

United States Department of Agriculture

Food Safety and Inspection Service

MLG 5C.01

Detection, Isolation, and Identification of Top Seven Shiga Toxin-Producing *Escherichia coli* (STEC) from Meat Products and Carcass and Environmental Sponges

This method describes the laboratory procedure for analysis of raw and ready-to-eat meat products, and laboratory environmental sponge samples for the seven targeted STEC serogroups (O157, O26, O45, O103, O111, O121 and O145).

Notice of Change

The FSIS Office of Public Health Science (OPHS) announces an update to the Agency confirmed criteria for *Escherichia coli* (*E. coli*) O157:H7. FSIS has aligned *E. coli* O157:H7 confirmation criteria, developed in 2008, with current FSIS laboratory confirmation criteria for non-O157 Shiga toxin-producing *E. coli* (STEC) developed in 2011. This alignment harmonizes *E. coli* O157 with non-O157 STECs for a unified STEC method and clarifies the FSIS STEC definition for public health partners and industry stakeholders. For a sample to identify as positive for STEC, the *E. coli* isolate must contain an *stx* gene, an *eae* gene, and genetically identify as one or more of the top seven serogroups (O26, O45, O103, O111, O121, O145, or O157). Harmonizing STEC confirmation testing creates a more efficient FSIS laboratory workflow where all regulated STECs are treated the same from initial screening to full isolate characterization. This update will not affect current FSIS laboratory flow leading to potential and presumptive positive results.

To incorporate the updated criteria, FSIS has revised the MLG 5C chapter with these details:

- The chapter is updated with a new user-friendly format with informational diagrams and graphics that clarify the procedure requirements.
- The criteria for potential, presumptive, and confirmed STECs are revised to align the top seven serogroups with the updated Agency definition:
 - i. Enriched sample broths which yield a positive when screened on real-time polymerase chain reaction (PCR) for all targeted genes (*stx*1/2, *eae*, and any of the seven serogroups) are defined as potential positive STECs for one or more of seven target STEC serogroups.
 - ii. One or more typical colonies on modified Rainbow® Agar (mRBA) that agglutinate with STEC latex agglutination reagents and are positive on the presumptive PCR assay are defined as presumptive positive STECs.
 - iii. A sample is considered positive for STEC when the *E. coli* isolate belongs to one of the seven targeted serogroups and contains an *stx* gene and an *eae* gene.
- STEC definitions are described in the appropriate section of the method and listed in red coded text boxes labeled “KEY DEFINITION.”
- Quality Control criteria are described in text boxes at the beginning of each section.
- Additional guidance and background information for the method is listed under “KEY FACT(S)” throughout the chapter.
- Sample preparation instructions for N60 trim samples are added.
- The confirmation procedure is updated to clarify testing required for isolate identification, serological identification, and genetic confirmation of STEC.
- The *fliC* H7 PCR for *E. coli* O157 is infrequently utilized as a classification test for inconclusive isolates. The H7 PCR is now archived and removed from the confirmation procedure, aligning *E. coli* O157 with the updated definition of a confirmed STEC positive. Any isolate with an inconclusive STEC confirmation result is submitted for whole genome sequencing by FSIS laboratories.

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Introduction

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. FSIS utilizes the following performance criteria when evaluating the suitability of an alternative laboratory method or product for a given analyte and sample matrix pair:

- Sensitivity, specificity and accuracy of 90% or greater.
- Positive predictive value and negative predictive value of 90% or greater.

Performance criteria are relative to the reference cultural method for that analyte and sample matrix as outlined in the corresponding MLG chapter. Method validation is necessary to demonstrate the equivalence of alternative tests as detailed in the document titled “FSIS Guidance for Evaluating Test Kit Performance.”

Method Design

The screening and isolation 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, modified Rainbow® Agar (mRBA).

Enriched sample broths which yield a positive result when screened on real-time PCR for all of the targeted genes (*eae*, *stx1/2*, and any of the seven serogroups) are defined as **POTENTIAL POSITIVE** for one or more of seven target STEC serogroups.

Colonies on mRBA are then tested for the presence of O antigens using an agglutination test and screened with a presumptive rapid screening PCR.

One or more typical colonies on mRBA that agglutinate with STEC latex agglutination reagents and are positive on the presumptive PCR assay are defined as **PRESUMPTIVE POSITIVE** STECs.

Agglutination positive mRBA colonies are then streaked onto tryptic soy agar with 5% sheep blood (SBA) for confirmation. Colonies on SBA are tested for the presence of O antigens using an agglutination test then identified using VITEK 2 and a confirmation rapid screening PCR.

A sample that confirms as an *E. coli* isolate containing an *stx* gene, an *eae* gene, and one of the target serogroups is defined as a **CONFIRMED STEC POSITIVE**.

Safety Precautions

STECs are categorized as a Biosafety Level 2 human pathogen with a low infectious dose (ingestion of 100 cells can cause disease). CDC guidelines for manipulating Biosafety Level 2 pathogens should be followed whenever live cultures of Shiga toxin positive *E. coli* are used. The use of gloves and eye protection is recommended for all post-enrichment viable culture work. Work surfaces shall be disinfected prior to and immediately after use. A Class II laminar flow biosafety cabinet is recommended for activities in which infectious aerosols or splashes may be created. The Safety Data Sheets (SDS) must 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 SDSs.

Equipment, Reagents, Media, and Cultures

Table 1: Equipment for MLG 5C

Equipment	Supplier	Purpose
Balance, sensitivity to at least ± 0.1 g	General lab supplier	Weigh samples
Blending/mixing equipment: Paddle blender or equivalent	General lab supplier	Mix samples
Incubators, static $42 \pm 1^\circ\text{C}$, $35 \pm 2^\circ\text{C}$	General lab supplier	Incubation of primary enrichment, mRBA plates, and SBA plates
iQ-Check	Bio-Rad	Automated DNA extraction and plate set-up
Vortexer	General lab supplier	Mix reagents in tubes
Magnetic stir plate	General lab supplier	Stir lysis reagent. Used for manual deep well plate preparation
Centrifuge	General lab supplier	Prepare DNA template positive PCR control
Heating block ($95\text{--}100^\circ\text{C}$)	General lab supplier	Prepare DNA template positive PCR control
Bio-Rad real-time PCR System (CFX96)	Bio-Rad	Perform VirX and SerO analyses
Cooling block ($2\text{--}8^\circ\text{C}$)	General lab supplier	Prepare sample DNA
Agitator-incubator for deep well plates (e.g., BioShake iQ Thermoshaker)	BioShake Catalog #1808-0506-1021	Temperature-controlled shaker for cell lysis–Manual Method
Ultraviolet (UV) light (long-wave)	VWR #36533-124	Examine SBA plates for cross-contamination
Infrared thermometer	General lab supplier	Measure temperature of agitator-incubator
Microcentrifuge tube rotating agitator	General lab supplier	Agitate IMS bead suspension
OctoMACS® Separation magnet & stand	Miltenyi Biotec #130-042-109 Miltenyi Biotec #130-042-303	Cell separation for serogroup filtration
Tray, autoclavable, approximately 28 cm x 15 cm for use with the OctoMACS®	VWR Catalog #62663-222	Use with cell separation magnet stand
Biochemical test kit and system, GN cards VITEK® 2 system	bioMérieux Vitek, Inc.	Biochemical identification
Optical density reader	General lab supplier	Measure culture suspension

Table 2: Test Kits

Reagent	Supplier	Purpose
iQ-Check STEC VirX	Bio-Rad	PCR kit to detect virulence genes <i>stx1/2</i> and <i>eae</i>
iQ-Check SerO	Bio-Rad	PCR kit to detect serogroups O157, O26, O45, O103, O145, O111, and O121
IMS Sero-specific Beads	General supplier	Target sero-specific STEC by conjugating beads to surface
Latex Kit	General supplier	Sero-specific latex will agglutinate suspected colonies

Table 3: Supplies

Material	Supplier	Purpose
Sterile plain, clear polypropylene bags with or without mesh (ca. 24" x 30 - 36"), or Whirl-Pak type bags or equivalent	General lab supplier	Enrichment
Hard-shell 96-Well PCR Plates, PCR strips or equivalent	Bio-Rad Catalog #HSP9955XTU	PCR
Pipets and sterile filter tips for the 5uL, 20uL, 150uL to 1mL volumes	General lab supplier	Add and mix reagents
1.5 ml microcentrifuge tubes	General lab supplier	Elute, dilute, and store DNA
iQ-Check™ Deep Well Microplates	Bio-Rad Catalog #3594900	Lysing cells during DNA extraction
Pre-Pierced sealing film	Bio-Rad Catalog #3600040	Seal deep well DNA extract
Microseal A Film	Bio-Rad Catalog #MSA5001	Seal 96-well PCR plate
Microseal B Film	Bio-Rad Catalog #MSB1001	Seal 96-well PCR plate
5mL Tube for PCR Mix	Bio-Rad Catalog #3594901	Used with iQ-Check prep
50ul Filter Tips	Bio-Rad Catalog #3594902	Used with iQ-Check prep
1000ul Filter Tips	Bio-Rad Catalog #3594903	Used with iQ-Check prep
Sterile inoculating loops, "hockey sticks" or spreaders, and needles	General lab supplier	Spread /streak plates
Filter unit, 0.2 µm, nylon, sterile	General lab supplier	Filter enrichment broth into 50 ml conical tube
Sterile disposable 12 x 75 mm polypropylene or polystyrene tubes	Fischer Catalog #14-956-1B or Falcon Catalog #352063	IMS bead solution dilution/separation
Sterile 50 ml conical tubes	Falcon Catalog #2070	Storage enrichment broth
Sterile 40um Cell strainer	Falcon Catalog #2340	Filter enrichment broth into 50 ml conical tube
MACS® Large cell separation columns	Miltenyi Biotec Catalog #422-02	Filter IMS bead solution on magnetic stand

Media

- Modified Tryptone Soya Broth (mTSB)
- E Buffer for IMS procedure
- Modified Rainbow[®] Agar (mRBA)
- Trypticase Soy Agar with 5% Sheep Blood (SBA)
- Nutrient or BHI agar slants for isolate storage
- Molecular Grade Water for sterility control

Cultures and Controls

- E. coli* O157:H7 reference strain that visibly fluoresces under UV light; from Microbiologics UV-BioTAG [TM] EC 43 derived from FDA ESC1177 catalog #01227UV-V or equivalent.
- E. coli* serogroups O26, O45, O103, O111, O121, and O145 meeting the following genetic characteristics: *stx*⁺ and *eae*⁺; from the American Type Culture Collection (ATCC), the STEC Center at The Michigan State University or the *E. coli* Reference Center at The Pennsylvania State University.

QUALITY CONTROL

Laboratory Quality Control Guidelines

The control cultures should be started at the same time and analyzed in the same manner as the samples. Confirm at least one isolate from the positive control sample. However, for safety considerations, toxin-attenuated or toxin-negative strains that have a typical appearance on mRBA 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. See Table 4 for Quality Control (QC) controls needed at each step of the procedure.

A tagged *E. coli* O157:H7 reference strain that visibly fluoresces under ultraviolet (UV) light is used as a positive control along with an uninoculated media control that serves as a negative control for the enrichment process. A kit positive control that contains the DNA of all 7 STEC strains (*stx*⁺, *eae*⁺) and a kit negative control is used based on the requirements of the screening technology.

If the screening technology used has no kit required controls that contain all 7 STEC strains (*stx*⁺, *eae*⁺) then prepare a DNA template positive PCR control including the DNA of all 7 STEC strains (*stx*⁺, *eae*⁺). The top 7 *stx*⁺ and *eae*⁺ STEC control strains shall include *E. coli* O157:H7, *E. coli* O26, *E. coli* O45, *E. coli* O103, *E. coli* O111, *E. coli* O121, and *E. coli* O145. Such strains can be obtained through reference culture collection centers including but not limited to the ATCC, the STEC Center at The Michigan State University and the *E. coli* Reference Center at The Pennsylvania State University.

Table 4. QC controls required for the STEC method

Step	Controls
Enrichment	<ul style="list-style-type: none"> • O157+ • media sterility
VirX	<ul style="list-style-type: none"> • O157+ • media sterility
SerO 1, 2, 3	<ul style="list-style-type: none"> • O157+ for SerO 1 only
IMS	<ul style="list-style-type: none"> • O157+ control used for all O groups on IMS
mRBA	<ul style="list-style-type: none"> • O157+ plated • streak other O groups+ to mRBA as needed • media sterility plate
Serology	<ul style="list-style-type: none"> • O group+ control from mRBA • use kit controls
Presumptive PCR	<ul style="list-style-type: none"> • O group+ control from mRBA • use kit controls • add PCR water sterility control as negative control
Confirmatory PCR	<ul style="list-style-type: none"> • O group+ control from SBA • use kit controls • add PCR water sterility control as negative control
VITEK	<ul style="list-style-type: none"> • O group+ control from SBA

Enrichment Procedure

QUALITY CONTROL

Preparation of Sample Enrichment Controls

A tagged *E. coli* O157:H7 reference strain that visibly fluoresces under UV light will differentiate QC culture control strains from true contaminants. This tagged *E. coli* O157:H7 control will be included with each sample batch as a positive enrichment broth control. The positive control culture will be inoculated into an appropriate matrix free of the target analyte at a low inoculum level, e.g., by preparing a test organism suspension in broth or saline, equivalent in turbidity to a 0.5 McFarland standard. Using a 1 µl loop, inoculate the broth or streak the plates to be tested. Alternatively, commercially prepared bacterial pellets may be used.

An uninoculated media control will be included with each sample batch as a negative enrichment broth control.

KEY FACTS

Sample temperature of $\leq 15^{\circ}\text{C}$ is required upon receipt.

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

FSIS regulatory programs require *Salmonella* testing in concurrence with STEC for raw beef products. mTSB serves as a universal media for culturing STEC and *Salmonella* from a single sample.

Sample Preparation and Primary Enrichment

- For beef trim/raw ground beef 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 enrichment broth), e.g., 325 ± 32.5 g sample with 975 ± 19.5 ml mTSB broth. Pummel, blend, or hand massage until clumps are dispersed.
- For N60 trim samples, sixty pieces of raw beef trim are utilized to prepare a sample. If the beef trim pieces are overweight, two sub-samples may be needed to process the sample. Using the sixty trim pieces, prepare a single 325 ± 32.5 g sample with 975 ± 19.5 ml mTSB broth. If raw beef trim remains after the first sample, use remaining meat to prepare a second sub-sample weighing a minimum of 65 ± 2 g and maximum of 325 ± 32.5 g. The final weight of this second sub-sample must be ≥ 63 g and ≤ 357.5 g. Enrich the second sub-sample at the same 1:4 dilution (one portion of product in three portions of enrichment broth).
- For environmental and carcass sponges with 10 ml of buffer, add 50 ± 5 ml of mTSB broth. For carcass sponges containing 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.
- Incubate all bags (static) with their contents for 15 to 24 h at $42 \pm 1^{\circ}\text{C}$ for mTSB or 15 to 22 h at $42 \pm 1^{\circ}\text{C}$ for products that use mTSB+n. Include a positive and uninoculated medium control for each group of samples tested.

Rapid Screening Procedure

QUALITY CONTROL

Rapid Screen PCR Controls

The STEC Rapid Screening PCR uses *stx/eae* PCR controls and O-group specific PCR controls.

- a. The *stx/eae* PCR controls for the iQ-Check™ STEC VirX screening include:
 - Positive enrichment control – enrichment inoculated with the *E. coli* O157:H7 reference control strain.
 - Negative enrichment control – uninoculated enrichment.
 - Kit positive control or DNA template positive PCR control including the DNA of all 7 STEC strains that are *stx*⁺ and *eae*⁺.
 - Kit negative control if required by the technology.
- b. Serogroup-specific PCR controls for the iQ-Check™ STEC® SerO tests include:
 - Positive control – enrichment inoculated with the *E. coli* O157:H7 reference control strain
NOTE: This is a positive control on the SerO 1 kit only for *E. coli* O157:H7; no positive serogroup-specific control available for SerO 2 or SerO 3 PCR kits.
 - Kit positive control or DNA template positive PCR control including the DNA of all 7 STEC strains that are positive for the O-groups of interest.
 - Kit negative control if required by the technology.
- c. To prepare a DNA template positive PCR control in the absence of a kit positive control:
Grow the top 7 STEC cultures on SBA and incubate at 35±2°C for 16-24 h. Pick typical colonies to create culture suspensions in molecular grade water corresponding to approximately 10⁹ CFU/ ml (approximately 0.5 McFarland reading on a densitometer). In one tube, add 1.0 ml from each suspension to 3.0 ml of molecular grade water to create a 10.0 ml cocktail of all 7 strains. This will provide approximately a 10⁸ CFU/ml cocktail using each strain. 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 may be used as the PCR positive control. DNA control template can be prepared as a batch, transferred to smaller volume tubes, and stored at ≤ -20°C for 1 year.

One Step Rapid Screening PCR for *stx/eae* and O-group

- a. Following incubation, screen all enriched samples on the iQ-Check™ STEC VirX screening (*stx*, *eae*) PCR Assay. Follow the rapid screening technology user guide for preparing reagents, performing the test, and reading the results. The equipment must be operated, and all records documented, according to laboratory work instructions.
- b. Samples that test negative for *stx* and/or *eae* on the STEC screening PCR shall be reported as **negative**. Samples that test positive for the STEC screening PCR (*stx* AND *eae*) will be further analyzed for serogroup identification using iQ-Check™ STEC® SerO test. **NOTE:** Store sample lysates at 2-8°C after loading onto instrument.

KEY FACT

The primary virulence factor for STEC is production of the Shiga toxins (*stx*1/2). Not all toxin-producing strains can cause hemolytic uremic syndrome indicating the potential role of other virulence factors in disease such as intimin (*eae*). The FSIS initial screening method detects these virulence genes plus genetically identifies the seven targeted serogroups. Any additional screening procedures should identify alternative targets in the enriched sample to confidently rule out or identify the presence of STEC.

- c. Samples that test positive for the STEC screening (*stx* AND *eae*) but negative for the 7 serogroups shall be reported as **negative**. If serogroup positive, the sample shall be reported as a **potential positive**. Proceed with the isolation procedure.
- d. Lysates may be sealed and stored for additional testing at 2-8°C for up to 1 week or at ≤20°C for one year.

KEY DEFINITION

Enriched samples that test positive for the STEC screening PCR (*stx* AND *eae*) and serogroup during this rapid screening procedure are reported as a **POTENTIAL POSITIVE**. Potential positive samples proceed to the IMS bead procedure and mRBA for isolation.

TROUBLESHOOTING GUIDE FOR RAPID SCREEN ANALYSIS

If the rapid screening analysis fails, reanalyze samples from the enrichment broth. If reanalysis from the enrichment broth is unsuccessful, repeat sample preparation from the sample reserve or discard the sample. Contact technical support from the manufacturer if experiencing equipment issues. If circumstances (e.g., a power outage or equipment failure) do not allow testing using the rapid screen system, the laboratory shall, if possible, continue cultural analysis of all affected samples.

For Inconclusive Screening Results:

- a. Samples with inconclusive results for the screening (*stx*, *eae*) may be tested again using STEC screening assays using either the same lysate or preparing new lysate tubes.
- b. For samples that are *stx/eae* positive but have an inconclusive result on the O-group assays, proceed to the isolation procedure and analyze for the inconclusive O-groups. Alternatively, the laboratory may investigate. Based on the findings, the laboratory may:
 - repeat the rapid screen analysis from the lysate step, or
 - prepare new rapid screen lysate tubes and repeat the analysis.

For Control Issues:

- a. In analytical runs where the positive control results are not positive, perform an investigation as all samples are affected. Based on the findings the laboratory may:
 - repeat the rapid screen analysis from the lysate step,
 - prepare new rapid screen lysate tubes and repeat the analysis, or
 - analyze all samples culturally.
- b. In analytical runs where the sterility control results are positive, perform an investigation as all samples are affected. Based on the finding the laboratory may:
 - identify the root cause, evaluate sterility of media,
 - cancel the STEC analysis for the samples in the batch if the sterility control fails,
 - repeat the rapid screen analysis from the lysate step, or
 - prepare new rapid screen lysate tubes and repeat the analysis.

Isolation Procedure

QUALITY CONTROL

Lab Quality Control Procedures for IMS Procedure and mRBA Plating

For the STEC IMS Control, use:

- Positive Control – For all serogroups, use the enrichment inoculated with the *E. coli* O157:H7 reference control strain.

For the mRBA Plating Controls, use:

- Positive Control for O157:H7 potential positive – Use the enrichment inoculated with the *E. coli* O157:H7 reference control strain for IMS.
- Positive Control for non-O157 potential positive – 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.
- Negative Control – the uninoculated mRBA.

Samples that are potentially positive from PCR screening 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 (e.g., anti-O157 will be used for samples with screen results positive for O157).

KEY FACT

The acid treatment step during the IMS isolation procedure is designed to reduce background flora in samples before plating on mRBA plates.

- Remove mRBA plates from 2-8°C storage, obtaining 4 plates for each screen-positive culture and two plates for the fluorescent *E. coli* O157:H7 control. Include the uninoculated mRBA for a negative control. Be sure the plates have no visible surface moisture at the time of use. NOTE: For non-O157 STECs, streak a control isolate from the serogroup(s) of interest (based on serogroup-specific screen results) onto mRBA and incubate along with the samples that have been treated with the IMS procedure.
- Remove a bottle of E Buffer from 2-8°C storage. Decant E Buffer for each positive serogroup identified in the sample and control into a sterile tube or bottle and allow it to warm to at least 18°C.
- For the O157:H7+ control and each screen-positive culture to be analyzed, prepare and label:
 - 50 ml conical centrifuge tube,
 - Two 1.5 ml microcentrifuge tubes, and
 - Four* 12 x 75 mm capped tubes filled with 0.9 ml E Buffer in 3 of the 4 tubes.*The acid treatment step is not performed on O157+ control so only two 12 x 75 mm capped tubes needed for positive control.
- Place a 40 µm Cell Strainer on a labeled 50-ml conical centrifuge tube. Pipet at least 5 ± 1 ml of each O157:H7+ control and enrichment culture into the respective Cell Strainer and collect at least 1.0 ml of filtrate. Additional amounts may be needed for multiple serogroups.
- Transfer 50.0 µl (or volume recommended by the manufacturer) of appropriate immunomagnetic capture beads determined by the serogroup PCR screen results to a sterile, labeled microcentrifuge tube.

NOTE: For *E. coli* O157:H7, prepare *E. coli* O157:H7 immunomagnetic bead suspension by following Table 5 below if using anti-*E. coli* O157 Dynabead® (Catalogue #71004 from Applied BioSystem). For equivalent products, follow manufacturer instructions. Include the positive control in the total number of cultures. Vortex the bead solution briefly (2-3 seconds), then add 50 µl to a labeled microcentrifuge tube, one for the control and each screen-positive culture. Use bead suspension immediately or hold these tubes at 2-8°C.

- f. Transfer 1.0 ml of a filtrate to corresponding microcentrifuge tube containing the immunomagnetic bead suspension and place in the clips of the LabQuake® tube agitator. Rotate the tubes for 10-15 min at 18-30°C.

- g. Attach the OctoMACS® Magnet to the Multistand.

- h. 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.

- i. 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.

- j. Transfer at least 0.5 ml E Buffer to the top of each column and let the buffer run through, then following agitation, transfer each culture and control to its corresponding column.

- k. 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.

- l. After the last wash has drained, remove the column from the OctoMACS® Magnet and insert the tip into the 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.

TABLE 5. *E. coli* O157 Immunomagnetic Bead Suspension Volumes

Immunomagnetic Bead Suspension Volumes for <i>E. coli</i> O157		
# of Cultures	µl of Beads*	µl of E-Buffer
1	15	135
2	20	180
3	25	225
4	30	270
5	35	315
6	40	360
7	45	405
8	50	450
9	55	495
10	60	540
11	65	585
12	70	630
13	75	675
14	80	720
15	85	765
16	90	810
17	95	855
18	100	900
19	105	945
20	110	990
*Dynal® anti- <i>E. coli</i> O157:H7 antibody-coated paramagnetic beads (vortex briefly before use)		
NOTE: Use a 1:10 dilution of stock beads for larger sample numbers		

- m. 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, vortex. 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.
- n. Vortex briefly to maintain beads in suspension and plate 0.1 ml from each tube (1:10 dilution and 1:100 dilutions) onto labeled mRBA plates. Use a hockey stick or spreader to spread plate the beads.
- o. **Acid Treatment:** For each sample, transfer 450 μ l of the undiluted bead suspension (MACS column eluant) to an empty labeled microcentrifuge tube. Add 25 μ l of 1N hydrochloric acid (HCl) to this bead suspension and vortex briefly. Note: For the *E. coli* O157 serogroup, the fluorescent *E. coli* O157:H7 control sample is excluded as it does not grow following acid treatment.
- p. Place the microcentrifuge tubes containing the acid treated suspension on a LabQuake[®] Agitator and rotate tubes for 1 hour at 18-30°C.
- q. Dilute the suspension by adding 475 μ l of E buffer.
- r. Vortex briefly to maintain beads in suspension and plate 0.1 ml of the diluted suspension onto a labeled mRBA plate. Use a hockey stick or spreader to spread plate the beads.
- s. Add 0.1 ml of the suspension to a labeled tube containing 0.9 ml E buffer and vortex briefly, representing a 1:10 dilution of the acid-treated cell suspension. Plate 0.1 ml of the diluted suspension onto a labeled mRBA plate.
- t. As soon as there is no visible moisture on the agar surface, invert plates and incubate for 20-24 h at 35 \pm 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.

Figure 1. Dilution Preparation for mRBA

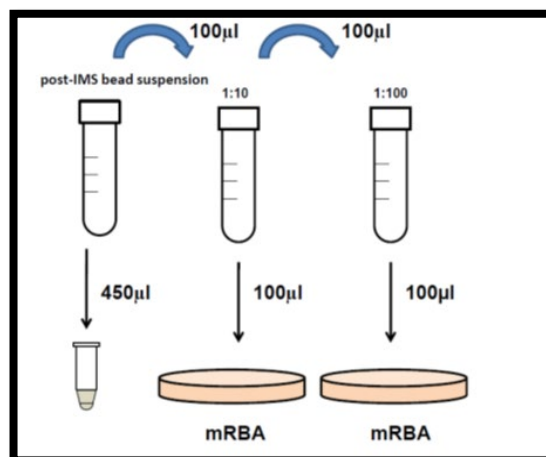
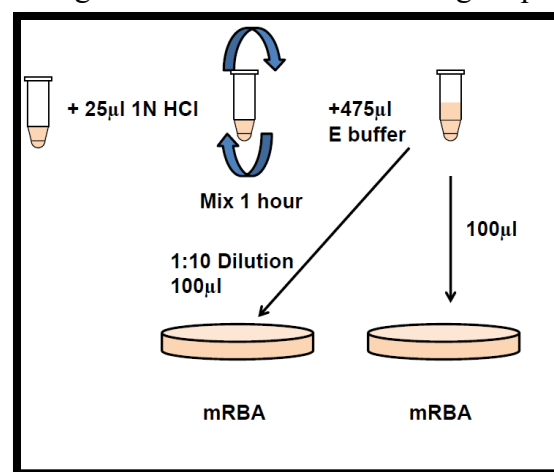


Figure 2. Acid Treatment Plating Step



Presumptive Identification of STECs

QUALITY CONTROL

Quality Control Procedures for Presumptive Identification

For Serology Controls on mRBA colonies, use:

- Positive Control – the serogroup(s) of interest (based on serogroup-specific PCR results) from mRBA,
- Kit positive and kit negative controls, and
- Autoagglutination kit control.

The presumptive PCR controls used for mRBA isolate screenings include:

- Positive control – Test an isolated colony from the serogroup(s) of interest (based on serogroup-specific PCR results),
- Negative control – Use uninoculated Molecular grade PCR Certified Water, and
- Kit positive control and kit negative control if required by the technology.

Examination of mRBA

After incubation, mRBA plates are to be examined for colonies that agglutinate with latex agglutination reagents specific for the serogroup of interest. Perform serological agglutination assays for STECs following manufacturer's instructions.

E. coli O157:H7 samples that have non-typical colonies on mRBA or typical colonies that are latex agglutination negative for O157 can be reported as negative. Colony colors from representative strains of STEC serogroups are listed in MLG 5C Appendix 2 "Morphologies of Representative Strains from Six non-O157 Shiga Toxin-Producing *Escherichia coli* (STEC) Grown on Modified Rainbow® Agar." Coloration of non-O157 *E. coli* colonies may vary based on proximity to other competitor colonies or medium discoloration due to competitor colony growth, so test at least one colony from each identified colony morphology found on the mRBA plate.

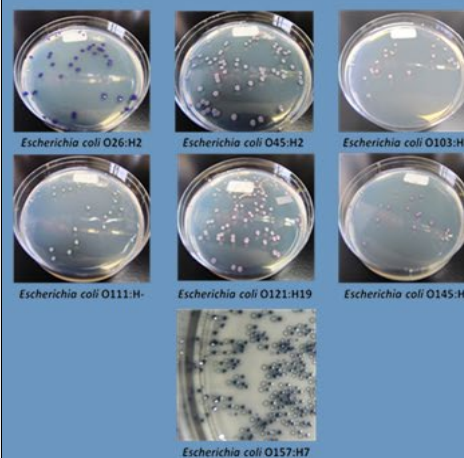
Samples that have no growth on mRBA or colonies that are agglutination negative for the serogroup identified by the rapid screen can be reported as negative for STEC. Samples that auto-agglutinate must be carried on as potential positive samples using further analysis with the presumptive rapid screen.

KEY FACTS

Latex agglutination tests consist of latex beads coated with antibody specific to the target serogroup. The beads bind to the pathogen and form complexes which appear as clumps in solution.

E. coli O157:H7 colonies typically 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. These colonies may also appear lighter, especially when plates are examined prior to 24 hours incubation.

Coloration of non-O157 *E. coli* colonies may vary. The following photographs show the variation of colors of control strains from each of the seven STEC serogroups grown in pure culture on modified Rainbow Agar plates.



Presumptive Rapid Screen Procedure

A presumptive rapid screen test is performed directly on agglutination positive colonies from the mRBA to verify presumptive positive colonies using the manufacturer's instructions for the iQ-Check™ Assay:

- Make a positive control suspension using the positive mRBA plate control. Use uninoculated Molecular Grade water for a sterility control. Include kit positive and kit negative controls.
- The sample is considered negative if the rapid screen is **negative** for the PCR targets.
- If an agglutination positive colony from mRBA is positive for *stx*, *eae*, and O group, the sample is considered a **presumptive positive** for the identified STEC.
- From the previous suspension, streak SBA for isolation. Also streak an *E. coli* STEC positive control to SBA using the serogroup of interest. Incubate inoculated SBA plates at $35 \pm 2^\circ\text{C}$ for 16-24 hours.

KEY DEFINITION

Presumptive positive STEC is defined as having one or more typical colonies on plating agar agglutinate with STEC latex agglutination reagents and positive on the presumptive PCR assay.

Confirmation of STECs

QUALITY CONTROL

Quality Control Procedures for Confirmation

For *E. coli* O157:H7 samples, examine SBA colonies using long-wave UV light to look for fluorescent organisms indicating O157 positive control contamination.

Use the serogroup positive control on SBA as the VITEK® 2 positive control for isolate identification.

Serology Controls for SBA isolates include:

- Positive Control – test the positive SBA control with the appropriate agglutination reagent for the serogroup(s) of interest (based on serogroup-specific PCR results),
- Kit positive and kit negative controls, and
- Autoagglutination kit control.

The confirmation PCR controls used for colony screenings include:

- Positive control – Test an isolated colony on SBA from the serogroup(s) of interest (based on serogroup-specific PCR results),
- Negative control – Use uninoculated Molecular grade PCR Certified Water, and
- Kit positive control and kit negative control if required by the technology.

Examination of SBA plates

After incubation, examine the SBA plates for both purity and evidence of contamination. Cross contamination with a positive fluorescent *E. coli* O157 control can be identified by using long wave UV light. Only the positive O157 control culture should fluoresce. If the SBA plates appear pure and uncontaminated, perform the confirmatory tests described below.

KEY FACT

The tagged *E. coli* O157:H7 reference strain visibly fluoresces under UV light to differentiate QC culture control strains from true contaminants.



Isolate Identification and Confirmation Tests

i. Isolate identification

To identify isolates for confirmation, inoculate VITEK® 2 GN cards (if using VITEK® 2 Compact) or equivalent. An isolate is positive on VITEK if biochemically identified as *E. coli*.

ii. Serological identification

To identify the absence or presence of O antigens, use a serological agglutination kit targeting the serogroup of interest. Use growth from the SBA plate. For inconclusive results, genetic testing may be necessary. An isolate is serologically positive for O antigens if latex agglutination occurs for the serogroup of interest.

iii. Genetic confirmation

Genetic confirmation is performed using the confirmatory PCR screen. The presence of the *stx* and *eae* genes in any STEC culture isolate(s) is confirmed by the iQ-Check™ STEC VirX *stx/eae* Screening assay. STECs are also confirmed by iQ-Check™ STEC® SerO Assay, targeting the serogroup of interest.

Confirmatory PCR Procedure

A confirmatory rapid test is performed directly on agglutination positive colonies from SBA to verify colonies for the presence of *stx*, *eae*, and serogroup, using manufacturer instructions for the iQ-Check™ Assay:

- After SBA incubation, perform the STEC serological agglutination test on colonies from the SBA plate.
- On the remainder of an agglutination positive colony from the SBA plate, confirm agglutination-positive colonies using iQ-Check™.
- Make a positive control suspension using the positive SBA plate control. Use uninoculated Molecular Grade water for a sterility control. Include kit positive and kit negative controls.
- Continue with the system protocol for the presence of *stx* and *eae* on the iQ-Check™ VirX Assay and the serogroup of interest on the iQ-Check™ SerO Assay.

CONFIRMATORY RESULTS

If an isolate or any additional presumptive positive colony picks from mRBA are **inconclusive** (e.g., inconclusive on serology or iQ-Check™), then the isolates are submitted for Whole Genome Sequencing.

If the isolate and any additional colony picks from mRBA are ultimately determined to be negative for *stx*, *eae*, or the 7 serogroup genes, the sample is **negative** for STEC.

If the agglutination positive isolate for the targeted STEC serogroups is positive for *stx* and *eae* genes, and positive for the targeted serogroup genes, then the isolate is **STEC positive**.

KEY DEFINITION

FSIS uses the following definition for reporting a **CONFIRMED POSITIVE STEC**:

A sample is considered positive for STEC when the *E. coli* isolate belongs to one of the seven targeted serogroups and contains a *stx* gene and an *eae* gene.

Storage of Cultures

Store “working” *E. coli* control cultures on nutrient agar slants or BHI agar slants. Transfer control stocks monthly onto duplicate nutrient or BHI agar slants, incubate overnight at $35 \pm 2^{\circ}\text{C}$, and then store them at $2-8^{\circ}\text{C}$. Use one of the slants as the working culture. Use the other slant for sub-culturing to reduce the chances of contamination.

After five transfers, the culture must be reconfirmed or a new culture initiated. For long term storage, freeze cultures in glycerol at $\leq -70^{\circ}\text{C}$ or less using cryo-beads, i.e., Cryostor™ or lyophilize.

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iQ-Check[™] STEC VirX User Guide, Test for the real-time PCR detection of virulence genes in Shiga Toxin Producing *E. coli*.

iQ-Check[™] STEC SerO User Guide, Test for the real-time PCR detection of 7 major serogroups in Shiga Toxin Producing *E. coli*.

For questions, please contact askfsis@usda.gov, and include “MLG5C” in the subject line.