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Standard Analytical Protocol for *Escherichia coli* O157:H7 in Water

Office of Research and Development
National Homeland Security Research Center

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The procedures described in this document are intended for use in laboratories when analyzing environmental samples in support of remediation efforts following a homeland security incident. The (culture-based) procedures provide viability determination and identification as either qualitative or quantitative results. The sample preparation procedures are deemed the most appropriate for the wide variety of matrices to be examined. To the extent possible, these procedures were developed to be consistent with other federal agency procedures. These procedures do not include the sample collection, rapid screening, field techniques, or molecular techniques that may accompany laboratory analysis.

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Contents

1.0
Scope and Application

1.1
This Standard Analytical Protocol (SAP) is for the identification, confirmation, and enumeration of *Escherichia coli* O157:H7 (*E. coli* O157:H7) in water samples using selective and non-selective media followed by biochemical characterization and serological confirmation.

1.2
This protocol has been adapted from the journal article "Evaluation of Techniques for Enrichment and Isolation of *Escherichia coli* O157:H7 from Artificially Contaminated Sprouts" by Weagant and Bound (Reference 15.1), and is for use by laboratories when analyzing samples in support of U.S. Environmental Protection Agency (EPA) homeland security efforts.

1.3
E. coli O157:H7 is the causative agent of enterohemorrhagic diarrhea. Due to the infectious nature of the bacterium and the potential for transmission to humans, all procedures should be performed in laboratories that use, at a minimum, biological safety level (BSL)-2 practices (Reference 15.2). Use of a biological safety cabinet (BSC) is recommended for any aerosol-generating procedures.

1.4
All sample handling, analysis, and reporting of results must be performed in accordance with established guidelines. Laboratories must have requisite resources in place prior to use of these procedures.

1.5
This protocol is not intended for use as a test for microorganisms other than *E. coli* O157:H7 and the matrices described.

2.0
Summary of Protocol

2.1
E. coli O157:H7 can be identified in a variety of water samples using selective media, and biochemical and serological analyses. Bacterial densities can be estimated using the most probable number (MPN) approach.

2.2
For qualitative results, samples are diluted 1:1 in double-strength modified buffered peptone water (mBPW). Samples are incubated at 36.0°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.

2.3
For quantitative results, samples are analyzed as received. All samples are analyzed using the MPN technique in mBPW and incubated at 36.0°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.

2.4
Broth cultures (MPN and qualitative analyses tubes) with positive growth (turbidity) are submitted to immunomagnetic separation (IMS) and sub-cultured onto tellurite cefixime sorbitol MacConkey (TC-SMAC) and Rainbow® agars.

2.5
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 biochemical and serological confirmation. Serological confirmation is by agglutination using *E. coli* O157 and H7 antiserum, followed by biochemical characterization using commercially available test strips (e.g., API 20E® or equivalent) or with a group of selected individual biochemical tests.

2.6
Broth cultures (MPN and qualitative analysis tubes) concentrated by IMS may be subjected to real-time polymerase chain reaction (PCR) confirmation in place of biochemical and serological confirmation.

2.7
Quantification of *E. coli* O157:H7 is determined using the MPN technique (Flowchart 16.1). Tubes that are confirmed positive for *E. coli* O157:H7 are used to determine MPN.

Note: *Typing by serology for the H7 antigen or PCR for the H7 gene was not conducted during the single-laboratory verification study. To confirm E. coli O157:H7, additional serological or PCR analyses would be necessary.*

3.0
Acronyms and Abbreviations

ASTM	ASTM International (formerly American Society for Testing and Materials)
ATCC®	American Type Culture Collection
BSC	Biological safety cabinet
BSL	Biological safety level
°C	Degrees Celsius
CT	Cefixime potassium tellurite
EPA	U.S. Environmental Protection Agency
g	Gram(s)
IMS	Immunomagnetic separation
L	Liter(s)
µL	Microliter(s)
µm	Micrometer(s)
mBPW	Modified buffered peptone water
mg	Milligram(s)
mL	Milliliter(s)
mm	Millimeter(s)
MPN	Most probable number
N	Normal
NCTC	National Collection of Type Cultures
NHSRC	National Homeland Security Research Center
NIST	National Institute of Standards and Technology
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PPE	Personal protective equipment, i.e., gloves, goggles, laboratory coat, etc.
psi	Pounds per square inch
QC	Quality control
SAP	Standard Analytical Protocol
TC-SMAC	Tellurite cefixime sorbitol MacConkey agar
TSA	Tryptic soy agar
w/v	Weight to volume ratio

4.0
Interferences and Contamination

4.1
Low recoveries of *E. coli* O157:H7 may be caused by the presence of either high numbers of competing or inhibitory organisms (e.g., other Enterobacteriaceae), or toxic substances (e.g., metals, organic compounds).

4.2
A viable but non-culturable state of *E. coli* O157:H7 may also account for lower recoveries (Reference 15.3).

5.0
Safety

5.1 Laboratory Hazards

To prevent transmission, disposable gloves should be worn when working with this organism. Hands should be washed immediately following removal of gloves. Direct and indirect contact of intact or broken skin with cultures and/or contaminated laboratory surfaces, accidental parenteral inoculation, and rarely, exposure to infectious aerosols are the primary hazards to laboratory personnel. Staff should apply safety procedures used for pathogens when handling all samples.

5.2 Recommended Precautions

5.2.1

E. coli O157:H7 is a BSL-2 pathogen and all procedures should be performed in laboratories that use, at a minimum, BSL-2 practices (Reference 15.2). This includes prohibiting eating, drinking, smoking, handling contact lenses, applying cosmetics, and storing food and drink in the laboratory.

5.2.2

A Class II BSC is recommended for sample manipulations where the risk of aerosol production is high. Production of aerosols should be avoided.

5.2.3

Disposable materials are recommended for sample manipulation.

5.2.4

Mouth-pipetting is prohibited.

5.2.5

The analyst must know and observe normal safety procedures required in a microbiology laboratory. These procedures must be followed while preparing, using, and disposing of media, cultures, reagents, and materials, and while operating sterilization equipment.

5.2.6

Personal Protective Equipment (PPE)

> ***5.2.6.1***
>
> Disposable nitrile gloves should be worn at all times to prevent contact with infectious materials. Gloves should be changed whenever they are visibly soiled. Aseptic technique should be used when removing gloves and other protective clothing.
>
> ***5.2.6.2***
>
> Protective goggles and/or non-breakable, chemical-resistant glasses should be worn, as appropriate.
>
> ***5.2.6.3***
>
> Protective laboratory coats covering arms and clothing and closed in the front should be worn at all times. Laboratory coats that become soiled should be changed.

5.2.7

This protocol does not address all safety issues associated with its use. Please refer to *Biosafety in Microbiological and Biomedical Laboratories (BMBL)*, 5th Edition (Reference 15.2) for additional safety information. A reference file of Material Safety Data Sheets should be available to all personnel involved in analyses.

6.0
Equipment and Supplies

6.1
Autoclave or steam sterilizer, capable of achieving 121°C (15 pounds per square inch [psi]) for 15 minutes

6.2
Autoclave bags, aluminum foil, or kraft paper

6.3
Balance, analytical, with ASTM Class S reference weights, capable of weighing 20 g ± 0.001 g

6.4
Balance, top loading, with Class S reference weights, capable of weighing 100 g ± 0.1 g

6.5
Bead rotator (Dynabeads® Sample Mixer or equivalent), optional

6.6
Beakers, glass or plastic (assorted sizes)

6.7
Biological safety cabinet, Class II (optional)

6.8
Borosilicate glass or plastic screw-cap, wide-mouth bottles, sterile (e.g., 250 mL)

6.9
Borosilicate glass culture tubes, with autoclavable screw or snap caps (25 × 150 mm)

6.10
Erlenmeyer flasks (500 mL, 1 L, 2 L)

6.11
Filters, for reagent sterilization, sterile (0.22 µm pore size), and filter syringes, sterile

6.12
Graduated cylinders (assorted sizes)

6.13
Gloves, sterile, nitrile or equivalent

6.14
Immunomagnetic separator (Dynal® Bead Separator or equivalent)

6.15
Incubators, microbiological type, maintained at 36.0°C ± 1.0°C and 42.0°C ± 0.5°C

6.16
Inoculation loops, sterile, disposable

6.17
Parafilm™ or equivalent

6.18
Petri dishes, sterile, plastic (15 × 100 mm)

6.19
pH meter

6.20
Pipettes, standard tip, sterile, plastic, disposable (assorted sizes)

6.21
Pipetting device (automatic or equivalent)

6.22
Stirring hotplates and stir bars

6.23
Test tube racks

6.24
Thermometer, National Institute of Standards and Technology (NIST)-traceable

6.25
Tissues, lint-free (Kimwipes® or equivalent)

6.26
Vortex

6.27
Waterbath, maintained at 45°C – 50°C

6.28
Weigh paper and boats

7.0
Reagents and Standards

7.1
Reagent-grade chemicals must be used in all tests. Unless otherwise indicated, reagents shall conform to the specifications of the Committee on Analytical Reagents of the American Chemical Society (Reference 15.4). For suggestions regarding the testing of reagents not listed by the American Chemical Society, see *AnalaR Standards for Laboratory Chemicals* (Reference 15.5) and *United States Pharmacopeia and National Formulary 24* (Reference 15.6).

7.2
Whenever possible, use commercially available culture media. The agar used in the preparation of culture media must be of microbiological grade. Storage requirements for prepared media and reagents are provided in Table 2 (Section 7.18).

7.3
Reagent-grade water must conform to specifications in *Standard Methods for the Examination of Water and Wastewater*, 21st Edition, Section 9020 (Reference 15.7).

7.4 Dilution Water
Dilution water is a phosphate buffer and is also commonly referred to as Butterfield buffer or phosphate buffered dilution water. Commercially prepared reagents are recommended (Hardy Diagnostics D699 or equivalent).

7.4.1
Composition of stock phosphate buffer solution:

Monopotassium phosphate (KH_2PO_4)	34.0 g
Reagent-grade water	0.5 L

7.4.2
Prepare stock phosphate buffer by dissolving 34.0 g monopotassium phosphate in 0.5 L of reagent-grade water. Adjust pH to 7.2 ± 0.5 with 1 N sodium hydroxide and dilute to 1.0 L with reagent-grade water. Sterilize by filtration or autoclave at 121°C (15 psi) for 15 minutes.

7.4.3
Composition of stock magnesium chloride solution:

Magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_2O$)	81.1 g
Reagent-grade water	1.0 L

7.4.4
Prepare stock magnesium chloride solution by dissolving 81.1 g of magnesium chloride hexahydrate in 1.0 L of reagent-grade water. Sterilize by filtration or autoclave at 121°C (15 psi) for 15 minutes.

7.4.5
After sterilization, store the stock solutions in the refrigerator. Handle aseptically. If evidence of mold or other contamination appears, the affected stock solution should be discarded and a fresh solution should be prepared.

7.4.6
Prepare phosphate buffered dilution water by adding 1.25 mL stock phosphate buffer solution and 5.0 mL stock magnesium chloride solution to 1.0 L of reagent-grade water. Autoclave at 121°C (15 psi) for 15 minutes.

7.5 Phosphate Buffered Saline (PBS)
Prepare reagent according to the following and store at <10°C and above freezing for a maximum of two weeks in tubes with loose caps or three months in screw-cap tubes.

7.5.1

Composition:

Monosodium phosphate (NaH_2PO_4)	0.58 g
Diosodium phosphate (Na_2HPO_4)	2.50 g
Sodium chloride	8.50 g
Reagent-grade water	1.0 L

7.5.2

Dissolve reagents in 1.0 L reagent-grade water, adjust pH to 7.4 ± 0.2 with 1.0 N hydrochloric acid or 1.0 N sodium hydroxide, and dispense appropriate volumes in screw-cap bottles or tubes and autoclave at 121°C (15 psi) for 15 minutes.

7.6 Modified Buffered Peptone Water (mBPW)

Prepare 1X, 2X, and 5X mBPW according to the following and store at <10°C and above freezing for a maximum of two weeks in tubes with loose caps or three months in screw-cap tubes.

7.6.1

Composition:

	1X	**2X**	**5X**
Peptone	10.0 g	20.0 g	50.0 g
Sodium chloride	5.0 g	10.0 g	25.0 g
Disodium phosphate (Na_2HPO4)	3.6 g	7.2 g	18.0 g
Monopotassium phosphate (KH_2PO4)	1.5 g	3.0 g	7.5 g
Casamino acids	5.0 g	10.0 g	25.0 g
Yeast extract	6.0 g	12.0 g	30.0 g
Lactose	10.0 g	20.0 g	50.0 g
Pyruvate	2.0 g	4.0 g	10.0 g
Reagent-grade water	1.0 L	1.0 L	1.0 L

7.6.2

Add reagents to 950 mL of reagent-grade water and mix thoroughly using a stir bar. Adjust pH to 7.2 ± 0.2 with 1.0 N hydrochloric acid or 1.0 N sodium hydroxide and bring up to 1.0 L. Dispense 10 mL (1X and 2X) or 5 mL (5X) aliquots in 25 × 150 mm tubes and autoclave at 121°C for 15 minutes. Cool to room temperature.

7.7 Tellurite Cefixime Sorbitol MacConkey Agar (TC-SMAC)

Commercially prepared media is recommended. Dehydrated medium (Oxoid CM0813 or equivalent), with cefixime and potassium tellurite (CT) supplement (Oxoid SR172 or equivalent), or prepared plates (Oxoid PO0702 or equivalent) may be used. If commercially prepared media are not available, prepare medium using procedures in Sections 7.7.1 through 7.7.4.

7.7.1

Composition:

Peptone	20.0 g
D-Sorbitol	10.0 g
Bile salts No. 3	1.5 g
Sodium chloride	5.0 g
Agar	15.0 g
Neutral red	0.03 g
Crystal violet	0.001 g
Reagent-grade water	1.0 L

7.7.2

Add reagents to 950 mL of reagent-grade water and mix thoroughly using a stir bar and hot plate. Boil for one minute with rapid stir bar agitation to dissolve completely. Adjust pH to 7.1 ± 0.2 with 1.0 N hydrochloric acid or 1.0 N sodium hydroxide and bring up to 1.0 L. Autoclave at 121°C (15 psi) for 15 minutes. Cool to 45°C – 50°C in a waterbath.

7.7.3

Prepare CT supplement according to manufacturer's instructions and filter sterilize.

7.7.4

Prepare TC-SMAC plates by adding two reconstituted CT supplement vials to the cooled medium, swirling to mix. Final concentrations for cefixime and tellurite are 0.05 mg/L and 2.5 mg/L, respectively. Aseptically pour 12 – 15 mL into each 15 × 100 mm sterile Petri plate.

7.8 Rainbow® Agar

Commercially prepared dehydrated medium (Biolog 80101 or equivalent) is recommended.

7.8.1

Add 60.0 g dehydrated reagent to 1.0 L of reagent-grade water and mix thoroughly using a stir bar and hot plate. Boil gently to dissolve and autoclave at 121°C (15 psi) for 15 minutes. Cool to 45°C – 50°C in a waterbath.

7.8.2

Prepare stock potassium tellurite (Sigma-Aldrich® P0677 or equivalent) solution by dissolving 0.008 g in 10.0 mL reagent-grade water and filter sterilizing. Prepare stock novobiocin (Sigma-Aldrich® N1628 or equivalent) by dissolving 0.010 g in 10 mL reagent-grade water and filter sterilizing.

7.8.3

Prepare plates by adding 1.0 mL each of tellurite and novobiocin stock solutions to the cooled medium, swirling to mix. Final concentrations should be 0.8mg/L and 10.0 mg/L, respectively, with medium pH of 7.9 – 8.3. Aseptically pour 12 – 15 mL into each 15 × 100 mm sterile Petri plate.

7.9 Tryptic Soy Agar (TSA)

Commercially prepared medium is recommended. Prepared plates (BBL™ 221803 or equivalent) or dehydrated media (BBL™ 211043 or equivalent) may be used. If commercially prepared media are not available, prepare media using procedures in Sections 7.9.1 and 7.9.2.

7.9.1

Composition:

Pancreatic digest of casein	15.0 g
Papaic digest of soybean meal	5.0 g
Sodium chloride	5.0 g
Agar	15.0 g
Reagent-grade water	1.0 L

7.9.2

Add reagents to 950 mL of reagent-grade water and mix thoroughly using a stir bar and hot plate. Heat to dissolve completely. Adjust pH to 7.3 ± 0.2 with 1.0 N hydrochloric acid or 1.0 N sodium hydroxide and bring up to 1.0 L. Autoclave at 121°C (15 psi) for 15 minutes and cool to 45°C – 50°C in a waterbath. Aseptically pour 12 – 15 mL into each 15 × 100 mm sterile Petri plate.

7.10

E. coli O157 IMS beads (Invitrogen™ 71004 or equivalent)

7.11

IMS wash buffer: Add 50 µL Tween® 20 to 100 mL of PBS, ensuring all of the Tween® 20 is expelled from the pipette tip; filter sterilize or autoclave at 121°C (15 psi) for 15 minutes.

7.12

Saline, physiological (0.85% w/v): Dissolve 0.85 g NaCl in 100 mL of reagent-grade water. Autoclave at 121°C (15 psi) for 15 minutes. Cool to room temperature.

7.13

E. coli O157 latex agglutination reagent (Oxoid DR0620M or equivalent)

7.14

E. coli H7 latex agglutination reagent (Wellcolex® *E. coli* O157:H7 Rapid Latex Agglutination Test or equivalent)

7.15

Biochemical test strip (bioMérieux API 20E® or equivalent)

7.16

Oxidase reagent (BD™ DrySlide™ 231746 or equivalent)

7.17

Positive and negative control cultures that are to be used in the procedures in Section 10.0 are listed in Table 1, below. Use of these controls is discussed in Section 9.0.

Table 1. Positive and Negative Control Cultures for Described Tests

Media/Tests	Positive Control	Negative Control
TC-SMAC	*E. coli* O157:H7 (ATCC® 700728™ / NCTC 12900)	*E. coli* (ATCC® 25922™)
Rainbow®	*E. coli* O157:H7 (ATCC® 700728™ / NCTC 12900)	*E. coli* (ATCC® 25922™)
O157 latex agglutination	*E. coli* O157:H7 (ATCC® 700728™ / NCTC 12900)	*E. coli* (ATCC® 25922™)
H7 serum agglutination (Optional)	*E. coli* O157:H7 (ATCC® 700728™ / NCTC 12900)	*E. coli* (ATCC® 25922™)
Biochemical test strip	*E. coli* O157:H7 (ATCC® 700728™ / NCTC 12900)	*Pseudomonas aeruginosa* (ATCC® 27853™)
Oxidase	*Pseudomonas aeruginosa* (ATCC® 27853™)	*E. coli* O157:H7 (ATCC® 700728™ / NCTC 12900)

7.18

Storage temperatures and times for prepared media and reagents are provided in Table 2. Follow manufacturers' guidelines for storage and expiration of all commercially prepared reagents.

Table 2. Storage Temperatures and Times for Prepared Media and Reagents[1]

Media/Reagents	Storage Temperature	Storage Time
Dilution water, PBS, saline (in screw-cap containers)	<10ºC and above freezing	3 months
Tubes: mBPW	<10ºC and above freezing	2 weeks in loose-cap tubes 3 weeks in screw-cap tubes
Plates: TC-SMAC, Rainbow®[2], TSA	<10ºC and above freezing	2 weeks

[1] If media/reagent is refrigerated, remove from refrigerator 1–1.5 hours prior to inoculation to ensure that it reaches room temperature prior to use

[2] Dehydrated medium is hygroscopic and light-sensitive; store tightly sealed and protected from light

8.0
Calibration and Standardization

8.1
Check temperature in incubators twice daily, a minimum of four hours apart, to ensure operation is within stated limits of the protocol. Record daily measurements in an incubator log book.

8.2
Check temperature in refrigerators/freezers at least once daily to ensure operation is within stated limits of the protocol. Record daily measurements in a refrigerator/freezer log book.

8.3
Calibrate thermometers and incubators annually against a NIST-certified thermometer or against a thermometer that meets the requirements of NIST Monograph SP 250-23 (Reference 15.8). Check mercury columns for breaks.

8.4
Calibrate pH meter prior to each use with two of three standards (e.g., pH 4.0, 7.0, or 10.0) closest to the range being tested.

8.5
Calibrate analytical and top-loading balances with ASTM Class S reference weights once per month, at a minimum. Check each day prior to use with Class S weights.

8.6
Calibrate micropipettors once per year. Spot-check micropipettor accuracy once per month by weighing a measured amount of reagent-grade water (1 µL = 1 mg).

8.7
Re-certify BSCs once per year. Re-certification must be performed by a qualified technician.

9.0
Quality Control

9.1 General

Each laboratory that uses this protocol is required to operate a formal quality assurance program that addresses and documents instrument and equipment maintenance and performance, reagent quality and performance, analyst training and certification, and records storage and retrieval. Specific quality control (QC) procedures for use with this protocol are discussed below.

Note: *Following testing and validation, this protocol will be updated to include QC criteria for initial and ongoing demonstration of capability as well as matrix spike/matrix spike duplicates.*

9.2 Negative Controls

9.2.1

The laboratory should analyze negative controls to ensure that all media and reagents are performing properly. Negative controls should be analyzed whenever a new batch of media or reagents is used. On an ongoing basis, the laboratory should analyze a negative control every day that samples are analyzed. Recommended negative control organisms are provided in Table 1 (Section 7.17), and descriptions of negative results are provided in Table 3 (Section 10.8).

9.2.2

Analysis of negative controls is conducted by inoculating media and performing biochemical tests with known negative control organisms as described in Section 10.0. The negative control is treated as a sample and submitted to the same analytical procedures.

9.2.3

If a negative control fails to exhibit the appropriate response, check and/or replace the associated media, reagents, and/or the respective negative control organism, and re-analyze the appropriate negative control and corresponding sample(s).

9.2.4

Viability of the negative controls should be demonstrated on a monthly basis, at a minimum, using a non-selective media (e.g., TSA).

9.3 Positive Controls

9.3.1

The laboratory should analyze positive controls to ensure that all media and reagents are performing properly. Positive controls should be analyzed whenever a new batch of media or reagents is used. On an ongoing basis, the laboratory should analyze a positive control every day that samples are analyzed. Recommended positive control organisms are provided in Table 1 (Section 7.17), and descriptions of positive results are provided in Table 3 (Section 10.8).

9.3.2

Analysis of positive controls is conducted by inoculating media and performing biochemical tests with known positive organisms as described in Section 10.0. The positive control is treated as a sample and submitted to the same analytical procedures.

9.3.3

If a positive control fails to exhibit the appropriate response, check and/or replace the associated media, reagents, and/or the positive control organism, and re-analyze the appropriate positive control and corresponding sample(s).

9.4 Method Blank

On an ongoing basis, every day that samples are analyzed, the laboratory should analyze a method blank using sterile dilution water or PBS (Sections 7.4 and 7.5, respectively) to verify the sterility of equipment, materials, and supplies. The method blank is treated as a sample and submitted to the same analytical procedures. Absence of growth indicates freedom from contamination.

9.5 Media Sterility Check

Test sterility of dilution water, PBS, mBPW, and TSA by incubating one unit (tube or plate) from each batch at 35.0°C – 37.0°C for 24 ± 2 hours and observing for growth. Test sterility of TC-SMAC and Rainbow® by incubating one unit (tube or plate) from each batch at 42.0°C ± 0.5°C for 24 ± 2 hours and observing for growth. Absence of growth indicates the media are sterile. On an ongoing basis, a media sterility check should be done every day that samples are analyzed.

10.0
Procedures

Process samples promptly upon receipt, allowing no more than six hours to elapse from the time of sample collection to the start of sample processing, which should be completed within two hours. *E. coli* O157:H7 is a pathogen, and all samples should be handled with caution, using appropriate BSL-2 procedures and PPE. A Class II BSC is recommended for sample manipulations where the risk of aerosol production is high, such as vigorous shaking.

10.1 Qualitative Sample Analysis

Add a sample volume (e.g., 200 mL) to an equal volume of double-strength mBPW (Section 7.6). Incubate at 36.0°C ± 1.0°C for 2.0 – 2.5 hours, non-shaking. Transfer samples to 42.0°C ± 0.5°C and continue incubation, non-shaking, for a total of 20 – 24 hours. After incubation, proceed to Section 10.4 for selective isolation of *E. coli* O157:H7.

10.2 Quantitative Sample Analyses

A multiple-tube assay incorporating differential sample volumes is used to estimate *E. coli* O157:H7 densities in undiluted or diluted samples. If low levels of *E. coli* O157:H7 are suspected, larger sample volumes (20.0 mL of original sample) should be used to inoculate the first row of tubes in the series. If high levels of *E. coli* O157:H7 are suspected, additional serial dilutions should be used. See Flowchart 16.1 for an overview of the sample dilution and inoculation scheme. A minimum sample volume of 156 mL is required if 20 mL volumes are used to inoculate the first row of tubes.

10.2.1 *Sample inoculation*

Arrange mBPW tubes in three rows (5 mL of 5X, 10 mL of 2X, and 10 mL of 1X) of five tubes each. Inoculate the first row of tubes (5 mL of 5X mBPW) with 20 mL of the undiluted sample. Inoculate 10 mL of the undiluted sample into each of the tubes in the second row (5 mL of 3X mBPW). Inoculate 1 mL from the initial sample into each of the tubes in the third row (1X mBPW). See Flowchart 16.1 for an overview of the sample inoculation scheme.

10.2.2 *Sample dilutions*

Samples may require serial dilution prior to inoculation due to high levels of *E. coli* O157:H7. If analyzing serially diluted samples, 1.0 mL of each dilution will be used to inoculate each tube of 1X mBPW, as appropriate.

10.2.3 *Growth*

Incubate tubes at 36.0°C ± 1.0°C for 2.0 – 2.5 hours, non-shaking. Transfer samples to 42.0°C ± 0.5°C and continue incubation, non-shaking, for a total of 20 – 24 hours. After incubation, proceed to Section 10.3 for selective separation and concentration of *E. coli* O157:H7.

10.3 Immunomagnetic Separation and Concentration

See Flowchart 16.2 for an overview of the colony identification procedures. From each tube with growth, conduct IMS as follows:

10.3.1

Suspend *E. coli* O157:H7 beads by vortexing and add 20 µL of the bead suspension to a sterile, 1.5 mL – 2.0 mL tube.

10.3.2

Gently swirl contents of mBPW tubes to mix, remove 1.0 mL of mBPW culture, add to tube with beads, and tightly cap. Mix for 10 minutes at room temperature using the tube rotator.

10.3.3

Place tubes in magnetic holder, inverting tubes several times to ensure that beads are concentrated in a pellet on the side of the tube; allow beads to settle for three minutes. Carefully aspirate liquid and discard.

10.3.4

Resuspend beads in 1.0 mL IMS wash buffer (Section 7.11) by inverting the tube several times. Place tubes in magnetic holder and allow beads to settle for three minutes. Aspirate wash buffer and resuspend the beads in 100 µL of IMS buffer.

Note: *IMS bead complex may be subjected to real-time PCR confirmation in place of, or in addition to, culture on selective media followed by biochemical and serological confirmation.*

10.4 Isolation on Selective Agars

10.4.1

Pipette 50 µL (half the beads) onto Rainbow® agar; use a sterile loop to spread the 50-µL aliquot over one-third of the plate then streak for isolation on the remaining portion of the plate. Repeat this procedure with the remaining 50 µL onto TC-SMAC.

10.4.2

Incubate Rainbow® and TC-SMAC plates for 18 – 24 hours at 42.0°C ± 0.5°C. On TC-SMAC, typical *E. coli* O157:H7 colonies are colorless and 2 – 3 mm in diameter. On Rainbow® agar, typical *E. coli* O157:H7 colonies are black/gray.

Note: *Colonies on Rainbow® agar tend to be much smaller and run together making it difficult to obtain well-isolated colonies for serological and biochemical confirmation. Prolonged incubation of TC-SMAC plates may result in colonies of E. coli O157:H7 losing their characteristic colorless appearance. Also, the color of sorbitol-positive colonies can fade, making them difficult to distinguish from sorbitol-negative colonies. Appropriate negative and positive controls should be analyzed to avoid confusion (Sections 9.2 and 9.3, respectively).*

10.5 Serological Analyses

The procedures for the use of latex agglutination kits are provided in Sections 10.5.1 and 10.5.2. Section 10.5.3 provides an optional procedure for the use of antiserum for serological confirmation.

10.5.1

E. coli O157 latex agglutination kit: Choose a well-isolated typical colony from each of the positive TC-SMAC and/or Rainbow® plates and emulsify growth using sterile physiological saline (Section 7.12). Evaluate according to manufacturer's instructions.

10.5.2

Repeat Section 10.5.1 using H7 latex agglutination reagent.

Note: *Identification of the H7 flagellar antigen is usually conducted by reference laboratories and may be difficult since isolates often require multiple passages in motility medium before the antigen is detected. PCR identification of the gene for the H7 antigen may be conducted in place of serology. Neither of these procedures was verified during the single-laboratory study.*

10.5.3

E. coli O157 or H7 antiserum (optional): Choose a well-isolated typical colony from each of the positive TC-SMAC and/or Rainbow® plates and emulsify growth using sterile physiological saline. Place two discrete drops of emulsified growth onto a slide. To the first drop of emulsified growth, add one drop of O157 antiserum. To the second drop of emulsified growth, add one drop of sterile saline (as a visual comparison).

Observe under magnification for an agglutination reaction, which indicates a positive result. *E. coli* O157:H7 is agglutination-positive for O157 antiserum. Results should be compared with those for positive and negative controls (Table 1) analyzed at the same time.

10.6 Isolation on TSA Plates

10.6.1

Streak one O157 serological-positive isolate onto a TSA plate from each serology-positive TC-SMAC or Rainbow® plate. For spiked samples with no background, a single serological-positive isolate from each dilution should be streaked onto a TSA plate. Incubate the plates at 36.0°C ± 1.0°C for 24 ± 2 hours.

10.6.2

Seal the TC-SMAC and Rainbow® plates with Parafilm™ and store at <10°C and above freezing for use as backup/archive plates. Use the TSA plates for biochemical analyses.

10.7 Biochemical Analyses

Use a single, isolated, large colony (2 – 3 mm diameter) from each TSA plate for biochemical test strip and oxidase analyses.

10.7.1 *Oxidase Test (BD™ DrySlide™ 231746 or equivalent)*

Following manufacturer's instructions, transfer a small amount of growth from an isolated colony to the slide. Oxidase-positive bacteria turn the reagent dark purple within 20 seconds. *E. coli* O157:H7 is oxidase-negative. Results should be compared with those for positive and negative controls (Table 3) analyzed at the same time.

10.7.2 *Biochemical Test Strips (API 20E® or equivalent)*

Emulsify the remainder of the colony in 0.85% NaCl. Follow manufacturers' instructions to inoculate wells and add appropriate reagents. Incubate test strip according to manufacturers' instructions. Add additional reagents, read, and record results.

Note: *Individual biochemical tests may be used instead of biochemical test strips to identify E. coli O157:H7.*

10.8 Summary of Positive and Negative Control, and *E. coli* O157:H7 Results

Typical results are provided in Table 3.

Table 3. Positive and Negative Result Descriptions and *E. coli* O157:H7 Results

Medium/Test	*E. coli* O157:H7 Results	Positive Control Result and Description	Negative Control Result and Description
TC-SMAC	Positive	Colorless colonies (sorbitol not fermented)	Pink to red colonies (sorbitol fermented)
Rainbow®	Positive	Black to gray colonies (glucoronidase-negative)	Pink to magenta colonies (glucoronidase-positive)
Oxidase	Negative	Purple to violet color change within 20 seconds	Colorless or very light pink color change over time
Biochemical test strip	Consult manufacturers' instructions		
O157 antiserum	Positive	Agglutination	No agglutination
H7 antiserum (Optional)	Positive	Agglutination	No agglutination

11.0
Data Analysis and Bacterial Density Calculation

11.1 Most Probable Number (MPN) Technique

Estimation of bacterial densities may be determined based on the number of tubes positive for *E. coli* O157:H7 by biochemical and serological or by PCR confirmation.

11.2 Calculation of MPN

If only three rows of tubes were analyzed, identify appropriate MPN value using either Table 5 or 6, depending on volumes assayed. If more than three rows of tubes were analyzed, the appropriate rows must be selected and MPN value calculated as described in Sections 11.2.1 and 11.2.2. Table 5 should only be used for volumes of 20.0 mL, 10.0 mL, and 1.0 mL. To select MPN values for volumes of 10.0 mL or less, use Table 6 (Sections 11.2.1 and 11.2.2).

11.2.1 Selection of Tubes

If more than three rows of tubes are inoculated with sample (e.g., volumes/dilutions), select the most appropriate rows of tubes according to the criteria listed in Sections 11.2.1.1 to 11.2.1.5. Examples of row selections and MPN/100 mL values are provided in Table 4.

11.2.1.1

Choose the smallest volume or the highest dilution giving positive results in all five tubes inoculated plus the two succeeding lower concentrations. In Example A from Table 4, 10 mL is a smaller volume than 20 mL and is the lowest volume giving positive results in all five tubes.

11.2.1.2

If the largest volume tested has less than five tubes with positive results, select it and the next two smaller volumes (Table 4, Examples B and C).

11.2.1.3

When a positive result occurs in a smaller volume than the three rows selected according to the rules above, change the selection to the largest volume that has less than five positive results and the next two smaller volumes (Table 4, Example D).

11.2.1.4

When the selection rules above have left unselected any smaller volumes with positive results, add those positive tubes to the row of tubes for the smallest volume selected (Table 4, Example E).

11.2.1.5

If there were not enough lower volumes analyzed to select three dilutions using the rules above, then select the three smallest volumes (Table 4, Example F).

Table 4. Examples of Appropriate Tube Selection and MPN/100 mL[1]

Example	20 mL	10 mL	1.0 mL	0.1 mL	Significant Dilutions	Table	MPN Index	MPN/100 mL
A	5/5	5/5	3/5	0/5	5-3-0	6	0.792	79.2
B	4/5	5/5	1/5	0/5	4-5-1	5	0.1524	15.24
C	0/5	1/5	0/5	0/5	0-1-0	5	0.0067	0.67
D	5/5	3/5	1/5	1/5	3-1-1	6	0.137	13.7
E	4/5	4/5	0/5	1/5	4-4-1	5	0.1181	11.81
F	5/5	5/5	5/5	2/5	5-5-2	6	5.422	542.2

[1] Appropriate volumes are underlined and the largest sample volumes analyzed are highlighted

11.2.2

For calculation of MPN/100 mL when additional dilutions are analyzed (e.g., 10^{-2}, 10^{-3}), obtain the MPN index value from Table 6 using the number of positive tubes in the three selected dilutions. Calculate MPN/100 mL using the equation below.

$$\text{MPN/100 mL} = \frac{\text{MPN Index from Table 6}}{\text{Middle volume analyzed in the series used for MPN determination}} \times 100$$

For example, a dilution series of 10^{-3}, 10^{-4}, 10^{-5}, with the following positive tubes 5, 1, 0, respectively would be:

$$\text{MPN /100 mL} = \frac{0.329}{10^{-4}} \times 100 = 3.29 \times 10^{5}$$

Table 5. MPN Index and 95% Confidence Limits for Various Combinations of Positive Results When Five Tubes are Used and Sample Inoculation Volumes are 20.0, 10.0, and 1.0 mL[1]

Combination of Positives	MPN Index	95% Confidence Limits		Combination of Positives	MPN Index	95% Confidence Limits	
		Lower	Upper			Lower	Upper
0-0-0	<0.006473	----	0.0223	1-3-0	0.0312	0.0055	0.0678
0-0-1	0.0065	0.0012	0.0223	1-3-1	0.0393	0.0092	0.0821
0-0-2	0.0130	0.0012	0.0352	1-3-2	0.0475	0.0132	0.0967
0-0-3	0.0195	0.0012	0.0472	1-3-3	0.0559	0.0173	0.1119
0-0-4	0.0262	0.0033	0.0589	1-3-4	0.0644	0.0216	0.1277
0-0-5	0.0328	0.0062	0.0706	1-3-5	0.0730	0.0260	0.1444
0-1-0	0.0067	0.0012	0.0228	1-4-0	0.0409	0.0099	0.0849
0-1-1	0.0134	0.0012	0.0360	1-4-1	0.0495	0.0141	0.1002
0-1-2	0.0202	0.0012	0.0483	1-4-2	0.0583	0.0185	0.1163
0-1-3	0.0270	0.0037	0.0604	1-4-3	0.0672	0.0231	0.1331
0-1-4	0.0339	0.0067	0.0725	1-4-4	0.0763	0.0277	0.1509
0-1-5	0.0408	0.0099	0.0847	1-4-5	0.0855	0.0324	0.1700
0-2-0	0.0138	0.0012	0.0367	1-5-0	0.0517	0.0152	0.1042
0-2-1	0.0208	0.0012	0.0495	1-5-1	0.0609	0.0199	0.1212
0-2-2	0.0279	0.0040	0.0619	1-5-2	0.0703	0.0247	0.1391
0-2-3	0.0350	0.0072	0.0745	1-5-3	0.0799	0.0296	0.1583
0-2-4	0.0422	0.0106	0.0871	1-5-4	0.0897	0.0346	0.1790
0-2-5	0.0494	0.0141	0.1001	1-5-5	0.0998	0.0397	0.2015
0-3-0	0.0215	0.0012	0.0507	2-0-0	0.0155	0.0012	0.0404
0-3-1	0.0288	0.0044	0.0636	2-0-1	0.0226	0.0018	0.0526
0-3-2	0.0362	0.0077	0.0766	2-0-2	0.0303	0.0051	0.0662
0-3-3	0.0437	0.0113	0.0898	2-0-3	0.0382	0.0087	0.0801
0-3-4	0.0512	0.0051	0.1243	2-0-4	0.0462	0.0125	0.0943
0-3-5	0.0588	0.0095	0.1428	2-0-5	0.0543	0.0165	0.1090
0-4-0	0.0299	0.0049	0.0654	2-1-0	0.0234	0.0022	0.0540
0-4-1	0.0375	0.0084	0.0789	2-1-1	0.0315	0.0056	0.0683
0-4-2	0.0453	0.0121	0.0927	2-1-2	0.0397	0.0094	0.0827
0-4-3	0.0531	0.0160	0.1069	2-1-3	0.0480	0.0134	0.0976
0-4-4	0.0611	0.0200	0.1216	2-1-4	0.0565	0.0177	0.1131
0-4-5	0.0691	0.0241	0.1369	2-1-5	0.0652	0.0221	0.1293
0-5-0	0.0390	0.0090	0.0814	2-2-0	0.0327	0.0062	0.0705
0-5-1	0.0470	0.0129	0.0958	2-2-1	0.0413	0.0101	0.0856
0-5-2	0.0553	0.0170	0.1107	2-2-2	0.0501	0.0144	0.1013
0-5-3	0.0636	0.0212	0.1262	2-2-3	0.0590	0.0189	0.1176
0-5-4	0.0720	0.0255	0.1425	2-2-4	0.0681	0.0236	0.1349
0-5-5	0.0806	0.0299	0.1596	2-2-5	0.0774	0.0283	0.1533
1-0-0	0.0072	0.0012	0.0241	2-3-0	0.0431	0.0110	0.0887
1-0-1	0.0139	0.0012	0.0369	2-3-1	0.0523	0.0155	0.1053
1-0-2	0.0209	0.0012	0.0497	2-3-2	0.0617	0.0203	0.1227
1-0-3	0.0281	0.0041	0.0623	2-3-3	0.0714	0.0252	0.1412
1-0-4	0.0353	0.0073	0.0749	2-3-4	0.0813	0.0303	0.1611
1-0-5	0.0425	0.0107	0.0878	2-3-5	0.0914	0.0354	0.1826
1-1-0	0.0144	0.0012	0.0377	2-4-0	0.0547	0.0168	0.1098
1-1-1	0.0217	0.0013	0.0509	2-4-1	0.0647	0.0218	0.1284
1-1-2	0.0290	0.0045	0.0640	2-4-2	0.0750	0.0271	0.1484
1-1-3	0.0365	0.0079	0.0771	2-4-3	0.0855	0.0325	0.1700
1-1-4	0.0441	0.0115	0.0905	2-4-4	0.0964	0.0380	0.1937
1-1-5	0.0517	0.0153	0.1043	2-4-5	0.1076	0.0436	0.2201
1-2-0	0.0224	0.0017	0.0523	2-5-0	0.0681	0.0235	0.1349
1-2-1	0.0301	0.0050	0.0658	2-5-1	0.0791	0.0292	0.1566
1-2-2	0.0379	0.0085	0.0795	2-5-2	0.0904	0.0349	0.1805
1-2-3	0.0457	0.0123	0.0935	2-5-3	0.1021	0.0409	0.2070
1-2-4	0.0537	0.0162	0.1079	2-5-4	0.1143	0.0469	0.2372
1-2-5	0.0618	0.0203	0.1229	2-5-5	0.1268	0.0531	0.2725

Table 5. MPN Index and 95% Confidence Limits for Various Combinations of Positive Results When Five Tubes are Used and Sample Inoculation Volumes are 20.0, 10.0, and 1.0 mL (cont.)[1]

Combination of Positives	MPN Index	95% Confidence Limits		Combination of Positives	MPN Index	95% Confidence Limits	
		Lower	Upper			Lower	Upper
3-0-0	0.0255	0.0028	0.0585	4-3-0	0.0797	0.0295	0.1579
3-0-1	0.0330	0.0063	0.0710	4-3-1	0.0937	0.0366	0.1877
3-0-2	0.0417	0.0103	0.0863	4-3-2	0.1086	0.0441	0.2228
3-0-3	0.0506	0.0147	0.1023	4-3-3	0.1245	0.0520	0.2656
3-0-4	0.0598	0.0193	0.1191	4-3-4	0.1414	0.0602	0.3218
3-0-5	0.0691	0.0241	0.1368	4-3-5	0.1595	0.0686	0.4067
3-1-0	0.0344	0.0069	0.0734	4-4-0	0.1012	0.0404	0.2049
3-1-1	0.0435	0.0112	0.0896	4-4-1	0.1181	0.0489	0.2476
3-1-2	0.0529	0.0159	0.1065	4-4-2	0.1364	0.0578	0.3038
3-1-3	0.0626	0.0207	0.1244	4-4-3	0.1563	0.0672	0.3890
3-1-4	0.0725	0.0258	0.1434	4-4-4	0.1780	0.0770	0.5273
3-1-5	0.0827	0.0310	0.1640	4-4-5	0.2015	0.0873	0.6411
3-2-0	0.0456	0.0122	0.0932	4-5-0	0.1304	0.0549	0.2836
3-2-1	0.0555	0.0171	0.1112	4-5-1	0.1524	0.0653	0.3687
3-2-2	0.0657	0.0223	0.1303	4-5-2	0.1769	0.0766	0.5210
3-2-3	0.0763	0.0277	0.1510	4-5-3	0.2046	0.0886	0.6528
3-2-4	0.0872	0.0333	0.1735	4-5-4	0.2357	0.1015	0.7516
3-2-5	0.0984	0.0390	0.1984	4-5-5	0.2708	0.1150	0.8426
3-3-0	0.0583	0.0186	0.1164	5-0-0	0.0549	0.0162	0.1116
3-3-1	0.0693	0.0241	0.1371	5-0-1	0.0637	0.0213	0.1265
3-3-2	0.0806	0.0299	0.1597	5-0-2	0.0763	0.0277	0.1510
3-3-3	0.0924	0.0359	0.1847	5-0-3	0.0896	0.0345	0.1787
3-3-4	0.1046	0.0421	0.2128	5-0-4	0.1037	0.0417	0.2107
3-3-5	0.1173	0.0484	0.2452	5-0-5	0.0953	0.0165	0.2234
3-4-0	0.0733	0.0262	0.1450	5-1-0	0.0678	0.0234	0.1344
3-4-1	0.0856	0.0325	0.1700	5-1-1	0.0816	0.0304	0.1618
3-4-2	0.0984	0.0390	0.1982	5-1-2	0.0963	0.0379	0.1936
3-4-3	0.1118	0.0457	0.2307	5-1-3	0.1121	0.0459	0.2316
3-4-4	0.1258	0.0526	0.2695	5-1-4	0.1291	0.0542	0.2796
3-4-5	0.1405	0.0597	0.3184	5-1-5	0.1293	0.0304	0.3090
3-5-0	0.0913	0.0354	0.1825	5-2-0	0.0879	0.0337	0.1751
3-5-1	0.1055	0.0426	0.2150	5-2-1	0.1046	0.0421	0.2128
3-5-2	0.1204	0.0500	0.2538	5-2-2	0.1227	0.0511	0.2605
3-5-3	0.1362	0.0577	0.3029	5-2-3	0.1427	0.0608	0.3267
3-5-4	0.1529	0.0656	0.3715	5-2-4	0.1646	0.0710	0.4385
3-5-5	0.1707	0.0738	0.4795	5-2-5	0.1767	0.0503	0.5230
4-0-0	0.0381	0.0082	0.0809	5-3-0	0.1151	0.0474	0.2394
4-0-1	0.0461	0.0125	0.0942	5-3-1	0.1368	0.0580	0.3050
4-0-2	0.0563	0.0175	0.1126	5-3-2	0.1614	0.0695	0.4183
4-0-3	0.0668	0.0229	0.1323	5-3-3	0.1895	0.0821	0.5899
4-0-4	0.0777	0.0284	0.1537	5-3-4	0.2216	0.0957	0.7101
4-0-5	0.0890	0.0342	0.1773	5-3-5	0.2527	0.0814	0.7971
4-1-0	0.0484	0.0136	0.0983	5-4-0	0.1571	0.0676	0.3935
4-1-1	0.0592	0.0190	0.1181	5-4-1	0.1907	0.0826	0.5954
4-1-2	0.0705	0.0248	0.1395	5-4-2	0.2319	0.0999	0.7409
4-1-3	0.0822	0.0308	0.1631	5-4-3	0.2834	0.1196	0.8726
4-1-4	0.0945	0.0370	0.1894	5-4-4	0.3475	0.1417	1.0160
4-1-5	0.1072	0.0434	0.2193	5-4-5	0.4256	0.1437	1.1800
4-2-0	0.0626	0.0207	0.1244	5-5-0	0.2398	0.0762	0.7629
4-2-1	0.0748	0.0269	0.1479	5-5-1	0.3477	0.1172	1.0160
4-2-2	0.0875	0.0335	0.1742	5-5-2	0.5422	0.1791	1.4190
4-2-3	0.1009	0.0403	0.2041	5-5-3	0.9178	0.2672	2.2010
4-2-4	0.1150	0.0473	0.2392	5-5-4	1.6090	0.3837	4.1030
4-2-5	0.1299	0.0546	0.2820	5-5-5	>1.6090	0.3837	----

[1]Table was developed using the MPN calculator developed by Albert Klee (Reference 15.9)

Table 6. MPN Index and 95% Confidence Limits for Various Combinations of Positive Results When Five Tubes are Used and Sample Inoculation Volumes are 10.0, 1.0, and 0.1 mL[1]

Combination of Positives	MPN Index	95% Confidence Limits		Combination of Positives	MPN Index	95% Confidence Limits	
		Lower	Upper			Lower	Upper
0-0-0	<0.018	---	0.063	1-3-0	0.083	0.012	0.196
0-0-1	0.018	0.003	0.063	1-3-1	0.104	0.020	0.243
0-0-2	0.036	0.003	0.101	1-3-2	0.125	0.029	0.296
0-0-3	0.054	0.003	0.137	1-3-3	0.147	0.038	0.364
0-0-4	0.072	0.008	0.174	1-3-4	0.169	0.048	0.460
0-0-5	0.091	0.015	0.212	1-3-5	0.191	0.057	0.566
0-1-0	0.018	0.003	0.063	1-4-0	0.105	0.021	0.245
0-1-1	0.036	0.003	0.101	1-4-1	0.127	0.030	0.300
0-1-2	0.055	0.003	0.138	1-4-2	0.148	0.039	0.370
0-1-3	0.073	0.008	0.175	1-4-3	0.170	0.048	0.468
0-1-4	0.091	0.015	0.214	1-4-4	0.193	0.058	0.575
0-1-5	0.110	0.023	0.256	1-4-5	0.215	0.067	0.657
0-2-0	0.037	0.003	0.102	1-5-0	0.128	0.030	0.303
0-2-1	0.055	0.003	0.139	1-5-1	0.150	0.040	0.375
0-2-2	0.074	0.008	0.176	1-5-2	0.172	0.049	0.477
0-2-3	0.092	0.015	0.215	1-5-3	0.195	0.058	0.583
0-2-4	0.111	0.023	0.258	1-5-4	0.217	0.068	0.664
0-2-5	0.129	0.031	0.307	1-5-5	0.240	0.077	0.731
0-3-0	0.056	0.003	0.140	2-0-0	0.045	0.003	0.119
0-3-1	0.074	0.009	0.177	2-0-1	0.068	0.006	0.164
0-3-2	0.093	0.016	0.217	2-0-2	0.091	0.015	0.213
0-3-3	0.112	0.023	0.260	2-0-3	0.115	0.025	0.269
0-3-4	0.130	0.031	0.310	2-0-4	0.139	0.035	0.338
0-3-5	0.149	0.039	0.372	2-0-5	0.164	0.046	0.437
0-4-0	0.075	0.009	0.179	2-1-0	0.068	0.006	0.166
0-4-1	0.094	0.016	0.219	2-1-1	0.092	0.015	0.216
0-4-2	0.112	0.024	0.263	2-1-2	0.116	0.025	0.272
0-4-3	0.131	0.032	0.313	2-1-3	0.141	0.036	0.343
0-4-4	0.150	0.040	0.377	2-1-4	0.166	0.046	0.447
0-4-5	0.169	0.048	0.462	2-1-5	0.192	0.057	0.571
0-5-0	0.094	0.016	0.221	2-2-0	0.093	0.016	0.218
0-5-1	0.113	0.024	0.265	2-2-1	0.118	0.026	0.276
0-5-2	0.133	0.032	0.317	2-2-2	0.143	0.036	0.349
0-5-3	0.152	0.040	0.382	2-2-3	0.168	0.047	0.456
0-5-4	0.171	0.048	0.470	2-2-4	0.194	0.058	0.581
0-5-5	0.190	0.056	0.563	2-2-5	0.221	0.069	0.675
1-0-0	0.020	0.003	0.068	2-3-0	0.119	0.026	0.279
1-0-1	0.040	0.003	0.108	2-3-1	0.144	0.037	0.355
1-0-2	0.060	0.003	0.149	2-3-2	0.170	0.048	0.467
1-0-3	0.081	0.011	0.191	2-3-3	0.197	0.059	0.591
1-0-4	0.101	0.019	0.236	2-3-4	0.223	0.070	0.683
1-0-5	0.122	0.028	0.287	2-3-5	0.251	0.082	0.759
1-1-0	0.040	0.003	0.109	2-4-0	0.146	0.038	0.361
1-1-1	0.061	0.003	0.150	2-4-1	0.172	0.049	0.477
1-1-2	0.081	0.011	0.192	2-4-2	0.199	0.060	0.600
1-1-3	0.102	0.019	0.238	2-4-3	0.226	0.072	0.692
1-1-4	0.123	0.028	0.290	2-4-4	0.254	0.083	0.768
1-1-5	0.144	0.037	0.354	2-4-5	0.282	0.094	0.836
1-2-0	0.061	0.003	0.151	2-5-0	0.174	0.050	0.488
1-2-1	0.082	0.012	0.194	2-5-1	0.201	0.061	0.610
1-2-2	0.103	0.020	0.240	2-5-2	0.229	0.073	0.700
1-2-3	0.124	0.029	0.293	2-5-3	0.257	0.084	0.776
1-2-4	0.146	0.038	0.359	2-5-4	0.286	0.095	0.845
1-2-5	0.167	0.047	0.451	2-5-5	0.315	0.107	0.910

Table 6. MPN Index and 95% Confidence Limits for Various Combinations of Positive Results When Five Tubes are Used and Sample Inoculation Volumes are 10.0, 1.0, and 0.1 mL (cont.)[1]

Combination of Positives	MPN Index	95% Confidence Limits		Combination of Positives	MPN Index	95% Confidence Limits	
		Lower	Upper			Lower	Upper
3-0-0	0.079	0.010	0.188	4-3-0	0.271	0.090	0.809
3-0-1	0.106	0.021	0.246	4-3-1	0.326	0.111	0.934
3-0-2	0.135	0.033	0.323	4-3-2	0.386	0.132	1.060
3-0-3	0.165	0.046	0.440	4-3-3	0.451	0.154	1.192
3-0-4	0.196	0.059	0.589	4-3-4	0.521	0.176	1.331
3-0-5	0.229	0.073	0.699	4-3-5	0.593	0.196	1.477
3-1-0	0.107	0.022	0.250	4-4-0	0.335	0.114	0.953
3-1-1	0.137	0.034	0.329	4-4-1	0.398	0.137	1.084
3-1-2	0.167	0.047	0.452	4-4-2	0.466	0.159	1.223
3-1-3	0.199	0.060	0.601	4-4-3	0.539	0.181	1.368
3-1-4	0.232	0.074	0.710	4-4-4	0.615	0.202	1.521
3-1-5	0.267	0.088	0.800	4-4-5	0.693	0.223	1.681
3-2-0	0.138	0.035	0.335	4-5-0	0.411	0.141	1.111
3-2-1	0.170	0.048	0.464	4-5-1	0.483	0.164	1.256
3-2-2	0.202	0.062	0.613	4-5-2	0.559	0.187	1.409
3-2-3	0.236	0.076	0.720	4-5-3	0.639	0.209	1.570
3-2-4	0.271	0.090	0.810	4-5-4	0.722	0.230	1.739
3-2-5	0.308	0.104	0.894	4-5-5	0.806	0.250	1.916
3-3-0	0.172	0.049	0.477	5-0-0	0.240	0.076	0.763
3-3-1	0.205	0.063	0.624	5-0-1	0.314	0.106	0.908
3-3-2	0.240	0.077	0.731	5-0-2	0.427	0.146	1.142
3-3-3	0.276	0.092	0.821	5-0-3	0.578	0.192	1.446
3-3-4	0.313	0.106	0.906	5-0-4	0.759	0.239	1.816
3-3-5	0.352	0.120	0.989	5-0-5	0.953	0.165	2.234
3-4-0	0.209	0.064	0.636	5-1-0	0.329	0.112	0.940
3-4-1	0.244	0.079	0.742	5-1-1	0.456	0.156	1.202
3-4-2	0.281	0.093	0.833	5-1-2	0.631	0.207	1.553
3-4-3	0.319	0.108	0.918	5-1-3	0.839	0.257	1.985
3-4-4	0.358	0.123	1.002	5-1-4	1.062	0.304	2.485
3-4-5	0.399	0.137	1.086	5-1-5	1.293	0.304	3.090
3-5-0	0.248	0.080	0.753	5-2-0	0.493	0.167	1.276
3-5-1	0.286	0.095	0.844	5-2-1	0.700	0.224	1.694
3-5-2	0.325	0.110	0.931	5-2-2	0.944	0.280	2.213
3-5-3	0.365	0.125	1.017	5-2-3	1.205	0.331	2.843
3-5-4	0.407	0.140	1.103	5-2-4	1.479	0.381	3.714
3-5-5	0.450	0.154	1.189	5-2-5	1.767	0.503	5.230
4-0-0	0.130	0.031	0.311	5-3-0	0.792	0.247	1.886
4-0-1	0.166	0.046	0.445	5-3-1	1.086	0.308	2.544
4-0-2	0.207	0.064	0.631	5-3-2	1.406	0.368	3.445
4-0-3	0.253	0.082	0.764	5-3-3	1.750	0.434	5.131
4-0-4	0.302	0.102	0.881	5-3-4	2.122	0.529	6.798
4-0-5	0.355	0.121	0.996	5-3-5	2.527	0.814	7.971
4-1-0	0.169	0.048	0.460	5-4-0	1.299	0.348	3.108
4-1-1	0.212	0.066	0.646	5-4-1	1.724	0.429	4.975
4-1-2	0.258	0.085	0.779	5-4-2	2.212	0.563	7.087
4-1-3	0.310	0.105	0.898	5-4-3	2.781	0.882	8.600
4-1-4	0.365	0.125	1.016	5-4-4	3.454	1.159	10.110
4-1-5	0.425	0.145	1.138	5-4-5	4.256	1.437	11.800
4-2-0	0.216	0.067	0.661	5-5-0	2.398	0.762	7.629
4-2-1	0.264	0.087	0.794	5-5-1	3.477	1.172	10.160
4-2-2	0.317	0.108	0.915	5-5-2	5.422	1.791	14.190
4-2-3	0.375	0.129	1.037	5-5-3	9.178	2.672	22.010
4-2-4	0.438	0.150	1.164	5-5-4	16.090	3.837	41.030
4-2-5	0.504	0.171	1.297	5-5-5	>16.090	3.837	-----

[1]Table was developed using the MPN calculator developed by Albert Klee (Reference 15.9)

12.0
Protocol Performance

Culture-based procedures were evaluated for *E. coli* O157 in a reference matrix (PBS) and two matrices of interest (drinking water, surface water) during a single-laboratory study. Based on workgroup discussion, a nine-tube MPN as opposed to the standard fifteen-tube MPN was utilized for study analyses. It is expected that the SAP will be implemented using a standard15-tube MPN. Details regarding laboratory method performance are provided in the study report (Reference 15.10). Summary results using the optimized (i.e., final) procedure from this study are provided in Table 7. Additional method performance data will be provided following testing in multiple laboratories.

Table 7. *E. coli* O157 Verification Results for Drinking Water and Surface Water Analyses using the "Optimized" Procedure

Date	Sample ID	Spike Level (CFU/100 mL)	MPN Combo	*E. coli* O157:H7 (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					
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:H7 (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 were subtracted from spiked surface water levels to calculate percent recovery

13.0
Pollution Prevention

13.1
The solutions and reagents used in this protocol pose little threat to the environment when recycled and managed properly.

13.2
Solutions and reagents should be prepared in volumes consistent with laboratory use to minimize the volume of expired materials to be disposed.

14.0
Waste Management

14.1
It is the laboratory's responsibility to comply with all federal, state, and local regulations governing waste management, particularly the biohazard and hazardous waste identification rules and land disposal restrictions, and to protect the air, water, and land by minimizing and controlling all releases from fume hoods and bench operations. Compliance with all sewage discharge permits and regulations is also required.

14.2
Samples, reference materials, and equipment known or suspected to have viable *E. coli* O157:H7 attached or contained must be sterilized prior to disposal.

14.3
For further information on waste management, consult *The Waste Management Manual for Laboratory Personnel* (Reference 15.11) and *Less Is Better: Laboratory Chemical Management for Waste Reduction* (Reference 15.12), both available from the American Chemical Society's Department of Government Relations and Science Policy, 1155 16th Street NW, Washington, DC 20036.

15.0 References

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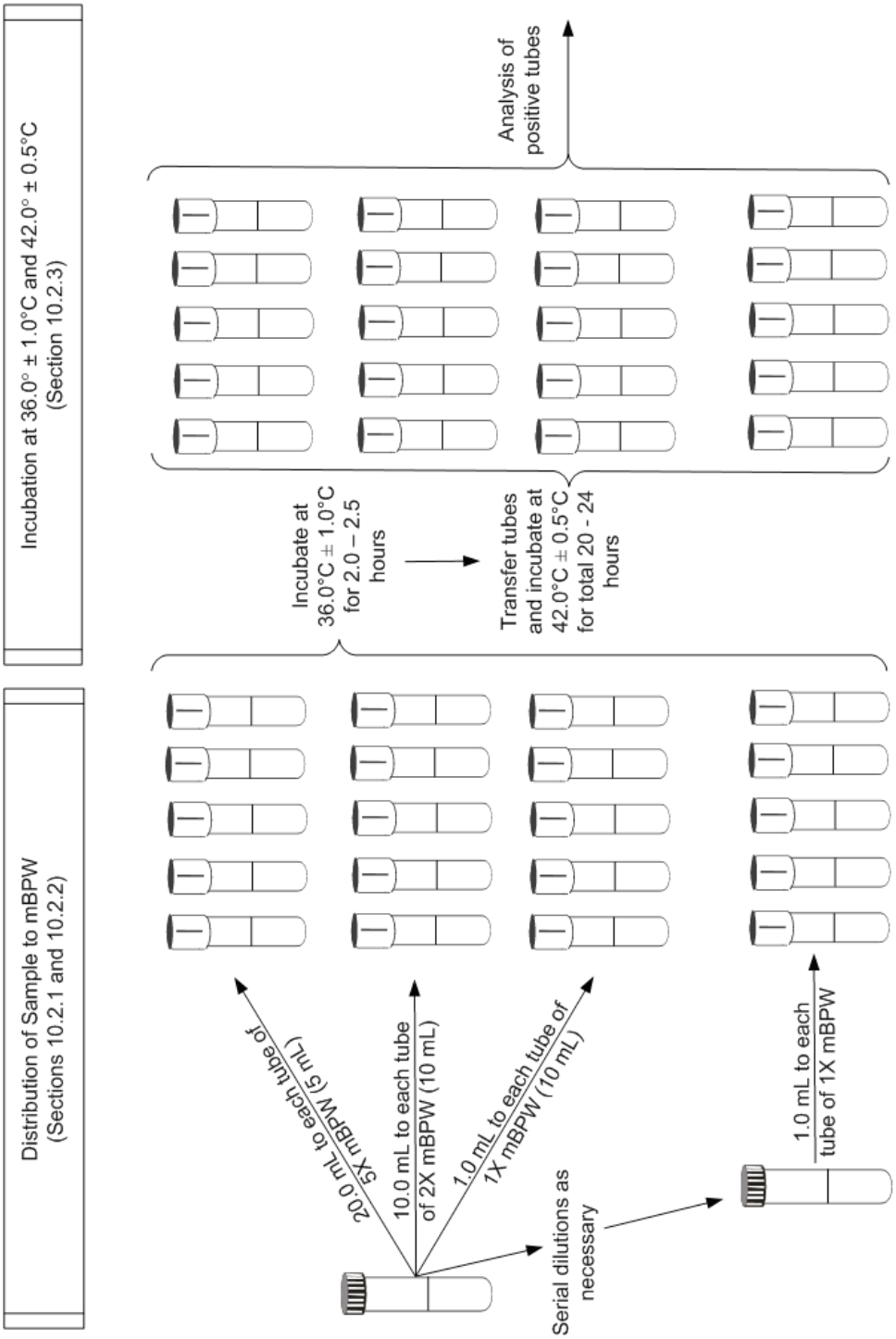
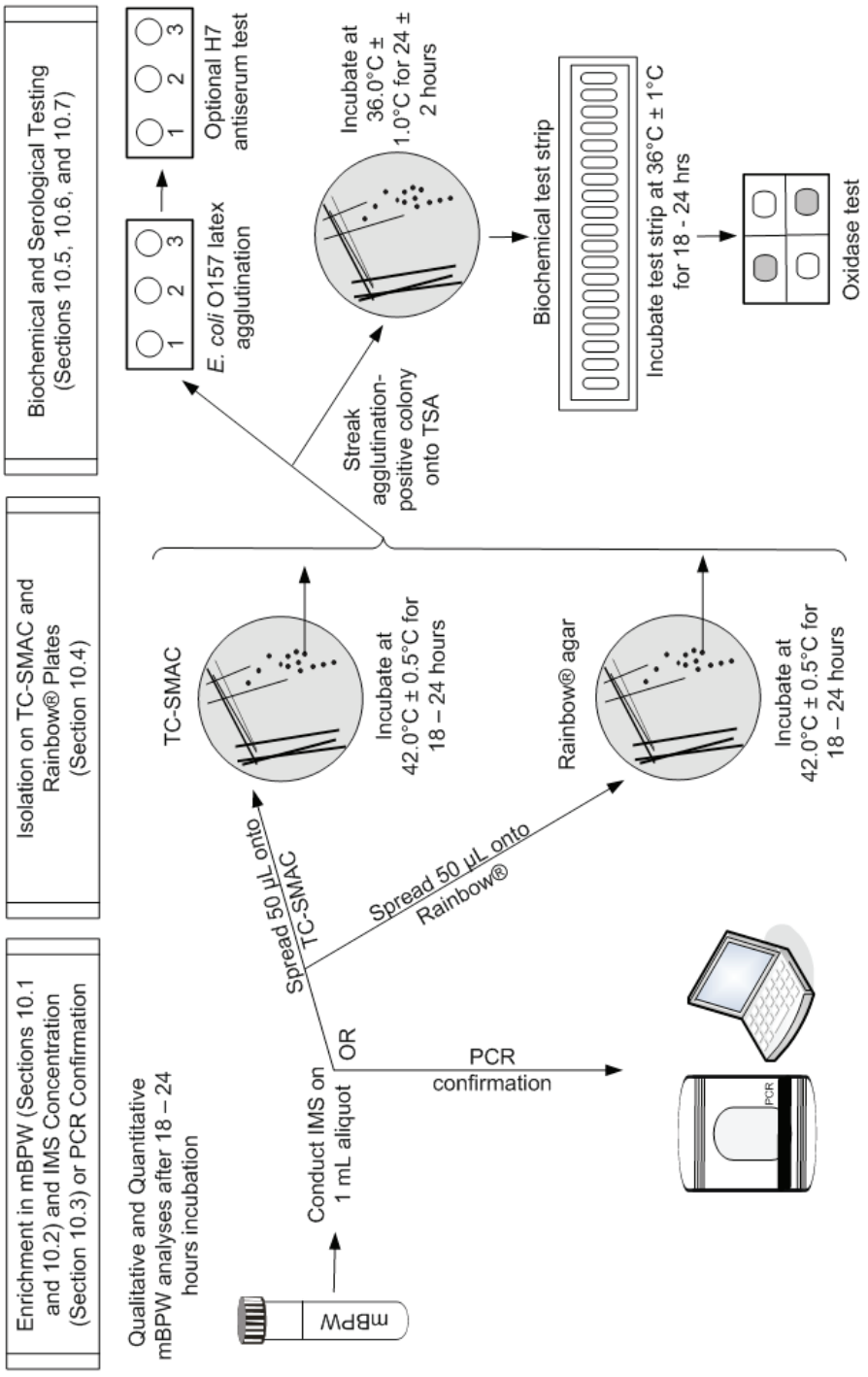
16.0
Flowcharts and Diagrams

16.1 Quantitative Analysis Dilution Scheme



16.2 Identification Flowchart



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