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

DEPARTMENT OF ENVIRONMENTAL PROTECTION LABORATORY
BIOLOGY PROGRAM
TALLAHASSEE, FLORIDA

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TABLE OF CONTENTS

1. SCOPE AND APPLICATION

2. SUMMARY OF METHOD

2.2. Interferences

2.3. Definitions

3. EQUIPMENT AND SUPPLIES

4. REAGENTS AND STANDARDS

5. SAMPLE PREPARATION PROCEDURE

6. SAMPLE ANALYSIS

7. CALCULATIONS AND PRECISION

8. SAFETY/HAZARDOUS WASTE MANAGEMENT

9. REFERENCES

APPENDIX OF CHANGES.

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1. SCOPE AND APPLICATION

1.1. The analysis of a clean sample matrix, fortified with a known quantity of the target organism from a manufactured standard such as a Quality Control (QC) standard or a Certified Reference Material (CRM), permits the monitoring of initial and ongoing method performance, as long as manufacturer instructions for use are followed. It is also useful as a demonstration of capability for analysts. The laboratory will assess performance for accuracy with the established acceptance criteria in the COA.

2. SUMMARY OF METHOD

2.1. Four spiked reference samples of either *Enterococcus faecalis* (*E. faecalis*), *Escherichia coli* (*E. coli*), or *Klebsiella variicola,* are prepared using vials of phosphate buffer, or Autoclaved DI water, and analyzed according to the respective method.

2.2. Interferences

2.2.1. Temperature of the reference bacteria pellet can affect its activity. Analysis should begin when the capped pellet has acclimated to room temperature (15-30°C).

2.2.2. Time of inoculation can affect bacterial activity. Analyze spiked sample within 30 minutes of reconstitution.

2.2.3. Aseptic techniques must be used to ensure that there is no contamination of the sample via the media, reagents, supplies, or the analyst.

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

2.3. Definitions

2.3.1. ***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.

2.3.2. ***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.

2.3.3. ***Klebsiella variicola* –** Gram-negative, non-spore forming, rod-shaped bacteria. One of several members of the family *Enterobacteriaceae.*

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

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3. EQUIPMENT AND SUPPLIES

3.1. 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. Plastic serological disposable pipettes (50 mL) sterile and individually wrapped, with pipette pump; calibration checked on each new lot per SOP MB-03.3.

3.5. Glass serological disposable pipettes (10 mL) sterile and individually wrapped, with pipette pump; calibration checked on each new lot per SOP MB-03.3.

3.6. 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.

3.7. Water baths TSWB 4 (S/N 300471596) and TSWB 5 (S/N 300472033) maintained at 35.0 ± 0.5 °C. Water baths TSWB 11 (S/N 300483022) and TSWB 7 (S/N 300483022) maintained at 41.0 ± 0.5 °C, and TSWB 8 (S/N 300536247) and TSWB 9 (S/N 300561398) maintained at 44.5 ±0.2 °C.

3.8. Colilert-18 snap pack media (for the detection of *E. coli* and *T. coli*)

3.9. Enterolert snap pack media (for the detection of *Enterococci*)

3.10. IDEXX Shrink-Banded Vessels without Sodium Thiosulfate

3.11. Antifoam dropper bottle

3.12. Quanti-Tray/2000 trays

3.13. UV light 365 nm, 6 watts or IDEXX UV Viewer Plus (S/N UVP1001734)

3.14. Colilert-18 Quanti-Tray/2000 comparators for *E. coli* and *T. coli*

3.15. Quanti-Tray rubber inserts for Quanti-Tray/2000

3.16. Quanti-Tray Sealer Model Plus (S/N QTP 13215001064 or QTP 13181503550)

3.17. Vacuum Filtration system

3.18. Membrane filtration unit, sterile and wrapped in foil

3.19. UV Sterilizer unit

3.20. Forceps, sterile, straight or curved with smooth tip

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3.21. Membrane filters, sterile, white gridded, 47 mm diameter, 0.45±0.02 µm pore size

3.22. Bunsen burner

3.23. Beaker, 30 mL

4. REAGENTS AND STANDARDS

4.1. Microbiological Reference Materials:

4.1.1. Certified Reference Material (CRM)

4.1.2. *Escherichia coli (E. coli) NCTC 9001*

4.1.3. *Klebsiella variicola (K. variicola)* ATCC 31488

4.1.4. *Enterococcus faecalis (E. faecalis)* NCTC 775

4.1.5. *Pseudomonas aeruginosa (P. aeruginosa)* NCTC 12951

4.1.6. *Streptococcus bovis (S. bovis)* NCTC 8177

4.2. Microbiologics Kwik-Stik™ Reference Culture Microorganisms

4.2.1. *Pseudomonas aeruginosa (P. aeruginosa)* ATCC® 10145

4.2.2. *Aerococcus viridans* (*A. viridans*) ATCC 10400

4.2.3. *Serratia marcescens* (*S. marcescens*) ATCC 43862

Note: Kwik-Stik cultures are prepared according to SOP MB-03.1

4.3. Dilu-Lok bottle with 99 mL Phosphate Buffer with $MgCl_2$ for membrane filtration method.

4.4. Autoclaved DI water for Quanti-Tray

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

5. SAMPLE PREPARATION PROCEDURE

5.1. Retrieve four vials of the target organism from the freezer and allow the capped vials to equilibrate to room temperature (15-30°C). Do not open vial until equilibration is complete. This should take about 15 minutes.

5.2. Once the sample pellet is at room temperature, open the vial and aseptically transfer the pellet to the hydration fluid. Shake vigorously to dissolve.

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5.3. Analyze spiked samples according to method requirements.

5.4. POSITIVE CONTROLS

5.4.1. For the detection of E. *coli* and *T. coli*, use a single pure *E. coli* pellet as the primary choice, or the Universal Microbe CRM as a secondary option. For *T. coli,* use a single pure *K. variicola* pellet. If *K. variicola* is not readily available, the Universal Microbe CRM or *E. coli pellet* can be used as an alternative for *T. coli* detection. For the detection of *Enterococci*, use a single pure *E. faecalis* pellet. Membrane filtration analysis requires four Dilu-Lok bottles, each spiked with the target bacteria, and analyzed according to SOP MB-01.1. Quanti-Tray analysis requires four spiked IDEXX vessels containing autoclaved DI water with the target bacteria, analyzed according to SOP MB-01.15.

5.5. NEGATIVE CONTROLS

5.5.1. For Colilert-18 Quanti-Tray analysis of *E. coli* and *T. coli*, use *P. aeruginosa*, either pellet or plated working cultures, and spike one IDEXX vessel containing 100 mL of Autoclaved DI water and analyze.

5.5.2. For Enterolert Quanti-Tray analysis of *Enterococci*, use *S. bovis* and *E. coli* pellets (or *E. coli* plated working culture) to spike one IDEXX vessel containing 100 mL Autoclaved DI water for each culture and analyze. Plated working cultures *A. viridans* and *S. marcescens,* can also be used as negative controls.

5.5.3. For membrane filtration, spike one Dilu-Lok bottle with *P. aeruginosa* and filter 0.1 mL.

6. SAMPLE ANALYSIS

6.1. For initial method performance (IPR), ongoing method performance (OPR), and initial demonstration of capability (IDOC) for analysts, prepare and analyze four Reference Samples by fortifying the corresponding hydration fluid with the target organism.

6.1.1. For membrane filtration, spike four Dilu-Lok bottles with a positive control and analyze according to SOP MB-01.1. Filter three primary dilution volumes of the spiked control, either Universal Water Microbe Cocktail or a single pure *E. coli* culture, to produce countable colonies within the control's COA quantifiable range. Include blanks and negative controls to verify method integrity.

6.1.2. For Quanti-Tray analysis, spike four IDEXX vessels containing 100 mL of autoclaved DI water with a positive control, either Universal Water Microbe Cocktail or a single pure *E. coli* culture, and analyze according to SOP MB-01.15. Include blanks and negative controls to verify method integrity.

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6.2. For continuing demonstration of capability (CDOC), analysts must complete an annual assessment to verify ongoing competency. CDOCs may be satisfied by Proficiency Tests (PTs), using the IDOC procedure, or through media QC that includes a method blank and duplicate dilutions to demonstrate precision. Otherwise, CDOCs can be satisfied as described below.

6.2.1. For membrane filtration, spike one Dilu-Lok with a positive control and analyze according to SOP MB-01.1. Filter three primary dilution volumes of the spiked control, either Universal Water Microbe Cocktail or a single pure *E. coli* culture, to produce countable colonies within the control's COA quantifiable range. Additional dilutions may be included if necessary to achieve countable results. Duplicate at least one dilution to demonstrate precision, and include a method blank to verify method integrity.

6.2.2. For Quanti-Tray analysis, spike one IDEXX vessel containing 100 mL of autoclaved DI water with a positive control, either Universal Water Microbe Cocktail, a single pure *E. coli* culture, or a single pure *E. faecalis* culture. Shake the vessel until the pellet is fully dissolved and analyze a 40 mL dilution with a duplicate. Include a method blank and follow SOP MB-01.15. A lower dilution volume may be used if necessary.

6.3. Internal Quantifiable Range. When the control's COA does not provide sufficient quantifiable limits, a qualified lab coordinator or supervisor shall establish an internal quantifiable range. All analyses using the control, including IDOCs, CDOCs, and QC, shall be evaluated against this range. Include blanks and negative controls to verify method integrity. All results and deviations must be documented. See the *Internal QC Criteria – Microbiology* Excel file in the folder: \\floridadep\Data\DEAR\Labs\Lab5\Biology\BenchCommon\Micro\Lab Established - Microbe QC Limits

7. CALCULATIONS AND PRECISION

7.1 Calculations for each spiked sample are performed according to SOP MB-01.1 for membrane filtration, and using the IDEXX MPN Generator (for calculations only, not for generating a Quanti-Tray Excel file) as explained in SOP MB-01.15 for Quanti-Tray. Averages are then calculated from these results for COA comparison.

7.2 Retrieve the Certificate of Analysis (COA) for the reference culture located in the following folder: \\floridadep\data\DEAR\Labs\Lab5\Biology\BenchCommon\COA\Micro supplies\cultures and check that the average results fall within the appropriate Acceptable Range specified.

7.3 Check precision, log range value and mean range, by comparing results against the microbiology Control Charts All Tests 032514 excel file, found in the following folder: \\floridadep\data\DEAR\Labs\Lab5\Biology\BenchCommon\Micro.

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7.4 Enter the highest and lowest result out of the four IDOC spikes into Control Charts to determine precision and accuracy. For CDOC, enter the result of the spike and its duplicate. Remove these numbers from control charts once precision results are collected, and enter them into the DOC excel file: IPR_OPR_data121823-w-form found in the following folder: X:\Labs\Lab5\Biology\BenchCommon\Micro\DOCs.

7.5 Record filter, incubation, read dates/times, analyst, buffered water and media used, QC record numbers for consumables used, and blanks and dups with their respective Test IDs for each sample spike in Prep Worksheet Manager per SOP BB-30. Use LIMS Prep Worksheet Manager to create a MICRO-QC worksheet. Laboratory → Technical → Prep Worksheet Manager→ Preparation ID: MICRO QC.

8. SAFETY/HAZARDOUS WASTE MANAGEMENT

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

8.2. Expired or unused IDEXX media is collected by hazardous waste.

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

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

8.5. Read SDS information for reagent handling and disposal.

9. REFERENCES

9.1. EPA Method 1600 (Sept 2014)

9.2. Standard Methods 9020 (2017)

9.3. Standard Methods 9222 (2017)

9.4. NELAC Quality Systems Manual

9.5. Difco™ & BBL™ Manual
\\floridadep\data\DEAR\Labs\Lab5\Biology\BenchCommon\Methods & Library Files

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

9.7. FDEP Laboratory Health and Safety Plan
https://floridadep.sharepoint.com/dear/Shared%20Documents/FDEP%20Health%20%20Safety%20Plan%20B%20-%20June%202021.pdf

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

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

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

9.11. IDEXX Quanti-Tray/2000 Colilert-18 method instructions

9.12. IDEXX Quanti-Tray/2000 Enterolert method instructions

9.13. IDEXX Quanti-Tray Sealer PLUS Manual

9.14. DEP SOP MB 01.1, *Membrane Filter Method for* Fecal coliforms

9.15. DEP SOP MB 01.15, *Quanti-Tray/2000 Colilert-18 Method for Escherichia coli,* Total coliforms*, and* Enterolert Method *for Enterococci*

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.
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.
12/28/2021	Updated link for the Difco™ & BBL™ Manual. Added a note to section 5.4.1, and updated reference to the flowchart.
12/21/2022	Updated SOP by adding the new protocol to determine initial method performance (IPR), ongoing method performance (OPR), and demonstration of capability (DOC) for analysts. Former procedure remains in the SOP, unedited,

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	to serve as an alternative method. Updated FDEP Laboratory Health and Safety Plan.
12/20/2023	Updated CDOC procedures for analysts. Updated hyperlinks. Added TSWB 9 to the equipment list. Updated Section 7 to include DOC calculations, location to LIMS modules for data entry and hyperlinks to data entry folders. Updated section 5 and 6 to clarify and remove redundancy on the procedure of analyzing negative controls. Section 10 was removed as it is no longer a procedure performed in the laboratory.
8/29/2024	Updated Section 6 CDOC Procedures. The language and structure were refined to enhance clarity and readability.
11/18/2025	Section 6 was revised to clarify procedural requirements, standardize terminology, and incorporate guidance for establishing in-house quantifiable ranges. The equipment and supplies section was updated. Section 5.4.1 was amended to permit the use of *E. coli* for *T. coli* DOCs, and the References section was revised accordingly.

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