United States Department of Agriculture
Food Safety and Inspection Service
Office of Public Health Science
Laboratory QA/QC Division
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# Laboratory Guidebook
# Notice of Change

Chapter new, **revised**, or archived: MLG 5B.03

Title: Detection and Isolation of non-O157 Shiga Toxin-Producing *Escherichia coli* (STEC) from Meat Products

Effective Date: 11/06/12

Description and purpose of change(s):

This revised chapter contains the addition of an optional presumptive PCR step to verify presumptive positive non-O157 colonies from the modified Rainbow Agar.

**The methods described in this guidebook are for use by the FSIS laboratories. FSIS does not specifically endorse any of the mentioned test products and acknowledges that equivalent products may be available for laboratory use. Method validation is necessary to demonstrate the equivalence of alternative tests. FSIS provides guidance at:**
**http://www.fsis.usda.gov/PDF/Validation_Studies_Pathogen_Detection_Methods.pdf**

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| Title: Detection and Isolation of non-O157 Shiga Toxin-Producing *Escherichia coli* (STEC) from Meat Products | | |
| Revision: 03 | Replaces: 02 | Effective: 11/06/12 |

**Procedure Outline**



## 5B.1 Introduction

Shiga toxin-producing *Escherichia coli* strains (STEC) of various serotypes have become an increasing public health concern since *E. coli* O157:H7 was first identified in 1982. STEC has been implicated in numerous outbreaks including development of hemolytic uremic syndrome (HUS) in some patients. Although *E. coli* O157:H7 has been most commonly identified as the cause of STEC infection, isolation of non-O157 STEC strains from clinical cases, outbreaks and environmental sources has been increasing (Posse *et al.*, 2008). A study at the Centers for Disease Control and Prevention showed that from 1983-2002 approximately 70% of non-O157 STEC infections in the United States were caused by strains from one of six major serogroups, including O26, O45, O103, O111, O121 and O145 (Brooks *et al.*, 2005). Virulence factors for non-O157 STEC include, but are not limited to, production of the shiga-like toxins 1 and/or 2 (Stx1, Stx2) and intimin (*eae*). Cattle and other

ruminants appear to be the main reservoir of non-O157 STEC, as well as the O157:H7 serotype (Arthur *et al.*, 2002). With carriage rates of non-O157 STEC in cattle being a public health concern, a method was devised to detect and isolate the six major non-O157 STEC serogroups (O26, O45, O103, O111, O121 and O145) in ground beef and beef trim. This method, developed by USDA-Agricultural Research Services Eastern Regional Research Center (USDA-ARS-ERRC) in Wyndmoor, Pennsylvania and the Outbreaks Section of the Eastern Laboratory (OSEL), utilizes multiplex Real-time PCR detection assays followed by cultural isolation. The screen multiplex Real-time PCR assay detects the presence of the shiga toxin (*stx*) and intimin (*eae*) genes. Note that while this assay detects shiga toxin gene sequences, it does not differentiate between *stx1* and *stx2*. Another set of three serogroup-specific multiplex PCR assays are used to identify genes within the O antigen gene cluster specific for each serogroup. Cultural isolation of non-O157 STEC from screen-positive enrichments (positive for *stx*, *eae* and top six O antigen gene cluster) proceeds using immunomagnetic separation (IMS) beads coated with serogroup-specific antibodies followed by plating onto modified Rainbow Agar (mRBA). A post-IMS acid treatment step is performed to help reduce background flora that grow on mRBA. Many strains of STEC have been reported to have acid tolerance at pH 2 while competitor organisms show pH sensitivity (Grant, 2004; Bagwhat *et al.*, 2005). Colonies on mRBA are tested for the presence of O antigens specific for the top six STEC serogroups using an agglutination test. Agglutination positive colonies are then streaked onto tryptic soy agar with 5% sheep blood (SBA) for confirmation using the multiplex PCR assays and biochemical identification.

## 5B.2 Safety Precautions

Similar to *E. coli* O157:H7, non-O157 STEC serotypes are human pathogens with a low infectious dose. The use of gloves, protective laboratory coats and eye protection is for all post enrichment viable culture work. Work surfaces must be disinfected prior to and immediately after use. Laboratory personnel must abide by CDC guidelines for manipulating Biosafety Class II pathogens. A Class II laminar flow biosafety cabinet is recommended for activities with potential for producing aerosols of pathogens. All available Material Safety Data Sheets (MSDS) shall be obtained from the manufacturer for the media, chemicals, reagents and microorganisms used in the analysis. The personnel who will handle the materials should read all MSDS sheets.

## 5B.3 Equipment, Reagents and Media

### 5B.3.1 Equipment and Materials

a. Balance, sensitivity ± 0.1 g
b. Incubators, static 42 ± 1°C and 35 ± 2°C
c. Micropipettors for culture plating to deliver volumes ranging from 15-1000 µl with sterile disposable filtered tips
d. Micropipettors for Real-time PCR to deliver volumes ranging from 0.50 µl to 1,000.00 µl with sterile, filtered tips
e. Applied Biosystems® ABI 7500 FAST PCR platform
f. Applied Biosystems® 7500 FAST MicroAMP™ Fast Optical 96-well Reaction Plate with Barcode (Part# 4346906)
g. Applied Biosystems® 7500 FAST MicroAMP™ Splash Free 96-Well Base (Part# 4312063)
h. Applied Biosystems® 7500 FAST Optical Adhesive Film (Part# 4311971)
i. Applied Biosystems® TaqMan® Environmental Master Mix 2.0 (Part# 4396838)
j. VITEK® 2 system
k. GN cards for VITEK® 2 system (bioMerieux Vitek, Inc.)
l. Heating block (95-99°C) or thermocycler for DNA preparation step
m. Vortexer
n. Centrifuge that holds microcentrifuge tubes and is capable of speeds up to 16,000 x g
o. Centrifuge plate adapter for the centrifugation of 96-well PCR plates
p. Disposable, sterile pipettes for volumes 1.0 ml and for 5.0 ml.
q. Sterile, inoculating loops, "hockey sticks" or spreaders, and needles
r. Rotating tube agitator with clips to hold microcentrifuge tubes
s. Sterile, disposable 12 x 75 mm polypropylene or polystyrene tubes
t. Sterile microcentrifuge tubes (1.5 - 2.0 ml)
u. Sterile 50 ml conical tubes
v. Sterile 40 µm Cell Strainer
w. MACS® Large Cell Separation Columns (Miltenyi Biotec # 422-02)
x. OctoMACS® Separation Magnet (Miltenyi Biotec # 421-09)
y. Multistand to support OctoMACS® Separation Magnet (Miltenyi Biotec # 423-03)
z. Tray, autoclavable, approximately 130 mm x 83 mm for use with the OctoMACS®

aa. Sterile filter or non filter bags
bb. Optical density reader

### 5B.3.2 Media and Reagents

a. Modified Tryptone Soya Broth supplemented with novobiocin (8.0 mg/L) plus casamino acids (mTSB+n)
b. Modified Rainbow Agar (Rainbow® Agar O157 Biolog Inc., Hayward California, 94545) containing 5.0 mg/L novobiocin, 0.05 mg/L cefixime trihydrate and 0.15 mg/L potassium tellurite
c. Cefixime trihydrate
d. Tryptic soy agar with 5% sheep blood [Sheep Blood Agar (SBA)]
e. 1.0 N Hydrochloric Acid (HCl)
f. Physiological saline solution (0.85% NaCl)
g. 1X Tris-EDTA (TE) Buffer
h. E Buffer, approximately 7 ml per sample (See Media and Reagents Appendix 1, Buffered Peptone Water, Bovine Albumin Sigma and Tween-20®)
i. Disinfectant (Lysol® I. C., 2.0%)
j. SDIX RapidChek® CONFIRM STEC Immunomagnetic Separation (IMS) Kit with anti-O26 antibody-coated paramagnetic beads, anti-O103 antibody-coated paramagnetic beads, anti-O111 antibody-coated paramagnetic beads, anti-O145 antibody-coated paramagnetic beads, anti-O45 antibody-coated paramagnetic beads, and anti-O121 antibody-coated paramagnetic beads
k. RNase free, DNase free PCR Certified Water
l. TaqMan® Environmental Mastermix 2.0, Applied Biosystems Catalog Number 4396838
m. Biochemical test kit and system, GN cards (VITEK® 2 system, bioMerieux Vitek, Inc., 595 Anglum Drive, Hazelwood, MO 63042-2395)
n. Primer and probe sequences detailed in MLG Chapter 5B Appendix 1 Primer and Probe Sequences and Reagent Concentrations for non-O157 Shiga Toxin-Producing *Escherichia coli* (STEC) Real-time PCR Assay, Integrated DNA Technologies (Coraville, Iowa)
o. Abraxis non-O157 STEC Latex Agglutination Test (LAT) Kits or equivalent specific for serogroups O26, O45, O103, O111, O121 and O145

## 5B.4 Quality Control

### 5B.4.1 General

a. Unless otherwise stated, weight and volume ranges and minutes have a tolerance of ±1%.
b. Modified Rainbow Agar (mRBA) plates have a shelf life of two weeks.
c. All media, plates and buffers shall be warmed to 18-35°C prior to use.
d. The top six non-O157 STEC control strains shall meet the following genetic characteristics: *stx*+ and *eae*+. Such strains can be obtained through reference culture collection centers including but not limited to the American Type Culture Collection (ATCC), the STEC Center at The Michigan State University and the *E. coli* Reference Center at The Pennsylvania State University. Non-O157 strains (*stx*+, *eae*+) must be used by FSIS Laboratories to prepare the DNA template positive PCR control. However, for safety considerations, toxin-attenuated or toxin-negative strains that have an appearance on mRBA typical of the non-O157 STEC may be used as controls on plating media for serological agglutination testing. The following non-O157 STEC control strains shall be used when stated in the method:

   i. *E. coli* O26, which shall be *stx* positive and *eae* positive
   ii. *E. coli* O45, which shall be *stx* positive and *eae* positive
   iii. *E. coli* O103, which shall be *stx* positive and *eae* positive
   iv. *E. coli* O111, which shall be *stx* positive and *eae* positive
   v. *E. coli* O121, which shall be *stx* positive and *eae* positive
   vi. *E. coli* O145, which shall be *stx* positive and *eae* positive

### 5B.4.2 Sample Enrichment Controls

Include with each sample batch, a positive growth control (*E. coli* O157:H7 strain 465-97 or other reference strain that is stx-, eae+) inoculated into a meat matrix free of the target analyte, and an uninoculated media (mTSB + casamino acid) control.

### 5B.4.3 DNA Extraction Control Preparation

Include with each sample batch, extract DNA from the positive growth control sample using the same extraction method as used to process the sample batch. This shall serve as positive DNA extraction controls.

### 5B.4.4 PCR Controls

a. ***stx/eae* screen PCR**
- DNA template from bioluminescent *E. coli* O157:H7 (DNA extraction *eae* positive control)
- DNA template from a cocktail of top six STEC cultures (PCR positive control) analyzed in triplicate
- No Template Control (NTC)

b. **Serogroup-specific screen PCR (O antigen gene cluster)**
- DNA template from a cocktail of top six STEC cultures (PCR positive control) analyzed in triplicate
- NTC

c. **Optional *stx/eae* presumptive PCR / *stx/eae* confirmatory PCR**
- DNA template from a cocktail of top six STEC cultures (PCR positive control) analyzed in triplicate
- NTC

d. **Optional serogroup-specific presumptive PCR (O antigen gene cluster) / Serogroup-specific confirmatory PCR (O antigen gene cluster)**
- DNA template from a cocktail of top six STEC cultures (PCR positive control) analyzed in triplicate
- NTC

To prepare PCR positive control template for the ABI® 7500 FAST, FSIS laboratories shall grow the top six STEC cultures on SBA and incubate at 35±2°C for 18-24 h. Colonies shall be used to create a culture suspension in PCR certified water corresponding to approximately $10^9$ CFU/ ml. In one tube, 1.0 ml from each suspension shall be added to 4.0 ml of PCR certified water to create a 10.0 ml cocktail of all six strains. This will provide approximately a $10^8$ CFU/ml cocktail using each strain. One hundred microliter aliquots of the suspension are then transferred to PCR tubes or microcentrifuge tubes and heated at 95-99°C for 10 minutes on a thermocycler or heating block. The tubes shall be centrifuged at 10,000 x g for 3 minutes to pellet cellular debris. The supernatant shall be used as the PCR positive control for all PCR assays. DNA control template can be prepared as a batch, aliquoted to smaller volume tubes, and stored at ≤-20°C for 1 year. Additional information on controls and troubleshooting based on control reactions is included in MLG 5B Appendix 3 PCR Platform Instructions, Data Analysis, and Control Results Interpretation for non-O157 Shiga Toxin-Producing *Escherichia coli* (STEC) Real-time PCR Assay.

### 5B.4.5 IMS Plating Controls

Streak the serogroup(s) of interest (based on serogroup-specific PCR results) onto mRBA and incubate along with the samples that have been treated with the IMS procedure.

## 5B.5 Sample Preparation and Primary Enrichment

Note: Disinfect the sample package prior to opening.

a. Place the 325±32.5g test portion per submitted sample into the sterile bag with mesh filter. Ensure that the entire test portion is on the same side of the mesh filter.
b. Add 975 ± 19.5 ml of modified TSB + casamino acids and 8 mg/L novobiocin (mTSB) to the test portion to provide a 1:4 dilution (one portion of product to three portions of broth). Pummel for approximately two minutes in a Stomacher™.
c. Incubate the test portion and the enrichment media at 42±1°C for 15-22 hours. Each group of samples should include a positive control enrichment (*E.* coli O157:H7 strain 465-97) and an uninoculated enrichment medium control.

## 5B.6 Screening Procedure using Real-time PCR

### 5B.6.1 DNA Extraction from Overnight Enrichments

a. Transfer 1.4 ± 0.1 ml of enrichment to a sterile microcentrifuge tube.
b. Centrifuge at 1,500 x g for 1 minute to pellet large debris (supernatant will still not be clear at this low speed but should no longer have large particles of meat).
c. Transfer the supernatant to a new sterile microcentrifuge tube. It is essential to ensure that none of the pelleted debris is carried over with the supernatant.
d. Centrifuge the supernatant at a setting of 10,000 *x* g for 5 minutes.
e. Discard the supernatant and add 500 ± 50 µl of 0.85% saline solution (PSS) to the pellet. Resuspend the pellet by either vortexing or using the pipet tip.
f. Centrifuge the tubes at a setting of 10,000 x g for 3 minutes. Remove the supernatant as completely as possible without disturbing the pellet.
g. Resuspend the pellet in 90 ± 9.0 µl of 1X TE Buffer. Use the pipet tip to thoroughly resuspend the pellet.
h. Heat at 97 ± 2$^{o}$C for 15 ± 1 minutes. Allow to cool at room temperature for approximately 2 minutes.

i. Centrifuge the tubes at a setting of 16,000 x g for 4 minutes.
j. Transfer the supernatant to a new sterile microcentrifuge tube without disturbing the pellet.
k. If the template is to be used within 24 hours, store at 2-8°C. For long-term storage, store at ≤ -20° C.

### 5B.6.2 Real-time PCR Procedure

Following DNA extraction from 15-22 h incubated enrichments, Real-time PCR will be used as a screen for the presence of *stx* toxin genes and the *eae* intimin gene. Samples with positive results (both gene targets) on the initial *stx/eae* PCR screen will be tested by three additional Real-time PCR assays to determine if a top six serogroup (O26, O45, O103, O111, O121 or O145) is present.

**Note:** The Real-time PCR assay described in this method has been optimized and validated specifically for the ABI® 7500 FAST. Use of other Real-time PCR platforms may require optimization with other probe quencher and reporter dyes. Additionally, the ABI® Environmental Mastermix has been validated and optimized for use on the ABI® 7500 FAST and would require additional optimization with reagent volumes and assay cycling parameters if using other Real-time PCR platforms. The assays have been validated for use with the Standard Chemistry, not the Fast Chemistry, on the ABI® 7500 FAST.

a. Set up the Real-time PCR assay on the ABI® 7500 FAST as described in MLG 5B Appendix 3 PCR Platform Instructions, Data Analysis, and Control Results Interpretation for non-O157 Shiga Toxin-Producing *Escherichia coli* (STEC) Real-time PCR Assay.
b. For the initial *stx* and *eae* PCR screen, prepare master mix with component volumes described in MLG 5B Appendix 1 Primer and Probe Sequences and Reagent Concentrations for non-O157 STEC Real-time PCR Assay.
c. Once the PCR mastermix is prepared, aliquot 20.0 µl of mastermix for each reaction into the wells of a sterile, 7500 FAST MicroAMP™ Fast Optical 96-well Reaction Plate or ABI® 7500 well strips. Keep the 96 well plate on the ABI® 7500 96-Well Base to ensure the PCR plate does not touch the bench.
d. Add 5.0 µl of DNA template from the DNA extraction step to the appropriate well. Follow Section 5B.4.4 for PCR controls to use for quality control. Seal the plate and centrifuge the plate briefly at approximately 2000 x g for 30

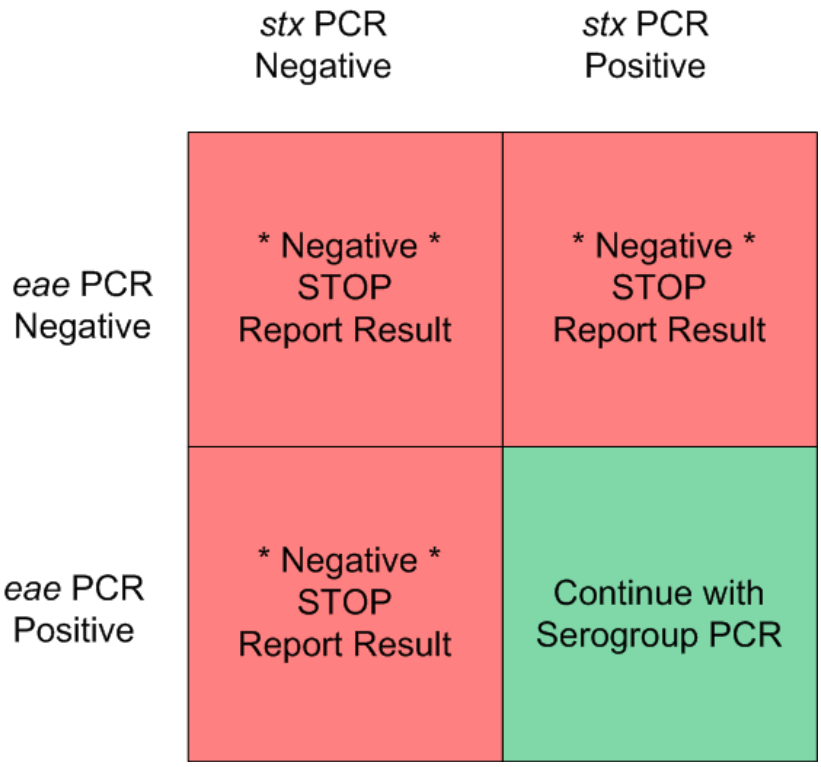
seconds prior to loading onto the ABI® 7500 FAST platform. This will ensure that no air bubbles are at the bottom of the wells.

e. Load the plate into the ABI® 7500 FAST and begin the assay.

f. At the end of the run, view the results to determine if samples are positive for *eae* and *stx* targets. Prior to the end of the run (45 cycles), a gene target is considered positive when the fluorescence crosses over the threshold that is auto-determined by the instrument. The auto-threshold determination is the default setting on the ABI® 7500 FAST and is automatically determined based on the mean baseline fluorescence. When the sample crosses the threshold for a particular target, a Cycle Threshold (Ct) value is given by the instrument. When the sample does not cross the threshold for a specific target, the instrument reports the sample as "Undetermined" for that target. The 16S rRNA internal control should be positive for every sample. The no template control (PCR certified water) should not yield a product (Ct>45) but may produce a trace 16S result in the 35-45 cycle threshold range; either case is acceptable. However, amplification of any other target other than 16S, or amplification of 16S below 35 cycles in the no template control well indicates cross-contamination, resulting in an invalid run. Consult MLG 5B Appendix 3 PCR Platform Instructions, Data Analysis, and Control Results Interpretation for non-O157 Shiga Toxin-Producing *Escherichia coli* (STEC) Real-time PCR Assay for additional controls and sample interpretation.

The dye channels for the ABI® 7500 FAST are as follows:
FAM = *stx*
VIC = *eae*
Cy5 = Internal Control (16S rRNA)
See Table 1 for PCR result interpretation.

**Table 1. Result interpretation for the *stx* and *eae* PCR assays.**




Samples negative for *stx* and/or *eae* targets are considered negative for non-O157 STEC. DNA extraction templates from any samples positive for both *stx* and *eae* targets will be further analyzed by PCR amplification of the serogroup specific gene targets using three multiplex Real-time PCR reactions.

g. For the serogroup-specific PCR screen, set up the Real-time PCR assay on the ABI® 7500 FAST as described in MLG 5B Appendix 3 PCR Platform Instructions, Data Analysis, and Control Results Interpretation for non-O157 Shiga Toxin-Producing *Escherichia coli* (STEC) Real-time PCR Assay and prepare master mix with component volumes described in MLG 5B Appendix 1 Primer and Probe Sequences and Reagent Concentrations for non-O157 STEC Real-time PCR Assay.

h. Once the PCR mastermix is prepared, aliquot 20.0 µl of mastermix for each reaction into the wells of a sterile, 7500 FAST MicroAMP™ Fast Optical 96-well Reaction Plate or ABI® 7500 well strips. Keep the 96 well plate on the

ABI® 7500 96-Well Base to ensure the PCR plate does not touch the bench. Add 5.0 µl of DNA template from the DNA extraction step to the appropriate well. Follow Section 5B.4.4 for PCR controls to use for quality control. Seal the plate and briefly centrifuge the plate at approximately 2000 x g for 30 seconds prior to loading onto the ABI® 7500 FAST platform. This will ensure that no air bubbles are at the bottom of the wells. Load the plate into the ABI® 7500 FAST and begin the assay.

i. At the end of the run, view the results to determine if samples are positive for the serogroup-specific targets. Prior to the end of the run (45 cycles), a gene target is considered positive when the fluorescence crosses over the threshold that is auto-determined by the instrument. The auto-threshold determination is the default setting on the ABI® 7500 FAST and is automatically determined based on the mean baseline fluorescence. When the sample crosses the threshold for a particular target, a Cycle Threshold (Ct) value is given by the instrument. When the sample does not cross the threshold for a specific target, the instrument reports the sample as "Undetermined" for that target. The no template control (PCR certified water) should not yield a product (Ct>45) but may produce a trace 16S result in the 35-45 cycle threshold range; either case is acceptable. However, amplification of any other target other than 16S, or amplification of 16S below 35 cycles in the no template control well indicates cross-contamination, resulting in an invalid run. The dye channels with their respective targets on the ABI® 7500 FAST are as follows:
FAM = Serogroup O26, Serogroup O45 and Serogroup O145
VIC = Serogroup O103, Serogroup O111 and Serogroup O121
Cy5 = Internal Control (16S rRNA)
The 16S rRNA internal control should be positive for every sample.

j. A sample that is negative for the serogroup targets is considered negative for the top six non-O157 STECs and the analysis for that sample stops. A sample with positive PCR results for *stx*, *eae* and serogroup(s) is considered a potential positive and the isolation procedure shall be continued.

## 5B.7 Isolation Procedure

Samples that are potentially positive by PCR screen results shall be plated onto modified Rainbow Agar (mRBA) following IMS. In the isolation procedure, IMS beads shall be used for the specific serogroup identified by the serogroup PCR reaction (i.e. anti-O26 will be used for samples with screen results positive for O26, anti-O45 for O45 PCR positive reactions, anti-O103 for O103 PCR positive reactions or anti-O121 for O121 PCR positive

reactions, anti-O111 for O111 PCR positive reactions and/or anti-O145 for O145 PCR positive reactions). A post-IMS acid treatment step has been added to reduce background flora on the mRBA plate. Following the one hour acid treatment step, samples are diluted 1:1 with E-buffer and 0.1 ml is spread plated onto mRBA. Additionally, the suspension is diluted 1:10 and 0.1 ml is spread plated onto mRBA.

### 5B.7.1 Immunomagnetic Separation and Culture Plating

a. Remove mRBA plates from 2-8°C storage, allowing 4 plates for each screen-positive culture and one plate for each serogroup control strain. Be sure that plates have no visible surface moisture at the time of use. If necessary, dry plates (e.g. for up to 30 minutes in a laminar flow hood with the lids removed) prior to use. Dried plates that are not used should be labeled "dried", placed in bags and returned to 2-8°C.

b. For each screen-positive culture, label two sterile microcentrifuge tubes (for step d and step m), one 50 ml conical centrifuge tube (for step c) and four 12 x 75 mm capped tubes (for steps i and j). For three of 12 x 75 mm tubes, add 0.9 ml E-Buffer and label one tube as 1:10, one tube as 1:100 and one tube as acid 1:10.

c. **Sample preparation from overnight enrichment:** For each serogroup that the sample is positive, transfer approximately 2-5 ml from overnight enrichment through a 40 µm Cell Strainer into a 50 ml conical centrifuge tubes.

d. **Binding of paramagnetic antibody beads to specific serogroup:** Transfer 50.0 µl (or volume recommended by the manufacturer) of appropriate immunomagnetic capture beads determined by the serogroup PCR screen results (O26, O45, O103, O111, O121 or O145) to a sterile, labeled microcentrifuge tube. Next, add 1 ml of enrichment filtrate to the appropriately labeled tube.

e. Place the microcentrifuge tubes containing enrichments and capture beads on LabQuake® Agitator and rotate tubes for 15 minutes at 18-30°C (or time recommended by the manufacturer).

f. For each sample, place one MACS® Large Cell Separation Columns onto the OctoMACS® Separation Magnet. Fill the tray below the separation magnet with disinfectant. Prime each separation column with at least 0.5 ml of E-buffer and allow the liquid to pass completely through before adding sample.

g. **Binding of beads to magnetic columns:** Once the liquid has passed through the column, add the 1.0 ml of enrichment plus IMS beads to each appropriately labeled column and allow liquid to completely pass through.

h. **Wash steps (4X):** Add 1.0 ml of E-buffer to each column allowing the liquid to pass completely through. Repeat 3 more times for a total of 4 washes.

i. **Elution step:** After the last wash has drained, remove the column from the OctoMACS® Magnet and insert the tip into an empty labeled 12 x 75 mm tube. Apply 1.0 ml of E Buffer to the column, and using the plunger supplied with the column, immediately flush out the beads into the tube. Use a smooth, steady motion to avoid splattering. Cap the tubes. Repeat this for each column.

j. Make a 1:10 dilution of each treated bead suspension by adding 0.1 ml of the bead suspension to a 12 x 75 mm labeled tube containing 0.9 ml E-Buffer. Make a 1:100 dilution by adding 0.1 ml of the 1:10 dilution to a 12 x 75 mm labeled tube containing 0.9 ml E Buffer.

k. Vortex briefly to maintain beads in suspension and plate 0.1 ml from each tube (1:10 dilution and 1:100 dilutions) onto a labeled mRBA plates. Use a hockey stick or spreader to spread plate the beads, being careful not to spread the beads against the edge of the plate.

l. As soon as there is no visible moisture on the agar surface, invert plates and incubate for 20-24 h at 35 ± 2°C.

m. **Acid Treatment:** For each sample, transfer 450 µl of the undiluted bead suspension (MACS column eluant) to an empty labeled microcentrifuge tube. Add 25 µl of 1N hydrochloric acid (HCl) to this bead suspension and vortex briefly. This will bring the pH to 2.0-2.5 using E-buffer.

n. Place the microcentrifuge tubes containing the acid treated suspension on a LabQuake® Agitator and rotate tubes for 1 hour at 18-30°C temperature.

o. After 1 hour, dilute the suspension by adding 475 µl of E-buffer.

p. Vortex briefly to maintain beads in suspension and plate 0.1 ml of the neutralized suspension onto a labeled mRBA plate. Use a hockey stick or spreader to spread plate the beads, being careful not to spread the beads against the edge of the plate.

q. Add 0.1 ml of the suspension to a labeled tube containing 0.9 ml E-buffer and vortex briefly. This shall represent a 1:10 dilution of the acid-treated cell suspension. Plate 0.1 ml of the diluted suspension onto an appropriately labeled mRBA plate.

r. As soon as there is no visible moisture on the agar surface, invert plates and incubate for 20-24 h at 35 ± 2°C.

## 5B.8 Identification and Confirmation

Following 20-24 h incubation of mRBA, plates will be examined for colonies that agglutinate with antisera specific for the serogroup of interest. Colonies colors from representative strains of each serogroup are listed in MLG 5B Appendix 2 Morphologies of Representative Strains from Top Six non-O157 Shiga Toxin-Producing *Escherichia coli* (STEC) Grown on Modified Rainbow Agar. However, the coloration of colonies described in MLG 5B Appendix 2 may vary based on proximity to other competitor colonies or medium discoloration due to competitor colony growth. Since the morphologies of the targeted STEC colonies may vary widely among strains and serogroups, test at least one colony from each identified colony morphology found on the mRBA plate. Samples that have no growth or only contain agglutination negative colonies on mRBA are negative for non-O157 STEC. Any sample with agglutination positive colonies for the serogroup of interest is a presumptive positive for non-O157 STEC. Agglutination positive colonies shall be streaked onto SBA for confirmation on the following day. An optional PCR assay may be performed on the agglutination positive colonies from mRBA to verify the sample is presumptive positive. Following 18-24 h incubation of the SBA, agglutination-positive colonies shall be confirmed with Real-time PCR and biochemical identification. The confirmatory PCR shall include the *stx* and *eae* multiplex PCR assay and the serogroup specific multiplex PCR assay. If no colony picks isolated from the mRBA confirm by PCR and VITEK® 2, the sample is negative for non-O157 STEC.

### 5B.8.1 Serological Agglutination Procedure

a. Use an inoculating loop or needle to transfer a portion of an isolated colony from the mRBA plate to serological agglutination reagent. Follow manufacturer's instructions on procedure and interpretation.
   **Control Reactions:** A reference strain from the serogroup of interest plated on mRBA shall be used as the positive culture control. For optional PCR screen from colonies isolated on mRBA, refer to section 5B.8.2 Optional Presumptive PCR Assay.
b. Transfer the remainder of an agglutination positive colony from the mRBA plate onto SBA for further biochemical and genetic confirmation. Streak up to 5 agglutination positive colonies onto SBA plates. Incubate plates at 35 ± 2°C for 18-24 hours.
c. Following SBA incubation, perform the agglutination test again on colonies from the SBA plate.
d. To confirm agglutination-positive colonies using PCR, prepare a DNA template by resuspending an agglutination positive colony from the SBA plate

in 50 µl of Molecular Grade Water and heating the suspension for 10 minutes at 95-99°C.

e. From the DNA template preparation, perform the *stx* and *eae* multiplex PCR assay and the serogroup specific PCR assay as previously described in Section 5B.6.2. For the serogroup specific PCR assay, it is necessary to only perform the multiplex assay for the serogroup of interest. Note: Each PCR assay shall include a NTC negative control and a positive control for each gene target. Refer to section 5B.4 Quality Control for details on expected controls.

f. Additionally, perform biochemical identification (VITEK® 2) on agglutination positive colonies from the incubated SBA. A positive isolate shall be identified biochemically as *E. coli*.

g. If the isolate is agglutination positive for top six STEC serogroups, PCR positive for *stx, eae,* and top six serogroup genes and biochemically identified as *E. coli*, the sample is positive for non-O157 STEC.

h. If the isolate and any additional colony picks from mRBA are ultimately determined to be PCR negative for *stx, eae,* top six serogroup genes, the sample is negative for non-O157 STEC.

### 5B.8.2 Optional Presumptive PCR Assay

An optional PCR test may be performed directly on agglutination positive colonies from the mRBA to verify presumptive positive colonies using the following procedure.

a. Transfer the remainder of an agglutination positive colony from the mRBA plate into 50 µl of Molecular Grade Water (for up to 5 colonies). Inoculate a SBA plate from each suspension and then heat the suspension for 10 minutes at 95-99°C.

b. Incubate inoculated SBA plates at 35 ± 2°C for 18-24 hours.

c. From the DNA template preparation, perform the *stx* and *eae* multiplex PCR assay and the serogroup specific PCR assay as described in Section 5B.6.2. For the serogroup specific PCR assay, it is only necessary to perform the multiplex assay for the serogroup of interest. Note: Each PCR assay shall include a NTC negative control and a positive control analyzed in triplicate for each gene target. Refer to section 5B.4. Quality Control for details on expected controls.

d. The sample is considered negative if any of the 3 PCR targets (*stx*, *eae* or serogroup) are negative AND the internal control is positive (16S Ct < 30). The inoculated SBA plates can then be discarded. If the internal control is negative (16S Ct >30), analyze colonies from the streaked SBA the next day.

e. If an agglutination positive colony is positive for O group, *stx* and *eae* targets, the sample is considered a presumptive positive for non-O157 STEC. Refer to section 5B.8.1 steps c-h for confirmation of the isolates as non-O157 STEC.

## 5B.9 Culture Storage

For storage requirements of the fluorescent *E. coli* O157:H7 strain (FSIS culture # EC 465-97 or the currently designated control strain) refer to MLG 5 Detection, Isolation and Identification of *Escherichia coli* O157:H7 from Meat Products, Section 5.3.c. Store other "working" non-O157 *E. coli* stock cultures on nutrient agar slants. Transfer stocks monthly onto duplicate nutrient agar slants, incubate overnight at 35± 2°C, and then store them at 2-8°C. Use one of the slants as the working culture. Use the other slant for sub-culturing to reduce the opportunity for contamination. Note: Cultures shall not be subcultured more than five times. After this period the culture must be re-confirmed biochemically or a new culture initiated. For long term storage, freeze cultures using cryo-beads i.e. Cryostor™ or lyophilize.

## 5B.10 Selected References

Applied Biosystems® 7500 Fast Real-time PCR System Guide

Arthur TM, Barkocy-Gallagher GA, Rivera-Betancourt M, Koohmaraie M (2002) Prevalence and characterization of non-O157 Shiga toxin-producing *Escherichia coli* on carcasses in commercial beef cattle processing plants. Appl Environ Microbiol. 68(10): 4847-52.

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