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Standard Practice for Characterization of *Bacillus* Spore Suspensions for Reference Materials[1]

This standard is issued under the fixed designation E2800; the number immediately following the designation indicates the year of original adoption or, in the case of revision, the year of last revision. A number in parentheses indicates the year of last reapproval. A superscript epsilon (ε) indicates an editorial change since the last revision or reapproval.

INTRODUCTION

Bacillus spp. are aerobic, rod-shaped, Gram positive bacteria that produce endospores under nutrient limiting conditions. The endospores are designed to persist in extreme environments and consequently are highly resistant to inactivation by heat, chemicals and irradiation. A few species of *Bacillus* are medically important because of their impact on human and animal health while others have important agricultural and industrial applications. Measurement of viable *Bacillus* spores present in a suspension can be performed using classical microbiology techniques, such as growth on nutrient medium. The spore suspension is diluted, an aliquot spread on solid nutrient medium, incubated at an appropriate temperature, and the resulting colonies counted. The selection of the type of growth medium and incubation temperature for the optimal growth of a particular *Bacillus* species should be determined by consultation of relevant literature or by comparison of different growth media and incubation temperatures.

Bacillus spore reference materials have many important applications in agriculture, basic research, medical diagnosis, detector validation, and sterility testing. Uniform methods for the characterization of spores will improve the comparison of different lots of materials and results between different laboratories.

1. Scope

1.1 This practice is focused on two basic measurements to characterize *Bacillus* reference materials, the enumeration of spores using growth of colonies on nutrient media and using phase contrast microscopy to determine spore quality and homogeneity. Additional information on advanced methods for characterization is provided in Appendix X1.

1.2 This document will provide the user with recommendations for measurement methods, and the details and conditions that should be employed to ensure reliable and high-quality data are obtained. The practice will help ensure that results obtained from the characterization are reported in a uniform manner. This will allow others to replicate the measurements and facilitate the comparison of different lots of *Bacillus* spore suspensions used as reference materials. It is important to note that the *Bacillus* species are a heterogeneous group and their specific requirements for growth and sporulation may vary. Users of this practice are encouraged to consult the literature for specific information on the species of *Bacillus* bacteria they are using (**1**).[2]

1.3 This standard practice does not provide guidance for the identification of unknown species of bacteria. The identification of *Bacillus* species has been traditionally based on colony morphology, growth on selective media, and biochemical tests, but more recently nucleic acid technologies have enabled the phylogenetic analysis of this group based on 16S DNA sequence similarities (**1**).

1.4 Some *Bacillus* spp. are pathogenic to humans and animals and the user is advised to adhere to safe laboratory procedures and practices for handling spores from these species (**2**).This standard does not purport to address all of the safety concerns, if any, associated with its use. It is the responsibility of the user of this standard to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use (**2**).

1.5 This practice assumes a basic knowledge of microbiology and molecular biology and access to the cited references.

[1] This practice is under the jurisdiction of ASTM Committee E54 on Homeland Security Applications and is the direct responsibility of Subcommittee E54.01 on CBRNE Sensors and Detectors.

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[2] The boldface numbers in parentheses refer to a list of references at the end of this standard.

1.6 The values stated in SI units are to be regarded as standard. No other units of measurement are included in this standard.

2. Referenced Documents

2.1 *ASTM Standards:*[3]

D1129 Terminology Relating to Water

D4455 Test Method for Enumeration of Aquatic Bacteria by Epifluorescence Microscopy Counting Procedure

D6974 Practice for Enumeration of Viable Bacteria and Fungi in Liquid Fuels—Filtration and Culture Procedures

E1873 Guide for Detection of Nucleic Acid Sequences by the Polymerase Chain Reaction Technique

E2197 Quantitative Disk Carrier Test Method for Determining Bactericidal, Virucidal, Fungicidal, Mycobactericidal, and Sporicidal Activities of Chemicals

E2414 Test Method for Quantitative Sporicidal Three-Step Method (TSM) to Determine Sporicidal Efficacy of Liquids, Liquid Sprays, and Vapor or Gases on Contaminated Carrier Surfaces

E2458 Practices for Bulk Sample Collection and Swab Sample Collection of Visible Powders Suspected of Being Biothreat Agents from Nonporous Surfaces

2.2 *Standard Methods for the Examination of Water and Wastewater:*[4]

Method 9218 Aerobic Endospores (2007)

Method 9215 Heterotrophic Plate Count (2004)

Environmental Protection Agency Standard Procedure for Enumeration of Bacterial Inocula on Carriers (Carrier Counts) for the Germicidal Spray Products as Disinfectants Test, Disinfectant Towelette Test, and the Tuberculocidal Activity of Disinfectants Test SOP Number: MD-04-05 Date Revised: 01-13-09[5]

2.3 *ISO Standards:*[6]

ISO 4833:2003 Microbiology of food and animal feeding stuffs -- Horizontal method for the enumeration of microorganisms – Colony-count technique at 30 degrees C

ISO 21528-1:2004 Microbiology of food and animal feeding stuffs -- Horizontal methods for the detection and enumeration of Enterobacteriaceae – Part 1: Detection and enumeration by MPN technique with pre-enrichment

2.4 *United States Pharmacopeia Standards:*

USP. 2006 Microbiological Best Laboratory Practices. USP 29 Suppl 2 pp. 3804-3807

USP. 2003 Good Microbiological Laboratory Practices. Pharmacopeial Forum 29(3):842-850.

USP. 2004 Microbiological Best Laboratory Practices. Pharmacopeial Forum 29(3):1713-1721

3. Terminology

3.1 *Definitions:*

3.1.1 *colony forming unit (CFU), n*—units for the number of viable particles present in a solution. A CFU can result from a single viable bacterial cell or from a clump of cells. **(D1129)**

3.1.2 *vortex mixing, v*—applying a tube containing a liquid sample to a special laboratory mixer that establishes a vigorous circular motion in the bottom of the tube.

3.1.2.1 *Discussion*—The circular mixing motion results in a vortex in the tube that ensures the complete suspension of the entire tube contents.

4. Summary of Practice

4.1 *Viable Spore Concentration by Plating on Nutrient Agar*—Plating bacteria on nutrient media is a well-established method for detection of bacteria in water **(3)**. Suspensions of spores are first mixed well by vortex mixing or pipetting up and down vigorously to insure homogeneity of the spore suspension and then serially diluted using an appropriate buffer. Three serial dilutions are prepared from a spore reference sample. An aliquot of the diluted spore suspension is placed on a nutrient agar plate and spread using aseptic techniques. After incubation, the colonies on the plates are counted and the numbers of viable spores are referred to as colony forming units (CFU). The average number of colonies obtained from the diluted suspension are used to calculate the concentration of the original stock solution and reported as CFU/mL. In order to obtain consistent results, careful attention must be paid to detail to ensure adequate dispersion of spores and avoiding losses during the process.

4.2 *Spore Quality and Homogeneity Determined by Phase Contrast Microscopy*—An inexpensive, rapid and effective method to determine quality and homogeneity of spore preparations. A drop of the spore sample is placed on a microscope slide and covered with a coverslip. The spore preparation is examined using a high power objective (typically 100×) with a phase contrast microscope. Phase bright spores, phase dark spores, phase dark vegetative cells, and spore clumps are counted either manually, using automated counting devices or by digital imaging and computer software techniques **(4)**. The percentage of phase bright spores in the sample is calculated and reported.

5. Significance and Use

5.1 Standard practices for the characterization of spores used as reference materials are important to ensure a uniform basis for testing the performance of detection devices and laboratory instruments. *Bacillus* spore suspensions can be used for a large variety of purposes including testing environmental sampling techniques, inactivation methods, decontamination methods and basic research.

5.2 The practice is intended for both manufacturers and end users of *Bacillus* spore suspensions. The results of the characterization measurements are presented in a report of analysis

[3] For referenced ASTM standards, visit the ASTM website, www.astm.org, or contact ASTM Customer Service at service@astm.org. For *Annual Book of ASTM Standards* volume information, refer to the standard's Document Summary page on the ASTM website.

[4] Available from American Public Health Association, Standard Methods for the Examination of Water and Wastewater, Washington, DC 20001, http://www.standardmethods.org/.

[5] Available from United States Environmental Protection Agency (EPA), Ariel Rios Bldg., 1200 Pennsylvania Ave., NW, Washington, DC 20460, http://www.epa.gov.

[6] Available from International Organization for Standardization (ISO), 1, ch. de la Voie-Creuse, Case postale 56, CH-1211, Geneva 20, Switzerland, http://www.iso.ch.

(ROA). The ROA should provide sufficient detail about the measurement technique to enable the customers to replicate the measurements, allowing them to determine if the properties of the spore suspension changed during shipping and storage.

5.3 The enumeration of the viable spores and determination of homogeneity by microscopic analysis are two basic measurements required for the minimal characterization of reference materials. Phase contrast microscopy does not require staining to distinguish the "phase bright" dormant spores from phase dark spores, dark vegetative cells and clumps. When spores germinate they appear phase dark under phase contrast imaging **(5)**. Germinated spores in a reference sample will soon die due to lack of nutrients. It is important in storing samples to prevent the premature germination of the spores. This standard practice includes the important steps for these measurements and includes guidance for advanced measurements. Additional guidance is given for advanced techniques to characterize spore suspensions that may be used to provide a higher level of characterized *Bacillus* spore reference samples.

5.4 The specific properties of the spores used for their intended application, such as susceptibility to disinfectant processes, should be determined in addition to the basic measurements covered in this practice. Additional information on the measurement of spore properties is located in the appendix.

6. Apparatus

6.1 *Pipettes,* fixed volume or adjustable. The performance of the pipettes should be checked to determine correct dispensing volume. (pipettors should be tested for proper performance frequently).

6.2 *Sterile Pipette Tips.*

6.3 *Vortex Mixer.*

6.4 *Incubator,* capable of maintaining 30 to 70 ± 2°C.

6.5 *Autoclave,* for preparing sterile media and sterilizing waste.

6.6 *Plate Spinner* (optional).

6.7 *Bunsen Burner or Alcohol Lamp* (optional).

6.8 *Sterile Glass or Sterile Plastic Disposable Spreader Rods.*

6.9 *Phase Contrast Microscope*—Low power (10 to 20×) and high power phase (40, 60, or 100×) objectives are preferred.

6.10 *Disposable Plastic (or Glass) Petri Dishes,* typically 100 mm in diameter and 15 mm deep, sterile.

6.11 *Dilution Tubes,* sterile.

6.12 *Glass Microscope Slides,* precleaned, typically 25 by 75 mm.

6.13 *Glass Coverslips.*

6.14 *Immersion Oil,* as recommended by microscope manufacturer suitable for objective used and coverslips.

7. Reagents

7.1 *Purity of Water*—Water used for preparation of solutions should be sterile and high purity; either reverse osmosis, de-ionized or distilled.

7.2 *Phosphate Buffered Saline (PBS)*—A typical composition is composed of 0.137 M NaCl, 0.0027 M KCl, 0.01 M sodium phosphate, pH 7.4. Other similar formulations may be used. The solution should be sterilized by autoclaving or filtration.

7.3 *Triton X100™ Stock Solution* (10 % vol./vol.), prepared in sterile water in a sterile container.

7.4 *Nutrient Agar Plates*—May be prepared in the laboratory or purchased. Plates should be stored and used within expiration date. Typically, laboratory prepared plates are stored at 4°C and used within 2 weeks. Specific media such as 5 % (vol./vol.) sheep blood agar plates may provide important colony morphology that can assist in the conformation of bacteria.

7.5 *Bleach,* freshly diluted, 10 % (vol./vol.). Confirm that the stock solution (commercial bleach, sodium hypochlorite) has not expired.

7.6 *Ethanol,* 70 % (vol./vol.), prepared in sterile water.

8. Hazards

8.1 Considerations for safe handling of spore suspensions. Some *Bacillus* spp. cause disease in human and animals. Prior to using these materials, the user must fully investigate the safety hazards associated with the particular *Bacillus* spp. and follow the appropriate safety guidelines. The correct training of personnel and the proper use of personal protective equipment (PPE) is essential. A good source for the information on laboratory safety is the publication "Biosafety in Microbiological and Biomedical laboratories" **(2)**. Route of infection and infectious dose and toxin production impact potential for infection/toxicity.

8.2 Use of good microbiology practices is important for safely working with the pathogenic *Bacillus* species. International guidelines for microbiological safety should be followed when appropriate **(6)**.

9. Sample Storage

9.1 Appropriate storage conditions are essential to preserve the properties of the spores. Preservation of spore viability (prevention of germination), sterility, and lack of clumping are the goals. Traditionally, spores have been stored in solutions of sterile water, 20 to 70 % (vol./vol.) ethanol or 1 % (wt./vol.) phenol in water at 4°C to prevent bacterial growth. Spores can be frozen, but the effect of freezing and thawing on the properties of the spores has to be determined.

9.2 It is essential to prevent premature germination of spores, clumping, and loss of spores during storage. One study used borosilicate glass vials with PTFE lined caps to store spores in a number of solutions including sterile deionized water, 20 % ethanol and 1 % phenol for periods up to several years at 4°C and found the spore viability for B. anthracis Sterne to be stable for over several years **(7)**.

9.3 It is important that the storage condition for each particular source of spores should be investigated to determine the effect of storage on specific properties and viability. Traditionally spore preparations are considered to be high quality if they contain greater than 90 % phase-bright viable spores as determined in the following sections.

10. Procedure

10.1 *Spore Viability and Concentration by Growth on Agar-Containing Media:*

10.1.1 Several important factors should be considered when using *Bacillus* spore suspensions, including the selection of the solution used for dilution, the containers used for dilution, and any treatment to reduce potential aggregates (clumps) of spores present in the samples. The choice of dilution media and container should be selected to reduce losses of the spores due to adhesion to the surfaces of the container (and pipette surfaces) during dilution. A suitable solution for dilution of spore suspensions is PBS containing Triton X-100™ (0.05% vol./vol) **(8)**. Other diluents and treatments can be used **(7-10)**, but their suitability for the spores from a particular species of Bacillus bacteria should be determined.

10.1.2 Spore suspensions have to be adequately mixed immediately before dilution and sampling because the spores will settle in the bottom of the tubes. Vortex mixing the tubes for 30 s before removing a sample is an effective step to suspend the spores.

10.1.3 At least three replicates of the serial dilutions should be done on the spore samples. A typical dilution would be to vortex mix the stock suspension (30 s), immediately remove 0.1 mL and add this to 0.9 mL of the diluents in a new tube, resulting in a 10^{-1} dilution. Repeating this process from the 10^{-1} dilution tube results in a 10^{-2} dilution, and so on. It is important to use the dilutions immediately after preparation to prevent loss of spores due to adhesion to the walls of the container. The number of serial dilutions necessary will be dependent upon the concentration of the initial sample. The optimal dilution will be to result in 30 to 300 colonies when spread on a plate (see sections below). New sterile pipettes should be used for each dilution.

10.1.4 The spread plate method is commonly used to enumerate bacteria present in water **(3)**. The diluted sample (as prepared above) is mixed and immediately dispensed (0.05 mL to 0.2 mL) onto the surface of the nutrient plate. It is desirable to have duplicate plates for each diluted sample. Sterile glass rods (L-shaped) or sterile plastic disposable spreaders are used to spread the inoculums evenly over the surface of the agar. A turntable device for spinning the plates speeds the process of spreading the inoculum on the agar surface of the plates. Plates are covered and are not moved until the liquid has been adsorbed. If the liquid is not absorbed, pre-dried plates may be used. The conditions for pre-drying the plates in an incubator overnight or under a sterile hood with the lid ajar should be determined for each type of agar media **(11)**.

10.1.5 After spreading, the covers are replaced and the plates are placed in an incubator at the desired temperature (typically 30 to 37 ± 2°C). The temperature should be measured with a NIST-traceable thermometer.

10.1.6 A good source of the composition and performance of growth media is the Difco™ & BBL™ **(12)**, Manual of Microbiological Culture Media, Remel™ catalog or the ASM Media Manual. Quality control of the growth media should be performed to ensure reliable results **(13)**. The storage condition and shelf life of growth media, as recommended by the manufacturer, should be followed.

10.1.7 The plates are removed from the incubator, typically after 18 h, but the optimal time should be established depending upon the species, temperature and media used. The bacterial colonies are counted either manually, with an electronic counter or imaged with a camera. Only those plates having between 30 and 300 colonies should be used for determining the concentration of the spores.

10.1.8 The colony forming units (CFUs) in the sample are calculated by dividing the actual colonies on a plate by the volume applied to the plate. This result is divided by the dilution factor to calculate CFU/mL. The average CFU values and the standard deviations of the measurement should be reported. An example of the calculation is shown in the appendix.

10.1.9 Low concentrations of spores- It is anticipated that spore suspensions used as reference materials will be sufficiently concentrated for plate count determinations or visualization by phase contrast microscopy. However, with low concentrations of spores, samples can be concentrated by either centrifugation or filtration as used for detection of spores in water systems **(14)**.

10.1.9.1 A short centrifugation, for example 12 000 × g for 5 min can be used. The spores can then be suspended in a smaller volume. Care must be taken to ensure this step has not resulted in losses or in the formation of spore clumps. Avoid excessive centrifugation or heating of samples during this step.

10.1.9.2 A large volume of spores can be concentrated on filters that can be directly placed on nutrient agar plates for enumeration of viable spores, such as the Standard Methods for the Examination of Water and Wastewater **(14)**.

10.1.10 Controls should be included to ensure the plates, spreaders, pipettes and solutions are not contaminated. Comparison of colony morphology on the plates to known spore samples can be a valuable indicator of possible contamination. Blank control samples (buffers without any added spores) are spread on agar plates and treated in the same manner as the other samples. The presence of colonies on the blank controls indicates the presence of contamination or poor aseptic technique. Sterilized solutions and materials should be used to avoid contamination.

10.1.11 It is important to prevent carry-over between different samples and dilutions. The spreading rods must either be single use (that is, sterile disposable plastic rods) or glass rods that can be sterilized between dilutions. Glass rods can be dipped in solutions of freshly diluted bleach (10 % vol/vol) for at least 10 min to inactivate any spores. The glass rods should then be rinsed in sterile water followed a rinse in 70 % (vol./vol.) ethanol followed by flaming using an alcohol lamp or gas burner. It is not necessary to change spreaders in between dilutions of the same sample as long as the plates are ordered from most dilute to least dilute when spreading. Glass

rods should be used for spread plates because bleach can cause corrosion of metal rods.

10.2 *Phase Contrast Microscopic Examination to Determine Homogeneity:*

10.2.1 Phase contrast microscopy requires special objectives and illumination. The specific directions for the microscope used should be followed to ensure that the optics and illumination system are correctly operating.

10.2.2 The spore suspension or a dilution is thoroughly mixed (vortex mixing for 30 s) and a drop (typically 0.01 mL) is removed and placed on a clean glass microscope slide. A cover slip is gently applied to the drop to evenly distribute the suspension under the coverslip. A pair of forceps can be used to aid in this process. The amount of solution used should form a thin layer covering most of the area under the coverslip. The correct amount of liquid will result in no liquid coming out from under the sides of the coverslip. Avoid pressure on the coverslip. Waiting a few minutes before applying the coverslip may help spores adhere to the glass slide.

10.2.3 If it is necessary to preserve the sample for future reference, the spores can be immobilized on the slide by embedding a sample of the spores in a thin layer of molten agar between the glass slide and the coverslip (**15**, **16**). Slides prepared this way can be sealed along the edges of the coverslip.

10.2.4 High magnification (typically 40 to 100× objectives) is required to visualize the spores in sufficient detail for identification. If an oil immersion objective is used, a drop of immersion oil is applied to the cover slip before the objective is moved into place. Care should be taken with wet mounts when oil immersion objectives are used to prevent loss of sample through compression of the coverslip.

10.2.5 The number of spores in a field should be between approximately 30 and 300. As a guide, the spores should be diluted to a concentration of at least 10^7 to 10^9 spores per mL for use with a 100× objective. If spores are too numerous to count accurately then a slide should be prepared with a diluted sample. If the number of spores is too low to count, then the spores will have to be concentrated by the methods discussed above.

10.2.6 Viable spores appear bright under phase contrast and vegetative cells and debris appear dark. The focus may need to be adjusted in a field of view to identify all of the spores. Clumps of phase bright spores should also be counted. A minimum of six fields of view should be chosen at random and the phase bright spores, the phase dark spores, the phase dark vegetative cells (including dark debris of similar size) and clumps of spores will be counted. The total number of spores counted per slide should be at least 300 to achieve a statistical validity.

10.2.7 The fraction of phase bright spores, phase dark spores, phase dark vegetative cells, and phase bright spore clumps should be reported as the percentage of total counted. Digital images can be captured from the microscope using a camera and computer software used to analyze the images and document results. A sample digital image of spores is shown in the appendix. A spore preparation that contains greater than 90 % phase bright spores is considered to be high quality.

10.2.8 The electronic images are useful for later reference and comparison to new lots or samples. The means of the values obtained from the slides should be averaged and a standard error of the mean reported along with the number of measurements (for example, N = 3 slides or dilutions).

11. Report

11.1 The report should express the concentration of viable spores as the average CFU/mL, including the standard deviation and the number of dilutions done.

11.2 The following details should be included for the enumeration: the composition of solution used for dilution, composition of growth media, temperature and time of incubation.

11.3 The results of phase contrast microscopy should be reported as the percentage of phase bright spores. The counts of phase bright spores, phase dark spores, phase dark vegetative cells, and clumps of phase bright spores can be recorded as illustrated in the work sheet below. The average of 6 fields should be reported and the standard deviation.

Field Number	Spores Phase Bright	Spores Phase Dark	Vegetative Cells Phase Dark	Clumps	Total	% Phase Bright Spores

12. Keywords

12.1 *Bacillus*; phase contrast microscopy; plate count; spores

APPENDIX

(Nonmandatory Information)

X1. ADVANCED METHODS FOR CHARACTERIZATION

X1.1 *Example of Viable Spore Concentration Calculation and Phase Contrast Image:*

X1.1.1 Table X1.1 shows a sample calculation, for three serial dilutions of a spore sample. For each serial dilution 0.2 mL was spread on to each of two duplicate plates. The colonies were counted after incubation and recorded. The average colonies concentration in CFU/mL was calculated by dividing the average colonies per dilution by the volume spread on the plate then dividing the result by the dilution factor. In the result from the three dilutions are used to calculate the mean and standard deviation. This result would be reported as CFU/mL = 1.5×10^6 (standard deviation 0.2×10^6, N=3).

X1.1.2 Example of phase contrast image of a *Bacillus* spore preparation (see Fig. X1.1). Only spores in focus are counted.

X1.2 *Additional Method to Characterize Spores:*

X1.2.1 Survival of spores in 2.5 N HCl for 5 min has been used to characterize spores (**4**). Vegetative cells and other bacteria do not survive such harsh conditions. The concentrations of viable spores are measured before and after exposure. Although relatively resistant to heat inactivation, *Bacillus* spores vary in their resistance depending upon the species and strain (**17, 18**). Measurement of the concentration of spores before and after heat treatment can be used to determine the quality of spore preparations. The spore concentration should not change after an appropriate heat treatment. The viability of spore preparations that contain a high proportion of germinated spores will decrease after the heat treatment.

X1.2.2 Traditional methods can also be used to stain spores using various dye methods that distinguish between spores and vegetative cells. Two methods commonly used are staining with carbolfuchsin and nigrosin (Dorner method) and Malachite green and safranin (Schaeffer-Fulton method) (**19**).

X1.2.3 Measurement of Genomic Equivalents-DNA. DNA can be extracted from spores and quantified using methods such as Quantitative PCR (QPCR) to calculate genomic equivalents (**7, 20-22**). Spores commonly have DNA associated with their surface that can be detected using sensitive PCR methods, and the extra-cellular DNA fraction is not stable with storage (**7**).

X1.2.4 Dipicolinic acid (DPA) is found in high concentrations of dormant spores, but not in vegetative cells. DPA is released from the interior of spores during germination. DPA release has been used for the detection of spores and as a measurement of heat resistance (**23**). DPA can be measured using absorbance after complexation with iron (**24**) or formation of a highly fluorescent compound by binding the lanthanide ion, Tb^{+3} (**25, 26**).

X1.2.5 Separation of *Bacillus* spores using density gradients is useful for determining the density of reference samples and can also be used for the preparation of pure fractions of spores. The density of wet and dry *Bacillus* spores has been determined by mass methods (**27**). Centrifugation in density gradients using organic solvents has been used for measurement of the dry density of *Bacillus* spores (**28, 29**). Measurement and preparation of wet spores have been done using a variety of density media, including Renografin (or Metrizamide) (**28, 29**), Percoll (**29, 30**), and sucrose.

X1.2.6 The size of *Bacillus* spore species can be measured using a variety of techniques. Size distributions can be rapidly determined using light scattering instruments (**30**). Transmission electron microscopy has been used to measure the dimension of *Bacillus* spores (**31**).

X1.2.7 Hydrophobicity and surface charge measurements of *Bacillus* spores can provide insight into the inter-spore interactions in solution and adhesion to environmental surfaces. Microbial adhesion to hydrocarbon solvents, hydrophobic interaction chromatography, and surface angle measurements have all been used for hydrophobicity estimations of bacteria (**32, 33**). Measurement of the electrophoretic mobility of bacteria can be used to calculate Zeta potential, an important measurement of surface charge of bacteria and spores (**34**).

TABLE X1.1 Sample Calculation for Three Serial Dilutions

Sample-dilution Set	Volume on plate (mL)	Dilution Factor	Colonies -plate 1	Colonies -plate 2	Average Colonies	CFU/mL
A-1	0.2	1.E-04	31	33	32	1.60E+06
A-2	0.2	1.E-04	42	23	32.5	1.63E+06
A-3	0.2	1.E-04	27	21	24	1.20E+06
Mean						1.48E+06
Standard Deviation						2.38E+05

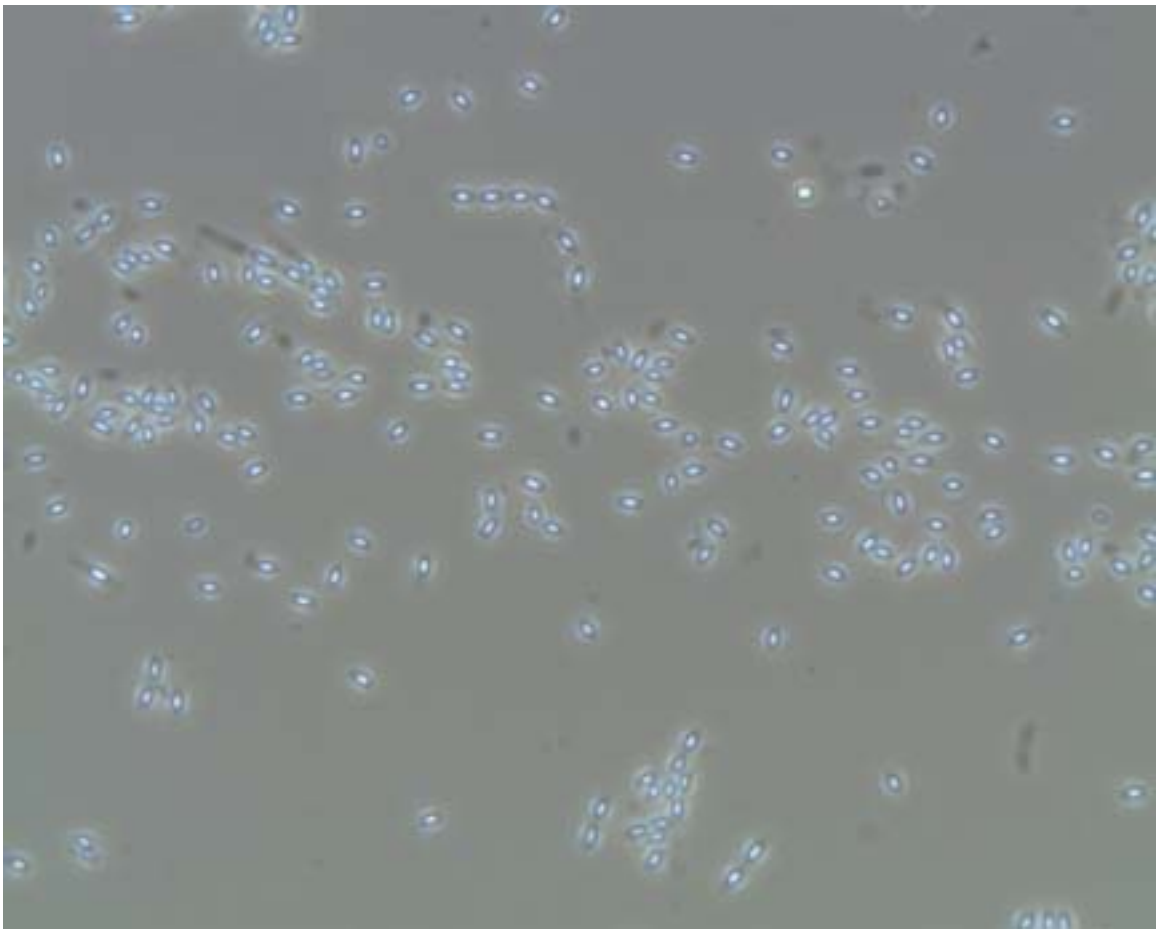

FIG. X1.1 *Bacillus* **Spore Preparation**

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