of BD FACSCount™ group was also excellent as evidenced by the tight Mean SDI and narrow SD (of the Mean SDI) noted in the longitudinal analysis (Figures 5.8-5.10). This excellent performance was maintained irrespective of whether "normal" or "low" material was used and a mean %CV of less than 10% CV were recorded for both normal and low CD4 absolute counts across three trials. In the same manner, for Normal and Low materials, a mean % CV of less than 8%CV were recorded for both normal and low CD4% of lymphocytes across three trials (Table 5.6).

In the first 2 trials, 2 results from FACSCount™ users were found out of ±2SD but in the subsequent trials their results were corrected and found within ±2SD. One error however, common to several BD FACSCount™ users, was related to incorrect pipetting and/ or the use of poorly calibrated pipettes, also reported elsewhere (7, 13). In this instance, the impact of advice given to improve pipetting skills by focusing on within-laboratory precision of individual technicians or advice on calibration of pipettes proved to be invaluable to these laboratories.

The other groups of users were BD FACSCalibur™ and Pima™ CD4 which were insufficient in number with a single user of Partec Cy Flow. Although the number of users was small, as a group, the laboratories using Pima™ CD4 method did not perform well. From these laboratories, 3 results were found out of ±2SD of the mean in the last 2 trials from the total number of eight Pima™ CD4 users in the same trials. The single Partec Cy Flow user results were within ±2SD in the first 2 trial but it was failed to participate in the last trial due to instrument problem which was associated with poorly developed on time maintenance. In trial 2, one result was found out of ±2SD from the total number of 3 BD FACSCalibur™ users.

A longitudinal statististical analysis was performed comprising all individual standardized SDI results, pooled across three trails, to assess mean SDI of laboratories grouped according to the CD4 methodology used (Figures 5.9, 5.10). BD FACSCount™, the most commonly used

methodology over the three trials, was analyzed using this model (Figures 5.9, 5.10). In contrast to the precision (%CV) within individual trials where data was trimmed (Figures 5.5, 5.6), this longitudinal analysis includes all results, as well as outliers, to give an overview on the true precision between laboratories using the CD4 methodology. It further facilitates insights into how much error is associated with the use of a particular technology and training needs (13, 20).

The group of laboratories that showed a mean (SDI) close to 0 and SD (Mean SDI) within the range of 0 - 1 were demonstrated to have the best accuracy and precision. In this analysis, two groups emerged: one group showing laboratories with tighter between laboratory precision suggesting less additional training needs (predominated by FACSCount™ users) and a few second group showing laboratories with wider between laboratory variations with more training needs (predominated by users of Pima™ CD4).

As confirmed in the individual trial analysis where within trial precision of the group was excellent (Figure 5.8), most of the laboratories using the BDS FACSCount™ technology showed mean SDI values close to 0 and SD of SDI values within the range of 0-1, indicating that these laboratories were able to consistently submit reliable and accurate results.

Overall, the performance of the CD4 EQAS laboratories in Addis Ababa is similar to that reported elsewhere (13, 7, 20). However, few of the CD4 methodologies specifically one Pima™ CD4 showed inconsistent result between trials. The probable reasons for this problem are several folds. In Addis Ababa, Pima™ CD4 users were small in number and it was difficult to evaluate as a group users.

In addition, these technologies were new and three of the Pima™ CD4 users were incorporated at the second trial of CD4 EQA scheme using In-house prepared PT panels. These technologies were allowed to give service for patients as they purchased and were not validated in house in comparison with other methods previously used for CD4 count in Ethiopia such as BD FACSCount™ (almost throughout the country) and BD FACSCalibur™ (in a few sites). The other difficulties with new coming methods is the laboratory technicians/technologists were not trained about the usage and maintenance of the machines rather deputies of the seller (vendor) company installs it in the laboratory and shows them how they perform at the first time of purchasing.

The findings obtained in this study are similar to findings reported in other studies (1, 4, 13, 15, 18, 20, 22, 26). Actually this programme was done in a short period of time (8 months) relative to other programmes done elsewhere (13, 15) but it was helpful in initiating improvement of individual and between laboratory variation among the participants as it was the first CD4 EQA scheme in Ethiopia.

In summary, CD4 EQA programme using In-house prepared PT panels was helpful in avoiding the challenges encountered during participation in external EQA providers like the high cost, logistical problems with sample transportation, feedback delay, and CD4 laboratory coverage.

7. LIMITATION OF THE STUDY

There are some limitations to this program. Firstly, unlike that of other CD4 EQA schemes done elsewhere with multiple number of trials, this local CD4 EQA programme was done in a short period of time (8 months). This was due to lack of sponsors since it needs a huge amount of budget for its continuity and sustainability.

Secondly, corrective action feedback was solely based on advising and providing guidelines but training was not given even if many requests were sent from participating laboratories. This was due to limited inputs and lack of sponsorship.

Thirdly: at the beginning of the study it was proposed to incorporate commercially donated Lymphosure stabilized materials in addition to in-house prepared PT panels in this programme but due to problems associated with transportation (import) it cannot carried out as was proposed.

8. CONCLUSION

The aim of the study was to assess the performance of participating CD4 laboratories using In-house prepared PT panels and establish the requirements for local CD4 EQA implementation in Addis Ababa, Ethiopia. The pilot-feasibility of preparation of in-house PT panel for CD4 enumeration was assessed and found to be satisfactory. Participant laboratory results were treated statistically to assess their performance in each trial and for each participant and longitudinal cumulative trial data were analyzed.

Over the three trials, the laboratories participating on the EQA Scheme using In-house prepared PT panels showed an average between laboratory precision/reproducibility of 10.87% CV and 5.14% CV for absolute CD4 counting and CD4% of Lymphocytes respectively. Most BD FACSCount users showed excellent accuracy and precision across the trials. The overall mean precision (%CV) was 9.13% and 5.15% for absolute CD4 counts and CD4% of lymphocytes respectively. Other group users were not sufficient in number to be treated statistically as a group.

Our CD4 EQA program using In-house prepared PT panels was helpful in early identifying of participant's gaps with regard to their CD4 count performance thereby in improving the reliability of CD4+T-lymphocyte determinations. This is becoming increasingly important as Ethiopia scales up its national ART access program for persons living with HIV/AIDS. CD4 EQA program using In-house prepared PT panels may be considered as a significant advancement for CD4 count laboratories in Addis Ababa and at national level by being used as a baseline, particularly as no such program had been available previously.

Above all, CD4 EQA programme using In-house prepared PT panels is helpful in avoiding being dependent externally. Because participation in externally provided EQA scheme is difficult in terms of cost, transportation, coverage of all laboratories, on time feedback, and communication. As a result, the scheme has been widely accepted by participant laboratories who have found that the CD4 EQAS scheme using In-house prepared PT panels help them to implement a better CD4 count service through early identification of their gaps regarding to their CD4 count performance.

To conclude, even if the scheme was carried out in short period of time relative to other EQAS like AFREQAS, it was helpful in avoiding being dependent externally and early identification of participant's gaps regarding to their CD4 count performance.

9. RECOMMENDATION

In Ethiopia, there is no established national CD4 EQA scheme; as a result, it is best to expand and continue the CD4 EQA programme using In-house prepared PT panels and by making it

stabilized at national level in a sustainable way by giving emphasis on its impact towards the improvement of CD4 laboratory results.

When laboratories install new CD4 count technologies, it is best to validate their new CD4 count technology in comparison with other reference CD4 methodology like BD FACSCount in order to obtain and release accurate and reliable CD4 count results.

CD4 EQA scheme using In-house prepared PT panel needs a huge amount of budget for its continuity as a sustainable programme; then government and other concerned body should give emphasis for its expansion and continuity at national level to help our HIV/AIDS patients by improving the CD4 count laboratories performance through regular and continued participation.

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11. ANNEXES

Annex A. principles and types of flow cytometry (48-51)

1. BD FACSCount System

Principle

The BD FACSCount system is designed to use unlysed whole blood, collected in EDTA. When whole blood is added to the tubes of a sample reagent pair, the fluorochrome-labeled antibodies bind specifically to antigens on the surface of lymphocytes, and a fluorescent nuclear dye binds to the nucleated blood cells. After a fixative solution is added to the reagent tubes, the sample is run on the instrument. The BD FACSCount system employs a two-colour immunofluorescence method for enumerating absolute lymphocyte counts (cells/µl whole blood) of CD3+ T-lymphocytes, CD3+ CD4+T-lymphocytes and CD3+CD8+ T-lymphocytes. CD3 cells fluoresce red and CD4 and CD8 cells fluoresce yellow when analysed on the instrument. In addition, the CD4:CD8 ratio is provided. A known number of reference beads is contained in each reagent tube and functions as fluorescence and quantitation standard for calculating the absolute counts T-lymphocytes.

Procedure for Double Reagent Pair

- Vortex reagent tubes for 5 seconds.
- Pipette 50µl of whole blood into each tube of the reagent pair and vortex.
- Incubate the tubes at room temperature for 60 – 120 minutes in dark.
- Add 50µl of fixative solution into each tube and vortex.
- Run the tubes on the instrument.

Note: Stained samples may be stored for up to 24 hours before running on the flow cytometer at temperature 20 to 25^0C

Procedure for Single Reagent Tube (CD3/CD4 only)

- Vortex reagent tubes for 5 seconds.
- Pipette 50µl of whole blood into the reagent tube and vortex.

- Incubate the tubes at room temperature for 60 – 120 minutes in dark.
- Add 50µl of fixative solution into each tube and vortex.
- Run the tubes on the instrument.

Note: Stained samples may be stored for up to 24 hours before running on the flow cytometer at temperature 20 to 25^0C.

2. BD FACSCalibur

Principle

BD FACSCalibur is a flow cytometer which is capable of measuring the scatter and the fluorescence parameter. It can detect the scatter parameter namely the forward and the side scatter which gives information about the size and granularity of the cell. The BD FACSCalibur can detect up to 3 fluorescence parameters. It can measure both absolute CD4 + T-lymphocyte count as well as % CD4 count.

Recommended antibody panels

BD TriTEST™ CD3 fluorescein isothiocyanate (FITC)/CD4 phycoerythrin (PE)/CD45 peridinin chlorophyll protein (PerCP) is a three-color direct immunofluorescence reagent to identify and determine the percentages and absolute counts of mature human T lymphocytes (CD3) and helper/inducer (CD3+CD4+) T-lymphocyte subsets in erythrocyte-lysed whole blood. When used with TruCOUNT™ Tubes, absolute counts of these populations can be enumerated from a single tube.

Procedure

- Take a trucount tube.
- Add 20 µl of tritest antibody.
- Add 50 µl of well mixed whole blood collected in K2 EDTA and vortex
- Incubate in dark for 15 minutes at room temperature.
- Add 450 µl of 1X lysing solution and vortex.
- Incubate in dark for 15 minutes
- Acquire on the BD FACSCalibur.

Annex B. Preparation of in-house PT panel procedures (28, 37, 42, 47)

An In-house PT panel was prepared and ready for shipment in Medical Biotech Laboratory according to standard guidelines as follows:

1. HIV-1 and -2, HBsAg, and HCV negative and with known $CD4^+$ count normal whole blood obtained from one donor (in 8-12 K3-EDTA BD vacutainer tubes) was pooled.
2. The above blood was splited in to two samples
3. Sample-A was checked for CD4 T-cell count by BD FACSCount and prepared in such a way that coincides with the normal CD4 count range for Ethiopians (753±227 for adult male) and a mean CD4 T cells counting of 775cells/µl according to Tsegaye et al, 1999
4. Sample-B was prepared by continuous dilution using PBS till desirable low CD4 count was reached (in a range of 100-300 cells/µl)
5. The above two samples in number 3 and 5 were thoroughly mixed in automated hematology-mixer machine
6. Low and normal CD4 count value was aliquoted in 1 ml volume in to their respective pre-labelled cryovials. Labels indicate trial number, low and normal CD4 panel code, and storage conditions
7. The two PT panels was capped and packed according to WHO recommended standard guidelines
8. PT panels was dispatched with all documents that indicates safety, panel handling and running instructions, and report form with provided code number

Annex C. questionnaire

Preliminary questionnaire for participating laboratories

Please complete this questionnaire regarding CD4 count laboratory practice and general quality measures in your laboratory to enable the External Quality Assessment (EQA) scheme to plan appropriate proficiency testing panels and developments to the scheme.

Part 1: Contact details

Name of laboratory: _____

Name of contact: _____

Address: _____

Telephone number: _____

Fax number: _____

E-mail address: _____

Part 2: CD4 count laboratory

1. Number of staff:
   - Full-time _____
   - Part-time _____
2. Number of these staff qualified in laboratory technology _____
3. How many blood samples are received and processed for CD4 count?

   ☐ Per day ☐ per week ☐ per month
4. Type of flow cytometers used in your laboratory (more than one option can be chosen)

   ❑ FACSCalibur

   ❑ FACSCan

   ❑ FACSCount

   ❑ Others*

State manufacturer for your choice: _____

* Specify: _____

5. Sample preparation (protocol) used during CD4 count:
   - ❑ Lyse and Wash
   - ❑ Lyse No Wash
   - ❑ No Lyse No Wash
   - ❑ Gradient separation of mononuclear cells
6. Which of the following gating strategy are included in your routine CD4 testing?

   ❑ Forward scatter – side ward scatter (FSC-SSC)

   ❑ CD45 – side ward scatter (CD45 - SSC)

   ❑ CD45 – CD14/back gating

   ❑ Forward scatter side ward scatter – CD45 – CD3 (FSC-SSC-CD45-CD3)

   ❑ FACSCount^{TM} (counting beads)

❑ Other (please specify)

7. During sample testing, is your laboratory abled to undertake the following?
   ❑ Daily QC running or with new reagent lot number
   ❑ Check-up of fluidic system and air bubbles
   ❑ Calibration before sample running
   ❑ Strict follow up of user's manual
8. What is your pipetting method?
   ❑ Manual micro-pipetting
   ❑ BD provided automated reverse pipetting
9. How often your pipettes are calibrated for precision and accuracy checkup?
   ❑ Regularly (specify the interval) _____. ❑ Not at all
10. Have you followed and practiced the manufacturer's procedures of your instrument during daily cleaning, shutdown, long cleaning, general cleaning, and maintenance procedures?
    ❑ Yes ❑ No
11. Please indicate when controls are set up to check system accuracy and linearity:
    ❑ With each batch of tests
    ❑ Daily, before you run patient samples
    ❑ With new reagent lot number
    ❑ Other (specify)
    ❑ Not used
12. CD4 count technique used in your laboratory
    ❑ Single plat form
    ❑ Dual plat form
13. If your laboratory uses dual plat form techniques, how do you obtain the absolute CD4 count?

    _____

14. FACSCount and FACSCalibur reagents used (more than one option can be chosen)
    ❑ Tube of CD4/CD3 reagents and reference beads containing Anti-CD4/CD3 (MAb)
    Reagent manufacturer: _____

❑ Tube of CD8/CD3 reagents and reference beads containing Anti-CD8/CD3 (MAb)

Reagent manufacturer: _____

❑ BD TriTEST™ CD3 FITC/CD4 PE/CD45 PerCP

Reagent manufacturer: _____

❑ Fixative solution (5%formaline)

Reagent manufacturer: _____

❑ Sheath fluid

Reagent manufacturer: _____

❑ BD FACSCount Control Beads

Reagent manufacturer: _____

15. BD FACSCount contro kit used for CD4 count testing

Manufacturer/supplier

❑ BD FACSCount contro (zero and low) _____

❑ BD FACSCount contro (medium and high) _____

16. Which of the following BD FACSCount CD4 reagents are used in your laboratory?
    - ❑ BD FACSCount CD4 reagent kit that enables simultaneous enumeration of absolute counts and determination of %CD4 in unlysed whole blood
    - ❑ Single-tube format BD FACSCount CD4/CD3 reagent kit that allow uncoupling of CD4 and CD3 enumerations from CD8 that delivers absolute CD4 count.
    - ❑ BD FACSCount Reagent kit which is provided in a 2-tube format that delivers absolute CD4, CD3, and CD8 counts, and a CD4/CD8 ratio.
17. If your laboratory uses FACSCalibur, do you run BD FACSComp software with BD Calibrite beads at the start of each workday to ensure the flow cytometer provides consistent results?

    ❑ Yes ❑ No

Part 3: Quality system

1. Is the organizational structure within your institution defined and documented?

   ❑ Yes ❑ No
2. Are job descriptions written for:

   ❑ All members of staff

❑ For some members of staff

❑ No

3. Is there a system for assessing the training needs of the staff: e.g. at appraisal?

   ❑ Yes ❑ No

4. Is there a staff training policy? ❑ Yes ❑ No

5. Is there a regular training offered for the person responsible for CD4 count?

   ❑ Yes ❑ Sometimes ❑ Not at all

6. Is competency assessment undertaken for all laboratory staff?

   ❑ Yes ❑ No

7. Are written standard operating procedures (SOPs) in place?

   ❑ For all tests and procedures

   ❑ For some tests and procedures ❑ No

8. Is there a system for equipment validation and calibration? ❑ Yes ❑ No

9. Is there a record of equipment maintenance and repair?

   ❑ Yes ❑ No

10. Is there a system for the evaluation of test kits and reagents?

    ❑ Yes ❑ No

11. Are all reagents used according to the manufacturers' instructions?

    ❑ Yes ❑ No

12. Are all reagents validated in-house? ❑ Yes ❑ No

13. Are all reagents used within their expiry date? ❑ Yes ❑ No

14. Is there a mechanism for reporting and investigating errors?

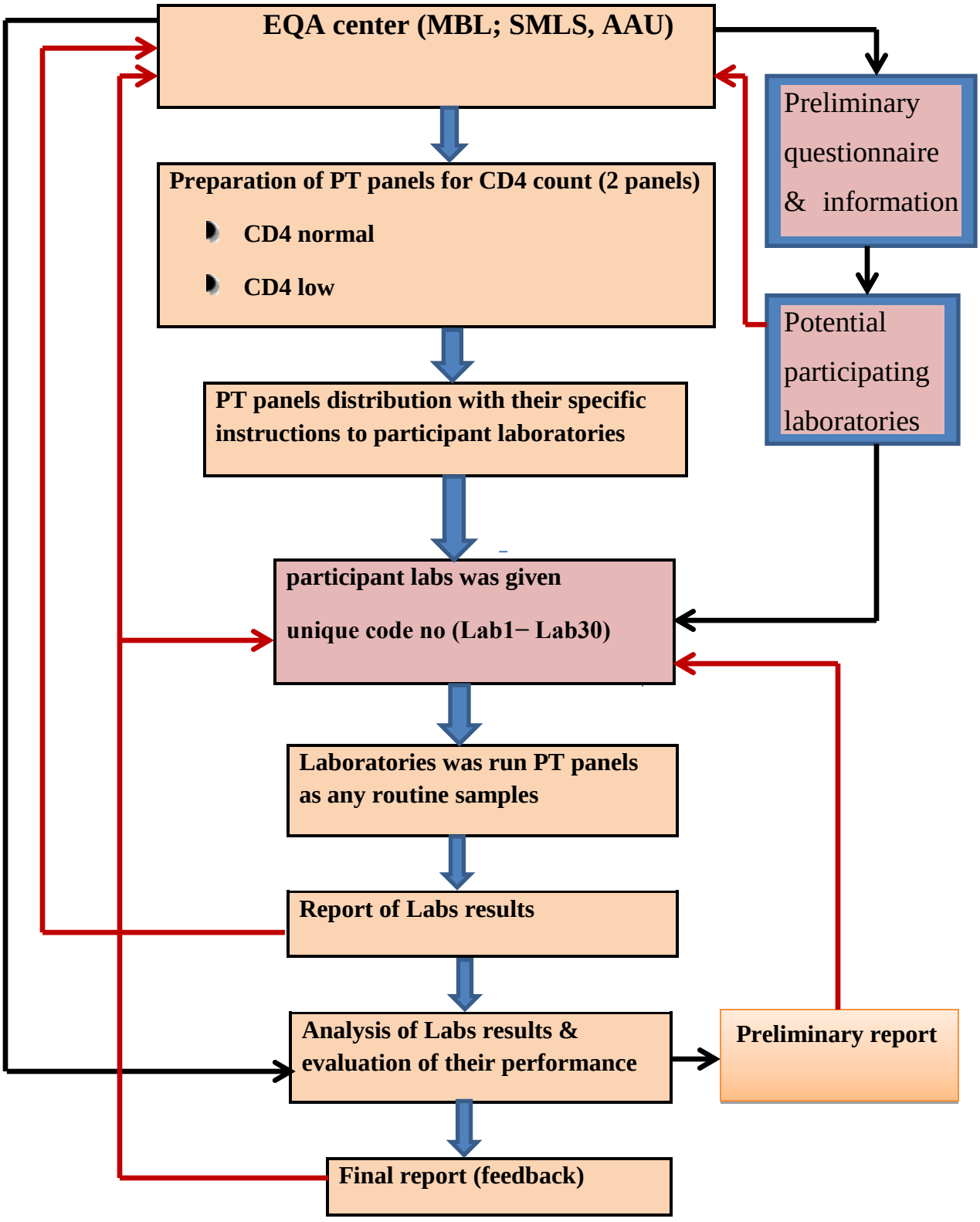
    ❑ Yes ❑ No

15. Does your laboratory have participated in external proficiency testing for CD4 count?

    ❑ Yes ❑ No ❑ If yes, for how many times _____

Thank you for completing this questionnaire. If you wish to expand on your answers or make any comments, please enclose a separate sheet.

Annex D. Work flow of the study/ Schema of the study



Annex E: Documentation required for the CD4 EQA scheme

Annex E1: Registration form for participating laboratories

External quality assessment scheme for CD4+ T-Lymphocyte count using in-house made Proficiency testing panels 2012/13

For EQAS use only

Registration form-participant code:

Please complete the following detailed information

1. Name and address of the person to whom test material is to be dispatched. A survey report and any queries will also be sent to this address.

Name of laboratory:									
Address:									
Phone number:									
Fax number:									
Name of contact:									
Position									
Department:									
Phone number:									
E-mail address:									
Type of laboratory (put √)	Public		Private		Clinic		Hospital		Commercial	
Room number:					Floor number:				
Street, Sub-city and local name									

2. Tests for which the laboratory wishes to register are:

❑ Absolute CD4, CD3, CD8 count, and CD4/CD8 ratios as well as CD4% values

❑ The above including %CD3 and CD8%

Name: ……. Signature: …….Date: ……..

Annex E2 : Participant unique code number

Dear participant,

Thank you for participating in our EQA for CD4+ T-cell count.

Your unique participant code number is the following:

Laboratory name ----------

Participant code number is ----------

Participant name and participant code number must be written on the results form.

Natnael kidanu

EQA for CD4+ count using in-house PT panels principal investigator

AAU and Medical Biotech laboratory

Annex E3: Laboratory Safety

A. Use universal precautions with all specimens
B. Adhere to the following safety practices and user's manual in your laboratory
    1. Wear laboratory coats and gloves when processing and analyzing specimens, including reading specimens on the flow cytometer
    2. Never pipette by mouse, use safety pipetting devices
    3. Handle and manipulate specimens (eg., aliquot and reagents, vortex, and aspirate) in a class I and II safety cabinet (if available).
    4. After working with specimens, remove gloves and wash hands with soap and water
    5. For stream-in-air flow cytometrys, follow the manufacturer's recommended procedures to eliminate the operator's exposure to any aerosols or droplets of sample materials
    6. Disinfect flowcytometer wastes. Before adding waste materials to the waste container, add a sufficient volume of undiluted house hold bleach (5% sodium

hypochlorite) when the container is full (eg., add 100ml of undiluted bleach to an empty 1000ml container).

7. Disinfect the flow cytometer as recommended by the manufacturer. One method is to flush the cytometer fluidic chambers with a 10% bleach solution for 5-10 minutes at the end of the day and then flush with water or saline for at least 10 minutes to remove excess bleach, which is corrosive.
8. Disinfect spills with house hold bleach or an appropriate dilution of mycobactericidal disinfectant. Note: organic matter will reduce the ability of bleach to disinfect infectious agent, NCCLS recommendations regarding how to disinfect specific areas should be followed. For use on smooth, hard surface, a 1% solution of bleach is usually adequate for dis infection; for porous surfaces, a 10% solution is needed.
9. Ensure that all samples have been properly fixed after staining and lysing, but before analysis. Note: some commercial reagents employ a single step, lyse and fix method that reduces the infectious activity of cell associated HIV by 3-4 logs, however, these reagents have not been evaluated for their effectiveness against other agents (eg., hepatitis virus). Cell free HIV can be inactivated with 1% paraformaldehyde within 30 minutes.

Adapted from Mandy et al, 2003.

Annex E4: PT materials Instructions and shipment

In-house made PT materials was packaged according to standards recommended by WHO and be shipped to participating laboratories (42, 47, 50)

Use of packaged material

This material was intended to represent clinical samples for the assessment of the performance of clinical laboratories undertaking routine CD4 count.

PT materials package

The PT materials package comprises a plastic transport bag containing documents and a sealed, clear plastic bag which contains:

- A polystyrene box within which vials of whole blood samples are held

- A pad that will absorb up to 50 ml of liquid: i.e. the entire contents of the package in the event of a breakage or leakage of all the vials.

Contents of this package

This polystyrene box contains two vials each of:

- One normal CD4 value
- One low CD4 value

Information required to control hazardous substances

Whole blood panels were prepared from one donor throughout the trials that have been tested and found negative for:

- HIV 1 and 2 antibodies
- HCV antibodies
- Ant-HBsAg.

Opening of vials

Remove the plastic seal and gently unscrew the cap from the vial.

Handling and disposal of packaged material

The source material from which the samples were obtained has been tested and found negative for HIV-1 and -2 antibodies, HCV antibodies and Anti-HBsAg. However, as with all preparations of human origin, the PT material cannot be assumed to be free from infectious agents. It should therefore be handled and discarded as if potentially infectious, in accordance with local practices and regulations. Samples should be stored at room temperature till analysis.

PT panel instructions

PT panel instructions for Sample A and B material provided

- Low CD4 count panels
- Normal CD4 panels

Instructions for testing and reporting results

Using your routine method, perform the tests for which you are registered on each PT material:

- ☐ Absolute CD4, CD3, and CD8 count as well as CD4/CD8 ratios
- ☐ Absolute CD4 count only

- ❑ Absolute CD4 count and CD4% values (%CD3 and %CD8 are options)

THE CLOSING DATE FOR PT panel Samples A1 and B1 was 22/12/2012

If you have any problems or queries regarding PT panel Sample A1 and B1, please contact the EQA scheme organizer:

Name of organizer: Natnael Kidanu

Title of organizer: EQAS for CD4 count using in-house PT panels

Address: Addis Ababa, Gulelie sub-city, around EHNRI

Telephone number: +251920050125

E-mail address: natnaelk1976@gmail.com

Annex E5: PT panels Result form for trial number 1

Panels sample A1 and B1

Laboratory registration code: _____

Dear Sir Participants: please attach your print out hard copy result with this form

1. Name of laboratory: _____
2. Date samples received: _____
3. Date samples tested/analyzed: _____
4. Date form completed: _____
5. Return date: _____
6. Sample quality/condition

Whole blood panels

	Sample A	Sample B
Satisfactory	❑	❑
Unsatisfactory	❑	❑

Reason out if unsatisfactory _____

1. The emphasis of this trial is EQA for CD4 count, and participants who report only a CD4 absolute count, and CD4 percentage of lymphocytes, are not compromised on this analysis by non-submission of CD3 or CD 8 results.

Please fill in your results in the space provided below

Results:

First write your PT panels code under PT panels code and fill your absolute and percent CD4 values on the space provided below

Absolute counts in (cells/µl) and CD4/8 ratios				
PT panels code	CD4 T-cells	CD8 Tcells	CD3+ T cells total	CD4/CD8 ratio
Sample A1				
Sample B1				

Lymphocyte percentages (%)			
PT panels code	CD4 T-cells	CD8 T-cells	CD3+ T-cells total
Sample A1			
Sample B1			

Flow cytometer used	
BDS FACSCalibur	
BDS FACSCount	
Partec Cy flow	
Pima™ CD4	
Others:	

Red cell lysis:	
Lysed no-wash	
Separated cells (eg. FICOLL)	
Lysed and washed	
No wash- no lyse	

To be filled in by new participants

Maintenance:	
Are your instrument regularly maintained?	
If so how often?	
Are your instruments regularly serviced?	
If so how often?	
Routine laboratory specimens	
How many CD4 specimens does your laboratory generate monthly? (approx)	
At the time of joining EQA for CD4 count	
At the current date	

Comments: _____

_____

Suggestions: _____

_____

Date: _____ **Signature: _____**

Name (please print): _____

Phone or e-mail back to Natnael Kidanu at:

E-MAIL: natnaelk1976@gmail.com Telephone no : +251920050125

Thank you for your participation

Annex E6: Participant performance report

$CD4^+$ T-Lymphocyte proficiency testing programme

Laboratory registration code: Lab 1

Results of survey number **sample A and B** of 2012/13 **trial 1**

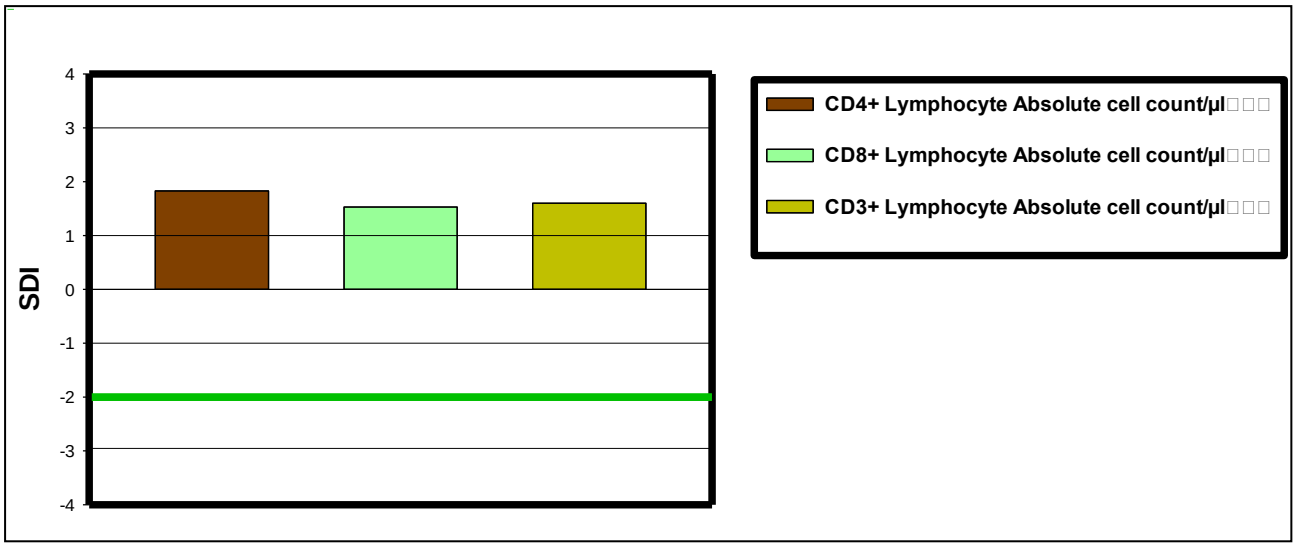
Date of report: 21/12/2012

Laboratory: Lab 1

Protocol: No lyse no wash

Instrument: BD FACSCount™

					All methodologies				
Test	**Results**	**Residual**	**SDI**	**> ± 2**	**Method**	**№**	**Mean**	**SD**	**CV**
CD4+ Lymphocyte Absolute cell count/µl	724	91.1	1.83	*	All	20	632.9	49.8	7.9
CD8+ Lymphocyte Absolute cell count/µl	606	72.7	1.53	*	All	10	533.3	47.6	8.9
CD3+ Lymphocyte Absolute cell count/µl	1,461	161	1.6	*	All	10	1,299.8	100.6	7.7
CD4 Lymphocyte %	NA				All	9	35.8	0.8	2.2



Annex F: Supplementary data

Annex F1: Longitudinal performance of individual laboratories

Longitudinal performances were graphically represented by plotting sequential SDI results from a specific participating laboratory on circular (radar) plots. This would reflect the accuracy and precision of testing over time. These radar plots demonstrate when a particular laboratory joined the EQA for CD4+ T-cells count scheme, instances when there are outlying results or where there are no submission of results, show when interventions improved performance, as well as overall accuracy and precision.

Performance with change of methodology could also be monitored. The selection of laboratories was representative of the performance across many participants which was specially chosen to represent either ideal performance or typical problems encountered through participation.

Annex G. Evaluation of results and statistical interpretations (28, 44, 47)

The analysis of results in this EQA scheme had three basic aims:

- To provide an overall summary of the total number of correct and incorrect results.
- To provide for each individual laboratory an analysis of its performance in each trial.
- To identify the causes of deviating results.

The procedure of evaluation requires the definition of target values and limits of acceptability of results

I. Establishing target values

A target value is the value which is used for assessing performance by the participants. A target value of an analyte in a PT material was established by longitudinal estimation of a consensus value using the results reported by the participants in the scheme. This is defined as an agreed majority view. It is the mean or median of the results obtained from all participants and is more practical in hematology.

If the results were statistically consistent with a Gaussian distribution the mean is calculated together with the standard deviation (SD) after elimination of obvious blunders and outliers (>3SD) in an initial statistical calculation, in order to obtain a trimmed SD. (i.e. S'). Mean is always related to accuracy or systematic error and the SD is related to precision or random error.

If, however, the data are perceived to be non-Gaussian, it is preferable to establish the median.

The SD can be estimated by relating it to the central 50% spread (between the 25th and 75th percentiles) by the formula:

$$S^m = \frac{central\ 50\%\ spread}{1.35}\ or\ \frac{Q3 - Q1\ (IQR)}{1.35} \qquad (1)$$

In the above calculations it may be appropriate to subdivide the data into method/instrument/system groups.

II. Limits of acceptability and performance standards

Using the results from all participants trimmed at 3 SD in an initial calculation

In the simplest procedure the reproducibility of results is assessed by a purely statistical analysis. The limits of acceptability are commonly set at 2 SD from the mean or median of all results. The results only describe the actual performance of the participants and therefore do not provide an incentive to quality improvement.

Evaluation procedure using a consensus value

SD was calculated from the distribution of all results

Results that are beyond ± 3 SD was excluded and a histogram was prepared

To obtain a trimmed target value the mean of results that show a normal (Gaussian) distribution was calculated.

If, however, the data show a non-Gaussian distribution, it was proceed as follows:

All results was ranked according to ascending order and the median (**P50**) is determined

The 25th and 75th percentiles (P25 and P75) or quartiles was determined and the interval

IQR = P75-P25 is calculated as well as the SD and CV was calculated as follows:

$$SD = \frac{IQR}{1.35}$$

$$CV = \frac{100SD}{P50} \qquad (2)$$

Histogram of all results in relation to the assigned target value and 2SD for visual inspection was prepared that includes the target value and the limits of acceptability (e.g. + 2 SD) for demonstration.

When results differ between groups of methods, calculate for each group, a group-based consensus value. The number of results in each group should be more than 10.

Statistical interpretation

Standard deviation index (SDI): This relates individual deviation from the mean or median to the SD, so that it is a measure of bias and calculated as follows:

$$SDI = \frac{Individual\ result\ (R) - Trimmed\ mean}{Trimmed\ SD\ (S')} \quad or \quad \frac{Individual\ result\ (R) - medin}{Estimated\ SD\ (S^m)} \qquad (3)$$

The target SDI is 0.00 but acceptable values are between ± 1.00

The SDI calculated from the mean is also known as the z-score. According to ISO Guide 43 the following judgment is made:

Results leading to $|\mathbf{Z}| \leq 2$ are satisfactory

Results leading to $|\mathbf{Z}| > 3$ are unsatisfactory

All other results ($2 < |\mathbf{Z}| < 3$ are questionable

Annex H. PT panel checklist

Procedure	Signature	Date
Request PT material		
Check that PT material meets specifications		
Perform pre-acceptance testing serology		
Review pre-acceptance results		
Process material		
Print labels for PT material vials		
Dispense into vials		
Perform post-dispensing serology		
Complete any registration amendments		
List participants for specific PT panel		
Make labels for posting PT material		
Print list used for packing and dispatch		
Print PT material instructions		
Print product insert		
Print result forms		
Collate and proof-read appropriate documentation		
Pack PT material and documentation		
Distribute PT panel to participants		
Distribute one self-addressed set of material to EQAS for post-distribution CD4 count		

Perform post-distribution CD4 count:		
❑ Day 1 ❑ Day 2 (stored at room temperature)		
Decide on correct results		
If necessary, review penalty scoring		
Transcribe results or enter into computer system		
Cross-check for transcription or data entry errors		
Edit results and re-check		
Double-check errors against results forms		
Check the number of errors		
Contact all participating laboratories with errors		
Analyze results		
Write preliminary report and comments		
Complete final proof-reading, revision and printing of reports		
Perform further analysis, if necessary		
Write report supplement, if necessary		
Write up log of errors and non-returns		
Check PT panel file is complete		

Annex I. Declaration

I, Natnael Kidanu declare that this thesis is my original work, sole author of this thesis and have never been presented in this or any other universities and that all the source material used for this thesis has been duly acknowledged

Natnael Kidanu

Signature:_____

Date of submission:_____

The thesis has been submitted for examination with my approval as a university advisor

1. Dawit Wolday (MD, MSc, PhD, Manager of Medical Biotech Laboratory)

   Signature: _____

   Date: _____

2. Samuel Kinde (MSc, Lecturer, SMLS, AAU)

   Signature: _____

   Date: _____

3. Gebru Mulugeta (MSc, Lecturer, AAU)

   Signature: _____

   Date:_____