Procedure for the Preparation and Analysis of Spiked Reference and Matrix Samples

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1. SCOPE AND APPLICATION

1.1. The evaluation of Spiked Reference Samples permits the monitoring of initial and ongoing method performance. It is also useful as a demonstration of capability for analysts. Sample matrix spikes make it possible to evaluate the effect of specific matrices on method recovery.

2. SUMMARY OF METHOD

2.1. A serial dilution of an undiluted spiking suspension (USS) of either *Enterococcus faecalis* (*E. faecalis*) or *Escherichia coli* (*E. coli*) is performed in vials of phosphate buffer water until it reaches a 10^{-6} dilution. Each dilution is then plated onto prepared TSA plates using 0.1 mL and 1.0 mL aliquots. Each volume is plated in triplicate. The plates are inverted and incubated for 20 ± 4 hours. At the end of the incubation period, count and record number of colonies per plate. The ideal colony count should be between 30 and 300 colonies for at least one dilution.

2.2. Interferences

2.2.1. TSA is not selective and can support the growth of many types of bacteria. Aseptic techniques must be used to ensure that there is no contamination of the sample via the media, reagents, supplies, or the analyst.

2.2.2. TSA should be tempered to 44-46 °C after melting to prevent it from re-solidifying before it is poured. Once TSA re-solidifies, it is no longer usable and should be disposed of properly.

2.2.3. TSA plates should be prepared 24 hours prior to use. Sample will not be absorbed properly if the media has not sufficiently solidified and bacteria will not grow properly if media has been allowed to over-dry.

2.2.4. Mix contents thoroughly after each inoculation and, again, before plating by vigorously agitating the vials. This breaks up clumps of bacteria and homogenizes the sample.

2.3. Definitions

2.3.1. **TSA -** Tryptic Soy Agar. A non-selective soybean and casein enriched agar medium used for the growth of aerobic and facultative anaerobic bacteria.

2.3.2. ***Enterococcus faecalis* –** Gram-positive cocci bacteria. One of several members of the genus *Enterococcus* that can be found in the feces of humans and other warm-blooded animals. Formerly of the Group D fecal streptococci group.

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2.3.3. ***Escherichia coli* –** Gram-negative, non-spore forming, rod-shaped bacteria. One of several bacteria species known as fecal coliforms. Fecal coliforms are commonly found in the feces of humans and warm-blooded animals.

3. EQUIPMENT AND SUPPLIES

3.1. Sterile gloves, latex or nitrile

3.2. Inoculating loops, sterile

3.3. Glass serological disposable pipettes (1 mL), sterile and individually wrapped, with pipette pump; calibration checked on each new lot per SOP MB-03.3.

3.4. Calibrated pipette (per SOP MB-03.3) with tips, for dispensing 0.1 mL

3.5. Thermo Fisher Scientific Heratherm IMH 60 Model 51028066(S/N 42511812) or Fisher Scientific Isotemp Incubator Model 650F (S/N 303N0034) maintained at 35 ± 0.5 °C

4. REAGENTS AND STANDARDS

4.1. *Enterococcus faecalis* (*E. faecalis*) ATCC #19433, working stock (See SOP MB-03.1.)

4.2. *Escherichia coli* (*E. coli*) ATCC #11775, working stock (See SOP MB-03.1.)

4.3. Dilu-Lok bottle with 99 mL Phosphate Buffer with $MgCl_2$, 5 bottles per bacteria species.

4.4. Tryptic Soy agar (TSA) - Prepare per SOP MB-02.33. Dry plates at room temperature for 24 hours prior to use.

4.4.1. Media can be poured into plates immediately after preparation, or it can be stored in milk dilution bottles in the Biology Walk-in Refrigerator or the Thermo Scientific Isotemp Refrigerator S/N 0162177801170112 located in B212 for up to 3 months and melted just before use. Melted prepared media can be tempered in a water bath at 44-46 °C for up to 3 hours. Appropriately discard any re-solidified media.

4.4.2. Each bottle of prepared media can pour up to 5 plates, including controls.

4.4.3. Number of plates needed per bacteria species: 9 plates for each volume being plated, plus 1 control plate for each bottle of media used.

4.5. Azide Dextrose broth (ADB) – Prepare per SOP MB-02.33.

4.6. Lauryl Tryptose Broth (LTB)—Prepare per SOP MB-02.33.

5. SPIKING SUSPENSION PROCEDURE (See Flowchart 8.1)

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5.1. Undiluted Spiking Suspension (USS)

5.1.1. For *E. faecalis* USS, add 1.0 mL of ADB to a 99 mL phosphate buffer bottle. For *E. coli* USS, add 1.0 mL of LTB to a 99 mL phosphate buffer bottle.

5.1.2. Add a loopful of *E. faecalis* to the ADB phosphate buffer bottle and shake vigorously. Similarly, add a loopful of *E. coli* to the LTB phosphate buffer bottle and shake vigorously.

5.1.3. Incubate at 35 ± 0.5 °C for 20 ± 4 hours.

5.2. Serial dilution of USS

5.2.1. After incubation, shake the USS vigorously and proceed to add 1.0 mL of the USS to a phosphate buffer bottle labeled with the species and the letter "A".

5.2.2. Thoroughly mix dilution "A". Withdraw 1.0 mL of sample "A" and add it to a second bottle of phosphate buffer labeled with the species and the letter "B".

5.2.3. Thoroughly mix dilution "B". Withdraw 11.0 mL of sample "B" and add to a phosphate buffer bottle labeled with the species and the letter "C".

5.2.4. Thoroughly mix dilution "C". Withdraw 11.0 mL of sample "C" and add it to a phosphate buffer bottle labeled with the species and the letter "D".

5.2.5. Thoroughly mix dilution "D".

5.3. Spread plates of spiking suspensions

5.3.1. Shake dilution "B" bottle a minimum of 25 times. Pipet 0.1 mL of the suspension onto a TSA plate labeled with the species and the volume. Use a bent inoculating needle to spread the suspension evenly over the surface, without tearing into the agar. Repeat for two more plates. Make triplicate plates in the same manner for dilutions "C" and "D".

5.3.2. For IPRs, OPRs, and DOCs, also make triplicate plates using 1 mL of the suspension, following the method in 5.3.1.

5.3.3. Allow the suspensions to absorb into the agar. Then, incubate inverted plates at 35 ± 0.5 °C for 20 ± 4 hours.

5.3.4. After incubation, count and record the number of colonies per plate.

5.4. Sample spiking

5.4.1. A Reference Sample is prepared by adding 3.0 mL of dilution "D" to a 99 mL phosphate buffer bottle and mixing thoroughly. Analyze a 5.0- and 10.0-mL volume of the Reference Sample by membrane filtration on appropriate

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media. Analyze 20.0 mL dilutions using the appropriate Quanti-Tray methods.

5.4.2. For initial method performance (IPR), prepare and analyze four Reference Samples.

5.4.3. For ongoing method performance (OPR), prepare and analyze one Reference Sample.

5.4.4. As demonstration of capability (DOC) for analysts, prepare and analyze four Reference Samples.

5.5. Matrix Spike (MS)

5.5.1. To prepare a matrix spike sample, add 3.0 mL of dilution "D" to 100 mL of sample being tested and mix thoroughly. Analyze appropriate volume of spiked sample by membrane filtration or Quanti-Tray using appropriate media.

5.5.2. Analyze the unspiked sample along with the spiked sample to obtain background concentration of sample for MS recovery calculations.

6. RECOVERY AND PRECISION CALCULATIONS

6.1. Concentration of USS (Use TSA plate results from Section 4.3 above.)

$$Enterococci_{USS} = \left(CFU_1 + CFU_2 + \mathsf{K} + CFU_n\right)/\left(V_1 + V_2 + \mathsf{K} + V_n\right)$$

Where,

$Enterococci_{USS}$ = CFU/mL of enterococci in undiluted spiking suspension (USS).

CFU = Use all counts from TSA plates that were between 30-300 CFU per plate.

V = Volume of undiluted sample on each TSA plate that had counts between 30-300 CFU. For example, a TSA plate from the "D" dilution has a volume of 10^{-7} mL.

n = Number of plates with counts within 30-300 CFU.

6.2. Concentration of spiked sample (From Section 4.4 and 4.5.)

$$T_{spiked} = \left(Enterococci_{USS}\right) \times \left(V_{spiked}\right)$$

Where,

T_{spiked} = True concentration (CFU/100mL) of spiked enterococci in sample.

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$Enterococci_{USS}$ = CFU/mL of enterococci in undiluted spiking suspension (USS). (From Section 5.1.)

V_{spiked} = Volume in mL of USS per 100 mL of sample. For example, the Reference Sample in Section 4.4.1 has a USS volume of 3.0 X 10^{-6} mL.

6.3. Percent Recovery of Spiked sample

$$R = 100 \times \frac{(N_S - N_U)}{T}$$

Where,

R = Percent recovery

N_S = Enterococci (CFU/100 mL) result from analysis of the spiked sample.

N_U = Enterococci (CFU/100 mL) result from analysis of the unspiked sample. (For matrix spikes.)

T = True concentration of enterococci in spiked sample. (From Section 5.2.)

6.4. Precision (Used for IPR and DOC)

$$RSD = \frac{SD}{A_R} \times 100$$

Where,

RSD = Precision as relative standard deviation

SD = Standard Deviation

A_R = Mean percent recovery

7. SAFETY/HAZARDOUS WASTE MANAGEMENT

7.1. Always autoclave any expired or unused bacterial cultures for 45 minutes before discarding in the trash.

7.2. Discard any unused melted agar in hazardous waste container. Do not reuse media that has re-solidified.

7.3. Refer to Bureau of Laboratories Health and Safety Plan for safety equipment, use of laboratory hoods, and chemical storage policies.

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7.4. Read MSDS information for reagent handling and disposal.

8. REFERENCES

8.1. EPA Method 1600 (Sept 2014)

8.2. Standard Methods 9020 (2017)

8.3. Standard Methods 9222 (2006)

8.4. NELAC Quality Systems Manual

8.5. Difco™ & BBL™ Manual \\fldep1\DEAR\Labs\Lab5\BenchCommon\Methods

8.6. FDEP Laboratory Quality Manual https://floridadep.gov/dear/florida-dep-laboratory/content/dep-laboratory-quality-assurance-manual-and-sops

8.7. FDEP Laboratory Health and Safety Plan http://publicfiles.dep.state.fl.us/DEAR/DEAR%20Safety/FDEP%20Health%20%20Safety%20Plan%20B%20-%20June%202016.pdf

8.8. DEP SOP MB-02.33, *Preparation of Dehydrated Culture Media*

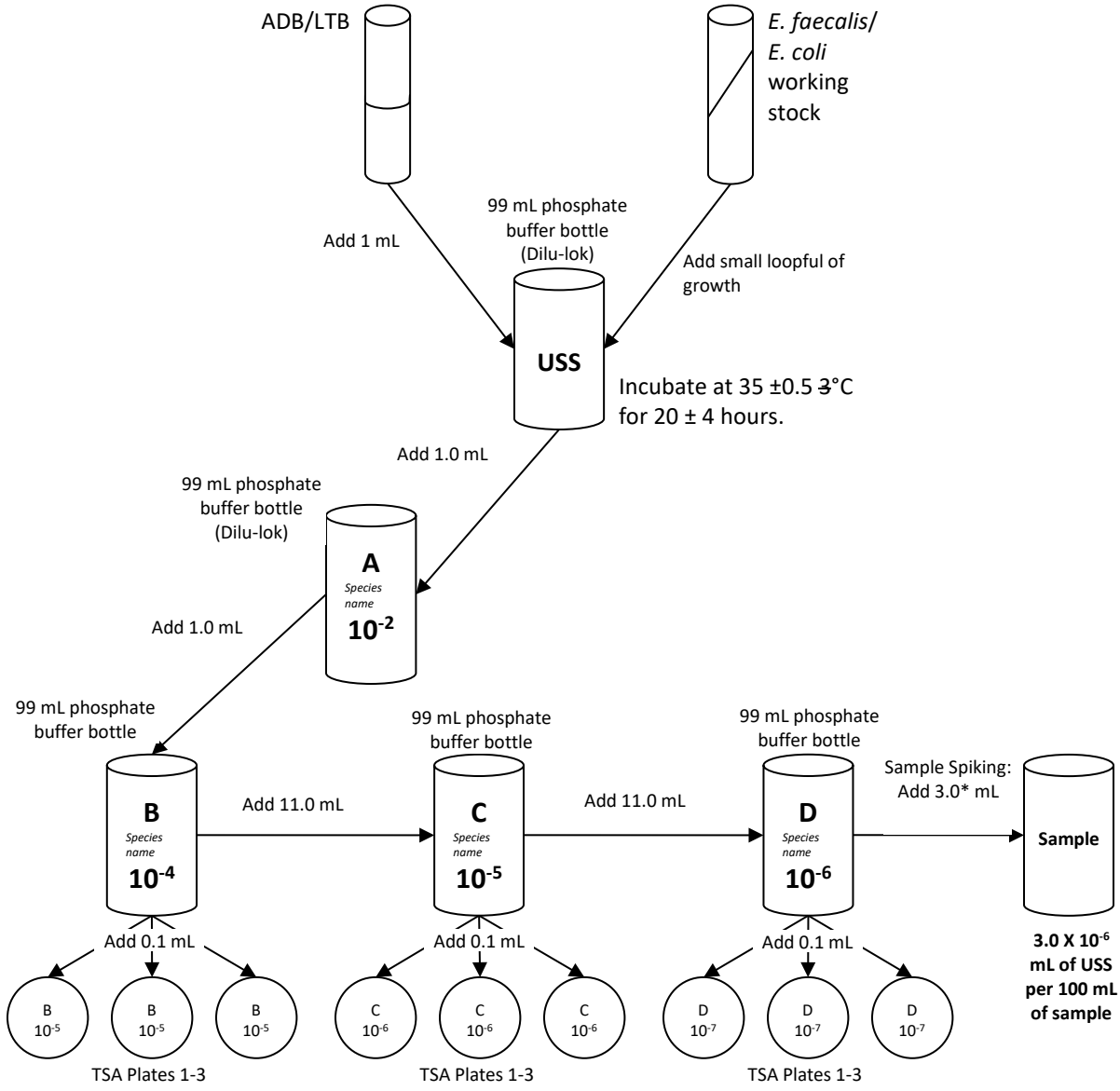
8.9. DEP SOP MB-03.1, *Maintenance of Bacterial Cultures*

8.10. DEP SOP MB-03.3, *Calibration and Use of Mechanical and Disposable Pipettes*

9. TABLES, DIAGRAMS

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Figure 9.1 – Flowchart of Spiking Suspension Procedure



*For analysts' DOCs, we take 0.5 ml of "B" of Enterococcus faecalis (E. faecalis) spike for Enterococcus Quanti-Tray test and 0.5 ml of "C" of E. coli spike for Colilert-18 Quanti-Tray and MF.

Appendix of Changes

02/19/2008 Added SOPs to references section and updated safety and hazardous waste management section.

02/11/2010 Added correct version of referenced method.

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02/02/2011 Updated references to include new versions of Quality Manual and Health and Safety Plan.

01/05/2012 Removed procedure for ADB broth and added reference to procedure in media SOP.

06/20/2016 Added Summary of Method, including Interferences and Definitions. In Equipment and Supplies, included calibration SOPs and Incubation Model and Serial Number. In Reagents and Standards, detailed preparation and use of TSA and included *Escherichia coli*. (Also included *E. coli* throughout the SOP.) In Sub-section Sample Spiking, included Quanti-Tray procedure. Added Difco™ & BBL™ Manual and Pipette Calibration SOP to References.

12/18/2017 Modified sentence structure in the following areas: Summary of Method, Interferences, Undiluted spiking suspension (USS) and Serial dilution of USS. Added one significant figure to volume amounts and added the words Dilu-lok, species name, and Sample Spiking to figure 8.1.

01/03/2019 Updated references to match current versions.

12/19/2019 Added new equipment references used for incubation and storage purposes. Updated Flowchart of Spiking Suspension Procedure for specific DOC tests.

12/17/2020 Updated equipment references for incubators in use. Updated links for Quality Manual and Health and Safety Plan.

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