© 2004 Wiley-Liss, Inc.

Cytometry Part A 62A:162–168 (2004)

# Production of Precise Microbiology Standards Using Flow Cytometry and Freeze Drying

**C. A. Morgan,[1,2]* P. Bigeni,[1] N. Herman,[1] M. Gauci,[1] P. A. White,[2] and G. Vesey[1,3]**

[1]BTF Pty. Ltd., North Ryde, New South Wales, Australia

[2]School of Biotechnology and Biomolecular Sciences, Faculty of Science, University of New South Wales, Sydney, New South Wales, Australia

[3]Institute for the Biotechnology of Infectious Diseases, University of Technology, Sydney, New South Wales, Australia

Received 30 September 2003; Revision Received 18 March 2004; Accepted 21 April 2004

**Background:** Quality control standards provide a quantity of microorganisms for routine use in microbiology to demonstrate the efficacy of testing methods and culture media. Standards are normally prepared by diluting a culture of microorganisms to obtain a suspension that contains an estimated number of colony-forming units per milliliter. The variability and inaccuracy of these standards increase the potential for false results. Flow cytometry has been used extensively to prepare precise standards of *Cryptosporidium* and *Giardia* that contain exact numbers of organisms in a volume of liquid (1). However, the same levels of accuracy have yet to be obtained for bacterial quality control standards.

**Methods:** A modification of a Becton Dickinson FACScalibur flow cytometer enabled 30 bacterial cells to be sorted into a single droplet, mixed with a cryoprotective solution within the droplet, and frozen in liquid nitrogen. The frozen droplets were then freeze dried for stable preservation of the viable bacterial cells.

**Results:** A freeze-dried sphere 3 mm in diameter was produced, which contained 30 microorganisms. The within-batch variation for these freeze-dried spheres was no greater than two standard deviations, and the between-batch variation was less than one standard deviation.

**Conclusions:** Bacterial reference controls can now be produced with consistent accuracy and unparalleled precision, thus enabling harmonization across the microbiological testing industry. © 2004 Wiley-Liss, Inc.

**Key terms:** precision; exact number of cells; cell sorting; bacteria; standard reference materials; BioBall

Reference materials and quality control standards are required for all aspects of scientific analysis, from beads of a specific fluorescent intensity for flow cytometric analysis to reference chemicals for high-performance liquid chromatographic techniques. To date, quality control standards for microbiology lack the precision and accuracy of other analytical sciences. To enable a reference material for bacteria to be produced, a methodology is required that can accurately count exact numbers of viable cells and then dispense the cells in a format that enables easy handling and long-term storage.

Current procedures for producing quality control preparations for bacteria depend on serial dilutions of cell cultures, where the initial numbers of cells is determined by colony growth on an agar plate (2). These techniques are open to operator error, risk of contamination, and a high degree of variation between replicates (3,4). More importantly, batch-to-batch variation is high, as no two bacterial cultures are identical in numbers. This variation limits comparative studies between laboratories. The availability of precise reference materials would allow regulatory authorities to apply strict quality standards across networks of laboratories or analysts.

Historically, 30 to 300 colonies are required on an agar plate to provide an accurate count. The upper limit is due to the difficulty in counting more than 300 colonies on a single agar plate, and the lower limit of 30 is set to limit the occurrence of zero counts. A precise quality control preparation with an average of 30 cells would enable quick and easy plate counting, without the risk of zero colonies on a plate.

The microbiology industry requires a precise reference material for bacterial microorganisms to provide a consistent number of cells per spores, with a reproducible amount of variation (<2 standard deviations [S.D.] from the mean). This standard needs to be in a format that is easy to use and does not require specialized storage conditions. Existing commercially available reference materials for the microbiology industry cannot deliver this level

*Correspondence to: C. A. Morgan, BTF Pty. Ltd., P.O. Box 599, North Ryde BC, NSW, 1670, Australia.

E-mail: contact@btfbio.com

Published online 31 October 2004 in Wiley InterScience (www.interscience.wiley.com).

DOI: 10.1002/cyto.a.20075

of precision. They are provided purely as a unknown quantity of freeze-dried cells in the form of an ampoule, vial, loop, or swab stick or as a semiquantitative pellet. The loops provide a user-friendly method for starting a culture that can then be diluted and used for long-term storage by freeze drying or freezing below −80°C. Semiquantitative reference cultures such as Quanti-cult (Oxoid, West Heidelberg, Australia) are quantified only within a one-log range of variation (100–1,000 colony-forming units [cfu]) and frequently require further dilution and aliquoting to obtain the desired number of cells. These reference cultures do not eliminate variation due to losses in dilution and handling, which can be the largest source of variation in preparing accurate reference standards.

Flow cytometric analyses of bacterial populations and single-cell sorting have been in use for several decades (5–7). The development of a mechanical sorting mechanism that employs a moving catcher tube within a closed fluidic system (8) has stabilized cell sorting technology to such an extent that no calibration or alignment is usually required. This type of cell sorter also has the advantage that aerosols are not created (9) and it has been commercially available for several years as a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA).

The FACSCalibur flow cytometer has been used previously to identify and sort accurate numbers of bioparticles (10,11); however, the collected volume required for 100 organisms is in excess of 1 ml. A volume of liquid containing exact numbers of organisms is routinely used as a quality control standard in the detection of *Cryptosporidium parvum* oocysts and *Giardia lamblia* cysts (10). There are disadvantages of using a liquid aliquot spike, including the possible losses encountered when the spike is added to a sample and the limited shelf life of a liquid preparation.

This report focuses on the development of a methodology to produce standard reference materials for precise numbers of bioparticles. The methodology incorporates several technical alterations to adapt a FACSCalibur flow cytometer to enable accurate quantities of viable bacteria to be sorted and dispensed into a single droplet of liquid. The single droplet dispensed from the modified flow cytometer can be snap frozen in liquid nitrogen and freeze dried to form a stable solid sphere containing the specified quantity of bioparticles.

## MATERIALS AND METHODS

### Bacterial Strains, Culturing Conditions, Media, and Spore Staining

*Listeria monocytogenes* NCTC 11994 and *Listeria innocua* ATCC 33090 were grown in a specially developed medium of brain–heart infusion broth (CM225, Oxoid) with 400 mM 3-[N-Morpholino] propane sulfonic acid (MOPS) buffer and 15% sucrose in a rotary shaking incubator at 37°C for 24 h. A cell density of 0.6 absorbance was required for *L. innocua* cultures, and a density between 0.8 and 1.0 absorbance at 600-nm wavelength was required for *L. monocytogenes* cultures.

*Escherichia coli* NCTC 9001 cells were grown for 24 h in a rotary shaking incubator at 37°C in medium consisting of 20% sucrose and 250 mM Hepes buffer in 1.6% (w/v) tryptone, 1% (w/v) yeast extract, and 0.5% (w/v) sodium chloride. A cell density between 1.2 and 1.3 absorbance at a wavelength of 600 nm was obtained. All cell cultures were sampled at 24 h, when cells were in the early stationary phase.

A 1-μl aliquot of each culture was diluted into 1 ml of phosphate buffered saline (PBS; Sigma Chemical Co., Castle Hill, Australia) immediately before flow cytometric analysis.

*Bacillus cereus* ATCC 10876 and *Bacillus subtilis* ATCC 6633 spores were grown on nutrient agar plates and incubated at 37°C for 48 h. The spores were harvested from the plates by washing the surface of the plates with 1 ml of filtered distilled water and then stored at 4°C. The fluorescent nucleic acid stain Syto 11 (Molecular Probes, Eugene, OR, USA) was used to separate the small percentage of vegetative cells from the spores within the preparation. Syto 11 has an absorption wavelength of 508 nm and an emission wavelength of 527 nm. In brief, Syto 11 was diluted 1:10,000 in filtered PBS, and 1 ml of the Syto 11 dilution was added to a 3-μl aliquot of the spore preparation. The spores were stained for 30 min at ambient temperature. A 400-μl aliquot of the stained spores was pipetted into a flow tube for flow cytometric analysis.

### Manual Preparation of Bacterial Cultures

Manual dilutions of bacteria were prepared for a comparison against the cytometer-counted standards. Cultures of *L. monocytogenes* were serially diluted in PBS, and the $10^{-6}$dilution of the culture was dispensed onto horse blood agar as single droplets through a Pasteur pipette.

### Modification of the Flow Cytometer Capture Tube

The flow cytometer used in this study was a FACSCalibur system (Becton Dickinson) with a 15-mW, 488-nm air cooled argon-ion laser. Scattered light and fluorescence signals are detected by a series of photomultiplier tubes; the three parameters used within this study were forward scatter, right-angle light scatter, and green fluorescence at 525 nm. Initial experiments to sort 30 fluorescent beads on a FACScalibur flow cytometer indicated that greater than 0.5 ml of sheath fluid had to be collected from the sort line to ensure that all 30 beads were captured. This volume of fluid was too large to form into a reproducible droplet. Examination of the sorting mechanism revealed that the volume within the capture tube is relatively large (~91 μl) and that this causes sorted particles to be spread throughout this volume of fluid. Three different modifications were made to the capture tube and tested in an attempt to reduce the internal volume and thus allow 30 sorted particles to be collected into a 25-μl droplet of fluid.

The first modification involved inserting a 70-mm length of hypodermic tubing with an external diameter of 0.4572 mm and an internal diameter of 0.254 mm (Small Parts, Inc., Miami Lakes, FL, USA) into the capture tube. The

hypodermic was held in place by the snug fit within the capture tube. A 115-mm length of silicon tubing with an internal diameter of 0.254 mm was threaded onto the hypodermic. The other end of silicon tubing was threaded onto a second length of hypodermic that was inserted into a droplet-forming nozzle (Precision Components, Fort Collins, CO, USA).

The second modification involved inserting into the capture tube a 153-mm length of hypodermic tubing with an external diameter of 0.4572 mm and an internal diameter of 0.254 mm (Small Parts, Inc.). The hypodermic was bent by 90 degrees approximately 10 mm after exiting the capture tube. Leaving a 100-mm length of straight tube, a second 90-degree bend was placed in the hypodermic to align the tube vertically. The droplet-forming nozzle was fitted to the end of the hypodermic.

The third modification involved inserting into the capture tube a 153-mm length of hypodermic tubing with an external diameter of 0.4572 mm and an internal diameter of 0.254 mm (Small Parts, Inc.). The hypodermic tube was curved through 180 degrees without exerting force onto the capture tube, and the end was inserted into the droplet-forming nozzle (Fig. 1).

### Lyoprotectant and Sheath fluid

Lyoprotectants are normally added to bacterial suspensions prior to freeze drying to maintain cell viability. In this study the lyoprotectant consisted of sheep serum (Starrate, Bethungra, Australia) that had been heat treated for 20 minutes at 60°C and then filter sterilised, using a 0.22μm syringe filter (Millipore, Bedford, MA, USA).

Initial experiments involved using the lyoprotectant, diluted 1:4 in PBS, as the sheath fluid. This resulted in considerable background noise and was abandoned (Fig. 2). PBS (Sigma Chemical Co.) was used as sheath fluid.

### Delivery of Lyoprotectant

A system was designed that enabled the lyoprotectant to be mixed with the sheath fluid as the sheath fluid exited the sort line (Fig. 1). The sort line hypodermic was inserted into the droplet nozzle until it rested flush with the droplet nozzle tip. A second hypodermic (internal diameter 0.254 mm) was inserted into the droplet nozzle and connected to a syringe pump through silicon tubing (internal diameter 0.254 mm). This second hypodermic enabled the lyoprotectant to be injected into the droplet as it formed. The injection line and hypodermic were cleaned after every use with a 10% sodium hypochlorite solution, followed by 0.22-μm filter sterilized distilled water.

Initial results indicated that the sheath fluid and the lyoprotectant were not mixing within the droplet. To overcome this, a mixing device was incorporated into the drop-forming nozzle by inserting a third hypodermic (0.254 mm in internal diameter; Small Parts, Inc.) containing a finer guidewire (0.2286 mm in diameter; Small Parts, Inc.) into the nozzle (Fig. 1). A 1-mm length of the fine guidewire was formed into a hook at the exit of the outer hypodermic. This wire was attached to an electric motor and spun at approximately 900 rpm. The action of the rotating hook formation at the end of the wire served to mix the suspension containing the sorted microorganisms, sheath fluid, and the lyoprotectant as the droplet formed.

### Droplet Control System

To control the initiation of droplet formation, a 6-psi microbore double-acting pneumatic cylinder (Asco, Frenchs Forest, NSW, Australia) was used with an attached polypropylene vacuum nozzle. The vacuum nozzle attached to the end of the shaft of the cylinder was held near the droplet nozzle exit point. The vacuum nozzle ensured that all liquids received from the droplet nozzle were sent to waste before droplet formation began. On activation of the double-acting cylinder, the vacuum nozzle was removed to allow a droplet to immediately form directly after removal. The pneumatic cylinder was controlled by a Programmable Logic Controller (PLC; model FC-30, Festo, Esslingen, Germany). The PLC also controlled the sorting process of the cytometer via a modified Macintosh mouse. In brief, the mouse was modified to accept a 0.1-s 24-V pulse signal from the PLC, which mimics a mouse click and begins the cytometer sorting process.

A push button switch was connected to the PLC. A program was written for the PLC that waited for the button to be pushed and then commenced a cycle that involved the following steps:

1. Activating the sort process through the mouse
2. Activating the turntable to turn and place the next cup containing liquid nitrogen under the droplet nozzle
3. Activating the pneumatic cylinder to remove the vacuum nozzle away from the fluid flow
4. Waiting 0.9 s to allow a single drop to form and break away
5. Activating the pneumatic cylinder to move the vacuum nozzle into the fluid flow

### Flow Cytometry

For analysis of bacteria, data were presented as a scatter plot of forward scatter against right-angle light scatter. For samples of stained spores, data were presented as a scatter plot of green fluorescence against right-angle light scatter. A sort region was created that enclosed the cells or spores, and sorting was performed in single-cell mode. The differential pressure was adjusted until a sort rate of 200 sorts/s was achieved. PBS was used as the sample buffer and the sheath fluid, and all samples were run on the “lo” setting for flow rate. The cytometer was cleaned with a 10% sodium hypochlorite solution through the sheath lines and the sample tube, followed by 0.22-μm filter sterilized distilled water.

### Droplet Collection and Freezing

The droplets formed by the automated cytometer process were collected systematically in 12 cups held on a rotating turntable. The cups were filled with liquid nitro-

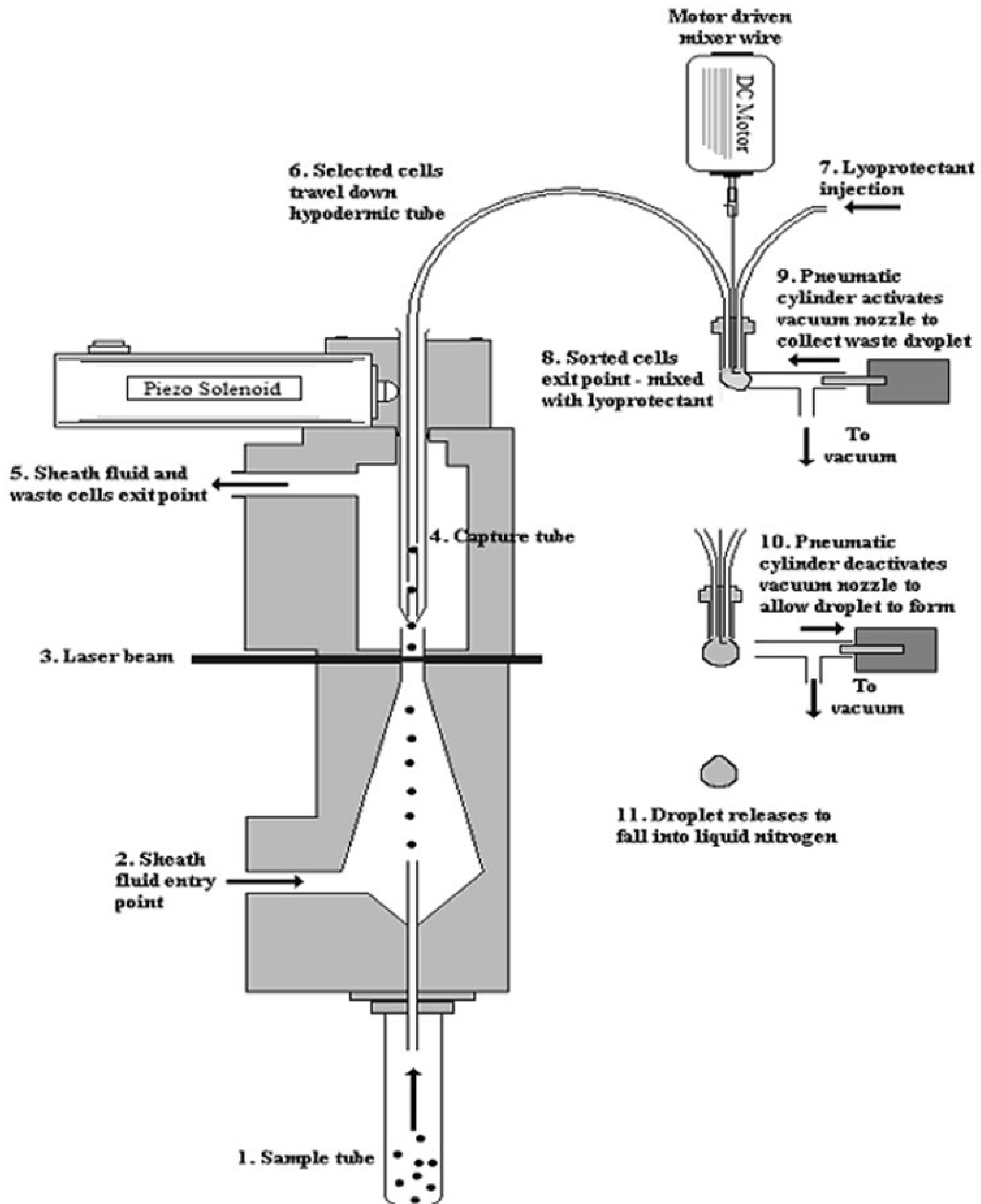


FIG. 1. Schematic diagram of the modified cytometer arrangement described as cytometer format 3. (1) Sample tube where the cells enter into the flow cytometer. (2) Sheath fluid enters the fluidic system of the flow cytometer and surrounds a single file stream of bacterial cells. (3) Laser beam scatters on each cell, allowing the detection and selection of the gated population required for sorting. (4) The capture tube, with the hypodermic tube inserted, is activated by the piezo solenoid to collect the selected cells. (5) Waste sheath fluid and waste cells exit the cytometer. (6) The selected cells travel down the hypodermic capture tube to the droplet nozzle. (7) Lyoprotectant is injected into the droplet nozzle through a secondary injection hypodermic tube. (8) The cells and lyoprotectant exit the cytometer at the droplet nozzle while being mixed by the mixer wire. (9) The pneumatic cylinder holds the vacuum nozzle to collect the waste droplets. (10) When deactivated, the vacuum nozzle pulls back and a droplet is allowed to form. (11) The droplet containing the sorted cells falls from the droplet nozzle and is frozen in liquid nitrogen.

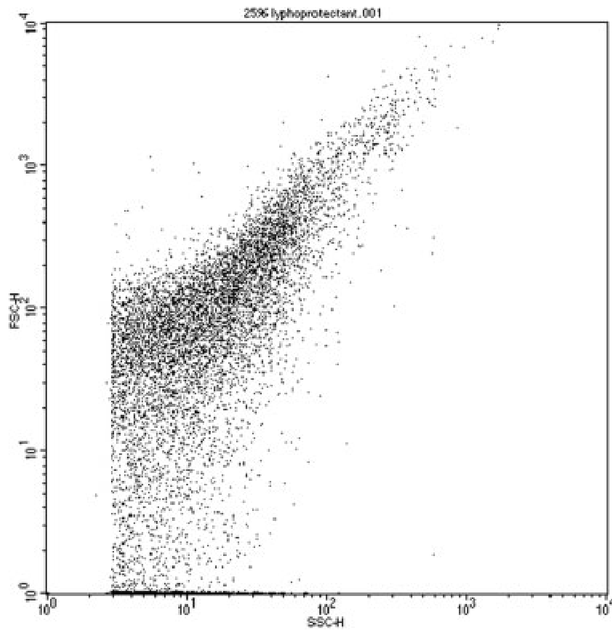


FIG. 2. Scatter plot showing the amount of scatter detected on the FACScalibur when 25% sheep serum in PBS is used as a sheath fluid. *Escherichia coli* cells are completely submerged by the scatter produced by the sheep serum sheath fluid.

gen at the beginning of the cycle and were kept topped up until droplet dispensing had finished. Once the required number of droplets were formed and frozen, the cups were removed from the turntable and the frozen spheres were placed into 1.5-ml freeze-drying vials (Crown Scientific, Brisbane, Australia) chilled with liquid nitrogen. One frozen sphere was transferred into each vial, and then a freeze-drying bung (Crown Scientific) that had been baked at 110°C for 3 h was carefully placed onto each vial to ensure the bung did not seal the vial.

### Freeze Drying

The freeze dryer (Telstar lyobeta 35, Terrassa, Spain) was prepared by lowering the shelf temperature to −10°C. The cycle was set to ramp the shelf temperature to 25°C and exert the maximum vacuum until 0.05 mbar was obtained. The cycle was 3 to 3.5 h long, depending on when the maximum vacuum level was reached. Once the cycle had finished, the vials were capped under vacuum within the freeze dryer. Before storage, the vials were crimped with aluminium crimps.

### Enumeration of Freeze-Dried Bacteria

To rehydrate the freeze-dried spheres, each sphere was tipped from its vial onto the surface of a nutrient agar plate for *B. cereus*, *E. coli*, and *B. subtilis* spheres or a Columbia blood agar for *L. monocytogenes* and *L. innocua* spheres. A 100-μl aliquot of 0.9% NaCl solution was used to dissolve the sphere before spreading. Plates were allowed to dry at ambient temperature and incubated at 37°C for 24 h, and colonies were enumerated and recorded.

## RESULTS

### Capturing 30 Sorted Bacteria Within a Single Drop

Experiments done to sort 30 bacteria showed that, with the normal configuration of the cytometer, the sorted particles spread out as they traveled through the capture tube and the silicon tubing (Table 1). The volume of sheath fluid that contained the bacteria would be too large to form a small freeze-dried ball. Three different modifications were made to the cytometer to reduce the volume of fluid required to capture 30 bacteria.

Cytometer format 1, with a small length of hypodermic tube inserted into the capture tube and silicon tubing connecting the hypodermic to the dispensing nozzle, produced 28 colonies in two droplets (Table 1). Cytometer format 2, using one hypodermic tube to connect the capture tube to the dispensing nozzle, produced 29 colonies; however, cells were still being detected in the second droplet. Cytometer format 3, using a curved hypodermic tube to connect the capture tube to the dispensing nozzle, produced 30 colonies in a single droplet. This format was therefore used for all further experiments (Fig. 1).

### Lyoprotectant

Serum, an efficient cryopreservative, was tested as a suitable sheath fluid for optimal freeze drying. Figure 2 shows a scatter plot obtained when a 25% sheep serum dilution was used as sheath fluid. The background scatter showed side scatter values of 2 to 1,000, thus completely obscuring the sort region for *E. coli* and most other unstained bacterial cells (Fig. 2). In contrast, filtered PBS as a sheath fluid did not produce any background interference and allowed accurate sorting of cell populations (Fig. 3). Two cell clusters were observed when sorting *E. coli* and other unstained bacteria; sorting from the two clusters showed that both contained 100% viable cells (data not shown); however, the region farthest away from the background scatter was chosen for sorting. The lyoprotectant was introduced into the droplet by reconfiguring the droplet nozzle as detailed in Materials and Methods and Figure 1.

Table 1
*Comparison of Escherichia coli NCTC 9001 cfu in Six Drops From the FACSCalibur With Three Different Formats of Hypodermic Insertion Into the Catcher Tube*[a]

| | Drop number | | | | | |
|---|---|---|---|---|---|---|
| Cytometer format | 1 | 2 | 3 | 4 | 5 | 6 |
| Standard FACScalibur | 0 | 10 | 12 | 4 | 2 | 1 |
| Format 1: hypodermic and silicon tubing | 25 | 3 | 0 | 0 | 0 | 0 |
| Format 2: bent hypodermic | 28 | 1 | 0 | 0 | 0 | 0 |
| Format 3: curved hypodermic | 30 | 0 | 0 | 0 | 0 | 0 |

[a]Thirty cells were sorted for each cytometer format.

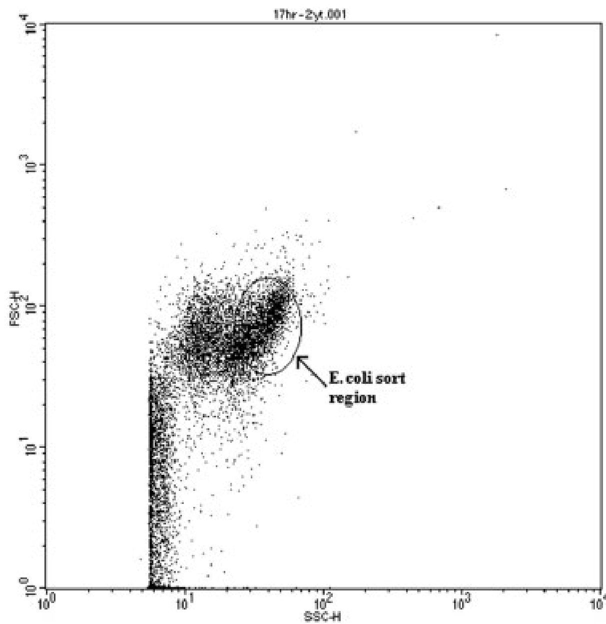


FIG. 3. FACSCalibur scatter plot from the analysis of *Escherichia coli* NCTC 9001 cells, indicating the sort region, and the background scatter obtained with a 0.2-μm filtered PBS sheath fluid. The *E. coli* sort region encloses only a proportion of the viable cell cluster to maximize the distance from the background scatter.

### Within-Batch and Between-Batch Variations

A direct comparison of batch variation was performed to indicate the difference in accuracy between a freeze-dried sphere and preparing manual dilutions of a cell culture. Figure 4 shows the difference between the two methods; the freeze-dried spheres produced a mean recovery of 29.7 cfu with an S.D. of 2.4. The manual dilutions produced a mean of 35.5 cfu with an S.D. of 7. The actual plate counts for the manual dilution method ranged from 22 to 52 cfu, and this range was three times greater than the 24- to 34-cfu range provided by the freeze-dried spheres.

Table 2 lists the amount of between-batch variation produced for five bacterial strains of freeze-dried spheres. The between-batch variation for each organism was no greater than 2 S.D. from the mean.

## DISCUSSION

The aim of this study was to produce a methodology for sorting accurate numbers of bacteria into a single droplet and subsequent preservation by freeze drying. The extensive development and alterations of the sort line of the flow cytometer have allowed this aim to be realized. The reduction of the catcher tube volume by the insertion of a hypodermic tube decreased the volume of sheath fluid exiting the flow cytometer, thus enabling 30 cells to be sorted in two droplets instead of five. This modification to the capture tube reduced the internal volume of the capture tube from 91 μl to 7 μl. The continual curved hypodermic needle used for this purpose was configured in a smooth curve to limit the flow restrictions within the sort line. This allowed the sorted cells to remain in a tight group rather than spread out as they traveled along the line to the dispensing nozzle. The continual curved hypodermic needle enabled the sorted cells to be dispensed in a single droplet.

Cryoprotectants and lyoprotectants such as serum and carbohydrates cause background interference when analyzed through a flow cytometer; by adding a secondary injection line into the droplet dispensing nozzle, a buffered sheath fluid of PBS could be used, and the preservatives for stabilizing and protecting the cells during freezing and freeze drying could be injected after the cell sorting had taken place. The addition of a mixing device also ensured that each sorted cell was exposed to the cryoprotectant within the droplet.

The freeze-dried spheres were easy to use; they could be tipped directly onto the agar surface, so there was no risk of losing bacteria due to handling. The spheres dissolved rapidly on an agar plate with 100 μl of sterile saline solution, and the simple spreading technique decreased the chance of contamination.

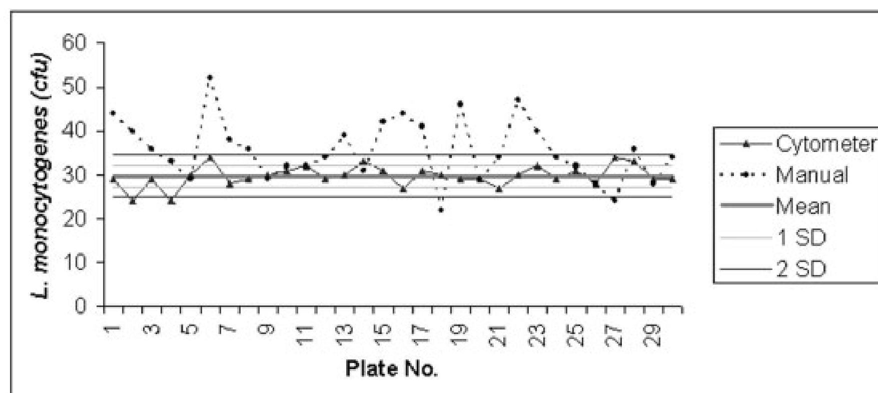


FIG. 4. Within-batch variation between 30 replicate horse blood agar plates (BioMerieux) prepared with a Pasteur pipette droplet of $10^{-6}$dilution of stationary phase *Listeria monocytogenes* cells grown in brain–heart infusion broth, 400 mM MOPS, and 15% sucrose (manual) compared with 30 replicate *L. monocytogenes* cytometer-produced spheres after rehydration onto horse blood agar (cytometer).

Table 2
*Recovery Data Showing Mean Colony Counts From Five Different Bacteria in Six Separate Batches and the Variation Between Batches*

| | Batch number | | | | | | Between batches | |
|---|---|---|---|---|---|---|---|---|
| Organism | 1 | 2 | 3 | 4 | 5 | 6 | Mean | S.D. |
| *Escherichia coli* NCTC 9001 | 29.7 | 32.4 | 32.4 | 33.2 | 31.8 | 28 | 31.3 | 2.0 |
| *Listeria monocyogenes* NCTC 11994 | 32.2 | 30.7 | 28.8 | | | | 30.6 | 1.7 |
| *Listeria innocua* ATCC 33090 | 30.1 | 29.0 | 29.6 | | | | 29.6 | 0.6 |
| *Bacillus subtilis* ATCC 6633 | 31.3 | 29.7 | 31.0 | 34.5 | 32.8 | 29.1 | 31.4 | 2.0 |
| *Bacillus cereus* ATCC 10876 | 31.2 | 29.6 | 29.4 | 29.3 | | | 29.9 | 0.9 |

Combining the precision of flow cytometric analysis and sorting with freeze drying provides an accurate reference quality control that can be reproducibly produced without batch-to-batch variation. Broth cultures set up with the strictest protocols did not produce the same counts of cells each time. By analyzing and sorting a culture with a flow cytometer, an accurate number of cells can be obtained with every batch, and multiple batches could be produced with previously unattainable levels of uniformity.

This sorting technology enables harmonization across the microbiology industry. Laboratories across the globe can use a precise reference material to test their processes and directly compare their results with those of any other laboratory using the same system. Recovery data for method validation or medium quality control, for example, can now be subject to less than 2 S.D. rather than an unmeasured amount of variation. The ability to harmonize analytical results has already been observed with the widespread use of flow-dispensed reference standards applied to the analysis of potable water for the human pathogens *Cryptosporidium* and *Giardia* (12). Regulatory bodies around the world now recommend that the quality control of this testing be carried out with precision reference materials (1); the same precision can now be applied to bacterial reference materials.

The ability to mechanically dispense a precise number of bacterial cells into a single droplet is a significant advance on the level of precision applied in microbiology today. Precise numbers of living cells can now be accurately dispensed into a 25-μl droplet of fluid that can be frozen, freeze dried, and then manipulated without losing any cells. The cytometer is simple to operate and does not need regular alignment or calibration.

The ability to count precise numbers of cells could also be applied to produce quantitative controls for polymerase chain reaction. Inserting a single specific DNA sequence into a bacterial genome and dispensing accurate numbers of these cells would provide a quantitative amount of the specific sequence of interest, e.g., 30. This low sequence copy number could provide a reference quality control for measuring inhibition during polymerase chain reaction amplification, thus enabling accurate quantification. This is especially important when small amounts of DNA need to be detected, e.g., detection of small quantities of genetically modified DNA.

## LITERATURE CITED


1. Drinking Water Inspectorate. Standard operating protocol for the monitoring of *Cryptosporidium* oocysts in treated water supplies to satisfy the water supply (water quality) regulations 2000, SI no. 3184 England. Part 2—laboratory and analytical procedures, 10 June 2003. London; Drinking Water Inspectorate. 2003.
2. Lattuada CP, Chiu JY, Dillard LH, Eye JG, Krumm GW. Quality control, USDA/FSIS microbiology laboratory guidebook. 3rd ed. Washington, DC: US Government Printing Office; 1998.
3. In't Veld PH. The use of reference materials in quality assurance programmes in food microbiology laboratories. Int J Food Microbiol 1998;45:35–41.
4. Piton C, Grappin R. A model for statistical evaluation of precision parameters of microbiological methods. J Assoc Off Anal Chem 1991; 74:92–103.
5. Nebe-von-Caron G, Stephens P, Hewitt C, Powell J, Badley R. Analysis of bacterial function by multi-colour fluorescence flow cytometry and single cell sorting. J Microsc Methods 2000;42:97–114.
6. Porter J, Edwards C, Morgan JAW, Pickup RW. Rapid, automated separation of specific bacteria from lake water and sewage by flow cytometry and cell sorting. Appl Environ Microbiol 1993;59:3327–3333.
7. Vesey G, Narai J, Ashbolt N, Williams KW, Veal D. Detection of specific microorganisms in environmental samples using flow cytometry. In: Darzynkiewicz Z, Robinson JP, Crissman HA, editors. Methods in cell biology. Volume 42: flow cytometry. 2nd ed. New York: Academic Press; 1994. p 490–519.
8. North HL. Method and apparatus for sorting particles with a moving catcher tube. US patent 5,030,002; 1991.
9. Shapiro HM. Practical flow cytometry. 3rd ed. New York: Wiley-Liss; 1995.
10. Reynolds DT, Slade RB, Sykes NJ, Jonas A, Fricker CR. Detection of *Cryptosporidium* oocysts in water: techniques for generating precise recovery data. J Appl Microbiol 1999;87:804–813.
11. Warnecke M, Weir C, Vesey G. Evaluation of an internal positive control for *Cryptosporidium* and *Giardia* testing in water samples. Lett Appl Microbiol 2003;37:244–248.
12. Vesey G, Hutton PE, Champion AC, Ashbolt NJ, Williams KL, Warton A, Veal DA. Application of flow cytometric methods for the routine detection of *Cryptosporidium* and *Giardia* in water. Cytometry 1994;1:1–6.