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Absolute Counts Using the ZE5 Cell Analyzer and Everest Software

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Introduction

This procedure allows you to calculate a standard curve that compares the performance of volumetric counting as calculated by the ZE5 Cell Analyzer (ZE5), to volumetric counting as calculated using an internal reference standard for a given set of conditions. Using this information, you can adjust your protocol and apply a calibration factor to adjust concentration, if needed. The best results are achieved by calibrating with the same samples, and at the same run conditions, that you will be using in your experiments. This procedure should be repeated after preventative maintenance or fluidics-related repair.

Important: The concentration statistic generated by the ZE5 is for informational use only and no claims are made for accuracy. Users should evaluate the counting performance based on the experimental conditions used, such as run speed, volume, sample, and so forth. This document contains a suggested guide for the counting procedure, but a mathematical calibration factor may be required to meet desired accuracy. In general, larger volumes and slower speeds provide the best performance.

Required Materials

- Beckman Coulter Flow Count Fluorospheres (Catalog No. 7547053), or Bio-Rad Flow Cytometry Absolute Count Standard (Catalog No. FCSC580) or equivalent

- Sample of cells to be used for experiment or Beckman Coulter CYTO-TROL Control Cells (Catalog No. 6604248) or equivalent

Instructions are based on using cells of interest or CYTO-TROL. Alternative biological or control materials can be used, but best results are obtained using the same sample type as used in the experiment.

Procedure

Calibration Beads Preparation

1. Vortex Flow-Count Fluorospheres for 10 to 12 secs (avoid excessive mixing to minimize air bubble formation).

2. Add 50 µL of Flow-Count Fluorospheres to an empty test tube or well for each serial dilution sample.
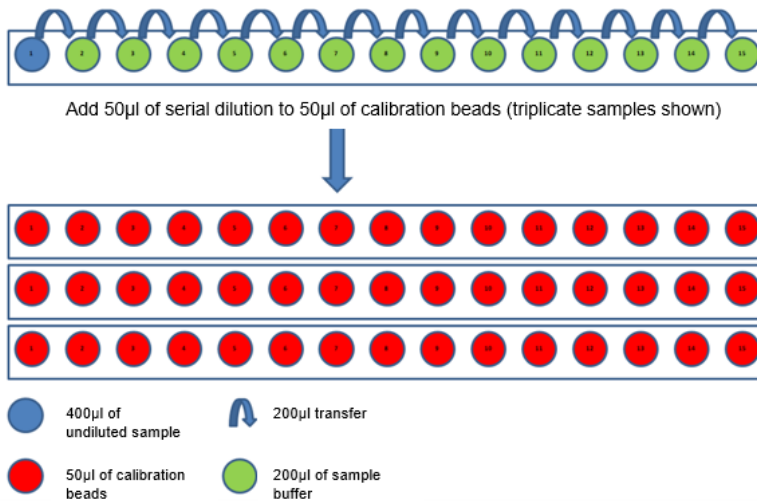
   Important: Do not pipette air bubbles. Proper counting depends on having the same volume of sample and beads; therefore, you should use the exact same pipette for transferring the beads as you use with cells.

10000158693 Ver A

Cell Preparation

1. Do one of the following

   ○ If using cells of interest, skip to step 4.

   ○ If using CYTO-TROL cells, pipette 1 mL of reconstitution buffer into a vial of cells. If a higher concentration is desired, you can suspend in 500 µl of reconstitution buffer.

2. Mix gently by inversion.

3. Allow cells to stabilize for 10 minutes before use.

4. Prepare serial dilutions of cells. Recommendation 1:2 for at least 15 dilutions.

   a. Place 400 µl of cell sample into the first tube.

   b. Place 200 µl of sample buffer in subsequent tubes.

   c. Transfer 100 µl of undiluted cells to the second tube containing 200 µl of buffer.

   d. Mix well and transfer 100 µl of that dilution to the next tube.

   e. Continue the dilution series until complete.

5. Transfer 50 µl of the diluted cells into the tubes or wells of a plate containing 50 µl of Flow-Count beads for each dilution. Set up three replicates.

   For best results, you should use the same pipette to dispense both cells and beads without adjusting volume in-between.

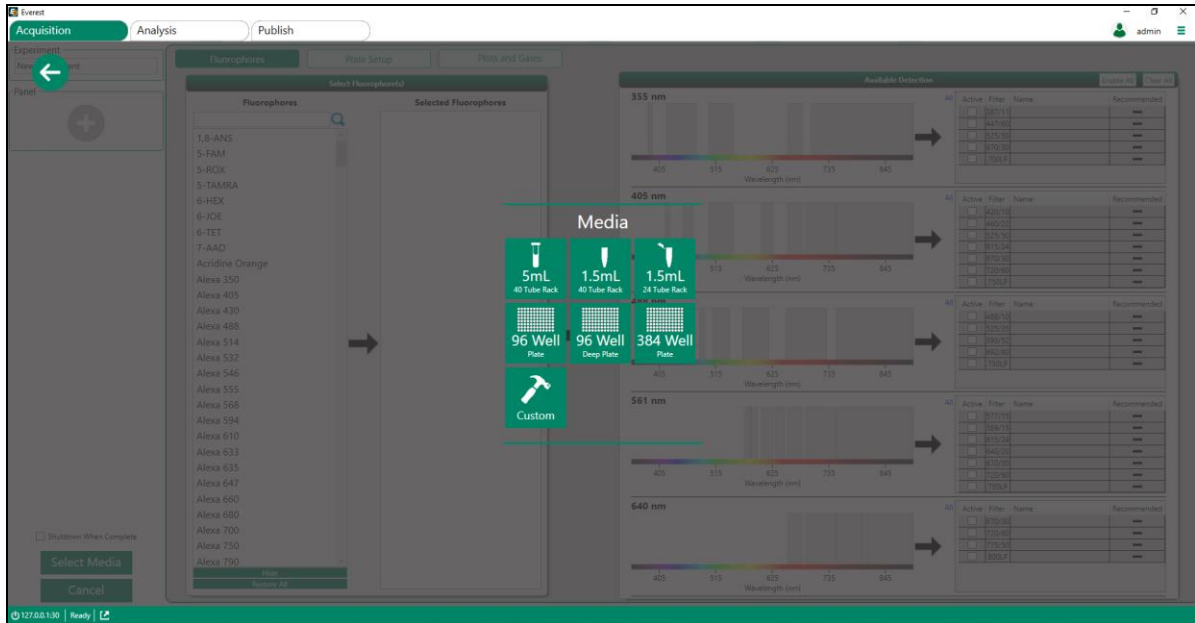**Preparation of serial dilution samples 1:2 final concentration**

Add 50µl of serial dilution to 50µl of calibration beads (triplicate samples shown)

● 400µl of undiluted sample	∩ 200µl transfer
● 50µl of calibration beads	● 200µl of sample buffer

Setup and Analysis in Everest Software

1. Log into Everest Software and click New Experiment.
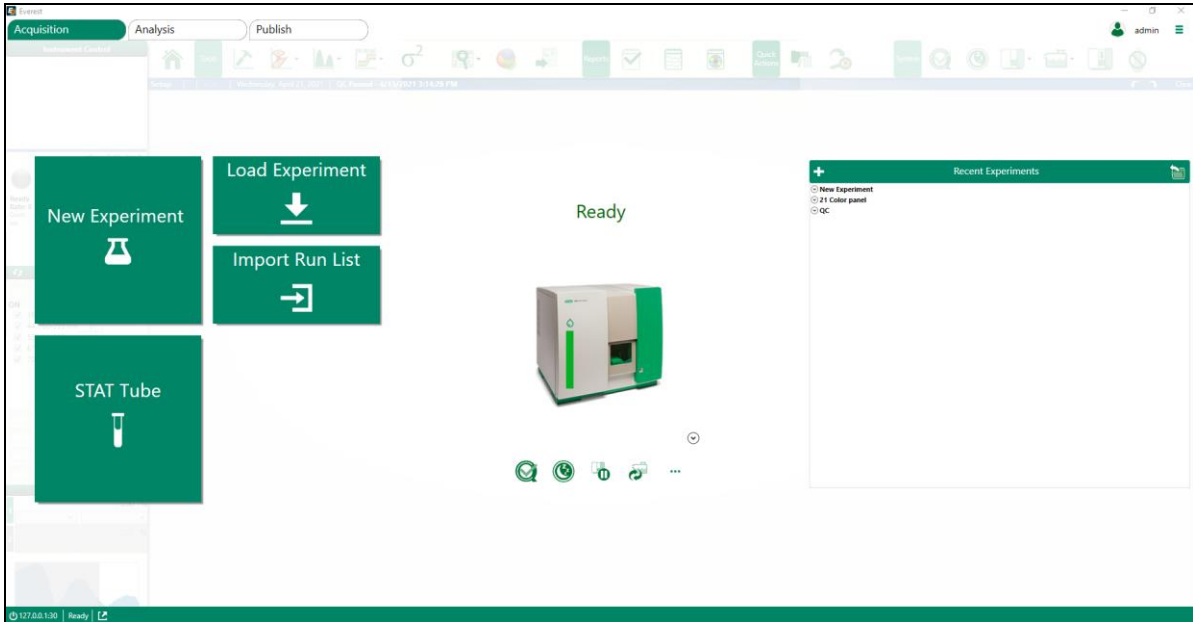
2. Select a media layout.

3. In the pane on the left, enter Absolute Counts as the panel name.

4. For your particular calibration beads, follow the manufacturer's recommended protocol for setting up gating for the fluorescent beads. On the Fluorophores tab, under Fluorophores, select the appropriate fluorophore for the counting beads you are using, and click the arrow to move it to Selected Fluorophores.
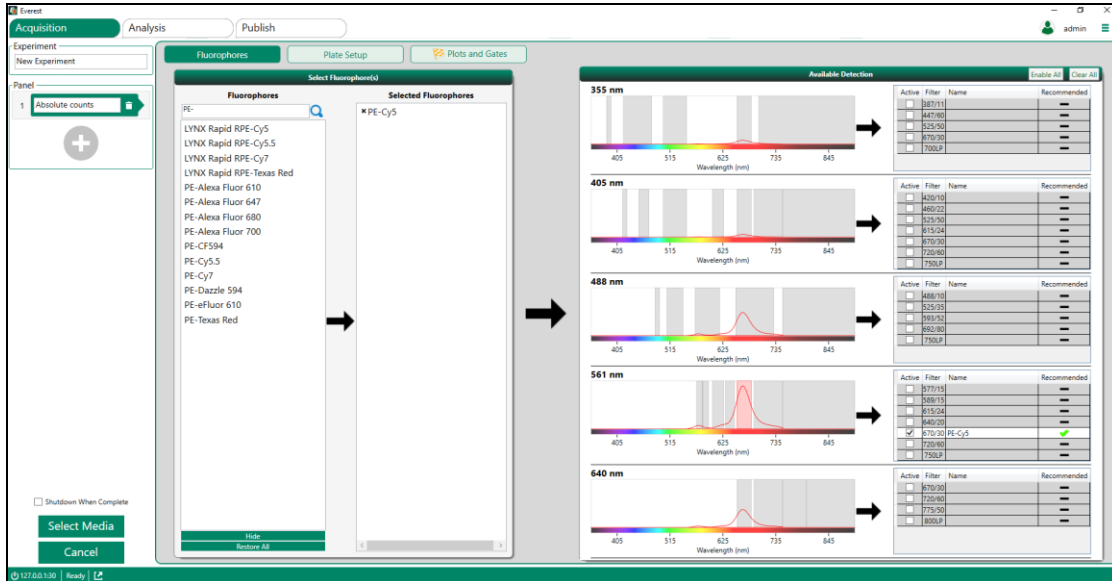
**Note:** PE-Cy5 is required for identification of Flow-Count beads.

5. Select the Plate Setup tab
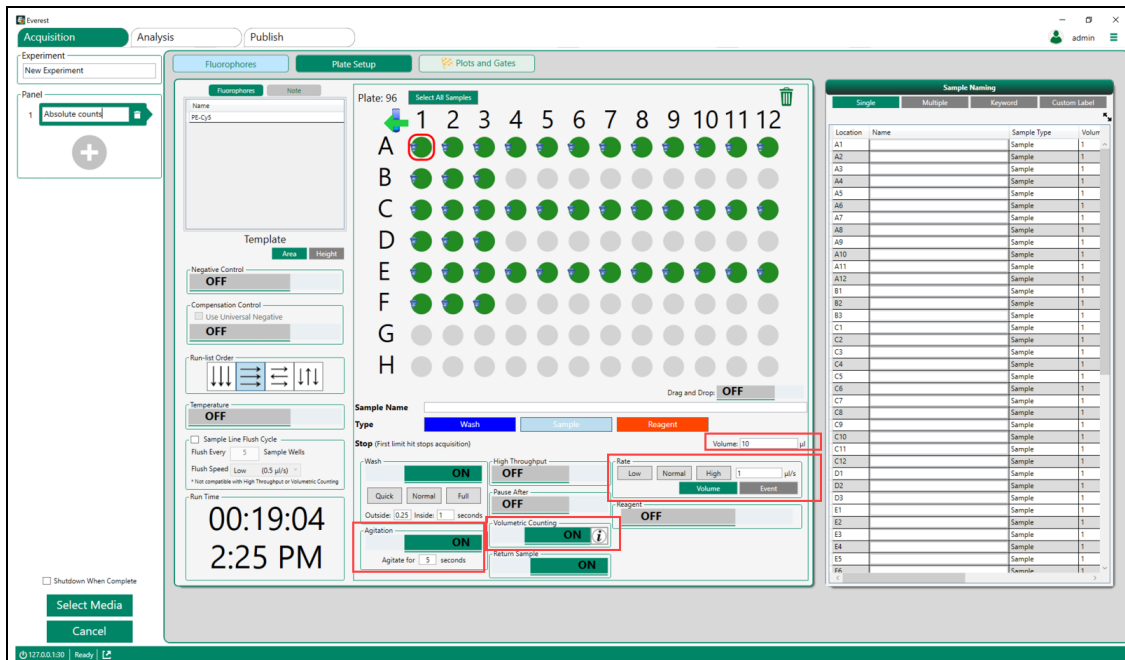
6. Select tube/well locations and select the Sample button below the plate layout.

7. Enable Agitation and Volumetric Counting.

Note: The best results are expected with volumetric counting, but you can use other modes.

8. Select the applicable Flow Rate (run speed) and enter the applicable Volume.

9. To facilitate setup, click the ⬅ icon to include a Stat Tube sample.

10. Click Plots and Gates to proceed to the next step.

11. Create plots for FSC A vs. FSC H and PE-Cy5 vs. FSC A., as follows:

    a. Create a region for cells (R1).

    b. Create a region around the Flow-Count beads. Label the beads as CAL.

    c. Create a gate to exclude doublets (R2) on the FSC A vs. FSC H and apply that gate/filter to the PE-Cy5 vs. FSC A plot.

    d. Click the ➡ icon in the lower-right corner to display the Acquisition screen.
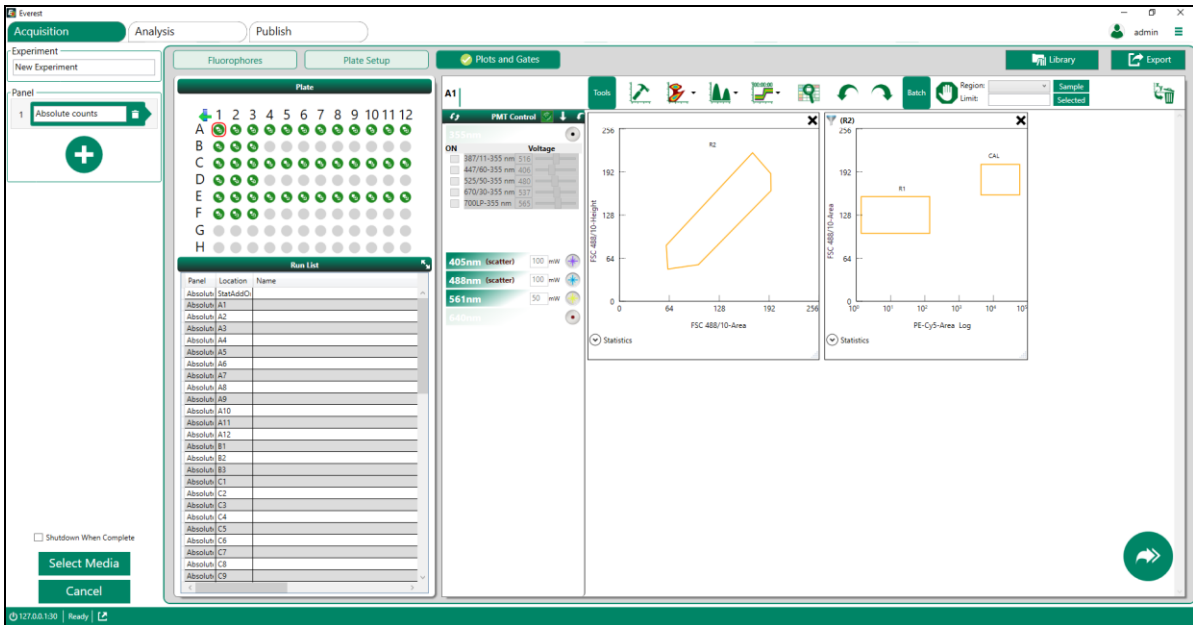
12. Using the Stat Tube in Setup mode, adjust FSC, SSC, and PE-Cy5 voltages so the Flow-Count and CYTO-TROL populations are on scale. Adjust gates accordingly.
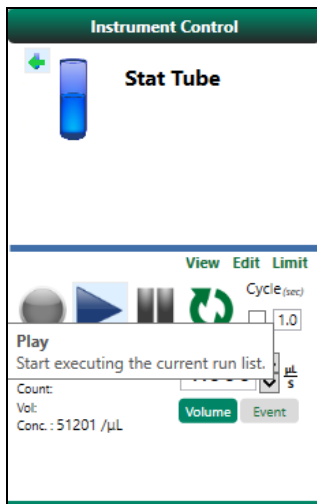
13. Switch to Acquisition mode, and ensure the first sample is selected (A1 in this case).

14. Press Play.
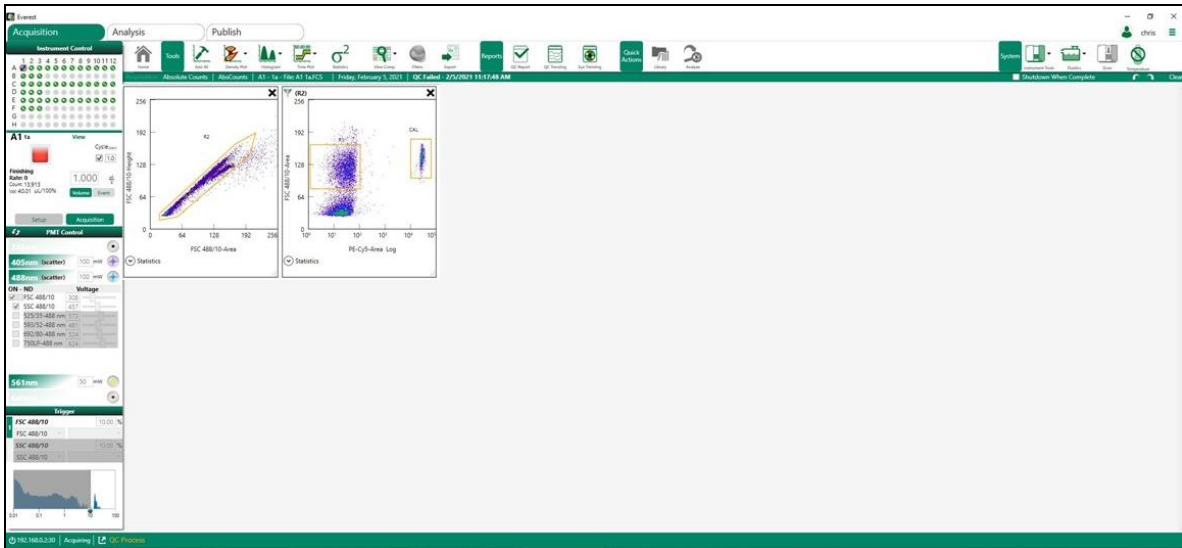
15. After all samples are acquired, plot the concentration of your cells, or CYTO-TROL, as determined by the concentration statistic in Everest Software vs the calculated concentration using the Flow-Count beads.

   Note: If applicable, you can use the slope of that line as a correction factor. Everest Software is not currently able to apply that correction factor, so you must manually adjust in whatever method you choose to display your results.

   Calculated Absolute Count (cells/mL) =

   [**Total number of your cells or CYTO-TROL counted** /

   (**Total number of calibration beads counted** × **the assayed calibration bead concentration provided by the vendor** × **0.5**)].

   Based on your results, you can define a concentration range and/or run conditions that provide the best performance.

Examples

Standard Acquisition Mode with 10,000 Event Limit

Sample volume varies when using an event limit. Inaccuracies are most apparent for high concentrations where the volume is low (~1 µl) or for heavily diluted samples, which have a larger volume but not enough counts.
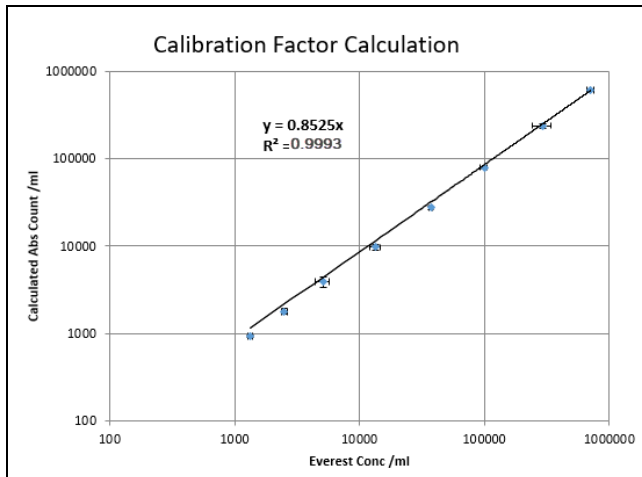
Figure A
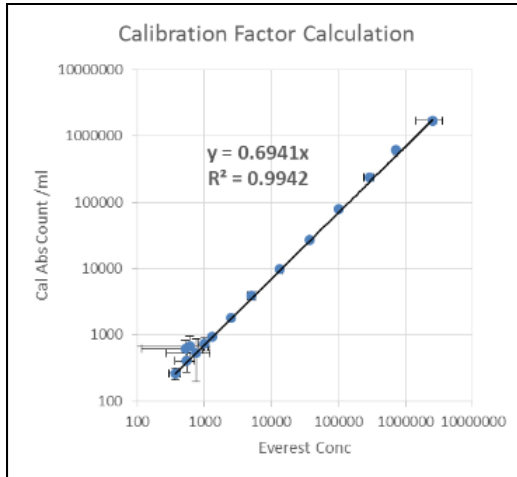
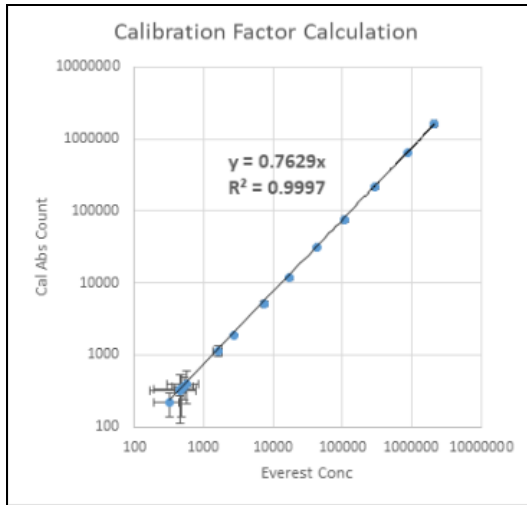| Acquisition conditions:	Triplicate samples Mean = ± SD Stop count = 10,000 events Volume range = 1.3 µl - 14.48 at 1.0 µl per sec
Results:	Average Everest cV = 27% Average beads cV = 18%

Figure B

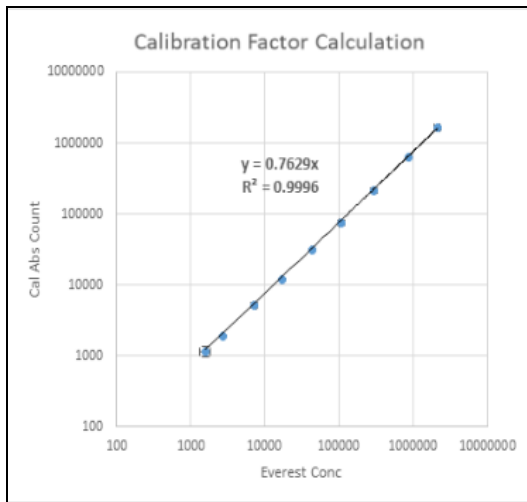| Acquisition conditions:	Same data as presented in Figure A, but high and low concentrations are removed to improve count accuracy and precision.
Results:	Average Everest cV = 8% Average beads cV = 6%

Volumetric Acquisition Mode

Figure C

Acquisition conditions:	Triplicate samples Mean = ± SD Volumetric = 20 µl at 1.0 µl per sec
	Note: Running a larger sample volume improves results at lower concentrations.
Results:	Average Everest cV = 20% Average beads cV = 21%

Figure D

Acquisition conditions:	Same data as presented in Figure C, but concentrations below 1000/ml are removed.
Results:	Average Everest cV = 6% Average beads cV = 6%

Contacting Technical Support

The Bio-Rad Technical Support department in the U.S. is open Monday through Friday, 5:00 AM to 5:00 PM, Pacific Time.

Phone: 1-800-424-6723, option 2
Email: Support@bio-rad.com (U.S./Canada Only)
For technical assistance outside the U.S. and Canada, contact your local technical support office or click the Contact Us link.

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For information on open source software used to develop Everest Software, see the ZE5 Cell Analyzer and Everest Software User Guide, Appendix D.

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