EPA/600/R-10/057 | July 2010 | www.epa.gov/ord

United States
Environmental Protection
Agency

Single-Laboratory Verification of Culture-Based Analytical Procedures for *Escherichia coli* O157:H7 in Water

STUDY REPORT

National Homeland Security Research Center Office of Research and Development
United States Environmental Protection Agency, Cincinnati, Ohio 45268

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U.S. ENVIRONMENTAL PROTECTION AGENCY
OFFICE OF RESEARCH AND DEVELOPMENT
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United States Environmental Protection Agency, Cincinnati, Ohio 45268

Acknowledgments

This report presents results of a laboratory study funded by the National Homeland Security Research Center (NHSRC) within the U.S. Environmental Protection Agency's (EPA's) Office of Research and Development (ORD) under the direction of Sanjiv R. Shah to verify culture-based analytical procedures for *E. coli* O157 in water. Computer Sciences Corporation (CSC) provided technical support and data evaluation under EPA contract EP-C-05-045.

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Acronyms

(*Included in the Study Report and/or Appendices*)

ACV	Acriflavin/cefsuludin/vancomycin
ANOVA	Analysis of variance
ASTM	American Society for Testing and Materials
ATCC®	American Type Culture Collection
BHI	Brain heart infusion
BSL	Biosafety level
°C	Degrees Celsius
CFU	Colony forming unit
CSC	Computer Sciences Corporation
EEB	EHEC enrichment broth
EHEC	Enterohemorrhagic *E. coli*
EPA	U.S. Environmental Protection Agency
HEPA	High efficiency particulate air
IMS	Immunomagnetic separation
LTB	Lauryl tryptose broth
MF	Membrane filtration
mBPW	Modified buffered peptone water
MPN	Most probable number
MUG	4-methylumbelliferyl-β-D-glucuronide
NCTC	National Collection of Type Cultures
NHSRC	National Homeland Security Research Center
OEM	Office of Emergency Management
ORD	Office of Research and Development
OSWER	Office of Solid Waste and Emergency Response
OW	Office of Water
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PSI	Pounds per square inch
QA	Quality assurance
QAPP	Quality assurance project plan
QC	Quality control
RSD	Relative standard deviation
SAP	Standard Analytical Protocol
SD	Standard deviation
SME	Subject matter expert

STEC	Shiga toxin-producing *E. coli*
TC-SMAC	Tellurite cefixime sorbitol MacConkey
TNTC	Too numerous to count
TSA	Tryptic soy agar
TSAYE	Tryptic soy agar with 0.6% yeast extract
TB	Tryptone broth

Section 1.0
Background

Subsequent to the anthrax attacks in the fall of 2001, federal and state personnel were tasked with a mission to provide response, recovery, and remediation for biological incidents. However, it is recognized that no standardized set of methods exists for collection, isolation, and analysis of these biological agents in environmental samples. This document presents results of the single-laboratory verification study (Study) of culture-based procedures for the identification and quantitation of *Escherichia coli* serotype O157 (*E. coli* O157) in water samples, a potential bioterrorism agent.

E. coli O157:H7 is a virulent member of the pathogenic enterohemorrhagic *E. coli* (EHEC) group. Members of the EHEC group, including *E. coli* O157:H7, that produce one or more Shiga toxins are often referred to as Shiga toxin-producing *E. coli* (STEC). Infection with STEC in humans has been associated with a spectrum of diseases, including gastroenteritis, hemorrhagic colitis, and hemolytic-uremic syndrome, the latter a potentially fatal complication in children less than 5 years of age and the elderly. EHEC/STEC is endemic in cattle and other domestic animals, and transmission to humans occurs primarily through contaminated water, vegetables contaminated by agricultural runoff, unpasteurized milk and juice, and undercooked meat products (Reference 8.1).

The culture-based procedures used to evaluate water matrices during the Study were adapted from *Standard Methods for the Examination of Water and Waste Water*, 21st Edition (Reference 8.2), and the journal article "Evaluation of Techniques for Enrichment and Isolation of *Escherichia coli* O157:H7 from Artificially Contaminated Sprouts" by Weagant and Bound (Reference 8.3).

The initial analytical procedure evaluated during the Study was based on EPA's "Draft Standardized Analytical Procedure for *Escherichia coli* O157:H7 in Environmental Samples (March 2008)." The procedure included the use of EHEC enrichment broth (EEB), tellurite cefixime sorbitol MacConkey (TC-SMAC) agar, and biochemical and serological confirmation. Results indicated that the procedure was not acceptable for either the reference matrix (phosphate buffered saline [PBS]), or the two matrices of interest (drinking water, surface water), and thus, the procedure was modified based on input from subject matter experts (SMEs) and workgroup guidance, and was optimized prior to verification.

In the final optimized procedure, a water sample (e.g., drinking water, surface water) is inoculated into modified buffered peptone water (mBPW) and incubated at 36°C ± 1.0°C for 2 – 2.5 hours followed by incubation at 42.0°C ± 0.5°C for a total of 20 – 24 hours. Tubes with growth (turbidity) are submitted to immunomagnetic separation (IMS) and sub-cultured onto TC-SMAC and Rainbow® agars. After growth at 42.0°C ± 0.5°C for 18 – 24 hours, TC-SMAC plates are examined for 2 – 3 mm colorless/gray colonies typical of *E. coli* O157:H7. On Rainbow® plates, typical colonies are black/gray. Isolated typical colonies are submitted to serological typing using *E. coli* O157 latex agglutination reagent (e.g., Oxoid DR0620M or equivalent), followed by biochemical characterization using commercially available test strips (e.g., API 20E® or equivalent). Quantification of *E. coli* O157:H7 is determined using the most probable number (MPN) technique. Tubes that confirmed positive for *E. coli* O157:H7 are used to determine MPN (Reference 8.4). Based on workgroup discussion, a nine-tube MPN as opposed to the standard fifteen-tube MPN was utilized to reduce the burden (2000 tubes and 4000 plates for a nine-tube MPN versus 3400 tubes and 6800 plates for a fifteen-tube MPN) on the laboratory once IMS, which was performed on each individual tube, was added to the procedure.

Note: Serological typing of the H7 antigen or polymerase chain reaction (PCR) for the H7 gene was not conducted during the single-laboratory verification Study. In order to confirm *E. coli* O157:H7, additional serological or PCR analyses would be necessary.

Section 2.0
Study Objectives and Design

The primary objective of this Study was to verify culture-based procedures for identification and enumeration of *E. coli* O157. As indicated in the Study Plan (Appendix B), this Study was originally designed to verify the procedures for water, solid, and particulate matrices. However, during this phase, only water matrices were evaluated, and only these are included in this report. A phased approach is being taken for evaluation of other matrices. Two sets of objectives were identified for the Study: Study objectives and data quality objectives.

Study Objectives

- Characterize analytical procedure performance (recovery and precision) for a reference matrix (PBS)
- Characterize analytical procedure performance (recovery and precision) for environmental matrices of interest (drinking water, surface water)
- Determine whether the analytical procedure requires revision prior to multi-laboratory validation

To accomplish these objectives, the Study was conducted in four phases, as described below.

- **Phase 1**. Identification of a qualified laboratory to participate in the Study, preparation of spikes, assessment of analytical procedures by preliminary analyses of water matrices (PBS [reference matrix], drinking water, surface water), review of data, identification of issues, and revision of protocol after consultation with SMEs
- **Phase 2**. Pilot analyses: Evaluation of water matrices using a modified procedure with antibiotics (acriflavin, cefsulodin, vancomycin), assessment of freeze-stressed cultures and unstressed (fresh cultures) laboratory-prepared spikes, review of data, identification and resolution of issues
- **Phase 3**. Optimization analyses: Evaluation of four treatment options (antibiotics, elevated incubation temperature, both, or neither) to optimize recoveries from PBS and surface water samples and resolve issues (e.g., inhibition of target) associated with high ambient/background microbial levels observed in surface water samples, statistical analyses of treatments, and revision of procedure
- **Phase 4**. Analyses of drinking water using the optimized procedure (elevated incubation temperature) according to the optimization instructions for elevated temperature

Data Quality Objective

Data produced under this Study were generated according to the analytical and quality assurance/quality control (QA/QC) procedures specified in the Study-specific instructions (Appendix A) and Standard Analytical Protocol (SAP). This ensured data integrity and validity for all matrices evaluated and allowed the Study workgroup to use the results to identify any necessary revisions of the SAP.

2.1 – Study Preparation

Prior to the Study, the following activities were completed, including identification of an appropriate laboratory and development and evaluation of the Spiking Protocol (Appendix C).

2.1.1 – Identification of Laboratory

A laboratory was identified that was (1) representative of the general user community, (2) had experience analyzing environmental samples for *E. coli* O157:H7, and (3) had access to representative matrices. To reduce Study costs, a volunteer laboratory was recruited. To reduce the burden on the laboratory and encourage participation, National Homeland Security Research Center (NHSRC) provided the media, reagents, and supplies needed for the Study. The requirements and responsibilities of the laboratory are detailed in Study-specific instructions (Appendix A) and the draft SAP.

2.1.2 – Preparation of Spiking Suspensions

The Study Plan (Appendix B) included the use of two spike types, BioBalls and laboratory-prepared. However, due to production difficulties with BioBalls, only laboratory-prepared spikes were used during the Study. During each week of the Study, *E. coli* O157:H7 (ATCC® 700728™/NCTC 12900) laboratory-prepared spiking suspensions were propagated in 1% lauryl tryptose broth (LTB) and incubated at 35.0°C ± 0.5°C for 20 ± 4 hours. Serially diluted spiking suspensions were used to spike samples. The laboratory enumerated spiking suspensions on the same day that samples were spiked and analyzed. Spiking suspensions were enumerated using spread plate technique (in triplicate) on tryptic soy agar (TSA) according to the Spiking Protocol (Appendix C).

2.2 – Sample Matrices

The laboratory analyzed water matrices to provide a means for evaluating and optimizing the performance of the analytical procedure. During the Study, a reference matrix (PBS) and two matrices of interest (drinking water and surface water) were evaluated. The reference matrix was analyzed to provide a means for assessing performance using a standard matrix that could be duplicated in the future during routine use of the method. The following water matrices were evaluated during the Study:

Reference Matrix

- PBS

Water Matrices

- Chlorinated drinking water (laboratory tap, dechlorinated with sodium thiosulfate)
- Surface water (reservoir)

2.3 – Sample Analyses

For preliminary analyses, a single unspiked and spiked sample was evaluated per matrix. For Study analyses samples, one unspiked PBS or two unspiked drinking or surface water samples, as appropriate, were evaluated by the procedure to determine background *E. coli* O157 concentrations. Results of preliminary analyses were used to identify and resolve issues. The laboratory conducted additional analyses to modify/optimize the protocol to improve recoveries. For each set of analyses, the data was evaluated by the workgroup prior to requesting the laboratory to conduct additional analyses. Table 1 summarizes the number and type of samples that were evaluated to meet the objectives listed in Section 2.

Table 1. Summary of Sample Analyses

Analysis Phase	Matrix	Spiking Description	Procedure/Treatment	No. of Replicates
Initial Analyses	Sterile PBS (Reference Matrix)	Unspiked	Original procedure: March 2008 draft document	1
		Lab-Prepared Spike		1
	Surface Water	Unspiked		1
		Lab-Prepared Spike		1
	Drinking Water	Unspiked		1
		Lab-Prepared Spike		1
Pilot Analyses: Stressed / Unstressed Spike	Sterile PBS (Reference Matrix)	Unspiked	Modified procedure with antibiotics (acriflavin, cefsulodin, & vancomycin), elevated incubation temperature (42.0°C), and IMS	1
		Lab-Prepared Spike		2
	Surface Water	Unspiked		1
		Lab-Prepared Spike		2
Pilot Analyses	Sterile PBS (Reference Matrix)	Unspiked	Modified procedure with antibiotics (acriflavin, cefsulodin, & vancomycin), elevated incubation temperature (42.0°C), and IMS	1
		Lab-Prepared Spike		4
	Surface Water	Unspiked		2
		Lab-Prepared Spike		4
	Drinking Water	Unspiked		2
		Lab-Prepared Spike		4
Pilot Follow-on Analyses	Sterile PBS (Reference Matrix)	Unspiked	With IMS and: Antibiotics (acriflavin, cefsulodin & vancomycin) Elevated incubation temperature (42.0°C)	1 per treatment
		Lab-Prepared Spike		2 per treatment
	Surface Water	Unspiked		1 per treatment
		Lab-Prepared Spike		2 per treatment
Optimization Analyses	Sterile PBS (Reference Matrix)	Unspiked	With IMS and: Antibiotics (cefsulodin & vancomycin) Elevated incubation temperature (42.0°C) Both (antibiotics and elevated incubation temperature) Neither (antibiotics nor elevated incubation temperature)	7 per treatment
		Lab-Prepared Spike		14 per treatment
	Surface Water	Unspiked		14 per treatment
		Lab-Prepared Spike		14 per treatment
Drinking Water Analyses	Sterile PBS (Reference Matrix)	Unspiked	With IMS and elevated incubation temperature (42.0°C)	1
		Lab-Prepared Spike		1
	Drinking Water	Unspiked		2
		Lab-Prepared Spike		4

2.4 – Quality Control Analyses

The participant laboratory performed the following QC analyses:

- **Method Blank**: The laboratory analyzed a sterile, unspiked PBS method blank during each week of analyses to verify the sterility of equipment, materials, and supplies.
- **Sterility Checks**: To evaluate the sterility of media and buffer, the laboratory incubated a representative portion of each batch (PBS, mBPW, TC-SMAC, Rainbow, and TSA) for 24 ± 2 hours at 35.0°C – 37.0°C or 42.0°C ± 0.5°C, as appropriate, and observed for growth. In addition, sterility checks were conducted each day samples were analyzed.
- **Positive and Negative Controls**: For the purpose of the Study, positive controls for selective agars and broths are those organisms that provide the characteristic growth and/or colony morphology of the target organism. Negative controls are those organisms that do not provide the characteristic target organism growth or reactions. For biochemical and serological analyses positive and negative controls are defined by their reaction (e.g., *Pseudomonas aeruginosa* is oxidase positive and *E. coli* O157:H7 is oxidase negative). The following positive and negative controls were evaluated during each week of the Study:
  - *E. coli* O157:H7 (ATCC® 700728™/ NCTC 12900): positive control (target organism)
  - *E. coli* (ATCC® 25922™): negative control (non-target organism)
  - *Pseudomonas aeruginosa* (ATCC® 27853™): positive or negative control for biochemical tests, as appropriate

Section 3.0
Study Management

This Study was designed under the direction of NHSRC within the U.S. Environmental Protection Agency (EPA's) Office of Research and Development (ORD) with consultation and input provided by workgroup members and SMEs. The EPA technical lead was Sanjiv R. Shah. Coordination of activities for the Study was conducted by Computer Sciences Corporation (CSC).

3.1 – Schedule

The duration of the Study was April 2008 to March 2009. The Study schedule is provided in Table 2. Analyses of additional matrices may be conducted at a later date.

Table 2. Schedule for Verification of *E. coli* O157 Culture-Based Procedures in Water Matrices

Analysis Phase	Date
Initial Analyses	April 7 – 12, 2008
Pilot Analyses	June 23, 2008 – September 13, 2008
Optimization Analyses	November 24, 2008 – January 6, 2009
Drinking Water Analyses	March 16 – 24, 2009

3.2 – Participant Laboratory

The participant laboratory was BioVir Laboratories, Inc., (Rick Danielson, Rosie Newton, and Jim Truscott), located at 685 Stone Road, Unit 6, Benicia, California 94510-1126.

Section 4.0
Data Reporting, Validation and Censoring

4.1 – Data Reporting

The laboratory submitted the following data to CSC for review and validation:

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

4.2 – Data Validation

CSC 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. The review for each sample confirmed the following:

- Original forms were submitted
- Incubation times were met
- Incubation temperatures were met
- Media sterility checks were performed and acceptable
- Positive and negative controls were performed and exhibited the appropriate response
- Samples were spiked with the appropriate dilution
- All procedures were performed according to Study-specific instructions and analytical procedures
- 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 were considered either (1) valid and acceptable or (2) invalid and unacceptable for inclusion in subsequent data analysis.

Optimization Analyses

- The extremely high spike level (10,200 colony forming units [CFU]/100 mL) for the November 24, 2008, analyses resulted in values above the analytical range (e.g., >1099) of the method and did not allow for accurate determination of recoveries. Thus, the data was considered invalid and not included in subsequent data analyses.
- Unspiked surface water replicates analyzed on December 8, 2008 resulted in MPN values of 1099 and 3.01. Due to this disparity and based on discussion with the laboratory, the 1099 value was considered invalid and not included in subsequent data analyses.

4.3 – Censored Data

In addition to the numerical sample results generated during this Study, low censored ("less than") results also were generated for unspiked samples that had non-detects.

The easiest approach to dealing with the low censored values for this Study would have been to eliminate them from data analysis. However, because the censored results provide valuable information, the censoring limit (the "less than" value itself [1.08]) was used in data analysis for these samples.

In addition to low censored values, 17 high censored (or "greater than") results (excluding November 24, 2008 results) were observed. The censoring limit, the "greater than" value itself, (e.g., >109.9 or >1099) obtained during optimization and drinking water analyses were used for data analysis.

Section 5.0
Results

This section includes results of unspiked and spiked samples for Initial Analyses (Section 5.1), Pilot Analyses (Section 5.2), Optimization Analyses (Section 5.3), Drinking Water Analyses (Section 5.4), and Combined Results of Verification of *E. coli* O157 Analyses in Drinking and Surface Water (Section 5.5). A summary of sample analyses is included in Section 2.3, Table 1. Only valid results are included in this section; a detailed description of invalid data is included in Section 4.2. Recoveries were based on the *E. coli* O157 concentrations in spiked samples minus ambient *E. coli* O157 concentrations in unspiked samples.

5.1 – Initial Analyses

Recoveries were 6%, 0%, and 13% for PBS, drinking water, and surface water, respectively using the original procedure described in the March 2008 document (enrichment in EEB and plating on TC-SMAC followed by biochemical and serological confirmation).

5.2 – Pilot Analyses

Results of the pilot analyses were used to determine if procedural modifications were appropriate. These modifications included enrichment in mBPW at 36.0°C ± 1.0°C for two hours, addition of antibiotics (acriflavin, cefsulodin, and vancomycin) and incubation at 42.0°C ± 0.5°C for an additional 20 – 24 hours. Tubes with growth were submitted to IMS and IMS beads were plated onto TC-SMAC and Rainbow® agars and incubated at 42.0°C ± 0.5°C for 18 – 24 hours followed by serological and biochemical confirmation. During the pilot analyses the workgroup thought it would be beneficial to evaluate the use of a freeze-stressed culture for spiking samples to potentially mimic organisms that may be stressed in the environment. Both freeze-stressed and fresh (non-stressed) cultures were evaluated. All other analyses conducted during the study utilized fresh cultures. Laboratory-specific results are provided in Tables 3 (non-stressed), 4 (stressed), and 5 (pilot analyses conducted the week of August 8, 2008). Due to very low recoveries observed during the analyses conducted the week of August 8, 2008, pilot analyses were repeated the week of September 1, 2008 (Table 6). Since recoveries observed for analyses conducted both weeks (August 8, 2008 and September 1, 2008) were low, additional analyses were conducted to evaluate the use of antibiotics and elevated incubation temperature independently. Results of this limited evaluation (follow-on analyses) are provided in Table 7.

Table 3. Pilot Analyses, Preliminary: Fresh (Non-Stressed) *E. coli* O157 Culture for Sample Spiking

Sample ID	Spike Level [1] (CFU/100 mL)	MPN Combo	E. coli O157 (MPN/100 mL)	Percent Recovery
PBS Samples				
Unspiked	NA	0-0-0	<1.08	
Spiked	3.2	1-1-0	2.6	48
	3.2	1-0-0	1.257	6
Surface Water Samples				
Unspiked	NA	0-0-0	<1.08	
Spiked	3.2	3-2-0	12.68	363
	3.2	2-1-0	4.647	111

[1] Observed spike level was one log lower than target spike level and may account for variability in recovery between replicates

To evaluate whether the analytical protocol would recover environmental (stressed) organisms, a freeze-stressed culture was used to spike samples prior to analyses (Table 4).

Table 4. Pilot Analyses, Preliminary: Freeze-Stressed *E. coli* O157 Culture for Sample Spiking

Sample ID	Spike Level [1] (CFU/100 mL)	MPN Combo	E. coli O157 (MPN/100 mL)	Percent Recovery
PBS Samples				
Unspiked	NA	0-0-0	<1.08	
Spiked	26	3-3-2	109.9	418
	26	3-3-3	>109.9	>418
Surface Water Samples				
Unspiked	NA	0-0-0	<1.08	
Spiked	26	3-3-3	>109.9	>418
	26	3-3-3	>109.9	>418

[1]Enumeration of the spikes on selective (TC-SMAC) and non-selective media demonstrated an approximate 10^3 reduction on the selective media, as anticipated. The spike level noted above was based on TC-SMAC enumeration.

Based on recoveries observed for analyses evaluating the modified procedure using a fresh culture (Table 3), the laboratory was asked to proceed with additional analyses to complete the verification study. The laboratory analyzed one unspiked PBS sample, two unspiked drinking water and surface water samples, and four spiked samples per matrix according to the modified procedure and Study instructions. Analyses were conducted the week of August 8, 2008. Results are provided in Table 5.

Table 5. Pilot Analyses: Fresh (Non-Stressed) *E. coli* O157 Culture for Sample Spiking

Sample ID	Spike Level (CFU/100 mL)	MPN Combo	E. coli O157 (MPN/100 mL)	Percent Recovery
PBS Samples				
Unspiked	NA	0-0-0	<1.08	
Spiked	2840	3-3-3	>109.9	4
		3-3-0	23.98	1
		3-3-0	23.98	1
		3-3-0	23.98	1
Drinking Water Samples				
Unspiked	NA	0-0-0	<1.08	
		0-0-0	<1.08	
Spiked	2840	3-3-0	23.98	1
		3-3-0	23.98	1
		3-3-0	23.98	1
		3-3-0	23.98	1
Surface Water Samples				
Unspiked	NA	0-0-0	<1.08	
		1-3-0	6.207	
Spiked	2840	3-3-1	46.22	1
		3-2-1	16.95	0
		3-3-0	23.98	1
		3-3-1	46.22	1

Due to the low recoveries observed for analyses conducted the week of August 8, 2008 (Table 5), the laboratory was asked to repeat the analyses the week of September 1, 2008 to determine which data set (Table 3 or 5) was indicative of the "true" performance of the analytical procedure.

Table 6. Pilot Analyses, Repeat: Fresh (Non-Stressed) E. coli O157 Culture for Sample Spiking [1]

Sample ID	Spike Level (CFU/100 mL)	MPN Combo	E. coli O157 (MPN/100 mL)	Percent Recovery
PBS Samples				
Unspiked	NA	0-0-0	<1.08	
Spiked	1490	3-3-1	46.22	3
		3-2-0	12.68	1
		3-3-2	109.9	7
		3-3-2	109.9	7
Drinking Water Samples				
Unspiked	NA	0-0-0	<1.08	
		0-0-0	<1.08	
Spiked	1490	3-3-1	46.22	3
		3-3-0	23.98	2
		3-3-0	23.98	2
		3-3-0	23.98	2
Surface Water Samples				
Unspiked	NA	3-3-2	109.9	
		3-3-2	109.9	
Spiked	1490	3-3-3	>109.9	0
		3-3-3	>109.9	0
		3-3-3	>109.9	0
		3-3-2	109.9	0

[1]Analyses stopped after mPBW

Recoveries observed for analyses conducted the week of September 1, 2008 were 7% or less, which was consistent with results from analyses conducted during the week of August 8, 2009 (Table 5). These results indicated that additional optimization of the analytical protocol would be required prior to conducting verification analyses. To determine if the use of both the antibiotic solution and elevated incubation temperature were inhibiting growth of the target organism, the laboratory conducted limited analyses to evaluate use of the antibiotic solution and elevated incubation temperature independently. Results for those analyses are provided in Table 7.

Table 7. Pilot Analyses, Follow-on: Independent Evaluation of Antibiotics and Elevated Temperature [1]

Sample ID	Spike Level (CFU/100 mL)	MPN Combo	E. coli O157 (MPN/100 mL)	Percent Recovery
PBS Samples - Option 1: Antibiotics (acriflavin, cefsulodin, & vancomycin) Only				
Unspiked	NA	0-0-0	<1.08	
Spiked	58	3-3-0	23.98	39
		3-3-1	46.22	78
Surface Water Samples - Option 1: Antibiotics (acriflavin, cefsulodin, & vancomycin) Only				
Unspiked	NA	0-0-0	<1.08	
Spiked	58	1-2-0	4.23	7
		3-3-0	23.98	39
PBS Samples - Option 2: Elevated Incubation Temperature Only				
Unspiked	NA	0-0-0	<1.08	
Spiked	58	3-3-0	23.98	39
		3-3-2	109.9	188
Surface Water Samples - Option 2: Elevated Incubation Temperature Only				
Unspiked	NA	3-3-0	23.98	
Spiked	58	3-3-0	46.22	38
		3-3-3	>109.9	>148

[1] Analyses were conducted the week of September 8, 2008

5.3 – Optimization Analyses

During Phase 3 (optimization analyses), the laboratory evaluated four treatments to determine which treatment was optimal. The four treatments evaluated were: (1) neither antibiotics nor elevated incubation temperature (42.0°C), (2) elevated incubation temperature, (3) antibiotics (cefsulodin and vancomycin [acriflavin was not used]), and (4) both antibiotics and elevated incubation temperature. During the optimization analyses each treatment was incorporated into the modified procedure: mBPW, IMS, plating on TC-SMAC and Rainbow® agars, followed by serological and biochemical confirmation, as appropriate. Seven runs were conducted, with 2 samples per treatment, per run, resulting in 14 spiked samples per treatment for each matrix (surface water and PBS). In addition, one unspiked or two unspiked samples were analyzed during each run for PBS and surface water, respectively. Summary results are provided in Tables 8 (spiked PBS), 9 (spiked surface water), and 10 (unspiked surface water). All unspiked PBS samples were negative (<1.08 MPN/100 mL). Table 10 presents *E. coli* O157 concentrations (MPN/100 mL) rather than recoveries because the samples were not spiked prior to analyses.

Table 8. Optimization Analyses, Results Summary: PBS Spiked with E. coli O157 [1, 2, 3]

Date	Spike Level (CFU/ 100 mL)	Mean Recovery (%)	Min Recovery (%)	Max Recovery (%)	SD (%)	RSD (%)	Overall Mean (%)	Overall SD (%)	Pooled within-run SD (%) [4]	Overall RSD (%)	Pooled within-run RSD (%) [5]
Neither (Antibiotics nor Elevated Incubation Temperature)											
12/1/2008	863	90	53	127	52	58	84	66	71	79	63
12/3/2008	963	114	114	114 [6]	0	0					
12/8/2008	1047	44	44	44	0	0					
12/15/2008	462	131	24	238	151	116					
12/22/2008	320	89	34	144	78	87					
1/6/2009	713	109	65	154	63	58					
Elevated Incubation Temperature (42.0ºC)											
12/1/2008	863	127	127	127	0	0	86	63	55	73	57
12/3/2008	963	69	25	114 [6]	63	91					
12/8/2008	1047	74	44	105	43	58					
12/15/2008	462	169	100	238	98	58					
12/22/2008	320	89	34	144	78	87					
1/6/2009	713	65	65	65	0	0					
Antibiotics (cefsulodin and vancomycin)											
12/1/2008	863	90	53	127	52	58	75	68	76	91	80
12/3/2008	963	48	48	48	0	0					
12/8/2008	1047	64	23	105	58	91					
12/15/2008	462	145	52	238	132	91					
12/22/2008	320	76	7	144	97	128					
1/6/2009	713	94	33	154	85	91					
Both (Antibiotics and Elevated Incubation Temperature)											
12/1/2008	863	90	53	127	52	58	97	93	91	96	65
12/3/2008	963	81	48	114 [6]	47	58					
12/8/2008	1047	105	105	105	0	0					
12/15/2008	462	169	100	238	98	58	97	93	91	96	65
12/22/2008	320	195	46	343 [6]	210	108					
1/6/2009	713	33	33	33	0	0					

[1] ">1099 MPN/100 mL" was replaced with "1099 MPN/100 mL" for calculation of summary statistics

[2] Two replicates were run per sample

[3] Samples were spiked with overnight (fresh) cultures

[4] Pooled within-run standard deviation (SD) determined by calculating the square root of the mean of the run variances

[5] Pooled within-run relative standard deviation (RSD) was determined by calculating the square root of the mean of the squared run RSDs

[6] Recovery calculated based on a high-censored (>) MPN value

Table 9. Optimization Analyses, Results Summary: Surface Water Spiked with E. coli O157 [1, 2, 3]

Date	Spike Level (CFU/ 100 mL)	Mean Recovery (%)	Min Recovery (%)	Max Recovery (%)	SD (%)	RSD (%)	Overall Mean (%)	Overall SD (%)	Pooled within-run SD (%) [4]	Overall RSD (%)	Pooled within-run RSD (%) [5]
Neither (Antibiotics nor Elevated Incubation Temperature)											
12/1/2008	863	64	2	127	88	137	87	94	102	109	87
12/3/2008	963	79	46	112 [6]	47	59					
12/8/2008 [7]	1047	33	23	44	15	45					
12/15/2008	462	168	99	237	98	58					
12/22/2008	320	148	-9	305 [6]	222	150					
1/6/2009	713	109	64	153	63	58					
Elevated Incubation Temperature (42.0ºC)											
12/1/2008	863	72	18	127	77	106	103	81	84	79	58
12/3/2008	963	114	114	114 [6]	0	0					
12/8/2008	1047	103	103	103 [6]	0	0					
12/15/2008	462	96	96	96	0	0					
12/22/2008	320	188	40	336 [6]	210	112					
1/6/2009	713	140	140	140	0	0					
Antibiotics (cefsulodin and vancomycin)											
12/1/2008	863	127	127	127 [6]	0	0	66	57	54	86	93
12/3/2008	963	26	5	47	30	116					
12/8/2008	1047	62	21	103 [6]	58	93					
12/15/2008	462	52	6	99	66	127					
12/22/2008	320	92	43	141	69	75					
1/6/2009	713	94	33	154	85	91					
Both (Antibiotics and Elevated Incubation Temperature)											
12/1/2008	863	64	1	127	89	138	66	74	83	113	90
12/3/2008	963	5	4	5	1	13					
12/8/2008	1047	74	44	105	43	58					
12/15/2008	462	124	9	238 [6]	162	131					
12/22/2008	320	94	46	143	69	73					
1/6/2009	713	94	33	154	85	91					

[1] ">1099 MPN/100 mL" was replaced with "1099 MPN/100 mL" for calculation of summary statistics

[2] Two replicates were run per sample

[3] Samples were spiked with overnight (fresh) cultures

[4] Pooled within-run SD determined by calculating the square root of the mean of the run variances

[5] Pooled within-run RSD was determined by calculating the square root of the mean of the squared run RSDs

[6] Recovery calculated based on a high-censored (>) MPN value

[7] The 1099 MPN/100 mL unspiked result was removed for calculation of summary statistics

Table 10. Optimization Analyses, Results Summary: Unspiked Surface Water [1, 2]

Date	Mean (MPN/ 100 mL)	Min (MPN/100 mL)	Max (MPN/100 mL)	SD (%)	RSD (%)	Overall Mean (MPN/100 mL)	Overall SD (%)	Pooled within-run SD (%) [3]	Overall RSD (%)	Pooled within-run RSD (%) [4]
Neither (Antibiotics nor Elevated Incubation Temperature)										
11/24/2008	5.90	1.08	10.72	6.82	115.51	25.11	65.24	64.05	259.86	86.79
12/1/2008	5.18	3.01	7.36	3.08	59.34					
12/3/2008	20.75	3.01	38.50	25.10	120.92					
12/8/2008 [5]	3.01	3.01	3.01	0.00	0.00					
12/15/2008	3.01	3.01	3.01	0.00	0.00					
12/22/2008	121.42	3.05	239.80	167.41	137.87					
1/6/2009	5.42	3.61	7.23	2.56	47.25					
Elevated Incubation Temperature (42.0ºC)										
11/24/2008	1.08	1.08	1.08	0.00	0.00	24.43	42.06	34.44	172.18	72.08
12/1/2008	3.01	3.01	3.01	0.00	0.00					
12/3/2008	3.01	3.01	3.01	0.00	0.00					
12/8/2008	23.17	3.61	42.73	27.66	119.39					
12/15/2008	17.10	14.69	19.50	3.40	19.90					
12/22/2008	22.87	3.01	42.73	28.09	122.82					
1/6/2009	100.77	42.73	158.80	82.07	81.45					
Antibiotics (cefsulodin and vancomycin)										
Date	**Mean (MPN/ 100 mL)**	**Min (MPN/100 mL)**	**Max (MPN/100 mL)**	**SD (%)**	**RSD (%)**	**Overall Mean (MPN/100 mL)**	**Overall SD (%)**	**Pooled within-run SD (%) [3]**	**Overall RSD (%)**	**Pooled within-run RSD (%) [4]**
11/24/2008	1.17	1.08	1.26	0.12	10.65	6.51	7.67	5.83	117.82	65.33
12/1/2008	2.04	1.08	3.01	1.36	66.65					
12/3/2008	6.88	1.08	12.68	8.20	119.20					
12/8/2008	19.31	10.99	27.63	11.77	60.93					
12/15/2008	3.01	3.01	3.01	0.00	0.00					
12/22/2008	10.96	7.23	14.69	5.27	48.10					
1/6/2009	2.18	1.08	3.28	1.55	71.29					
Both (Antibiotics and Elevated Incubation Temperature)										
11/24/2008	1.17	1.08	1.26	0.12	10.65	1.66	0.96	0.62	57.89	31.51
12/1/2008	1.08	1.08	1.08	0.00	0.00					
12/3/2008	1.08	1.08	1.08	0.00	0.00					
12/8/2008	1.87	1.14	2.60	1.03	55.31					
12/15/2008	1.08	1.08	1.08	0.00	0.00					
12/22/2008	3.48	3.01	3.96	0.67	19.23					
1/6/2009	1.84	1.08	2.60	1.07	58.36					

[1] The "<" values were replaced with actual value (e.g., <1.08 was replaced with 1.08) for calculation of summary statistics

[2] Two replicates were run per sample

[3] Pooled within-run SD was determined by calculating the square root of the mean of the run variances

[4] Pooled within-run RSD was determined by calculating the square root of the mean of the squared run RSDs

[5] The 1099 MPN/100 mL result was removed for calculation of summary statistics

After the results of the optimization analyses were analyzed and evaluated, the workgroup determined that treatment two (elevated incubation temperature) was the optimal treatment option. The final culture-based, optimized procedure is as follows: (1) enrich samples in mBPW at 36.0°C ± 1.0°C for 2 – 2.5 hours followed by incubation at 42.0°C ± 0.5°C for an additional 20 – 24 hours, (2) submit tubes with growth to IMS, (3) plate IMS beads onto TC-SMAC and Rainbow® agars and incubate at 42.0°C ± 0.5°C for 18 – 24 hours, and (4) submit isolates to serological and biochemical test-based identification/confirmation.

5.4 – Drinking Water Analyses

Based on workgroup acceptance of the procedure, the laboratory was asked to evaluate two, unspiked and four, spiked drinking water samples using the optimized procedure. Results are provided in Table 11.

Table 11. Drinking Water Analyses, Results: Verification of the Optimized Procedure for E. coli O157

Sample ID	Spike Level (CFU/100 mL)	MPN Combo	E. coli O157 (MPN/100 mL)	Percent Recovery
PBS Samples				
Unspiked	NA	0-0-0	<1.081	
Spiked	438	3-3-1	462.2	105
		3-3-1	462.2	105
Drinking Water Samples				
Unspiked	NA	0-0-0	<1.081	
		0-0-0	<1.081	
Spiked	438	3-3-3	>1099	250
		3-3-2	1099	250
		3-3-2	1099	250
		3-3-1	462.2	105

5.5 – Combined Results of Verification of *E. coli* O157 Analyses in Drinking and Surface Water

Results for both drinking water and surface water samples using the "optimized" procedure (pre-enrichment in mBPW at 36°C, incubation at 42.0°C, IMS, plating on TC-SMAC and Rainbow® agars, and serological and biochemical confirmation) are summarized in Table 12. Surface water results are from Tables 9 and 10 and drinking water and PBS results are from Table 11. Verification results were compiled for drinking water and surface water to facilitate comparison of recoveries for both matrices. Please note not all PBS verification results are provided in Table 12.

Table 12. Drinking Water and Surface Water Analyses, Results: Verification of the "Optimized" Procedure

E. coli O157

Date	Sample ID	Spike Level (CFU/100 mL)	MPN Combo	E. coli O157 (MPN/100 mL)	Percent Recovery (corrected for ambient concentrations [1])
PBS Samples					
3/16/2009	Unspiked	NA	0-0-0	<1.08	
	Spiked	438	3-3-1	462.2	105
			3-3-1	462.2	105
Drinking Water Samples					
3/16/2009	Unspiked	NA	0-0-0	<1.08	
			0-0-0	<1.08	
	Spiked	438	3-3-3	>1099	250
			3-3-2	1099	250
			3-3-2	1099	250
			3-3-1	462.2	105
Surface Water Samples					
Date	**Sample ID**	**Spike Level (CFU/100 mL)**	**MPN Combo**	**E. coli O157 (MPN/100 mL)**	**Percent Recovery (corrected for ambient concentrations [1])**
12/1/2008	Unspiked	NA	0-0-0	<3.008	
			0-0-0	<3.008	
	Spiked	863	3-3-2	1099	127
			3-1-3	158.8	18
12/3/2008	Unspiked	NA	0-0-1	3.008	
			0-0-1	3.008	
	Spiked	963	3-3-2	1099	114
			3-3-3	>1099	114
12/8/2008	Unspiked	NA	3-1-0	42.73	
			1-0-0	3.61	
	Spiked	1047	3-3-2	1099	103
			3-3-2	1099	103

Date	Sample ID	Spike Level (CFU/100 mL)	MPN Combo	E. coli O157 (MPN/100 mL)	Percent Recovery (corrected for ambient concentrations [1])
12/15/2008	Unspiked	NA	2-1-0	14.69	
			1-2-2	19.5	
	Spiked	462	3-3-1	462.2	96
			3-3-1	462.2	96
12/22/2008	Unspiked	NA	3-1-0	42.73	
			0-0-1	3.008	
	Spiked	320	3-2-1	149.4	40
			3-3-2	1099	336
1/6/2009	Unspiked	NA	3-1-3	158.8	
			3-1-0	42.73	
	Spiked	713	3-3-2	1099	140
			3-3-2	1099	140

[1] Background levels of E. coli O157 in unspiked surface water samples (Table 10) were subtracted from spiked surface water levels to calculate percent recovery

Using the optimized procedure, results indicate that elevated incubation temperature (42.0°C) combined with IMS sufficiently reduced background organisms in surface water samples, resulting in good recoveries. Recoveries for the reference matrix (PBS) and drinking water were also good with this procedure. As there was no evidence of background organisms in drinking water samples, IMS may not be necessary for this matrix.

Section 6.0
Data Analysis and Discussion

After lengthy evaluation of multiple analytical procedures for identification and enumeration of *E. coli* O157 in water matrices, a culture-based procedure was optimized to overcome high microbial background in surface water samples. While not evaluated during this study, water quality parameters (e.g., pH, turbidity) may be collected during the use of this procedure. As indicated in Section 2, solid and particulate matrices were not evaluated during this phase of the effort.

6.1 – Initial Analyses

Preliminary water analyses were conducted using a laboratory-prepared *E. coli* O157:H7 spiking suspension and the procedure in the draft March 2008 document (enrichment in EEB, plating on TC-SMAC, and biochemical and serological confirmation). Problems of overgrowth with background organisms were encountered with surface water but not with PBS or drinking water samples. However, recoveries were poor for all three matrices: PBS, drinking water, and surface water (See Section 5.1).

SMEs were consulted regarding the high background in surface water samples. Potential options for method optimization were discussed including the use of mBPW as the primary enrichment media, elevated incubation temperature (42.0°C), antibiotics (acriflavin, cefsulodin, and vancomycin), IMS, and plating on both TC-SMAC and Rainbow® agars. The SMEs recommended mBPW because it has been used successfully to recover *E. coli* O157:H7 in environmental matrices. IMS was recommended as a step to separate *E. coli* O157 from the background flora, and antibiotics and elevated temperature were recommended to reduce background flora. The use of the two plating media was recommended to ensure that as many *E. coli* O157:H7 strains as possible could be detected by the method. Since *E. coli* O157:H7 is not as thermotolerant as other *E. coli* strains, the SMEs recommended a 42.0°C incubation as opposed to a 44.5°C incubation utilized for other *E. coli* strains.

6.2 – Pilot Analyses

Based on workgroup recommendation, analyses were conducted to evaluate SME suggested modifications, including the use of mBPW as the pre-enrichment medium, antibiotics (acriflavin, cefsulodin, and vancomycin), elevated temperature (42.0°C), and IMS. Initial results indicated the modifications were needed (see Tables 3 and 4). However, additional analyses of all three matrices (PBS, drinking water, surface water) resulted in recoveries ranging from 0 – 7%, indicating that further optimization would be required (see Tables 5 and 6). Limited pilot analyses with the modified method (mBPW and IMS) utilizing either antibiotics (acriflavin, cefsulodin, and vancomycin) or elevated temperature (42.0°C) independently (Table 7) provided improved recoveries, in comparison to samples analyzed using both (Tables 5 and 6). As a result, the workgroup recommended that the method should be optimized with regard to the use of antibiotics and elevated temperature.

6.3 – Optimization Analyses

Based on pilot analyses, it was suspected that the synergistic effect of both elevated temperature and antibiotics was detrimental to recovery of *E. coli* O157. SMEs and workgroup also speculated that acriflavin might be too harsh, especially when used in combination with additional antibiotics; therefore, optimization analyses were conducted with only cefsulodin and vancomycin. As a result, based on the workgroup recommendation, optimization analyses were conducted to evaluate four different treatment options: (1) neither antibiotics nor elevated incubation temperature (42.0°C), (2) elevated incubation temperature, (3) antibiotics (cefsulodin and vancomycin [acriflavin was not used]), and (4) both antibiotics and elevated incubation temperature. Results of spiked surface water and PBS samples are described in Section 6.3.1 and results of unspiked surface water samples are discussed in Section 6.3.2.

6.3.1 – Evaluation of Results for Spiked Surface Water and PBS Samples

Comparisons of the effect of the different treatment options on spiked surface water and PBS sample recovery were performed using two-way Analysis of Variance (ANOVA) models. Sample run was included in the models to control for between-run variability. A run-by-treatment interaction was also assessed for each matrix. Due to the high frequency of high-censored results (>1099 MPN/100 mL), analyses were performed with recoveries for high-censored samples calculated using (1) the censoring value (1099 MPN/100 mL) as the value when calculating recoveries for high-censored data, (2) the next possible MPN value (4622 MPN/100 mL, assuming one additional positive tube, if a 0.01 dilution had also been evaluated) as the value when calculating recoveries for high-censored data, and (3) all high-censored data removed. The high spike level

(10,200 CFU/100 mL) for the first run (November 24, 2008) resulted in some values above the analytical range (e.g., >1099), such that no uncensored result would produce a percent recovery greater than 11%. As a result, statistical analyses were also performed with and without data from this run. Analyses were also run with and without log-transforming the recoveries as the data did not give a clear indication of how the results were distributed within a treatment, matrix, and run. In all cases, based on the ANOVA models, 1) no run-by-treatment interaction was observed (i.e., the effect of treatment on recovery did not differ significantly between runs) and 2) no significant difference was observed between optimization treatments for either matrix.

6.3.2 – Evaluation of Results for Unspiked Surface Water

Since *E. coli* O157 was observed in the unspiked surface water samples for all seven runs, statistical analyses on unspiked surface water sample results were also performed, providing an unexpected opportunity to assess method performance on ambient *E. coli* O157 (Table 10). Comparisons of the effect of the different treatment options were also performed for the MPN/100 mL results observed in unspiked surface water samples using two-way ANOVA models. Sample run was included in the models to control for between-run variability. Run-by-treatment interaction was also assessed for each matrix. MPN values were log-transformed prior to analyses. Based on the ANOVA models, no run-by-treatment interaction was observed (i.e., the effect of treatment on MPN/100 mL did not differ significantly between runs). Interestingly, a significant difference in log MPN/100 mL was observed between the four different optimization treatments ($p = 0.0032$). To identify which specific treatments differed from which other treatments, comparisons between treatments were performed using Tukey's pairwise comparisons. Based on Tukey's:

- mean unspiked surface water results were significantly higher when "neither" treatment (101.8 mean MPN/100 mL) was applied, compared to when "both elevated temperatures and antibiotics were applied" (1.7 mean MPN/100 mL),
- mean unspiked surface water results were significantly higher when "elevated incubation temperature" (24.4 mean MPN/100 mL) was applied, compared to when "both elevated temperature and antibiotics were applied" (1.7 mean MPN/100 mL), and
- "use of antibiotics" (6.5 mean MPN/100 mL) was not significantly different from any of the other treatments.

It should be noted that the mean MPN/100 mL for "neither" is being driven by a single 1099 MPN/100 mL result observed on December 8, 2008. The laboratory could not readily identify any issues, and they also expressed concern regarding the high value. In recognition that this single result had a substantial impact on the mean for that treatment, the statistical analyses described above were repeated with that data point removed. Again, a significant difference in log MPN/100 mL was observed between the four different treatments ($p = 0.0014$) and, although the mean result for "neither" dropped from a mean of 101.8 MPN/100 mL to 25.1 MPN/100 mL, Tukey's comparisons revealed the same statistical results, as noted above; that is, the exclusion of the 1099 MPN/100 mL had no effect on the result of the statistical analyses (Table 10).

Although the "neither" treatment provided the higher mean recovery (25.1 MPN/100 mL vs. 24.4 MPN/100 mL for elevated temperature), it was determined that some element of selectivity was required in the procedure (i.e., elevated incubation temperature) to address issues with background organisms in some surface water matrices. Results indicated that elevated incubation temperature sufficiently reduced background organisms in the surface water matrix (background organisms were not observed in PBS and drinking water matrices). Thus, based on the optimization results and workgroup recommendations, the procedure was revised to include the use of elevated incubation temperature (42.0°C), along with mBPW, and IMS.

6.4 – Drinking Water Analyses using Optimized Procedure

Since optimization analyses were conducted using PBS and surface water matrices, follow-on analyses were conducted using the optimized procedure (mBPW, elevated incubation temperature, and IMS) to verify that the procedure performed acceptably with drinking water. The estimated percent recoveries, ranging from 105% to 250% were considered acceptable. It should be noted that while 250% recovery may seem high, this is not surprising given the variability in the 1099 MPN value (the 95% confidence interval for this value is 225 – 3075). It should also be noted that while using a 15-tube MPN set-up would increase laboratory burden and reduce the number of samples that could be processed per day, it would also result in tighter confidence intervals and potentially minimize the impact of an additional positive tube (e.g., a 5-5-0 combination is 240 MPN/100 mL and a 5-5-1 combination is 350 MPN/100 mL). It should also be noted that an MPN value is not considered absolute quantitation, as a direct plate count would be, because values are based on the probability of tube being positive.

6.5 – Rainbow® Agar

The use of Rainbow® agar was problematic throughout the study for the following reasons. The agar is only available from a single vendor and was often on backorder during the study. In addition, the laboratory indicated that it was very difficult to obtain isolated colonies on Rainbow® agar and in many instances when typical colonies were observed on TC-SMAC, there were no typical colonies on the corresponding Rainbow® agar plates. In addition, the participant laboratory commented on the varying quality of the media from lot-to-lot. It is recommended that a panel of different *E. coli* O157:H7 strains should be evaluated as a preliminary step to the multi-laboratory validation study, to confirm whether the use of Rainbow® agar is necessary.

Section 7.0
Conclusion

Based on the results of the Study, the optimized culture-based procedure has acceptable method performance for identification and enumeration of *E. coli* O157 in PBS, drinking water, and surface water samples. The optimized procedure includes: (1) cultures are enriched in mBPW at 36.0°C ± 1.0°C for 2 – 2.5 hours followed by incubation at 42.0°C ± 0.5°C for an additional 20 – 24 hours, (2) tubes with growth are submitted to IMS, (3) IMS beads are plated onto TC-SMAC and Rainbow® agars and incubated at 42.0°C ± 0.5°C for 18 – 24 hours, and (4) isolates are then submitted to serological and biochemical confirmation. Time to results for the procedure as verified is approximately 96 hours from receipt of samples. However, time to results would be approximately 48 hours if only serological confirmation were conducted (e.g., no biochemical confirmation) or 72 hours if alternate biochemical analyses were used (e.g., Vitek®).

As a result, the optimized procedure merits multi-laboratory validation to assess method performance and set quantitative QC criteria, so that the procedure can be considered for potential implementation on a national scale. The draft SAP will be revised to include the optimized procedure prior to multi-laboratory validation. Although the method was evaluated using IMS for all three water matrices, consideration may be given to evaluating drinking water samples both with and without the IMS step during preliminary phases of the multi-laboratory evaluation to confirm its necessity in this matrix. Due to issues associated with Rainbow® agar, we recommend, as a preliminary step to the multi-laboratory validation study, evaluating a panel of different *E. coli* O157:H7 strains to determine whether the use of Rainbow® agar is necessary.

Section 8.0
References

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