United States Department of Agriculture
Food Safety and Inspection Service
Office of Public Health Science
Laboratory QA Staff
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Laboratory Guidebook
Notice of Change

Chapter new, **revised**, or archived: MLG 5B.05

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

Effective Date: 06/29/14

Description and purpose of change(s):

The method has been extended for use on raw ground beef mixed with raw pork and/or raw poultry products.

A centrifuge step was added for analyzing raw beef mixes containing poultry on the post-enrichment PCR rapid screens.

The incubation time of sheep blood agar plates inoculated for biochemical tests was expanded from 18-24 hours to 16-24 hours at 35 ± 2°C.

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 as detailed in the document titled "FSIS Guidance for Evaluating Test Kit Performance" available on the FSIS website.

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Title: Detection and Isolation of non-O157 Shiga Toxin-Producing *Escherichia coli* (STEC) from Meat Products and Carcass and Environmental Sponges		
Revision: 05	Replaces: 04	Effective: 06/29/14

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 utilizes the BAX® STEC suite followed by cultural isolation. The BAX® System Real-time PCR Screening Assay for *stx* and *eae* 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*. Two additional BAX® real-time PCR assays, STEC Suite Panel 1 and Panel 2, 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 mRBA. A post-IMS acid treatment step is performed to help reduce background microflora 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 BAX® real-time 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 Safety Data Sheets (SDS) 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 SDS.

5B.3 Equipment, Reagents and Media

5B.3.1 Equipment and Materials

a. Balance, sensitivity ± 0.1 g
b. Blending/mixing equipment: Paddle blender, Sterile Osterizer-type blender with sterilized cutting assemblies, and blender jars or equivalent and adapters for use with Mason jars
c. Sterile plain, clear polypropylene bags (ca. 24" x 30 - 36"), or Whirl-Pak™ type bags (or equivalent)
d. Incubators, static 42 ± 1°C and 35 ± 2°C
e. PCR tube holder (Qualicon or equivalent).
f. Cell lysis tube cooling block (Qualicon or equivalent) held at 5 ± 3ºC
g. PCR cooling block (Qualicon or equivalent) held at 5 ± 3ºC
h. Heating block set at 37 ± 2ºC
i. Heating block set at 95 ± 3ºC
j. Repeating pipettor to deliver 200 ± 20 µl and sterile tips
k. Pipettor to deliver 20 ± 1µl, and sterile disposable filtered tips
l. Pipettor to deliver 150 ± 15 µl, and sterile disposable filtered tips
m. Eight-channel pipettor to deliver 30 ± 3 µl, and sterile disposable tips
n. Pipettor to deliver 5 ± 1 µl, and sterile disposable tips.
o. 12 X 75 mm Falcon 352063, or equivalent, tubes
p. Cell lysis tubes and caps, cell lysis tube rack and box (Genemate® 8 strip tubes, ISC Bioexpress, T-3120-5 or equivalent)
q. Pipettor or pipettes to deliver 5 ml
r. Dupont Qualicon BAX® System Q7 Instrument
s. BAX® System Real–time PCR Assay STEC Screening (Part # D14642964) held at 5 ± 3ºC
t. BAX® System Real–time PCR Assay STEC Panel 1 (Part # D14642970) held at 5 ± 3ºC
u. BAX® System Real–time PCR Assay STEC Panel 2 (Part # D14642987) held at 5 ± 3ºC
v. Micropipettors for culture plating to deliver volumes ranging from 15-1000 µl with sterile disposable filtered tips
w. VITEK® 2 system
x. GN cards for VITEK® 2 system (bioMerieux Vitek, Inc.)
y. Heating block (95-99°C) or thermocycler for DNA preparation step

z. Vortexer
aa. Centrifuge that holds microcentrifuge tubes and is capable of speeds up to 16,000 x g
bb. Centrifuge plate adapter for the centrifugation of 96-well PCR plates
cc. Disposable, sterile pipettes for volumes 1.0 ml and for 5.0 ml.
dd. Sterile, inoculating loops, "hockey sticks" or spreaders, and needles
ee. Rotating tube agitator with clips to hold microcentrifuge tubes
ff. Sterile, disposable 12 x 75 mm polypropylene or polystyrene tubes
gg. Sterile microcentrifuge tubes (1.5 - 2.0 ml)
hh. Sterile 50 ml conical tubes
ii. Sterile 40 µm Cell Strainer
jj. MACS® Large Cell Separation Columns (Miltenyi Biotec # 422-02)
kk. OctoMACS® Separation Magnet (Miltenyi Biotec # 421-09)
ll. Multistand to support OctoMACS® Separation Magnet (Miltenyi Biotec # 423-03)
mm. Tray, autoclavable, approximately 130 mm x 83 mm for use with the OctoMACS®
nn. Sterile filter or non-filter bags
oo. Optical density reader

5B.3.2 Media and Reagents

a. Modified Tryptone Soya Broth (mTSB)
b. Modified Rainbow Agar (mRBA) [Rainbow® Agar O157 Biolog Inc., Hayward California, 94545] containing 5.0 mg/L sodium 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. Romer Labs 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. Biochemical test kit and system, GN cards (VITEK® 2 system, bioMerieux Vitek, Inc., 595 Anglum Drive, Hazelwood, MO 63042-2395)

m. 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 ±2%.

b. All media, plates and buffers shall be warmed to 18-35°C prior to use.

c. 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. In the absence of a positive test sample, control cultures may be terminated at the same point as the sample analyses. 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

Note: In the absence of a positive test sample, control cultures may be terminated at the same point as the sample analyses.

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) control.

5B.4.3 BAX® Real-time PCR Controls

a. ***stx/eae* screen PCR**
- 20 µl enrichment from bioluminescent *E. coli* O157:H7 strain 465-97 (growth control)
- DNA template (5 µl) from a cocktail of top six STEC cultures (PCR positive control)
- Uninoculated mTSB medium (20 µl)

b. **Serogroup-specific screen PCR (Panel 1 and Panel 2)**
- DNA template (5 µl) from a cocktail of top six STEC cultures (PCR positive control)
- Uninoculated mTSB medium (20 µl)

c. **Optional *stx/eae* presumptive PCR / *stx/eae* confirmatory PCR**
- DNA template (5 µl) from a cocktail of top six STEC cultures (PCR positive control)

d. **Optional serogroup-specific presumptive PCR (Panel 1 and Panel 2) / Serogroup-specific confirmatory PCR (Panel 1 and Panel 2)**
- DNA template (5 µl) from a cocktail of top six STEC cultures (PCR positive control)

To prepare PCR positive control DNA template, FSIS laboratories shall grow the top six STEC cultures on SBA and incubate at 35±2°C for 16-24 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, transferred to smaller volume tubes, and stored at ≤ -20°C for 1 year.

5B.4.4 IMS Plating Controls

Streak an isolate from 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. For raw beef, raw beef mixes, beef trim, and trim components, 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. Add 975 ± 19.5 ml of mTSB to the test portion to provide a 1:4 dilution (one portion of product to three portions of broth). Pummel, blend or hand massage until well mixed.

   Incubate the test portion and the enrichment media at 42±1°C for 15-24 hours. Each group of samples should include a positive control enrichment (*E. coli* O157:H7 strain 465-97) and an uninoculated enrichment medium control.

b. For environmental sponges and carcass sponges with 10 ml of buffer, add 50 ± 5 ml of mTSB broth. For carcass sponges with more buffer, use a 1:6 ratio of mTSB (for example, a swab with 25 ml of buffer will use 125 ml of enrichment broth) to each bagged sponge sample. Pummel, blend or hand massage until well mixed.

5B.6 Screening Procedure using BAX® Real-time PCR

5B.6.1 Procedure

Following incubation, perform the rapid screen using 20 µl of mTSB sample enrichment for all matrices except raw beef mixes containing poultry. Follow the current BAX® System User's Guide for preparing reagents, performing the STEC screening PCR, Panel 1 and Panel 2 PCR, and interpreting results, if applicable. The

real-time PCR assay developed for the ABI 7500 FAST is an alternative screen described in MLG 5B Appendices 1 and 3.

Following incubation of raw beef mixes containing poultry, a centrifuge step must be performed prior to BAX® screening:

- Dispense 200 ± 20 µl lysis reagent to each cell lysis tube.
- Heat the filled lysis tubes for 20 ±1 minute at 37 ± 2°C. Aseptically transfer 1 ml of the poultry mix enrichment sample to a sterile 1.5 ml microcentrifuge tube.
- Centrifuge at a setting of 1,500 x g for 1 minute (at speed) to pellet large debris. Supernatant will still not be clear at this low speed but should no longer have large particles of meat suspended.
- Transfer the supernatant to a new sterile 1.5 ml microcentrifuge tube. It is essential to ensure that none of the pelleted debris is carried over with the supernatant.
- Centrifuge supernatant at 10,000 x g for 5 minutes.
- Discard the supernatant from the centrifuge tube, leaving a little of the supernatant if necessary so the pellet is not disturbed during this step.
- Suspend the pellet in 100 µl of PCR grade water either by vortexing or using the pipet tip.
- Add 5 µl of the suspension directly to the pre-heated lysis buffer that was prepared during the initial steps.
- Heat the inoculated lysis tubes for 10 ± 1 minute at 95 ± 3 °C. Perform remainder of the PCR test according to manufacturer's instructions.

5B.6.2 Interpretation of Results

a. Samples that test negative for the BAX® STEC screening PCR (*stx*, *eae*) shall be reported as negative. Samples that test positive for the STEC screening PCR (*stx*, *eae*) will be further analyzed by using the positive BAX® lysate in the Panel 1 and Panel 2 tests. Samples must remain chilled at 2-8°C until loaded into the instrument. Remaining lysate may be sealed and stored for additional testing with other BAX® System STEC suite assays. Lysates may be stored at 2-8°C for up to 7 days or at -20 ± 3°C for up to 14 days.
Note: For Panel 1 and Panel 2 results, each well must be clicked individually and the results for each individual O-group should be recorded.

b. Samples that test positive for the STEC screening PCR (*stx, eae*) but negative for both Panel 1 and Panel 2 shall be reported as negative. If any of the O-groups from Panel 1 or Panel 2 are positive, the sample shall be reported as a potential positive. Proceed with the isolation procedure as described in Section 5B.7.

c. Samples that are indeterminate or have an invalid result for the BAX® STEC screening PCR (*stx, eae*) should be tested again using STEC screening PCR and Panels 1 and 2 assays using either the same lysate or preparing new lysate tubes.

Samples that are BAX® STEC screening PCR (*stx, eae*) positive but indeterminate or have an invalid result on one or both Panel 1 and 2 assays proceed to Section 5B.7 Isolation Procedure and analyze for the indeterminate O groups.

Alternatively, the laboratory may review the cause and perform a correction. Based on the findings, the laboratory may:

- repeat the BAX® analysis from the rack loading step or
- prepare new BAX® tubes and repeating the analysis.

d. In analytical runs where the positive control tests BAX® -negative, indeterminate, or has a signal-error result, the entire batch of samples is affected and a review of the cause and a correction shall be performed. Based on the findings the laboratory may:

- repeat the BAX® analysis from the rack loading step
- prepare new BAX® tubes and repeating the analysis
- analyze all of the samples culturally.

If reanalysis of a sample with indeterminate or invalid BAX® results is unsuccessful then use the alternative screen, perform cultural analysis, prepare fresh analytical portions from the sample reserve, or discard the sample.

5B.7 Isolation Procedure

Samples that are potentially positive by PCR screen results shall be plated onto 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 the 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 latex agglutination reagents specific for the serogroup of interest. Colony 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 mRBA. 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.

Following a restreak of presumptive colonies and 16-24 h incubation of the SBA, agglutination-positive colonies shall be confirmed with BAX® real-time PCR and biochemical identification. The confirmatory BAX® real-time PCR shall include the Screening assay (*stx* and *eae*) and the O-group Panel which includes the serogroup that the colony had a positive agglutination reaction (i.e. Panel 1 for O26, O111, O121, and Panel 2 for O45, O103, and O145). If no colony picks isolated from the mRBA confirm by PCR and VITEK® 2, the sample is negative for non-O157 STEC. If a FSIS Laboratory has confirmatory test results insufficient to allow identification (i.e. confirmatory PCR positive but biochemically negative), then the isolate is transferred to the Outbreaks Section of the Eastern Laboratory Microbiology Branch (OSEL), or current FSIS reference laboratory, for further testing prior to reporting.

5B.8.1 Presumptive PCR Assay

A PCR test may be performed directly on agglutination positive colonies from the mRBA to verify presumptive positive colonies using the following procedure. The presumptive PCR assay is optional for non-FSIS laboratories.

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).
b. Add 5 µl from the suspension to a BAX® lysis tube. Heat lysate at 37 ± 2°C for 20 ± 1 minute then 95 ± 3°C for 10 ± 1 minute then cool for 5-30 minutes in cooling block. Add 30 µl of the lysate to the BAX® Real-time Screening assay (*stx*/*eae*) reaction tube and the appropriate Panel reaction tube each on a cooling block. **Note:** Each PCR assay shall include a positive control as described in Quality Control section 5B.4.
c. The sample is considered negative if any of the 3 PCR targets (*stx*, *eae* or serogroup) are negative.
d. If an agglutination positive colony from mRBA is positive for O group, *stx a*nd *eae* targets, the sample is considered a presumptive positive for non-O157 STEC. Refer to section 5B.8.2 for confirmation of the isolates as non-O157 STEC.
e. From the previous suspension, streak SBA for isolation. Incubate inoculated SBA plates at 35 ± 2°C for 16-24 hours.

5B.8.2 Serological Agglutination and Confirmation PCR 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 presumptive PCR screen from colonies isolated on mRBA, refer to section 5B.8.2 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 16-24 hours.
c. Following SBA incubation, perform the agglutination test again on colonies from the SBA plate.
d. To confirm agglutination-positive colonies using BAX® real-time PCR, prepare a template by suspending an agglutination positive colony from the SBA plate in 50 µl of Molecular Grade Water and adding 5 µl of this suspension to BAX® lysis buffer.

e. Continue with the BAX® system protocol from the "Perform Lysis" step. The BAX® lysate will then be used for the STEC Screening assay and the appropriate Panel assay Note: Each PCR assay shall include a positive control as described in Quality Control section 5B.4.

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, BAX® real-time 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 BAX® real-time PCR negative for *stx, eae,* top six serogroup genes, the sample is negative for 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. For long term storage, freeze cultures using cryo-beads, i.e., Cryostor™ or lyophilize.

5B.10 Selected References

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