United States Department of Agriculture

Food Safety and Inspection Service

MLG 5C.04

Detection, Isolation, and Identification of Top Seven Shiga Toxin-Producing *Escherichia coli* (STEC) from Meat Products, 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 top 7 STEC serogroups (O157, O26, O45, O103, O111, O121 and O145).

Notice of Change

The method was revised to update the pathogen detection system for STEC samples with the GENE-UP® Thermocycler as part of laboratory technology modernization efforts. A pathogenic *E. coli* (PEC) PCR test was added to determine the presence of Enterohemorrhagic *Escherichia coli* (EHEC) in an enriched sample with higher confidence at the initial screening stage. This laboratory tool adds efficiency by employing a novel molecular diagnostic target to rapidly identify samples containing STEC (i.e. *stx* and *eae* in the same cell) earlier in the workflow. It is expected that adoption of this new screening method will reduce the number of potential and presumptive positive STEC results that do not confirm positive. Updates to major sections referencing the new detection system include the Rapid Screen Procedure, Presumptive Rapid Screen Procedure, and Confirmatory PCR Procedure.

Immuno-concentration of the enrichment using VIDAS® *E. coli* serogroups (ESPT) replaces immunomagnetic separation prior to plating onto modified Rainbow® Agar (mRBA). The automated process concentrates multiple serogroups, using one set of mRBA plates, creating workflow efficiencies.

The STEC Method flow chart is included as part of the method, instead of as an appendix. The revision also includes a biosafety chart to further explain safety precautions regarding MLG 5C.

The updates to the method do not affect the communication of potential positive, presumptive positive, or confirmed positive of STEC results to stakeholders.

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Isolation and Identification of Shiga Toxin-Producing *Escherichia coli* (STEC) Method Flow Chart

Day 0

Sample Collection

Inspectors collect samples.

Day 1

Sample Prep

Laboratory receives samples.

Prepare samples using mTSB and incubate.

Day 2

Screening

Screen enrichment for the *stx* and *eae*, and pathogenic *Escherichia coli* (PEC)

Screen for serogroup detection

Conduct isolation procedure for the serogroup of interest and incubate mRBA

Samples that are *stx* (-), *eae* (-), PEC (-), or all serogroups (-) report as **NEGATIVE**

Samples that are *stx* (+), *eae* (+), PEC (+), and at least one serogroup (+) report as **POTENTIAL POSITIVE**

Day 3

Isolation

Select and test well-isolated mRBA colonies to:

1) Perform latex agglutination test for serogroup of interest

2) Perform PCR assay to detect *stx*, *eae*, and serogroup of interest

Transfer presumptive positive samples onto SBA and incubate

Samples that are latex (-), *stx* (-), *eae* (-), or serogroup of interest (-) report as **NEGATIVE**

Samples that are latex (+), *stx* (+), *eae* (+), serogroup (+) report as **PRESUMPTIVE POSITIVE**

Day 4

Confirmation

Select and test well-isolated SBA colonies to:

1) Perform latex agglutination test for serogroup of interest

2) Perform PCR assay to detect *stx*, *eae*, and serogroup of interest

3) Perform proteomic confirmation on Bruker MALDI Biotyper

Samples that are latex (-), *stx* (-), *eae* (-), or serogroup of interest (-) report as **NEGATIVE**

Samples that are latex (+), *stx* (+), *eae* (+), serogroup (+) report as **CONFIRMED POSITIVE**

Introduction

The methods described in this guidebook are for use by 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 detection 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 Reporting Workflow

The screening and isolation method is based on enrichment in a selective broth medium, application of a rapid screening test, immuno-concentration from enrichment broth, 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 the targeted genes (*eae*, *stx*1/2, 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.

Presumptive positive mRBA colonies are then streaked onto trypticase 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 Bruker® MALDI Biotyper (MBT) and a confirmation rapid screening PCR.

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

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 will 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 handle the materials are to read all SDSs. See the Biosafety Chart at the end of the chapter for more information.

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 ± 1°C, 35 ± 2°C	General lab supplier	Incubation of primary enrichment, mRBA plates, and SBA plates
Vortex Mixer	General lab supplier	Mix reagents in tubes
Plate Mixer and Rack Adaptor	General lab supplier	Lyse samples
Plate Centrifuge	General lab supplier	Mix lysis and reagents in PCR tube
Heating block (95 – 100°C)	General lab supplier	Prepare DNA template positive PCR control
GENE-UP® Thermocycler	bioMérieux #414056	DNA detection and amplification
Ultraviolet (UV) light (long-wave)	General lab supplier	Examine SBA plates for cross-contamination
VIDAS®	bioMérieux	Immuno-concentration of enrichment broth
Bruker® MALDI Biotyper	Bruker Inc.	Proteomic confirmation
Biochemical test kit and system, GN cards VITEK® 2 system	bioMérieux Vitek, Inc.	Biochemical confirmation
Optical density reader	General lab supplier	Measure culture suspension
Timer	General lab supplier	Measuring time for critical steps

Table 2: Test Kits

Reagent	Supplier	Purpose
GENE-UP® Lysis Kit	bioMérieux #414057	Lyses cells of all target microorganisms
GENE-UP® STEC - *stx* & *eae* (EH1)	bioMérieux #423109	PCR kit to detect virulence genes *stx*1/2 and *eae*
GENE-UP® Pathogenic *E. coli* (PEC)	bioMérieux #424143	PCR kit to detect presence of EHEC
GENE-UP® *E. coli* O157:H7 (ECO)	bioMérieux #423108	PCR kit to detect serogroup O157
GENE-UP® STEC – Top 6 (EH2)	bioMérieux #414154	PCR kit to detect serogroup O26, O45, O103, O111, O121, and O145

VIDAS® UP *E. coli* Serogroups (ESPT)	bioMérieux #30229	Immuno-selective kit to detect sero-specific STEC
Latex Kit	General supplier	Sero-specific latex will agglutinate suspected colonies
Bruker® MALDI Biotyper reagents	Bruker Inc. or General supplier	Proteomic confirmation

Table 3: Supplies

Material	Supplier	Purpose
Cloth sampling Device	Fremonta Catalog # MT-S100 or General supplier	Raw beef trim sample collection device
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
Pipets and sterile filter tips for the 1 µL, 5 µL, 20 µL, 150 µL to 1 mL volumes	General lab supplier	Add and mix reagents
1.5 mL microcentrifuge tubes	General lab supplier	Elute, dilute, and store DNA
10 µL Biotix filter pipette tip	bioMérieux #419194	Transfer lysate to PCR pellet
Sterile inoculating loops, "hockey sticks" or spreaders, and needles	General lab supplier	Spread /streak plates
Sterile disposable 12 x 75 mm polypropylene or polystyrene tubes	General lab supplier	IMS bead solution dilution/ separation
Sterile 50 mL conical tubes	General lab supplier	Storage enrichment broth
Sterile 40 µm Cell strainer	General lab supplier	Filter enrichment broth into 50 mL conical tube

Media

Media formulations are available in MLG Appendix 1, Media and Reagents.

a. Modified Tryptone Soy Broth (mTSB)
b. Modified Rainbow® Agar (mRBA)
c. Trypticase Soy Agar with 5% Sheep Blood (SBA)
d. Nutrient or Brain Heart Infusion (BHI) Agar slants for isolate storage
e. Molecular Grade Water
f. Physiological saline (PSS)

Cultures and Controls

a. *E. coli* O157:H7 reference strain that visibly fluoresces under UV light; from Microbiologics UV-BioTAG [TM] EC43 derived from FDA ESC1177 catalog #01227UV-V or equivalent.

b. *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

Include at least one positive culture and one uninoculated medium control. Incubate the controls along with the samples and analyze in the same manner as the samples. Confirm at least one isolate from the positive control sample. 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.

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.

A positive PCR control that contains the DNA of all 6 non-O157 STEC strains (*stx*+, *eae*+) and a negative media sterility control is used based on the requirements of the screening technology. If the screening technology used has no required kit controls that contain all 6 non-O157 STEC strains (*stx*+, *eae*+), then prepare a PCR positive control including the DNA of all 6 non-O157 STEC strains (*stx*+, *eae*+).

Table 4. QC Controls Required for the STEC Method

Step	Controls
Enrichment	• Positive control – enrichment inoculated with the *E. coli* O157:H7 reference control strain • Negative control – uninoculated enrichment
Rapid Screening Procedure: *stx*, *eae*, Pathogenic *E. coli*	• Positive control – enrichment inoculated with the *E. coli* O157:H7 reference control strain • Negative control – uninoculated enrichment
Rapid Screening Procedure: Top 7 Serogroups	• Positive control – enrichment inoculated with the *E. coli* O157:H7 reference control strain • PCR positive control including the DNA of the 6 non-O157 STEC strains that are positive for the O-groups of interest • Negative control – uninoculated enrichment
Isolation	• Positive Control – For all serogroups, use the enrichment inoculated with the *E. coli* O157:H7 reference control strain
mRBA Plating	• Positive Controls o For all serogroups, use the Positive Control from the Isolation step (*E. coli* O157:H7 reference control strain) o 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 isolation procedure • Negative Control – the uninoculated mRBA.
Presumptive Serology	• Positive Control – the serogroup(s) of interest (based on serogroup-specific PCR results) from mRBA • Kit positive and kit negative controls • Autoagglutination kit control
Presumptive PCR	• Positive control – Test an isolated colony on mRBA from the serogroup(s) of interest (based on serogroup-specific PCR results), • Negative control –uninoculated Molecular grade PCR Certified Water
Confirmation Serology	• Positive Control – serogroup(s) of interest (based on serogroup-specific PCR results) from SBA • Kit positive and kit negative controls • Autoagglutination kit control
Confirmation PCR	• Positive control – Test an isolated colony on SBA from the serogroup(s) of interest (based on serogroup-specific PCR results) • Negative control – uninoculated Molecular grade PCR Certified Water
Bruker Biotyper	• Bruker® MALDI Biotyper controls • Positive control – serogroup of interest (based on serogroup-specific positive PCR results) 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. Include this tagged *E. coli* O157:H7 control with each sample batch as a positive enrichment broth control. Prepare a test organism suspension in broth or saline, equivalent in turbidity to a 0.5 McFarland standard. Using a 1 µL loop, inoculate approximately 100-150 mL broth. Alternatively, commercially prepared bacterial pellets may be used.

Include an uninoculated enrichment broth sample that will serve as the negative media control with each batch.

KEY FACTS

Sample temperature of ≤ 15°C is required upon receipt as warmer temperatures can allow competing bacteria to outgrow the targets.

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

1) Prepare samples for enrichment.
   a) *Beef trim/raw ground beef components, Raw ground beef and raw beef/pork or Poultry mixes*
      i) Disinfect the surface of intact sample package(s) prior to opening.
      ii) In a sterile strainer bag, prepare a single sample using 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. Stomach or hand massage until clumps are dispersed.

   b) *N60 trim samples*
      i) 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.
         (1) Using the sixty trim pieces, prepare a single 325 ± 32.5 g sample with 975 ± 19.5 mL mTSB broth.
         (2) 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.
      ii) Enrich the second sub-sample at the same 1:4 dilution (one portion of product in three portions of enrichment broth). Stomach or hand massage until clumps are dispersed.

   c) *Environmental and carcass sponges with 10 mL of buffer*
      i) Add 50 ± 5 mL of mTSB broth. Mix well.
      ii) 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.

   d) *Cloth samples pre-moistened with 25 mL of buffer*
      i) Tare the balance with a dry cloth then bring total weight of sample up to 200 ± 4 g with mTSB for enrichment.
      ii) Aseptically manipulate the cloth to ensure it is submerged into the mTSB before stomaching or hand massaging and incubation.

2) Incubate all bags with their contents for 15 to 24 h at 42 ± 1°C. Include a positive and uninoculated medium control for each group of samples tested.

Rapid Screening Procedure

QUALITY CONTROL

Preparing PCR Positive Control

To prepare a PCR positive control:

Grow the top 6 non-O157 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 a 0.5 McFarland reading on an optical density reader. In one tube, add 1.0 mL from each suspension to 4.0 mL of molecular grade water to create a 10.0 mL cocktail of the six non-O157 STEC strains and vortex the suspension. 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 will be centrifuged at 10,000 x g for 3 minutes to pellet cellular debris. The supernatant is used as the PCR positive control. The PCR positive control can be prepared as a batch, confirmed on screening technology for quality control, transferred to smaller volume tubes, and stored at ≤ -20°C for 1 year.

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

1) Screen all enriched samples using the *stx* and *eae*, and the pathogenic *E. coli* (PEC) rapid screening PCR assays. Follow the rapid screening technology user guide for preparing reagents, performing the test, and reading the results. Store sample lysates at 2 – 8°C after loading onto instrument.
   a) Samples that test negative for *stx* and/or *eae* on the STEC screening PCR are reported as **negative.**
   b) For samples that test positive for the STEC screening PCR (*stx AND eae*), use the results from the PEC PCR to determine the presence of Enterohemorrhagic *Escherichia coli* (EHEC).
      i) Samples that test negative on the PEC PCR are reported as **negative**.
      ii) Samples that test positive on the PEC screening are analyzed for serogroup identification.

2) Screen all *stx, eae,* and PEC positive samples for the top 7 serogroups.
   a) Samples that test positive for the STEC screening (*stx AND eae*) and PEC but negative for the 7 serogroups are reported as **negative**.
   b) If one or more serogroup is positive, the sample is reported as a **potential positive**. Proceed with the Isolation Procedure.

KEY FACT

The FSIS STEC detection and isolation laboratory method targets the *stx* gene, which encodes Shiga toxin, and the *eae* gene, which allows STEC to attach to human intestinal cells: these are both "virulence genes" that are essential for STEC to cause human disease. The rapid screening protocol detects these virulence genes and genetically identifies the seven targeted serogroups. Any additional screening procedures should identify alternative gene targets in the enriched sample to confidently rule out or identify the presence of STEC.

TROUBLESHOOTING GUIDE FOR RAPID SCREEN ANALYSIS

For Inhibited Sample Screening Results:

- repeat the rapid screen analysis from the lysate step, or
- prepare new rapid screen lysate tubes and repeat the analysis, or
- analyze all inhibited samples culturally, or
- reanalyze using the GENE-UP® inhibition protocol.
  1) When diluting samples, controls are added and must be diluted also.
  2) Add 10 µL of control buffer into a microcentrifuge tube.
  3) Add 5 µL lysate to 10µL control buffer.
  4) Pipette 10 µL of 1:3 lysate to PCR tube.
  5) If the diluted sample is still inhibited or positive, proceed to isolation procedure.

For Control Issues:

- 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, or
  - prepare new rapid screen lysate tubes from the enrichment broth and repeat the analysis, or
  - analyze all samples culturally.
- 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, or
  - analyze sterility sample culturally and add an additional sterility sample to act as a process control.

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 will, if possible, continue cultural analysis for all affected samples.

Isolation Procedure

Samples that are potentially positive from PCR screening are immuno-concentrated on the VIDAS® *E. coli* Serogroups (ESPT) kit by using the ESP2 Protocol. Filtrates and dilutions from the immuno-concentrate are used to streak the mRBA plates.

1) Remove mRBA plates from 2 – 8ºC storage to allow time for the moisture to evaporate. Be sure the plates have no visible surface moisture at the time of use.
   i) Six plates are needed for each screen-positive culture and the fluorescent *E. coli* O157:H7 control.
   ii) One uninoculated mRBA plate serves as a negative control
   iii) One mRBA plate is needed for each non-O157 STEC control isolate from the serogroup(s) of interest (based on serogroup-specific screen results).

2) Place a 40 µm cell strainer on a labeled 50 mL conical centrifuge tube. Pipet each enrichment culture and *E. coli* O157:H7 control into the respective cell strainer and collect at least 3 mL of filtrate.

3) Pipet 800 µL of enrichment filtrate into the fourth well on a labeled ESPT strip. Follow manufacturer instructions to perform analysis. After the ESP2 Protocol is complete, pipet 30 µL of the immuno-concentrate from the first well onto mRBA. Plate in duplicate. Use a hockey stick or spreader to spread plate.

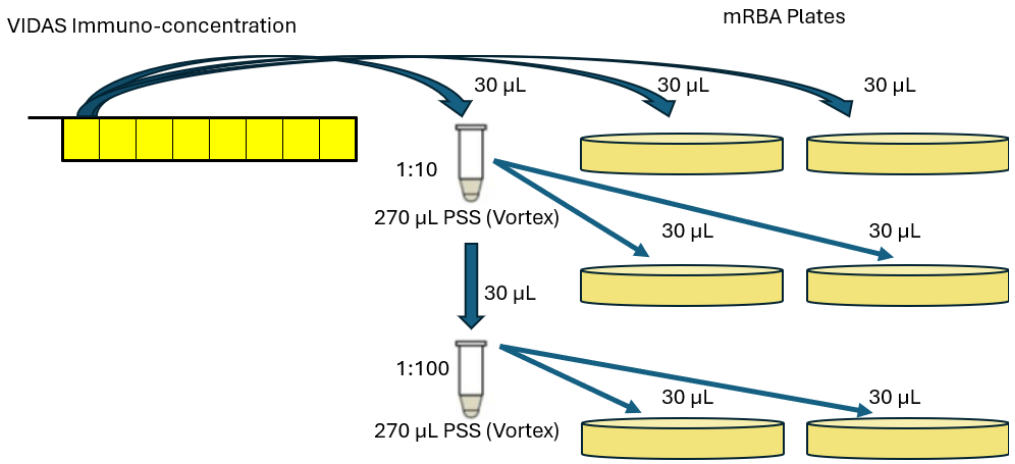
4) Make a 1:10 dilution by adding 30 µL of the immuno-concentrate to a microcentrifuge tube containing 270 µL physiological saline (PSS) and vortex.

5) Make a 1:100 dilution by adding 30 µL of the 1:10 dilution to a microcentrifuge tube containing 270 µL PSS. Vortex briefly and plate 30 µL from each tube (1:10 dilution and 1:100 dilutions) onto labeled mRBA plates in duplicate. Use a hockey stick or spreader to spread plate.

6) Streak a non-O157 STEC 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 isolation procedure.

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

Figure 1. Dilution Preparation for mRBA Plates



Presumptive Identification of STECs

Examination of mRBA

1) Examine mRBA plates for typical colonies.
2) Perform serological agglutination assays for STECs following manufacturer's instructions.

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; test at least one colony from each identified colony morphology found on the mRBA plate.

An mRBA plate with no growth, non-typical colonies, or typical colonies that are latex agglutination negative for the serogroup identified by the rapid screen can be reported as negative for STEC.

An mRBA plate with typical colonies that are latex agglutination positive or auto-agglutinate for the serogroup identified by the rapid screen must be carried on for further analysis with the presumptive rapid screen procedure.

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. Refer to MLG 5C Appendix 2 for examples of 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

Perform a presumptive rapid screen test directly on agglutination positive colonies from the mRBA plates. Use the manufacturer's instructions for the GENE-UP® PCR assays.

KEY DEFINITION

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

1) Make a cell suspension from agglutination positive colonies and positive controls by transferring a part of the typical colony to microcentrifuge tube with 200 µL of Molecular Grade water. Use 200 µL uninoculated Molecular Grade water for a sterility control.
2) Perform lysis by transferring 20 µL of prepared cell suspension to Gene-Up Lysis tube and follow manufacturer instructions to lyse the culture.
3) Prepare a dilution of 1:100 using the lysed culture (10 µL) and Molecular Grade water (990 µL).
4) Perform PCR using the diluted extract:

i) Use 10 µL of the diluted extract for the presence of *stx* and *eae*, and
ii) Use 10 µL of the diluted extract for the serogroup of interest

5) Presumptive Results
   a) Samples that test **negative** for *stx*, *eae,* **or** the O group of interest is reported as **negative**.
   b) Samples that test **positive** for *stx*, *eae,* **and** O group are reported as **presumptive positive** for the identified STEC. Proceed with the inoculating SBA plates and the Confirmation of STECs.
6) From the undiluted cell suspension, streak the sample and an *E. coli* STEC positive control containing the serogroup of interest to SBA for isolation.
7) Incubate inoculated SBA plates at 35 ± 2°C for 16 – 24 h.

Confirmation of STECs

Examination of SBA plates

Examine the SBA plates for both purity and evidence of contamination. If the colonies on an SBA plate exhibit different morphologies, the plate is mixed and is not pure. 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.

1) If the plates are determined to be a mixed culture, perform serological testing on isolated colonies. Transfer remainder of agglutination positive colony to both mRBA and SBA. Incubate as instructed in previous sections.
   a. Use mRBA to determine purity.
   b. Perform confirmatory testing on SBA.
2) If the SBA plates appear pure and uncontaminated, perform the testing described below.

Isolate Confirmation and Serological Identification Tests

Use growth from the SBA plate to conduct the following isolation confirmation and serological identification tests.

i. Isolate Confirmation
Perform proteomic confirmatory tests using a pure culture. Analyze samples using commercially available test systems such as Bruker® MALDI Biotyper or validated equivalent systems. Exercise caution when interpreting the identification of *E. coli*, as these systems do not distinguish between *E. coli* and *Shigella* spp. We recommend the use of confirmatory PCR and latex-based assays as described in this method in combination with the Bruker® MALDI Biotyper to confirm those organisms.

Refer to manufacturer's instructions to use the instrument, prepare reagents, and troubleshooting guidance.

The Bruker® MALDI Biotyper allows for the use of each available preparation method (direct, extended direct, and tube extraction) as needed to identify organisms. Refer to the manufacturer's documentation for a full list of organism coverage and thresholds.

ii. Serological identification
A serological agglutination kit targets the serogroup of interest to identify the absence or presence of O antigens.

1) Perform the STEC serological agglutination test on a portion of a colony from the SBA plate.
   a. For inconclusive results, genetic testing may be necessary.

b. An isolate is serologically positive for O antigens if latex agglutination occurs for the serogroup of interest and must be carried on for further analysis with the Confirmatory PCR Procedure.

iii. Confirmatory PCR Procedure

Perform a confirmatory rapid testing directly on agglutination positive colonies from the SBA plates. Use manufacturer instructions for the GENE-UP® Assay.

1) On the remainder of an agglutination positive colony from the SBA plate, confirm agglutination-positive colonies using GENE-UP®.
2) Prepare agglutination positive colonies and controls for confirmatory PCR. Make a positive control suspension using the positive SBA plate control.
   a) Create cell suspension from agglutination positive colonies and positive controls by transferring part of the typical colony from the SBA plate to a microcentrifuge tube with 200 µL of Molecular Grade water. Use 200 µL uninoculated Molecular Grade water for a sterility control.
   b) Perform lysis by transferring 20 µL of prepared cell suspension to Gene-Up Lysis tube and follow manufacturer instruction to lyse the culture.
   c) Prepare dilution of 1:100 using the lysed culture (10 µL) and Molecular Grade water (990 µL).
   d) Perform PCR using the diluted extract:
      i) Use 10 µL of the diluted extract for the presence of *stx* and *eae*, and
      ii) Use 10 µL of the diluted extract for the serogroup of interest

Confirmatory Results

If an isolate or any additional presumptive positive colony picks from mRBA are **inconclusive** (e.g., inconclusive on serology or GENE-UP®), then Whole Genome Sequencing is used to confirm the isolate.

KEY DEFINITION

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.

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.**

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 ± 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 five transfers, the culture must be reconfirmed or a new culture initiated. For long term storage, freeze cultures in glycerol at ≤ -70°C using cryo-beads, i.e., Cryostor™ or lyophilize.

Appendix: Alternative Methods

Biochemical Confirmation

FSIS laboratories may elect to use biochemical confirmation methods (VITEK® 2) for reasons including: Bruker® MALDI MBT is unavailable, interruption in reagent supply chain, or results comparison.

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

Biosafety Chart

Safety Information and Precautions

1. Recommended protective equipment: Nitrile or latex gloves, lab coat, and safety glasses
2. Hazards

Procedure Step	*Hazard*	*Recommended Safety Procedures*
DNA Isolation- Extraction of Shiga Toxin-Producing *Escherichia coli* (STEC)	If ingested, STEC infections can cause severe illness or death. A portion of STEC infections will develop hemolytic uremic syndrome (HUS).	Follow CDC guidelines for manipulating Biosafety Level 2 (BSL-2) pathogens.
Confirmation of STECs- Examination of SBA plates using UV light	Using UV lamps can cause eye irritation and/or damage if eyes are directly exposed. The cornea of the eye can become inflamed or burned. Prolonged UV lamp exposure can also cause skin irritation and/or damage such as burns.	Refrain from staring directly at the UV lamp when in use. UV blocking eyewear should be worn. Use gloves when fluorescing samples under the UV lamp. Turn off the UV lamp when not in use.

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GENE UP User Guide, GENE-UP® Primary Assays The Basics.

Bruker® MALDI Biotyper User Guide. Proteomic isolate confirmation testing.

VITEK® 2 Compact User Guide. Biochemical isolate confirmation testing.

Contact Information and Inquiries

Inquiries about methods can be submitted through the USDA website via the "Ask USDA" portal at https://ask.usda.gov or please contact:

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This method has been validated, reviewed, approved, and deemed suitable and fit for purpose for use in the USDA FSIS Field Service Laboratories.

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