AccuCheck COUNTING BEADS

Product code: PCB100 (100 tests)

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 hematology analyzer, or a single-platform technique using flow cytometry alone. The single-platform technique is the most 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 analyzed blood volume can be determined by either a volumetric or a fluorescent bead-based method.

DESCRIPTION

Developed by Dr. Alberto Orfao and colleagues in Spain, AccuCheck Counting Beads 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 beads (A and B beads). These two fluorospheres are used as a double internal standard for blood volume calculation. A known volume of AccuCheck Counting Beads 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 bead unknown, the number of cells per microliter (the absolute count) is obtained by relating the number of cells counted to the total number of fluorescent bead events. The cell number is then multiplied by the number of total fluorospheres per unit of volume. As the Pipette Check Counting Bead 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 describes in the following process:

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

Beads A and B have been designed such that their unique characteristics allow the determination of whether they have been homogenously sampled. Bead A is a 6.4µM sphere that in flow cytometry presents a low forward scatter (FSC) signal, a lower side scatter (SSC) signal, and emits broadly when excited with a 488 nm argon laser; however, bead A does not fluoresce when excited with either a 633 nm HeNe or 635 nrn red diode laser. Bead B is a 6.36 µm sphere that presents a low FSC signal, a slightly higher SSC signal and a higher fluorescent signal when excited with a 488 nm argon laser. Unlike bead A, bead B fluoresces when excited by a longer wavelength red laser.

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 bead events must be acquired to ensure the accuracy of the assay.

For research use only. CAUTION: Not intended for human or animal therapeutic or diagnostic use.

ADVANTAGES

1. AccuCheck Counting Beads are the only available single-platform reagent for absolute cell 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 AccuCheck Counting Beads 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 manufacturer's indicated proportion.
2. AccuCheck Counting Beads are easy to acquire and analyze as they avoid side scatter adjustments in the clear visualization of all positively stained cells as well as the two-fluorescent bead clusters. AccuCheck Counting Beads 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. The production of AccuCheck Counting Beads is strictly controlled to guarantee the initial bead quantity or concentration of total fluorospheres, the proportion of type A and type B and the stability of the product.
4. AccuCheck Counting Beads are suspended in a solution containing bovine serum albumin in the suspension media to prevent beads from 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 (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 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 stop. This will leave some residual sample in the pipette tip. It is recommended that the first sample taken for dispensing (dry tip dispensing) be discarded. 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 100 µl of blood into each tube.

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

A.4 Immediately prior to use, carefully mix AccuCheck Counting Beads for 30-45 seconds manually (do not vortex). With the same pipette used for the sample dispensing, add100 µl (same value as sample) of AccuCheck Counting Beads to each tube of lysed sample using the reverse pipetting technique.

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

* For additional information please refer to the Cal-Lyse™ No-Wash Staining Procedure on Cal-Lyse ™ Lysing solution (Cat# GAS010) data Sheet available on Invitrogen web site, www.invitrogen.com.

B. **Flow Cytometer Set Up**

B.1 Follow manufacturer's instrument setup procedure and run protocol compatible with color combination used. Run an unstained and lysed tube, setting **a low value for threshold or discriminator in the FSC channel**. No additional modification of FSC and SSC detectors is needed to acquire these beads. AccuCheck Counting Beads can be detected on a FSC vs. SSC dot plot as well as in the appropriate fluorescence channel.

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

C. **Acquisition of Data**

C.1 To obtain reliable values collect a minimum of 1,000 beads. 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 vs. SSC dot plot (Region R1), Figure 1.
- Display only region R1 on a FL2/SSC dot plot. A different channel should be used if the staining of cells interferes with bead analysis. Create a new gate (Region R2) to select all beads, making sure to exclude debris, Figure 2.
- Display a FL2/SSC dot plot gated on R1 and R2. Create two new gates (Regions 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 manufacturer's indicated proportions.
- Note the number of total beads for later calculations.

Figure 1

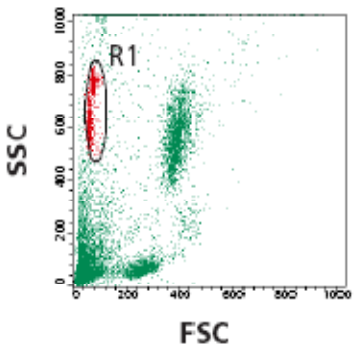


Figure 2

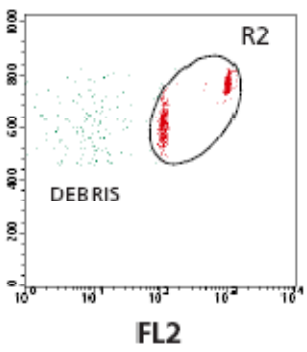
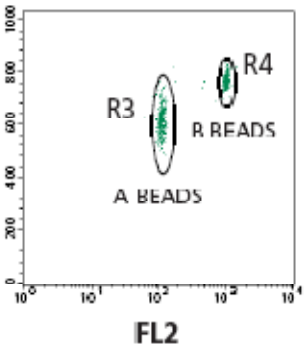


Figure 3



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:

Absolute Count (cells/µl) = $\frac{\text{number of cells counted}}{\text{Total number of beads counted}}$ **x** **Number of AccuCheck Counting Beads / Beads per µl**

STORAGE CONDITIONS

Store at + 4^{0}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 (NaN_3).
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

See COA

Volume/Tests:

PCB-100: 10 ml/ 100 test
Cal-Lyse™ Lysing Solution:
Code GAS-010 - 25 ml - 250 tests
Code GAS-010s - 100 ml - 1000 tests

Explanation of symbols

Symbol	Description	Symbol	Description
REF	Catalogue Number	LOT	Batch code
RUO	Research Use Only	IVD	*In vitro* diagnostic medical device
	Use by		Temperature limitation
	Manufacturer	EC REP	European Community authorised representative
[-]	Without, does not contain	[+]	With, contains
	Protect from light		Consult accompanying documents
	Directs the user to consult instructions for use (IFU), accompanying the product.		

For research use only. CAUTION: Not intended for human or animal therapeutic or diagnostic use.

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