U.S. FOOD & DRUG ADMINISTRATION

Bacteriological Analytical Manual

Chapter 4A: Diarrheagenic *Escherichia coli*

July 2020 Edition

July 2020

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Revision History

- July 2020: Update to section R.1.c for use of instrumental methods to screen for *E. coli* and correction to Table 5.
- October 2018: Sec. R. #2. Procedure for confirmation of non-O157 STEC isolates has been clarified with instructions to perform WGS and PFGE on all STEC isolates.
- October 2017: Sec. Q.1.c. The 5P PCR and LIB 3811 methods used for confirming O157:H7 isolates have been deleted and archived. Also, instructions were added to do PFGE and WGS on all O157:H7 isolates.
- October 2017: Additional instructions and cautionary statements have been added to Sec. R.1.c on isolation of STEC. Specifically, the number of colony picks has been increased and with specific instructions.
- August 2016: The Lightcycler method has been archived and replaced with the AB 7500 Fast platform. Extensive Modifications to Section K: Screening method for *E. coli* Serotype O157:H7/STEC from Foods; Section L: Equipment and Materials; Section M: Media and Reagents, Section N: Sample Preparation and Enrichment Procedure, and Section O: Real-time PCR Screening.
- November 2015: Section R: The 13-plex Luminex suspension array: STEC Molecular Serotyping and Virulence Profiling Protocol replaces the 11-plex STEC Molecular Serotyping Protocol, which will be archived, when reagents for the 13-plex assay becomes available. The new Luminex based suspension array can now identify 11 clinically relevant STEC O serogroups and detect two STEC adherence factor genes: *eae* and *aggR*.
- July 2014: Section N: A new sample preparation for leafy produce (except cilantro and parsley) has been added; the sample preparation for cilantro and parsley has been modified.
- July, 2013: Section R: STEC Molecular Serotyping Protocol: Luminex-based suspension array to identify 11 STEC O serogroups has been added.
- December 2012: Section R: The testing for non-O157 STEC has been changed from optional to required. All samples that are PCR positive for *Stx*1 and *Stx*2 but negative for *uid*A, must be followed up with additional testing to isolate the STEC organism. In Section R.1.a. the SHIBAM agar has been added to facilitate the isolation of STEC from enrichment samples.
- February 2011: Section M: Addition of primers in Table 2; Section Q: addition of 5P Multiplex PCR for confirmation of O157:H7 isolates
- July 2009: Section M modified to include the real-time PCR screening method.

Introduction

Escherichia coli is one of the predominant species of facultative anaerobes in the human gut and usually harmless to the host; however, a group of pathogenic *E. coli* has emerged that causes diarrheal disease in humans. Referred to as Diarrheagenic *E. coli* (28) or commonly as pathogenic *E. coli*, these groups are classified based on their unique virulence factors and can only be identified by these traits. Hence, analysis for pathogenic *E. coli* often requires that the isolates be first identified as *E. coli* before testing for virulence markers. The pathogenic groups include enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), diffusely adherent *E. coli* (DAEC) and perhaps others that are not yet well characterized (21, 28). Of these, only the first 4 groups have been implicated in food or water borne illness. Some properties and symptoms of these 4 subgroups are discussed below and summarized in Table 1.

ETEC is recognized as the causative agent of travelers' diarrhea and illness is characterized by watery diarrhea with little or no fever. ETEC infections occurs commonly in under-developed countries but, in the U.S., it has been implicated in sporadic waterborne outbreaks as well as due to the consumption of soft cheeses, Mexican-style foods and raw vegetables. Pathogenesis of ETEC is due to the production of any of several enterotoxins. ETEC may produce a heat-labile enterotoxin (LT) that is very similar in size (86 kDa), sequence, antigenicity, and function to the cholera toxin (CT). ETEC may also produce a heat stable toxin (ST) that is of low molecular size (4 kDa) and resistant to boiling for 30 min. There are several variants of ST, of which ST1a or STp is found in *E. coli* isolated from both humans and animals, while ST1b or STh is predominant in human isolates only. The infective dose of ETEC for adults has been estimated to be at least 10^8 cells; but the young, the elderly and the infirm may be susceptible to lower levels. Because of its high infectious dose, analysis for ETEC is usually not performed unless high levels of *E. coli* have been found in a food. Also, if ETEC is detected, levels should also be enumerated to assess the potential hazard of the contaminated food. Production of LT can be detected by Y-1 adrenal cell assays (28) or serologically by commercial reverse passive latex agglutination assay and ELISA (see Appendix 1). The production of ST can also be detected by ELISA or by infant mouse assay (35). Both LT and ST genes have also been sequenced and PCR (37, 41) and gene probe assays (see chapter 24) are available. Analysis of colonies on plating media using gene probe/colony hybridization also allows enumeration of ETEC in foods.

EIEC closely resemble *Shigella* and causes an invasive, dysenteric form of diarrhea in humans (7). Like *Shigella*, there are no known animal reservoirs; hence the primary source for EIEC appears to be infected humans. Although the infective dose of *Shigella* is low and in the range of 10 to few hundred cells, volunteer feeding studies showed that at least 10^6 EIEC organisms are required to cause illness in healthy adults. Unlike typical *E. coli*, EIEC are non-motile, do not decarboxylate lysine and do not ferment lactose, so they are anaerogenic. Pathogenicity of EIEC is primarily due its ability to invade and destroy colonic tissue. The invasion phenotype, encoded by a high molecular weight plasmid, can be detected by invasion assays using HeLa or Hep-2 tissue culture cells (7, 25) or by PCR and probes specific for invasion genes (see chapter 24).

EPEC causes a profuse watery diarrheal disease and it is a leading cause of infantile diarrhea in developing countries. EPEC outbreaks have been linked to the consumption of contaminated drinking water as well as some meat products. Through volunteer feeding studies the infectious

dose of EPEC in healthy adults has been estimated to be 106 organisms. Pathogenesis of EPEC involves intimin protein (encoded by eae gene) that causes attachment and effacing lesions (14); but it also involves a plasmid-encoded protein referred to as EPEC adherence factor (EAF) that enables localized adherence of bacteria to intestinal cells (36). Production of EAF can be demonstrated in Hep-2 cells and the presence of eae gene can be tested by PCR assays (28).

EHEC are recognized as the primary cause of hemorrhagic colitis (HC) or bloody diarrhea, which can progress to the potentially fatal hemolytic uremic syndrome (HUS). EHEC are typified by the production of verotoxin or Shiga toxins (*Stx*). Although *Stx1* and *Stx*2 are most often implicated in human illness, several variants of *Stx*1 and *Stx*2 exist. There are many serotypes of *Stx*-producing *E. coli* (STEC), but only those that have been clinically associated with HC are designated as EHEC. Of these, O157:H7 is the prototypic EHEC and most often implicated in illness worldwide (3, 13, 19, 28). The infectious dose for O157:H7 is estimated to be 10 - 100 cells; but no information is available for other EHEC serotypes. EHEC infections are mostly food or water borne and have implicated undercooked ground beef (3, 13), raw milk (31), cold sandwiches (19), water (34), unpasteurized apple juice (2) and sprouts and vegetables (4, 17). EHEC O157:H7 are phenotypically distinct from *E. coli* in that they exhibit slow or no fermentation of sorbitol and do not have glucuronidase activity (see chapter 4. LST-MUG for details); hence, these traits are often used to isolate this pathogen from foods. The production of *Stx*1 and *Stx*2 can be tested by cytotoxicity assays on vero or HeLa tissue culture cells or by commercially available ELISA or RPLA kits (see Appendix 1). Gene probes (see chapter 24) and PCR assays specific for *stx1* and *stx2* and other trait EHEC markers are also available (12, 15) (and see below).

Table 1. Some properties and symptoms associated with pathogenic E. coli subgroups.

Properties/Symptoms	ETEC	EPEC	EHEC	EIEC
Toxin	LT/ST[a]	-	Shiga or Vero toxin (*Stx* or VT)	-
Invasive	-	-	-	+
Intimin	-	+	+	-
Enterohemolysin	-	-	+	-
Stool	Watery	Watery, Bloody	Watery, very bloody	Mucoid, bloody
Fever	Low	+	-	+
Fecal leukocytes	-	-	-	+
Intestine involved	Small	Small	Colon	Colon, lower small
Serology	Various	O26, O111 & others	O157:H7, O26, O111 & others	Various
I_D[b]	High	High	Low	High

[a] LT, labile toxin; ST, stable toxin.
[b] I_D, infective dose.

Isolation and Identification of Pathogenic *Escherichia coli* - Except STEC and EHEC of serotype 0157:H7 (see Section K)

Since pathogenic *E. coli* are identified based on its unique virulence properties, the analytical procedure for these pathogens in foods generally requires the isolation and identification of the organisms as *E. coli* before testing for the specific virulence traits. Following is a general procedure for enrichment and isolation of pathogenic *E. coli* from food (25).

A. Equipment and materials

1. Balance, ≥ 2 kg with 0.1 g sensitivity
2. Blender, Waring or equivalent model with low speed operation at 8000 rpm, with 1 liter glass or metal jar
3. Incubators, 35 ± 0.5°C and 44 ± 1°C
4. Petri dishes 20 × 150 mm
5. Pipets, Pasteur
6. pH test paper, range 6.0-8.0

B. Media

1. Tryptone phosphate (TP) broth (M162)
2. Brain heart infusion (BHI) broth (M24)
3. Levine's eosin-methylene blue (L-EMB) agar (M80)
4. MacConkey agar (M91)
5. Triple sugar iron (TSI) agar (M149)
6. Blood agar base (BAB) (M21)
7. Tryptone (tryptophane) broth (M164)
8. Bromcresol purple broth (M26) supplemented individually with 0.5% (w/v) of each: glucose, adonitol, cellobiose, sorbitol, arabinose, mannitol, and lactose
9. Urea broth (M171)
10. Lysine decarboxylase broth, Falkow (M87).
11. Potassium cyanide (KCN) broth (M126)
12. MR-VP broth (M104)
13. Indole nitrite medium (tryptic nitrate) (M66)
14. Acetate agar (M3)
15. Mucate broth (M105)
16. Mucate control broth (M106)
17. Malonate broth (M92)
18. Koser's citrate broth (M72)

C. Reagents: inorganic, organic, and biological

1. Sodium bicarbonate solution, 10%, aqueous (sterile) (R70)
2. ONPG (*o*-nitrophenyl-β-D-galactopyranoside) disks (R53)
3. Phosphate buffered saline solution, (sterile) (PBS) (R60), or Butterfield's Phosphate-buffered dilution water (BPBW) (R11).
4. Kovac's reagent (R38)
5. VP reagents (R89)
6. Oxidase test reagent (R54)
7. Nitrite detection reagents (R48)
8. Mineral oil, heavy sterile (R46)
9. Gram stain reagents (R32)

D. Enumeration

Generic *E. coli* can be enumerated by MPN but enumeration is seldom done for pathogenic *E. coli* strains. Also, no pathogenic *E. coli* enumeration methods have been validated.

E. Enrichment for Pathogenic E. coli – except STEC and O157:H7 (See Section K)

The approach recommended here permits qualitative determination of the presence of pathogenic *E. coli*. Aseptically weigh 25 g of sample into 225 ml of BHI broth (dilution factor of 1:10). If necessary, sample size may deviate from 25 g depending on availability of the sample, as long as the diluent is adjusted proportionally. Blend or stomach briefly. Incubate the homogenate for 10 min at room temperature with periodic shaking then allow the sample to settle by gravity for 10 min. Decant medium carefully into a sterile container and incubate for 3 h at 35°C to resuscitate injured cells. Transfer contents to 225 mL double strength TP broth in a sterile container and incubate 20 h at 44.0 ± 0.2°C. After incubation, streak to L-EMB and MacConkey agars. Incubate these agars for 20 h at 35°C.

F. Selection

Typical lactose-fermenting colonies on L-EMB agar appear dark centered and flat, with or without metallic sheen. Typical colonies on MacConkey agar appear brick red. Lactose non-fermenting biotypes on both agars produce colorless or slightly pink colonies.

NOTE: EIEC do not ferment lactose and there may also be atypical non-lactose fermenting strains in the other pathogenic *E. coli* groups; therefore, as many as 20 colonies (10 typical and 10 atypical) should be picked for further characterization.

G. Conventional Biochemical Screening and Identification (8, 30)

Use the procedures described in Chapter 4 for biochemical and morphological identification of *E. coli*. However, because many enteric bacteria can also grow in the TP enrichment broth, plus anaerogenic, non-motile and slow or lactose non-fermenting strains of *E. coli* must also be considered, additional tests may need to be performed. Some of these new or modified reactions are discussed here.

1. **Primary screening**. Transfer suspicious colonies to TSI agar, BAB slant, tryptone broth, arabinose broth, and urea broth. Incubate 20 h at 35°C. Reject H_2S-positive, urease-positive, arabinose non-fermenting, and indole-negative strains. To test for the ONPG reaction, suspend growth from TSI in 0.85% saline to give detectable turbidity. Add an ONPG-impregnated disk and incubate 6 h at 35°C. Yellow color indicates positive

reaction. Reject ONPG-negative, aerogenic cultures. Some Alkalescens-Dispar strains (i.e., anaerogenic *Escherichia*) are ONPG-negative.

2. **Secondary screening** (48 h incubation at 35°C unless otherwise specified). To identify cultures, test additional reactions shown in Table 1, Chapter 4, to subdivide *Escherichia* spp. Since it is not known whether these additional species are of pathogenic significance to humans, strains giving typical reactions for *E. coli* should be further investigated. To differentiate *E. coli* from *Shigella*, examine anaerogenic, non-motile, slow lactose fermenters for lysine decarboxylase, mucate, and acetate reactions. *Shigella sonnei*, which may grow in the same enrichment conditions, is anaerogenic and non-motile. It also produces a negative indole reaction and shows slow or non-fermentation of lactose. The biochemical-physiological characteristics of *E. coli* are summarized in Table 2, chapter 4.

3. Alternatively, use API20E or the automated VITEK biochemical assay to identify the organism as *E. coli*.

H. Tests for Enterotoxigenic E. coli (ETEC)

When *E. coli* levels in foods exceed 10^4 cells/g, perform enumeration for ETEC by colony hybridization analysis using DNA probes for LT and ST (Chapter 24). If biological activity assays are necessary, LT can be detected by the Y-l tissue culture test (28) and ST can be detected by the infant mouse test (28) (For detailed procedures of these assays, see Chapter 4, BAM, Edition 8, Revision A /1998). There are also commercially available RPLA and ELISA tests to detect LT and ST toxins as well as PCR assays (41).

I. Tests for Enteroinvasive *E. coli* (EIEC)

If an isolate is suspected to be EIEC, the invasive potential of the isolates may be tested by the Sereny test or the Guinea pig keratoconjunctivitis assay (28) (For detailed procedures on Sereny test, see Chapter 4, BAM, Edition 8, Revision A /1998). Invasive potential of the isolates can also be determined by the HeLa tissue culture cell assay as described (25), or with the *in vitro* staining technique using acridine orange to stain intracellular bacteria in HeLa monolayers (26). Alternatively, since the *invA* gene sequence of EIEC closely resembles that of *Shigella*, DNA probe and PCR assays for *inv* gene of *Shigella* will also work for EIEC.

Caution: Since both EIEC and *Shigella* will give positive probe and PCR reactions, it is critical that the organisms are identified first as *E. coli*.

J. Tests for Enteropathogenic *E. coli* (EPEC)

EPEC strains are identified based on 3 key traits: attachment and effacing lesion (A/E), localized adherence on cells and the lack of Shiga toxin (*Stx*) production. This last trait is also used to distinguished strains of EPEC from EHEC. Phenotypically, A/E and localized adherence are tested using Hep-2 or HeLa tissue cells. Absence of Stx can be determined using tests outlined for EHEC (see below). There are also PCR and probes for the EAF plasmid that encodes for localized adherence and the *eae* gene that encodes for the intimin that causes the A/E phenotype.

Caution: There are several variants of *eae* gene and some EPEC strains carry *eae* variants identical to EHEC serotypes; hence, these tests will detect strains from both pathogenic groups. For specific virulence assays for EPEC, see Nataro and Kaper, 1998 (28) for detailed procedures..

K. Screening method for *E. coli* STEC and Serotype O157:H7 from Foods

In the previous eBAM, food samples may be screened for O157:H7 using either the SmartCycler II or LightCycler® 2.0 platforms (16, 17, 42). These methods used modified Buffered Peptone Water with pyruvate (mBPWp), which contained several anti-microbial reagents that effectively suppressed normal flora growth and non-target competitors, yet allowed the growth of viable O157:H7 cells (including other STEC) and is capable of detecting <1 cfu/g in foods. Improved recovery of pathogenic *E. coli* from difficult matrices such as salad mixes and sprouts was achieved with the mBPWp enrichment (32, 38).

The real-time PCR assay configured for the SmartCycler II platform (16, 17, 42), is specific for the *stx1* and *stx2* genes and the +93 single nucleotide polymorphism in the *uidA* gene that encode for the β-D-glucuronidase (GUD) enzyme (11). The +93 SNP is highly conserved in O157:H7 and O157:H- strains that produce *Stx* and is an accurate identification marker for O157:H7 strains (9). The *stx1* and *stx2* markers on the real-time PCR assays also enabled the detection of other STEC strains, some of which are known human pathogens (see Section R).

Since these assays had been implemented, some have noted that occasionally, the +93 *uidA* target does not come up as positive or are very late positives (high Ct values), especially in samples that contained high generic *E. coli* levels. Generic *E. coli* do not have the +93 *uidA* SNP, so will not be detected by the +93 *uidA* probe, but it does have the *uidA* gene, and so will be amplified by the PCR primers. We speculate that high levels of generic *E. coli* in the sample may be depleting the reagents and thereby affecting the outcomes of some of the delicate targets, like the *uidA* SNP. To rectify this problem, primers specific for the *wzy* gene of the O157 antigen was used as an alternative to the +93 *uidA* SNP for testing for O157:H7. The O157 wzy primers were combined with the same *stx*1 and *stx*2 primers and the assay was also formatted to use the AB7500 platform, which has high-throughput capacity. Although the O157 *wzy* primers will detect all O157 serogroup strains, including non-H7 strains that are not STEC or pathogenic, many of these strains may be suppressed by the selective enrichment medium and therefore, not impact the outcome of the PCR assay. The premise of detecting both *stx* and O157 markers in a sample is a fairly good indication that O157:H7 strains may be present. The AB7500 assay was validated for various foods by the FERN labs.

With the approval of the BAM Council and the FDA MMVA, the LightCycler method in Chap. 4a was archived and replaced with the ABI7500 assay, which also has the capability for high-throughput analytical testing.

It should be cautioned, that when using multiplex PCR assays to test mixed culture samples, such as food or environmental enrichment broths, it is not unusual to see instances where the various targets detected are not from a single strain, but from different isolates. Hence, is critical that PCR positive samples are confirmed to show that all targets (*stx* and O157) are within the same isolate. Confirmation involves plating PCR-positive samples onto differential agars to isolate the strain and test it by biochemical, serological and genetic assays. If PCR results are

positive for *stx* only, follow the procedure in Section R. for STEC isolation. It is advisable that isolates are tested again by PCR for *stx*1 and *stx*2 genes to confirm their toxigenic potential.

NOTE: If users do not have access to real-time PCR equipment, follow procedure in Section P for cultural enrichment and isolation.

L. Equipment and materials

1. Balance, 1-500 g with 0.1 g sensitivity
2. Sterile glass jars for enrichment
3. Optional: Stomacher with appropriate plastic bags for 250 ml vol.
4. Large resealable plastic bags to accommodate 200 g of product (for analysis of leafy produce)
5. Incubators: 36°C ± 1°C and 42°C ± 1°C
6. Petri dishes 20 × 150 mm
7. Microcentrifuge tubes (0.5 to 2.0 mL)
8. Conical centrifuge tubes (50.0 mL)
9. Micropipettors (e.g., 0.5-20 µL, 20-200 µL, 200-1000 µL)
10. Microcentrifuge (capable of spinning at 15000 × g)
11. Pipets (1 to 10 ml volume)
12. Pipet tips (0.2 to 1000 µl volume) (aerosol resistant tips)
13. Vortex Mixer
14. Filter Paper
15. Waterbath or heat block capable of maintaining 100°C.
16. SmartCycler II PCR thermolcycler (Cepheid, Sunnyvale, CA) capable of performing cycling parameters described below and simultaneous real-time sequence detection for FAM, TET, Texas Red and Cy5 dyes.
17. SmartCycler PCR reaction tubes (minimum reaction volume of 25 µl) and racks compatible with PCR thermolcycler
18. Applied Biosystems 7500 Fast Real-time PCR thermocycler (SDS version 1.4) (Applied Biosystems, Thermo-Fisher Scientific Brand)
19. AB 7500 Fast Plates (AB# 4346906)
20. Optical Adhesive Film for 7500 Fast Plates (AB# 4311971)
21. 8-well strips; alternative tp plates (AB# 4311971)
22. Appropriate ABI 7500 Fast Plate Holder (specific for 96-well tray or 8-strip well tubes)
23. Ice bucket and ice
24. Sterile tongs
25. Gloves

M. Media and Reagents

1. Modified Buffered Peptone water with pyruvate (mBPWp) and Acriflavin-Cefsulodin-Vancomycin (ACV) Supplement (M192a)

2. STEC/O157 primers and probes listed in Tables 2 and 3 are specific to real-time PCR platform being used.

    a. Primers - 10 µM working solution of each primer listed in Table 2 or 3. Stock and Working solutions can be prepared from commercially synthesized primers with

basic desalt 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 µM working solution of each probe listed in Tables 2 or 3. Dual Hybridization Probes should be purchased as RP HPLC-purified and labeled as indicated in Tables 2 and 3. Stock and working solutions can be prepared from commercially synthesized probes 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. Exogenous Internal Amplification Control (IAC)

Smart Cycler Platform: (IAC) Full length sequence as described in U.S. Patent Application 0060166232: cgcatgtggt cacagccctg acgaagctgt catcaagttc ataatgacat cgatatgggt gccgttcgag cagtttagcc ctaaatcacc ctaccggcag acgtatgtca cattcaccag ggagacgcat gagattggat gctgttgtgc gccctcaaca atgtaacgaa tggctgcatc gtctcgcgaa tattgtcgta ccatcatctg acttggctca tgtctgcaag aggcttcgca ctgggctttatg

Prepare a stock solution of the IAC DNA sequence from commercially synthesized product and titer to a stock solution where a known amount will reliably provide a Ct between 25-35 PCR cycles.

NOTE: Alternatively, the primers, probes and IAC internal control assay components are also available as a lyophilized bead product for use on the SmartCycler II orIAC full length template (BioGX, Birmingham, AL).

Table 2. Primer/probe sequences for use on SmartCycler II platform.

Primers[1]	**GenBank #**	**Bases**	**5' → 3' Sequence**
Stx1F934		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

Probes[1]	**GenBank #**	**Bases**	**5' → 3' Sequence**
stx1P990	M19473	31	TxRd-TgA TgA gTT TCC TTC TAT gTg TCC ggC AgA 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]		26	Cy5- TCT CAT gCg TCT CCC Tgg TgA ATg Tg-BHQ2

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

[2,3] IAC primers and probes are covered by U.S. Patent Application 0060166232 and sited in: Nordstrom, *et al.* 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 (14); and Angelo DePaola, *et al.* 2010. Bacterial and Viral Pathogens in Live Oysters: 2007 United States Market Survey. *Appl. Environ. Microbiol*. **76**:2754-2768 (15).

AB 7500 Fast Platform: TaqMan® Exogenous Internal Positive Control (Applied Biosystems, Carlsbad, CA. #4308323)

Table 3. Primer and probe sequences for use on the AB 7500 Fast 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
wzyF1831	AF061251	24	CTC gAT AAA TTg CgC ATT CTA TTC
wzyR1936	AF061251	23	CAA TAC ggA gAg AAA Agg ACC AA

Probes[1]	GenBank #	Bases	5' → 3' Sequence
stx1P990	M19473	31	Cy5-TgA TgA gTT TCC TTC TAT gTg TCC ggC AgA T-BHQ2
stx2P1249	X07865	25	TAMRA-TCT gTT AAT gCA ATg gCg gCg gAT T-BHQ2
wzyP1881	AF305917	25	6FAM - ACT TAg Tgg CTg ggA ATg CAT Cgg C – BHQ1

[1] Primer/Probe name composed of target gene (*stx*1, *stx*2 or *uid*A), forward primer (F), reverse primer (R) or probe (610LC, 670LC, 705LC, or FL P), 5' base position of oligonucleotide in the respective gene sequence specified in column 2.

3. Additional Real-time PCR additional reagents depending on real-time platform being used:

    a. SmartCycler II Platform - OmniMix-HS or SmartMix HM PCR Reagent Beads (Cepheid, Sunnyvale, CA. Also available through Fisher).

    b. AB 7500 Fast Platform - Express qPCR Supermix Universal Taq (Invitrogen, Carlsbad, CA. #11785200)

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

5. Chromogenic selective agar:
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 (contains 10 mg/L novobiocin plus 0.8 mg/L potassium tellurite).

6. Levine's Eosin-Methylene Blue (L-EMB) agar (M80) (Section R only)

7. Trypticase Soy Agar with Yeast Extract (TSAYE) (M153).

8. Butterfield's Phosphate Buffer(R11) (pH 7.2 ± 0.2. Sterilize by autoclaving)

9. Sterile distilled water, molecular grade water or equivalent

10. Physiological saline (0.85% NaCl)

11. Kovac's Reagent (R38)

12. ColiComplete Discs - fluorogenic MUG substrate for GUD and chromogenic X-gal for GAL (BioControl, Bellevue, WA)

13. Anti-O157 and anti-H7 latex reagent (ThermoScientific Remel 4, Lenexa, KS, or equivalent)

14. API20E or VITEK GNI (BioMerieux, St. Louis, MO).

15. STEC heart infusion washed blood agar with Mitomycin-C (SHIBAM) (M195)

N. Sample Preparation and Enrichment Procedure

1. Sample Preparation

    a. Leafy Produce (except cilantro and parsley) – to 200g of sample, add 450 ml of 1× mBPWp. Do not blend or stomach.

    b. Cilantro and parsley – to 25g of sample, add 225 ml of 1× mBPWp. Do not blend or stomach.

    c. Juice, milk or other turbid beverage samples – aseptically centrifuge 200 ml of sample at 10,000 × g for 10 min. After decanting the supernatant, resuspend the pellet material in 225 ml of mBPWp.

    d. Bottled water or other non-turbid liquids – weigh 125 ml into 125 ml of 2× mBPWp. Also, use this approach with liquids in which a visible pellet would not be produced after centrifugation.

    e. All other foods – weigh 25 g of food into 225 ml of mBPWp. Blend or stomach briefly as necessary.

2. Enrichment
   Incubate homogenate at 37°C ± 1°C static for 5 hours, then add 1ml each of the ACV supplements per 225 ml of mBPWp (see Section M2) and incubate at 42°C ± 1 °C static overnight (18-24 h).

Enrichment Control Strains - 465-97 USDA (*stx*1-, *stx*2- *uid*A+). Alternatively, use ATCC43890 (*stx*1+, *stx*2-, *uid*A+), ATCC 43888 (*stx*1-, *stx*2- *uid*A+) 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.

OPTIONAL: When O157:H7 contamination is suspected, particularly in foods with high levels of competing microbial flora, such as sprouts or raw meats, the use of immunomagnetic separation (IMS) prior to screening can be helpful. Several IMS procedures are available, including Dynabeads® anti-*E.coli* O157 (ThermoFisher Scientific, Waltham, MA) and Pathatrix Immunocapture system *E.coli* O157 kit (ThermoFisher Scientific, Waltham, MA) and some of these have been tested on selected foods. Perform IMS on the 5 hr or overnight enrichment

(depending on the IMS system used). From the final IMS bead suspension (approximately 100 µl), plate and assay by real-time PCR as described below in section O. For specific details on performing IMS on enrichment samples, contact: Karen Jinneman, FDA-PRLNW (karen.jinneman@fda.hhs.gov)

O. Real-time PCR Screening

Real-time PCR assembly and data analysis protocols are described below for two instrument platforms; SmartCycler II and AB 7500 Fast. Contact: Karen Jinneman, FDA-PRLNW (karen.jinneman@fda.hhs.gov)

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 broths, refer to section P for cultural analysis of all enrichments.

1. DNA Template Preparation:

    a. Transfer 1ml of overnight enrichment to a microcentrifuge tube and centrifuge 12,000 × g for 3 min.

    b. Remove supernatant and completely resuspend pellet in 1 ml 0.85% NaCl.

    c. Centrifuge 12,000 × g for 3 min.

    d. Remove supernatant and completely resuspend pellet in 1 ml sterile water.

    e. Place in waterbath or heat block capable of maintaining 100°C for 10 min.

    f. Centrifuge 12,000 × g for 1 minute, remove and save supernatant as DNA template (This may be frozen, minimum -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 minimum -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 (*stx*1+, *stx*2+ and *uid*A+).

    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. **Smart Cycler II** - Reaction assembly and data analysis protocol:

a. Prepare a PCR Master Mix from the reaction components and final concentrations for STEC/O157 listed in Table 4. Keep all thawed reagents and reactions on ice. Alternatively a PCR Master Mix may be prepared by following package insert for STEC/O157 CSR bead and OmmiMix HS or SmartMix HM bead.

b. Add 24 µl of Master Mix to each SmartCycler tube and cap loosely.

c. Add 1 µl of sample or control template and snap cap tightly.

d. Briefly centrifuge to bring all liquid to bottom of tube and place in thermalcycler.

e. 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 4. Smart Cycler II Protocol Amplification Reaction Components[1]

Volume/rxn	Component	Final Concentration
qsi to 25 µl [2]	Sterile Distilled Water	
0.625 µl	Primer stx1F934 (10 µM Work Solution)	0.25 µM
0.625 µl	Primer stx1R1042 (10 µM Work Solution)	0.25 µM
0.625 µl	Primer stx2F1218 (10 µM Work Solution)	0.25 µM
0.375 µl	Primer IAC55F (10 µM Work Solution)	0.15 µM
0.625 µl	Primer stx2R1300 (10 µM Work Solution)	0.25 µM
0.625 µl	Primer uidAF241 (10 µM Work Solution)	0.25 µM
0.625 µl	Primer uidAR383 (10 µM Work Solution)	0.25 µM
0.375 µl	Primer IAC186R (10 µM Work Solution)	0.15 µM
0.375µl	Probe stx1PTxRd990 (10 µM Work Solution)	0.15 µM
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 IC DNA primers and probes are lyophilized in the STEC/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 control nucleic acid molecule as described in U.S. Patent Application 0060166232. The amount of IAC template needs to be adjusted based on the prepared stock concentration to report Cycle threshold at about 25-35 PCR cycles when no inhibition is present in the reaction.

Qualitative data analysis

On the SmartCycler II Instrument, set the following Analysis Settings for FAM,

TET, TxRd and Cy5 channels (32). Update analysis settings if they are changed before recording results.

NOTE: Internal Control (IC) cycle threshold (Ct) is CSR Lot specific (Ct = 25-35). If Ct is consistently not present for the IC (Cy5, channel 4) target in the negative control tube only at 15 fsu, contact method authors. Lot specific recommendations for Cy5 manual threshold setting may be issued.

Usage: Assay
Curve Analysis: Primary
Threshold Setting: Manual
Manual Threshold Fluorescence Units: 15.0
Auto Min Cycle: 5
Auto Max Cycle: 10
Valid Min Cycle: 3
Valid Max. Cycle: 60
Background subtraction: ON
Boxcar Avg. Cycles: 0
Background Min. Cycle: 5
Background Max. Cycle: 40

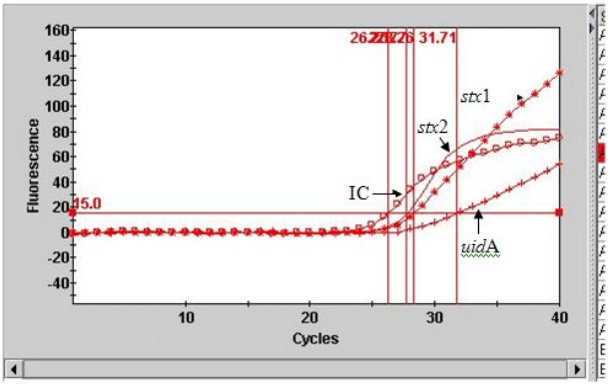
Primary fluorescence curves that cross the threshold will be recorded as "POS" and the cycle number when it crossed the threshold will be displayed in the Results Table view (Figure 1A). Negative results are shown as "NEG". The FAM, TET, TxRd and Cy5 channels correlate to *stx*2, +93 *uid*A, *stx*1 and IC 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 Smart Cycler 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



4. **AB 7500 Fast (SDS ver.1.4)**

I. Reaction Assembly:

a. Prepare a Master Mix containing all of the components and final concentrations for STEC O157 listed in Table 5 (below).
b. Aliquot 28 µl or Master Mix to each reaction tube.
c. Add 2µl sample or control template for a final volume to 30 µl
d. Cap tubes or seal plate carefully to avoid we-to-well contact.
e. Briefly centrifuge 8-strip tubes or 96-well plates in appropriate instrument to bring contents to bottom of tube.
f. Place tubes into AB 7500 Fast Instrument. NOTE: Ensure appropriate tube is used for 96-well plate or 8-strip well.

Table 5. AB 7500 Fast E. coli O157/STEC Reaction Components

Volume/rxn	Component	Final Concentration
0.75 µl	Primer stx1F934 (10 µM Work Solution)	0.25 µM
0.75 µl	Primer stx1R1042 (10 µM Work Solution)	0.25 µM
0.75 µl	Primer stx2F1218 (10 µM Work Solution)	0.25 µM
0.75 µl	Primer stx2R1300 (10 µM Work Solution)	0.25 µM
0.90 µl	Primer wzyF1831 (10 µM Work Solution)	0.3 µM
0.90 µl	Primer wzyR1936 (10 µM Work Solution)	0.3 µM
0.60 µl	Probe stx1P990 Cy5 (10 µM Work Solution)	0.20 µ M
0.45 µl	Probe stx2P1249 TAMRA (10 µM Work Solution)	0.15 µM
0.45 µl	Probe wzyP1881 FAM (10 µM Work Solution)	0.15 µM
3.00 µl	Internal Positive Control Primer/Probe Mix[1]	
0.60 µl	IPC DNA[1]	
15 µl	Express qPCR Supermix Universal[2]	
0.06 µl	ROX dye[2]	
3.04 µl	Molecular Grade Water	
2.0 µl	Template (Sample or control)	
30 µl	Total Reaction Volume	

[1] Included in TaqMan® Exogenous Internal Positive Control Kit (Applied Biosystems, Carlsbad, CA. #4308323).
[2] Included in Express qPCR Supermix Universal Taq (Invitrogen, Calrsbad, CA. #11785200).

g. Create new document on AB 7500 Fast (SDS ver. 1.4).

i. Give each run a unique name

ii. Define run parameters including
Assay: Standard Curve (Absolute Quantitation)
Container: 96-Well Clear
Run Mode: Fast 7500

iii. From the select detectors window highlight and add appropriate detectors for the *E. coli* assay as follows. Note assigning color assists in quick visual interpretation of results. (If detectors have not already been created, make it use the "New Detector" option in the new document wizard on the AB7500 instrument.)

Parameter Settings for Detectors

Target Name	Reporter	Quencher	Color
stx1	Cy5	(none)	Red
stx2	TAMRA	(none)	Blue
wzy	FAM	(none)	Green
IPC	VIC	(none)	Black

iv. Select ROX as the Passive Reference dye then "Finish" to create the document.

v. The next screens will be used to set up sample plate.

vi. In the "Plate" tab, highlight sites to be used, right-click and use well inspector to associate all four detectors with each site.

vii. Highlight individual sample sites and type in sample information.

viii. After sample information is entered, click on "Instrument tab".

ix. Input the instrument settings for the *E. coli* O157/STEC assay to the following:

Stage 1: 95.0 °C; 1 min
Stage 2: 45 cycles
    Step 1: 94°C; 10 sec
    Step 2: 63°C; 40 sec
Sample Volume: 30 µL
Run Mode: Fast 7500
Date Collection: Stage 2, Step 2, (63.0@0:40)

x. When finalized, "Start" run.

xi. When prompted, click the "Save and Continue" button.

xii. Save SDS file as per local laboratory protocol. Run time is approximately 1 h.

Qualitative data analysis and viewing results on the AB 7500 fast

a. From the "Results" tab, select "Analysis". then from the dropdown menu then "Analysis Settings"

b. Set "Analysis Settings" for all detectors to:

Manual Ct Threshold of **0.05**

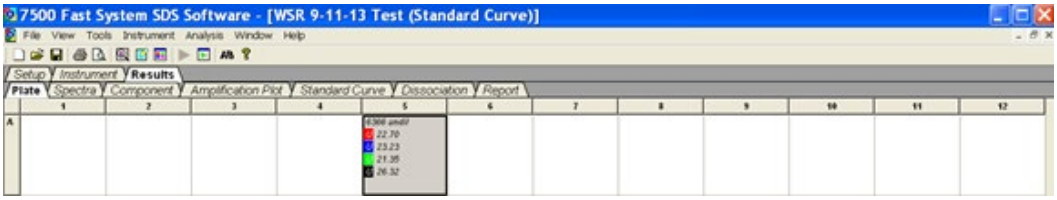
Manual Baseline is set to cycles **3** and **15** and click the "OK" button.

c. "Analyze" the run from the "Analyze" option on the "Analysis" dropdown menu.

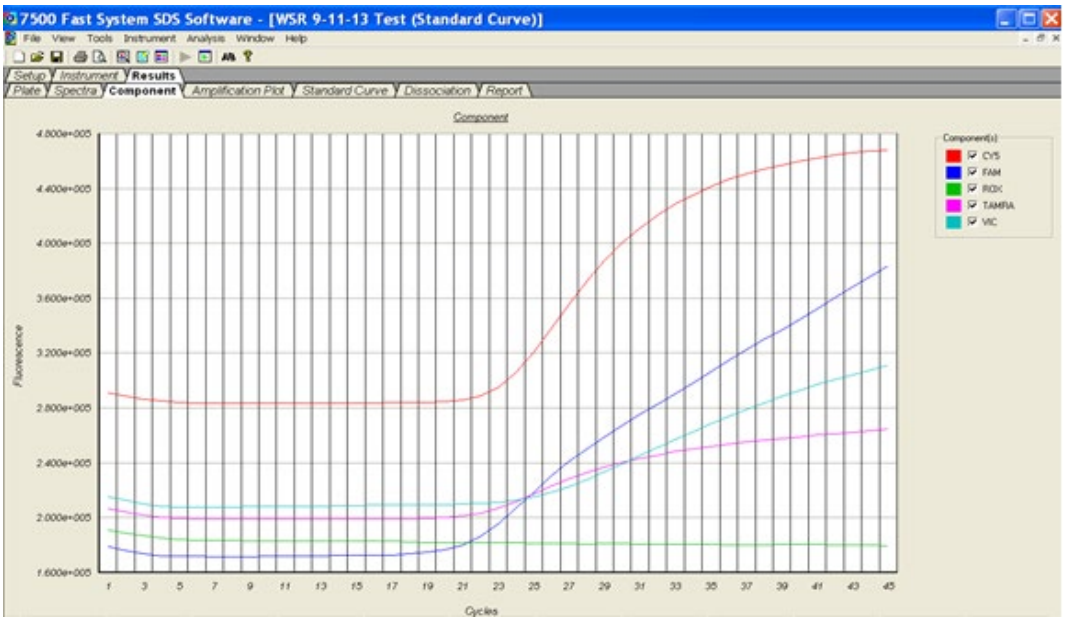
d. View the Ct results on the Results Plate, by selecting "Display" and then "Ct" from the "Analysis" dropdown menus. Image3 "Report" tab view.

Figure 1. Example of result output from AB7500 Fast. a. "Plate tab V=view, b. "Component" tab view, c. "Amplification Plot" view, and d. "Report" view.

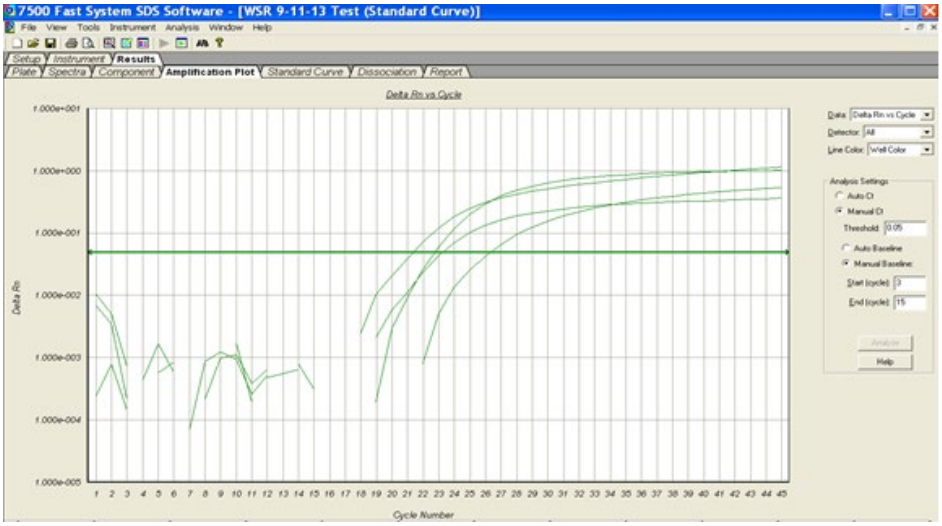
"Plate" tab view.



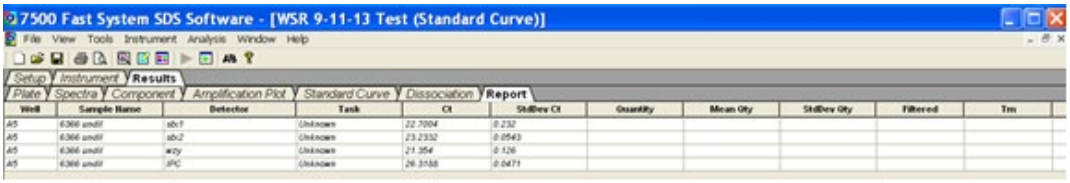
"Component tab" view.



"Amplification Plot" view



"Report" view.



Interpretation of real-time PCR Results (Smart Cycler II and AB17500)

Ensure that for each run the PCR positive control is positive for all gene specific targets; IC may be positive or negative for a valid result and PCR negative control is negative for all specific gene targets and positive for IC component. Interpret samples as follows:

Negative samples:
All three targets – *stx1*, *stx*2, and +93 *uid*A SNP or O157 (*wzy*) depending on instrument platform used are "negative" (no Ct value) and the internal control (IC) is positive, indicating that the reactions worked correctly. Sample is negative, no further analysis is needed.

Probable positive O157 samples:
NOTE: With the SmartCycler assay, if the +93 *uid*A SNP or O157 target (*wzy*) is positive by itself or in combination with positive *stx*1, *stx*2 or both. Go to sec. P for culture isolation and confirmation of E. coli O157: H7. Be aware that there are O157 non-H7 strains that will also be positive with the O157 target (*wzy*), but these will be negative with the +93 *uid*A SNP.

NOTE: With the SmarCycler assay, if the +93 *uidA* SNP target (*wzy*) is negative but either or both *stx*1 and *stx*2 are positive, it is possible that the +93 *uid*A SNP target was not effectively amplified above background, perhaps due to high generic *E. coli* levels in the sample. Continue with section P for isolation of *E. coli* O157:H7 in parallel with isolation of non-O157 STEC as described in section R.

Probable positive STEC samples:
If the +93 *uid*A SNP target or the O157 target (*wzy*) are is negative but *stx*1 and *stx*2 are positive, the sample may contain a non-O157 STEC. See sec. R, for additional information and isolation procedure.

NOTE: It is possible to have an IC negative when one or more gene targets are positive, as the amplification of the target genes may out-compete the IC for available reagents. In those cases, the analysis is still valid as long as the amplification of the target gene has crossed the threshold to indicate successful PCR. Continue to section P or R for isolation of O157:H7 or STEC, respectively. However, if all the target genes are negative as well as the spiked food control or IC, it invalidates the PCR analysis. Troubleshoot the reaction and rerun the assay or streak enrichments as decribed in section P.

The Screening method for *E. coli* Serotype O157:H7/STEC from Foods on the Light Cycler 2.0 Instrument Platform is available here. (**NOTE: Archived Content**)

P. O157:H7 – Cultural Isolation and Presumptive Isolate Screening

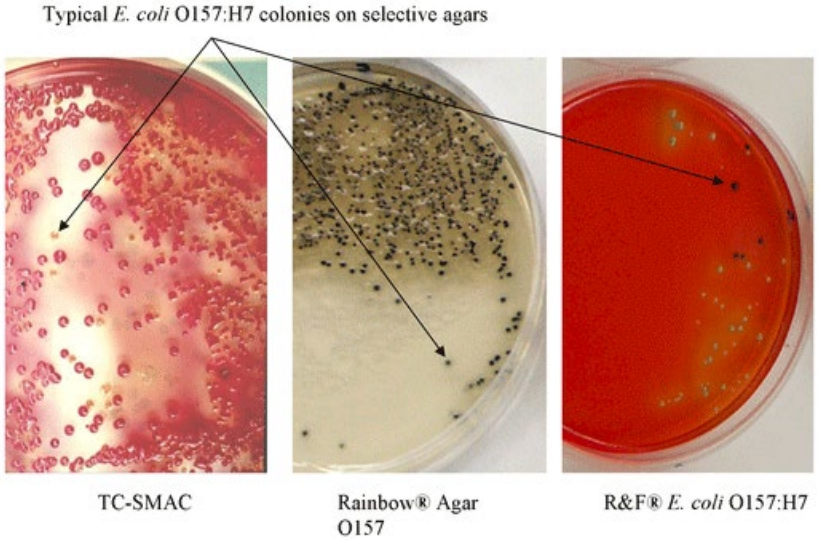
For overnight enrichment samples that are found probable positive by the real-time PCR assay, cultural confirmation is required. Similarly, for samples that have not been screened by real-time PCR follow these procedures for culture isolation.

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°C ± 1°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 3. Appearance of typical *E. coli* O157:H7 on TC-SMAC, Rainbow® Agar O157 and R&F® *E. coli* O157:H7 agars.

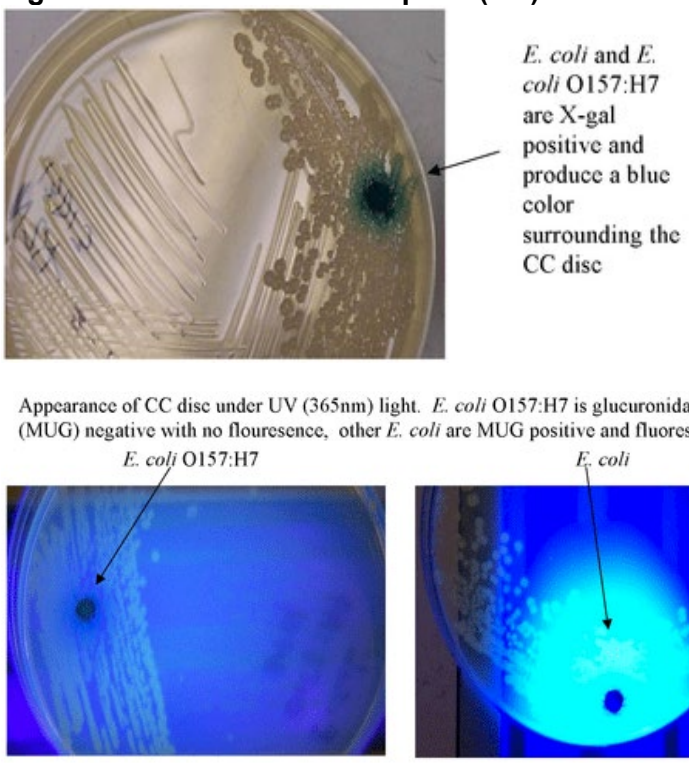
    c. Screen typical colonies by picking a portion of each isolated suspect colony from the isolation agar and testing for O157 antigen by latex agglutination (Remel kit).

    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 ±

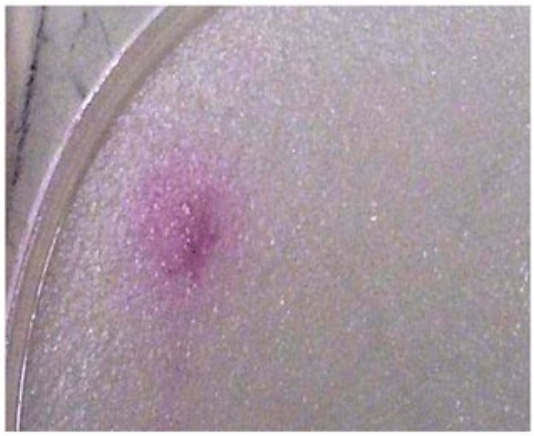
1°C. CC has a chromogenic assay for galactopyranosidase (X-gal) and a fluorogenic assay for glucuronidase (MUG) on the same disc. The positive control should show blue color on and around the disc (indicative for coliforms) and blue fluorescence around the disc under long wave UV (365 nm) light (indicative of *E. coli*). Strains of O157:H7 are X-gal (+) but MUG (-).

Figure 4. 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 5. Indole positive result of typical *E. coli* O157:H7.



Q. Isolate Confirmation Tests

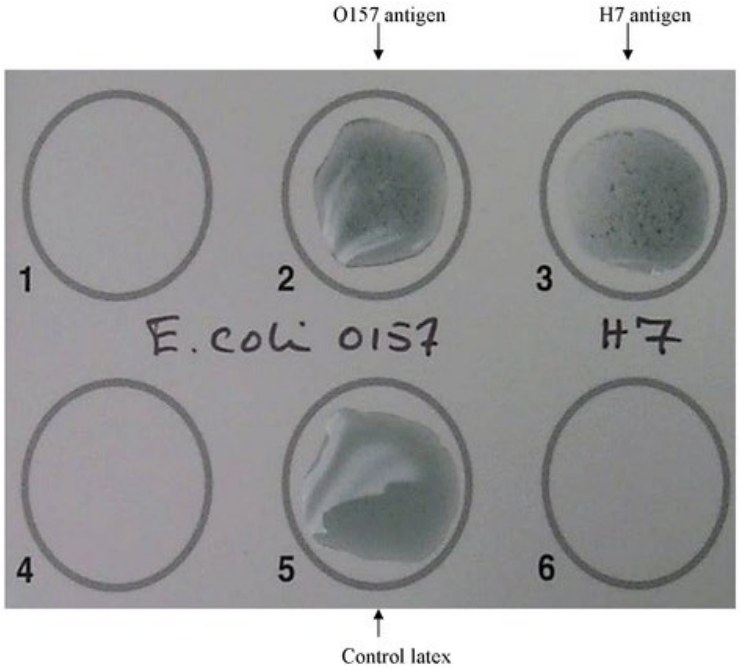
1. For typical colonies shown to be X-gal positive, MUG negative and indole positive, perform the following confirmation tests from the isolated colony on TSAYE.

    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-Scientific, Remel, Lenexa, KS, 800-255-6730) or equivalent, gives satisfactory results.

    NOTE: If the isolate is O157 and H7 positive, it is evidence 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 1st with the O157 reagent as other non-O157 *E. coli* serotypes can also carry the H7 antigen.

    Figure 6. 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 (Smart Cycler II or AB17500) used in screening the enrichment.

   NOTE: An O157:H7 and O157:NM isolate that carry stx are considered pathogenic. However, an O157:NM strain that does not carry stx or other EHEC virulence factors is probably non-pathogenic. There are many E. coli O157 serotypes that carry other than H7 antigen (ie: H3, H12, H16, H38, H45, etc), and these often do not carry EHEC virulence factors. But, NM variants of these have been isolated (10).

d. Further characterize the isolate by Pulsed Field Gel Electrophoresis (PFGE) using standardized PulseNet PFGE protocols (1) and perform whole genome sequencing (WGS) on all confirmed isolates using standardized GenomeTrakr methods and submit data to GenomeTrakr.

   Archived Method: PCR for Confirmation of *E. coli* Serotype O157:H7 is available here. (PDF, 195Kb).

R. Screening method for non-O157 STEC

Both the *stx1* and *stx2* genes and allelic forms of these genes are carried by ~ 300 STEC serotypes, but many of these have not been implicated in illness and may be found in the intestinal flora of healthy humans. Be aware that the real-time PCR assay, described in section O, will detect these STEC strains as well. So, a +93 *uidA* (-) but *stx1* and/or *stx2* (+) real-time PCR result is only indicative that the sample possibly contains an STEC, but should not be interpreted that it is a pathogenic STEC.

As a group, EHEC, are a subset of STEC and comprised of pathogenic strains, of which O157:H7 is the prototypic strain (seropathotype A) (20) . There are several well known EHEC strains that have caused illness worldwide, ie: O26, O111, O121, O103, O145, O45, etc (seropathotype B). In the past, FDA regulatory position has focused only on O157:H7, however, the presence of any pathogenic STEC in products regulated by the agency are of concern, so it is essential to isolate the STEC for additional testing to discern EHEC from STEC strains that have not been implicated in illness and may not be pathogenic. The following section describes isolation and confirmation procedures for non-O157 STEC and provides follow up guidance when STEC are detected.

NOTE: The enrichment procedure and real-time PCR screening assay described in sections N and O have also been validated for detection and recovery of other non-O157 STEC as well. Refer to sections K-O for necessary equipment, media and reagents, sample preparation, enrichment and real-time PCR screening procedures.

1. Isolation procedure.

   For overnight enrichment samples that are found probable positive for STEC (Positive for one or both of the *stx* gene targets) by the real-time PCR assay, cultural confirmation is required. Similarly, for samples that have not been screened by real-time PCR due to lack of instrumentation, follow these procedures for culture isolation.

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 L-EMB agar (M80) and a chromogenic agar as described in section P. Optionally, a streak plate may also be included.

   An additional agar, STEC heart infusion washed blood agar with Mitomycin-C (SHIBAM) (M195), could also be included (Lin et al., 2012). SHIBAM combines modifications to Beutin's Washed Sheep's Blood Agar from Sugiyama et al. (2001), (addition of Mitomycin C) and Kimura et al. (1999), (optimal washed blood and base agar) to better isolate shiga toxin producing *E. coli*. Most STECs produce enterohemolysin and appear hemolytic on SHIBAM plates and are easily distinguishable from background microbiota (Fig. 9). Be aware that some *E. coli* produce α-hemolysin, which is distinct from the STEC enterohemolysin. On blood agar plates, colonies of *E. coli* strains that produce α-hemolysin show a large clear zone of hemolysis (Fig. 10, right), which is distinct from that of enterohemolysin (Fig. 9). SHIBAM may be prepared (M195) or purchased from Hardy Diagnostics (Catalog #A146).

b. Incubate plates at 37°C ± 1°C for 18 - 24 h. On L-EMB, typical *E. coli* colonies appear as dark centered and flat, with or without a metallic sheen. On SHIBAM, STEC colonies will appear grey to off-white and flat, with or without a clear zone of hemolysis ranging from narrow to several mm. (Figure 9).

c. Pick both typical and atypical *E. coli* colonies from the chromogenic agar, L-EMB and both enterohemolytic and non-enterohemolytic colonies on SHIBAM (see **NOTE** below). Pick up to 12 colonies total (preferably two typical and two atypical colonies from the same plate of each media type) and streak onto TSAYE with CC (see Section P.1.e.). *E. coli* will be X-gal (+), but may be MUG (+) or (-). See the Table below for the typical colonial morphologies of O157:H7 and STEC on various types of plating media.

   Alternatively, other instrumental methods are acceptable to rapidly screen for *E. coli* colonies if they have been validated per FDA's Microbiological Methods Validation Guidelines, AOAC's Appendix J, or ISO 16140-6:2019 if approved for use by FDA's Microbiology Methods Validation Subcommittee.

d. **NOTE**: The STEC group is very large and complex and therefore, can be difficult to isolate and identify. For example, there are atypical STEC that do not ferment lactose and will not give typical colonies on lactose based medium like L-EMB. These strains will also appear as X-gal (-) with CC. Eg: STEC O121:H19 strains have been found to exhibit slow or no lactose fermentation unless it has been induced by exposure to lactose-containing media. Similarly, studies showed that between 11 to 40% of STEC do not have the *ehxA* gene and therefore, not show enterohemolytic zone on SHIBAM. Conversely, some generic *E. coli* and some ETEC can have *ehxA*. So, when picking colonies from SHIBAM, be aware that not all enterohemolytic colonies may be STEC and conversely, some non-enterohemolytic colonies may be STEC.

   Also, be mindful that PCR screening data should be a strong indicator for the Stx profile among recovered isolates. In other words, if the rt-PCR screening assay shows good positive signals for both *stx1* and *stx2* genes but only STEC strains

that carried one of these genes is isolated, it is likely that isolates with the other STEC profile may still be present in the enrichment. In those cases, pick additional 12 colonies from the plates, including a few atypical colonies, streak to TSAYE + CC and retest to find the other STEC strains.

General appearance of *E. coli* O157:H7 and other STEC on selective and differential agars

	TC-SMAC (Sorbitol)	**Rainbow O157**	**R&F O157**	**CHROMagar O157**	**L-EMB**	**SHIBAM**
O157:H7	Neutral/gray with smokey center	Black or gray	Black to dark blue	Mauve	Dark purple center with or without green sheen	White with hemolytic zone
Non O157 STEC	Fuschia pink to tan	Many shades from blue to purple	Green to dark green with brownish center	Blue to blue green	Dark purple center with or without green sheen	White with or without hemolytic zone

e. Test X-gal positive isolates with the Spot Indole Test (Section P.1.f.).

Figure 8. Typical apperance of *E. coli* colonies on L-EMB agar

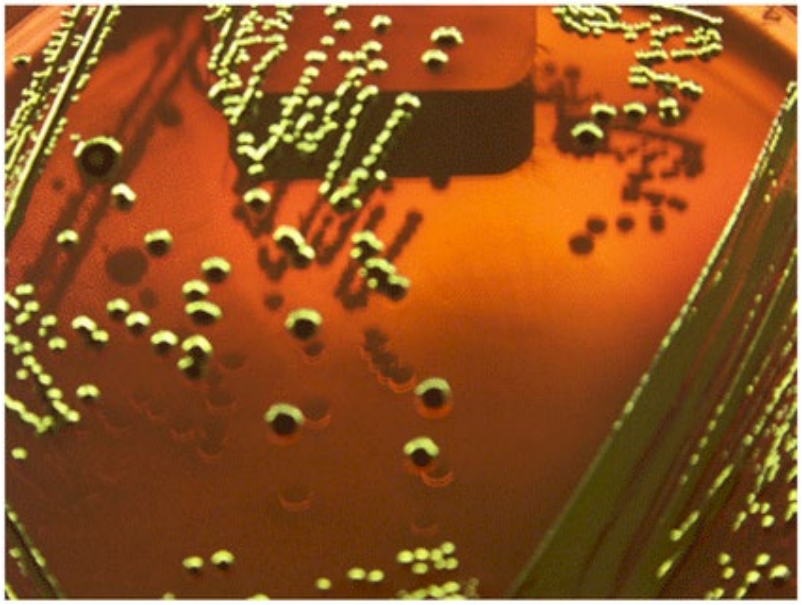


Figure 9. Typical appearance of hemolytic STEC on SHIBAM among background microbial flora from cilantro. Photo taken illuminated on light box to highlight hemolysis.

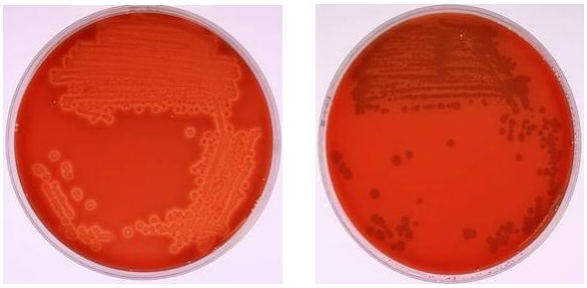


Figure 10. Typical appearance of a hemolytic O45 STEC strain (left) and a non-pathogenic *E. coli* (ATCC 8739) (right) on SHIBAM. Photos taken illuminated on light box to highlight hemolysis. Please note: ATCC 25922 is hemolytic on SHIBAM (most likely due to α-hemolysin)

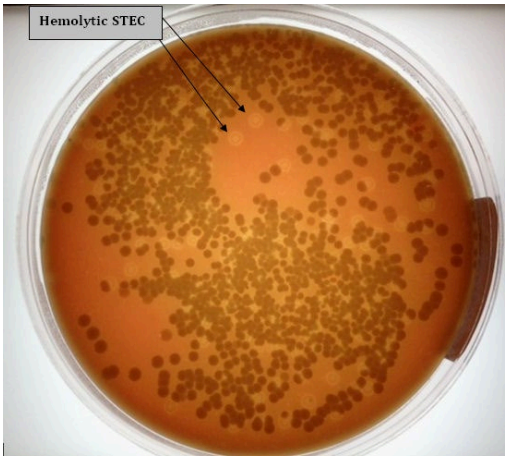
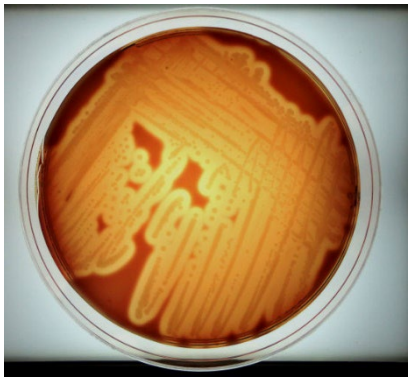


Figure 11. Example of hemolysis on SHIBAM caused by an *E. coli* strain carrying the alpha-(α)hemolysin (*α-hly*) gene. Note that the zone of hemolysis is wider than that seen with strains that carry the enterohemolysin gene (Figures 9 and 10). α-hemolysin is often found in uropathogenic *E. coli* and in certain ETEC, EPEC and STEC strains.



2. Isolate Confirmation Tests

   Confirm isolates that were picked as described in section R.1.c and are X-gal (+), MUG (+) or (-) and indole (+).

   a. Identify the isolates as *E. coli* using API20E or VITEK, following manufacturer instructions.

   b. Retest the *E. coli* isolates to confirm presence of stx genes (see Q.1.c.).

   c. Characterize all STEC isolate by Pulsed Field Gel Electrophoresis (PFGE) using standardized PulseNet PFGE protocols (1).

   d. Do whole genome sequencing (WGS) using standardized GenomeTrakr methods. Submit data to GenomeTrakr, additional bioinformatic programs may be used for health risk analysis.

      Note: Selected STEC serotypes have caused severe illnesses, so it is important to identify the serotype of STEC strains. Adherence factors are required for STEC to cause severe illness and therefore, essential to test for adherence genes to better assess potential for severe risks. All these data can be obtained from bioinformatic analysis of WGS data. Other options: The 13-Plex assay (Luminex-based Suspension Array to Identify STEC O serogroups O26, O45, O91, O103, O104, O111, O113, O121, O128, O145, O157, eae, and aggR) (PDF, 1.2 Mb) will identify 11 of the most clinically relevant STEC O serogroups and detect the presence of adherence factor genes eae and aggR. In outbreaks, where one of these 11 O types is the suspected causative agent, the 13-Plex assay maybe useful to screen large number of isolates to identify the pathogen. For information on 13-Plex, contact: Julie Kase, CFSAN. The FDA E. coli Identification (ECID) array has also been used to obtain data on serotypes and adherence genes for health risk analysis (21).

      Contact: Peter Feng, CFSAN (240-402-1650) for any questions.

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