Detection of total aerobic counts in industrial water and biologics protein production: Sample Concentration Method Analysis utilizing portable dried reagents (DuraClone) and a bench-top flow cytometer (CytoFLEX)

Carley Ross, Jeffrey Cobb, Shankar Pattabhiraman, and James Tung

Beckman Counter Inc., Life Sciences, Miami, FL, United States

ABSTRACT

Industrial cooling towers and water supply require constant monitoring for bacterial presence to avoid or mitigate issues, such as corrosion and health threats, caused by biologic organisms. The current analysis methods require up to 5 days for results. Customers are required to take 6 one-liter samples, from the water system and concentrate them prior to analysis. We analyzed four concentration methods: centrifugation, disposable filtration, glass filtration and concentrating pipet. Each method was analyzed for time to answer, recovery, and per test and capital cost. Staining procedures were optimized with E. coli and S. aureus and improved using DuraClone dried reagents. The stained samples were then analyzed on the CytoFLEX VSSC for total aerobic bacteria counts (TAB). Industrial samples from cooling towers and biopharmaceutical protein production processes were analyzed.

Results: The TAB recovery was highest with the CP (71%) with lower recovery values for DF>GF>Cent. The time required for a 1 L sample to answer was fastest (16 min) CP>GF>DF>Cent. Total cost per test was DF>CP>GF>Cent. The TO and PI staining provided the highest signal to noise for live/dead on bacteria.

Conclusion: Presented here is an optimized sample to answer method for counting TAB via flow cytometry. The CytoFLEX provides effective detection of bacteria and can be easily adapted into a mobile testing unit with DuraClone reagents. The assay measured a detection limit lower than 45 colony forming units per ml and reduced assay time from 5 days to 16 minutes. Combining the sample concentration method, DuraClone reagents and the CytoFLEX flow cytometer, customers are able to quickly respond to bacteria contamination.

MATERIALS AND METHOD

Sample concentration:

Recovery was first tested with Polysciences, 700 nm YG beads in 1 L DI H2O, then E. coli and S. aureus. Method 1: Centrifugation (Cent) in the Beckman Coulter Allegra 30-x required a slow spin at 500 g, 20 min and then a capture spin at 3750 g for 20 min. Method 2: Pall Sentino pump with disposable microfunnel (20 µm PCTE, 0.45 µm) were used in tandem to remove debris and capture bacteria (DF). Method 3: glass filtration (Pall, Sterlitech) was used with 20 µm PCTE pre-filter and 0.45 µm PCTE capture filter (GF). Method 4: a concentrating pipet (CP, Innovaprep) with a 330 µm pipet sleeve and 0.45 µm polysulfone filter pipet with PBS elution buffer were used with a 2-step elution. Staining: Bacteria were stained with Syto9 (Thermo), PI, CFSE, Thiazole orange (Sigma), SYBR Green (Thermo), Propidium Iodie (PI), 7AAD, and HO33342 with a PBS, 1 mM EDTA and 0.1% Tween 20 solution. DuraClone dried reagent TO and PI were tested in the final analysis with Beckman Coulter flow count beads. Flow cytometry: The samples were analyzed on a CytoFLEX with a VSSC trigger, fluorescence detection for TO, PI and Flow count beads

Viability and bacteria staining:

Stock solution: E. Coli or S. aureus were suspended in PBS so that the suspension was slightly turbid. Half of the stock solution volumes were heated in a 70°C water bath for 20 minutes to create non-viable bacteria populations. The heat killed bacteria were allowed to cool to room temperature and remixed with the viable bacteria. The mixed bacteria solutions were diluted 1:10 in PBS

A 0.1 mL aliquot of the diluted bacteria was incubated each individual viability dye and every possible combination of either 7-AAD or Propidium Iodide with the remaining 6 permeable dyes for 30 minutes. All FDA and CFSE incubations were done in a 37°C water bath. Incubations were at the final concentrations shown here: Carboxyfluorescein diacetate succinimidyl ester (CFSE) at 5µM; Syto 9 at 10 µM; Thiazole Orange (TO) at 3.4 µM; SYBR Green at 100X; Hoechst 33342 at 88.5 µM; Fluoroscein diacetate (FDA) at 10 µM; Propidium Iodide at 136 µM; and 7-AAD at 9 µM. After 30 min, 0.9 mL of PBS was added to the stained bacteria. Bacteria suspensions were analyzed on the CytoFLEX flow cytometer.

Limit of Detection.

Stock solution: E. Coli were suspended in PBS so that the suspension was slightly turbid. The stock solutions were diluted 1:10 in PBS working solution. A 0.1 mL aliquot of the diluted bacteria was incubated with Syto 9 for 30 minutes at the following bacteria dilutions: Working solution, a 1:10 of working solution, and serial 1 to 2 dilutions up to a 1:1280 dilution of bacteria in PBS. All dilutions were brought up to 1mL analysis volume including the addition of FlowCount beads before being analyzed on a CytoFLEX instrument equipped with Violet Side Scatter detection. 10 µL of the three lowest dilution samples were plated on agar plate as predicate to determine viable bacteria count.

Figure 1 Recovery work flow of different concentration and filtration methods for total aerobic bacteria count (TAB)

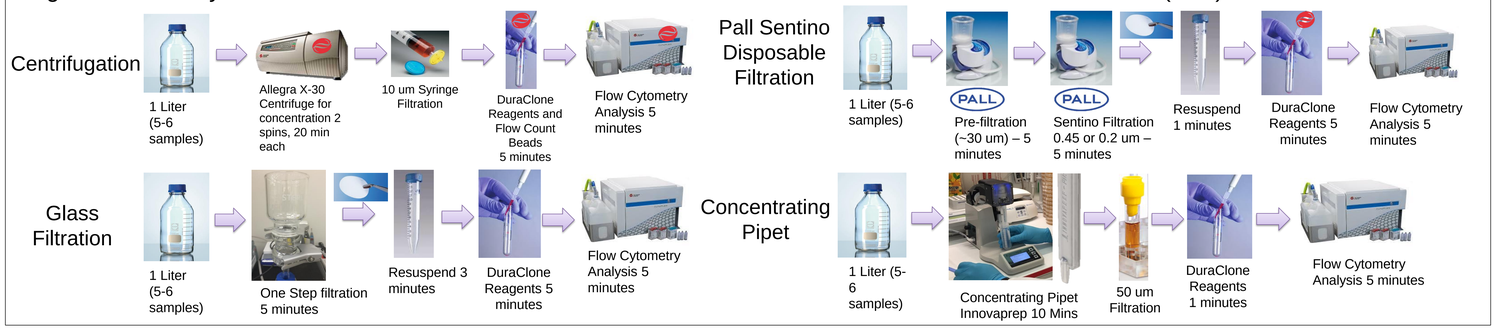


Table 1	Centrifugation	Glass Filtration	Pall Sentino Disposable Filtration	Concentrating Pipet
Recovery	47%	50%	53%	71%
Total Time (6 samples) (hr)	7:09	2:48	2:15	1:36

Figure 1 Recovery work flow of different concentration and filtration methods for total aerobic bacteria count (TAB). E. coli in 1-liter of water was filtered and concentrated following the four different methods, Centrifugation, Glass Filtration, Pall Sentino Disposable filtration, and concentrating pipet. These four method were run based on the workflow shown above. The processing time and recovery with each filtration/concentration method was recorded and analyzed. The final process time and recover cost per test are shown in **Table 1**.

Figure 2 SSC vs Violet SSC

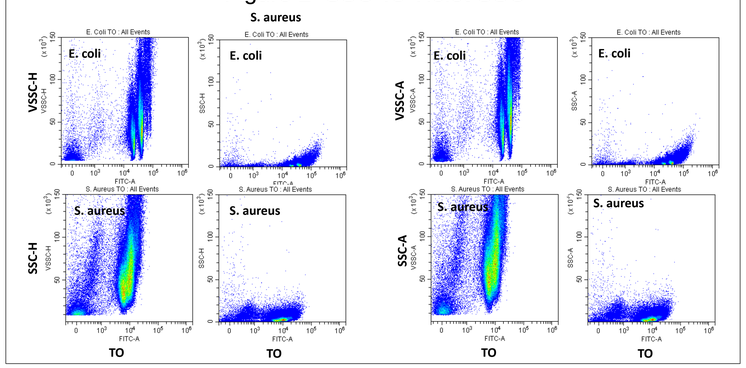


Figure 2 SSC vs Violet SSC. CytoFLEX instruments configured with Violet Side Scatter (VSSC) has better resolution for E. coli and S. aureus compared to the standard Side Scatter (SSC) from the 488nm laser.

Figure 3 E. coli viability staining. E. coli bacteria were stained with membrane permeable dye to identify intact bacteria and membrane impermeable dyes to identify membrane-compromised cells.

Figure 4 Sample and dye Carryover. All samples showed cell and dye carryover events. The observation of carryover extent for each dye is listed in Figure 4.

Figure 5. E. coli limits of detection. Stock E. coli solution was diluted 1:10 and serially diluted at 1:2 until dilution reaches 1:1280. The dilution was stained with Syto9 without viability stain. FlowCount beads were added to the samples before analysis on CytoFLEX. The 3 lowest dilutions were plated on agar plate as predicate to provide viable bacteria count for comparison.

Figure 3 E. coli Viability staining

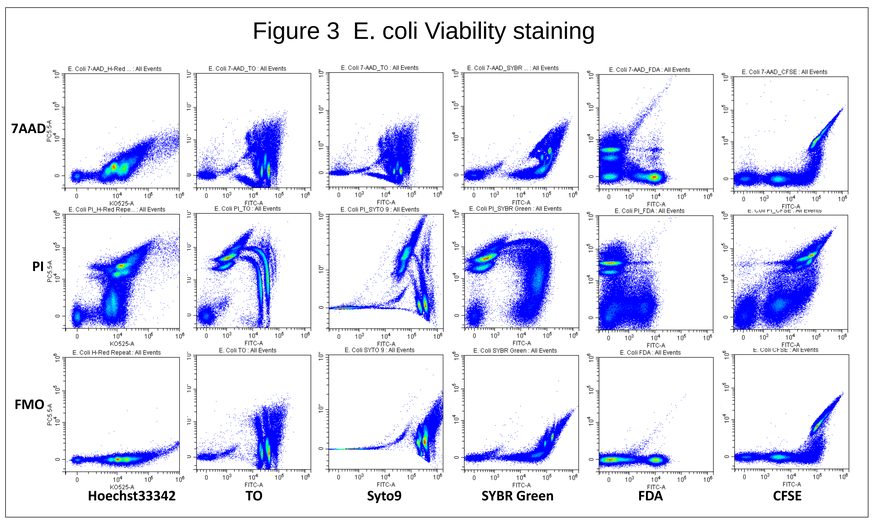
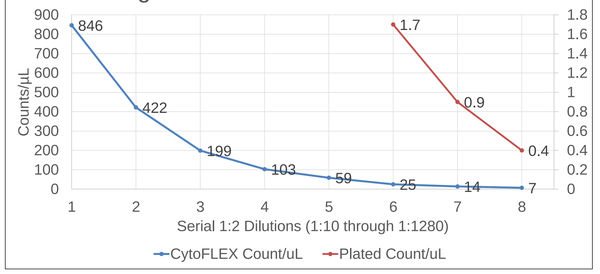


Figure 4 Sample and dye carryover

	7-AAD	Propidium Iodide	Thiazole Orange	Hoechst 33342	FDA	CFSE	Syto 9	SYBR Green
E. coli	Low	High	High	High	Low	Low	High	Low
S. aureus	Low	High	High	High	Low	Moderate	High	Moderate

Figure 5 E. coli Limits of Detection



BECKMAN COULTER Life Sciences