



CERTIFICATION

AOAC Research Institute *Performance Tested Methods*SM

Certificate No.
071902

The AOAC Research Institute hereby certifies the method known as:

Molecular Detection Assay 2 – STEC Gene Screen (MDA2-STXEAE)

manufactured by

Neogen Corporation
620 Leshar Place
Lansing, Michigan 48912
USA

This method has been evaluated and certified according to the policies and procedures of the AOAC *Performance Tested Methods*SM Program. This certificate indicates an AOAC Research Institute Certification Mark License Agreement has been executed which authorizes the manufacturer to display the AOAC Research Institute *Performance Tested Methods*SM certification mark on the above-mentioned method for the period below. Renewal may be granted by the Expiration Date under the rules stated in the licensing agreement.

A handwritten signature in black ink, appearing to read "Bradley A. Stawick".

Bradley A. Stawick, Senior Director
Signature for AOAC Research Institute

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METHOD NAME Neogen® Molecular Detection Assay 2 – STEC Gene Screen (MDA2-STXEAE) Formerly 3M™ Molecular Detection Assay 2 – STEC Gene Screen (MDA2-STXEAE)	CATALOG NUMBER MDA2STXEAE48
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INDEPENDENT LABORATORY Q Laboratories, Inc.* 1930 Radcliff Drive Cincinnati, OH 45204 USA SGS Vanguard Sciences, Inc. 224 North Derby Lane North Sioux, SD 57049 USA	MODIFICATION JANUARY 2022 TEQ Analytical Laboratories, Inc. Aurora, CO, USA 80045 USA	APPLICABILITY OF METHOD Target Organisms – Shiga-toxin producing enterohemorrhagic <i>E. coli</i> (<i>E. coli</i> strains containing <i>Escherichia coli</i> genes <i>stx</i> 1 (codes for Shiga toxin type 1) and/or <i>stx</i> 2 (codes for Shiga toxin type 2, and <i>eae</i> gene (codes for intimin). Matrixes – Fresh raw beef trim (375 g, ~75% lean), fresh raw ground beef (375 g, ~73% lean), fresh raw spinach (200 g) MODIFICATION MARCH 2020 – (MLG 5C.00) fresh raw ground beef (25 g, ~75% lean), fresh raw ground pork (375 g, ~70% lean), fresh raw poultry parts (375 g), and (BAM 4A) sprouts (25 g) MODIFICATION JANUARY 2022 – (AOAC SMPR 2020:012; 10 g) – Dried cannabis flower[>0.3% delta 9-tetrahydrocannabinol (THC)] and dried hemp flower (≤0.3% THC) MODIFICATION DECEMBER 2022 – (ISO/TS 13136:2012) mechanically separated chicken (25 g) Performance claims – Performance equivalent to that of U. S. Department of Agriculture-Food Safety and Inspection Service Microbiology Laboratory Guidebook (MLG), 5C.00, Detection, Isolation and Identification of Top Seven Shiga Toxin-Producing <i>Escherichia coli</i> (STECs) from Meat Products and Carcass and Environmental Sponges for fresh raw beef trim (375 g ~75 lean), fresh raw ground beef (375 g ~73% lean, 25 g ~75 lean), fresh raw ground pork (375 g, ~70% lean), fresh raw poultry parts (375 g) (2), and to the U.S. Food and Safety Administration Bacteriological Analytical Manual (BAM) Chapter 4A, Diarrheagenic <i>Escherichia coli</i> for spinach (200 g) and sprouts (25 g) (3). MODIFICATION JANUARY 2022 – Performance met the acceptance criteria established in the <i>Standard Method Performance Requirement (SMPR®)</i> for Detection of Shiga Toxin-Producing <i>Escherichia coli</i> in Cannabis and Cannabis Products (AOAC SMPR 2020:012; 6) for dried cannabis flower (>0.3% THC) and dried hemp flower (<0.3% THC) at a 10 g test portion size. MODIFICATION DECEMBER 2022 – The study data were unable to detect a significant statistical difference in results between the Molecular Detection Assay 2 – STEC Gene Screen (<i>stx</i> and <i>eae</i>) and ISO/TS 13136:2012 <i>Microbiology of food and animal feed — Real-time polymerase chain reaction (PCR)-based method for the detection of food-borne pathogens — Horizontal method for the detection of Shiga toxin-producing Escherichia coli (STEC) and the determination of O157, O111, O26, O103 and O145 serogroups</i> (8) for mechanically separated chicken.
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ORIGINAL CERTIFICATION DATE July 03, 2019	CERTIFICATION RENEWAL RECORD Renewed through December 2026.
METHOD MODIFICATION RECORD	SUMMARY OF MODIFICATION
<ol style="list-style-type: none"> 1. January 2020 Level 1 2. March 2020 Level 2 3. October 2021 Level 1 4. January 2022 Level 2 5. December 2022 Level 2 6. January 2024 Level 1 7. February 2024 Level 2 	<ol style="list-style-type: none"> 1. Editorial changes. 2. Matrix Extension to add fresh raw ground beef (~75% lean), fresh raw ground pork (~70% lean), fresh raw poultry parts, and sprouts. 3. Editorial changes. 4. Matrix Extension to add dried cannabis flower (>0.3% THC) and dried hemp flower (≤0.3% THC) 5. Matrix Extension to add mechanically separated chicken. 6. Editorial changes to rebrand method from 3M to Neogen Corporation. 7. Manufacturing location change from Columbia, Missouri to Lansing, Michigan.
Under this AOAC Performance Tested MethodsSM License Number, 071902 this method is distributed by: NONE	Under this AOAC Performance Tested MethodsSM License Number, 071902 this method is distributed as: NONE



PRINCIPLE OF THE METHOD (1)

The Neogen® Molecular Detection Assay 2 - STEC Gene Screen (*stx* and *eae*) is used with the Neogen Molecular Detection System for the rapid and specific screening of *Escherichia coli* genes *stx1* and/or *stx2* and *eae* in enriched food samples. The Molecular Detection Assays use loop-mediated isothermal amplification to rapidly amplify nucleic acid sequences with high specificity and sensitivity, combined with bioluminescence to detect the amplification.

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

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

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

DISCUSSION OF THE VALIDATION STUDY (1)

The results of the inclusivity and exclusivity testing show that all 50 out of 50 inclusivity strains tested were detected by the Molecular Detection Assay 2 - STEC Gene Screen (*stx* and *eae*). Zero out of the 40 exclusivity strains tested were detected by the 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 Molecular Detection Assay 2 - STEC Gene Screen (*stx* and *eae*) 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 hours of enrichment for fresh spinach. A summary of POD analyses are presented in Table 4.

Feedback from laboratory analysts from an independent matrix study highlighted additional strengths of the 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 hours post incubation for up to 45 sample replicates. Another benefit mentioned was the simplicity of the method; with only 2 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 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.

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

Number	Strain Source	Strain ID	Genus	species	Serogroup	stx1 ^a	stx2 ^a	eae ^a	Isolation Source	MDA2 - STEC (stx and eae) results
1	<i>E. coli</i> Reference Center at Penn State University ^b	10.2360	<i>Escherichia</i>	<i>coli</i>	O45:H2	+	-	+	Unknown	positive
2	STEC Center at Michigan State University ^c	TW04257	<i>Escherichia</i>	<i>coli</i>	O111:-	+	+	+	Washington - BD ^d Fever Vomit	positive
3	STEC Center at Michigan State University	TW07991	<i>Escherichia</i>	<i>coli</i>	O103:NM	+	-	+	Ohio - Unknown	positive
4	Minnesota Department of Health ^e	2011024930-1	<i>Escherichia</i>	<i>coli</i>	O103:H2	+	-	+	Minnesota - Stool/Diarrhea	positive
5	STEC Center at Michigan State University	TW08101	<i>Escherichia</i>	<i>coli</i>	103:H2	+	-	+	Denmark - Feces	positive
6	Minnesota Department of Health	2011027007-3	<i>Escherichia</i>	<i>coli</i>	O103:H2	+	-	+	Minnesota - Stool/Diarrhea	positive
7	Minnesota Department of Health	2011032087-1	<i>Escherichia</i>	<i>coli</i>	O26:H11	+	-	+	Minnesota - Stool/Diarrhea	positive
8	STEC Center at Michigan State University	TW08039	<i>Escherichia</i>	<i>coli</i>	O121	-	+	+	Montana - Unknown	positive
9	<i>E. coli</i> Reference Center at Penn State University	99.0723	<i>Escherichia</i>	<i>coli</i>	O26	+	-	+	Unknown	positive
10	<i>E. coli</i> Reference Center at Penn State University	99.0704	<i>Escherichia</i>	<i>coli</i>	O26	+	-	+	Unknown	positive
11	STEC Center at Michigan State University	TW07814	<i>Escherichia</i>	<i>coli</i>	O26:H11	+	+	+	Idaho - HUS ^f	positive
12	STEC Center at Michigan State University	TW07705	<i>Escherichia</i>	<i>coli</i>	O26:H46	+	+	+	Utah - Watery Stool	positive
13	USDA ARS ^g	96-3285	<i>Escherichia</i>	<i>coli</i>	O45:H2	+	-	+	CDC ^h -Human Stool	positive
14	STEC Center at Michigan State University	TW14003	<i>Escherichia</i>	<i>coli</i>	O45:H2	+	-	+	Michigan - Unknown	positive
15	STEC Center at Michigan State University	TW11239	<i>Escherichia</i>	<i>coli</i>	103:H25	+	-	+	Washington - Unknown	positive
16	STEC Center at Michigan State University	TW05997	<i>Escherichia</i>	<i>coli</i>	103:N	+	-	+	Idaho - Unknown	positive
17	STEC Center at Michigan State University	TW07990	<i>Escherichia</i>	<i>coli</i>	O103:NM	+	-	+	Washington - Feces	positive
18	ATCC ⁱ	BAA179	<i>Escherichia</i>	<i>coli</i>	O111:H8	+	+	+	Alabama - HUS	positive
19	ATCC	BAA181	<i>Escherichia</i>	<i>coli</i>	O111:H8	+	+	+	South Dakota - HUS	positive
20	STEC Center at Michigan State University	TW07931	<i>Escherichia</i>	<i>coli</i>	O121:H19	-	+	+	Massachusetts - Bloody Diarrhea	positive
21	USDA ARS	08023	<i>Escherichia</i>	<i>coli</i>	O121:H19	-	+	+	FDA ^j - Human	positive
22	ATCC	BAA-2129	<i>Escherichia</i>	<i>coli</i>	O145:H28	-	+	+	Germany - Diarrhea	positive
23	USDA ARS	05-6544	<i>Escherichia</i>	<i>coli</i>	O26:H11	+	-	+	Human PHAC ^k	positive
24	USDA ARS	TB285	<i>Escherichia</i>	<i>coli</i>	O26:H2	+	-	+	Human University of Washington	positive
25	USDA ARS	93-3118	<i>Escherichia</i>	<i>coli</i>	O26:H11	+	-	+	Human PHAC	positive
26	USDA ARS	96-1415	<i>Escherichia</i>	<i>coli</i>	O26:H11	+	-	+	Human PHAC	positive
27	USDA ARS	96-001	<i>Escherichia</i>	<i>coli</i>	O26:H11	+	-	+	Human PHAC	positive
28	USDA ARS	b8026 C1	<i>Escherichia</i>	<i>coli</i>	O45:H2	+	-	+	Calif, CDC	positive
29	USDA ARS	05-6545	<i>Escherichia</i>	<i>coli</i>	O45:H2	+	-	+	Human, PHAC	positive
30	USDA ARS	SJ7	<i>Escherichia</i>	<i>coli</i>	O45:H2	+	-	+	Human CDC	positive
31	USDA ARS	SJ8	<i>Escherichia</i>	<i>coli</i>	O45:H2	+	-	+	Human CDC	positive
32	USDA ARS	SJ9	<i>Escherichia</i>	<i>coli</i>	O45:H2	-	+	+	Human CDC	positive
33	USDA ARS	B8227 C8	<i>Escherichia</i>	<i>coli</i>	O45	+	-	+	Calif, CDC	positive
34	USDA ARS	97-3112	<i>Escherichia</i>	<i>coli</i>	O103:H25	+	-	+	Human CDC	positive
35	USDA ARS	tb154	<i>Escherichia</i>	<i>coli</i>	O103:H6	+	-	+	Human, University of Washington	positive
36	USDA ARS	03-2444	<i>Escherichia</i>	<i>coli</i>	O103:H25	+	-	+	Human PHAC	positive
37	USDA ARS	04162	<i>Escherichia</i>	<i>coli</i>	O103:H6	+	-	+	Human FDA	positive
38	USDA ARS	96-3166	<i>Escherichia</i>	<i>coli</i>	O111:NM	+	+	+	Human CDC	positive
39	USDA ARS	TB226	<i>Escherichia</i>	<i>coli</i>	O111:HN	+	+	+	Human, University of Washington	positive
40	USDA ARS	01387	<i>Escherichia</i>	<i>coli</i>	O111:H8	+	-	+	Human FDA	positive
41	USDA ARS	96-1585	<i>Escherichia</i>	<i>coli</i>	O121:H19	+	-	+	Human, PHAC	positive
42	USDA ARS	97-3068	<i>Escherichia</i>	<i>coli</i>	O121:H19	-	+	+	Human, CDC	positive
43	USDA ARS	03-4064	<i>Escherichia</i>	<i>coli</i>	O121:NM	-	+	+	Human, PHAC	positive
44	USDA ARS	DA-1	<i>Escherichia</i>	<i>coli</i>	O121	-	+	+	Human STEC Center MSU	positive
45	USDA ARS	07865	<i>Escherichia</i>	<i>coli</i>	O145:H28	-	+	+	Cow feces, FDA	positive
46	USDA ARS	FSIS 258-93	<i>Escherichia</i>	<i>coli</i>	O157:H7	+	+	+	Beef Patty Outbreak	positive



47	USDA ARS	FSIS 298-94	<i>Escherichia coli</i>	O157:H7	+	-	+	Ground Beef outbreak	positive
48	USDA ARS	FSIS 012-89	<i>Escherichia coli</i>	O157:H7	+	+	+	Beef Brisket	positive
49	Minnesota Department of Health	2011020423-1	<i>Escherichia coli</i>	O45:H2	+	+	+	Minnesota - Stool/Diarrhea	positive
50	Minnesota Department of Health	2011027121-2	<i>Escherichia coli</i>	O111:NM	+	-	+	Minnesota - Stool/Diarrhea	positive

^aPCR Gene Characterization (internally screened at Neogen using primers from appendix 4C of USDA MLG FSIS 5C.00).
^b*E. coli* Reference Center at Penn State University = The *E. coli* Reference Center at Pennsylvania State University, University Park, PA.
^cSTEC Center at Michigan State University = Thomas S. Whittam STEC Center at Michigan State University, East Lansing, MI.
^dBD = Bloody Diarrhea.
^eMinnesota Department of Health, St. Paul, MN.
^fHUS = Hemolytic Uremic Syndrome.
^gUSDA ARS = U.S. Department of Agriculture, Agricultural Research Service, Washington, DC.
^hCDC = Centers for Disease Control and Prevention, Atlanta, GA.
ⁱATCC = American Type Culture Collection, Manassas, VA.
^jFDA = U.S. Food and Drug Association, College Park, MD.
^kPHAC = Public Health Agency of Canada, Ottawa, Ontario, Canada.

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

Number	Source	Strain ID	Genus	Species	Source	Serogroup ^a	MDA2STEC (<i>stx</i> and <i>eae</i>) results
1	ATCC ^b	9372	<i>Bacillus</i>	<i>atrophaeus</i>	Unknown		Negative
2	ATCC	8090	<i>Citrobacter</i>	<i>freundii</i>	Unknown		Negative
3	ATCC	43162	<i>Citrobacter</i>	<i>brakii</i>	Clinical isolate, California		Negative
4	ATCC	27156	<i>Citrobacter</i>	<i>koseri</i>	type strain of <i>Citrobacter diversus</i>		Negative
5	ATCC	25944	<i>Cronobacter</i>	<i>sakazakii</i>	Child's throat		Negative
6	ATCC	51816	<i>Enterobacter</i>	<i>amnigenus</i>	Milk, Minnesota		Negative
7	ATCC	49464	<i>Enterococcus</i>	<i>raffinosis</i>	Clinical isolate		Negative
8	ATCC	11303	<i>Escherichia</i>	<i>coli</i>	Unknown		Negative
9	ATCC	10799	<i>Escherichia</i>	<i>coli</i>	Unknown		Negative
10	ATCC	25922	<i>Escherichia</i>	<i>coli</i>	clinical isolate		Negative
11	ATCC	8739	<i>Escherichia</i>	<i>coli</i>	Feces		Negative
12	ATCC	10536	<i>Escherichia</i>	<i>coli</i>	Unknown		Negative
13	BEI Resources, NIAID, NIH ^c	5.3169	<i>Escherichia</i>	<i>coli</i>	Human isolate	O25:H4	Negative
14	BEI Resources, NIAID, NIH ^c	85.1284	<i>Escherichia</i>	<i>coli</i>	Human isolate, 1985	O6:H31	Negative
15	BEI Resources, NIAID, NIH ^c	U9-41	<i>Escherichia</i>	<i>coli</i>	Human urine isolate	O2:K1:H4	Negative
16	BEI Resources, NIAID, NIH ^c	F11119-41	<i>Escherichia</i>	<i>coli</i>	Unknown	O16:K1:H-	Negative
17	BEI Resources, NIAID, NIH ^c	E3b	<i>Escherichia</i>	<i>coli</i>	Human peritoneum	O75:K95:H5	Negative
18	ATCC	51815	<i>Hafnia</i>	<i>alvei</i>	Milk, Minnesota		Negative
19	ATCC	51817	<i>Klebsiella</i>	<i>oxytoca</i>	Milk, Minnesota		Negative
20	ATCC	13438	<i>Klebsiella</i>	<i>pneumoniae</i>	Water		Negative
21	ATCC	13882	<i>Klebsiella</i>	<i>pneumoniae</i>	Moto, starter of sake		Negative
22	ATCC	9595	<i>Lactobacillus</i>	<i>rhamnosus</i>	Unknown		Negative
23	ATCC	8014	<i>Lactobacillus</i>	<i>plantarum</i>	Unknown		Negative
24	ATCC	19111	<i>Listeria</i>	<i>monocytogenes</i>	Poultry, England		Negative
25	ATCC	19119	<i>Listeria</i>	<i>ivanovii</i>	Sheep, Bulgaria		Negative
26	ATCC	4698	<i>Micrococcus</i>	<i>luteus</i>	Unknown		Negative
27	ATCC	49143	<i>Moraxella</i>	<i>catarrhalis</i>	Clinical isolate		Negative
28	ATCC	43071	<i>Proteus</i>	<i>mirabilis</i>	Rectum, Georgia		Negative
29	ATCC	13315	<i>Proteus</i>	<i>vulgaris</i>	Unknown		Negative
30	ATCC	27853	<i>Pseudomonas</i>	<i>aeruginosa</i>	Blood culture		Negative
31	ATCC	13525	<i>Pseudomonas</i>	<i>fluorescens</i>	Pre-filter tanks, England		Negative
32	ATCC	51812	<i>Salmonella</i>	<i>Typhimurium</i>	Human blood, Minnesota, United States		Negative
33	ATCC	6962	<i>Salmonella</i>	<i>newport</i>	Food poisoning fatality, England		Negative
34	ATCC	51741	<i>Salmonella</i>	<i>infantis</i>	Pasta		Negative
35	ATCC	12022	<i>Shigella</i>	<i>flexneri</i>	Unknown		Negative
36	ATCC	8700	<i>Shigella</i>	<i>boydii</i>	Unknown		Negative
37	ATCC	25931	<i>Shigella</i>	<i>sonnei</i>	Feces, human		Negative
38	ATCC	6538	<i>Staphylococcus</i>	<i>aureus</i>	Human lesion		Negative
39	ATCC	33317	<i>Streptococcus</i>	<i>bovis</i>	Cow dung		Negative
40	ATCC	23715	<i>Yersinia</i>	<i>enterocolitica</i>	Human blood, petechiae, from anterior chamber of the eye, 47-year-old female, Missouri, USA, 1968		Negative

^aIf applicable.
^bATCC = American Type Culture Collection, Manassas, VA.
^cBEI = BEI Resources, NIAID, NIH, Manassas, VA.



Table 3. Matrix Study: Molecular Detection Assay 2 - STEC Gene Screen (*stx* and *eae*) Results, Presumptive vs. Confirmed – POD Results (1)

Matrix	MPN/portion ^a (or inoculation level)	N ^b	MDA2 – STEC Gene Screen (<i>stx</i> and <i>eae</i>) presumptive			MDA2 - STEC Gene Screen (<i>stx</i> and <i>eae</i>) confirmed			dPOD _{CP} ^f	95% CI ^g
			x ^c	POD _{CP} ^d	95% CI	x	POD _{CC} ^e	95% CI		
Fresh Raw Beef Trim ^h , 75% lean (375 g) <i>E. coli</i> O157:H7 MDP 28	N/A ⁱ	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
Fresh Raw Ground Beef ^h , 73% lean (375 g) <i>E. coli</i> O26 ATCC BAA-1653	N/A	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	N/A	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

^aMPN = Most Probable Number is calculated using the LCF MPN calculator, with 95% confidence interval.

^bN = Number of test portions.

^cx = Number of positive test portions.

^dPOD_{CP} = Candidate method presumptive positive outcomes divided by the total number of trials.

^ePOD_{CC} = Candidate method confirmed positive outcomes divided by the total number of trials.

^fdPOD_{CP} = Difference between the candidate method presumptive result and candidate method confirmed result POD values.

^g95% CI = If the confidence interval of a dPOD does not contain zero, then the difference is statistically significant at the 5% level.

^h10 and 18 h enrichment time points produced identical results.

ⁱN/A = Not applicable.



Table 4. Matrix Study: Unpaired Analysis, Molecular Detection Assay 2 - STEC Gene Screen (*stx* and *eae*) Results, Candidate vs. Reference – POD Results (1)

Matrix ^a	Strain	MPN ^b /test portion	N ^c	MDA2STEC (<i>stx</i> and <i>eae</i>) results			Reference method ^g results			dPOD _C ^h	95% CI ⁱ
				X ^d	POD _C ^e	95% CI	x	POD _R ^f	95% CI		
Raw beef trim ^j (375 g)	<i>E. coli</i> O157:H7 MDP 28	N/A ^k	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
Raw ground beef ^j (375 g)	<i>E. coli</i> O26 BAA-1653	N/A	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
Spinach (200 g)	<i>E. coli</i> O111 C4-61-1	N/A	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

^aPortions were analyzed as an unpaired study

^bMPN = Most Probable Number is calculated using the LCF MPN calculator, with 95% confidence interval.

^cN = Number of test portions.

^dx = Number of positive test portions.

^ePOD_C = Candidate method confirmed positive outcomes divided by the total number of trials.

^fReference method = MLG 5C.00 for raw beef trim, raw ground beef; BAM 4A spinach.

^gPOD_R = Reference method confirmed positive outcomes divided by the total number of trials.

^hdPOD_C = Difference between the confirmed candidate method result and reference method confirmed result POD values.

ⁱ95% CI = If the confidence interval of a dPOD does not contain zero, then the difference is statistically significant at the 5% level

^j10 and 18 h enrichment time points produced identical results.

^kN/A = Not applicable.

DISCUSSION OF THE MODIFICATION STUDY APPROVED MARCH 2020 (4)

The Molecular Detection Assay 2 STEC Gene Screen (*stx* and *eae*) method successfully recovered STEC from fresh raw ground beef (~75% lean), fresh raw ground pork (~70% lean), and fresh raw poultry parts after 10 and 18 h enrichment time points in Neogen BPW ISO; as well as STEC from sprouts after an 18–24 h enrichment time point in Neogen BPW ISO. 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 all samples tested.

The Molecular Detection Assay 2 STEC Gene Screen (*stx* and *eae*) method is quick and simple to perform, providing results in less than 2 h post incubation for 30 sample replicates. The methods offer the benefit of minimal possible contamination since only 2 sample transfers are needed to perform the methods. The small footprint and ability to link multiple Molecular Detection Systems to a single laptop computer offer high throughput. The MDS software is user friendly with the ability to track assay lot information and sample identification quickly and with ease. The added benefit of the real time curves also improves any troubleshooting issues that may arise.

Table 1. Matrix Study: Molecular Detection Assay 2 - STEC Gene Screen (*stx* and *eae*) Results, Presumptive vs. Confirmed – POD Results (4)

Matrix	Strain	MPN/portion ^a (or inoculation level)	N ^b	MDA2 – STEC Gene Screen (<i>stx</i> and <i>eae</i>) presumptive			MDA2 - STEC Gene Screen (<i>stx</i> and <i>eae</i>) confirmed			dPOD _{CP} ^f	95% CI ^g
				x ^c	POD _{CP} ^d	95% CI	x	POD _{CC} ^e	95% CI		
Fresh raw ground beef ^h 75% lean (25 g)	<i>E. coli</i> O103 MSU ⁱ TW07697	N/A ^j	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	-0.47, 0.47
		0.51 (0.26, 0.87)	20	8	0.40	0.22, 0.61	8	0.40	0.22, 0.61	0.00	-0.13, 0.13
		1.97 (0.91, 4.27)	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	-0.47, 0.47
Fresh raw ground pork ^h 70% lean (375 g)	<i>E. coli</i> O145 MSU TW07596	N/A	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	-0.47, 0.47
		0.49 (0.25, 0.84)	20	8	0.40	0.22, 0.61	8	0.40	0.22, 0.61	0.00	-0.13, 0.13
		1.97 (0.91, 4.27)	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	-0.47, 0.47
Fresh raw poultry parts ^h (375g)	<i>E. coli</i> O157:H7 ATCC ^k BAA-460	N/A	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	-0.47, 0.47
		0.68 (0.39, 1.12)	20	9	0.45	0.22, 0.61	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
Sprouts (25 g)	<i>E. coli</i> O157:H7 ATCC BAA-460	N/A	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	-0.47, 0.47
		0.63 (0.35, 1.04)	20	8	0.40	0.26, 0.66	9	0.45	0.26, 0.66	0.00	-0.13, 0.13
		2.58 (1.15, 5.78)	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	-0.47, 0.47

^aMPN = Most Probable Number is calculated using the LCF MPN calculator, with 95% confidence interval.

^bN = Number of test portions.

^cx = Number of positive test portions.

^dPOD_{CP} = Candidate method presumptive positive outcomes divided by the total number of trials.

^ePOD_{CC} = Candidate method confirmed positive outcomes divided by the total number of trials.

^fdPOD_{CP} = Difference between the candidate method presumptive result and candidate method confirmed result POD values.

^g95% CI = If the confidence interval of a dPOD does not contain zero, then the difference is statistically significant at the 5% level.

^h10 and 18 h meat enrichment time points produced identical results.

ⁱMSU = Michigan State University, Lansing, MI.

^jN/A = Not applicable.

^kATCC = American Type Culture Collection, Manassas, VA.

Table 2. Matrix Study: Unpaired Analysis, Molecular Detection Assay 2 - STEC Gene Screen (*stx* and *eae*) Results, Candidate vs. Reference – POD Results (4)

Matrix ^a	Strain	MPN ^b /test portion	N ^c	MDA2STEC (<i>stx</i> and <i>eae</i>) results			Reference method ^d results			dPOD _C ^h	95% CI ⁱ
				X ^d	POD _C ^e	95% CI	x	POD _R ^f	95% CI		
Fresh raw ground beef ^f 75% lean (25 g)	<i>E. coli</i> O103 MSU ^k TW07697	N/A ^j	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	-0.43, 0.43
		0.51 (0.26, 0.87)	20	8	0.40	0.22, 0.61	7	0.35	0.18, 0.57	0.05	-0.23, 0.32
		1.97 (0.91, 4.27)	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	-0.43, 0.43
Fresh raw ground pork/ 70% lean (375 g)	<i>E. coli</i> O145 MSU TW07596	N/A	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	-0.43, 0.43
		0.49 (0.25, 0.84)	20	8	0.40	0.22, 0.61	7	0.35	0.18, 0.57	0.05	-0.23, 0.32
		1.97 (0.91, 4.27)	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	-0.43, 0.43
Fresh raw poultry parts/ (375g)	<i>E. coli</i> O157:H7 ATCC ^m BAA-460	N/A	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	-0.43, 0.43
		0.68 (0.39, 1.12)	20	9	0.45	0.26, 0.66	8	0.40	0.22, 0.61	0.05	-0.24, 0.33
		2.58 (1.15, 5.78)	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	-0.43, 0.43
Sprouts (25 g)	<i>E. coli</i> O157:H7 ATCC BAA-460	N/A	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	-0.43, 0.43
		0.63 (0.35, 1.04)	20	8	0.40	0.22, 0.61	8	0.40	0.22, 0.61	0.00	-0.28, 0.28
		2.58 (1.15, 5.78)	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	-0.43, 0.43

^aPortions were analyzed as an unpaired study

^bMPN = Most Probable Number is calculated using the LCF MPN calculator, with 95% confidence interval.

^cN = Number of test portions.

^dx = Number of positive test portions.

^ePOD_C = Candidate method confirmed positive outcomes divided by the total number of trials.

^fReference method = MLG 5C.00 for raw beef trim, raw ground beef; BAM 4A sprouts.

^gPOD_R = Reference method confirmed positive outcomes divided by the total number of trials.

^hdPOD_C = Difference between the confirmed candidate method result and reference method confirmed result POD values.

ⁱ95% CI = If the confidence interval of a dPOD does not contain zero, then the difference is statistically significant at the 5% level

^j10 and 18 h meat enrichment time points produced identical results.

^kMSU = Michigan State University, Lansing, MI. ^lN/A = Not applicable.

^mN/A = Not applicable.

ⁿATCC = American Type Culture Collection, Manassas, VA.



DISCUSSION OF THE MODIFICATION STUDY APPROVED JANUARY 2022 (5)

The Molecular Detection Assay 2 – STEC Gene Screen (*stx* and *eae*) successfully detected the target STEC species in dried cannabis flower and dried hemp flower at a 10 g sample size. Difference in POD analysis for the presumptive versus confirmed positives showed no statistically significant differences, with all ranges of the 95% confidence intervals containing the zero (0) point. There was one presumptive positive result in the dried cannabis flower after 32 h of enrichment that was not confirmed. It is possible that a very low level of a STEC organism was present in the sample, but no STEC was detected culturally.

The independent laboratory reported that processing samples was very user friendly with a standard heat dependent lysis step and transfer into pre-aliquoted lyophilized pellets in reagent tube wells. The assay was able to be run from a single set of lysis tubes. A short run time of roughly 60 minutes, with presumptive positive results displaying quickly in the run, was a very beneficial aspect.

The Molecular Detection Assay 2 - STEC Gene Screen (*stx* and *eae*) allows users to obtain presumptive positive results after 28 h of incubation and one to two hours of processing and assay run time. Presumptive results are easily visualized, denoted by a plus or minus sign within the software.

Table 1. Molecular Detection Assay – STEC Gene Screen (*stx* and *eae*) presumptive vs. confirmed results in dried cannabis flower (>0.3% THC) and dried hemp flower (≤0.3% THC) (5)

Matrix and Inoculum	Enrichment time	MPN ^a / Test Portion	N ^b	x ^c	Presumptive		x	Confirmed		dPOD _{cp} ^f	95% CI ^g
					POD _{cp} ^d	95% CI		POD _{cc} ^e	95% CI		
Dried cannabis flower 10 g	28 h	NA ⁱ	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	(-0.47, 0.47)
		0.88 (0.40, 2.02)	20	10	0.50	0.30, 0.70	10	0.50	0.30, 0.70	0.00	(-0.13, 0.13)
		2.96 (1.54, 9.78)	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	(-0.47, 0.47)
<i>E. coli</i> O157:H7 (ATCC ^h 43895)	32 h	NA	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	(-0.47, 0.47)
		0.88 (0.40, 2.02)	20	11	0.55	0.34, 0.74	10	0.50	0.30, 0.70	0.05	(-0.11, 0.21)
		2.96 (1.54, 9.78)	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	(-0.47, 0.47)
Dried hemp Flower 10 g	28 h	NA	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	(-0.47, 0.47)
		1.15 (0.61, 2.45)	20	10	0.50	0.30, 0.70	10	0.50	0.30, 0.70	0.00	(-0.13, 0.13)
		2.96 (1.54, 9.78)	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	(-0.47, 0.47)
<i>E. coli</i> O26 (CDC ^j 03-3014)	32 h	NA	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	(-0.47, 0.47)
		1.15 (0.61, 2.45)	20	10	0.50	0.30, 0.70	10	0.50	0.30, 0.70	0.00	(-0.13, 0.13)
		2.96 (1.54, 9.78)	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	(-0.47, 0.47)

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

^bN = Number of test portions.

^cx = Number of positive test portions.

^dPOD_{cp} = Candidate method presumptive positive outcomes divided by the total number of trials.

^ePOD_{cc} = Candidate method confirmed positive outcomes divided by the total number of trials.

^fdPOD_{cp} = Difference between the candidate method presumptive result and candidate method confirmed result POD values.

^g95% CI = If the confidence interval of a dPOD does not contain zero, then the difference is statistically significant at the 5% level.

^hAmerican Type Culture Collection, Manassas, VA.

ⁱNot applicable.

^jCenters for Disease Control and Prevention, Atlanta, GA.

DISCUSSION OF THE MODIFICATION STUDY APPROVED DECEMBER 2022 (7)

The Molecular Detection Assay 2 – STEC Gene Screen (*stx* and *eae*) successfully detected STEC in fresh mechanically separated chicken. Using POD analysis, no statistically significant differences were observed between the number of positive test portions detected by the candidate methods and the reference method at all time points (10 h, 18 h, and 24 h) for all materials. The Molecular Detection Assay 2 – STEC Gene Screen (*stx* and *eae*) is quick and easy to perform. The dehydrated pellets dissolve quickly when lysate is added to the PCR tubes which removes an extra step of ensuring the pellets have been thoroughly dissolved. The Molecular Detection System software is user friendly including assay kit lot information and real-time presumptive results. Run times are short at 60 min and presumptive positive results appear when they are detected in real time which can further shorten the time to results. All test results are interpreted and displayed automatically at the end of the run without requiring input by the user.

Table 1. Molecular Detection Assay 2 – STEC Gene Screen (*stx* and *eae*), Candidate vs. Reference – POD Results (7)

Matrix	MPN ^a / Test Portion	N ^b	Candidate method results ^c			Reference method results ^f			dPOD _c ^h	95% CI ⁱ
			x ^d	POD _c ^e	95% CI	x	POD _R ^g	95% CI		
Mechanically separated chicken (25 g) ^k	NA ⁱ	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	-0.43, 0.43
	0.40 (0.18, 0.72)	20	7	0.35	0.18, 0.57	7	0.35	0.18, 0.57	0.00	-0.28, 0.28
	2.77 (1.85, 9.26)	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	-0.43, 0.43

^aMPN = Most Probable Number is calculated using the LCF MPN calculator ver. 2.0 provided by AOAC RI, with 95% confidence interval.

^bN = Number of test portions.

^cIdentical results were obtained for both the Neogen Molecular Detection Assay 2 – STEC Gene Screen (*stx* and *eae*) and STEC Gene Screen (*stx*) at all time points tested (10 h, 18 h, and 24 h).

^dx = Number of positive test portions.

^ePOD_c = Candidate method presumptive positive outcomes confirmed positive divided by the total number of trials.

^fReference method = ISO/TS 13136:2012 Microbiology of food and animal feed — Real-time polymerase chain reaction (PCR)-based method for the detection of food-borne pathogens — Horizontal method for the detection of Shiga toxin-producing *Escherichia coli* (STEC) and the determination of O157, O111, O26, O103 and O145 serogroups.

^gPOD_R = Reference method confirmed positive outcomes divided by the total number of trials.

^hdPOD_c = Difference between the confirmed candidate method result and reference method result POD values.

ⁱ95% CI = If the confidence interval of a dPOD does not contain zero, then the difference is statistically significant at the 5% level.

^jNA = Not applicable.

^kLow and high level test portions inoculated with American Type Culture Collection (ATCC) strain 43895, an *E. coli* O157:H7 strain positive for *stx1*, *stx2*, and *eae* genes

Table 2. Molecular Detection Assay 2 – STEC Gene Screen (*stx* and *eae*), Presumptive vs. Confirmed – POD Results (7)

Matrix	MPN ^a / Test Portion	Candidate method presumptive result ^c				Candidate method confirmed result ^h				
		N ^b	x ^c	POD _{CP} ^d	95% CI	x	POD _{CC} ^e	95% CI	dPOD _{CP} ^f	95% CI ^g
Mechanically separated chicken (25 g) ^k	NA ⁱ	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	-0.47, 0.47
	0.40 (0.18, 0.72)	20	7	0.35	0.18, 0.57	7	0.35	0.18, 0.57	0.00	-0.13, 0.13
	2.77 (1.85, 9.26)	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	-0.47, 0.47

^aMPN = Most Probable Number is calculated using the LCF MPN calculator ver. 2.0 provided by AOAC RI, with 95% confidence interval.

^bN = Number of test portions.

^cIdentical results were obtained for both the Neogen Molecular Detection Assay 2 – STEC Gene Screen (*stx* and *eae*) and STEC Gene Screen (*stx*) at all time points tested (10 h, 18 h, and 24 h).

^dx = Number of positive test portions.

^ePOD_{CP} = Candidate method presumptive positive outcomes divided by the total number of trials.

^fPOD_{CC} = Candidate method confirmed positive outcomes divided by the total number of trials.

^gdPOD_{CP} = Difference between the candidate method presumptive result and candidate method confirmed result POD values.

^h95% CI = If the confidence interval of a dPOD does not contain zero, then the difference is statistically significant at the 5% level.

ⁱNA = Not applicable.

^kLow and high level test portions inoculated with American Type Culture Collection (ATCC) strain 43895, an *E. coli* O157:H7 strain positive for *stx1*, *stx2*, and *eae* genes



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