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Article

MICROBIOLOGICAL METHODS

Validation of the Applied Food Diagnostics, Inc. Simultaneous Multiplex Real Time PCR (SIMUL-qPCR) *Listeria* Species and *Monocytogenes* Assay in Selected Foods and Environmental Surfaces: AOAC Performance Tested Method[SM] 062001

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Abstract

Background: The Simultaneous Multiplex Real Time PCR (SIMUL-qPCR) *Listeria* species and *monocytogenes* Assay is a quick, reliable method for detecting *Listeria* species and *monocytogenes* in environmental and food samples. The assay multiplexes several targets in one run to properly identify *Listeria* species and *monocytogenes*. The assay uses proprietary medium, *Listeria* Recovery and Enrichment Broth (LREB), for enrichment purposes. LREB was specifically formulated to improve the recovery and growth of *Listeria* while inhibiting competing background flora.
Objective: This report details the method validation study to validate frankfurters, ready-to-eat (RTE) sliced turkey, soft fresh raw cheese, chicken salad, ice cream, cooked eggs, pasteurized milk, and frozen/cooked shrimp, as well as environmental surface sponges and swabs for stainless steel, plastic, rubber, ceramic tile, and sealed concrete.
Method: Matrix studies, inclusivity/exclusivity, product consistency/stability, and robustness testing were conducted to assess the method's performance.
Results: There were no statistically significant differences found between the candidate and reference methods in the matrix studies. Inclusivity/exclusivity testing showed that the assay was able to detect both *Listeria* species and *monocytogenes* strains while excluding the non-*Listeria* isolates. Small variations in critical test parameters (enrichment time, extraction reagent volume, and extracted sample volume) did not adversely affect the assay's performance, and stability testing indicated consistent results for at least 1 year.
Conclusions: The data presented in this report show that this a reliable method for detecting *Listeria* species and *monocytogenes*.
Highlights: This assay allows for one sample to be tested for both *Listeria* species and *monocytogenes* with one PCR test.

The Applied Food Diagnostics, Inc. (AFD) Simultaneous Multiplex Real Time PCR (SIMUL-qPCR) *Listeria* species and *monocytogenes* assay is a rapid and reliable method for detecting *Listeria* species and *monocytogenes* in environmental samples and food products. All SIMUL-qPCR System assays are designed to have the same instrument run time, allowing simultaneous identification of all SIMUL-qPCR System assays. In addition, each assay utilizes the power of multiplexing several targets

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during the same run. The SIMUL-qPCR *Listeria* species and *monocytogenes* Assay incorporates a multiplex approach to identifying both *Listeria* species and *monocytogenes* in the same run.

Principle of the Method

This protocol is a multifaceted approach to the detection of *Listeria* species in a variety of food products and environmental samples. Specifically formulated media are utilized for enriching samples, followed by cultural (detection plate) and rapid (quantitative real-time PCR) detection procedures. *Listeria* Recovery and Enrichment Broth (LREB) combines nutritional components with additional ingredients that are necessary to selectively improve the recovery and growth of *Listeria*. The selective agents present in LREB have been optimized to efficiently inhibit competing normal bacterial flora without affecting the growth of *Listeria* species. LREB is formulated for buffering capacity to ensure growth in a variety of matrices.

The sample is enriched at a specific temperature. Detection procedures can occur after a specified minimal enrichment time.

During PCR amplification, forward and reverse primers hybridize to unique sequences of *Listeria* species and *monocytogenes* 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. Two unique and specific primers and probe mixtures are present in this assay.

Scope of Method

(a) *Analyte(s)*.—*Listeria* species and *Listeria monocytogenes*.
(b) *Matrixes*.—Frankfurters (125 g), ready-to-eat (RTE) sliced turkey (125 g), soft fresh raw cheese (25 g), chicken salad (25 g), ice cream (25 g), cooked eggs (25 g), pasteurized milk (25 g), frozen/cooked shrimp (25 g), stainless steel (4 in. × 4 in., 1 in. × 1 in.), plastic (1 in. × 1 in.), rubber (1 in.× 1 in.), ceramic tile (1 in. × 1 in.), and sealed concrete (1 in. × 1 in.).
(c) *Summary of validated performance claims*.—Performance comparable to that of the US Department of Agriculture Food Safety and Inspection Service (FSIS) *Microbiology Laboratory Guidebook* (MLG), Section 8.10, Isolation and Identification of *Listeria monocytogenes* from Red Meat, Poultry and Egg Products, and Environmental Samples (1) for frankfurters, RTE sliced turkey, and cooked eggs, and the US Food and Drug Administration (FDA) *Bacteriological Analytical Manual* (BAM), Chapter 10 (2), for chicken salad, ice cream, pasteurized milk, frozen shrimp, and environmental surface sponges/swabs.

Definitions

(a) *Probability of detection (POD)*.—The proportion of positive analytical outcomes for a qualitative method for a given matrix at a given analyte level or concentration. POD is concentration dependent. Several POD measures can be calculated: POD_R (reference method POD), POD_C (confirmed candidate method POD), POD_{CP} (candidate method presumptive result POD), and POD_{CC} (candidate method confirmation result POD).
(b) *Difference of probabilities of detection (dPOD)*.—This is the difference between any two POD values. If the confidence interval of a dPOD does not contain zero, then the difference is statistically significant at the 5% level.

Materials and Methods

Test Kit Information

(a) *Kit name*.—Simultaneous Multiplex Real Time PCR (SIMUL-qPCR) *Listeria* species and *monocytogenes* Assay.
(b) *Cat. No*.—SMRT-LSLM-096.
(c) *Ordering information*.—Applied Food Diagnostics, Inc., 18 Industrial Drive, Bloomsburg, PA 17815Telephone: 570-450-7995.

Test Kit Components

(a) SIMUL-qPCR *Listeria* species and *monocytogenes* Assay Kit.
  (1) *Lysis buffer*.—Two 25 mL bottles.
  (2) *PCR tubes*.—Twelve strips of eight tubes/caps.

Additional supplies and reagents required but not provided:

(b) *AFD LREB*.—AFD proprietary media, DMR-LREB-1KG.
(c) *Sterile sampling bags*.—AFD LC-SSB3000-CTN or equivalent.
(d) *1.5 mL microcentrifuge tubes or equivalent PCR-grade plastics for cell lysis*.—AFD LC-MCTB015-CTN or equivalent.

Apparatus

If an item number is not listed, this apparatus is considered to be routinely found in a microbiology laboratory or can be supplied by multiple vendors.

(a) *MyGo Pro real-time PCR instrument and installed MyGo Pro software v3.4*.—Available from Azura Genomics Inc.
(b) *Autoclave*.
(c) *Incubator*.—30 ± 1°C.
(d) *Incubator*.—35 ± 1°C.
(e) *Heating blocks with inserts*.—SH1004 and SW1500 or equivalent. Range of 5°C to 130°C. Available from Southwest Science.
(f) *Vortex*.—SBV1000 or equivalent. Available from Southwest Science.
(g) *Calibrated thermometer*.—Range of −50°C to 70°C.
(h) *Adjustable mechanical pipettes*.—Dispensing volume of 2–20, 20–200 μL, and 100–1000 μL.
(i) *Multi-channel pipettes*.—Dispensing volume of 5–50 μL.
(j) *Microcentrifuge tube racks*.—1148C70 or equivalent. Available from Thomas Scientific.
(k) *Mini-centrifuge (optional)*.—SC1012 or equivalent. Available from Southwest Science.

Standard Reference Materials

Reference cultures are sourced from the following locations:

(a) American Type Culture Collection (ATCC), Manassas, VA.
(b) *Salmonella* Genetic Stock Centre (SGSC, University of Calgary, Canada.
(c) United States Department of Agriculture Eastern Regional Research Center (USDA ERRC), Wyndmoor, PA.
(d) BEI Resources, Manassas, VA.
(e) Michigan State University STEC Center, East Lansing, MI.

Safety Precautions

This product is for in vitro diagnostic use only. Do not ingest, inhale, or allow to come into contact with skin. Observe approved biohazard precautions and aseptic techniques. Biosafety level 2 procedures should be exercised (3). Extreme care should be taken in handling test samples and enrichment broths. All enrichment broths may contain various pathogens whether or not they contain *Listeria*. The kit is to be used only by adequately trained and qualified laboratory personnel in a laboratory setting. All laboratory specimens should be considered infectious and handled accordingly.

General Preparation

(a) Prepare all media and use all confirmation kits according to the manufacturer's directions.
(b) Prewarm prepared LREB to35 ± 1°C.
(c) Turn on the heating blocks to 95 ± 3°C as measured by a calibrated thermometer.
(d) Power on the qPCR instrument and create a run file from the SIMUL-qPCR template. The SIMUL-qPCR template contains the required cycle.

Sample Preparation

To prepare frankfurters and RTE sliced turkey for testing, aseptically sample 125 g and place it in a sterile bag. Add 1 L ± 50 mL of pre-warmed LREB to the sample. Hand mix by massaging each sample that is in the sealed bag for approximately 1 min to homogenize each sample. Incubate the samples at 30 ± 1°C for 30–36 h.

For soft fresh raw cheese, chicken salad, ice cream, cooked eggs, pasteurized milk, and frozen/cooked shrimp, aseptically sample 25 g and place it in a sterile bag. Add 225 ± 15 mL of prewarmed LREB to the sample. Hand mix by massaging or mechanically homogenize bystomaching each sample that is in the sealed bag for approximately 1 min to homogenize each sample. Incubate the samples at 30 ± 1°C for 30–36 h.

For environmental sponges, add 90 ± 10 mL of prewarmed LREB to the sponge. Hand mix by massaging each sample that is in the sealed bag for approximately 1 min. Incubate the samples at 30 ± 1°C for 30–36 h.

For environmental swabs, add 9 ± 1 mL of prewarmed LREB to the sponge. Hand mix by massaging each sample that is in the sealed bag for approximately 1 min. Incubate the samples at 30 ± 1°C for 30–36 h.

Lysis of Samples

After incubation, label one 1.5 mL microcentrifuge tube or equivalent PCR-grade plastic tube per sample using a waterproof marker, and aseptically pipette 400 µL of lysis buffer into each labeled tube. Return the lysis buffer to storage (2–8°C). Pipette 5 µL of the enrichment broth into the prepared tube. Cap the tube(s).

Heat the closed tubes for 10 min at 95 ± 3°C in the heat block. Remove the closed tubes from the heat block and allow the tubes to cool for 5 min at room temperature. If needed, lysate may be held in a refrigerator (2–8°C) for up to 48 h before proceeding to SIMUL-qPCR assay.

Analysis

(a) *PCR setup*.— The qPCR setup and data entry should be completed prior to transferring the samples. Refer to the AFD PCR User Guide and the MyGo Pro PCR Software manual (4) for detailed instructions. Select the AFD Template files to begin the run configuration. The AFD template file contains all of the PCR machine settings required to perform the run. Do not change any settings under the "Experiment," "Run Profile," or "Data" tab. Under the "Samples" tab, populate the sample fields according to the well placement/position. Include the kit lot number in the "Notes" field. Add the targets to the sample(s). After cell lysis and loading, click "Start Run".

(b) *Assay setup*.— Select the PCR tubes of the assay(s) for the desired testing being performed. Assays can be run individually or concurrently. Arrange strips of PCR tubes according to your run file. Remove the caps from the strip of tubes, and pipette 20 µL of lysate into the sample wells of the PCR test strip, ensuring the pellet is hydrated. PCR pellets must be hydrated and resealed within 10 min after removing the caps from the PCR tubes. Place a cap onto each tube and press down to seal each lid. Make sure each lid is tightly secured before running the tubes on the PCR machine. If air bubbles are present, carefully flick the reaction tubes until no air bubbles remain. Briefly spin down the reaction tubes in a mini-centrifuge. Load the qPCR instrument and start the run.

Calculations, Interpretation, and Test Result Report

Once the run is complete, results are analyzed automatically by the software. The software analyzes any DNA amplification data and will display a C_q value for any sample that amplifies. The C_q value refers to the PCR cycle number for which DNA amplification (specifically the fluorescence emitted when the DNA is amplified) is detected by the PCR software program. Only a C_q value that has a typical sigmoidal curve or the beginning of the curve is considered positive for the target. When a C_q value is not obtained, the result is negative for the target provided a C_q value is present in the CAL Fluor® Red 610 channel for the IAC.

Confirmation

All positive results are potential positives and confirmation is recommended. Enriched samples can be confirmed using the most current version of either the FSIS MLG method or the FDA BAM method using the enrichment broth, stored at 2–8°C.

Validation Study

This validation study was conducted under the AOAC Research Institute (RI) *Performance Tested Method*℠ program and the *AOAC INTERNATIONAL Methods Committee Guidelines for Validation of Microbiological Methods for Food and Environmental Surfaces* (5). Method developer studies were conducted in the laboratories of AFD, and included the inclusivity/exclusivity studies, matrix studies for all claimed matrixes, product consistency and stability studies, and robustness testing. The independent laboratory study for product testing was conducted by WBA Analytical Laboratories (Springdale, AR), and included a matrix study for frankfurters and fresh soft raw cheese. For environmental surfaces, the independent laboratory study was conducted by Q Laboratories (Cincinnati, OH), and included a matrix study for stainless steel.

Method Developer Studies

(a) *Inclusivity study*.—A total of 50 isolates of *L. monocytogenes* strains and 25 non-*L. monocytogenes Listeria* species strains were analyzed with the SIMUL-qPCR *Listeria* species and

monocytogenes Assay. The non-*L. monocytogenes Listeria* species tested were *L. grayi*, *L. innocua*, *L. ivanovii*, *L. seeligeri*, *L. welshimeri*, *L. aquatica*, *L. marthii*, and *L. riparia*. Each strain was cultured per the instructions outlined in the Assay kit insert. Strains were obtained from ATCC, USDA ERRC, and BEI Resources.

The testing was performed by removing isolates from slants stored in a −20°C freezer and placing a loopful of each isolate into LREB medium. Each strain was incubated at 30 ± 1°C for 30–36 h and then diluted with sterile LREB medium to approximately 100 times the LOD_{50} of the SIMUL-qPCR *Listeria* species and *monocytogenes* Assay (approximately 10^4 CFU/mL). Data from the inclusivity testing are detailed in Table 1.

(b) *Exclusivity testing*.—A total of 30 isolates of non-*Listeria* strains were tested with the SIMUL-qPCR *Listeria* species and *monocytogenes* Assay following the kit instructions. The strains were obtained from ATCC, BEI Resources, the *Salmonella* Genetic Stock Centre at the University of Calgary, Canada, Michigan State University STEC Center, and the USDA ERRC. Exclusivity testing was conducted by taking colonies grown on Tryptic Soy Agar (TSA) slants stored in a 2–4°C refrigerator and growing the strains in brain heart infusion (BHI) broth incubated at 37 ± 2°C for 20–24 h. All strains were tested undiluted after incubation using the SIMUL-qPCR *Listeria* species and *monocytogenes* Assay. Data from the exclusivity testing are detailed in Table 2.

(c) *Matrix study*.—All the matrixes were obtained from either local grocery stores or retail warehouses and were prescreened for *Listeria* species and *L. monocytogenes* using the Hygiena BAX *Listeria* species kit and Hygiena BAX *Listeria monocytogenes* kit prior to testing to determine if any natural contamination was present.

For frankfurters (beef and pork blend), RTE sliced turkey (presliced store-bought deli turkey), and cooked eggs, a 25 g portion was taken and enriched in 225 ± 5 mL of University of Vermont (UVM) Medium and stomached for 2 min. Each sample was incubated at 30 ± 2°C for 20–26 h. After incubation, 0.1 ± 0.02 mL of the enrichment was transferred into 10 ± 0.5 mL of supplemented Fraser broth. Inoculated Fraser broth tubes were incubated at 35 ± 2°C for 24–28 h. Tubes were examined after incubation for darkening. If no darkening occurred, tubes were reincubated for a total incubation time of 46–50 h. Any tubes that exhibited a darkening in color were then streaked to Modified Oxford Agar (MOX) plates. Plates were incubated for 24–28 h at 35 ± 2°C. UVM enrichments were also streaked onto MOX plates and incubated at 35 ± 2°C for 24–28 h. Colonies were streaked onto Sheep Blood Agar (SBA) plates for purity before being confirmed using *Listeria* API strips. A 25 g sample for each matrix was also plated for total plate count. Results are reported in Table 3.

For fresh soft raw cheese (ricotta), chicken salad, ice cream, pasteurized milk, and frozen shrimp, a 25 g portion of each was added to a sterile container. A volume of 225 mL of Buffered *Listeria* Enrichment Broth (BLEB) containing pyruvate was added to the container and thoroughly blended. For environmental sponges, 10 mL of Dey-Engley(D/E)broth was added to the sponges before swabbing the environment. Environmental surfaces were swabbed by swabbing vertically 10 times, flipping the swab and then swabbing horizontally 10 times, and diagonally 10 times, applying even pressure. Ninety milliliters of BLEB with pyruvate was added to the sponges. Samples were incubated at 30°C for 4 h. After the initial incubation, three filter-sterilized selective agents were aseptically added to the BLEB to achieve the final concentrations of 10 mg/L acriflavin, 40 mg/L cyclohexamide, and 50 mg/L sodium nalidixic acid. After mixing, the samples were put back in the 30°C incubator and incubated for 24–48 h. After 24 h and 48 h, BLEB enrichments were streaked to MOX plates and ALOA plates. Plates were incubated at 35°C for 48 h. Plates were examined after 24 h. MOX plates were streaked for purity onto Tryptone Soya Yeast Extract Agar plates and incubated at 30°C for 24–48 h. Remaining colony growth was stabbed into 5% sheep blood agar plate and incubated at 35°C for 24–48 h. Colonies were confirmed using *Listeria* API strips. A 25 g portion from each food matrix was also tested for total plate count.

For all matrixes, an unpaired study was conducted due to the difference in enrichment media used between the candidate and reference methods. For frankfurters, RTE sliced turkey, and cooked eggs, samples were processed using either the SIMUL-qPCR *Listeria* species and *monocytogenes* Assay or the USDA FSIS MLG Section 8.10 method. For soft fresh raw cheese, chicken salad, ice cream, pasteurized milk, frozen/cooked shrimp, and environmental surfaces, all samples were processed using either the SIMUL-qPCR *Listeria* species and *monocytogenes* Assay or the FDA BAM Chapter 10 method. All enrichments, including samples that were not inoculated with *Listeria*, were processed for cultural confirmation regardless of the results obtained by the SIMUL-qPCR method. Colonies were confirmed using *Listeria* API strips.

To prepare the materials for testing, each product for each food matrix was aseptically combined into a large sterile sampling bag and hand massaged to thoroughly mix the product together. The product was spiked using strains sub-cultured in BHI at 37 ± 2°C for 20–24 h. Frankfurters were inoculated with *L. monocytogenes* 1/2b (USDA ERRC B-33258). RTE sliced turkey was spiked with *L. ivanovii* (USDA ERRC B-33017), and cooked eggs were spiked with *L. seeligeri* (USDA ERRC B-33019). Soft fresh raw cheese was inoculated with *L. monocytogenes* 4b (USDA ERRC B-33000), the chicken salad was spiked with *L. monocytogenes* 4e (USDA ERRC B-33120), and the ice cream was spiked with *L. welshimeri* (USDA ERRC B-33194). The pasteurized milk samples were spiked with *L. monocytogenes* 4c (BEI NR-111), and the frozen/cooked shrimp samples were spiked with *L. monocytogenes* 1/2a (BEI NR-13229).

Each matrix was artificially contaminated with the indicated strains at two contamination levels: a low level to achieve a fractional response of 5–15 positive test portions out of 20 replicate portions tested (approximately 0.2–2 CFU/25 g), and a high level to achieve five positive test portions out of five replicate portions tested (approximately 5–10 CFU/25 g). Samples were mixed well to create homogenous mixtures.

For frankfurters, RTE sliced turkey, cooked eggs, ice cream, pasteurized milk, and frozen/cooked shrimp, the strains were heat-stressed before spiking. The liquid culture was heated to 55°C for 10–20 min to achieve 50–80% injury. Heat-stressed strains were plated onto LM agar and Standard Methods Agar (SMA) to determine the percent injury.

For frankfurters and RTE sliced turkey, 25 g portions of each contamination level (including the uncontaminated level) were weighed into a sterile sampling bag. For the candidate method, an additional 100 g of non-inoculated product was added to bring the weight of each portion to 125 g. The portions were held at 2–8°C for 48–72 h to allow for stabilization of the microorganisms in the food environment.

For cooked eggs, soft fresh raw cheese, chicken salad, ice cream, pasteurized milk, and frozen/cooked shrimp, 25 g portions of each contamination level (including the uncontaminated level) were weighed into a sterile bag for both the candidate and reference method. The portions of cooked eggs,

Table 1. Inclusivity list—*Listeria* strains

No.	Genus	Species	Serotype	Source	Origin	SIMUL-qPCR Result
1	*Listeria*	*grayi*		ATCC[a] 25401	Standing corn stalks and leaves	+
2	*Listeria*	*welshimeri*		USDA[b] ERRC B-33266	Florida, USA	+
3	*Listeria*	*grayi*		ATCC 19120	Animal feces	+
4	*Listeria*	*grayi*		USDA ERRC B-33214	Wheat-processing plant	+
5	*Listeria*	*seeligeri*		USDA ERRC B-57212	Unknown	+
6	*Listeria*	*innocua*		USDA ERRC B-33314	Unknown	+
7	*Listeria*	*ivanovii* subsp. *Ivanovii*		USDA ERRC B-33165	Bovine	+
8	*Listeria*	*marthii*		BEI[c] NR-9581	Run-off water	+
9	*Listeria*	*marthii*		BEI NR-9582	Stream water	+
10	*Listeria*	*marthii*		BEI NR-9579	Soil	+
11	*Listeria*	*marthii*		BEI NR-9580	Standing water puddle	+
12	*Listeria*	*seeligeri*		USDA ERRC B-33019	Soil	+
13	*Listeria*	*ivanovii*		USDA ERRC B-33017	Sheep	+
14	*Listeria*	*welshimeri*		USDA ERRC B-33020	Decaying vegetation	+
15	*Listeria*	*innocua*		USDA ERRC B-33003	California, USA	+
16	*Listeria*	*innocua*		ATCC 33091	Human feces	+
17	*Listeria*	*ivanovii* subsp. *londoniensis*		ATCC BAA-139	Washing water	+
18	*Listeria*	*welshimeri*		ATCC 35897	Decaying plant material	+
19	*Listeria*	*innocua*	6a	ATCC 33090	Cow brain	+
20	*Listeria*	*grayi*		USDA ERRC B-33023	Chinchilla feces	+
21	*Listeria*	*seeligeri*		ATCC 35967	Soil	+
22	*Listeria*	*aquatica*		USDA ERRC B-57629	Running water	+
23	*Listeria*	*riparia*		USDA ERRC B-57632	Running water	+
24	*Listeria*	*welshimeri*		USDA ERRC B-33194	Wheat-processing plant	+
25	*Listeria*	*grayi*		ATCC 700545	Unknown	+
26	*Listeria*	*monocytogenes*	4b	USDA ERRC B-33000	Cheese	+
27	*Listeria*	*monocytogenes*	1/2b complex	USDA ERRC B-33045	Turkey, pork, beef hot dogs	+
28	*Listeria*	*monocytogenes*	1/2b	USDA ERRC B-33258	Smoked boneless ham	+
29	*Listeria*	*monocytogenes*	1/2b	USDA ERRC B-33272	Environmental isolates	+
30	*Listeria*	*monocytogenes*	1/2b	USDA ERRC B-33273	Environmental isolates	+
31	*Listeria*	*monocytogenes*	1/2b	USDA ERRC B-33254	Roast beef	+
32	*Listeria*	*monocytogenes*	1/2b	USDA ERRC B-33046	Chicken	+
33	*Listeria*	*monocytogenes*	1/2b	USDA ERRC B-33073	Bovine	+
34	*Listeria*	*monocytogenes*	1/2a	USDA ERRC B-33106	Raw milk	+
35	*Listeria*	*monocytogenes*	4d	USDA ERRC B-33116	Sheep	+
36	*Listeria*	*monocytogenes*	4e	USDA ERRC B-33120	Chicken	+
37	*Listeria*	*monocytogenes*	1/2b	USDA ERRC B-33130	Bovine milk	+
38	*Listeria*	*monocytogenes*	1/2b	USDA ERRC B-33162	Bovine	+
39	*Listeria*	*monocytogenes*	1/2b complex	BEI NR-108	Human	+
40	*Listeria*	*monocytogenes*	3a	BEI NR-110	Human cerebrospinal fluid	+
41	*Listeria*	*monocytogenes*	4b	BEI NR-111	Chicken	+
42	*Listeria*	*monocytogenes*	4c	BEI NR-112	Sheep	+
43	*Listeria*	*monocytogenes*	4d	BEI NR-113	Chicken	+
44	*Listeria*	*monocytogenes*	1/2a	BEI NR-13233	Soil	+
45	*Listeria*	*monocytogenes*	1/2a	BEI NR-13229	Human	+
46	*Listeria*	*monocytogenes*	1/2b	BEI NR-13237	Bovine abortion	+
47	*Listeria*	*monocytogenes*	4c	BEI NR-13232	Bovine	+
48	*Listeria*	*monocytogenes*	4b	BEI NR-13231	Trout	+
49	*Listeria*	*monocytogenes*	1/2b	BEI NR-13230	Human	+
50	*Listeria*	*monocytogenes*		BEI NR-4098	Human meningitis	+
51	*Listeria*	*monocytogenes*	4a	BEI NR-109	Ruminant tissue	+
52	*Listeria*	*monocytogenes*		USDA ERRC B-33259	Chicken	+
53	*Listeria*	*monocytogenes*		USDA ERRC B-33260	Beef sausage links	+
54	*Listeria*	*monocytogenes*		USDA ERRC B-33261	Beef jerky	+
55	*Listeria*	*monocytogenes*		USDA ERRC B-33264	Sliced cooked beef	+
56	*Listeria*	*monocytogenes*		USDA ERRC B-33274	Florida, USA	+
57	*Listeria*	*monocytogenes*		USDA ERRC B-33276	Chicken	+
58	*Listeria*	*monocytogenes*	1/2a	USDA ERRC B-33814	Clinical isolate	+

(continued)

Table 1. (continued)

No.	Genus	Species	Serotype	Source	Origin	SIMUL-qPCR Result
59	*Listeria*	*monocytogenes*	3a	USDA ERRC B-33225	Unknown	+
60	*Listeria*	*monocytogenes*		USDA ERRC B-33282	Duck breast	+
61	*Listeria*	*monocytogenes*	3c	USDA ERRC B-33226	Unknown	+
62	*Listeria*	*monocytogenes*		USDA ERRC B-33238	Beef jerky	+
63	*Listeria*	*monocytogenes*	1/2b complex	USDA ERRC B-33239	Beef/pork franks	+
64	*Listeria*	*monocytogenes*	1/2b complex	USDA ERRC B-33240	Beef/pork franks	+
65	*Listeria*	*monocytogenes*		USDA ERRC B-33241	Cooked apple sausage	+
66	*Listeria*	*monocytogenes*	1/2b complex	USDA ERRC B-33242	Roast beef	+
67	*Listeria*	*monocytogenes*		USDA ERRC B-33243	Cooked beef	+
68	*Listeria*	*monocytogenes*	1/2b complex	USDA ERRC B-33245	Environmental isolates	+
69	*Listeria*	*monocytogenes*		USDA ERRC B-33246	White chicken salad	+
70	*Listeria*	*monocytogenes*		USDA ERRC B-33247	Roast beef	+
71	*Listeria*	*monocytogenes*	1/2b complex	USDA ERRC B-33248	BBQ Chicken	+
72	*Listeria*	*monocytogenes*	1/2b complex	USDA ERRC B-33250	Boneless smoked ham steak	+
73	*Listeria*	*monocytogenes*		USDA ERRC B-33253	Cooked ham	+
74	*Listeria*	*monocytogenes*		BEI HM-1048	Human	+
75	*Listeria*	*monocytogenes*	4c	USDA ERRC B-33115	Arabian oryx	+

[a] American Type Culture Collection, Manassas, VA.
[b] American Genetic Stock Centre, University of Calgary, Canada.
[c] BEI Resources, Manassas, VA.

soft fresh raw cheese, chicken salad, and pasteurized milk were held at 2–8°C for 48–72 h, while the ice cream and frozen cooked shrimp were held at −20°C for 2 weeks to allow for stabilization of the microorganisms in the food environment.

Stainless steel was inoculated with *L. monocytogenes* 4a (BEI NR-109), ceramic with *L. ivanovii* (USDA ERRC B-33165), plastic with *L. innocua* (USDA ERRC B-33003), concrete with *L. marthii* (BEI NR-9580), and rubber with *L. grayi* (USDA ERRC B-33214). Strains were grown in BHI at 34–36°C for 18–24 h and then diluted in 0.1% peptone water to two contamination levels: a low level to achieve a fractional response of 5–15 positive test portions out of 20 replicate portions tested, and a high level to achieve five positive test portions out of five replicate portions tested. Stainless steel was co-inoculated with *Enterococcus faecalis* (BEI NR-31884), grown in BHI at 34–36°C for 18–24 h and then diluted in 0.1% peptone water to a level approximately 10 times the concentrations of *Listeria*. Each surface was inoculated with approximately 0.25 mL for 4 in. × 4 in. and 0.1 mL for 1 in. × 1 in. Surfaces were dried at room temperature for 16–24 h. Surfaces were checked to make sure the surfaces were visibly dry before sampling, and both candidate and reference method swabbing occurred on the same day.

For all food matrixes, most probable number (MPN) analyses were conducted on the high and low contamination levels. Test portions from the reference method analysis plus additional portions from each contamination level were used to create a minimum five-replicate, three-level MPN. For frankfurters, RTE sliced turkey, and cooked eggs, the FSIS MLG 8.10 reference method was used. For soft fresh raw cheese, chicken salad, ice cream, pasteurized milk, and frozen/cooked shrimp, the FDA BAM Chapter 10 reference method was used.

All MPN results were calculated using the Least Cost Formulations MPN calculator program (6). The matrix study results are detailed in Tables 4 and 5.

(d) *Real-time stability testing.*—Three lots of SIMUL-qPCR *Listeria* species and *monocytogenes* Assay (lot numbers 09118, 27418, and 09119) were examined for lot-to-lot variability and product stability over the 12 month shelf-life period. The study compared lots near expiration, lots at the middle of the shelf life, and lots that were recently manufactured.

A pure culture of *L. monocytogenes* 1/2b (USDA ERRC B-33258) was grown in LREB medium at 30 ± 1°C for 30–36 h. The culture was diluted in LREB to a level to yield fractional results (2–8 positive results out of 10 replicate portions tested). A pure culture of *E. faecalis* (BEI NR-31884) was also grown in BHI at 37°C for 20–24 h. This culture was not diluted for the test. Each lot of SIMUL-qPCR *Listeria* assays were tested with 10 replicates of diluted *L. monocytogenes* and 10 replicates of undiluted *E. faecalis*. The samples were blind-coded and randomized before being tested.

Test results were analyzed by POD statistical analysis to 95% confidence intervals to determine any variance between lots and time points. Results are shown in Tables 6 and 7.

(d) *Robustness study.*—A robustness study was conducted on frankfurters to evaluate the ability of the method to remain unaffected by small variations in method parameters that might occur if the method was performed by an end user. Upper and lower limits of three method parameters were evaluated using a factorial design; enrichment time (24 and 48 h), volume of extraction reagent 380 and 420 µL), and volume of extracted DNA sample (15 and 25 µL). The nominal test conditions for the assay are 30 h enrichment time, 400 µL extraction reagent, and 20 µL of extracted sample.

Frankfurters were inoculated with *L. monocytogenes* 1/2b (USDA ERRC B-33258) at a level to yield fractional results (2–8 positive results out of 10 replicate portions tested). Ten replicate 125 g portions of inoculated frankfurters and 10 replicate 125 g portions of non-inoculated frankfurters were inoculated with LREB at 30 ± 1°C. After enrichment, aliquots from each enriched test portion were taken at each time point and analyzed using the SIMUL-qPCR *Listeria* species and *monocytogenes* Assay. POD values and 95% confidence intervals were calculated, and data were analyzed for any variance that occurred due to changes in the parameter settings. Results are presented in Table 8.

(e) *Independent laboratory study.*—WBA Analytical Laboratories performed the independent laboratory study for the food product

Table 2. Exclusivity list—non-*Listeria* strains

No.	Genus	Species	Source	Origin	SIMUL-qPCR result
1	*Alcaligenes*	*faecalis* subsp. *faecalis*	USDA ERRC[a] B-170	USDA, Beltsville, MD	–
2	*Citrobacter*	*koseri*	SGSC[b] 5610	Unknown	–
3	*Bacillus*	*subtilus*	BEI[c] NR-607	Unknown	–
4	*Bacillus*	*cereus*	BEI NR-608	Laboratory isolate	–
5	*Citrobacter*	*freundii*	ATCC[d] 43864	Unknown	–
6	*Cronobacter*	*sakazakii*	ATCC BAA-894	Human clinical specimen	–
7	*Klebsiella*	*ozaenae*	SGSC 2810	Unknown	–
8	*Escherichia*	*fergusonii*	SGSC 5718	Human feces	–
9	*Escherichia*	*coli* O75: K95: H5	BEI NR-17715	Human	–
10	*Enterobacter*	*taylorae*	SGSC 5283	Unknown	–
11	*Providencia*	*stuartii*	SGSC 5639	Unknown	–
12	*Ewingella*	*americana*	SGSC 5640	Human feces	–
13	*Hafnia*	*alvei*	SGSC 5583	Unknown	–
14	*Klebsiella*	*oxytoca*	SGSC 5366	Unknown	–
15	*Klebsiella*	*pneumoniae* subsp. *pneumoniae*	SGSC 5926	Unknown	–
16	*Lactobacillus*	*lactis*	ATCC 19257	Unknown	–
17	*Serratia*	*marcescens*	SGSC 5354	Unknown	–
18	*Serratia*	*odorifera*	SGSC 5576	Unknown	–
19	*Shigella*	*sonnei*	SGSC 5576	Unknown	–
20	*Shigella*	*flexneri*	SGSC 5577	Unknown	–
21	*Staphylococcus*	*aureus*	ATCC 29213	Wound	–
22	*Pseudomonas*	*aeruginosa*	BEI NR-48982	Human	–
23	*Yersinia*	*enterocolitica*	USDA ERRC B-41479	Ground beef	–
24	*Morganella*	*morganii*	SGSC 5435	Unknown	–
25	*Proteus*	*mirabilis*	SGSC 5445	Unknown	–
26	*Escherichia*	*coli* O157: H7	ATCC 43888	Human feces	–
27	*Escherichia*	*coli* O121	MSU[e] TW08004	Human	–
28	*Escherichia*	*coli* O111	MSU TW05150	Cow	–
29	*Salmonella*	*enterica* subsp. *enterica* Enteritidis	SGSC 2475	Unknown, Connecticut	–
30	*Salmonella*	*enterica* subsp. *enterica* Typhimurium	SGSC 2522	Human, Mexico	–

[a] United States Department of Agriculture Eastern Regional Research Center, Windsor, PA.
[b] *Salmonella* Genetic Stock Centre, University of Calgary, Canada.
[c] BEI Resources, Manassas, VA.
[d] American Type Culture Collection, Manassas, VA.
[e] Michigan State University STEC Center, East Lansing, MI.

Table 3. Aerobic plate count results of food matrixes

No.	Food	Aerobic plate count
1	Frankfurters	20 CFU/g
2	RTE sliced turkey	10 CFU/g
3	Cooked eggs	<10 CFU/g
4	Fresh raw soft cheese	10 CFU/g
5	Frozen/cooked shrimp	<10 CFU/g
6	Chicken salad	540 CFU/g
7	Ice cream	20 CFU/g
8	Pasteurized milk	50 CFU/g
9	Frankfurters—independent laboratory	20 CFU/g
10	Fresh raw soft cheese—independent laboratory	60 CFU/g

validation. Q Laboratories performed the independent laboratory study for the surface validation. Matrix studies were conducted on frankfurters, fresh raw soft cheese, and stainless steel environmental surfaces. The SIMUL-qPCR *Listeria* method was compared to the USDA FSIS MLG method for detection of *Listeria* in frankfurters, and to the FDA BAM method for the detection of *Listeria* in fresh raw soft cheese and on stainless steel surfaces. Frankfurters were inoculated with *L. monocytogenes* 1/2b (ATCC BAA839). Soft raw fresh cheese was inoculated with *L. monocytogenes* 4b (ATCC 19115). Stainless steel surfaces were co-inoculated with *L. monocytogenes* 4a (ATCC 19114) and *E. faecalis* (ATCC 29212). Each matrix was inoculated with the indicated organism to achieve two contamination levels: a fractional level (5–15 positive results out of 20 replicate portions tested), and a high level (approximately five positive results out of five replicate portions tested).

Frankfurters were obtained from a fully cooked processing facility and combined into 1 lot. Using aseptic technique, 100 g portions were weighed into sterile bags and kept stored in the freezer until samples were ready for inoculation. Two bulk-sized lots were weighed into sterile bags to be inoculated at a fractional level and a high level with *L. monocytogenes* 1/2b (heat-stressed). Once inoculation occurred and the portion was homogenized by hand-mixing, it was held at 2–8°C for 48 h to stabilize before samples were prepared and testing was initiated. Uninoculated frankfurters were screened for *L. monocytogenes* using the Hygiena BAX Real Time *Listeria monocytogenes*

Table 4. SIMUL-qPCR *Listeria* presumptiveversus confirmed results

				SIMUL-qPCR *Listeria* species and *monocytogenes* presumptive			SIMUL-qPCR *Listeria* species and *monocytogenes* confirmed				
Matrix	Strain	MPN[a]/test portion	n[b]	x[c]	POD_{CP}[d]	95% CI	x	POD_{CC}[e]	95% CI	$dPOD_{CP}$[f]	95% CI[g]
Frankfurters	*L. monocytogenes* 1/2b (USDA ERRC	N/A[h]	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	–0.43, 0.43
125 g	B-33258)	0.91 (0.54, 1.50)	20	9	0.45	0.26, 0.66	9	0.45	0.26, 0.66	0.00	0.28, 0.28
		5.01 (2.46, 10.2)	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	–0.43, 0.43
Frankfurters[j]	*L. monocytogenes* 1/2b (ATCC BAA839)	N/A	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	–0.43, 0.43
125 g		0.25 (0.10, 0.48)	20	11	0.55	0.34, 0.74	13	0.65	0.43, 0.82	–0.10	–0.37, 0.19
		1.13 (0.49, 2.59)	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	–0.43, 0.43
RTE sliced turkey	*L. ivanovii* (USDA ERRC B-33017)	N/A	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	–0.43, 0.43
125 g		1.19 (0.77, 2.08)	20	10	0.50	0.30, 0.70	8	0.40	0.22, 0.61	0.10	–0.19, 0.37
		4.79 (2.51, 9.17)	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	–0.43, 0.43
Cooked eggs	*L. seeligeri* (USDA ERRC B-33019)	N/A	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	–0.43, 0.43
25 g		0.72 (0.47, 1.07)	20	15	0.75	0.53, 0.89	15	0.75	0.53, 0.89	0.00	–0.26, 0.26
		9.37 (5.07, 1.00E + 12)[i]	5	4	0.80	0.38, 1.00	4	0.80	0.38, 1.00	0.00	–0.47, 0.47
Fresh raw soft cheese	*L. monocytogenes* 4b (USDA ERRC B-33000)	N/A	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	–0.43, 0.43
25 g		0.57 (0.31, 0.96)	20	10	0.50	0.30, 0.70	10	0.50	0.30, 0.70	0.00	–0.28, 0.28
		9.37 (5.07, 1.00E + 12)[i]	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	–0.43, 0.43
Fresh raw soft cheese[j]	*L. monocytogenes* 4b (ATCC 19115)	N/A	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	–0.43, 0.43
25 g		1.21 (0.79, 1.93)	20	13	0.65	0.43, 0.82	14	0.70	0.48, 0.85	–0.05	–0.32, 0.23
		4.92 (2.27, 10.7)	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	–0.43, 0.43
Frozen/cooked shrimp	*L. monocytogenes* 1/2a (BEI NR-13229)	N/A	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	–0.43, 0.43
25 g		1.27 (0.81, 2.08)	20	16	0.80	0.58, 0.92	17	0.85	0.64, 0.95	–0.05	–0.29, 0.19
		6.45 (2.88, 14.5)	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	–0.43, 0.43
Chicken salad 25 g	*L. monocytogenes* 4e (USDA ERRC B-33120)	N/A	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	–0.43, 0.43
		0.78 (0.47, 1.23)	20	5	0.25	0.11, 0.47	4	0.20	0.08, 0.42	0.05	–0.21, 0.30
		4.79 (2.51, 9.17)	5	4	0.80	0.38, 1.00	4	0.80	0.38, 1.00	0.00	–0.47, 0.47
Ice cream	*L. welshimeri* (USDA ERRC B-33194)	N/A	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	–0.43, 0.43
25 g		0.56 (0.30, 0.96)	20	7	0.35	0.18, 0.57	7	0.35	0.18, 0.57	0.00	–0.28, 0.28
		9.37 (5.07, 1.00E + 12)[i]	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	–0.43, 0.43
Pasteurized milk	*L. monocytogenes* 4c (BEI NR-111)	N/A	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	–0.43, 0.43
25 g		0.86 (0.51, 1.40)	20	16	0.80	0.58, 0.92	16	0.80	0.58, 0.92	0.00	–0.25, 0.25
		3.63 (1.74, 7.57)	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	–0.43, 0.43
Stainless steel	*L. monocytogenes* 4a (BEI NR-109)/10×	N/A	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	–0.43, 0.43
1 in. × 1 in.	*E. faecalis* (NR-31884)	N/A	20	15	0.75	0.53, 0.89	15	0.75	0.53, 0.89	0.00	–0.26, 0.26
AFD swab		N/A	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	–0.43, 0.43
Stainless steel	*L. monocytogenes* 4a (BEI NR-109)/10×	N/A	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	–0.43, 0.43
4 in. × 4 in.	*E. faecalis* (NR-31884)	N/A	20	12	0.60	0.39, 0.78	15	0.75	0.53, 0.89	–0.15	–0.40, 0.13
Sponge		N/A	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	–0.43, 0.43
Stainless steel[j]	*L. monocytogenes* 4a (ATCC 19114)/10×	N/A	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	–0.43, 0.43
4 in. × 4 in.	*E. faecalis* (ATCC 29212)	N/A	20	7	0.35	0.18, 0.57	7	0.35	0.18, 0.57	0.00	–0.13, 0.13
sponge		N/A	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	–0.43, 0.43

(continued)

Table 4. (continued)

Matrix	Strain	MPN[a]/test portion	n[b]	SIMUL-qPCR *Listeria* species and *monocytogenes* presumptive			SIMUL-qPCR *Listeria* species and *monocytogenes* confirmed				
				x[c]	POD_{CP}[d]	95% CI	x	POD_{CC}[e]	95% CI	$dPOD_{CP}$[f]	95% CI[g]
Plastic	*L. innocua* (USDA ERRC B-33003)	N/A	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	–0.43, 0.43
1 in. × 1 in.		N/A	20	11	0.55	0.34, 0.74	9	0.45	0.26, 0.66	0.10	–0.19, 0.43
AFD swab		N/A	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	–0.43, 0.43
Rubber	*L. grayi* (USDA ERRC B-33214)	N/A	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	–0.43, 0.43
1 in. × 1 in.		N/A	20	12	0.60	0.39, 0.78	12	0.60	0.39, 0.78	0.00	–0.28, 0.28
AFD swab		N/A	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	–0.43, 0.43
Ceramic tile	*L. ivanovii* (USDA ERRC B-33165)	N/A	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	–0.43, 0.43
1 in. × 1 in.		N/A	20	9	0.45	0.26, 0.66	9	0.45	0.26, 0.66	0.00	–0.28, 0.28
AFD swab		N/A	5	4	0.80	0.38, 1.00	4	0.80	0.38, 1.00	0.00	–0.47, 0.47
Sealed concrete	*L. marthii* (BEI NR-9580)	N/A	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	–0.43, 0.43
1 in. × 1 in.		N/A	20	6	0.30	0.15, 0.52	8	0.40	0.22, 0.61	–0.10	–0.36, 0.18
AFD swab		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 portions.
[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] NA =Not applicable.
[i] All test portions in the MPN were positive, leading to the high value.
[j] Matrix tested by the independent laboratory.

Table 5. SIMUL-qPCR *Listeria* species and *monocytogenes* assay versus MLG 4.09 and BAM Ch. 5

				SIMUL-qPCR *Listeria* species and *monocytogenes* results			Reference method results[e]				
Matrix	Strain	MPN[a]/test portion	n[b]	x[c]	POD_C[d]	95% CI	x	POD_R[f]	95% CI	$dPOD_C$[g]	95% CI[h]
Frankfurters	*L. monocytogenes* 1/2b (USDA ERRC	N/A[h]	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	–0.43, 0.43
125 g	B-33258)	0.91 (0.54, 1.50)	20	9	0.45	0.26, 0.66	13	0.65	0.43, 0.82	–0.20	–0.46, 0.10
		5.01 (2.46, 10.2)	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	–0.43, 0.43
Frankfurters[j]	*L. monocytogenes* 1/2b (ATCC BAA839)	N/A	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	–0.43, 0.43
125 g		0.25 (0.10, 0.48)	20	11	0.55	0.34, 0.74	6	0.30	0.15, 0.52	0.25	–0.05, 0.50
		1.13 (0.49, 2.59)	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	–0.43, 0.43
RTE sliced turkey	*L. ivanovii* (USDA ERRC B-33017)	N/A	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	–0.43, 0.43
125 g		1.19 (0.77, 2.08)	20	8	0.40	0.22, 0.61	12	0.60	0.39, 0.78	–0.20	–0.46, 0.10
		4.79 (2.51, 9.17)	5	5	1.00	0.57, 1.00	4	0.80	0.38, 1.00	0.20	–0.28, 0.62
Cooked eggs	*L.seeligeri* (USDA ERRC B-33019)	N/A	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	–0.43, 0.43
25 g		0.72 (0.47, 1.07)	20	15	0.75	0.53, 0.89	15	0.75	0.53, 0.89	0.00	–0.26, 0.26
		9.37 (5.07, 1.00E + 12)[i]	5	4	0.80	0.38, 1.00	5	1.00	0.57, 1.00	–0.20	–0.62, 0.28
Fresh raw soft cheese	*L. monocytogenes* 4b (USDA ERRC B-33000)	N/A	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	–0.43, 0.43
25 g		0.57 (0.31, 0.96)	20	10	0.50	0.30, 0.70	8	0.40	0.22, 0.61	0.10	–0.19, 0.37
		9.37 (5.07, 1.00E + 12)[i]	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	–0.43, 0.43
Fresh raw soft cheese[j]	*L. monocytogenes* 4b (ATCC 19115)	N/A	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	–0.43, 0.43
25 g		1.21 (0.79, 1.93)	20	13	0.65	0.43, 0.82	14	0.70	0.48, 0.85	–0.05	–0.32, 0.23
		4.92 (2.27, 10.7)	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	–0.43, 0.43
Frozen/cooked shrimp	*L. monocytogenes* 1/2a (BEI NR-13229)	N/A	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	–0.43, 0.43
25 g		1.27 (0.81, 2.08)	20	16	0.80	0.58, 0.92	13	0.65	0.43, 0.82	0.15	–0.12, 0.40
		6.45 (2.88, 14.5)	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	–0.43, 0.43
Chicken salad 25 g	*L. monocytogenes* 4e (USDA ERRC B-33120)	N/A	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	–0.43, 0.43
		0.78 (0.47, 1.23)	20	4	0.20	0.08, 0.42	7	0.35	0.18, 0.57	–0.15	–0.40, 0.12
		4.79 (2.51, 9.17)	5	4	0.80	0.38, 1.00	4	0.80	0.38, 1.00	0.00	–0.47, 0.47
Ice cream	*L. welshimeri* (USDA ERRC B-33194)	N/A	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	–0.43, 0.43
25 g		0.56 (0.30, 0.96)	20	7	0.35	0.18, 0.57	7	0.35	0.18, 0.57	0.00	–0.28, 0.28
		9.37 (5.07, 1.00E + 12)[i]	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	–0.43, 0.43
Pasteurized milk	*L. monocytogenes* 4c (BEI NR-111)	N/A	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	–0.43, 0.43
25 g		0.86 (0.51, 1.40)	20	16	0.80	0.58, 0.92	12	0.60	0.39, 0.78	0.20	–0.08, 0.44
		3.63 (1.74, 7.57)	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	–0.43, 0.43
Stainless steel	*L. monocytogenes* 4a (BEI NR-109)/10×	N/A	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	–0.43, 0.43
1 in. × 1 in.	*E. faecalis* (NR-31884)	N/A	20	15	0.75	0.53, 0.89	7	0.35	0.18, 0.57	0.40	0.09, 0.62
AFD swab		N/A	5	5	1.00	0.57, 1.00	4	0.80	0.38, 1.00	0.20	–0.28, 0.62
Stainless steel	*L. monocytogenes* 4a (BEI NR-109)/10×	N/A	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	–0.43, 0.43
4 in. × 4 in.	*E. faecalis* (NR-31884)	N/A	20	12	0.60	0.39, 0.78	7	0.35	0.18, 0.57	0.25	–0.05, 0.50
Sponge		N/A	5	5	1.00	0.57, 1.00	4	0.80	0.38, 1.00	0.20	–0.28, 0.62
Stainless steel[j]	*L. monocytogenes* 4a (ATCC 19114)/10×	N/A	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	–0.43, 0.43
4 in. × 4 in.	*E. faecalis* (ATCC 29212)	N/A	20	7	0.35	0.18, 0.57	6	0.30	0.15, 0.52	0.10	–0.23, 0.32
Sponge		N/A	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	–0.43, 0.43

(continued)

Table 5. (continued)

Matrix	Strain	MPN[a]/test portion	n[b]	SIMUL-qPCR *Listeria* species and *monocytogenes* results			Reference method results[e]				
				x[c]	POD_C[d]	95% CI	x	POD_R[f]	95% CI	$dPOD_C$[g]	95% CI[h]
Plastic	*L. innocua* (USDA ERRC B-33003)	N/A	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	–0.43, 0.43
1 in. × 1 in.		N/A	20	9	0.45	0.26, 0.66	7	0.35	0.18, 0.57	0.10	–0.19, 0.43
AFD swab		N/A	5	5	1.00	0.57, 1.00	4	0.80	0.38, 1.00	0.20	–0.28, 0.62
Rubber	*L. grayi* (USDA ERRC B-33214)	N/A	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	–0.43, 0.43
1 in. × 1 in.		N/A	20	12	0.60	0.39, 0.78	14	0.70	0.48, 0.85	–0.10	–0.36, 0.18
AFD swab		N/A	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	–0.43, 0.43
Ceramic tile	*L. ivanovii* (USDA ERRC B-33165)	N/A	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	–0.43, 0.43
1 in. × 1 in.		N/A	20	9	0.45	0.26, 0.66	9	0.45	0.26, 0.66	0.00	–0.28, 0.28
AFD swab		N/A	5	4	0.80	0.38, 1.00	4	0.80	0.38, 1.00	0.00	–0.47, 0.47
Sealed concrete	*L. marthii* (BEI NR-9580)	N/A	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	–0.43, 0.43
1 in. × 1 in.		N/A	20	6	0.30	0.15, 0.52	10	0.50	0.30, 0.70	–0.20	–0.45, 0.10
AFD swab		N/A	5	4	0.80	0.38, 1.00	5	1.00	0.57, 1.00	–0.20	–0.62, 0.28

[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 portions.
[c] x = Number of positive test portions.
[d] POD_C = Candidate method presumptive positive outcomes that confirmed positive divided by the total number of trials.
[e] Reference method = MLG 8.10 for frankfurters, RTE sliced turkey, cooked eggs; FDA BAM Ch. 5 for fresh raw soft cheese, frozen/cooked shrimp, chicken salad, ice cream, pasteurized milk, environmental surfaces.
[f] POD_R = Reference method positive outcomes divided by the total number of trials.
[g] $dPOD_C$ = Difference between the candidate method result and reference method result POD values.
[h] 95% CI = If the confidence interval of a dPOD does not contain zero, then the difference is statistically significant at the 5% level.
[i] Not applicable.
[j] All test portions in the MPN were positive, leading to the high value.
[k] Matrix tested by the independent laboratory.

Table 6. SIMUL-qPCR *Listeria* species and *monocytogenes* assay stability results: 0 month versus 6 month

		SIMUL-qPCR *Listeria* species and *monocytogenes* 0 month kit			SIMUL-qPCR *Listeria* species and *monocytogenes* 6 month kit				
Strain	n[a]	x[b]	POD_0[c]	95% CI	x	POD_6[d]	95% CI	$dPOD_C$[e]	95% CI[f]
L. monocytogenes 1/2b (USDA ERRC[g] B-33258)	10	6	0.60	0.31, 0.83	6	0.60	0.31, 0.83	0.00	–0.37, 0.37
E. faecalis (BEI[h] NR-31884)	10	0	0.00	0.00, 0.28	0	0.00	0.00, 0.28	0.00	–0.28, 0.28

[a] n = Number of test portions.
[b] x = Number of positive test portions.
[c] POD_0 = Positive outcomes of the 0 month kit divided by the total number of trials.
[d] POD_6 = Positive outcomes of the 6 month kit divided by the total number of trials.
[e] $dPOD_C$ = Difference between the 0 month kit and the 6 month kit 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.
[g] United States Department of Agriculture Eastern Regional Research Center, Windsor, PA.
[h] BEI Resources, Manassas. VA.

Table 7. SIMUL-qPCR *Listeria* species and *monocytogenes* assay stability results: 0 month versus 12 month

		SIMUL-qPCR *Listeria* species and *monocytogenes* 0 month kit			SIMUL-qPCR *Listeria* species and *monocytogenes* 12 month kit				
Strain	n[a]	x[b]	POD_0[c]	95% CI	x	POD_{12}[d]	95% CI	$dPOD_C$[e]	95% CI[f]
L. monocytogenes 1/2b (USDA ERRC[g] B-33258)	10	6	0.60	0.31, 0.83	6	0.60	0.31, 0.83	0.00	–0.37, 0.37
E. faecalis (BEI[h] NR-31884)	10	0	0.00	0.00, 0.28	0	0.00	0.00, 0.28	0.00	–0.28, 0.28

[a] n = Number of test portions.
[b] x = Number of positive test portions.
[c] POD_0 = Positive outcomes of the 0 month kit divided by the total number of trials.
[d] POD_{12} = Positive outcomes of the 12 month kit divided by the total number of trials.
[e] $dPOD_C$ = Difference between the 0 month kit and the 12 month kit 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.
[g] United States Department of Agriculture Eastern Regional Research Center, Windsor, PA.
[h] BEI Resources, Manassas. VA.

test. Screening samples yielded negative results. Two uninoculated 25 g samples were plated for aerobic plate count (APC) on the same day that 100 g uninoculated portions were prepared. The results for APC are in Table 3.

Ricotta cheese was purchased from a local grocery store. All packages of ricotta were combined and mixed thoroughly. Two lots were weighed into sterile bags to be inoculated, one for fractional inoculation and one for high inoculation. Uninoculated ricotta was screened for *L. monocytogenes* using the Hygiena BAX Real Time *Listeria monocytogenes* test. Screening samples yielded negative results. Two uninoculated 25 g samples were plated for APC on the same day that the two lots were weighed to be inoculated. The results for APC are in Table 3.

An 18 GA 300 series, brush-finish, NSF-certified stainless steel surface was used for the study; 4 in. × 4 in. squares were utilized for each sample. The stainless-steel surface was thoroughly sanitized and cleaned before inoculation with *L. monocytogenes* and 10× *E. faecalis*.

For the frankfurters, 25 g portions of each contamination level (including the uncontaminated level) were weighed into a sterile sampling bag. For the candidate method, an additional 100 g of non-inoculated product was added to bring the weight of each portion to 125 g. The portions were held at 2–8°C for 48–72 h to allow for stabilization of the microorganisms in the food environment. All portions were randomized and blind-coded prior to testing.

For the fresh raw soft cheese study, 25 g portions of each contamination level (including the uncontaminated level) were weighed into a sterile sampling bag. The portions were held at room temperature (20–25°C) for 2 weeks to allow for stabilization of the microorganisms in the food environment. All portions were randomized and blind-coded prior to testing.

The stainless-steel surface was inoculated by distributing 250 µL of the appropriate inoculation levels of *Listeria* and *E. faecalis* directly onto the surface and spreading to a 4 in. × 4 in. area using sterile L-shaped spreader bars for even distribution. The stainless-steel sheet was left at room temperature to dry for approximately 16–24 h.

For the SIMUL-qPCR *Listeria* method, 1 L of prewarmed (30 ± 1°C) LREB medium was added to each 125 g test portion of frankfurters. The samples were homogenized and then incubated at 30 ± 1°C. Testing was conducted on individual test portions at 30 h. The SIMUL-qPCR *Listeria* species and *monocytogenes* Assay Kit Insert was followed to test the candidate samples. All samples were confirmed at 30 h regardless of results. For the reference method for frankfurters, 225 mL of UVM was added to

Table 8. Robustness variants versus SIMUL-qPCR *Listeria* species and *monocytogenes* assay

		SIMUL-qPCR *Listeria* species and *monocytogenes* Test conditions			SIMUL-qPCR *Listeria* species and *monocytogenes* Recommended conditions				
Test conditions[a]	n[b]	x[c]	POD_{TC}[d]	95% CI	x	POD_R[e]	95% CI	$dPOD_{TC}$[f]	95% CI[g]
				Frankfurters, artificially contaminated with *Listeria monocytogenes* 1/2b (USDA ERRC B-33258[h])					
24 h, 380 µL, 15 µL	10	7	0.70	0.40, 0.89	7	0.70	0.40, 0.89	0.00	–0.36, 0.36
24 h, 380 µL, 25 µL	10	7	0.70	0.40, 0.89	7	0.70	0.40, 0.89	0.00	–0.36, 0.36
24 h, 420 µL, 15 µL	10	7	0.70	0.40, 0.89	7	0.70	0.40, 0.89	0.00	–0.36, 0.36
24 h, 420 µL, 25 µL	10	7	0.70	0.40, 0.89	7	0.70	0.40, 0.89	0.00	–0.36, 0.36
48 h, 380 µL, 15 µL	10	7	0.70	0.40, 0.89	7	0.70	0.40, 0.89	0.00	–0.36, 0.36
48 h, 380 µL, 25 µL	10	7	0.70	0.40, 0.89	7	0.70	0.40, 0.89	0.00	–0.36, 0.36
48 h, 420 µL, 15 µL	10	7	0.70	0.40, 0.89	7	0.70	0.40, 0.89	0.00	–0.36, 0.36
48 h, 420 µL, 25 µL	10	7	0.70	0.40, 0.89	7	0.70	0.40, 0.89	0.00	–0.36, 0.36
						Frankfurters, uncontaminated			
24 h, 380 µL, 15 µL	10	0	0.00	0.00, 0.28	0	0.00	0.00, 0.28	0.00	–0.28, 0.28
24 h, 380 µL, 25 µL	10	0	0.00	0.00, 0.28	0	0.00	0.00, 0.28	0.00	–0.28, 0.28
24 h, 420 µL, 15 µL	10	0	0.00	0.00, 0.28	0	0.00	0.00, 0.28	0.00	–0.28, 0.28
24 h, 420 µL, 25 µL	10	0	0.00	0.00, 0.28	0	0.00	0.00, 0.28	0.00	–0.28, 0.28
48 h, 380 µL, 15 µL	10	0	0.00	0.00, 0.28	0	0.00	0.00, 0.28	0.00	–0.28, 0.28
48 h, 380 µL, 25 µL	10	0	0.00	0.00, 0.28	0	0.00	0.00, 0.28	0.00	–0.28, 0.28
48 h, 420 µL, 15 µL	10	0	0.00	0.00, 0.28	0	0.00	0.00, 0.28	0.00	–0.28, 0.28
48 h, 420 µL, 25 µL	10	0	0.00	0.00, 0.28	0	0.00	0.00, 0.28	0.00	–0.28, 0.28

[a] Test conditions: enrichment time, volume of extraction reagent, volume of extracted DNA sample. Recommended condition; 10 h enrichment, 400 µL extraction reagent, 20 µL extracted sample.
[b] n = Number of test portions.
[c] x = Number of positive test portions.
[d] POD_{TC} = Test condition combination positive outcomes divided by the total number of trials.
[e] POD_R = Recommended test condition positive outcomes divided by the total number of trials.
[f] $dPOD_{TC}$ = Difference between the test condition combination and the recommended test condition POD values.
[g] 95% CI = If the confidence interval of a dPOD does not contain zero, then the difference is statistically significant at the 5% level.
[h] United States Department of Agriculture Eastern Regional Research Center, Windsor, PA.

the 25 g test portions and homogenized in a stomacher for 30 s prior to incubation at 30 ± 1°C for 20 h. All samples were confirmed using the FSIS MLG 8.10 method.

For the fresh raw soft cheese, 225 mL of prewarmed (30 ± 1°C) LREB medium was added to each 25 g test portion. Samples were homogenized before being incubated for 30 h at 30 ± 1°C. Each sample was tested with the SIMUL-qPCR *Listeria* species and *monocytogenes* kit as per the assay instructions. All test portions were confirmed using the BAM reference method regardless of the presumptive result. For the reference method for fresh raw soft cheese, 225 mL of BLEB medium with pyruvate was added to the 25 g test portions and homogenized in a stomacher for 30 s prior to incubation at 30 ± 1°C for 4 h. After the 4 h incubation, 1.1 mL of filter sterilized selective agents (10 mg/L acriflavine, 40 mg/L cycloheximide, and 50 mg/L sodium nalidixic acid) was added to each sample, mixed and incubation continued at 30 ± 1°C to give a total incubation of 24 h. All samples were confirmed using the FDA BAM Chapter 10 method.

For the stainless steel, the sponge was premoistened with 10 mL of D/E neutralizing broth and used to swab the 4 in. × 4 in. test area using the following technique: 10× vertically, 10× horizontally, and 10× diagonally while rolling the swab between the fingers. The swab was placed into a sterile test tube with expression solution and held for 2 h at room temperature prior to adding 90 mL LREB and incubating at 30 ± 1°C for 30–36 h as per the SIMUL-qPCR *Listeria* protocol. Regardless of presumptive result, all test portions were confirmed as described below using the BAM reference method. For the reference method, the 4 in. × 4 in. test area was swabbed 10× vertically, 10× horizontally, and 10× diagonally using a premoistened sponge. The sponge was placed in a Whirl-Pak bag with enough Dey–Engley neutralizing broth to cover it and left to stand at room temperature for 2 h prior to analysis. After 2 h, each sample sponge was transferred to 225 mL of BLEB with pyruvate, homogenized for 2 min, and incubated at 30 ± 1°C for 4 h ± 30 min. Following 4 h of incubation, selective supplements acriflavine (10 mg/L), sodium nalidixic acid (50 mg/L), and cycloheximide (40 mg/L) were added to each test portion, mixed, and incubated for the remainder of the 24 h enrichment period. All samples were confirmed using the FDA BAM Chapter 10 method.

MPN analyses were conducted on the high and low contamination levels for frankfurters and fresh raw soft cheese. Test portions from the reference method analysis plus additional portions from each contamination level were used to create a minimum five-replicate, three-level MPN. For frankfurters, the FSIS MLG 8.10 reference method was used. For fresh raw soft cheese, the FDA BAM Chapter 10 reference method was used. All MPN results were calculated using the Least Cost Formulations MPN calculator program. The matrix study results are detailed in Tables 4 and 5.

Discussion

Inclusivity and Exclusivity

Of the 75 inclusivity strains analyzed by the SIMUL-qPCR *Listeria* species and *monocytogenes* Assay, all 75 inclusivity strains were

correctly detected by the SIMUL-qPCR *Listeria* assay kit. The kit also properly identified all 50 *L. monocytogenes* strains.

Of the 30 exclusivity strains, none were detected by the SIMUL-qPCR method. All the samples were negative for both the *Listeria* species channel and the *L. monocytogenes* channel.

Real-Time Stability Study

The results from the real-time product consistency and stability study showed that there were 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 1 year shelf life of the SIMUL-qPCR *Listeria* species and *monocytogenes* Assay.

Robustness Study

The results from the robustness study showed that there was no statistically significant difference by POD analysis when small alterations were made to the protocol. Increasing or decreasing enrichment time, volume of the extraction reagent, and volume of the extracted DNA sample slightly did not affect the performance of the *Listeria* species and *monocytogenes* Assay.

Matrix Study

Results from both the method developer and independent studies of the SIMUL-qPCR *Listeria* species and *monocytogenes* Assay for the food and environmental samples are outlined in Tables 4 and 5. 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. Environmental samples proved challenging in trying to recover cells off the surface at low levels due to the humidity in the environment. Multiple trials were run for some of the surfaces to achieve fractional levels.

During confirmation testing for the raw products, the method developer laboratory had issues with competing flora growing on plates. Colonies had to be re-streaked for further isolation to obtain the intended target. Method developer results are reported here.

For frankfurters, there were nine presumptive positive results and nine confirmed positives by the SIMUL-qPCR method in the low contamination level. The FSIS MLG 8.10 reference method had 13 positive portions. POD analysis showed that the differences in results were not statistically significant. All portions in the high level were positive (presumptive and confirmed) for both methods.

For the RTE turkey, there were 10 presumptive positive results in the low contamination level for the SIMUL-qPCR method and eight portions were confirmed positive. The background bacteria made it difficult to culturally confirm some of the positives. For the FSIS MLG 8.10 reference method, 12 portions were positive. POD analysis showed no statistically significant differences between the candidate and reference methods. All portions in the high level were positive (presumptive and confirmed) for both methods.

For the cooked eggs, there were no differences in results between the candidate individual and FSIS MLG 8.10 reference methods for the low contamination level. Fifteen portions were presumptive positive and confirmed positive. At the high contamination level, four portions were presumptive positive and four confirmed positive. For the reference method, five samples were positive. POD analysis showed no significant difference in the statistical results.

For the cheese matrix, all 10 of the presumptive positive portions in the low level confirmed positive. For the reference method, eight portions confirmed positive, compared to the 10 confirmed by the SIMUL-qPCR method. All portions in the high level were positive (presumptive and confirmed) for both methods. There were no statistical differences shown by POD analysis.

For the frozen/cooked shrimp, 16 portions were presumptive positive and 17 confirmed positive for the individual samples. For the FDA BAM Chapter 10 reference method, 13 samples were positive. All portions in the high level were positive (presumptive and confirmed) for individual and reference methods. No statistical differences between methods were indicated by POD analysis.

For chicken salad, there were five presumptive positive portions in the low contamination level, but only four samples confirmed positive. This matrix had a high APC count relative to the target contamination level, which made confirmation difficult. There were seven positive portions for the reference method. For the high contamination level, there were four positive results (presumptive and confirmed) for the individual and reference methods. POD analysis showed no statistically significant difference between the candidate and reference methods.

For ice cream, seven portions were positive (presumptive and confirmed) for the SIMUL-qPCR method and for the reference method in the low contamination level. For the high contamination level, there were five positive results (presumptive and confirmed) for the individual and reference methods. POD analysis showed no statistically significant difference between the candidate and reference methods.

The pasteurized milk had 16 presumptive positive results and all confirmed positive. The FDA BAM Chapter 10 reference method had 12 confirmed positives. For the high contamination level, there were five positive results (presumptive and confirmed) for the individual and reference methods. POD analysis showed no statistically significant difference between the candidate and reference methods.

For stainless steel, a 1 in. × 1 in. area was tested using a swab, and a 4 in. × 4 in. was tested using a sponge via the candidate method. For the low contamination level, the swab detected 15 presumptive positive results in the 1 in. × 1 in. area, and all 15 were confirmed positive. The sponge detected 12 presumptive positive results, but 15 confirmed positive. The low number of target cells led to some portions not being detected by the candidate method. For the high contamination level, the swab detected all five high contamination level portions, and all confirmed positive. The sponge also detected five of the five high contamination level portions, of which all five confirmed positive. The FDA BAM Chapter 10 method reported four of the five high contamination level positive portions as well as seven positive results for the low contamination level set. For the 1 in. × 1 in. swabs, the low contamination level showed a statistical difference in results favoring the SIMUL-qPCR method; otherwise there were no statistically significant differences between the methods.

The plastic was swabbed in a 1 in. × 1 in. area. Eleven portions at the low contamination level were presumptive positive, and nine portions were confirmed positive. It is possible that the PCR method detected dead cells from the swab that were not able to be confirmed culturally. All five portions at the high contamination level confirmed positive. Seven portions at the low contamination level and four portions at the high contamination level were positive for the FDA BAM method, leading to no statistically significant differences between the two methods.

The rubber was also swabbed in a 1 in. × 1 in. area. For the low contamination level, 12 portions were presumptive positive by the SIMUL-qPCR, and all 12 portions were confirmed positive. For the FDA BAM Chapter 10 method, 14 portions were positive. For the high contamination level, there were five positive results (presumptive and confirmed) for the individual and reference methods. POD analysis showed no statistically significant difference between the candidate and reference methods.

For ceramic, a 1 in. × 1 in. area was swabbed. For the low contamination level, the candidate method had nine presumptive positive results, and all nine confirmed positive. The FDA BAM Chapter 10 method had nine positive results. For the high contamination level, both the candidate and reference methods had four positive results (presumptive and confirmed for the candidate method). POD analysis showed no statistical difference.

For concrete, a 1 in. × 1 in. surface area was also swabbed. For the low contamination level, the candidate method had six presumptive positive results, and eight portions confirmed positive. The reference method had 10 positive results. For the high contamination level, the candidate method had four presumptive positive results, and all four confirmed positive. The reference method had five positive results. POD analysis did not show any statistically significant differences in the methods.

Independent Laboratory Studies

For the SIMUL-qPCR method on frankfurters, at the 30 h enrichment time point for the individual set, 11 out of 20 portions were presumptive positive, and 13 confirmed positive. For the reference method, six portions were positive. For the high contamination level of the individual set, 5 out of 5 samples were presumptive positive and confirmed positive for the candidate method. The reference method had 4 out of 5 positive portions.

For fresh raw soft cheese, 13 out of 20 test portions were presumptive positive. Fourteen samples were confirmed positive by culture. For the reference method, 14 out of 20 test portions were positive. All five portions at the high contamination level were for both the candidate and reference methods.

For the stainless steel sponges, 7 out of 20 test portions were presumptive positive, and all seven confirmed positive. For the reference method, 6 out of 20 samples were positive. For both the candidate and reference methods, all five portions at the high contamination level were positive.

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 for any of the matrixes.

Conclusions

The data outlined in this report confirms that the SIMUL-qPCR *Listeria* species and *monocytogenes* Assay is suitable for detecting *Listeria* species and *monocytogenes* in frankfurters, RTE sliced turkey, cooked eggs, cheese, chicken salad, ice cream, pasteurized milk, frozen/cooked shrimp, as well as on stainless steel, plastic, rubber, ceramic tile, and sealed concrete. POD analysis showed that there were no statistically significant differences between the candidate method and the USDA FSIS MLG 8.10 and FDA BAM Chapter 10 reference method for any of the matrixes. The study also demonstrated that the assay is capable of detecting *Listeria monocytogenes* and *Listeria* species strains but excluded strains similar to non-*Listeria* strains. Small variations in parameters will not affect the validity of the results obtained by the method, and the assay collection is proven to perform adequately during its 1 year shelf life.

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