



PerfectCount™ Cell Counting System (Patent Pending)

INSTRUCTION MANUAL

CATALOG NUMBERS X1271K AND X1272K

BACKGROUND

In recent years, the enumeration of absolute cell counts has become relevant in different research and clinical settings. The enumeration of absolute levels of cells and their subsets in clinical samples is an established technique in enumeration of CD4+ and CD8+ T-lymphocyte for monitoring of patients with human immunodeficiency virus (HIV+), enumeration of CD34+ hematopoietic stem and progenitor cells in patients candidates for autotransplantation, and in enumeration of residual leukocytes in evaluating leukoreduced blood products for transfusions.

Absolute cell subset counts by flow cytometry can be performed using dual-platform technique (flow cytometry combined with a hematology analyzer) and single-platform technique (flow cytometry alone). The single-platform technique is the most frequently used method for absolute cell counting since it avoids wide interlaboratory variations and underestimation's, allowing to positively identify the cells of interest and exclude contaminating cells. The identified cell subsets are then directly related to the original blood volume. The analyzed blood volume can be determined by either a volumetric or a fluorescent microsphere-based method.

PerfectCount™ Microspheres are an efficient single-platform method for absolute counts which 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. Known volumes of PerfectCount Microspheres are added to the same known volume of stained blood in a lyse-no-wash technique and the beads are counted along with cells. Since the **concentration of fluorospheres is known**, the number of cells per microliter (absolute counting) is obtained by relating the number of cells to the number of each one of the two fluorescent microsphere events and multiplying by the number of each fluorosphere per unit of volume. **As the PerfectCount microspheres system contains two different fluorospheres in a known proportion, assuring the accuracy of the assay by verifying the proportion of both types of beads, and the final absolute count is determined as is described in the following process:**

Final Absolute Count = (number of cells counted / total number of fluorospheres counted (A+B)) x number of fluorospheres per μ l (Known Concentration)

Microspheres type A and B have been chosen on the basis of their different characteristics which assures a homogeneous acquisition of the sample. Both types of microspheres contain multiple fluorophores stable for long period that can be detected in FL1, FL2, FL3 and FL4 but type A and B microspheres present different fluorescence intensities which permits their differentiation.

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Exalpha Biologicals, Inc., 86 Rosedale Rd. Watertown, MA 02472
Tel: 800.395.1137 or 617.924.3400, Fax: 866.924.5100 or 617.924.5100,
Web:www.exalpha.com

PROCEDURE

This method is independent of lysing procedures and antibody volumes. High-precision dispensing is required only in the blood and fluorosphere pipetting step(s) therefore a reverse pipetting technique must be used (see Procedure section, Step A2 for details of this technique).

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

- Relevant cells are defined by their immunofluorescence.
- Pipette precision is the main variability factor. 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 lower end of 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 same fluorescence channel that includes all the positively stained cells and the two fluorosphere clusters.
- At least 1,000 events of each bead must be acquired to ensure an adequate accuracy of the assay.

ADVANTAGES

1. PerfectCount™ Microspheres are the only single platform for absolute count system available with a double internal standard represented by two different types of beads (type A and type B), which determines if acquisition of the sample by the flow cytometer is performed homogeneously. PerfectCount™ Microspheres contain 2 types of beads which float at different levels in the tube. The accuracy of the assay is verified by the proportion of both types of beads after acquisition of the sample with the manufacture's indicated proportion.
2. PerfectCount™ Microspheres are easier to acquire and analyze since the procedure avoids side scatter adjustments in order to visualize clearly all the positively stained cells and the two fluorescence clusters.
3. PerfectCount™ Microspheres supply one value of absolute counts for cell populations in a single tube, which offers a more accurate, precise and reproducible method to determine final absolute cell counts.
4. PerfectCount™ Microspheres are strictly quality-controlled by Exalpa Biologicals to guarantee initial bead quantity or concentration of total fluorospheres and proportion between type A and type B and stability of the product for long period.
5. PerfectCount™ Microspheres contain bovine albumin in suspension media to prevent bead sticking to the tube walls.

PROTOCOL

IMPORTANT TIP: Accurate pipetting is required for accurate test results. Please use the pipetting instructions provided in step A.2 for pipetting of blood and PerfectCount™ samples.

1. Preparation of the sample

A.1 Verify the accuracy of the pipette. Pipette calibration can be performed using distilled water (1 µl distilled water = 1 mg) and a precision weighing scale.

Generally, fresh samples are easier to analyze therefore it is recommended to use samples 48 hour old or less. The use of cryopreserved samples is not recommended.

Homogenize the sample (no Vortex)

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- A.2 The primary sample pipetting step plays the major role in influencing measurement precision and accuracy, therefore reverse pipetting must be used. 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 stop, leaving some residual sample in the pipette tip. It is recommended do not use the first sample taken for dispensing (dry tip dispensing). In order to perform a wet tip dispensing draw sample at 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 wall of the tube.
Pipette by reverse pipetting technique 100 µl blood into each polystyrene tube.
- A.3 Stain cells by adding monoclonal antibodies of interest (or Lymphogram™ as described in Technical Bulletin) to each tube. Mix gently and incubate for 10 minutes at room temperature in dark. Add 2 ml of lysing solution (QuickLyse, Cat. No. QL250) in each tube. Mix and leave in dark for 10 minutes at room temperature.
- A.4 Immediately prior to use, carefully mix PerfectCount™ Microspheres for 30-45 seconds manually (do not vortex). With the same pipette instrument used for the sample dispensing, add 100µL (same volume as original sample) of PerfectCount™ Microspheres to each tube of lysed sample. Use of the same pipette instrument with a clean tip is imperative as this instrument has been calibrated for this experiment.
- A.5. Cover the sample with Parafilm and homogenize for 30 seconds before acquisition

2. Set up instrument

- B.1 Follow manufacture's instrument setup procedure and run protocol compatible with color combination used. Run an unstained and lysed tube setting **at low value of threshold or discriminator on the FSC**, no additional modification on FSC and SSC detectors are needed to acquire these beads. PerfectCount™ Microspheres are detected on FSC/SSC dot plot and in fluorescences FL1, FL2, FL3 and FL4.
- B.2 Draw a region beads FSC/SSC dot plot which includes leukocytes subpopulations and the two fluorescence cluster.

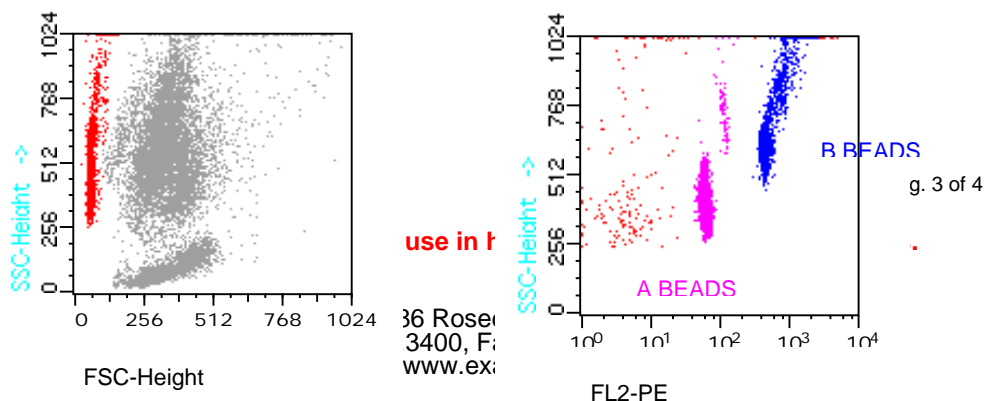
3. Acquire data

- C.1 To obtain a reliable absolute count value, collect a minimum of 1000 microspheres. At the same time, collect appropriate number of cells.

4. Analysis of results

D.1 Analysis of type A and B beads proportion:

- Create a gate selecting all beads (type A and type B) on FSC/SSC dot plot (Region A or R1)
- Display only region A or R1 on a SSC/FL2 dot plot. Create two new gates to select



and differentiate beads type A and type B.

- Verify on gate statistic table that proportion of each type of gated beads after acquisition of the sample coincide with the manufacture indicated proportion.
- Note number of total beads for later calculations

D.3 Create gating regions to select cell subsets of interest in each study.

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

D.4 Calculate absolute number of cell population

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

$$\text{Absolute Count (cells/}\mu\text{l)} = \frac{\text{Number of Cells Counted}}{\text{Total Number of Beads Counted}} \times \text{Number of PerfectCount microspheres per } \mu\text{l}$$

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