CERTIFICATION

AOAC Research Institute

Performance Tested Methods[SM]

Certificate No.

091301

The AOAC Research Institute hereby certifies the method known as:

BAX® System Real-Time PCR Assay Suite for STEC

manufactured by

Hygiena
2 Boulden Circle
New Castle, DE 19720
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.

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

Issue Date	December 11, 2024
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MODIFICATION JANUARY 2022: Nisha Corrigan, Casey Simmons, Leo Lorine, and Alex Tudor
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SUBMITTING COMPANY
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ESL Building 400
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CURRENT SPONSOR
Hygiena
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METHOD NAME
BAX® System Real-Time PCR Assay Suite for STEC
Formerly DuPont™ BAX® System Real-Time PCR Assay Suite for STEC

CATALOG NUMBERS
BAX® Screening Kit KIT2021 (D14642964), BAX® System Panel 1 KIT2008 (D14642970), BAX® System Panel 2 KIT2009 (D14642987)

INDEPENDENT LABORATORY
Aegis Food Testing Laboratories, Inc.
224 N. Derby Lane
North Sioux City, SD 57049
USA

INDEPENDENT LABORATORY MODIFICATION JANUARY 2022
TEQ Analytical Laboratories, Inc.
12635 E. Montview Blvd.
Suite 175
Aurora, CO 80045 USA

APPLICABILITY OF METHOD
Target organism
STEC Screening Assay – *stx* and *eae* virulence genes
STEC Panel 1 assay – *E. coli* O26, O111, O121
STEC Panel 2 assay – *E. coli* O45, O103, O145

Matrixes – raw beef trim (375g), raw ground beef (325 g, 375g), raw ground beef plus soy (325g).
MODIFICATION DECEMBER 2017 – raw ground beef (25 g) and all-purpose flour (25 g).
MODIFICATION JANUARY 2022 – (AOAC SMPR 2020.12; 10 g) dried cannabis flower [>0.3% delta 9-tetrahydrocannabinol (THC)] and dried hemp flower (≤0.3% THC).
MODIFICATION APRIL 2023 – Sampling cloths swabbed from 375 g beef trim portions.

***Performance claims* – Sensitivity equal to or better than the corresponding reference method.**
MODIFICATION JANUARY 2022: BAX System Real-Time PCR Assay for STEC Suite test kit is effective in screening for STEC species (O26, O111, O121, O45, O103, O145) in dried cannabis flower (>0.3% THC) and dried hemp flower (≤0.3% THC) at a 10 g test portion size and met the requirements of *Standard Method Performance Requirements* (SMPRs®) for Detection of Shiga toxin-producing *Escherichia coli* in Cannabis and Cannabis Products (AOAC SMPR 2020.012; 8).
MODIFICATION APRIL 2023: The study data were unable to find a statistically detectable difference in results from zero between the BAX System Real-Time PCR Assay for STEC Suite and the United States Department of Agriculture Food Safety and Inspection Service MLG 5C.03 Detection, Isolation, and Identification of Top Seven Shiga Toxin-Producing Escherichia coli (STEC) from Meat Products, Carcass, and Environmental Sponges (8) from sampling cloths swabbed from 375 g beef trim test portions in 8–24 h using MP media or modified Tryptic Soy Broth with casamino acids (mTSB +caa).

REFERENCE METHODS

Least Cost Formulations, Ltd., MPN Calculator-Version 1.6 (http://www.lcfltd.com/customer/LCFMPNCalculator.exe) (2)

USDA FSIS (2011) Microbiology Laboratory Guidebook (3)

US FDA (2017) *FDA Bacteriological Analytical Manual,* Chapter 4A, Diarrheagenic *Escherichia coli* (6)

USDA FSIS (2014) *Microbiology Laboratory Guidebook,* Chapter 5B.05, Detection and Isolation of non-O157 Shiga Toxin-Producing *Escherichia coli* (STEC) from Meat Products and Carcass and Environmental Sponges (7)

***Standard Method Performance Requirements* (SMPRs®) for Detection of Shiga toxin-producing *Escherichia coli* in Cannabis and Cannabis Products (AOAC SMPR 2020.012) (8)**

United States Department of Agriculture Food Safety and Inspection Service MLG 5C.03 Detection, Isolation, and Identification of Top Seven Shiga Toxin-Producing Escherichia coli (STEC) from Meat Products, Carcass, and Environmental Sponges (10)

BAX® System Real-Time PCR Assay Suite for STEC, AOAC *Performance Tested Methods℠* Certification Number 091301

ORIGINAL CERTIFICATION DATE	CERTIFICATION RENEWAL RECORD
September 24, 2013	Renewed through December 2025.

METHOD MODIFICATION RECORD	SUMMARY OF MODIFICATION
1. March 2017 Level 1	1. Name change from DuPont Nutrition & Health to Qualicon Diagnostics LLC., a Hygiena company.
2. December 2017 Level 1	2. Inserts, manuals, and labels updated to Hygiena.
3. December 2017 Level 2	3. Matrix extension to add raw ground beef (25 g) and all-purpose flour (25 g).
4. January 2018 Level 1	4. Editorial changes to insert and labels to update Hygiena.
5. May 2019 Level 1	5. Editorial insert updates and corporate address.
6. December 2019 Level 1	6. Editorial changes.
7. December 2021 Level 1	7. Editorial changes.
8. January 2022 Level 2	8. Matrix extension to include dried cannabis flower (>0.3% THC) and dried hemp flower (≤0.3% THC).
9. April 2023 Level 2	9. Matrix extension to include sampling cloths for 375 g beef trim portions.
10. January 2024 Level 1	10. Editorial changes.
11. December 2024 Level 1	11. Editorial changes.

Under this AOAC *Performance Tested Methods℠* License Number, 091301 this method is distributed by:	Under this AOAC *Performance Tested Methods℠* License Number, 091301 this method is distributed as:
NONE	NONE

PRINCIPLE OF THE METHOD (1)

PCR Amplification - The BAX® System uses the Polymerase Chain Reaction (PCR) to amplify specific fragments of bacterial DNA, which are stable and unaffected by growth environment. The fragments are genetic sequences that are unique to each of the *E. coli* serotypes and the associated *stx* and *eae* virulence factors, providing a highly reliable indicator that the target organisms are present in the sample. The BAX System simplifies the PCR process by combining the requisite primers, polymerase and nucleotides into a stable, dry, manufactured tablet already packaged inside the PCR tubes. After amplification, these tubes remain sealed for the detection phase, thus significantly reducing the potential for contamination with one or more molecules of amplified PCR product.

Fluorescent Real-Time Detection - This automated BAX System method uses fluorescent detection to analyze PCR products. One PCR primer for each associated target (*stx* and *eae* in the STEC Screening assay and three of the O-types of interest in each of the STEC Panel assays) and one for the internal DNA control target contains a fluorescent dye (a different one for each target) as a constituent of the primer as well as a quencher (the uni-molecular combination of a primer and fluorescent dye with an associated quencher either on the same molecule or on a separate oligonucleotide constitute a Scorpion™ Probe). When incorporated into a PCR product, the dye and quencher are spatially separated at the temperature at which detection occurs, which causes an increase in emission signal. The BAX® System instrument measures the magnitude and characteristics of fluorescent signal change from cycle to cycle of the PCR process. An analysis by the BAX® System software algorithm then evaluates that data to determine a positive or negative result which is displayed as described below.

The BAX System real-time STEC suite is a two-stage screening method. After appropriate sample enrichment, the Screening assay is used to determine the presence or absence of the Shiga toxin genes (*stx* encoding genes) and intimin (*eae* encoding genes) and clear negative samples quickly. If the Screening assay returns positive results for both virulence factors, then the two multiplex panel assays are run to detect and differentiate the top six STEC serogroups of public health concern which are regulated by the United States Department of Agriculture Food Safety and Inspection Service (USDA-FSIS) as adulterants in beef.

DISCUSSION OF THE VALIDATION STUDY (1)

For the internal and independent laboratory validation studies, POD analysis for all food indicated that the test method performed in a fashion not statistically different than the reference method, with the exception of one beef trim replicate (spiked with an O26 strain). The one replicate that produced a statistically distinguishable difference in method performance indicated that the BAX System method had a greater recovery of the target pathogen than the reference method. The results of the inclusivity and exclusivity studies demonstrate 100% inclusivity and exclusivity for the BAX® System real-time STEC suite for detecting both the stx and eae virulence genes and identifying the six targeted serogroups.

Table 3. Method comparison results POD (1)

Matrix	Strain	MPN[a]/test portion	N[c]	Test Method			Reference Method			dPOD$_c$[g]	95% CI[h]
				x[d]	POD$_c$[e]	95% CI	x	POD$_R$[f]	95% CI		
Beef Trim / MP Media (11 and 24 hours)	O26:H11	7.9 (3.0, 21)	5	5	1.0	(0.57, 1.0)	5	1.0	(0.57, 1.0)	0	(-0.43, 0.43)
		0.51 (0.24, 0.90)	20	17	0.85	(0.64, 0.95)	7	0.35	(0.18, 0.57)	0.50	(0.20, 0.30)
		Negative Control	5	0	0	(0, 0.43)	0	0	(0, 0.43)	0	(-0.43, 0.43)
Beef Trim / MP Media (11 and 24 hours)	O145:H-	10 (3.9, 26)	5	5	1.0	(0.57, 1.0)	5	1.0	(0.57, 1.0)	0	(-0.43, 0.43)
		0.42 (0.19, 0.73)	20	7	0.35	(0.18, 0.57)	7	0.35	(0.18, 0.57)	0	(-0.28, 0.28)
		Negative Control	5	0	0	(0, 0.43)	0	0	(0, 0.43)	0	(-0.43, 0.43)
Ground beef / mTSB + 2 mg/L novobiocin (11 and 24 hours)	O103:H11	18 (7.3, 54)	5	5	1.0	(0.57, 1.0)	5	1.0	(0.57, 1.0)	0	-(0.43, 0.43)
		0.78 (0.41, 1.3)	20	15	0.75	(0.53, 0.89)	8	0.40	(0.22, 0.061)	0.35	(-0.05, 0.58)
		Negative Control	5	0	0	(0, 0.43)	0	0	(0, 0.43)	0	(-0.43, 0.43)
Ground beef with soy /mTSB+caa+n (12 and 24 hours)	O26:H11	0.87 (0.54, 1.4)	30	19	0.63	(0.46, 0.78)	19	0.63	(0.46, 0.78)	0	(-0.25, 0.25)
		Negative Control	5	0	0	(0, 0.43)	0	0	(0, 0.43)	0	(-0.45, 0.45)
Ground beef with soy /mTSB+caa+n (12 and 24 hours)	O111:H-	0.91 (0.57, 1.4)	30	18	0.60	(0.42, 0.75)	18	0.60	(0.42, 0.75)	0	(-0.23, 0.23)
		Negative Control	5	0	0	(0, 0.43)	0	0	(0, 0.43)	0	(-0.45, 0.45)
Beef Trim / MP Media 11 hrs[i]	O145:H28	3.01	5	4	0.80	(0.38, 1.00)	5	1.00	(0.57, 1.00)	-0.2	(-0.62, 0.28)
		0.56	20	6	0.30	(0.15, 0.52)	9	0.45	(0.26, 0.66)	-0.15	(-0.41, 0.14)
		Negative Control	5	0	0.00	(0.00, 0.44)	0	0.00	(0.00, 0.44)	0.00	(-0.44, 0.44)
Beef Trim / TSB Media 11 hrs[i]	O145:H28	3.01	5	5	1.00	(0.57, 1.00)	5	1.00	(0.57, 1.00)	0.00	(-0.44, 0.44)
		0.56	20	7	0.35	(0.18, 0.57)	9	0.45	(0.26, 0.66)	-0.1	(-0.37, 0.19)
		Negative Control	5	0	0.00	(0.00, 0.44)	0	0.00	(0.00, 0.44)	0.00	(-0.44, 0.44)
Beef Trim / TSB Media 24 hrs[i]	O145:H28	3.01	5	5	1.00	(0.57, 1.00)	5	1.00	(0.57, 1.00)	0.00	(-0.44, 0.44)
		0.56	20	7	0.35	(0.18, 0.57)	9	0.45	(0.26, 0.66)	-0.1	(-0.37, 0.19)
		Negative Control	5	0	0.00	(0.00, 0.44)	0	0.00	(0.00, 0.44)	0.00	(-0.44, 0.44)
Beef Trim / mTSB + caa +n 24 hrs[i]	O145:H28	3.01	5	5	1.00	(0.57, 1.00)	5	1.00	(0.57, 1.00)	0.00	(-0.43, 0.43)
		0.56	20	9	0.45	(0.26, 0.66)	9	0.45	(0.26, 0.66)	0.00	(-0.25, 0.25)
		Negative Control	5	0	0.00	(0.00, 0.44)	0	0.00	(0.00, 0.44)	0.00	(-0.44, 0.44)

Table 4. Inclusivity Results for STEC Screening (*stx* and eae containing strains) (1)

Strain ID	Other ID	*E. coli* serotype	Source	BAX result *eae*	BAX result *stx*	*stx*$_1$ gene	*stx*$_2$ gene	*eae* gene
DEC 101	-	O145:H16	MSU>USDA	Pos	Pos	Present	Absent	Present
DD13417	CDC 85-337	O4:HNM	US CDC>USDA	Pos	Pos	Present	Present	Present
DD13418	CDC 95-3209	O14:HNM	US CDC>USDA	Pos	Pos	Absent	Present	Present
DD1450	-	O157:H7	Human Clinical	Pos	Pos	Absent	Present	Present
DD13430	CDC 86-3153	O125:HNM	US CDC>USDA	Pos	Pos	Present	Absent	Present
DD13435	CDC 88-3001	O165:H25	US CDC>USDA	Pos	Pos	Absent	Present	Present
DD13439	PHAC 03-2682	O5:HNM	PHAC>USDA	Pos	Pos	Present	Absent	Present
DD13440	PHAC 05-0376	O55:H7	PHAC>USDA	Pos	Pos	Present	Absent	Present
DD13448	SJ87	O63:HNM	US CDC>USDA	Pos	Pos	Present	Absent	Present
TD8136		O157:H7	Cattle	Pos	Pos	Present	Present	Present
DD13459	FDA BB2	O55:H7	US FDA>USDA	Pos	Pos	Present	Absent	Present
DD13461	FDA DD4	O177:H25	US FDA>USDA	Pos	Pos	Absent	Present	Present
DD13462	FDA EE5	O111:H8	US FDA>USDA	Pos	Pos	Present	Present	Present
DD13464	GG7	O103:H2	US FDA>USDA	Pos	Pos	Present	Absent	Present
DD13465	HH8	O26:H11	US FDA>USDA	Pos	Pos	Present	Absent	Present
DD13368	SJ18	O121:H19	US CDC>USDA	Pos	Pos	Present	Present	Present
MA6	-	O157:H7 (rough)	FDA	Pos	Pos	Present	Present	Present
05-6545	-	O45:H2	US CDC>USDA	Pos	Pos	Present	Absent	Present
BCL 17	-	O5:N	MSU>USDA	Pos	Pos	Present	Absent	Present
DD640	ATCC 43889	O157:H7	ATCC	Pos	Pos	Absent	Present	Present
DD641	ATCC 43890	O157:H7	ATCC	Pos	Pos	Present	Absent	Present
DD642	ATCC 43895	O157:H7	ATCC	Pos	Pos	Present	Present	Present
DD914	ATCC 43894	O157:H7	ATCC	Pos	Pos	Present	Present	Present
DD916	ATCC 35150	O157:H7	ATCC	Pos	Pos	Present	Present	Present
DD8297	C984	O157:H7	Korea	Pos	Pos	Present	Unknown	Present
DD8298	86-24	O157:H7	Human Clinical	Pos	Pos	Absent	Present	Present

HC = Health Canada , US CDC = United States Centers for Disease Control and Prevention
PHAC = Public Health Agency of Canada, USDA = United States Department of Agriculture,
US FDA = United States Food and Drug Administration

Table 5. Inclusivity Results for STEC Panel 1 (*E. coli* O26, O111, O121) (1)

Strain ID	*E. coli* serotype	Source	BAX result	Strain ID	*E. coli* serotype	Source	BAX result
DD1720	O26	Unknown	POS O26	DD1858	O111	Unknown	POS O111
DD1807	O26	USDA	POS O26	DD1927	O111	Unknown	POS O111
DD1831	O26	USDA	POS O26	R70	O111	Human Clinical	POS O111
DD1913	O26	USDA	POS O26	R71	O111	Human Clinical	POS O111
DD5902	O26	Unknown	POS O26	R72	O111	Human Clinical	POS O111
DD5903	O26	Unknown	POS O26	DD13362	O121	USDA	POS O121
DD5904	O26	Unknown	POS O26	DD13363	O121	USDA	POS O121
DD5905	O26	Unknown	POS O26	DD13364	O121	USDA	POS O121
DD9704	O26	Unknown	POS O26	DD13365	O121	USDA	POS O121
DD9705	O26	Unknown	POS O26	DD13366	O121	USDA	POS O121
DD9706	O26	Unknown	POS O26	DD13367	O121	USDA	POS O121
DD9707	O26	Unknown	POS O26	DD13368	O121	USDA	POS O121
R144	O26	USDA MDP	POS O26	DD13370	O121	USDA	POS O121
R58	O26	USDA MDP	POS O26	DD2440	O121	Unknown	POS O121
R59	O26	USDA MDP	POS O26	DD2460	O121	Unknown	POS O121
R60	O26	USDA MDP	POS O26	R184	O121	USDA	POS O121
DD133400	O111	Unknown	POS O111	R185	O121	USDA	POS O121
DD133401	O111	Unknown	POS O111	R186	O121	USDA	POS O121
DD133402	O111	Unknown	POS O111	R187	O121	USDA	POS O121
DD133403	O111	Unknown	POS O111	R188	O121	USDA	POS O121
DD1729	O111	Unknown	POS O111	R75	O121	USDA	POS O121
DD1808	O111	Unknown	POS O111	R76	O121	USDA	POS O121
DD1809	O111	Unknown	POS O111	R84	O121	USDA	POS O121

Table 6. Inclusivity Results for STEC Panel 2 (*E. coli* O45, O103, O145) (1)

Strain ID	*E. coli* serotype	Source	BAX result	Strain ID	*E. coli* serotype	Source	BAX result
DD13349	O45	USDA	POS O45	DD13388	O103	University of Washington	POS O103
DD13350	O45	USDA	POS O45	DD13389	O103	University of Washington	POS O103
DD13351	O45	USDA	POS O45	DD2521	O103	Unknown	POS O103
DD13352	O45	USDA	POS O45	DD2530	O103	Unknown	POS O103
DD13353	O45	USDA	POS O45	R163	O103	USDA	POS O103
DD13354	O45	USDA	POS O45	R164	O103	USDA	POS O103
DD13355	O45	USDA	POS O45	R165	O103	USDA	POS O103
DD13358	O45	USDA	POS O45	R166	O103	USDA	POS O103
DD13390	O145	USDA	POS O45	R167	O103	USDA	POS O103
DD13361	O45	USDA	POS O45	R168	O103	USDA	POS O103
DD2450	O45	Unknown	POS O45	R66	O103	Human Clinical	POS O103
R62	O45	Human Clinical	POS O45	R67	O103	Human Clinical	POS O103
R63	O45	Human Clinical	POS O45	R68	O103	Human Clinical	POS O103
R64	O45	Human Clinical	POS O45	DD13391	O145	USDA	POS O145
DD13373	O103	USDA	POS O103	DD13392	O145	USDA	POS O145
DD13374	O103	USDA	POS O103	DD13393	O145	USDA	POS O145
DD13375	O103	USDA	POS O103	DD13394	O145	USDA	POS O145
DD13376	O103	USDA	POS O103	DD13395	O145	USDA	POS O145
DD13377	O103	USDA	POS O103	DD13397	O145	USDA	POS O145
DD13378	O103	USDA	POS O103	DD13398	O145	USDA	POS O145
DD13379	O103	USDA	POS O103	DD2439	O145	Unknown	POS O145
DD13380	O103	USDA	POS O103	DD2483	O145	Unknown	POS O145
DD13381	O103	USDA	POS O103	DD2526	O145	Unknown	POS O145
DD13382	O103	USDA	POS O103	R198	O145	USDA	POS O145
DD13383	O103	USDA	POS O103	R77	O145	Human Clinical	POS O145
DD13384	O103	USDA	POS O103	R78	O145	Human Clinical	POS O145
DD13386	O103	USDA	POS O103	R79	O145	Human Clinical	POS O145
DD13387	O103	USDA	POS O103	R80	O145	Human Clinical	POS O145

Table 7. Exclusivity Results for Non-*E. coli* Strains (1)

Strain name	Strain ID	STEC Screening result	STEC Panel 1 result	STEC Panel 2 result
Citrobacter freundii	DD2558	NEG	NEG	NEG
Citrobacter freundii	DD383	NEG	NEG	NEG
Enterobacter amnigenus	DD13186	NEG	NEG	NEG
Enterobacter amnigenus	DD13187	NEG	NEG	NEG
Enterobacter asburiae	DD13161	NEG	NEG	NEG
Enterobacter cloacae	DD13135	NEG	NEG	NEG
Enterobacter hormaechei	DD13162	NEG	NEG	NEG
Enterobacter sakazakii	DD13094	NEG	NEG	NEG
Enterobacter sakazakii	DD13099	NEG	NEG	NEG
Enterobacter sakazakii	DD13134	NEG	NEG	NEG
Enterobacter turicensis	DD13163	NEG	NEG	NEG
Escherichia hermanii	DD13151	NEG	NEG	NEG
Hafnia alvei	DD5588	NEG	NEG	NEG
Klebsiella oxytoca	DD658	NEG	NEG	NEG
Klebsiella ozaenae	DD657	NEG	NEG	NEG
Klebsiella pneumoniae	DD373	NEG	NEG	NEG
Morganella morganii	DD13142	NEG	NEG	NEG
Morganella morganii	DD3064	NEG	NEG	NEG
Listeria monocytogenes	DD1309	NEG	NEG	NEG
Bacillus subtilis	DD1939	NEG	NEG	NEG
Enterococcus faecalis	DD2425	NEG	NEG	NEG
Carnobacterium divergens	DD2539	NEG	NEG	NEG
Citrobacter diversus	DD2561	NEG	NEG	NEG
Pantoea agglomerans	DD2599	NEG	NEG	NEG
Vibrio vulnificus	DD2633	NEG	NEG	NEG
Cronobacter sakazaki	DD2847	NEG	NEG	NEG
Lactococcus lactis	DD3590	NEG	NEG	NEG
Stapylococus epidermis	DD3624	NEG	NEG	NEG
Streptococcus equi	DD3998	NEG	NEG	NEG
Leuconostoc mesenteroides	DD4001	NEG	NEG	NEG
Carnobacterium gallinarum	DD4063	NEG	NEG	NEG
Pediococcus damnosus	DD4303	NEG	NEG	NEG
Shigella sonnei	DD6832	NEG	NEG	NEG
Yersinia entercolitica	DD7120	NEG	NEG	NEG
Edwardsiella tarda	DD13139	NEG	NEG	NEG
Kluyvera georgiana	DD13261	NEG	NEG	NEG
Yersinia enterocolitica	DD7120	NEG	NEG	NEG
Burkholderia cepacia	DD11946	NEG	NEG	NEG
Xanthomonas maltophilia	DD6263	NEG	NEG	NEG
Providencia alcalofaciens	DD960	NEG	NEG	NEG
Shigella boydii	DD1081	NEG	NEG	NEG
Shigell flexneri	DD1083	NEG	NEG	NEG
Brocothrix thermosphacta	DD666	NEG	NEG	NEG
Hafnia alvei	DD2389	NEG	NEG	NEG

Table 8. Exclusivity Results for Non-Target *E. coli* Strains (1)

E. coli serogroup	Strain ID	stx/eae presence	STEC Screening result	STEC Panel 1 result	STEC Panel 2 result
O1	DD2434	No/No	Neg	Neg	Neg
O104	DD13427	No/No	Neg	Neg	Neg
O113	DD13437	No/No	Neg	Neg	Neg
O113	DD13450	No/No	Neg	Neg	Neg
O113	DD13451	No/No	Neg	Neg	Neg
O113	DD13452	No/No	Neg	Neg	Neg
O113	DD13463	No/No	Neg	Neg	Neg
O113	DD2445	No/No	Neg	Neg	Neg
O114	DD1721	No/No	Neg	Neg	Neg
O115	DD1770	No/No	Neg	Neg	Neg
O117	DD13428	No/No	Neg	Neg	Neg
O117	DD2441	No/No	Neg	Neg	Neg
O118	DD2438	No/No	Neg	Neg	Neg
O119	DD13429	No/No	Neg	Neg	Neg
O125	DD1836	No/No	Neg	Neg	Neg
O126	DD13431	No/No	Neg	Neg	Neg
O126	DD1861	No/No	Neg	Neg	Neg
O127	DD1835	No/No	Neg	Neg	Neg
O128	DD13432	No/No	Neg	Neg	Neg
O128	DD13445	No/No	Neg	Neg	Neg
O128	DD13446	No/No	Neg	Neg	Neg
O128	DD13460	No/No	Neg	Neg	Neg
O128	DD1718	No/No	Neg	Neg	Neg
O137	DD13433	No/No	Neg	Neg	Neg
O139	DD1769	No/No	Neg	Neg	Neg
O143	DD1732	No/No	Neg	Neg	Neg
O146	DD13434	No/No	Neg	Neg	Neg
O152	DD1889	No/No	Neg	Neg	Neg

DISCUSSION OF THE MODIFICATION STUDY APPROVED DECEMBER 2017 (4)

The results of the BAX STEC Suite were compared to the results of the reference culture methods using probability of detection (POD) and difference in probability of detection (dPOD), according to the AOAC Micro Guidelines Appendix J. For flour samples enriched using the BAX System method, the real-time PCR assays (STEC Screening and Panel 1) detected *stx*, *eae* and the O121 serogroup for 8/20 low spiked samples and 5/5 high spiked samples at 24 h enrichment. All BAX System results were confirmed by culture (Table 1). The corresponding unpaired samples enriched using the FDA BAM reference method resulted in 7/20 culture positives for the low spike samples and 5/5 culture positives for the high spike samples. All uninoculated controls were negative. Inoculation levels were determined to be 0.43 CFU/test portion for the low level, and 4.3 CFU/portion for the high level, as determined using the BAM Ch. 4A method with the Least Cost Formulations (LCF) MPN calculator (2). At each inoculation level, the BAX STEC Suite method and the reference method demonstrated no significant statistical difference as indicated by POD analysis (the 95% confidence interval of the dPOD included 0 in all cases) as indicated in Table 2.

For raw ground beef samples enriched using the BAX System method, the real-time PCR assays detected *stx*, *eae* and the O126 serogroup for 11/20 low spiked samples and 5/5 high spiked samples at both 10 and 24 h enrichment times. All BAX® System results were confirmed by culture. The corresponding unpaired samples enriched using the USDA-FSIS culture reference method resulted in 8/20 culture positives for the low spike samples and 5/5 culture positives for the high spike samples. All uninoculated controls were negative. In addition, the BAX STEC suite was run on the reference method enriched test portions (mTSB), with results of 8/20 for the low spiked samples and 5/5 for the high spiked samples at both 10 and 24 h, matching the reference method results (see Tables 1 and 2). Inoculation levels were determined to be 0.51 CFU/test portion for the low level, and 3.36 CFU/portion for the high level, as determined using the USDA-FSIS MLG 5B.05 method with the LCF MPN calculator. At each inoculation level, the BAX STEC Suite method and the reference method, regardless of enrichment, demonstrated no significant statistical difference as indicated by POD analysis.

The results of these statistical analyses demonstrate no significant difference in the presumptive results compared to the confirmed results for flour and ground beef using either enrichment (BPW or mTSB) (Tables 1 and 2). While the flour and ground beef matrixes (enriched in BPW) produced more positives with the BAX System method than with the reference methods (Table 2), the differences were not statistically significant.

Table 1. BAX System STEC Presumptive vs. Confirmed Results for 25 g of All-Purpose Flour and Ground Beef (4)

Sample Type	Strain	MPN[a]/test portion	N[b]	BAX Presumptive			BAX Confirmed			dPOD$_{CP}$[f]	95% CI[g]
				X[c]	POD$_{CP}$[d]	95% CI	X	POD$_{CC}$[e]	95% CI		
Flour (25 g)	STEC O121 DD13363	0	5	0	0	(0.00, 0.43)	0	0	(0.00, 0.43)	0	(-0.45, 0.45)
		0.43	20	8	0.4	(0.22, 0.61)	8	0.4	(0.22, 0.61)	0	(-0.28, 0.28)
		4.3	5	5	1	(0.57, 1.00)	5	1	(0.57, 1.00)	0	(-0.43, 0.43)
Ground Beef (25 g) in BPW[h]	STEC O26 MSU TW00971	0	5	0	0	(0.00, 0.43)	0	0	(0.00, 0.43)	0	(-0.43, 0.43)
		0.51	20	11	0.55	(0.34, 0.74)	11	0.55	(0.34, 0.74)	0	(-0.28, 0.28)
		3.36	5	5	1	(0.57, 1.00)	5	1	(0.57, 1.00)	0	(-0.43, 0.43)
Ground Beef (25 g) in mTSB[h]	STEC O26 MSU TW00971	0	5	0	0	(0.00, 0.43)	0	0	(0.00, 0.43)	0	(-0.43, 0.43)
		0.51	20	8	0.4	(0.22, 0.61)	8	0.4	(0.22, 0.61)	0	(-0.28, 0.28)
		3.36	5	5	1	(0.57, 1.00)	5	1	(0.57, 1.00)	0	(-0.43, 0.43)

[a]MPN/test portion = Most Probable Number is based on the POD of reference method test portions using the Least Cost Formulations MPN calculator.
[b]N = Number of test portions.
[c]X = Number of positive test portions.
[d]POD$_{CP}$ = BAX® method presumptive positive results divided by the total number of test portions.
[e]POD$_{CC}$ = BAX® method confirmed positive results divided by the total number of test portions.
[f]dPOD$_{CP}$ = Difference between the BAX® method presumptive result and BAX® method confirmed result 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]Portions were tested by the BAX STEC Suite System at 10 and 24 h with no difference in results.

Table 2. BAX System method vs. the Reference method for the Detection of non-O157 STEC in 25g Flour and Ground Beef

Sample Type	Strain	MPN[a]/test portion	N[b]	BAX Method			Reference Method			dPOD$_{C}$[f]	95% CI[g]
				X[c]	POD$_{C}$[d]	95% CI	X	POD$_{R}$[e]	95% CI		
Flour (25 g)	STEC O121 DD13363	0	5	0	0	(0.00, 0.43)	0	0	(0.00, 0.43)	0	(-0.45, 0.45)
		0.43	20	8	0.4	(0.22, 0.61)	7	0.35	(0.18, 0.57)	0.05	(-0.23, 0.32)
		4.3	5	5	1	(0.57, 1.00)	5	1	(0.57, 1.00)	0	(-0.43, 0.43)
Fresh Raw Ground Beef (25 g) BPW[h]	STEC O26 MSU TW00971	0	5	0	0	(0.00, 0.43)	0	0	(0.00, 0.43)	0	(-0.43, 0.43)
		0.51	20	11	0.55	(0.34, 0.74)	8	0.4	(0.22, 0.61)	0.15	(-0.15, 0.41)
		3.36	5	5	1	(0.57, 1.00)	5	1	(0.57, 1.00)	0	(-0.43, 0.43)
Fresh Raw Ground Beef (25 g) mTSB[h]	STEC O26 MSU TW00971	0	5	0	0	(0.00, 0.43)	0	0	(0.00, 0.43)	0	(-0.43, 0.43)
		0.51	20	8	0.4	(0.22, 0.61)	8	0.4	(0.22, 0.61)	0	(-0.28, 0.28)
		3.36	5	5	1	(0.57, 1.00)	5	1	(0.57, 1.00)	0	(-0.43, 0.43)

[a]MPN = Most Probable Number is based on the POD of reference method test portions using the Least Cost Formulations MPN calculator.
[b]N/A = Not applicable.
[c]N = Number of test potions.
[d]X = Number of positive test portions.
[e]POD$_{C}$ = Confirmed candidate method positive outcomes divided by the total number of trials.
[f]POD$_{R}$ = Confirmed reference method positive outcomes divided by the total number of trials.
[g]dPOD$_{C}$ = Difference between the candidate method and reference method 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.
[h]Portions were tested by the BAX STEC Suite System at 10 and 24 h with no difference in results.

DISCUSSION OF THE MODIFICATION STUDY APPROVED JANUARY 2022 (7)

The BAX System Real-time PCR 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 point. There was one unconfirmed BAX presumptive positive in the STEC screen PCR assay in dried hemp flower that could not be confirmed for any of the Top 7 STEC. It's possible that there was a non-Top 7 STEC in the material, but further investigation was not conducted by the independent laboratory. According to independent laboratory feedback, processing samples for these assays was very user friendly with a standard heat dependent lysis step and transfer into pre-aliquoted lyophilized pellets in PCR wells. Short run times on the instrument helped improve throughput for processing samples.

The BAX Real-time PCR Assays for detecting STEC species allow users to obtain presumptive positive results after 24 h of incubation and 1–2 h of processing and assay run time for STEC analysis. Presumptive results are easily visualized, denoted by a plus or minus sign within the software.

Table 1. Matrix study: BAX Real-time PCR Assays for STEC Suite and *E. coli* O157:H7 Exact (used together for Top 7 STEC results) presumptive vs. confirmed results in dried cannabis flower (>0.3% THC) and dried hemp flower (≤0.3% THC) (7)

	MPN[a] / Test			Presumptive			Confirmed			
Matrix and Inoculum	Portion	N[b]	x[c]	POD_{cp}[d]	95% CI	x	POD_{cc}[e]	95% CI	$dPOD_{cp}$[f]	95% CI[g]
Dried cannabis	NA[i]	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	(-0.47, 0.47)
flower 10 g (*E. coli*	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)
O157:H7 ATCC[h]	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)
43895)										
Dried hemp Flower	NA	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	(-0.47, 0.47)
10 g (*E. coli* O157:H7	1.48 (0.77, 3.74)	20	14	0.70	0.48, 0.86	14	0.70	0.48, 0.86	0.00	(-0.13, 0.13)
ATCC 43890)	6.77 (3.95, 16.2)	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 potions.
[c]x = Number of positive test portions.
[d]POD_{CP} = Candidate method presumptive positive outcomes divided by the total number of trials.
[e]POD_{CC} = Candidate method confirmed positive outcomes divided by the total number of trials.
[f]$dPOD_{CP}$ = Difference between the candidate method presumptive result and candidate method confirmed result 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]American Type Culture Collection, Manassas, VA.
[i]Not applicable.

Table 3. Matrix study: BAX Real-time PCR Assays for STEC Suite presumptive vs. confirmed results in dried hemp flower (≤0.3% THC) (7)

Matrix and	MPN[a] / Test			Presumptive			Confirmed			
Inoculum	Portion	N[b]	x[c]	POD_{cp}[d]	95% CI	x	POD_{cc}[e]	95% CI	$dPOD_{cp}$[f]	95% CI[g]
Dried hemp Flower	NA[i]	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	(-0.47, 0.47)
10 g (*E. coli* O26	1.15 (0.61, 2.45)	20	11	0.55	0.34, 0.74	10	0.50	0.30, 0.70	0.05	(-0.11, 0.21)
CDC[h] 03-3014)	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)

[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 potions.
[c]x = Number of positive test portions.
[d]POD_{CP} = Candidate method presumptive positive outcomes divided by the total number of trials.
[e]POD_{CC} = Candidate method confirmed positive outcomes divided by the total number of trials.
[f]$dPOD_{CP}$ = Difference between the candidate method presumptive result and candidate method confirmed result 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]Centers for Disease Control and Prevention, Atlanta, GA.
[i]Not applicable.

DISCUSSION OF THE MODIFICATION STUDY APPROVED APRIL 2023 (10)

The BAX System Real-time PCR Assays for STEC Suite successfully detected the target STEC species in beef trim sampling cloths at a 375 g test portion size. Although the initial study MP media data set showed 9 presumptive positives at 8 h but 10 presumptive positives at 10 and 24 h, subsequent repeat studies demonstrated the method's ability to detect all presumptive positives at 8, 10 and 24 h. All presumptive positives in these second sets of data also confirmed positive. The study data were unable to find a statistical difference between the BAX STEC method's presumptive and confirmed results, nor between the BAX STEC and the MLG 5C.03 reference method results with 95% confidence.

Table 1. Matrix study: BAX Real-time PCR Assay for STEC Suite presumptive vs. confirmed results in beef trim (375 g) sampling cloths (10)

Matrix and Inoculum	cfu[a]/ Test Portion	N[b]	x[c]	Presumptive		Confirmed			dPOD$_{cp}$[f]	95% CI[g]
				POD$_{cp}$[d]	95% CI	x	POD$_{cc}$[e]	95% CI		
Beef trim Sampling cloth (*E. coli* O26:H11 DD 9703[h]) 8 h, MP media	NA[i]	5	0	0.00	0.00, 0.00	0	0.00	0.00, 0.00	0.00	(-0.47, 0.47)
	0.64	20	9	0.45	0.26, 0.66	9	0.45	0.26, 0.66	0.00	(-0.13, 0.13)
	4.76	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	(-0.47, 0.47)
Beef trim Sampling cloth (*E. coli* O26:H11 DD 9703[h]) 10 h, MP media	NA	5	0	0.00	0.00, 0.00	0	0.00	0.00, 0.00	0.00	(-0.47, 0.47)
	0.64	20	9	0.45	0.26, 0.66	9	0.45	0.26, 0.66	0.00	(-0.13, 0.13)
	4.76	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	(-0.47, 0.47)
Beef trim Sampling cloth (*E. coli* O26:H11 DD 9703[h]) 24 h, MP media	NA	5	0	0.00	0.00, 0.00	0	0.00	0.00, 0.00	0.00	(-0.47, 0.47)
	0.64	20	9	0.45	0.26, 0.66	9	0.45	0.26, 0.66	0.00	(-0.13, 0.13)
	4.76	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	(-0.47, 0.47)

[a]cfu/test portion = Inoculating strain was grown overnight, then serially diluted and plated in triplicate to determine appropriate concentration for inoculation.
[b]N = Number of test potions.
[c]x = Number of positive test portions.
[d]POD$_{CP}$ = Candidate method presumptive positive outcomes divided by the total number of trials.
[e]POD$_{CC}$ = Candidate method confirmed positive outcomes divided by the total number of trials.
[f]dPOD$_{CP}$ = Difference between the candidate method presumptive result and candidate method confirmed result 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]Hygiena Culture Collection, New Castle, DE.
[i]Not applicable.

Table 2. BAX Real-time PCR Assay for STEC Suite method vs. reference method results in beef trim (375 g) sampling cloths (10)

Matrix and Inoculum	cfu[a]/ Test Portion	N[b]	x[c]	BAX Method		Reference Method			dPOD$_c$[f]	95% CI[g]
				POD$_c$[d]	95% CI	x	POD$_g$[e]	95% CI		
Beef trim Sampling cloth (*E. coli* O26:H11 DD 9703[h]) 8 h, MP media[i]	NA[j]	5	0	0.00	0.00, 0.00	0	0.00	0.00, 0.00	0.00	(-0.43, 0.43)
	0.64	20	9	0.45	0.26, 0.66	13	0.65	0.43, 0.82	-0.20	(-0.46, 0.10)
	4.76	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	(-0.43, 0.43)
Beef trim Sampling cloth (*E. coli* O26:H11 DD 9703[h]) 10 h, MP media	NA	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	(-0.43, 0.43)
	0.64	20	9	0.45	0.26, 0.66	13	0.65	0.43, 0.82	-0.20	(-0.46, 0.10)
	4.76	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	(-0.43, 0.43)
Beef trim Sampling cloth (*E. coli* O26:H11 DD 9703[h]) 24 h, MP media	NA	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	(-0.43, 0.43)
	0.64	20	9	0.45	0.26, 0.66	13	0.65	0.43, 0.82	-0.20	(-0.46, 0.10)
	4.76	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	(-0.43, 0.43)
Beef trim Sampling cloth (*E. coli* O26:H11 DD 9703[h]) 8 h, mTSB+caa[k]	NA	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	(-0.47, 0.47)
	0.64	20	13	0.65	0.43, 0.82	13	0.65	0.43, 0.82	0.00	(-0.13, 0.13)
	4.76	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	(-0.47, 0.47)
Beef trim Sampling cloth (*E. coli* O26:H11 DD 9703[h]) 24 h, mTSB+caa	NA	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	(-0.47, 0.47)
	0.64	20	13	0.65	0.43, 0.82	13	0.65	0.43, 0.82	0.00	(-0.13, 0.13)
	4.76	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	(-0.47, 0.47)

[a]cfu/portion = Inoculating strain was grown overnight, then serially diluted and plated in triplicate to determine appropriate concentration for inoculation.
[b]N = Number of test potions.
[c]X = Number of positive test portions.
[d]POD$_C$ = Confirmed candidate method presumptive positive outcomes confirmed positive divided by the total number of trials.
[f]POD$_R$ = Confirmed reference method positive outcomes divided by the total number of trials.
[e]dPOD$_C$ = Difference between the candidate method and reference method POD values.
[f]95% CI = If the confidence interval of a dPOD does not contain zero, then the difference is statistically significant at the 5% level.
[h]Hygiena Culture Collection, New Castle, DE.
[i]Results calculated using unpaired POD statistical analysis.
[i]Not applicable.
[k]Results calculated using paired POD statistical analysis.

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