*CountBright™ Absolute Counting Beads *for flow cytometry**

Quick Facts

Storage upon receipt:

- 2–6ºC
- Protect from light

Fluorescence

- excitation: UV to 635 nm
- emission: 385 nm to 800 nm

Nominal microsphere concentration:

50,000 beads in 50 µL (Lot specific concentration provided on label)

Introduction

Flow cytometry provides a rapid method to quantify cell characteristics, however most flow cytometers cannot directly provide the cell concentration or absolute count of cells in a sample. Absolute cell counts have been widely used in quantifying cell populations and disease progression, including studies of stem cells and HIV/AIDS.[1-5] Absolute cell counts are generally obtained either by combining a separate cell concentration determination from a hematology analyzer with flow cytometric population data (multiple platform testing) or by adding an internal microsphere counting standard to the flow cytometric sample (single platform testing). The single platform method is preferred as it is technically less complicated and more accurate than multiple platform testing.[6]

CountBright™ absolute counting beads are a calibrated suspension of microspheres that are brightly fluorescent across a wide range of excitation and emission wavelengths and contain a known concentration of microspheres. For absolute counts, a specific volume of the microsphere suspension is added to a specific volume of sample, so that the ratio of sample volume to microsphere volume is known. The volume of sample analyzed can be calculated from the number of microsphere events, and can be used with cell events to determine cell concentration. In general, at least 1,000 bead events should be acquired to assure a statistically significant determination of sample volume.

CountBright™ absolute counting beads can be used with any sample type, including no-wash/lysed whole blood. The microspheres in the reagents are approximately 7 µm in diameter and have settling properties similar to lymphocytes. Sample preparation steps that can lead to cell or microsphere loss, such as washes, should be avoided. For antibody protocols, CountBright™ absolute counting beads should be used with reagents titered for no-wash staining.

CountBright™ absolute counting beads can be used with either a scatter or fluorescence threshold. When using a scatter threshold, the microsphere signal should be above the threshold. The microspheres can be gated by a single parameter, but a combination of parameters can be used to resolve microspheres from cells and other events.

Materials

Kit Component

CountBright™ absolute counting beads, 5 mL in 0.1% Tween 20, 2 mM sodium azide

The kit provides sufficient reagents for 100 flow cytometry assays, each using 50 µL of counting beads per test.

Spectral Characteristics

CountBright™ absolute counting beads are broadly fluorescent. Fluorescence can be excited by wavelengths from UV to 635 nm; fluorescence emission can be read between 385 nm and 800 nm. The fluorescence intensity of the microspheres has been adjusted to be about 5–50 times brighter than the anticipated intensities of typically stained cells.

Storage and Handling

Upon receipt, store the kit at 2–6˚C, protected from light. The CountBright™ absolute counting beads should be stable for at least 12 months.

Experimental Protocol for CountBright™ Absolute Counting Beads

Note: The accuracy of cell counts based on CountBright™ absolute counting beads depends on sample handling and the precise delivery of the volume of beads. The CountBright™ absolute counting beads must be mixed well to assure a uniform suspension of microspheres; vortex for 30 seconds before removing an aliquot. The microsphere suspension can be pipetted by standard techniques, but more viscous solutions, such as blood, require reverse pipetting for accurate volume delivery. Cell suspensions may be diluted, but should be assayed without wash steps.

2.1 Allow the CountBright™ absolute counting beads to come to room temperature. Gently vortex the microsphere suspension for 30 seconds to completely resuspend.

2.2 Immediately after vortexing the counting bead suspension add 50 µL of counting beads to the stained cells and vortex.

Note: It is important to record the volume of cells before the addition of the counting beads and to use a volume of at least 300 µL. At this dilution, the small amount of Tween 20 and sodium azide contributed by the CountBright™ absolute counting beads has not been noted to affect cell staining or viability. In some cases, volumes of counting beads other than 50 µL may be appropriate. Ensure the equation used to calculate cell numbers is adjusted to account for the different volume.

2.3 Run the sample on the flow cytometer. Set the forward scatter threshold low enough to include the microspheres on the forward scatter vs linear side scatter plot. Gate on the CountBright™ absolute counting beads using a forward vs linear side scatter plot. Verify that the microsphere gate includes the highest side scatter gate (Figure 1). If using CD45+ vs log side scatter gating, the CountBright™ absolute counting beads can be distinguished from cells for gating (Figure 2).

Note: A fluorescence threshold may also be used to analyze cells and microspheres. Collect at least 1,000 bead events to assure a statistically significant determination of sample volume.

2.4 The counting beads will appear in the upper right region of all fluorescence dot plots (Figures 3, 4, and 5), and can be gated accordingly.

Note: If the CountBright™ absolute counting beads cannot be resolved from cells using a particular emission parameter combination, use a different combination of emission parameters to gate the counting beads.

CountBright™ absolute counting beads can be used with a variety of reagents and kits. Examples of the use of CountBright™ beads with our Vybrant® Apoptosis Assay Kit #4 (V13243) and our LIVE/DEAD® Viability/Cytotoxicity Kit (L3224) are shown in Figures 4 and 5, respectively.

Calculation of cell concentration:

$$\frac{A}{B} \times \frac{C}{D} = \text{concentration of sample as cells/µL}$$

Where:
A = number of cell events
B = number of bead events
C = assigned bead count of the lot (beads/50 µL)
D = volume of sample (µL)

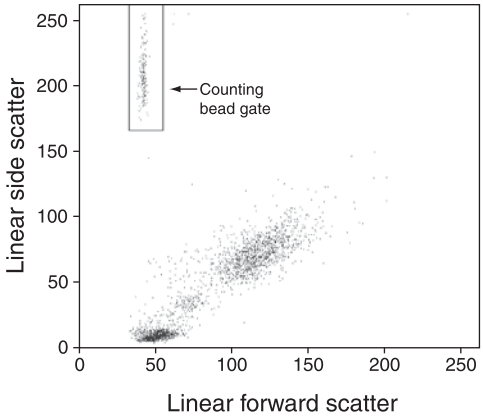

Figure 1. Counting bead gating with no-wash/lysed whole blood. Peripheral blood was lysed with Caltag Cal-Lyse™ Lysing Solution before adding CountBright™ absolute counting beads. A forward scatter vs linear side scatter plot with forward scatter threshold set to exclude debris was used for analysis. The counting bead gate was adjusted to include the last channel in side scatter.

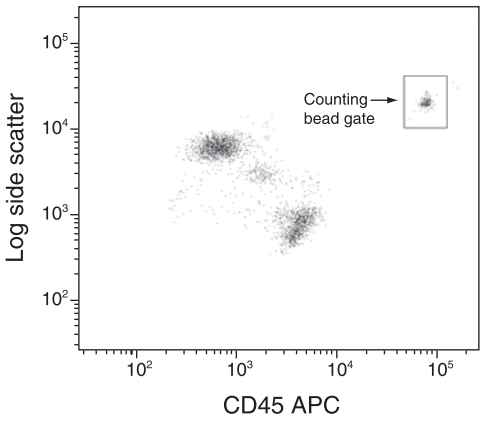

Figure 2. Counting bead gating with no-wash/lysed whole blood and CD45 gating. Peripheral blood lysed with Caltag Cal-Lyse™ Lysing Solution was stained in a no-wash assay format with anti-CD45 allophycocyanin before adding CountBright™ absolute counting beads. A plot of CD45-positive cells vs logarithmic side scatter shows the counting bead gate.

Example calculation: A 1,000 µL volume of cells was stained. Afterwards, 50 µL of CountBright™ absolute counting beads was added.

$$\frac{1{,}700 \text{ cells}}{1{,}030 \text{ beads}} \times \frac{49{,}500 \text{ beads}/50 \text{ µL}}{1{,}000 \text{ µL}} = 81.7 \text{ cells/µL}$$

Note: The calculation should be corrected if the sample is diluted or if a different volume of CountBright™ absolute counting beads is used.

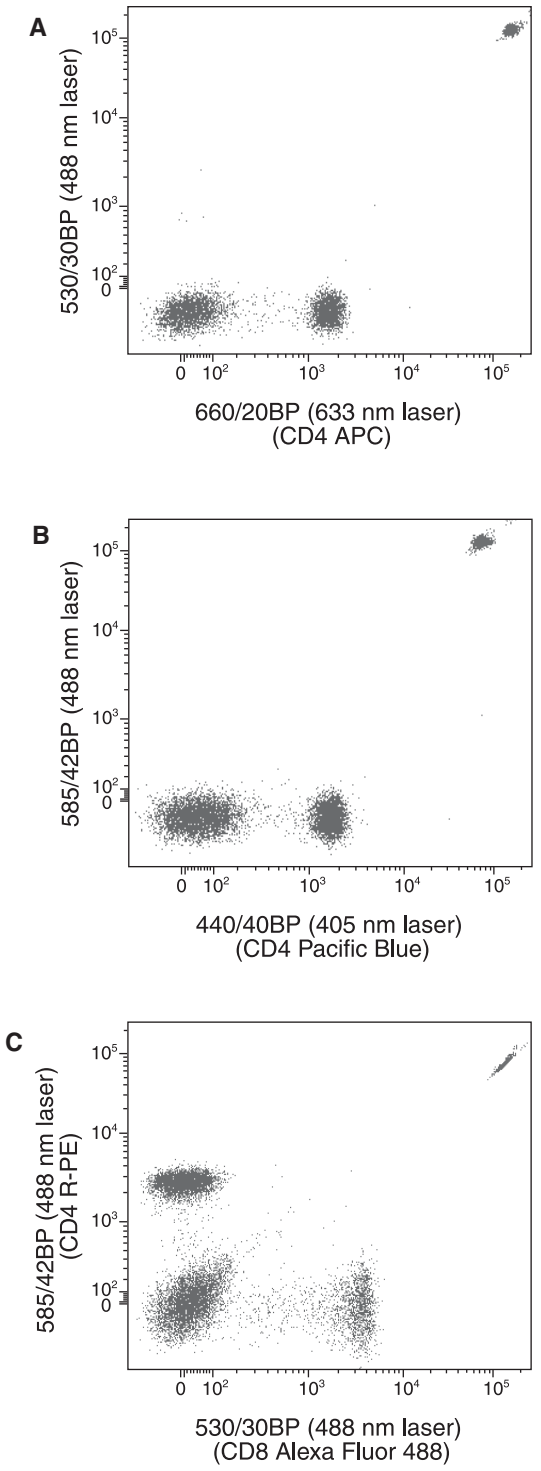

Figure 3. Stained no-wash/lysed whole blood, gated on lymphocytes with the addition of counting beads. A) Plot of cells stained with an anti-CD4 allophycocyanin (APC) conjugate and collected through a 660/20 bandpass filter with 633 nm excitation vs a 530/30 bandpass filter with 488 nm excitation. B) Plot of cells stained with anti-CD4 Pacific Blue™ and collected through a 450/50 bandpass filter with 405 nm excitation vs a 585/42 bandpass filter with 488 nm excitation. C) Dual parameter plot of cells stained with anti-CD4 R-phycoerythrin (R-PE) collected through a 585/42 bandpass filter and anti-CD8 Alexa Fluor® 488 collected through a 530/30 bandpass filter with 488 nm excitation.

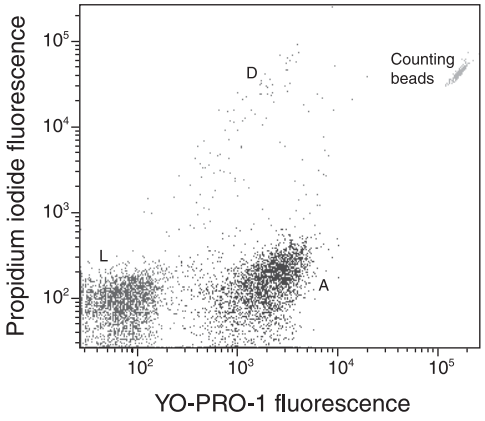

Figure 4. Plot of YO-RPO®-1 fluorescence collected with 530/30 bandpass filter vs propidium iodide fluorescence collected with 585/42 bandpass filter, showing apoptotic (A), live (L), and dead (D) cells, as well as counting beads. Jurkat cells (human T-cell leukemia) treated with 10 µM camptothecin for four hours. Cells were then treated with the reagents in the Vybrant® Apoptosis Assay Kit #4 (V13243). Counting beads were added and the sample was analyzed by flow cytometry using 488 nm excitation.

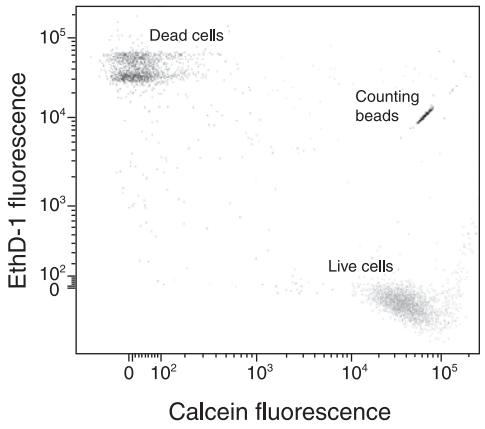

Figure 5. Plot of calcein fluorescence collected with 530/30 bandpass filter vs ethidium homodimer-1 fluorescence collected with 610/20 bandpass filter, showing clear separation of live and dead cells, as well as counting beads. A mixture of live and heat-killed Jurkat cells (human T-cell leukemia) were treated with the reagents in the LIVE/DEAD® Viability/Cytotoxicity Kit (L3224), counting beads were added and the sample analyzed by flow cytometry using 488 nm excitation.

References

1. Cytotherapy 5, 55 (2003); **2.** Br J Haematol 106, 1059 (1999); **3.** Clin Diagn Lab Immunol 7, 336 (2000); **4.** J Acquir Immune Defic Syndrom 39, 32 (2005); **5.** Br J Haematol 115, 953 (2001); **6.** MMWR Recomm Rep 52,1 (2003).

Product List *Current prices may be obtained from our website or from our Customer Service Department.*

Cat #	Product Name	Unit Size
C36950	CountBright™ absolute counting beads *for flow cytometry* *100 assays*	5 mL

Contact Information

Further information on Molecular Probes products, including product bibliographies, is available from your local distributor or directly from Molecular Probes. Customers in Europe, Africa and the Middle East should contact our office in Paisley, United Kingdom. All others should contact our Technical Service Department in Eugene, Oregon.

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