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Results of the Interlaboratory Validation of EPA Method 1600 (mEI) for Enterococci in Wastewater Effluent

February 2004

U.S. Environmental Protection Agency
Office of Water (4303T)
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Volunteer Research Laboratory

- EPA Office of Research and Development, National Risk Management Research Lab: Mark C. Meckes

Volunteer Participant Laboratories

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Volunteer Verification Laboratory

- City of Los Angeles Bureau of Sanitation: Farhana Mohamed, Ann Dalkey, Ioannice Lee, Genevieve Espineda, and Zora Bahariance

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Executive Summary

This report presents the results of the U.S. Environmental Protection Agency's (EPA's) interlaboratory validation study (the "Study") of a membrane filtration procedure for the analysis of enterococcus in wastewater: EPA Method 1600 which uses membrane-enterococcus indoxyl-β-D-glucoside agar medium (mEI). The September 2002 version of the Method (EPA-821-R-02-022) was followed during the Study. The purposes of the Study were to characterize method performance (sensitivity, specificity, precision, and recovery) across multiple laboratories and disinfected wastewater matrices and to develop quantitative quality control (QC) acceptance criteria.

Twelve volunteer participant laboratories, an enterococci verification laboratory, and a research laboratory participated in the Study which was conducted during the week of September 22, 2003. Each laboratory spiked samples with BioBall™ *Enterococcus faecalis* (*E. faecalis*) spikes and laboratory-prepared *E. faecalis* spikes. Samples were spiked in accordance with the Method 1600 spiking protocol (the "Spiking Protocol," **Appendix A**). Results from samples spiked with BioBall™ spikes were used to assess method performance and for the development of (QC) acceptance criteria to support future assessments of method and laboratory performance for disinfected wastewater matrices. QC criteria were also developed based on results of the laboratory-prepared spikes (in addition to criteria developed based on BioBalls™) to ensure that QC criteria will be available for these methods, if BioBalls™ become unavailable.

Results submitted by laboratories were validated using a standardized data review process to confirm that results were generated in accordance with study-specific instructions and the September 2002 version of EPA Method 1600. Method 1600 recovery of enterococci was acceptable, with mean laboratory-specific recoveries of enterococci from disinfected wastewater samples spiked with BioBalls™ ranging from 77.1% to 114.9%, with an overall mean recovery of 90.8%. Laboratory-specific relative standard deviations (RSDs) ranged from 0% to 69.5%, with an overall pooled, within-laboratory RSD of 22.6%.

False positive rates were also acceptable, with laboratory-specific false positive rates for unspiked disinfected/secondary results combined, ranging from 0% - 27.8%. For secondary wastewater (excluding disinfected results), only 11 of 132 typical colonies submitted to verification were non-enterococci, resulting in a false positive rate of 8.3% for secondary wastewater. For disinfected wastewater (excluding secondary results), only three of 69 typical colonies submitted to verification were non-enterococci, resulting in a false positive rate of 4.3% for disinfected wastewater.

In contrast, laboratory-specific false negative rates for unspiked disinfected/secondary results combined, ranged from 13.3% - 100.0%. For secondary wastewater (excluding disinfected results), 62 of 118 atypical colonies submitted to verification were identified as enterococci, resulting in a false negative rate of 52.5% for secondary wastewater. For disinfected wastewater (excluding secondary results), eight of 12 atypical colonies submitted to verification were identified as enterococci, resulting in a false negative rate of 66.7% for disinfected wastewater.

Results of this study indicate that Method 1600 precision, recovery, and false positive rates are acceptable for the determination of enterococci in disinfected wastewater. However, false negative rates observed during this study were high and should be taken into consideration when using results from this method. When evaluating wastewater using Method 1600, it is recommended that the false negative rate for each matrix be evaluated through biochemical confirmation and results adjusted accordingly, especially if large numbers of atypical colonies are observed in a particular matrix. If very few atypical colonies are observed in samples for a particular matrix, the high false negative rates observed during this study may be less of a concern.

SECTION 1.0 BACKGROUND

The enterococci test is recommended as a measure of recreational water quality. Epidemiological studies have led to the development of criteria which can be used to promulgate recreational water standards based on established relationships between health effects and water quality. Method 1600 was recently approved for monitoring ambient waters for enterococci (68 FR 43272, July 21, 2003). Prior to this study, none of the approved enterococci methods had been validated for wastewater analyses. National Pollutant Discharge Elimination System (NPDES) permit holders and others have requested that EPA validate one or more enterococci methods for evaluation of wastewater effluents.

1.1 Summary of the Method

Method 1600 is a membrane filtration procedure for the detection of enterococci in water samples. In Method 1600, a water sample is filtered through a membrane (0.45 µm pore-size), which retains the bacteria. After filtration, the membrane containing the bacteria is placed on a selective medium, mEI agar, and incubated at 41°C ± 0.5°C for 24 hours. All colonies that produce a blue halo (regardless of color) are considered enterococci.

SECTION 2.0 STUDY OBJECTIVES AND STUDY DESIGN

2.1 Study Objectives

The following objectives were established for the Study:

- Generate at least six sets of useable, valid data to characterize method performance
- Characterize Method 1600 sensitivity and specificity across multiple laboratories and disinfected wastewater matrices through the assessment of false positive and negative rates
- Characterize Method 1600 accuracy (recovery and precision) across multiple laboratories and disinfected wastewater effluents
- Establish Method 1600 quantitative Quality Control (QC) acceptance criteria for initial and ongoing laboratory and method performance assessments
- Establish Method 1600 quantitative QC acceptance criteria for matrix spikes

To accomplish these objectives, this Study was conducted in five phases (the technical approach for each phase is described below):

- **Phase 1** involved identification of qualified laboratories to participate in the Study, including a research laboratory to confirm that the spiking approach for the Study was acceptable; participant laboratories to analyze both BioBall™ spikes and laboratory-prepared spiking suspensions; and a verification laboratory to speciate colonies from the participant laboratories for the assessment of false positive and negative rates.
- **Phase 2** involved enumeration and use of BioBall™ (BTF Pty Ltd, Sydney, Australia) spikes of *E. faecalis* ATCC #19433 (Manassas, VA) at each of the participant laboratories. BioBalls are pre-packaged, water-soluble balls containing a precise number of bacteria. BioBall™ products are freeze-dried and have a shelf life of 6 months when stored at -20°C. BioBalls™ were selected for this study because they minimize the burden on the participant laboratories and because BioBalls™ are typically very precise spikes.
- **Phase 3** involved enumeration and preparation of a laboratory-prepared spiking suspension of *E. faecalis* ATCC #19433 at each of the participant laboratories.
- **Phase 4** involved the analysis of unspiked/spiked wastewater samples and unspiked/spiked sterile phosphate buffered saline (PBS) samples at the participant laboratories.

The following data quality objective was established for this Study:

- Data produced under this Study were required to be generated according to the analytical and quality assurance (QA)/QC procedures in the September 2002 version of Method 1600 (EPA-821-R-02-022) or approved changes to these procedures as provided to participant laboratories during the course of the Study. This data quality objective was developed to ensure that data were of known and reliable quality, thereby allowing EPA to use the results of the Study to identify the need for further revision of the method.

2.2 Phase 1 Technical Approach: Identification of Laboratories

The Study required three types of laboratories: a research laboratory, participant laboratories, and a centralized verification (identification) laboratory.

2.2.1 Research Laboratory

EPA's National Risk Management Research Laboratory (NRMRL) in Cincinnati, OH, served as the research laboratory for this Study. Prior to sample analysis at the participant laboratories, the research laboratory evaluated the procedure for preparation of the *E. faecalis* spiking suspensions that would be used as the laboratory-prepared spiking suspensions during the Study. The research laboratory (**Section 3.1**) confirmed that appropriate spike levels could be obtained by growing *E. faecalis* in 1% azide dextrose broth as specified in the Method 1600 Spiking Protocol (**Appendix A**).

2.2.2 Participant Laboratories

Participant laboratories analyzed samples to provide EPA with the data necessary to assess method performance and develop QC acceptance criteria. The participant laboratories also provided typical and atypical colonies to the verification laboratory for identification. Participant laboratories (**Section 3.3**) were representative of the general user community, with experience analyzing wastewater or ambient water samples for enterococci using membrane filtration techniques. Laboratory availability was also considered. A detailed Wastewater Laboratory Capabilities Checklist (**Appendix B**) was used to collect information from laboratories and screen potential participants to ensure that laboratories were qualified. Participants also needed to have access to representative disinfected and secondary treated wastewater effluents from the same facility.

Qualified volunteer laboratories were recruited in an effort to reduce costs. To reduce the burden on participant laboratories, EPA provided the media, reagents, and supplies necessary for the Study.

2.2.3 Verification Laboratory

The City of Los Angeles Bureau of Sanitation Microbiology Laboratory in Playa del Rey, CA served as the centralized verification laboratory. To assess false positive and negative rates, the verification laboratory speciated all typical and atypical colonies submitted by the participant laboratories. Colonies were identified using the Vitek® automated identification system. A detailed Wastewater Laboratory Capabilities Checklist (**Appendix B**) was used to collect information from laboratories and screen potential verification laboratories to ensure the centralized verification laboratory was qualified.

The verification laboratory was also recruited as a volunteer in an effort to reduce costs. To reduce the burden on the verification laboratory, EPA provided all necessary verification media and supplies.

2.3 Phase 2 Technical Approach: BioBall™ Spikes

Phase 2 involved enumeration and preparation of BioBall™ spikes of *E. faecalis* ATCC #19433 according to the Method 1600 Spiking Protocol (**Appendix A**). Results from samples spiked with BioBalls™ were used to assess inter- and intra-laboratory precision and recovery (method performance) and to develop QC acceptance criteria. The "lot mean value" provided by the manufacturer was used as the "true spike concentration." In addition participant laboratories enumerated BioBalls™ on the day of analysis in triplicate, using the spread plate

technique and tryptic soy agar (TSA) as described in the Method 1600 Spiking Protocol (**Appendix A**), to confirm stability of the BioBalls™.

2.4 Phase 3 Technical Approach: Lab-Prepared Spiking Suspensions

Phase 3 involved each laboratory preparing and enumerating spiking suspensions of *E. faecalis* ATCC #19433 according to the Method 1600 Spiking Protocol (**Appendix A**). To ensure that QC criteria are available for this method if BioBalls™ become unavailable, QC criteria were also developed based on results of the laboratory-prepared spikes. Results from samples spiked with laboratory-prepared spiking suspensions were not used to assess method performance.

Spiking suspensions were enumerated in triplicate, using the spread plate technique and TSA as described in the Method 1600 Spiking Protocol (**Appendix A**). To estimate the "true spike concentration," the participant laboratories enumerated the laboratory-prepared spiking suspensions on the same day that the validation study samples were spiked and analyzed.

2.5 Phase 4 Technical Approach: Sample Analysis

Phase 4 entailed the use of Method 1600 at multiple laboratories to analyze unspiked/spiked wastewater samples and PBS samples for *E. faecalis*.

The following objectives were established for Phase 4:

- Generate false positive and negative rate data for Method 1600 in disinfected wastewater effluents for the assessment of sensitivity and specificity. It should be noted that while the objective of the Study was to assess sensitivity and specificity for Method 1600 in disinfected wastewater, this was not possible because of the low numbers of colonies from disinfected wastewater samples. As a result, colonies from secondarily treated wastewater were also used to assess sensitivity and specificity of the Method. It was not possible to assess sensitivity/specificity solely in the matrix of interest (disinfected wastewater) during this study.

- Generate precision and recovery data for Method 1600 in disinfected wastewater effluents

- Develop quantitative QC acceptance criteria for Method 1600 in sterile PBS to support future assessments of laboratory performance

- Develop quantitative QC acceptance criteria for Method 1600 in the matrix of interest (disinfected wastewater) to support future assessments of method performance

- Generate a minimum of six sets of useable (as recommended by ASTM for method validation), valid data from the interlaboratory validation study to characterize method performance

Table 1 summarizes the number of samples that were evaluated to meet the objectives listed above. A detailed discussion is included in **Sections 2.5.1** through **2.5.5** below.

Table 1. Method 1600 Validation Study Analyses Performed by Each Laboratory

Matrix	Spiking Description	Sample Number	Verification	Purpose of Analysis	Results Provided in the Following Tables
Disinfected wastewater	Unspiked	N/A	N/A [b]	Range-finding (**Section 2.5.1**)	N/A
Secondary	Unspiked	N/A	N/A	Range-finding (**Section 2.5.1**)	N/A
Disinfected wastewater	Unspiked	1 - 4	5 typical and 5 atypical	Evaluation of ambient enterococci concentrations (**Section 2.5.2**)	4
				False positive and negative rates (**Section 2.5.2**)	5 and 6
Secondary [a]	Unspiked	5 - 6	5 typical and 5 atypical	False positive and negative rates (**Section 2.5.2**)	5 and 6
Disinfected wastewater	BioBalls™	7 - 8	N/A	Assessment of method accuracy (**Section 2.5.3**)	7
				Assessment of matrix spike QC criteria (**Section 2.5.5**)	
Disinfected wastewater	Lab-prepared	9 - 10	N/A	Assessment of matrix spike QC criteria (**Section 2.5.5**)	8
Sterile PBS	BioBalls™	11 - 14	N/A	Develop quantitative QC criteria for IPR [c] and OPR [d] (**Section 2.5.4**)	9
Sterile PBS	Lab-prepared	15 - 18	N/A	Develop quantitative QC criteria for IPR and OPR (**Section 2.5.4**)	10

[a] Colonies from these samples were only submitted to verification when a sufficient number of colonies from disinfected samples were not available

[b] N/A: Not applicable

[c] IPR: Initial Method/Laboratory Performance Assessment

[d] OPR: Ongoing Method/Laboratory Performance Assessment

2.5.1 Range-finding Analyses

Range-finding analyses were conducted on the secondary and disinfected wastewater effluents during the week of validation study analyses. These analyses were conducted one day prior to the analysis of validation study samples to determine the filtration volume(s) necessary to obtain plates within the optimum counting range for the method.

2.5.2 Assessment of Method Sensitivity and Specificity

Sensitivity and specificity of Method 1600 was assessed through the evaluation of false positive and false negative rates. Each of the 12 participant laboratories evaluated four unspiked disinfected wastewater samples for false positive/negative results by submitting five typical and five atypical colonies from each of the four disinfected wastewater samples to verification through biochemical evaluation. Because very few colonies were expected to be available for verification from the unspiked disinfected samples, two unspiked secondary effluent samples were also filtered to ensure that a sufficient number of colonies were available for verification during the

Study. Colonies from the secondary samples were only submitted to verification when a sufficient number of colonies from disinfected samples were not available. As a result, the study was designed to verify a total of 240 typical and 240 atypical colonies (including colonies from disinfected and secondary samples).

For each colony submitted to verification, the laboratory streaked the colony onto an mEI agar plate for isolation, inverted the plate, and incubated at 41°C ± 0.5°C for 24 ± 2 hours. Plates were labeled with sample identification information and colony type. To prepare plates for shipping, the laboratory wrapped the edges of the plates with parafilm, wrapped the stack of plates associated with each sample with bubble wrap, placed the plates into a cooler lined with a trash bag, and surrounded the plates with ice packs. Plates were shipped to the verification laboratory via Federal Express Priority Overnight Service.

Upon receipt at the verification laboratory, a single isolated colony was picked from each mEI plate and streaked for growth onto a tryptic soy agar (TSA) plate with 5% sheep blood (one TSA plate for every mEI plate) and incubated at 35°C ± 0.5°C for 18 to 24 hours. A suspension was prepared by placing growth from the blood agar plates in 3 mL of physiological saline. The suspensions were then evaluated using a Vitek® (bioMerieux, Hazelwood, Missouri), which is an automated biochemical identification system that utilizes test cards with either 30 or 45 microwells containing identification substrates and antimicrobials. For identification of enterococci during the Study, gram positive identification (GPI) cards were filled with the suspension using the Vitek®'s automated card filler and placed into the card reader/incubator. Readings were taken by the Vitek® every 15 minutes until identification (speciation) was complete.

2.5.3 Assessment of Method Accuracy (Precision and Recovery)

Method bias was evaluated through the analysis of two disinfected wastewater samples spiked with BioBalls™ at each of the participant laboratories. Each sample was spiked with a single BioBall™ containing a lot mean value (provided by the manufacturer) of 32.1 *E. faecalis* ATCC #19433 colony forming units (CFU). Recovery was assessed by comparing *E. faecalis* concentrations in the spiked samples (minus the ambient concentrations assessed from the analysis of the unspiked disinfected samples from **Section 2.5.2**) to the BioBall™ lot mean value. Precision was assessed based on the relative standard deviation of the two replicate recoveries.

2.5.4 Development of Quantitative QC Criteria for Initial (IPR) and Ongoing (OPR) Method/Laboratory Performance Assessments

To collect the data necessary to develop quantitative QC recovery and precision criteria for use in initial and ongoing method and laboratory performance, each participant laboratory analyzed four sterile 100-mL PBS samples spiked with BioBalls™ and four sterile 100-mL PBS samples spiked with laboratory-prepared spiking suspensions using ATCC #19433 in both cases. Samples spiked with BioBalls™ were spiked with approximately 32.1 CFU per sample and samples spiked with laboratory-prepared spiking suspensions were spiked with approximately 68.5 CFU per sample. QC criteria were developed based on results of the laboratory-prepared spikes, in addition to developing criteria based on BioBalls™, to ensure that QC criteria are available for Method 1600, if BioBalls™ become unavailable.

2.5.5 Development of Quantitative QC Criteria for Matrix Spikes (MS)

Quantitative QC criteria for matrix spikes were developed for use in assessing matrix interferences, should the methods be implemented for use in disinfected wastewater by EPA. To collect the data necessary to develop these criteria, each participant laboratory analyzed two disinfected wastewater samples spiked with BioBalls™ and two disinfected wastewater samples spiked with laboratory-prepared spiking suspensions using ATCC #19433 in both cases. Samples spiked with BioBalls™ were spiked with approximately 32.1 CFU per aliquot filtered and samples spiked with laboratory-prepared spiking suspensions were spiked with approximately 68.5 CFU per aliquot filtered. It should be noted that several laboratories were instructed to spike less than 100 mL of disinfected wastewater because of either turbidity or high enterococci concentrations in the samples (see **Section 4.1**). It should be noted that the same two disinfected wastewater samples spiked with BioBalls™ were used to assess method accuracy (**Section 2.5.3**) and to develop quantitative QC criteria for matrix spikes.

2.5.6 Quality Control (QC) Analyses

Participating laboratories completed the following QC requirements: media sterility checks, dilution water sterility checks, method blanks (sterile unspiked PBS), filtration blanks, positive controls, and negative controls. *E. faecalis* (ATCC #19433) served as the positive control and *E. coli* (ATCC #11775) as the negative control.

2.5.7 Minimum Validation Study Requirements

The Study met or exceeded the ASTM D2777-98 (Reference 10.2) method validation recommendations with every respect except number of concentrations. Only one spike concentration (instead of three) was evaluated because the Study was designed to evaluate samples spiked at levels similar to what was expected to be observed in disinfected wastewater samples at most laboratories. **Table 2** presents a comparison of the Study with ASTM D2777-98 validation study requirements.

Table 2. Comparison of ASTM Recommendations and the Method 1600 Study

Minimum ASTM Recommendations [a]	Method 1600 Study
6 participant laboratories	12 participant laboratories
1 matrix type plus reference matrix (typically reagent water)	1 matrix type plus reference matrix (PBS)
1 matrix type plus reference matrix (typically reagent water)	Samples from a total of 12 facilities plus reference matrix (PBS)
~3 concentrations	1 concentration
36 spiked reagent water samples (6 samples at 6 laboratories)	96 IPR samples (for each of 12 participants, 4 replicate PBS samples spiked with BioBalls™ and 4 replicate samples spiked with lab-prepared spikes)
36 spiked matrix samples (6 samples at 6 laboratories)	48 MS samples (for each of 12 participants, 2 duplicate MS samples spiked with BioBalls™ and 2 duplicate MS samples spiked with lab-prepared spikes)

[a] ASTM. Standard Practice for Determination of Precision and Bias of Applicable Test Methods of Committee D-19 on Water (ASTM D2777-98), October 1998.

SECTION 3.0 STUDY IMPLEMENTATION

3.1 Study Management

This study was designed under the direction of the Office of Science and Technology, Engineering and Analysis Division within the U.S. Environmental Protection Agency's (EPA's) Office of Water (OW). The EPA technical lead was Robin K. Oshiro. Coordination of activities for the Study were performed by DynCorp/CSC Biology Studies Group. Evaluation of the 1% azide dextrose broth and *Enterococcus faecalis* (ATCC #19433) following the Method 1600 Spiking Protocol (Appendix A) was performed by the EPA's Office of Research and Development (ORD), National Risk Management Laboratory (NRMRL).

3.2 Schedule

The Study schedule was as follows: practice analyses occurred the week of August 25, 2003 and the actual validation study analyses occurred the week of September 22, 2003. Range-finding analyses were conducted on Monday, September 22nd. Isolates were received at the verification laboratory on September 26, 2003. Verifications were started on October 2, 2003 and completed on October 30, 2003.

3.3 Research, Participant, and Verification Laboratories

The 12 participating laboratories, verification laboratory, and research laboratory involved in the Study are shown in **Table 3**.

Table 3. Laboratories Participating in the Interlaboratory Validation of Method 1600 [a]

City of Los Angeles Bureau of Sanitation Farhana Mohamed, Ann Dalkey, Ioannice Lee, Genevieve Espineda, and Zora Bahariance Hyperion Treatment Plant 12000 Vista del Mar, Playa del Rey, CA 90293	**Massachusetts Water Resources Authority** Steve Rhode and Mariya Gofnsteyn 190 Tafts Avenue, Winthrop, MA 02152
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County Sanitation Districts of L.A. County (SJC) Shawn Thompson and Julie Millenbach 1965 South Workman Mill Road, Whittier, CA 90601	**Texas A&M University** Suresh Pillai and Reema Singh 418D Kleberg Center, 2472 TAMUS, College Station, TX 77843
Environmental Associates Ltd. Susan Boutros and John Chandler 24 Oakbrook Drive, Ithaca, NY 14850	**University of Iowa, Hygienic Laboratory** Nancy Hall and Cathy Lord Oakdale Campus # H101 OH, Iowa City, IA 52242
Hampton Roads Sanitation District Anna Rule, Paula Hogg, and Bob Maunz 1432 Air Rail Avenue, Virginia Beach, VA 23471	**Wisconsin State Laboratory of Hygiene** Jon Standridge, Sharon Kluender, Linda Peterson, and Jeremy Olstadt 2601 Agriculture Drive, Madison, WI 53718
Hoosier Microbiological Laboratories Don Hendrickson, Katy Bilger and Lindsey Shelton 912 West McGalliard, Muncie, IN 47303	**Utah Department of Health** Sanwat Chaudhuri and Devon Cole 46 North Medical Drive, Salt Lake City, UT 84113
Verification laboratory:	**City of Los Angeles Bureau of Sanitation, Microbiology Laboratory** Farhana Mohamed, Ann Dalkey, Ioannice Lee, Genevieve Espineda, and Zora Bahariance Hyperion Treatment Plant, 12000 Vista del Mar, Playa del Rey, CA 90293
Research laboratory:	**EPA Office of Research and Development, National Risk Management Research Lab** Mark C. Meckes 26 West Martin Luther King Dr., Cincinnati, OH 45268-1320

[a] No endorsement of these laboratories is implied, nor should any be inferred. Participant laboratories have been randomly assigned numbers for purposes of presenting data in this report.

SECTION 4.0 DATA REPORTING AND VALIDATION

4.1 Data Reporting

Laboratories submitted the following data to DynCorp for review and validation:

- Completed cover sheet with sample collection and QC information
- Completed sample-specific reporting forms
- Documentation of any additional information that would assist in evaluating the data

4.2 Data Validation

DynCorp used data review checklists to ensure that each data package was complete and to ensure that each sample result met the study-specific and method-specific requirements. Items reviewed for each sample included the following:

- Confirmation that original forms were submitted
- Confirmation that incubation times were met
- Confirmation that incubation temperatures were met
- Confirmation that pre-filtration blank and phosphate buffer blank tested negative for enterococci
- Confirmation that media sterility checks were performed and acceptable
- Confirmation that positive and negative controls were performed and exhibited the appropriate response
- Confirmation that samples were spiked with the appropriate dilution
- Confirmation that calculations were correct

This process was performed independently by two data reviewers, each of whom entered the results into separate spreadsheets designed for data review and validation for this study. The results were compared to verify consistency and identify potential data entry errors.

Based on data review, the data from **Laboratory 2** were considered invalid and unacceptable for inclusion in subsequent data analysis because the laboratory did not perform all of the required quality control checks (pre-filtration blank and negative control) during the validation study.

It should be noted that several laboratories were instructed to spike less than 100 mL of disinfected wastewater because of either turbidity or high enterococci concentrations in the samples (based on range-finding analyses). Spiking and filtering a smaller volume of sample helped ensure that plates within the optimum counting range were obtained. Adjustments were made as follows:

- **Laboratory 1:** Due to high enterococci concentrations, Laboratory 1 was instructed to spike 1 mL of disinfected wastewater per sample.

- **Laboratory 9:** Laboratory 9 spiked 100 mL of disinfected effluent and filtered three volumes (i.e., 50, 30, and 10 mL) to obtain countable plates.

- **Laboratory 10:** Laboratory 10 could not filter volumes greater than 25 mL due to the turbidity of the disinfected effluent samples. As a result, Laboratory 10 was instructed to spike 25 mL of disinfected wastewater per sample. *Note:*Results from Laboratory 10, Sample 8 (disinfected wastewater spiked with a BioBall™) were not available because of confluent growth.

- **Laboratories 11 and 12:** Due to high enterococci concentrations, Laboratories 11 and 12 were instructed to spike 10 mL of disinfected wastewater per sample.

Results from **Laboratory 10**, Sample 8 (disinfected wastewater spiked with a BioBall™) were not available

because of confluent growth.

4.3 Censored Data

During the evaluation of validation study samples, results below the analytical range of the method (less-than results, also referred to as left-censored) were observed for some of the unspiked disinfected effluent samples. Left-censored results were observed with the following frequency: <1 enterococci per 100 mL, 18 results; <4 enterococci per 100 mL, four results; <10 enterococci per 100 mL, one result; and <100 enterococci per 100 mL, one result. The censor limit was replaced with one half of the "less than" value for subsequent data analyses for these samples. It should be noted that at first glance, replacing the <100 enterococci per 100 mL observed in Laboratory 1's unspiked disinfected wastewater with 50 enterococci per 100 mL seems unreasonable. However, that laboratory's other results for unspiked disinfected wastewater were 700, 300, and 300 enterococci per 100 mL. Since the other results were much higher than the <100 value, simply removing the <100 from the data set for estimating the concentration of background enterococci in the disinfected sample was deemed inappropriate and half the censor limit was used instead.

SECTION 5.0 RESULTS

All of the results included in this section were considered valid. Please see **Section 4** for detailed data invalidation information. Results of unspiked wastewater samples and false positive/negative rates are provided in **Section 5.1**, results of spiked disinfected wastewater samples are provided in **Section 5.2**, and results of spiked PBS samples are provided in **Section 5.3**. Please see **Section 6** for the development of QC acceptance criteria and **Section 7** for a discussion of method performance.

5.1 Unspiked Sample Results

Results from unspiked disinfected wastewater sample analyses are provided in **Table 4**. These data were used to estimate the background concentration of enterococci in disinfected wastewater samples.

Results of the verification analysis (assessment of false positive and negative rates based on unspiked disinfected and secondary confirmations) were used to assess method performance (see discussion in **Section 7**). Valid verification results from unspiked disinfected and secondary wastewater effluent samples are summarized in **Table 5**. Valid, laboratory-specific verification results are summarized in **Table 6**. Any typical colony that was identified as non-enterococci by the Vitek® was considered a false positive result. Any atypical colony that was identified as an enterococci by the Vitek® was considered a false negative result. It should be noted that some of the isolates submitted to verification did not exhibit growth on one of the two verification streak plates (mEI or blood agar). Isolates that did not grow on blood agar at the verification laboratory were treated as if they had not been submitted to verification and eliminated from subsequent data analyses. Colonies that did not grow after streaking for isolation on mEI agar plates, were considered to be non-enterococci and included in data analyses as non-enterococci colonies. The decision to include the colonies that exhibited no growth on the mEI as non-enterococci was based on the selective nature of the medium, as the mEI was considered the first step in the verification process.

Table 4. Summary of Enterococci Results from Unspiked Disinfected Wastewater Samples (see Table 1 for cross-reference to sample number)

Lab	Sample number (CFU [a]/100 mL)				Mean CFU/100 mL	SD [b]
	1	2	3	4		
1	<100	700	300	300	337.50	268.87
2	Invalid [c]					
3	<1	<1	2	<1	0.88	0.75
4	<1	<1	<1	<1	0.50	0.00
5	<1	1	<1	<1	0.63	0.25
6	<1	<1	<1	<1	0.50	0.00
7	4	6	4	10	6.00	2.83
8	<1	<1	<1	<1	0.50	0.00
9	84	72	76	73	76.25	5.44
10	<4	<4	<4	<4	2.00	0.00
11	30	40	80	40	47.50	22.17
12	10	10	10	<10	8.75	2.50
Overall (n = 44)					**43.73**	**81.4 [d]**

[a] Colony forming units

[b] Standard deviation

[c] Data validation discussed in Section 4.2

[d] Pooled within-lab standard deviation was determined by calculating the square root of the mean of the lab variances

Table 5. Summary of False Positive and False Negative Rates Associated with Unspiked Disinfected and Unspiked Secondary Wastewater Effluents

Matrix (Sample No.)	False Positive Assessment			False Negative Assessment		
	Typical colonies submitted	Number of false positives	False positive rate (%)	Atypical colonies submitted	Number of false negatives	False negative rate (%)
Unspiked Disinfected (Samples 1 - 4)	69	3	4.3	12	8	66.7
Unspiked Secondary (Samples 5, 6)	132	11	8.3	118	62	52.5
Unspiked Disinfected/Secondary (Samples 1 - 6)	201	14	7.0	130	70	53.9

Table 6. Laboratory-Specific False Positive and False Negative Rates Associated with Unspiked Wastewater Effluents (Disinfected and Secondary Results Combined, Samples 1-6, see cross-reference in Table 1)

Lab	False Positive Assessment			False Negative Assessment		
	Typical colonies submitted	Number of false positives	False positive rate (%)	Atypical colonies submitted	Number of false negatives	False negative rate (%)
1	20	0	0.0	15	14	93.3
2	Invalid [a]					
3	14	0	0.0	3	3	100.0
4	17	0	0.0	6	6	100.0
5	18	0	0.0	17	17	100.0
6	20	2	10.0	Not Assessed [b]		
7	17	0	0.0	11	4	36.4
8	18	5	27.8	15	2	13.3
9	20	0	0.0	18	8	44.4
10	19	4	21.1	14	4	28.6
11	20	3	15.0	18	7	38.9
12	18	0	0.0	13	5	38.5

[a] Data validation discussed in Section 4.2

[b] Atypical colonies were only observed on filters with colonies that were too numerous to count (TNTC)

5.2 Spiked Disinfected Sample Results

Results from disinfected wastewater samples spiked with BioBalls™ (**Table 7**) were used to assess method performance (see discussion in **Section 7**) and develop QC acceptance criteria for matrix spikes for use in assessing matrix interferences (see discussion in **Section 6**). Results from disinfected wastewater samples spiked with laboratory-prepared spiking suspensions (**Table 8**) were used to develop QC acceptance criteria (**Section 6**, these data were not used to assess method performance).

Table 7. Summary of Enterococci Results from Disinfected Samples Spiked with BioBalls™ (see Table 1 for cross-reference to sample number)

Lab	Spike Level (CFU/100 mL) [a, b]	Percent Recovery by Sample		Mean Percent Recovery	SD [c]	RSD [d] (%)
		7	8			
1	3210	95.4	95.4	95.4	0.0	0.0
2	Invalid [e]					
3	32.1	87.6	87.6	87.6	0.0	0.0
4	32.1	76.3	91.9	84.1	11.0	13.1
5	32.1	82.2	91.5	86.8	6.6	7.6
6	32.1	95.1	73.2	84.1	15.4	18.3
7	32.1	96.6	90.3	93.5	4.4	4.7
8	32.1	101.2	85.7	93.5	11.0	11.8
9	32.1	58.4	171.3	114.9	79.9	69.5
10	128.4	82.6	Invalid [e]	82.6	Not assessed	
11	321	81.8	72.4	77.1	6.6	8.6
12	321	93.8	97.0	95.4	2.2	2.3
Overall (n = 21)				**90.8**	**25.2 [f]**	**22.6 [g]**

[a] Colony forming units

[b] Spike level is based on the lot mean value provided by the manufacturer and the volume of sample that was spiked (it was necessary to spike <100 mL at some laboratories because of either high turbidity or high background concentrations of enterococci)

[c] Standard deviation

[d] Relative standard deviation

[e] Data review and validation discussed in Section 4.2

[f] Pooled within-lab standard deviation was determined by calculating the square root of the mean of the lab variances

[g] Pooled within-lab relative standard deviation was determined by calculating the square root of the mean of the squared lab RSDs

Table 8. Summary Enterococci Results from Disinfected Samples Spiked with Laboratory-Prepared Spiking Suspensions (see Table 1 for cross-reference to sample number)

Lab	Spike Level (CFU/100 mL)[a,b]	Percent Recovery by Sample		Mean Percent Recovery	SD[c]	RSD[d] (%)
		9	10			
1	3440	26.5	25.1	25.8	1.0	4.0
2	Invalid[e]					
3	55.2	87.2	103.5	95.3	11.5	12.1
4	64.2	75.5	81.8	78.7	4.4	5.6
5	44	105.4	78.1	91.8	19.3	21.0
6	193	43.8	46.4	45.1	1.8	4.1
7	60.7	42.8	41.2	42.0	1.2	2.8
8	62.52	90.4	71.2	80.8	13.6	16.8
9	32	84.6	58.6	71.6	18.4	25.7
10	114	107.0	86.0	96.5	14.9	15.4
11	1080	74.3	81.7	78.0	5.2	6.7
12	710	28.3	28.3	28.3	0.0	0.0
Overall (n = 22)				**66.7**	**10.9[f]**	**13.1[g]**

[a] Colony forming units

[b] Spike level is based on laboratory enumeration of spiking suspension and the volume of sample that was spiked (it was necessary to spike <100 mL at some laboratories because of either high turbidity or high background concentrations of enterococci)

[c] Standard deviation

[d] Relative standard deviation

[e] Data validation discussed in Section 4.2

[f] Pooled within-lab standard deviation was determined by calculating the square root of the mean of the lab variances

[g] Pooled within-lab relative standard deviation was determined by calculating the square root of the mean of the squared lab RSDs

5.3 Spiked PBS Results

Results from PBS samples spiked with BioBalls™ (**Table 9**) and PBS samples spiked with laboratory-prepared spiking suspensions (**Table 10**) were used to develop QC acceptance criteria for use in assessing initial and on-going method/laboratory performance (see discussion in **Section 6**).

Table 9. Summary of Enterococci Results from PBS Samples Spiked with BioBalls™ (see Table 1 for cross-reference to sample number)

Lab	Spike Level (CFU/100 mL) [a,b]	Percent Recovery by Sample				Mean Percent Recovery	SD [c]	RSD [d] (%)
		11	12	13	14			
1	32.1	93.5	99.7	96.6	102.8	98.1	4.0	4.1
2	Invalid [e]							
3	32.1	99.7	96.6	109.0	93.5	99.7	6.7	6.8
4	32.1	102.8	96.6	90.3	84.1	93.5	8.0	8.6
5	32.1	102.8	102.8	99.7	115.3	105.1	6.9	6.6
6	32.1	90.3	87.2	93.5	96.6	91.9	4.0	4.4
7	32.1	87.2	99.7	71.7	93.5	88.0	12.0	13.7
8	32.1	87.2	87.2	96.6	84.1	88.8	5.4	6.1
9	32.1	99.7	93.5	105.9	77.9	94.2	12.0	12.8
10	32.1	93.5	93.5	96.6	99.7	95.8	3.0	3.1
11	32.1	90.3	87.2	99.7	109.0	96.6	9.9	10.2
12	32.1	96.6	99.7	90.3	102.8	97.4	5.3	5.5
Overall (n = 44)						**95.4**	**7.6 [f]**	**8.1 [g]**

[a] Colony forming units

[b] Spike level is based on the lot mean value provided by the manufacturer

[c] Standard deviation

[d] Relative standard deviation

[e] Data validation discussed in Section 4.2

[f] Pooled within-lab standard deviation was determined by calculating the square root of the mean of the lab variances

[g] Pooled within-lab relative standard deviation was determined by calculating the square root of the mean of the squared lab RSDs

Table 10. Summary of Enterococci Results from PBS Samples Spiked with Laboratory-Prepared Spiking Suspensions (see Table 1 for cross-reference to sample number)

Lab	Spike Level (CFU/100 mL) [a,b]	Percent Recovery by Sample				Mean Percent Recovery	SD [c]	RSD [d] (%)
		15	16	17	18			
1	34.4	49.4	26.2	34.9	20.3	32.7	12.6	38.7
2	Invalid [e]							
3	55.2	90.6	123.2	83.3	65.2	90.6	24.2	26.7
4	64.2	76.3	77.9	84.1	82.6	80.2	3.7	4.6
5	44	81.8	86.4	84.1	118.2	92.6	17.1	18.5
6	193	46.1	51.8	51.3	43.0	48.1	4.2	8.8
7	60.7	37.9	44.5	34.6	32.9	37.5	5.1	13.6
8	62.5	76.8	120.0	96.0	88.0	95.2	18.3	19.2
9	32	93.8	93.8	125.0	81.3	98.4	18.7	19.0
10	28.5	80.7	77.2	98.2	77.2	83.3	10.1	12.1
11	108	86.1	80.6	82.4	82.4	82.9	2.3	2.8
12	71	42.3	26.8	23.9	36.6	32.4	8.5	26.3
Overall (n = 44)						**70.4**	**13.4** [f]	**20.0** [g]

[a] Colony forming units

[b] Spike level is based on laboratory enumeration of spiking suspension on tryptic soy agar (TSA) plates

[c] Standard deviation

[d] Relative standard deviation

[e] Data validation discussed in Section 4.2

[f] Pooled within-lab standard deviation was determined by calculating the square root of the mean of the lab variances

[g] Pooled within-lab relative standard deviation was determined by calculating the square root of the mean of the squared lab RSDs

SECTION 6.0 DEVELOPMENT OF QC ACCEPTANCE CRITERIA

All data analyses described below were performed using the results of disinfected wastewater and PBS samples spiked with either BioBalls™ or laboratory-prepared spiking suspensions. Separate QC acceptance criteria were calculated for BioBall™ and laboratory-prepared spike results, to ensure that QC criteria are available for this method if BioBalls™ become unavailable.

6.1 Outlier Analyses

Valid results from samples spiked with BioBalls™ and laboratory-prepared spiking suspensions were screened for outliers in accordance with the procedures described in American Society for Testing and Materials (ASTM) guidance D2777-98 (**Reference 8.2**). Outlying data were identified and removed in two steps: identification of outlying laboratories, followed by identification of individual sample results. First, outlying laboratories were identified using Youden's laboratory ranking test (**Reference 8.2**). For this test, laboratories were ranked and screened to identify laboratories with significantly higher or lower results than the other laboratories. The second test for identification of outlying data is the Grubbs test (**Reference 8.2**), which identifies individual sample results for outlying observations.

It should be noted that outlier analyses were only performed for development of QC acceptance criteria (**Section 6**). Outlier analyses were not conducted for the assessment of method performance (**Section 7**), as all valid data were included in the assessment of method performance.

6.1.1 Youden's Laboratory Ranking Test

The valid data were first tested for the presence of an outlying laboratory using Youden's laboratory ranking test. For this test, results were stratified by spike type (BioBalls™ vs laboratory-prepared), using recoveries from both disinfected wastewater and PBS samples. Youden's test was conducted separately by spike type, because separate criteria were to be calculated for BioBall™ and laboratory-enumerated spiked samples. Sample results for each laboratory were sorted based on sample number. Prior to conducting the Youden's laboratory ranking test, one missing result was replaced by the mean of the non-missing results for the given spike type, matrix, and laboratory. This replacement value was only used for the Youden test and was not used in any other data analyses. Based on the Youden's test, two laboratories were removed for laboratory-prepared sample analyses. These two laboratories (**Laboratories 1 and 12**) were both biased low compared to the remaining laboratories, and were not used in the development of QC acceptance criteria. (The low recoveries from the laboratory-prepared spiking suspensions may be related to spiking suspensions not being sufficiently homogenized prior to spiking samples or enumerating the spiking suspensions.) No laboratories were removed for BioBall™ spiked sample analyses.

6.1.2 Grubbs Test for Individual Outlying Sample Results

After removing outlying laboratories identified using Youden's lab ranking test, the remaining data were then tested for the presence of individual outlying results using Grubbs test. Grubbs test was run separately for each matrix (disinfected wastewater and PBS) and spike type. Grubbs test was run without performing any data transformations. This was because statistical and graphical evaluations did not reveal any major departures from the assumption of the data following a Normal distribution. Application of Grubbs test resulted in the removal of one BioBall™ disinfected wastewater sample result analyzed by Laboratory 9 with a recovery of 171%. This result was not used in the development of QC acceptance criteria.

6.2 Initial Precision and Recovery (IPR) and Ongoing Precision and Recovery (OPR)

QC acceptance criteria for initial precision and recovery (IPR) and ongoing precision and recovery (OPR) were developed based on the results from PBS (reference matrix) samples spiked with BioBalls™ and laboratory-prepared spiking suspensions during the Study, as these QC tests will be performed using PBS as the reference matrix by laboratories using the method during monitoring. Again, separate QC acceptance criteria were calculated for BioBall™ and laboratory-prepared spike results

The IPR and OPR recovery criteria were calculated based on within and between laboratory variance components. These variance components were calculated with PROC MIXED from the SAS version 8 program using the maximum likelihood method of estimation on the recovery results. Details on the maximum likelihood estimation can be found in the user's guide for this program (**Reference 8.3**).

Estimates of between laboratory variance and within laboratory variance were labeled s^2_L and s^2_w, respectively.

The combined standard deviation for IPR (is_c) is:

$$is_c = \sqrt{(1+\frac{\sum_{i=1}^{L} n_i^2}{n_T^2})s_L^2 + (\frac{1}{4}+\frac{1}{n_T})s_w^2}$$

Where:
L = number of labs for the given spiking procedure
n_i = number of PBS sample results for laboratory i for the given spiking procedure
n_T = total number of PBS results from all laboratories for the given spiking procedure

Upper and lower limits for IPR samples were then calculated as:

$$X_{Mean} \pm t_{(0.975;\, idf)} * is_c$$

Where idf is calculated using Satterthwaite's estimate as given below:

$$idf = \frac{is_c^4}{\frac{\left[\left(1+\frac{\sum_{i=1}^{L} n_i^2}{n_T^2}\right) * s_L^2\right]^2}{L-1} + \frac{\left[\left(\frac{1}{4}+\frac{1}{n_T}\right) * s_w^2\right]^2}{n_T - L}}$$

The combined standard deviation (os_c) for OPR is:

$$os_c = \sqrt{(1+\frac{\sum_{i=1}^{L} n_i^2}{n_T^2})s_L^2 + (1+\frac{1}{n_T})s_w^2}$$

Where:
L = number of labs for the given spiking procedure
n_i = number of PBS sample results for laboratory i for the given spiking procedure
n_T = total number of PBS results from all laboratories for the given spiking procedure

Upper and lower limits for OPR samples were then calculated as:

$$X_{Mean} \pm t_{(0.975;odf)} * os_c$$

Where odf is calculated using Satterthwaite's estimate as given below:

$$odf = \frac{os_c^4}{\frac{\left[\left(1+\frac{\sum_{i=1}^{L} n_i^2}{n_T^2}\right) * s_L^2\right]^2}{L-1} + \frac{\left[\left(1+\frac{1}{n_T}\right) * s_w^2\right]^2}{n_T - L}}$$

The precision criterion for IPR samples was calculated as a maximum relative standard deviation (RSD). The RSD for each laboratory and each spiking procedure was calculated by dividing the standard deviation of the recoveries by the mean of the recoveries for that laboratory and procedure. The RSDs were pooled directly, rather than calculating a pooled RSD by pooling standard deviations and dividing by an overall mean, because the pooled RSD calculated using the latter approach was unduly affected by laboratories which exhibited high bias and low variability.

The pooled RSD was then calculated as:

$$RSD_{pool} = \sqrt{\frac{1}{n_T - L}\sum_{i=1}^{L} (n_i - 1) * RSD_i^2}$$

Where:

L =	*number of laboratories for the given procedure*
n_i =	*number of PBS sample results for laboratory i for the given spiking procedure*
n_T =	*total number of PBS sample results for all laboratories for the given spiking procedure*
RSD_i =	*RSD calculated using the recoveries for laboratory i for the given spiking procedure*

The maximum RSD was then calculated as:

$$RSD_{Max} = \sqrt{F_{(0.95;3,n_T-L)}} * RSD_{Pool}$$

Where:
n_T = *total number of IPR and OPR results from all laboratories*
L = *number of laboratories*

The calculated IPR and OPR QC acceptance criteria are provided in **Table 11.**

Table 11. Calculated Initial and Ongoing Precision and Recovery (IPR and OPR) Acceptance Criteria

Performance test	BioBall™ acceptance criteria	Lab-prepared spike acceptance criteria
Initial precision and recovery (IPR)		
• Mean percent recovery	85% - 106%	31% - 127%
• Precision (as maximum relative standard deviation)	14%	28%
Ongoing precision and recovery (OPR) as percent recovery	78% - 113%	27% - 131%

6.3 Matrix Spike (MS) and Matrix Spike Duplicate (MSD) Recovery and Precision

QC acceptance criteria for matrix spikes (MS) and matrix spike duplicates (MSD) were developed based on data from the spiked disinfected wastewater matrices used in the validation study. Separate QC acceptance criteria were calculated for BioBall™ and laboratory-prepared spike results

Recovery criteria were based on estimates of each variance component (between laboratory and within laboratory) and were calculated using PROC MIXED from SAS version 8 using the maximum likelihood method of estimation on the recovery results. Details on the maximum likelihood estimation can be found in the user's guide for this program (**Reference 8.3**). Between matrix variability could not be separated from between laboratory variability because each laboratory analyzed a different disinfected wastewater sample, and therefore the estimate of between laboratory variance also includes matrix variability.

Estimates of between laboratory variance and within laboratory variance were labeled s^2_L and s^2_w, respectively.

The combined standard deviation for MS/MSD (s_c) is:

$$s_c = \sqrt{(1+\frac{\sum_{i=1}^{L} n_i^2}{n_T^2})s_L^2 + (1+\frac{1}{n_T})s_w^2}$$

Where:
L = number of labs for the given spiking procedure
n_i = number of disinfected sample results for laboratory i for the given spiking procedure
n_T = total number disinfected sample results from all laboratories for the given spiking procedure

Upper and lower limits for MS/MSD samples were then calculated as:

$$X_{Mean} \pm t_{(0.975;df)} * s_c$$

Where:
X_{mean} = the mean recovery of all disinfected wastewater samples, and for the given spiking procedure

df is calculated using Satterthwaite's estimate as given below:

$$df = \frac{s_c^4}{\frac{\left[\left(1+\frac{\sum_{i=1}^{L} n_i^2}{n_T^2}\right) * s_L^2\right]^2}{L-1} + \frac{\left[\left(1+\frac{1}{n_T}\right) * s_w^2\right]^2}{n_T - L}}$$

The precision criterion for MS/MSD samples was calculated as a maximum relative percent difference (RPD). The RSD for each laboratory and matrix was calculated by dividing the standard deviation of the two recoveries by the mean of those recoveries for that lab and matrix. The RSDs were pooled directly, rather than calculating a pooled RSD by pooling standard deviations and dividing by an overall mean, because the pooled RSD calculated using the latter approach was unduly affected by laboratories/matrices which exhibited high bias and low variability.

The pooled RSD was then calculated as:

$$RSD_{pool} = \sqrt{\frac{1}{L}\sum_{i=1}^{L} RSD_i^2}$$

Where:

L = *number of laboratories for the given spiking procedure, and*

RSD_i = *RSD calculated using the recoveries for laboratory i for the given spiking procedure*

The maximum RPD was then calculated as:

$$RPD_{Max} = \sqrt{2 * F_{(0.95;1,L)}} * RSD_{Pool}$$

Where:

L = *the total number of laboratories for the given spiking procedure, and*

2 = *a constant used to convert an RSD to an RPD (when calculated using 2 values, the RSD and RPD differ by a factor of* $\sqrt{2}$ *).*

The calculated MS/MSD QC acceptance criteria are listed in **Table 12.**

Table 12. Calculated Matrix Spike Precision and Recovery Acceptance Criteria

Performance test	BioBall™ acceptance criteria	Lab-prepared acceptance criteria
Mean percent recovery for MS or MS/MSD	63% - 110%	29% - 122%
Precision (as maximum relative percent difference of MS/MSD)	28%	47%

SECTION 7.0 ASSESSMENT OF METHOD PERFORMANCE: DISCUSSION AND CONCLUSIONS

Results of this Study enabled assessment of the Method's performance in PBS and disinfected wastewater, and enabled development of quality control (QC) acceptance criteria that can be used to confirm acceptable laboratory and method performance on an ongoing basis in disinfected wastewater monitoring surveys and other studies. Method performance was evaluated through the assessment of false positive and negative rates in unspiked disinfected and unspiked secondary wastewater samples and through the evaluation of precision and recovery in disinfected wastewater samples spiked with BioBalls™. (Results from samples spiked with laboratory-prepared spiking suspensions were not used to assess method performance.) It should be noted that outlier analyses were not conducted for the assessment of method performance, as all valid data were included in the assessment of method performance.

Results from the laboratory enumerations of the BioBalls™ indicate that BioBall™ spikes of *E. faecalis* (ATCC #19433) were stable and precise, as laboratory enumeration of the BioBalls™ estimated a mean of 29.9 CFU per BioBall™ and a pooled within-laboratory RSD of 10.5%, compared to the manufacturer's lot mean value of 32.1 CFU per BioBall™ and RSD of 7.8%. In contrast, the laboratory-prepared spiking suspensions (used for development of QC acceptance criteria) were significantly less precise (based on an F-test comparing the pooled within-laboratory RSDs at α=0.05), with a pooled within-laboratory RSD of 24.0%.

Method 1600 recovery of enterococci was acceptable, with mean laboratory-specific recoveries of enterococci from disinfected wastewater samples spiked with BioBalls™ ranging from 77.1% to 114.9%, with an overall mean recovery of 90.8%. Laboratory-specific RSDs ranged from 0% to 69.5%, with a pooled, within-laboratory RSD of 22.6%.

False positive rates were also acceptable, with laboratory-specific false positive rates for unspiked disinfected/secondary results combined, ranging from 0% - 27.8%. For secondary wastewater (excluding disinfected results), only 11 of 132 typical colonies submitted to verification were non-enterococci, resulting in a false positive rate of 8.3% for secondary wastewater. For disinfected wastewater (excluding secondary results), only three of 69 typical colonies submitted to verification were non-enterococci, resulting in a false positive rate of 4.3% for disinfected wastewater.

In contrast, laboratory-specific false negative rates for unspiked disinfected/secondary results combined, ranged from 13.3% - 100.0%. For secondary wastewater (excluding disinfected results), only 62 of 118 atypical colonies submitted to verification were identified as enterococci, resulting in a false negative rate of 52.5% for secondary wastewater. For disinfected wastewater (excluding secondary results), eight of 12 atypical colonies submitted to verification were identified as enterococci, resulting in a false negative rate of 66.7% for disinfected wastewater.

Results of this study indicate that Method 1600 precision, recovery, and false positive rates are acceptable for the determination of enterococci in disinfected wastewater. However, false negative rates observed during this study were high and should be taken into consideration when using results from this method. When evaluating wastewater using Method 1600, it is recommended that the false negative rate for each matrix be evaluated through biochemical confirmation and results adjusted accordingly, especially if large numbers of atypical colonies are observed in a particular matrix. If very few atypical colonies are observed in samples for a particular matrix, the high false negative rates observed during this study may be less of a concern.

SECTION 8.0 REFERENCES

8.1 American Public Health Association, American Water Works Association, and Water Environment Federation. 1995. *Standard Methods for Water and Wastewater.* 20th Edition. Sections: 9020, 9221, 9222.

8.2 American Society for Testing and Materials. 1998. Annual Book of ASTM Standards, Vol. 11.01. *Standard Practice for Determination of Precision and Bias of Applicable Test Methods of Committee D-19 on Water*, ASTM D2777-98, October 1998.

8.3 SAS Institute Inc. 1994. SAS/STAT User's Guide, Volume 2, GLM-VARCOMP. Version 6, 4th Edition, June 1994.

8.4 USEPA. 2002. *EPA Method 1600: Enterococci in Water by Membrane Filtration Using membrane-Enterococcus Indoxyl-β-D Glucoside Agar (mEI)*, EPA-821-R-02-022, September 2002.

SECTION 9.0 ACRONYMS

CFU	Colony forming unit
IPR	Initial precision and recovery
MS	Matrix spike
MSD	Matrix spike duplicate
OPR	Ongoing precision and recovery
QA	Quality assurance
QC	Quality control
RPD	Relative percent difference
RSD	Relative standard deviation
SAS	Statistical analysis software
SD	Standard deviation

Appendix A:
Method 1600 Spiking Protocol

Enterococci Spiking Protocol
Interlaboratory Wastewater Validation Study of Method 1600

(August 20, 2003)

The purpose of this protocol is to provide laboratories with enterococci spiking procedures for the interlaboratory wastewater validation study of Method 1600. During this study, laboratories will spike samples using laboratory-prepared spiking solutions and with BioBalls™ (a commercially available product from BioTechnology Frontiers, Sydney, Australia). The following sections are included in this protocol:

Laboratory-Prepared Spiking Solutions

Section 1: Preparation of Laboratory-Prepared Spiking Suspensions
Section 2: Laboratory-Prepared Sample Spiking and Spiking Suspension Enumeration
Section 3: Calculation of Laboratory-Prepared Spike Percent Recovery

BioBalls™

Section 4: BioBall™ Sample Spiking and BioBall™ Enumeration
Section 5: Calculation of BioBall™ Spike Percent Recovery

1.0 Preparation of Laboratory-Prepared Spiking Suspensions

1.1 **Stock Culture.** Prepare a stock culture by inoculating a trypticase soy agar (TSA) slant (or other non-selective media) with *Enterococcus faecalis* ATCC #19433 and incubating at 35°C ± 3°C for 20 ± 4 hours. This stock culture may be stored in the dark at room temperature for up to 30 days.

1.2 **1% Azide Dextrose Broth.** Prepare a 1% solution of azide dextrose broth by combining 99 mL of sterile phosphate buffered saline (Method 1600, Section 7.4) and 1 mL of sterile single strength azide dextrose broth in a sterile screw cap bottle or re-sealable dilution water container. Shake to mix.

1.3 **Spiking Suspension (Undiluted).** From the stock culture of *Enterococcus faecalis* ATCC #19433 in Section 1.1, transfer a small loopful of growth to the 1% Azide dextrose broth solution and vigorously shake a minimum of 25 times. Incubate at 35°C ± 3°C for 20 ± 4 hours. The resulting spiking suspension contains approximately 1.0×10^6 to 1.0×10^7 enterococci colony forming units (CFU) per mL. This is referred to as the "undiluted spiking suspension." ***Note:*** *During the Method 1600 validation study, growth of spiking suspensions will begin on Monday, so the spiking suspensions are ready to be spiked into the samples on Tuesday.*

1.4 Proceed to Section 2.0 for sample spiking and enumeration of spiking suspension.

2.0 Laboratory-Prepared Sample Spiking and Spiking Suspension Enumeration

Since the objective of spiking the sample is to establish percent recovery, it is necessary to determine the number of enterococci in the undiluted spiking suspension prepared in Section 1.3. This section provides instructions for sample spiking (Section 2.1) and spiking suspension enumeration (2.2).

2.1 Sample spiking

2.1.1 Dilute spiking suspension

2.1.1.1 Mix the spiking suspension by vigorously shaking the bottle a minimum of 25 times. Use a sterile pipette to transfer 1.0 mL of the undiluted spiking suspension (from Section 1.3 above) to 99 mL of sterile phosphate buffered saline (Method 1600, Section 7.4), cap, and mix by vigorously shaking the bottle a minimum of 25 times. This is spiking suspension dilution "A". A 1.0-mL volume of dilution "A" is 10^{-2} mL of the original undiluted spiking suspension.

2.1.1.2 Use a sterile pipette to transfer 1.0 mL of spiking suspension dilution "A" (from Section 2.1.1.1 above) to 99 mL of sterile phosphate buffered saline (Method 1600, Section 7.4), cap, and mix by vigorously shaking the bottle a minimum of 25 times. This is spiking suspension dilution "B". A 1.0-mL volume of dilution "B" is 10^{-4} mL of the original undiluted spiking suspension.

2.1.1.3 Use a sterile pipette to transfer 11.0 mL of spiking suspension dilution "B" (from Section 2.1.1.2 above) to 99 mL of sterile phosphate buffered saline (Method 1600, Section 7.4), cap, and mix by vigorously shaking the bottle a minimum of 25 times. This is spiking suspension dilution "C". A 1.0-mL volume of dilution "C" is 10^{-5} mL of the original undiluted spiking suspension.

2.1.1.4 Use a sterile pipette to transfer 11.0 mL of spiking suspension dilution "C" (from Section 2.1.1.3 above) to 99 mL of sterile phosphate buffered saline (Method 1600, Section 7.4), cap, and mix by vigorously shaking the bottle a minimum of 25 times. This is spiking suspension dilution "D". A 1.0-mL volume of dilution "D" is 10^{-6} mL of the original undiluted spiking suspension.

2.1.2 Spike sample(s)

2.1.2.1 To spike sample, add 3.0 mL of spiking suspension dilution "D" (from Section 2.1.1.4 above) to 100-mL of unspiked sample and mix by vigorously shaking the bottle a minimum of 25 times. This is the "spiked" sample. The volume (mL) of undiluted spiking suspension added to each 100 mL of sample is 3.0 x 10^{-6} mL per 100 mL [(3.0 mL x 10^{-6} mL) per 100 mL of sample], which is referred to as $V_{\text{spiked per 100 mL sample}}$ in Section 3.2 below. This is the "spiked" sample. Analyze the spiked sample according to the instructions provided in Method 1600, Section 11.0.

2.2 Enumeration of undiluted spiking suspension (prepared in Section 1.3)

2.2.1 Prepare trypticase soy agar (TSA) according to manufacturer's directions, add 10 - 15 mL of TSA per 100 x 15 mm petri dish, and allow to solidify. Ensure that agar surface is dry. ***Note:*** *Agar plates must be dry and free from condensation prior to use. To ensure that the agar surface is dry, plates should be made several days in advance and stored inverted at room temperature or dried using a laminar-flow hood.*

2.2.2 Each of the following will be conducted in triplicate, resulting in the evaluation of nine spread plates:

- Pipet 0.1 mL of dilution "B" (Section 2.1.1.2) onto surface of pre-dried TSA plate [10^{-5} mL (0.00001) of the original spiking suspension].
- Pipet 0.1 mL of dilution "C" (Section 2.1.1.3) onto surface of pre-dried TSA plate [10^{-6} mL (0.000001)of the original spiking suspension].
- Pipet 0.1 mL of dilution "D" (Section 2.1.1.4) onto surface of pre-dried TSA plate [10^{-7} mL (0.0000001)of the original spiking suspension].

2.2.3 For each spread plate, using a sterile bent glass rod or spreader, distribute inoculum over the surface of medium by rotating the dish by hand or on a turntable. ***Note:*** *Please ensure that the*

inoculum is evenly distributed over the entire surface of the plate.

2.2.4 Allow inoculum to absorb into the medium completely.

2.2.5 Invert plates and incubate at 35°C ± 0.5°C for 20 ± 4 hours.

2.2.6 Count and record number of colonies per plate. Refer to Section 3.0 for calculation of spiking suspension concentration.

3.0 Calculation of Spiked Enterococci Percent Recovery

Spiked enterococci percent recovery will be calculated in three steps as indicated in Sections 3.1 through 3.3 below. *Note: The example calculated numbers provided in the tables below have been rounded at the end of each step. If your laboratory recalculates the examples using a spreadsheet and rounds only after the final calculation (Step 3), the percent recoveries may be slightly different.*

3.1 Step 1: Calculate Concentration of Enterococci (CFU / mL) in Undiluted Spiking Suspension

3.1.1 The number of enterococci (CFU / mL) in the undiluted spiking suspension (prepared in Section 1.3 above) will be calculated using all TSA plates from Section 2.2 yielding counts within the ideal range of 30 to 300 CFU per plate.

3.1.2 If the number of colonies exceeds the upper range (i.e., >300) or if the colonies are not discrete, results should be recorded as "too numerous to count" (TNTC).

3.1.3 Calculate the concentration of enterococci (CFU / mL) in the undiluted spiking suspension according to the following equation. (Example calculations are provided in Table 1 below.)

$$\text{Enterococci}_{\text{undiluted spike}} = (CFU_1 + CFU_2 + \ldots + CFU_n) / (V_1 + V_2 + \ldots + V_n)$$

Where,

$\text{Enterococci}_{\text{undiluted spike}}$	=	Enterococci (CFU / mL) in undiluted spiking suspension
CFU	=	Number of colony forming units from TSA plates yielding counts within the ideal range of 30 to 300 CFU per plate
V	=	Volume of undiluted sample on each TSA plate yielding counts within the ideal range of 30 to 300 CFU per plate
n	=	Number of plates with counts within the ideal range

TABLE 1. EXAMPLE CALCULATIONS OF ENTEROCOCCI SPIKING SUSPENSION CONCENTRATION

Examples	CFU / plate (triplicate analyses) from TSA plates in Section 2.2.5			Enterococci CFU / mL in undiluted spiking suspension (Enterococci $_{undiluted\ spike}$)*
	10^{-5} mL plates	10^{-6} mL plates	10^{-7} mL plates	
Example 1	94, 106, 89	9, 11, 28	1, 0, 4	$(94+106+89) / (10^{-5}+10^{-5}+10^{-5}) =$ $289 / (3.0 \times 10^{-5}) = 9{,}633{,}333 =$ **9.6×10^{6} CFU / mL**
Example 2	32, 55, 72	8, 5, 3	0, 0, 0	$(32+55+72) / (10^{-5}+10^{-5}+10^{-5}) =$ $159 / (3.0 \times 10^{-5}) = 5{,}300{,}000 =$ **5.3×10^{6} CFU / mL**

*Enterococci $_{undiluted\ spike}$ is calculated using all plates yielding counts within the ideal range of 30 to 300 CFU per plate

3.2 Step 2: Calculate "True" Spiked Enterococci (CFU / 100 mL)

3.2.1 Calculate true concentration of spiked enterococci (CFU / 100 mL) according to the following equation. Example calculations are provided in Table 2 below.

$$T_{Spiked\ Enterococci} = (Enterococci_{\ undiluted\ spike}) \times (V_{\ spiked\ per\ 100\ mL\ sample})$$

Where,

$T_{Spiked\ Enterococci}$	=	Number of spiked Enterococci (CFU / 100 mL)
$Enterococci_{\ undiluted\ spike}$	=	Enterococci (CFU / mL) in undiluted spiking suspension (calculated in Section 3.1.3)
$V_{\ spiked\ per100\ mL\ sample}$	=	mL of undiluted spiking suspension per 100 mL sample (Section 2.1.2.1)

Table 2. Example Calculations of Spiked Enterococci

Enterococci $_{undiluted\ spike}$ (Table 1 above)	$V_{\ spiked\ per\ 100\ mL\ sample}$ (Section 2.1.2.1 above)	$T_{Spiked\ Enterococci}$
9.6×10^{6} CFU / mL	3.0 X 10^{-6} mL per 100 mL of sample	$(9.6 \times 10^{6}$ CFU / mL) x $(3.0 \times 10^{-6}$ mL / 100 mL) = **28.8 CFU / 100 mL**
5.3×10^{6} CFU / mL	3.0 X 10^{-6} mL per 100 mL of sample	$(2.8 \times 10^{6}$ CFU / mL) x $(3.0 \times 10^{-6}$ mL / 100 mL) = **8.4 CFU / 100 mL**

3.3 Step 3: Calculate Percent Recovery

3.3.1 Calculate percent recovery (R) using the following equation.

$$R = 100 \times \frac{(N_s - N_u)}{T}$$

Where,

R	=	Percent recovery
N_s	=	Enterococci (CFU / 100 mL) in the spiked sample (Method 1600, Section 12)
N_u	=	Enterococci (CFU / 100 mL) in the unspiked sample (Method 1600, Section 12)
$T_{\text{Spiked Enterococci}}$	=	True spiked Enterococci (CFU / 100 mL) in spiked sample (Section 3.2, above)

Note: During the validation study, N_u (unspiked sample) is the mean enterococci (CFU / 100 mL) of the 4 unspiked disinfected wastewater samples.

3.3.2 Example percent recovery calculations are provided in Table 3.

Table 3. Example Percent Recovery Calculations

N_s (CFU / 100 mL)	N_u (CFU / 100 mL)	$T_{\text{Spiked Enterococci}}$ (CFU / 100 mL)	Percent recovery (R)
42	<1	28.8	100 x (42 - 1) / 28.8 = **142%**
34	10	28.8	100 x (34 - 10) / 28.8 = **83%**
16	<1	8.4	100 x (16 - 1) / 8.4 = **179%**
10	<1	8.4	100 x (10 - 1) / 8.4 = **107%**

4.0 BioBall™ Sample Spiking and Enumeration

During the validation study, each laboratory will enumerate enterococci in the BioBalls™ so that percent recovery can be evaluated. This section provides instructions for sample spiking (Section 4.1) and spiking suspension enumeration (4.2).

After receipt at your laboratory, the BioBalls™ should be stored at -20°C. Preparation of the BioBalls™ prior to spiking is not necessary, as they can be spiked directly into the sample once the vial is opened.

4.1 Sample spiking

4.1.1 Open BioBall™ vial by removing the crimp and cap. To spike a sample, aseptically add 1 BioBall™ to 100 mL of unspiked sample and mix by vigorously shaking the bottle a minimum of 25 times. This is the "spiked" sample. Analyze the spiked sample according to the instructions provided in Method 1600, Section 11.0.

4.2 Enumeration of BioBall™ (used in Section 4.1 above)

4.2.1 Prepare trypticase soy agar (TSA) according to manufacturer's instructions, add 10 - 15 mL of TSA per 100 x 15 mm petri dish, and allow to solidify. Ensure that agar surface is dry. ***Note:*** *Agar plates must be dry and free from condensation prior to use. To ensure that the agar surface is dry, plates should be made several days in advance and stored inverted at room temperature or dried using a laminar-flow hood.*

4.2.2 Each of the following will be conducted in triplicate, resulting in the evaluation of three spread plates:

- Open BioBall™ vial by removing the crimp and cap. Aseptically place one BioBall™ onto the center of each pre-dried TSA plate by tipping the vial over the medium.
- Immediately pipette 100 µl of sterile phosphate buffered saline solution (Method 1600, Section 7.4) directly onto the BioBall™.
- Allow the BioBall™ to dissolve.

4.2.3 For each spread plate, using a sterile bent glass rod or spreader, distribute the BioBall™ inoculum over surface of medium by rotating the dish by hand or on a turntable. ***Note:*** *Please ensure that the inoculum is evenly distributed over the entire surface of the plate.*

4.2.4 Allow inoculum to absorb into the medium completely.

4.2.5 Invert plates and incubate at 35°C ± 3°C for 20 ± 4 hours.

4.2.6 Count and record number of colonies per plate. Refer to Section 5.0 for calculation of the concentration of enterococci in the BioBall™.

5.0 Calculation of BioBall™ Spike Percent Recovery

Spiked BioBall™ percent recovery will be calculated following the steps indicated below. ***Note:*** *The example calculated numbers provided in the tables below have been rounded at the end of each step. If your laboratory recalculates the examples using a spreadsheet and rounds only after the final calculation (Step 3), the percent recoveries may be slightly different.*

5.1 Step 1: Calculate Mean Enterococci per BioBall™ and "True" Spiked Enterococci (CFU / 100 mL)

The mean concentration of enterococci (CFU) in the BioBalls™ will be calculated using all three TSA plates from Section 4.2. Since one BioBall™ is spiked per 100 mL sample, use the mean number of enterococci per BioBall™ as the "true" spiked enterococci per 100 mL sample. For example,

$$T_{\text{Spiked Enterococci}} \text{ (CFU / 100 mL)} = (30 + 38 + 28) / 3 = 32 \text{ per 100 mL}$$

Where,

$T_{\text{Spiked Enterococci}}$ = True spiked Enterococci (CFU / 100 mL) in spiked sample

5.2 Step 2: Calculate Percent Recovery

Calculate percent recovery (R) using the following equation.

$$R = 100 \times \frac{(N_s - N_u)}{T}$$

Where,

R	=	Percent recovery
N_s	=	Enterococci (CFU / 100 mL) in the spiked sample (Method 1600, Section 12)
N_u	=	Enterococci (CFU / 100 mL) in the unspiked sample (Method 1600, Section 12)
$T_{\text{Spiked Enterococci}}$	=	True spiked Enterococci (CFU / 100 mL) in spiked sample (Section 5.2, above)

Note: During the validation study, N_u (unspiked sample) is the mean enterococci (CFU / 100 mL) of the 4 unspiked disinfected wastewater samples.

Example percent recovery calculations are provided in Table 4.

Table 4. Example Percent Recovery Calculations

N_s (CFU / 100 mL)	N_u (CFU / 100 mL)	T (CFU / 100 mL)	Percent recovery (R)
24	<1	32	100 × (24 - 1) / 32 = **72%**
36	10	32	100 × (36 - 10) / 32 = **81%**

Appendix B:
Wastewater Laboratory Capabilities Checklist

Wastewater Laboratory Capabilities Checklist
(June 17, 2003)

EPA plans to invite 11 laboratories (10 participants and 1 referee) to participate in a study to validate the modified mTEC (*E. coli*) and mEI (enterococci) methods in wastewater effluent. EPA will provide all media and disposable materials for the study and will also cover all shipping costs. Volunteers will be acknowledged in both the method and validation study reports.

The schedule will include two weeks (range-finding and validation study) of analyses per method with an additional week of practice analyses prior to the study. The study is tentatively scheduled to begin in September, with some potential analyses being conducted by the referee laboratory in August.

If your laboratory is interested in participating in the validation study as either a participant laboratory or the referee, please provide the requested information below and fax the signed, completed checklist to Mike Chicoine at 703.461.8056 by **Thursday July 3**. In addition, please send the form electronically to Mike Chicoine at mike.chicoine@dyncorp.com.. Mike will confirm receipt of the checklist. If you have any questions pertaining to the information requested below or the validation study, please do not hesitate to contact Yildiz Chambers at 703.461.2165 or yildiz.chambers@dyncorp.com.

Section 1. Laboratory Capabilities and Experience

a. Please complete the requested capabilities and experience information below. The information requested in Table 1 pertains to experience with a given method, **regardless of matrix (i.e., surface water, wastewater) analyzed**.

Table 1. Analyst Experience

Analyst	Years of experience or estimated number of samples analyzed						
	Methods to be validated		Other membrane filter methods				
	modified mTEC	mEI	mEndo or LES Endo	mFC	NA-MUG	mTEC	mE/EIA

b. Primary analyst's name: _____

c. Primary analyst's years of experience performing wastewater analyses: _____

d. What certifications does your laboratory have for microbial analyses? _____

_____

e. Additional comments: _____

Section 2. Background Information

a. Does your laboratory have access to wastewater samples? Yes No
If yes, please indicate in Table 2 or Table 3, as appropriate, the types of wastewater to which you have access.

b. If your laboratory has experience analyzing wastewater samples for *E. coli* and/or enterococci, in **Table 2**, place a check "✓" next to the wastewater type(s) to which you have access and indicate the method(s) used for analysis and typical concentrations of each analyte. If your laboratory does not have experience analyzing wastewater samples for *E. coli* and/or enterococci, please complete Table 3.

Table 2. *E. coli* and Enterococci

Access?	Wastewater type	Monitoring frequency	*E. coli*		Enterococci	
			Methods	Typical range	Methods	Typical range
Example ✓	**Primary treated**	**1 per month**	**SM 9221B/F**	**30×10^5**	**SM 9230B**	**12×10^3**
	Raw					
	Primary treated					
	Secondary treated					
	Tertiary treated					
	Chemically disinfected					

c. If your laboratory has experience analyzing wastewater samples for total coliforms, fecal coliforms, fecal streptococci, or other indicator organisms, in **Table 3**, place a check "✓" next to the wastewater type(s) to which you have access and indicate the method(s) used for analysis and typical ranges.

Table 3. Other Indicator Organisms

Access ?	Wastewater type	Monitoring frequency	Other indicator organisms		
			Organism(s)	Methods	Typical range
	Raw				
	Primary treated				
	Secondary treated				
	Tertiary treated				
	Chemically disinfected				

d. How many membrane filtration funnels will be available for use during the study? _____

e. How many funnels may be used at one time (i.e., the size of the manifold that will be used to analyze samples 3, 6, etc.)? _____

f. In Table 3, below, please indicate the medium used for isolation prior to inoculation of each type of verification procedure used in your laboratory.

Table 3. Verification Procedures

Verification procedure	Isolation medium
API 20E®	
VITEK®	
BIOLOG	
BBL Crystal™	
Other (please describe below)*	

*If other, please describe: _____

g. Is your laboratory potentially interested in verifying isolates from other laboratories? Yes No

Section 3. Referee Laboratory

If your laboratory is interested in participating in this study as the referee laboratory, please respond to the following questions.

a. Has your laboratory served as a referee laboratory for other studies? If yes, please briefly describe the study or studies.

b. Does your laboratory have experience preparing and enumerating spiking suspensions? *(Please provide a brief description including organism(s) and associated study.)*

b. Does your laboratory have experience isolating and propagating environmental (wild type) strains from environmental samples? *(Please provide a brief description including organism(s) and associated sample(s).*

c. Is your laboratory's shipping and receiving department familiar with the shipment of dangerous goods? *(Dangerous goods shipping containers and associated shipping documentation will be provided.)*

d. If preparation of spiking suspensions is necessary, does your laboratory have sufficient personnel, supplies (e.g., flasks, etc.), and equipment (e.g., incubator space) to propagate spiking suspensions (*E. coli* and enterococci) and ship the suspensions to the participant laboratories?

e. If the participant laboratories spike samples with wastewater from their own facility, does your laboratory have sufficient personnel, supplies and equipment to enumerate spikes in triplicate for each participant?

f. Additional comments: _____

_____

_____

I certify that the information provided above is accurate and complete:

Primary Analyst or Lab Manager: _____

Laboratory name: _____

Signature: _____

Date: _____