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Laboratory Guidebook
Notice of Change

Chapter new, **revised**, or archived: MLG 5.09

Title: Detection, Isolation and Identification of *Escherichia coli* O157:H7 from Meat Products and Carcass and Environmental Sponges

Effective Date: 01/15/15

Description and purpose of change(s):

A Real-time PCR has been added as a confirmatory test for Shiga toxin gene detection with the Shiga toxin detection assay as an alternative confirmation test.

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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Procedure Outline

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5.1 Introduction

The following method is used for the analysis of raw and ready-to-eat meat products, and laboratory environmental sponge samples for *Escherichia coli* O157:H7. The method is based on enrichment in a selective broth medium, application of a rapid screening test, immunomagnetic separation (IMS) in paramagnetic columns, and plating on a highly selective medium.

Unless otherwise stated all measurements cited in this method have a tolerance of ± 2%.

5.2 Safety

E. coli O157:H7 is a human pathogen with a low infectious dose (ingestion of 100 cells can cause disease). The use of gloves and eye protection is mandatory 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) should 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.

5.3 Quality Control Practices

a. All media and E-Buffer must be pre-warmed to 18-35ºC prior to use.

b. The fluorescent strain of *E. coli* O157:H7 is used in this procedure to monitor for cross contamination. The protocol for the use of the fluorescent strain of *E. coli* O157:H7 as a positive control follows:

Wild-type strains of *E. coli* O157:H7 transformed with pGFP produce a green fluorescent protein. As a result of this transformation, fluorescent strains of *E. coli* O157:H7 possess the unique property of expressing bright green fluorescence visible in the dark when illuminated by long-wave UV light. This property, which sets them apart from typical *E. coli* O157:H7, makes them useful positive controls for analyses of meat samples for *E. coli* O157:H7. At different steps in the procedure, both test samples and (fluorescent) positive controls can be tested for the bright green fluorescence as a quality control measure to make sure that positive sample isolates actually came from the test sample and not from accidental contamination by the positive control cultures.

Fluorescent cultures can be subjected to *E. coli* O157:H7 isolation and identification procedures without losing their fluorescent properties. These strains retain their fluorescent properties when grown in SOB media with added ampicillin (SOB + A). These cultures must be transferred every 7 days to fresh SOB + A media, according to the protocol outlined below. The fluorescent colonies are ready to be used as positive controls on day 3 of the following protocol, and for the next 6 consecutive days without losing their fluorescent properties. If these cultures are not needed on a continuous basis, they can be stored at refrigeration temperatures on SOB + A agar plates in zip-lock bags or sealed with parafilm® for 1 month and then transferred, or started up again 2 days before needed. *Strict adherence* to the protocol described below is essential, in order to ensure that the fluorescent strains do not lose their ability to express green fluorescence.

i. Test the fluorescent *E. coli* O157:H7 strain (FSIS culture # EC 465-97 or the currently designated control strain) on SOB + A agar plate for fluorescence by illuminating colonies under long-wave UV light in the dark.

ii. Select only fluorescent colonies and inoculate a 10 ml tube of SOB+A broth. Incubate at 35 ± 2°C overnight.

iii. Streak the culture from the SOB + A broth onto a SOB + A agar plate. Incubate at 35 ± 2°C overnight.

iv. Examine colonies on the plate for fluorescence. The fluorescent colonies are ready to be inoculated into Modified Tryptone Soya Broth (mTSB) or Modified Tryptone Soya Broth + novobiocin (mTSB+n) at this stage. These cultures on SOB + A agar plates can be stored refrigerated and be used as positive controls for 6 more days. Incubate the inoculated enrichment broth positive control culture at 42 ± 1°C overnight, along with the test samples.

v. Continue analysis per Sections 5.5-5.7 and test the Tryptic soy agar with 5% sheep blood (SBA) Plates of the fluorescent positive controls and any positive sample cultures for fluorescence.

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

5.4 Equipment, Materials, Media, Reagents and Test Kits

5.4.1 Equipment

a. Balance, sensitivity to 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 with or without mesh (ca. 24" x 30 - 36"), or Whirl-Pak™ type bags (or equivalent)
d. Incubators, static 42 ± 1°C, 35 ± 2°C, and 22 ± 2°C
e. Micropipettors to deliver 15-1000 µl with sterile disposable filtered micropipette tips
f. Mechanical Pipettor with 1.0 ml, 5.0 ml, 10.0 ml sterile pipettes
g. Inoculating loops, "hockey sticks" or spreaders, and needles
h. UV light (long-wave, e.g. VWR # 36553-124)
i. Filter unit, 0.2 µm, nylon, sterile
j. Infrared thermometer
k. LabQuake® Agitator with clips to hold microcentrifuge tubes
l. Sterile disposable 12 x 75 mm polypropylene tubes (e.g. Fisher # 14-956-1B)
m. Microcentrifuge and sterile 1.5 ml microcentrifuge tubes
n. Sterile 50 ml conical tubes (e.g. Falcon® # 2070) or sterile bottles
o. Sterile 40 µm Cell Strainer (Falcon® # 2340)
p. MACS® Large Cell Separation Columns (Miltenyi Biotec # 422-02)

q. OctoMACS® Separation Magnet (Miltenyi Biotec # 421-09)
r. Multistand to support OctoMACS® Separation Magnet (Miltenyi Biotec # 423-03)
s. Tray, autoclavable, approximately 130 mm x 83 mm (e.g. VWR # 62663-222) for use with the OctoMACS®
t. VITEK® 2 system

5.4.2 Media, Reagents and Cultures

a. Modified Tryptone Soya Broth (mTSB)
b. Modified Tryptone Soya Broth with Novobiocin (mTSB+n)
c. Modified Rainbow® Agar (mRBA) [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
d. Tryptic soy agar with 5% sheep blood (SBA)
e. SOB + A Medium
f. E Buffer, approximately 7 ml per sample (Buffered Peptone Water, Bovine Albumin Sigma # A7906-500G and Tween-20®)
g. Disinfectant (Lysol® I. C., 2.0%)
h. Dynal® # 710.04 anti-*E. coli* O157 antibody-coated paramagnetic beads (Dynal Inc., Lake Success, NY 11042)
i. *E. coli* O157:H7 strain 465-97 (positive control used throughout method)
j. *E.coli* ATCC strain 25922 (optional negative control for the latex agglutination assay)

5.4.3 Test Kits

a. *E. coli* O157:H7 latex agglutination test kit (RIM® *E. coli* O157:H7 Latex Test Kit, REMEL, 12076 Santa Fe Drive, Lenexa, KS 66215)
b. Biochemical test kit and system, GN cards (VITEK® 2 system, bioMerieux Vitek, Inc., 595 Anglum Drive, Hazelwood, MO 63042-2395)
c. BAX® System Real–time PCR Assay STEC Screening (Part # D14642964) held at 5 ± 3ºC, or
d. Shiga toxin test kit [Premier® EHEC, cat. # 608096 (Meridian Diagnostics, Inc., 3471 River Hills Dr., Cincinnati, OH, 45244)

5.5 Detection Procedure

a. Sample Preparation

Note: Disinfect the surface of intact sample package(s) prior to opening.

i. For beef trim/trim components, raw ground beef and raw beef/pork or poultry mixes, prepare in a sterile strainer bag a single sample in enrichment broth with a 1:4 dilution (one portion of product in three portions of medium), e.g., 325 ± 32.5 g sample with 975 ± 19.5 ml mTSB broth. Pummel, blend or hand massage until clumps are dispersed.

Unless analyzing the entire submitted sample, prepare a representative sample of the product by taking approximately equal portions from a variety of locations.

ii. For environmental 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 sponge with 25 ml of buffer will use 125 ml of enrichment broth) to each sponge sample. Pummel, blend or hand massage until well mixed.

iii. For outbreak-related samples:
Raw Product: randomly collect thirteen 25 ± 1 g sub-samples (total of 325 ± 13 g) that are representative of the entire sample. When possible avoid the exterior surfaces of non-intact samples. Place each 25 ± 1 g sub-sample in a sterile strainer bag and add 225 ± 4.5 ml of mTSB for a 1:10 dilution. Pummel, blend or hand massage until clumps are dispersed.

Fermented Sausage or Cooked Meat Products: randomly collect thirteen 25 ± 1 g sub-samples (total of 325 ± 13 g) that are representative of the entire sample. When possible avoid the exterior surfaces of non-intact samples. Place each sub-sample in a sterile strainer bag and add 225 ± 4.5 ml (1:10 dilution) of mTSB+n. Pummel, blend or hand massage until clumps are dispersed.

iv. For Most Probable Numbers (MPN) determination, follow MPN instructions given in the specific program protocol or see MLG Appendix 2, Most Probable Number Procedure and Tables.

b. Incubate all bags (static) with their contents for 15 to 24 h at 42 ± 1°C for mTSB or 15 to 22 h at 42 ± 1°C for mTSB+n. Include a positive and uninoculated medium control for each group of samples tested. Use the fluorescent *E. coli* O157:H7 strain (FSIS culture # EC 465-97) as a positive control.

c. From the enrichment cultures in the sterile bags, the laboratory may perform the screening test for *E. coli* O157:H7 as described in MLG 5A. The enrichment culture may be analyzed immediately upon removal from the incubator without waiting for tempering to room temperature. To prevent clogging the pipette tip, be sure to collect the appropriate size sample from the enrichment culture outside the inner strainer bag.

d. Samples negative by the screening test can be reported as negative for *E. coli* O157:H7.

e. Begin isolation procedures from the enrichment culture for screen positive samples and controls.

5.6 Isolation Procedure

Note: Steps a.- i. may be performed in a sequence that is convenient to the laboratory personnel.

a. Prepare E Buffer by mixing 0.5 g Bovine Albumin and 50 µl Tween-20® into 100 ml Buffered Peptone Water (BPW). Filter sterilize (0.2 µm) and store at 2-8°C.

b. Remove mRBA plates from 2-8°C storage, allowing 4 plates for each screen-positive culture and each control, with the exception of 2 plates for the fluorescent *E. coli* O157:H7 control. 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.

c. Remove a bottle of E Buffer from 2-8°C storage. Decant 7 ml of E Buffer for each culture and each control into a sterile tube or bottle and allow it to warm to at least 18°C. (Return the stock E Buffer to 2-8°C.)

d. For each positive control and screen-positive culture to be analyzed, keep in order and label 50 ml conical centrifuge tubes so that the positive control is first, followed by all cultures. Maintain this order for subsequent steps.

e. For each positive control and screen-positive culture, label two sterile 1.5 ml microcentrifuge tubes (for step g and step t), one 50 ml conical centrifuge tube (for

step h) and four 12 x 75 mm capped tubes (one for step p). For each set of 12 x 75 mm tubes, label one tube and add 0.9 ml E Buffer to three of the four tubes (step q).

f. Prepare the Dynal® #710.04 *E. coli* O157:H7 immunomagnetic bead suspension by following Table 1 below. Be sure to include the positive control in the total number of cultures. Use the bead suspension immediately (step g), or hold at 2-8°C. Return the stock vial of Dynal® #710.04 *E. coli* O157:H7 immunomagnetic beads to 2-8°C.

g. Vortex the bead solution briefly (2-3 seconds), then add 50 µl to a labeled microcentrifuge tube (from step e), one for the control and screen-positive culture. Use immediately or hold these tubes at 2-8°C.

h. Place a 40 µm Cell Strainer on a labeled 50-ml conical centrifuge tube (from step e). Pipet 5 ± 1 ml of each control and enrichment culture into the respective Cell Strainer and collect at least 1.0 ml of filtrate.

i. Do not proceed with more than the number of tubes that the OctoMacs® magnet(s) will hold. Transfer 1.0 ml of a filtrate (step h) to the corresponding microcentrifuge tube containing the immunomagnetic bead suspension (step g) and place in the clips of the LabQuake® tube agitator. Rotate the tubes for 10-15 min at 18-30°C.

j. Attach the OctoMACS® Magnet to the Multistand.

k. Position a tray on the base of the Multistand so that it will collect the filtrate passing through the columns. Add approximately 300 ml of 2% Lysol® I. C. disinfectant to cover the bottom of the tray.

l. Label and place the appropriate number of Large Cell Separation columns on the OctoMACS® Magnet. Insert columns from the front making sure the column tips do not touch any surfaces. Leave the plungers in the bags at this time to maintain sterility.

m. Transfer at least 0.5 ml E Buffer to the top of each column and let the buffer run through.

n. Resuspend, then transfer each culture and control from step i. to its corresponding column.

o. After a culture or control has drained through, wash the column by applying 1.0 ml of E Buffer to each column and allow to drain. Repeat 3 more times for a total of 4 washes.

p. 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 (from step e.). 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. If the OctoMACS® magnet is to be used for a second set of cultures, it must be decontaminated as described in step u, below. Repeat steps j-s for the additional cultures.

q. 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.

r. 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.

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

t. **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. Note: The fluorescent *E. coli* O157:H7 control sample is excluded as it does not grow following acid treatment.

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

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

w. 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.

x. 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.

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

z. *Optional*: Streak *E. coli* ATCC strain 25922 to TSA with 5% Sheep's Blood Agar for use as a latex negative control.

aa.. Decontaminate the OctoMACS® Magnet by applying 2% Lysol® I. C. disinfectant directly to the surface. After approximately ten minutes, rinse with deionized or tap water. Allow the unit to air-dry or use absorbent paper towels to dry the unit.

Table 1. Immunomagnetic Bead Suspension Volumes

# of Cultures	*ul of Beads**	*ul of E-Buffer*	*# of Cultures*	*ul of Beads**	*ul of E-Buffer*
1	15	135	26	145	1305
2	20	180	27	150	1350
3	25	225	28	155	1395
4	30	270	29	160	1440
5	35	315	30	165	1485
6	40	360	31	175	1575
7	45	405	32	180	1620
8	50	450	33	185	1665
9	55	495	34	190	1710
10	60	540	35	195	1755
11	65	585	36	200	1800
12	70	630	37	205	1845
13	75	675	38	210	1890
14	80	720	39	215	1935
15	85	765	40	220	1980
16	90	810	41	230	2070
17	95	855	42	235	2115
18	100	900	43	240	2160
19	105	945	44	245	2205
20	110	990	45	250	2250
21	120	1080	46	255	2295
22	125	1125	47	260	2340
23	130	1170	48	265	2385
24	135	1215	49	270	2430
25	140	1260	50	275	2475

* Dynal® anti-*E. coli* O157:H7 antibody-coated paramagnetic beads (vortex briefly before use)

5.7 Identification and Confirmation

a. After incubation, *E. coli* O157:H7 colonies have black or gray coloration on modified Rainbow® Agar. When *E. coli* O157:H7 colonies are surrounded by pink or magenta colonies, they may have a bluish hue. Mark colonies typical of *E. coli* O157:H7 and perform latex agglutination assays for O157, following manufacturer's instructions. Samples that have non-typical colonies on mRBA or typical colonies that are latex agglutination negative for O157 can be reported as negative for *E. coli* O157:H7. Streak all latex positive colonies, up to a total of five from each sample (one per sub-sample, if possible) onto SBA plates. Incubate SBA plates for 16-24 h at 35 ± 2°C.

b. After incubation, examine the SBA plates for purity under visible light, and evidence of cross contamination with the positive control by using long wave UV light. Only the positive control culture, *E. coli* O157:H7 strain 465-97, should fluoresce. If the SBA plates appear pure and uncontaminated, perform the following confirmatory tests:

i. Biochemical confirmation.
Inoculate VITEK® 2 GN cards (if using VITEK® 2 Compact).

ii. O157 and H7 confirmation.
To confirm the absence or presence of O157 and H7 antigens, use an *E. coli* O157:H7 latex test agglutination kit (RIM® *E. coli* O157:H7 Latex Test Kit). Use growth from the SBA plate (from step b). For inconclusive results, genetic testing (e.g. PCR) may be necessary.

iii. Shiga toxin/toxin genes confirmation.
The presence of Shiga toxin(s) in a culture isolate(s) is confirmed by the use of a toxin assay, e.g., Meridian Premier® EHEC Kit. When Shiga toxin(s) is (are) not demonstrated, detection of one or more toxin genes by PCR is used for confirmation. NOTE: The positive control culture, *E. coli* O157:H7 (FSIS culture # EC 465-97), is toxin-negative.

Alternatively, the toxin gene PCR assay, e.g., BAX® System Real–time PCR Assay STEC Screening (*stx, eae*) may be performed in lieu of the toxin assay.

c. To perform confirmation of O157 and H7 antigens by serological agglutination and Shiga toxin gene(s) by BAX® System Real–time PCR Assay STEC Screening (*stx, eae*) use the following procedure:

- After SBA incubation, perform the *E. coli* O157:H7 latex test agglutination kit agglutination test on colonies from the SBA plate.
- To confirm agglutination-positive colonies using BAX® real-time PCR Assay STEC Screening (*stx, eae*), 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.
- Continue with the BAX® system protocol. The BAX® lysate will then be used for the PCR assay.
- Additionally, perform biochemical identification (VITEK® 2) on agglutination positive colonies from the incubated SBA.
- If the isolate is serologically positive for "O157", BAX® real-time PCR positive for the *stx/eae* gene and biochemically identified as *E. coli*, the sample is positive.

d. FSIS uses the following definitions for reporting *E. coli* O157:H7:

- Potential Positive - a sample that causes a positive reaction with the screen test
- Presumptive Positive - a sample that has typical colonies, observed on modified Rainbow® Agar, and reacts specifically with O157 antiserum
- Confirmed Positive – a biochemically identified *Escherichia coli* isolate that is serologically or genetically determined to be "O157" that meets at least one of the following criteria:
    1) Positive for Shiga toxin (ST) production
    2) Positive for Shiga toxin gene(s) *(stx*)
    3) Genetically determined to be "H7"

e. If an FSIS Laboratory has an isolate or any additional presumptive positive colony picks from mRBA that are ultimately determined to be BAX® Real-time PCR negative or indeterminate for *stx/eae*, then the isolates are submitted to the Outbreaks Section of the Eastern Laboratory Microbiology Branch (OSEL), for further Shiga toxin gene and H7 gene PCR testing.

f. If a laboratory performs a toxin assay and the isolate fails to express Shiga toxin (toxin negative result), but a follow-up PCR assay detects the genes necessary to express Shiga toxin, then it is considered by the agency to be *E. coli* O157:H7 confirmed positive.

g. If the isolate was H7 negative and found to be Shiga toxin and gene negative (by EHEC test and genetic test for Shiga toxin genes) additional PCR test(s) for H7

gene(s) are performed. If the H7 PCR test is positive, the isolate is considered *E. coli* O157:H7 confirmed positive.

h. If the isolate is *E. coli* O157 presumptive positive, but additional tests show it to be H7 negative (by latex agglutination and PCR) and Shiga toxin negative (by EHEC test and genetic test for Shiga toxin genes), then the isolate would be reported as *E. coli* O157:H7 negative.

5.8 Storage of Cultures

For storage requirements of the fluorescent *E. coli* O157:H7 strain (FSIS culture # EC 465-97 or the currently designated control strain), refer to Section 5.3.c. of this chapter.

Store other "working" *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 chances of contamination. 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.

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