OXFORD

MICROBIOLOGICAL METHODS

Validation of the 3M™ Molecular Detection Assay 2 - STEC Gene Screen (*stx*) for the Detection of Shiga Toxin Gene (*stx*1 and/or *stx*2) in Additional Matrixes: AOAC *Performance Tested Method*™ 071903

Micki L. Rosauer ⓘ ,[1,*] Christina Barnes ⓘ ,[1] Karen M. Silbernagel ⓘ ,[1] Kateland M. Koch ⓘ ,[2] and Leslie Thompson-Strehlow ⓘ [3]

[1]3M Company, Food Safety Department, St. Paul, MN 55144-1000, USA, [2]Q Laboratories, Microbiology Research and Development Laboratory, Cincinnati, OH 45204, USA, [3]SGS Vanguard Sciences Inc., Research and Development, North Sioux City, SD, USA

*Corresponding author's e-mail: mlrosauer@mmm.com

Abstract

Background: The 3M™ Molecular Detection Assay 2 - STEC Gene Screen (*stx*) method is based on gene amplification by the use of real-time loop-mediated isothermal amplification when used with the 3M Molecular Detection System for the rapid and specific detection of Shiga toxin gene (*stx*1 and/or *stx*2) from Shiga toxin-producing enterohemorrhagic *Escherichia coli* (STEC) in enriched foods.
Objective: The 3M Molecular Detection Assay 2 - STEC Gene Screen (*stx*) method was evaluated as a Level 2 method modification to add new matrixes to the certified claim: 25 g fresh raw ground beef (approximately 75% lean), 375 g raw beef trim (approximately 75% lean), 375 g fresh raw ground pork (approximately 70% lean), 375 g fresh raw poultry parts, and 25 g sprouts.
Methods: Matrix studies were conducted to assess the method's performance compared to the US Department of Agriculture Food Safety and Inspection Service *Microbiology Laboratory Guidebook*, 5C.00 for meat and poultry, and to the US Food and Drug Administration *Bacteriological Analytical Manual*, Ch. 4A for sprouts, using an unpaired study design.
Results: The 3M Molecular Detection Assay 2 - STEC Gene Screen (*stx*) method demonstrated no significant differences between presumptive and confirmed results or between candidate and reference method results for any of the matrixes tested.
Conclusion and Highlights: The data collected in these studies demonstrate that the 3M Molecular Detection Assay 2 - STEC Gene Screen (*stx*) is a reliable method for the rapid and specific detection of STEC in fresh raw ground beef (approximately 75% lean), fresh raw beef trim (approximately 75% lean), fresh raw ground pork (approximately 70% lean), fresh raw poultry parts, and sprouts.

General Information

Escherichia coli (*E. coli*) is found in the environment, in foods (notably in meat, pork, raw milk, unpasteurized dairy products, and unpasteurized juices), and in the intestines of people and animals. Most *E. coli* strains are harmless and are important commensals in the human intestinal tract; however, some strains can cause diarrhea or other illnesses.

Infection with *E. coli* which produce a toxin known as Shiga toxin can cause illness; these *E. coli* strains are often transmitted through water and food contaminated from contact with animals or animal waste. These strains are collectively called Shiga toxin-producing *E. coli* (STEC). Symptoms of illnesses caused by STEC can include abdominal cramps, diarrhea (which may progress to bloody diarrhea), fever, and vomiting. In 5 to 15% of patients, it can lead to hemolytic uremic syndrome (HUS), characterized by thrombocytopenia (low blood platelet levels), hemolytic anemia, and acute renal failure (1). In fact, STEC-related HUS is the leading cause of acute renal failure in young children (2).

As with many foodborne illnesses, health ministries and other health-related organizations estimate that STEC-related illnesses are significantly underreported. In the United States, for instance, there were 8672 cases of STEC reported to the National Notifiable Diseases Surveillance System (NNDSS) for the United States and US Territories in 2017 (3). However, the estimate for STEC illness each year in the United States is more than 265 000 (4). The global incidence of STEC-related illness has been estimated to be 2 801 000 acute illnesses annually (5).

The major sources of STEC infections are related to the consumption of undercooked or raw meat, raw milk and unpasteurized dairy products, and increasingly, ready-to-eat foods such as fresh fruits and vegetables (1, 6).

There are several actions that can be taken to avoid STEC infection, including washing of hands after handling raw meat, cooking meats like ground beef thoroughly to an internal temperature of 71°C/160°F, and separating food preparation areas to prevent cross-contamination (7).

Principle

The 3M™ Molecular Detection Assay 2 - STEC Gene Screen (*stx*) is used with the 3M™ Molecular Detection System for the rapid and specific screening of *E. coli* genes *stx*1 and/or *stx*2 in enriched food samples. The 3M Molecular Detection Assays use loop-mediated isothermal amplification to rapidly amplify nucleic acid sequences with high specificity and sensitivity, combined with bioluminescence to detect the amplification.

An algorithm interprets the light output curve resulting from the detection of the nucleic acid amplification. Results are analyzed automatically by the software and are color-coded based on the result. A positive or negative result is determined by analysis of a number of unique curve parameters. Presumptive positive results are reported in real-time while negative results will be displayed after the run is completed. Presumptive positive samples should be confirmed as per the laboratory standard operating procedures or by following the appropriate reference method confirmation, MLG 5C.00 (8) or BAM Chapter 4A (9) as relevant to the matrix.

The 3M Molecular Detection Assay 2 - STEC Gene Screen (*stx*) is intended for use in a laboratory environment by professionals trained in laboratory techniques. 3M has not documented the use of this product in industries other than food. For example, 3M has not documented this product for testing pharmaceutical, cosmetics, clinical, or veterinary samples. The 3M Molecular Detection

Assay 2 - STEC Gene Screen (*stx*) has not been evaluated with all possible food products, food processes, testing protocols, or with all possible strains of bacteria. The 3M Molecular Detection Instrument is intended for use with samples that have undergone heat treatment during the assay lysis step, which is designed to destroy organisms present in the sample. Samples that have not been properly heat treated during the assay lysis step may be considered a potential biohazard and should *not* be inserted into the 3M Molecular Detection Instrument.

As with all test methods, the source of enrichment medium can influence the results. The 3M Molecular Detection Assay 2 - STEC Gene Screen (*stx*) has been evaluated for use with the 3M Buffered Peptone Water (ISO Formulation; 3M BPW ISO) enrichment broth.

Scope of Method

(a) *Target organisms.*—Shiga toxin-producing *E. coli* (STEC) strains containing genes *stx*1 (codes for Shiga toxin type 1) and/or *stx*2 (codes for Shiga toxin type 2).

(b) *Matrixes.*—Current claim: fresh raw ground beef (375 g, approximately 73% lean), fresh raw spinach (200 g). Matrix extension: fresh raw ground beef (approximately 75% lean, 25 g), fresh raw beef trim (approximately 73% lean, 375 g), fresh raw ground pork (approximately 70% lean, 375 g), fresh poultry parts (375 g), and sprouts (25 g).

(c) *Summary of validated performance claims.*—Performance comparable to that of US Department of Agriculture Food Safety and Inspection Service *Microbiology Laboratory Guidebook* (USDA/FSIS MLG), 5C.00, Detection, Isolation and Identification of Top Seven Shiga Toxin-Producing *Escherichia coli* (STECs) from Meat Products and Carcass and Environmental Sponges (8) for fresh raw ground beef, fresh raw beef trim, fresh raw ground pork, and fresh poultry parts, and to the US Food and Drug Administration *Bacteriological Analytical Manual* (FDA-BAM) Ch. 4A, Diarrheagenic *Escherichia coli* (9) for spinach and sprouts.

Definitions

(a) *Probability of detection (POD).*—The proportion of positive analytical outcomes for a qualitative method for a given matrix at a given analyte level or concentration. POD is concentration dependent. Several POD measures can be calculated: POD_R (reference method POD), POD_C (confirmed candidate method POD), POD_{CP} (candidate method presumptive result POD), and POD_{CC} (candidate method confirmation result POD).

(b) *Difference of probabilities of detection (dPOD).*—Difference of probabilities of detection is the difference between any two POD values. If the confidence interval of a dPOD does not contain zero, then the difference is statistically significant at the 5% level.

Materials and Methods

Test Kit Information

(a) *Kit name.*—3M Molecular Detection Assay 2 - STEC Gene Screen (*stx*).

(b) *Cat. No.*—MDA2STX96.

(c) *Ordering information.*—https://www.3m.com/.

Test Kit Components

(a) *STEC Gene Screen (stx) Reagent Tubes.*—96 orange tubes (4 pouches; containing 3 strips of 8 tubes).
(b) *Lysis Solution Tubes.*—96 clear tubes (12 strips of 8 tubes, with each tube containing 580 µL lysis solution).
(c) *Reagent Control (RC).*—16 individual clear flip-top tubes (2 pouches of 8 individual flip-top tubes).
(d) *Extra reagent tube caps.*—96 orange caps.
(e) *Product instructions.*

Additional Supplies and Reagents

(a) *3M Molecular Detection System Matrix Control.*—Cat. No. MDMC96.
(b) *3M Buffered Peptone Water (ISO Formulation; 3M BPW ISO).*—Cat. No. BPW500.
(c) *3M Molecular Detection System Instrument.*—Cat. No. MDS100.
(d) *Laptop with 3M Molecular Detection System Software.*—Version 2.4.0.0.
(e) *3M Molecular Detection Speed Loading Tray.*—Cat. No. MDSSLT.
(f) *Empty lysis tube rack.*
(g) *3M Molecular Detection Cap/Decap Tool—Lysis.*—Cat. No. MDSCDL.
(h) *3M Molecular Detection Cap/Decap Tool—Reagent.*—Cat. No. MDSCDR.
(i) *3M Molecular Detection Chill block insert.*—Cat. No. MDSCBIN.
(j) *3M Molecular Detection Heat block Insert.*—Cat. No. MDSHBIN.

Apparatus

(a) *Incubators.*—Capable of maintaining 37 ± 1°C and 41.5 ± 1°C.
(b) *Filter laboratory blender bags.*
(c) *Serological pipette bulbs (automatic pipette).*—For sampling and delivering of 1–10 mL.
(d) *Serological pipettes.*—Aerosol resistant.
(e) *Precision pipettors.*—For sampling and delivering of 10 µL and 20 µL.
(f) *Micropipette tips.*—Aerosol resistant.
(g) *Multi-channel pipette.*—Capable of 20 µL.
(h) *Dry bath incubator.*—Capable of maintaining a temperature of 100 ± 1°C.
(i) *Calibrated thermometer.*—Capable of measuring a temperature of 100 ± 1°C.
(j) *Calibrated timer.*
(k) *Refrigerator.*—Capable of maintaining 2–8°C, for storing the lysates.

Safety Precautions

Follow the protocol and perform the tests exactly as stated in the product instructions. Failure to do so may lead to inaccurate results. Perform pathogen testing in a properly equipped laboratory under the control of personnel trained in current proper testing techniques: for example, good laboratory practices, ISO/IEC 17025 ([10]), or ISO 7218 ([11]). Incubated enrichment media and equipment or surfaces that have come into contact with incubated media may contain pathogens at levels sufficient to cause risk to human health. Always follow standard laboratory safety practices, including wearing appropriate protective apparel and eye protection while handling reagents and contaminated samples. Avoid contact with the contents of the enrichment media and reagent tubes after amplification. Dispose of enriched samples according to current local/regional/national regulations and industry standards. Samples that have not been properly heat treated during the assay lysis step may be considered a potential biohazard and should *not* be inserted into the 3M Molecular Detection Instrument. Do not exceed the recommended temperature setting on the heater. Do not exceed the recommended heating time. Use an appropriate, calibrated thermometer to verify the 3M Molecular Detection Heat Block Insert temperature (e.g., a partial immersion thermometer or digital thermocouple thermometer, not a total immersion thermometer). The thermometer must be placed in the designated location in the 3M Molecular Detection Heat Block Insert.

General Preparation

Follow all instructions carefully. Failure to do so may lead to inaccurate results. Decontaminate laboratory benches and equipment (pipettes, cap/decap tools, etc.) periodically with a 1–5% (v/v) household bleach in water solution or DNA removal solution. Prepare 3M BPW ISO as per product instructions. Store prepared broth at 2–8°C if it will not be immediately used after preparation. Ensure enrichment media is pre-warmed to 41.5 ± 1°C before use. For all meat and highly particulate samples, the use of filter bags is recommended.

Sample Preparation

Note: Sample preparation instructions below are excerpted from the full product "Instructions for Use" and so include references to full categories of raw ground beef, pieces and trim, and leafy produce. For this matrix study, fresh raw ground beef, pieces, trim, and fresh sprouts were tested.

(a) *Raw ground beef, pieces, and trim.*— For 25 g test portions, add 225 ± 5 mL pre-warmed 41.5 ± 1°C 3M BPW ISO broth; for 375 g test portions, add 1125 ± 5 mL pre-warmed 41.5 ± 1°C 3M BPW ISO broth. Hand massage the beef (ground beef, pieces, and trim) samples for 30–60 s to disperse and break apart clumps.
(b) *Raw meat (pork, poultry, lamb, bison).*—375 g test portions: add 1125 ± 5 mL pre-warmed 41.5 ± 1°C 3M BPW ISO broth. Hand massage the raw meat (ground pork, poultry, and non-beef meat) samples by hand for 30–60 s to disperse and break apart clumps.
(c) *Leafy produce.*—Aseptically transfer 200 g sample to a sterile bag and add 450 mL 3M BPW ISO, pre-warmed to 41.5 ± 1°C. Rinse enrichment broth over leaves and agitate gently for 30–60 s. Do not massage or homogenize leaves.
(d) *Sprouts (25 g test portions).*—Add 225± 5 mL pre-warmed 41.5 ± 1°C 3M BPW ISO broth and rinse over sprouts for 30–60 s and do not massage or homogenize.

Sample Enrichment

(a) Incubate the bag aerobically at 41.5 ± 1°C.
(b) Incubate raw meats 10–18 h. Incubate leafy produce and sprouts 18–24 h.

Analysis

(a) Preparation of the 3M Molecular Detection Speed Loader Tray:

(1) Wet a cloth or disposable towel with a 1–5% (v/v) household bleach in water solution and wipe the 3M Molecular Detection Speed Loader Tray.

(2) Rinse the 3M Molecular Detection Speed Loader Tray with water.

(3) Use a disposable towel to wipe the 3M Molecular Detection Speed Loader Tray dry.

(4) Ensure the 3M Molecular Detection Speed Loader Tray is dry before use.

(b) Place the 3M Molecular Detection Chill Block Insert directly on the laboratory bench: The 3M Molecular Detection Chill Block Tray is not used. Use the block at ambient laboratory temperature (20–25°C).

(c) Place the 3M Molecular Detection Heat Block Insert in a dry double block heater unit. Turn on the dry block heater unit and set the temperature to allow the 3M Molecular Detection Heat Block Insert to reach and maintain a temperature of 100 ± 1°C. *Note*: Depending on the heater unit, allow approximately 30 min for the 3M Molecular Detection Heat Block Insert to reach temperature. Using an appropriate, calibrated thermometer (e.g., a partial immersion thermometer, digital thermocouple thermometer, not a total immersion thermometer) placed in the designated location, verify that the 3M Molecular Detection Heat Block Insert is at 100 ± 1°C.

(d) Launch the 3M Molecular Detection Software and log in. Contact your 3M Food Safety representative to ensure you have the most updated version of the software.

(e) Turn on the 3M Molecular Detection Instrument.

(f) Create or edit a run with data for each sample. Refer to the 3M Molecular Detection System User Manual for details. *Note*: The 3M Molecular Detection Instrument must reach and maintain "Ready" state before inserting the 3M Molecular Detection Speed Loader Tray with reaction tubes. This heating step takes approximately 20 min and is indicated by an orange light on the instrument's status bar. When the instrument is ready to start a run, the status bar will turn green.

Lysis

(a) Allow the 3M Lysis Solution tubes to warm up by setting the rack at ambient temperature (20–25°C) overnight (16–18 h). Alternatives to equilibrate the 3M Lysis Solution tubes to ambient temperature are to set the 3M Lysis Solution tubes on the laboratory bench for at least 2 h, incubate the 3M Lysis Solution tubes in a 37 ± 1°C incubator for 1 h, or place them in a dry double block heater for 30 s at 100 ± 1°C.

(b) Invert the capped tubes to mix. Proceed to next step within 4 h after inverting.

(c) Remove the enrichment broth from the incubator.

(1) Gently massage the bottom of the enrichment bag before transferring the sample to the 3M Lysis Solution tube.

(2) Additional sample may be required for re-testing or confirmatory steps.

(d) One 3M Lysis Solution tube is required for each sample and the negative control (NC) sample (sterile enrichment medium).

(1) 3M Lysis Solution tube strips can be cut to desired tube number. Select the number of individual 3M Lysis Solution or 8-tube strips needed. Place the 3M Lysis Solution tubes in an empty rack.

(2) To avoid cross-contamination, decap one 3M Lysis Solution tube strip at a time and use a new pipette tip for each transfer step.

(3) Transfer enriched sample to 3M Lysis Solution tube. Transfer each enriched sample into an individual 3M Lysis Solution tube first. Transfer the NC last.

(4) Use the 3M Molecular Detection Cap/Decap Tool-Lysis to decap one Lysis Solution tube strip—one strip at a time.

(5) Discard the 3M Lysis Solution tube cap—if lysate will be retained for retest, place the caps into a clean container for re-application after lysis.

(6) Transfer 20 μL sample into a 3M Lysis Solution tube. *Warning*: Should you choose to use neutralizing buffer that contains aryl sulfonate complex as a hydrating solution for environmental sponge samples, it is necessary to perform a 1:2 dilution (1 part sample into 1 part sterile enrichment broth) of the enriched environmental sample before testing in order to reduce the risks associated with a false-negative result leading to the release of contaminated product. Another option is to transfer 10 μL neutralizing buffer enrichment into the 3M Lysis Solution tubes.

(e) When all samples have been transferred, transfer 20 μL NC (sterile enrichment medium) into a 3M Lysis Solution tube. Do not use water as a NC.

(f) Verify that the temperature of the 3M Molecular Detection Heat Block Insert is at 100 ± 1°C.

(g) Place the uncovered rack of 3M Lysis Solution Tubes in the 3M Molecular Detection Heat Block Insert and heat for 15 ± 1 min. During heating, the 3M Lysis Solution will change from pink (cool) to yellow (hot). Samples that have not been properly heat treated during the assay lysis step may be considered a potential biohazard and should *not* be inserted into the 3M Molecular Detection Instrument.

(h) Remove the uncovered rack of 3M Lysis Solution Tubes from the heating block and allow to cool in the 3M Molecular Detection Chill Block Insert for at least 5 min and a maximum of 10 min. The 3M Molecular Chill Block Insert, used at ambient temperature (20–25°C) without the 3M Molecular Detection Chill Block Tray, should sit directly on the laboratory bench. When cool, the lysis solution will revert to a pink color.

(i) Remove the rack of 3M Lysis Solution Tubes from the 3M Molecular Detection Chill Block Insert.

Amplification

(a) One 3M Molecular Detection Assay 2 - STEC Gene Screen (*stx*) Reagent Tube is required for each sample and the NC.

(1) 3M Molecular Detection Assay 2 - STEC Gene Screen (*stx*) Reagent Tube strips can be cut to desired tube number. Select the number of individual Reagent Tubes or 8-tube strips needed.

(2) Place Reagent Tubes in an empty rack.

(3) Avoid disturbing the reagent pellets from the bottom of the tubes.

(b) Select one 3M Reagent Control Tube and place in rack.

(c) To avoid cross-contamination, decap one 3M Molecular Detection Assay 2 - STEC Gene Screen (*stx*) Reagent Tube strip at a time and use a new pipette tip for each transfer step.

(d) Transfer 20 µL of each sample lysate into individual 3M Molecular Detection Assay 2 - STEC Gene Screen (*stx*) Reagent Tubes first followed by the NC. Hydrate the 3M Reagent Control Tube last.

(e) Use the 3M Molecular Detection Cap/Decap Tool-Reagent to decap one 3M Molecular Detection Assay 2 -STEC Gene Screen (*stx*) Reagent Tubes-strip at a time. Discard cap.

   (1) Transfer 20 µL sample lysate from the upper half of the liquid (avoid precipitate) in the 3M Lysis Solution Tube into corresponding 3M Molecular Detection Assay 2 - STEC Gene Screen (*stx*) Reagent Tube. Dispense at an angle to avoid disturbing the pellets. Mix by gently pipetting up and down five times.

   (2) Repeat until individual sample lysate has been added to a corresponding 3M Molecular Detection Assay 2 - STEC Gene Screen (*stx*) Reagent Tube in the strip.

   (3) Cover the 3M Molecular Detection Assay 2 - STEC Gene Screen (*stx*) Reagent Tubes with the provided extra caps and use the rounded side of the 3M Molecular Detection Cap/Decap Tool-Reagent to apply pressure in a back-and-forth motion ensuring that the cap is tightly applied.

   (4) Repeat steps as needed, for the number of samples to be tested.

   (5) When all sample lysates have been transferred, transfer 20 µL NC lysate into a 3M Molecular Detection Assay 2 - STEC Gene Screen (*stx*) Reagent Tube.

   (6) Transfer 20 µL NC lysate into a RC tube. Dispense at an angle to avoid disturbing the pellets. Mix by gently pipetting up and down five times.

(f) Load capped tubes into a clean and decontaminated 3M Molecular Detection Speed Loader Tray. Close and latch the 3M Molecular Detection Speed Loader Tray lid.

(g) Review and confirm the configured run in the 3M Molecular Detection Software.

(h) Click the "Start" button in the software and select instrument for use. The selected instrument's lid automatically opens.

(i) Place the 3M Molecular Detection Speed Loader Tray into the 3M Molecular Detection Instrument and close the lid to start the assay. Results are provided within 60 min, although positives may be detected sooner.

(j) After the assay is complete, remove the 3M Molecular Detection Speed Loader Tray from the 3M Molecular Detection Instrument and dispose of the tubes by soaking in a 1–5% (v/v) household bleach in water solution for 1 h and away from the assay preparation area.

(k) *Note*: To minimize the risk of false positives due to cross-contamination, never open reagent tubes containing amplified DNA. This includes 3M Molecular Detection Assay 2 - STEC Gene Screen (*stx*) Reagent, 3M Reagent Control, and 3M Matrix Control Tubes. Always dispose of sealed reagent tubes by soaking in a 1–5% (v/v) household bleach in water solution for 1 h and away from the assay preparation.

Results and Interpretation

An algorithm interprets the light output curve resulting from the detection of the nucleic acid amplification. Results are analyzed automatically by the software and are color-coded based on the result. A positive or negative result is determined by analysis of a number of unique curve parameters. Presumptive positive results are reported in real-time while negative results will be displayed after the run is completed. *Note*: Even a negative sample will not give a zero reading as the system and 3M Molecular Detection Assay 2 - STEC Gene Screen (*stx*) amplification reagents have a "background" relative light unit (RLU) reading.

Confirmation

Presumptive positive samples should be confirmed as per the laboratory standard operating procedures or by following the appropriate reference method confirmation, USDA/FSIS MLG 5C.00 or FDA-BAM Ch 4A as relevant to the matrix, beginning with transfer from the primary enrichment broth to selective plates, to confirmation of isolates using appropriate biochemical, microscopic, and serological methods. For matrixes specified by USDA/FSIS MLG 5C, immunomagnetic separation (IMS) should be done prior to plating on selective medium.

In the rare event of any unusual light output, the algorithm labels this as "Inspect". 3M recommends the user to repeat the assay for any Inspect samples. If the result continues to be Inspect, proceed to confirmation test using USDA/FSIS MLG 5C.00 or FDA-BAM Ch. 4A.

In the event of discordant results (presumptive positive with the 3M Molecular Detection Assay 2 - STEC Gene Screen (*stx*), non-confirmed by USDA/FSIS MLG 5C.00 or FDA-BAM Ch. 4A), the laboratory should follow their established standard operating procedures to report their results.

Validation Study

This matrix extension study was conducted under the *Performance Tested Method*[SM] program according to the AOAC INTERNATIONAL *Methods Committee Guidelines for Validation of Microbiological Methods for Food and Environmental Surfaces* ([12]). Matrix studies were conducted independently by Q Laboratories (Cincinnati, OH) and SGS Vanguard Sciences, Inc. (North Sioux City, SD); both American Association for Laboratory Accreditation (A2LA) accredited to ISO/IEC 17025:2005 (10) for *E. coli* O157: H7 and for non-O157 STEC.

Matrix Study

(a) *Methodology.*—Bulk samples of all matrices studied (fresh raw beef trim 75% lean, fresh raw ground beef 75% lean, fresh raw ground pork 70% lean, fresh raw poultry parts, and spouts) were purchased from local supplier(s). Per supplier agreement, the raw beef trim was not prescreened for natural contamination with STEC organisms. The remaining fresh raw meat matrixes were prescreened for natural STEC contamination following the USDA/FSIS MLG 5C.00 reference method, and the sprouts were pre-screened following FDA-BAM Ch. 4A. No natural contamination by the target organism was detected in those products screened. Each matrix was artificially contaminated with an STEC strain at low and high contamination levels, while a sample of each matrix was set aside to run as an uncontaminated control. Thirty test portions of each matrix were analyzed in the unpaired comparison: 20 portions at a low contamination level, 5 portions at a high contamination level, and 5 portions non-contaminated. The low contamination level was targeted at a level to achieve a fractional response (5–15 positive results/20 replicates portions tested), while the high contamination level was targeted 10 times higher to produce 5 positive results/5 replicate portions tested. A 15-tube most probable number (MPN) was performed for both the high and low load samples to determine the final concentration of target per sample.

E. coli strains from the Microbiological Data Program (MDP), US Meat Animal Research Center, Clay Center, NE, the American Type Culture Collection (ATCC), Manassas, VA, or from the Michigan State University Culture Collection (MSU), Lansing, MI, were used to artificially contaminate the matrixes. All matrixes were artificially contaminated with a liquid culture of Shiga toxin-producing *E. coli*. For fresh raw beef trim, *E. coli* O157: H7 (MDP 28) was used; the culture was grown in brain heart infusion broth (BHI) overnight at 35–37°C. The culture was stored at 2–8°C for 24 h while the inoculum level was determined. Serial dilutions were prepared in tubes of sterile distilled water to achieve an inoculation level of 0.2–2 colony forming units (CFU)/25g or 2–10 CFU/25g. Fresh raw ground beef (approximately 75% lean) was inoculated with *E. coli* O103 MSU TW07697, fresh raw ground pork (approximately 70% lean) with *E. coli* O145 MSU TW07596, and fresh raw poultry parts and sprouts with *E. coli* O157: H7 ATCC BAA-460. Each of these cultures was propagated on tryptic soy agar with 5% sheep blood (SBA) from a stock culture stored at 70°C. SBA was incubated for 24 ± 2 h at 35 ± 1°C. The pure culture was transferred to BHI broth and incubated for 24 ± 2 h at 35 ± 1°C. Following incubation, each culture was diluted to a target level using BHI as the diluent and added to the matrix at an appropriate amount where the low-level inoculated samples would yield 0.2–2 CFU/25g and the high-level inoculated samples would yield 2–10 CFU/25 g.

Inoculated matrix was mixed to ensure homogeneous distribution of the organisms within the matrix and was held for 48–72 h at 2–8°C to allow for equilibration of the organism. To create 375 g test portions, 25 g from each contamination level was combined with 350 g uninoculated matrix on the day of analysis. For 25 g test portions, 25 g replicates for each level of contamination were transferred to sterile filter laboratory blender bags on the day of analysis.

3M Molecular Detection Assay 2 - STEC Gene Screen (stx)

Fresh raw beef trim (approximately 75% lean, 375 g test portions), fresh raw ground beef (approximately 75% lean, 25 g test portions), fresh raw pork (approximately 70% lean, 375 g test portions), fresh raw poultry parts (375 g test portions), and sprouts (25 g test portions) were enriched and incubated according to the *Sample Preparation* and *Sample Enrichment* sections above. After 10 and 18 h enrichment time points, each enriched test portion of the fresh raw meat samples was screened using the 3M Molecular Detection Assay 2 - STEC Gene Screen (*stx*) method. Each sprout test portion was screened using the 3M Molecular Detection Assay 2 - STEC Gene Screen (*stx*) method after 18–24 h of enrichment. Regardless of presumptive results, all fresh raw meat test portions were confirmed using the modified USDA/FSIS MLG 5C.00 reference method, and all sprout test portions were confirmed using the FDA-BAM Ch. 4A reference method, after the 18 h enrichment time point. For all test portions, final confirmation was obtained by the bioMérieux VITEK GN2 (Durham, NC) OMA **2011.17**.

Reference Methods USDA/FSIS MLG 5C.00 and FDA-BAM Ch. 4A

All media for the reference method and cultural confirmations were prepared according to USDA/FSIS MLG 5C.00 or FDA-BAM Ch. 4A methods.

USDA/FSIS MLG 5C.00 Method

A modified version of the USDA/FSIS MLG 5C.00 was conducted as the 3M Molecular Detection Assay 2 - STEC Gene Screen (*stx*)

method is not able to determine the serogroup of *E. coli* present. For the modified USDA/FISIS MLG 5C.00 reference method, 25 g test portions of raw beef trim (approximately 75% lean), fresh raw ground beef (approximately 75% lean), fresh raw ground pork (approximately 70% lean), and poultry parts were enriched with 225 ± 4.5 mL modified tryptic soy broth (mTSB). Samples were massaged by hand for 20–30 s to disperse clumps and incubated for 15–24 h at 42 ± 1°C.

After incubation all samples were screened for *stx* and *eae* genes using the iQ-Check™ VirX (Hercules, CA) following the user guide and product instructions. Samples that screened negative for the *stx* and/or *eae* gene were reported as negative, inconclusive results were repeated, and all samples that screened positive for *stx* and *eae* genes were screened for serogroup using the iQ-Check SerO following the user guide and product instructions. Positive samples were confirmed by IMS isolation with anti-*E. coli* magnetic beads for the specific serogroup identified and plated onto modified rainbow agar (mRBA). mRBA plates were incubated at 35 ± 2°C for 20–24 h. Following incubation mRBA plates were examined for colonies and latex agglutination was performed for the specific serogroup to confirm *E. coli* colonies. Samples that had no growth or were agglutination negative were reported as negative. Colonies that were agglutination positive were further confirmed by streaking to SBA for isolation. SBA plates were incubated at 35 ± 2°C for 20–24 h. Following incubation latex agglutination was performed on colonies. Colonies with positive agglutination were screed for the *stx* and *eae* genes using the iQ-Check VirX following the user guide and product instructions. Final confirmation was obtained by bioMérieux VITEK2 GN OMA **2011.17**.

FDA-BAM Ch. 4A Method

For the FDA-BAM Ch. 4A reference method, 25 g test portions of sprouts were enriched with 225 ± 5 mL modified Buffered Petone water with pyruvate and acriflavin, cefsulodin, and vancomycin supplement. All test portions were stomached for 2 min at medium speed and incubated for 24 h at 42 ± 1°C with shaking at 140 revolutions per min.

Following enrichment, all test portions were serially diluted 1:10 in phosphate buffered water to 10^{-2} through 10^{-4}. A 100 μL aliquot of the serial dilutions was plated in duplicate onto Levine's eosin-methylene blue, MacConkey agar with sorbitol, cefixime, and tellurite, and Rainbow® agar in order to achieve isolated colonies. All plates were incubated for 18–24 h at 37 ± 1°C. After incubation plates containing typical colonies were screened for the appropriate STEC by latex agglutination. Up to 10 isolated colonies that screened positive were streaked to SBA and tryptic soy agar with yeast (TSAYE) and incubated for 18–24 h at 37 ± 1°C. Following incubation, a ColiComplete (CC) disc was placed into the heaviest area of growth on the TSAYE plates and incubated for an additional 18–24 h at 37 ± 1°C. The CC discs on the TSAYE plates were observed for typical reactions (blue color change with no fluorescence under long-wave UV) and a spot indole test was conducted. Final biochemical confirmations were obtained by bioMérieux VITEK2 GN OMA **2011.17**.

(a) *Results*.—As per criteria outlined in Appendix J ([12](12)), fractional positive results were obtained for both 10 and 18 h enrichment time points for the 3M Molecular Detection Assay 2 - STEC Gene Screen (*stx*) method for fresh raw beef trim, raw ground beef, fresh raw ground pork, and fresh raw poultry parts. Fractional positive results were also obtained for the 3M

Table 1. Matrix study: 3M Molecular Detection Assay 2 - STEC Gene Screen (stx) results, presumptive versus confirmed—POD results

| Matrix | Strain | MPN/portion[b] | n[c] | 3M MDA2[a] - STEC Gene Screen (stx) presumptive | | | 3M MDA2 - STEC Gene Screen (stx) confirmed | | | dPOD$_{CP}$[g] | 95% CI[h] |
				x[d]	POD$_{CP}$[e]	95% CI	x	POD$_{CC}$[f]	95% CI		
Fresh raw ground beef[i], 75% lean (25 g)	E. coli O103 MSU[j] TW07697	NA[k]	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	−0.47, 0.47
		0.51, (0.26, 0.87)	20	8	0.40	0.22, 0.61	8	0.40	0.22, 0.61	0.00	−0.13, 0.13
		1.97, (0.91, 4.27)	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	−0.47, 0.47
Fresh raw beef trim 73% lean (375 g)	E. coli O157: H7 MDP[l] 28	NA	5	10 h: 1	0.20	0.00, 0.62	0	0.00	0.00, 0.43	0.20	−0.36, 0.76
				18 h: 2	0.40	0.12, 0.77	0	0.00	0.00, 0.43	0.40	−0.21, 1.00
		0.84, (0.14, 0.49)	20	10 h: 14	0.70	0.48, 0.85	14	0.40	0.22, 0.61	0.00	−0.13, 0.13
				18 h: 15	0.70	0.53, 0.89	14	0.40	0.22, 0.61	0.05	−0.11, 0.21
		2.84, (1.38, 5.87)	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	−0.47, 0.47
Fresh raw ground pork[i], 70% lean (375 g)	E. coli O145 MSU TW07596	NA	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	−0.47, 0.47
		0.49, (0.25, 0.84)	20	8	0.40	0.22, 0.61	8	0.40	0.22, 0.61	0.00	−0.13, 0.13
		1.97, (0.91, 4.27)	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	−0.47, 0.47
Fresh raw poultry parts[i] (375g)	E. coli O157: H7 ATCC[m] BAA-460	NA	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	−0.47, 0.47
		0.68, (0.39, 1.12)	20	9	0.45	0.22, 0.61	9	0.44	0.22, 0.61	0.00	−0.13, 0.13
		2.58, (1.15, 5.78)	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	−0.47, 0.47
Sprouts (25 g)	E. coli O157: H7 ATCC BAA-460	NA	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	−0.47, 0.47
		0.63, (0.35, 1.04)	20	8	0.40	0.26, 0.66	8	0.40	0.26, 0.66	0.00	−0.13, 0.13
		2.58, (1.15, 5.78)	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	−0.47, 0.47

[a] MDA2 = Molecular Detection Assay 2.

[b] MPN = Most Probable Number is based on the POD of reference method test portions using the Least Cost Formulations MPN calculator, with 95% confidence interval.

[c] n = Number of test potions.

[d] x = Number of positive test portions.

[e] POD$_{CP}$ = Candidate method presumptive positive outcomes divided by the total number of trials.

[f] POD$_{CC}$ = Candidate method confirmed positive outcomes divided by the total number of trials. Candidate enrichments were confirmed using the appropriate reference procedure: MLG 5C.00 for fresh raw meat; FDA BAM Chapter 4A for fresh sprouts.

[g] dPOD$_{CP}$ = Difference between the candidate method presumptive result and candidate method confirmed result POD values.

[h] 95% CI = If the confidence interval of a dPOD does not contain zero, then the difference is statistically significant at the 5% level.

[i] 10 and 18 h enrichment time points produced identical results.

[j] MSU = Michigan State University Culture Collection, Lansing, MI.

[k] NA = Not applicable.

[l] MDP = Microbiological Data Program, US Meat Animal Research Center, Clay Center, NE.

[m] ATCC = American Type Culture Collection, Manassas, VA.

Table 2. Matrix study: Unpaired analysis, 3M™ Molecular Detection Assay 2 - STEC Gene Screen (*stx*) results versus reference method results

Matrix[c]	Strain	MPN[d]/test portion	n[e]	x[f]	POD$_{CP}$[g]	95% CI	x	POD$_{CC}$[h]	95% CI	dPOD$_{CP}$[i]	95% CI[j]
				3M MDA2[a]—STEC Gene Screen (*stx*) results			Reference method[b] results				
Fresh raw ground beef[k] 75% lean (25 g)	*E. coli* O103 MSU[l] TW07697	NA*m*	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	−0.47, 0.47
		0.51, (0.26, 0.87)	20	8	0.40	0.22, 0.61	8	0.40	0.22, 0.61	0.00	−0.13, 0.13
		1.97, (0.91, 4.27)	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	−0.47, 0.47
Fresh raw beef trim 73% lean (375 g)	*E. coli* O157: H7 MDP[n] 28	NA	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	−0.43, 0.43
		0.84, (0.14, 0.49)	20	14	0.70	0.48, 0.85	10	0.5	0.18, 0.57	−0.20	−0.10, 0.45
		2.84, (1.38, 5.87)	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	−0.43, 0.43
Fresh raw ground pork[k] 70% lean (375 g)	*E. coli* O145 MSU TW07596	NA	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	−0.43, 0.43
		0.49, (0.25, 0.84)	20	8	0.40	0.22, 0.61	7	0.35	0.18, 0.57	0.05	−0.23, 0.32
		1.97, (0.91, 4.27)	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	−0.43, 0.43
Fresh raw poultry parts[k] (375g)	*E. coli* O157: H7 ATCC[o] BAA-460	NA	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	−0.43, 0.43
		0.68, (0.39, 1.12)	20	9	0.45	0.26, 0.66	8	0.40	0.22, 0.61	0.05	−0.24, 0.33
		2.58, (1.15, 5.78)	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	−0.43, 0.43
Sprouts (25 g)	*E. coli* O157: H7 ATCC BAA-460	NA	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	−0.43, 0.43
		0.63, (0.35, 1.04)	20	8	0.40	0.22, 0.61	8	0.40	0.22, 0.61	0.00	−0.28, 0.28
		2.58, (1.15, 5.78)	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	−0.43, 0.43

[a] MDA2 = Molecular Detection Assay 2.
[b] Reference method = MLG 5C.00 for raw beef trim, raw ground beef, raw ground pork, raw poultry parts; BAM 4A for sprouts.
[c] Portions were analyzed as an unpaired study.
[d] MPN = Most Probable Number is based on the POD of reference method test portions using the LCF MPN calculator, with 95% confidence interval.
[e] n = Number of test portions.
[f] x = Number of positive test portions.
[g] POD$_{CP}$ = Candidate method presumptive positive outcomes divided by the total number of trials.
[h] POD$_{CC}$ = Candidate method confirmed positive outcomes divided by the total number of trials.
[i] dPOD$_{CP}$ = Difference between the candidate method presumptive result and candidate method confirmed result POD values.
[j] 95% CI = If the confidence interval of a dPOD does not contain zero, then the difference is statistically significant at the 5% level.
[k] Both 10 and 18 h enrichment time points produced identical results.
[l] MSU = Michigan State University Culture Collection (MSU), Lansing, MI.
[m] NA = Not applicable.
[n] MDP = Microbiological Data Program, US Meat Animal Research Center, Clay Center, NE.
[o] ATCC = American Type Culture Collection, Manassas, VA.

Molecular Detection Assay 2 - STEC Gene Screen (*stx*) method for sprouts at an 18 h enrichment.

The POD and dPOD statistical analyses for paired studies and unpaired studies were calculated according to Appendix J, Annex C (12). The matrix study POD and dPOD results are presented in Tables 1 and 2. Background aerobic microbial counts for fresh raw beef trim, ground beef, ground pork, and poultry parts, conducted per USDA/FSIS MLG 3.02 *Quantitative Analysis of Bacteria in Foods as Sanitary Indicators* method (13), were 5.4×10^3, 4.2×10^6, 3.6×10^6, and 4.5×10^6 CFU/g, respectively. FDA-BAM Manual, Ch. 3 (14) was used to determine the background aerobic microbial count for fresh sprouts, 2.8×10^7 CFU/g. The final inoculum level in the low-level and high-level inoculum test portions was determined by MPN on the day of analysis using the Least Cost Formulation MPN Calculator v2.0 (Least Cost Formulations, Ltd, Virginia Beach, VA).

Paired dPOD analyses were used to compare the 3M Molecular Detection Assay 2 - STEC Gene Screen (*stx*) presumptive results from the 3M BPW ISO enrichment broth to the 3M method confirmed results as described above. Data are shown in Table 1. All dPOD comparisons for all matrixes showed no significant statistical difference at the 5% level between presumptive and confirmed results. Additionally, there were no 3M method negative results that confirmed positive.

For the raw beef trim, there were three STEC Gene Screen (*stx*) method presumptive test samples that did not confirm as positive per the USDA/FSIS MLG confirmation: one uninoculated sample at both 10 and 18 h of enrichment, a second uninoculated sample after 18 h enrichment, and one low-level sample after 18 h of enrichment. Because the USDA/FSIS MLG confirmation defines both *stx* and *eae* gene detection, further investigation was performed with the USDA/FSIS MLG 5C Appendix 3.00 PCR (USDA PCR) method on the broth from these samples (15). USDA PCR method results showed one of the uninoculated samples as *stx* positive/*eae* negative and the other as *stx* negative/*eae* positive. The low-level inoculum sample that did not confirm showed USDA PCR results of *stx* positive/*eae* positive. The samples were also run through the USDA/FSIS MLG confirmation process again looking for *stx* positive/*eae* negative colonies, and none were found.

Unpaired dPOD analyses were used to compare the 3M Molecular Detection Assay 2 - STEC Gene Screen (*stx*) confirmed results from the 3M BPW ISO broth to the reference method enriched cultural results. Data are shown in Table 2. There were no significant statistical differences seen between the 3M Molecular Detection Assay 2 - STEC Gene Screen (*stx*) confirmed results from the 3M BPW ISO enrichment broth and the USDA/FSIS MLG 5C.00 reference method confirmed results for fresh raw ground beef, fresh raw beef trim, fresh raw ground pork, or fresh raw chicken parts, or between the 3M Molecular Detection Assay 2 - STEC Gene Screen (*stx*) confirmed results and the FDA-BAM Ch. 4A method confirmed results for sprouts.

Discussion

The POD analysis between the 3M Molecular Detection Assay 2 - STEC Gene Screen (*stx*) and the appropriate reference method for each matrix indicated that there was no significant difference at the 5% level between the number of positive results by the two methods at any time points tested (10 and 18 h of enrichment for raw beef trim, raw ground beef, raw ground pork, and raw chicken pieces, and 18 h of enrichment for fresh sprouts).

Paired dPOD analyses between the 3M Molecular Detection Assay 2 - STEC Gene Screen (*stx*) presumptive results and confirmed results using the appropriate confirmation method by matrix type show dPOD values of 0.00 (no difference) for all matrixes and levels with the exception of raw beef trim at the uninoculated and low levels. Additional testing on unconfirmed presumptive sample broths suggests that there may have been a low level of natural STEC contamination in the matrix. The *E. coli* O26 ATCC BAA-1653 strain used to inoculate raw beef trim is positive for both *stx* and *eae* genes; retesting one of the uninoculated unconfirmed presumptive samples with the USDA/FSIS MLG 5C.00 PCR method showed a *stx* positive/*eae* negative result. A second unconfirmed presumptive sample retested with the USDA/FSIS MLG 5C.00 PCR method showed a *stx* positive/*eae* positive result, so Shiga toxin was identified by two separate molecular methods for a second "unconfirmed presumptive" sample. Further, since no pre-screening was done for STEC with this matrix per supplier agreement, a low-level natural contamination would not have been identified before matrix testing commenced.

Feedback from laboratory analysts from independent matrix studies highlighted additional strengths of the 3M Molecular Detection Assay 2 - STEC Gene Screen (*stx*) method. Analysts found the method quick and simple to perform, providing results in less than 2 h post incubation for up to 90 sample replicates. Another benefit mentioned was the simplicity of the method; with only two sample transfers, risks of possible contamination are minimized. The small footprint and ability to link multiple 3M Molecular Detection Systems to a single laptop computer, offering high throughput, was noted. Analysts also found the 3M Molecular Detection System software to be user friendly, with the ability to track assay lot information and sample identification quickly and with ease, with real-time curves allowing for improvement of any troubleshooting issues that may arise.

Conclusions

The 3M Molecular Detection Assay 2 - STEC Gene Screen (*stx*) method successfully Shiga toxin genes (*stx*1/*stx*2) from STECs in fresh raw ground beef (25 g test samples), fresh raw beef trim (375 g test samples), fresh raw ground pork (375 g test samples), and fresh chicken parts (375 g test samples) after 10 and 18 h of enrichment, and from sprouts (25 g test samples) after 18 h of enrichment, using 3M BPW ISO as the enrichment medium. Using POD analysis, no statistically significant differences were observed between the number of positive samples detected by the candidate method and the appropriate reference method for any of the samples tested, at any of the time points tested. The data collected demonstrate that the 3M Molecular Detection Assay 2 - STEC Gene Screen (*stx*) method is suitable for extension of *Performance Tested Method*[SM] certification for rapid and specific detection of Shiga toxin-producing *E. coli* to cover fresh raw ground beef, fresh raw beef trim, fresh raw ground pork, fresh raw chicken parts, and sprouts.

Acknowledgments

Independent Laboratories
Benjamin Bastin, M. Joseph Benzinger, Jr., Erin Crowley, and James Agin
Q Laboratories
Microbiology Research and Development Laboratory
1930 Radcliff Dr
Cincinnati, OH 45204
USA

Leslie Thompson-Strehlow
SGS Vanguard Sciences, Inc.
Research and Development
224 North Derby Ln
North Sioux City, SD 57049
USA

Reviewer
Yi Chen
U.S. Food and Drug Administration
College Park, MD 20740
USA

Conflict of Interest

None declared.

References

1. World Health Organization (2018) *E. coli*, https://www.who.int/news-room/fact-sheets/detail/e-coli (accessed July 2019)
2. Tarr, P., Gordon, C., & Chandler, W. (2005) *Lancet* **365**, 1073–1086. doi:10.1016/S0140-6736(05)71144-2
3. CDC Division of Health Informatics and Surveillance (2018) National Notifiable Diseases Surveillance System, 2017 Annual Tables of Infectious Disease Data, TABLE 2m. Reported Cases of Notifiable Diseases, by Region and Reporting Area, https://wonder.cdc.gov/nndss/static/2017/annual/2017-table2m.html (accessed July 2019)
4. National Center for Emerging and Zoonotic Infectious Diseases, Centers for Disease Control and Prevention (2006) Estimates of Foodborne Illness in the United States, Reference CS228412, https://www.cdc.gov/foodborneburden/pdfs/scallan-estimated-illnesses-foodborne-pathogens.pdf (accessed July 2019)
5. Majowicz, S., Scallan, E., Jones-Bitton, A., Sargeant, J., Stapleton, J., Angulo, F., Yeung, D., & Kirk, M. (2014) *Foodborne Pathog. Dis.* **11**, 447–455. doi:10.1089/fpd.2013.1704
6. Kintz, E., Brainard, J., Hooper, L., & Hunter, P. (2017) *Int. J. Hyg. Environ. Health*. **220**, 57–67. doi:10.1016/j.ijheh.2016.10.011
7. Stop Foodborne Illness (2019) What is Food Poisoning? http://stopfoodborneillness.org/awareness/what-is-foodborne-illness/ (accessed July 2019)
8. U.S. Department of Agriculture Food Safety and Inspection Service (2019) Microbiology Laboratory Guidebook, Ch. 5C.00, Detection, Isolation and Identification of Top Seven Shiga Toxin-Producing Escherichia coli (STEC) from Meat Products and Carcass and Environmental Sponges, https://www.fsis.usda.gov/sites/default/files/media_file/2021-08/MLG-5C.02.pdf (accessed version 5C.00 June 2019)
9. U.S. Food and Safety Administration (2018) *Bacteriological Analytical Manual,* Ch. 4A, Diarrheagenic Escherichia coli, https://www.fda.gov/food/laboratory-methods-food/bam-chapter-4a-diarrheagenic-escherichia-coli (accessed June 2019)
10. ISO/IEC 17025:2017: General Requirements for the Competence of Testing and Calibration Laboratories
11. ISO 7218:2007: Microbiology of Food and Animal Feeding Stuffs – General Rules for Microbiological Examination
12. Official Methods of Analysis (2019) 21st Ed., AOAC INTERNATIONAL, Rockville, MD, Appendix J, http://www.eoma.aoac.org/app_j.pdf (accessed June 2019)
13. U.S. Department of Agriculture Food Safety and Inspection Service (2019) Microbiology Laboratory Guidebook, Ch. 3.02, Quantitative Analysis of Bacteria in Foods as Sanitary Indicators, https://www.fsis.usda.gov/sites/default/files/media_file/2021-03/MLG-3.pdf (accessed July 2019)
14. U.S. Food and Drug Administration (2001) *Bacteriological Analytical Manual,* Ch. 3, Aerobic Plate Count, https://www.fda.gov/food/laboratory-methods-food/bam-chapter-3-aerobic-plate-count (accessed July 2019)
15. U.S. Department of Agriculture Food Safety and Inspection Service (2019) Microbiology Laboratory Guidebook, Ch. 5C, Appendix 4.00: Primer and Probe Sequences and Reagent Concentrations for non-O157 Shiga Toxin-Producing Escherichia coli (STEC) Real-Time PCR Assay, https://www.fsis.usda.gov/sites/default/files/media_file/2021-03/mlg-5-appendix-4.pdf (accessed July 2019)