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Microbial Whole Cell Characterizations

In Support of the NIST Rapid Microbial Testing Methods Consortium – Reference Materials Working Group

Kirsten Parratt
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Abstract

Validation of rapid sterility testing technologies is critical for their implementation in supporting safety of cell and gene therapy products. However, many rapid sterility testing methods rely on newer measurands versus traditional growth-based methods, and no ground-truth, fit-for-purpose reference materials currently exist for rapid methods, especially molecular methods based on nucleic acid measurements. Microbial cell count and genome copy count are key properties that can support the adoption of molecular methods but are challenging to quantify. In the feasibility studies described herein, NIST performed multiple characterizations of commercially available microbial cell materials commonly used for qualification and validation of sterility testing to identify potential paths forward for additional certified values on these materials. Samples consisted of viable, single-strain microbial reference materials certified by the manufacturer for number of colony-forming units (CFU). NIST performed cell count and characterization experiments using CFU (culturable cells), Coulter counting (total cells), fluorescence flow cytometry (total cells, total genome copies), and impedance flow cytometry (total cells) methods. Only materials with relatively high cell concentrations (cell number > 10^5 per mL) were found to be suitable for the methods performed herein, and acellular material varied across products and sometimes interfered with measurements. It was concluded that factors including acellular material, cell aggregation, and dead cells contributed to discrepancies among CFU counts, total cell counts, and genome copy counts, likely stemming from both strain-specific cell characteristics and manufacturer-specific processes. Based on these results, a draft roadmap on optimizing characterization methods for a given material was developed to assist stakeholders interested in assigning total cell count and genome copy count to microbial cell reference materials. The results and lessons learned were shared with the Working Group 1 – Reference Materials of the NIST Rapid Microbial Testing Methods (RMTM) Consortium to support understanding of challenges and opportunities related to adding new certified values to existing reference materials. Experimental details and a results summary are provided here to help inform all RMTM stakeholders developing or using microbial cell reference materials for molecular rapid microbial testing methods.

Keywords

Cell Counting; CFU; Enumeration; Genome Copies; Microbial Cells; Molecular Methods; Rapid Microbial Testing Methods; Reference Materials; Sterility Testing.

Table of Contents

Executive Summary **1**
1. Introduction **2**
2. Materials and Methods **5**
2.1. Materials 5
2.1.1. Reagents 5
2.1.2. Cells 5
2.2. Methods 6
2.2.1. Fixation 7
2.2.2. CFU 7
2.2.3. Fluorescence Flow Cytometry (FFC) 8
2.2.4. Impedance Flow Cytometry (IFC) 9
2.2.5. Coulter Counting (CC) 9
2.2.6. Microscopy 10
3. Results and Discussion **11**
3.1. Product Differences Were Observed for Identical Starting Strains 11
3.2. Matrix Material is Detectable across Strains and Manufacturers 13
3.3. Total Cell Counts are Feasible Across Materials 16
3.4. Total Genome Copy Count Methods Need Additional Development 18
4. A Roadmap to New Value Assignment **22**
5. Conclusion **23**
Appendix A. Workflow for Total Cell Count **24**
Appendix B. Workflow for Total Genome Copy Count **25**
References **26**

List of Tables

Table 1: Cell and Matrix Materials Used **6**
Table 2: Methods, Measurands, and Instruments Used **7**
Table 3: CFU Experimental Conditions **8**

List of Figures

Figure 1: Overview of Measurements by Strain and Method. The number of measurements (y-axis) for each strain (x-axis) is shown for each measurement method (color-coded) **11**
Figure 2: Differing *E. coli* Cell Characteristics Across Products. Representative images from different *E. coli* products (one product per column: A/E, B/F, C/G, and D/H). Top row: Cells are identifiable as the

high NUV450-H signals on the right side of each plot (indicated by red dashed ovals added to aid the reader). The NUV450-H signal (x-axis) corresponds to Hoechst33342 staining, the VSSC-H signal (y-axis) corresponds to scattering, and the color of the datapoints corresponds to event density (blue – sparse to red – concentrated). Bottom row: Cells are identifiable as the high diameter events on the right-hand side of each histogram (red dashed lines). The equivalent diameter (x-axis) corresponds to the diameter of a sphere equivalent to the volume displaced by the event, and the y-axis reports event counts in each bin. 12

Figure 3: Matrix Detected in *P. aeruginosa* Samples. Representative images from different *P. aeruginosa* products (columns). Top row: Cells are identifiable as the high NUV450-H signals on the right side of each plot (indicated by red dashed ovals added to aid the reader), unstained cells with low signal (indicated by black arrow), and potential stained matrix with medium signals (indicated by red arrows). The NUV450-H signal (x-axis) corresponds to Hoechst33342 staining, the VSSC-H signal (y-axis) corresponds to scattering, and the color of the datapoints corresponds to event density (blue – sparse to red – concentrated). Bottom row: Cells are identifiable as the high diameter events on the right-hand side of one histogram (indicated by red dashed lines added to aid the reader). The equivalent diameter (x-axis) corresponds to the diameter of a sphere equivalent to the volume displaced by the event, and the y-axis reports event counts in each bin. 14

Figure 4: Flow Cytometry Comparisons of Diluent, Preservation "Blank", and Microbial Material Across Manufacturers. Representative images from two different products (A and B, colored backgrounds). Within each product, three samples were measured using the same settings (left – diluent, middle – preservation media "blank", right – microbes in preservation media) and are shown in two ways to facilitate comparisons (top – VSSC-H vs FSC-H, bottom – VSSC-H vs NUV450-H). The NUV450-H signal (x-axis) corresponds to Hoechst33342 staining, the VSSC-H signal (y-axis) corresponds to side scattering, the FSC-H signal (y-axis) corresponds to forward scattering, and the color of the datapoints corresponds to event density (blue – sparse to red – concentrated). 15

Figure 5: Comparison of Count Methods Across Species. Average measured counts relative to the CFU given on the CoA (y-axis, log scale) were compared across viable (top) and total (bottom) count methods (color-coding) for several different species (x-axis). CFU – Colony-forming unit, CC – Coulter counting, FFC – Fluorescence flow cytometry, IFC – Impedance flow cytometry. 17

Figure 6: Ratio of Coulter and FFC-Total Measurements. Average measured counts from Coulter relative to counts from FFC-Total (y-axis, log scale) were compared for several different species (x-axis). Error bars are the standard deviation for each material of a particular species. CC – Coulter counting, FFC – Fluorescence flow cytometry. 18

Figure 7: Fluorescence Microscopy of Live and Formaldehyde-Fixed Microbial Cells. Representative results from DIC (grey) and fluorescent (black/blue) images from *S. aureus* (top row), *B. spizizenii* (middle row), and *A. brasiliensis* (bottom row) samples which were stained with Hoechst33342 before (left column) and after fixation with formaldehyde (right column). All samples were imaged under the same conditions and presented on the same fluorescent scale, apart from the *S. aureus* which was significantly brighter and thus presented on a larger fluorescent scale. The *S. aureus* fixed sample was not measured because the microscopy and flow cytometry showed complete staining of the live sample. The scale bar is 20 µm. 20

Figure 8: Fluorescence Flow Cytometry of Live and Formaldehyde-Fixed Microbial Cells. Representative results from *S. aureus* (top row), *B. spizizenii* (middle row), and *A. brasiliensis* (bottom row) samples which were stained with Hoechst33342 before (left column) and after fixation with formaldehyde (right column). All samples were analyzed under the same conditions and shown with no gating. Cells are identifiable as the high NUV450-H signals on the right-hand side of each cytogram.

The NUV450-H signal (x-axis) corresponds to Hoechst33342 staining, the VSSC-H signal (y-axis) corresponds to scattering, and the colors correspond to event density (blue – sparse to red – concentrated). 21

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Author Contributions

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Executive Summary

The purpose of this report is to describe feasibility studies undertaken by NIST in support of priorities for the NIST Rapid Microbial Testing Methods (RMTM) Consortium, Working Group 1 – Reference Materials. The goal of the studies was not to compare products or make recommendations, but to evaluate the feasibility of measuring and applying new certified values to commercially available microbial cell reference materials intended for use in sterility testing according to U.S. Pharmacopeia (USP) <71> and other standards. Expanding the certification to include values such as total cell count or total genome copies would facilitate use of existing materials in new applications including development and validation of non-culture based RMTMs, such as molecular methods. The feasibility studies indicated that measurands beyond CFU could be measured on these products, with measurement technologies detecting differences among the products not evident by CFU measurements alone. These results are not surprising, as currently available materials were designed for CFU certification only. This work raises awareness within the RMTM community regarding the technical possibilities and challenges in measuring additional properties of cell materials. It also highlights the types of differences present across available materials that would be critical to characterize when using the materials to support a broadening range of sterility testing technologies. Properly accounting for reference material differences can help reduce measurement bias and support manufacturers and end-users in their goal of increasing advanced therapy safety. Based on the results, NIST developed a draft roadmap to assist stakeholders in optimizing methods to measure total cell and total genome copy number on existing materials to provide fit-for-purpose certified values (and complement the existing CFU data) for molecular-based RMTMs. This report provides details of the feasibility studies, including the materials, methods, results, and findings.

1. Introduction

Before therapies can be administered to patients, the manufacturer must demonstrate that the product is sterile, meaning there are no viable microbes present in the product. In the United States, this requirement is described in the Code of Federal Regulations (21 CFR Part 610).(1) Traditionally, this requirement is met by "compendial testing" according to U.S. Pharmacopeia (USP) <71>, which is based on the ability of contaminating microbes to grow to a visibly discernible level under specified growth conditions.(2) Medicinal product manufacturers validate their sterility testing regimes with a specified set of microbial organisms deemed to be representative of potential contaminants, including pathogens and commensal species, that serve as a positive control to confirm that growth of the microbes would be detectable within the specified 14-day culture period. These "compendial" microbes can be purchased as live microbial reference materials from multiple manufacturers.

Such sterility tests are regularly performed for traditional medicinal products (e.g., small-molecule drugs) but are not suitable for newer short shelf-life therapies, such as cell therapy products (e.g., chimeric antigen receptor cells) that are manufactured and delivered to patients before results from a traditional test are available.(3) Rapid microbial testing methods (RMTMs) address this need by detecting contaminating microbes within a few hours or days via measurements that do not rely on visible growth. RMTMs are relatively new and critical for many regenerative medicine products, but manufacturers can encounter roadblocks to their implementation related to the investment in time, interpretation, and approvals to change methods.(4) In addition, a considerable challenge to the use of RMTMs is the difficulty of demonstrating the equivalency of these new alternative methods to compendial tests.

In 2020, NIST launched the NIST RMTM Consortium (referred to as "the Consortium") to help facilitate validation and adoption of rapid sterility methods for advanced therapy products. The Consortium is divided into working groups focused on supporting different elements of RMTM adoption, including Working Group 1 dedicated to reference materials. Microbial cell reference materials play a critical role in RMTM qualification and validation but typically only have a certified value based on colony-forming units (CFU). Many RMTMs rely on alternative, non-culture-based measurands to detect microbes, but CFU does not have a known universally predictable relationship with other measurands, making it challenging to use these reference materials for some RMTMs. New certified values are needed for these reference materials to be better suited for RMTMs.

Total cell number and total genome copy number were identified as values important for the implementation of molecular-based RMTMs such as polymerase chain reaction (PCR) and next-generation sequencing (NGS). These RMTMs are based on nucleic acid measurements, not culturability. It is often assumed that each colony forms from a single starting cell, and that cell carries a single genome with a single copy associated with a particular gene, but these assumptions are often invalid for microbial cells. Microbial cell genomes often include multiple copies of particular genes (including the commonly used 16S and 18S regions), and microbial cells may include entire replicate genomes depending on their biology, environment, and state of replication or growth. Thus, if a reference material contains cells with on average more than one genome copy per cell or aggregated cells, a highly efficient molecular method RMTMs with

minimal sample loss may overestimate the number of CFUs present. It is also possible that molecular methods may underestimate the CFU present if the workflow has significant losses, such as a DNA extraction step that does not fully lyse the cells to obtain all the encapsulated DNA.(5) Other challenges are the potential presence of extracellular DNA or a large proportion of dead cells, both of which are likely to be included in molecular method results. Total cell count is a useful measurement to accompany CFU and genome copy measurements because it can help identify sources of discrepancy and bias due to variables such as cell lysing efficiency and dead cell counts.

Ideally, new certified values could be added to existing reference materials to support their use in RMTMs. As reference material manufacturers do not currently assign these values, end-users must generate their own assignments for commercially available reference materials or materials produced in-house for RMTMs. It would be useful to share these data to reduce duplicate efforts and harmonize methods used to assign values. Additional data also benefit reference material manufacturers who might be interested in adding new values to their products but have not performed these characterizations due to financial or other considerations.

NIST therefore undertook several small-scope studies regarding the feasibility of measuring total cells and genome copies in commercially available microbial cell reference materials developed for traditional sterility testing (e.g., USP <71>) and other culture-based applications. The motivation behind sharing these data is to document the range of characteristics observed in current reference materials and lessons learned relevant to the characterization methods. These studies were not comprehensive and had limited numbers of biological and technical replicates. Studies were performed *ad hoc* based on availability of the materials and NIST staff time. Methods and protocols varied among the studies depending on the characteristics of the materials being measured. Results were presented to all interested Consortium members with manufacturers and products identified, and some information was shared publicly during Consortium Workshops and in Interlaboratory Study Reports. Methods and anonymized results are discussed in this report.

Reference materials consisting of different microbial strains were obtained from several manufacturers. Measurements were performed using technologies that could potentially assign new certified values of total cell number or total genome copy number to the materials. Specifically, these technologies were fluorescence flow cytometry, impedance flow cytometry, and Coulter counter, in addition to CFU. It is important to emphasize that these are methods for material characterization and **are not RMTMs**. Results were compiled from a combination of studies to capture overarching conclusions about the range of observed material characteristics that could be important for their use in RMTM applications and for the certification of additional values. These conclusions include:

1. Product Differences Were Observed for Identical Starting Strains
2. Matrix Material is Detectable across Strains and Manufacturers
3. Total Cell Counts are Feasible Across Materials
4. Total Genome Copy Count Methods Need Additional Development

It is expected that the data summary and discussion herein will be useful to the RMTM community and inform discussion around the use of existing reference materials for RMTM development, validation, and equivalency demonstration. Based on these results, NIST has concluded that it is unlikely that any one measurement protocol to quantify total cell number or genome copies will be equally appropriate for all commercially available materials or all microbes. This conclusion is supported by literature on flow cytometry and DNA extraction methods, which show dependencies on the microbial cell materials measured.(6, 7) However, from a metrology perspective, it is entirely appropriate to use material-specific characterization methods to assign ground-truth values to reference materials and, in turn, use these materials to validate RMTM method performance.

It is expected that complete optimization of a characterization method will need to be undertaken by each reference material manufacturer in order to properly account for the specifics of their product. As a start, NIST's feasibility studies have laid the groundwork to enable the successful application of these characterization methods by surveying a broad range of materials and microbes and building a draft "roadmap" to guide method development to quantify these new values. During the studies described herein, common workflows for cell count (Appendix A) and genome copy count (Appendix B) were developed and optimized for internal use as new challenges were discovered (Section 3). Each workflow compares results from multiple characterization methods and describes potential mitigations for situations where results diverge. The workflow drafts were included here to provide a summary of current NIST thinking and to spark discussions with stakeholders. Significant additional investment from the stakeholder community is required to transform these drafts into formal, standardized procedures that include quantitative determination of method concordance to guide decision making. If these quantitative workflows were established, they would increase confidence in cell and genome copy enumerations for microbial cell reference materials used with RMTMs and across a broad range of microbiology applications.

In addition to these Consortium efforts, NIST's Microbial Metrology Program works on microbial cell enumeration for a variety of microbes and stakeholder groups and is working to develop reference materials with new certified values and methods appropriate for characterization of different microbes.

2. Materials and Methods

Certain equipment, instruments, software, or materials are identified in this paper in order to specify the experimental procedure adequately. Such identification is not intended to imply recommendation or endorsement of any product or service by NIST, nor is it intended to imply that the materials or equipment identified are necessarily the best available for the purpose.

2.1. Materials

2.1.1. Reagents

Phosphate-buffered saline (PBS, Corning, #46-013-CM) was used as rehydration media and as the diluent for further dilutions of materials.

Agar plate types were selected based on the identity of the strains and formulated with 1.5 % by mass Difco Agar (BD Bacto, catalogue # 214010). Media included Tryptic Soy Broth (TSA, BD Bacto, # 211825), Brain-Heart Infusion (BHI, MilliporeSigma, #53286), Nutrient Broth (NA, Acumedia, #7146A), Reinforced Clostridial Agar (Acumedia, #7714A), and Difco Sabourauds agar modified (BD Bacto, #274720).

Multisizer experiments used 0.9% (by mass) sodium chloride irrigation solution (Baxter, catalogue # 2F7124) or Coulter Isoton Diluent (Beckman Coulter, #8547148) as diluent.

Fixation was performed with formaldehyde (ThermoFisherScientific, #119690010).

2.1.2. Cells

Products obtained are listed in **Table 1**. Materials were stored according to manufacturer's recommendations. Before use, materials were suspended in the manufacturer's rehydration solution (if provided) or PBS. Some materials were "blanks" that contained preservation media without cells. The ATCC MiniPacks are glycerol cell stocks without an associated count value. These materials were centrifuged to remove the supernatant and resuspended in PBS immediately before use. In a separate experiment, these materials were also used to grow fresh cultures.

Table 1: Cell and Matrix Materials Used

Manufacturer	Product Line	Species	Strain	CoA CFU[1]
ATCC	MINIPACK	*Aspergillus brasiliensis*	ATCC 16404	NA
ATCC	MINIPACK	*Bacillus subtilis*	ATCC 6633	NA
ATCC	MINIPACK	*Candida albicans*	ATCC 10231	NA
ATCC	MINIPACK	*Escherichia coli*	ATCC 8739	NA
ATCC	MINIPACK	*Pseudomonas aeruginosa*	ATCC 9027	NA
ATCC	MINIPACK	*Staphylococcus aureus*	ATCC 6538	NA
ATCC	MINIPACK	*Clostridium sporogeneses*	ATCC 19404	NA
ATCC	MINIPACK	*Salmonella enterica*	ATCC 14028	NA
ATCC	MINIPACK	*Staphylococcus epidermidis*	ATCC 12228	NA
ATCC	MINIPACK	*Klebsiella pneumonia*	ATCC 10031	NA
Biomerieux	Bioball	Blank	Blank	NA
Biomerieux	Bioball	*Aspergillus brasiliensis*	NCPF 2275	1.09E+08
Biomerieux	Bioball	*Bacillus subtilis*	NBRC 13722	9.03E+07
Biomerieux	Bioball	*Burkholderia cepacia*	NCTC 10743	1.47E+08
Biomerieux	Bioball	*Candida albicans*	NCPF 3179	1.09E+08
Biomerieux	Bioball	*Pseudomonas aeruginosa*	NCTC12924	1.04E+08
Biomerieux	Bioball	*Staphylococcus aureus*	NCTC 10788	1.33E+08
Biomerieux	Bioball	*Escherichia coli*	NCTC 12923	5.50E+02
Biomerieux	Bioball	*Escherichia coli*	NCTC 9001	1.0E+04
Biomerieux	Bioball	*Escherichia coli*	NCTC 12923	1.10E+08
Millipore-Sigma	Vitroid	Blank	Blank	NA
Millipore-Sigma	Vitroid	*Escherichia coli*	CECT 434	1.0E+02 to 1.0E+04
Millipore-Sigma	Vitroid	*Escherichia coli*	CECT 516	5.0E+04 to 1.5E+05
Millipore-Sigma	Vitroid	*Pseudomonas aeruginosa*	CECT 111	1.10E+05
Microbiologics	Custom	Blank	Blank	NA
Microbiologics	EZ-AccuShot	*Escherichia coli*	ATCC 8739	< 5.2E+01
Microbiologics	Custom	*Pseudomonas aeruginosa*	ATCC 9027	1.70E+09
Microbiologics	Custom	*Staphylococcus aureus*	ATCC 6538	1.70E+09
Microbiologics	Custom	*Burkholderia humptydooensis*	*sp. nov. MSMB121*	NA
Stratix	Instant Inoculator	Blank	Blank	NA
Stratix	Instant Inoculator	*Escherichia coli*	ATCC 8739	> 1.0E+06
Stratix	Instant Inoculator	*Pseudomonas aeruginosa*	ATCC 9027	> 1.0E+05
Stratix	Instant Inoculator	*Aspergillus brasiliensis*	ATCC 16404	> 1.0E+06

[1] CFU from the Certificate of Analysis (CoA) is listed for all materials. “NA” indicates that no CFU value is assigned.

2.2. Methods

General protocols are given here for the methods and instruments (**Table 2**), and any important deviations are noted for the relevant datasets. Dilution volumes were varied depending on the

starting concentration of the microbes or the resuspension instructions provided by the material manufacturer.

Table 2: Methods, Measurands, and Instruments Used

Method (abbreviation)	**Measurand**	**Instrument**
Coulter Counting (CC)	Particle count via impedance (total)	Multisizer 4
Impedance Flow Cytometry (IFC)	Particle count via impedance (intact, total)	BactoBox
Fluorescence Flow Cytometry (FFC)	Particle count via scattered light and fluorescence (total, genomes, viable)	CytoFLEX LX
Growth (CFU)	Colony forming units (viable)	Growth at 37°C

2.2.1. Fixation

Fixation buffer was freshly prepared immediately before use by mixing 37 % (by volume) formaldehyde solution with 10X PBS and nuclease-free water to generate a final concentration of 3.7 % (by volume) formaldehyde in 1X PBS. Cells were added to 1 mL of fixation buffer and placed on a rotator for 60 min at room temperature. After fixation, cells were washed two times. For each wash, cells were pelleted via centrifugation (5 minutes, 4500 x g, at room temperature), the supernatant disposed of, and the pellet resuspended in 1 mL PBS.

2.2.2. CFU

CFU assays were performed by drip plating 10 μL volumes or spread plating 100 μL volumes, depending on the experiment. In drip plating a multichannel pipette was used to deposit a row of droplets at the top of a square agar plate held parallel to the ground. The plate was then carefully tilted vertically such that the droplets rolled to the bottom of the agar plate, each creating a line of sample. Observations were performed on concentrations ranging from 10^0 times to 10^8 times more dilute than the starting materials, depending on the experiment. A negative control plate was also prepared from the diluent used in each experiment. Samples were incubated per conditions in **Table 3**Table 3. These same growth conditions were used to generate freezer stocks of the MINIPACK cells for “in-house” samples. Colonies were manually counted, and CFU was determined by selecting dilution(s) with countable colonies (spread-plate: 20 colonies to 200 colonies, drip plate: 5 colonies to 50 colonies). If multiple countable dilutions were present, the geometric mean was used to calculate CFU.

Table 3: CFU Experimental Conditions

Strain[1]	Agar Type	Incubation Time (h)	Incubation Temperature (°C)	Incubation Atmosphere
ATCC 6538, CECT 111, ATCC 9027	TSA	24	30	Aerobic
ATCC 6538, ATCC 8739, CECT 516, NCTC 12923, CECT 111, ATCC 9027, ATCC 12228	TSA	24	37	Aerobic
MSMB 121	TSA	48	30	Aerobic
ATCC 6633	BHI	24	30	Aerobic
ATCC 8739, CECT 516, NCTC 12923	BHI	24	37	Aerobic
ATCC 19615	BHI	24	37	5 % CO_2
CECT 111, ATCC 9027	NA	24	30	Aerobic
CECT 111, ATCC 9027, ATCC 8739, CECT 516, NCTC 12923, NCTC 12924, NCTC 10788, NBRC 13722, NCTC 10743, NCPF 3179, ATCC 14028, ATCC 10031	NA	24	37	Aerobic
ATCC 19404	Reinforced clostridial agar	24	37	Anaerobic
ATCC 10231, ATCC 16404	Sabourauds agar	24 to 48	25	Aerobic

[1] Different culture conditions were used depending on the material and experiment. Recommended conditions from the manufacturer were used when possible. Some materials were cultured under different conditions in separate experiments.

2.2.3. Fluorescence Flow Cytometry (FFC)

In preparation for FFC, higher concentration materials were diluted to an approximate cell concentration of 10^7 per mL based on the CFU certified value. Lower concentration materials were diluted to reach a minimum working volume of 100 µL. Materials were incubated with fluorescent probes immediately before measurement.

Hoechst33342 was used in all samples and is a cell-permeant probe that primarily binds double stranded DNA. Hoechst-positive events were classified as cells. Hoechst33342 working stock was prepared by diluting 20 mmol/L Hoechst33342 (Thermo Fisher Scientific, # 62249) to 2 mmol/L in PBS. The Hoechst working stock was further diluted to a final concentration of (4 to 40) µmol/L for incubation with the cells. A subset of samples were stained with viability probes: MitoView633 (MV, Biotium, cat# #70055), DiBac4(3) (Bis-(1,3-Dibutylbarbituric Acid)Trimethine Oxonol (DiBac, Biotium, catalogue # 61011), and CellTrackerRed (Invitrogen, #C34552). MV is a cell-permeable probe that accumulates in the mitochondria in eukaryotic cells and in the cell

interior for prokaryotes. MV was reconstituted per manufacturer's instructions to 200 µmol/L and diluted into a final concentration in the samples of 0.2 µmol/L. DiBac is a cell membrane potential probe that permeates cells with compromised membranes. DiBac was prepared in dimethylsulfoxide (DMSO) at 2 mmol/L and diluted to a final concentration in the samples of 0.2 µmol/L. CellTrackerRed is a cell-permeable probe that reacts with thiol groups such as those on glutathione. Stock solution was prepared by dissolving CellTrackerRed in DMSO to a concentration of 10 mmol/L and diluted to a final concentration in the samples of 20 µmol/L.

After probes had been added to samples, tubes were incubated at room temperature or 37 °C, statically or shaking at 120 rpm, for at least 15 min before analysis on the flow cytometer. Conditions varied across experiments.

System startup and quality control were performed on the flow cytometer (CytoFLEX LX, Beckman Coulter) per manufacturer's instructions. Additional dilutions of the stained samples were performed in PBS if necessary. Samples were measured for 2 min to 4 min at 10 µL/min. Laser settings for each channel (indicated according to manufacturer's labels) varied by experiment but were generally set as FSC = 1000, SSC = 20, VSSC = 1, NUV450 = 20 to 100, B525 = 100, and R660 = 100.

Each sample was measured multiple times and recorded as FCS files. Depending on the experiment, analysis was performed in one of several ways, using CytExpert (Beckman Coulter), RStudio (Posit), or FlowJo v10 (FlowJo). Gates were drawn manually or algorithmically, generally starting with FSC and VSSC, followed by NUV450. FlowJo was used to generate representative flow cytograms for this report. When necessary, files were concatenated to reach a minimum cell count of 10 000 for visualization purposes.

2.2.4. Impedance Flow Cytometry (IFC)

BactoBox (SBT Instruments, hardware version 7.3) was utilized per manufacturer's protocols. PBS was diluted to 0.11X and 0.22 µm filtered before use to achieve a conductivity within the operational range of the instrument. For each replicate observation, 101 µL of sample was added to 10 mL PBS in a 15 mL conical tube, vortexed, and measured. Additional dilutions were performed if necessary. The total particle number and intact cell number were recorded for each sample. Disinfection with 70 % (by volume) isopropanol was performed after every 20 measurements per instrument requirements.

All experiments were conducted with the default analysis program, meaning thresholds were not modified to optimize performance for specific strains or materials. Count values below 10 000 were not quantified by BactoBox and were reported as "NA" for analysis.

2.2.5. Coulter Counting (CC)

Multisizer 4 (Beckman Coulter) experiments were conducted on samples diluted in saline or ISOTON II. Volumes of diluent and cells varied depending on the experiment and the starting concentration of the material. The instrument was started and calibrated per manufacturer's instructions using size calibration beads (Beckman Coulter 2.0 µm Coulter CC Size Standard L2, #

6602794). Accuvette™ (Beckman Coulter) sample holders were prepared with 10 mL to 20 mL of 0.1 µm filtered saline or ISOTON II. The instrument was set up using a 20 µm aperture tube for most experiments and changed to a 50 µm aperture tube for larger cells (e.g. *A. brasiliensis*). Immediately before each acquisition, the sample was vortexed briefly, a volume ranging from 20 µL to 1 mL was added to a prepared Accuvette, and the sample was mixed. Samples were measured with acquisition volumes ranging from (100 to 300) µL. Between samples, the aperture tube was flushed with saline and immersed in an Accuvette filled with saline for rinsing.

The Multisizer software was used to calculate sample concentration. The "Coincident Correction" feature was applied in the software to account for single events that were deemed likely to consist of two or more particles. The software automatically calculates the number of objects per mL for the original (undiluted) sample replicate based on the acquired volume, dilution performed, and user-selected size range for analysis. The Multisizer measures the volume displaced by each particle via impedance and reports the measurement as the diameter of a sphere of equivalent volume ("size"). Selected size ranges depended on the sample and were ≈ 0.5 µm to 4 µm for smaller cells and ≈ 2.5 µm to 20 µm for larger cells, and all particles in the selected ranges were assumed to be cells.

2.2.6. Microscopy

Samples were prepared for imaging by concentrating via centrifugation (for samples with starting cell concentrations lower than ≈ 10^7 per mL), incubation with fluorescent probes, and mounting on slides. Fluorescent probes and staining protocols for microscopy were similar to those for fluorescence flow cytometry. Slides (Premium Frosted Microscope Slides, Fisher Scientific, #12-544-2) were prepared by cleaning and pretreatment. For cleaning, slides were sonicated for 30 min in 2 mol/L potassium hydroxide (Sigma-Aldrich, #PG1767-500g) in 50 % (by volume) ethanol and then sonicated for 30 min in distilled water, air dried, and stored at room temperature. Immediately before use, slides were pretreated with 0.05 % by mass poly-L-lysine (Sigma-Aldrich, #P8920-100mL) in 50 % (by volume) ethanol and air dried. Samples were mounted on slides by depositing a small volume, generally 3 µL, onto a prepared slide and allowing the sample to dry. Then, 3.5 µL PBS was pipetted on top of the dried sample, and an (18 x 18) mm, #1 coverslip (VWR VistaVision, #16004-308) was applied. Coverslips were sealed to the slide by applying CoverGrip Coverslip Sealant (Biotium, #23005).

Samples were imaged using a Nikon Eclipse Ti-2 with an ORCA Flash 4.0 camera (Hammamatsu) and SOLA light engine (Lumencor). Images were collected using a 60X 1.2 numerical aperture (NA) water objective in differential interference contrast (DIC) (source intensity = 10 %, integration time = 90 ms) and fluorescence (DAPI filter cube, ex: 350/50 nm, em: 460/50 nm, 400 nm dichroic mirror, SOLA intensity = 20 %, integration time = 400 ms). Images were recorded in Nikon format (nd2) and viewed using ImageJ (8).

3. Results and Discussion

Materials were evaluated in six separate studies. To avoid association of specific products with results, which could be misunderstood as comparing products, raw data have not been made available, and results are partially blinded. Most studies included multiple materials and spanned several different experiments. When possible, a single starting stock of each material was prepared and divided among all methods performed on that date. The absolute number of measurements collected is grouped by strain identification and color-coded by characterization method (**Figure 1**).

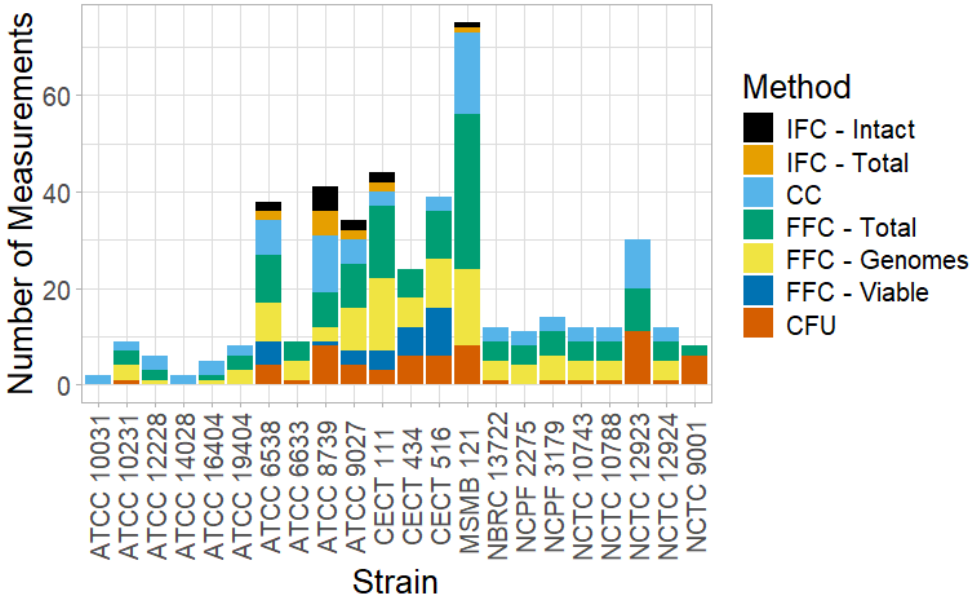


Figure 1: Overview of Measurements by Strain and Method. The number of measurements (y-axis) for each strain (x-axis) is shown for each measurement method (color-coded).

3.1. Product Differences Were Observed for Identical Starting Strains

CFU-certified reference materials can be compared by CFU (e.g., 10 CFU in one material should be comparable to 10 CFU in another material), but that comparison does not necessarily extend to newer measurands such as total cells or total genome copies (e.g., the total cell number in a material with 10 CFU may not equal the total cell number in a different material with 10 CFU). In this work, the ratios between CFU and other properties such as total cell number and total genome copies were found to vary across materials.

A portion of the studies characterized samples from nominally the "same" microbial strain but produced by different vendors to provide insight into this issue. As one example, *Escherichia coli* (*E. coli*) ATCC 8379/NCTC 12923/CECT 516 cells are the basis of many reference materials with certified CFU values. Herein, differences in the number of total cells or genome copies relative to the certified CFU values were observed across *E. coli* materials of this strain (discussed further in Section 3.3). Likely these different ratios are due to different preparation

and processing methods used across manufacturers. An awareness of the possibility of each reference material having unique characteristics is important when using these materials in RMTMs and should be considered when interpreting data from validation and comparability demonstrations.

For example, in these studies, cells produced by different manufacturers appeared to have different average genome copies per cell. This was determined by staining cells with Hoechst33342, which binds double-stranded DNA, and measuring the resulting distribution using fluorescence flow cytometry (**Figure** 2, top row). Cells were apparent as higher-intensity NUV450-H subpopulations on the right-hand side of each plot. Within the cell gate, genome copy counts were calculated based on distinct subpopulations if detectable (e.g., **Figure 2B**). In materials without clear peaks in the distribution of DNA per cell, subpopulations with distinct genome copy counts could not be extracted (e.g., **Figure 2D**), and cells were estimated to have one genome copy each.

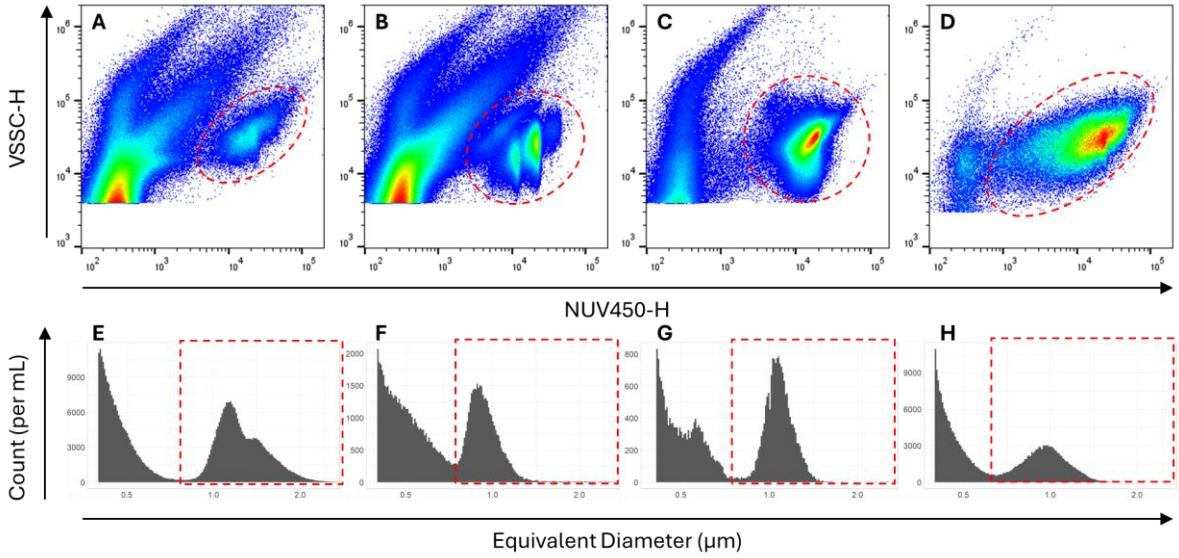


Figure 2: Differing *E. coli* Cell Characteristics Across Products. Representative images from different *E. coli* products (one product per column: A/E, B/F, C/G, and D/H). Top row: Cells are identifiable as the high NUV450-H signals on the right side of each plot (indicated by red dashed ovals added to aid the reader). The NUV450-H signal (x-axis) corresponds to Hoechst33342 staining, the VSSC-H signal (y-axis) corresponds to scattering, and the color of the datapoints corresponds to event density (blue – sparse to red – concentrated). Bottom row: Cells are identifiable as the high diameter events on the right-hand side of each histogram (red dashed lines). The equivalent diameter (x-axis) corresponds to the diameter of a sphere equivalent to the volume displaced by the event, and the y-axis reports event counts in each bin.

The particle characterization techniques also suggest that the cell size distribution differs across products (**Figure 2E-H**). Although all four samples are nominally centered around an equivalent diameter close to 1 µm, differences in object volume distribution and range are evident. Additionally, the size discrepancy is potentially mirrored in the fluorescence flow cytometry, as the VSSC signal can be an approximation for the size of small objects such as microbial cells (more details and limitations of this statement are discussed in detail elsewhere (9)).

Cell size is unlikely to greatly impact the detection of the material with molecular RMTMs, but RMTMs based on other measurands (e.g. absorbance, ATP concentration) may be influenced by cell size or other cell properties that may potentially correlate with cell size. Therefore, these phenotypical differences across manufacturers are of potential importance to the end user.

3.2. Matrix Material is Detectable across Strains and Manufacturers

Microbial cell reference materials are not just cells – they typically contain a background matrix for cell stabilization, and many particle characterization technologies used to count total cells or genome copies are able to detect matrix particles present in the materials. Here "matrix" is meant to encompass multiple types of additional material that could be present including extracellular matrix produced by the cells during culture, byproducts or debris from cells that may have been disrupted during processing, and preservation media added by the manufacturer to support cell viability. The matrix amount and properties could interfere with characterization of a material's cell count and genome copies. Based on the data herein, two types of matrices were apparent in the materials tested: biologically derived and preservation media derived.

The first type of background matrix detected is likely produced by the cells during culture (potentially it could also be cell byproducts). This matrix is species or strain dependent (rather than manufacturer dependent), since some cells naturally produce more extracellular matrix than others, such as *P. aeruginosa*. Matrix was apparent in quantification of *P. aeruginosa* materials from multiple manufacturers using fluorescence flow cytometry and Coulter counting (**Figure 3**). In some materials, it was challenging to detect the cells over the background matrix signal, particularly with Coulter counting (e.g., Figure 3E-H), whereas the fluorescent flow cytometry could better distinguish some cells from the matrix (Figure 3A-D). These data also provide another example of how different manufacturing processes can result in different cell phenotypes for the same nominal strain.

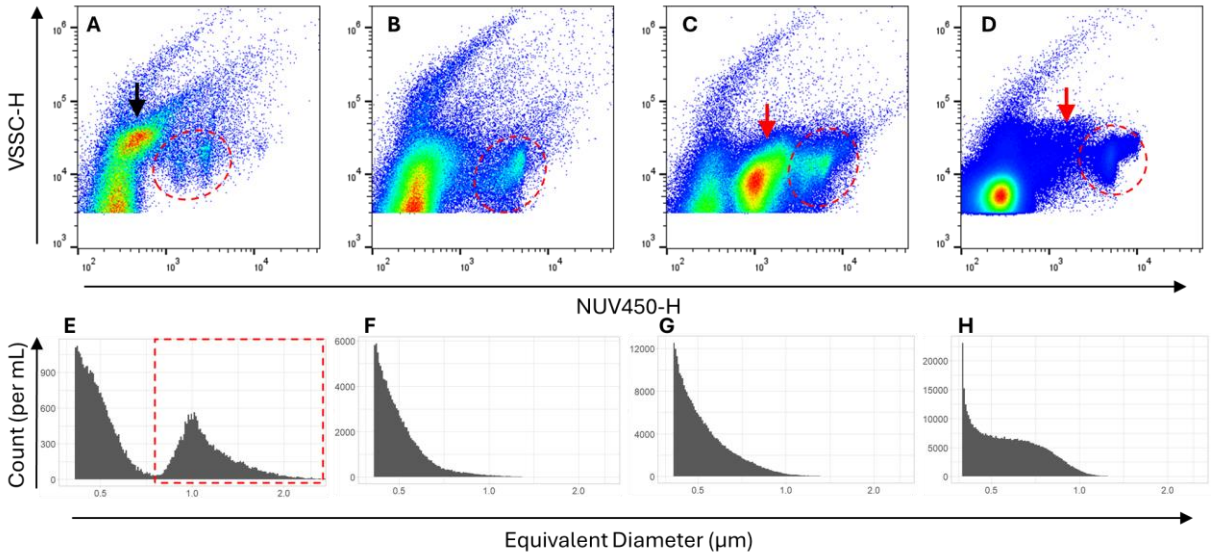


Figure 3: Matrix Detected in *P. aeruginosa* Samples. Representative images from different *P. aeruginosa* products (columns). Top row: Cells are identifiable as the high NUV450-H signals on the right side of each plot (indicated by red dashed ovals added to aid the reader), unstained cells with low signal (indicated by black arrow), and potential stained matrix with medium signals (indicated by red arrows). The NUV450-H signal (x-axis) corresponds to Hoechst33342 staining, the VSSC-H signal (y-axis) corresponds to scattering, and the color of the datapoints corresponds to event density (blue – sparse to red – concentrated). Bottom row: Cells are identifiable as the high diameter events on the right-hand side of one histogram (indicated by red dashed lines added to aid the reader). The equivalent diameter (x-axis) corresponds to the diameter of a sphere equivalent to the volume displaced by the event, and the y-axis reports event counts in each bin.

The second type of matrix detected is hypothesized to be preservation media added by the manufacturers. For some materials tested here, “blank” materials (cell-free preservation media controls) were measured and compared to the cell-containing materials. These data are not provided in full, because a comprehensive study was not performed, blanks were not available for all samples, and measurements were collected using different settings across experiments, such that they cannot be compared appropriately. However, some generalizations on the preservation matrix can be made. In some cases, the low intensity events in the flow cytometry measurements for the blanks and the corresponding cell materials were similar, suggesting that the low intensity events in the cell materials were from the matrix background and can be detected (**Figure 4**). Hoechst33342 staining of both preservation media examples seemed to be minimal as the “matrix” events do not emit fluorescence in NUV450 (low x-axis values), indicating that the Hoechst fluorescence staining can be used to differentiate matrix and cell events for these materials. Therefore, the impact of preservation media on flow cytometry counts in these studies is expected to be minimal. The impact of matrix on counts derived from Coulter measurements is expected to be greater, since there could be overlap between large matrix particles/aggregates and small cells. These results highlight the challenges that particles from the preservation media present to characterization of reference materials, and additional studies would be needed to evaluate matrix effects across materials and characterization methods.

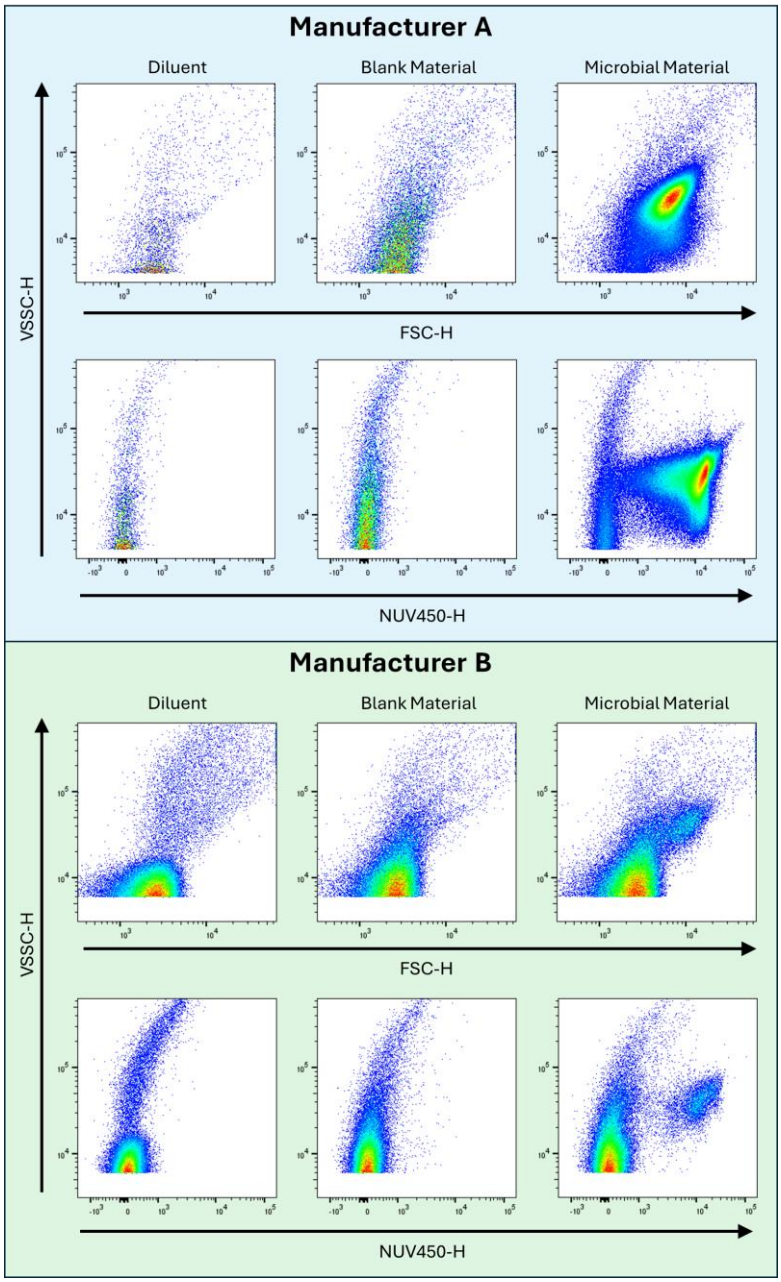


Figure 4: Flow Cytometry Comparisons of Diluent, Preservation "Blank", and Microbial Material Across Manufacturers. Representative images from two different products (A and B, colored backgrounds). Within each product, three samples were measured using the same settings (left – diluent, middle – preservation media "blank", right – microbes in preservation media) and are shown in two ways to facilitate comparisons (top – VSSC-H vs FSC-H, bottom – VSSC-H vs NUV450-H). The NUV450-H signal (x-axis) corresponds to Hoechst33342 staining, the VSSC-H signal (y-axis) corresponds to side scattering, the FSC-H signal (y-axis) corresponds to forward scattering, and the color of the datapoints corresponds to event density (blue – sparse to red – concentrated).

For the typical CFU-based characterization of cell reference materials, the presence and composition of this matrix is not typically expected to impact CFU results or growth of the cells in a compendial sterility test. However, given this matrix was detectable by impedance flow

cytometry, fluorescence flow cytometry, and Coulter counting, low signal matrix events could partially overlap with the quantification region for the cells of interest and interfere with characterization methods beyond CFU. Additionally, the matrix may potentially interact with reactions performed in RMTMs, such as DNA extractions or PCR amplifications, with unknown impact on the detection of the microbial cells. If a manufacturer used the same preservation media for all microbial cell reference materials, effects of the matrix on the characterization methods or RMTM detection may become apparent, but this would not be the case if a manufacturer selected preservation media based on the cell being preserved. For any cell counting measurement, the impact of matrix counts will depend on the ratio of matrix counts to cell counts for a given characterization method and the analyses applied to differentiate matrix particles and cells.

3.3. Total Cell Counts are Feasible Across Materials

Total cell count is an important value that could help facilitate the comparison of results from CFU and molecular measurements, especially if other confounding values (e.g., dead cells, extracellular DNA) can be measured as well. Many reference materials may contain non-viable cells that did not survive the preservation process. These cells would not be measurable by CFU but could be measurable by molecular methods if the genomic DNA is still present. These additional genome copies could result in higher-than-expected detection via PCR or sequencing. Similarly, a single CFU could consist of multiple aggregated cells, which would also result in additional genome copies. To identify potential discrepancies, the number of cells was measured via several methods that quantify total or viable cell counts. Results are reported here as the average count measured by each method (color-coded) divided by the CFU given on the material's CoA (**Figure 5**). For some materials only a CFU range was given so the median of the stated limits was used as the reference value.

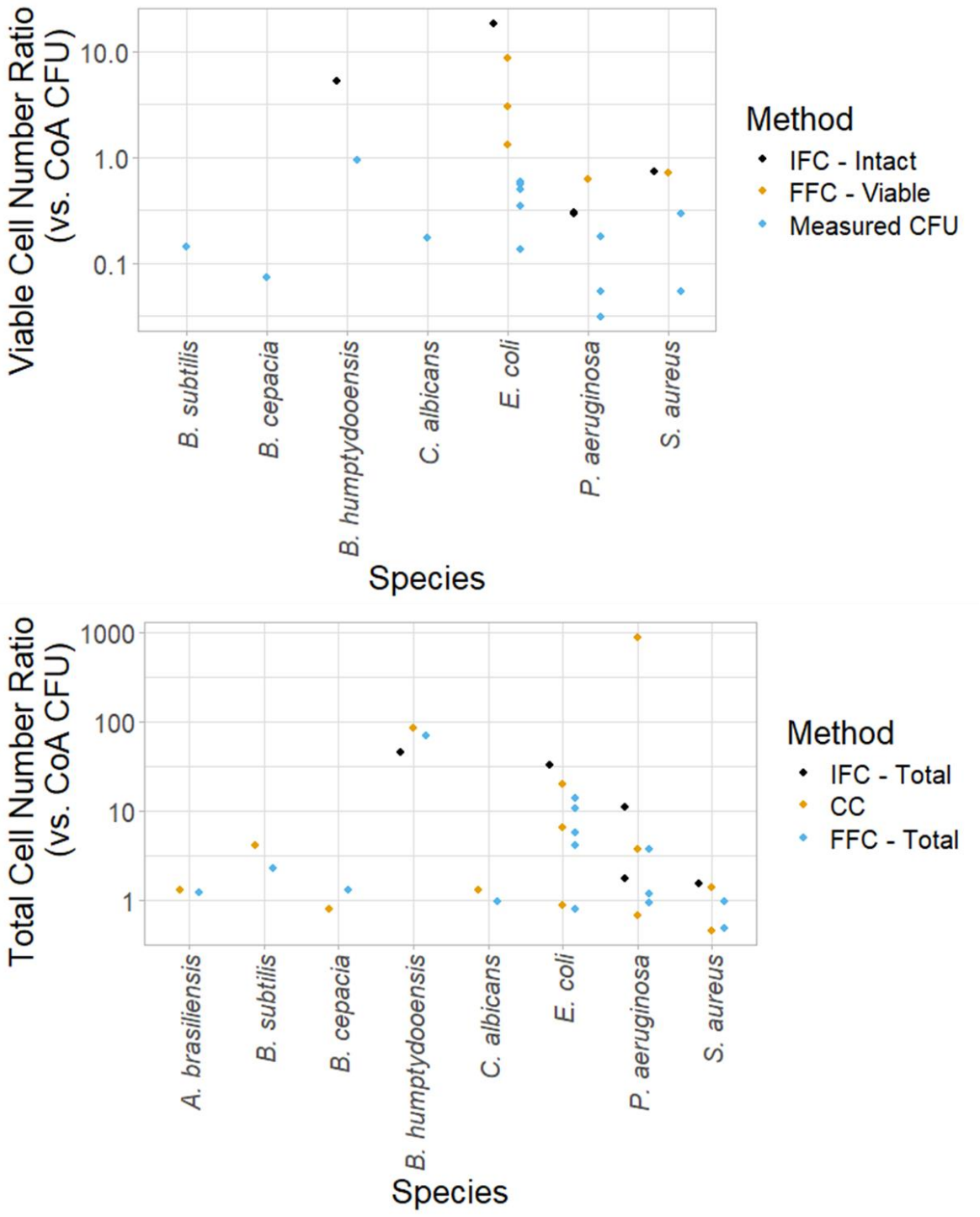


Figure 5: Comparison of Count Methods Across Species. Average measured counts relative to the CFU given on the CoA (y-axis, log scale) were compared across viable (top) and total (bottom) count methods (color-coding) for several different species (x-axis). CFU – Colony-forming unit, CC – Coulter counting, FFC – Fluorescence flow cytometry, IFC – Impedance flow cytometry.

Average measurements from Coulter were also compared with average measurements from FFC-Total to generate ratios for those two total count methods (**Figure 6**).

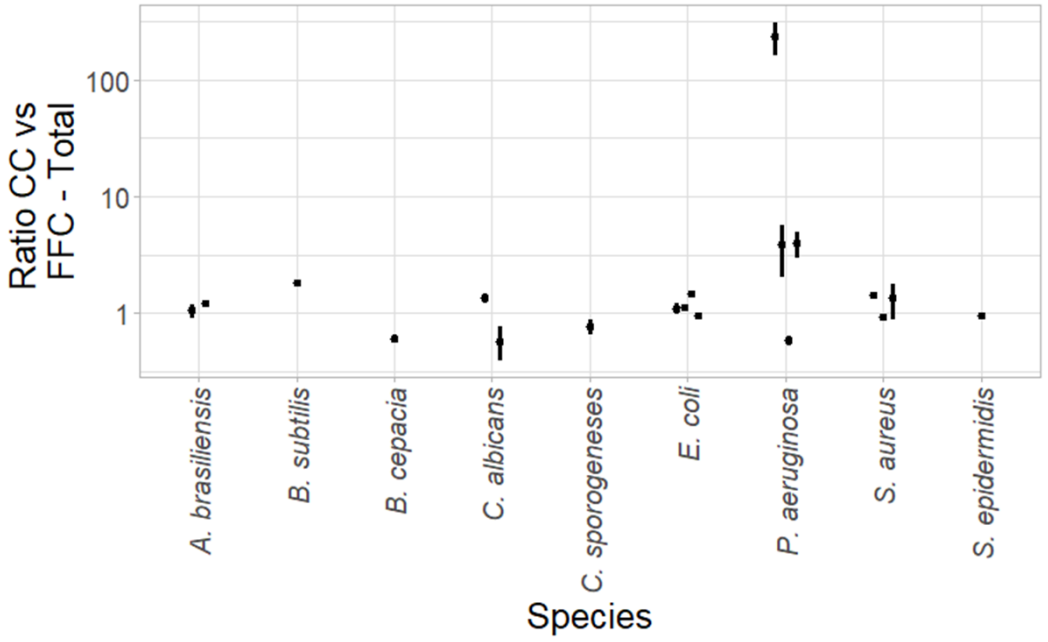


Figure 6: Ratio of Coulter and FFC-Total Measurements. Average measured counts from Coulter relative to counts from FFC-Total (y-axis, log scale) were compared for several different species (x-axis). Error bars are the standard deviation for each material of a particular species. CC – Coulter counting, FFC – Fluorescence flow cytometry.

3.4. Total Genome Copy Count Methods Need Additional Development

Genome or gene copy count certified values would be most appropriate for molecular methods that enumerate nucleic acid sequences if they could be measured confidently. It would be straightforward to assign values using the molecular methods themselves, for example, via ddPCR. However, doing so assumes that the DNA was fully extracted from the cells, which is unlikely unless methods were optimized for each material of interest.

Therefore, using an additional, orthogonal method to assign genome copy numbers will support measurement assurance. NIST has developed protocols to measure fluorescence from Hoechst33342 bound to intracellular double-stranded DNA and therefore calculate the genome copy number per cell without extraction. Fluorescence microscopy can be used to confirm that all cells exhibit intracellular staining and sometimes resolve individual genome copies. Fluorescence flow cytometry can be used to measure a large number of individual cells to estimate the distribution of genome copies per cell across the population.

However, the Hoechst33342 staining method also requires optimization for each material, as different cell types and phenotypes can exhibit different staining characteristics that could bias the measured fluorescence. For example, the genome copy count for the *E. coli* shown in **Figure 3** can be relatively easy to estimate if distinct subpopulations are present (upper right panel) or

difficult in cases with a single, broad population (lower right panel). These are likely true biological differences in genome copy number due to the growth conditions selected for manufacturing each product; NIST has separately investigated this possibility with in-house cultures (data not shown). **Figure 4C, D** show another situation, in which matrix may be binding the stain or preventing it from accessing the cells' genomes leading to the poorly defined subpopulations with high intensity NUV450 intensity. Additionally, robust cells (e.g. fresh from culture) or cells with robust membranes (e.g. spores) are capable of actively pumping the stain out or resisting entry into the cytoplasm.

For these studies, microscopy was used to determine whether the Hoechst33342 had successfully reached the genomes. Cells were identified via DIC and/or a cell body stain (CellTracker Red, not shown). Measurements were also made on formaldehyde-fixed cells to determine if the live cells had been fully stained with Hoechst. The fixation is expected to inhibit membrane pump activity and potentially help permeabilize the membrane. **Figure 7** shows examples of the three scenarios encountered when testing the compendial species. First, *S. aureus* (top row) seemingly exhibited complete staining before fixation and would be a good candidate for genome copy enumeration using the Hoechst method. Second, *B. subtilis* (middle row) stained while live but was brighter after fixation. Third, *A. brasiliensis* (bottom row) did not stain even after fixation. Similar fluorescence intensity comparisons were made with flow cytometry on the same samples (**Figure 8**).

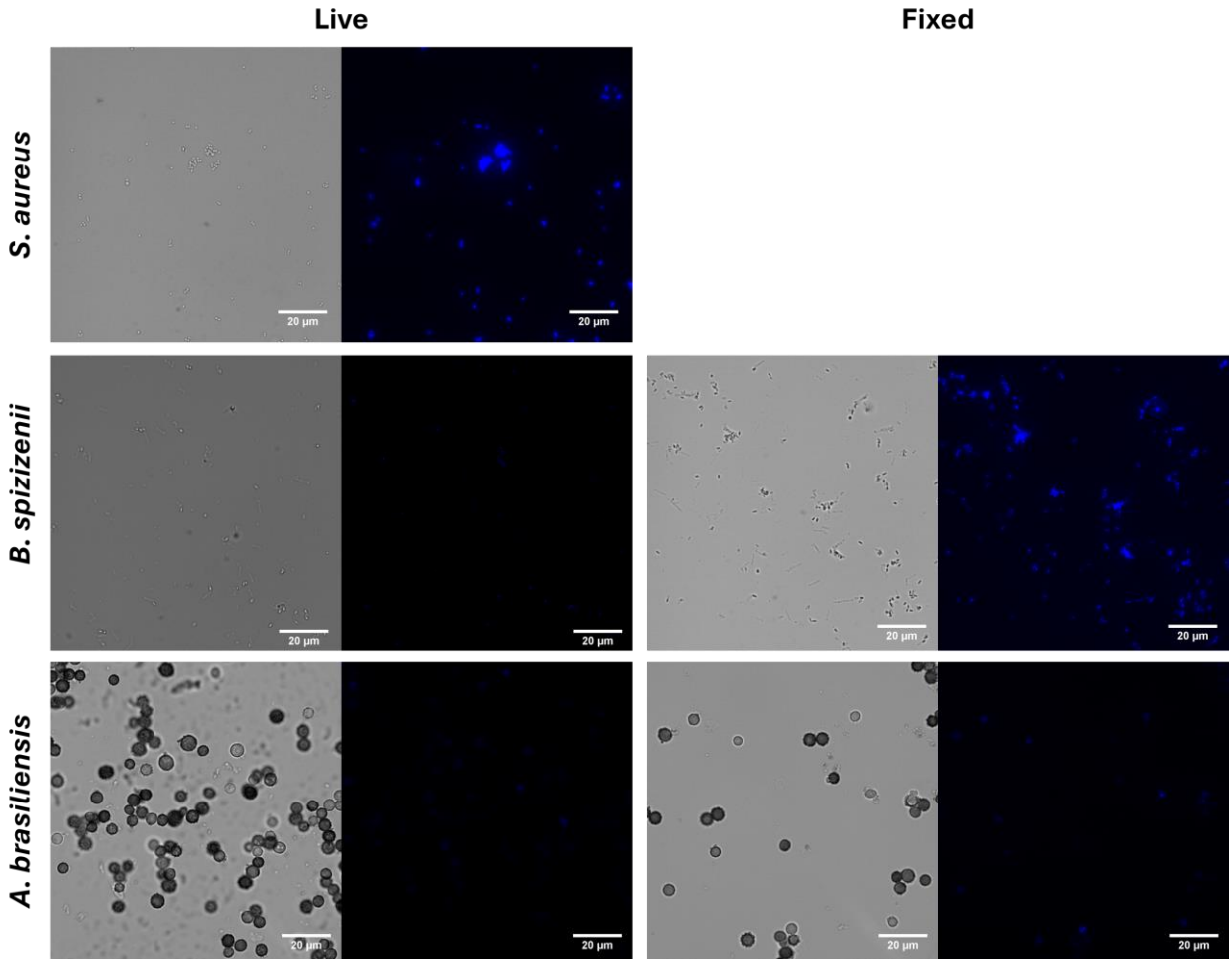


Figure 7: Fluorescence Microscopy of Live and Formaldehyde-Fixed Microbial Cells. Representative results from DIC (grey) and fluorescent (black/blue) images from *S. aureus* (top row), *B. spizizenii* (middle row), and *A. brasiliensis* (bottom row) samples that were stained with Hoechst33342 before (left column) and after fixation with formaldehyde (right column). All samples were imaged under the same conditions and presented on the same fluorescent scale, apart from the *S. aureus* which was significantly brighter and thus presented on a larger fluorescent scale. The *S. aureus* fixed sample was not measured because the microscopy and flow cytometry showed complete staining of the live sample. The scale bar is 20 µm.

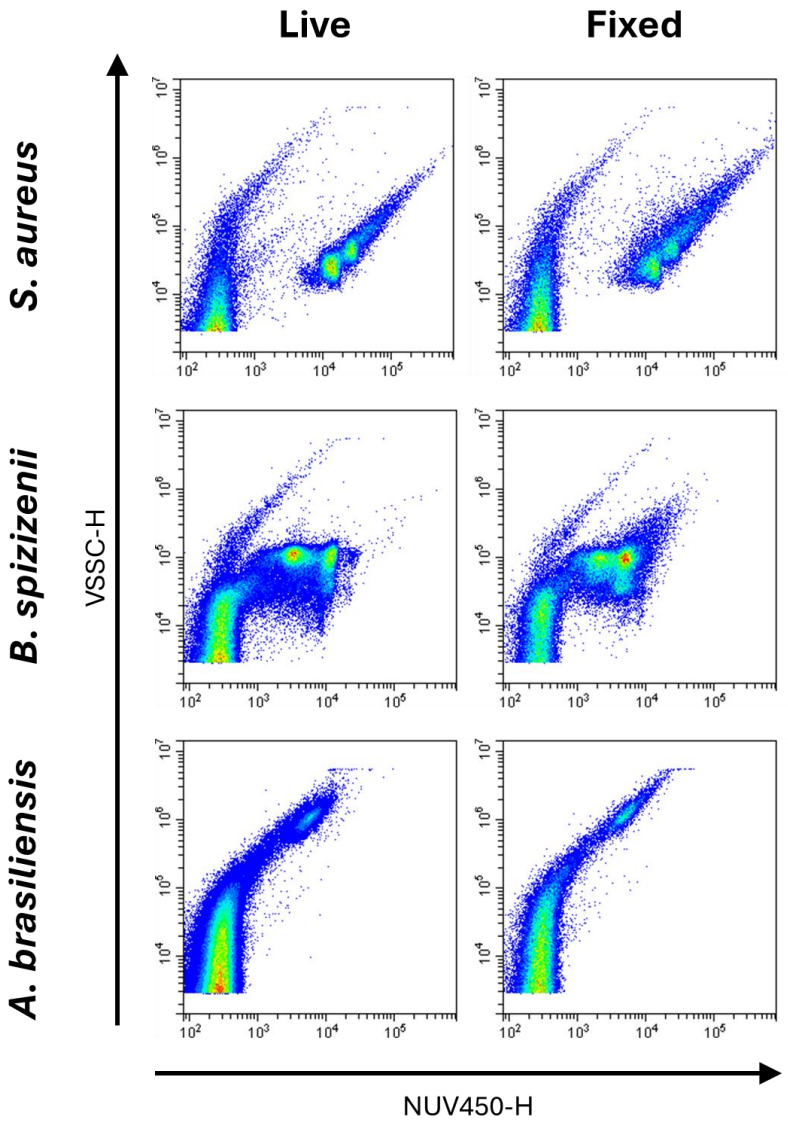


Figure 8: Fluorescence Flow Cytometry of Live and Formaldehyde-Fixed Microbial Cells. Representative results from *S. aureus* (top row), *B. spizizenii* (middle row), and *A. brasiliensis* (bottom row) samples stained with Hoechst33342 before (left column) and after fixation with formaldehyde (right column). All samples were analyzed under the same conditions and shown with no gating. Cells are identifiable as the high NUV450-H signals on the right-hand side of each cytogram. The NUV450-H signal (x-axis) corresponds to Hoechst33342 staining, the VSSC-H signal (y-axis) corresponds to scattering, and the colors correspond to event density (blue – sparse to red – concentrated).

4. A Roadmap to New Value Assignment

A common challenge encountered during these studies was identifying methods that could be applied across materials that had significant variability in microbial characteristics, cell number, and product composition. For example, differences in fluorescent signal across microbes were observed when using a single staining protocol (**Figure 8, Figure 9**). This is not dissimilar from challenges with culturing microbes, since different microbes generally have specific nutrient requirements. Over time the sterility testing field has identified media and growth conditions that are suitable for all compendial testing, but this process is still on-going for newer characterization methods.

For the immediate future, it was concluded that assigning new values to existing whole cell reference materials will require a "Roadmap" with varying levels of method modification needed to optimize the measurement of different materials. Continuing the above example, one method of genome copy count assignment might be fluorescent flow cytometry for which the *S. aureus* live material could be stained and counted directly. However, the *B. spizizenii* might require counting the live material, fixing the material to stain the genome, recounting the fixed material to account for cell loss during the fixation process, and back-calculating the genome copy number. These considerations were summarized into two draft workflows (Appendix A & B) for total cell count and total genome count value assignment and shared with the Consortium during the 2024 NIST RMTM Consortium workshop.

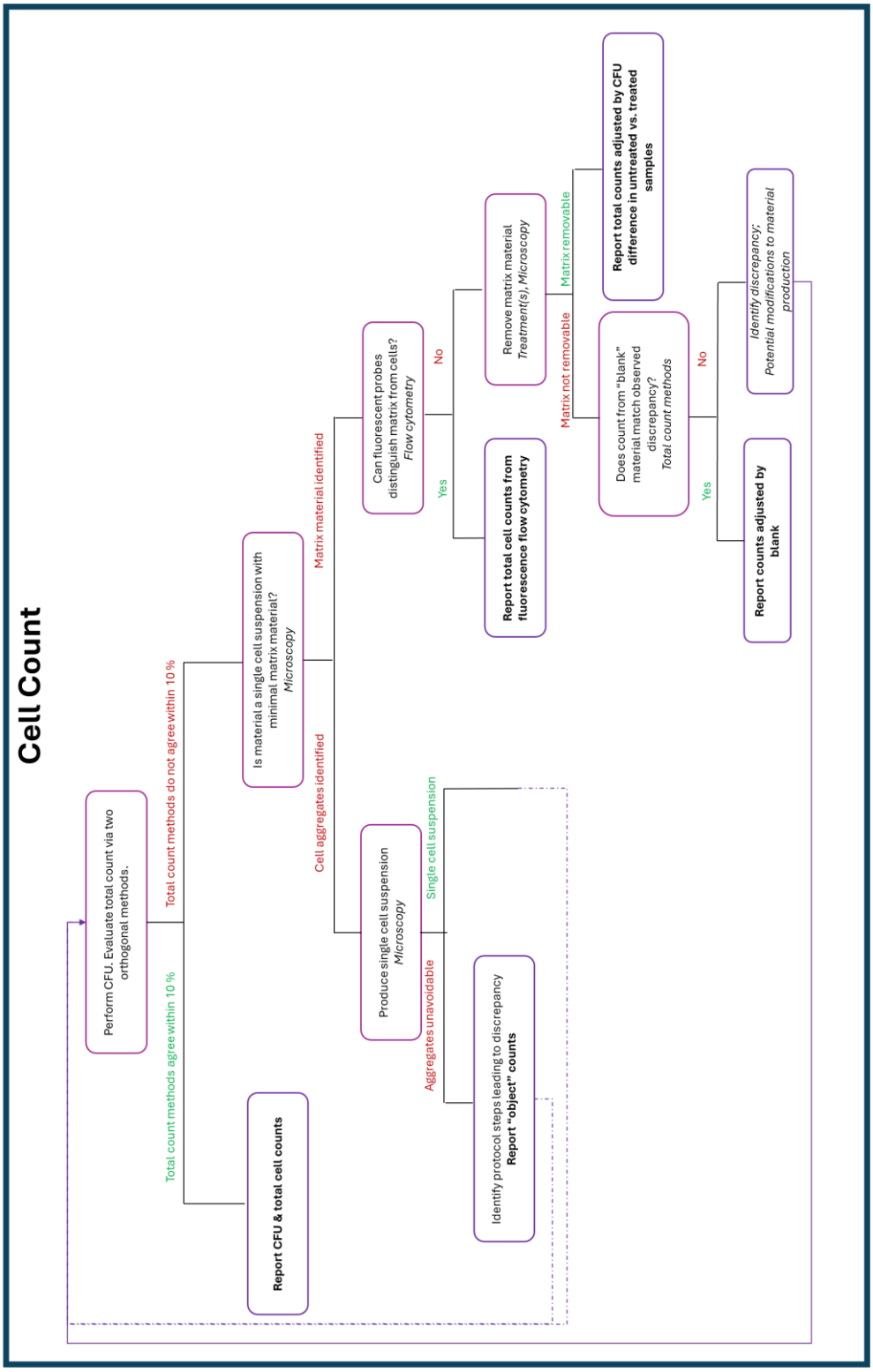
More work should be done to further refine these workflows such that they would be applicable for any microbial reference material and support decision-making for real materials. Likely this would involve testing the workflow for a large number of different reference materials and providing openly accessible data to demonstrate the process. The resulting workflows could then be used by any manufacturer or end-user interested in assigning total cell or genome copy counts and support the creation of fit-for-purpose reference materials that could better support regulatory oversight for RMTMs.

5. Conclusion

The goal of these studies was to evaluate the feasibility of adding new certified values to existing microbial whole cell reference materials. Though the collected datasets had numerous limitations due to the nature of the work (as discussed in the introduction, methods, and discussion), potentially useful quantitative and qualitative evaluations of material characteristics could still be made for some studies. Key observations were presented with representative data to inform reference material users and manufacturers about the types of differences observed across material strains and manufacturers when using newer quantification methods. Brief discussions of how the measured values could be important for molecular RMTMs were also included, as well as ideas for future work.

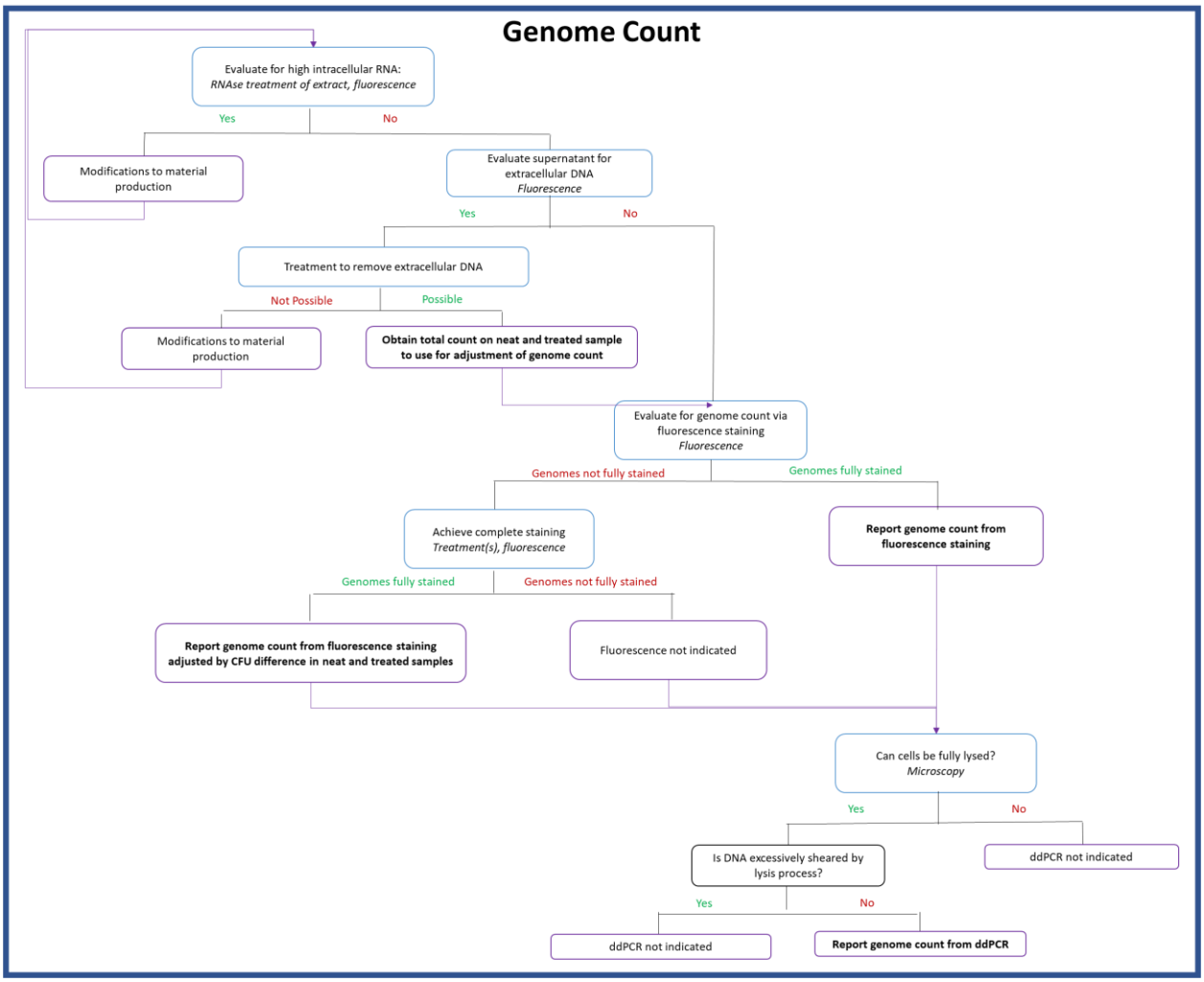
The feasibility studies have shown that many existing materials are likely suitable for cell count value assignment and some even for genome copy count assignment. Either of these values are likely to be more fit-for-purpose than CFU when evaluating the performance of molecular RMTMs and could be used in tandem to provide measurement assurance. To facilitate this process, NIST has drafted methodologic workflows to support a Roadmap that stakeholders could use to guide cell count and genome copy value assignment to their own materials. These workflows are expected to be broadly useful, and thus stakeholders in RMTM as well as other microbiology applications are invited to provide feedback to help advance microbial enumeration metrology.

Appendix A. Draft Workflow for Total Cell Count



Bolded text indicates steps where results can be reported. Italicized text indicates measurements suggested for the step.

Appendix B. Draft Workflow for Total Genome Copy Count



Bolded text indicates steps where results can be reported. Italicized text indicates measurements suggested for the step.

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