Evaluation of an Acoustic Focusing Flow Cytometer for Enumeration of Probiotic Organisms

Andrzej A. Benkowski* and Jean L. Schoeni
Covance Food Solutions, Madison, Wisconsin *Corresponding author: andrzej.benkowski@covance.com

Introduction

The probiotics industry estimated that in 2016 probiotic consumption retailed at $39.9 billion; $4.3 billion was attributed to dietary supplements.[1] In a sector where 38% growth is expected from 2016 to 2021,[1] stakeholders are demanding more information about quantity, quality and identity of organisms in probiotic products.

Enumeration provides the most basic required information with methodologies evolving to provide improved accuracy, precision, and turnaround times. Flow cytometry has shown promise in rapidly providing precise, quantitative and qualitative probiotic information. Acoustic-assisted hydrodynamic flow cytometry incorporates the advance of using sound waves to align the particles in the fluid stream for counting. This reduces the time to result.

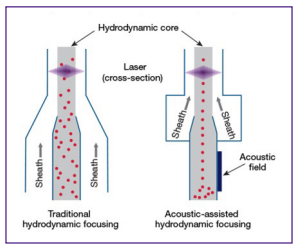

Figure 1. Rapid, improved alignment with acoustic focusing.

Objective

To validate an acoustic focusing flow cytometer, verify its use in performing ISO 19344:2015(E)/IDF 232:2015(E) to enumerate probiotic organisms in freeze dried and microencapsulated freeze dried powders and extend use of the method to more complex matrices.

Materials and Methods

USP 39 <621> was used to validate the Invitrogen Attune NxT acoustic focusing flow cytometer (Life Science Technologies; Figure 2). Two preparations of calibrated Liquid Counting Beads (Becton Dickinson) were analyzed. The data generated were compared to the theoretical value.

Figure 2. The Attune NxT flow cytometer.

Verification of ISO 19344:2015(E)/IDF 232:2015(E), Protocol B, followed USP 40 <1225>/ICH Q2(R1)[2] and was performed using the validated cytometer. Freeze dried powders of *Lactobacillus acidophilus* (HOWARU), *Bifidobacterium lactis* (BL-04), and *Streptococcus thermophilus* (ST-21) cultures were used to evaluate accuracy, precision (repeatability), intermediate precision (ruggedness), range, limit of quantitation, specificity, linearity and robustness.

ISO method 19344 is a basic flow cytometry procedure where the initial suspensions are homogenized by stomaching for two minutes and then allowed to hydrate for an additional 10 minutes. The initial suspensions and/or dilutions are then stained as per one of three protocols. Protocol B incorporates dual staining that targets nucleic acid; propidium iodide (PI) and SYTO®24. The stained samples are evaluated for numbers of live, injured and dead cells based on stain uptake and emissions produced.

Flow cytometry counts from accuracy and precision testing were compared to cultural plating and direct microscopic counting (with trypan blue staining) derived from like sample preparations. Plate counts were generated following: ISO 20128:2006/IDF 192:2006 (*L. acidophilus*), ISO 29981:2010/IDF 220:2010 (*B. lactis*) and ISO 7889:2003/IDF 117:2003 (*S. thermophilus*). Acceptance criteria for the method verification were based on AOAC Appendix K, "Guidelines for Dietary Supplements and Botanicals".[3]

Use of the ISO flow cytometry method for enumeration of probiotics was extended to lipid microencapsulated *L. acidophilus* freeze dried powders based on matrix extension guidance included in USP 40 <1225>/ICH Q2(R1)[2] and the acceptance criteria described in AOAC Appendix K.[3] Some procedural modifications were required for the detection of live, injured and dead cells in these samples. Sodium borate rather than sodium chloride-peptone was used to prepare the initial dilution; homogenization by stomaching was increased from two minutes to four minutes; and no hydration hold was required.

Results

Instrument Validation

Table 1. System Validation Results for the Attune NXT Flow Cytometer

Calibrated Bead Preparation	Replicates	% Recovery (Acceptance ≥ 70 %)	% RSD (Acceptance ≤ 15 %)
1	5	91	3
2	5	91	2
Overall	10	91	2

Method Verification

Table 2. Accuracy of Counts Obtained by Flow Cytometry Enumeration

	Mean % Recovery at Specified Dilution (Acceptance ≥ 70 % of Cultural Plate Count)		
Test Organism	10^{-6}	10^{-7}	10^{-8}
L. acidophilus	107	117	160
B. lactis	81	82	121
S. thermophilus	82	101	90
Overall	94	100	127

Table 3. Evaluation of Precision and Intermediate Precision for Each Test Organism

	% RSD (Acceptance ≤ 15 %)	
Test Organism	Precision (Repeatability) n = 6	Intermediate Precision (Ruggedness) n = 12
L. acidophilus	7	7
B. lactis	10	7
S. thermophilus	4	13

The range of concentration that can be assayed using this method is 10^5 to 10^9 active fluorescent units (AFU)/g.

The limit of quantitation (LOQ) is 10^4 AFU/g.

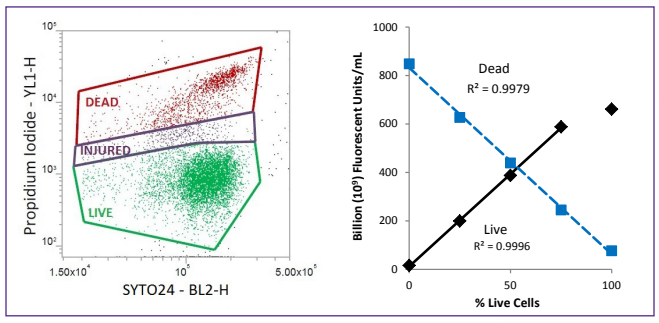

Figure 3. Example of specificity testing showing differentiated live and dead cell populations. Left: flow cytometry scatter plot showing different populations. Right: plot of resulting test data.

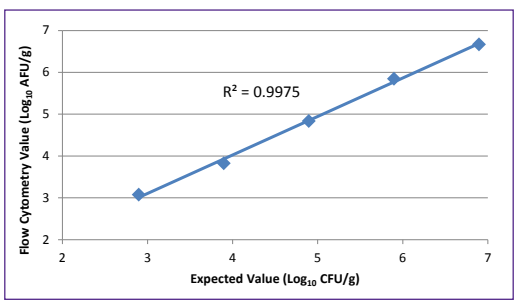

Figure 4. Linearity of *S. thermophilus* data. *Lactobacillus acidophilus* (R^2 = 0.9984) and *B. lactis* (R^2 = 0.9988) produced similar results.

Table 4. Reference and Most Extreme Procedure Modification Results Used to Evaluate the Robustness of Method

	Conditions Evaluated						Resulting Flow Count (10^9 AFU/g) Acceptance ≥ 70 % Recovery Compared to Reference		
	Hold Time (minutes)		Dye Incubation Time (minutes)		Temperature of Peptone Salt				
Sample Preparation	10	60	15	30	Ambient	4 °C	*L. acidophilus*	*B. lactis*	*S. thermophilus*
Reference	X		X		X		310	576	709
Modification No. 8 (Extreme)		X		X		X	329	432	705

Table 5. Test Method Comparisons

	% RSD for Mean Counts (n = 15 per method per test organism)			
Test Organism	Flow Live	Plate Culturable	Flow Total	Microscopic Total
L. acidophilus	6	14	7	16
B. lactis	7	10	15	17
S. thermophilus	12	26	14	8

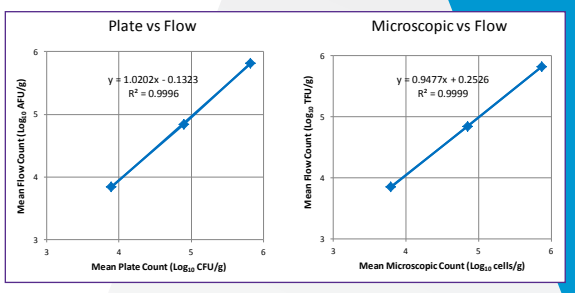

Figure 5. An example of comparable results produced by flow cytometry and standard plate counting (left) and flow cytometry and direct microscopic counting (right).

Matrix Extension for Microencapsulated Probiotic Powders

Table 6. Accuracy, Precision and Intermediate Precision Data

Accuracy			Precision	Intermediate Precision
Mean % Recovery at Specified Dilution (Acceptance ≥ 70 % of Cultural Plate Count)			% RSD n = 9 (Acceptance ≤ 15 %)	
10^{-6}	10^{-7}	10^{-8}		
117	116	128	8	7

Table 7. Comparison of Enumeration Results by % RSD

	% RSD for Mean Counts	
Matrix	Flow Cytometry Live (n = 21)	Plating Colony (n = 3)
Microencapsulated Freeze Dried Probiotic Powder	7	7

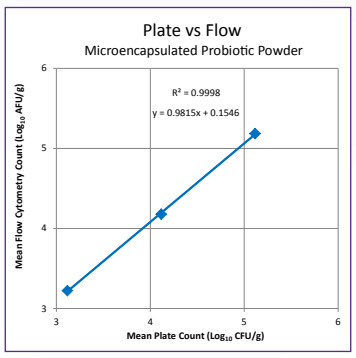

Figure 6. Comparable enumeration results were produced for microencapsulated probiotic powder by flow cytometry and standard plate counting.

Conclusions

Our results verified that a validated acoustic focusing flow cytometer produces reliable, accurate and reproducible enumeration data for lyophilized probiotic cultures following ISO 19344:2015(E)/IDF 232:2015(E). Furthermore, flow cytometry generates results that are comparable to cultural plate and direct microscopic counting. The methodology is applicable to both freeze dried and microencapsulated probiotic powders.

Advantages of flow cytometry methodology over classical methods have been demonstrated as:

- **Superior precision** Approximately one-half the RSD of other enumeration techniques
- **Faster delivery** Results in <8 hours compared to 2-3 days by culture.
- **More information** Discernment between live, dead and injured cells informs product formulation, stability and guides development

References

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