SPHERO™ Technical Note **STN-15 Rev B 082107**

MEASURING ABSOLUTE CELL COUNT USING SPHERO™ ACCUCOUNT FLUORESCENT PARTICLES

Introduction

Assays for cell counting using flow cytometry and calibrated fluorescent particles are rapid and accurate. The single platform method that enumerates T-cells by counting the identifier cells in either a precise known cell volume or an internal 'spike' of a known number of calibrated fluorescent particles by flow cytometry is simple and efficient (1). These assays allow the counting of T-cells during anti-T-cell globulin treatment of cardiac, lung, and renal transplant patients (2). In addition, laboratories can determine the absolute count of CD4 and CD8 T-cells to estimate HIV disease progression with the single platform method (3). Calibrated fluorescent particles and flow cytometry are also used to count platelets in a wide range of murine models of platelet disorders (4). It is also possible to count other various cell types with flow cytometry and calibrated fluorescent particles.

The accurate quantitation of cells in blood is crucial for precise determination of treatment procedures during clinical care. Flow cytometry and calibrated fluorescent particles have improved cell counting methods over predicated methods. In the past, multi-platform methods were used to quantitate T-cells. The conventional multi-platform method uses cell marker percentages from the flow cytometer and white blood cell count, percent lymphocytes, and absolute lymphocyte count from a hematology analyzer to enumerate T-cells in blood. Substantial variations in data have resulted between different laboratories using multi-platform methods. These variations are due to an error created in each independent measurement which multiplies at consecutive steps during calculations. It is assured that the single-platform CD4 and CD8 T-cell determination technique is an acceptable alternative to the multi-platform method. Single-platform assays using a known number of reference particles resulted in significant and substantial improvements in laboratory-to-laboratory and within-laboratory precision over multi-platform methods for T-cell enumeration (1).

The method for absolute cell counting that employs flow cytometry and calibrated fluorescent particles is known as the ratio-metric method of absolute counting. This method uses only light scattering parameters for cell gating on the flow cytometer. Forward angle light scatter measures the size of the cells. Side scatter, right angle light scatter, measures the granularity of the cells. Cells viewed under these parameters simultaneously produce a distinct cell group on the dot plot. During T-cell counting, a gate is placed around the lymphocytes as the sample is viewed with forward angle light scattering parameters vs. right angle light scattering parameters with the flow cytometer.

The number of lymphocytes then is measured as the events are gated on a CD3-FITC vs. CD4-RPE dot plot. Next, the total number of calibration fluorescence particles is counted when the events from RPE-Cy5 vs. forward angle light scatter parameters are gated. The absolute CD4+ T-cell count is equal to the ratio of CD4+ cells counted to the number of calibrated particles counted, multiplied by the concentration of the calibration particles considering that the concentration of the calibration particles is known (5). Other cells types can be counted using similar flow cytometer parameters.

The SPHERO™ AccuCount Particles are designed to be used as reference particles during cell enumeration. The absolute cell number can be determined using the SPHERO™ AccuCount Particles since the number of particles per mL is known (4). The SPHERO™ AccuCount Particles are very easy to use and are cost effective. The AccuCount Fluorescent Particles are fluorescent in FITC, PE, PE-Cy5 and APC channel. Both AccuCount

Fluorescent and AccuCount Blank (nonfluorescent) Particles are available in 5, 7 and 10µm particles size to accommodate the size of the cells to be counted. These particles are designed for research use only and are not approved for clinical use.

Procedure

To obtain accurate absolute cell counts, the SPHERO™ AccuCount Particles are used in conjunction with flow cytometry. The SPHERO™ AccuCount Particles have a concentration of approximately $1x10^6$ particles/mL. The actual concentration is listed on the Technical Data Sheet for the product. The first step during sample preparation is to add the monoclonal antibody to 100µL of the test sample. The sample is then incubated, lysed, washed, and resuspended in 1 to 2 mL of phosphate buffer saline, 0.1M, pH 7.4. If staining and lysing are not necessary, add a known volume of test sample to the 1 to 2 mL of phosphate buffer saline. Washing the AccuCount Particles with the sample prior to analysis is discommended because a reduction in the number of reference particles will occur. Next, add exactly 50µL of the AccuCount Particle to the suspension. The precision during pipetting of the AccuCount Particles is absolutely critical. The sample is then analyzed by flow cytometry. The bead and cell population are gated on the fluorescence and/or side scatter channel. Record the number of events for the AccuCount Particles and the test sample. The absolute cell count is then determined with the following equation.

CALCULATION:
(A/B) x (C/D) = Number of Cell per µL

Where:
A = number of events for the Test sample
B = number of events for the AccuCount Particles
C = number of AccuCount Particles per 50µL
D = volume of test Sample initially used in µL

Conclusion

The SPHERO™ AccuCount Particles are used to accurately and rapidly obtain absolute cell counts. SPHERO™ AccuCount Particles are used as reference particles during cell enumeration. T-cell counts using the flow cytometry and calibration fluorescence particles gives better reproducibility and agreement for replicated samples compared to multi-platform methods. The precision of single-platform absolute T-cell counts over multi-platform methods is increased with careful and precise pipette measurements (2). The use of fluorspheres for routine clinical assessment of CD4 and CD8 absolute cell counts is recommended (3).

References

1. Reimann, Keith A., O'Gorman, Maurice R.G., Spritzler, John, Wilkening, Cynthia L., Sabath, Daniel E., Helm, Karen, Campbell, Donald E., and the DAIDS/NIAID New Technology Evaluation Group. 'A multisite comparison of CD4 and CD8 T lymphocyte counting by single vs. multiple platform methodologies: Evaluation of Beckman Coulter® Flow-Count™ Fluorospheres and tertaONE™ SYSTEM', The Adult AIDS Clinical Trials Group. 1999. (WWW) http://aactg.s-3.com/pub/download/imm/IQA/flowcount.pdf (accessed 11 March 2002)

2. Shenton, B.K., Manas, D., Dark, J.H., Talbot, D., Clark, K.R., and Bell, Alison. 'Immune monitoring of Anti-Thymocyte Globulin (ATG) treatment in transplant patients', Clinical and Applied Immunology Reviews, 1, 347-371 (2001)

3. Schnizlein-Bick, Carol T., Spritzler, John, Wilkening, Cynthia L., Nicholson, Janet K.A., O'Gorman, Maurice R.G., Site Investigators, and the DAIDS/NIAID New Technology Evaluation Group. 'Evaluation of TruCount™ Absolute-Count Tubes for Determining CD4 and CD8 Cell Numbers in Human Immunodeficiency Virus-Positive Adults', The Adult AIDS Clinical Trials Group. (WWW) http://aactg.s-3.com/pub/download/imm/IQA/trucount.pdf (accessed 11 March 2002)

4. Alugupalli, Kishore R., Michelson, Alan A., Barnard, Marc R. and Leong, John M. 'Serial Determination of Platelet Counts in the Same Mouse by Flow Cytometry', Thrombosis and Haemostasis, 86, 668-671 (2001)

5. O'Gorman, Maurice. 1998, 'Evaluation of Single-Platform Technologies for Absolute CD4+ and CD8+ Cells', paper presented at the Workshop Session-HIV Disease Prognostic Indicators at the 1998 Conference on the Laboratory Science of HIV, (WWW) http://www.phppo.cdc.gov/mlp/pdf/labhiv/MO'Gorman.pdf (accessed 20 March 2002)