

Application Note 10

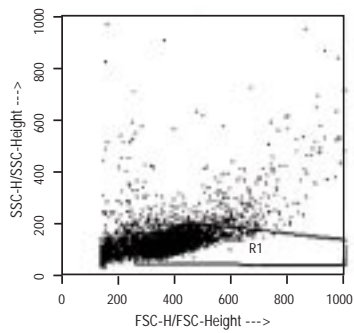
Measurement of Cell Recovery

After Sorting with a Catcher-Tube-Based

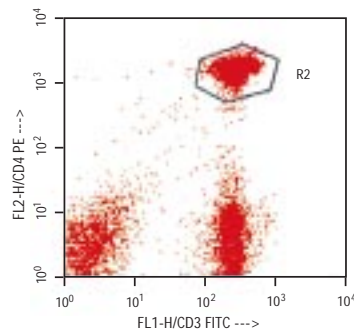
Cell Sorter

Introduction

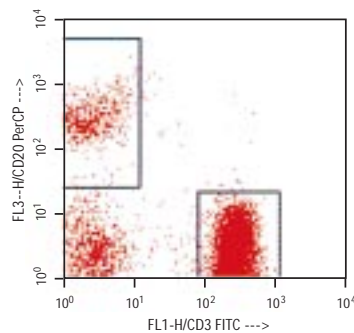
In many experiments using sorted cells, it is important to be able to count the number of cells recovered from the collection tube in addition to measuring sample purity. We present here a method for simultaneously counting cells and measuring sample purity quickly and reproducibly by adding a known number of beads to a sample, then analyzing by flow cytometry. A new method of analysis can be performed rapidly and easily using *Attractors*[™] software, now available from Becton Dickinson. We demonstrate that at least 75% of the sorted cells can be recovered from the collection tubes with a purity >97% when using this procedure.[†]



1a

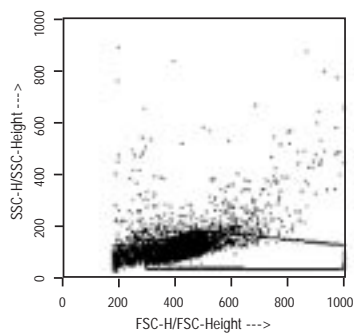


1b

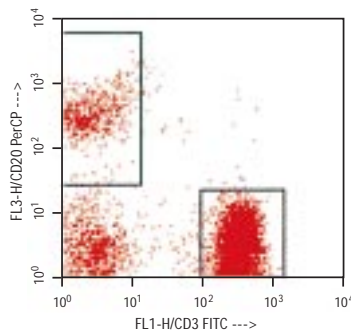


1c

Figure 1 Dot plots of (1a) forward scatter versus side scatter, (1b) FL1 versus FL2 gated on lymphocytes, and (1c) FL1 versus FL3 gated on lymphocytes, before sorting. A logical combination (R1 AND R2 AND R3) of the lymphocyte gate (R1) and two fluorescence gates (R2, R3) was used to sort the CD3⁺ CD4⁺ population.



2a



2b

Figure 2 Dot plots of (2a) forward scatter versus side scatter, and (2b) FL1 versus FL3 gated on lymphocytes, before sorting. A logical combination (R1 AND R5) of the lymphocyte gate (R1) and a fluorescence gate (R5) was used to sort the CD3⁻ CD20⁺ population.

Materials and Methods

Cell Preparation and Staining

Twenty mL of whole blood was collected into a VACUTAINER® K₃ EDTA blood collection tube (Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ). Peripheral blood mononuclear cells (PBMCs) were isolated using LeucoPREP® separation tubes (Becton Dickinson Labware [BDL], Lincoln Park, NJ) as instructed in the package insert. The PBMCs were washed with Ca⁺⁺/Mg⁺⁺-free Dulbecco's phosphate-buffered saline (DPBS; ICN Biomedicals, Costa Mesa, CA) containing 0.5% bovine serum albumin (BSA) (Sigma Chemical Company, St. Louis, MO), and resuspended at 1 x 10⁷ cells/mL. An aliquot of unstained cells was retained for an autofluorescence control to optimize the instrument. Twenty million cells were stained with 400 µL each of CD3 (Leu™-4) FITC, CD4 (Leu-3) PE,[‡] and CD20 (Leu-16) PerCP[§] (Becton Dickinson Immunocytometry Systems [BDIS], San Jose, CA) for 20 minutes on ice. The stained cells were washed twice, and resuspended at 2 x 10⁷ cells/mL in DPBS-BSA for sorting. Three compensation control samples containing only one fluorochrome each were prepared by staining 100 µL of cells with either 20 µL of CD3 FITC, 20 µL of CD4 PE, or 20 µL of CD20 PerCP. After a 20-minute incubation, the samples were washed with DPBS-BSA, then resuspended in 500 µL DPBS-BSA. A sample containing 1 x 10⁶ cells/mL was prepared by adding 50 µL of the 2 x 10⁷ suspension to 950 µL of DPBS-BSA, for use later as a cell count control. The cells were stored at 4°C until after the sort.

Flow Cytometry

The FACSsort was set up using AutoCOMP™ software (BDIS) and CaliBRITE™ beads (BDIS). Amplifiers were set with the scatter channels in linear mode and the fluorescence channels in log mode. Detector settings were adjusted to bring the unstained cell population on scale in all scatter and fluorescence parameters. The compensation networks were adjusted to remove any spectral overlap between the fluorescence channels using the compensation control samples. For the first sort, the cells were sorted using a logical combination (R1 AND R2 AND R3) of the lymphocyte scatter region (R1), the CD3⁺CD4⁺ fluorescence region (R2) and the CD3⁺CD20⁻ fluorescence region (R3) as shown in Figure 1. This population comprised approximately 40% of the sample. For the second sort, the cells were sorted using the logical combination (R1 AND R5) of the lymphocyte population (R1) and the CD3⁻CD20⁺ fluorescence region (R5) as shown in Figure 2. This population comprised approximately 8.5% of the sample.

A 10,000-event presort data file and a data file of the unstained cells were collected, then the cells were sorted in Single Cell mode using the LO flow rate. The CD3⁺CD4⁺ cells were sorted at a rate of approximately 250 cells/s with an FSC threshold rate of ~3000 cells/s. One hundred thousand cells were sorted into each of three 50-mL polypropylene conical tubes (BDL) that had been coated overnight at 4°C with 4% BSA-DPBS.¹ After rinsing the sort lines by running a tube of PBS on the sample injection port and allowing the fluid to drip for 1 minute into each of the three collection stations, the CD3⁻CD20⁺ cells were sorted at a rate of ~50 cells/s, with 25,000 cells sorted into each of two 50-mL coated tubes. Tubes were kept at 4°C until the sorts were complete. The cells were concentrated by centrifuging the 50-mL tubes for 10 minutes at 300 x g. Approximately 500 µL was left in the tubes after aspiration of the excess supernatant. The cell pellets were resuspended and the cell suspensions were transferred to separate 12 x 75-mm tubes for reanalysis. We will refer to these tubes as the *recovery tubes*.

Analysis of Sorted Cells

To confirm the counting method was valid, count control tubes were prepared from the sample containing 10⁶ cell/mL. The cell count in this tube was first confirmed by hemacytometer count, then dilutions were prepared as follows: 10⁵ cells/tube (100 µL cells + 200 µL PBS), 5 x 10⁴ cells/tube (50 µL cells + 250 µL PBS), and 10⁴ cells/tube (10 µL cells + 290 µL PBS). Three replicate tubes of each dilution were prepared.

Unlabeled CaliBRITE beads were used as a reference for determining the cell counts. Two hundred microliters of well-mixed beads were diluted 1:4 in 600 µL of PBS. The beads were then counted on a hemacytometer.

Twenty-five microliters of counted CaliBRITE beads were added to each count control and recovery tube. The samples were resuspended, then run on the FACSort after lowering the FSC threshold to allow for acquisition of the CaliBRITE beads. An FSC vs SSC dot plot of the cells plus beads is shown in Figure 3. A count gate of 1000 events was set on R1 to ensure that an adequate number of cell events were acquired for each sample, and data was collected for each tube. Three data files were saved for each of the 50-mL recovery tubes to obtain triplicate readings, and a data file of the beads only was also collected.

Data Analysis

The count control data files were analyzed using an FSC vs SSC dot plot in Lysys II software. (A similar procedure can be followed in CELLQuest software.) Regions were set to include all the cell events and all the bead events (see Figure 3). By viewing the region statistics, the number of cell events and the number of bead events could be determined. The number of cells in each tube was determined using the following calculation (number of beads was determined by the hemacytometer count):

$$\# \text{ cells} = \frac{\# \text{ cell events}}{\# \text{ bead events}} \times \# \text{ beads}$$

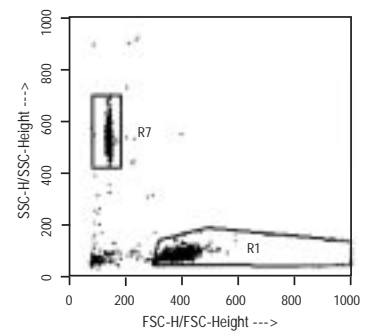


Figure 3 Dot plot of forward scatter versus side scatter showing the cells (R1) and the unlabeled CaliBRITE beads (R7). These are also the regions used to determine the number of cells and beads.

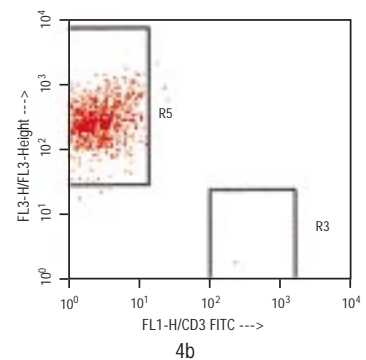
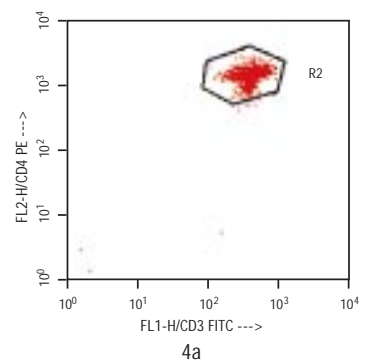


Figure 4 Dot plots showing the post-sort purities gated on cell events. The CD3⁺CD4⁺ sort is shown on the FL1 versus FL2 plot (4a). The average purity of this sort was 99.6%. The CD3⁻CD20⁺ sort is shown on the FL1 versus FL3 plot (4b). The average purity of this sort was 97.9%.

The same method was used to determine the number of cells in the recovery tubes. The percent recovery was calculated by dividing the calculated cell number by the number of cells sorted (as given by the Sort Total in the FACSSort window) and multiplying by 100. The percent purity was obtained by viewing the quadrant statistics of the fluorescent populations through a gate that excluded the beads (NOT R7) or that excluded all noncellular events (R1). Post-sort dot plots are shown in Figure 4.

Data Analysis Using *Attractors™* Software

Attractors is an advanced flow cytometry data analysis software that does not use the traditional gate and quadrant analysis to calculate results. Instead, an attractor—a cluster-seeking object that automatically adapts to user-defined populations—identifies the populations of interest and calculates statistics for those populations. Attractors is a Macintosh-based automated software that allows quick and accurate analysis of data, without operator intervention.

To analyze the recovery data** using Attractors software, an attractor set was created defining the bead population, the lymphocyte population and the two subset populations that were sorted. An example attractor set is shown in Figure 5. By saving the attractor set after it was created, it can be opened for future recovery experiments. The batch analysis function of Attractors software was used for simple data analysis. The values from the spreadsheet created by the batch analysis were used for the same calculation as in the LYSYS II analysis to determine the number of cells recovered from the sort. The purity was calculated by the software and reported in the spreadsheet as the Membership % for the attractors of the sorted populations.

Another feature of Attractors software, the absolute count function, may also be used for this analysis. For more information on this and other features of Attractors software, please refer to the *Attractors User's Guide*.

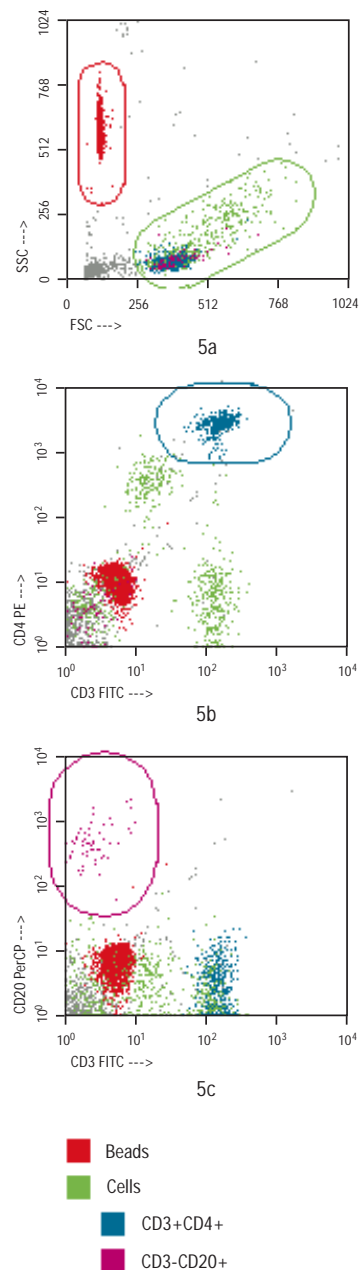


Figure 5 Attractor set for the analysis of the recovered cells. There is a bead attractor and a cell attractor with two sort subattractors.

Results

The graph in Figure 6 shows the excellent correlation of the expected cell counts versus the cell counts calculated using this method. The method is consistent for the replicates at each dilution and yields cell counts that are >90% of the cell counts obtained using a hemacytometer.

For the CD3⁺CD4⁺ sort, the average recovery was approximately 95%. For the CD3⁺CD20⁺ sort, the average recovery was approximately 75%. The purities for both sorts were >97% when gated on cell events. The results for each sorted tube are shown in Table 1. Although comparison data is not shown, we have observed that coating the collection tubes with BSA before use is critical for achieving maximal recovery. Elimination of the precoating step can reduce the recovery to as low as 10%.

Recovery Tube	Average* Cell Number ±SEM)**	Average Recovery Number ±SEM)**	Average Purity ±SEM)**
CD3 ⁺ CD4 ⁺ #1	85,433 ±1451	85.4% ±1.5	99.6% ±0.1
CD3 ⁺ CD4 ⁺ #2	95,533 ±1963	95.5% ±2.0	99.7% ±0.1
CD3 ⁺ CD4 ⁺ #3	101,000 ±577	101.0% ±0.5	99.6% ±0.3
CD20 ⁺ #1	17,933 ±554	71.5% ±2.2	97.0% ±0.3
CD20 ⁺ #2	19,564 ±993	78.2% ±4.0	98.7% ±0.8

* Averages were taken from triplicate flow cytometric analyses of each tube
** Standard Error of the Mean

Discussion and Summary

In this application note we have described a method for counting cells by flow cytometry using a calibrated bead population as a reference. With this method we have shown it is possible to obtain accurate counts on samples with as few as 10,000 cells/tube. Two factors key for obtaining accurate results with this method are: the numbers from the count control samples and the reference beads must be accurate, and a standard curve has to be generated to verify the accuracy of the calculated cell counts.

We have also demonstrated that with this method, recovery and purity can be measured simultaneously. A variation of the methodology presented here is to analyze only a measured aliquot of the sorted sample for recovery and purity, then apply a correction factor to the equation in the Data Analysis section. Using an aliquot rather than the entire sample adds another source for error in calculating cell counts; therefore, you must ensure the sample and aliquot volumes are measured accurately.

Finally, we have shown it is possible to recover at least 70% of the cells after sorting with a FACSORT, even when the target population comprises a relatively small percentage of the original cell suspension.[†]

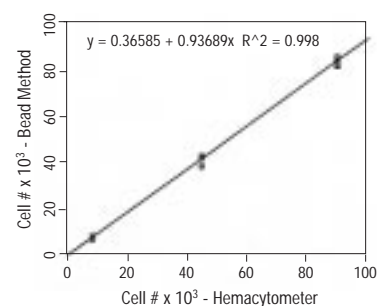


Figure 6 The cell number as counted on the hemacytometer versus the cell number as calculated using the CalIBRITE bead method.

Table 1 The average cell number, average recovery, and average purity when gated on cell events for each sorted tube. The number of cells counted was ~1,000 for each of the samples, and the number of beads added to each tube was 139,500.

BD publishes this method

as a service to investigators.

Detailed support for non-flow

cytometric aspects of this

procedure may not be

available from BDIS.

** This application is for*

research use only. Not

for use in diagnostic or

therapeutic procedures.

Hints

- When analyzing the sorted sample, decrease the FSC threshold to detect all the CaliBRITE bead events, and use the LO flow rate to obtain a tight bead population distribution. These steps ensure all the bead events will be enumerated when you draw the region around the population.
- Use a reliable electronic counter if possible, to count your bead standard and the initial dilution of your cell count controls. This eliminates an additional hemacytometer count.
- If your count controls are calculated to be less than 90% of the expected number, you may use a correction factor as derived from the standard curve (Figure 6) to get a better estimation of the recovered cell number. You should then try to determine the reason for the discrepancy.
- To determine the viability of the cells after acquiring your count and purity file, add an equal volume of PBS containing 2 µg/mL propidium iodide (PI) to each tube, incubate for 10 minutes, and acquire another data file. The FL2 bright population is the dead cell population.
- Use a staining strategy that includes one reagent that does not stain your target cells. Dead or highly autofluorescent cells are often positive for all parameters.
- Gate on the cellular events when calculating purity to ensure debris is not a factor.
- When using the Absolute Count function of Attractors software, be sure to have very accurate bead counts and sample volume measurements to ensure correct results. Resuspending the sorted samples in a specific volume rather than an estimated volume will help ensure the volume is correct.

Contributors

Erin Handsfield Scholz and Donna Gandour
Becton Dickinson Immunocytometry Systems, San Jose, CA

† It is important to note that many factors can affect the number of cells recovered from a given sort. Factors such as cell type, length of sort, sort fraction handling, and sample preparation can affect results.

‡ US Patent No. 4,520,110.

§ US Patent No. 4,876,190.

¶ US Patent No. 5,232,828.

** Data files acquired on the Hewlett Packard platform must be transferred to the Macintosh using FACSNet™ or Consort™ File Exchange. The files must also be converted to FCS 2.0 format using FACSConvert. Please consult the appropriate user's guide for details.

**BECTON
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United States
Becton Dickinson
Immunocytometry Systems
2350 Cume Drive
San Jose, CA 95131-1807
Ordering Information
(800) 223-8226
Customer Support Center
Tel (800) 448-BDIS (2347)
Fax (408) 954-BDIS (2347)
www.bdfacs.com

Becton Dickinson Canada, Inc
2464 South Sheridan Way
Mississauga, Ontario
L5J 2M8
Canada
Tel (905) 822-4820
Fax (905) 822-2644

Becton Dickinson European HQ
Denderstraat 24
B-9320 Erembodegem-Aalst
Belgium
Tel (32) 53-720211
Fax (32) 53-720450

Nippon Becton Dickinson
Company Ltd
DS Bldg
5-26, Akasaka 8-chome
Minato-ku, Tokyo 107
Japan
Tel (81) 3-5413-8251
Fax (81) 3-5413-8155

Becton Dickinson and Company
Asia Pacific Division
30 Tuas Avenue #2
Singapore 639461
Tel: (65) 860-1478
Fax: (65) 860-1590



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