KCMC Biotechnology Laboratory	STANDARD OPERATING PROCEDURE	Effective Date 02-10-2006	SOP-Number FLOW003.01
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Title: Flow Cytometry Staining, Acquisition and Analysis using CD45 Gating-MultiTEST Reagents with TruCOUNT Tubes

SOP References: **Supersedes:**

Author/Date: **Moses Sichangi, M.S**

Approvals/Date:

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Chris Drakeley, Ph.D.
KCMC Biotechnology Laboratory Director

_____

John A. Bartlett, M.D.
ISAAC Program Director

_____

Guido Ferrari, M.D.
ISAAC Pathogenesis Program Co-Director

This SOP has been read and understood by:

Name	Date
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Annual Review		
Reviewed by:	**Review Date**	**Signature**

Document History:

Version Number	Reason for Changes	Date
FLOW003.01	Initial	16-08-2006

Abbreviations and Definitions

ACTG	Aids Clinical Trials Group
APC	Allophycocyanin
BD	Becton Dickinson Biosciences
BSL-2	Biosafety Level -2
CD	Cluster Designation
EDTA	Ethylene Diamine Tetraacetic Acid
FACS	Fluorescence Activated Cell Sorter
FITC	Fluoresceine Isothiocyanate
FL	Fluorescence
IATA	International Association of Air Transport
ID	Identification Number
ISAAC	International Studies of AIDS- Associated Co-infections
KCMC	Kilimanjaro Christian Medical Centre
PE	Phycoerythrin
PerCP	Peridinin Chlorophyll Protein
QC	Quality Control
QNS	Quantity not sufficient
SD	Standard Deviation
SOP	Standard Operating Procedure
SSC	Side Scatter

1. Method Summary

This procedure outlines the process for staining and processing whole blood samples for CD4/CD8 analysis. It involves staining whole blood samples using BD MultiTEST reagent (anti CD3/CD8/CD45/CD4) in TruCOUNT Tubes. When whole blood is added to the reagent, the fluorochrome-labeled antibodies in the reagent bind specifically to leukocyte surface antigens. The stained samples are treated with FACS Lysing Solution to lyse erythrocytes. During acquisition, the cells travel past the laser beam and scatter the laser light. The stained cells fluoresce. These scatter and fluorescence signals, detected by the instrument, provide information about the cells' sizes, internal complexity, and relative fluorescence intensity.

MultiTEST reagents employ fluorescence triggering, allowing direct fluorescence gating of the lymphocyte population to reduce contamination by unlysed or nucleated red blood cells in the gate. This allows analyses of a relatively cleaner population of cells.

2. Scope

This SOP applies to all staff at the KCMC Biotechnology laboratory who use the FACSCalibur. Only authorized staff may use the FACSCalibur.

3. Safety Precautions

3.1. Standard Safety precautions for handling blood should be used under BSL-2 conditions.

3.3. Wear disposable gloves and lab coat.

3.4. All procedures should be done inside certified biosafety cabinets.

3.5. The lasers in the FACSCalibur emit laser beams which can be harmful to the eyes. Avoid contact with naked eyes when the top cover of the FACSCalibur is opened.

4. Waste Generation, Handling and Disposal

Follow all relevant Standard Operating Procedures (SOP) in disposing biohazard waste generated by this method. Follow KCMC SOP for waste storage and disposal (Biohazard Safety SOP [SFT.01]).

5. Specimen Collection, Transport and Handling

5.1. Specimen collection

5.1.1. Obtain peripheral blood in EDTA anticoagulant (4ml) for adults and 2 ml microtainers for children.

5.1.2. Ensure to obtain 4ml of blood sample in 4ml vacutainer and 2ml in the 2ml microtainer in order to account for the amount of anticoagulant and. This prevents diluting the blood further. Ensure the blood sample level gets to the indicated mark on the sample collection tube.

5.1.3. All specimens should be regarded as potentially infectious, 'standard precautions' for blood collection and handling should be properly followed.

5.1.4. Specimen Transport [Outside of lab]:

5.1.4.1. Specimens should be placed in well stopper tubes (EDTA vacutainers) and should be placed with adequate absorbent material, preferably in a closed leak proof container for delivery.

5.1.4.2. Refer to Tanzanian and IATA regulations for domestic and international shipping of specimens.

5.1.4.3. Specimens should be maintained at room temperature ($18^\circ - 25^\circ$ C) during transportation and storage.

5.1.5. Testing must be performed within 24 hours from the time of collection.

5.2. Specimen labeling and request submission:

5.2.1. Each patient sample must be properly labeled with the following information: Sample ID number, Study ID number, Date and time of sample collection.

5.2.2. Each sample should be accompanied by a study ID number and a patient ID number according to the KCMC rules for samples labeling and protection of patient's rights.

6. Rejection Criteria

6.1. Unlabeled or incorrectly labeled specimens

6.2. Specimen quantity not sufficient (QNS).

6.3. Clotted specimen.

6.4. Hemolysed blood specimen.

6.5. Specimen more than 24 hours old.

6.6. Specimen exposed to cold temperatures (transported on ice/ice packs).

7. Equipment and Materials/Reagents

7.1 Equipment and Materials

7.1.1. Disposable 5 ml 12x75-mm Falcon capped polystyrene test tubes, (Cat. # 352058, BD Biosciences)

7.1.2. Micropipette with tips capable of delivering 20µl for antibody reagent

7.1.3. Micropipettor with tips (BD Electronic Pipette, Cat# 343208) for pipetting 50µl blood sample.

7.1.4. Bulk dispenser or pipette (450µl) for dispensing 1X FACS Lysing solution

7.1.5. FACSCalibur (BD Biosciences)

7.1.6. MultiSET Software (BD Biosciences)

7.1.7. Vortex mixer

7.1.8. Printer

7.2. Reagents

7.2.1. MultiTEST reagent with TruCOUNT Tubes (Cat. # 342-447, BD Biosciences):

7.2.1.1. MultiTEST CD3 FITC/CD8PE/CD45PerCP/CD4 APC

7.2.1.2. TruCOUNT Tubes containing freeze-dried pellet of fluorescent beads.

7.2.2 FACS Lysing solution 10X (Cat. # 349202, BD Biosciences)

7.2.3 CaliBRITE (3) Beads (Cat. # 340-486, BD Biosciences)

7.2.4. APC CaliBRITE Beads (Cat. # 340-487, BD Biosciences)

7.2.5. BD FACSFlow Sheath fluid, (Cat. # 342-003, BD Biosciences)

7.2.6. TruCOUNT Controls (BD Cat. # 340335)

7.2.7. BD Multi-Check Control (2.5 ml vial Cat. # 340911)

7.2.8. Reagent-grade (distilled or deionized) water.

8. Staining Procedure

8.1. Dilute the 10X FACS Lyse concentrate 1:10 with room temperature (18° – 25°C) distilled water. The prepared solution is stable for 1 month when stored in a glass container at room temperature.

8.2 For each set of samples, label a TruCOUNT Tube with assay date and experiment number to be used as assay control. This tube will be used to stain a sample with known ranges of CD4 and CD8 values to determine any technical errors in the assay procedure.

8.3. For each patient sample, label one TruCOUNT Tube with the sample identification number and date.

8.4. Pipette 20 µl of the MultiTEST CD3 FITC/CD8 PE/45 PerCP/CD4 APC reagent into the bottom of each tube for each patient and assay control tube. Pipette just above the stainless steel retainer. Do not touch the pellet.

8.5. Pipette 50µl of well-mixed EDTA anticoagulated whole blood into the bottom of the corresponding tube for each patient. **NOTE:** Avoid smearing blood down the side of the tube as it will not be stained.

8.6. Pipette 50µl of Multi-Check Normal control or other approved control into the assay control tube.

8.7. Cap the tubes and vortex gently to mix. Incubate for 15 minutes in the dark at room temperature (18^{o}C -25^{o}C).

8.8. Add 450µl 1 X FACS Lysing solution to each tube.

8.9. Cap the tubes and vortex gently to mix. Incubate for 15 minutes in the dark at room temperature. The samples are now ready to be analyzed on the flow cytometer.

8.10. If samples are not to be analyzed immediately after preparation, store them in the dark at room temperature ($18^{\circ} - 25^{\circ}C$). Samples must be analyzed within 24 hours.

9. Set-Up for Acquisition and Analysis

9.1. Run FACSComp on "Lyse /No Wash" settings (See SOP# FLOW00.01). Check to ensure all the parameters on FACSComp report have passed.

9.2. Choose MultiSET from the Apple menu.

9.3 Double click the MultiSET icon to launch MultiSET software.

9.4. The application window will appear. Sign in your name as the operator. Click "Accept " to continue to the Set Up View.

9.5. At the Set Up View window verify the following: make sure the choices are checked:

9.5.1. Data Source; select 'From cytometer–Acquisition with analysis'. For entry level, file name prefix; select sample ID.

9.5.2. Folders for automatic data saving options, select data files location as follows; Laboratory Report, Physician Report, Summary Report, Export Document, Use Date Generated File Name.

9.5. Click "Accept" if satisfactory.

9.6. The FACSComp view appears. Either launch into FACSComp or click "Skip FACSComp" if already done. This is the option to run FACSComp within MultiSET.

9.7. Proceed to the Test Preference view window.

9.8. Verify the following sections have been made (checked):

9.8.1. All subset results are chosen (i.e. CD45+CD3+% Lymph, CD45+CD3+ Abs, CD3+CD4+ % Lymph, CD3+CD4+ Abs, CD3+CD8+ %Lymph, CD3+CD8+ Abs)

9.8.2. Select; Report Reference Ranges (User defined range)

9.8.3. Select; Quality Control (QC) Message for out of Normal Range

9.8.4. Select; Laboratory Report Choices as Report Percent and absolute counts

9.8.5. Summary Report ID; Select sample name and sample ID

9.8.6. Physician Report Choices; Select CD3+CD4+% T-Lymph, CD3+CD8+% Lymph, CD3+% Lymph, CD3+CD4+CD8+% T-Lymph, CD3+ Abs Cnt, CD3+CD4+ Abs Cnt, CD3+CD8+ Abs Cnt

9.8.7. Subset Ranges; make sure the normal Reference values are current

9.8.8. Panel Tools; Choose MultiTEST TruC and ACTG CD4 PANEL

9.8.9. Reagent Tools; Select MultiTEST TruC as reagent type and CD3/CD8/CD45/CD4 TruC as the reagent. The number of dot plots to be displayed on laboratory report and fluorescence parameter information changes automatically with selected reagent type above.

9.8.10. Acquisition Target Information; enter the minimum events to be acquired as 2500. Otherwise use default setting of 2000.

9.8.11. Click on lot IDs; Select reagent lot and enter the lot number of the current antibody reagent being used. Select Absolute count beads and enter the lot ID and beads count per pellet as it appears on the current lot being used. Note that these numbers will be changed whenever a new lot of reagents are received. When running control beads, click on control beads lot IDs; enter the lot ID, Low beads count, Medium beads count, High beads count and the respective SD. Review these entries and click on accept.

10. Acquisition and Analysis

10.1. Sample View window appears. Enter the following information; sample name, sample ID and case number. Sample information should be entered following the order in which they will be acquired (run on the FACSCalibur). Enter the correct panel name for antibody combination for each tube.

10.2. Download saved instrument settings from the sample view before advancing to the acquisition. Click on cytometer from the menu options, and choose instrument settings.

When open select lyse/no wash (LNW) option. Click on 'set' to install the current instrument settings, then click on 'done' to exit.

10.3. Click RUN TEST to start acquisition.

10.4. Vortex and install the stained sample tube labeled 'control' onto the cytometer. Press the 'RUN' button on the cytometer control panel. A pre-acquisition phase is displayed. Inspect the dot plots. To adjust threshold click on cytometer from the menu options and choose Threshold. Adjust FL3 (CD45 PerCP) threshold to get rid of most debris. If it is satisfactory click "Acquire".
Important note: Vortex each sample tube before installing on the cytometer

10.5. When acquisition is complete, proceed to view the laboratory report. Click manual gate to display the plots. Check that the expert gate is in the right location gating the CD45 PerCP bright population with low on SSC. Adjust the gate if necessary. View and inspect the CD3+/SSC plot and check the CD3+ attractor. Adjust if necessary to gate the CD3+ population.

10.6. Click 'Analyze' to confirm the changes and re-analyse results. Click on 'next' to view the physician report. Click 'next' to acquire the next sample. Run (acquire) the rest of the samples until the whole set is complete.

10.7. When acquisition is complete, proceed to view the analysis. Verify that QC values are acceptable. Values should be verified as follows:

10.7.1. The Multi-Check (assay control) values obtained for each set (run) should fall within the established range values provided with the control. The values to be checked for will include CD45+CD3+%, CD3+CD4+%, CD3+CD8+%, CD45+CD3+ Abs Cnt, CD3+CD4+ Abs Cnt, CD3+CD8+Abs Cnt.

10.7.2. If the values for the Multicheck control or the alternative control used fall outside the expected ranges then the patient results should not be reported. If values are within the manufacturer's ranges, then patient results can be reported.

10.7.3. The difference between the sum of CD3+CD4+ and CD3+CD8+ should be less than 5% of the total CD3+ cells.

10.7.4. Report the current normal values for the requested subset of T-lymphocytes on the requisition form. This information is in the table 1 below. This information is also reported on the physician report that should accompany the completed requisition form.

Table1: T-Lymphocyte Normal Reference Values for Moshi Population

ANALYTE	ABS Cnt/uL	PERCENTAGE
CD45+CD3+	769-2798	53.7-82.6
CD3+CD4+	405-1500	27-55
CD3+CD8+	261-1033	15-41

10.8. After verifying, results should be reported on the requisition form with the reference normal values. A copy of the completed requisition form should be filed under lock in the laboratory. Send the completed requisition form and the physician report form to the requesting physician by end of the day or where not possible the following morning. The testing technician should initialize the results and signed by verifying supervisor before releasing.

11. Quality Control Measures

11.1. Run FACSComp daily before running patient samples on the FACSCalibur. FACSComp report should be inspected to check if all parameters have passed for lyse /no wash procedure. This indicates whether the instrument settings are in correct settings including the fluorochrome compensation.

11.2. An assay control with known values of CD4+ and CD8+ cells (e.g MultiCheck, or CD-Chex Plus) should be included to check for technical errors in the procedure.

11.3. A sample from a reference laboratory should be run using this method at least twice a year to check on comparability of the method results with other laboratories both regionally and or internationally.

11.4. Perform parallel testing on at least two samples using new reagents received and current reagents in use. In addition, the assay control sample with known T-lymphocyte values should be processed using the new reagents and current reagents. The results should have a correlation of $\geq$ 90%.

11.5. Each technician performing this assay should run TruCOUNT Control beads (low, medium and high) at least every month to determine precision in pipetting.

11.6. Each technician performing this assay should read SOP for quality control (FLOW004.01)

12. Results Interpretation

12.1. Before releasing patient results check that the assay control values are within the expected ranges.

12.1. Results should be interpreted as low, normal or high on the basis of the current subset ranges in table 1. Patient results should not be released until verified by the laboratory supervisor. Submit both the completed requisition form and the physician report printout for each patient sample to the physician.

11. References:

1. Becton Dickinson MultiSET Software User's Guide. Manual Part Number: 11-10870-01 Rev A December 1998

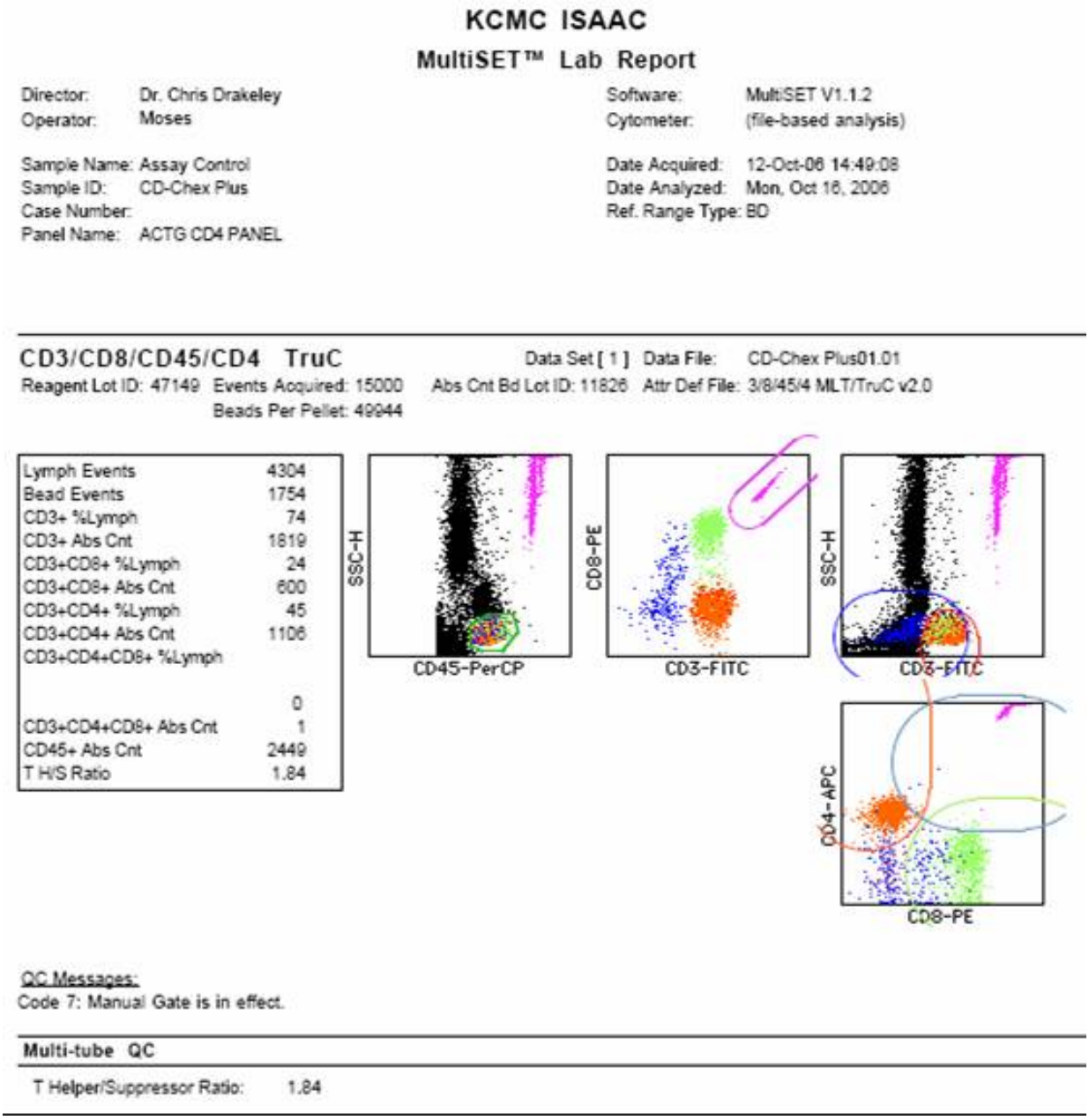
2. Centers for Disease Control. Revised guidelines for performing CD4+ T-cell determinations in persons with human immunodeficiency virus (HIV), MMWR 1997; 46(No. RR-2): I-29.

3. Nicholson JKA, Jones BM, Hubbard M. CD4 T-lymphocyte determination on whole blood specimens using a single-tube, three-color assay, Cytometry 1993; 14:685-689

4. Nicholson J, Kidd P, Mandy F; Livnat D, Kagan J. Three-color supplement to the NIAID DAIDS, Guideline for flow cytometric immunophenotyping, Cytometry, 1996; 26:227-230.

Appendices:

Appendix 1: Flow Cytometry Laboratory Report



KCMC ISAAC

MultiSET™ Lab Report

Director: Dr. Chris Drakeley
Operator: Moses

Software: MultiSET V1.1.2
Cytometer: (file-based analysis)

Sample Name: Assay Control
Sample ID: CD-Chex Plus
Case Number:
Panel Name: ACTG CD4 PANEL

Date Acquired: 12-Oct-06 14:49:08
Date Analyzed: Mon, Oct 16, 2006
Ref. Range Type: BD

CD3/CD8/CD45/CD4 TruC — Data Set [1] Data File: CD-Chex Plus01.01

Reagent Lot ID: 47149 Events Acquired: 15000 Abs Cnt Bd Lot ID: 11826 Attr Def File: 3/8/45/4 MLT/TruC v2.0

Beads Per Pellet: 49944

Lymph Events	4304
Bead Events	1754
CD3+ %Lymph	74
CD3+ Abs Cnt	1819
CD3+CD8+ %Lymph	24
CD3+CD8+ Abs Cnt	600
CD3+CD4+ %Lymph	45
CD3+CD4+ Abs Cnt	1106
CD3+CD4+CD8+ %Lymph	0
CD3+CD4+CD8+ Abs Cnt	1
CD45+ Abs Cnt	2449
T H/S Ratio	1.84

QC Messages:

Code 7: Manual Gate is in effect.

Multi-tube QC

T Helper/Suppressor Ratio: 1.84

Appendix 2: Flow Cytometry Physician Report

KCMC ISAAC

MultiSET™ Physician Report

Director: Dr. Chris Drakeley
Operator: Moses

Software: MultiSET V1.1.2
Cytometer: (file-based analysis)

Sample Name: Assay Control
Sample ID: CD-Chex Plus
Case Number:
Panel Name: ACTG CD4 PANEL

Date Acquired: 12-Oct-06 14:49:08
Date Analyzed: Mon, Oct 16, 2006
Reference Range Type: BD

Result Name	%/Ratio	Abs Cnt (cells/µL)	Reference Range
T Lymphs % of Lymphs (CD3+/CD45+)	74		55% – 84%
T Lymphs (CD3+) Abs Cnt		1819	690 – 2540
T Suppressor % of Lymphs (CD3+CD8+/CD45+)	24		13% – 41%
T Suppressor Lymphs (CD3+CD8+) Abs Cnt		600	190 – 1140
T Helper % of Lymphs (CD3+CD4+/CD45+)	45		31% – 60%
T Helper Lymphs (CD3+CD4+) Abs Cnt		1106	410 – 1590
CD3+CD4+CD8+ % of Lymphs (CD3+CD4+CD8+/CD45+)	0		
CD3+CD4+CD8+ Abs Cnt		1	

Multi-tube QC

T Helper/Suppressor Ratio: 1.84

Comments:

Laboratory Director: