United States Department of Agriculture
Agricultural Marketing Service, Science & Technology
Microbiological Data Program

SOP No: MDP-MTH-11		Page 1 of 16
Title: Real-time PCR Detection of Shiga-toxin Producing *Escherichia coli* (STEC), Serotype O157 and non-O157 in Fresh Produce and Food with non-O157 Isolation and Identification		
Revision: 01	Replaces: 7/1/2010	Effective: 9/1/2011

1. Purpose

To provide a standard procedure for the detection of Shiga-toxin producing *Escherichia coli* (STEC), serotype O157 and non-O157 in commodities analyzed for the USDA, AMS, Microbiological Data Program (MDP).

2. Scope

This standard operating procedure (SOP) shall be followed by all laboratories conducting microbiological studies for MDP, including support laboratories conducting non-routine activities. This SOP represents minimum MDP requirements and is presented as a general guideline. Each laboratory shall have written procedures that provide specific details concerning how the procedure has been implemented in that laboratory.

3. Principle

STECs are detected by a multiplex real-time PCR assay specific for the *stx*-1, *stx*-2 genes and the +93 *uidA* mutation unique to *E. coli* O157:H7. This assay screens for the presence of STECs, including *E. coli* O157:H7 in foods using the Cepheid Smart Cycler II platform.

4. Safety

E. coli O157:H7 is a human pathogen with a low infectious dose. Laboratory personnel should utilize Biosafety Level II (BSL-2) practices for microbiological manipulations of known and potential pathogens. A BSL-2 laminar flow biosafety cabinet is recommended for activities with potential for producing aerosols of pathogens. Material Safety Data Sheets (MSDS) should be obtained from manufacturers for media, chemicals and reagents used in the analysis and personnel who will handle the materials should know the location of and have ready access to the MSDS sheets for reference.

5. Outline of Procedures

6. References

- SOP No: FERN-MIC.0003.00E, Procedures for the Detection of Shiga-toxin *Escherichia coli* (STEC), serotype O157 and non-O157 in Food – Detection by Real-time PCR using Cepheid Smart Cycler II. Effective: 08-13-07
- BAM Online, Chapter 4a: Diarrheagenic *Escherichia coli.* Last updated: 08/2009. http://www.fda.gov/Food/ScienceResearch/LaboratoryMethods/BacteriologicalAnalyticalManualBAM/UCM070080 (last accessed 06/2010)
- BAM Online, Media Preparation, m192A
- USDA Microbiological Data Program (MDP) 2010 Multi-Laboratory Method Verification Study: Realtime PCR assays for detecting shiga toxin DNA sequences of *E. coli* (STEC) O157:H7 and/or non-O157 serotypes. May 2010.
- Biosafety in Microbiological and Biomedical Laboratories (BMBL), 5th Edition. http://www.cdc.gov/od/ohs/biosfty/bmbl5/bmbl5toc.htm. U.S. Department of Health and Human Services, Public Health Service, Centers for Disease Control and Prevention and National Institutes of Health:
- SOP MDP-LABOP-02, Sample Receipt, Elution, Pre-enrichment and DNA Extraction
- SOP MDP-DATA-01, Record Keeping and Results Reporting
- SOP MDP-QA-03, Quality Assurance (QA) Controls
- VITEK® Users Manual, bioMérieux

7. Specific Procedures

7.1 Equipment and Materials

- Smart Cycler II system, capable of performing cycling parameters described in this SOP and simultaneous real-time detection for FAM, TET, Texas Red and Cy5 fluorophores, Cepheid.
- Smart Cycler reaction tubes, 25 µl minimum capacity, Cepheid
- Smart Cycler reaction tube cooling block, Cepheid
- Smart Cycler reaction tube centrifuge
- VITEK® System or VITEK® 2 Compact System, bioMérieux
- VITEK® GNI+ Card or GN cards, bioMérieux
- Vortex mixer
- Appropriate micropipettors
- Pipet tips, sterile, filter, appropriate sizes
- Microcentrifuge tubes, 1.5 ml
- Incubators: 35 ± 2°C, 42 ± 2°C and 44 ± 2°C

7.2 Media and Reagents

- TP Broth
- Modified Buffered Peptone Water plus pyruvate (mBPWp) with ACV
- Chromagenic agar plates (Example: DRG Chromagenic agar, etc.)
- L-EMB Agar
- MacConkey Agar
- LST broth
- PCR grade water, nuclease free
- OmniMix® HS PCR master mix reagent beads, or equivalent, containing 3 units TaKaRa hot start Taq polymerase, 200 µM dNTPs, 4 mM MgCl2, and 25 mM HEPES pH 8.0 ± 0.1.
- 10 µM Working Solution of each primer and probe listed in Table 1 or use CSR bead available from BioGX. The CSR bead contains all of the primers and probes listed in Table 1. (http://www.biogx.com/BioGX_Home/BioGX_Products.html).
    - Stock and Working probe and primer solutions can be prepared using commercially synthesized primers with basic desalt purification (Fisher/Genosys or equivalent) by rehydrating with sterile distilled water

to appropriate concentrations. Store frozen until use.

- Probes should be purchased as RP HPLC purified and labeled as follows:
  - *Stx*-1 probe is 5′ Texas Red and 3′BHQ2 labeled (Integrated DNA Technologies Inc., or equivalent).
  - *Stx*-2 probe is 5′ FAM and 3′ BHQ1 labeled (Integrated DNA Technologies Inc., or equivalent).
  - *uidA* probe is 5′ TET and 3′ MGB-NFQ labeled, Applied Biosystems, or equivalent
  - Working solutions of probes should be aliquoted into small portions and kept frozen until use.

Table 1: STEC Primer/Probe Sequences

Primer/Probe Name	Sequence (5′ → 3′)
Stx1F934	GTGGCATTAATACTGAATTGTCATCA
Stx1R1042	GCGTAATCCCACGGACTCTTC
Stx2F1218	GATGTTTATGGCGGTTTTATTTGC
Stx2R1300	TGGAAAACTCAATTTTACCTTTAGCA
uidAF241	CAGTCTGGATCGCGAAAACTG
uidAR383	ACCAGACGTTGCCCACATAATT
UidAP266	TET-ATTGAGCAGCGTTGG-MGB/NFQ
Stx1P990	Texas Red – TGATGAGTTTCCTTCTATGTGTCCGGCAGAT – BHQ2
Stx2P1249	6FAM-TCTGTTAATGCAATGGCGGCGGATT-BHQ1
Internal Control55F	No sequence available Contained in STEC CSR bead (BioGX)
Internal Control186R	No sequence available Contained in STEC CSR bead (BioGX)
Internal Control Cy5	No sequence available Contained in STEC CSR bead (BioGX)

7.3 List of Controls (Specific strains are listed in SOP MDP-QA-03)

7.3.1 Carry all cultural controls from all screening methods previously completed through this entire procedure. Refer to SOP MDP-LABOP-02 for control setup. If any of the controls fail to yield a satisfactory result refer to SOP MDP-QA-03.

- No-template Control: Transfer 20 µl of Master Mix and 5 µl of PCR grade water.
- Negative Culture Control: DNA from MDP-017
- Positive Cultural Control: DNA from MDP-004
- Positive Produce Control: DNA from inoculated produce culture control (MDP-004) from SOP MDP LABOP-02
- Media Control for Cultural Confirmation
- Positive DNA Control: MDP-019: grow culture and extract DNA prior to assay setup

7.4 Template and Master Mix Preparation

7.4.1 Use extracted DNA from overnight UPB enriched samples from SOP MDP-LABOP-02.

7.4.2 Prepare master mix containing all of the components, except template, and in sufficient volume to run all samples and controls.

7.4.2.1 When using the OmniMix™ HS reagent beads, they contain reagents for **two** 25µl reactions, therefore they need to be rehydrated accordingly. The CSR beads contain all the primer, probe and internal control reagents for performing **four** 25µl PCR amplifications. Two OmniMix™ HS beads per CSR bead is required to provide the necessary reagents for four PCR amplifications.

7.4.2.2 To prepare master mix, determine the number of reactions needed. Add PCR grade water equal to the number of reactions (n) times 20µl (volume of master mix added to each reaction tube). Add the appropriate number of OmniMix HS beads followed by the appropriate number of CSR beads. Briefly mix the master mix. Store the prepared master mix in the dark at 2-8^{o}C prior to use. Prepared master mix can be stored not more than 48 hours. *(Example: For 16*

PCR reactions, add 320µl PCR grade water to a DNA/RNA free sterile microfuge tube. Add 8 OmniMix HS beads to the tube. Add 4 CSR beads to the tube.)

The preparation of the Master Mix should be performed in a clean hood. Add the PCR grade water to a sterile microcentrifuge tube, add all of the beads, and let it sit in a cold block until they fully dissolve.

7.4.2.3 Aliquot 20µL of Master Mix to each of the Smart Cycler reaction tubes using the Smart Cycler cold block. Then add 5uL of undiluted DNA sample (template) or control template for a final volume of 25µl in appropriate reaction tube.

7.4.2.4 Close the reaction tubes and briefly centrifuge using the Smart Cycler centrifuge to bring all liquid to the bottom of the reaction tube. Avoid touching the bottom of the Smart Cycler tubes as fingerprints and debris could affect results.

7.4.2.5 Keep all thawed reagents and reagents on ice. Protect CSR beads from light.

7.4.2.6 Load the reaction tubes in to the Smart Cycler block and analyze according to pre-set protocol specified in step 7.5 of this SOP.

7.5 Real-time PCR Analysis

7.5.1 Create run on SmartCycler II

7.5.2 Give each run a unique run name.

7.5.3 Select Dye set FTTC25

7.5.4 Create and save the following protocol and Select sites used in run. The parameters of this cycle are as follows:

7.5.4.1 A 2-step PCR protocol is used with initial activation hold of 60 sec at

95°C, followed by 40 cycles of 10 sec at 94°C, (optics off) then 40 sec at 63°C, (optics on).

7.5.4.2 Set analysis settings on SmartCycler® II instrument, for FAM, TET and Texas Red channels, and if using CSR beads, include the Cy5 channel. Manual Threshold Fluorescence Units for the Cy5 IC is Lot dependent. If no Ct is present for the IC in the negative control at default 15.0 fsu, refer to Lot specific recommendations.

(1) Usage: Assay
(2) Curve Analysis: Primary
(3) Threshold Setting: Manual
(4) Manual Threshold Fluorescence Units: 15.0
(5) Auto Min Cycle: 5
(6) Auto Max Cycle: 10
(7) Valid Min. Cycle: 3
(8) Valid Max. Cycle: 60
(9) Background subtraction: ON
(10) Boxcar Avg. Cycles: 0
(11) Background Min. Cycle: 5
(12) Background Max. Cycle: 40

7.5.4.3 Ensure the Manual Threshold Fluorescence Units have been changed to 15.0. Update analysis settings if they are changed before recording results.

7.5.5 Start Run.

7.5.6 Primary fluorescence curves that cross the threshold will be recorded as POS and the cycle when the sample crossed the threshold will be recorded in the Results Table view (Figure 1A).

7.5.7 Results can be viewed graphically (Figure 1B): A report can be generated or a screen capture of the results table view can be used to record data. Table 3 shows spectral characteristics of the dyes employed in the assay as well as the available optical channels on the Smart Cycler II system. Fluorescence in each channel is recorded separately on the instrument

Table 2: Spectral properties of dyes and available channels on the Smart Cycler II

Fluorescent Dye (nm) Dye	Absorption Max. (nm)	Emission Max.	
FAM	494	518	
TET	522	538	
TxRd	598	615.5	
Cy5	649	670	
Smart Cycler II	Excitation (nm)	Emission (nm)	Dye used in Assay
Ch. 1	450-495	510-527	FAM
Ch. 2	500-550	565-590	TET
Ch. 3	565-590	606-650	TxRd
Ch. 4	630-650	670-750	Cy5

Stx2 presence is recorded in FAM channel (Channel 1)
***uid*A** *E. coli* O157:H7 clonal type is recorded in TET channel (Channel 2)
***stx*1** presence recorded in TxRd channel (Channel 3)
IC (Internal Control) presence recorded in Cy5 channel (Channel 4)

Figure 1A: Results Table View

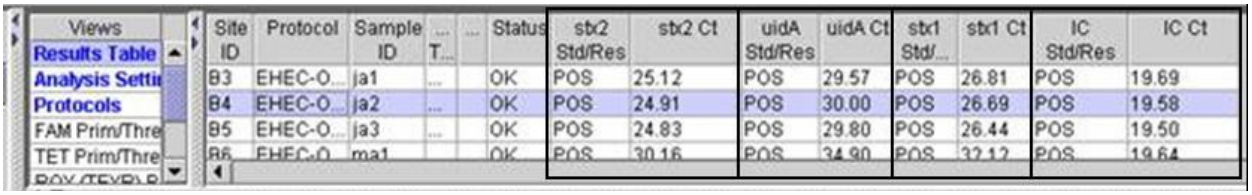


Figure 1A - Results table view. For each channel the final result, positive or negative, is reported in the first column and the Ct at which the positive values crossed the threshold is reported in the second column. The FAM, TET, TxRd and Cy5 channels correlate to the *stx*2, *E.coli*O157:H7, *uid*A clonal group, *stx*1 and IC targets respectively.

Figure 1B: Results Graphical View

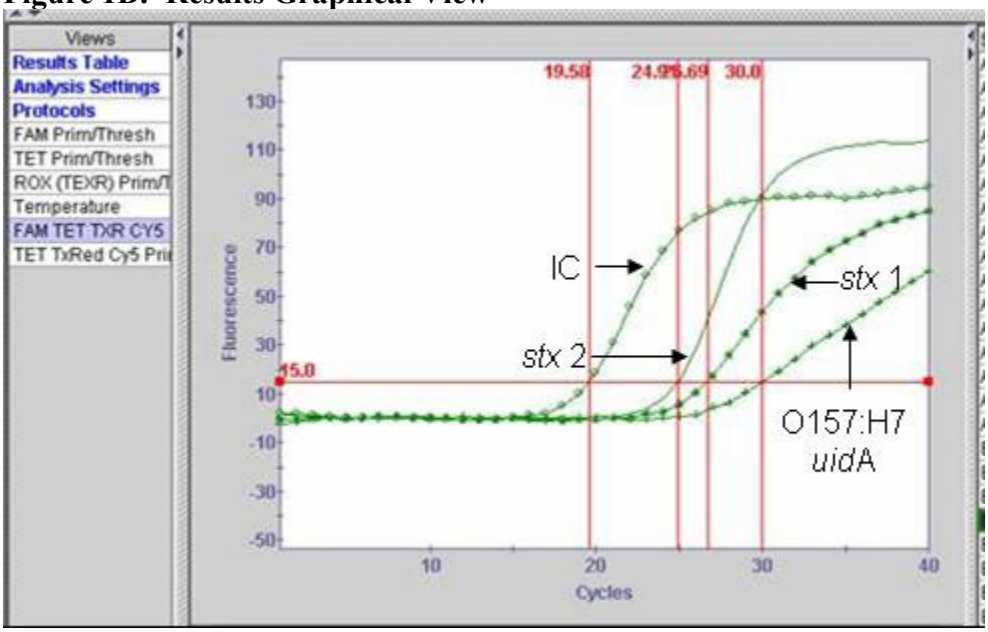


Figure 1B is a graphical view for the four channels for isolat*e E.coli* O157:H7 ATCC 43895.

7.6 Interpretation of Results

7.6.1 A preliminary positive result for STEC is indicated by DNA detected for a respective gene target (*stx*-1, *stx*-2, *uidA*) if indicated as "POS".

7.6.1.1 If a pooled sample is positive for any one of the targets, extract DNA from individual samples as per SOP MDP-LABOP-02.

7.6.1.2 Setup PCR reactions for the previously extracted pooled and the individual samples as per sections 7.2 through 7.5 of this SOP. Also, run same samples following SOP MDP MTH-12.

7.6.1.3 Samples that show positive *stx*-1 or *stx*-2 (and are negative for MDP MTH-12) are considered suspect positive for non-O157 STECs and shall be culturally confirmed as per section 7.7 in this SOP.

7.6.1.4 Samples that show positive *uidA* results (regardless of *stx*-1 or *stx*-2 results) by SOP MDP MTH-11 and/or positive results by SOP MDP-MTH-12 are considered presumptive positive for *E. coli* O157:H7 and shall be confirmed according to SOP MDP MTH-06.

7.6.2 A negative result for STEC is indicated when no DNA is detected for a respective gene target (*stx*-1, *stx*-2, *uidA*) if indicated as "NEG" and no cycle threshold value is present.

7.7 Isolation and Identification of non-O157 STEC

Due to the complex nature of isolation, laboratories are encouraged to use best professional judgment and experience to obtain a pure culture of the target organism. Isolation and identification can be carried out from the pooled sample and the individual sample that tested positive in 7.6.1.3

7.7.1 For each STEC positive pooled sample (except for alfalfa sprouts), transfer 10 mL of pooled UPB enriched sample to one 90 mL aliquot of mBPWp (with ACV added immediately)and incubate at 42 ± 2°C for 18-24 hours. In addition, transfer 10 mL of pooled UPB enriched sample to 90 mL TP broth and incubate at 44 ± 2°C for 18-24 hours.

Note: 10 mL of the pooled sample is left over and if required can be used for additional analyses (ex: IMS of the pooled UPB if sample is positive on MDP MTH-12.

7.7.2 In addition, transfer 25 mL of the positive individual UPB enriched samples into 225 mL of mBPWp (with ACV added immediately) and incubate at 42 ± 2°C for 18-24 hours. Also, transfer 25 mL of each of the positive individual UPB enriched samples into 225 mL of TP broth and incubate at 44 ± 2°C for 18-24 hours. For sprouts, transfer 25 mL of the individual positive sample accordingly.

7.7.3 Following incubation, streak duplicate sets of plates of chromogenic agar, L-EMB, and MacConkey plates. Incubate one set of plates overnight at 35 ± 2°C and incubate the other set of plates overnight at 42 ± 2°C.

Note: Use professional judgment and experience in deciding the number of plates needed for picking minimum number of 20 isolated colonies

7.7.4 Examine plates and pick at least 20 typical isolated colonies or swipes from any of the selective agar plates (chromogenic, L-EMB, and MacConkey) to appropriate media (e.g., LST, BHI, etc.). It is advised to pool multiples of isolates and screen on Rt-PCR to determine what isolate(s) that are positive (e.g., pick 20 colonies and pool into 2 groups; example: if you have 20 LST samples, split into two groups containing 10 each) and perform DNA extraction according to SOP MDP-LABOP-02 and perform Rt-PCR. ***Document procedures used accordingly.***

Note: Use professional judgment and experience in deciding the maximum number of colonies required for screening which depends on the extent of contamination, the background microflora level, type of commodity and post harvest handling.

Typical Colony Characteristics of pathogenic *E. coli*		**Other Organisms of Interest**
Medium	**Colony Characteristics**	
MacConkey	Red to pink	
L-EMB	Blue-black and green w/metallic sheen	
Chromogenic agar	refer to manufacturer's user guide	refer to manufacturer's user guide

7.7.5 Identify the individual SmartCycler® Rt-PCR positive culture. Streak the culture to selective agar plates for isolation. Incubate overnight at 35 ± 2°C.

7.7.6 Examine plates and if selective agar plates contain typical colonies, streak the typical colony onto BA plate and incubate overnight at 35 ± 2°C. Perform VITEK® using growth off BA or other appropriate plating media. (If VITEK® identifies the organism as a possible *E. coli* O157:H7, further identification based on cultural tests and serotype is required as per SOP-MDP-MTH-06. MPO shall be notified per SOP MDP-DATA-01.) Pick 3-5 individual typical colonies to LST or non-specific rich broth.

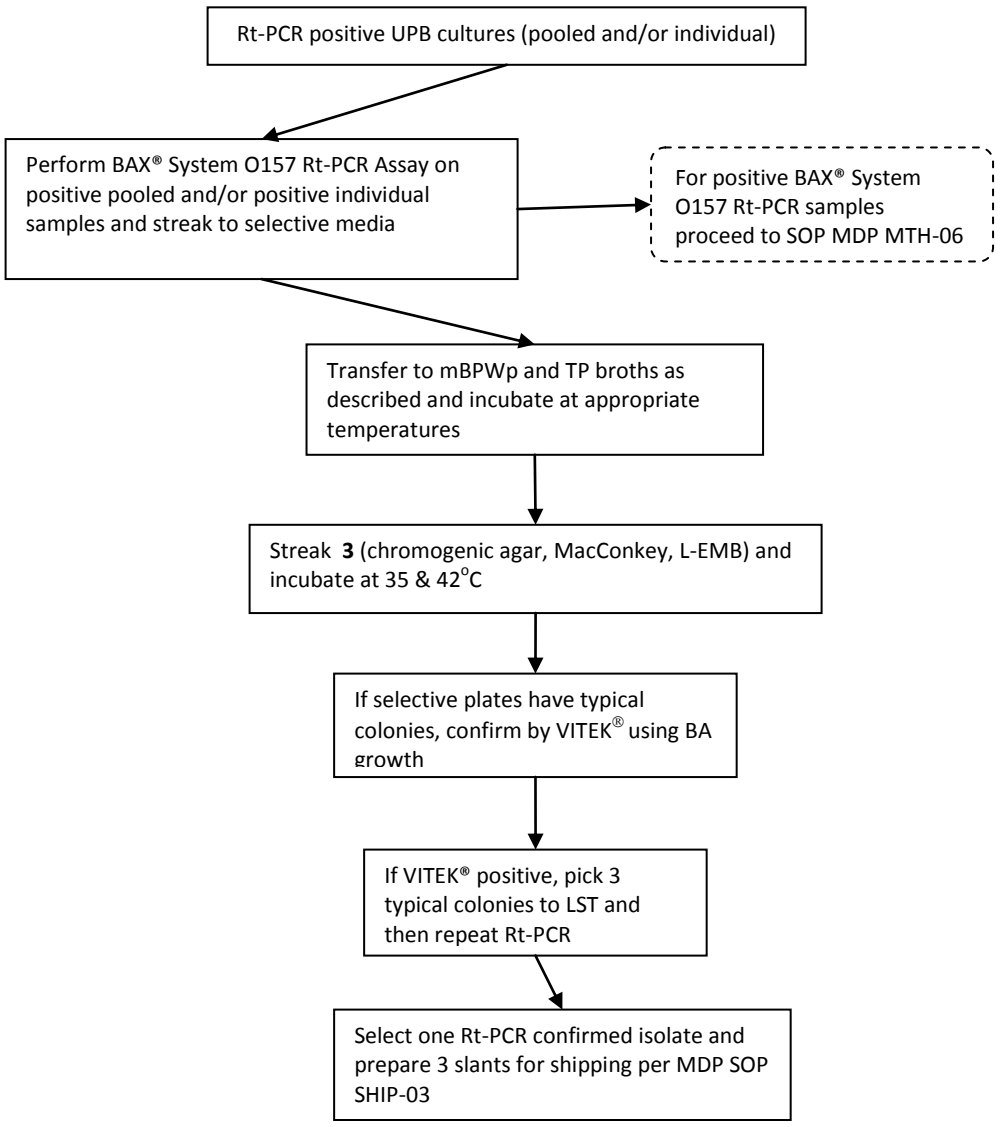
7.7.7 Repeat Rt-PCR on 3 isolates. Choose one isolate that is identified as *E. coli* and possesses toxin gene(s) for archiving and shipping according to SOP MDP-SHIP-03.

7.8 Tryptone Phosphate (TP) Broth - Autoclave for 15 minutes at 121°C. Final pH should be 7.0 ± 0.2.

Tryptone	20.0 g
K_2HPO_4	2.0 g
KH_2PO_4	2.0 g
NaCl	5.0 g
Tween 80	1.5 mL
Deionized Water	1.0 L

7.9 Reporting - A "preliminary STEC" is defined as an isolated organism that is shown by Rt-PCR to possess the DNA sequences associated with the stx1 and/or stx2 genes and to NOT possess the *E. coli* O157:H7 DNA sequence (secondary analysis per MDP-MTH-12). The isolate(s) slants are to be shipped to the repository (per MDP-SHIP-03 SOP) for archival and/or additional testing. If additional testing is performed by an outside agency, upon receipt of PFGE/serological results, MPO will provide test data to repository and originating laboratory. Report results according to SOP MDP-DATA-01.

Flowchart:
STEC Isolation and Identification



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Revision 01 June 2011 Monitoring Programs Division

- Revised SOP Title to read "…with non-O157 Isolation and Identification"
- Updated References, Section 6
- Added VITEK® and cards information, Section 7.1
- Replaced "mEC+n" with "mBPWp (with ACV added immediately)" entire SOP
- Replaced "CHROMager™ E. coli plates" with "chromagenic agar plates", entire SOP
- Deleted italicized "SmartMix "note at end of Section 7.2
- Increased volumes from "…4 µl of Master Mix and 1 µl of…" to "…20 µl of Master Mix and 5 µl of…", Section 7.3.1
- Deleted "SmartMix™HM", Section 7.4.2.1
- Increased volume from "…4 µl of Master Mix…" to "…20 µl of Master Mix…", Section 7.4.2.2
- Deleted old Section 7.4.2.3
- Increased volume from "4 µl" to "20 µl" and increased volume from "1 µl" to "5 µl", old Section 7.4.2.4
- Deleted "1:10", changed "diluted" to "undiluted", and added "DNA", old Section 7.4.2.4
- Replaced the word "suspect" with "preliminary", Section 7.6.1
- Deleted the words "2 separate" and "one" and "35±2°C and the second at", Section 7.7.2
- Added Notes, Sections 7.7.3 and 7.7.4
- Added bold words in "Examine plates and **pick at least 20** typical isolated colonies or swipes from **any of the** selective agar plates (chromogenic, L-EMB, and MacConkey) to appropriate media (e.g., LST, BHI, etc.)", Section 7.7.4
- Revised "Typical Colony Characteristics of pathogenic *E. coli"* Table to reflect chromagenic agar information, Section 7.7.4
- Deleted mEC+n recipe, Section 7.8
- Deleted sentence from TP broth recipe, "For double strength TP broth, follow the instructions above, except add 500ml of deionized water." , Section 7.8
- Replaced word "Suspect" with "Preliminary", section 7.9
- Revised STEC Isolation and Identification Flowchart