



**Testing Methodologies for *E. coli*
O157:H7 and *Salmonella* species in
Spent Sprout Irrigation Water (or
Sprouts)**

October 2015, Version 1

**U.S Food and Drug Administration
Center for Food Safety and Applied Nutrition
5100 Paint Branch Parkway
College Park, Maryland 20740**

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I. Introduction

The methods described here are for the purposes of testing spent sprout irrigation water (or sprouts) for *E. coli* O157:H7 and *Salmonella* species. These methods have been utilized in FDA's Compliance Programs for testing samples of spent sprout irrigation water and sprouts and are based on methods and procedures described in the Bacteriological Analytical Manual (BAM)^{1, 2}.

II. Detection of *E. coli* O157:H7

This section describes a method where enrichments of sprouts/spent irrigation water samples are screened by real-time PCR to quickly rule out negative samples or establish the presumptive presence of *E. coli* O157:H7 in 24 h. Confirmation of presumptive positive results can be completed within 3 days. This screening method uses modified Buffered Peptone Water with pyruvate (mBPWp), which contain anti-microbial reagents that effectively suppress normal flora growth and non-target competitors, yet allows the growth of viable O157:H7 cells and is capable of detecting the presence of *E. coli* O157:H7 in food at a level of <1 cfu/g. Samples that are positive by real-time PCR screening will require cultural confirmation which involves plating onto differential agars to establish the strain's inability to ferment sorbitol, identification of the isolate as *E. coli*, and serologically typing for the O157 and the H7 antigens. It is advisable that isolates are tested again by PCR for *stx1* and *stx2* genes to confirm their toxigenic potential.

A. Equipment and materials

1. Balance, 1-500 g with 0.1 g sensitivity
2. Sterile glass jars for enrichment
3. Incubators: 36°C ± 1°C and 42°C ± 1°C
4. Petri dishes 20 × 150 mm

¹Bacteriological Analytical Manual, Chapter 4A. Diarrheagenic *Escherichia coli*, Version of February 2011, Current content as of September 25, 2015

²Bacteriological Analytical Manual, Chapter 5. *Salmonella*, Version of May 2014, Current content as of September 25, 2015

5. Microcentrifuge tubes (0.5 to 2.0 ml)
6. Conical centrifuge tubes (50.0 ml)
7. Micropipettors (e.g., 0.5-20 μ l, 20-200 μ l, 200-1000 μ l)
8. Microcentrifuge (capable of spinning at 15000 \times g)
9. Pipets (1 to 10 ml volume)
10. Pipet tips (0.2 to 1000 μ l volume) (aerosol resistant tips)
11. Vortex Mixer
12. Filter Paper
13. Waterbath or heat block capable of maintaining 100°C
14. SmartCycler II PCR Thermalcycler (Cepheid, Sunnyvale, CA) capable of performing cycling parameters described below and simultaneous real-time sequence detection for FAM, TET, Texas Red and Cy5 dyes
15. SmartCycler PCR reaction tubes (minimum reaction volume of 25 μ l) and racks compatible with PCR thermalcycler
16. Ice bucket and ice
17. Sterile tongs
18. Latex gloves or equivalent

B. Media and reagents

1. Modified Buffered Peptone water with pyruvate (mBPWp) and Cefsulodin-Vancomycin (CV) Supplement (M192a).
2. O157 primers and probes listed in Tables 1 and 2 are specific to the SmartCycler II PCR platform.
 - a. Primers - 10 μ M working solution of each primer listed in Table 1. Stock and Working solutions can be prepared from commercially synthesized primers with basic desalting purification (Fisher/Genosys or equivalent) by rehydrating with sterile distilled water to appropriate concentrations. Store at -20°C to -70°C in a non-frost-free freezer.
 - b. Probes - 10 μ M working solution of each probe listed in Table 2. Dual Hybridization Probes should be purchased as RP HPLC-purified and labeled as indicated in Table 2. Stock and working solutions can be prepared from commercially synthesized probes by mixing with molecular grade sterile distilled water. Working solutions should be aliquoted in small amounts and stored frozen (-20 to -70°C) and away from light until use to avoid fluorophore degradation.
 - c. IAC exogenous nucleic acid molecule: Full length sequence as described in U.S. Patent Application 0060166232: cgcattgtgt cacagccctg acgaagctgt catcaagttc ataatacat cgatatgggt gccgttcgag cagtttagcc ctaaataacc ctaccggcag acgtatgtca

cattcaccag ggagacgcat gagattggat gctgtgtgc gccctcaaca atgtaacgaa tggctgcatc
gtctcgcgaa tattgtcgta ccatcatctg acttggetca tgctgcaag aggcttcgca ctgggetttag

Titer to a stock solution where a known amount will provide a reliable Ct (threshold cycle) between 25-35 PCR cycles.

Note: Alternatively the primers, probes and internal control assay components are also available as a lyophilized bead product for use on the SmartCycler II.

Table 1. Primer sequences for use on SmartCycler II platform.

Primers ¹	GenBank #	Bases	5' → 3' Sequence
Stx1F934	M19473	26	gTg gCA TTA ATA CTg AAT TgT CAT CA
Stx1R1042	M19473	21	gCg TAA TCC CAC ggA CTC TTC
Stx2F1218	X07865	24	gAT gTT TAT ggC ggT TTT ATT TgC
Stx2R1300	X07865	26	Tgg AAA ACT CAA TTT TAC CTT Tag CA
UidAF241	AF305917	21	CAg TCT ggA TCg CgA AAA CTg
UidAR383	AF305917	22	ACC AgA CgT TgC CCA CAT AAT T
IAC55F2		17	ATg ggT gCC gTT CgA gc
IAC186R ^{2,3}		19	Cga gaC gAT gCa gCC aTT C

¹ Primer/Probe name composed of target gene (*stx1*, *stx2* or *uidA*), forward primer (F), reverse primer (R) or probe (P), 5' base position of oligonucleotide in the respective gene sequence specified in column 2.

² Internal Amplification Control (IAC) primers and probes are covered by U.S. Patent Application 0060166232.

³ Nordstrom, J.L., M.C.L. Vickery, G.M. Blackstone, S.L. Murray and A. DePaola. 2007. Development of a multiplex real-time PCR assay with an internal amplification control for the detection of total and pathogenic *Vibrio parahaemolyticus* bacteria in oysters. *Appl. Environ. Microbiol.* 73:5840-5847 and DePaola, A., J.L. Jones, J. Woods, W. Burkhardt III, K.R. Calci, J.A. Krantz, J.C. Bowers, K. Kasturi, R.H. Byars, E. Jacobs, D. Williams-Hill, and K. Nabe. 2010. Bacterial and viral pathogens in live oysters: 2007 United States Market Survey. *Appl. Environ. Microbiol.* 76:2754-2768.

Table 2. Probe sequences for use on SmartCycler II platform. (refer to footnotes in Table 1)

Probes ¹	GenBank #	Bases	5' → 3' Sequence
Stx1P990	M19473	31	TxRd-TgA TgA gTT TCC TTC TAT gTg TCC ggC AgA

Probes ¹	GenBank #	Bases	5' → 3' Sequence
			T-BHQ2
Stx2P1249	X07865	25	6FAM-TCT gTT AAT gCA ATg gCg gCg gAT T-BHQ1
UidAP266	AF305917	15	TET-ATT gAg CAg CgT Tgg-MGB/NFQ
ICP-Cy5 ^{2,3}		26	Cy5- TCT CAT gCg TCT CCC Tgg TgA ATg Tg-BHQ2

3. Real-time PCR additional reagents (For the SmartCycler II PCR platform):
 - a. OmniMix-HS or SmartMix HM PCR Reagent Beads (Cepheid, Sunnyvale, CA. Also available through Fisher).
4. Tellurite Cefixime – Sorbitol MacConkey Agar (TC-SMAC) (M194)
5. Chromogenic selective agar:
 - a. R&F[®] *E. coli* O157:H7 agar (R&F Laboratories, Downers Grove, IL), prepared according to manufacturer instructions. Rainbow[®] Agar O157 (BIOLOG, Hayward, CA) prepared according to manufacturer instructions for high background flora containing 10 mg/L novobiocin plus 0.8 mg/L potassium tellurite.
6. Trypticase Soy Agar with Yeast Extract (TSAYE) (M153)
7. Butterfield's Phosphate Buffer (R11) (pH 7.2 ± 0.2. Sterilize by autoclaving)
8. Sterile distilled water, molecular grade water or equivalent
9. Physiological saline (0.85% NaCl)
10. Kovac's Reagent (R38)
11. ColiComplete Discs - contains fluorogenic MUG substrate for GUD and chromogenic X-gal for GAL (BioControl, Bellevue, WA)
12. Anti-O157 and anti-H7 latex reagent (RIM *E. coli* O157:H7 Latex Test (Thermo Fisher Scientific, Remel Products, Lenexa, KS) or equivalent)
13. API20E or VITEK GNI (BioMerieux, St. Louis, MO).

C. Sample preparation and enrichment procedure

1. Sample Preparation
 - a. **Spent sprout irrigation water** – aseptically transfer 100 ml of the spent sprout irrigation water into a sterile 1 L flask containing 100 ml of 2× mBPWp + CV (M192a). Swirl to mix thoroughly.
 - b. **Sprouts** – aseptically weigh 25 g of sprouts into a stomacher bag. Add 225 ml of mBPWp + CV (M192a). Mix the samples in a stomacher at medium speed for 2 min.

2. Enrichment

- a. Incubate the enrichment broth mixtures for 24 h at 42°C (107.6°F), with shaking at 140 RPM for spent sprout irrigation water (a) and sprouts (b) samples.

Enrichment Control Strains - 465-97 USDA (*stx1*-, *stx2*- *uidA*+). Alternatively, use ATCC43890 (*stx1*+, *stx2*-, *uidA*+), ATCC 43888 (*stx1*-, *stx2*- *uidA*+) or equivalent if 465-97 is not available. Note: these strains do not have both *stx* genes and should not be used as controls in PCR.

D. Real-time PCR screening

Real-time PCR assembly and data analysis protocols are described below for the SmartCycler II platform. Use of other platforms and protocols must first be validated. Alternately, if equipment and/or reagents are not available for real-time PCR screening of enrichment cultures, refer to section E for cultural analysis of all enrichments.

1. DNA Template Preparation:

- a. Transfer 1ml of overnight enrichment to a microcentrifuge tube and centrifuge at 12,000 × g for 3 min.
- b. Remove supernatant and completely resuspend the pellet in 1 ml 0.85% NaCl.
- c. Centrifuge at 12,000 × g for 3 min.
- d. Remove supernatant and completely resuspend the pellet in 1 ml sterile water.
- e. Place in waterbath or heat block capable of maintaining 100°C for 10 min.
- f. Centrifuge at 12,000 × g for 1 min, remove and save supernatant as DNA template (This may be frozen at -20°C for future PCR tests).
- g. Make a 1:10 dilution of this template and use 1 µl for testing by real time-PCR.
- h. For pure cultures (including control cultures), 1 ml of broth culture or colony growth from agar plate suspended in 0.85% saline maybe prepared as in steps a-g above. Templates may be frozen at -20°C for future use.

2. PCR Controls:

- a. For a positive PCR control include template prepared from *E. coli* O157:H7, such as ATCC strain 43895 (EDL 933) or ATCC strain 43894 both possessing all three gene targets (*stx1*, *stx2* and *uidA*).
- b. If no internal amplification control is incorporated into the reaction, prepare a reaction tube including 1 µl of a 1:10 dilution of EDL 933 control template and 1 µl of a 1:10 dilution of the food sample enrichment template.
- c. Always include a no template (water) negative control tube in every run.

3. SmartCycler II - reaction assembly and data analysis protocol:

- a. Reaction assembly

- i. Prepare a PCR Master Mix from the reaction components and final concentrations for O157 listed in Table 3. Keep all thawed reagents and reactions on ice. Alternatively a PCR Master Mix may be prepared by following package insert for O157 CSR bead and OmimiMix HS or SmartMix HM bead.
- ii. Add 24 μ l of Master Mix to each SmartCycler tube and cap loosely.
- iii. Add 1 μ l of sample or control template and snap cap tightly.
- iv. Briefly centrifuge to bring all liquid to bottom of tube and place in thermocycler.
- v. Create a "run" on SmartCycler II. Give each run a unique run name, select Dye set FTTC25, select 2-step PCR protocol as described below and assign appropriate sites on SC block.

Step	Criteria
Initial Activation	60 sec at 95°C
40 cycles	10 sec at 94°C, (optics off)
40 cycles	40 sec at 63°C, (optics on)

Table 3. SmartCycler II protocol amplification reaction components¹

Volume/rxn	Component	Final Concentration
qsi to 25 μ l ²	Sterile Distilled Water	
0.625 μ l	Primer stx1F934 (10 μ M Work Solution)	0.25 μ M
0.625 μ l	Primer stx1R1042 (10 μ M Work Solution)	0.25 μ M
0.625 μ l	Primer stx2F1218 (10 μ M Work Solution)	0.25 μ M
0.375 μ l	Primer IAC55F (10 μ M Work Solution)	0.15 μ M
0.625 μ l	Primer stx2R1300 (10 μ M Work Solution)	0.25 μ M
0.625 μ l	Primer uidAF241 (10 μ M Work Solution)	0.25 μ M
0.625 μ l	Primer uidAR383 (10 μ M Work Solution)	0.25 μ M
0.375 μ l	Primer IAC186R (10 μ M Work Solution)	0.15 μ M
0.375 μ l	Probe stx1PTxRd990 (10 μ M Work Solution)	0.15 μ M

Volume/rxn	Component	Final Concentration
0.0625 µl	Probe stx2PFAM1249 (10 µM Work Solution)	0.025 µM
0.25 µl	Probe uidAPTET-MGB266 (10 µM Work Solution)	0.1 µM
0.375 µl	Probe ICPCy5 (10 µM Work Solution)	0.15 µM
	IAC Template DNA ³	
0.5 bead	OmniMix-HS or SmartMix-HS	
1-5 µl	Template (Sample or control)	

¹ All primers, probes, and Internal Amplification Control (IAC) DNA primers and probes are lyophilized in the O157 CSR Bead

² Appropriate amount of sterile distilled water is added depending on sample template volume being used.

³ Full length (252 bp) internal amplification control DNA as described in U.S. Patent Application 0060166232. The amount of IAC template needs to be adjusted based on the prepared stock concentration so that the Cycle threshold (Ct) values will be within the range of 25-35 PCR cycles when no inhibition is present in the reaction.

4. Qualitative data analysis.

On the SmartCycler II Instrument, set the following Analysis Settings for FAM, TET, TxRd and Cy5 channels (Smart Cycler Operator Manual, 1999. USA). Update analysis settings if they are changed before recording results. **Note:** The cycle threshold (Ct) for Internal Amplification Control (IAC) is CSR lot specific (Ct = 25-35).

- a. Usage: Assay
- b. Curve Analysis: Primary
- c. Threshold Setting: Manual
- d. Manual Threshold Fluorescence Units: 15.0
- e. Auto Min Cycle: 5
- f. Auto Max Cycle: 10
- g. Valid Min Cycle: 3
- h. Valid Max. Cycle: 60
- i. Background subtraction: ON
- j. Boxcar Avg. Cycles: 0
- k. Background Min. Cycle: 5
- l. Background Max. Cycle: 40

Primary fluorescence curves that cross the threshold will be recorded as "POS" and the cycle number when it crossed the threshold will be displayed in the Results Table view

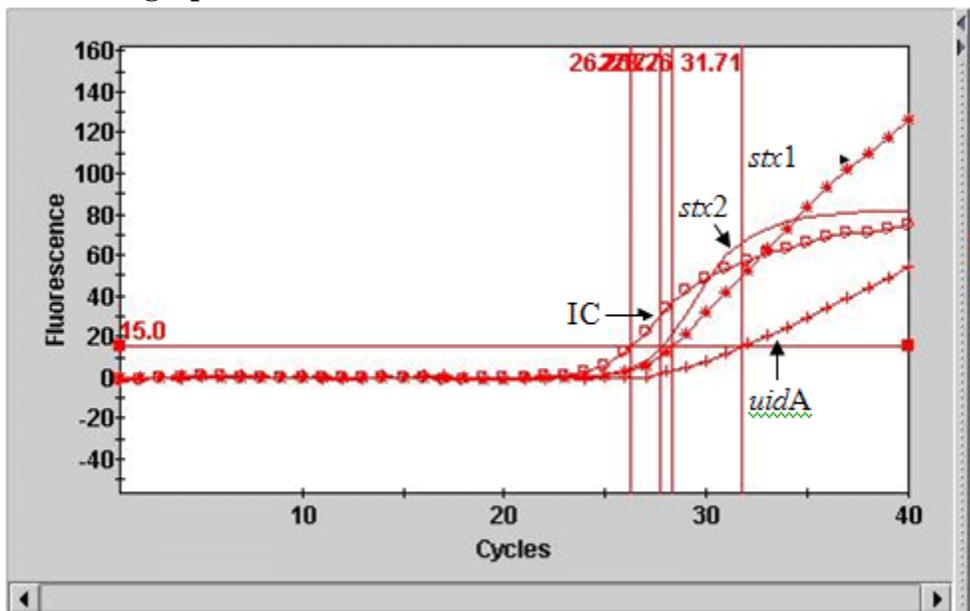
(Figure 1A). Negative results are shown as "NEG". The FAM, TET, TxRd and Cy5 channels correlate to *stx2*, +93 *uidA*, *stx1* and IAC targets, respectively. Results can also be viewed graphically. For example, Figure 1B is a graphical view for the four channels for isolate *E. coli* O157:H7 strain EDL 933 that carries all 3 targets.

Figure 1. Example of result output from SmartCycler II. A. Results viewed in table form, B. Graphical representation of results.

A. Results table view

Views	Site ID	Protocol	Sample ID	Sample Type	Notes	Status	stx2 Std/...	stx2 Ct	uidA Std/...	uidA Ct	stx1 Std/R...	stx1 Ct	IC Std/...	IC Ct
Results Table	A4	EHEC-...	-2	UNKN	4321099	OK	POS	24.40	POS	27.30	POS	24.58	POS	25.74
Analysis Settings	A5	EHEC-...	-2	UNKN	4321099	OK	POS	24.52	POS	27.16	POS	24.86	POS	25.74
Protocols	A6	EHEC-...	-3	UNKN	4321099	OK	POS	27.92	POS	31.53	POS	28.33	POS	26.36
4 PLEX	A7	EHEC-...	-3	UNKN	4321099	OK	POS	27.77	POS	31.71	POS	28.26	POS	26.25

B. Results graphical view



5. Interpretation of real-time PCR Results (SmartCycler II)

a. Negative samples:

All three targets – *stx1*, *stx2*, and +93 *uidA* SNP are "negative" and the spiked PCR control is positive for one or more targets. If an internal control (IC) is incorporated in the reaction as in the CSR bead, and is positive, this would also indicate that the reactions worked correctly.

- No further analysis is needed.

b. Probable positive O157 samples:

If the +93 *uidA* SNP target is positive by itself or in combination with positive in either *stx1*, *stx2* or both. Proceed to section E for culture isolation and confirmation

NOTE: It is possible to have an IC be negative when one or more gene targets are positive, as the amplification of the target genes may out-compete the Internal Control for available reagents. In those instances, the analysis is still valid as long as the amplification of the target gene has crossed the threshold to indicate successful PCR. Continue with section E for isolation. However, if all the target genes and the spiked food control (or IC) are negative, this is considered an invalid PCR analysis. Troubleshoot the reaction and rerun the assay or streak enrichments as described in section E.

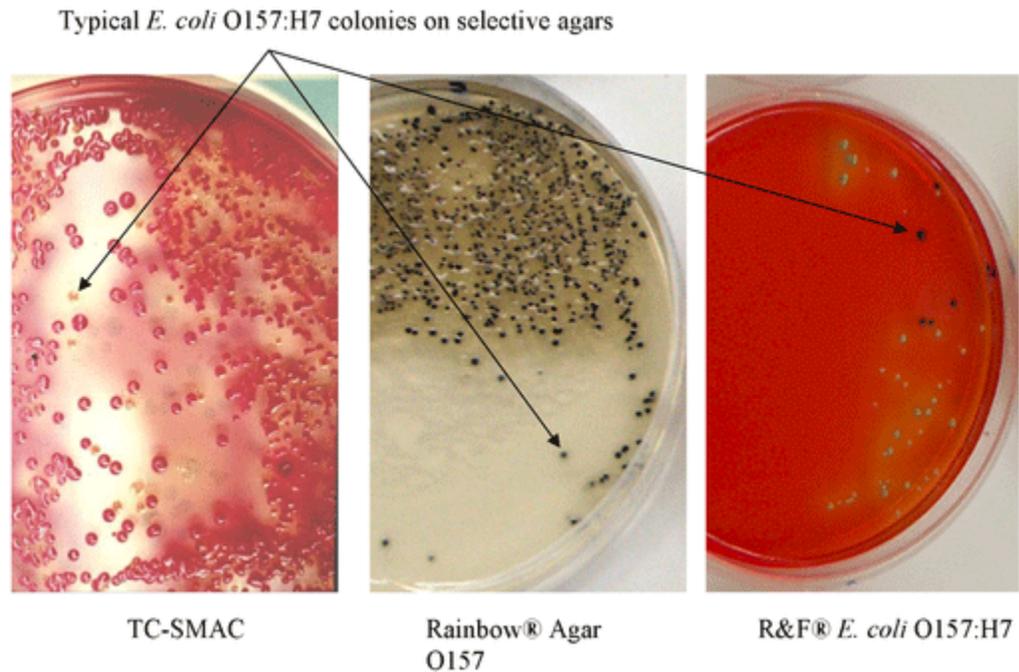
E. Cultural isolation and presumptive isolate screening

For overnight enrichment samples that are found probable positive by the real-time PCR assay, cultural confirmation is required. Similarly, for samples that have not been screened by real-time PCR follow these procedures for culture isolation.

1. Isolation procedure

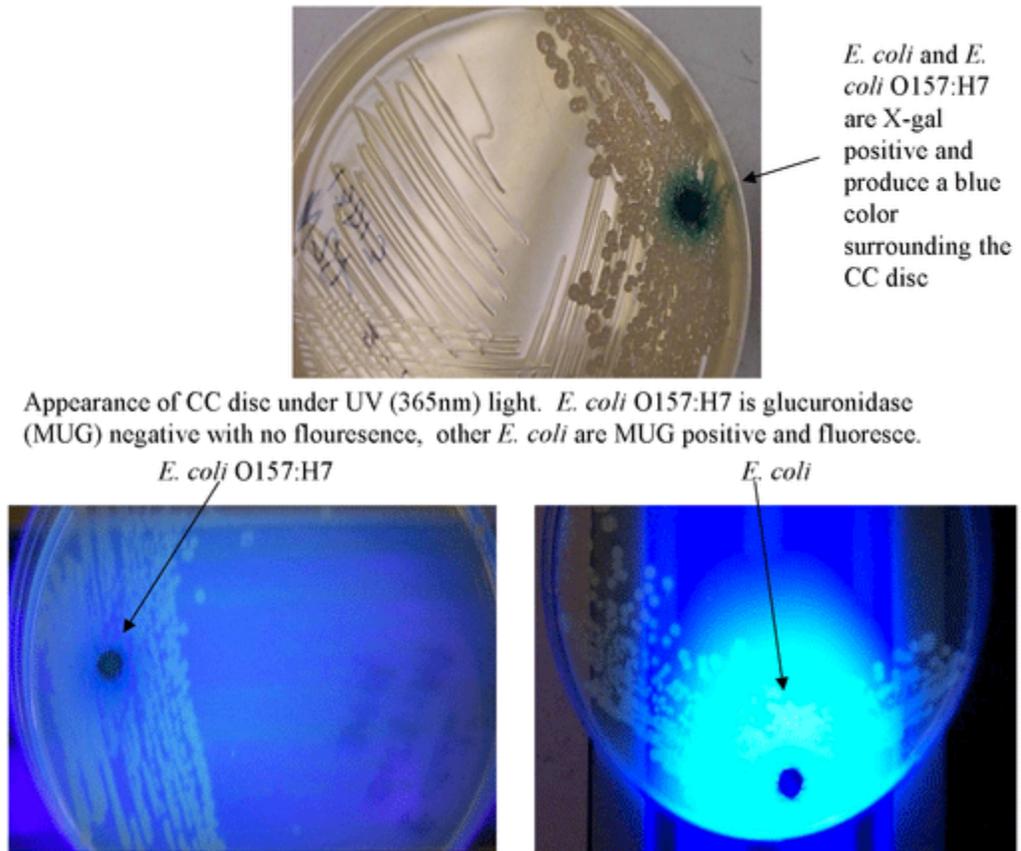
- a. Serially dilute the overnight sample enrichment in Butterfield's phosphate buffer (R11) and spread-plate appropriate dilutions (usually 0.05 ml of 10^{-2} and 10^{-4} dilutions should yield approximately 100-300 isolated colonies) in duplicate onto TC-SMAC and one chromogenic agar (Rainbow[®] Agar O157 or R&F[®] *E. coli* O157:H7 agar). Optionally, a streak plate may also be included.
- b. Incubate plates at $37^{\circ}\text{C} \pm 1^{\circ}\text{C}$ for 18-24 h. On TC-SMAC, typical O157:H7 colonies are colorless or neutral/gray with a smoky center and 1-2 mm in diameter. Sorbitol-fermenting bacteria such as most *E. coli* appear as pink to red colonies. On Rainbow[®] Agar O157 or R&F[®] *E. coli* O157:H7 agar, *E. coli* O157H7 colonies should appear as black to blue-black colonies.

Figure 2. Appearance of typical *E. coli* O157:H7 on TC-SMAC, Rainbow® Agar O157 and R&F® *E. coli* O157:H7 agars.



- c. Screen typical colonies by picking a portion of each suspect colony from the isolation agar and testing for O157 antigen by latex agglutination (RIM *E. coli* O157:H7 Latex Test (Thermo Fisher Scientific, Remel Products, Lenexa, KS)).
- d. Pick all typical colonies that screen positive (up to 10, if >10 are present) from isolation agars and streak onto TSAYE plates to check for purity.
- e. Place a ColiComplete (CC) disc (BioControl, Bellevue, WA) in the heaviest streak area on the TSAYE plate. Prepare a similar TSAYE plate using a known MUG-positive *E. coli* strain as positive control. Incubate the plates 18-24 h at 37°C ± 1°C. CC has a chromogenic assay for galactopyranosidase (X-gal) and a fluorogenic assay for glucuronidase (MUG) on the same disc. The positive control should show blue color on and around the disc (indicative of coliforms) and blue fluorescence around the disc under long wave UV (365 nm) light (indicative of *E. coli*). Strains of O157:H7 are X-gal (+) but MUG (-).

Figure 3. Results of ColiComplete (CC) disc for *E. coli* and *E. coli* O157:H7



- f. Spot Indole Test: Spot growth from TSAYE plate to a filter wetted with Kovac's reagent. *E. coli* O157:H7 are indole positive.

Figure 4. Indole positive result of typical *E. coli* O157:H7.



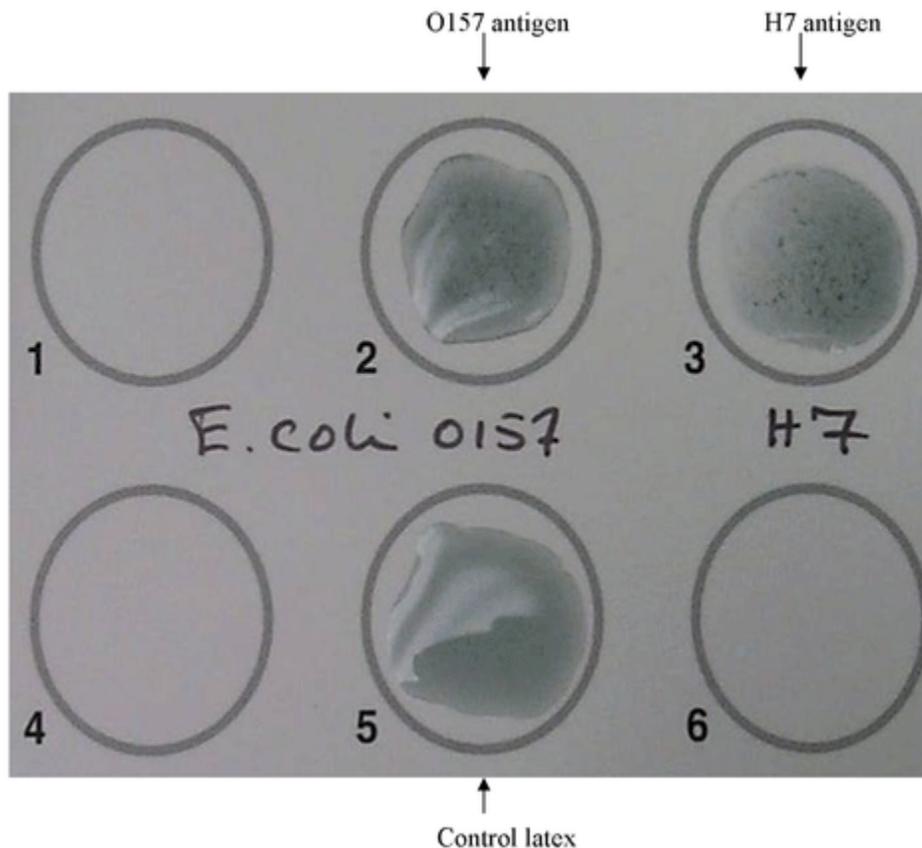
F. Isolate confirmation tests

1. For typical colonies shown to be X-gal positive, MUG negative and indole positive, perform the following confirmation tests on the isolated colony from TSA/YE.
 - a. Confirm the presence of the O157 and H7 antigens using commercial antisera and following manufacturer's instructions. RIM *E. coli* O157:H7 Latex Test (Thermo Fisher Scientific, Remel Products, Lenexa, KS) or equivalent, gives satisfactory results.

NOTE: If the isolate is O157 and H7 positive, it is evident that the isolate is of the O157:H7 serotype. But if the isolate is O157 (+) but H7 (-), proceed with confirmation steps below, as it may be a non-motile variant (O157:NM), hence needs to be tested by PCR to determine its toxigenic potential. The isolate can also be subcultured to blood agar to induce motility and the H7 reaction retested.

Caution: Be sure to test the isolate with the control latex provided with the kit, to rule out the possibility of autoagglutinating strains of *E. coli* that will react with both reagents. Also, do not use H7 latex reagent without testing first with the O157 reagent as other non-O157 *E. coli* serotypes can also carry the H7 antigen.

Figure 5. Typical *E. coli* O157:H7 latex agglutination result.



- b. Test O157 and H7 positive strains with API20E or VITEK to identify as *E. coli*.

NOTE: An isolate that is sorbitol (-), indole (+), MUG (-), serologically (+) for O157 and H7 and is identified as *E. coli* is a confirmed positive for *E. coli* O157:H7.

- c. Isolates that have been confirmed to be O157:H7 as well as isolates that are O157 (+) but H7 (-), need to be retested to verify their toxigenic potential using. The real-time PCR assay (SmartCycler II) is described in this document.

G. Media preparations: See Appendix 1

III. Detection of *Salmonella*

This section describes the method for the detection of *Salmonella* species in sprouts and spent sprout irrigation water samples. Procedures for the screening, isolation, cultural confirmation as well as serological tests are presented.

A. Equipment and materials

1. Sterile, 6 L Erlenmeyer flasks, optionally, containers of appropriate capacity to accommodate composited samples
2. Balance, with weights; 2000 g capacity, sensitivity of 0.1 g
3. Balance, with weights; 120 g capacity, sensitivity of 5 mg
4. Incubator, 35.0 ± 2 °C
5. Refrigerated incubator or laboratory refrigerator, 5-8°C
6. Water bath, 49.0 ± 1 °C
7. Water bath, circulating, thermostatically-controlled, 43.0 ± 0.2 °C
8. Water bath, circulating, thermostatically-controlled, 42.0 ± 0.2 °C
9. Sterile spoons or other appropriate instruments for transferring food samples
10. Sterile culture dishes, 15 x 100 mm, glass or plastic
11. Sterile pipets, 1 ml, with 0.01 ml graduations; 5 and 10 ml, with 0.1 ml graduations
12. Inoculating needle and inoculating loop (about 3 mm id), nichrome, platinum-iridium, chromel wire, or sterile plastic
13. Sterile test or culture tubes, 16 x 150 mm and 20 x 150 mm; serological tubes, 10 x 75 mm or 13 x 100 mm
14. Test or culture tube racks
15. Vortex mixer
16. Lamp (for observing serological reactions)
17. Fisher or Bunsen burner
18. pH test paper (pH range 6-8) with maximum graduations of 0.4 pH units per color change

B. Media and reagents

For preparation of media and reagents, refer to Methods 967.25-967.28 in *Official Methods of Analysis*³.

1. Lactose broth (M74)
 2. Brain Heart Infusion (BHI) broth (M24)
 3. Tetrathionate (TT) broth (M145)
 4. Rappaport-Vassiliadis (RV) medium (M132). NOTE: RV medium must be made from its individual ingredients. Commercial formulations are not acceptable.
 5. Xylose lysine desoxycholate (XLD) agar (M179)
 6. Hektoen enteric (HE) agar (M61)
 7. Bismuth sulfite (BS) agar (M19)
 8. Triple sugar iron agar (TSI) (M149)
 9. Tryptone (tryptophane) broth (M164)
 10. Trypticase (tryptic) soy broth (M154)
 11. MR-VP broth (M104)
 12. Simmons citrate agar (M138)
 13. Urea broth (M171)
 14. Urea broth (rapid) (M172)
 15. Malonate broth (M92)
 16. Lysine iron agar (LIA) (Edwards and Fife) (M89)
 17. Lysine decarboxylase broth (M87)
 18. Motility test medium (semisolid) (M103)
 19. Potassium cyanide (KCN) broth (M126)
 20. Phenol red carbohydrate broth (M121)
 21. Purple carbohydrate broth (M130)
 22. MacConkey agar (M91)
 23. Tryptose blood agar base (M166)
 24. Universal preenrichment broth (M188)
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³ AOAC INTERNATIONAL. 2000. *Official Methods of Analysis*, 17th ed., Methods 967.25-967.28, 978.24, 989.12, 991.13, 994.04, and 995.20. AOAC INTERNATIONAL, Gaithersburg, MD.

25. Kovacs' reagent (R38)
26. Voges-Proskauer (VP) test reagents (R89)
27. 1 N Sodium hydroxide solution (R73)
28. 1 N Hydrochloric acid (R36)
29. Bromocresol purple dye solution, 0.2% (R9)
30. Methyl red indicator (R44)
31. Physiological saline solution, 0.85% (sterile) (R63)
32. Formalinized physiological saline solution (R27)
33. *Salmonella* polyvalent somatic (O) antiserum
34. *Salmonella* polyvalent flagellar (H) antiserum
35. *Salmonella* somatic group (O) antisera: A, B, C₁, C₂, C₃, D₁, D₂, E₁, E₂, E₃, E₄, F, G, H, I, Vi, and other groups, as appropriate
36. *Salmonella* Spicer-Edwards flagellar (H) antisera

C. Preparation of test portion for isolation of *Salmonella*

1. **Spent sprout irrigation water.** Add 375 ml spent sprout irrigation water directly to a 6 L Erlenmeyer flask (or any appropriate flask) containing 3,375 ml lactose broth. Swirl to mix thoroughly. Allow flasks to stand for 60 ± 5 minutes at room temperature. Mix well and determine the pH with test paper. Adjust pH, if necessary, to 6.8 ± 0.2 with sterile 1 N NaOH or 1N HCl. Loosely covered with sterile aluminum foil. Incubate at $35 \pm 2^\circ\text{C}$ for 24 ± 2 hours and continue as in D1-D8 below.
2. **Sprouts.** Add 375 g sprouts directly to a 6 L Erlenmeyer flask (or any appropriate flask) containing 3,375 ml universal preenrichment broth. Mix well by hand for 2 minutes. Do not blend or homogenize. Loosely covered with sterile aluminum foil. Incubate at $35 \pm 2^\circ\text{C}$ for 24 ± 2 hours and continue as in D1-D8, below.

D. Isolation of *Salmonella*

1. Gently shake incubated sample.
2. Transfer 0.1 ml mixture to 10 ml Rappaport-Vassiliadis (RV) medium. Vortex.
3. Transfer 1 ml mixture to 10 ml tetrathionate (TT) broth. Vortex.
4. Incubate RV medium 24 ± 2 h at $42.0 \pm 0.2^\circ\text{C}$ (circulating, thermostatically-controlled, water bath). Incubate TT broth 24 ± 2 h at $43.0 \pm 0.2^\circ\text{C}$ (circulating, thermostatically-controlled, water bath).
5. After incubation, streak 3 mm loopful (10 μl) incubated TT broth on bismuth sulfite (BS) agar, xylose lysine desoxycholate (XLD) agar, and Hektoen enteric (HE) agar. **Prepare BS plates the day before streaking and store in dark at room temperature until streaked.**

6. Repeat with 3 mm loopful (10 μ l) of RV medium.
7. Incubate plates 24 ± 2 h at 35°C .
8. Examine plates for presence of colonies that may be *Salmonella*.

TYPICAL *Salmonella* COLONY MORPHOLOGY

Pick 2 or more colonies of *Salmonella* from each selective agar after 24 ± 2 h incubation. Typical *Salmonella* colonies are as follows:

- a. **Hektoen enteric (HE) agar.** Blue-green to blue colonies with or without black centers. Many cultures of *Salmonella* may produce colonies with large, glossy black centers or may appear as almost completely black colonies.
- b. **Xylose lysine desoxycholate (XLD) agar.** Pink colonies with or without black centers. Many cultures of *Salmonella* may produce colonies with large, glossy black centers or may appear as almost completely black colonies.
- c. **Bismuth sulfite (BS) agar.** Brown, gray, or black colonies; sometimes they have a metallic sheen. Surrounding medium is usually brown at first, but may turn black in time with increased incubation, producing the so-called halo effect.

If typical colonies are present on the BS agar after 24 ± 2 h incubation, then pick 2 or more colonies. Irrespective of whether or not BS agar plates are picked at 24 ± 2 h, re-incubate BS agar plates an additional 24 ± 2 h. After 48 ± 2 h incubation, pick 2 or more typical colonies, if present, from the BS agar plates, only if colonies picked from the BS agar plates incubated for 24 ± 2 h give atypical reactions in triple sugar iron agar (TSI) and lysine iron agar (LIA) that result in culture being discarded as not being *Salmonella*. See sections D-10 and D-11, below, for details in interpreting TSI and LIA reactions.

ATYPICAL *Salmonella* COLONY MORPHOLOGY

In the absence of typical or suspect *Salmonella* colonies, search for atypical *Salmonella* colonies as follows:

- a. **HE and XLD agars.** Atypically a few *Salmonella* serotypes produce yellow colonies with or without black centers on HE and XLD agars. In the absence of typical *Salmonella* colonies on HE or XLD agars after 24 ± 2 h incubation, pick 2 or more atypical *Salmonella* colonies.
- b. **BS agar.** Atypically some serovars produce green colonies with little or no darkening of the surrounding medium. If typical or suspect colonies are not present on BS agar after 24 ± 2 h, then do not pick any colonies but reincubate the plates for an additional 24 ± 2 h. If typical or suspect colonies are not present after 48 ± 2 h incubation, then pick 2 or more atypical colonies.

SUGGESTED CONTROL CULTURES

In addition to the positive control cultures (typical *Salmonella*), 3 additional *Salmonella* cultures are recommended to assist in the selection of atypical *Salmonella* colony morphology on selective agars. These cultures are a lactose-positive, H_2S -positive *S. diarizonae* (ATCC 12325) and a lactose-negative, H_2S -negative *S. abortus equi* (ATCC 9842); **OR** a lactose-positive, H_2S -negative *S. diarizonae* (ATCC 29934). These cultures

may be obtained from the American Type Culture Collection, 10801 University Boulevard, Manassas, VA 20110-2209.

9. Lightly touch the very center of the colony to be picked with sterile inoculating needle and inoculate TSI slant by streaking slant and stabbing butt. Without flaming, inoculate LIA slant by stabbing butt twice and then streaking slant. Since lysine decarboxylation reaction is strictly anaerobic, the LIA slants must have deep butt (4 cm). Store picked selective agar plates at 5-8°C.
10. Incubate TSI and LIA slants at 35°C for 24 ± 2 h. Cap tubes loosely to maintain aerobic conditions while incubating slants to prevent excessive H₂S production. *Salmonella* in culture typically produces alkaline (red) slant and acid (yellow) butt, with or without production of H₂S (blackening of agar) in TSI. In LIA, *Salmonella* typically produces alkaline (purple) reaction in butt of tube. Consider only distinct yellow in butt of tube as acidic (negative) reaction. Do not eliminate cultures that produce discoloration in butt of tube solely on this basis. Most *Salmonella* cultures produce H₂S in LIA, thus blackening of the LIA slant is commonly observed. Some non-*Salmonella* cultures produce a brick-red reaction in LIA slants.
11. All cultures that give an alkaline butt in LIA, regardless of TSI reaction, should be retained as potential *Salmonella* isolates and submitted for biochemical and serological tests. Cultures that give an acid butt in LIA and an alkaline slant and acid butt in TSI should also be considered potential *Salmonella* isolates and should be submitted for biochemical and serological tests. Cultures that give an acid butt in LIA and an acid slant and acid butt in TSI may be discarded as not being *Salmonella*. Test retained, presumed-positive TSI cultures as directed in D-12, below, to determine if they are *Salmonella* species, including *S. arizonae*. If TSI cultures fail to give typical reactions for *Salmonella* (alkaline slant and acid butt), pick additional suspect colonies from selective agar plates not giving presumed-positive culture and inoculate TSI and LIA slants as described in D-9, above.
12. Apply biochemical and serological identification tests to:
 - a. Three presumptive TSI cultures recovered from set of plates streaked from RV medium, if present, and 3 presumptive TSI slants recovered from plates streaked from TT broth, if present.
 - b. If 3 presumptive-positive TSI cultures are not isolated from one set of agar plates, test other presumptive-positive TSI slants, if isolated, by biochemical and serological tests. Examine a minimum of 6 TSI cultures for each 25 g analytical unit or each 375 g composite.

E. Identification of *Salmonella*

1. **Mixed cultures.** Streak TSI agar cultures that appear to be mixed on MacConkey agar, HE agar, or XLD agar. Incubate plates 24 ± 2 h at 35°C. Examine plates for presence of colonies suspected to be *Salmonella*.

- a. **MacConkey agar.** Typical colonies appear transparent and colorless, sometimes with dark center. Colonies of *Salmonella* with clear areas of precipitated bile caused by other organisms sometimes present.
- b. **Hektoen enteric (HE) agar.** See D-8a, above.
- c. **Xylose lysine desoxycholate (XLD) agar.** See D-8b, above. Transfer at least 2 colonies suspected to be *Salmonella* to TSI and LIA slants as described in D-8, above, and continue as in D-10, above.

2. Pure cultures

- a. **Urease test (conventional).** With sterile needle, inoculate growth from each presumed-positive TSI slant culture into tubes of urea broth. Since occasional, uninoculated tubes of urea broth turn purple-red (positive test) on standing, include uninoculated tube of this broth as control. Incubate 24 ± 2 h at 35°C .
- b. **Optional urease test (rapid).** Transfer two 3-mm loopfuls of growth from each presumed-positive TSI slant culture into tubes of rapid urea broth. Incubate 2 h in $37.0 \pm 0.5^{\circ}\text{C}$ water bath. Discard all cultures giving positive test. Retain for further study all cultures that give negative test (no change in color of medium).

3. Serological polyvalent flagellar (H) test

- a. Perform the polyvalent flagellar (H) test at this point, or later, as described in E-5, below. Inoculate growth from each urease-negative TSI agar slant into either 1) BHI broth and incubate 4-6 h at 35°C until visible growth occurs (to test on same day); or 2) trypticase soy-tryptose broth and incubate 24 ± 2 h at 35°C (to test on following day). Add 2.5 ml formalinized physiological saline solution to 5 ml of either broth culture.
- b. Select 2 formalinized broth cultures and test with *Salmonella* polyvalent flagellar (H) antisera. Place 0.5 ml of appropriately diluted *Salmonella* polyvalent flagellar (H) antiserum in 10 x 75 mm or 13 x 100 mm serological test tube. Add 0.5 ml formalinized broth culture to be tested. Prepare saline control by mixing 0.5 ml formalinized physiological saline solution with 0.5 ml formalinized antigen. Incubate mixtures in a water bath set at $48\text{-}50^{\circ}\text{C}$. Observe at 15 min intervals and record final results in 1 h.

Positive -- agglutination in test mixture and no agglutination in control.

Negative -- no agglutination in test mixture and no agglutination in control.

Nonspecific -- agglutination in both test mixture and control. Test the cultures giving such results with Spicer-Edwards antisera.

4. **Spicer-Edwards serological test.** Use this test as an alternative to the polyvalent flagellar (H) test. It may also be used with cultures giving nonspecific agglutination in polyvalent flagellar (H) test. Perform Spicer-Edwards flagellar (H) antisera test as described in E-3b, above. Perform additional biochemical tests (E-5a to 5c, below) on cultures giving positive flagellar test results. If both formalinized broth cultures are negative, perform serological tests on 4 additional broth cultures (E-3a, above). If possible, obtain 2 positive cultures for additional biochemical testing (E-5a to 5c, below).

If all urease-negative TSI cultures from sample give negative serological flagellar (H) test results, perform additional biochemical tests (E-5a to 5c, below).

5. Testing of urease-negative cultures

- a. **Lysine decarboxylase broth.** If LIA test was satisfactory, it needs not be repeated. Use lysine decarboxylase broth for final determination of lysine decarboxylase if culture gives doubtful LIA reaction. Inoculate broth with small amount of growth from TSI slant suspicious for *Salmonella*. Replace cap tightly and incubate 48 ± 2 h at 35°C but examine at 24 h intervals. *Salmonella* species cause alkaline reaction indicated by purple color throughout medium. Negative test is indicated by yellow color throughout medium. If medium appears discolored (neither purple nor yellow) add a few drops of 0.2% bromcresol purple dye and re-read tube reactions.
- b. **Phenol red dulcitol broth or purple broth base with 0.5% dulcitol.** Inoculate broth with small amount of growth from TSI culture. Replace cap loosely and incubate 48 ± 2 h at 35°C , but examine after 24 h. Most *Salmonella* species give positive test, indicated by gas formation in inner fermentation vial and acid pH (yellow) of medium. Production of acid should be interpreted as a positive reaction. Negative test is indicated by no gas formation in inner fermentation vial and red (with phenol red as indicator) or purple (with bromcresol purple as indicator) color throughout medium.
- c. **Tryptone (or tryptophane) broth.** Inoculate broth with small growth from TSI agar culture. Incubate 24 ± 2 h at 35°C and proceed as follows:
 - i. **Potassium cyanide (KCN) broth.** Transfer 3 mm loopful of 24 h tryptophane broth culture to KCN broth. Heat rim of tube so that good seal is formed when tube is stoppered with wax-coated cork. Incubate 48 ± 2 h at 35°C but examine after 24 h. Interpret growth (indicated by turbidity) as positive. Most *Salmonella* species do not grow in this medium, as indicated by lack of turbidity.
 - ii. **Malonate broth.** Transfer 3 mm loopful of 24 h tryptone broth culture to malonate broth. Since occasional uninoculated tubes of malonate broth turn blue (positive test) on standing, include uninoculated tube of this broth as control. Incubate 48 ± 2 h at 35°C , but examine after 24 h. Most *Salmonella* species cultures give negative test (green or unchanged color) in this broth.
 - iii. **Indole test.** Transfer 5 ml of 24 h tryptophane broth culture to empty test tube. Add 0.2-0.3 ml Kovacs' reagent. Most *Salmonella* cultures give negative test (lack of deep red color at surface of broth). Record intermediate shades of orange and pink as \pm .
 - iv. **Serological flagellar (H) tests for *Salmonella*.** If either polyvalent flagellar (H) test (E-3, above) or the Spicer-Edwards flagellar (H) test tube test (E-4, above) has not already been performed, either test may be performed here.
 - v. Discard, as not *Salmonella*, any culture that shows either positive indole test and negative serological flagellar (H) test, or positive KCN test and negative lysine decarboxylase test.

6. Serological somatic (O) tests for *Salmonella*.

(Pre-test all antisera to *Salmonella* with known cultures.)

- a. **Polyvalent somatic (O) test.** Mark off 2 sections about 1 x 2 cm each on inside of glass or plastic petri dish (15 x 100 mm) with a wax pencil. Commercially available sectioned slides may be used. Emulsify 3 mm loopful of culture from 24-48 h TSI slant or, preferably, tryptose blood agar base (without blood) with 2 ml 0.85% saline. Add 1 drop of culture suspension to upper portion of each rectangular marked section. Add 1 drop of saline solution to lower part of one section only. Add 1 drop of *Salmonella* polyvalent somatic (O) antiserum to other section only. With clean sterile transfer loop or needle, mix culture suspension with saline solution for one section and repeat for other section containing antiserum. Tilt mixtures in back-and-forth motion for 1 min and observe against dark background in good illumination. Consider any degree of agglutination a positive reaction. Classify polyvalent somatic (O) test results as follows:

Positive -- agglutination in test mixture; no agglutination in saline control.

Negative -- no agglutination in test mixture; no agglutination in saline control.

Nonspecific -- agglutination in test and in control mixtures. Perform further biochemical and serological tests as described in *Edwards and Ewing's Identification of Enterobacteriaceae*⁴.

- b. **Somatic (O) group tests.** Test as in E-6a, above, using individual group somatic (O) antisera including Vi, if available, in place of *Salmonella* polyvalent somatic (O) antiserum. For special treatment of cultures giving positive Vi agglutination reaction, refer to sec. 967.28B in *Official Methods of Analysis*³. Record cultures that give positive agglutination with individual somatic (O) antiserum as positive for that group. Record cultures that do not react with individual somatic (O) antiserum as negative for that group.

7. **Additional biochemical tests.** Classify as *Salmonella* those cultures which exhibit typical *Salmonella* reactions for tests 1-11, shown in Table 1. If one TSI culture from 25 g analytical unit is classified as *Salmonella*, further testing of other TSI cultures from the same 25 g analytical unit is unnecessary. Cultures that contain demonstrable *Salmonella* antigens as shown by positive *Salmonella* flagellar (H) test but do not have biochemical characteristics of *Salmonella* should be purified (E-1, above) and retested, beginning with E-2, above.

Perform the following additional tests on cultures that do not give typical *Salmonella* reactions for tests 1-11 in Table 1 and that consequently do not classify as *Salmonella*.

- a. **Phenol red lactose broth or purple lactose broth.**

⁴ Ewing, W.H. 1986. *Edwards and Ewing's Identification of Enterobacteriaceae*, 4th ed. Elsevier, New York.

- i. Inoculate broth with small amount of growth from unclassified 24-48 h TSI slant. Incubate 48 ± 2 h at 35°C , but examine after 24 h.

Positive -- acid production (yellow) and gas production in inner fermentation vial. Consider production of acid only as positive reaction. Most cultures of *Salmonella* give negative test result, indicated by no gas formation in inner fermentation vial and red (with phenol red as indicator) or purple (with bromocresol purple as indicator) throughout medium.

- ii. Discard as not *Salmonella*, cultures that give positive lactose tests, except cultures that give acid slants in TSI and positive reactions in LIA, or cultures that give positive malonate broth reactions. Perform further tests on these cultures to determine if they are *S. arizonae*.
- b. **Phenol red sucrose broth or purple sucrose broth.** Follow procedure described in E-7a-i, above. Discard as not *Salmonella*, cultures that give positive sucrose tests, except those that give acid slants in TSI and positive reactions in LIA.
 - c. **MR-VP broth.** Inoculate medium with small amount of growth from each unclassified TSI slant suspected to contain *Salmonella*. Incubate 48 ± 2 h at 35°C .
 - i. Perform Voges-Proskauer (VP) test at room temperature as follows: Transfer 1 ml 48 h culture to test tube and incubate remainder of MR-VP broth for an additional 48 h at 35°C . Add 0.6 ml α -naphthol and shake well. Add 0.2 ml 40% KOH solution and shake. To intensify and speed up the reaction, add a few crystals of creatine. Read results after 4 h; development of pink-to-ruby red color throughout medium is considered a positive test. Most cultures of *Salmonella* are VP-negative, indicated by absence of development of pink-to-red color throughout broth.
 - ii. Perform methyl red test as follows: To 5 ml of 96 h MR-VP broth, add 5-6 drops of methyl red indicator. Read results immediately. Most *Salmonella* cultures give positive test, indicated by diffuse red color in medium. A distinct yellow color is negative test. Discard, as not *Salmonella*, cultures that give positive KCN and VP tests and negative methyl red test.
 - d. **Simmons citrate agar.** Inoculate this agar, using needle containing growth from unclassified TSI agar slant. Inoculate by streaking slant and stabbing butt. Incubate 96 ± 2 h at 35°C . Read results as follows:

Positive -- presence of growth, usually accompanied by color change from green to blue. Most cultures of *Salmonella* are citrate-positive.

Negative -- no growth or very little growth and no color change.

8. **Classification of cultures.** Classify, as *Salmonella*, cultures that have reaction patterns of Table 1. Discard, as not *Salmonella*, cultures that give results listed in any subdivision of Table 2. Perform additional tests described in *Edwards and Ewing's Identification of Enterobacteriaceae*⁴ to classify any culture that is not clearly identified as *Salmonella* by classification scheme in Table 1 or not eliminated as not being *Salmonella* by test reactions in Table 2. If neither of 2 TSI cultures carried through biochemical tests

confirms the isolate as *Salmonella*, perform biochemical tests, beginning with E-5, on remaining urease-negative TSI cultures from the same 25 g analytical unit.

Table 1. Biochemical and serological reactions of *Salmonella*

#	Test or substrate	Positive result	Negative result	<i>Salmonella</i> species reaction ^(a)
1.	Glucose (TSI)	yellow butt	red butt	+
2.	Lysine decarboxylase (LIA)	purple butt	yellow butt	+
3.	H ₂ S (TSI and LIA)	blackening	no blackening	+
4.	Urease	purple-red color	no color change	–
5.	Lysine decarboxylase broth	purple color	yellow color	+
6.	Phenol red dulcitol broth	yellow color and/or gas	no gas; no color change	+ ^(b)
7.	KCN broth	growth	no growth	–
8.	Malonate broth	blue color	no color change	– ^(c)
9.	Indole test	red color at surface	yellow color at surface	–
10.	Polyvalent flagellar test	agglutination	no agglutination	+
11.	Polyvalent somatic test	agglutination	no agglutination	+
12.	Phenol red lactose broth	yellow color and/or gas	no gas; no color change	– ^(c)
13.	Phenol red sucrose broth	yellow color and/or gas	no gas; no color change	–
14.	Voges-Proskauer test	pink-to-red color	no color change	–
15.	Methyl red test	diffuse red color	diffuse yellow color	+
16.	Simmons citrate	growth; blue color	no growth; no color change	V

^a +: 90% or more positive in 1 or 2 days; –: 90% or more negative in 1 or 2 days;
v: variable.

^b Majority of *S. arizonae* cultures are negative.

^c Majority of *S. arizonae* cultures are positive.

Table 2. Criteria for discarding non-*Salmonella* cultures

#	Test or substrate	Results
1.	Urease	positive (purple-red color)
2.	Indole test and Polyvalent flagellar (H) test; or Indole test and Spicer-Edwards flagellar test	positive (red color at surface) negative (no agglutination) positive (red color at surface) negative (no agglutination)
3.	Lysine decarboxylase and KCN broth	negative (yellow color) positive (growth)
4.	Phenol red lactose broth	positive (yellow color and/or gas) ^{(a), (b)}
5.	Phenol red sucrose broth	positive (yellow color and/or gas) ^(b)
6.	KCN broth, Voges-Proskauer test, and Methyl red test	positive (growth) positive (pink-to-red color) negative (diffuse yellow color)

^a Test malonate broth positive cultures further to determine if they are *S. arizonae*.

^b Do not discard positive broth cultures if corresponding LIA cultures give typical *Salmonella* reactions; test further to determine if they are *Salmonella* species.

9. **Presumptive generic identification of *Salmonella*.** As alternative to conventional biochemical tube system, use any of 5 commercial biochemical systems (API 20E, Enterotube II, *Enterobacteriaceae* II, MICRO-ID, or Vitek 2 GN) for presumptive generic identification of foodborne *Salmonella*. Choose a commercial system based on a demonstration in analyst's own laboratory of adequate correlation between commercial system and biochemical tube system delineated in this identification section. Commercial biochemical kits should not be used as a substitute for serological tests³. Assemble supplies and prepare reagents required for the kit. Inoculate each unit according to Method 978.24 (API 20E, Enterotube II, and *Enterobacteriaceae* II), sec. 989.12 (MICRO-ID), and Method 2011.17 (Vitek 2 GN) in *Official Methods of Analysis*³, incubating for time and temperature specified. Add reagents, observe, and record results. For presumptive identification, classify cultures, according to ref. 3 (see footnote 3 on p.15), as *Salmonella* or not *Salmonella*.

For confirmation of cultures presumptively identified as *Salmonella*, perform the *Salmonella* serological somatic (O) test (E-6, above) and the *Salmonella* serological flagellar (H) test (E-3, above) or the Spicer-Edwards flagellar (H) test (E-4, above), and classify cultures according to the following guidelines:

- a. Report as *Salmonella* those cultures classified as presumptive *Salmonella* with commercial biochemical kits when the culture demonstrates positive *Salmonella* somatic (O) test and positive *Salmonella* (H) test.
 - b. Discard cultures presumptively classified as not *Salmonella* with commercial biochemical kits when cultures conform to AOAC criteria³ for classifying cultures as not *Salmonella*.
 - c. For cultures that do not conform to a or b, classify according to additional tests specified in E-2 to E-7, above, or additional tests as specified by Ewing⁴, or send to reference typing laboratory for definitive serotyping and identification.
10. **Treatment of cultures giving negative flagellar (H) test.** If biochemical reactions of certain flagellar (H)-negative culture strongly suggest that it is *Salmonella*, the negative flagellar agglutination may be the result of nonmotile organisms or insufficient development of flagellar antigen. Proceed as follows: Add motility test medium in petri dish. With a small amount of growth from TSI slant, inoculate by stabbing medium once about 10 mm from edge of plate to depth of 2-3 mm. Do not stab to bottom of plate or inoculate any other portion. Incubate 24 h at 35°C. If organisms have migrated 40 mm or more, retest as follows: Transfer 3 mm loopful of growth that migrated farthest to trypticase soy-tryptose broth. Repeat either polyvalent flagellar (H) (E-3, above) or Spicer-Edwards (E-4, above) serological tests. If cultures are not motile after the first 24 h, incubate an additional 24 h at 35°C; if still not motile, incubate for up to 5 days at 25°C. Classify culture as nonmotile if above tests are still negative.

G. Media preparations: See Appendix 1

Appendix 1: Media Preparations

I. *E. coli* O157:H7

1. R11: Butterfield's Phosphate-Buffered Dilution Water

KH ₂ PO ₄	34 g
Distilled water	500 ml

Adjust pH to 7.2 with 1 N NaOH. Bring volume to 1 liter with distilled water. Sterilize 15 min at 121°C. Store in refrigerator.

Dilution blanks: Take 1.25 ml of above stock solution and bring volume to 1 liter with distilled water. Dispense into bottles to 90 or 99 ± 1 ml. Sterilize 15 min at 121°C.

Note: This same formulation is referred to as Buffered Dilution Water in “Recommended Procedures for the Examination of Seawater and Shellfish”, American Public Health Association, 4th ed., Washington, DC., p14-15, 1970.

2. R38: Kovacs' Reagent

p-Dimethylaminobenzaldehyde	5 g
Amyl alcohol (normal only)	75 ml
HCl (concentrated)	25 ml

Dissolve p-dimethylaminobenzaldehyde in normal amyl alcohol. Slowly add HCl. Store at 4°C. To test for indole, add 0.2-0.3 ml reagent to 5 ml of 24 h bacteria culture in tryptone broth. Dark red color in surface layer is a positive test for indole.

3. Modified M192a: Modified Buffered Peptone water with pyruvate (mBPWp) and Cefsulodin-Vancomycin(CV) Supplement

a. Modified Buffered Peptone water with pyruvate (mBPWp)

Peptone	10.0 g
NaCl	10.0 g
Na ₂ HPO ₄	3.6 g
KH ₂ PO ₄	1.5 g
Casamino acids	5.0 g
Yeast extract	6.0 g
Lactose	10.0 g
Sodium Pyruvate	1.0 g
Distilled water	1000 ml (Use 500 ml for 2× strength)

b. Cefsulodin-Vancomycin (CV) Supplement for mBPWp

Supplement	Conc. in mBPWp	Conc. in stock	Amt. of stock/ 225ml
Cefsulodin	10 mg/L	1.125 g/500 ml	1 ml
Vancomycin	8 mg/L	0.90 g/500 ml	1 ml

Filter, sterilize. Add 1ml of each to 225 ml mBPWp for selective overnight enrichment.

4. M194: Tellurite Cefixime – Sorbitol MacConkey Agar (TC-SMAC)

a. Prepare Sorbitol-MacConkey Agar (M-139) as directed.

Peptone or gelysate	17.0 g
Protease peptone No. 3 or polypeptone	3.0 g
Sorbitol	10.0 g
Bile salts, purified	1.5 g
NaCl	5.0 g
Agar	13.5 g
Neutral red	0.03 g
Crystal violet	0.001 g
Distilled water	1 liter

Dissolve ingredients in distilled water by heating with stirring. Autoclave 15 min at 121°C. Final pH, 7.1 ± 0.2.

b. Once SMAC has been autoclaved and tempered, then add the 2 filter-sterilized additives below to obtain the final concentrations shown.

Potassium tellurite	2.5 mg/L (250 µl of 1% solution)*
Cefixime	0.05 mg/L (1ml of 50 mg/L solution in 95% EtOH)

Note: 1% Potassium tellurite may be store at 4° C for 1 month. Cefixime may be stored at -20°C for 1 year.

5. M153: Trypticase Soy Agar with 0.6% Yeast Extract (TSAYE)

Trypticase soy agar	40 g
Yeast extract	6 g
Distilled water	1 liter

Weigh ingredients, add water, mix, and autoclave 15 min at 121°C. Final pH, 7.3 ± 0.2. After autoclaving, swirl to disperse molten agar.

II. *Salmonella*

1. M19, Bismuth Sulfite Agar (Wilson and Blair)

Polypeptone (or peptone)	10 g
Beef extract	5 g
Dextrose	5 g
Na ₂ HPO ₄ (anhydrous)	4 g
FeSO ₄ (anhydrous)	0.3 g
Bismuth sulfite (indicator)	8 g
Brilliant green	0.025 g

Agar	20 g
Distilled water	1 liter

Mix thoroughly and heat with agitation. Boil for about 1 min to obtain uniform suspension. (Precipitate will not dissolve.) Cool to 45-50°C. Suspend precipitate by gentle agitation, and pour 20 ml portions into sterile 15 × 100 mm petri dishes. Let plates dry for about 2 h with lids partially open; then close lids. Final pH, 7.7 ± 0.2

DO NOT AUTOCLAVE. Prepare plates on day before streaking and store in dark. Selectivity decreases in 48h.

2. M24, Brain Heart Infusion (BHI) Broth and Agar (Equivalent media are available from Difco and BBL)

a. Medium 1:

Calf brain, infusion from	200 g
Beef heart, infusion from	250 g
Proteose peptone (Difco) or polypeptone (Bioquest)	10 g
NaCl	5 g
Na ₂ HPO ₄	2.5 g
Dextrose	2.0 g
Distilled water	1 liter

Dissolve ingredients of **Medium 1** in distilled water with gentle heat.

b. Medium 2:

Brain heart-infusion	6.0 g
Peptic digest of animal tissue	6.0 g
NaCl	5.0 g
Dextrose	3.0 g
Pancreatic digest of gelatin	14.5 g
Na ₂ HPO ₄	2.5 g
Distilled water	1 liter

Suspend ingredients of **Medium 2** in distilled water and boil for 1 min to completely dissolve.

c. For both **Medium 1** and **Medium 2**, dispense broth into bottles or tubes for storage. Autoclave 15 min at 121°C. Final pH, 7.4 ± 0.2. Commercially available BHI is acceptable.

d. To prepare brain heart infusion agar, add 15 g agar to 1 liter BHI broth. Heat to dissolve agar before dispensing into bottles or flasks. Autoclave 15 min at 121°C.

3. R9: Bromcresol Purple Dye Solution. 0.2%

Bromcresol purple dye	0.2 g
Distilled water (sterile)	100 ml

Dissolve 0.2 g dye in 100 ml sterile distilled water.

4. R27: Formalinized Physiological Saline Solution

Formaldehyde solution (36-38%)	6 ml
NaCl	8.5 ml

Distilled water 1 liter

Dissolve 8.5 g NaCl in 1 liter distilled water. Autoclave 15 min at 121°C. Cool to room temperature. Add 6 ml formaldehyde solution. Do not autoclave after addition of formaldehyde.

5. M61: Hektoen Enteric (HE) Agar

Peptone	12 g
Yeast extract	3 g
Bile salts No. 3	9 g
Lactose	12 g
Sucrose	12 g
Salicin	2 g
NaCl	5 g
Sodium thiosulfate	5 g
Ferric ammonium citrate	1.5 g
Bromthymol blue	0.065 g
Acid fuchsin	0.1 g
Agar	14.0 g
Distilled water	1 liter

Heat to boiling with frequent agitation to dissolve. Boil for no longer than 1 min. Do not overheat. Cool in water bath. Pour 20 ml portions into sterile 15 × 100 mm petri dishes. Let dry 2 h with lids partially open. Final pH, 7.5 ± 0.2.

Storage: May be stored up to 30 days under refrigeration (4 ± 2°C).

6. R36: 1 N Hydrochloric Acid

HCl (concentrated)	89 ml
Distilled water to make 1 liter	

7. R38: Kovacs' Reagent

<i>p</i> -Dimethylaminobenzaldehyde	5 g
Amyl alcohol (normal only)	75 ml
HCl (concentrated)	25 ml

Dissolve *p*-dimethylaminobenzaldehyde in normal amyl alcohol. Slowly add HCl. Store at 4°C. To test for indole, add 0.2-0.3 ml reagent to 5 ml of 24 h bacteria culture in tryptone broth. Dark red color in surface layer is a positive test for indole.

8. M74: Lactose Broth

Beef extract	3 g
Peptone	5 g
Lactose	5 g
Distilled water	1 liter

Dispense 225 ml portions into 500 ml Erlenmeyer flasks. After autoclaving 15 min at 121°C and just before use, aseptically adjust volume to 225 ml with the addition of sterile distilled water. Final pH, 6.9 ± 0.2.

9. M87: Lysine Decarboxylase Broth (Falkow) (for *Salmonella*)

Gelysate or peptone	5 g
Yeast extract	3 g
Glucose	1 g
L-Lysine	5 g
Bromcresol purple	0.02 g
Distilled water	1 liter

Heat until dissolved. Dispense 5 ml portions into 16 × 125 mm screw-cap tubes. Autoclave loosely capped tubes 15 min at 121°C. Screw the caps on tightly for storage and after inoculation. Final pH, 6.8 ± 0.2.

10. M89: Lysine Iron Agar (Edwards and Fife)

Gelysate or peptone	5 g
Yeast extract	3 g
Glucose	1 g
L-Lysine hydrochloride	10 g
Ferric ammonium citrate	0.5 g
Sodium thiosulfate (anhydrous)	0.04 g
Bromcresol purple	0.02 g
Agar	15 g
Distilled water	1 liter

Heat to dissolve ingredients. Dispense 4 ml portions into 13 × 100 mm screw-cap tubes. Autoclave 12 min at 121°C. Let solidify in slanted position to form 4 cm butts and 2.5 cm slants. Final pH, 6.7 ± 0.2.

11. M92: Malonate Broth

Yeast extract	1 g
(NH ₄) ₂ SO ₄	2 g
K ₂ HPO ₄	0.6 g
KH ₂ PO ₄	0.4 g
NaCl	2 g
Sodium malonate	3 g
Glucose	0.25 g
Bromthymol blue	0.025 g
Distilled water	1 liter

Dissolve by heating, if necessary. Dispense 3 ml portions into 13 × 100 mm test tubes. Autoclave 15 min at 121°C. Final pH, 6.7 ± 0.2.

12. R44: Methyl Red Indicator

Methyl red	0.10 g
Ethanol, 95%	300 ml
Distilled water	To make 500 ml

Dissolve methyl red in 300 ml ethanol. Bring volume to 500 ml with distilled water.

13. M103: Motility Test Medium (Semisolid)

Beef extract	3 g
Peptone or gelysate	10 g
NaCl	5 g
Agar	4 g
Distilled water	1 liter

Heat with agitation and boil 1-2 min to dissolve agar. Dispense 20 ml portions into 20 × 150 mm screw-cap tubes, replacing caps loosely. Autoclave 15 min at 121°C. Cool to 45°C after autoclaving. Tighten caps, and refrigerate at 5-8°C. To use, re-melt in boiling water or flowing steam, and cool to 45°C. Aseptically dispense 20 ml portions into sterile 15 × 100 mm petri plates. Cover plates and let solidify. **Use on the same day as prepared.** Final pH, 7.4 ± 0.2.

14. M104: MR-VP Broth

a. Medium 1

Buffered peptone-water powder (Difco or BBL)	7 g
Glucose	5 g
K ₂ HPO ₄	5 g
Distilled water	1 liter

b. Medium 2

Pancreatic digest of casein	3.5 g
Peptic digest of animal tissue	3.5 g
Dextrose	5.0 g
Potassium phosphate	5.0 g
Distilled water	1 liter

Dissolve ingredients in water with gentle heat if necessary. Dispense 10 ml into 16 × 150 mm test tubes and autoclave 15 min at 118-121°C. Final pH, 6.9 ± 0.2.

c. Medium 3

Peptone	5.0 g
Glucose	5.0 g
Phosphate buffer	5.0 g
Distilled water	1 liter

Dissolve ingredients in water. Dispense 10 ml into 16 × 150 mm test tubes and autoclave 15 min at 121°C. Final pH, 7.5 ± 0.2.

15. M91: MacConkey Agar

Proteose peptone or polypeptone	3 g
Peptone or gelysate	17 g

Lactose	10 g
Bile salts No. 3 or bile salts mixture	1.5 g
NaCl	5 g
Neutral red	0.03 g
Crystal violet	0.001 g
Agar	13.5 g
Distilled water	1 liter

Suspend ingredients and heat with agitation to dissolve. Boil 1-2 min. Autoclave 15 min at 121°C, cool to 45-50°C, and pour 20 ml portions into sterile 15 × 100 mm petri dishes. Dry at room temperature with lids closed.

DO NOT USE WET PLATES. Final pH, 7.1 ± 0.2.

16. M121: Phenol Red Carbohydrate Broth

Trypticase or proteone peptone No. 3	10 g
NaCl	5 g
Beef extract (optional)	1 g
Phenol red (7.2 ml of 0.25% phenol red solution)	0.018 g
Distilled water	1 liter
Carbohydrate*	

*Dissolve either 5 g dulcitol, 10 g lactose, or 10 g sucrose (as specified in the *Salmonella* test) in this basal broth.

Dispense 2.5 ml portions into 13 × 100 mm test tubes containing inverted 6 × 50 mm fermentation tubes. Autoclave 10 min at 118°C. Final pH, 7.4 ± 0.2.

Alternatively, dissolve ingredients, omitting carbohydrate, in 800 ml distilled water with heat and occasional agitation. Dispense 2.0 ml portions into 13 × 100 mm test tubes containing inverted fermentation tubes. Autoclave 15 min at 118°C and let cool. Dissolve carbohydrate in 200 ml distilled water and sterilize by passing solution through bacteria-retaining filter. Aseptically add 0.5 ml sterile filtrate to each tube of sterilized broth after cooling to less than 45°C. Shake gently to mix. Final pH, 7.4 ± 0.2.

17. R63: Physiological Saline Solution 0.85% (Sterile)

NaCl	8.5 g
Distilled water	1 liter

Dissolve 8.5 g NaCl in water. Autoclave 15 min at 121°C. Cool to room temperature.

18. M126: Potassium Cyanide (KCN) Broth

*Potassium cyanide (KCN)	0.5 g
Proteose peptone No. 3 or polypeptone	3 g
NaCl	5 g
KH ₂ PO ₄	0.225 g
Na ₂ HPO ₄	5.64 g

Distilled water 1 liter

Dissolve above ingredients **except potassium cyanide** and autoclave 15 min at 121°C. Cool and refrigerate at 5-8°C. Final pH, 7.6 ± 0.2.

Prepare KCN stock solution by dissolving 0.5 g KCN in 100 ml sterile distilled water cooled to 5-8°C.

Using bulb pipetter, add 15 ml cold KCN stock solution to 1 liter cold, sterile base.

***DO NOT PIPET BY MOUTH!!! Use protective equipment/gloves when handling!**

Mix and aseptically dispense 1.0-1.5 ml portions to 13 × 100 mm sterile tubes. Using aseptic technique, stopper tubes with No. 2 corks impregnated with paraffin. Prepare corks by boiling in paraffin for about 5 min. Place corks in tubes so that paraffin does not flow into broth but forms a seal between rim of tubes and cork. Store tubes at 5-8°C for **no longer than 2 weeks before use.**

19. M130: Purple Carbohydrate Fermentation Broth Base

Proteose peptone No. 3	10 g
Beef extract	1 g
NaCl	5 g
Bromcresol purple	0.02 g
Distilled water	1 liter

Prepare as for **phenol red carbohydrate broth (M121)**. Final pH, 6.8 ± 0.2.

20. M132: Rappaport-Vassiliadis Medium

a. Broth Base

Tryptone	5 g
NaCl	8 g
KH ₂ PO ₄	1.6 g
Distilled water	1 liter

b. Magnesium chloride solution

MgCl ₂ ·6H ₂ O	400 g
Distilled water	1 liter

c. Malachite green oxalate solution

Malachite green oxalate	0.4 g
Distilled water	100 ml

To prepare the complete medium, combine 1000 ml broth base, 100 ml magnesium chloride solution, and 10 ml malachite green oxalate solution (total volume of complete medium is 1110 ml). Broth base must be prepared on same day that components are combined to make complete medium. Magnesium chloride solution may be stored in a dark bottle at room temperature for up to 1 year. To prepare solution, dissolve entire contents of MgCl₂·6H₂O from newly opened container according to formula, because this salt is very hygroscopic. Malachite green oxalate solution may be stored in a dark bottle at room temperature for up to 6 months. Merck analytically pure malachite green oxalate is recommended because other brands may not be equally effective. Dispense 10

ml volumes of complete medium into 16 × 150 mm test tubes. Autoclave 15 min at 115°C. Final pH, 5.5 ± 0.2. Store in refrigerator and use within 1 month.

This medium must be made from its individual ingredients. Use of commercially available dehydrated media is not recommended. Users of this medium should be aware that commercial preparations may have different formulations and may suggest different incubation temperatures from those recommended in this manual.

Storage Conditions: Refrigerator [4-8°C]

21. M138: Simmons Citrate Agar

Sodium citrate	2 g
NaCl	5 g
K ₂ HPO ₄	1 g
NH ₄ H ₂ PO ₄	1 g
MgSO ₄	0.2 g
Bromthymol blue	0.08 g
Agar	15 g
Distilled water	1 liter

Heat gently with occasional agitation. Boil 1-2 min until agar dissolves. Fill 13 × 100 or 16 × 150 mm screw-cap tubes 1/3 full. Autoclave 15 min at 121°C. Before medium solidifies, incline tubes to obtain 4-5 cm slants and 2-3 cm butts. Final pH, 6.8 ± 0.2.

22. R73: 1 N Sodium Hydroxide Solution

NaOH	40 g
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Add distilled water to make 1 liter
Use for adjusting pH of culture media.

23. M145: Tetrathionate (TT) Broth

Polypeptone	5 g
Bile salts	1 g
Calcium carbonate	10 g
Sodium thiosulfate·5H ₂ O	30 g
Distilled water	1 liter

Suspend ingredients in 1 liter distilled water, mix, and heat to boiling. **DO NOT AUTOCLAVE.** (Precipitate will not dissolve completely.) Cool to less than 45°C. Store at 5-8°C. Final pH, 8.4 ± 0.2.

24. M149: Triple Sugar Iron Agar (TSI)

a. Medium 1

Polypeptone	20 g
NaCl	5 g
Lactose	10 g
Sucrose	10 g
Glucose	1 g

Fe(NH ₄) ₂ (SO ₄) ₂ •6H ₂ O	0.2 g
Na ₂ S ₂ O ₃	0.2 g
Phenol red	0.025 g
Agar	13 g
Distilled water	1 liter

b. **Medium 2**

Beef extract	3 g
Yeast extract	3 g
Peptone	15 g
Proteose peptone	5 g
Glucose	1 g
Lactose	10 g
Sucrose	10 g
FeSO ₄	0.2 g
NaCl	5 g
Na ₂ S ₂ O ₃	0.3 g
Phenol red	0.024 g
Agar	12 g
Distilled water	1 liter

These two media are interchangeable for general use.

Suspend ingredients of **Medium 1** in distilled water, mix thoroughly, and heat with occasional agitation. Boil for about 1 min to dissolve ingredients. Fill 16 × 150 mm tubes 1/3 full and cap or plug to maintain aerobic conditions. Autoclave **Medium 1** for 15 min at 118°C.

Prepare **Medium 2** in the same manner as **Medium 1**, except autoclave 15 min at 121°C. Before the media solidify, incline tubes to obtain 4-5 cm slant and 2-3 cm butt. Final pH, 7.3 ± 0.2 for **Medium 1** and 7.4 ± 0.2 for **Medium 2**.

25. M154: Trypticase (Tryptic) Soy Broth

Trypticase peptone	17 g
Phytone peptone	3 g
NaCl	5 g
K ₂ HPO ₄	2.5 g
Glucose	2.5 g
Distilled water	1 liter

Heat with gentle agitation to dissolve. Dispense 225 ml into 500 ml Erlenmeyer flasks. Autoclave 15 min at 121°C. Final pH, 7.3 ± 0.2.

26. M160: Trypticase Soy-Tryptose Broth

Trypticase soy broth (commercial, dehydrated)	15 g
Tryptose broth (commercial, dehydrated)	13.5 g
Yeast extract	3 g

Distilled water 1 liter

Dissolve ingredients in 1 liter water. Heat gently to dissolve. Dispense 5 ml portions into 16 × 150 mm test tubes. Autoclave 15 min at 121°C. Final pH, 7.2 ± 0.2.

27. M164: Tryptone (Tryptophane) Broth, 1%

Tryptone or trypticase 10 g
Distilled water 1 liter

Dissolve and dispense 5 ml portions into 16 × 125 or 16 × 150 mm test tubes. Autoclave 15 min at 121°C. Final pH, 6.9 ± 0.2.

28. M166: Tryptose Blood Agar Base

Tryptose 10 g
Beef extract 3 g
NaCl 5 g
Agar 15 g
Distilled water 1 liter

Suspend ingredients in distilled water, mix thoroughly, and heat with occasional agitation. Boil for about 1 min. Fill 16 × 150 mm tubes 1/3 full and cap or plug to maintain aerobic conditions. Autoclave 15 min at 121°C. Before media solidify, incline tubes to obtain 4-5 cm slant and 2-3 cm butt.

29. M179, Xylose Lysine Desoxycholate (XLD) Agar

Yeast extract 3 g
L-lysine 5 g
Xylose 3.75 g
Lactose 7.5 g
Sucrose 7.5 g
Sodium desoxycholate 2.5 g
Ferric ammonium citrate 0.8 g
Sodium thiosulfate 6.8 g
NaCl 5 g
Agar 15 g
Phenol red 0.08 g
Distilled water 1 liter

Heat with agitation just until medium boils. Do not overheat. Pour into plates when medium has cooled to 50°C. Let dry about 2 h with covers partially open. Then close lids. Final pH, 7.4 ± 0.2.

Storage: May be stored up to 30 days under refrigeration (4 ± 2°C).

30. M188, Universal Preenrichment Broth

Tryptone 5 g
Proteose peptone 5 g

KH ₂ PO ₄	15 g
Na ₂ HPO ₄	7 g
NaCl	5 g
Dextrose	0.5 g
MgSO ₄	0.25 g
Ferric ammonium citrate	0.1 g
Sodium pyruvate	0.2 g
Distilled water	1.0 liter

Heat with gentle agitation to dissolve. Autoclave 15 min at 121°C. Final pH, 6.3 ± 0.2.

31. M171: Urea Broth

Urea	20 g
Yeast extract	0.1 g
Na ₂ HPO ₄	9.5 g
KH ₂ PO ₄	9.1 g
Phenol red	0.01 g
Distilled water	1 liter

Dissolve ingredients in distilled water. DO NOT HEAT. Sterilize by filtration through 0.45 µm membrane. Aseptically dispense 1.5-3.0 ml portions to 13 × 100 mm sterile test tubes. Final pH, 6.8 ± 0.2.

32. M172: Urea Broth (Rapid)

Urea	20 g
Yeast extract	0.1 g
KH ₂ PO ₄	0.091 g
Na ₂ HPO ₄	0.095 g
Phenol red	0.01 g
Distilled water	1 liter

Prepare as for urea broth (M171).

33. R89: Voges-Proskauer (VP) Test Reagents

a. Solution 1

α-Naphthol	5 g
Alcohol (absolute)	100 ml

b. Solution 2

Potassium hydroxide	40 g
Distilled water to make 100 ml	

c. Voges-Proskauer (VP) test

Transfer 1 ml of 48 h culture to test tube and add 0.6 ml solution 1 and 0.2 ml solution 2. Shake after adding each solution. To intensify and speed up reaction, add a few creatine crystals to mixture. Let stand at room temperature. Read results 4 h after adding reagents.