

Introduction

In recent years, the determination of absolute cell counts has been shown to be relevant in different research and clinical settings. The enumeration of absolute levels of cells and cell subsets in clinical samples is an established technique in the quantification of CD4⁺ and CD8⁺ T-lymphocytes for the monitoring of patients with human immunodeficiency virus (HIV⁺) infection, and in the measurement of CD34⁺ haematopoietic stem and progenitor cells in patients who are candidates for bone marrow autotransplantation. This technique has also been used to quantitate the number of residual leukocytes in evaluating leukoreduced blood products for transfusion.

Absolute cell subset counts determined by flow cytometry can be gained using either a dual-platform technique combining flow cytometry with a haematology analyser, or a single-platform technique using flow cytometry alone. The single-platform technique is the more frequently used method for absolute cell enumeration as it avoids wide interlaboratory variations and underestimations. The identified cell subsets are then directly related to the original blood volume. The analysed blood volume can be determined by either a volumetric or a fluorescent microsphere-based method.

Description

Developed by Dr. Alberto Orfao and colleagues in Spain, **Perfect-Count Microspheres** are an efficient single-platform method for absolute cell counts that combines the advantages of direct flow cytometric immunophenotyping with the use of two different fluorescent microspheres (A and B beads). These two fluorospheres are used as a double internal standard for blood volume calculation. A known volume of Perfect-Count Microspheres is added to the same known volume of stained blood in a lyse-no-wash technique. The beads are counted along with cells. Since the **concentration of fluorospheres is known**, the number of cells per microliter (the absolute count) is obtained by relating the number of cells counted to the total number of fluorescent microsphere events. The cell number is then multiplied by the number of total fluorospheres per unit of volume. **As the Perfect-Count Microspheres system contains two different fluorospheres in a known proportion, we can first assure the accuracy of the assay by verifying the proportion of both types of beads. The final absolute count is determined as described in the following process:**

Final Absolute Count= (number of cells counted/ total number of Microspheres counted (A+B)) x number of Microspheres per µl (known concentration)

Microspheres type A and B have been chosen on the basis of their different characteristics. These differences allow the determination of whether the microspheres have been homogeneously sampled. Both types of microspheres contain multiple fluorospheres stable for a long period. These

fluorospheres can be detected in FL1, FL2, FL3 and FL4; however, type A and B microspheres present different fluorescence intensities. This permits their differentiation.

Procedure

This method is not affected by the use of different lysing reagents or varying antibody volumes. High-precision dispensing is limited only to the blood and fluorospheres pipetting step. Here, a reverse pipetting technique must be used.

The major essential requirements for this absolute counting method are the following:

- Relevant cells are defined by their immunofluorescence
- Pipette precision represents the main area for potential variability. For this reason a reverse pipetting technique should be used. Briefly, the pipette plunger is pressed to the second stop, the fluid is aspirated in slight excess and the aspirated sample is dispensed against the lower end of the wall of the tube until the first pipette stop, leaving some residual sample in the pipette tip.
- A threshold or discriminator can be set on the fluorescence channel that includes all the positively stained cells and the two fluorosphere bead clusters.
- At least 1,000 events of each bead must be acquired to ensure the accuracy of the assay.

Advantages

1. *Perfect-Count Microspheres* are the only available single platform reagent for absolute counts with a double internal standard represented by two different types of beads (type A and type B). This allows a determination of whether acquisition of the sample by the flow cytometer is performed homogeneously. The *Perfect-Count Microsphere* system contains 2 types of beads that float at different levels in the tube. The accuracy of the assay is checked by verifying that the proportion of both types of beads after acquisition of the sample agrees with the manufactures indicated proportion.
2. *Perfect-Count Microspheres* are easy to acquire and analyse as they avoid side scatter adjustments in the clear visualisation of all positively stained cells as well as the two-fluorescent bead clusters. *Perfect-Count Microspheres* supply one value of absolute counts for cell populations in a single tube. That is a more accurate, precise and reproducible method for determining final absolute cell counts.
3. *Perfect-Count Microsphere* is a product strictly controlled by Cytognos S.L. and our quality control guarantees initial bead quantity or concentration of total fluorospheres, the proportion between type A and type B and the stability of the product.
4. *Perfect-Count Microspheres* are suspended in a solution containing bovine serum albumin in the suspension media to prevent bead sticking to the tube walls.

Protocol

A. Preparation of the sample

A.1. Verify the accuracy of the pipette. Pipette calibration can be performed using distilled water (1µl distilled water= 1mg) and a precision weighing scale. Generally, fresh samples are easier to analyse. Therefore, it is recommended to use samples that are less than 48 hours old. The use of cryopreserved samples is not recommended. Mix the sample well without vortexing.

A.2. The primary sample pipetting step plays the major role in influencing measurement precision and accuracy, therefore reverse pipetting must be used. To reverse pipette, the pipette plunger is pressed to the second stop, the fluid is aspirated in slight excess, and the aspirated sample is dispensed against the dry round bottom of the test tube until the first pipette stops. This will leave some residual sample in the pipette tip. It is recommended not to use the first sample taken for dispensing (dry tip dispensing). In order to perform wet tip dispensing, draw sample to the second pipette stop, do two or three gentle dispense cycles at the first stop, keeping the pipette tip within the sample; and finally dispense at the first stop against the lower end of the wall of the tube. Pipette by reverse pipetting technique 100µl of blood into each tube.

A.3. Stain cells adding the monoclonal antibodies of interest to each tube. Mix gently and incubate for 10minutes at room temperature in dark. Add 100µl of Cal-Lyse™ (Product codes GAS-010 and GAS-010S-100) lysing solution (kept at room temperature) to each tube, vortex each tube. Mix and incubate in the dark for 10minutes at room temperature. Add 1ml of deionised water (kept at room temperature), incubate for 5 minutes and vortex*.

A.4. Immediately prior to use, carefully mix *Perfect-Count Microspheres* for 30-45seconds manually (do not vortex). With the same pipette used for the sample dispensing, add 100µl (same value as sample) of *Perfect-Count Microspheres* to each tube of lysed sample using the reverse pipetting technique.

A.5. Cover sample tube with Parafilm and mix thoroughly for 30seconds just before acquisition on a flow cytometer.

*For additional information please see Cal-Lyse No Wash Staining procedure on the Cytomark website: [http://cytomark.co.uk/downloads/Caltag - CalLyse application protocol.pdf](http://cytomark.co.uk/downloads/Caltag_-_CalLyse_application_protocol.pdf)

B. Instrument Setup:

B.1. Follow manufacturer's instrument setup procedure and run protocol compatible with colour combination used. Run an unstained and lysed tube, setting a **low value of threshold or discriminator in the forward scatter (FSC) channel**. No additional modification of FSC and side scatter (SSC) detectors are needed to acquire these beads. Perfect-Count Microspheres can be detected on FSC/SSC dot plot and in FL1, FL2, FL3 and FL4.

B.2. Draw a region on the FSC/SSC dot plot that includes the leukocyte subpopulations and the two fluorescent bead clusters.

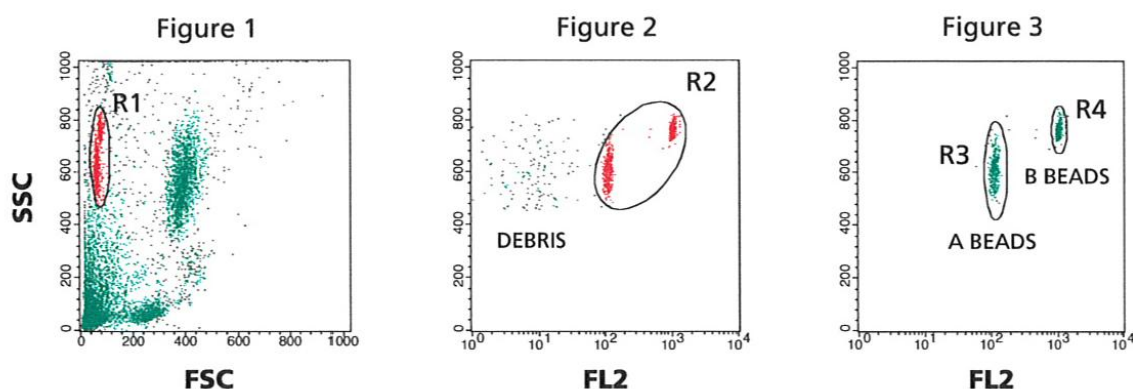
C. Acquire data

C.1 To obtain reliable values collect a minimum of 1000 microspheres. And at the same time collect an appropriate number of cells.

D. Analysis of results

D.1 Analysis of A and B bead proportion:

- Create a gate selecting all beads (type A and type B) on FSC/SSC dot plot (Region A or R1), Figure 1
- Display only region A or R1 on a FL2/SSC dot plot. FL1, FL3 or FL4 may be used if the staining of cells interferes with bead analysis. Create a new gate (Region B or R2) to select all beads, making sure to exclude debris, Figure 2.
- Display only region B or R2 on a FL2/SSC dot plot. Create two new gates (Regions C and D or R3 and R4) to select and differentiate type A (LOWER INTENSITY FOR FL2) and type B beads (HIGHER INTENSITY FOR FL2), Figure 3.
- Verify on the gate statistics table that the proportion of each type of gated beads, agrees with the manufacturers indicated proportions.
- Note the number of total beads for later calculations.



D.2 Create gating regions to select cell subsets of interest in each study.

- Note number of events in regions of interest for later calculations.

D.3 Calculate absolute number of any gated cell population.

- Calculate the absolute number of cell population of interest according to the following formula:

$$\text{Absolute Count (cells / } \mu\text{l)} = \frac{\text{Number of cells counted}}{\text{Total number of fluorospheres counted}} \times \text{Number of Perfect count Microspheres per } \mu\text{L}$$

STORAGE CONDITIONS:

Store at 4°C. DO NOT FREEZE.

This product is photosensitive and should be protected from light.

Reagents are stable for the period shown on the vial label when stored properly.

HEALTH AND SAFETY INFORMATION:

This product contains 0.1% sodium azide (NaN₃).

EWG-Nr. 247-852-1. R22: harmful if swallowed.

S46: If swallowed, seek medical advice immediately and show this container or label.

LOT SPECIFIC DATA:**Volume/Tests:**

PCB-100: 10ml/100 tests

Perfect Count beads are manufactured for Caltag Medsystems Ltd by:

Cytognos

37007-SALAMANCA (SPAIN)

CAL-LYSE Lysing Solution :

Product Code GAS-010 – 25ml for 250 tests

Product Code GAS-010s – 100ml for 1000 tests

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