TITLE: TRU-COUNT ENUMERATION OF T-CELLS IN WHOLE

PRINCIPLES OF THE PROCEDURE:

When whole blood is added to the reagent, the fluorochrome labeled antibodies in the reagent bind specifically to leukocyte surface antigens. 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 cell's size, internal complexity, and relative fluorescence intensity. These reagents employ fluorescence triggering, allowing direct fluorescence gating of the lymphocyte population (1-3) to reduce contamination of unlysed or nucleated red blood cells in the gate.

When TruCount tubes are used, a known volume of sample is stained directly in these tubes. The lyophilized pellet in the tube dissolves, releasing a known number of fluorescent beads. During analysis, the absolute number (cells/uL) of positive cells in the sample can be determined by comparing cellular events to bead events. If appropriate software is used, absolute counts are determined by the software. If manually performing data analysis, simply divide the number of positive cellular events by the number of bead events, and then multiply by the TruCount bead concentration.

$$\frac{\text{\# of events in region containing cell population}}{\text{\# Of events in absolute count bead region}} \times \frac{\text{\# of beads per test (found on label)}}{\text{test volume}}$$

CLINICAL APPLICATIONS OF THE PROCEDURE:

Suppressor / cytotoxic lymphocytes are a subset of T lymphocytes (CD3+) that are CD8+. Helper / inducer lymphocytes are a subset of T lymphocytes (CD3+) that are CD4+. CD3+ CD8+ and CD3+ CD4+ percentages or counts are used to characterize and monitor some forms of immunodeficiency and autoimmune diseases.
Determining percentages or counts of helper / inducer T lymphocytes can be useful in monitoring human immunodeficiency virus (HIV) infected individuals. Individuals with HIV typically exhibit a steady decrease of helper / inducer T lymphocyte counts as the infection progresses.

The percentage of suppressor / cytotoxic lymphocytes lies outside the normal reference range in some autoimmune diseases (4). The relative percentage of the CD8+ subset is elevated in many patients with congenital or acquired immune deficiencies such as severe combined immunodeficiency (SCID) or acquired immune deficiency syndrome (AIDS).

The Center for Disease Control (CDC) recommends using reagent combinations containing CD3 antibodies for determining the percentage of T lymphocyte subsets in HIV infected subjects. The combination CD3/CD8/CD45/CD4 allows helper / inducer T lymphocytes to be identified and enumerated separately from contaminating CD3-CD4+ monocytes.

NK lymphocytes identified as CD3- and CD16+ and/or CD56+ have been shown to mediate cytotoxicity against certain tumors or virus infected cells (5). NK mediated cytotoxicity does not require class I or class II major histocompatibility complex (MHC) molecules to be present on the target cell (6).

Total T and B lymphocytes are used to characterize and monitor some forms of immunodeficiency (7-9) and autoimmune diseases (10, 11).

SPECIMEN REQUIREMENTS:

Collect blood aseptically by venipuncture (12, 13) into a sterile EDTA (ethylenediaminetetraacetic acid) or Sodium Heparin vacutainer blood collection tube. A minimum of 0.5 mL of whole blood is required for this procedure. Anticoagulated blood stored at room temperature (20-25 C) must be stained within 48 hours of draw and then analyzed within 24 hours of staining. Whole blood samples refrigerated before staining may give aberrant results. Samples obtained from patients taking immunosuppressive drugs can yield poor resolution (14). Blast cells may interfere with test results.

Samples will be rejected if they are hemolyzed, clotted, frozen, fixed, not properly labeled, or greater than 48 hours old when received in the lab.

REAGENTS/SUPPLIES:

Multitest antibody reagents CD3/CD8/CD45/CD4 and CD3/CD16CD56/CD45/CD19, FACS Lysing solution (10x), TruCount tubes, pipette tips, reagent grade (distilled or deionized) water, calibrate beads, APC beads, blood sample, sheath fluid, Streck normal and low controls, 12 x 75-mm polystyrene test tubes with cap

FACSLyse preparation: Dilute 1 part 10x FACSLyse with 9 parts of room temperature deionized water. Label as 1x FACSLyse with date and time of preparation, store at room temperature and discard after 24 hours.

EQUIPMENT & INSTRUMENTATION:

Vortex mixer, micropipettor, flow cytometer, bulk dispenser or pipettor

Flow Cytometer (for RPCI Calibur).

PROCEDURE:

SAMPLE PREPARATION:

1. For each patient and control sample label a TruCount tube with identification number and reagent name.
    a. Note: Before use, verify that the TruCount bead pellet is intact and within the metal retainer at the bottom of the tube. If this is not the

examine the desiccant each time the pouch is opened. If the desiccant has turned from blue to lavender, discard the remaining tubes.

2. Pipette 20uL of Multitest CD3/CD8/CD45/CD4 into the bottom of each appropriately labeled tube. Pipette just above the stainless steel retainer, do not touch the pellet. Repeat for the Multitest reagent CD3/CD16CD56/CD45/CD19.
3. Pipette 50uL of well mixed, anticoagulated whole blood into the bottom of the tube.
    a. Note: Avoid smearing blood down the side of the tube. Accuracy is critical, use reverse pipetting(15) technique to pipette sample onto the side of the tube just above the retainer. Use care to protect tubes from direct light.
4. Cap the tube and vortex gently to mix. Incubate for 15 minutes in the dark at room temperature (20-25C).
5. Add 450uL of 1x Facs lysing solution to the tube.
    a. Note: Dilute the 10x concentrate 1:10 with room temperature deionized water.
6. Cap the tube and vortex gently to mix. Incubate for 15 minutes in the dark at room temperature (20-25C). The sample is now ready to be analyzed on the flow cytometer.

INSTRUMENTATION

Prepare all bead suspensions immediately prior to use. Mix bead vials by gentle inversion or very gentle vortexing prior to use. Label 2 12x75 –mm polystyrene tubes Tube A and Tube B. Dispense 1mL of sheath fluid into Tube A. Dispense 3ml sheath fluid into Tube B. Gently mix the Calibrite bead vials, then add 1 drop unlabeled beads and 1 drop of APC beads to Tube A. Add 1 drop each of unlabeled, FITC, Pe, PerCP, and APC beads to Tube B. Note: Invert bead vials completely when adding a drop to the tube. Make sure to obtain a full drop of beads. The drop should be cloudy, indicating the beads are properly mixed.

Using FACSComp software adjust PMT voltage settings using Tube A. Adjust fluorescence compensation using Tube B. Perform a Sensitivity Test using Tube B. Generate a printout of the sensitivity test results and keep the printouts in a log book. Record PMT voltages and channel separations obtained for each parameter in a daily log sheet. Optimize settings for your sample, as needed. Instrument settings might need to be manually optimized before running cells. Visually inspect dot plots for proper PMT gains, compensation, and FS threshold.

PROCEDURAL NOTES:

Calibrite beads are recommended for use with FACSComp software on a BD FACSCalibur flow cytometer. In some cases the software may not be able to automatically set up the instrument. If this occurs, manually adjust the settings.

QUALITY CONTROL GUIDELINES:

As per the procedure the control tubes are run along side the patient sample.

EXPECTED VALUES:

The reference ranges for CD3/CD8/CD45/CD4 shown in table 1 were determined at three clinical investigation centers in the United States. Subjects were hematologically normal adults between the ages of 18 and 65 years.

Subset	n	Mean	95% Range
Helper/inducer T lymphocytes (%)	164	45	33-58
Suppressor/cytotoxic T lymphocytes (%)	164	24	13-39
Total T lymphocytes (%)	164	72	56-86
Helper/inducer T lymphocytes (cells/uL)	164	941	404-1612
Suppressor/cytotoxic T lymphocytes (cells/uL)	164	511	220-1129
Total T lymphocytes (cells/uL)	164	1513	723-2737

REPORTING RESULTS & CALCULATIONS:

All calculations are preformed on the excel worksheet.

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APPROVED BY		pkw					