Probiotic Enumeration by Flow Cytometry: Method Scope Extension of ISO 19344

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Introduction

The 2016 retail value of probiotic consumption was estimated to be $39.9 billion; $4.3 billion was attributed to dietary supplements.[1] In a sector where 38% growth is expected between 2016 and 2021,[1] probiotic dietary supplement stakeholders are demanding more information about quantity, quality and identity of organisms in probiotic products. Increasingly, flow cytometry is being used to address manufacturers' and consumers' demands and has shown promise in rapidly providing precise, quantitative and qualitative probiotic information.

Figure 1. The Attune NxT flow cytometer.

A flow cytometer uses laser light to detect particles in a fluid stream. Emissions scatter at different angles depending on particle size and internal complexity. A newer innovation, acoustic-assisted hydrodynamic flow cytometry, uses sound waves to align the particles in the fluid stream, which reduces the time to result.

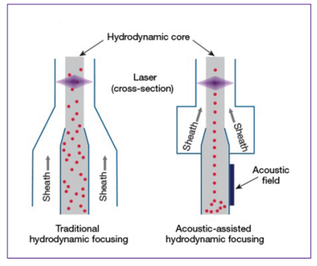

Figure 2. Rapid, improved alignment with acoustic focusing.

Previously, we verified ISO 19344:2015(E)/IDF 232:2015(E) for enumerating probiotics in lyophilized powders using acoustic focusing flow cytometry by following USP 40-NF 35 <1225>/ICH Q2 (R1).[2,3,4,5] The study showed that cytometric accuracy, precision and turnaround times were comparable to or better than those of plate and microscopic counting. Here, the utility of flow cytometry is expanded by applying ISO 19344 (Protocol B)[3] to additional probiotic matrices and its performance with those matrices is established.

Materials

Instrument

Invitrogen™ Attune™ NxT acoustic flow cytometer (Thermo Fisher Scientific, Inc.; Figure 1)

Matrices

- Frozen Non-Fat Yogurt (Frozen NFY)
- Lipid-Coated Microencapsulated Freeze-Dried *Lactobacillus acidophilus* (Microencap)
- Non-Fat Yogurt (NFY)
- White Chocolate Chips (WCC)

Methods

Matrix Extension Guidance

We followed USP 40-NF 35 <1225>[4]/ICH Q2 (R1)[5] to determine accuracy, precision and intermediate precision of the assay with the test matrices. Acceptance criteria for the method verification were based on AOAC Appendix K, "Guidelines for Dietary Supplements and Botanicals."[6]

Flow cytometry results for accuracy and precision testing were compared with cultural plating derived from like sample preparations. Plate counts were generated following: ISO 27205:2010(E)/IDF 149:2010(E)[7] (modified) and ISO 7889:2003(E)/IDF 117:2003(E).[8]

Assay

ISO 19344:2015(E)/IDF 232:2015(E), Protocol B,[3] with matrix-specific modifications was evaluated for its ability to provide rapid, precise and accurate enumeration of live, injured and dead cells (Figure 3). It should be noted that ISO/IDF considers stained cells as presumed live, injured or dead and refers to them as active, damaged or non-active, respectively.

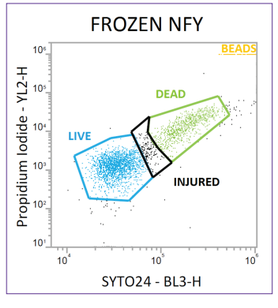

Figure 3. Example of a scatter plot showing differentiated live, injured and dead cell populations.

Assay Modifications (Figure 4)

- Frozen NFY – Stomached sample passed through a 70 µm mesh.
- Microencap – Sample stomached four minutes in sodium borate buffer. No hold time.
- NFY – Stomached sample passed through 70 µm mesh.
- WCC – Initial salt peptone diluent tempered to 37°C. Hold five minutes at room temperature and five minutes at 45°C.

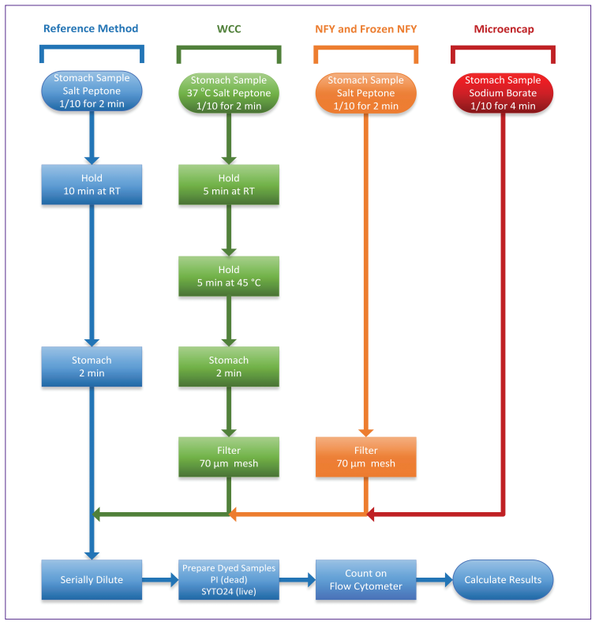

Figure 4. Flow diagram of ISO 19344, Protocol B with modification steps specified.

Results

Accuracy

The mean % recovery for the matrices tested ranged from 109-122 (Table 1).

Table 1. Accuracy Demonstrated by Mean Recovery

	Mean % Recovery at Specified Dilution (Accceptance range 70–125 %)				
Matrix	**10^{-4}**	**10^{-5}**	**10^{-6}**	**10^{-7}**	**Overall**
Frozen NFY	109	121	136	ND[a]	122
Microencap	117	116	128	ND	120
NFY	ND	100	116	111	109
WCC	112	112	115	ND	113

[a] ND; not determined

Precision

Percent relative standard deviation (% RSD) for all matrices met the specified acceptance criterion (≤ 15%).

Table 2. Precision and Intermediate Precision Determined by Evaluating Matrices Using Modified ISO 19344 Methodology

	% RSD (Acceptance ≤ 15 %)	
Matrix	**Precision (n = 9)**	**Intermediate Precision (n = 9)**
Frozen NFY	13	13
Microencap	8	7
NFY	12	12
WCC	15	14

Platform Comparison

Similar results were obtained from both methods.

Table 3. Comparable % RSD Observed Between Flow Cytometry and Plate Count Results

	% RSD for Mean Counts	
Matrix	**Flow Cytometry Live (n = 21)**	**Plating Colony (n = 3)**
Frozen NFY	12	9
Microencap	7	7
NFY	11	11
WCC	14	17

Conclusions

A validated acoustic focusing flow cytometer produces reliable, accurate and reproducible enumeration data for multiple, different matrices. With slight modifications, ISO 19344:2015(E)/IDF 232:2015(E) can be used to enumerate probiotics contained in Frozen NFY, Microencap, NFY and WCC. Furthermore, for these matrices, flow cytometry generates results that are comparable to cultural plate counting.

Flow cytometry, formerly validated, demonstrated advantages over classical methods:

- 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

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