



CERTIFICATION

AOAC Research Institute *Performance Tested Methods*SM

Certificate No.
071903

The AOAC Research Institute hereby certifies the method known as:

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

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 - STX) Formerly 3M™ Molecular Detection Assay 2 – STEC Gene Screen (MDA2 - STX)	CATALOG NUMBER MDA2STX96
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INDEPENDENT LABORATORY Original Validation and Modification December 2022 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 <i>E. coli</i> (<i>E. coli</i> strains containing <i>Escherichia coli</i> genes <i>stx1</i> (codes for Shiga toxin type 1) and/or <i>stx2</i> (codes for Shiga toxin type 2). Matrixes – fresh raw ground beef (375g, ~73% lean), fresh raw spinach (200 g) MODIFICATION MARCH 2020 – (MLG 5C.00) fresh raw ground beef (25 g, ~75% lean), fresh raw beef trim (~75 % lean, 375 g), 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 ground beef (375g ~73% lean, 25 g, ~75% lean), fresh raw beef trim (~75 % lean, 375 g), 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; 1) 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 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 <ol style="list-style-type: none"> 1. January 2020 Level 1 2. March 2020 Level 2 3. January 2022 Level 2 4. December 2022 Level 2 5. January 2024 Level 1 6. February 2024 Level 2 	SUMMARY OF MODIFICATION <ol style="list-style-type: none"> 1. Editorial review. 2. Matrix Extension to add fresh raw ground beef (~75% lean), raw beef trim (~75% lean), fresh raw ground pork (~70% lean), fresh raw poultry parts, and sprouts. 3. Matrix Extension to add dried cannabis flower (>0.3% THC) and dried hemp flower (≤0.3% THC). 4. Matrix Extension to add mechanically separated chicken. 5. Editorial changes to rebrand method from 3M to Neogen Corporation. 6. Manufacturing location change from Columbia, Missouri to Lansing, Michigan.
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PRINCIPLE OF THE METHOD (1)

The Neogen® Molecular Detection Assay 2 - STEC Gene Screen (*stx*) is used with the Neogen Molecular Detection System for the rapid and specific screening of *Escherichia coli* genes *stx1* and/or *stx2* 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.

The Molecular Detection Assay 2 - STEC Gene Screen (*stx*) 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*) has not been evaluated with all possible food products, food processes, testing protocols or with all possible strains of bacteria. The 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*) has been evaluated for use with the Neogen Buffered Peptone Water (ISO Formulation) (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*). Zero out of the 45 exclusivity strains tested were detected by the Molecular Detection Assay 2 - STEC Gene Screen (*stx*).

As per criteria outlined in Appendix J of the Official Methods of Analysis Manual for evaluations in matrixes, fractional positive results were obtained for both 10 and 18 h enrichment time points for fresh raw ground beef, and at the 18 h enrichment time point for fresh spinach. The probability of detection (POD) was calculated as the number of positive outcomes divided by the total number of trials. The POD was calculated for the candidate presumptive results, POD_{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 the appropriate reference method for each matrix indicated that there was no significant difference at the 5% level between the number of positive results by the two methods at any time points tested (10 and 18 h of enrichment for fresh raw ground beef, and 18 h of enrichment for fresh spinach). A summary of POD analyses are presented in Tables 4 and 5.

Results from the robustness study and product stability and lot-to-lot variability study results both indicate the rigor of the method, since small variations in test parameters (enrichment time, lysis time and lysed sample reaction volume), age of the product, and variations between product lots do not adversely affect the Molecular Detection Assay 2 - STEC Gene Screen (*stx*) performance.

Feedback from laboratory analysts from independent matrix studies highlighted additional strengths of the Molecular Detection Assay 2 - STEC Gene Screen (*stx*) method. Analysts found the method quick and simple to perform, providing results in less than 2 h post incubation for up to 90 sample replicates. Another benefit mentioned was the simplicity of the method; with only 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) (1)

Number	Strain Source	Strain ID	Genus	species	Serogroup	stx1 ^a	stx2 ^a	Isolation Source	MDA2STEC (stx) results
1	<i>E. coli</i> Reference Center (PSU) ^b	10.2360	<i>Escherichia</i>	<i>coli</i>	O45:H2	+	-	Unknown	positive
2	MSU STEC Center ^c	TW04257	<i>Escherichia</i>	<i>coli</i>	O111:-	+	+	Washington - BD ^d Fever Vomit	positive
3	MSU STEC Center	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	MSU STEC Center	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	MSU STEC Center	TW08039	<i>Escherichia</i>	<i>coli</i>	O121	-	+	Montana - Unknown	positive
9	<i>E. coli</i> Reference Center (PSU)	99.0723	<i>Escherichia</i>	<i>coli</i>	O26	+	-	Unknown	positive
10	<i>E. coli</i> Reference Center (PSU)	99.0704	<i>Escherichia</i>	<i>coli</i>	O26	+	-	Unknown	positive
11	MSU STEC Center	TW07814	<i>Escherichia</i>	<i>coli</i>	O26:H11	+	+	Idaho - HUS ^f	positive
12	MSU STEC Center	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	MSU STEC Center	TW14003	<i>Escherichia</i>	<i>coli</i>	O45:H2	+	-	Michigan - Unknown	positive
15	MSU STEC Center	TW11239	<i>Escherichia</i>	<i>coli</i>	O103:H25	+	-	Washington - Unknown	positive
16	MSU STEC Center	TW05997	<i>Escherichia</i>	<i>coli</i>	O103:N	+	-	Idaho - Unknown	positive
17	MSU STEC Center	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	MSU STEC Center	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	+	-	Calf, 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	+	-	Calf, 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</i>	<i>coli</i>	O157:H7	+	-	Ground Beef outbreak	positive
48	USDA ARS	FSIS 012-89	<i>Escherichia</i>	<i>coli</i>	O157:H7	+	+	Beef Brisket	positive
49	Minnesota Department of Health	2011020423-1	<i>Escherichia</i>	<i>coli</i>	O45:H2	+	+	Minnesota - Stool/Diarrhea	positive
50	Minnesota Department of Health	2011027121-2	<i>Escherichia</i>	<i>coli</i>	O111:NM	+	-	Minnesota - Stool/Diarrhea	positive

^aPCR Gene Characterization (internally screened with PCR using primers from appendix 4C of USDA MLG FSIS 5C.00).

^b*E. coli* Reference Center (PSU) = The *E. coli* Reference Center at Pennsylvania State University, University Park, PA.

^cMSU STEC Center = 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, Ontario, Canada.

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

Number	Source	Strain ID	Genus	Species	Source	Serogroup ^o	MDA2STEC (stx) 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 ^c	5.3169	<i>Escherichia</i>	<i>coli</i>	Human isolate	O25:H4	negative
14	BEI	85.1284	<i>Escherichia</i>	<i>coli</i>	Human isolate, 1985	O6:H31	negative
15	BEI	U9-41	<i>Escherichia</i>	<i>coli</i>	Human urine isolate	O2:K1:H4	negative
16	BEI	F11119-41	<i>Escherichia</i>	<i>coli</i>	Unknown	O16:K1:H-	negative
17	BEI	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>	Typhimurium	Human blood, Minnesota, United States		negative
33	ATCC	6962	<i>Salmonella</i>	Newport	Food poisoning fatality, England		negative
34	ATCC	51741	<i>Salmonella</i>	Infantis	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
41	BEI	E2348/69	<i>Escherichia</i>	<i>coli</i>	Infant diarrhea outbreak, Taunton, England.	O127:H6	negative
42	BEI	TB182A	<i>Escherichia</i>	<i>coli</i>	Infant diarrhea, Seattle Washington	O55:H7	negative
43	BEI	RDEC-1	<i>Escherichia</i>	<i>coli</i>	Rabbit diarrhea	O15:H2	negative
44	<i>E. coli</i> Reference Center (PSU) ^d	95.0187	<i>Escherichia</i>	<i>coli</i>	Unknown	O145	negative
45	USDA ARS ^e	5.0623	<i>Escherichia</i>	<i>coli</i>	Unknown	O45:H2	negative

^aIf applicable.^bATCC = American Type Culture Collection, Manassas, VA.^cBEI = BEI = BEI Resources, NIAID, NIH, Manassas, VA.^d*E. coli* Reference Center (PSU) = The *E. coli* Reference Center at Pennsylvania State University, University Park, PA.^eUSDA ARS = U.S. Department of Agriculture, Agricultural Research Service, Washington, DC.

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

Matrix	MPN/portion ^a (or inoculation level)	N ^b	MDA2 – STEC Gene Screen (stx) presumptive			MDA2 - STEC Gene Screen (stx) confirmed				
			x ^c	POD _{CP} ^d	95% CI	x	POD _{CC} ^e	95% CI	dPOD _{CP} ^f	95% CI ^g
Fresh Raw Ground Beef ^h , 73% lean (375 g) <i>E. coli</i> O26 ATCC ⁱ 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	0.80	0.58, 0.92	0.00	-0.13, 0.13
	7.43 (3.08, 17.94)	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	-0.47, 0.47

^aMPN (if applicable) = 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. Candidate enrichments were confirmed using the appropriate reference procedure: MLG 5C.00 for fresh raw ground beef; FDA BAM Chapter 4A for fresh spinach.

^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.

ⁱATCC = American Type Culture Collection, Manassas, VA.

^jN/A = Not applicable.

Table 4. Matrix Study: Unpaired Analysis, Molecular Detection Assay 2 – STEC Gene Screen (stx) results vs. Reference Method results (1)

Matrix ^a	Strain	MPN ^b /test portion	N ^c	MDA2STEC (stx) results			Reference method ^g results			dPOD _{CP} ^h	95% CI ⁱ
				X ^d	POD _{CP} ^e	95% CI	x	POD _{CC} ^f	95% CI		
Raw ground beef ^f (375 g)	<i>E. coli</i> O26 ATCC ^k 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	0.35	0.18, 0.57	0.05	-0.23, 0.32
		2.58 (1.15, 5.78)	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	-0.43, 0.43
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	-0.43, 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 based on the POD of reference method test portions using the LCF MPN calculator, with 95% confidence interval.

^cN = Number of test portions

^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.

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

^hdPOD_{CP} = Difference between the candidate method presumptive result and candidate 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.

^jBoth 10 and 18 h enrichment time points produced identical results.

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

^lN/A = Not applicable

DISCUSSION OF THE MODIFICATION STUDY APPROVED MARCH 2020 (4)

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

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

Feedback from laboratory analysts from independent matrix studies highlighted additional strengths of the Molecular Detection Assay 2 - STEC Gene Screen (*stx*) method. Analysts found the method quick and simple to perform, providing results in less than 2 h post incubation for up to 90 sample replicates. Another benefit mentioned was the simplicity of the method; with only 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.

MODIFICATION DATA APPROVED MARCH 2020 (4)

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

Matrix	Strain	MPN/portion ^a	N ^b	MDA2 – STEC Gene Screen (<i>stx</i>) presumptive			MDA2 - STEC Gene Screen (<i>stx</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 , 73% lean (25 g)	<i>E. coli</i> O103 MSU ⁱ	N/A ^j	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	-0.47, 0.47
	TW07697	0.51, (0.26, 0.87)	20	8	0.40	0.22, 0.61	8	0.40	0.22, 0.61	0.00	-0.13, 0.13
		1.97, (0.91, 4.27)	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	-0.47, 0.47
Fresh raw beef trim 73% lean (375 g)	<i>E. coli</i> O157:H7 MDP ^k 28	N/A	5	10 h: 1	0.20	0.00, 0.62	0	0.00	0.00, 0.43	0.20	-0.36, 0.76
				18 h: 2	0.40	0.12, 0.77	0	0.00	0.00, 0.43	0.40	-0.21, 1.00
		0.84, (0.14, 0.49)	20	10 h: 14	0.70	0.48, 0.85	14	0.40	0.22, 0.61	0.00	-0.13, 0.13
				18 h: 15	0.70	0.53, 0.89	14	0.40	0.22, 0.61	0.05	-0.11, 0.21
		2.84, (1.38, 5.87)	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	-0.47, 0.47
Fresh raw ground pork ^h , 70% lean (375 g)	<i>E. coli</i> O145 MSU	N/A	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	-0.47, 0.47
	TW07596	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 ^l 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	9	0.44	0.22, 0.61	0.00	-0.13, 0.13
		2.58, (1.15, 5.78)	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	-0.47, 0.47
Sprouts (25 g)	<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	8	0.40	0.26, 0.66	0.00	-0.13, 0.13
		2.58, (1.15, 5.78)	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	-0.47, 0.47

^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. Candidate enrichments were confirmed using the appropriate reference procedure: MLG 5C.00 for fresh raw ground beef; FDA BAM Chapter 4A for fresh sprouts.

^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.

ⁱMSU = Michigan State University Culture Collection, Lansing, MI.

^jN/A = Not applicable.

^kMDP = Microbiological Data Program, U.S. Meat Animal Research Center, Clay Center, NE.

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

Table 2. Matrix Study: Unpaired Analysis, Molecular Detection Assay 2 – STEC Gene Screen (stx) results vs. Reference Method results (4)

Matrix ^a	Strain	MPN ^b /test portion	N ^c	MDA2STEC (stx) results			Reference method ^g results			dPOD _{CP} ^h	95% CI ⁱ
				X ^d	POD _{CP} ^e	95% CI	x	POD _{CC} ^f	95% CI		
Fresh raw ground beef ^j 73% lean (25 g)	<i>E. coli</i> O103 MSU ^k TW07697	N/A ^l	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	-0.47, 0.47
		0.51, (0.26, 0.87)	20	8	0.40	0.22, 0.61	8	0.40	0.22, 0.61	0.00	-0.13, 0.13
		1.97, (0.91, 4.27)	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	-0.47, 0.47
Fresh raw beef trim 73% lean (375 g)	<i>E. coli</i> O157:H7 MDP ^m 28	N/A	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	-0.43, 0.43
		0.84, (0.14, 0.49)	20	14	0.70	0.48, 0.85	10	0.5	0.18, 0.57	-0.20	-0.10, 0.45
		2.84, (1.38, 5.87)	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	-0.43, 0.43
Fresh raw ground pork ^j 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 ^j (375g)	<i>E. coli</i> O157:H7 ATCC ⁿ 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 based on the POD of reference method test portions using the LCF MPN calculator, with 95% confidence interval.

^cN = Number of test portions

^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.

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

^hdPOD_{CP} = Difference between the candidate method presumptive result and candidate 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.

^jBoth 10 and 18 h enrichment time points produced identical results.

^kMSU = Michigan State University Culture Collection (MSU) – Lansing, MI^kN/A = Not applicable.

^lN/A = Not applicable.

^mMDP = Microbiological Data Program, U.S. Meat Animal Research Center, Clay Center, NE.

ⁿATCC = American Type Culture Collection, Manassas, VA.

DISCUSSION OF THE MODIFICATION STUDY APPROVED JANUARY 2022 (5)

The Molecular Detection assays 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 strain 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) 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) 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 ⁱ 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) 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 method 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), Candidate vs. Reference – POD Results (7)

Matrix	MPN ^a / Test Portion	Candidate method results ^c				Reference method results ^f				
		N ^b	x ^d	POD _c ^e	95% CI	x	POD _R ^g	95% CI	dPOD _c ^h	95% CI ⁱ
Mechanically separated chicken (25 g) ^k	NA ^j	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) (AOAC RI PTM 071902) and STEC Gene Screen (stx) (AOAC RI PTM 071903) 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), 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 ^j	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) (AOAC RI PTM 071902) and STEC Gene Screen (stx) (AOAC RI PTM 071903) 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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