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CERTIFICATION

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Certificate No.

022001

The AOAC Research Institute hereby certifies the test kit known as:

Simultaneous Multiplex Real Time PCR (SIMUL-qPCR) Top7 STEC Assay Collection

manufactured by

Applied Food Diagnostics, Inc.
387 Hazle Street
Nuremberg, PA
18421

This method has been evaluated as a single-site method in the AOAC® *Performance Tested Methods*SM Program and found to perform as stated by the manufacturer contingent to the comments contained in the manuscript. This certificate means that an AOAC® Certification Mark License Agreement has been executed which authorizes the manufacturer to display the AOAC *Performance Tested* SM certification mark along with the statement - "THIS METHOD'S PERFORMANCE WAS REVIEWED BY AOAC RESEARCH INSTITUTE AND WAS FOUND TO PERFORM TO THE MANUFACTURER'S SPECIFICATIONS" - on the above mentioned method for a period of one calendar year from the date of this certificate (February 05, 2020 – December 31, 2020). Renewal may be granted at the end of one year under the rules stated in the licensing agreement.

Scott Coates

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Scott Coates, Senior Director
Signature for AOAC Research Institute

February 05, 2020
Date

METHOD AUTHORS
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SUBMITTING COMPANY
Applied Food Diagnostics, Inc.
387 Hazle Street
Nuremburg, PA 18241 USA

KIT NAME(S)
Simultaneous Multiplex Real Time PCR (SIMUL-qPCR) Top7 STEC Assay Collection (Single source service-based method)

CATALOG NUMBERS
SMRT-T7-032, SMRT-T7-096

INDEPENDENT LABORATORY
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Springdale, AR 72762

AOAC EXPERTS AND PEER REVIEWERS
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APPLICABILITY OF METHOD
Target Organisms - Shiga toxin (stx) genes, Intimin genes (eae), *E. coli* Serotypes O157, O26, O45, O1O3, O111, O121 and O145.

Matrices – Fresh raw ground beef (~75% lean), fresh raw beef trim (~75%), and beef carcass sampling sheets (spunbonded polyolefin sampling sheet)

Performance claims - Performance comparable to that of the U. S. Department of Agriculture-Food Safety and Inspection Service *Microbiology Laboratory Guidebook* (MLG), 5.09 (2), Detection, Isolation and Identification of *Escherichia coli* O157:H7 from Meat Products and Carcass and Environmental Sponges for fresh raw ground beef and MLG 5B.05 (3), Detection and Isolation of non-O157 Shiga Toxin-Producing *Escherichia coli* (STEC) from Meat Products and Carcass and Environmental Sponges for fresh raw beef trim and beef carcass sampling sheets.

REFERENCE METHODS
U.S. Department of Agriculture-Food Safety and Inspection Service (2015) *Microbiology Laboratory Guidebook*, 5.09, Detection, Isolation and Identification of *Escherichia coli* O157:H7 from Meat Products and Carcass and Environmental Sponges https://www.fsis.usda.gov/wps/wcm/connect/51507fdb-dded-47f7-862d-ad80c3ee1738/MLG-5.pdf?MOD=AJPERES (3)
Off **U. S. Department of Agriculture-Food Safety and Inspection Service (2014)** *Microbiology Laboratory Guidebook*, 5B.05, Detection and Isolation of non-O157 Shiga Toxin-Producing *Escherichia coli* (STEC) from Meat Products and Carcass and Environmental Sponges http://www.fsis.usda.gov/wps/wcm/connect/7ffc02b5-3d33-4a79-b50c-81f208893204/MLG-5B.pdf?MOD=AJPERES (3)

ORIGINAL CERTIFICATION DATE
February 05, 2020

CERTIFICATION RENEWAL RECORD
New Approval

METHOD MODIFICATION RECORD
NONE

SUMMARY OF MODIFICATION
NONE

Under this AOAC® *Performance Tested*^SM License Number, 022001 this method is distributed by:
NONE

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PRINCIPLE OF THE METHOD (1)
Enterohemorrhagic *E. coli* Recovery and Enrichment Broth (EREB) is a proprietary medium, optimized for single-step recovery and enrichment of Enterohemorrhagic *E. coli* (EHEC) from raw beef trim, raw ground beef, and beef carcass sampling sheets. The medium can also be used for the recovery and enrichment of *Salmonella* as a single-enrichment method alongside EHEC in beef samples.
During PCR amplification, forward and reverse primers hybridize to unique sequences of the target's genomic DNA. A fluorogenic probe is included in the same reaction mixture which consists of a DNA probe labeled with a 5`-dye and a 3`-quencher. During PCR amplification, the probe is cleaved, and the reporter dye and quencher are separated. The resulting increase in fluorescence can be detected on the real-time PCR instrument. The kit is composed of 5 unique PCR reaction tubes with multiple STEC targets which are to be run simultaneously but may be processed individually.

DISCUSSION OF THE VALIDATION STUDY (1)

Inclusivity and Exclusivity

Of the 50 inclusivity strains analyzed by the SIMUL-qPCR Top 7 STEC Assay, all 50 inclusivity strains were correctly detected by the appropriate PCR target within the Top 7 STEC assay kit collection. Additionally, all 50 strains were correctly identified to contain either the intimin gene, the shiga toxin-producing gene, or both.

Of the 30 exclusivity strains, none were detected by the SIMUL-qPCR method. Initially three of the exclusivity isolates gave a positive PCR call (*Bacillus pumilis, Ewingella americana,* and *Providencia staurtii*), but the blank well also called. An examination of the run data indicated a possibility of cross-contamination among the wells, and so a repeat test was conducted. After a rerun of these isolates and the blank well starting from the beginning of the lysing process, all results were negative.

Real-Time Stability Study

The results from the real-time product consistency and stability study showed that there was no statistically significant differences by POD analysis between the recently manufactured lots and those halfway through the expiration period or the recently manufactured lots and those nearing expiration. The study verified the one-year shelf life of the SIMUL-qPCR Top 7 STEC Assay.

Robustness Study

The results from the robustness study showed that there was no statistically significant difference by POD analysis when small alterations are made to the protocol. Increasing or decreasing enrichment time, volume of the extraction reagent, and volume of extracted DNA sample slightly had no effect the performance of the Top 7 STEC Assay.

Matrix Study

Results from both the method developer and independent studies of the SIMUL-qPCR Top 7 STEC Assay for both raw beef trim, raw ground beef and beef carcass sampling sheets are outlined in Tables 3–6. Throughout the study, the method developer had difficulty achieving fractional results, and repeat attempts had to be made to spike at the correct inoculation levels. The method developer also saw issues with pooling at fractional levels. During the pooling process, it was determined that the pooled samples had to incubate for an additional two hours for both matrixes in order to achieve the fractional results comparable to the reference method.

During confirmation testing, the method developer laboratory had issues with competing flora growing on plates. Colonies had to be re-streaked for further isolation to obtain the correct result.

For both raw beef trim and raw ground beef, the candidate method had some presumptive PCR results that did not culturally confirm. It was found that the presumptive positives that did not confirm had late C_q calls on the PCR. Those late C_q calls were determined to be difficult to culturally confirm due to the low levels of the *E. coli* target cells as well as high levels of competing background flora. It was found that the pooled data for both the 12 h and 18 h time points for both matrixes did not have much difference in the presumptive PCR results than the individual samples at the 10 h and 18 h time points. The candidate presumptive PCR results were not statistically different by POD analysis compared to the candidate confirmed results (using PCR, agglutination, and biochemical confirmation) for both the individual samples as well as pooled samples. Also, no statistically significant difference was found between the candidate confirmed method and the USDA FSIS MLG 5.09 and 5B.05 reference method for both individual and pooled samples.

For the sampling sheets, there was no difference in the number of presumptive positives at any of the time points, including the pooled samples. All presumptive samples confirmed positive. The reference method had 3 confirmed positives for the high inoculation level and 6 confirmed positives for the low inoculation level. By comparison, the candidate method had 4 confirmed positives for the high inoculation level and 4 confirmed positives for the low inoculation level. The difference between the candidate and reference method results was not statistically significant.

Independent Laboratory Study

For the SIMUL-qPCR method, at the 10 h individual enrichment point, 6 out of 20 samples were positive. At the 12 h and 18 h pooled enrichment points, 9 out of 20 samples were positive. At the 18 h individual enrichment time point, 10 out of 20 samples were positive. Nine out of 20 test portions confirmed positive. For the reference method, 8 out of 20 test portions confirmed positive. All five of the high inoculation set were positive and all five of the uninoculated sets were negative for both the candidate and reference methods. There were no statistically significant differences found between the candidate presumptive versus confirmed results at any time point, and no significant differences found between the candidate and reference methods.

Data from validation study
Table 1. Inclusivity List – Top 7 STEC Strains (1)

No.	Species	Serotype	stx	eae	Source	Origin	SIMUL-qPCR Top 7 Result
1	*E. coli*	O157:H7	Pos	Pos	ATCC[a] 43895	Raw hamburger meat	+
2	*E. coli*	O157:H7	Pos	Pos	PSU ECRC[b] 99.0238	Deer	+
3	*E. coli*	O157:H7	Pos	Pos	PSU ECRC 10.2268	Cow	+
4	*E. coli*	O157:H7	Pos	Pos	ATCC 35150	Human	+
5	*E. coli*	O157:H7	Pos	Pos	PSU ECRC 10.2550	Unknown	+
6	*E. coli*	O157:H7	Pos	Pos	BEI[c] NR-8	Human	+
7	*E. coli*	O157:H7	Pos	Pos	BEI NR-7	Human	+
8	*E. coli*	O103	Pos	Pos	MSU[d] TW01675	Human	+
9	*E. coli*	O103	Pos	Pos	MSU TW07881	Human	+
10	*E. coli*	O103	Pos	Pos	MSU TW07975	Human	+
11	*E. coli*	O103	Pos	Pos	MSU TW07990	Human	+
12	*E. coli*	O103	Pos	Pos	MSU TW04162	Human	+

13	*E. coli*	O103	Pos	Pos	PSU ECRC 17.2641	Unknown	+
14	*E. coli*	O103:H2	Pos	Pos	BEI NR-17625	Horse	+
15	*E. coli*	O103:H2	Pos	Pos	BEI NR-17626	Human	+
16	*E. coli*	O111	Pos	Pos	MSU TW07926	Human	+
17	*E. coli*	O111	Pos	Pos	MSU TW07813	Human	+
18	*E. coli*	O111	Pos	Pos	MSU TW05608	Human	+
19	*E. coli*	O111	Pos	Pos	MSU TW04257	Human	+
20	*E. coli*	O111	Pos	Pos	MSU TW07870	Cow	+
21	*E. coli*	O111	Pos	Pos	MSU TW05150	Cow	+
22	*E. coli*	O111	Pos	Pos	PSU ECRC 0.1079	Cow	+
23	*E. coli*	O121	Pos	Pos	MSU TW08004	Human	+
24	*E. coli*	O121	Pos	Pos	PSU ECRC 17.2643	Unknown	+
25	*E. coli*	O121	Pos	Pos	MSU TW08039	Human	+
26	*E. coli*	O121	Pos	Pos	MSU TW07972	Human	+
27	*E. coli*	O121	Pos	Pos	MSU TW08969	Human	+
28	*E. coli*	O121	Pos	Pos	MSU TW08970	Human	+
29	*E. coli*	O121:H19	Pos	Pos	BEI NR-17630	Human	+
30	*E. coli*	O145	Pos	Pos	MSU TW07596	Human	+
31	*E. coli*	O145	Pos	Pos	MSU TW03064	Rabbit	+
32	*E. coli*	O145	Pos	Pos	MSU TW08087	Human	+
33	*E. coli*	O145	Pos	Pos	MSU TW09153	Human	+
34	*E. coli*	O145	Pos	Pos	PSU ECRC 4.0967	Rabbit	+
35	*E. coli*	O145	Pos	Pos	MSU TW09356	Human	+
36	*E. coli*	O145:H2	Pos	Pos	BEI NR-17633	Rabbit	+
37	*E. coli*	O26	Pos	Pos	MSU TW08998	Human	+
38	*E. coli*	O26	Pos	Pos	PSU ECRC 5.2217	Human	+
39	*E. coli*	O26	Pos	Pos	MSU TW09156	Human	+
40	*E. coli*	O26	Pos	Pos	MSU TW04270	Human	+
41	*E. coli*	O26	Pos	Pos	MSU TW07936	Human	+
42	*E. coli*	O26	Pos	Pos	MSU TW07948	Human	+
43	*E. coli*	O26	Pos	Pos	PSU ECRC 6.1592	Unknown	+
44	*E. coli*	O45	Pos	Pos	MSU TW00965	Human	+
45	*E. coli*	O45	Pos	Pos	MSU TW03068	Cow	+
46	*E. coli*	O45	Pos	Pos	PSU ECRC 11.1079	Goat	+
47	*E. coli*	O45	Pos	Pos	MSU TW03070	Cow	+
48	*E. coli*	O45	Pos	Pos	MSU TW01589	Cow	+
49	*E. coli*	O45	Pos	Pos	MSU TW07947	Human	+
50	*E. coli*	O45	Pos	Pos	MSU TW09183	Human	+

^aAmerican Type Culture Collection, Manassas, VA.
^bThe Pennsylvania State University *E. coli* Reference Center, University Park, PA.
^cBEI Resources, Manassas, VA.
^dMichigan State University STEC Center, East Lansing, MI.

Table 2. Exclusivity List – Non-STEC Strains (1)

No.	Genus	species	Source	Origin	SIMUL-qPCR Top 7 Result
1	*Citrobacter*	*freundii*	SGSC[a] 5346	Unknown	-
2	*Citrobacter*	*koseri*	SGSC 5610	Unknown	-
3	*Citrobacter*	*amalonaticus*	SGSC 5616	Unknown	-
4	*Cronobacter*	*sakazakii*	ATCC[b] BAA-894	Human clinical specimen	-
5	*Ewingella*	*americana*	SGSC 5640	Human feces	-
6	*Enterobacter*	*taylorae*	SGSC 5283	Unknown	-
7	*Enterobacter*	*cloacae*	SGSC 5330	Unknown	-
8	*Enterobacter*	*aerogenes*	SGSC 5347	Unknown	-
9	*Enterobacter*	*intermedius*	SGSC 5584	Bean sprouts	-
10	*Enterococcus*	*faecalis*	BEI[c] NR-31884	Human blood	-
11	*Escherichia*	*coli*	ATCC 10536	Unknown	-
12	*Escherichia*	*blattae*	SGSC 5712	Cockroach	-
13	*Escherichia*	*hermanii*	SGSC 5715	Human	-
14	*Escherichia*	*vulneris*	SGSC 5716	Human	-
15	*Escherichia*	*fergusonii*	SGSC 5718	Human feces	-
16	*Hafnia*	*alvei*	USDA ERRC[d] B-41102	Ground beef	-
17	*Klebisella*	*oxytoca*	SGSC 5367	Unknown	-
18	*Klebsiella*	*pneumoniae subsp. pneumoniae*	SGSC 5926	Unknown	-
19	*Klebsiella*	*ozaenae*	SGSC 2810	Unknown	-
20	*Klebsiella*	*terrigena*	SGSC 5928	Drinking water	-
21	*Klebsiella*	*planticola*	SGSC 5929	Radish root	-
22	*Morganella*	*morganii*	SGSC 5435	Unknown	-
23	*Proteus*	*mirabilis*	ATCC 7002	Urine of patient with kidney stones	-
24	*Providencia*	*stuartii*	SGSC 5639	Unknown	-
25	*Serratia*	*marcescens*	SGSC 5354	Unknown	-
26	*Shigella*	*boydii*	SGSC 5579	Unknown	-
27	*Shigella*	*flexneri*	USDA ERRC B-51067	Ground beef	-
28	*Shigella*	*sonnei*	USDA ERRC B-41922	Ground beef	-
29	*Staphylococcus*	*aureus*	ATCC 29213	Wound	-
30	*Yersinia*	*enterocolitica*	USDA ERRC B-41479	Ground beef	-
31	*Salmonella*	*enterica subsp. enterica* Enteritidis	ATCC 13076	Unknown	-
32	*Carnobacterium*	*divergens*	ATCC 43887	Vacuum Packed Minced Beef	-

[a]*Salmonella* Genetic Stock Centre, University of Calgary, Canada.
[b]American Type Culture Collection, Manassas, VA.
[c]BEI Resources, Manassas, VA.
[d]United States Department of Agriculture Eastern Regional Research Center, Wyndmoor, PA.

Matrix	Strain	MPN^a/25g	N^b	x^c	POD$_{CP}{}^d$	95% CI	x	POD$_{CC}{}^e$	95% CI	dPOD$_{CP}{}^f$	95% CI^g
					SIMUL-qPCR Top 7 STEC Presumptive			SIMUL-qPCR Top 7 STEC Confirmed			
Ground beef 10 h	*E. coli* O157:H7 ATCC^h 43895	N/A^i	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	-0.43, 0.43
		1.69 (1.08, 2.90)	20	10	0.50	0.30, 0.70	11	0.55	0.34, 0.74	-0.05	-0.33, 0.24
		7.28 (3.36, 15.7)	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	-0.43, 0.43
Ground beef^j 10 h	*E. coli* O157:H7 ATCC 35150	N/A	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	-0.43, 0.43
		0.60 (0.33, 0.99)	20	6	0.30	0.15, 0.52	9	0.45	0.26, 0.66	-0.15	0.10, 0.35
		4.92 (2.43, 10.7)	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	-0.43, 0.43
Beef trim 10 h	*E. coli* O26 MSU^k TW08998	N/A	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	-0.43, 0.43
		0.57 (0.30, 0.94)	20	6	0.30	0.15, 0.52	6	0.30	0.15, 0.52	0.00	-0.27, 0.27
		0.81 (0.33, 1.44)	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	-0.43, 0.43
Sampling Sheets 10 h	*E. coli* O121 MSU^k TW08004	N/A	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	-0.43, 0.43
		N/A	20	4	0.20	0.08, 0.42	4	0.20	0.08, 0.42	0.00	-0.25, 0.25
		N/A	5	4	0.80	0.38, 1.00	4	0.80	0.38, 1.00	0.00	-0.47, 0.47
Ground beef 18 h	*E. coli* O157:H7 ATCC 43895	N/A^h	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	-0.43, 0.43
		1.69 (1.09, 2.89)	20	12	0.60	0.39, 0.78	11	0.55	0.34, 0.74	0.05	-0.24, 0.33
		7.28 (3.36, 15.7)	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	-0.43, 0.43
Ground beef^j 18 h	*E. coli* O157:H7 ATCC 35150	N/A	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	-0.43, 0.43
		0.60 (0.33, 0.99)	20	10	0.50	0.30, 0.70	9	0.45	0.26, 0.66	0.08	-0.11, 0.21
		4.92 (2.43, 10.7)	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	-0.43, 0.43
Beef trim 18 h	*E. coli* O26 MSU TW08998	N/A	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	-0.43, 0.43
		0.57 (0.30, 0.94)	20	11	0.55	0.34, 0.74	6	0.30	0.15, 0.52	0.25	-0.05, 0.50
		0.81 (0.33, 1.44)	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	-0.43, 0.43
Sampling Sheets 18 h	*E. coli* O121 MSU^k TW08004	N/A	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	-0.43, 0.43
		N/A	20	4	0.20	0.08, 0.42	4	0.20	0.08, 0.42	0.00	-0.25, 0.25
		N/A	5	4	0.80	0.38, 1.00	4	0.80	0.38, 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 LCF 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}ATCC = American Type Culture Collection, Manassas, VA.

^{i}Not applicable.

^{j}Matrix tested by the independent laboratory.

^{k}MSU = Michigan State University, East Lansing, MI.

Table 4. SIMUL-qPCR Top 7 STEC Assay vs. Reference Method Results – Individual samples (1)

Matrix	Strain	MPN[a]/25g	N[b]	x[c]	POD$_C$[d]	95% CI	x	POD$_R$[e]	95% CI	dPOD$_C$[f]	95% CI[g]
					SIMUL-qPCR Top 7 STEC Results Confirmed			Reference Method Results			
Ground beef 10 h	E. coli O157:H7 ATCC[h] 43895	N/A[i]	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	-0.43, 0.43
		1.69 (1.09, 2.90)	20	10	0.50	0.30, 0.70	15	0.75	0.53, 0.89	-0.25	-0.49, 0.05
		7.28 (3.36, 15.7)	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	-0.43, 0.43
Ground beef[j] 10 h	E. coli O157:H7 ATCC 35150	N/A	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	-0.43, 0.43
		0.60 (0.33, 0.99)	20	6	0.30	0.15, 0.52	8	0.40	0.22, 0.61	-0.10	-0.36, 0.18
		4.92 (2.43 10.7)	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	-0.43, 0.43
Beef trim 10 h	E. coli O26 MSU[k] TW08998	N/A	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	-0.43, 0.43
		0.57 (0.30, 0.94)	20	6	0.30	0.15, 0.52	7	0.35	0.18, 0.57	-0.05	-0.32, 0.23
		0.81 (0.33, 1.44)	5	5	1.00	0.57, 1.00	3	0.60	0.23, 0.88	0.40	-0.12, 0.77
Sampling Sheets 10 h	E. coli O121 MSU[k] TW08004	N/A	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	-0.43, 0.43
		N/A	20	4	0.20	0.08, 0.42	6	0.30	0.15, 0.52	-0.10	-0.35, 0.17
		N/A	5	4	0.80	0.38, 1.00	3	0.60	0.23, 0.88	0.20	-0.31, 0.62
Ground beef 18 h	E. coli O157:H7 ATCC 43895	N/A	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	-0.43, 0.43
		1.69 (1.09, 2.90)	20	11	0.55	0.34, 0.74	15	0.75	0.53, 0.89	-0.20	-0.45, 0.09
		7.28 (3.36, 15.7)	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	-0.43, 0.43
Ground beef[j] 18 h	E. coli O157:H7 ATCC 35150	N/A	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	-0.43, 0.43
		0.60 (0.33, 0.99)	20	9	0.45	0.26, 0.66	8	0.40	0.22, 0.61	0.05	-0.24, 0.33
		4.92 (2.43, 10.7)	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	-0.43, 0.43
Beef trim 18 h	E. coli O26 MSU TW08998	N/A	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	-0.43, 0.43
		0.57 (0.30, 0.94)	20	6	0.30	0.15, 0.52	7	0.35	0.18, 0.57	-0.05	-0.32, 0.23
		0.81 (0.33, 1.44)	5	5	1.00	0.57, 1.00	3	0.60	0.23, 0.88	0.40	-0.12, 0.77
Sampling Sheets 18 h	E. coli O121 MSU[k] TW08004	N/A	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	-0.43, 0.43
		N/A	20	4	0.20	0.08, 0.42	6	0.30	0.15, 0.52	-0.10	-0.35, 0.17
		N/A	5	4	0.80	0.38, 1.00	3	0.60	0.23, 0.88	0.20	-0.31, 0.62

[a]MPN = Most Probable Number is based on the POD of reference method test portions using the LCF MPN calculator, with 95% confidence interval.

[b]N = Number of test potions.

[c]x = Number of positive test portions.

[d]POD$_C$ = Confirmed candidate method positive outcomes divided by the total number of trials.

[e]POD$_R$ = Confirmed reference method positive outcomes divided by the total number of trials.

[f]dPOD$_C$ = Difference between the candidate method and reference method 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]ATCC = American Type Culture Collection, Manassas, VA.

[i]Not applicable.

[j]Matrix tested by the independent laboratory.

[k]MSU = Michigan State University, East Lansing, MI.

Table 5. SIMUL-qPCR Top 7 STEC Assay Presumptive vs. Confirmed Results – Pooled samples (1)

Matrix	Strain	MPN[a]/25g	N[b]	x[c]	POD$_{CP}$[d]	95% CI	x	POD$_{CC}$[e]	95% CI	dPOD$_{CP}$[f]	95% CI[g]
					SIMUL-qPCR Top 7 STEC Presumptive			SIMUL-qPCR Top 7 STEC Confirmed			
Ground beef 12 h	*E. coli* O157:H7 ATCC[h] 43895	N/A[i]	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	-0.43, 0.43
		1.69 (1.09, 2.90)	20	11	0.55	0.34, 0.74	11	0.55	0.34, 0.74	0.00	-0.28, 0.28
		7.28 (3.36, 15.7)	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	-0.43, 0.43
Ground beef[j] 12 h	*E. coli* O157:H7 ATCC 35150	N/A	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	-0.43, 0.43
		0.60, (0.33, 0.99)	20	9	0.45	0.26, 0.66	9	0.45	0.26, 0.66	0.07	-0.13, 0.13
		4.92 (2.43, 10.7)	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	-0.43, 0.43
Beef trim 12 h	*E. coli* O26 MSU[k] TW08998	N/A	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	-0.43, 0.43
		0.57 (0.30, 0.942)	20	7	0.35	0.18, 0.57	6	0.30	0.15, 0.52	0.05	-0.23, 0.32
		0.81 (0.33, 1.44)	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	-0.43, 0.43
Sampling Sheets 12 h	*E. coli* O121 MSU[k] TW08004	N/A	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	-0.43, 0.43
		N/A	20	4	0.20	0.08, 0.42	4	0.20	0.08, 0.42	0.00	-0.25, 0.25
		N/A	5	4	0.80	0.38, 1.00	4	0.80	0.38, 1.00	0.00	-0.47, 0.47
Ground beef 18 Hour	*E. coli* O157:H7 ATCC 43895	N/A	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	-0.43, 0.43
		1.69 (1.09, 2.90)	20	12	0.60	0.39, 0.78	11	0.55	0.34, 0.74	0.05	-0.24, 0.33
		7.28 (3.37, 15.7)	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	-0.43, 0.43
Ground beef[j] 18 h	*E. coli* O157:H7 ATCC 35150	N/A	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	-0.43, 0.43
		0.60, (0.33, 0.99)	20	9	0.45	0.26, 0.66	9	0.45	0.26, 0.66	0.07	-0.13, 0.13
		4.92 (2.43, 10.7)	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	-0.43, 0.43
Beef trim 18 h	*E. coli* O26 MSU TW08998	N/A	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	-0.43, 0.43
		0.57 (0.30, 0.94)	20	10	0.50	0.30, 0.70	6	0.30	0.15, 0.52	0.25	-0.10, 0.45
		0.81 (0.33, 1.44)	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	-0.43, 0.43
Sampling Sheets 18 h	*E. coli* O121 MSU[k] TW08004	N/A	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	-0.43, 0.43
		N/A	20	4	0.20	0.08, 0.42	4	0.20	0.08, 0.42	0.00	-0.25, 0.25
		N/A	5	4	0.80	0.38, 1.00	4	0.80	0.38, 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 LCF 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]ATCC = American Type Culture Collection, Manassas, VA.

[i]Not applicable.

[j]Matrix tested by the independent laboratory.

[k]MSU = Michigan State University, East Lansing, MI

Table 6. SIMUL-qPCR Top 7 STEC Assay vs. Reference Method Results – Pooled samples (1)

Matrix	Strain	MPN[a]/25g	N[b]	x[c]	POD$_C$[d]	95% CI	x	POD$_R$[e]	95% CI	dPOD$_C$[f]	95% CI[g]
					SIMUL-qPCR Top 7 STEC Results Confirmed			Reference Method Results			
Ground beef 12 h	E. coli O157:H7 ATCC[h] 43895	N/A[i]	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	-0.43, 0.43
		1.69 (1.09, 2.90)	20	11	0.55	0.34, 0.74	15	0.75	0.53, 0.89	-0.20	-0.45, 0.09
		7.28 (3.36, 15.7)	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	-0.43, 0.43
Ground beef[j] 12 h	E. coli O157:H7 ATCC 35150	N/A	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	-0.43, 0.43
		0.60 (0.33, 0.99)	20	9	0.45	0.26, 0.66	8	0.40	0.22, 0.61	0.05	-0.24, 0.33
		4.92 (2.43, 10.7)	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	-0.43, 0.43
Beef trim 12 h	E. coli O26 MSU[k] TW08998	N/A	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	-0.43, 0.43
		0.57 (0.30, 0.94)	20	6	0.30	0.15, 0.52	7	0.35	0.18, 0.57	-0.05	-0.32, 0.23
		0.81 (0.33, 1.44)	5	5	1.00	0.57, 1.00	3	0.60	0.23, 0.88	0.40	-0.12, 0.77
Sampling Sheets 12 h	E. coli O121 MSU[k] TW08004	N/A	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	-0.43, 0.43
		N/A	20	4	0.20	0.08, 0.42	6	0.30	0.15, 0.52	-0.10	-0.35, 0.17
		N/A	5	4	0.80	0.38, 1.00	3	0.60	0.23, 0.88	0.20	-0.31, 0.62
Ground beef 18 h	E. coli O157:H7 ATCC 43895	N/A	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	-0.43, 0.43
		1.69 (1.09, 2.90)	20	11	0.55	0.34, 0.74	15	0.75	0.53, 0.89	-0.20	-0.45, 0.09
		7.28 (3.36, 15.7)	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	-0.43, 0.43
Ground beef[j] 18 h	E. coli O157:H7 ATCC 35150	N/A	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	-0.43, 0.43
		0.60 (0.33, 0.99)	20	9	0.45	0.26, 0.66	8	0.40	0.22, 0.61	0.05	-0.24, 0.33
		4.92 (2.43, 10.7)	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	-0.43, 0.43
Beef trim 18 h	E. coli O26 MSU TW08998	N/A	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	-0.43, 0.43
		0.57 (0.30, 0.94)	20	6	0.30	0.15, 0.52	7	0.35	0.18, 0.57	-0.05	-0.32, 0.23
		0.81 (0.33, 1.44)	5	5	1.00	0.57, 1.00	3	0.60	0.23, 0.88	0.40	-0.12, 0.77
Sampling Sheets 18 h	E. coli O121 MSU[k] TW08004	N/A	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	-0.43, 0.43
		N/A	20	4	0.20	0.08, 0.42	6	0.30	0.15, 0.52	-0.10	-0.35, 0.17
		N/A	5	4	0.80	0.38, 1.00	3	0.60	0.23, 0.88	0.20	-0.31, 0.62

[a]MPN = Most Probable Number is based on the POD of reference method test portions using the LCF MPN calculator, with 95% confidence interval.
[b]N = Number of test potions.
[c]x = Number of positive test portions.
[d]POD$_C$ = Confirmed candidate method positive outcomes divided by the total number of trials.
[e]POD$_R$ = Confirmed reference method positive outcomes divided by the total number of trials.
[f]dPOD$_C$ = Difference between the candidate method and reference method 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]ATCC = American Type Culture Collection, Manassas, VA.
[i]Not applicable.
[j]Matrix tested by the independent laboratory.
[k]MSU = Michigan State University, East Lansing, MI.

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