OXFORD

Journal of AOAC INTERNATIONAL, 2026, 1–13

https://doi.org/10.1093/jaoacint/qsag015
Advance Access Publication Date: 3 April 2026
Research Article

Microbiological Methods

The SureFast® Listeria 3plex ONE Kit and the SureFast® PREP Bacteria Kit in a Variety of Foods and Environmental Samples: Multi-Laboratory Validation, First Action 2025.04

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Abstract

Background: The SureFast® Listeria 3plex ONE Kit uses the real-time polymerase chain reaction (PCR) technique for multiplex detection of *Listeria* species and *Listeria monocytogenes* in foods and environmental samples. The SureFast Listeria 3plex ONE Kit can also be used with the SureFast PREP Bacteria Kit to provide a second DNA extraction option and can be analyzed on multiple thermocyclers.

Objective: The kit was evaluated using 25 g test portions in an unpaired study design for a single matrix, ready-to-eat smoked deli turkey, as part of a harmonized validation protocol with MicroVal Certification.

Methods: The matrix was compared to the *International Organization for Standardization Technical* (ISO) 11290–1:21017 reference standard; 17 participants from 16 laboratories located within Austria, Germany, Italy, Spain, and the United States were solicited for the multi-laboratory validation (MLV), with only 10 participants submitting data. Three levels of contamination were evaluated: an uninoculated control level (0 colony-forming units (CFU)/test portion), a low inoculum level (0.2–2 CFU/test portion), and a high inoculum level (2–10 CFU/test portion). Statistical analysis was conducted according to the Probability of Detection (POD) statistical model.

Results: Results obtained for the low inoculum level test portions produced a dLPOD value with 95% confidence interval of −0.01 (−0.04, 0.02). The dLPOD results indicate equivalence between the candidate method and reference method for the matrix evaluated and the method demonstrated acceptable multi-laboratory reproducibility as determined in the MLV. False positive and false negative rates were determined for the matrix and produced values of 0.0% and 0.8%, respectively.

Conclusions: Based on the data generated, the method demonstrated equivalence to the ISO reference method in a MLV study.

Highlights: The SureFast ONE 3plex method is a rapid and reliable alternative for the multiplex detection of *Listeria* species and *Listeria* monocytogenes in a broad range of foods and environmental surfaces.

Listeria species, including *Listeria monocytogenes*, are Gram-positive, rod-shaped bacteria that are ubiquitous in soil, water, and several animals intended for consumption. These bacteria are the causative agent of listeriosis. Listeriosis is usually caused by eating food that has been contaminated. The infection is most likely to sicken pregnant women and their newborns, adults aged 65 or older, and people with weakened immune systems. Of most concern are infections occurring in pregnant women where the infection can lead to miscarriage, stillbirth, premature delivery, or even life-threating infections such as sepsis or meningitis in the newborn. Most people will become infected with *Listeria* after consuming contaminated food, with the majority of recalls associated with ready-to-eat food products. The most recent outbreak involved sliced deli meat in 2024. The outbreak, which occurred over 19 states within the United States, led to a total of 60 hospitalizations and resulted in 10 deaths (1).

The SureFast® Listeria 3plex ONE Kit detects and differentiates *Listeria* spp. and *L. monocytogenes* in a variety of foods and one environmental surface, stainless steel. The method consists of four steps: cultural enrichment, DNA extraction, specific real-time PCR detection, and interpretation of results. A second DNA extraction kit, SureFast® PREP Bacteria Kit, can also be used with SureFast Listeria 3plex ONE Kit to provide an alternative option for DNA extraction (2).

Received: 19 November 2025. **Revised:** 13 February 2026. **Accepted:** 13 February 2026

The SureFast Listeria 3plex ONE Kit real-time PCR assay can be performed with commonly used real-time PCR instruments, equipped for detection of four fluorescence emissions in the channels VIC/HEX, ROX, and Cy5 at the same time. Each PCR reaction contains an internal amplification control (IAC). If the DNA extract contains PCR-inhibiting substances, the signal of the amplification control can be affected, or the amplification will be suppressed. Examples for PCR-inhibiting substances are alcohols (e.g., ethanol, isopropanol), surfactants (e.g., cetrimonium bromide, SDS, Triton X-100), and salts (e.g., sodium chloride). In addition, spices, herbs, algae, cocoa and other sample matrixes can have PCR-inhibiting effects (2).

Single-Laboratory Validation (SLV) Study

Before the multi-laboratory validation (MLV), the SureFast Listeria 3plex ONE Kit was validated according to the current AOAC Guidelines (3) in an AOAC INTERNATIONAL *Performance Tested Method*^{SM} (PTM) study. The SureFast Listeria 3plex ONE Kit is pending PTM certification for the following matrixes; frankfurter sausage (25 g), deli ham (25 g), smoked deli turkey (25 g), smoked salmon (25 g), frozen cooked shrimp (25 g), liquid whole egg (25 g), raw milk (25 g), pasteurized whole milk (25 g), ice cream (25 g), gorgonzola cheese (25 g), Gouda cheese (25 g), Brie cheese (25 g), bagged salad (25 g), canned enoki mushroom (25 g), ground soy vegan meat substitute (25 g), guacamole (25 g), process rinse water (25 mL), stainless-steel surfaces (4″ × 4″, sponges), and stainless-steel surfaces (1″ × 1″, swab). *See* Tables 1 and 2 for an overview of the validated matrixes conducted in the SLV for PTM certification.

Multi-Laboratory Validation (MLV) Study

The purpose of this MLV was to compare the reproducibility of the SureFast Listeria 3plex ONE Kit to the *International Organization for Standardization Technical Standard* 11290–1:2017: Microbiology of food chain—Horizontal method for the detection and enumeration of *Listeria monocytogenes* and of *Listeria* spp. – Part 1: Detection method (ISO 11290–1:2017) (4), for ready-to-eat (RTE) smoked deli turkey (25 g).

Study Design

In this MLV study, RTE smoked deli turkey was used to evaluate the rapid method. The matrix was obtained from a local retailer and screened for the presence of *Listeria* spp. and *L. monocytogenes* by the ISO 11290–1:20170 standard. The RTE smoked deli turkey was artificially contaminated with a heat-stressed culture of *L. monocytogenes* 1/2A CWD 1609 (source: University of Vermont; origin, Turkey Frank Factory). The matrix was inoculated at two levels of contamination: a high inoculation level of approximately 2–10 colony-forming units (CFU)/test portion and a low inoculation level of approximately 0.2–2 CFU/test portion. A set of uninoculated control test portions (0 CFU/test portion) were also included. The inoculated test portions were held for 96 h before initiating testing.

Eight replicates at the high inoculation level and uninoculated control level, along with 12 replicates at the low level of inoculation were evaluated by each participant. Due to different enrichment conditions, unpaired samples were used. A total of 56 samples, 28 for the SureFast Listeria 3plex ONE Kit (25 g test portions) and 28 for the reference method (25 g test portions) were sent to each participant. Collaborators were also sent an additional uninoculated test portion for determining the total aerobic plate count (APC) using the *International Organization for Standardization Technical Standard* 4833–1 (2013) Microbiology of the food chain—Horizontal method for the enumeration of microorganisms—Part 1: Colony count at 30 °C by the pour plate technique [ISO 4833–1 (2013)] (5) on the day samples were received.

A detailed MLV study packet outlining all necessary information related to the study including media preparation, test portion preparation, and documentation of results was sent to each collaborating laboratory before the initiation of the study.

Preparation of Inoculum and Test Portions

The *L. monocytogenes* strain used in this evaluation was propagated onto Tryptic Soy Agar with 5% Sheep Blood (SBA) from a Q Laboratories (Cincinnati, OH) frozen stock culture stored at −70°C. The organism was incubated for 24 ± 2 h at 37 ± 2 °C. Isolated colonies were picked to 10 mL of Brain Heart Infusion (BHI) broth and incubated for 18–24 h at 37 ± 2 °C.

After incubation, the strain was heat stressed by heating the culture at 50 ± 0.2 °C for 10 min. The heat stressed culture was then plated onto a nonselective agar (TSA/YE) and a selective agar (MOX) and incubated for 18–24 h at 37 ± 1 °C. After incubation, each agar was enumerated and the % injury was determined using the following formula. The inoculating culture had to have a % injury of 50–80:

$$\left(1-\frac{n_{\text{select}}}{n_{\text{nonselect}}}\right)\times 100$$

where n_{select} = number of colonies on selective agar and $n_{\text{nonselect}}$ = number of colonies on nonselective agar.

The % injury for the culture used to inoculate the RTE smoked deli turkey was 58%. The heat-stressed culture was diluted in Brain Heart Infusion (BHI) broth to the target level and added to the matrix to yield a low level (0.2–2 CFU/25 g) expected to produce fractional positive results and a high level (2–10 CFU/25 g) expected to produce all positive results. The inoculated matrix was mixed with a sterile spatula to ensure homogeneous distribution of the inoculum within the matrix.

To determine the level of *L. monocytogenes* in the test matrix, a 5-tube Most Probable Number (MPN) was conducted on the first day of analysis following the ISO 11290–1:2017 standard. The MPN for each contamination level was determined by analyzing 5 × 50 g test portions, the reference method test portions from the collaborating laboratories that analyzed sample sets (120 × 25 g) and 5 × 10 g test portions. The MPN and 95% confidence intervals were calculated using the Least Cost Formulations MPN Calculator, version 1.6) (6).

Test Portion Distribution

All samples were labeled with a randomized, blind-coded three-digit number affixed to the sample container. All test portions were shipped on a Friday according to the Category B Dangerous Goods shipment regulations set forth by the International Air Transport Association (IATA). Upon receipt, samples were held by the collaborating laboratory at refrigerated temperature (2–8 °C) until Wednesday or Thursday when analysis was initiated after a total equilibration time of 96 h. A temperature probe was included in each shipment in order to track the temperature of the package during transit. Participants were instructed to obtain the temperature of their package upon receipt and document the results on the Sample Receipt Confirmation form provided and fax or email it back to the study director.

Table 1. PTM validation study summary for the SureFast Listeria 3plex ONE Kit *Listeria* spp. claim

Claim	Matrixes	Sample size	Enrichment media/dilution	Enrichment temp., °C	Enrichment time SureFast PREP Bacteria Kit DNA extraction, h	Enrichment time SureFast Listeria 3plex ONE DNA extraction, h
Listeria spp.	Frankfurter sausage	25 g	Half-Fraser/1:10	37 ± 1	18–20	26–28
	Deli ham	25 g	Half-Fraser/1:10	37 ± 1	18–20	26–28
	Smoked deli turkey	25 g	Half-Fraser/1:10	37 ± 1	18–20	26–28
	Smoked salmon	25 g	Half-Fraser/1:10	37 ± 1	18–20	26–28
	Frozen cooked shrimp	25 g	Half-Fraser/1:10	37 ± 1	18–20	26–28
	Liquid whole egg	25 mL	Demi Fraser/1:10	37 ± 1	18–20	26–28
	Raw milk	25 mL	Primary: Half-Fraser/1:10 Secondary: Fraser Broth (w/o ferric ammonium citrate)/1:10	Primary: 37 ± 1 Secondary: 37 ± 1	Primary: 18–20 Secondary: 24 ± 2	Primary: 26–28 Secondary: 24 ± 2
	Pasteurized whole milk	25 mL	Primary: Half-Fraser/1:10 Secondary: Fraser Broth (w/o ferric ammonium citrate)/1:10	Primary: 37 ± 1 Secondary: 37 ± 1	Primary: 18–20 Secondary: 24 ± 2	Primary: 26–28 Secondary: 24 ± 2
	Ice cream	25 g	Primary: Half-Fraser/1:10 Secondary: Fraser Broth (w/o ferric ammonium citrate)/1:10	Primary: 37 ± 1 Secondary: 37 ± 1	Primary: 18–20 Secondary: 24 ± 2	Primary: 26–28 Secondary: 24 ± 2
	Gorgonzola cheese	25 g	Primary: Half-Fraser/1:10 Secondary: Fraser Broth (w/o ferric ammonium citrate)/1:10	Primary: 37 ± 1 Secondary: 37 ± 1	Primary: 18–20 Secondary: 24 ± 2	Primary: 26–28 Secondary: 24 ± 2
	Gouda cheese	25 g	Primary: Half-Fraser/1:10 Secondary: Fraser Broth (w/o ferric ammonium citrate)/1:10	Primary: 37 ± 1 Secondary: 37 ± 1	Primary: 18–20 Secondary: 24 ± 2	Primary: 26–28 Secondary: 24 ± 2
	Brie cheese	25 g	Primary: Half-Fraser/1:10 Secondary: Fraser Broth (w/o ferric ammonium citrate) 1:10	Primary: 37 ± 1 Secondary: 37 ± 1	Primary: 18–20 Secondary: 24 ± 2	Primary: 26–28 Secondary: 24 ± 2
	Bagged salad	25 g	Half-Fraser/1:10	37 ± 1	18–20	26–28
	Canned enoki mushroom	25 g	Half-Fraser/1:10	37 ± 1	18–20	26–28
	Ground soy vegan meat substitute	25 g	Half-Fraser/1:10	37 ± 1	18–20	26–28
	Guacamole	25 g	Half-Fraser/1:10	37 ± 1	18–20	26–28
	Stainless-steel surface (1″ × 1″ test area)	Swabs	Half-Fraser/1:10	37 ± 1	18–20	26–28
	Stainless-steel surface (4″ × 4″ test area)	Sponges	Half-Fraser/1:10	37 ± 1	18–20	26–28
	Process rinse water	25 mL	Half-Fraser/1:10	37 ± 1	18–20	26–28

Test Portion Analysis

Collaborators were instructed to follow the appropriate preparation and analysis as outlined in the study protocol. Two separate sets of 28 test portions (8 high, 12 low, and 8 uninoculated controls for each method) were analyzed due to the unpaired study design. The SureFast Listeria 3plex ONE Kit test portions (25 g) were enriched with 225 mL of Half-Fraser Broth homogenized for 30 s and incubated at 37 ± 1 °C for 18–20 h for use with the SureFast PREP Bacteria Kit DNA extraction, or 26–28 h for use with the SureFast Listeria 3plex ONE DNA extraction. The samples were analyzed using the SureFast Listeria 3plex ONE Kit on either the Bio-Rad CFX96 Deep Well (CFX), Bio-Rad Opus Deep Well (Opus), or -Biopharm RIDA®CYCLER RT-PCR systems following the instructions for use. All three RT-PCR systems have been included in the PTM evaluations. Table 3 presents a summary of the collaborator participation along with RT-PCR system and DNA extraction protocol used.

Regardless of the presumptive result, all test portions were confirmed following the ISO 11290–1:2017 standard beginning with a transfer to the secondary enrichment Fraser Broth (FB), and by isolating a 10 µL aliquot of sample enrichment directly to Modified Oxford Agar (MOX), PALCAM Agar, and/or Agar Listeria according to Ottaviani and Agosti (ALOA). The FB tubes and plates were incubated at 37 ± 2 °C for 24 ± 2 h. After incubation, the FB was also isolated to MOX, PALCAM Agar, and/or ALOA and incubated at 37 ± 2 °C for 24 ± 2 h. Typical isolated colonies from the selective agars were transferred to Tryptic Soy Agar with 6% Yeast Extract (TSAYE) plates and incubated for 18–24 h at 37 ± 1 °C. Final confirmation for all samples was obtained by microscopic examination, beta-hemolysis reaction, and utilization of L-Rhamnose and D-Xylose.

For the reference method test portions, 25 g samples were enriched in Demi Fraser Broth and analyzed according to the procedures in the ISO 11290–1:2017 standard as described in the preceding paragraph.

Table 2. PTM validation study summary for the SureFast Listeria 3plex ONE Kit *Listeria monocytogenes* claim

Claim	Matrixes	Sample size	Enrichment media/dilution	Enrichment temp., °C	Enrichment time SureFast PREP Bacteria Kit DNA extraction, h)	Enrichment time SureFast Listeria 3plex ONE DNA extraction, h
L. monocytogenes	Frankfurter sausage	25 g	Half-Fraser/1:10	37 ± 1	18–20	26–28
	Deli ham	25 g	Half-Fraser/1:10	37 ± 1	18–20	26–28
	Smoked deli turkey	25 g	Half-Fraser/1:10	37 ± 1	18–20	26–28
	Frozen cooked shrimp	25 g	Half-Fraser/1:10	37 ± 1	18–20	26–28
	Liquid whole egg	25 mL	Half-Fraser/1:10	37 ± 1	18–20	26–28
	Raw milk	25 mL	Primary: Half-Fraser/1:10Secondary: Fraser Broth (w/o ferric ammonium citrate)/1:10	Primary: 37 ± 1 Secondary: 37 ± 1	Primary: 18–20 Secondary: 24 ± 2	Primary: 26–28 Secondary: 24 ± 2
	Pasteurized whole milk	25 mL	Primary: Half-Fraser/1:10 Secondary: Fraser Broth (w/o ferric ammonium citrate)/1:10	Primary: 37 ± 1 Secondary: 37 ± 1	Primary: 18–20 Secondary: 24 ± 2	Primary: 26–28 Secondary: 24 ± 2
	Gouda cheese	25 g	Primary: Half-Fraser/1:10 Secondary: Fraser Broth (w/o ferric ammonium citrate)/1:10	Primary: 37 ± 1 Secondary: 37 ± 1	Primary: 18–20 Secondary: 24 ± 2	Primary: 26–28 Secondary: 24 ± 2
	Brie cheese	25 g	Primary: Half-Fraser/1:10 Secondary: Fraser Broth (w/o ferric ammonium citrate)/1:10	Primary: 37 ± 1 Secondary: 37 ± 1	Primary: 18–20 Secondary: 24 ± 2	Primary: 26–28 Secondary: 24 ± 2
	Bagged salad	25 g	Half-Fraser/1:10	37 ± 1	18–20	26–28
	Canned enoki mushroom	25 g	Half-Fraser/1:10	37 ± 1	18–20	26–28
	Ground soy vegan meat substitute	25 g	Half-Fraser/1:10	37 ± 1	18–20	26–28
	Stainless-steel surface (1″ × 1″ test area)	Swabs	Half-Fraser/1:10	37 ± 1	18–20	26–28
	Stainless-steel surface (4″ × 4″ test area)	Sponges	Half-Fraser/1:10	37 ± 1	18–20	26–28

Table 3. Participation of each collaborating laboratory

Laboratory	Analyst	Participated[a]	DNA extraction used	RT-PCR system used
1[b]	1	N	NA[c]	NA
2	1	Y	ONE	RIDACYCLER
3[b]	1	N	NA	NA
4[b]	1	N	NA	NA
5[b]	1	N	NA	NA
6[b]	1	N	NA	NA
7	1	Y	ONE	CFX 96
8	1	Y	PREP	RIDACYCLER
9	1	Y	PREP	CFX 96
10	1	Y	ONE	Opus
11[b]	1	N	NA	NA
12[b]	1	N	NA	NA
13	1	Y	ONE	CFX 96
14	1	Y	ONE	CFX 96
15A	1	Y	ONE	RIDACYCLER
15B	2	Y	PREP	Opus
16	1	Y	PREP	Opus

[a] Y = Collaborator analyzed the food type; N = Collaborator did not analyze the food type.
[b] Collaborator received samples after the deadline due to customs delay and was unable to participate.
[c] NA = Not applicable.

Statistical Analysis

Each collaborating laboratory reported the test results on the data sheets provided. The data sheets were submitted to the study director at the end of testing for statistical analysis. Data for each contamination level was analyzed using the probability of detection (POD) statistical model (7) and conducted using the AOAC Binary Data Interlaboratory Study Workbook (version 5.1) (8). For each laboratory, the POD was calculated for the candidate presumptive results, POD_{CP}, the candidate confirmatory results (including false negative results), POD_{CC}, presumptive candidate results that confirmed positive (excluding false negative results), POD_C, and the reference method, POD_R. LPOD values, POD values obtained from combining all valid collaborator POD data, were determined for candidate presumptive results, $LPOD_{CP}$, candidate confirmed results (including false negative results) $LPOD_{CC}$, presumptive candidate results that confirmed positive (excluding false negative results), $LPOD_C$, and reference method results, $LPOD_R$. Using LPOD values, the $dLPOD_{CP}$, the difference in the candidate presumptive and confirmatory results from all collaborators and the $dLPOD_C$, the difference in the confirmed candidate and reference methods were calculated. A $dLPOD_C$ confidence interval not containing the point zero would indicate a statistically significant difference between the SureFast Listeria

3plex ONE Kit and the reference standard at the 5% probability level. In addition to POD and LPOD values, the repeatability standard deviation (s_r), the among-laboratory repeatability standard deviation (s), the reproducibility standard deviation (s_R), and the P_T value were calculated (8). The s_r provides the standard deviation of data within one laboratory, the s_L provides the difference in standard deviation between laboratories, and the s_R provides the standard deviation in data between different laboratories. The P_T value provides information on the homogeneity of the test portions sent to the laboratories.

AOAC Official Method 2025.04
***Listeria* species and *L. monocytogenes* in a Broad Range of Foods and Environmental Samples**
SureFast® Listeria 3plex ONE Kit and the SureFast® PREP Bacteria Kit
First Action 2025

(Applicable to detection of *Listeria* species in the following: frankfurter sausage [25 g], deli ham [25 g], smoked deli turkey [25 g], smoked salmon [25 g], frozen cooked shrimp [25 g], liquid whole egg [25 ml], raw milk [25 ml], pasteurized whole milk [25 mL], ice cream [25 g], Gorgonzola cheese [25 g], Gouda cheese [25 g], Brie cheese [25 g], bagged salad [25 g], canned enoki mushroom [25 g], ground soy vegan meat substitute [25 g], guacamole [25 g], stainless-steel surface [swab, 1″ × 1″ test area], stainless-steel surface [sponge, 4″ × 4″ test area], process rinse water [25 ml]).

Applicable to detection of *Listeria monocytogenes* in the following: Frankfurter Sausage (25 g) Deli Ham (25 g), Smoked Deli Turkey (25 g), Frozen Cooked Shrimp (25 g), Liquid Whole Egg (25 mL), Raw Milk (25 mL), Pasteurized whole milk (25 mL), Gouda Cheese (25 g), Brie Cheese (25 g), Bagged Salad (25 g), Canned Enoki Mushroom (25 g), Ground Soy Vegan Meat Substitute (25 g), Guacamole (25 g), Stainless-Steel Surface (swab, 1″ × 1″ Test Area), Stainless-Steel Surface (sponge, 4″ × 4″ Test Area)

See Table 2025.04A for a summary of results of the multilaboratory study.

See Table 2025.04B for detailed results of the multilaboratory study.

A. Principle

The SureFast Listeria 3plex ONE Kit detects and differentiates *Listeria* spp. and *L. monocytogenes* in a variety of foods and one environmental surface, stainless steel. The method consists of four steps: cultural enrichment, DNA extraction, specific real-time PCR detection, and interpretation of results. A second DNA extraction kit, SureFast PREP Bacteria Kit, can also be used with SureFast Listeria 3plex ONE Kit to provide an alternative option for DNA extraction.

The SureFast Listeria 3plex ONE Kit real-time PCR assay can be performed with commonly used real-time PCR instruments, equipped for detection of four fluorescence emissions in the channels VIC/HEX, ROX, and Cy5 at the same time. Each PCR reaction contains an internal amplification control (IAC). If the DNA extract contains PCR-inhibiting substances, the signal of the amplification control can be affected, or the amplification will be suppressed. Examples for PCR-inhibiting substances are alcohols (e.g., ethanol, isopropanol), surfactants (e.g., cetrimonium bromide, SDS, Triton X-100), and salts (e.g., sodium chloride). In addition, spices, herbs, algae, cocoa, and other sample matrixes can have PCR-inhibiting effects.

B. Apparatus, Reagents, and Supplies

Items available from R-Biopharm from the following website: https://food.r-biopharm.com/ (R-Biopharm Inc., 870 Vossbrink Drive, Washington, MO 63090)

(a) *SureFast Listeria 3plex ONE Kit.*—Cat. No. F5217.
- (1) *Reaction Mix (1).*—Two tubes with a yellow lid containing 1050 µL of reaction mix.
- (2) *Taq Polymerase (2).*—One tube with a dark red lid containing 80 µL of Taq polymerase.
- (3) *Positive Control (3).*—One tube with a light blue lid containing 200 µL of positive control.
- (4) *Lysis Buffer (L).*—Two bottles with a clear lid containing 25 mL of lysis buffer.

(b) *SureFast PREP Bacteria Kit.*—Cat. No. F1021.
- (1) *Lysis buffer (L).*—One bottle containing 25 mL of lysis buffer.
- (2) *Binding buffer (B).*—One bottle containing 15 mL of binding buffer, after isopropanol (≥99.7% purity) has been added.
- (3) *Wash buffer (W).*—One bottle containing 60 mL of wash buffer, after ethanol (≥96% purity) has been added.
- (4) *Elution buffer (E).*—One bottle containing 10 mL of elution buffer.
- (5) *Receiver tubes (R).*—Fifty (50) clear 2.0 mL tubes.
- (6) *Receiver tubes (T).*—Fifty (50) clear 1.5 mL tubes.
- (7) *Spin filter (S).*—Fifty (50) clear spin filters Detection Kit.—Cat. No. 601500720.

Additional Items Required

(a) *Half (Demi) Fraser Broth.*—Any formulation equivalent to ISO formulation.
(b) *Ethanol.*—Any with ≥96% purity.
(c) *Isopropanol.*—Any with >99.7% purity.
(d) *Real-Time PCR Instrument.*—Bio-Rad CFX96 Deep Well DX, Bio-Rad Opus Deepwell, or R-Biopharm RIDA®CYCLER.
(e) *Incubator.*—Capable of maintaining 37 ± 1 °C.
(f) *Filter laboratory blender bags.*
(g) *Laboratory paddle blender.*—Seward 400, or equivalent for sample homogenization.
(h) *Vortex mixer.*
(i) *Microcentrifuge tubes.*—Free from DNA and DNase, any 1.5 and 2.0 mL, with lock cap.
(j) *Microcentrifuge.*—Capable of maximum speed 12 000 rpm.
(k) *Dry bath incubator.*—Capable of maintaining 60 and 95 °C.
(l) *Thermomixer.*—Capable of maintaining 99 °C with high-speed agitation.
(m) *Precision pipettors.*—For sampling and delivering of 0.7 µL–1000 µL.
(n) *Micropipet tips.*—Aerosol resistant.

C. Safety Precautions

(a) *SureFast Listeria 3plex ONE Kit.*—The assay components are nonhazardous. All materials should be used following good laboratory practices. Materials and reagents must be discarded according to appropriate waste procedures used in the laboratory, and in accordance with local, state, and federal regulations. *Listeria* spp. and *L. monocytogenes* are Bio-Safety Level 2 organisms. All samples should be handled as potentially infectious, and proper personal protective equipment should be used while handling samples,

Table 2025.04A. Summary of results for the SureFast Listeria 3plex ONE Kit for the detection of *Listeria species* and *L. monocytogenes*

Method[a]	SureFast Listeria 3plex ONE Kit		
Inoculation Level	**Uninoculated**	**Low**	**High**
Candidate presumptive positive/total no. of samples analyzed	0/80	55/120	80/80
Candidate presumptive LPOD (CP)	0.00 (0.00, 0.05)	0.46 (0.36, 0.56)	1.00 (0.95, 1.00)
s_r[b]	0.00 (0.00, 0.21)	0.49 (0.44, 0.52)	0.00 (0.00, 0.21)
s_L[c]	0.00 (0.00, 0.21)	0.07 (0.00, 0.25)	0.00 (0.00, 0.21)
s_R[d]	0.00 (0.00, 0.29)	0.50 (0.45, 0.52)	0.00 (0.00, 0.29)
P[e]	1.0000	0.2820	1.0000
Candidate confirmed positive/total no. of samples analyzed	0/80	56/120	80/80
Candidate confirmed LPOD (CC)	0.00 (0.00, 0.05)	0.46 (0.36, 0.58)	1.00 (0.95, 1.00)
s_r	0.00 (0.00, 0.21)	0.49 (0.44, 0.52)	0.00 (0.00, 0.21)
s_L	0.00 (0.00, 0.21)	0.10 (0.00, 0.28)	0.00 (0.00, 0.21)
s_R	0.00 (0.00, 0.29)	0.50 (0.45, 0.52)	0.00 (0.00, 0.29)
P	1.0000	0.1692	1.0000
Positive reference samples/total no. of samples analyzed	0/80	53/120	80/80
Reference LPOD	0.00 (0.00, 0.05)	0.44 (0.31, 0.57)	1.00 (0.95, 1.00)
s_r	0.00 (0.00, 0.21)	0.48 (0.42, 0.52)	0.00 (0.00, 0.21)
s_L	0.00 (0.00, 0.21)	0.15 (0.00, 0.35)	0.00 (0.00, 0.21)
s_R	0.00 (0.00, 0.29)	0.50 (0.45, 0.52)	0.00 (0.00, 0.29)
P	1.0000	0.0320	1.0000
dLPOD (candidate vs. reference)[f]	0.00 (−0.03, 0.03)	0.02 (−0.13, 0.17)	0.00 (−0.03, 0.03)
dLPOD (candidate presumptive vs. candidate confirmed)[f]	0.00 (−0.05, 0.05)	−0.01 (−0.04, 0.02)	0.00 (−0.05, 0.05)

[a] Results include 95% confidence intervals.
[b] Repeatability standard deviation.
[c] Among-laboratory standard deviation.
[d] Reproducibility standard deviation.
[e] P = Homogeneity test of laboratory PODs.
[f] A confidence interval for dLPOD that does not contain the value 0 indicates a statistically significant difference between the two methods.

enrichments, or assay components. Due to the significantly increased risk of infection and mortality, direct contact with *L. monocytogenes* samples is strongly discouraged for pregnant women and people with weakened immune systems.

(b) *SureFast PREP Bacteria Kit.*—The assay components are non-hazardous; however, the binding and lysis buffer may cause serious eye irritation. Wear protective clothing, gloves, and eye or face protection. If contact with your eyes occurs, rinse with water for several minutes. All materials should be used following good laboratory practices. Materials and reagents must be discarded according to appropriate waste procedures used in the laboratory, and in accordance with local, state, and federal regulations. *Listeria* spp. and *L. monocytogenes* are Bio-Safety Level 2 organisms. All samples should be handled as potentially infectious and proper personal protective equipment should be used while handling samples, enrichments, or assay components.

D. Sample Enrichment

(a) *All food matrixes excluding dairy products (25 g or 25 mL test portions).*—For 25 g test portions, add 225 mL Half (Demi) Fraser Broth that has been prewarmed to room temperature before use. Homogenize by laboratory paddle blender for 30 s and incubate enrichments at 37 ± 1°C. Incubate samples for 18–20 h for use with the SureFast PREP Bacteria Kit DNA extraction, or 26–28 h for use with the SureFast Listeria 3plex ONE DNA extraction.

(b) *Raw and pasteurized dairy products.*—For 25 g test portions, add 225 mL Half (Demi) Fraser Broth that has been prewarmed to room temperature before use. Homogenize by laboratory paddle blender for 30 s and incubate at 37 ± 1°C. Incubate samples for 18–20 h for use with the SureFast PREP Bacteria Kit DNA extraction, and 26–28 h for use with the SureFast Listeria 3plex ONE DNA extraction. After incubation, transfer 0.1 mL of enrichment to 10 mL of Fraser broth (without ferric ammonium citrate), mix well by vortex and incubate at 37 ± 1°C for 24 ± 2 h. After incubation, proceed with the appropriate DNA extraction.

(c) *Environmental samples (1″ × 1 ″ swabs and 4″ × 4 ″ sponges).*—Premoisten swabs with 1 mL of Hi-Cap Neutralizing Broth and sponges with 10 mL of Hi-Cap Neutralizing Broth. Sample the environmental surface and hold the swab and/or sponge at room temperature for 2 h. Add the swabs to test tubes containing 9 mL of Half (Demi) Fraser Broth and add the sponges to 90 mL of Half (Demi) Fraser Broth. Incubate samples at 37 ± 1°C for 18–20 h when using the SureFast PREP Bacteria Kit DNA extraction, or 26–28 h when using the SureFast Listeria 3plex ONE DNA extraction.

E. Test Portion Analysis

(a) *DNA Extraction—SureFast Listeria 3plex ONE Kit Method*

(1) After incubation, mix the enrichment before removing an aliquot for DNA extraction. Allow sample enrichment to settle for 5–10 min.

(2) Add 500 µL of lysis buffer into a 2.0 mL microcentrifuge tube.

Table 2025.04B. Comparative results for the detection of *Listeria species* and *L. monocytogenes* in 25 g RTE smoked deli turkey test portions by the SureFast Listeria 3plex ONE Kit vs. ISO 11290–1:2017 in a multi-laboratory study[a]

			Candidate presumptive (CP)			Candidate confirmed (CC)			Candidate result (C)			Reference method (R)			C vs. R	CP vs. CC
Statistic	Matrix	Collaborator	N	X	POD (CP)	N	X	POD (CC)	N	X	POD (C)	N	X	POD (R)	dLPOD (C, R)	dLPOD (CP, CC)
Uninoculated control																
	RTE smoked deli turkey	1[b]	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
		2	8	0	0.00	8	0	0.00	8	0	0.00	8	0	0.00	0.00	0.00
		3[b]	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
		4[b]	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
		5[b]	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
		6[b]	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
		7	8	0	0.00	8	0	0.00	8	0	0.00	8	0	0.00	0.00	0.00
		8	8	0	0.00	8	0	0.00	8	0	0.00	8	0	0.00	0.00	0.00
		9	8	0	0.00	8	0	0.00	8	0	0.00	8	0	0.00	0.00	0.00
		10	8	0	0.00	8	0	0.00	8	0	0.00	8	0	0.00	0.00	0.00
		11[b]	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
		12[b]	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
		13	8	0	0.00	8	0	0.00	8	0	0.00	8	0	0.00	0.00	0.00
		14	8	0	0.00	8	0	0.00	8	0	0.00	8	0	0.00	0.00	0.00
		15A	8	0	0.00	8	0	0.00	8	0	0.00	8	0	0.00	0.00	0.00
		15B	8	0	0.00	8	0	0.00	8	0	0.00	8	0	0.00	0.00	0.00
	MPN/test portion	16	8	0	0.00	8	0	0.00	8	0	0.00	8	0	0.00	0.00	0.00
Estimate	0.00	All	80	0	0.00	80	0	0.00	80	0	0.00	80	0	0.00	0.00	0.00
LCL	0.00				0.00			0.00			0.00			0.00	−0.03	−0.05
UCL	0.00				0.05			0.05			0.05			0.05	0.03	0.05
s_r[c]					0.00			0.00			0.00			0.00		
LCL					0.00			0.00			0.00			0.00		
UCL					0.21			0.21			0.21			0.21		
s_L[d]					0.00			0.00			0.00			0.00		
LCL					0.00			0.00			0.00			0.00		
UCL					0.21			0.21			0.21			0.00		
s_R[e]					0.00			0.00			0.00			0.00		
UCL					0.00			0.00			0.00			0.29		
LCL					0.29			0.29			0.29			0.00		
P_T[f]					1.0000			1.0000			1.0000			1.0000		
Low inoculum level																
	RTE deli turkey	1[b]	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
		2	12	5	0.42	12	5	0.42	12	5	0.42	12	3	0.25	0.00	0.17
		3[b]	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
		4[b]	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
		5[b]	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
		6[b]	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
		7	12	8	0.67	12	9	0.75	12	9	0.67	12	7	0.58	−0.08	0.08
		8	12	6	0.50	12	6	0.50	12	6	0.50	12	4	0.33	0.00	0.17
		9	12	7	0.58	12	7	0.58	12	7	0.58	12	5	0.42	0.00	0.17

(continued)

Table 2025.04B. (continued)

			Candidate presumptive (CP)			Candidate confirmed (CC)			Candidate result (C)			Reference method (R)			C vs. R	CP vs. CC
Statistic	**Matrix**	**Collaborator**	**N**	**X**	**POD (CP)**	**N**	**X**	**POD (CC)**	**N**	**X**	**POD (C)**	**N**	**X**	**POD (R)**	**dLPOD (C, R)**	**dLPOD (CP, CC)**
		Uninoculated control														
		10	12	7	0.58	12	7	0.58	12	7	0.58	12	9	0.75	0.00	−0.17
		11[a]	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
		12[b]	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
		13	12	3	0.25	12	3	0.25	12	3	0.25	12	5	0.42	0.00	−0.17
		14	12	7	0.58	12	7	0.58	12	7	0.58	12	6	0.50	0.00	0.08
		15A	12	6	0.50	12	6	0.50	12	6	0.50	12	9	0.75	0.00	−0.25
		15B	12	3	0.25	12	3	0.25	12	3	0.25	12	3	0.25	0.00	0.00
	MPN/test portion	16	12	3	0.25	12	3	0.25	12	3	0.25	12	2	0.17	0.00	0.08
Estimate	0.70	All	120	55	0.46	120	56	0.46	120	56	0.46	120	53	0.44	0.02	−0.01
LCL	0.52				0.37			0.36			0.37			0.33	−0.13	−0.04
UCL	0.90				0.55			0.58			0.55			0.56	0.17	0.02
s_r[c]					0.49			0.49			0.49			0.44		
LCL					0.44			0.44			0.44			0.31		
UCL					0.52			0.52			0.52			0.57		
s_L[d]					0.07			0.10			0.10			0.15		
LCL					0.00			0.00			0.00			0.00		
UCL					0.25			0.28			0.28			0.35		
s_R[e]					0.50			0.50			0.50			0.50		
UCL					0.45			0.45			0.45			0.45		
LCL					0.52			0.52			0.52			0.52		
P_T[f]					0.2820			0.1692			0.1692			0.0320		
		High inoculum level														
	RTE deli turkey	1[b]	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
		2	8	8	1.00	8	8	1.00	8	8	1.00	8	8	1.00	0.00	0.00
		3[b]	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
		4[b]	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
		5[b]	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
		6[b]	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
		7	8	8	1.00	8	8	1.00	8	8	1.00	8	8	1.00	0.00	0.00
		8	8	8	1.00	8	8	1.00	8	8	1.00	8	8	1.00	0.00	0.00
		9	8	8	1.00	8	8	1.00	8	8	1.00	8	8	1.00	0.00	0.00
		10	8	8	1.00	8	8	1.00	8	8	1.00	8	8	1.00	0.00	0.00
		11[b]	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
		12[b]	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
		13	8	8	1.00	8	8	1.00	8	8	1.00	8	8	1.00	0.00	0.00
		14	8	8	1.00	8	8	1.00	8	8	1.00	8	8	1.00	0.00	0.00
		15A	8	8	1.00	8	8	1.00	8	8	1.00	8	8	1.00	0.00	0.00
		15B	8	8	1.00	8	8	1.00	8	8	1.00	8	8	1.00	0.00	0.00

(continued)

Table 2025.04B. (continued)

			Uninoculated control													
			Candidate presumptive (CP)			Candidate confirmed (CC)			Candidate result (C)			Reference method (R)			C vs. R	CP vs. CC
Statistic	Matrix	Collaborator	N	X	POD (CP)	N	X	POD (CC)	N	X	POD (C)	N	X	POD (R)	dLPOD (C, R)	dLPOD (CP, CC)
	MPN/test portion	16	8	8	1.00	8	8	1.00	8	8	1.00	8	8	1.00	0.00	0.00
Estimate	9.25	All	80	80	1.00	80	80	1.00	80	80	1.00	80	80	1.00	0.00	0.00
LCL	4.00				0.95			0.95			0.95			0.95	−0.03	−0.05
UCL	22.20				1.00			1.00			1.00			1.00	0.03	0.05
s_r[c]					0.00			0.00			0.00			0.00		
LCL					0.00			0.00			0.00			0.00		
UCL					0.21			0.21			0.21			0.21		
s_L[d]					0.00			0.00			0.00			0.00		
LCL					0.00			0.00			0.00			0.00		
UCL					0.21			0.21			0.21			0.00		
s_R[e]					0.00			0.00			0.00			0.00		
UCL					0.00			0.00			0.00			0.29		
LCL					0.29			0.29			0.29			0.00		
P_T[f]					1.0000			1.0000			1.0000			1.0000		

[a] N = Number of test portions; X = number of positive test portions; LCL = lower confidence limit; UCL = upper confidence limit.
[b] NA = Collaborator did not complete testing.
[c] Repeatability standard deviation.
[d] Among-laboratory standard deviation.
[e] Reproducibility standard deviation.
[f] PT-value = Homogeneity test of laboratory PODs.

(3) Transfer 200 µL from the upper third of sample enrichment into 2.0 mL microcentrifuge tube containing the lysis buffer.
(4) Vortex the microcentrifuge tube briefly and place into heating block of a dry bath incubator. Incubate tube in heating block at 95 °C for 10 min without shaking.
(5) Remove the tube from heating block and allow to cool at room temperature for 1 min.
(6) After allowing the tubes to cool to room temperature, proceed to DNA extraction.
(7) The lysate is ready-to-use for PCR. If it is not used immediately, it can be stored at 2–8 °C for up to 4 h. If it is intended for a longer storage, transfer 100 µL of the lysate in a new tube (not provided with the kit) and store at −20 °C.

Note: It is necessary to ensure that the food particulate is not stirred up and no particles are pipetted in the PCR reaction. If the particles do not sediment, the lysate should be centrifuged for 1 min at 12 000 rpm. Afterwards transfer 100 µL of the supernatant into a new 2 mL microcentrifuge tube.

(b) *DNA Extraction—SureFast PREP Bacteria Kit Method*

(1) Before analysis, add 11 mL of isopropanol (≥99.7% purity) to the Binding Buffer and mix thoroughly.
(2) After adding isopropanol, the shelf life of the Binding Buffer is as indicated on the label, if stored at 14–25 °C.
(3) Before analysis, add 42 mL of ethanol (≥96% purity) to the Wash Buffer and mix thoroughly.
(4) After adding ethanol, the shelf life of the Wash Buffer is as indicated on the label, if stored at 14–25 °C.
(5) Transfer the needed amount of elution buffer into a new 1.5 mL microcentrifuge tube (100 µL per sample, suggested to also include a 10% excess). Preheat the elution buffer to 60 °C in a dry bath incubator.
(6) Mix enrichment. Allow sample enrichment to settle for 5–10 min.
(7) Transfer 1.0 mL of sample enrichment into a 1.5 mL microcentrifuge tube.
(8) Centrifuge tubes for 5 min at 12 000 rpm.
(9) Discard the supernatant by either disposing or removing by aspirating with a micropipet.
(10) Add 400 µL of Lysis Buffer; cap tube. Mix briefly by vortexing. Place tubes in thermomixer with heating block. Incubate at 99 °C for 10 min with continual shaking.
(11) Centrifuge sample lysate for 1 min at 12 000 rpm.
(12) Transfer 300 µL of the supernatant into a new 1.5 mL microcentrifuge tube.

Note: Depending on the nature of the sample, a smaller amount of liquid may be obtained after centrifugation. In this case, transfer the actual volume for the following steps or increase the volume of the Lysis Buffer before lysis.

(13) Add 200 µL of Binding Buffer to the supernatant; cap tube. Mix briefly by vortexing.
(14) Place a Spin Filter into a 2.0 mL Receiver tube. Transfer the entire mixed sample from step (13) directly onto the filter. Incubate at room temperature for 1 min.
(15) Centrifuge Spin Filter with Receiver Tube for 1 min at 12000 rpm.
(16) After centrifugation, discard the filtrate and place the Spin Filter back into the Receiver tube.
(17) Add 550 µL of Wash Buffer to the Spin Filter and centrifuge for 1 min at 12 000 rpm. Discard the filtrate and place the Spin Filter back into the Receiver Tube.
(18) Add 550 µL of Wash Buffer again to the Spin Filter and centrifuge for 1 min at 12 000 rpm. Discard the filtrate and place the Spin Filter back into the Receiver Tube.
(19) Remove the residual ethanol by centrifuging for 2 min at 12 000 rpm.
(20) Place the Spin Filter into a 1.5 mL Receiver tube and add 100 µL of the preheated (60 °C) Elution Buffer directly onto the filter. Incubate Spin Filter device at room temperature for 3 min.
(21) Centrifuge the Elution Buffer from step (q) for 1 min at 10 000 rpm. After centrifugation, discard the Spin Filter.
(22) The eluted DNA is ready-to-use for PCR analysis. The DNA can be used immediately or stored for up to 24 h at 2–8 °C. For a storage time of more than 24 h, it should be kept at −20 °C.

(c) *Real-Time PCR Detection—SureFast Listeria 3plex ONE Kit*

(1) Calculate the total number of reactions needed to analyze the DNA lysates and the controls. Prepare the real-time PCR mix. *See* Table 2025.04C for the calculation of the master mix.

Required control reactions for the specific PCR assay: negative control, extraction control, and positive control. For the analysis of enrichments, additional controls are needed: zero control (enrichment before incubate) and medium control. *Note*: It is recommended to prepare the mix with a 10% additional volume to compensate for any reagent loss.

(2) Pipet 20 µL of the master mix into appropriate PCR tubes/wells.
(3) Close the negative control well (it is ready for PCR without any addition).
(4) Pipet 5 µL of sample DNA into the designated PCR tubes/wells and close them.
(5) Pipet 5 µL of positive control into the designated tubes/wells and close them.
(6) Centrifuge all tubes/plates briefly at low speed (500 rpm for 10 s).
(7) Place tubes/plates into the real-time PCR instrument (Bio-Rad CFX96 Deep Well, Bio-Rad Opus Deep Well, or R-Biopharm RIDA CYCLER) and start the run according to the setup as provided in the instructions for use (IFU).

Note: Description of the controls:

Negative control.—No additional material aside from master-mix.

Extraction control.—The extraction is performed without the sample—use only Lysis Buffer.

Positive control.—Master-mix and within the kit's provided Positive Control.

Zero control.—Master-mix and sample prior incubate enrichment.

Table 2025.04C. Example for the calculation and preparation of 10 real-time PCR reactions

Components of the master mix	Amount per reaction	10 reactions (including 10% excess)
Reaction mix, µL	19.3	212.3
Taq polymerase, µL	0.7	7.7
Total vol., µL	20	220

Medium control.—Only medium—no sample.

(d) *Interpretation and test result report.*

(1) When the analysis is complete, the result interpretation must be made according to the usual analysis program recommended by the real-time PCR instrument manufacturer.

(2) The control reactions must show the correct results. *Listeria* spp. are detected in the ROX channel. *L. monocytogenes* is detected in the Cy5 channel. The amplification control is detected in the VIC/HEX channel. Detection of *L. monocytogenes* must also detect *Listeria* spp. in ROX channel.

(3) A sample is positive for the respective parameter if the sample shows amplification in the respective channel. High amplicon concentrations can result in a weak or absent signal of the internal amplification control (IAC). A Cp value for the IAC is not needed to obtain a positive result of the positive control.

(4) A sample is negative for the respective parameter if the sample DNA shows no amplification in the respective channel and if the internal control (VIC/HEX channel) of the sample is positive.

(5) If the sample DNA in the VIC/HEX channel shows no amplification or sigmoidal amplification curve in the VIC/HEX channel, this indicates that inhibitors are present in the sample DNA that inhibit the PCR. Under these circumstances, DNA isolation and purification of the sample needs to be improved. It is recommended to centrifuge the lysate before the PCR analysis or to repeat the extraction process. Alternatively, the DNA can be diluted (recommendation is 1:2 in PCR–water) and analyzed again for inhibition.

Note: The dilution factor also affects the detection limit of the specific *Listeria* spp. and *Listeria monocytogenes* PCR assay.

(6) Final interpretation of results is summarized as outlined in Table 2025.04D:

F. Confirmation

Confirm all enrichments according to the ISO 11290-1:2017 reference standard.

G. Result Reporting

Report all results as either target (*Listeria* spp. and/or *L. monocytogenes*) detected or not detected per test portion analyzed. Follow the interpretation of results as outlined previously in section **E(d)** above.

Results

Results of Multi-Laboratory Validation Study

The MLV study involved a method comparison evaluation of the SureFast Listeria 3plex ONE Kit to the ISO 11290-1:2017 reference standard for RTE smoked deli turkey. A total of 17 participants throughout Austria, Germany, Italy, Spain, and the United States were sent samples. However, only 10 participants submitted data. For one of the laboratories, two separate analysts analyzed two sets of samples, whereas the remaining laboratories each had one participating analyst. Out of the 17 participants, 10 participants submitted data. There were seven laboratories that did not receive samples due to customs issues and were unable to participate. For the laboratories that received samples, each participant analyzed 28 unpaired test portions for the SureFast Listeria 3plex ONE Kit and ISO 11290-1:2017 reference standard: 8 inoculated with a high level of *L. monocytogenes*, 12 inoculated with a low level of *L. monocytogenes*, and 8 uninoculated controls. In addition to the test portions, all participants set up an aerobic plate count (APC) to determine the total microbial load of test matrix. The average APC result obtained by the collaborators was 1.9×10^9 CFU/g. The highest count documented out of all of the participants was 1.2×10^{10} CFU/g and the lowest was 3.0×10^5 CFU/g.

Five replicate 25 g test portions (randomly sampled from 50% of the total packages used in the analysis) were screened following the ISO 11290-1:2017 reference standard for the presence of *Listeria* spp. and *L. monocytogenes*. All test portions produced negative results for the target analyte.

Table 2025.04A summarizes the multi-laboratory results. In accordance with criteria outlined in Appendix J of the AOAC Validation Guidelines, fractional positive results were obtained. Detailed results for each laboratory are presented in Table 2025.04B. The level of *L. monocytogenes* was determined by MPN on the day of initiation of analysis by the coordinating laboratory. The MPN levels obtained, with a 95% confidence interval, were 0.70 CFU/test portion (0.52, 0.90) for the low inoculum level and 9.25 CFU/test portion (4.00, 22.20) for the high inoculum

Table 2025.04D. Final interpretation of real-time PCR results

Result in the respective channel			
ROX channel *Listeria* spp.	**Cy5 channel *L. monocytogenes***	**VIC/HEX channel IAC**	**Interpretation**
Positive	Negative	Positive/negative	*Listeria* spp. DNA detected
Positive	Positive	Positive/negative	*Listeria monocytogenes* DNA detected
Negative	Negative	Positive	Negative, *Listeria* DNA not detected
Negative	Positive	Positive/negative	Invalid
Negative	Negative	Negative	Invalid
Negative	Positive	Positive	Questionable[a] for *Listeria* monocytogenes—DNA

[a] Result for *Listeria monocytogenes* is questionable and analysis for this sample will be repeated. This can appear with low levels of target at the limit of detection.

level. MPN results are presented in the second column of Table 2025.04B.

RTE Smoked Deli Turkey

Detailed results of the LPOD statistical analysis are presented in Table 2025.04B.

For the low inoculation level, 55 out of 120 test portions ($LPOD_{CP}$ of 0.46) were reported as presumptive positive by the SureFast Listeria 3plex ONE Kit with 56 out of 120 test portions ($LPOD_{CC}$ of 0.46) confirming positive. For the reference method, 53 out of 120 test portions were reported as positive ($LPOD_R$ of 0.44). A $dLPOD_C$ value of 0.02 with 95% confidence interval of (−0.13, 0.17) was obtained between the candidate and reference method, indicating no statistically significant difference between the two methods. A $dLPOD_{CP}$ value of −0.01 with 95% confidence intervals of (−0.04, 0.02) was obtained between presumptive and confirmed results indicating no statistically significant difference between the presumptive and confirmed results.

For the high inoculation level, 80 out of 80 test portions ($LPOD_{CP}$ of 1.00) were reported as presumptive positive by the SureFast Listeria 3plex ONE Kit. There were 80 out of 80 reported test portions ($LPOD_{CC}$ of 1.00) that confirmed positive. For samples that produced presumptive positive results by the SureFast Listeria 3plex ONE Kit, 80 out of 80 samples confirmed positive ($LPOD_C$ of 1.00). For the reference method, 80 out of 80 test portions were reported as positive ($LPOD_R$ of 1.00). A $dLPOD_C$ value of 0.00 with 95% confidence interval of (−0.03, 0.03) was obtained between the candidate and reference method, indicating no statistically significant difference between the two methods. A $dLPOD_{CP}$ value of 0.00 with 95% confidence intervals of (−0.05, 0.05) was obtained between presumptive and confirmed results, indicating no statistically significant difference between the presumptive and confirmed results.

For the uninoculated controls, 0 out of 80 samples ($LPOD_{CP}$ of 0.00) produced a presumptive positive result by the SureFast Listeria 3plex ONE Kit with 0 out of 80 test portions ($LPOD_{CC}$ of 0.00) confirming positive. There were 0 out of 80 samples that produced a presumptive positive result by the SureFast Listeria 3plex ONE Kit that confirmed positive ($LPOD_C$ of 0.00). For the reference method, 0 out of 80 test portions were reported as positive ($LPOD_R$ of 0.00). A $dLPOD_C$ value of 0.00 with 95% confidence interval of (−0.03, 0.03) was obtained between the candidate and reference method, indicating no statistically significant difference between the two methods. A $dLPOD_{CP}$ value of 0.00 with 95% confidence intervals of (−0.05, 0.05) was obtained between presumptive and confirmed results, indicating no statistically significant difference between the presumptive and confirmed results.

Discussion

No negative feedback was provided with regard to the performance of SureFast Listeria 3plex ONE Kit by the collaborators. One laboratory, Laboratory 7, reported negative results for both the *Listeria* spp. and *L. monocytogenes* channels for one low-level sample.

Out of the 280 samples analyzed, only one result was discrepant. The discrepant sample was reextracted and analyzed with a new lysate. In addition, a third extraction from the secondary Fraser broth enrichment was also conducted. The repeat analysis reproduced the original results from the primary enrichment. However, the analysis from the secondary enrichment produced a presumptive positive result. Because a presumptive positive result was obtained only from the secondary enrichment, the final result was considered a false negative. Results of the reanalysis are shown in Table 2025.XXE.

Table 2025.04E. Results of the reanalysis of one low inoculation level sample conducted in laboratory 7 during the multi-laboratory study.

Original lysate	New lysate (from HF)	New lysate (from FB)	Confirmed result
−/−	−/−	+/+ (23.90 LS and 24.90 LM)	*L. monocytogenes*

Overall, the data generated during this evaluation demonstrate the reproducibility of this method. No statistically significant differences were observed between the presumptive method and the confirmed results.

CRediT Author Statement

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Acknowledgments

We would like to extend a sincere thank you to these collaborators for their dedicated participation in this study: Bill Centrella, AFL Texas, Grand Prairie, Texas, USA; Izaskun Martínez Pizarro, ALKEMI S.A., AGQ Labs, Madrid, Spain; Ute Pfähler, Analytisches Labor BOSTEL GmbH, Stuttgart, Germany; Christine Wind, Oliver Wach, Else Brand, Chemisches und Veterinäruntersuchungsamt Karlsruhe Abt.6/MIB, Karlsruhe, Germany; Remigio Berto, EPTA NORD, Italy; Cristina Borghetti, Giuseppe Citterio Salumificio spa., Italy; Jean Pierre Studer, INLAB Solution S.R.L.B7, Italy; Kathrin Amplatz, Institut für Lebensmittelsicherheit Innsbruck AGES, Österreichische Agentur für Gesundheit und Ernährungssicherheit GmbH, Austria; Mikel Arzubialde, ITSAS NATURA-NATURALEZA MARINA S.L., Spain; María Delia Fernández Gutiérrez, LABORATORIOS MICROAL S.L., Spain; Alessandro Pierasco, Dott.ssa Claudia Caneto, Lifeanalytics, Italy; Antonia Clippard, Stephanie Kahms, Peter Sanders, R-Biopharm AG Darmstadt, Darmstadt, Germany; Pilar Docio Solé, SEGURALIMENT S.L., Spain; Leslie Thompson-Strehlow and Jesus Sanchez, SGS North America, North Sioux City, SD, USA; Jerri Lynn Pickett, Tyson Foods, Inc. Corporate Food Safety Lab, Springdale, AR, USA; Ben Bastin, Q Laboratories, Inc., Cincinnati, OH, USA. We would like to extend special thanks to all team members of the Microbiology Research and Development Laboratory at Q Labs, LLC for their incredible efforts during the MLV study.

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Journal of AOAC International, 2026, 00, 1–13
https://doi.org/10.1093/jaoacint/qsag015
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