Application Note

Rapid Counting of Somatic Cells in Dairy Milk Using the Scepter™ 2.0 Cell Counter, Following Spin-Wash Sample Preparation

Amedeo Cappione, Ph.D. and Janet Smith
EMD Millipore

Introduction

Mastitis is an inflammatory change in the mammary gland characterized by pathological changes in mammary tissue. This potentially fatal infection is the most common and most costly disease facing the United States dairy industry[1]. Milk from sick cows exhibits increased somatic cell counts (SCC) due to the release of white blood cells into the gland to combat infection. Samples displaying SCC ≤ 200,000 cells/mL are considered healthy, values ≥ 300,000 cells/mL are indicative of early stages of infection (subclinical mastitis), and counts ≥ 750,000 cells/mL denote full-blown mastitis. Given that subclinical cases present the greatest cost burden due to a lack of physical indications, research efforts designed at targeting rapid detection of elevated SCC counts as well as therapeutic interventions are critical to sustaining the dairy market.

Milk is an emulsion of fat globules in a water-based fluid. Milk fat globules possess a number of attributes which severely hinder accurate somatic cell counting.

Characteristics of Milk Fat Globules Hindering Somatic Cell Counting:

- Significantly more abundant than cells. Depending on the sample, this difference can be on the order of 5000X.
- Stick to cells, forming aggregates that must be dispersed prior to sized-based detection.
- Diameter range: 0.1-20 µm; globule size overlaps with the average somatic cell diameter (8.5-10 µm)
- Content varies significantly, both cow-to-cow and day-to-day

Most current methodologies for measuring SCC in milk involve the use of cell-specific fluorescent dyes to analyze samples using flow cytometry and microscopy.

The Scepter handheld, automated cell counter provides a rapid, reliable alternative to traditional SCC analysis. The device combines the ease of automated instrumentation and the accuracy of the Coulter principle of impedance-based particle detection in an affordable, handheld format. The Scepter cell counter uses a combination of analog and digital hardware for sensing, signal processing, data storage, and graphical display. The precision-made, consumable polymer sensor has a laser-drilled aperture in its cell sensing zone that enables the instrument to use the Coulter principle to discriminate cell diameter and volume at submicron and sub-picoliter resolution, respectively.

The spin-wash protocol, when used in conjunction with the Scepter cell counter and 40 µm aperture sensor, enables rapid, precise somatic cell counting with results comparable to fluorescence-based platforms.

EMD Millipore is a division of Merck KGaA, Darmstadt, Germany

Materials and Methods

Samples – Fresh milk samples (40-50 mL) were routinely acquired from a local dairy farm, Dunajski Dairy (Peabody, MA). Samples were stored at 4 °C and used within 48 hours before disposal. Prolonged storage resulted in appreciable changes to both the fat and cell content.

The Spin-wash protocol for dairy milk sample preparation

Upon standing for 12 to 24 hours, fresh milk separates into a high-fat "cream" layer and a larger, low-fat milk layer. The fat globules rise to the top because fat is less dense than water. Separation can also be accomplished rapidly through centrifugation. The protocol is as follows:

1. Allow milk and phosphate-buffered saline (PBS) to come to room temperature.
2. Add 500 µL PBS to 500 µL milk in a 1.5 mL microfuge tube.
3. Mix quickly by vortexing.
4. Centrifuge at 1000 g for 2 minutes in a tabletop centrifuge.
5. Remove upper cream layer using a cotton swab, pour off remaining skim layer.
    a. The cream layer is very viscous and will stick to the sides of the tube. Attempt to remove as much as possible by gently swirling the cotton swab.
    b. A white pellet containing cells and fat should be visible at the bottom of the tube.
6. Add 1 mL PBS to the tube. Do not resuspend pellet.
7. Repeat steps 4-6 twice.
    a. Certain samples may require additional spin cycles to achieve sufficient removal of fat for cell detection.
8. After the final spin, resuspend the cell pellet in 500 µL PBS by gently pipetting up and down.
9. Acquire cell concentration and cell size data using a Scepter cell counter equipped with a 40 µm aperture sensor.
    a. The 40 µm sensor has an upper detection limit of 1.5×10^6 cells/mL. For this reason, certain samples may need to be further diluted for accurate counting.

SSC testing on a guava easyCyte™ flow cytometer

Following spin-wash, aliquots of each sample were mixed with ViaCount® reagent (EMD Millipore). The reagent contains a cell-permeant nuclear dye that preferentially labels cells.

Stained samples were analyzed on the guava easyCyte flow cytometer (EMD Millipore). For each sample, 10,000 total events were acquired.

External SCC testing

For each milk sample, SCCs were verified by Agri-Mark Dairy Cooperative (Lawrence, MA). Samples were tested using a Fossomatic™ cell counter (Foss, Denmark), a fluorescent-based flow cytometry platform.

Percent recovery studies

Jurkat cells were labeled using CellTracker Green CFMDA (5-chloromethylfluorescein diacetate, Life Technologies). Dye was resuspended in DMSO to 10 mM. Cells were labeled by incubating 1 e6/mL in fresh culture media at 37 °C for 30 min with 1:1000 dilution of dye. Cells were washed 2X with PBS prior to use. Cells were added to milk samples at known concentrations. Following a short equilibration time, milk samples were processed and analyzed using the guava easyCyte.

Results

To determine the effectiveness of fat globule removal by the spin-wash method, samples were processed as follows: (1) 1:1 dilution in PBS, (2) One spin-wash, and (3) Three spin-washes. Processed samples were analyzed with the Scepter cell counter using 40 µm sensors. The results presented in Figure 1 are from a sample with high SCC. The top row displays diameter histogram plots acquired on the Scepter cell counter. Initial attempts at Scepter analyses of whole milk proved unsuccessful; over half the samples resulted in sensor failure due to either "Lost Start" errors (due to air bubbles or insufficiently submerged sensors) or blocked apertures. Samples that could be read displayed concentration values that greatly overestimated true SCC values due to fat globule interference. For samples treated with three spin-wash cycles, the 40 µm sensor showed a clear, distinguishable peak corresponding to the somatic cell fraction. While three spin-wash cycles were sufficient to permit visualization of somatic cells in most samples, additional wash steps were required for samples with extremely high fat content.

Somatic cell detection was also confirmed through fluorescent labeling using ViaCount reagent and the guava easyCyte cytometer. The middle row of histograms shows a distinct peak of fluorescently labeled cells (**red**) even in the unwashed fraction. As 10,000 total events were collected for each sample, the change in relative size of the fat (**green**) and cell (**red**) peaks with increased wash cycles confirms the removal of fat globules. In the bottom row, Foward Scatter (FSC) vs. Side Scatter (SSC) dot plots revealed cells (**black**) being released from fat aggregates as well as the relative decrease in number and size of fat globules (**red**) with successive washes.

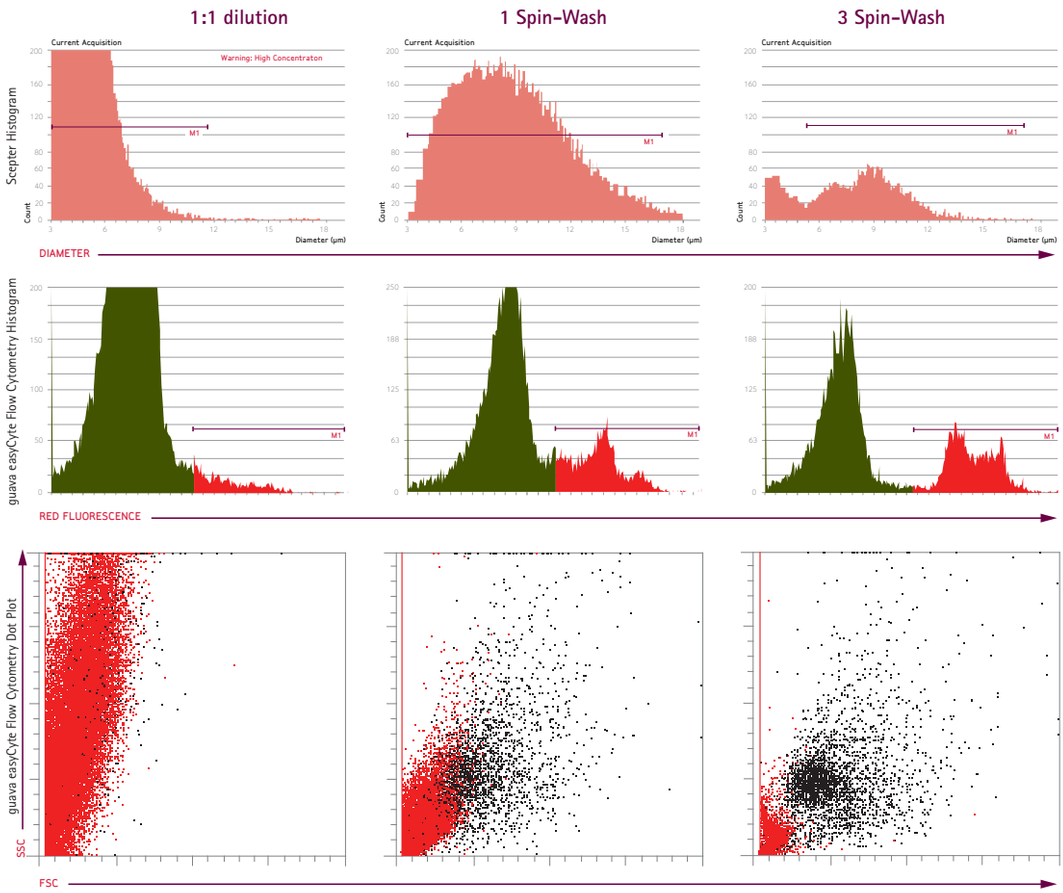

Figure 1. Spin-wash treatment effectively removes fat globules from dairy milk samples.

The concentration of somatic cells in milk samples can vary greatly depending on diet, time relative to pregnancy, and overall health status. To test the performance range of the spin-wash method, we assessed a number of samples from both healthy cows and those presenting with mastitis. The spin-wash method was equally successful at enriching for the somatic cell fraction across a wide range of somatic cell counts. Representative examples of low (healthy), medium, and high SCC (mastitis) milk samples are displayed in Figure 2. For Scepter-derived histograms, a distinct peak was seen for all three sample types. In each case, the mean diameter of this peak was consistent with the reported size range for bovine somatic cells. Cell counts were confirmed by flow cytometry. Results presented in the dot plots showed a significant increase in the frequency of detected cells (**black**) in high SCC samples as compared to low SCC samples. These increases paralleled the larger peak sizes demonstrated by the Scepter histograms.

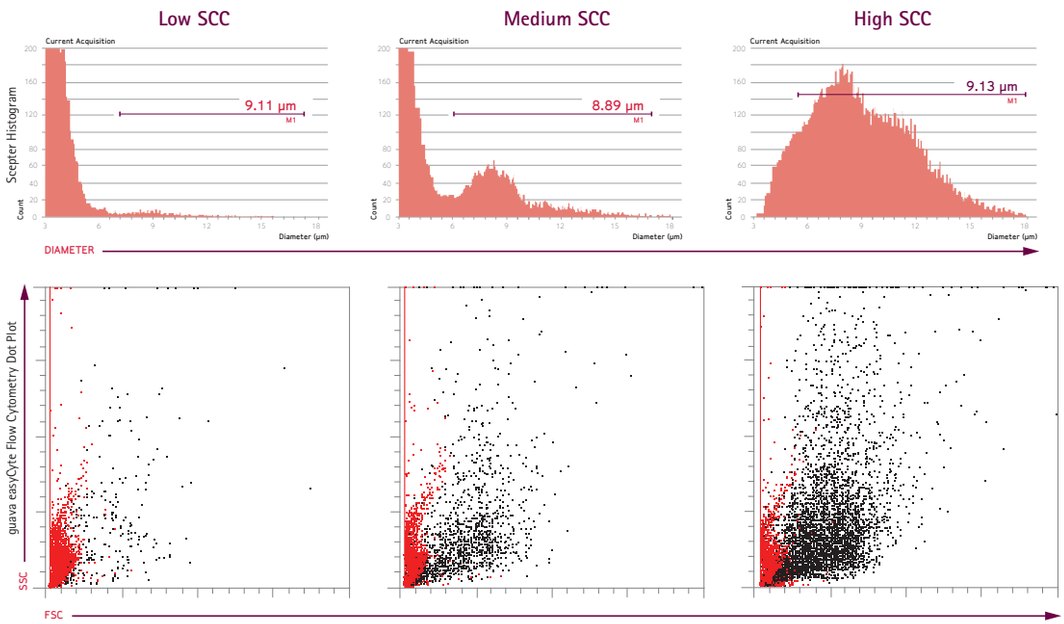

Figure 2. Scepter cell counting and guava easyCyte flow cytometry provide interpretable SCC data for dairy milk samples containing low, medium and high numbers of somatic cells.

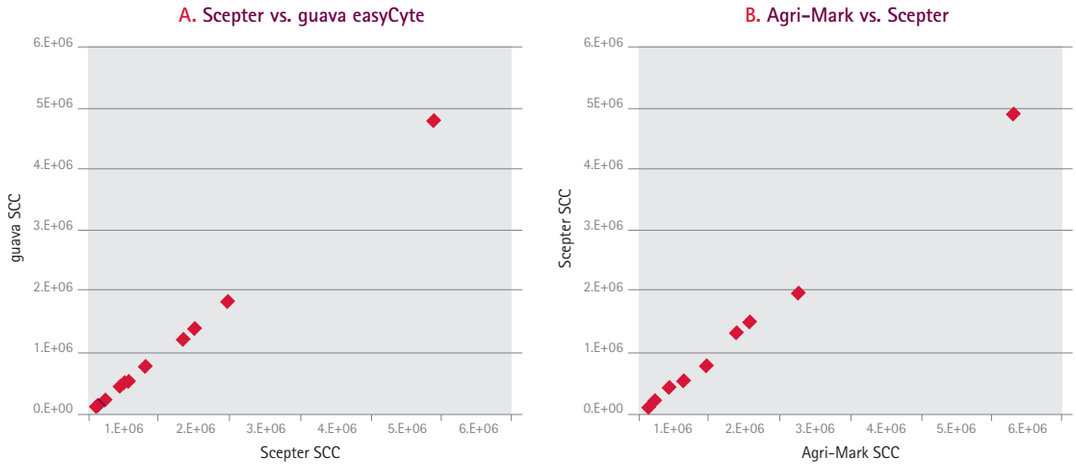

Figure 3. Highly correlated SCC data between three cell counting platforms

A. guava easyCyte values are derived from the number of fluorescently labeled cells following ViaCount staining.
B. Agri-Mark is an external SCC testing facility.

Twelve samples were processed and analyzed (Figure 3). Overall, the Scepter-derived SCC showed good agreement with values acquired using flow cytometry (Figure 3A). However, in nearly all cases, Scepter cell counts were slightly higher than flow cytometry values; this difference may be due to greater distinction of cells from fat afforded by fluorescent labeling for flow cytometry. The plotted values represent the mean of 3 replicates; both platforms showed high reproducibility in sampling results with average percent coefficient of variation (%CV) of 4.5 (Scepter cell counter) and 5.0 (guava easyCyte flow cytometer). Although slightly lower in all cases, results from both systems were also consistent with those determined externally (Agri-Mark; Figure 3B) on whole milk, a result which may be due to cell loss during the washing protocol.

Table 1. Spin-wash sample preparation leads to limited cell loss in dairy milk samples.

	% CELL RECOVERY		
JURKAT CELL INPUT	1-SPIN	2-SPIN	3-SPIN
50000	95.6	91.3	85.9
200000	97.9	92.7	88.7
500000	97.3	93.7	90.8
1000000	97.7	94.1	90.1
3000000	98.8	94.8	91.5

To quantify this possible cell loss, fluorescently labeled Jurkat cells were added to milk samples at known concentrations. Samples were subjected to multiple rounds of spin-washes and analyzed by flow cytometry to determine the degree of cell loss (Table 1). While we observed a slightly greater cell loss with decreasing starting number, losses were no greater than 15% in any sample after three rounds of washes. These findings help substantiate the difference in SCC values calculated using the Scepter or guava easyCyte platforms and external testing.

Conclusion

The Scepter cell counter offers the accuracy of impedance -based particle detection in an intuitive, as well as affordable, handheld format. The new 40 µm aperture sensor offers expanded sensitivity for discrimination of smaller cells and particles. This platform, when used in conjunction with the Spin-wash protocol, provides a quick and reliable method for accurate somatic cell counting in milk samples.

1. Viguier, C., Arora, S., Gilmartin, N., Welbeck, K., and O'Kennedy, R. (2009) Mastitis Detection: Current Trends and Future Perspectives. Trends in Biotechnology. 27(8):486-93.

EMD MILLIPORE

www.millipore.com/CellularAnalysis

Fisher Scientific

In the United States:
For customer service, call 1-800-766-7000.
To fax an order, use 1-800-926-1166.
To order online: www.fishersci.com

In Canada:
For customer service, call 1-800-234-7437.
To fax an order, use 1-800-463-2996.
To order online: www.fishersci.ca