# Rare Event detection using Acoustic Cytometry

**April Anderson, Justin Hicks, and Jolene A. Bradford**
**Flow Cytometry Systems, Life Technologies, Eugene, Oregon 97402 USA**

Background: Detection of rare events includes populations of cells comprising less than 5% of the total cells, which includes the detection of stem cells, minimal residual disease, tetramers, NKT cells and fetomaternal hemorrhage. Analysis of rare cell populations requires the collection of high numbers of events in order to attain a reliable measure of accuracy, leading to long acquisition times.[1] Acoustic cytometry is a new technology that allows dilute samples to be processed quickly.[2] Conventional cytometers attain a static volumetric ratio of sheath to sample flow of typically greater than 100. By combining acoustic and hydrodynamic focusing, ratios can be reduced by several orders of magnitude and sample injection rates can be increased up to 6 fold (1ml/min) compared to pressure driven cytometers, allowing higher throughput with dilute samples. The collection times and percentage of rare events detected of the Applied Biosystems® Attune™ Acoustic Focusing Cytometer are compared to a conventional cytometer that uses hydrodynamic focusing.

Methods: One hundred microliters of whole blood collected from a normal donor was stained with 5µL CD45 Pacific Blue™ conjugate (Invitrogen) and 5µL CD34 phycoerythrin conjugate (Invitrogen). The sample was then incubated at room temperature for 20 minutes. Red blood cells were then lysed by the addition of 2mL High-Yield Lyse (Invitrogen) solution and incubated at room temperature for 10 minutes. SYTOX® AADvanced™ Dead Cell Stain (Invitrogen) was added at a concentration of 1µM and incubated for 5 minutes. Data was acquired on both the Applied Biosystems® Attune™ Acoustic Focusing Cytometer and the BD™ LSRII flow cytometer. Events were acquired using the high sample flow rate on the conventional cytometer and a sample flow rate of 1000 µl/min on the Attune™ acoustic cytometer. Dead cells were eliminated from the analysis by gating on SYTOX® AADvanced™ negative cells and then looking at SSC vs. CD34 events. The number of CD34-positive events were analyzed, acquisition time recorded, and the results compared across the instruments tested.

Results: Rare CD34-positive populations were detected on all instruments, giving equivalent results. The time of acquisition was substantially less using acoustic cytometry compared to conventional flow cytometry.

Conclusions: Acoustic cytometry allows faster sample acquisition times as compared to conventional hydrodynamic focusing instruments. Acquisition times can be reduced by 5-10 fold using the acoustic cytometer compared to a traditional pressure driven flow cytometer that uses hydrodynamic focusing. Comparable results in rare event detection were demonstrated between both instrument platforms. Acoustic cytometry allows for high throughput of samples without the need for sample concentration, making this platform ideal for no-wash techniques such as the detection of CD34 positive cells. Additionally, absolute counts can be obtained on the Attune™ cytometer without the need for an external counting source, such as counting beads.

Table 1. Table 1. A comparison of the theoretical maximum flow rates for commercially available cytometers across cell/particle concentrations

| Sample Concentration (particles/mL) | Sample Flow Rate (µl/min) 10 | 30 | 60 | 100 | 120 | 150 | 200 | 500 | 1000 |
|---|---|---|---|---|---|---|---|---|---|
| 1.00E+04 | 2 | 5 | 10 | 17 | 20 | 25 | 33 | 83 | 167 |
| 5.00E+04 | 8 | 25 | 50 | 83 | 100 | 125 | 167 | 417 | 833 |
| 1.00E+05 | 17 | 50 | 100 | 167 | 200 | 250 | 333 | 833 | 1,667 |
| 5.00E+05 | 83 | 250 | 500 | 833 | 1,000 | 1,250 | 1,667 | 4,167 | 8,333 |
| 1.00E+06 | 167 | 500 | 1,000 | 1,667 | 2,000 | 2,500 | 3,333 | 8,333 | |
| 5.00E+06 | 833 | 2,500 | 5,000 | 8,333 | 10,000 | | | | |
| 1.00E+07 | 1,667 | 5,000 | 10,000 | | | | | | |


When looking at the maximum flow rate of a given instrument along with the sample concentration, the maximum event rate is shown in particles per second. For example, a sample with a concentration of $1x10^6$ particles/mL at a flow rate of 100 µl/ml, would have a theoretical maximum event rate of 1,667 events/second when acquired on a Beckman Coulter Cell Lab Quanta™ SC (100 µl/min). The same sample acquired on the Attune™ Acoustic Cytometer would have a theoretical maximum event rate of 8,333 events/second at 500 µl/min. This is possible because cells are focused by sound and independent of hydrodynamic focusing which is rate dependent. The Attune™ Acoustic Cytometer is able to achieve event rates more than an order of magnitude higher than what can be achieved using conventional hydrodynamic focusing cytometry.

## Single-platform assay for absolute CD34+ cells without beads

A lyse/no-wash staining procedure is used to prevent loss of cells due to washing. Conventionally, counting beads are used in a single-platform technique that incorporates a known number of counting beads in the assay, and allows a calculated CD34+ count by calculating the ratio between the number of beads and CD34+ cells counted. The Attune™ Acoustic Cytometer allows for direct absolute cell counts without the need for counting beads. This technique has several advantages: i) it eliminates the variability resulting from using an assayed counting bead, ii) it may contribute to a higher level of standardization between laboratories, iii) it avoids the added cost of the bead reagent, iv) uses a streamlined process, and v) eliminates a potential source of error with gating and calculations using the beads.

Figure 1. Comparison of absolute CD34 positive cell counts from the Attune™ and LSRII cytometers.

Peripheral blood from a normal donor was spiked with CD34 positive Stem-Trol™ cells (Beckman Coulter) and stained per the protocol detailed in methods and materials. Before samples were collected and analyzed, 50µL of CountBright™ Absolute Counting Beads (Invitrogen) were added and used to calculate absolute CD34 counts. The sample was collected and analyzed on the Attune™ Acoustic Cytometer and the BD™ LSR II conventional cytometer.

Plots A and B are examples from the BD™ LSR II system, and plots C and D are from the Attune™ cytometer; plots A and C are gated on live cells (SYTOX® AADvanced™ negative cells) while plots B and D are gated on live CD45 positive events. Counting beads are gated on total events.

We compared the results obtained using counting beads to those measured directly by volumetric displacement on the Attune™ cytometer. These results were also compared to absolute counts obtained on the BD™ LSRII using counting beads. All absolute cell count results are within 5%.

Acquisition times are calculated from the time recording starts to when the stopping gate has been achieved.

| Instrument | Acquisition Time | CD34+ cells/µl derived count using beads | CD34+ cells/µl direct measurement |
|---|---|---|---|
| BD™ LSR II | 13 min 49 sec | 8.01 | The LSR II does not give direct measurement |
| Attune™ Acoustic Cytometer | 1 min 17 sec | 7.91 | 8 |

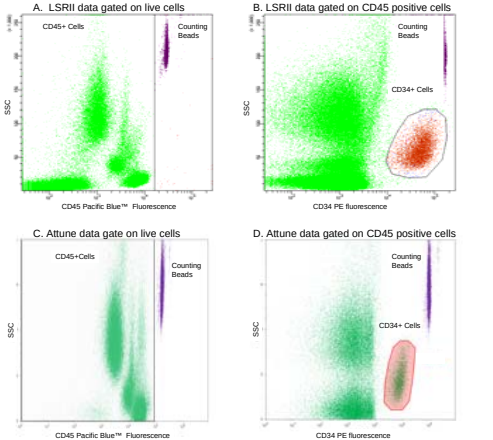
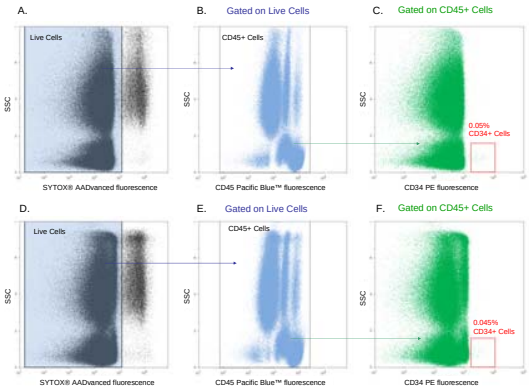



Figure 2. CD34+ cells in circulating peripheral blood

The range of circulating CD34+ cells in peripheral blood from a healthy donor is ~0.013 - 0.11% of CD45 positive cells[7]. Detection of rare events, such as CD34+ cells, requires the acquisition of hundreds of thousands cells to achieve statistically relevant accuracy. Here peripheral blood from a normal donor was stained according to the method protocol and run on the Attune™ Acoustic cytometer at a flow rate of 500 µl/minute (Plots A, B, & C) and 1000 µl/minute (Plots D, E, & F) with a stop gate set at 500,000 total cells. Plots A and D display total cells stained with SYTOX® AADvanced™ dead cell stain and show the live cell gate; plots B and E are gated on live cells; plots C and F are gated on live CD45 positive cells.




| Sample Flow Rate (µl/min) | Acquisition Time | Percentage of CD34+ Cells of Leukocytes | CD34+ cells/µl direct measurement |
|---|---|---|---|
| 500 | 6 min 26 sec | 0.050% | 0.07 |
| 1000 | 4 min 28 sec | 0.045% | 0.05 |

***References:***

1. *Current Protocols in Cytometry* (2003) 6.4.1-6.4.23 *2. Current Protocols in Cytometry (2009)* 49:1.22.1-1.22.12. *3.* www.bdbiosciences.com *4.* www.accuricytometers.com *5.* www.coulterflow.com *6. www.appliedbiosystems.com* 7. BD Procount Progenitor Cell Enumeration Kit, Technical Data Sheet