Results of the Interlaboratory Testing Study for the Comparison of Methods for Detection and Enumeration of Enterococci and *Escherichia coli* in Combined Sewer Overflows (CSOs)

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Volunteer Referee Laboratory

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Volunteer Participant Laboratories

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Disclaimer

Mention of company names, trade names, or commercial products does not constitute endorsement or recommendation for use.

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Executive Summary

In 2001, EPA proposed several methods for both enterococci and *Escherichia coli* (*E. coli*) (FR 66: 45811) evaluation in ambient waters. During the comment period for this proposal, National Pollutant Discharge Elimination System (NPDES) permit holders and others requested that EPA promulgate one or more methods for these organisms for the evaluation of wastewater effluents (effluents) and combined sewer overflows (CSOs). We promulgated Enterococci and *E. coli* methods for effluents in 2007 (FR 72:14220), but not for discharges that include CSOs. This report describes our evaluation of the promulgated methods applied to CSO matrices. Previously there was no data on method performance in CSO matrices. Now we have data from five laboratories that may be used as a starting point for end users. These data include quality control criteria for CSO matrices and PBS samples spiked at low and high levels.

During rain events, a combined sewer overflow (CSO) event may occur. A CSO is defined as a discharge from a combined sewer system (i.e., a wastewater collection system owned by a State or municipality which conveys sanitary wastewaters and storm water through a single-pipe system to a publicly owned treatment works (POTW)) at a point prior to the POTW treatment plant. CSOs are point sources that, compared to typical wastewater effluent, often contain high levels of pathogenic microorganisms because they contain stormwater, untreated human and industrial waste, toxic materials, and debris. These CSO discharges are subject to NPDES permit requirements.

Method 1106.1 [Enterococci in Water by Membrane Filtration Using membrane-Enterococcus-Esculin Iron Agar (mE-EIA)], Method 1600 [Enterococci in Water by Membrane Filtration Using membrane-Enterococcus Indoxyl-β-D Glucoside Agar (mEI)], Method 1103.1 [*Escherichia coli* (*E. coli*) in Water by Membrane Filtration Using membrane-Thermotolerant *Escherichia coli* Agar (mTEC)], and Method 1603 [*Escherichia coli* (*E. coli*) in Water by Membrane Filtration Using Modified membrane-Thermotolerant *Escherichia coli* Agar (Modified mTEC)] were followed during the study. The purposes of the Study were to characterize method performance (precision and recovery) across multiple laboratories and Combined Sewer Overflow matrices, and develop quantitative quality control (QC) acceptance criteria.

Seven volunteer participant laboratories, two verification laboratories, and one referee laboratory participated in the Study which was conducted over a period of almost three years from March 2005 through December 2007. Usable data sets were obtained from five of the seven labs. During the study, each laboratory spiked samples with laboratory-prepared *Enterococcus faecalis* (ATCC #19433) and *Escherichia coli* (ATCC #11775) suspensions. High (500,000 *E. faecalis* or 3,000,000 *E. coli* CFU/100 mL) and low (3000 CFU/100 mL) spike levels were used, depending on the CSO type: disinfected CSO matrices were spiked with the low level spike, while total bypass CSO samples were spiked with the high-level spike. Samples were spiked in accordance with study-specific spiking protocols. Results from unspiked and spiked CSO and PBS samples were used to assess method performance.

The mean percent recovery of enterococci for Method 1106.1 in disinfected wastewater and CSO's were equivalent (86.3% and 86.8%, respectively). For the *Enterococcus* Method 1600, the mean percent recovery was higher in disinfected wastewater (90.8%) than in CSO's (66.3%). Recovery using either *E. coli* method 1103.1 (57.8% in disinfected wastewater, 81% in CSO) or 1603 (67% in disinfected wastewater, 91.7% CSO) was better when the method was used for disinfected wastewater than in CSO's.

Section 1.0 Background

Combined sewer overflow (CSO) events are discharges from municipal sewer systems or treatment plants that occur when the volume of wastewater exceeds the system's capacity due to periods of heavy rainfall or snow melt. During periods of increased flows treatment processes may be altered to maintain the plants' integrity. Enterococci and *Escherichia coli* (*E. coli*) analyses are recommended as an indication of recreational water quality. Epidemiological studies have led to the development of criteria which have been used to establish recreational water standards based on established relationships between health effects and water quality. Methods for monitoring these bacterial water quality indicators have recently been approved for disinfected wastewaters and/or ambient/recreational waters. National Pollutant Discharge Elimination System (NPDES) permit holders and others have requested that EPA validate one or more methods for the evaluation of enterococci and *E. coli* in CSO effluents (discharges). The methods evaluated included EPA Methods 1106.1 (mE/EIA) and 1600 (mEI) and for enterococci, and 1103.1 (mTEC) and 1603 (modified mTEC) for *E. coli* (References 9.1– 9.4).

1.1 Summary of Methods

1.1.1 Enterococci Test Methods

In EPA Method 1106.1 (Reference 9.1), a water sample is filtered through a 0.45 µm pore-size membrane. Following filtration, the membrane is placed on a selective medium, mE agar, and incubated at 41.0ºC ± 0.5ºC for 48 ± 3 hours. Following incubation, the filter is transferred to a differential medium, EIA agar, and incubated at 41.0ºC ± 0.5ºC for an additional 20-30 minutes. Pink to red colonies that develop a black or reddish-brown precipitate on the underside of the filter are considered enterococci.

In EPA Method 1600 (Reference 9.2), a water sample is filtered through a 0.45 µm pore-size membrane. After filtration, the membrane is placed on a selective medium, mEI agar, and incubated at 41.0°C ± 0.5°C for 24 ± 2 hours. All colonies greater than 0.5 mm in size that produce a blue halo (regardless of color) are considered enterococci.

1.1.2 *E. coli* Test Methods

In EPA Method 1103.1 (Reference 9.3), a water sample is filtered through a 0.45 µm pore-size membrane. After filtration, the membrane is placed on a selective medium, mTEC, incubated at 35.0°C ± 0.5°C for 2 ± 0.5 hours to resuscitate injured or stressed bacteria, and then incubated at 44.5°C ± 0.2°C for 22 ± 2 hours. Following incubation, the filter is transferred to a filter pad saturated with urea substrate and left at room temperature for 15-20 minutes. All yellow, yellow-green or yellow-brown colonies are considered *E. coli*.

In EPA Method 1603 (Reference 9.4), a water sample is filtered through a 0.45 µm pore-size membrane. After filtration, the membrane is placed on a selective medium, modified mTEC agar, incubated at 35.0°C ± 0.5°C for 2 ± 0.5 hours, and then incubated at 44.5°C ± 0.2°C for 22 to 24 hours. All red and magenta colonies are considered *E. coli*.

Section 2.0 Study Objectives and Study Design

2.1 Study Objectives

The following objectives were established for the Study:

- Characterize the stability of *E. faecalis* and *E. coli* laboratory-prepared spiking suspensions.
- Characterize the sensitivity and specificity of each individual method across multiple laboratories and CSO matrices through the assessment of false positive and negative rates.
- Characterize the accuracy (recovery and precision) of each individual method across multiple laboratories and CSO matrices.
- Establish quantitative QC acceptance criteria for CSO matrix spike recoveries for each method.
- Compare performance of the enterococci methods (1106.1 and 1600).
- Compare performance of the *E. coli* methods (1103.1 and 1603).

The following data quality objectives were established for the Study:

- Generate a minimum of 6 sets of valid data from participant laboratories for each method.
- Data produced under this study must be generated according to the analytical and QA/QC procedures in each of the analytical methods or approved changes to these procedures to ensure that data is of known and reliable quality, and allows EPA to use the results of the study to identify any need for further revision of the method.

2.2 Technical Approach

Details on the technical approach for conducting this study are provided in Sections 2.2.1 to 2.2.5, below.

2.2.1 Laboratory-Prepared Spike Stability Assessment

Laboratory-prepared spiking suspension stability was assessed by the referee-laboratory prior to the CSO validation study to determine viability of the suspensions over a four-day period. The purpose of this assessment was to determine how frequently laboratories need to propagate laboratory-prepared spiking suspensions in preparation for a potential CSO event. Assessment of laboratory-prepared spiking suspension stability involved the enumeration of triplicate *E. faecalis* (ATCC #19433) and *E. coli* (ATCC #11775) laboratory-prepared spiking suspensions at 24, 48, 72, and 96 hours after inoculation.

E. coli. The laboratory enumerated three replicate laboratory-prepared *E. coli* spiking suspensions by the following procedures.

- Spread plate technique using tryptic soy agar (TSA) plates (CSO validation study spiking protocol)
- EPA Method 1603 (modified mTEC)
- EPA Method 1103.1 (mTEC)

E. faecalis. The laboratory enumerated three replicate laboratory-prepared *E. faecalis* spiking suspensions by the following procedures:

- Spread plate technique using TSA plates (CSO validation study spiking protocol)
- EPA Method 1600 (mEI)
- EPA Method 1106.1 (mE/EIA)

2.2.2 Identification of Qualified Analytical Laboratories

Participant laboratories were chosen to be representative of the general user community, with experience analyzing wastewater or ambient water samples for enterococci and *E. coli* using membrane filtration techniques, and with access to representative CSO matrices. A detailed Laboratory Capabilities Checklist was used to collect this information from laboratories and to screen potential participants to ensure that laboratories were qualified.

2.2.2.1 Referee Laboratory [EPA Office of Research and Development (ORD)/National Risk Management Research Laboratory (NRMRL)]

Prior to the validation study the referee laboratory evaluated the stability of *E. coli* and *E. faecalis*. Based on these results laboratories were instructed to propagate a "fresh" spiking suspension every fourth day.

2.2.2.2 Participant Laboratories

Participant laboratories analyzed samples to provide EPA with the data necessary to assess method performance and develop QC acceptance criteria. Participant laboratories were representative of the general user community, with some experience analyzing CSO samples using Methods 1600, 1106.1, 1603 and 1103.1. Participants also needed to have access to a representative CSO matrix within driving distance (2 hours) of the laboratory to ensure that holding times were met. A detailed Laboratory Capabilities Checklist (Appendix A) was used to collect information from laboratories and screen potential participants to ensure that laboratories were qualified. Laboratory availability was also considered.

2.2.2.3 Verification Laboratories

Verification laboratories were required to have access to a Vitek® and experience characterizing either isolates using the Vitek® system and gram-negative plus (GNI+) and/or gram-positive (GPI) cards.

To reduce cost, volunteer laboratories were recruited. To reduce the burden on participant laboratories and to encourage volunteer participants, EPA provided the media, reagents, and disposable supplies needed for stability assessment, validation and verification. Unlike other EPA method validation studies, prepared plates (mE, EIA, mEI, mTEC, and modified mTEC) were provided to the laboratories to ensure media was readily available when a CSO event occurred and to reduce the burden on the laboratories. Use of dehydrated media would have required laboratories to prepare fresh media every two weeks.

2.2.3 Sample Collection

Samples were collected at the point of plant effluent discharge for this study. For untreated discharges, a 2-L bulk sample was collected and for disinfected discharges, a 3-L bulk sample was collected. Samples were held at <10ºC and above freezing prior to analysis and analyzed within 6 hours of sample collection. Total bypass samples are those that were discharged without any treatment and secondary bypass disinfected samples are those that went through primary treatment, disinfected, and then discharged.

2.2.4 Laboratory-Prepared Spiking Suspensions

Every fourth day that there was potential for a CSO event, participant laboratories propagated fresh spiking suspensions of *E. coli* (ATCC #11775) and *E. faecalis (*ATCC #19433) to ensure a culture would be available when a CSO event occurred. After incubation, laboratory-prepared suspensions were stored in the refrigerator at <10ºC and above freezing until a new suspension was propagated.

To determine the "true spike concentration" during the validation study, the participant laboratories were directed to enumerate *E. coli* (ATCC #11775) and *E. faecalis (*ATCC #19433) laboratory-prepared spiking suspensions on the same day that the validation study samples were spiked and analyzed. Samples were spiked with laboratory-prepared spiking suspensions according to the CSO Spiking Protocol (Appendix B).

- For <u>disinfected</u> CSO matrices, a 100-mL aliquot of each replicate was spiked with 3×10^{-4} mL of undiluted spiking suspension of *E. faecalis* (ATCC #19433) or with 3×10^{-5} mL of undiluted spiking suspension of *E. coli* (ATCC #11775), resulting in an approximate spike of 3000 CFU/100 mL.

- For <u>total bypass</u> CSO matrices, a 100-mL aliquot of each replicate was spiked with 5.0×10^{-2} mL of undiluted spiking suspension of *E. faecalis* (ATCC #19433) or with 3.0×10^{-2} mL of undiluted spiking suspension of *E. coli* (ATCC #11775), resulting in an approximate spike of 500,000 CFU or 3,000,000 CFU/100 mL, respectively.

  For sterile PBS samples spiked at a low-level, a 100-mL aliquot of each replicate was spiked with 3×10^{-4} mL of undiluted spiking suspension of *E. faecalis* (ATCC #19433) or with 3×10^{-5} mL of undiluted spiking suspension of *E. coli* (ATCC #11775), resulting in an approximate spike of 3000 CFU/100 mL .

- For sterile PBS samples spiked at a high-level, a 100-mL aliquot of each replicate was spiked with 5.0×10^{-2} mL of undiluted spiking suspension of *E. faecalis* (ATCC #19433) or with 3.0×10^{-2} mL of undiluted spiking suspension of *E. coli* (ATCC #11775), resulting in an approximate spike of 500,000 or 3,000,000 CFU/100 mL, respectively.

2.2.5 Validation Study Sample Analyses

During the validation study, all four methods (1103.1, 1106.1, 1600 and 1603) were used to analyze unspiked and spiked CSO and PBS samples at multiple laboratories. **Table 1** summarizes the number and type of samples that were evaluated to meet the objectives listed in Section 2.

Table 1. Number of Sample Analyses per Laboratory and Method for the Combined Sewer Overflow (CSO) Validation Study

Matrix	Spiking Description	No. of Samples per Method	Filters per Sample	Isolate Verification	Purpose of Analysis
Disinfected Wastewater	Unspiked	1	5	N/A	Preliminary analyses
Disinfected Wastewater	Lab-prepared	1	5	N/A	Preliminary analyses
Sterile PBS[1]	BioBalls	4	1	N/A	QC check for initial precision and recovery (IPR)
Sterile PBS[1]	Lab-prepared	1	3	N/A	Preliminary analyses
CSO	Unspiked	4	3 - 5	20 typical & 20 atypical per sample	False positive and negative rates
					Evaluation of ambient background bacteria concentrations
CSO	Lab-prepared (high or low spike level, dependent on CSO type)	4	3 - 5	N/A	Assessment of method performance and development of QC criteria
Sterile PBS	Lab-prepared: low-level spike	3	3	N/A	Assessment of method and laboratory performance
	Lab-prepared: high-level spike	3	5	N/A	Assessment of method and laboratory performance
Sterile PBS	Unspiked	1	1	N/A	QC check

[1] Phosphate buffered saline

2.2.5.1 Preliminary Analyses

Preliminary analyses were conducted using unspiked disinfected effluent, spiked disinfected effluent (low level spike) and spiked PBS samples (low and high level spike) prior to the start of the validation study. In addition, laboratories enumerated referee-prepared *E. faecalis* and *E. coli* spiking suspensions using TSA plates and the spread plate technique.

2.2.5.2 Quality Control (QC) Analyses

Participating laboratories completed the following QC analyses: media sterility checks, dilution water sterility checks, filter sterility check, filtration blanks, positive controls, and negative controls.

- **Methods 1600 and 1106.1**. *E. faecalis* (ATCC #19433) served as the positive control and *E. coli* (ATCC #11775) as the negative control.
- **Methods 1603 and 1103.1**. *E. coli* (ATCC #11775) served as the positive control and *E. faecalis* (ATCC #19433) as the negative control.

2.2.5.3 Assessment and Comparison of Method Sensitivity and Specificity

The sensitivity and specificity of each method was assessed through the evaluation of false positive and false negative rates. Each of the participant laboratories evaluated four unspiked CSO samples for false positive/negative results by submitting five typical and five atypical colonies from each of the four CSO samples analyzed by each method to verification through biochemical evaluation.

The verification procedure is as follows:

- For each colony submitted to verification, the laboratory streaked the colony onto a TSA slant and incubated the slant at 35.0ºC ± 0.5ºC for 24 ± 2 hours. Tubes were labeled with sample identification information and colony type. Sample identification, colony type, and morphology were recorded on the colony-specific tracking form provided to the laboratories.

- To prepare slants for shipping, participant laboratories wrapped the edges of the tubes with parafilm and wrapped the stack of tubes associated with each sample with bubble wrap. Tubes were placed into a cooler lined with a trash bag and were surrounded by blue ice. The cooler was sealed with shipping tape. FedEx shipping documents were provided and the cooler was shipped to the appropriate verification laboratory which conducted verifications using the Vitek® automated identification system.

- To minimize verification laboratory burden, verifications were conducted at two laboratories. One laboratory verified all *E. coli* isolates and the other verified all enterococci isolates. Each verification laboratory verified typical and atypical colonies for each method using the Vitek®.

Note: The Vitek® automated identification system is a fully automated system that performs bacterial identification of isolates using fluorescent technology. After primary isolation, an isolated colony is prepared at a known optical density in saline and inoculated into the Vitek® system. The gram negative card contains 41 fluorescent biochemical tests that are read every 15 minutes. Algorithms are used for organism identification.

The false positive rates were calculated as the percentage of positive results submitted to confirmation for which the target organism was confirmed to not be present. The false negative rates were calculated as the percentage of negative results submitted to confirmation for which the target organism was confirmed to be present.

2.2.5.4 Assessment and Comparison of Method Accuracy (Precision and Recovery)

Method precision and recovery were evaluated through the analysis of CSO and PBS samples spiked with laboratory-prepared spikes. All laboratories spiked three PBS samples at each of two levels, high and low, as described below. Each laboratory spiked four CSO samples at either a high or low level, dependent on the type of discharge (e.g. secondary bypass disinfected, total bypass) and utility-specific historical data.

Recoveries were assessed by comparing spike recovery (concentration in the spiked samples minus the ambient/unspiked concentration) to the “true” spiked value. Precision was assessed based on the relative standard deviation of the four replicate recoveries.

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

One of the goals of this study was to develop quantitative QC criteria for matrix spikes for use in assessing CSO matrix interferences, for use in CSO samples. To collect the data necessary to develop these criteria, each participant laboratory analyzed four CSO samples per method spiked with laboratory-prepared spiking suspensions. The spiking approach was determined based on the type of CSO being evaluated (e.g. secondary bypass disinfected, total bypass) and utility-specific historical data. Typically, 3-5 filters were analyzed per sample.

However, due to the limited number of valid data sets generated during this study, QC criteria were not established.

(Note that for each method, the same four CSO samples spiked with laboratory-prepared spiking suspensions were used to assess method accuracy and to obtain data to develop quantitative QC criteria for matrix spike recoveries.)

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 was performed by a contractor to EPA, the CSC Microbiology and Biochemistry Studies Group.

3.2 Schedule

Each laboratory analyzed initial precision and recovery (IPR) samples and met criteria for all four methods prior to conducting Study analyses. Study analyses could not be scheduled because sample collection/analyses were dependent on weather events. When laboratories analyzed for both analytes (enterococci and *E. coli*), the CSO sample analyzed was from a single event.

The duration of the study was March 2005 to December 2007. After almost three years the study was considered completed, as CSO events were so sporadic.

3.3 Research and Participant Laboratories

The participating laboratories involved in the Study are shown in **Table 2**.

Table 2. Combined Sewer Overflow (CSO) Study Participant Laboratories [a]

Lacey, Olympia, Turnwater, and Thurston (LOTT) Alliance Paula Williamson and Paul Jue 500 Adams St, NE, Olympia, WA 98501	**Massachusetts Water Resources Authority** Steve Rhode and Mariya Gofshteyn 190 Tafts Ave, Winthrop, MA 02152
Narragansett Bay Commission John Motta, Lisa Andrade, and Walter Palm 1 Service Road, Laboratory Building, Providence, RI 02905	**Pittsburgh Water and Sewer Authority** Stanley States and Jay Kuchta 900 Freeport Rd, Pittsburgh, PA 15238
University of Iowa Hygienic Laboratory Nancy Hall and Cathy Lord Hygienic Laboratory 102, Oakdale Campus #H101 OH Iowa City, IA 52242	**Virginia Consolidated Laboratories** Debbie Paul, Bob Sulouff, Tom York, Jim Pearson, and Grier Mills 600 N 5th Street, Richmond, VA 23219
Wisconsin State Laboratory of Hygiene Sharon Kluender, Linda Peterson, and Jeremy Olstadt 435 Stovall Building 465 Henry Mall, Madison, WI 53706	
Referee Laboratory:	**EPA Office of Research and Development (ORD)** **National Risk Management Research Laboratory (NRMRL)** Mark C. Meckes and Laura Boczek 26 West Martin Luther King Drive, Cincinnati, OH 45268
Verification Laboratory A:	**City of Los Angeles, Bureau of Sanitation, Environmental Monitoring Division** Gerald McGowen, Stan Asato, Ioannice Lee, Hung Pham, Pauline Nguyen, and Marieta Ravelo Hyperion Treatment Plant 12000 Vista del Mar, TSF Rm 452, Playa del Rey, CA 90293
Verification Laboratory B:	**Orange County Sanitation District (OCSD)** Charlie McGee, Michael von Winckelman and Kim Patton 10844 Ellis Ave, Fountain Valley, CA 92708-7018

[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 CSC Microbiology and Biochemistry Studies Group for review and validation:

- Completed cover sheet with sample collection and QC information
- Completed sample-specific reporting forms
- Completed calculations spreadsheets
- Colony-specific tracking forms for typical and atypical isolates
- Printouts from Vitek® analyses to confirm colony identity
- Documentation of any additional information that would assist in evaluating the data

4.2 Data Validation

The CSC Microbiology and Biochemistry Studies Group used data review checklists to ensure that each data package was complete and 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 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 all procedures were performed according to each method and study-specific instructions
- 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 described below were noted and considered either valid and acceptable or invalid and unacceptable for inclusion in subsequent data analysis.

General Issues

In some instances replacement plates from the media vendor did not arrive prior to a CSO event due to manufacturing issues (e.g., back orders, custom orders) requiring laboratories to use expired media to analyze samples or wait for another event. In these cases QC checks (positive and negative controls) were evaluated to determine if data was considered valid or invalid. Please see below for specific data validation issues including expired media.

Enterococci (Methods 1106.1 and 1600)

Laboratory 1

- Although mE and EIA prepared plates exceeded the manufacturer's expiration date by 13 and 15 days, respectively, all positive and negative controls exhibited appropriate responses, and thus data was considered valid and included in subsequent data analyses.

Laboratory 4

- TSA plates were incubated for 46 hours, 22 hours longer than specified in the spiking protocol. Given that the TSA plate counts were consistent with previous results and other laboratories and many protocols using TSA require a 48 ± 3 hour incubation, TSA enumerations were considered valid and included in subsequent data analyses.
- Although mE and mEI prepared plates exceeded the manufacturer's expiration date by 5 and 6 days, respectively, all positive and negative controls exhibited appropriate responses, and thus data was considered valid and included in subsequent data analyses.

Laboratory 7

- The pH of PBS used (6.94) was below the accepted range for the methods (7.4 ± 0.2). However, positive and negative controls showed appropriate responses; therefore, data were considered valid and included in the subsequent data analyses.

E. coli **(Methods 1103.1 and 1603)**

Laboratory 1

- Although modified mTEC prepared plates exceeded the manufacturer's expiration date by 13 days, all positive and negative controls exhibited appropriate responses, and thus data was considered valid and included in subsequent data analyses.

Laboratory 6

- Dilutions evaluated for unspiked CSO samples did not produce reliable counts due to high levels of background organisms and therefore could not be used to accurately characterize target concentrations. Therefore data was considered invalid and not included in subsequent data analyses.

Laboratory 7

- The pH of the PBS used (6.94) was below the accepted range (7.4 ± 0.2). However, positive and negative controls showed appropriate responses; therefore data were considered valid and included in the subsequent data analyses.

Section 5.0 Results

This section includes results for Methods 1106.1 and 1600 unspiked (Section 5.1) and spiked (Section 5.2) Combined Sewer Overflow (CSO) matrices, Methods 1103.1 and 1603 unspiked (Section 5.3) and spiked (Section 5.4) CSO matrices, and results for Methods 1106.1 and 1600 (Section 5.5) and Methods 1103.1 and 1603 (Section 5.6) for spiked PBS matrices. Only valid results are included in this section; a detailed description of data invalidation information is included in Section 4.

5.1 Enterococci: Method 1106.1 and 1600 Unspiked Combined Sewer Overflow Sample Results

Results from unspiked CSO sample analyses are provided in **Table 3**. The unspiked CSO data were used to estimate the background concentration of enterococci in CSO samples.

Results of the verification analyses were used to assess method performance (see discussion in Section 6). Laboratory-specific verification results are summarized in **Tables 4** and **5**. Any typical colony that was identified as non-enterococci by the Vitek® was considered a false positive confirmation result. Any atypical colony that was identified as enterococci by the Vitek® was considered a false negative confirmation result. Colonies that did not grow after streaking for growth onto TSA slants and isolates that did not grow at the verification laboratory were treated as if they had not been submitted to verification and eliminated from subsequent data analyses.

Table 3. Summary of Valid, Enterococci Results for Unspiked Combined Sewer Overflow (CSO) Samples

Method	Lab	[a] CFU/100 mL by Sample				Mean CFU/100 mL	SD [b]	RSD [c] (%)
		1	2	3	4			
1106.1	1	200	1300	1200	1500	1050	580.2	55.3
	4	340,000	430,000	330,000	350,000	362,500	45,734.7	12.6
	7	1200	1500	1800	2100	1650	387.3	23.5
1600	1	800	630	1000	900	833	157.8	19
	4	270,000	240,000	260,000	270,000	260,000	14,142.1	5.4
	7	9000	16,000	12,000	16,000	13,250	3403.4	25.7
1106.1 Overall (n = 12)						**121,733**	**30,493.4 [d]**	**40.9 [e]**
1600 Overall (n = 12)						**91,361**	**9697.8 [d]**	**22.6 [e]**

[a] Colony forming unit
[b] Standard deviation
[c] Relative standard deviation
[d] Pooled within-lab standard deviation was determined by calculating the square root of the mean of the lab variances
[e] Pooled within-lab relative standard deviation was determined by calculating the square root of the mean of the squared lab RSDs

Table 4. Method 1106.1 Laboratory-Specific False Positive and False Negative Confirmation Rates for Unspiked Combined Sewer Overflow (CSO) Samples

Laboratory	Typical Colonies Submitted	No. False Positive Colonies	False Positive Confirmation Rate (%)	Atypical Colonies Submitted	No. False Negative Colonies	False Negative Confirmation Rate (%)
1	20	0	0	0	0	
4	20	3	15	20	7	35
7	20	0	0	20	0	0
Overall	60	3	**5**	40	7	**17.5**

Table 5. Method 1600 Laboratory-Specific False Positive and False Negative Confirmation Rates for Unspiked Combined Sewer Overflow (CSO) Samples

Laboratory	Typical Colonies Submitted	No. False Positive Colonies	False Positive Confirmation Rate (%)	Atypical Colonies Submitted	No. False Negative Colonies	False Negative Confirmation Rate (%)
1	18	0	0	0	0	
4	19	0	0	13	4	30.8
7	20	0	0	19	8	42.1
Overall	57	0	**0**	32	12	**37.5**

5.2 Enterococci: Method 1106.1 and 1600 Spiked Combined Sewer Overflow Sample Results

Results from CSO samples spiked with laboratory-prepared *E. faecalis* (ATCC #19433) suspensions (**Table 6**) were used to assess method performance (see discussion in Section 6).

Table 6. Summary of Valid, Enterococci Results for Spiked Combined Sewer Overflow (CSO) Samples

Method	Lab	Spike Level (CFU/100 mL) [a]	Percent Recovery by Sample				Mean Percent Recovery	SD [b]	RSD [c] (%)
			5	6	7	8			
1106.1	1	2800	16	9	9	5	9.8	4.5	45.6
	4	313,333	108	12	172	235	131.7	95.3	72.4
	7	1,850,000	108	130	119	119	118.8	8.8	7.4
1600	1	2800	24	17	17	24	20.3	4.1	20.4
	4	313,333	109	77	109	13	76.6	45.1	58.9
	7	1,850,000	107	102	97	102	102	4.4	4.3
1106.1 Overall (n = 12)							**86.8**	**63.9**	**57.3**
1600 Overall (n = 12)							**66.3**	**30.4**	**41.7**

[a] Colony forming unit
[b] Standard deviation
[c] Relative standard deviation
[d] Pooled within-lab standard deviation was determined by calculating the square root of the mean of the lab variances
[e] Pooled within-lab relative standard deviation was determined by calculating the square root of the mean of the squared lab RSDs

5.3 *E. coli*: Method 1103.1 and 1603 Unspiked Combined Sewer Overflow Sample Results

Results from unspiked CSO sample analyses are provided in **Table 7**. The unspiked CSO data were used to estimate the background concentration of enterococci in CSO samples.

Results of the verification analyses were used to assess method performance (see discussion in Section 6). Laboratory-specific verification results are summarized in **Tables 8** and **9**. Any typical colony that was identified as non- *E. coli* by the Vitek® was considered a false positive confirmation result. Any atypical colony that was identified as *E. coli* by the Vitek® was considered a false negative confirmation result. Colonies that did not grow after streaking for growth onto TSA slants and isolates that did not grow at the verification laboratory were treated as if they had not been submitted to verification and eliminated from subsequent data analyses.

Table 7. Summary of Valid, *E. coli* Results for Unspiked Combined Sewer Overflow (CSO) Samples

Method	Lab	[a] CFU/100 mL by sample				Mean CFU/100 mL	SD [b]	RSD[c] (%)
		1	2	3	4			
1103.1	1	13,200	10,700	11,900	12,100	11,975	1024.3	9
	4	420,000	430,000	430,000	380,000	415,000	23,804.8	6
	5	15	6	15	16	13	4.7	36
	7	30,000	10,000	30,000	20,000	22,500	9574.3	43
1603	1	20,400	13,200	12,700	15,300	15,400	3518.5	23
	4	570,000	640,000	680,000	630,000	630,000	45,460.6	7
	5	8	5	8	13	8.5	3.3	39
	7	30,000	10,000	30,000	20,000	22,500	9574.3	43
1103.1 Overall (n = 16)						**112,372**	**14,590.9 [d]**	**33 [e]**
1603 Overall (n = 16)						**166,977**	**26,899.3 [d]**	**36 [e]**

[a] Colony forming unit
[b] Standard deviation
[c] Relative standard deviation
[d] Pooled within-lab standard deviation was determined by calculating the square root of the mean of the lab variances
[e] Pooled within-lab relative standard deviation was determined by calculating the square root of the mean of the squared lab RSDs

Table 8. Method 1103.1 Laboratory-Specific False Positive and False Negative Confirmation Rates for Unspiked Combined Sewer Overflow (CSO) Samples

Laboratory	Typical Colonies Submitted	No. False Positive Colonies	False Positive Confirmation Rate (%)	Atypical Colonies Submitted	No. False Negative Colonies	False Negative Confirmation Rate (%)
4	15	3	20	20	0	0
5	20	4	20	20	1	5
7	20	3	15	20	1	5
Overall	55	10	**18.2**	60	2	**3.3**

Table 9. Method 1603 Laboratory-Specific False Positive and False Negative Confirmation Rates for Unspiked Combined Sewer Overflow (CSO) Samples

Laboratory	Typical Colonies Submitted	No. False Positive Colonies	False Positive Confirmation Rate (%)	Atypical Colonies Submitted	No. False Negative Colonies	False Negative Confirmation Rate (%)
1	20	0	0	20	0	0
4	20	4	20	20	1	5
7	20	0	0	20	0	0
Overall	60	4	**6.7**	60	1	**1.7**

5.4 *E. coli*: Method 1103.1 and 1603 Spiked Combined Sewer Overflow Sample Results

Results from CSO samples spiked with laboratory-prepared *E. coli* (ATCC #11775) suspensions (**Table 10**) were used to assess method performance (see discussion in Section 6).

Table 10. Summary of Valid, *E. coli* Results for Spiked Combined Sewer Overflow (CSO) Samples

Method	Lab	Spike Level (CFU/100 mL) [a]	Percent Recovery by Sample				Mean Percent Recovery	SD [b]	RSD[c] (%)
			5	6	7	8			
1103.1	1	2010	4379	7364	3832	8857	6108.2	2401.6	39.3
	4	1,630,000	79	85	67	30	65	24.7	38
	5	3500	88	71	51	88	74.6	17.7	23.7
	7	4,670,000	117	113	79	104	103.4	17.3	16.7
1603	1	2010	6697	2219	8189	2816	4980.1	2918.4	58.6
	4	1,630,000	72	35	96	29	58	31.8	54.9
	5	3500	128	128	128	145	132.6	8.6	6.5
	7	4,670,000	72	81	96	89	84.6	10.3	12.1
1103.1 Overall (n = 16)							**1587.8**	**1386.7 [d]**	**35.7[e]**
1603 Overall (n = 16)							**1313.8**	**1685.1 [d]**	**47 [e]**

[a] Colony forming unit
[b] Standard deviation
[c] Relative standard deviation
[d] Pooled within-lab standard deviation was determined by calculating the square root of the mean of the lab variances
[e] Pooled within-lab relative standard deviation was determined by calculating the square root of the mean of the squared lab RSDs

5.5 Enterococci: Method 1106.1 and 1600 Low- and High-Level Spiked PBS Sample Results

Results from PBS samples spiked with laboratory-prepared *E. faecalis* (ATCC #19433) suspensions (**Tables 11** ad **12**) were used to assess method performance (see discussion in Section 6).

Table 11. Summary of Valid, Enterococci Results for PBS Samples Spiked with Low-Level Spikes

Method	Lab	Spike Level (CFU/100 mL) [a]	Percent Recovery by Sample			Mean Percent Recovery	SD [b]	RSD [c] (%)
			9	10	11			
1106.1	1	2800	29	29	25	27.4	2.1	7.5
	4	1880	64	80	85	76.2	11.1	14.5
	7	11,100	72	126	108	102.1	27.5	27
1600	1	2800	24	29	29	27	2.7	9.9
	4	1880	69	90	85	81.6	11.1	13.6
	7	11,100	72	108	162	114.1	45.3	39.7
1106.1 Overall (n = 9)						**68.6**	**21 [d]**	**22.3 [e]**
1600 Overall (n = 9)						**74.2**	**33.1 [d]**	**30.5 [e]**

[a] Colony forming unit
[b] Standard deviation
[c] Relative standard deviation
[d] Pooled within-lab standard deviation was determined by calculating the square root of the mean of the lab variances
[e] Pooled within-lab relative standard deviation was determined by calculating the square root of the mean of the squared lab RSDs

Table 12. Summary of Valid, Enterococci Results for PBS Samples Spiked with High-Level Spikes

Method	Lab	Spike Level (CFU/100 mL) [a]	Percent Recovery by Sample			Mean Percent Recovery	SD [b]	RSD [c] (%)
			12	13	14			
1106.1	1	466,667	21	19	24	21.4	2.1	10
	4	313,333	105	80	86	90.4	13.3	14.7
	7	1,850,000	108	114	108	109	3.1	2.8
1600	1	466,667	30	41	21	30.7	9.7	31.5
	4	313,333	109	73	112	97.9	21.3	21.7
	7	1,850,000	108	70	119	99.1	25.5	25.8
1106.1 Overall (n = 9)						**73.9**	**9.7 [d]**	**12.7 [e]**
1600 Overall (n = 9)						**75.9**	**24.5 [d]**	**32.6 [e]**

[a] Colony forming unit
[b] Standard deviation
[c] Relative standard deviation
[d] Pooled within-lab standard deviation was determined by calculating the square root of the mean of the lab variances
[e] Pooled within-lab relative standard deviation was determined by calculating the square root of the mean of the squared lab RSDs

5.6 *E. coli*: Method 1103.1 and 1603 Low- and High-Level Spiked PBS Sample Results

Results from PBS samples spiked with laboratory-prepared *E. coli* (ATCC #11775) suspensions (**Tables 13** and **14**) were used to assess method performance (see discussion in Section 6).

Table 13. Summary of Valid, *E. coli* Results for PBS Samples Spiked with Low-Level Spikes

Method	Lab	Spike Level (CFU/100 mL) [a]	Percent Recovery by Sample			Mean Percent Recovery	SD [b]	RSD [c] (%)
			9	10	11			
1103.1	1	2010	6	6	7	6.6	0.8	11.5
	4	1630	86	55	80	73.6	16.2	22.1
	5	3500	60	49	89	65.7	20.6	31.4
	6	1620	62	68	130	86.4	37.6	43.5
	7	4670	94	90	120	101.4	16.2	16
1603	1	2010	6	5	5	5.8	0.6	9.9
	4	1630	61	49	86	65.4	18.7	28.6
	5	3500	149	146	114	136.2	19	14
	6	1620	43	123	68	78	41.4	53
	7	4670	84	109	71	87.8	19.6	22.4
1103.1 Overall (n =15)						**66.8**	**26.6 [d]**	**33.5 [e]**
1603 Overall (n = 15)						**74.6**	**29 [d]**	**36.4 [e]**

[a] Colony forming unit
[b] Standard deviation
[c] Relative standard deviation
[d] Pooled within-lab standard deviation was determined by calculating the square root of the mean of the lab variances
[e] Pooled within-lab relative standard deviation was determined by calculating the square root of the mean of the squared lab RSDs

Table 14. Summary of Valid, *E. coli* Results for PBS Samples Spiked with High-Level Spikes

Method	Lab	Spike Level (CFU/100 mL) [a]	Percent Recovery by Sample			Mean Percent Recovery	SD [b]	RSD [c] (%)
			12	13	14			
1103.1	1	2,010,000	1	1	1	1.2	0.1	8.6
	4	1,630,000	67	92	55	71.6	18.7	26.2
	5	3,500,000	100	109	140	116.2	21.1	18.1
	6	1,620,000	87	302	57	149	133.8	89.8
	7	4,670,000	92	94	88	91.4	3.3	3.6
1603	1	2,010,000	1	1	1	0.7	0.03	4.3
	4	1,630,000	80	86	98	87.9	9.4	10.7
	5	3,500,000	120	117	154	130.5	20.7	15.8
	6	1,620,000	41	414	401	285.2	211.8	74.3
	7	4,670,000	101	84	96	93.5	8.9	9.5
1103.1 Overall (n = 15)						**85.9**	**74.9 [d]**	**52.4 [e]**
1603 Overall (n =15)						**119.6**	**116.8 [d]**	**42.4 [e]**

[a] Colony forming unit
[b] Standard deviation
[c] Relative standard deviation
[d] Pooled within-lab standard deviation was determined by calculating the square root of the mean of the lab variances
[e] 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

This section describes the development of quantitative QC acceptance criteria in a reference matrix (PBS) and the matrix of interest (CSO) to support future assessments of laboratory and method performance. All data analyses described below were performed using the results of PBS and CSO samples spiked with laboratory-prepared suspensions.

6.1 Outlier Analyses

Valid results from samples spiked with laboratory-prepared suspensions were screened for outliers in accordance with the procedures described in American Society for Testing and Materials (ASTM) guidance D2777-98 (Reference 9.5). Due to the small number of laboratories participating in this study, the data could not be screened for outlying laboratories. Grubbs test (Reference 9.5) was performed, which evaluates individual sample results for outlying observations, as described below.

The PBS and CSO data were tested for the presence of individual outlying recoveries using Grubbs test, which was run separately for each matrix, method and spike level without performing any data transformations. Application of Grubbs test resulted in the removal of a single outlying result. This recovery (a high-spiked PBS result determined using Method 1103.1 by laboratory 6) was high-biased compared to the other recoveries, and was not used in the development of QC criteria.

Outlier analyses were only performed for development of QC acceptance criteria (Section 6.2 and 6.3). 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 (i.e., outliers were not removed).

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 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. Separate criteria were determined for low-level and high-level PBS spikes.

The IPR and OPR recovery criteria were calculated based on within and between laboratory variance components (Reference 9.6). 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 9.7).

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 samples (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 laboratories for the given spike level and method
n_i = number of PBS sample results for laboratory i for the given spike level and method
n_T = total number PBS sample results from all laboratories for the given spike level and method

Upper and lower limits for the mean recovery of four IPR samples were then calculated as:

$$X_{mean} \pm t_{0.975;idf)} * is_c$$

Where:

X_{mean} = the mean recovery of all PBS samples, and for the given spiking procedure

df 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 samples 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 laboratories for the given spike level and method
n_i = number of PBS sample results for laboratory i for the given spike level and method
n_T = total number PBS sample results from all laboratories for the given spike level and method

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

$$X_{mean} \pm t_{0.975;odf)} * s_c$$

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) of four PBS sample results. The pooled RSD for each laboratory was calculated by dividing the pooled within-laboratory standard deviation calculated previously by the overall mean recovery for that laboratory and matrix, as shown below:

$$RSD_{pool} = \sqrt{\frac{s_w^2}{X_{mean}}}$$

Where:

S_w and X_{mean} are the pooled within-lab standard deviation and mean recovery calculated above

The maximum RSD was then calculated as:

$$RSD_{\max} = \sqrt{F_{(0.95;3,L)}} * RSD_{pool}$$

Where:

L = the total number of laboratories for the given spike level and method.

The calculated IPR QC acceptance criteria are provided in **Tables 15** and **16**.

Table 15. Calculated Initial Precision and Recovery (IPR) and Ongoing precision and Recovery (OPR) Acceptance Criteria *for E. coli* Methods

Performance test	Method 1103.1 acceptance criteria	Method 1603 acceptance criteria
Low level spike		
IPR		
• Mean percent recovery	detect[1] - 156%	detect – 194%
• Precision (as maximum relative standard deviation of 4 samples)	63%	62%
OPR	detect – 159%	detect – 194%
• percent recovery		
High level spike		
IPR		
• Mean percent recovery	detect - 184%	detect – 349%
• Precision (as maximum relative standard deviation of 4 samples)	43%	154%
OPR	detect – 184%	detect – 397%
• percent recovery [2]		

[1] The term "detect" is used to indicate that the calculated lower limit was negative.
[2] In cases where the OPR recovery criteria were calculated to be tighter than the IPR recovery criteria, the OPR criteria were set to the calculated IPR criteria

Table 16. Calculated Initial Precision and Recovery (IPR) and Ongoing precision and Recovery (OPR) Acceptance Criteria *for Enterococci* Methods

Performance test	Method 1106.1 acceptance criteria	Method 1600 acceptance criteria
Low level spike		
IPR		
• Mean percent recovery	detect[1] - 200%	detect – 210%
• Precision (as maximum relative standard deviation of 4 samples)	55%	80%
OPR	detect – 200%	detect – 210%
• percent recovery [1]		
High level spike		
IPR		
• Mean percent recovery	detect - 259%	detect – 206%
• Precision (as maximum relative standard deviation of 4 samples)	24%	58%
OPR	detect – 259%	detect – 206%
• percent recovery [1]		

[1] The term "detect" is used to indicate that the calculated lower limit was negative.
[2] In cases where the OPR recovery criteria were calculated to be tighter than the IPR recovery criteria, the OPR criteria were set to the calculated IPR criteria

Matrix Spike (MS) Recovery

QC acceptance criteria for matrix spikes (MS) were developed based on laboratory-spiked sample data from the CSO matrices used in the validation study. Separate QC acceptance criteria were calculated for each method.

Recovery criteria were based on estimates of each variance component (between laboratory and within laboratory) and were calculated using PROC MIXED from SAS version 9 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 9.7). For each matrix/spike level, between sample variability could not be separated from between laboratory variability because each laboratory analyzed a different CSO sample, and therefore the estimate of between laboratory variance also includes sample 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 samples (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 laboratories for the given method
n_i = number of CSO sample results for laboratory i for the given method
n_T = total number CSO sample results from all laboratories for the given method

Upper and lower limits for the recovery of MS samples were then calculated as:

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

Where:

X_{mean} = the mean recovery of all CSO samples for the given method, and

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 calculated MS QC acceptance criteria are listed in **Tables 17** and **18.**

Table 17. Calculated Matrix Spike Recovery Acceptance Criteria for *E. coli* Methods

Performance test	Method 1103.1 acceptance criteria	Method 1603 acceptance criteria
Percent Recovery for MS samples	23 – 139%	detect[1] – 206%

[1] The term "detect" is used to indicate that the calculated lower limit was negative.

Table 18. Calculated Matrix Spike Recovery Acceptance Criteria for Enterococci Methods

Performance test	Method 1106.1 acceptance criteria	Method 1600 acceptance criteria
Percent Recovery for MS samples	detect[1] – 275%	detect – 187%

[1] The term "detect" is used to indicate that the calculated lower limit was negative.

Section 7.0 Assessment and Discussion of Method Performance

Method performance was evaluated through the evaluation of precision and recovery in CSO samples and PBS samples spiked with laboratory-prepared spiking suspensions and through the assessment of false positive and false negative rates in unspiked CSO samples. Outlier analyses were not conducted for the assessment of method performance, therefore all valid data were included in the assessment of method performance.

7.1 Enterococci Methods

7.1.1 Method 1106.1

Method 1106.1 Recovery and Precision

Method 1106.1 recovery was characterized by mean laboratory-specific recoveries of enterococci from spiked CSO samples ranging from 9.8% to 131.7%, with an overall mean recovery of 86.8%. Laboratory-specific RSDs for spiked CSO samples ranged from 7.4% to 72.4%, with a pooled, within-laboratory RSD of 57.3%.

Mean laboratory-specific recoveries of enterococci from PBS spiked with low-level spikes ranged from 27.4% to 102.1%, with an overall mean recovery of 68.6%. Laboratory-specific RSDs for PBS samples spiked with low-level spikes ranged from 7.5% to 27.0%, with a pooled, within-laboratory RSD of 22.3%. Mean laboratory-specific recoveries of enterococci from PBS spiked with high-level spikes ranged from 21.4% to 109.9%, with an overall mean recovery of 73.9%. Laboratory-specific RSDs for PBS samples spiked with high-level spikes ranged from 2.8% to 14.7%, with a pooled, within-laboratory RSD of 12.7%.

- Method 1106.1 mean recoveries for Laboratory 1 were considerably lower for all three matrix/spike type combinations with mean recoveries of 9.8% (CSO), 27.4% (PBS low-level), and 21.4% (PBS high-level), skewing overall means.

Method 1106.1 False Positive and Negative Assessment

Laboratory-specific rates false positive confirmation rates ranged from 0% to 15.0%, with an overall false positive confirmation rate of 5%. In contrast, the false negative confirmation rates were higher ranging from 0% to 35%, with an overall false negative confirmation rate of 17.5%. It should be noted that all 7 of the atypical colonies that were identified as a false negative were from one lab, and therefore, from a single matrix. It should also be noted that Laboratory 1 did not have any atypical colonies to submit to verification.

7.1.2 Method 1600

Method 1600 Recovery and Precision

Method 1600 recovery was characterized by mean laboratory-specific recoveries of enterococci from spiked CSO samples ranging from 20.3% to 102%, with an overall mean recovery of 66.3%. Laboratory-specific RSDs for spiked CSO samples ranged from 4.3% to 58.9%, with a pooled, within-laboratory RSD of 41.7%.

Mean laboratory-specific recoveries of enterococci from PBS spiked with low-level spikes ranged from 27% to 114.1%, with an overall mean recovery of 74.2%. Laboratory-specific RSDs for PBS samples spiked with spiked low-level spikes ranged from 9.9% to 39.7%, with a pooled, within-laboratory RSD of 30.5%. Mean laboratory-specific recoveries of enterococci from PBS

spiked with high-level spikes ranged from 30.7% to 99.1%, with an overall mean recovery of 75.9%. Laboratory-specific RSDs for PBS samples spiked with high-level spikes ranged from 21.7% to 31.5%, with a pooled, within-laboratory RSD of 32.6%.

- Method 1600 mean recoveries for Laboratory 1 were considerably lower for all three matrix/spike type combinations with mean recoveries of 20.3% (CSO), 27% (PBS low-level), and 30.7% (PBS high-level), skewing overall means.

Method 1600 False Positive and Negative Assessment

The false positive confirmation rate for all laboratories was 0%. In contrast, laboratory-specific false negative confirmation rates ranged from 30.8% to 42.1%. Eight of the twelve colonies that were identified as a false negative were from one lab, and therefore, from a single matrix. Laboratory 1 did not have any atypical colonies to submit to verification.

Similar to the results observed for unspiked secondary wastewater samples during the validation of Method 1600 in disinfected wastewater (2003), many of the false negatives (atypical colonies submitted to verification which identified as enterococci) were pink to red in color but simply lacked a blue halo (Reference 9.8). The predecessor to EPA Method 1600 for enterococci is EPA Method 1106.1, which uses mE and EIA media. For EPA Method 1106.1, pink to red colonies on mE, which produce a brown precipitate after transfer to EIA are considered positive for enterococci. Tetrazolium chloride (TTC), the reagent responsible for producing pink to red enterococci colonies on mE, is also included as a reagent in mEI.

When evaluating CSO matrices using Method 1600, some pink to red colonies without a halo may be enterococci. These colonies should be verified, especially if large numbers of these colonies are observed in a particular matrix. If very few pink to red colonies are observed in samples from a particular matrix, the high false negative rates observed during this study may be less of a concern.

7.1.3 Comparison of Enterococci Method Performance

Table 19 summarizes results of valid, spiked PBS and spiked CSO results for both Methods 1106.1 and 1600.

Table 19. Summary of Method 1106.1 and 1600 Enterococci Recoveries for Spiked PBS and Combined Sewer Overflow Samples

Method	PBS Low-level Spike			PBS High-level Spike			CSO		
	Mean Recovery (%)	SD [a] (%)	RSD [b] (%)	Mean Recovery (%)	SD [a] (%)	RSD [b] (%)	Mean Recovery (%)	SD [a] (%)	RSD [b] (%)
1106.1	68.6	21	22.3	73.9	40.9	12.7	86.7	63.9	57.3
1600	74.2	33.1	30.5	75.9	38.1	32.6	66.3	30.4	41.7

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

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

Comparison of Methods 1106.1 and 1600 Recovery

Mean recoveries of the two methods were compared using two-way ANOVA models fit separately for each organism, matrix and spike type (i.e., spike source and level). An F-test was run first for each organism, matrix and spike type to test whether there was a significant interaction between laboratory and method (i.e., whether the effect of method on mean recovery differed significantly between laboratories). Where there was no significant laboratory-by-method interaction, an overall F-test was used to assess whether there was a significant difference in mean recovery between methods, controlling for variability between laboratories. Where there was a significant laboratory-by-method interaction, a separate one-way ANOVA model was fit for each laboratory.

There was no significant interaction between lab and method for any of the three matrix/spike type combinations. Therefore, a single comparison of mean recoveries could be made for each matrix and spike type. For all three matrix/spike type combinations, mean recovery did not differ significantly between methods.

Comparison of Methods 1106.1 and 1600 Precision

Comparisons of recovery variability for samples spiked with laboratory-prepared *E. faecalis* spiking suspensions observed for Methods 1106.1 and 1600 were evaluated using F-tests, based on pooled within-laboratory variances. F-tests were performed separately for each matrix (PBS and CSO) and spike type. Based on these analyses, within-laboratory precision was significantly better for Method 1600 (pooled within-laboratory standard deviation equaling 30.4%) than for Method 1106.1 (pooled within-laboratory standard deviation equaling 63.9%) in CSOs. In contrast the within-laboratory variability was better for Method 1106.1 (pooled within-laboratory standard deviation equaling 9.7%) than for Method 1600 (pooled within-laboratory standard deviation 24.2%) in PBS spiked with high-level spikes. The within laboratory variability did not differ significantly for PBS spiked with low-level spikes.

Comparison of Methods 1106.1 and 1600 False Positive and False Negative Confirmation Rates

For CSO matrices, the false negative confirmation rates were higher for Method 1600 (37.5%) compared to Method 1106.1 (17.5%). In contrast, the false positive confirmation rate was higher for Method 1106.1 (5%) compared to Method 1600 (0%).

Table 20. Comparison of Methods 1106.1 and 1600 False Positive and False Negative Confirmation Rates for Unspiked CSO Matrices

Matrix	Method 1106.1		Method 1600	
	False Positive Confirmation Rate (%)	False Negative Confirmation Rate (%)	False Positive Confirmation Rate (%)	False Negative Confirmation Rate (%)
CSO	5.0	17.5	0	37.5

7.2 *E. coli* Methods

7.2.1 Method 1103.1

Method 1103.1 Recovery and Precision
Method 1103.1 was characterized by mean laboratory-specific recoveries of *E. coli* from spiked CSO samples ranging from 65% to 6108.2%, with an overall mean recovery of 1587.8%. Laboratory-specific RSDs for spiked CSO samples ranged from 16.7% to 39.3%, with a pooled, within-laboratory RSD of 35.7%.

Mean laboratory-specific recoveries of *E. coli* from PBS spiked with low-level spikes ranged from 6.6% to 101.4%, with an overall mean recovery of 66.8%. Laboratory-specific RSDs for PBS samples spiked with low-level spikes ranged from 11.5% to 43.5%, with a pooled, within-laboratory RSD of 33.5%. Mean laboratory-specific recoveries of *E. coli* from PBS spiked with high-level spikes ranged from 1.2% to 149%, with an overall mean recovery of 85.9%. Laboratory-specific RSDs for PBS samples spiked with high-level spikes ranged from 3.6% to 89.8%, with a pooled, within-laboratory RSD of 52.4%.

- Method 1103.1 mean recoveries for Laboratory 1 were considerably lower for PBS spiked with low- and high-level spikes 6.6% and 1.2%, respectively, skewing overall means.
- Although Method 1103.1 overall mean recovery in CSO samples was very high, individual laboratory Method 1103.1 mean recoveries for 3 of 4 laboratories were considerably lower, ranging from 65% to 103.4%. Laboratory 1 observed a very high mean recovery of 6108.2%, skewing the overall recoveries. The high recoveries observed by Laboratory 1 could have been due to inaccurate determination of ambient concentrations or an error in spiking the CSO samples.

Method 1103.1 False Positive and Negative Assessment
Laboratory-specific rates false positive confirmation rates ranged from 15% to 20%, with an overall false positive confirmation rate of 18.2%. In contrast, the false negative confirmation rates were lower ranging from 0% to 5%, with an overall false negative confirmation rate of 3.3%.

7.2.2 Method 1603

Method 1603 Recovery and Precision
Method 1603 recovery was characterized by mean laboratory-specific recoveries of *E. coli* from spiked CSO samples ranging from 58% to 4980.1%, with an overall mean recovery of 1313.8%. Laboratory-specific RSDs for spiked CSO samples ranged from 6.5% to 58.6%, with a pooled, within-laboratory RSD of 47%.

Mean laboratory-specific recoveries of *E. coli* from PBS spiked with low-level spikes ranged from 5.8% to 136.2%, with an overall mean recovery of 74.6%. Laboratory-specific RSDs for PBS samples spiked with low-level spikes ranged from 9.9% to 53%, with a pooled, within-laboratory RSD of 36.4%. Mean laboratory-specific recoveries of *E. coli* from PBS spiked with high-level spikes ranged from 0.7% to 285.2%, with an overall mean recovery of 119.6%. Laboratory-specific RSDs for PBS samples spiked with high-level spikes ranged from 4.3% to 74.3%, with a pooled, within-laboratory RSD of 42.4%.

- Method 1603 mean recoveries for Laboratory 1 were considerably lower for PBS spiked with low- and high-level spikes 5.8% and 0.7%, respectively, skewing overall means.

- Although Method 1603 overall mean recovery in CSO samples was very high, individual laboratory Method 1603 mean recoveries for 3 of 4 laboratories were considerably lower, ranging from 58% to 132.6%. Laboratory 1 observed a very high mean recovery of 4980.1%, skewing the overall recoveries. The high recoveries observed by Laboratory 1 could have been due to inaccurate determination of ambient concentrations or an error in spiking the CSO samples.

Method 1603 False Positive and Negative Assessment

Laboratory-specific rates false positive confirmation rates ranged from 0% to 20%, with an overall false positive confirmation rate of 6.7%. In contrast, the false negative confirmation rates were lower ranging from 0% to 5%, with an overall false negative confirmation rate of 1.7%.

7.2.3 Comparison of *E. coli* Method Performance

Table 21 summarizes results of valid, spiked PBS and spiked CSO results for both Methods 1103.1 and 1603.

Table 21. Summary of Method 1103.1 and 1603 *E. coli* Recoveries for Spiked PBS and Combined Sewer Overflow Samples

Method	PBS Low-level Spike			PBS High-level Spike			CSO		
	Mean Recovery (%)	SD [a] (%)	RSD [b] (%)	Mean Recovery (%)	SD [a] (%)	RSD [b] (%)	Mean Recovery (%)	SD [a] (%)	RSD [b] (%)
1103.1	68.6	21	22.3	73.9	9.74	12.7	1587.8	6795.7	35.7
1603	74.2	33	30.5	75.9	24.5	32.6	1313.8	1685.1	47

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

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

Comparison of Methods 1103.1 and 1603 Recovery

Mean recoveries of the two methods were compared following the same methodology as used in comparing enterococci method performance (Section 6.1.3).

There was a significant interaction between lab and method for high-level laboratory-prepared spiking suspensions in PBS samples. Therefore, a separate comparison of mean recoveries was performed for each of the five laboratories. Based on these separate comparisons, a significant difference in mean recovery between the methods was observed in samples analyzed by Laboratory 5, with mean recovery being significantly greater for samples analyzed by Method 1603. No significant difference in mean recovery between methods was observed in samples analyzed by the other laboratories.

For CSO samples and low-level laboratory-prepared spiking suspensions in PBS samples, no significant interaction between lab and method was observed. Therefore, a single comparison of mean recoveries could be made for these two matrix/spike type combinations. In each case, mean recovery did not differ significantly between methods.

Comparison of Methods 1103.1 and 1603 Precision

Comparisons of recovery variability for samples spiked with laboratory-prepared *E. coli* spiking suspensions observed for Methods 1103.1 and 1603 were evaluated using F-tests, based on pooled within-laboratory variances. F-tests were performed separately for each matrix (PBS and CSO) and spike type. Based on these analyses, within-laboratory precision was not significantly different for any matrix/spike type combination.

Comparison of Methods 1103.1 and 1603 False Positive and False Negative Confirmation Rates

For CSO matrices, the false positive confirmation rates were higher for Method 1103.1 (18.2%) compared to Method 1603 (6.7%). The false negative confirmation rate was slightly higher for Method 1103.1 (3.3%) compared to Method 1600 (1.7%).

Table 22. Comparison of Methods 1103.1 and 1603 False Positive and False Negative Confirmation Rates for Unspiked CSO Matrices

Matrix	Method 1103.1		Method 1603	
	False Positive Confirmation Rate (%)	False Negative Confirmation Rate (%)	False Positive Confirmation Rate (%)	False Negative ConfirmationRrate (%)
CSO	18.2	3.3	6.7	1.7

Section 8.0 Conclusion

With data sets from five laboratories, quality control criteria for CSO matrices and PBS samples spiked at low- and high-levels were developed. Previously there was no data on method performance in CSO matrices. Now we have data from five laboratories that may be used as a starting point for end users.

Section 9.0 References

9.1 USEPA. 2002. *EPA Method 1106.1: Enterococci in Water by Membrane Filtration Using membrane-Enterococcus-Esculin Iron Agar (mE-EIA),* EPA-821-R-04-022, September 2002.

9.2 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.

9.3 USEPA. 2004. *EPA Method 1103.1: Escherichia coli (E. coli) in Water by Membrane Filtration Using membrane-Thermotolerant Escherichia coli Agar (mTEC),* EPA-821-R-04-024, September 2004.

9.4 USEPA. 2002. *EPA Method 1603: Escherichia coli (E. coli) in Water by Membrane Filtration Using Modified membrane-Thermotolerant Escherichia coli Agar (Modified mTEC),* EPA-821-R-02-023, September 2002.

9.5 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.

9.6 Neter, John, W. Wasserman, and M. H. Kutner. Applied Linear Statistical Models. 3rd Edition. Richard D. Irwin, Inc. Burr Ridge, IL, 1990. Pages 619-620.

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

9.8 USEPA. 2005. *Results of the Interlaboratory Validation of EPA Method 1600 (mEI) for Enterococci in Wastewater Effluent*. EPA-821-R-04-019. February 2005.

Section 10.0 Acronyms

CFU	Colony forming unit
CSO	Combined sewer overflow
IPR	Initial precision and recovery
MS	Matrix spike
OPR	Ongoing precision and recovery
PBS	Phosphate buffered saline
QA	Quality assurance
QC	Quality control
RPD	Relative percent difference
RSD	Relative standard deviation
SD	Standard deviation
TNTC	Too numerous to count

Appendix A:

Laboratory Capabilities Checklist

Laboratory Capabilities Checklist
Combined Sewer Overflow (CSO) Method Validation Study

(May 18, 2006)

EPA plans to invite 13 laboratories (12 participants and 1 verification) to participate in a study to validate methods for the detection and enumeration of *E. coli* and enterococci in CSOs. The methods to be evaluated include EPA Methods 1603 (modified mTEC) and 1103.1 (mTEC) for *E. coli,* and Methods 1600 (mEI) and 1106.1 (mE/EIA) for enterococci. Each of these methods will require filtration of diluted samples and subsequent incubation on selective media prior to target analyte detection and enumeration. EPA will provide all media and disposable materials for the study and will also cover all shipping costs. Volunteer laboratories and participants will be acknowledged in the validation study reports and in the final versions of each method. The study is tentatively scheduled to begin late spring.

If your laboratory is interested in participating in the validation study as a participant or verification laboratory, please provide the requested information below and fax the signed, completed checklist to Ruth Grunerud at 703.461.8056 by **Friday, May 26th**. In addition, please send the form electronically to Ruth Grunerud at rgrunerud@csc.com. Ruth 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 ychambers@csc.com. *Note:* If it is more convenient for your laboratory, we are happy to contact the wastewater facility directly, just let us know.

In addition to collecting information for the upcoming CSO validation study, EPA has also requested that a limited amount of information on sanitary sewer overflows (SSOs) and storm runoff be collected.

Section 1. Laboratory Capabilities and Experience

a. Please complete the requested capabilities and experience information below, if this information has not been previously provided to CSC. The information requested in **Table 1** pertains to experience with a given method, regardless of matrix (e.g., 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	mTEC	mE/EIA	mEI	mEndo or LES Endo	mFC	NA-MUG

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:

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	1106.1	12×10^3
	Raw					
	Primary treated					
	Secondary treated					
	Tertiary treated					
	Disinfected					
	Combined Sewer Overflow (CSO)					
	Sanitary Sewer Overflow (SSO)					
	Storm Runoff					

Section 2. Background Information

a. Does your laboratory have access to CSO samples? Yes No
Please indicate in Table 2, below.

b. Has your laboratory ever participated in a wastewater and/or CSO study? Yes No

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

d. If your laboratory has experience analyzing wastewater/CSO/SSO/storm runoff samples for total coliforms, fecal coliforms, fecal streptococci, or other indicator organisms, please place a check " ✓" next to the wastewater type(s) that you have access to and indicate the method(s) used for analysis and typical analyte ranges observed in Table 3, below.

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				
	Combined Sewer Overflow (CSO)				
	Sanitary Sewer Overflow				
	Storm Runoff				

e. If you indicated (Table 2 and Table 3) that your laboratory has access to and/or experience analyzing Combined Sewer Overflow (CSO) samples, please provide as much information regarding these matrices as possible (below). This information will assist in the evaluation matrix suitability.

e.1. Please characterize the composition of the CSO matrices that your laboratory has analyzed (e.g., % agricultural, % industrial, % residential, other- please specify)?

e.2. CSC recognizes that your laboratory may have access to multiple facilities with potential CSO matrices. For each facility, please describe the treatment process (e.g., total bypass-no disinfection, primary/secondary bypass-disinfected, other-please describe)? Please be as descriptive, as possible.

Facility 1:

Facility 2:

Facility 3:

e.3. What is the frequency of CSO events at each of these facilities?

e.4. What amount of rainfall generally triggers a CSO event at each of these facilities?

e.5. If available, please provide historical bacterial data (e.g., total coliforms, fecal coliforms, *E. coli*, enterococci, other) from the CSO matrices?

e.6 Will your laboratory be prepared to collect and analyze CSO samples within the 8-hour holding time?

Yes No

e.7. Please provide as much information as possible regarding the potential treatment facilities where your laboratory may obtain CSO samples:

Facility Name	Contact Name	Contact Phone	Contact Email	Address

Additional comments:

Section 3. General Information.

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

b. 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.)? _____

c. How does your laboratory disinfect filtration assemblies/funnels between sample filtrations?

Section 4. Verification Laboratory

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

Table 3. Verification Procedures

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

*If other, please describe:

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

Primary Analyst or Lab Manager (please print): _____

Laboratory name: _____

Signature: _____

Date: _____

Appendix B:

CSO Enterococci Spiking Protocol

Enterococci Spiking Protocol for Interlaboratory Validation of Methods 1106.1 (mE-EIA) and 1600 (mEI) in Combined Sewer Effluents (CSE)

(April 11, 2005)

The purpose of this protocol is to provide laboratories with enterococci spiking procedures for the interlaboratory validation of Methods 1106.1 and 1600 in combined sewer effluents (CSE). The following sections are included in this protocol:

Section 1: Preparation of Spiking Suspension
Section 2: Spiking Suspension Dilution and Enumeration
Section 3: Sample Spiking
Section 4: Calculation of Percent Recovery

1.0 Preparation of Spiking Suspension

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 (Methods 1106.1 and 1600, Section 7.5) 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 thoroughly.

1.3 Spiking Suspension (Undiluted). From the stock culture of *E. 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: *After incubation, the spiking suspension may be held at 6°C ± 2°C for a maximum of 72 hours. In anticipation of a potential CSE event, spiking suspensions should be propagated every fourth day to ensure that a viable suspension is available for sample spiking during the validation study. (For example, if the suspension was propagated on Monday, a new spiking suspension should be propagated on Friday.)*

2.0 Dilution and Enumeration of Spiking Suspension

Since one of the objectives of spiking the sample is to assess 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 dilution (Section 2.1) and enumeration (Section 2.2) of the spiking suspension.

Note: *Please be sure to thoroughly mix each of the spiking suspensions prior to performing the dilutions in the steps below, as homogeneous suspensions are critical for accurate dilution and enumeration.*

2.1 Dilution of spiking suspension

Please note: *The approach to diluting spiking suspensions was revised based on the practice week results.*

2.1.1 Mix the spiking suspension by vigorously shaking the bottle a minimum of 25 times. Use a sterile pipette to transfer **11.0 mL** of the undiluted spiking suspension (from Section 1.3 above) to 99 mL of sterile phosphate buffered saline (PBS), 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^{-1} mL of the original undiluted spiking suspension.

2.1.2 Use a sterile pipette to transfer **11.0 mL** of spiking suspension dilution "A" (from Section 2.1.1 above) to 99 mL of sterile PBS, 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^{-2} mL of the original undiluted spiking suspension.

2.1.3 Use a sterile pipette to transfer **1.0 mL** of spiking suspension dilution "B" (from Section 2.1.2 above) to 99 mL of sterile PBS, 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^{-4} mL of the original undiluted spiking suspension.

2.1.4 Use a sterile pipette to transfer **11.0 mL** of spiking suspension dilution "C" (from Section 2.1.3 above) to 99 mL of sterile PBS, 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^{-5} mL of the original undiluted spiking suspension

2.1.5 Use a sterile pipette to transfer **11.0 mL** of spiking suspension dilution "D" (from Section 2.1.4 above) to 99 mL of sterile PBS, cap, and mix by vigorously shaking the bottle a minimum of 25 times. This is spiking suspension dilution "E". A 1.0-mL volume of dilution "E" is 10^{-6} mL of the original undiluted spiking suspension

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

2.2.1 Prepare tryptic soy agar (TSA) according to manufacturer's instructions, add 12 - 15 mL of TSA per 100 × 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 prior to use, 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:

- Mix dilution **"C"** by vigorously shaking the bottle a minimum of 25 times. Pipet 0.1 mL of dilution **"C"** (Section 2.1.3) onto surface of pre-dried TSA plate [10^{-5} mL (0.00001) of the original spiking suspension].
- Mix dilution **"D"** by vigorously shaking the bottle a minimum of 25 times. Pipet 0.1 mL of dilution **"D"** (Section 2.1.4) onto surface of pre-dried TSA plate [10^{-6} mL (0.000001) of the original spiking suspension].
- Mix dilution **"E"** by vigorously shaking the bottle a minimum of 25 times. Pipet 0.1 mL of dilution **"E"** (Section 2.1.5) onto surface of pre-dried TSA plate [10^{-7} mL (0.0000001) of the original spiking suspension].

3.0 Sample Spiking

PBS samples will be spiked at two levels, low (Section 3.1.1) and high (Section 3.1.2). For each spike level, three PBS samples will be spiked. Four CSE samples will be spiked at *either* a low or high spike level, dependent on whether the effluent sample is disinfected or untreated, as described in Section 3.2.

3.1 Spiked PBS Samples

3.1.1 Low Spiking Concentration

- Add 3.0 mL of spiking suspension dilution **"C"** (from Section 2.1.3, above) to each of **three** 100-mL sterile PBS samples and mix each sample by vigorously shaking the bottle a minimum of 25 times. The volume (mL) of undiluted spiking suspension added to each 100 mL sample is 3.0 mL x 10^{-4} mL per 100 mL, which is referred to as V_{spiked} per 100 mL in Section 4.2 below.
- Filter the following aliquots from each spiked PBS sample prepared above: 10 mL, 1.0 mL, and 0.1 mL and analyze according to the instructions provided in Methods 1106.1 and 1600, Section 11.

Note: *When analyzing smaller sample volumes (e.g., <20 mL), 20-30 mL of PBS should be added to the funnel or an aliquot of sample should be dispensed into a 20-30 mL dilution blank prior to filtration. This will allow even distribution of the sample on the membrane.*

3.1.2 High Spiking Concentration

- Add 0.5 mL of spiking suspension dilution **"A"** (from Section 2.1.1) to each of **three** 100-mL sterile PBS samples and mix each sample by vigorously shaking the bottle a minimum of 25 times. The volume (mL) of undiluted spiking suspension added to each 100 mL sample is 5.0 × 10^{-2} mL per 100 mL [(0.5 mL × 10^{-1} mL) per 100 mL of sample], which is referred to as V_{spiked} per 100 mL in Section 4.2 below.
- Prepare the following serial dilutions from each spiked PBS sample prepared above: 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4} mL. Labs may use 99 mL or 9 mL dilution blanks to prepare the dilution series. Filter 1 mL of each serial dilution and analyze according to the instructions provided in Methods 1106.1 and 1600, Section 11.

Note: *When analyzing smaller sample volumes (e.g., <20 mL), 20-30 mL of PBS should be added to the funnel or an aliquot of sample should be dispensed into a 20-30 mL dilution blank prior to filtration. This will allow even distribution of the sample on the membrane.*

3.2 Spiked CSE Samples

3.2.1 Low Spiking Concentration (only for use by laboratories that are spiking disinfected effluent)

- Add 3.0 mL of spiking suspension dilution **"C"** (from Section 2.1.3, above) to each of the **four** 100-mL disinfected CSE samples and mix by vigorously shaking the bottle a minimum of 25 times. The volume (mL) of undiluted spiking suspension added to each 100 mL sample is 3.0×10^{-4} mL per 100 mL, which is referred to as V_{spiked} per 100 mL in Section 4.2 below.
- Filter the following aliquots from each of the spiked disinfected CSE samples prepared above:10 mL, 1.0 mL, 0.1 (10^{-1}) mL, and 0.01 (10^{-2}) mL, and analyze according to the instructions provided in Methods 1106.1 and 1600, Section 11.

Note: *When analyzing smaller sample volumes (e.g., <20 mL), 20-30 mL of PBS should be added to the funnel or an aliquot of sample should be dispensed into a 20-30 mL dilution blank prior to filtration. This will allow even distribution of the sample on the membrane.*

3.2.2 High Spiking Concentration (only for use by laboratories that are spiking untreated effluent)

- Add 0.5 mL of spiking suspension dilution **"A"** (from Section 2.1.1) to each of **four** 100-mL untreated CSE samples and mix by vigorously shaking the bottle a minimum of 25 times. The volume (mL) of undiluted spiking suspension added to each 100 mL sample is 5.0×10^{-2} mL per 100 mL [(0.5 mL × 10^{-1} mL) per 100 mL of sample], which is referred to as V_{spiked} per 100 mL in Section 4.2 below.
- Prepare the following serial dilutions from each of the spiked CSE samples prepared above: 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7} mL. Labs may use 99 mL or 9 mL dilution blanks to prepare the dilution series. Filter 1 mL of each serial dilution and analyze according to the instructions provided in Methods 1106.1 and 1600, Section 11.0.

Note: *When analyzing smaller sample volumes (e.g,, <20 mL), 20-30 mL of PBS should be added to the funnel or an aliquot of sample should be dispensed into a 20-30 mL dilution blank prior to filtration. This will allow even distribution of the sample on the membrane.*

4.0 Calculation of Percent Recovery

Note: *This section was added per laboratory request and is for information purposes only. Laboratories are not required to calculate percent recovery during this study.*

Spiked enterococci percent recovery will be calculated in three steps as indicated in Sections 4.1 through 4.3 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.

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

4.1.1 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.

4.1.2 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).

4.1.3 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.)

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

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 2.2.2			Enterococci CFU / mL in undiluted spiking suspension (Enterococci $_{\text{undiluted spike}}$)*
	10^{-5} mL plates	10^{-6} mL plates	10^{-7} mL plates	
Example 1	94, 106, 89	10, 0, 4	0,0,0	$(94+106+89) / (10^{-5}+10^{-5}+10^{-5})$ = $289 / (3.0 \times 10^{-5}) = 9{,}633{,}333 =$ **9.6×10^{6} CFU / mL**
Example 2	169, 209, 304	24, 30, 28	0, 2, 0	$(169+209+30) / (10^{-5}+10^{-5}+10^{-6})$ = $408 / (2.1 \times 10^{-5}) = 19{,}428{,}571$

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

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

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

Where,

$T_{\text{Spiked Enterococci}}$	=	Number of spiked enterococci (CFU / 100 mL)
$\text{Enterococci}_{\text{undiluted spike}}$	=	Enterococci (CFU / mL) in undiluted spiking suspension (calculated in Section 4.1.3)
$V_{\text{spiked per100 mL sample}}$	=	mL of undiluted spiking suspension per 100 mL sample (Section 3)

Table 2. Example Calculations of Spiked Enterococci

$\text{Enterococci}_{\text{undiluted spike}}$ (Table 1 above)	**$V_{\text{spiked per 100 mL sample}}$**	**$T_{\text{Spiked Enterococci}}$**
9.6×10^6 CFU / mL	3.0×10^{-4} mL per 100 mL of sample **(low)**	(9.6×10^6 CFU / mL) x (3.0×10^{-4} mL / 100 mL) = **2880 CFU / 100 mL**
1.9×10^7 CFU / mL	5.0×10^{-2} mL per 100 mL of sample **(high)**	(1.9×10^7 CFU / mL) x (5.0×10^{-2} mL / 100 mL) = **9.5×10^5 CFU / 100 mL**

4.3 Step 3: Calculate Percent Recovery

4.3.1 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 (Methods 1106.1/1600, Section 12)
N_u	=	Enterococci (CFU / 100 mL) in the unspiked sample (Methods 1106.1/1600, Section 12)
$T_{\text{Spiked Enterococci}}$	=	True spiked Enterococci (CFU / 100 mL) in spiked sample (Section 4.2, above)

Note: *During the validation study, N_u (unspiked sample) is the mean Enterococci (CFU / 100 mL) of the 4 unspiked effluent samples.*

4.3.2 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)	$T_{Spiked\ Enterococci}$ (CFU / 100 mL)	Percent recovery (R)
3700	120	2880	100 x (3700 - 120) / 2880 = **124%**
2600	300	2880	100 x (2600 - 300) / 2880 = **80%**
1.6×10^6	1.2×10^3	9.5×10^5	100 x (1.6×10^6 - 1.2×10^3) / 9.5 × 10^5 = **168%**
5.9×10^5	5.5×10^1	9.5×10^5	100 x (5.9×10^5 - 5.5×10^1) / 9.5 × 10^5 = **62%**

Appendix C:

CSO *E. coli* Spiking Protocol

E. coli Spiking Protocol for Interlaboratory Validation of Methods 1103.1 (mTEC) and 1603 (modified mTEC) in Combined Sewer Effluents (CSE)

(April 11, 2005)

The purpose of this protocol is to provide laboratories with *E. coli* spiking procedures for the interlaboratory validation of Methods 1103.1 and 1603 in combined sewer effluents (CSE). The following sections are included in this protocol:

Section 1: Preparation of Spiking Suspensions
Section 2: Spiking Suspension Dilution and Enumeration
Section 3: Sample Spiking
Section 4: Calculation of Percent Recovery

1.0 Preparation of Spiking Suspension

1.1 Stock Culture. Prepare a stock culture by inoculating a trypticase soy agar (TSA) slant (or other non-selective media) with *Escherichia coli* ATCC #11775 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% Lauryl Tryptose Broth (LTB). Prepare a 1% solution of LTB by combining 99 mL of sterile phosphate buffered saline (Methods 1103.1 and 1603, Section 7.5) and 1 mL of sterile single strength LTB 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 *E. coli* ATCC #11775 in Section 1.1, transfer a small loopful of growth to the 1% LTB 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 1.0×10^7 to 1.0×10^8 *E. coli* colony forming units (CFU) per mL. This is referred to as the "undiluted spiking suspension."

Note: *After the spiking suspension is incubated, it may be held at 6°C ± 2°C for a maximum of 72 hours. In anticipation of a potential CSE event, spiking suspensions should be propagated every fourth day to ensure that a viable suspension is available for sample spiking during the validation study. (For example, if the suspension was propagated on Monday, a new spiking suspension should be propagated on Friday.)*

2.0 Dilution and Enumeration of Spiking Suspension

Since one of the objectives of spiking the sample is to assess percent recovery, it is necessary to determine the number of *E. coli* in the undiluted spiking suspension prepared in Section 1.3. This section provides instructions for dilution (Section 2.1) and enumeration (Section 2.2) of the spiking suspension.

Note: *Please be sure to thoroughly mix each of the spiking suspensions prior to performing the dilutions in the steps below, as homogeneous suspensions are critical for accurate dilution and enumeration.*

2.1 Dilution of spiking suspension

2.1.1 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 (PBS), 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.

2.1.2 Use a sterile pipette to transfer **1.0 mL** of spiking suspension dilution "A" (from Section 2.1.1 above) to 99 mL of sterile PBS, 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.

2.1.3 Use a sterile pipette to transfer **11.0 mL** of spiking suspension dilution "B" (from Section 2.1.2 above) to 99 mL of sterile PBS, 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.

2.1.4 Use a sterile pipette to transfer **11.0 mL** of spiking suspension dilution "C" (from Section 2.1.3 above) to 99 mL of sterile PBS, 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.

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

2.2.1 Prepare tryptic soy agar (TSA) according to manufacturer's instructions, add 12 - 15 mL of TSA per 100 × 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 prior to use, 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:

- Mix dilution **"B"** by vigorously shaking the bottle a minimum of 25 times. Pipet 0.1 mL of dilution **"B"** (Section 2.1.2) onto surface of pre-dried TSA plate [10^{-5} mL (0.00001) of the original spiking suspension].
- Mix dilution **"C"** by vigorously shaking the bottle a minimum of 25 times. Pipet 0.1 mL of dilution **"C"** (Section 2.1.3) onto surface of pre-dried TSA plate [10^{-6} mL (0.000001) of the original spiking suspension].
- Mix dilution **"D"** by vigorously shaking the bottle a minimum of 25 times. Pipet 0.1 mL of dilution **"D"** (Section 2.1.4) onto surface of pre-dried TSA plate [10^{-7} mL (0.0000001) of the original spiking suspension].

3.0 Sample Spiking

PBS samples will be spiked at two levels, low (Section 3.1.1) and high (Section 3.1.2). For each spike level, three PBS samples will be spiked. Four CSE samples will be spiked at *either* a low or high spike level, dependent on whether the effluent sample is disinfected or untreated, as described in Section 3.2.

Note: *Please be sure to thoroughly mix each of the spiking suspensions prior to performing the dilutions in the steps below, as homogeneous suspensions are critical for accurate dilution and enumeration.*

3.1 Spiked PBS Samples

3.1.1 Low Spiking Concentration

- Add 3.0 mL of spiking suspension dilution **"C"** (from Section 2.1.3 above) to each of **three** 100-mL sterile PBS samples and mix each sample by vigorously shaking the bottle a minimum of 25 times. The volume (mL) of undiluted spiking suspension added to each 100 mL sample is 3.0×10^{-5} mL per 100 mL, which is referred to as V_{spiked} per 100 mL in Section 4.2 below.
- Filter the following aliquots from each spiked PBS sample prepared above: 10 mL, 1.0 mL, and 0.1 mL, and analyze according to the instructions provided in Methods 1103.1 and 1603, Section 11.0.

Note: *When analyzing smaller sample volumes (e.g., <20 mL), 20-30 mL of PBS should be added to the funnel or an aliquot of sample should be dispensed into a 20-30 mL dilution blank prior to filtration. This will allow even distribution of the sample on the membrane.*

3.1.2 High Spiking Concentration

- Add 3.0 mL of spiking suspension dilution **"A"** (from Section 2.1.1 above) to each of **three** 100-mL sterile PBS samples and mix by vigorously shaking the bottle a minimum of 25 times. The volume (mL) of undiluted spiking suspension added to each 100 mL sample is 3.0×10^{-2} mL per 100 mL, which is referred to as V_{spiked} per 100 mL in Section 4.2 below.
- Prepare the following serial dilutions from each spiked PBS sample prepared above: 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4}. Labs may use 99 mL or 9 mL dilution blanks to prepare the dilution series. Filter 1 mL of each serial dilution and analyze according to the instructions provided in Methods 1103.1 and 1603, Section 11.0.

Note: *When analyzing smaller sample volumes (e.g., <20 mL), 20-30 mL of PBS should be added to the funnel or an aliquot of sample should be dispensed into a 20-30 mL dilution blank prior to filtration. This will allow even distribution of the sample on the membrane.*

3.2 Spiked CSE Samples

3.2.1 Low Spiking Concentration (only for use by laboratories that are spiking disinfected effluent)

- Add 3.0 mL of spiking suspension dilution **"C"** (from Section 2.1.3 above) to each of the **four,** 100-mL disinfected CSE samples and mix by vigorously shaking the bottle a minimum of 25 times. The volume (mL) of undiluted spiking suspension added to each 100 mL sample is 3.0×10^{-5} mL per 100 mL, which is referred to as V_{spiked} per 100 mL in Section 4.2 below.
- Filter the following aliquots from each of the spiked disinfected CSE samples prepared above: 10 mL, 1.0 mL, 0.1 (10^{-1}) mL, and 0.01 (10^{-2}) mL, and analyze according to the instructions provided in Methods 1103.1 and 1603, Section 11.0.

Note: *When analyzing smaller sample volumes (e.g., <20 mL), 20-30 mL of PBS should be added to the funnel or an aliquot of sample should be dispensed into a 20-30 mL dilution blank prior to filtration. This will allow even distribution of the sample on the membrane.*

3.2.2 High Spiking Concentration (only for use by laboratories that are spiking untreated effluent)

- Add 3.0 mL of spiking suspension dilution **"A"** (from Section 2.1.1 above) to each of **four** 100-mL untreated CSE samples and mix by vigorously shaking the bottle a minimum of 25 times. The volume (mL) of undiluted spiking suspension added to each 100 mL sample is 3.0×10^{-2} mL per 100 mL, which is referred to as V_{spiked} per 100 mL in Section 4.2 below.
- Prepare the following serial dilutions from each of the spiked CSE samples prepared above: 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7} mL. Labs may use 99 mL or 9 mL dilution blanks to prepare the dilution series. Filter 1 mL of each serial dilution and analyze according to the instructions provided in Methods 1103.1 and 1603, Section 11.0.

Note: *When analyzing smaller sample volumes (e.g., <20 mL), 20-30 mL of PBS should be added to the funnel or an aliquot of sample should be dispensed into a 20-30 mL dilution blank prior to filtration. This will allow even distribution of the sample on the membrane.*

4.0 Calculation of Percent Recovery

Note: *This section was added per laboratory request and is for information purposes only. Laboratories are not required to calculate percent recovery during this study.*

Spiked *E. coli* percent recovery will be calculated in three steps as indicated in Sections 4.1 through 4.3 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.

4.1 Step 1: Calculate Concentration of *E. coli* (CFU / mL) in Undiluted Spiking Suspension

4.1.1 The number of *E. coli* (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.

4.1.2 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).

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

$$E.\ coli_{\text{undiluted spike}} = (CFU_1 + CFU_2 + ... + CFU_n) / (V_1 + V_2 + ... + V_n)$$

Where,

E. coli undiluted spike	=	*E. coli* (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 *E. coli* spiking suspension concentration

Examples	CFU / plate (triplicate analyses) from TSA plates (Section 2.2.2)			*E. coli* CFU / mL in undiluted spiking suspension ($EC_{\text{undiluted spike}}$)*
	10^{-5} mL plates	10^{-6} mL plates	10^{-7} mL plates	
Example 1	TNTC, TNTC, TNTC	94, 106, 89	10, 0, 4	$(94+106+89) / (10^{-6}+10^{-6}+10^{-6})$ = $289 / (3.0 \times 10^{-6}) = 96{,}333{,}333$ –
Example 2	269, 289, 304	24, 30, 28	0, 2, 0	$(269+289+30) / (10^{-5}+10^{-5}+10^{-6})$ = $588 / (2.1 \times 10^{-5}) = 28{,}000{,}000$

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

4.2 Step 2: Calculate "True" Spiked *E. coli* (CFU / 100 mL)

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

$$T_{\text{Spiked } E.\ coli} = (E.\ coli_{\text{ undiluted spike}}) \times (V_{\text{ spiked per 100 mL sample}})$$

Where,

$T_{\text{Spiked } E.\ coli}$	=	Number of spiked *E. coli* (CFU / 100 mL)
$E.\ coli_{\text{ undiluted spike}}$	=	*E. coli* (CFU / mL) in undiluted spiking suspension (calculated in Section 4.1.3)
$V_{\text{ spiked per100 mL sample}}$	=	mL of undiluted spiking suspension per 100 mL sample (Section 3)

Table 2. Example Calculations of Spiked *E. coli*

EC $_{\text{undiluted spike}}$ (Table 1 above)	**V $_{\text{spiked per 100 mL sample}}$**	**$T_{\text{Spiked } E.\ coli}$**
9.6×10^7 CFU / mL	3.0×10^{-5} mL per 100 mL of sample **(low)**	(9.6×10^7 CFU / mL) × (3.0×10^{-5} mL / 100 mL) = **2880 CFU / 100 mL**
2.8×10^7 CFU / mL	3.0×10^{-2} mL per 100 mL of sample **(high)**	(2.8×10^7 CFU / mL) × (3.0×10^{-2} mL / 100 mL) = **8.4×10^5 CFU / 100 mL**

4.3 Step 3: Calculate Percent Recovery

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

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

Where,

R	=	Percent recovery
N_s	=	*E. coli* (CFU / 100 mL) in the spiked sample (Methods 1103.1/1603, Section 13)
N_u	=	*E. coli* (CFU / 100 mL) in the unspiked sample (Methods 1103.1/1603, Section 13)
$T_{\text{Spiked } E.\ coli}$	=	True spiked *E. coli* (CFU / 100 mL) in spiked sample (Section 4.2, above)

Note: *During the validation study, N_u (unspiked sample) is the mean E. coli (CFU / 100 mL) of the 4 unspiked effluent samples.*

4.3.2 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)	$T_{Spiked\ E.\ coli}$ (CFU / 100 mL)	***Percent recovery (R)***
3700	120	2880	100 × (3700 - 120) / 2880 = 124%
2600	300	2880	100 × (2600 - 300) / 2880 = **80%**
1.6×10^6	1.2×10^3	8.4×10^5	100 x (1.6×10^6 - 1.2×10^3) / 8.4×10^5 = **190%**
5.9×10^5	5.5×10^1	8.4×10^5	100 x (5.9×10^5 - 5.5×10^1) / 8.4×10^5 = **70%**