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Laboratory Guidebook
Notice of Change

Chapter new, **revised**, or archived: MLG 5.05

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

Effective Date: 10/01/2010

Description and purpose of change(s):

Section 5.2 *Safety Precautions* was clarified and Section 5.1.2 *Limits of Detection* was removed. An alternative technique for sample preparation was validated for Section 5.5.a. Raw beef mixed with other meat or poultry product was validated as an additional matrix. A single 325 g portion can be used as a sample preparation option to test raw ground beef, cooked meat patties, raw beef trim, and raw ground beef mixed with poultry or pork. In Section 5.7.b *Identification and Confirmation*, an additional test kit system was validated as an option for performing biochemical confirmations.

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.

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Title: Detection, Isolation and Identification of *Escherichia coli* O157:H7 from Meat Products		
Revision: 05	Replaces: MLG 5.04	Effective: 10/01/10

Procedure Outline

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 Material Safety Data Sheets (MSDS) 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 MSDS sheets.

5.3 Quality Control Practices

a. Rainbow® Agar plates have a shelf life of 2 weeks.

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

c. 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 TSB 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 mTSB+n

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.

5.4 Equipment, Materials, Media, Reagents and Test Kits

5.4.1 Equipment

a. Balance, sensitivity to 0.1 g
b. Stomacher™ 400 or 3500 with appropriate sizes of sterile Stomacher™ bags, with or without mesh (Tekmar Co., Cincinnati, Ohio), Bagpage® + 3500 (Interscience Lab. Inc., Boston MA) or equivalent bag mixer and bags
c. Incubators, static 42 ± 1°C, 35 ± 2°C, and 22 ± 2°C
d. Micropipettors to deliver 15-1000 µl with sterile disposable filtered micropipet tips
e. Mechanical Pipettor with 1.0 ml, 5.0 ml, 10.0 ml sterile pipettes
f. Inoculating loops, "hockey sticks" or spreaders, and needles
g. UV light (long-wave, e.g. VWR # 36553-124, or equivalent)
h. Filter unit, 0.2 µm, nylon, sterile
i. Infrared thermometer
j. LabQuake® Agitator (or equivalent) with clips to hold microcentrifuge tubes
k. Sterile disposable 12 x 75 mm polypropylene tubes (e.g. Fisher # 14-956-1B, or equivalent)
l. Microcentrifuge and sterile 1.5 ml microcentrifuge tubes
m. Sterile 50 ml conical tubes (e.g. Falcon® # 2070, or equivalent) or sterile bottles
n. Sterile 40 µm Cell Strainer (Falcon® # 2340, or equivalent)
o. MACS® Large Cell Separation Columns (Miltenyi Biotec # 422-02, or equivalent)
p. OctoMACS® Separation Magnet (Miltenyi Biotec # 421-09, or equivalent)
q. Multistand to support OctoMACS® Separation Magnet (Miltenyi Biotec # 423-03, or equivalent)
r. Tray, autoclavable, approximately 130 mm x 83 mm (e.g. VWR # 62663-222, or equivalent) for use with the OctoMACS®
s. VITEK® system, VITEK® 2 system, or equivalent

5.4.2 Media, Reagents and Cultures

a. Modified TSB broth with novobiocin plus casaminoacids (mTSB+n) Oxoid, or equivalent
b. Rainbow® Agar O157 (Biolog Inc., Hayward California, 94545) containing 10 mg/L novobiocin plus 0.8 mg/L potassium tellurite, or equivalent selective medium
c. Tryptic soy agar with 5% sheep blood (SBA)
d. SOB + A Medium
e. E Buffer, approximately 7 ml per sample (Buffered Peptone Water, Bovine Albumin Sigma # A7906-500G, or equivalent and Tween-20®, or equivalent)
f. Disinfectant (Lysol® I. C., 2.0%, or equivalent)
g. Dynal® # 710.04 anti-*E. coli* O157 antibody-coated paramagnetic beads (Dynal Inc., Lake Success, NY 11042), or equivalent
h. *E. coli* O157:H7 strain 465-97 (positive control used throughout method)
i. *E. coli* ATCC strain 25922 (negative control for bead capture and screen tests)

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), or equivalent

b. Biochemical test kit and system, GNI and GNI Plus cards (VITEK® system, bioMerieux Vitek, Inc., 595 Anglum Drive, Hazelwood, MO 63042-2395), GN cards (VITEK® 2 system), or equivalent

c. Shiga toxin test kit [Premier® EHEC, cat. # 608096 (Meridian Diagnostics, Inc., 3471 River Hills Dr., Cincinnati, OH, 45244), or equivalent

5.5 Detection Procedure

a. Sample Preparation

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

i. For raw ground beef and raw ground beef mixed with other meat or poultry product, prepare a single sample with a 1:4 ratio of product to enrichment broth in a sterile Strainer Stomacher™ bag (i.e. 325 ± 32.5 g sample with 975 ± 19.5 ml mTSB+n broth). Pummel for approximately two minutes in a Stomacher™.

Alternatively, prepare a sample with a 1:10 ratio of sample to enrichment broth using five 65 ± 2 g randomly collected subsamples representative of the entire sample in a sterile Strainer Stomacher™ bag (i.e. each 65 ± 2 g sub-sample with 585 ± 11.7 ml mTSB+n broth). Pummel for two minutes in a Stomacher™.

ii. For cooked meat patties, prepare a single sample with a1:4 ratio of product to enrichment broth in a sterile Strainer Stomacher™ bag (i.e. 325 ± 32.5 g sample with 975 ± 19.5 ml mTSB+n broth). Pummel for approximately two minutes in a Stomacher™.

Alternatively, prepare a sample with a 1:10 ratio of sample to enrichment broth using five 65 ± 2 g randomly collected subsamples representative of the entire sample in a sterile Strainer Stomacher™ bag (i.e. each 65 ± 2 g sub-samples with 585 ± 11.7 ml mTSB+n broth). Pummel for two minutes in a Stomacher™.

When testing fermented sausage products, prepare a sample with a 1:10 ratio of sample to enrichment broth using five 65 ± 2 g subsamples containing representative portions from both the outer surface (shell) and inner section (core).

iii. For beef trim/trim components, prepare a single sample with a 1:4 ratio sample of product to enrichment broth in a sterile Strainer Stomacher™ bag (i.e. 325 ± 32.5 g product with 975 ± 19.5 ml mTSB+n broth). Pummel for two minutes in a Stomacher™.

Alternatively, prepare a sample with a 1:10 ratio of sample to enrichment broth using five 65 ± 2 g randomly collected subsamples representative of the entire sample in a sterile Strainer Stomacher™ bag (i.e. each 65 ± 2 g sub-samples with 585 ± 11.7 ml mTSB+n broth). Pummel for two minutes in a Stomacher™.

Unless analyzing the entire submitted sample, prepare a representative sample of the product by taking approximately equal portions from a variety of locations. For non-comminuted components, FSIS inspectors submit samples that generously represent the surface of trimmings. For intact chunk beef or cuts, favor sampling of the surface to mimic submitted beef trim product and to provide the best opportunity for detection.

iv. For environmental sponge samples, add 50 ± 5 ml of mTSB+n broth to each bagged sponge sample. Pummel for two minutes in a Stomacher™.

v. For outbreak-related samples, 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 Stomacher™ bag and add 225 ± 4.5 ml of mTSB+n broth. Pummel for 2 minutes in a Stomacher™.

vi. 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 22 h at 42 ± 1°C. Include a positive, negative, 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 and *E. coli* ATCC strain 25922 as the negative control.

c. From the enrichment cultures in the Stomacher™ bags, the laboratory may perform the screening test for *E. coli* O157:H7. Refer to 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 and discarded.

e. Samples positive by the screening test should be reported as potential positives. Begin isolation procedures from the enrichment culture in the Stomacher™ bag.

5.6 Isolation Procedure

Note: Steps a.-l. 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 Rainbow® Agar plates from 2-8°C storage, allowing 3 plates for each screen-positive culture and each 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, negative control and screen-positive culture to be analyzed, order and label 50 ml conical centrifuge tubes so that the positive control is first, followed by the negative control, then all cultures. Maintain this order for subsequent steps.

e. For each positive control, negative control, and screen-positive culture, label two sterile 1.5 ml microcentrifuge tubes (for step g and step s), one 50 ml conical centrifuge tube (for step h.) and two 12 x 75 mm capped tubes (one for step p.). For each pair of 12 x 75 mm tubes, label one tube and add 0.9 ml E Buffer (for 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 and negative controls 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 each 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. (or equivalent) 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 0.5ml 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. Vortex the tubes from step p. briefly to resuspend the beads. 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 (from step e.).

r. Vortex briefly to maintain beads in suspension and plate 0.1 ml from each tube (from step p. and step q.) onto a labeled Rainbow® Agar 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.

s. Vortex the tubes containing undiluted beads (from step p.) and transfer to a labeled microfuge tube (from step e.) and centrifuge at least one minute using a bench-top microcentrifuge to concentrate the beads. Withdraw and discard the supernatant without disturbing the beads. Add 0.1 ml of E Buffer to the beads, resuspend the beads and transfer the beads to a labeled Rainbow® Agar plate. Spread plate the beads as described in step r.

t. As soon as there is no visible moisture on the agar surface, invert plates and incubate for 24-26 h at 35 ± 2°C.

u. Decontaminate the OctoMACS® Magnet by applying 2% Lysol® I. C. (or equivalent)

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.

# 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 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. 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.

Note: If no typical colonies are present, hold the original Rainbow® plates at 20-24°C for an additional 6-24 h then re-examine for typical colonies.

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-GNI or GNI Plus cards (if using VITEK® system), VITEK 2 GN cards (if using VITEK® 2 Compact), or an equivalent biochemical identification testing system. The cytochrome oxidase and gram stain tests are optional.

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, or equivalent). 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 is confirmed by the use of a toxin assay, e.g., Meridian Premier® EHEC Kit, or equivalent. 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.

c. 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 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"

If an FSIS Laboratory has confirmatory test results insufficient to allow identification, 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.

For example, if a presumptive positive isolate is tested in a FSIS laboratory for Shiga toxin and it is Shiga toxin negative, then the isolate is transferred to OSEL for further testing.

A Shiga toxin gene PCR test is performed in OSEL. If the isolate fails to express Shiga toxin (toxin negative result), but has the genes necessary to express Shiga toxin, then it is considered by the agency to be *E. coli* O157:H7 confirmed positive.

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.

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 opportunity for contamination. Cultures may be subcultured up to 5 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.

5.9 Selected References

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