Designation: E3226 – 19

Standard Test Method for Processing Cellulose Sponge-wipes to Detect *Bacillus anthracis* Spores Sampled from Environmental Surfaces[1]

This standard is issued under the fixed designation E3226; 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.

1. Scope

1.1 This test method covers a standardized method of processing cellulose wipes in a biosafety level 3 (BSL3) laboratory in order to detect and provide a semi-quantitative estimate of *Bacillus anthracis* contamination after sampling of non-porous surfaces. Sampling may be conducted to characterize the extent of the contamination, or for area clearance after decontamination.

1.2 The laboratory procedures should be performed in a BSL3 laboratory by those trained for BSL3 microbiological techniques.

1.3 This test method is specific to *B. anthracis*, but could be adapted for use with other organisms.

1.4 The interlaboratory study was conducted with cellulose sponge wipes pre-moistened with neutralizing buffer. All reproducibility, sensitivity, and specificity data are based on the performance of these wipes. A review was conducted by subcommittee in 2019, and re-confirmed these ILS data are valid.

1.5 *Units*—The values stated in SI units are to be regarded as standard. The values given in parentheses after SI units are provided for information only and are not considered standard.

1.6 *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, health, and environmental practices and determine the applicability of regulatory limitations prior to use.*

1.7 *This international standard was developed in accordance with internationally recognized principles on standardization established in the Decision on Principles for the Development of International Standards, Guides and Recommendations issued by the World Trade Organization Technical Barriers to Trade (TBT) Committee.*

2. Referenced Documents

2.1 *ASTM Standards:*[2]

E177 Practice for Use of the Terms Precision and Bias in ASTM Test Methods

E691 Practice for Conducting an Interlaboratory Study to Determine the Precision of a Test Method

E2756 Terminology Relating to Antimicrobial and Antiviral Agents

3. Terminology

3.1 For definitions of general terms used in this test method, refer to E2756 Terminology Relating to Antimicrobial and Antiviral Agents

3.2 *Definitions:*

3.2.1 *accuracy, n*—the closeness of the agreement between the result of a measurement and a true value of the quantity that is being measured.

3.2.2 *eluate, n*—an eluent, which may contain the recovered organism (s).

3.3 *Definitions of Terms Specific to This Standard:*

3.3.1 *eluent, n*—any solution that is harmless to the organism(s) recovered from a surface, and that is added to a wipe to recover the organism(s) from it.

3.3.2 *laboratory response network (LRN), n*—a national network of local, state and federal public health, food testing, veterinary diagnostic, and environmental testing laboratories that provide the laboratory infrastructure and capacity to respond to biological and chemical terrorism, and other public health emergencies.

3.3.3 *non-porous, adj*—describes a surface that is resistant to absorption of liquid.

3.3.4 *quality control (QC), v*—the operational techniques and the activities, which sustain a quality of material, product, system, or service that will satisfy given needs; also the use of such techniques and activities.

[1] This test method is under the jurisdiction of ASTM Committee E35 on Pesticides, Antimicrobials, and Alternative Control Agents and is the direct responsibility of Subcommittee E35.15 on Antimicrobial Agents.

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[2] 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.

3.3.4.1 *Discussion*—The quality control techniques and activities are to ensure the precision of the method, and identify if any cross-contamination occurs in the field or in the laboratory.

3.3.5 *wipe, n*—a sponge, gauze or cloth that is used to sample a surface for the purpose of collecting organisms from that surface.

3.4 *Abbreviations:*

BSC = Biosafety Cabinet
BSL3 = Biosafety Level three
CDC = Centers for Disease Control and Prevention, Dept. of Health and Human Services, United States Federal Agency
CFU = colony forming units
LRN = laboratory response network
MCE = mixed cellulose esters
PBS = phosphate buffered saline
PBST = phosphate buffered saline containing 0.02 % polysorbate 80
PCR = polymerase chain reaction
TNTC = too numerous to count
TSA II = trypticase soy agar with 5% sheep blood
TSB = trypticase soy broth

4. Summary of Test Method

4.1 The sampling test method is based on traditional culture methods, since determining the presence of culturable organisms is important in an environmental investigation.

4.1.1 This test method describes the elution of spores from a wipe, and the culturing for isolation of colonies.

4.1.2 Real time polymerase chain reaction (PCR) confirmation of colonies and/or presumptive identification of *B. anthracis* in eluate and broth can only be performed at a laboratory response network (LRN) affiliated laboratory, using LRN validated reagents and methods, and therefore communication with a local LRN laboratory is essential.

4.2 Spores are eluted from the wipe using a circulating stomacher while submerged in phosphate buffered saline (PBS) containing a surfactant.

4.2.1 An aliquot of the eluate is diluted in series and spread onto agar plates, another aliquot is filtered and the filter placed onto agar plates.

4.2.2 The wipe and the remaining eluate is placed into broth to maximize the detection of low numbers of *B. anthracis* spores.

4.2.3 An aliquot of the eluate is analyzed by real-time PCR, to provide rapid, yet only presumptive results.

4.3 Following overnight incubation, suspect *B. anthracis* colonies on agar plates are identified by colony morphology and confirmed by a *B. anthracis* real-time PCR assay.

4.3.1 If no growth is observed on the plates, the overnight growth in the broth is sub-cultured to plates and presumptive colonies tested using real-time PCR.

4.3.2 If no suspect colonies are found on any agar plates, broth cultures are analyzed via real time-PCR to presumptively identify the presence *B. anthracis* below the quantification limit.

4.4 A semi-quantitative estimate of the amount of contamination is derived based upon growth on the plates, on the filter or in the broth.

4.5 See Annex A1 for work flow diagram.

5. Significance and Use

5.1 This procedure describes a standardized method of processing cellulose wipes in a biosafety level 3 laboratory in order to detect and provide a semi-quantitative estimate of *B. anthracis* contamination after sampling of non-porous surfaces. Sampling may be conducted to characterize the extent of contamination or for clearance of an area after decontamination.

6. General Equipment and Labware

6.1 Biological safety cabinet (BSC),

6.2 Seward Stomacher 400 Circulator[3]

6.3 Seward Stomacher closure bags,

6.4 Centrifuge with rotors and sealable centrifuge buckets to hold 50 mL conical tubes,

6.5 40 kHz Ultrasonic bath,

6.6 Vortex mixer,

6.7 Pipettors for 1 mL and 100 µL,

6.8 Automatic pipettors for 5mL, 25 mL pipettes and 50 mL pipettes,

6.9 Vacuum filtration manifold,

6.10 Vacuum pump or vacuum line with vacuum gauge, and

6.11 Incubator set to appropriate temperature for target organism (35-37 °C for *B. anthracis*).

7. Reagents and Materials[4]

7.1 Laboratory marking pen.

7.2 Disposable surgical gown.

7.3 Disposable gloves.

7.4 Sterile disposable 10 µL loops.

7.5 Sterile Cell spreaders.

7.6 Laboratory tissue wipes.

7.7 Sterile screw-capped microcentrifuge tubes.

7.8 Sterile, plastic, screw-cap 50 mL centrifuge tubes.

7.9 Racks for 50 ml centrifuge tubes.

[3] This instrument was used in the multi-lab study to determine the performance of the method. It is the only stomacher known to this committee at the time of method development that performs circulation as well as stomaching of the sample. If similar products are known and are commercially available, please inform ASTM committee E35.15.

[4] *ACS Reagent Chemicals, Specifications and Procedures for Reagents and Standard-Grade Reference Materials*, American Chemical Society, Washington, DC. For suggestions on the testing of reagents not listed by the American Chemical Society, see *Analar Standards for Laboratory Chemicals*, BDH Ltd., Poole, Dorset, U.K., and the *United States Pharmacopeia and National Formulary*, U.S. Pharmacopeial Convention, Inc. (USPC), Rockville, MD.

7.10 Sterile pipette tips with aerosol filter for 1 mL and 100 µL pipettors.

7.11 Sterile disposable 5mL, 25 mL and 50 mL pipettes.

7.12 Sterile forceps.

7.13 Disposable filter funnel containing 47 mm diameter, 0.45 µm mixed cellulose esters (MCE) membrane.

7.14 Sterile Specimen Cups (4.5 oz).

7.15 Specimen cup rack.

7.16 Sterile phosphate buffered saline with 0.02% polysorbate 80 (PBST)—Prepare by, dissolving 8 g NaCl, 0.2 g KCl , 1.44 g Na2HPO4, and 0.24 g of KH2PO4, and 2 mL polysorbate 80 in deionized water. Adjust pH to 7.4 ± 0.2 with 0.1 N NaOH or 0.1 N HCl and bring to volume to 1 L with deionized water. Autoclave for 20 min at 121 °C.

7.17 9 mL serial dilution tubes containing PBST.

7.18 Trypticase soy agar + 5% sheep blood plates (TSA II), prepare TSA by adding 40 g powdered media to 1L deionized water, autoclave at 121 °C for 15 min. Cool to 50 °C and add 50 mL sterile defibrinated sheep blood. Can also be purchased commercially.

7.19 Trypticase Soy broth (TSB), prepared according to manufacturer's directions, or purchased commercially.

7.20 Disposable plastic 1 L beakers containing 200 mL 1:10 dilution of standard household bleach (final concentration 0.525%) for tip and spreader discard.

7.21 Disposable Bleach wipes for clean-up of surfaces after possible exposure to *B. anthracis* spores.

7.21.1 Products proven effective for surface decontamination of *B. anthracis* spores can be found in the document; Surface Decontamination Methodologies for a Wide-area *B. anthracis* Incident (**1**).[5]

7.22 Absorbent workbench pads.

7.23 Wipe with 10 mL neutralizing Buffer (3M Sponge Stick; catalog # SSL10NB or unused wipe of the type accepted for processing, for internal process control).

8. Hazards

8.1 These procedures should be performed in a BSL3 laboratory. Refer to Procedure for Laboratory Safety and Decontamination and the Biosafety in Microbiological and Biomedical Laboratories (BMBL) (**2**).

9. Sampling, Test Specimens, and Test Units

9.1 *Acceptable sample types*—Wipes of non-porous environmental surfaces suspected of contamination with *B. anthracis* spores.

9.1.1 Efficiency data based on use of a cellulose sponge-wipe on a stick pre-moistened with a neutralizing buffer (such as Sponge-Stick[6] with neutralizing buffer, 3M; catalog #SSL-10NB).

NOTE 1—The performance characteristics of this procedure are based on the 3M cellulose sponge-wipe. If another wipe is used, the efficiency may not be the same and the repeatability limits based on testing using this wipe will not apply (**3**).

9.2 *Sampling area*—smooth non-porous surface of 645 cm^2 (100 $in.^2$).

9.3 *Sampling technique*—refer to Surface Sampling Procedures for *B. anthracis* Spores from Smooth, Non-porous Surfaces (**4**) for sampling procedures.

9.4 *Provide documentation with complete sample description:*

9.4.1 Unique identifier,

9.4.2 Facility sampled,

9.4.3 Specific site within the facility,

9.4.4 Item sampled or surface description, or both,

9.4.5 Surface area sampled,

9.4.6 Date, time and identity of the person collecting the sample,

9.4.7 Name of submitter, and

9.4.8 Sealed container.

9.5 *Rejection criteria:*

9.5.1 Incomplete labeling or documentation, or both.

9.5.2 Compromised primary containment of samples

10. Shipping, Transport, and Storage

10.1 Refer to the following websites for information regarding the shipping of infectious substances and biological agents:

10.1.1 International Air Transport Association: http://www.iata.org/publications/dgr/Pages/index.aspx

10.1.2 Department of Transportation: http://hazmat.dot.gov/

10.1.3 American Society for Microbiology: http://www.asm.org/

10.1.4 American Biological Safety Association: http://www.absa.org/

10.1.5 The laboratory's LRN affiliate may also refer to the LRN website for additional shipping guidance.

10.1.6 If intentional contamination is suspected, initiate chain-of-custody procedures and documentation, if not already initiated.

10.2 *Storage*—Samples shall be stored/maintained in a BSL-3 laboratory. Samples must be stored as shipped at 2-8 °C for up to 48 h after collection, in a secured refrigerator (**5**). When ready to process, all samples must be processed on the same day as other samples in the shipment. Culture plates may be maintained in the laboratory until PCR analysis is completed.

11. Preparation of Apparatus

11.1 Clean the workspace (BSC) by wiping surfaces with 10 % bleach, followed by deionized water, and lastly with 70 % isopropyl alcohol (or equivalent). Wipe with a disposable towel to remove any excess liquid. Place an absorbent pad in the BSC prior to beginning work with samples.

11.2 Assemble equipment in BSC as needed: Stomacher, vortex, filtration manifold, automatic pipettors, racks, etc.

11.3 Assemble extra supplies and reagents near BSC.

11.4 Unpack shipping containers directly into a BSC.

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

[6] Trademarked by 3M.

11.5 Label one Stomacher[3] bag for each wipe and place in a bag rack.

11.6 Label one specimen cup for each wipe sample.

11.7 Label two sterile 50 mL centrifuge tubes for each wipe sample and place in tube rack.

11.8 For each sample, label eleven TSA II plates and 2 dilution tubes (containing 9 mL PBST) with the sample number and dilution factors as seen in Table 1.

12. Quality Control

12.1 Include one Internal Process Control (3M Sponge-Stick with 10 mL neutralizing Buffer, or unused wipe of the type used for sampling) along with each set of samples processed, preferably at least one for every 10 samples (10 %). If samples are batched by area sampled, include an internal process control with each batch, even if the number of samples is less than 10.

12.1.1 This process control will consist of placing a clean, unused 3M sponge-stick (or wipe of the same type used for sampling) into a specimen cup.

12.1.2 The cup and wipe will be processed the same way as the wipe samples. Running this process control is a check for cross contamination in the laboratory.

12.2 *Field blank*—provide a blank (clean unused) wipe alongside samples that were collected from the field.

12.2.1 This field blank shall be processed the same as the unknown samples to check for cross contamination during the collection process.

12.2.2 If no field blank is included, proceed without including this control.

12.3 *Positive control*—a positive control is not required for this method, as introducing *B. anthracis* spores into the laboratory is considered a hazard.

13. Procedure

13.1 *Dislodge spores from the sample wipes:—*

13.1.1 Put on gloves and disposable protective clothing (BSL-3 PPE). All subsequent procedures involving manipulation of wipes or spore suspensions must be carried out in a BSC.

13.1.2 If plastic handle remnant is present, remove from between folds of sponge. Manipulate the sponge through the bag, tear each side of the sponge away from the plastic (Fig. 1 and Fig. 2).

13.1.3 Aseptically push the sponge to the top of the bag close to the opening and use sterile forceps to transfer the wipe to a Stomacher® bag.

13.1.4 Place the forceps back into their sterility packaging to use in step 13.1.9.

TABLE 1 Sample Number and Dilution Factors

Dilution Tubes (9 mL PBST)	TSA II plates	
	Dilution	Number of Plates
	10^{0}	2
	10^{-1}	3
10^{-1}	10^{-2}	3
10^{-2}	10^{-3}	3

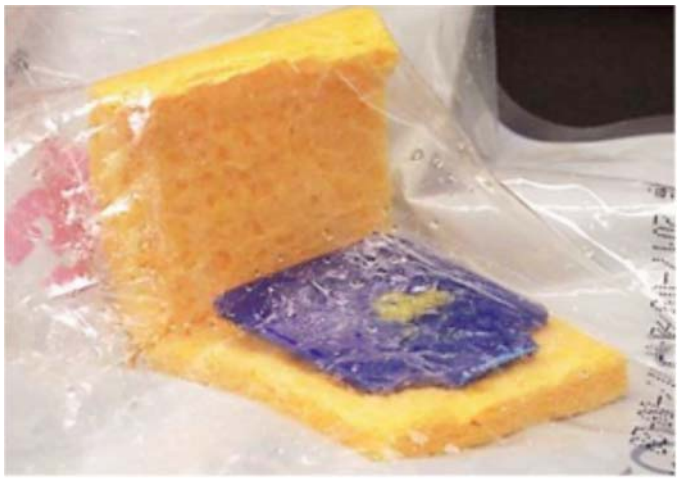


FIG. 1 Sponge Stick Head in Transport Bag, with Plastic Handle Remnant Between the Folds of the Sponge

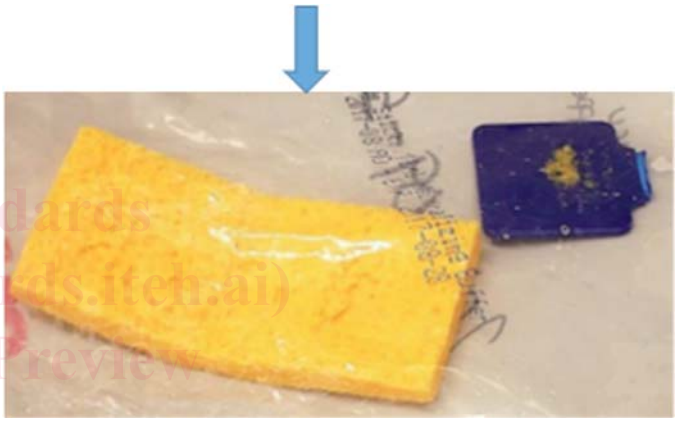


FIG. 2 Sponge Stick Head in Transport Bag, with Plastic Handle Remnant Removed from Sponge

13.1.5 Add 90 mL of PBST to each bag that contains a wipe.

13.1.6 Place the bag containing the wipe into the Stomacher[3], with the sponge unfolded, so the wipe rests evenly between the homogenizer paddles.

13.1.7 Stomach each wipe for 1 min at 260 RPM.

13.1.8 Open the door of the Stomacher[3] and remove the bag containing the wipe.

13.1.9 Keeping the bag closed, manipulate the wipe to the top of the bag while using your gloved hands to squeeze excess liquid from the wipe. Open the bag and, using forceps from step 13.1.4, lift the wipe and place it into a labeled specimen cup and set aside for step 13.8.

13.1.10 Repeat steps 13.1.2 through 13.1.9 for all samples, changing gloves and forceps between samples.

13.1.11 Allow bags to sit for 10 min to allow elution suspension foam to settle.

13.2 *Concentrate wipe elution suspension:*

13.2.1 Gently mix elution suspension up and down with a 50 mL pipette three times.

13.2.2 Split elution suspension volume equally, remove half of the suspension volume (~45 mL) with a sterile 50 mL pipette

and place it in a 50 mL screw capped centrifuge tube. Place remaining suspension (~45 mL) into a second 50 mL tube.

13.2.3 Repeat steps 13.2.1 through 13.2.2 for all samples.

13.2.4 *In BSC*—Place tubes into sealing centrifuge buckets.

13.2.5 Decontaminate sealed centrifuge buckets by wiping with 1:10 bleach before removing from the BSC.

13.2.6 Centrifuge tubes at 3500 × g for 15 min. Do not use the brake option on the centrifuge to slow the rotor, as re-suspension of pellet may occur.

13.2.7 Remove supernatant from each tube with a 50 mL pipette and discard the supernatant, to leave approximately 3 mL in each tube. The pellet may be easily disturbed and not visible, so place pipette tip away from the tube bottom.

13.3 *Vortex and sonicate tubes, measure volume:*

13.3.1 Set vortex mixer to high intensity level and touch activation.

13.3.2 Turn on sonicating water bath.

13.3.3 Vortex tubes for 30 s.

13.3.4 Transfer tubes to ultrasonic bath and sonicate for 30 s.

13.3.5 Repeat vortex and sonication cycles two additional times.

13.3.6 Remove suspension (approximately 3 mL) from one tube with a sterile 5 mL pipette and place it in the other tube of the same sample.

13.3.7 Measure final volume (approximately 6 mL) of suspension with 10 mL pipette and record on tube and data sheet (see Appendix X1).

13.4 Perform serial dilution of the spore elution suspension in 9 mL PBST.

13.4.1 Vortex elution suspension on high for 30 s.

13.4.2 Remove 1 mL of spore elution suspension (10^0) and place in one tube (9 mL) of PBST. This is the 10^{-1} suspension. Recap the PBST tube and vortex on high for 30 s.

13.4.3 Open cap of the 10^{-1} suspension and remove 1 mL of this suspension and place in a new 9 mL tube of PBST. This is the 10^{-2} suspension. Recap the PBST tube and vortex on high for 30 s.

13.4.4 This will result in three spore suspensions: the initial wipe elution suspension (no dilution=10^0) and two serial dilutions of this suspension in PBST (10^{-1} and 10^{-2}).

13.4.5 Repeat steps (13.4.1) through (13.4.3) for all samples.

13.5 Culture diluted spore suspensions on TSA II: See Annex A2 for a diagram of the plating described below.

13.5.1 After vortexing tubes well, remove 100 µL from the 10^{-2} suspension with the P100 pipette and place on to a plate of TSA II labeled 10^{-3}.

NOTE 2—The plating of 100 µL is an additional 1:10 dilution of the 10^{-2} suspension resulting in a 10^{-3} dilution on the plate. Repeat two more times for a total of three inoculated plates.

13.5.2 Spread the inoculum on each of the three 10^{-3}-labeled TSA II plates with one cell spreader. Discard spreader.

13.5.3 After vortexing tubes well, remove 100 µL from the 10^{-1} suspension with the P100 pipette and place on to a plate of TSA II labeled 10^{-2}.

NOTE 3—The plating of 100 µL is an additional 1:10 dilution of the 10-1 suspension resulting in a 10^{-2} dilution on the plate. Repeat 2 more times for a total of three inoculated plates.

13.5.4 Spread the inoculum on each of the three 10^{-2}-labeled TSA II plates with one cell spreader. Discard spreader.

13.5.5 After vortexing tubes well, remove 100 µL from the initial wipe elution suspension (10^0) with the P100 pipette and place on to a plate of TSA II labeled 10-1.

NOTE 4—The plating of 100 µL is an additional 1:10 dilution of the initial swab elution suspension (10^0) resulting in a 10^{-1} dilution on the plate. Repeat 2 more times for a total of three inoculated plates.

13.5.6 Spread the inoculum on each of the three 10^{-1}-labeled TSA II plates with one cell spreader. Discard spreader.

13.5.7 Place all plates in an incubator set at 36 °C ± 1 for 18-24 h.

13.6 Rapid screening for detection of presumptive *B. anthracis* in sample

NOTE 5—Real-time PCR must be performed at a LRN laboratory utilizing LRN standard procedure "Rapid Preparation of Cell Lysates from Culture-grown Bacteria for Subsequent Testing by Real-time PCR Assay" and "Detection of Bacillus anthracis DNA."

13.6.1 Remove 200 µL of undiluted eluate (10^0) and place in a pre-labeled tube.

13.6.2 Send to LRN laboratory or perform DNA extraction using a LRN-approved extraction method and send extract to LRN laboratory for real time PCR following the current LRN-approved PCR procedure.

NOTE 6—Culture procedures are priority and must be initiated prior to testing this sample by PCR. The result of PCR testing of undiluted eluate is presumptive. A negative PCR result at this stage of testing does not ensure absence of viable *B. anthracis* spores. A positive PCR result indicates the presence of *B. anthracis* DNA but does not determine viability

13.7 Capture spores on filter funnel membranes and culture the membrane with spores on TSA II plates.

13.7.1 For each sample, label two TSA II plates with the sample number and 10^0.

13.7.2 Place two 0.45 µm filter funnels on the vacuum manifold.

13.7.3 Moisten filter funnel membranes with 5 mL PBST, open vacuum and vacuum through the filter. All filtering should be done with a vacuum pressure <20 cm Hg.

13.7.4 With the vacuum valve closed, place 10 mL of PBST into each filter cup.

13.7.5 Add 1.0 mL of 10^0 wipe elution suspension from 13.3.7 to each filter cup.

NOTE 7—If volume other than 1.0 mL is used, calculations will need to be adjusted accordingly.

13.7.6 Open valves and vacuum the suspension through the filter.

13.7.7 Rinse the walls of each filter funnel cup with 10 mL of PBST and vacuum through the filter.

13.7.8 Squeeze the walls of the filter funnel cup gently and separate the walls from the base holding the filter. Remove each filter membrane with sterile forceps and place grid-side up on a TSA II plate labeled 10^0. Make sure that the filter is in good contact with the surface of the agar. If an air pocket

occurs under the filter, use the sterile forceps to lift the edge of the filter to release the air pocket for better contact with the agar.

13.7.9 Repeat steps (13.7.1) through (13.7.8) for each sample.

13.8 *Broth Enrichment*—Add the remaining 100 elution suspension from 13.3.7 for each sample to the specimen cup containing the corresponding wipe (from 13.1.9). Add 30 mL TSB to each specimen cup. Repeat for each sample.

13.9 Incubate TSA II plates with filter membranes and TSB specimen cups at 36 ± 1 °C for 18–24 h.

13.10 *Count colony-forming units (CFU):*

13.10.1 Examine the TSA II plates from the serially diluted spore suspensions and the filter membrane for colonies that are consistent with *B. anthracis* colony morphology. Count colony-forming units (CFU) for each suspect *B. anthracis* type.

NOTE 8—Colony characteristics of *B. anthracis* (Refer to Fig. 3 and Fig. 4): After incubation of TSA II plates for 18-24 hours at 36 °C ± 1, colonies of *B. anthracis* are generally 2-5 mm in diameter, but may be smaller in size after exposure to disinfectants or other inhibitors. The flat or slightly convex colonies are irregularly round, with edges that are slightly undulate (irregular, wavy border) and have a ground-glass appearance. Comma-shaped projections from the colony edge may be present. In contrast to colonies of *B. cereus* and *B. thuringiensis*, colonies of *B. anthracis* are not β-hemolytic. For additional colony morphology descriptions and photos, refer to the American Society for Microbiology (ASM) Sentinel Laboratory Procedures for Identification of *Bacillus anthracis* and *B. cereus biovar anthracis* (**6**).

13.10.2 Record the number of colony-forming units (CFU) of each suspect *B. anthracis* colony type (if more than one colony type observed is suspicious) on the supplied data sheet (Fig. X1.1). If the CFU is <250/plate, record actual number. If the CFU is >250/plate, record as "too numerous to count" (TNTC). If no growth of suspect *B. anthracis* colonies record as "None detected." For Real-time PCR confirmation of *B. anthracis* representative colonies, see 13.12.

13.11 *Evaluate the TSB Enrichment:*

13.11.1 If broth is not cloudy after 18-24 h incubation, record as no growth (NG) and incubate for an additional 24 h.

13.11.2 If broth is cloudy, record as positive growth (G+) and proceed to next step.

13.11.3 Hand mix TSB specimen cup with positive growth for 30 s. Remove some broth from the specimen cup with a 10 µL loop and streak on a TSA II plate for isolation. Repeat 2 times for a total of three TSA II isolation plates.

13.11.4 Incubate the isolation plates for 18-24 h at 36 °C ± 1.

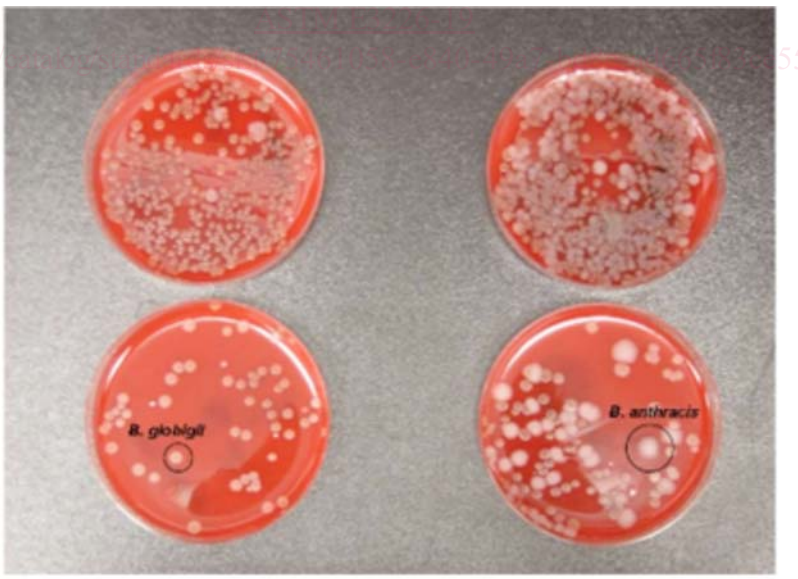
NOTE 9—If presence/absence results are needed quickly, PCR can be performed on cloudy TSB media while waiting for streaks to incubate. Results from PCR of the TSB media are presumptive and require the completion of the entire procedure. Analysis of the TSA II streaks followed by PCR on isolated suspect colonies is necessary to confirm the presence of *B. anthracis* as well as viability.

13.11.5 Examine plates for *B. anthracis*-suspect colonies. If any colonies are isolated, go to 13.12 for PCR confirmation.

13.12 *Real-time PCR Confirmation:*

13.12.1 If the serial dilution plates are countable, designate 2-5 *B. anthracis* representative colonies from the three counted replicate dilution plates to be used for PCR confirmation and go to 13.12.4. Colonies should not be combined but assayed in separate PCR runs.

13.12.2 If serial dilution plates are not countable (<25 CFU/plate), designate 2-5 *B. anthracis* representative colonies



Left side: Example of environmental organisms including *B. atrophaeus* var. globigii
Right side: Example of environmental organisms with *B. anthracis* colony circled

FIG. 3 Colonies of *B. atrophaeus* var. globigii, and *B. anthracis*, and a consortia of environmental organisms on TSA II after 24 hr incubation.