

## MICROBIOLOGICAL METHODS

# Validation of the 3M™ Molecular Detection Assay 2 - STEC Gene Screen (stx) for the Detection of Shiga Toxin Gene (stx1 and/or stx2) in Fresh Raw Ground Beef and Fresh Spinach: AOAC Performance Tested Method<sup>SM</sup> 071903

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## 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 (stx1 and/or stx2) from Shiga toxin-producing *Escherichia coli* (STEC) in enriched foods. The stx assay does not differentiate between stx1 and stx2 but detects the presence of stx1 and/or stx2.

**Objective:** The 3M Molecular Detection Assay 2 - STEC Gene Screen (stx) method was evaluated for AOAC® Performance Tested Methods<sup>SM</sup> certification.

**Methods:** Matrix studies, inclusivity/exclusivity, robustness testing, product stability, and lot-to-lot variability testing were conducted to assess the method's performance.

**Results:** The 3M Molecular Detection Assay 2 - STEC Gene Screen (stx) demonstrated equivalent results to the United States Department of Agriculture/Food Safety and Inspection Service Microbiology Laboratory Guidebook Chapter 5C.00 reference method for fresh raw ground beef, and the U.S. Food and Drug Administration *Bacteriological Analytical Manual* Chapter 4A reference method for fresh spinach. The 3M Molecular Detection Assay

2 - STEC Gene Screen (stx) detected all STEC *E. coli* strains (*E. coli* strains with stx1 and/or stx2 genes) and did not detect any of the 45 strains from the exclusivity panel. Robustness testing indicated that small variations in critical test parameters did not adversely affect the assay's performance. Product consistency and stability testing demonstrated no differences between the lots evaluated.

**Conclusion:** 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 Shiga toxin-producing *E. coli* in raw ground beef and spinach.

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**Highlights:** The 3M Molecular Detection Assay 2 - STEC Gene Screen (stx) method is suitable for the rapid and specific detection of Shiga toxin-producing *E. coli* in fresh raw ground beef, and spinach.

## 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, 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 *stx1* and/or *stx2* 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.

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

- Target organisms.*—Shiga toxin-producing *E. coli* (strains containing *E. coli* gene *stx1*, coding for Shiga toxin type 1, and/or *stx2*, coding for Shiga toxin type 2).
- Matrixes.*—Fresh raw ground beef (375 g, approximately 73% lean), fresh raw spinach (200 g).
- Summary of validated performance claims.*—Performance equivalent to that of U.S. Department of Agriculture-Food Safety and Inspection Service *Microbiology Laboratory Guidebook* (USDA/FSIS MLG), Chapter 5C.00, Detection, isolation and identification of top seven shiga toxin-producing *Escherichia coli* (STECs) from meat products and carcass and environmental sponges for fresh raw ground beef (8), and to the U.S. Food and Safety Administration *Bacteriological Analytical Manual* (FDA BAM) Chapter 4A, Diarrheagenic *Escherichia coli* for spinach (9).

## Definitions

- 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).
- 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

- 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 (four pouches; containing three strips of eight tubes).
- (b) Lysis Solution Tubes.—96 clear tubes (12 strips of eight tubes, with each tube containing 580  $\mu$ L lysis solution).
- (c) Reagent Control (RC).—16 individual clear flip-top tubes (two pouches of eight individual flip-top tubes).
- (d) Extra reagent tube caps.—96 orange caps.
- (e) Product Instructions.

#### Additional Supplies and Reagent

- (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<sup>TM</sup> Molecular Detection Heat block Insert.—Cat. No. MDSHBIN.
- (j) 3M<sup>TM</sup> Molecular Detection Chill Block Insert.—Cat. No. MDSCBIN.

#### Apparatus

- (a) Incubators.—Capable of maintaining  $37 \pm 1^\circ\text{C}$  and  $41.5 \pm 1^\circ\text{C}$ .
- (b) Filter laboratory blender bags.
- (c) Serological pipette bulbs (automatic pipette).—For sampling and delivering 1–10 mL.
- (d) Serological pipettes.—Aerosol resistant.
- (e) Precision pipettors.—For sampling and delivering 10  $\mu$ L and 20  $\mu$ L.
- (f) Micropipette tips.—Aerosol resistant.
- (g) Multi-channel pipette.—Capable of 20  $\mu$ L.
- (h) Dry bath incubator.—Capable of maintaining a temperature of  $100 \pm 1^\circ\text{C}$ .
- (i) Calibrated thermometer.—Capable of measuring a temperature of  $100 \pm 1^\circ\text{C}$ .
- (j) Calibrated timer.
- (k) Refrigerator.—Capable of maintaining  $2\text{--}8^\circ\text{C}$ , 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 in water) household bleach solution or DNA removal solution. Prepare 3M BPW-ISO as per product instructions. Store prepared broth at  $2\text{--}8^\circ\text{C}$  if it will not be immediately used after preparation. Ensure enrichment media is pre-warmed to  $41.5 \pm 1^\circ\text{C}$  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 and fresh spinach were tested.

- (a) Raw ground beef.—Aseptically transfer 375 g sample to a sterile bag and add 1125 mL 3M BPW-ISO, pre-warmed to  $41.5 \pm 1^\circ\text{C}$ . Hand massage the samples for 30–60 s to disperse and break apart clumps after adding BPW-ISO.
- (b) Leafy produce.—Aseptically transfer 200 g sample to a sterile bag and add 450 mL 3M BPW-ISO, pre-warmed to  $41.5 \pm 1^\circ\text{C}$ . Rinse enrichment broth over leaves and agitate gently for 30–60 s. Do not massage or homogenize leaves.

#### Sample Enrichment

- (a) Incubate the bag aerobically at  $41.5 \pm 1^\circ\text{C}$ .
- (b) Incubate raw ground beef for 10–18 h. Incubate leafy produce for 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 in water) household bleach 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  $100 \pm 1^\circ\text{C}$ . *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 (i.e., a partial immersion thermometer or digital thermocouple thermometer, not a total immersion thermometer) placed in the designated location, verify that the 3M Molecular Detection Heat Block Insert is at  $100 \pm 1^\circ\text{C}$ .
  - (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  $37 \pm 1^\circ\text{C}$  incubator for 1 h, or place them in a dry double block heater for 30 s at  $100 \pm 1^\circ\text{C}$ .
  - (b) Invert the capped tubes to mix. Proceed to the 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. After collecting the sample, roll down bag to minimize headspace and reduce exposure of the enrichment to air. If confirmation of presumptive results is required, proceed to confirmatory steps as soon as presumptive result is obtained.
  - (d) One 3M Lysis Solution tube is required for each sample and the negative control (NC) sample (sterile enrichment medium).
    - (1) 3M Lysis Solution Tubes tube strips can be cut to desired tube number. Select the number of individual 3M Lysis Solution or eight-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  $\mu\text{L}$  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  $\mu\text{L}$  of the neutralizing buffer enrichment into the 3M Lysis Solution tubes.
- (e) When all samples have been transferred, transfer 20  $\mu\text{L}$  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  $100 \pm 1^\circ\text{C}$ .
  - (g) Place the uncovered rack of 3M Lysis Solution Tubes in the 3M Molecular Detection Heat Block Insert and heat for  $15 \pm 1$  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 eight-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 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 Tube strip at a time. Discard cap.
  - (1) Transfer 20  $\mu$ L sample lysate from the upper half of the liquid (avoid precipitate) in the 3M Lysis Solution Tube into a 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 all individual sample lysates have 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 extra caps provided 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  $\mu$ L NC lysate into a 3M Molecular Detection Assay 2 - STEC Gene Screen (stx) Reagent Tube.
  - (6) Transfer 20  $\mu$ L 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 in water) household bleach 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 in water) household bleach 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 Chapter 5C.00 or FDA BAM Chapter 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 matrices specified by USDA/FSIS MLG Chapter 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 Chapter 5C.00 or FDA BAM Chapter 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 Chapter 5C.00 or FDA BAM Chapter 4A), the laboratory should follow their established standard operating procedures to report their results.

### Validation Study

This validation study was conducted under the AOAC Research Institute *Performance Tested Method*<sup>SM</sup> (PTM) program according to the AOAC *Methods Committee Guidelines for Validation of Microbiological Methods for Food and Environmental Surfaces* (12). The inclusivity/exclusivity, robustness, and stability testing were performed by 3M. Matrix studies were conducted independently by Q Laboratories (Cincinnati, OH, USA), and SGS Vanguard Sciences, Inc. (North Sioux City, SD, USA); both American Association for Laboratory Accreditation (A2LA) accredited to ISO/IEC 17025:2005 (10) for *E. coli* O157: H7 and for non-O157 Shiga toxin-producing *E. coli* (STEC).

### Inclusivity/Exclusivity

- (a) **Methodology.**—Inclusivity and exclusivity testing were conducted to ensure that the 3M Molecular Detection Assay 2 - STEC Gene Screen (stx) specifically detects Shiga toxin-producing *E. coli* strains, while not reacting with non-target strains and species. For inclusivity, 50 *E. coli* strains containing the *stx1* and/or *stx2* genes were tested (Table 1); these strains were previously characterized in external collections as STEC strains. Additionally, strains were screened by PCR using primer sequences for *stx1* and *stx2* outlined in Appendix 4.00 of USDA/FSIS MLG Chapter 5C.00 (13). Each strain was cultured in 3M BPW-ISO for 10 h at  $41.5 \pm 1^\circ\text{C}$  and diluted 1000-fold before testing. Fifty exclusivity strains, consisting of closely related non-*E. coli* species and *E. coli* non-STEC strains, were grown in BPW-ISO and incubated at  $37 \pm 2^\circ\text{C}$  for 24 h or until turbid (Table 2). Exclusive cultures were diluted one log when visually turbid and the average CFU/mL tested on the Molecular

Table 1. Inclusivity testing results for 3M Molecular Detection Assay 2 - STEC Gene Screen (stx)

Number	Strain source	Strain ID	Genus	Species	Serogroup	stx1 <sup>a</sup>	stx2 <sup>a</sup>	Isolation source	3M MDA2 <sup>1</sup> - STEC (stx) results
1	E. coli Reference Center (PSU) <sup>b</sup>	10.2360	Escherichia	coli	O45: H2	+	-	Unknown	Positive
2	MSU STEC Center <sup>c</sup>	TW04257	Escherichia	coli	O111:-	+	+	Washington—BD <sup>d</sup> fever vomit	Positive
3	MSU STEC Center	TW07991	Escherichia	coli	O103: NM	+	-	Ohio—unknown	Positive
4	Minnesota Department of Health <sup>e</sup>	2011024930-1	Escherichia	coli	O103: H2	+	-	Minnesota—stool/Diarrhea	Positive
5	MSU STEC Center	TW08101	Escherichia	coli	O103: H2	+	-	Denmark—Feces	Positive
6	Minnesota Department of Health	2011027007-3	Escherichia	coli	O103: H2	+	-	Minnesota—Stool/Diarrhea	Positive
7	Minnesota Department of Health	2011032087-1	Escherichia	coli	O26: H11	+	-	Minnesota—Stool/Diarrhea	Positive
8	MSU STEC Center	TW08039	Escherichia	coli	O121	-	+	Montana—Unknown	Positive
9	E. coli Reference Center (PSU)	99.0723	Escherichia	coli	O26	+	-	Unknown	Positive
10	E. coli Reference Center (PSU)	99.0704	Escherichia	coli	O26	+	-	Unknown	Positive
11	MSU STEC Center	TW07814	Escherichia	coli	O26: H11	+	+	Idaho—HUS <sup>f</sup>	Positive
12	MSU STEC Center	TW07705	Escherichia	coli	O26: H46	+	+	Utah—Watery Stool	Positive
13	USDA ARS <sup>g</sup>	96-3285	Escherichia	coli	O45: H2	+	-	CDC <sup>h</sup> -Human Stool	Positive
14	MSU STEC Center	TW14003	Escherichia	coli	O45: H2	+	-	Michigan—Unknown	Positive
15	MSU STEC Center	TW11239	Escherichia	coli	O103: H25	+	-	Washington—Unknown	Positive
16	MSU STEC Center	TW05997	Escherichia	coli	O103: N	+	-	Idaho—Unknown	Positive
17	MSU STEC Center	TW07990	Escherichia	coli	O103: NM	+	-	Washington—Feces	Positive
18	ATCC <sup>i</sup>	BAA179	Escherichia	coli	O111: H8	+	+	Alabama—HUS	Positive
19	ATCC	BAA181	Escherichia	coli	O111: H8	+	+	South Dakota—HUS	Positive
20	MSU STEC Center	TW07931	Escherichia	coli	O121: H19	-	+	Massachusetts—Bloody Diarrhea	Positive
21	USDA ARS	08023	Escherichia	coli	O121: H19	-	+	FDJ—Human	Positive
22	ATCC	BAA-2129	Escherichia	coli	O145: H28	-	+	Germany—Diarrhea	Positive
23	USDA ARS	05-6544	Escherichia	coli	O26: H11	+	+	PHAK <sup>k</sup> —Human	Positive
24	USDA ARS	TB285	Escherichia	coli	O26: H2	+	-	University of Washington—Human	Positive
25	USDA ARS	93-3118	Escherichia	coli	O26: H11	+	-	Human, PHAC	Positive
26	USDA ARS	96-1415	Escherichia	coli	O26: H11	+	-	PHAC—Human	Positive
27	USDA ARS	96-001	Escherichia	coli	O26: H11	+	-	PHAC—Human	Positive
28	USDA ARS	b8026 C1	Escherichia	coli	O45: H2	+	-	CDC—Calf	Positive
29	USDA ARS	05-6545	Escherichia	coli	O45: H2	+	-	PHAC—Human	Positive
30	USDA ARS	SJ7	Escherichia	coli	O45: H2	+	-	CDC—Human	Positive
31	USDA ARS	SJ8	Escherichia	coli	O45: H2	+	-	CDC—Human	Positive
32	USDA ARS	SJ9	Escherichia	coli	O45: H2	-	+	CDC—Human	Positive
33	USDA ARS	B8227 C8	Escherichia	coli	O45	+	-	CDC—Calf	Positive
34	USDA ARS	97-3112	Escherichia	coli	O103: H25	+	-	CDC—Human	Positive
35	USDA ARS	tb154	Escherichia	coli	O103: H6	+	-	University of Washington—Human	Positive
36	USDA ARS	03-2444	Escherichia	coli	O103: H25	+	-	PHAC—Human	Positive
37	USDA ARS	04162	Escherichia	coli	O103: H6	+	-	FDA—Human	Positive
38	USDA ARS	96-3166	Escherichia	coli	O111: NM	+	+	CDC—Human	Positive
39	USDA ARS	TB226	Escherichia	coli	O111: HN	+	+	University of Washington—Human	Positive
40	USDA ARS	01387	Escherichia	coli	O111: H8	+	-	FDA—Human	Positive
41	USDA ARS	96-1585	Escherichia	coli	O121: H19	+	-	PHAC—Human	Positive
42	USDA ARS	97-3068	Escherichia	coli	O121: H19	-	+	CDC—Human	Positive
43	USDA ARS	03-4064	Escherichia	coli	O121: NM	-	+	PHAC—Human	Positive
44	USDA ARS	DA-1	Escherichia	coli	O121	-	+	STEC Center MSU—Human	Positive

(continued)

Table 1. (continued)

Number	Strain source	Strain ID	Genus	Species	Serogroup	stx1 <sup>a</sup>	stx2 <sup>a</sup>	Isolation source	3M MDA2 <sup>1</sup> - STEC (stx) results
45	USDA ARS	07865	Escherichia	coli	O145: H28	-	+	FDA—Cow feces	Positive
46	USDA ARS	FSIS 258-93	Escherichia	coli	O157: H7	+	+	Beef Patty Outbreak	Positive
47	USDA ARS	FSIS 298-94	Escherichia	coli	O157: H7	+	-	Ground Beef outbreak	Positive
48	USDA ARS	FSIS 012-89	Escherichia	coli	O157: H7	+	+	Beef Brisket	Positive
49	Minnesota Department of Health	2011020423-1	Escherichia	coli	O45: H2	+	+	Minnesota—Stool/Diarrhea	Positive
50	Minnesota Department of Health	2011027121-2	Escherichia	coli	O111: NM	+	-	Minnesota—Stool/Diarrhea	Positive

<sup>a</sup> PCR gene characterization (internally screened with PCR using primers from Appendix 4C of USDA/FSIS MLG Chapter 5C.00).

<sup>b</sup> E. coli Reference Center (PSU) = The E. coli Reference Center at Pennsylvania State University, University Park, PA, USA.

<sup>c</sup> MSU STEC Center = Thomas S. Whittam STEC Center at Michigan State University, East Lansing, MI, USA.

<sup>d</sup> BD = Bloody diarrhea.

<sup>e</sup> Minnesota Department of Health, St. Paul, MN, USA.

<sup>f</sup> HUS = Hemolytic uremic syndrome.

<sup>g</sup> USDA ARS = U.S. Department of Agriculture, Agricultural Research Service, Washington, DC, USA.

<sup>h</sup> CDC = Centers for Disease Control and Prevention, Atlanta, GA, USA.

<sup>i</sup> ATCC = American Type Culture Collection, Manassas, VA, USA.

<sup>j</sup> FDA = U.S. Food and Drug Administration, College Park, MD, USA.

<sup>k</sup> PHAC = Public Health Agency of Canada, ON, Canada.

<sup>l</sup> Molecular Detection Assay 2.

Detection System was  $9.32 \times 10^7$  CFU/mL. Inclusivity and exclusivity cultures were randomized and blind-coded for testing on the 3M Molecular Detection Assay 2 - STEC Gene Screen (stx).

- (b) **Results.**—The results of the inclusivity and exclusivity testing are presented in Tables 1 and 2. Fifty out of 50 inclusivity strains tested were detected by the 3M Molecular Detection Assay 2 - STEC Gene Screen (stx). Zero out of 45 exclusivity strains tested were detected by the 3M Molecular Detection Assay 2 - STEC Gene Screen (stx).

### Matrix Study

- (a) **Methodology.**—Bulk samples of the matrices studied (fresh raw ground beef, approximately 73% lean, and fresh spinach) were purchased from local supplier(s). The fresh raw ground beef was prescreened for natural contamination with STEC organisms following the USDA/FSIS MLG Chapter 5C.00 reference method, and the spinach was prescreened following FDA BAM Chapter 4A: no natural contamination by the target organism was detected during product screening. 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, five portions at a high contamination level, and five portions uncontaminated. 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 and determined using the Least Cost Formulation MPN Calculator v2.0, Least Cost Formulations, Ltd, Virginia Beach, VA.

E. coli strains from the American Type Culture Collection (ATCC; Manassas, VA, USA) were used to artificially contaminate the matrices. Fresh raw ground beef samples were inoculated with E. coli O26 BAA 1653. The culture was grown in brain heart infusion (BHI) broth at  $35 \pm 1^\circ\text{C}$  for  $24 \pm 2$  h. Following incubation, the 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/25 g 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  $2–8^\circ\text{C}$  to allow for equilibration of the organism. For the 375 g test portions analyzed by the 3M Molecular Detection Assay 2 - STEC Gene Screen (stx) method, 25 g from each contamination level was combined with 350 g uninoculated matrix on the day of analysis. For 25 g test portions analyzed by the USDA/FSIS MLG Chapter 5C.00 method, 25 g replicates for each level of contamination were transferred to sterile filter laboratory blender bags on the day of analysis.

Fresh spinach was inoculated with E. coli O111 strain C4-61-1. The culture was grown in BHI overnight at  $35–37^\circ\text{C}$ , then serial dilutions were prepared in tubes of sterile distilled water to achieve an inoculation level of 0.2–2 CFU/200 g for the low level, and 2–10 CFU/200 g for the high level. The raw spinach

Table 2. Exclusivity testing results for 3M Molecular Detection Assay 2 - STEC Gene Screen (stx)

Number	Strain source	Strain ID	Genus	Species	Isolation source	Serogroup <sup>a</sup>	3M MDA2 <sup>f</sup> - STEC (stx) results
1	ATCC <sup>b</sup>	9372	Bacillus	<i>atrophaeus</i>	Unknown	— <sup>8</sup>	Negative
2	ATCC	8090	Citrobacter	<i>freundii</i>	Unknown	—	Negative
3	ATCC	43162	Citrobacter	<i>brakii</i>	Clinical isolate, California	—	Negative
4	ATCC	27156	Citrobacter	<i>koseri</i>	Type strain of <i>Citrobacter diversus</i>	—	Negative
5	ATCC	25944	Cronobacter	<i>sakazakii</i>	Child's throat	—	Negative
6	ATCC	51816	Enterobacter	<i>amnigenus</i>	Milk, Minnesota	—	Negative
7	ATCC	49464	Enterococcus	<i>raffinosis</i>	Clinical isolate	—	Negative
8	ATCC	11303	Escherichia	<i>coli</i>	Unknown	—	Negative
9	ATCC	10799	Escherichia	<i>coli</i>	Unknown	—	Negative
10	ATCC	25922	Escherichia	<i>coli</i>	Clinical isolate	—	Negative
11	ATCC	8739	Escherichia	<i>coli</i>	Feces	—	Negative
12	ATCC	10536	Escherichia	<i>coli</i>	Unknown	—	Negative
13	BEI <sup>c</sup>	5.3169	Escherichia	<i>coli</i>	Human isolate	O25: H4	Negative
14	BEI	85.1284	Escherichia	<i>coli</i>	Human isolate, 1985	O6: H31	Negative
15	BEI	U9-41	Escherichia	<i>coli</i>	Human urine isolate	O2: K1: H4	Negative
16	BEI	F1119-41	Escherichia	<i>coli</i>	Unknown	O16: K1: H-	Negative
17	BEI	E3b	Escherichia	<i>coli</i>	Human peritoneum	O75: K95: H5	Negative
18	ATCC	51815	Hafnia	<i>alvei</i>	Milk, Minnesota	—	Negative
19	ATCC	51817	Klebsiella	<i>oxytoca</i>	Milk, Minnesota	—	Negative
20	ATCC	13438	Klebsiella	<i>pneumoniae</i>	Water	—	Negative
21	ATCC	13882	Klebsiella	<i>pneumoniae</i>	Moto, starter of sake	—	Negative
22	ATCC	9595	Lactobacillus	<i>rhamnosus</i>	Unknown	—	Negative
23	ATCC	8014	Lactobacillus	<i>plantarum</i>	Unknown	—	Negative
24	ATCC	19111	Listeria	<i>monocytogenes</i>	Poultry, England	—	Negative
25	ATCC	19119	Listeria	<i>ivanovii</i>	Sheep, Bulgaria	—	Negative
26	ATCC	4698	Micrococcus	<i>luteus</i>	Unknown	—	Negative
27	ATCC	49143	Moraxella	<i>catarrhalis</i>	Clinical isolate	—	Negative
28	ATCC	43071	Proteus	<i>mirabilis</i>	Rectum, Georgia	—	Negative
29	ATCC	13315	Proteus	<i>vulgaris</i>	Unknown	—	Negative
30	ATCC	27853	Pseudomonas	<i>aeruginosa</i>	Blood culture	—	Negative
31	ATCC	13525	Pseudomonas	<i>fluorescens</i>	Pre-filter tanks, England	—	Negative
32	ATCC	51812	Salmonella	<i>Typhimurium</i>	Human blood, Minnesota	—	Negative
33	ATCC	6962	Salmonella	<i>Newport</i>	Food poisoning fatality, England	—	Negative
34	ATCC	51741	Salmonella	<i>Infantis</i>	Pasta	—	Negative
35	ATCC	12022	Shigella	<i>flexneri</i>	Unknown	—	Negative
36	ATCC	8700	Shigella	<i>boydii</i>	Unknown	—	Negative
37	ATCC	25931	Shigella	<i>sonnei</i>	Feces, human	—	Negative
38	ATCC	6538	Staphylococcus	<i>aureus</i>	Human lesion	—	Negative
39	ATCC	33317	Streptococcus	<i>bovis</i>	Cow dung	—	Negative
40	ATCC	23715	Yersinia	<i>enterocolitica</i>	Human blood, petechiae, from anterior chamber of the eye, 47-year-old female, Missouri, 1968	—	Negative

(continued)

Table 2. (continued)

Number	Strain source	Strain ID	Genus	Species	Isolation source	Serogroup <sup>a</sup>	3M MDA <sup>2</sup> - STEC (stx) results
41	BEI	E2348/69	Escherichia	coli	Infant diarrhea outbreak, Taunton, England.	O127: H6	Negative
42	BEI	TB182A	Escherichia	coli	Infant diarrhea, Seattle, Washington	O55: H7	Negative
43	BEI	RDEC-1	Escherichia	coli	Rabbit diarrhea	O15: H2	Negative
44	E. coli Reference Center (PSU) <sup>d</sup>	95.0187	Escherichia	coli	Unknown	O145	Negative
45	USDA ARS <sup>e</sup>	5.0623	Escherichia	coli	Unknown	O45: H2	Negative

<sup>a</sup>If applicable.<sup>b</sup>ATCC = American Type Culture Collection, Manassas, VA, USA.<sup>c</sup>BEI = BEI Resources, NIAID, NIH, Manassas, VA, USA.<sup>d</sup>E. coli Reference Center (PSU) = The E. coli Reference Center at Pennsylvania State University, University Park, PA, USA.<sup>e</sup>USDA ARS = U.S. Department of Agriculture, Agricultural Research Service, Washington, DC, USA.<sup>f</sup>Molecular Detection Assay 2.<sup>g</sup>Not available.

was inoculated in bulk, thoroughly mixed, and then separated into 200 g samples. The product was stored at 2–8°C for 72–96 h. A portion remained uninoculated to serve as an uninoculated negative control.

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

The 375 g test portions of raw ground beef were prepared by mixing the 25 g pre-inoculated sample with 350 g uncontaminated material. Each 375 g test portion was aseptically transferred to a sterile bag and combined with 1125 mL pre-warmed 41.5 ± 1°C BPW-ISO enrichment media and hand massaged for 30–60 s to disperse and break apart clumps. For fresh spinach, 450 mL pre-warmed 41.5 ± 1°C BPW-ISO was added to each 200 g test portion, and the liquid was gently rinsed over the leaves for 30–60 s without massaging or homogenizing. The fresh raw ground beef samples were incubated at 41.5 ± 1°C for 10–18 h, and the fresh spinach samples were incubated at 41.5 ± 1°C for 18 h. After incubation, all the samples were analyzed by 3M Molecular Detection Assay 2 - STEC Gene Screen (stx) as described in Analysis. All samples, regardless of screening result, were confirmed using the corresponding reference method.

### USDA/FSIS MLG Chapter 5C.00 and FDA BAM Chapter 4A

All media for the reference method and cultural confirmations were prepared according to USDA/FSIS MLG Chapter 5C.00 or FDA BAM Chapter 4A methods. For raw ground beef samples, 25 g sample test portions were aseptically added to sterile bags, then 225 ± 4.5 mL modified tryptic soy broth (mTSB) was added to each bag. Samples were massaged by hand for 30–60 s to disperse clumps and then were incubated for 15–24 h at 42 ± 1°C.

For spinach samples, 200 g sample test portions were aseptically added to sterile bags, then 450 mL of 1x modified Buffered Petone Water with pyruvate was added to each 200 g test portion. Bags were gently pressed to facilitate the flow of enrichment broth over the leaves. Bags were incubated at 37 ± 1°C for 5 h, then 1 mL each of the Acriflavin, Cefsulodin, and Vancomycin supplements was added per 225 mL mBPWP. Samples were further incubated at 42 ± 1°C for an additional 18–24 h.

Following the 18 h enrichment time point, all matrix samples, regardless of presumptive results, were culturally confirmed. For fresh raw ground beef, confirmation was completed per the USDA/FS ISMLG Chapter 5C.00 method; final confirmation was determined by VITEK2 GN Official Method 2011.17. For fresh spinach, confirmation was completed by plating on Levine's eosin-methylene blue, MacConkey Agar with Sorbitol, Cefixime, and Tellurite and Rainbow® Agar, and subsequent analysis of biochemical and serological characteristics of suspect colonies according to the FDA BAM Chapter 4A methods.

(a) **Results.**—The POD and dPOD statistical analyses for paired studies and unpaired studies were calculated according to Appendix J, Annex C (10). Background aerobic microbial counts for fresh raw ground beef, conducted per USDA/FSIS MLG Chapter 3.02 Quantitative analysis of bacteria in foods as sanitary indicators reference method, were 6.51 CFU/g. For fresh spinach, the background aerobic microbial counts were 6.14 CFU/g per FDA BAM Chapter 3 Aerobic plate count (14, 15).

Paired dPOD analyses were used to compare the 3M Molecular Detection Assay 2 - STEC Gene Screen (stx) presumptive

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

Matrix	MPN/portion <sup>a</sup>	n <sup>b</sup>	3M MDA2 - STEC Gene Screen (stx) presumptive			3M MDA2 - STEC Gene Screen (stx) confirmed			dPOD <sub>CP</sub> <sup>f</sup>	95% CI <sup>g</sup>
			x <sup>c</sup>	POD <sub>CP</sub> <sup>d</sup>	95% CI	x	POD <sub>CC</sub> <sup>e</sup>	95% CI		
Fresh raw ground beef <sup>h</sup> , 73% lean (375 g) <i>E. coli</i> O26 ATCC <sup>i</sup> BAA-1653	NA <sup>j</sup>	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, 0.85)	20	8	0.40	0.22, 0.61	8	0.40	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
Fresh spinach (200 g) <i>E. coli</i> O111 ATCC C4-61-1	NA	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	-0.47, 0.47
	1.20 (0.85, 2.24)	20	16	0.80	0.58, 0.92	16	0.80	0.58, 0.92	0.00	-0.13, 0.13
	7.43 (3.08, 17.94)	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	-0.47, 0.47

<sup>a</sup>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.

<sup>b</sup>n = Number of test portions.

<sup>c</sup>x = Number of positive test portions.

<sup>d</sup>POD<sub>CP</sub> = Candidate method presumptive positive outcomes divided by the total number of trials.

<sup>e</sup>POD<sub>CC</sub> = Candidate method confirmed positive outcomes divided by the total number of trials. Candidate enrichments were confirmed using the appropriate reference procedure: USDA/FSIS MLG Chapter 5C.00 for fresh raw ground beef; FDA BAM Chapter 4A for fresh spinach.

<sup>f</sup>dPOD<sub>CP</sub> = Difference between the candidate method presumptive result and candidate method confirmed result POD values.

<sup>g</sup>95% CI = If the confidence interval of a dPOD does not contain zero, then the difference is statistically significant at the 5% level.

<sup>h</sup>10 and 18 h enrichment time points produced identical results.

<sup>i</sup>ATCC = American Type Culture Collection, Manassas, VA, USA.

<sup>j</sup>NA = Not applicable.

**Table 4.** Matrix study: Unpaired analysis, 3M Molecular Detection Assay 2 - STEC Gene Screen (stx) results versus reference method results

Matrix <sup>a</sup>	Strain	MPN/test portion <sup>b</sup>	n <sup>c</sup>	3M MDA2 - STEC (stx) results			Reference method results <sup>g</sup>			dPOD <sub>CP</sub> <sup>h</sup>	95% CI <sup>i</sup>
				x <sup>d</sup>	POD <sub>CP</sub> <sup>e</sup>	95% CI	x	POD <sub>CC</sub> <sup>f</sup>	95% CI		
Fresh raw ground beef <sup>f</sup> (375 g)	<i>E. coli</i> O26	NA	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	-0.43, 0.43
	ATCC <sup>k</sup> BAA-1653	0.49 (0.25, 0.85)	20	8	0.40	0.22, 0.61	8	0.35	0.18, 0.57	0.05	-0.23, 0.32
		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
Fresh spinach (200 g)	<i>E. coli</i> O111	NA	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	-0.43, 0.43
	ATCC C4-61-1	1.20 (0.85, 2.24)	20	16	0.80	0.58, 0.92	14	0.70	0.48, 0.85	0.10	-0.17, 0.35
		7.43 (3.08, 17.94)	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	-0.43, 0.43

<sup>a</sup>Portions were analyzed as an unpaired study.

<sup>b</sup>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.

<sup>c</sup>n = Number of test portions.

<sup>d</sup>x = Number of positive test portions.

<sup>e</sup>POD<sub>CP</sub> = Candidate method presumptive positive outcomes divided by the total number of trials.

<sup>f</sup>POD<sub>CC</sub> = Candidate method confirmed positive outcomes divided by the total number of trials.

<sup>g</sup>Reference method = USDA/FSIS MLG Chapter 5C.00 for raw ground beef; FDA BAM 4A for spinach.

<sup>h</sup>dPOD<sub>CP</sub> = Difference between the candidate method presumptive result and candidate method confirmed result POD values.

<sup>i</sup>95% CI = If the confidence interval of a dPOD does not contain zero, then the difference is statistically significant at the 5% level.

<sup>j</sup>Both 10 and 18 h enrichment time points produced identical results.

<sup>k</sup>ATCC = American Type Culture Collection, Manassas, VA, USA.

<sup>l</sup>NA = Not applicable.

results from the 3M BPW-ISO enrichment broth to the 3M method confirmed results as described above. Data are shown in Table 3.

For the low contamination level of fresh raw ground beef, the eight presumptive positive results at both 10 and 18 h confirmed as positive, with a dPOD of 0.00 and confidence interval (CI) of (-0.13, 0.13). For the low contamination level of fresh spinach, there were 16 presumptive positives at 18 h, with all 16 confirming as positive, with a dPOD of 0.00 and CI of (-0.13, 0.13). All other portions for each matrix lot and contamination level that were presumptive positive with the 3M Molecular Detection Assay 2 - STEC Gene Screen (stx) confirmed as positive. There were no 3M method negative results that confirmed

positive. All dPOD comparisons showed no significant statistical difference.

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 4. 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 Chapter 5C.00 reference method confirmed results for fresh raw ground beef, or between the 3M Molecular Detection Assay 2 - STEC Gene Screen (stx) confirmed results and the FDA BAM Chapter 4A method confirmed results for fresh spinach.

**Table 5.** Robustness: 3M Molecular Detection Assay 2 - STEC Gene Screen (stx) results: test conditions versus nominal condition

Test condition	n <sup>a</sup>	Test condition results			Nominal condition results <sup>d</sup>			dPOD <sup>f</sup>	95% CI <sup>g</sup>
		x <sup>b</sup>	POD <sub>TC</sub> <sup>c</sup>	95% CI	x	POD <sub>NV</sub> <sup>e</sup>	95% CI		
Ground beef samples inoculated with <i>Escherichia coli</i> O157 culture (FSIS 258-93) <sup>h</sup>									
9.5 h <sup>i</sup> , 13 min <sup>j</sup> , 18 $\mu$ L <sup>k</sup>	10	6	0.60	(0.31, 0.83)	6	0.60	(0.31, 0.83)	0.00	(-0.25, 0.25)
9.5 h, 13 min, 22 $\mu$ L	10	6	0.60	(0.31, 0.83)	6	0.60	(0.31, 0.83)	0.00	(-0.25, 0.25)
9.5 h, 17 min, 18 $\mu$ L	10	6	0.60	(0.31, 0.83)	6	0.60	(0.31, 0.83)	0.00	(-0.25, 0.25)
9.5 h, 17 min, 22 $\mu$ L	10	6	0.60	(0.31, 0.83)	6	0.60	(0.31, 0.83)	0.00	(-0.25, 0.25)
26 h, 13 min, 18 $\mu$ L	10	6	0.60	(0.31, 0.83)	6	0.60	(0.31, 0.83)	0.00	(-0.25, 0.25)
26 h, 13 min, 22 $\mu$ L	10	6	0.60	(0.31, 0.83)	6	0.60	(0.31, 0.83)	0.00	(-0.25, 0.25)
26 h, 17 min, 18 $\mu$ L	10	6	0.60	(0.31, 0.83)	6	0.60	(0.31, 0.83)	0.00	(-0.25, 0.25)
26 h, 17 min, 22 $\mu$ L	10	6	0.60	(0.31, 0.83)	6	0.60	(0.31, 0.83)	0.00	(-0.25, 0.25)
Uninoculated ground beef samples									
9.5 h <sup>i</sup> , 13 min <sup>j</sup> , 18 $\mu$ L <sup>k</sup>	10	0	0.00	(0, 0.28)	6	0.00	(0, 0.28)	0.00	(-0.25, 0.25)
9.5 h, 13 min, 22 $\mu$ L	10	0	0.00	(0, 0.28)	6	0.00	(0, 0.28)	0.00	(-0.25, 0.25)
9.5 h, 17 min, 18 $\mu$ L	10	0	0.00	(0, 0.28)	6	0.00	(0, 0.28)	0.00	(-0.25, 0.25)
9.5 h, 17 min, 22 $\mu$ L	10	0	0.00	(0, 0.28)	6	0.00	(0, 0.28)	0.00	(-0.25, 0.25)
26 h, 13 min, 18 $\mu$ L	10	0	0.00	(0, 0.28)	6	0.00	(0, 0.28)	0.00	(-0.25, 0.25)
26 h, 13 min, 22 $\mu$ L	10	0	0.00	(0, 0.28)	6	0.00	(0, 0.28)	0.00	(-0.25, 0.25)
26 h, 17 min, 18 $\mu$ L	10	0	0.00	(0, 0.28)	6	0.00	(0, 0.28)	0.00	(-0.25, 0.25)
26 h, 17 min, 22 $\mu$ L	10	0	0.00	(0, 0.28)	6	0.00	(0, 0.28)	0.00	(-0.25, 0.25)

<sup>a</sup>n = Number of test portions.<sup>b</sup>x = Number of positive test portions.<sup>c</sup>POD<sub>TC</sub> = Test condition parameter positive outcomes divided by the total number of trials.<sup>d</sup>Nominal condition results = 22 h enrichment, 15 min lysis time, and 20  $\mu$ L lysate into reaction tube.<sup>e</sup>POD<sub>NV</sub> = Nominal value positive outcomes divided by the total number of trials.<sup>f</sup>dPOD = Difference between the test condition and the nominal condition POD values.<sup>g</sup>95% CI = If the confidence interval of a dPOD does not contain zero, then the difference is statistically significant at the 5% level.<sup>h</sup>United States Department of Agriculture, Food Safety and Inspection Service, Agricultural Research Service, Washington, DC, USA.<sup>i</sup>Enrichment time.<sup>j</sup>Lysis heating time.<sup>k</sup>Volume of lysate into reaction tube.

### Robustness Study

(a) **Methodology.**—The robustness study was conducted to determine whether variations to the method parameters, specifically those that may occur when performed by an end user, affect the 3M Molecular Detection Assay 2 - STEC Gene Screen (stx) method performance. Upper and lower limits of three method parameters were evaluated; enrichment time (9.5 and 26 h), lysis heating time (13 and 17 min), and volume of lysate to reaction tube (18 and 22  $\mu$ L). The nominal conditions for the assay are 10.5 h enrichment time, 15 min lysis heating time, and 20  $\mu$ L lysate to reaction tube, and were run alongside the test conditions in a factorial design. Ground beef samples were used in the robustness evaluation. Samples were prepared by distributing 375 g 85% ground beef into 96 oz filter enrichment bags and adding 1125 mL pre-warmed BPW-ISO to each sample. Samples were homogenized by hand. Ten of the rinse samples were inoculated at a fractional level with Shiga toxin-producing *E. coli* O157 strain FSIS 258-93. This culture was obtained from the USDA Agriculture Research Service (ARS; Washington, DC, USA). Ten additional samples were left non-inoculated. Samples were incubated aerobically at 41.5°C for 9.5, 10.5, and 26 h. At each assessment time, samples were subjected to 13 and 17 min of heat treatment in lysis at 100°C. The third robustness parameter, volume of lysate to reaction tube, was evaluated by analyzing replicates by transferring 18 or 22  $\mu$ L lysate into each 3M Molecular Detection Assay 2 - STEC Gene Screen (stx) reagent tube.

(b) **Results.**—Results of the robustness study are presented in Table 5. Six of the 10 replicate fractional-level inoculated portions were positive by the nominal parameters, and by each test condition. All non-inoculated portions were negative. The dPOD values comparing the test conditions to the nominal parameters were zero in all cases, indicating that small variations in these test parameters (enrichment time, lysis time, and lysed sample reaction volume) do not adversely affect the 3M Molecular Detection Assay 2 - STEC Gene Screen (stx) performance.

### Product Stability and Lot-to-Lot Variability Study

(a) **Methodology.**—The stability and lot-to-lot variability of three different test kits were evaluated. Two lots were at eight months of real-time shelf life, which were the oldest available at the time of the product stability study. These lots were prepared in the same timeframe but varied by lots of raw materials used in production. The third lot was at one month of real-time shelf life. The three lots were evaluated by testing with pure culture. Ten replicate samples of each lot were tested with a fractional level preparation of target organism, Shiga toxin-producing *E. coli* O157: H7 (FSIS 258-93), and ten replicate samples of a non-diluted, related organism, *Salmonella* Typhimurium ATCC 14028.

The target STEC *E. coli* strain was grown for 10 h in BPW-ISO at 41.5  $\pm$  1°C and diluted with sterile BPW-ISO before testing. The non-target *S. Typhimurium* strain was grown in

**Table 6.** Product consistency and stability study: 3M Molecular Detection Assay 2 - STEC Gene Screen (stx) results

Lot No.	Age of lot at time of testing, months	n <sup>a</sup>	Pure culture; Shiga toxin-producing <i>Escherichia coli</i> O157 (FSIS 258–93) <sup>b</sup> ; fractional (low) level			n	Pure culture; <i>Salmonella</i> Typhimurium (ATCC 14028) <sup>c</sup> ; undiluted		
			x <sup>c</sup>	POD <sub>T</sub> <sup>d</sup>	95% CI		x	POD <sub>NT</sub> <sup>f</sup>	95% CI
100418–1	8	10	7	0.70	(0.40, 0.89)	10	0	0.00	(0.00, 0.28)
100418–2	8	10	7	0.70	(0.40, 0.89)	10	0	0.00	(0.00, 0.28)
040319	1	10	7	0.70	(0.40, 0.89)	10	0	0.00	(0.00, 0.28)

<sup>a</sup>n = Number of test portions.

<sup>b</sup>United States Department of Agriculture, Food Safety and Inspection Service, Agricultural Research Service, Washington, DC, USA.

<sup>c</sup>x = Number of positive test portions.

<sup>d</sup>POD<sub>T</sub> = Target strain positive outcomes divided by the total number of trials.

<sup>e</sup>American Type Culture Collection, Manassas, VA, USA.

<sup>f</sup>POD<sub>NT</sub> = Non-target strain positive outcomes divided by the total number of trials.

BPW-ISO at 37°C for 24 h and was not diluted before testing. Samples were blind-coded and randomized for the assessment.

- (b) **Results.**—Results of the product stability and lot-to-lot variability study are presented in Table 6. Seven of the 10 replicate fractional-level inoculated portions were positive by each of the three lots of the 3M Molecular Detection Assay 2 - STEC Gene Screen (stx). All non-inoculated portions were negative. Results indicate that the age and lot-to-lot variability of 3M Molecular Detection Assay 2 - STEC Gene Screen (stx) product tested do not adversely affect assay performance.

## Discussion

The results of the inclusivity and exclusivity testing show that all 50 out of 50 inclusivity strains tested were detected by the 3M Molecular Detection Assay 2 - STEC Gene Screen (stx). Zero out of the 45 exclusivity strains tested were detected by the 3M Molecular Detection Assay 2 - STEC Gene Screen (stx).

As per criteria outlined in Appendix J of the *Official Methods of Analysis Manual* for evaluations in matrixes, fractional positive results were obtained for both 10 and 18 h enrichment time points for fresh raw ground beef, and at the 18 h enrichment time point for fresh spinach. The probability of detection (POD) was calculated as the number of positive outcomes divided by the total number of trials. The POD was calculated for the candidate presumptive results, POD<sub>CP</sub>, the candidate confirmatory results, POD<sub>CC</sub>, the difference in the candidate presumptive and confirmatory results, dPOD<sub>CP</sub>, presumptive candidate results that confirmed positive, POD<sub>C</sub>, the reference method, POD<sub>R</sub>, and the difference in the confirmed candidate and reference methods, dPOD<sub>C</sub>. 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 fresh raw ground beef, and 18 h of enrichment for fresh spinach). A summary of POD analyses is presented in Tables 4 and 5.

Results from the robustness study and product stability and lot-to-lot variability study results both indicate the rigor of the method, since small variations in test parameters (enrichment time, lysis time, and lysed sample reaction volume), age of the product, and variations between product lots do not adversely

affect the 3M Molecular Detection Assay 2 - STEC Gene Screen (stx) performance.

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 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 recovered STEC from fresh raw ground beef after 10 and 18 h of enrichment, and from fresh spinach 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.

Matrix studies, inclusivity/exclusivity, robustness testing, product stability, and lot-to-lot variability testing were conducted to assess the performance of the 3M Molecular Detection Assay 2 - STEC Gene Screen (stx) method. The data collected in these studies demonstrate that the 3M Molecular Detection Assay 2 - STEC Gene Screen (stx) method is suitable for *Performance Tested Method*<sup>SM</sup> certification for rapid and specific detection of Shiga toxin-producing *E. coli* in fresh raw ground beef, and spinach.

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#### Conflict of Interest

None declared.

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