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Research Article

MICROBIOLOGICAL METHODS

Validation of the 3M™ Molecular Detection Assay 2 - STEC Gene Screen (*stx* and *eae*) for the Detection of Shiga Toxin Gene (*stx* and *eae*) in Fresh Raw Beef Trim, Fresh Raw Ground Beef and Fresh Spinach: AOAC *Performance Tested Method*℠ 071902

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Abstract

Background: The 3M™ Molecular Detection Assay 2 - STEC Gene Screen (*stx* and *eae*) method is based on gene amplification using real time loop-mediated isothermal amplification with the 3M Molecular Detection System for the rapid and specific detection of Shiga toxin gene (*stx1* and/or *stx2*) and intimin gene (*eae*) from Shiga toxin-producing enterohemorrhagic *Escherichia coli* (STEC) in enriched foods.
Objective: The 3M Molecular Detection Assay 2 - STEC Gene Screen (*stx* and *eae*) method was evaluated for AOAC® *Performance Tested Methods*℠ certification.
Methods: Matrix studies, inclusivity/exclusivity, robustness, 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* and *eae*) demonstrated equivalent results to the US Department of Agriculture–Food Safety and Inspection Service *Microbiology Laboratory Guidebook* 5C.00 for fresh raw beef trim and fresh raw ground beef, and to the US Food and Drug Administration *Bacteriological Analytical Manual* Chapter 4A for fresh spinach. The 3M Molecular Detection Assay 2 - STEC Gene Screen (*stx* and eae) detected 50 of 50 *E. coli* strains with *stx*1 and/or *stx*2 genes, and the *eae* gene, and detected zero of 40 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 demonstrates that the 3M Molecular Detection Assay 2 - STEC Gene Screen (*stx* and *eae*) is a reliable method for the rapid and specific detection of STEC in raw beef trim, raw ground beef, and spinach.

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Highlights: The 3M Molecular Detection Assay 2 - STEC Gene Screen (*stx* and *eae*) method is suitable for the rapid and specific detection of STECs in raw beef trim, 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). Additionally, some of these strains may also harbor *eae*, a gene which codes for intimin, a protein involved with formation of lesions in the gut. *E. coli* strains that contain both *stx* and *eae* genes are described as enterohemorrhagic *E. coli* (EHEC). Symptoms of illnesses caused by STEC can include abdominal cramps, diarrhea (which may progress to bloody diarrhea), fever, and vomiting. In 5–15% 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 source of STEC infections is 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* and *eae*) is used with the 3M Molecular Detection System for the rapid and specific screening of *E coli* genes *stx*1 and/or *stx*2 and *eae* 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, U.S. Department of Agriculture–Food Safety and Inspection Service, *Microbiology Laboratory Guidebook* (USDAFSIS/MLG) 5C.00 (8) or U.S. Food and Drug Administration, *Bacteriological Analytical Manual* (FDA-BAM) Chapter 4A (9) as relevant to the matrix.

The 3M Molecular Detection Assay 2 - STEC Gene Screen (*stx* and *eae*) 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* and *eae*) 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* and *eae*) has been evaluated for use with 3M Buffered Peptone Water (ISO Formulation: 3M BPW ISO) enrichment broth.

Scope of Method

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

(b) *Matrixes*.—Fresh raw beef trim (375 g, approximately 75% lean), fresh raw ground beef (375 g, approximately 73% lean), fresh raw spinach (200 g).

(c) *Summary of validated performance claims*.—Performance equivalent to that of 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 beef trim and fresh raw ground beef (8), and to the FDA-BAM Chapter 4A: Diarrheagenic *Escherichia coli* for spinach (9).

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* and *eae*).
(b) *Cat. No*.—MDA2STXEAE48.
(c) *Ordering information*.—https://www.3m.com/

Test Kit Components

(a) *STEC Gene Screen (stx) Reagent Tubes*.—48 orange tubes (two pouches; containing three strips of eight tubes).
(b) *STEC Gene Screen (eae) Reagent Tubes*.—48 red tubes (two pouches; containing three strips of eight tubes).
(c) *Lysis Solution Tubes*.—96 clear tubes (12 strips of eight tubes, with each tube containing 580 μL lysis solution).
(d) *Reagent Control (RC)*.—16 individual clear flip-top tubes (two pouches of eight individual flip-top tubes).
(e) *Extra reagent tube caps*.—96 orange caps, 96 red caps.
(f) *Product instructions*.

Additional Supplies and Reagents

(a) *3M Molecular Detection System Matrix Control*.—Cat. No. MDMC96NA.
(b) *3M Buffered Peptone Water (ISO Formulation)*.—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*.—Cat. No. MDSCBIN.
(j) *3M Molecular Detection Heat Block*.—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 1–10 mL.
(d) *Serological pipettes*.—Aerosol resistant.
(e) *Precision pipettors*.—For sampling and delivering 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, in water) household bleach 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, fresh raw beef trim, and fresh spinach were tested.

(a) *Raw ground beef, pieces and trim*.—Aseptically transfer 375 g sample to a sterile bag and add 1125 mL 3M BPW-ISO, pre-warmed to 41.5 ± 1°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 ± 1°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 ± 1°C.**
(b) Incubate raw ground beef, pieces and trim, 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 ± 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 (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 ± 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. Selection of the STXEAE-2 icon in the software selects two adjacent wells (A1, A2, B1, B2, etc.), one for *stx* and the other for *eae* reagent tube, as each sample is run with two assays. The Negative Control (NC) is set up for each of the reagent tubes, and one RC is set up for the kit. 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 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 NC sample (sterile enrichment medium).
   (1) 3M Lysis Solution Tubes tube strips can be cut to the 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 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 of the 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*) and one 3M Molecular Detection Assay 2—STEC Gene Screen (*eae*) Reagent Tube is required for each sample and the NC.
   (1) Tube strips can be cut to desired tube number. Select the number of individual 3M Molecular Detection Assay 2—STEC Gene Screen (*stx*) and 3M Molecular Detection Assay 2—STEC Gene Screen (*eae*) Reagent Tubes or eight-tube strips needed.
   (2) Place 3M Molecular Detection Assay 2 - STEC Gene Screen (*stx*) tubes in an empty rack in one column.
   (3) Place 3M Molecular Detection Assay 2 - STEC Gene Screen (*eae*) tubes in the adjacent right column.
   (4) 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* or *eae*) Reagent Tube strip at a time and use a new pipette tip for each transfer step.

(d) Transfer each of the lysates to 3M Molecular Detection Assay 2 - STEC Gene Screen (*stx* or *eae*) Reagent Tubes as described below.
   (1) First, transfer20 μL from a sample lysate to a 3M Molecular Detection Assay 2 - STEC Gene Screen (*stx*) Reagent Tube.
   (2) Second, transfer 20 μL of the same sample lysate to a 3M Molecular Detection Assay 2 - STEC Gene Screen (*eae*) Reagent Tube in the adjacent right column.
   (3) Repeat steps 1 and 2 above for each of the remaining sample lysates.
   (4) After all sample lysate transfers, add NC lysate to each of 3M Molecular Detection Assay 2 - STEC Gene Screen (*stx*) Reagent Tubes and 3M Molecular Detection Assay 2 - STEC Gene Screen (*eae*) Reagent Tubes.
   (5) Transfer NC lysate last to the Reagent Control Tubes. Dispense at an angle to avoid disturbing the pellets. Mix by gently pipetting up and down five times.

(e) 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.

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

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

(h) 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.

(i) 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.

(j) *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* and *eae*) 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* and *eae*) 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.00, 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* and *eae*), 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*^{SM} (PTM) program according to the *AOAC Methods Committee Guidelines for Validation of Microbiological Methods for Food and Environmental Surfaces*, Appendix J (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 STEC.

Inclusivity/Exclusivity

(a) *Methodology*.—Inclusivity and exclusivity testing were conducted to ensure that the 3M Molecular Detection Assay 2 -

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

Number	Strain source	Strain ID	Genus	Species	Serogroup	*stx*1[a]	*stx*2[a]	*eae*[a]	Isolation source	3M MDA2[b] - STEC (*stx* and *eae*) results
1	*E. coli* Reference Center at Penn State University[c]	10.2360	*Escherichia*	*coli*	O45: H2	+	−	+	Unknown	Positive
2	STEC Center at Michigan State University[d]	TW04257	*Escherichia*	*coli*	O111:-	+	+	+	Washington—BD[d], fever vomit	Positive
3	STEC Center at Michigan State University	TW07991	*Escherichia*	*coli*	O103: NM	+	−	+	Ohio—unknown	Positive
4	Minnesota Department of Health[f]	2011024930-1	*Escherichia*	*coli*	O103: H2	+	−	+	Minnesota—stool/diarrhea	Positive
5	STEC Center at Michigan State University	TW08101	*Escherichia*	*coli*	103: 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	STEC Center at Michigan State University	TW08039	*Escherichia*	*coli*	O121	−	+	+	Montana—unknown	Positive
9	*E.coli* Reference Center at Penn State University	99.0723	*Escherichia*	*coli*	O26	+	−	+	Unknown	Positive
10	*E.coli* Reference Center at Penn State University	99.0704	*Escherichia*	*coli*	O26	+	−	+	Unknown	Positive
11	STEC Center at Michigan State University	TW07814	*Escherichia*	*coli*	O26: H11	+	+	+	Idaho—HUS[g]	Positive
12	STEC Center at Michigan State University	TW07705	*Escherichia*	*coli*	O26: H46	+	+	+	Utah—watery stool	Positive
13	USDA ARS[h]	96-3285	*Escherichia*	*coli*	O45: H2	+	−	+	CDC[i]- human stool	Positive
14	STEC Center at Michigan State University	TW14003	*Escherichia*	*coli*	O45: H2	+	−	+	Michigan—unknown	Positive
15	STEC Center at Michigan State University	TW11239	*Escherichia*	*coli*	103: H25	+	−	+	Washington—unknown	Positive
16	STEC Center at Michigan State University	TW05997	*Escherichia*	*coli*	103: N	+	−	+	Idaho—unknown	Positive
17	STEC Center at Michigan State University	TW07990	*Escherichia*	*coli*	O103: NM	+	−	+	Washington—feces	Positive
18	ATCC[j]	BAA179	*Escherichia*	*coli*	O111: H8	+	+	+	Alabama—HUS	Positive
19	ATCC	BAA181	*Escherichia*	*coli*	O111: H8	+	+	+	South Dakota—HUS	Positive
20	STEC Center at Michigan State University	TW07931	*Escherichia*	*coli*	O121: H19	−	+	+	Massachusetts—BD	Positive
21	USDA ARS	08023	*Escherichia*	*coli*	O121: H19	−	+	+	FDA[k]human	Positive
22	ATCC	BAA-2129	*Escherichia*	*coli*	O145: H28	−	+	+	Germany—diarrhea	Positive
23	USDA ARS	05-6544	*Escherichia*	*coli*	026: H11	+	−	+	PHAC[l] –Human	Positive
24	USDA ARS	TB285	*Escherichia*	*coli*	026: H2	+	−	+	University of Washington – Human	Positive
25	USDA ARS	93-3118	*Escherichia*	*coli*	026: H11	+	−	+	PHAC – Human	Positive
26	USDA ARS	96-1415	*Escherichia*	*coli*	026: H11	+	−	+	PHAC – Human	Positive
27	USDA ARS	96-001	*Escherichia*	*coli*	026: H11	+	−	+	PHAC – Human	Positive
28	USDA ARS	b8026 C1	*Escherichia*	*coli*	045: H2	+	−	+	CDC – Calf	Positive
29	USDA ARS	05-6545	*Escherichia*	*coli*	045: H2	+	−	+	PHAC – Human	Positive
30	USDA ARS	SJ7	*Escherichia*	*coli*	045: H2	+	−	+	CDC – Human	Positive
31	USDA ARS	SJ8	*Escherichia*	*coli*	045: H2	+	−	+	CDC – Human	Positive
32	USDA ARS	SJ9	*Escherichia*	*coli*	045: H2	−	+	+	CDC – Human	Positive
33	USDA ARS	B8227 C8	*Escherichia*	*coli*	045	+	−	+	CDC – Calf	Positive
34	USDA ARS	97-3112	*Escherichia*	*coli*	0103: H25	+	−	+	CDC – Human	Positive
35	USDA ARS	tb154	*Escherichia*	*coli*	0103: H6	+	−	+	University of Washington – Human	Positive
36	USDA ARS	03-2444	*Escherichia*	*coli*	0103: H25	+	−	+	PHAC –Human	Positive
37	USDA ARS	04162	*Escherichia*	*coli*	0103: H6	+	−	+	FDA – Human	Positive
38	USDA ARS	96-3166	*Escherichia*	*coli*	0111: NM	+	+	+	CDC –vHuman	Positive
39	USDA ARS	TB226	*Escherichia*	*coli*	0111: HN	+	+	+	University of Washington – Human	Positive
40	USDA ARS	01387	*Escherichia*	*coli*	0111: H8	+	−	+	FDA – Human	Positive
41	USDA ARS	96-1585	*Escherichia*	*coli*	0121: H19	+	−	+	PHAC – Human	Positive
42	USDA ARS	97-3068	*Escherichia*	*coli*	0121: H19	−	+	+	CDC – Human	Positive
43	USDA ARS	03-4064	*Escherichia*	*coli*	0121: NM	−	+	+	PHAC – Human	Positive

(continued)

a. (continued)

Number	Strain source	Strain ID	Genus	Species	Serogroup	*stx1*[a]	*stx2*[a]	*eae*[a]	Isolation source	3M MDA2[b] - STEC (*stx* and *eae*) results
44	USDA ARS	DA-1	*Escherichia*	*coli*	0121	–	+	+	STEC Center MSU – Human	Positive
45	USDA ARS	07865	*Escherichia*	*coli*	0145: H28	–	+	+	FDA – Cow feces	Positive
46	USDA ARS	FSIS 258-93	*Escherichia*	*coli*	0157: H7	+	+	+	Beef Patty Outbreak	Positive
47	USDA ARS	FSIS 298-94	*Escherichia*	*coli*	0157: H7	+	–	+	Ground Beef outbreak	Positive
48	USDA ARS	FSIS 012-89	*Escherichia*	*coli*	0157: 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

[a] PCR gene characterization (internally screened at 3M using primers from Appendix 4C of USDA/FSIS MLG Chapter 5C.00).
[b] Molecular Detection Assay 2.
[c] *E. coli* Reference Center at (PSU) = The *E. coli* Reference Center at Pennsylvania State University, University Park, PA, USA.
[d] MSU STEC Center = Thomas S. Whittam STEC Center at Michigan State University, East Lansing, MI, USA.
[e] BD = Bloody diarrhea.
[f] Minnesota Department of Health, St. Paul, MN, USA.
[g] HUS = Hemolytic uremic syndrome.
[h] USDA ARS = US Department of Agriculture (USDA), Agricultural Research Service (ARS), Washington, DC, USA.
[i] CDC = Centers for Disease Control and Prevention, Atlanta, GA, USA.
[j] ATCC = American Type Culture Collection, Manassas, VA, USA.
[k] FDA = US Food and Drug Administration, College Park, MD, USA.
[l] PHAC = Public Health Agency of Canada, Ottawa, ON, Canada.

STEC Gene Screen (*stx* and *eae*) specifically detects Shiga toxin-producing, enterohemorrhagic *E. coli* strains, while not reacting with non-target strains and species. For inclusivity, 50 *E. coli* strains containing the *stx1* and/or *stx2* and the *eae* gene were tested (Table 1). These strains were identified as STECs in external strain collections and were additionally screened by PCR using primer sequences for *stx1*, *stx2*, and *eae* 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 ± 1°C and diluted 1000-fold before testing. Forty 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 ± 2°C for 20–24 h (Table 2). Exclusive cultures were diluted one log when visually turbid, and the average CFU/mL tested on the 3M Molecular Detection System was 9.32×10^7 CFU/mL. Inclusivity and exclusivity cultures were randomized and blind-coded for testing on the 3M Molecular Detection Assay 2 - STEC Gene Screen (*stx* and *eae*).

(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* and *eae*). Zero out of 40 exclusivity strains tested were detected by the 3M Molecular Detection Assay 2 - STEC Gene Screen (*stx* and *eae*).

Matrix Study

(a) *Methodology*.—Bulk samples of all matrices studied (fresh raw beef trim approximately 75% lean, 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 pre-screened following FDA-BAM Chapter 4A; no natural contamination by the target organism was detected during product screening. Per supplier agreement, the fresh raw beef trim was not pre-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, 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 Formulation,Ltd, (Virginia Beach, VA).

E. coli strains from the American Type Culture Collection (ATCC) were used to artificially contaminate the matrices. Fresh raw beef trim was inoculated with *E. coli* O157: H7 strain MDP 28. The culture was grown in brain heart infusion broth (BHI) overnight at 35–37°C, then serial dilutions were prepared in tubes of sterile distilled water diluent to achieve an inoculation level of 0.2–2 CFU/25g or 2–10 CFU/

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

Number	Strain source	Strain ID	Genus	Species	Isolation source	Serogroup[a]	3M MDA2[b] - STEC (*stx and eae*) results
1	ATCC[c]	9372	*Bacillus*	*atrophaeus*	Unknown	–[e]	Negative
2	ATCC	8090	*Citrobacter*	*freundii*	Unknown	–	Negative
3	ATCC	43162	*Citrobacter*	*brakii*	Clinical isolate, California	–	Negative
4	ATCC	27156	*Citrobacter*	*koseri*	Type strain of *Citrobacter diversus*	–	Negative
5	ATCC	25944	*Cronobacter*	*sakazakii*	Child's throat	–	Negative
6	ATCC	51816	*Enterobacter*	*amnigenus*	Milk, Minnesota	–	Negative
7	ATCC	49464	*Enterococcus*	*raffinosus*	Clinical isolate	–	Negative
8	ATCC	11303	*Escherichia*	*coli*	Unknown	–	Negative
9	ATCC	10799	*Escherichia*	*coli*	Unknown	–	Negative
10	ATCC	25922	*Escherichia*	*coli*	Clinical isolate	–	Negative
11	ATCC	8739	*Escherichia*	*coli*	Feces	–	Negative
12	ATCC	10536	*Escherichia*	*coli*	Unknown	–	Negative
13	BEI Resources, NIAID, NIH[d]	5.3169	*Escherichia*	*coli*	Human isolate	O25: H4	Negative
14	BEI Resources, NIAID, NIH[d]	85.1284	*Escherichia*	*coli*	Human isolate, 1985	O6: H31	Negative
15	BEI Resources, NIAID, NIH[d]	U9-41	*Escherichia*	*coli*	Human urine isolate	O2: K1: H4	Negative
16	BEI Resources, NIAID, NIH[d]	F11119-41	*Escherichia*	*coli*	Unknown	O16: K1: H-	Negative
17	BEI Resources, NIAID, NIH[d]	E3b	*Escherichia*	*coli*	Human peritoneum	O75: K95: H5	Negative
18	ATCC	51815	*Hafnia*	*alvei*	Milk, Minnesota	–	Negative
19	ATCC	51817	*Klebsiella*	*oxytoca*	Milk, Minnesota	–	Negative
20	ATCC	13438	*Klebsiella*	*pneumoniae*	Water	–	Negative
21	ATCC	13882	*Klebsiella*	*pneumoniae*	Moto, starter of sake	–	Negative
22	ATCC	9595	*Lactobacillus*	*rhamnosus*	Unknown	–	Negative
23	ATCC	8014	*Lactobacillus*	*plantarum*	Unknown	–	Negative
24	ATCC	19111	*Listeria*	*monocytogenes*	Poultry, England	–	Negative
25	ATCC	19119	*Listeria*	*ivanovii*	Sheep, Bulgaria	–	Negative
26	ATCC	4698	*Micrococcus*	*luteus*	Unknown	–	Negative
27	ATCC	49143	*Moraxella*	*catarrhalis*	Clinical isolate	–	Negative
28	ATCC	43071	*Proteus*	*mirabilis*	Rectum, Georgia	–	Negative
29	ATCC	13315	*Proteus*	*vulgaris*	Unknown	–	Negative
30	ATCC	27853	*Pseudomonas*	*aeruginosa*	Blood culture	–	Negative
31	ATCC	13525	*Pseudomonas*	*fluorescens*	Pre-filter tanks, England	–	Negative
32	ATCC	51812	*Salmonella*	*Typhimurium*	Human blood, Minnesota, United States	–	Negative
33	ATCC	6962	*Salmonella*	*newport*	Food poisoning fatality, England	–	Negative
34	ATCC	51741	*Salmonella*	*infantis*	Pasta	–	Negative
35	ATCC	12022	*Shigella*	*flexneri*	Unknown	–	Negative
36	ATCC	8700	*Shigella*	*boydii*	Unknown	–	Negative
37	ATCC	25931	*Shigella*	*sonnei*	Feces, human	–	Negative
38	ATCC	6538	*Staphylococcus*	*aureus*	Human lesion	–	Negative
39	ATCC	33317	*Streptococcus*	*bovis*	Cow dung	–	Negative
40	ATCC	23715	*Yersinia*	*enterocolitica*	Human blood, petechiae, from anterior chamber of the eye, 47-year-old female, Missouri, USA, 1968	–	Negative

[a] If applicable.
[b] Molecular Detection Assay 2.
[c] ATCC = American Type Culture Collection, Manassas, VA, USA.
[d] BEI = BEI Resources, NIAID, NIH, Manassas, VA, USA.
[e] – = Data not available.

25g. The raw beef trim was inoculated in bulk with a target of 0.2–2 CFU/sample, thoroughly mixed, and then separated into 25 g sample portions for the beef trim and stored at 2–8°C for 72–96 h. A portion remained uninoculated to serve as uninoculated negative control. Prior to initiation of testing, 25 g inoculated raw beef trim samples were combined with 350 g of uninoculated product to form a 375 g sample.

Fresh raw ground beef samples were inoculated with *E. coli* O26 BAA 1653. The culture was grown in BHI broth at 35 ± 1°C for 24 ± 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/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. For the 375 g test portions analyzed by the 3M Molecular Detection Assay 2 - STEC Gene Screen (*stx* and *eae*) method, 25 g from each contamination level was combined with 350 g uninoculated matrix on the day of analysis before being transferred to sterile filter laboratory blender bags. For 25 g test portions analyzed by the UDSA/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°C, then serial dilutions were prepared in tubes of sterile distilled water to achieve an inoculation level of 0.2–2 CFU/200g for the low level, and 2–10 CFU/200g for the high level. The raw spinach 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 and eae)

The 375 g test portions of fresh raw beef trim and fresh 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 30–60s 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 raw beef trim and 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 the 3M Molecular Detection Assay 2 - STEC Gene Screen (*stx* and *eae*) 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 beef trim and 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 peptone water with pyruvate (mBPWp) 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-Vancomycin (ACV) supplements was added per 225 mL of mBPWp. Samples were then 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 beef trim and fresh raw ground beef, confirmation was completed per USDA/FSIS-MLG Chapter 5C.00; final biochemical confirmation was determined by VITEK2 GN *Official Method* **2011.17**. For fresh spinach, confirmation was completed by plating on Tellurite Cefixime – Sorbitol MacConkey (TC-SMAC) agar, Rainbow O157 agar, and Levine's eosin-methylene blue (L-EMB) 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 *AOAC Methods Committee Guidelines for Validation of Microbiological Methods for Food and Environmental Surfaces*, Appendix J, Annex C. The matrix study POD and dPOD results are presented in Tables 3 and 4. Matrix study POD and dPOD graphs are shown in Figures 1–12. Background aerobic microbial counts in fresh raw beef trim and fresh raw ground beef were 3.73 $\log_{10}$ CFU/g and 6.51 $\log_{10}$ CFU/g, respectively, as determined by USDA/FSIS MLG Chapter 3.02 (14). The background aerobic microbial counts in fresh spinach were 6.14 $\log_{10}$ CFU/g as determined by FDA-BAM Chapter 3 (15).

Paired dPOD analyses were used to compare the 3M Molecular Detection Assay 2 - STEC Gene Screen (*stx* and *eae*) presumptive results from the 3M BPW-ISO enrichment broth to the 3M method confirmed results. All test portions grown in the 3M BPW-ISO enrichment broth were confirmed using the appropriate cultural reference method procedure. Data are shown in Table 3.

For the low contamination level of fresh raw beef trim, there were 14 presumptive positive samples after both 10 and 18 h of incubation, and all 14 confirmed as positive, with a dPOD of 0.00 and confidence interval (CI) of (−0.13, 0.13). For fresh raw ground beef, the 8 presumptive positive results at 10 and 18 h of incubation confirmed as positive, with a dPOD of 0.00 and 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* and *eae*) 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* and *eae*) confirmed results from the 3M BPW-ISO enrichment broth to the reference method enriched cultural results.

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

Matrix/inoculating organism	MPN/portion[a]	N[b]	3M MDA2[c] - STEC Gene Screen (*stx* and *eae*) presumptive			3M MDA2 - STEC Gene Screen (*stx* and *eae*) confirmed				
			x[d]	POD_{CP}[e]	95% CI	x	POD_{CC}[f]	95% CI	$dPOD_{CP}$[g]	95% CI[h]
Fresh raw beef trim[i], 75% lean (375 g)/ *E. coli* O157: H7	NA[l]	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	−0.47, 0.47
	0.80 (0.49, 1.36)	20	14	0.70	0.48, 0.85	14	0.70	0.48, 0.85	0.00	−0.13, 0.13
	2.29 (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
MDP[j] 28										
Fresh raw ground beef[i], 73% lean (375 g)/*E. coli* O26 ATCC[k] BAA-1653	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, 0.85)	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)/*E. coli* 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	0.08	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

[a] 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.
[b] N = Number of test portions.
[c] Molecular Detection Assay 2.
[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.
[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] Ten and 18 h enrichment time points produced identical results.
[j] MDP = Microbiological Data Program, US Meat Animal Research Center, Clay Center, NE, USA.
[k] ATCC = American Type Culture Collection, Manassas, VA, USA.
[l] NA = Not applicable.

Table 4. Matrix study: unpaired analysis, 3M Molecular Detection Assay 2 - STEC Gene Screen (*stx* and *eae*) results, candidate versus reference—POD results

Matrix/inoculating organism	MPN[a]/test portion	N[b]	3M MDA2[c] - STEC (*stx* and *eae*) results			Reference method[f] results				
			x[d]	POD_C[e]	95% CI	x	POD_R[g]	95% CI	$dPOD_C$[h]	95% CI[i]
Fresh raw beef trim[j], 75% lean (375 g)/ *E. coli* O157: H7	NA[m]	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	−0.43, 0.43
	0.80 (0.49, 1.36)	20	14	0.70	0.48, 0.85	10	0.50	0.30, 0.70	0.20	−0.10, 0.45
	2.29 (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
MDP[k] 28										
Fresh raw ground beef[j], 73% lean (375 g)/*E. coli* O26 ATCC[l] BAA-1653	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, 0.85)	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
Fresh spinach (200 g)/*E. coli* O111 ATCC C4-61-1	NA	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	−0.43, 0.43
	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

[a] MPN = Most probable number is calculated using the LCF MPN calculator, with 95% confidence interval.
[b] N = Number of test portions.
[c] Molecular Detection Assay 2.
[d] x = Number of positive test portions.
[e] POD_C = Candidate method confirmed positive outcomes divided by the total number of trials.
[f] Reference method = USDA/FSIS MLG Chapter 5C.00 for raw beef trim, raw ground beef; FDA-BAM Chapter 4A spinach.
[g] POD_R = Reference method confirmed positive outcomes divided by the total number of trials.
[h] $dPOD_C$= Difference between the confirmed candidate method result and reference method confirmed result POD values.
[i] 95% CI = If the confidence interval of a dPOD does not contain zero, then the difference is statistically significant at the 5% level.
[j] Ten and 18 h enrichment time points produced identical results.
[k] MDP = Microbiological Data Program, US Meat Animal Research Center, Clay Center, NE, USA.
[l] ATCC = American Type Culture Collection, Manassas, VA, USA.
[m] NA = Not applicable.

Data are shown in Table 4. There were no significant statistical differences seen between the 3M Molecular Detection Assay 2 - STEC Gene Screen (*stx* and *eae*) confirmed results from the 3M BPW-ISO enrichment broth and the USDA/FSIS MLG Chapter 5C.00 reference method confirmed results for fresh raw beef trim or fresh raw ground beef, or between the 3M Molecular Detection Assay 2 - STEC Gene Screen (*stx* and *eae*) confirmed results and the FDA-BAM Chapter 4A method confirmed results for fresh spinach.

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* and *eae*) 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 μL). The nominal conditions for the assay are 10.5 h enrichment time, 15 min lysis heating time, and 20 μL 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 73% lean 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. 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 transferring 18 or 22 μL lysate into each 3M Molecular Detection Assay 2 - STEC Gene Screen (*stx* and *eae*) 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 3M Molecular Detection Assay 2 - STEC Gene Screen (*stx* and *eae*) 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* and *eae*) 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 enterohemorrhagic *E. coli* O157: H7 (FSIS 258-93), and 10 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 ± 1°C and diluted with sterile BPW-ISO before testing. The non-target *S. typhimurium* strain was grown in TSB

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

		Test condition results			Nominal condition[d] results				
Test condition	N[a]	x[b]	POD_{TC}[c]	95% CI	x	POD_{NV}[e]	95% CI	dPOD[f]	95% CI[g]
Ground beef samples inoculated with *Escherichia coli* O157 culture (FSIS 258–93)[h]									
9.5 h[I], 13 min[j], 18 μL[k]	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 μL	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 μL	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 μL	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 μL	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 μL	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 μL	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 μL	10	6	0.60	(0.31, 0.83)	6	0.60	(0.31, 0.83)	0.00	(−0.25, 0.25)
Non-inoculated ground beef samples									
9.5 h[I], 13 min[j], 18 μL[k]	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 μL	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 μL	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 μL	10	0	0.00	(0, 0.28)	6	0.00	(0, 0.28)	0.00	(−0.25, 0.25)
26 h, 13 min, 18 μL	10	0	0.00	(0, 0.28)	6	0.00	(0, 0.28)	0.00	(−0.25, 0.25)
26 h, 13 min, 22 μL	10	0	0.00	(0, 0.28)	6	0.00	(0, 0.28)	0.00	(−0.25, 0.25)
26 h, 17 min, 18 μL	10	0	0.00	(0, 0.28)	6	0.00	(0, 0.28)	0.00	(−0.25, 0.25)
26 h, 17 min, 22 μL	10	0	0.00	(0, 0.28)	6	0.00	(0, 0.28)	0.00	(−0.25, 0.25)

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

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

Lot No.	Age of lot at time of testing, months	N[a]	Pure culture; Shiga toxin-producing *Escherichia coli* O157 (FSIS 258–93)[b]; fractional (low) level				Pure culture; *Salmonella* Typhimurium (ATCC 14028)[e]; undiluted			
			x[c]	POD_T[d]	95% CI		N	x	POD_{NT}[f]	95% CI
Lot 100418–1	8	10	7	0.70	(0.40, 0.89)		10	0	0.00	(0.00, 0.28)
Lot 100418–2	8	10	7	0.70	(0.40, 0.89)		10	0	0.00	(0.00, 0.28)
Lot 100419	1	10	7	0.70	(0.40, 0.89)		10	0	0.00	(0.00, 0.28)

[a] N = Number of test portions.
[b] United States Department of Agriculture, Food Safety and Inspection Service, Agriculture Research Service, Washington, DC, USA.
[c] x = Number of positive test portions.
[d] POD_T = Target strain positive outcomes divided by the total number of trials.
[e] American Type Culture Collection, Manassas, VA, USA.
[f] POD_{NT} = Non-target strain positive outcomes divided by the total number of trials.

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* and *eae*). All non-inoculated portions were negative. Results indicate that the age and lot-to-lot variability of the 3M Molecular Detection Assay 2 - STEC Gene Screen (*stx* and *eae*) products tested do not adversely affect assay performance.

Results and 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* and *eae*). Zero out of the 40 exclusivity strains tested were detected by the 3M Molecular Detection Assay 2 - STEC Gene Screen (*stx* and *eae*).

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 beef trim and 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, $POD_{CP,}$ the candidate confirmatory results, POD_{CC}, the difference in the candidate presumptive and confirmatory results, $dPOD_{CP,}$ presumptive candidate results that confirmed positive, $POD_{C,}$ the reference method, POD_R, and the difference in the confirmed candidate and reference methods, $dPOD_C$. The POD analysis between the 3M Molecular Detection Assay 2 - STEC Gene Screen (*stx* and *eae*) and the reference method indicated that there was no significant difference at the 5% level between the number of positive results by the two methods at all time points tested (10 and 18 h of enrichment) for fresh raw beef trim and fresh raw ground beef, and at 18 h of enrichment for fresh spinach. A summary of POD analyses are presented in Tables 4 and 5.

Robustness, product stability, and lot-to-lot variability study results indicate the rigor of the method, demonstrating that small variations in test parameters (enrichment time, lysis time, and lysed sample reaction volume), age of the product, and variations between product lots did not adversely affect the 3M Molecular Detection Assay 2 - STEC Gene Screen (*stx* and *eae*) performance.

Feedback from laboratory analysts from an independent matrix study highlighted additional strengths of the 3M Molecular Detection Assay 2 - STEC Gene Screen (*stx* and *eae*) method. Analysts found the method quick and simple to perform, providing results in less than 2 h post incubation for up to 45 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* and *eae*) method successfully recovered STEC from fresh raw beef trim and 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 methods and the reference methods for any of the samples tested, at any of the time points tested.

Matrix studies, inclusivity/exclusivity, robustness testing, and 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* and *eae*) method. The data collected in these studies demonstrate that the 3M Molecular Detection Assay 2 - STEC Gene Screen (*stx* and *eae*) method is suitable for *Performance Tested Method*[SM] certification for rapid and specific detection of Shiga toxin-producing enterohemorrhagic *E. coli* in fresh raw beef trim, fresh raw ground beef, and spinach.

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Conflict of Interest

None declared.

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