Validation of the PhageDx™ Listeria Assay for Detection of Listeria spp. on Stainless Steel and Ceramic Environmental Surfaces AOAC Performance Tested MethodSM 102005

Minh Nguyen¹, Jose Gil², Wendy Hahn¹, Thai Dong¹, John Paulson¹, Dustin Stevens¹, Henriett Zahn¹, Brinna Zimmer¹, and Steve Erickson¹

¹Laboratory Corporation of America®/MedTox, 402 County Road D West, St. Paul, MN 55112
²Laboratory of America Corporation®/National Genetics Institute, 2440 Sepulveda Blvd., Suite 235, Los Angeles, CA 90064

Corresponding author’s e-mail: Nguyem5@LabCorp.com

Abstract

Background: The PhageDx™ Listeria Assay is a simple, specific, and sensitive assay based on the infection of Listeria spp. by selected bacteriophages and the resultant expression of a luciferase reporter gene. Results are generated in as little as 24.5 h for stainless steel and ceramic environmental surfaces.

Objective: An AOAC Performance Tested MethodsSM (PTM) study was conducted to validate the PhageDx™ Listeria Assay for the detection of Listeria on stainless steel and ceramic surfaces.

Methods: The performance of the PhageDx™ method was compared to that of the FDA/ BAM Ch. 10. Inclusivity/exclusivity, product consistency and stability, and robustness testing also were conducted.
**Results**: Inclusivity testing demonstrated that the reporter bacteriophages were specific for *Listeria* ssp. and detected 58/61 *Listeria* strains tested, including all 34 *Listeria monocytogenes* strains. The reporter bacteriophage also was shown to not detect 46/47 non-*Listeria* bacteria in exclusivity testing. Robustness testing showed that the method performed well with specific deviations from the standard protocol. Consistency and stability testing demonstrated that the recombinant phage gave consistent results across three production lots and was stable when stored under appropriate conditions for at least 6 months. Matrix studies on stainless steel and ceramic surfaces showed that there was no significant difference between the PhageDx™ *Listeria* Assay and the FDA/BAM Chapter 10 reference methods.

**Conclusions and Highlights**: The validation study demonstrates that the PhageDx™ *Listeria* Assay is an effective method for the detection of *Listeria* spp. on stainless steel and ceramic environmental surfaces and meets the qualifications for AOAC PTM status.

**General Information**

*Listeria monocytogenes* is a bacterial pathogen that can be found in moist environments such as soil, water, and decaying vegetation and when ingested, most commonly through eating contaminated food, can cause listeriosis. In the U.S., listeriosis is estimated to affect approximately 1600 people resulting in about 260 deaths annually. Symptoms of infection include diarrhea, vomiting, nausea, headaches, stiff neck, confusion, loss of balance, convulsions, fever and muscle aches. Populations that are particularly susceptible to the effects of listeriosis are pregnant women and their newborns, older adults (65 + years) and people with weakened immunity and where infections can result in death. Infections in
pregnant women can also result in miscarriage, stillbirth, premature delivery or life threatening infant infections (1, 2).

Common food sources of Listeria contamination include deli meats, dairy products, and produce. *Listeria* spp. can survive in a wide range of conditions typically used in food preservation such as extremes in temperature and pH and high salt concentrations. Food contamination commonly occurs via transfer from contaminated surfaces that the food comes in contact with (1, 2). Thus, an essential measure to prevent Listeria contamination is to monitor sanitation of food preparation areas. The PhageDx™ *Listeria* Assay provides all the reagents necessary for sample infection, lysis, and luciferase detection. After a 20–24 h enrichment of the test portions in Buffered Listeria Enrichment Broth (BLEB) at 35 ± 1°C for stainless steel and ceramic surfaces (4" x 4" test area). A portion of the enriched sample is placed in a well of a 96-well break-away plate and infected with the PhageDx™ *Listeria* Recombinant Phage at 30°C for 4 hours. Then, lysis/luciferase substrate master mix is added to the infected sample and read on a luminometer for signal measurement. Results are expressed as Relative Light Units or RLU. Total sample handling time is approximately 30 minutes.

**Principle of the Method**

The PhageDx™ *Listeria* Assay is a simple, specific, and sensitive diagnostic test for *Listeria* on ceramic and stainless steel environmental surfaces. The kit is designed to be used with a Promega luminometer and the clear results indicate which samples contain viable *Listeria*. The assay consists of 5 basic steps; sample collection, enrichment, phage infection, substrate addition, and signal read. Presumptive results are available in 24.5 h which includes a 20–24 h enrichment step and a 4 h infection step. The PhageDx™
Listeria Assay is designed to be performed by qualified laboratory personnel in laboratories performing microbiological analysis.

The PhageDx™ Listeria Assay is based on the infection of Listeria spp. by bacteriophages and replication of the infecting bacteriophages within their specific hosts. Bacteriophages demonstrate a high specificity for their bacterial host and are capable of replicating within their host quickly to high numbers. The recombinant phages used in the PhageDx™ Listeria Assay also express a luciferase reporter during replication. The presence of Listeria spp. is determined by incubating the lysate with the appropriate luciferase substrate and detecting emitted light in a luminometer. An absence of detected light above background indicates that no viable Listeria are present in that sample.

Scope of method

(a) Target organism.—Listeria spp. [L. monocytogenes (1/2a, 1/2b, 1/2c, 3a, 4a, 4b, 4c, 4d, 4e), L. innocua, L. ivanovii, L. seeligeri, L. welshimeri, L. grayi].

(b) Matrix.—Stainless steel and ceramic surfaces (4” X 4”).

(c) Summary of Validated Performance Claims.—Performance comparable to that of the U.S. Food and Drug Administration Bacterial Analytical Manual Chapter 10, Detection of Listeria monocytogenes in Foods and Environmental Samples, and Enumeration of Listeria monocytogenes in Foods (FDA/BAM Chapter 10; 3).

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: $\text{POD}_R$ (reference method POD), $\text{POD}_C$ (confirmed candidate method POD), $\text{POD}_{cp}$ (candidate method presumptive result POD) and $\text{POD}_{cc}$ (candidate method confirmation result POD).

(b) **Difference of Probabilities of Detection (dPOD).**—Difference of probabilities of detection 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.**—PhageDx™ Listeria Assay.

(b) **Catalog Number.**—5013.

(c) **Ordering Information.**—Not applicable. For internal use at Laboratory Corporation of America only.

**Test Kit Components**

(a) **PhageDx™ Listeria recombinant phage.**—Part No. 3401, 12 tubes containing 100 µL Phage solution.
(b) **Lysis buffer.**—Part No. 3010, 12 tubes containing 150 µL Lysis buffer.

(c) **Assay buffer.**—Part No. 3003, 12 tubes containing 500 µL Assay buffer.

(d) **Luciferase substrate.**—Part No. 3004, 12 tubes containing 10 µl Luciferase substrate.

(e) **96-well break-apart plate.**—Part No. 3005, one pouch containing white break-apart plate (8 wells × 12 strips).

(f) **Plate sealing tape.**—Part No. 3011, 2 pieces per kit.

(g) **One package insert.**—Part No. 3402.

**Additional supplies and reagents**

(a) **Sample sponge/bag.**—Polyurethane sponge with Letheen, EZ Reach™ Polyurethane Sponge Sampler with Letheen (World Bioproducts, Cat. No. EZ18FR-10LET-PUR).

(c) **Racks for holding sample bag.**

(d) **Buffered Listeria Enrichment Broth.**—(Thermo Scientific Remel™ Listeria Enrichment Broth, Cat. No. R453692)

(e) **Sterile filtered tips.**—For sampling and delivering of 10 µL–1000 µL.

(f) **Appropriate personal protective equipment.**—See [https://www.cdc.gov/labs/pdf/CDC-BiosafetyMicrobiologicalBiomedicalLaboratories-2009-P.PDF](https://www.cdc.gov/labs/pdf/CDC-BiosafetyMicrobiologicalBiomedicalLaboratories-2009-P.PDF).

For confirmation (optional)

(g) **RAPID’ Listeria plates.**—Bio-Rad RAPID’ L. mono Agar, Cat. No. 3564293; RAPID’ L. mono supplement 1, Cat. No. 3564294; RAPID’ L. mono supplement 2, Cat. No. 3564746.
Apparatus

(a) **Adjustable single channel pipettors.**—For sample collection and delivery (10 µL–1000 µL)

(b) **Vortex mixer.**

(c) **Air incubators capable of 30 ± 1°C, 35 ± 1°C, and 37 ± 1°C.**

(d) **Promega GloMax® 96 or Navigator luminometer.**

(e) **Personal computer for luminometer control and data analysis.**

Safety Precautions

(a) The PhageDx™ *Listeria* Assay involves the enrichment of samples which may contain human pathogenic *Listeria* and have the potential for contamination with subsequent handling of those samples. This method should be conducted by properly trained laboratory personnel in a suitable microbiology laboratory in accordance with “Biosafety in Microbiological and Biomedical Laboratories”, U.S. Department of Health and Human Services, [https://www.cdc.gov/labs/pdf/CDC-BiosafetyMicrobiologicalBiomedicalLaboratories-2009-P.PDF](https://www.cdc.gov/labs/pdf/CDC-BiosafetyMicrobiologicalBiomedicalLaboratories-2009-P.PDF). Care should be taken when handling the sample and reagents while performing the method.

(b) Materials and reagents provided in the PhageDx™ *Listeria* Assay are not considered hazardous if used according to the assay method. Please review the Material Safety Data Sheet prior to performing the assay.

(c) Follow all relevant guidelines and laboratory protocols while performing the assay and manufacturer’s equipment instructions.
**General Preparation**

(a) Prepare BLEB media according to manufacturer’s instructions.

(b) Before using the reagents, flick or spin the tube to collect all of the solution at the bottom of the tube.

(c) Before adding the pre-warmed BLEB to the sample, confirm that the media and incubator are warmed to 35 ± 1°C. Do not allow the pre-warmed media to cool before adding to the sample. Maintain the media at 35 ± 1°C in an incubator or water bath if preparing multiple samples. It is important to maintain the temperature of the sample and BLEB media used in the enrichment incubation.

(d) Prepare the Promega luminometer by following the manufacturer’s cleaning procedure and make sure there are no signal “hot spots” that will affect the sample results by reading an empty plate. All signals should be less than 20 Relative Light Units (RLU). Set up the luminometer to read the appropriate wells on the plate using the pre-programmed PhageDx™ Assay protocol or alternatively, set-up a read protocol with a 3 minute or 180 second delay between starting the program and the start of the signal reads and 2 plate read runs with a 1 second integration per sample.

**Sample Preparation**

(a) For each surface type, use the EZ Reach Polyurethane Sponge Sampler to sample each test area. Swab environmental surfaces using firm and even pressure vertically (approximately 10 times), then flip the sampler and use the other side to swab horizontally (approximately 10 times) and
diagonally (approximately 10 times). Replace sponge back into sample bag and break off and discard handle. Store at room temperature (20–25°C) for 2 h prior to enrichment.

(b) Add 20 mL of BLEB media pre-warmed to 35 ± 1°C and massage sponge for 10–20 s. Incubate sponge at 35 ± 1°C for 20–24 h.

(c) Remove the enriched samples from the incubator and massage the sponge in media for 10–20 s. Keeping the sponge in the bag, move sponge away from media and squeeze media from sponge and gently mix media in the bag. **Note:** It is critical that the analyte is released into the media and evenly distributed throughout the sample.

(d) Immediately after mixing the media, using a single channel pipettor and fresh sterile tip for each sample, transfer 150 µL of the enriched sample into a well of the 96-well plate, one well per sample. **Note:** If continuing to confirmation assay, replace sponge into the media and continue to enrich at 35°C ± 1 for an additional 4-8 hours for a total of 24-28 ± 2 hours.

(e) Remove one tube containing the recombinant phage solution for each 8 well strip used. Flick or spin the tube to collect all of the solution at the bottom of the tube. Add 10 µL of recombinant phage to the 150 µL sample and mix thoroughly by gently pipetting up and down, being careful not to introduce bubbles. Cover samples with plate sealing tape to prevent evaporation and cross contamination and place the 96-well plate in the 30 ± 1°C incubator for 4 h.

(f) Remove one tube containing the lysis buffer, assay buffer, and substrate for each 8 well strip used and thaw to room temperature. Flick or spin the tubes to collect all of the solution at the bottom of the tubes. Prepare the lysis/luciferase master mix by transferring the entire contents of assay buffer (0.5 mL) and lysis buffer (150 µL) tubes to the substrate tube (10 µL) and mix well by either vortexing or inverting the tube several times. **NOTE:** Use within 1 h of preparation.
(g) Using a clean tip for each sample, add 65 µL of the lysis/luciferase master mix to each well using a single channel pipettor. Mix thoroughly by pipetting up and down, being careful not to introduce bubbles.

(h) Once all of the samples have received the lysis/luciferase master mix, place the sample plate in the luminometer, close the lid, and initiate the read program.

Interpretation and Test Result Report

(a) The luminometer program will display the results on the screen as relative light unit (RLU) values corresponding to the well positions of the break-apart plate.

(b) Samples positive for *Listeria* will have a reading value of 300 RLU or greater. Negative samples will be less than 300 RLU.

(c) Once all of the samples have been run and analyzed, remove the plate from the luminometer and follow the manufacturer’s instructions for cleaning the instrument and shut down.

*Note: In some cases, the PhageDx™ Listeria Assay will generate a very high signal and result in adjacent wells to read as a false positive due to the bleed over signal from the well with high signal. If a sample well is positive and has a 1000X lower signal than the adjacent sample well with a higher signal, (typically in the range of 300-2000 RLU), this could be a result of bleed over signal. In these cases, we recommend that the contents of the potential false positive well (lower RLU sample) be transferred to a*
new well at least 2-3 well distance from the high signal well or to a new strip and re-read to confirm that the signal is from the sample and not a result of bleed over signal.

**Confirmation**

We recommend that presumptive positives from the phage assay be confirmed.

(a) To confirm, continue to enrich sponge samples for an additional 4–8 h for a total of 24–28 h at 35 ± 1°C. This is can be done concurrently with the 4 h phage infection step. Sponge samples that were positive with the phage assay can then proceed to the next steps.

(b) Massage sponge and contents of sample bag vigorously for 10-20 s to mix contents well. Remove a 100 µL aliquot and streak onto RAPID’ *Listeria* plates (Bio-Rad RAPID’ *L. mono* Agar, Cat. No. 3564293; RAPID’ *L. mono* supplement 1, Cat. No. 3564294; RAPID’ *L. mono* supplement 2, Cat. No. 3564746) and incubate for an additional 24-48 h at 37 ± 1°C. *L. monocytogenes* forms blue (pale blue, grey-blue, or dark blue) colonies. *L. ivanovii* forms blue-green colonies with a yellow halo. Other *Listeria* spp. form white colonies with or without a yellow halo. Refer to manufacturer’s product insert for detailed description.

(c) Confirm plate test results by testing individual colonies using approved qPCR tests. Alternatively, the user may use another approved reference method confirmation procedure.

**Validation Study**

This validation study was conducted under the AOAC Research Institute *Performance Tested*
Method\textsuperscript{SM} program and the AOAC INTERNATIONAL Methods Committee Guidelines for Validation of Microbiological Methods for Food and Environmental Surfaces, Appendix J (4). Method developer studies were conducted in the laboratories of Laboratory Corporation of America Holdings, and included the inclusivity/exclusivity study, product consistency and stability studies, and robustness testing. The Independent Laboratory Study was conducted by Q Laboratories, Inc., and included inclusivity and exclusivity studies for selected strains and matrix studies for all claim matrixes.

Method Developer Studies

Inclusivity and Exclusivity Studies

Inclusivity (Listeria) and exclusivity strains were obtained from academic, governmental, and commercially available sources. A total of 61 Listeria strains were tested for the inclusivity study, 54 strains by LabCorp and an additional 7 by Q Laboratories. For inclusivity testing done at LabCorp, 54 Listeria species covering \textit{L. monocytogenes} serotypes 1/2a, 1/2b, 1/2c, 3a, 4a, 4b, 4c, 4d, and 4e, \textit{L. innocua}, \textit{L. ivanovii}, \textit{L. seeligeri}, \textit{L. welshimeri}, \textit{L. grayi} strains were examined. Each strain was grown at 35 ± 1°C for 20–24 h in BLEB medium. The cultures were then diluted to approximately 100 times the LOD\textsubscript{50} of the PhageDx™ Listeria Assay transferred to a 96-well plate.

A total of 47 non-Listeria strains were tested for the exclusivity study, 45 strains by LabCorp and 3 by Q Laboratories (\textit{Enterococcus faecalis} ATCC 29212 tested by both LabCorp and Q Laboratories). For exclusivity testing done at LabCorp, 45 different strains of non-Listeria species and strains were grown under optimal enrichment conditions suitable to the strain type (medium and temperature) for a
minimum of 20–24 h. One hundred microliters of the undiluted cultures were transferred to 96-well plate.

For both inclusivity and exclusivity studies, samples were infected with 10 µL of PhageDx™ *Listeria* phage reagent at 30°C for 4 h. After infection, 65 µL of lysis/luciferase master mix was added to the wells and samples were read on a luminometer. Samples with a signal of ≥ 300 RLU were considered positive.

**Results.**—Of the 54 *Listeria* inclusivity strains tested at LabCorp, 51 were detected and 3 were not detected (Table 1). All 34 *L. monocytogenes* strains tested were detected, while those strains that were not detected included 1 strain each of *L. innocua* (ATCC BAA-680), *L. seeligeri* (ATCC 51335) and *L. welshimeri* (ATCC 43551) (Table 1). Those strains that were not detected could be divided into 2 categories. The first category, is one in which the strain would be detected with higher cell numbers. *L. innocua* (ATCC BAA-680) and *L. welshimeri* (ATCC 43551) were detected with 20 h enrichment, or within the recommended enrichment time frame for environmental samples (data not shown). *L. seeligeri* (ATCC 51335), however, remained negative with extended enrichment times.

Of the 45 exclusivity strains examined by LabCorp, 44 of the 45 strains were negative (Table 2). One strain, *Lactobacillus plantarum* ATCC 14917, was reported as positive; however, the signal generated was significantly lower than that of *Listeria* strains (data not shown).

**Product Consistency (lot-to-lot) and Stability Studies**

Three separate production lots of PhageDx™ *Listeria* recombinant phage were prepared according to written manufacturing documents and tested according to quality control procedures. Quality control
procedures verified that each lot when diluted to working concentration had the same titer, background, and level of detection. Recombinant phage reagents were aged between 0 and 6 months when assayed for stability.

Consistency and stability were done according to AOAC guidance, where a sample was inoculated with *L. monocytogenes* (ATCC 7302) to give fractional positives. Ten replicates were run in the PhageDx™ Assay, and the RLU values analyzed. A set of stability studies was also conducted using the non-target bacterium *Enterococcus faecalis* (ATCC 29212). Overnight cultures of *E. faecalis* were used directly in the assay. Results are shown in Table 3.

**Results.**—The PhageDx™ *Listeria* recombinant phages can be manufactured consistently and are stable for at least 6 months when stored at 4°C. Manufactured lots were made on 2/20/20, 4/20/20, and 8/20/20 according to written manufacturing documents. Each lot produced similar results when tested according to QC tests for bacteriophage concentration, background signal, and limit of detection. Stability tests of each lot were performed to determine the shelf life of the recombinant phage. These tests demonstrated that lots produced 0 months prior to testing showed no significant difference from lots produced at least 6 months prior to testing. Additionally, no variation in exclusivity was observed with these three recombinant phage lots in tests with *E. faecalis*.

**Robustness Study**

Three parameters were varied to demonstrate assay robustness: infection time (± 30 m), recombinant phage concentration (± 20%) and lysis/luciferase master mix amount (± 5 µL). Briefly, stainless steel surfaces were inoculated with *L. monocytogenes* (ATCC 19115) at a low level to generate
partial positives or 100X *E. faecalis* (ATCC 29212) and allowed to dry and sit for 16–24 h at room
temperature (20–25°C). The PhageDx™ *Listeria* Assay protocol was followed with the variations in
infection time, recombinant phage concentration, and lysis/substrate master mix amounts as indicated
in Table 4. Samples with RLU values of 300 or greater were considered positive. Samples were confirmed
by allowing samples to enrich for a total of 24–28 h and plating on RAPID’ *L. monocytogenes* plates and
incubating at 37°C for an additional 24–48 h. The presence of blue (pale blue, grey-blue, or dark blue)
colonies on selective plates indicated a positive result for *L. monocytogenes*. A summary of the testing is
presented in Table 4.

**Results.**—Robustness testing of the PhageDx™ *Listeria* Assay demonstrated that variations in
infection time, recombinant phage concentration, and lysis/luciferase master mix amounts do not alter
the results compared to the standard protocol. Infection times of 3.5 and 4.5 h, recombinant phage
volumes of 8 and 12 µL and lysis/luciferase master mix volumes of 60 and 70 µL produced identical
results to the standard protocol of 4 h infection, 10 µL of recombinant phage and 65 µL of
lysis/luciferase master mix in both uninoculated and low inoculum test samples (Table 4). These results
indicate that these deviations from the PhageDx™ *Listeria* Assay protocol did not alter the final results.

**Independent Laboratory Validation Study**

**Inclusivity**

The inclusivity and exclusivity study evaluated two strains of *Listeria grayi*, three strains of *Listeria
seeligeri*, two strains of *Listeria welshimeri*, one strain of *Enterococcus faecalis*, one strain of

---

**Page 15 of 25**

ScholarOne Support phone: 434-964-4100 email: ts.mcsupport@thomson.com
Enterococcus faecium, and one strain of Streptococcus pyogenes. All cultures evaluated were propagated from a stock culture stored at -70 °C to trypticase soy agar with 5% sheep blood (SBA) and incubated for 24 ± 2 h at 35 ± 1°C. After incubation of the target organisms, a single colony from SBA was transferred to a 9 mL aliquot of BLEB for 20–24 h at 35 ± 1 °C. Exclusivity strains were transferred to non-selective media and incubated under conditions for optimal growth. Inclusivity and exclusivity testing was done in the same manner as method developer’s inclusivity and exclusivity studies. All inclusivity and exclusivity strains were blind coded and randomized. Tests results were decoded and reported as either positive or negative.

Results.—For the inclusivity and exclusivity study of the seven strains of Listeria spp. tested, seven were correctly included, and for the three exclusivity strains all three were correctly excluded. Results are included in Tables 1 and 2.

Matrix Study

The independent laboratory evaluation included matrix studies for 4” X 4” test areas of stainless steel (18 GA 300 series, brush finish, NSF certified stainless steel) and ceramic (unglazed) surfaces, comparing the PhageDx™ Listeria Assay to 2017 FDA/BAM Chapter 10. Within each sample set, there were 5 uninoculated samples (0 CFU/test portion), 20 low-level inoculated samples (0.2–2 CFU/test portion), and 5 high-level inoculated samples (2–10 CFU/test portion). The low inoculation level was
designed to produce fractional positive results in which the candidate or reference method produced 5–15 positive results (25–75%).

All samples were analyzed by the PhageDx™ Listeria Assay following enrichment with pre-warmed (35 ± 1°C) BLEB and incubated for 20–24 h at 35 ± 1°C, analysis was conducted after 20 h. Regardless of presumptive results, all samples were culturally confirmed by the FDA/BAM Chapter 10 reference method. In addition, all samples were confirmed following the alternative confirmation as described in section “Confirmation”. Final confirmation for all samples was obtained by Bruker MALDI Biotyper following AOAC OMA 2017.10 (5).

Organism Preparation and Inoculation

For ceramic surfaces, 4” x 4” areas were inoculated with 0.25 mL of diluted L. innocua ATCC 43547 culture and sampled using sampling sponges pre-moistened in letheen broth. For stainless steel surfaces, 4” x 4” areas were inoculated with 0.25 mL of diluted L. monocytogenes 4a CWD 1554. In addition to the L. monocytogenes culture, stainless steel surface was co-inoculated with a competitor organism, Enterococcus faecalis ATCC 29212, at 10 x the level of the target organism. All cultures utilized were propagated from a stock culture stored at -70 °C to SBA and incubated for 24 ± 2 h at 35 ± 1°C. After incubation, a single colony from SBA was transferred to a 9 mL aliquot of BHI broth for 20-24 h at 35 ± 1°C. Each culture was then diluted to the target concentration by performing serial dilutions as 0.1% peptone water as the diluent. For the uninoculated test portions, sterile 0.1% peptone water was applied to the test area. Each surface was dried for 16–24 h at room temperature (24 ± 2°C) prior to
sampling. To determine the inoculation level for the environmental surfaces, aliquots of each inoculum were plated onto Tryptic Soy agar (TSA) in triplicate.

_FDA/BAM Chapter 10 Detection of Listeria monocytogenes in Foods and Environmental Samples, and Enumeration of Listeria monocytogenes in Foods_

For environmental samples, sponges were pre-moistened with 10 mL of D/E neutralizing broth. Following addition of D/E neutralizing broth surfaces were swabbed using firm and even pressure 10 times vertically and horizontally. All environmental samples were then stored at 22 ± 2°C for 2 h ± 15 minutes. Swabs were enriched with 90 mL of BLEB, massaged by hand for 2 min and incubated at 30 ± 1°C for 4 h. Following 4 h of incubation, selective supplements acriflavine (10 mg/L), sodium nalidixate (40 mg/L) and cycloheximide (50 mg/L) were added to each test portion and samples were re-incubated for 24–48 h.

After 24 h of total incubation, the enriched samples were streaked to MOX agar plates and incubated at 35 ±1°C for 24–48 h. In addition to MOX each enriched sample was streaked to the chromogenic selective agar Brilliance Listeria Agar (BLA) and incubated at 37 ± 1°C for 24 ± 2 h. The enriched samples were re-incubated for an additional 24 h at 30 ± 1°C and then streaked to a second MOX and BLA agar plate which was incubated for 24–48 h at 35 ± 1°C and 37 ± 1°C for 24 ± 2 h, respectively. MOX and BLA agar plates were examined for suspect colonies, and if present, at least 5 colonies were streaked to Tryptic Soy Agar containing 0.6% yeast extract (TSA/YE). The TSA/YE plates were incubated at 35 ± 1°C for 24–48 h and then examined for purity. Pure colonies were tested for catalase reactivity and a Gram stain was conducted. A pure _Listeria monocytogenes_ colony was
transferred to Trypticase Soy Broth containing 0.6% yeast extract (TSB/YE). The TSB/YE cultures were incubated at 25 ± 1°C overnight, or until the broth was turbid, indicating sufficient growth. Catalase-positive organisms were stabbed into SBA plates and incubated at 35 ± 1°C for 24–48 h. The TSB/YE tubes incubated at 25 ± 1°C were used to prepare a wet mount slide to determine motility pattern. After incubation, the SBA plates were examined for hemolysis. Final confirmation was conducted using Bruker MALDI Biotyper following AOAC OMA 2017.10.

*PhageDx™ Listeria Assay*

Sponges from ceramic (4” x 4” test area) and stainless steel (4” x 4” test area) swabbed surfaces were enriched according to the protocol described previously in “Organism Preparation and Inoculation”.

PhageDx™ Listeria Assay was performed as described in section “Sample preparation”. Briefly, using a polyurethane sponge pre-moistened with letheen, surfaces were swabbed and the sponge placed into the sample bag and held for 22 ± 2°C for 2 h ± 15 m. Swabs were enriched with 20 mL of pre-warmed BLEB, massaged by hand for 10–20 s and incubated at 35 ± 1°C for 20 h. Following enrichment, 150 µL sample aliquots were transferred to a 96-well plate. Ten microliters of phage reagent was added to each sample and samples were incubated at 30 ± 1°C for 4 h. After infection, 65 µL of lysis/luciferase substrate master mix was added to the samples and then read on a luminometer. All samples were culturally confirmed by the FDA/BAM Chapter 10 reference method. All samples were also confirmed by an alternate confirmation described previously in “Confirmation” section. Final confirmation for all samples was obtained by Bruker MALDI Biotyper following AOAC OMA 2017.09.
Results.—As per criteria outlined in Appendix J of the *Official Methods of Analysis* Manual, fractional positive results were obtained for the PhageDx™ *Listeria* Assay (4). For ceramic and stainless steel (4” x 4” test areas) environmental samples the inoculum recovered for the target *L. innocua* and *L. monocytogenes* strains fell within the targeted levels, while the competitor organism was at a level at least 10 times higher. The probability of detection (POD) was calculated as the number of positive outcomes divided by the total number of trials (4). Comparisons of the PhageDx™ presumptive results to the confirmed results using the FDA/BAM Chapter 10 reference method showed no significant difference between the two (Table 5). No significant difference was found when comparing the results of the PhageDx™ presumptive results and FDA/BAM Chapter 10 results in an unpaired study (Table 6). There was also no significant difference in the PhageDx™ presumptive results and results of the PhageDx™ confirmation method (Table 7). Finally, there was no difference between the presumptive PhageDx™ results confirmed using the PhageDx™ confirmation method and the FDA/BAM reference method (Table 8). In summary, POD analysis between the PhageDx™ *Listeria* Assay method and the reference method indicated that there was no significant difference at the 5% level between the number of positive results by the two methods for both matrixes. The POD analysis between the PhageDx™ *Listeria* Assay presumptive and confirmed results indicated that there was no significant difference at the 5% level for the method for both matrixes. A summary of POD analyses (7) are presented in Tables 5–8.

Discussion
The results of this validation study show that the PhageDx™ Listeria Assay is an effective alternative to the FDA/BAM Chapter 10 for the detection of Listeria on stainless steel and ceramic surfaces.

In inclusivity and exclusivity testing, the method was shown to be specific for Listeria, correctly identifying 58/61 Listeria target strains including all 34 L. monocytogenes strains tested. The PhageDx™ Listeria Assay was unable to detect 3 strains within the inclusivity panel, 1 strain each of L. innocua (ATCC BAA-680), L. seeligeri (ATCC 51335) and L. welshimeri (ATCC 43551). It is unclear as to exactly why these strains failed to generate a positive result since the PhageDx™ Listeria assay was able to detect other members of the species. One possible explanation is that these strains do not have the receptor(s) or the optimal receptors required for efficient recognition by the phage (8). Possessing a receptor that was sufficiently similar to Listeria also may explain why Lactobacillus plantarum generated a false positive result. Another possibility for a false negative may be that the strain has a mechanism that prevents the phage from producing luciferase. These mechanisms may include, but are not limited to preventing phage absorption, preventing phage DNA entry, or the cutting phage nucleic acids (9).

The recombinant phage can be produced consistently and is stable for 6 months when stored appropriately. Robustness testing of the PhageDx™ Listeria Assay indicated that the method works well when the assay parameters (infection time, recombinant phage concentration and substrate amount) were varied from the stated protocol.

Independent laboratory testing demonstrated that the PhageDx™ Listeria Assay was able to detect Listeria at low levels and in the presence of competing bacteria on stainless steel and ceramic surfaces. These studies also demonstrated that the performance of the PhageDx™ Listeria Assay was not statistically different from that of FDA/BAM Chapter 10 for environmental surfaces. Additionally, the
PhageDx™ confirmation procedure was shown to produce results identical to the FDA/BAM Chapter 10 reference method confirmation procedure.

The PhageDx™ *Listeria* Assay has a number of advantages over the FDA/BAM Chapter 10 reference method. In addition to being a specific assay, the results are easy to interpret as an RLU endpoint is used to determine the outcome of the assay. Another advantage is that PhageDx™ provides a presumptive positive result in as little as 24.5 h compared to 72+ h in the case of the FDA/BAM Chapter 10 reference method. PhageDx™ is also a simple test that involves only five basic steps: enrichment, sampling, infection, substrate addition, and signal readout. Finally, PhageDx™ Assay is a rapid method that offers a considerable time savings alternative compared to the FDA/BAM Chapter 10 reference method.

**Conclusion**

Results of this validation study support the claim that the PhageDx™ *Listeria* Assay is a specific, sensitive, fast, and simple method for the detection of *Listeria* on stainless steel and ceramic surfaces and is statistically comparable to the FDA/BAM Chapter 10 reference method. By using a luciferase-expressing recombinant bacteriophage, the PhageDx™ assay was able to detect a positive sample after 20 h enrichment and a 4 h infection for environmental surfaces. The PhageDx™ *Listeria* Assay thus offers a significant shorter time-to-results compared with the FDA/BAM Chapter 10 reference method.
Acknowledgments

Submitting Company

Laboratory Corporation of America®, 531 South Spring Street, Burlington, NC 27215

Independent Laboratory

Kateland Koch, Benjamin Bastin, M. Joseph Benzinger, Jr. and James Agin

Q Laboratories, Inc., 1930 Radcliff Drive, Cincinnati, OH USA 45204

Reviewer(s)

Yi Chen

U.S. Food and Drug Administration, Center for Food Safety and Applied Nutrition, 5100 Paint Branch Pkwy., College Park, MD 20740

Maria Cristina Fernandez

Maimonides University, Hidalgo 775 (esq. Felipe Vallese), C1405BCK Buenos Aires, Argentina

Michael Brodsky

Brodsky Consultants, 73 Donnamora Crescent, Thornhill, Ontario L3T 4K6, Canada

References

(1) Centers for Disease Control and Prevention: Listeria (Listeriosis). Updated March 10, 2020. (Accessed July 2020)
https://www.cdc.gov/listeria/index.html

(2) U.S Food & Drug Administration: Listeria (Listeriosis). Updated March 29, 2019. (Accessed August 2020)

https://www.fda.gov/food/foodborne-pathogens/listeria-listeriosis#Home

(3) Food and Drug Administration Bacteriological Analytical Manual Chapter 10: Detection of Listeria monocytogenes in Foods and Environmental Samples, and Enumeration of Listeria monocytogenes in Foods. October 2017. (Accessed July 2020)

https://www.fda.gov/food/laboratory-methods-food/bam-chapter-10-detection-listeria-monocytogenes-foods-and-environmental-samples-and-enumeration

(4) Official Methods of Analysis (2019), 21st Ed., Appendix J, AOAC INTERNATIONAL, Rockville, MD, http://www.eoma.aoac.org/app_j.pdf (Accessed July 2020)

(5) Confirmation and Identification of Listeria monocytogenes, Listeria spp. and Other Gram Positive Organisms by the Bruker MALDI Biotyper Method: Collaborative Study. First Action OMA 2017.10

(6) Wehling, P., LaBudde, R., Brunelle, S., Nelson, M. Probability of Detection (POD) as a Statistical Model for the Validation of Qualitative Methods. Journal of AOAC International, Vol. 94, No. 1, 2011.

(7) Least Cost Formulations, Ltd., AOAC Binary Data Interlaboratory Study Workbook Version 2.3 (2014) -http://lcfltd.com/aoac/aoac-binary-v2-2.xls (Accessed July 2020)

(8) Bielmann, R., Habann, M., Eugster, M.R., Lurz, R., Calendar, R., Klumpp, J., Loessner, M.J. Receptor binding protein of Listeria monocytogenes bacteriophages A118 and P35 recognize serovar-specific teichoic acids. Virology 477; (2015) 110-118.
(9) Labrie, S. J., Samson, J. E., Moineau, S. Bacteriophage resistance mechanisms. Nature Reviews: Microbiology Vol. 8 (2010) 317-327
| No. | Species                  | Serogroup | Source            | Origin  | PhageDx Result |
|-----|--------------------------|-----------|-------------------|---------|----------------|
| 1   | Listeria monocytogenes   | 1/2a      | ATCC 15313        | Rabbit  | Positive       |
| 2   | Listeria monocytogenes   | 1/2a      | ATCC 51774        | Human   | Positive       |
| 3   | Listeria monocytogenes   | 1/2a      | ATCC 35152        | Guinea Pig | Positive     |
| 4   | Listeria monocytogenes   | 1/2a      | ATCC BAA-679      | Rabbit  | Positive       |
| 5   | Listeria monocytogenes   | 1/2a      | ATCC 51775        | Cheese  | Positive       |
| 6   | Listeria monocytogenes   | 1/2a      | ATCC 19111        | Poultry | Positive       |
| 7   | Listeria monocytogenes   | 1/2a      | ATCC 51772        | Cheese  | Positive       |
| 8   | Listeria monocytogenes   | 1/2b      | ATCC BAA-751      | Not available | Positive |
| 9   | Listeria monocytogenes   | 1/2b      | ATCC BAA-839      | Not available | Positive |
| 10  | Listeria monocytogenes   | 1/2b      | ATCC 51780        | Cheese  | Positive       |
| 11  | Listeria monocytogenes   | 1/2c      | ATCC 51779        | Cheese  | Positive       |
| 12  | Listeria monocytogenes   | 2         | ATCC 9525         | Not available | Positive     |
| 13  | Listeria monocytogenes   | 1/2C      | ATCC 19112        | Human   | Positive       |
| 14  | Listeria monocytogenes   | 3a        | ATCC 19113        | Human   | Positive       |
| 15  | Listeria monocytogenes   | 3a        | ATCC 51782        | Cheese  | Positive       |
| 16  | Listeria monocytogenes   | 4         | ATCC 51781        | Not available | Positive |
| 17  | Listeria monocytogenes   | 4a        | ATCC 19114        | Sheep   | Positive       |
| 18  | Listeria monocytogenes   | 4b        | ATCC 19115        | Human   | Positive       |
| 19  | Listeria monocytogenes   | 4b        | Cornell FSL-11-225 | Not available | Positive |
| 20  | Listeria monocytogenes   | 4b        | ATCC 51776        | Cheese  | Positive       |
| 21  | Listeria monocytogenes   | 4b        | ATCC 51787        | Cheese  | Positive       |
| 22  | Listeria monocytogenes   | 4b        | ATCC 13932        | Human   | Positive       |
| 23  | Listeria monocytogenes   | 4b        | ATCC 51777        | Cheese  | Positive       |
| 24  | Listeria monocytogenes   | 4c        | ATCC 19116        | Chicken | Positive       |
| 25  | Listeria monocytogenes   | 4d        | ATCC 19117        | Sheep   | Positive       |
| 26  | Listeria monocytogenes   | 4e        | ATCC 19118        | Chicken | Positive       |
| 27  | Listeria monocytogenes   |           | ATCC 700402       | Not available | Positive |
| 28  | Listeria monocytogenes   |           | ATCC 7302         | Not available | Positive |
| 29  | Listeria monocytogenes   |           | ATCC 7644         | Not available | Positive |
| 30  | Listeria monocytogenes   |           | ATCC 23074        | Not available | Positive |
| 31  | Listeria monocytogenes   |           | ATCC 23073        | Not available | Positive |
| 32  | Listeria monocytogenes   |           | ATCC 43256        | Not available | Positive |
| 33  | Listeria monocytogenes   |           | ATCC 984          | Not available | Positive |
| 34  | Listeria monocytogenes   | 4a        | UVM CWD 1554      | Not available | Positive |
| 35  | Listeria grayi           |           | ATCC 19120        | Not available | Positive |
| 36  | Listeria grayi           |           | ATCC 25401        | Not available | Positive |
| 37  | Listeria grayi           |           | ATCC 70054       | Not available | Positive |
| 38  | Listeria grayi<sup>a</sup> |           | QL 30911.12      | Environmental sample | Positive |
| 39  | Listeria grayi serovar Murray<sup>a</sup> |           | NCTC 10814       | Not available | Positive |
|   |        |     |       |           |               |       |     |
|---|--------|-----|-------|----------|---------------|-------|-----|
| 40 | Listeria innocua 6a | ATCC 33090 | Not available | Positive |
| 41 | Listeria innocua | ATCC S1742 | Not available | Positive |
| 42 | Listeria innocua 6b | ATCC 33091 | Not available | Positive |
| 43 | Listeria innocua 6b | ATCC 43547 | Not available | Positive |
| 44 | Listeria innocua 6a | ATCC BAA-680 | Not available | Negative* |
| 45 | Listeria ivanovii | ATCC 19119 | Not available | Positive |
| 46 | Listeria ivanovii | ATCC 49953 | Not available | Positive |
| 47 | Listeria ivanovii 5 | ATCC BAA-678 | Not available | Positive |
| 48 | Listeria ivanovii | ATCC BAA-753 | Not available | Positive |
| 49 | Listeria ivanovii | ATCC 49954 | Not available | Positive |
| 50 | Listeria seeligeri | ATCC 35967 | Not available | Positive |
| 51 | Listeria seeligeri | ATCC 51334 | Not available | Positive |
| 52 | Listeria seeligeri 4a | ATCC 51335 | Not available | Negative |
| 53 | Listeria seeligeri* | QL 031011.2 | Creamer | Positive |
| 54 | Listeria seeligeri* | QL 031011.5 | Frozen vegetables | Positive |
| 55 | Listeria seeligeri* | Cornell FSL-S4-035 | Not available | Positive |
| 56 | Listeria welshimeri 6b | ATCC 35897 | Not available | Positive |
| 57 | Listeria welshimeri 6a | ATCC 43551 | Not available | Negative* |
| 58 | Listeria welshimeri 6a | ATCC 43549 | Not available | Positive |
| 59 | Listeria welshimeri 1/2b | ATCC 43580 | Not available | Positive |
| 60 | Listeria welshimeri* | QL 030911-8 | Beef | Positive |
| 61 | Listeria welshimeri* | UVM LW001 | Not available | Positive |

*Serogroup listed if applicable.
*American Type Culture Collection, Manassas, VA.
Cornell University, Ithaca, NY.
University of Vermont, Burlington, VA.
*Q Laboratories, Cincinnati, OH.
*National Collection of Type Cultures, Proton Down, Salisbury UK.
20 h enrichment generated positive result.
*Testing done by Q Laboratories.
| No. | Species                                      | Source      | PhageDx Result |
|-----|---------------------------------------------|-------------|----------------|
| 1   | Acinetobacter baumannii                      | ATCC 19606  | Negative       |
| 2   | Acinetobacter calcoaceticus                 | ATCC 23055  | Negative       |
| 3   | Bacillus cereus                              | ATCC 14579  | Negative       |
| 4   | Bacillus cereus                              | ATCC 13061  | Negative       |
| 5   | Bacillus circulans                           | ATCC 61     | Negative       |
| 6   | Bacillus coagulans                           | ATCC 7050   | Negative       |
| 7   | Bacillus licheniformis                       | ATCC 9789   | Negative       |
| 8   | Bacillus megaterium                          | ATCC 14581  | Negative       |
| 9   | Bacillus mycoides                            | ATCC 6462   | Negative       |
| 10  | Bacillus pumilus                             | ATCC 700814 | Negative       |
| 11  | Bacillus subtilis                            | ATCC 23857  | Negative       |
| 12  | Bacillus subtilis subsp. subtilis            | ATCC 6051   | Negative       |
| 13  | Bacillus weihenstephanensis                  | ATCC 12826  | Negative       |
| 14  | Citrobacter braakii                          | ATCC 51113  | Negative       |
| 15  | Citrobacter freundii                         | ATCC 8090   | Negative       |
| 16  | Citrobacter koseri                           | ATCC 25408  | Negative       |
| 17  | Cronobacter muytjensii                       | ATCC 51329  | Negative       |
| 18  | Cronobacter sakazakii                        | ATCC 12868  | Negative       |
| 19  | Escherichia coli                             | ATCC 9637   | Negative       |
| 20  | Edwardsiella tarda                           | ATCC 15947  | Negative       |
| 21  | Enterobacter aerogenes                       | ATCC 13048  | Negative       |
| 22  | Enterobacter cloacae, subsp cloacae          | ATCC 13047  | Negative       |
| 23  | Enterobacter kobei                           | ATCC BAA-260| Negative       |
| 24  | Enterococcus faecalis                        | ATCC 19433  | Negative       |
| 25  | Enterococcus faecalis                        | ATCC 29212  | Negative       |
| 26  | Enterococcus faecium                         | ATCC 19434  | Negative       |
| 27  | Enterococcus faecium<sup>a</sup>             | ATCC 700221 | Negative       |
| 28  | Escherichia fergusoni                        | ATCC 35469  | Negative       |
| 29  | Escherichia hermanni                         | ATCC 33650  | Negative       |
| 30  | Hafnia alevi                                 | ATCC 13337  | Negative       |
| 31  | Klebsiella oxytoca                           | ATCC 43165  | Negative       |
| 32  | Klebsiella pneumonia                         | ATCC 4352   | Negative       |
| 33  | Lactobacillus plantarum                      | ATCC 14917  | Positive       |
| 34  | Lactobacillus rhamnosus                      | ATCC 7469   | Negative       |
| 35  | Morganella morganii : subsp. Maorganii M11   | ATCC 25830  | Negative       |
| 36  | Pseudomonas aeruginosa; Strain Boston 41401 | ATCC 27853  | Negative       |
|   | Species                        | ATCC   | Result   |
|---|-------------------------------|--------|----------|
|40 | *Serratia marcescens*         | 13880  | Negative |
|41 | *Shigella flexneri*           | 12022  | Negative |
|42 | *Shigella sonnei*             | 9290   | Negative |
|43 | *Staphylococcus aureus*       | 27660  | Negative |
|44 | *Staphylococcus epidermidis*  | 14990  | Negative |
|45 | *Staphylococcus haemolyticus* | 29970  | Negative |
|46 | *Streptococcus pyogenes*      | 19615  | Negative |
|47 | *Yersinia enterocolitica*     | 23715  | Negative |

*a* American Type Culture Collection, Manassas, VA.

*b* Testing done by Q Laboratories, Cincinnati, OH.

*c* Testing done by both LabCorp and Q Laboratories
Table 3. Stability and Consistency (lot-to-lot) of PhageDx™ Listeria Recombinant Phage – POD Comparison

| Phage lot # | Lot age, months | N | x | PODA | 95% CI | Phage lot # | Lot age, months | N | x | PODB | 95% CI | dPODAB | 95% CI |
|-------------|-----------------|---|---|------|--------|-------------|-----------------|---|---|------|--------|--------|--------|
| Listeria monocytogenes (target) | | | | | | | | | | | | | |
| B | 0 | 10 | 4 | 0.4 | 0.17, 0.69 | C | 0 | 10 | 6 | 0.6 | 0.31, 0.83 | -0.20 | -0.53, 0.21 |
| A | 6 | 10 | 4 | 0.4 | 0.17, 0.69 | C | 0 | 10 | 6 | 0.6 | 0.31, 0.83 | -0.20 | -0.53, 0.21 |
| A | 6 | 10 | 4 | 0.4 | 0.17, 0.69 | B | 4 | 10 | 4 | 0.4 | 0.17, 0.69 | 0.00 | -0.37, 0.37 |
| Enterococcus faecalis (non-target) | | | | | | | | | | | | | |
| B | 4 | 10 | 0 | 0.0 | 0.0, 0.28 | C | 0 | 10 | 0 | 0.0 | 0.0, 0.28 | 0.0 | -0.28, 0.28 |
| A | 6 | 10 | 0 | 0.0 | 0.0, 0.28 | C | 0 | 10 | 0 | 0.0 | 0.0, 0.28 | 0.0 | -0.28, 0.28 |
| A | 6 | 10 | 0 | 0.0 | 0.0, 0.28 | B | 4 | 10 | 0 | 0.0 | 0.0, 0.28 | 0.0 | -0.28, 0.28 |

aN = Number of test portions.
bx = Number of positive test portions.
cPODA = Positive outcomes divided by the total number of trials first member of pair.
dPODB = Positive outcomes divided by the total number of trials second member of pair.
edPODAB = Difference in POD between the paired comparison.
f95% CI = If the confidence interval of a dPOD does not contain zero, then the difference is statistically significant at the 5% level.
gLot A was produced 2/20/20.
hLot B was produced 04/20/20.
iLot C was produced 08/20/20.
Table 4. Robustness study: Impact of Varying Infection time, Phage Concentration, Lysis/Luciferase Master Mix Concentration on PhageDx™ Listeria Assay Results – POD Comparison

| Test Condition | Test Parameters | Test Condition Results | Nominal Condition Results |
|----------------|----------------|------------------------|--------------------------|
|                | Infection time | Volume phage | Volume substrate | x | POD± | 95% CI | x | POD± | 95% CI | dPOD± | 95% CI |
| Stainless steel surface – inoculated with *L. monocytogenes* ATCC 19115 (target) 55 CFU/test area |
| 1              | 3.5 h          | 8 µL            | 60              | 10 | 4    | 0.40 | 0.17, 0.69 | 4  | 0.40 | 0.17, 0.69 | 0.0  | -0.25, 0.25 |
| 2              | 3.5 h          | 8 µL            | 70              | 10 | 4    | 0.40 | 0.17, 0.69 | 4  | 0.40 | 0.17, 0.69 | 0.0  | -0.25, 0.25 |
| 3              | 3.5 h          | 12 µL           | 60              | 10 | 4    | 0.40 | 0.17, 0.69 | 4  | 0.40 | 0.17, 0.69 | 0.0  | -0.25, 0.25 |
| 4              | 3.5 h          | 12 µL           | 70              | 10 | 4    | 0.40 | 0.17, 0.69 | 4  | 0.40 | 0.17, 0.69 | 0.0  | -0.25, 0.25 |
| 5              | 4.5 h          | 8 µL            | 60              | 10 | 4    | 0.40 | 0.17, 0.69 | 4  | 0.40 | 0.17, 0.69 | 0.0  | -0.25, 0.25 |
| 6              | 4.5 h          | 8 µL            | 70              | 10 | 4    | 0.40 | 0.17, 0.69 | 4  | 0.40 | 0.17, 0.69 | 0.0  | -0.25, 0.25 |
| 7              | 4.5 h          | 12 µL           | 60              | 10 | 4    | 0.40 | 0.17, 0.69 | 4  | 0.40 | 0.17, 0.69 | 0.0  | -0.25, 0.25 |
| 8              | 4.5 h          | 12 µL           | 70              | 10 | 4    | 0.40 | 0.17, 0.69 | 4  | 0.40 | 0.17, 0.69 | 0.0  | -0.25, 0.25 |

Stainless steel surface – inoculated with *E. faecalis* ATCC 29212 (non-target) 4950 CFU/test area

| Test Condition | Test Parameters | Test Condition Results | Nominal Condition Results |
|----------------|----------------|------------------------|--------------------------|
|                | Infection time | Volume phage | Volume substrate | x | POD± | 95% CI | x | POD± | 95% CI | dPOD± | 95% CI |
| 1              | 3.5 h          | 8 µL            | 60              | 10 | 0    | 0.0  | 0.00, 0.28 | 0  | 0.0  | 0.00, 0.28 | 0.0  | -0.25, 0.25 |
| 2              | 3.5 h          | 8 µL            | 70              | 10 | 0    | 0.0  | 0.00, 0.28 | 0  | 0.0  | 0.00, 0.28 | 0.0  | -0.25, 0.25 |
| 3              | 3.5 h          | 12 µL           | 60              | 10 | 0    | 0.0  | 0.00, 0.28 | 0  | 0.0  | 0.00, 0.28 | 0.0  | -0.25, 0.25 |
| 4              | 3.5 h          | 12 µL           | 70              | 10 | 0    | 0.0  | 0.00, 0.28 | 0  | 0.0  | 0.00, 0.28 | 0.0  | -0.25, 0.25 |
| 5              | 4.5 h          | 8 µL            | 60              | 10 | 0    | 0.0  | 0.00, 0.28 | 0  | 0.0  | 0.00, 0.28 | 0.0  | -0.25, 0.25 |
| 6              | 4.5 h          | 8 µL            | 70              | 10 | 0    | 0.0  | 0.00, 0.28 | 0  | 0.0  | 0.00, 0.28 | 0.0  | -0.25, 0.25 |
| 7              | 4.5 h          | 12 µL           | 60              | 10 | 0    | 0.0  | 0.00, 0.28 | 0  | 0.0  | 0.00, 0.28 | 0.0  | -0.25, 0.25 |
| 8              | 4.5 h          | 12 µL           | 70              | 10 | 0    | 0.0  | 0.00, 0.28 | 0  | 0.0  | 0.00, 0.28 | 0.0  | -0.25, 0.25 |

*a Each test condition is being compared to the nominal test condition.

*b N = Number of test portions per condition.

*x = Number of positive test portions per condition.
POD = Positive outcomes divided by the total number of trials per condition.
Nominal condition = 4 h infection, 10 µL phage, 65 µL lysis/luciferase master mix substrate.
POD_N = Positive outcomes divided by the total number of trials per nominal condition.
dPOD_{TN} = Difference in POD between the test condition and nominal condition.
95% CI = If the confidence interval of a dPOD does not contain zero, then the difference is statistically significant at the 5% level.
### Table 5. PhageDx™ Listeria Results: Presumptive vs. Confirmed using the reference method procedure

| Matrix            | Strain                              | CFU/test area | Nr  | POD<sub>CP</sub> | 95% CI      | POD<sub>CC</sub> | 95% CI      | dPOD<sub>CP</sub> | 95% CI |
|-------------------|-------------------------------------|---------------|-----|-----------------|--------------|-----------------|--------------|------------------|--------|
| Stainless steel   | L. monocytogenes 4a (CWD<sup>h</sup> 1554)/E. faecalis ATCC 29212 | 48 & 510      | 20  | 0.40            | 0.22, 0.61   | 0.40            | 0.22, 0.61   | 0.00             | -0.13, 0.13 |
|                   |                                     | 160 & 1800    | 5   | 1.00            | 0.57, 1.00   | 1.00            | 0.57, 1.00   | 0.00             | -0.47, 0.47 |
| Ceramic           | L. innocua (ATCC 43547 6b)         | 60            | 20  | 0.45            | 0.26, 0.66   | 0.45            | 0.26, 0.66   | 0.00             | -0.13, 0.13 |
|                   |                                     | 200           | 5   | 1.00            | 0.57, 1.00   | 1.00            | 0.57, 1.00   | 0.00             | -0.47, 0.47 |

*a* CFU = Colony forming units applied per test area.

*b* N = Number of test portions.

*c* x = Number of positive test portions.

*d* POD<sub>CP</sub> = Candidate method presumptive positive outcomes divided by the total number of trials.

*e* POD<sub>CC</sub> = Candidate method confirmed positive outcomes (confirmed using the reference method procedure) divided by the total number of trials.

*f* dPOD<sub>CP</sub> = 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* CWD = University of Vermont, Burlington, VT.

*i* ATCC = American Type Culture Collection, Manassas, VA.

*j* N/A = Not applicable.

---

*ScholarOne Support phone: 434-964-4100 email: ts.mcsupport@thomson.com*
**Table 6. Method Comparison Results: PhageDx™ *Listeria* vs. BAM Ch. 10**

| Matrix                  | Strain                        | CFU/test area     | N<sup>b</sup> | x<sup>c</sup> | POD<sub>C</sub><sup>d</sup> | 95% CI    | BAM Ch. 10 | x | POD<sub>R</sub><sup>e</sup> | 95% CI | dPOD<sub>C</sub><sup>f</sup> | 95% CI<sup>g</sup> |
|-------------------------|-------------------------------|-------------------|--------------|--------------|-----------------|----------|------------|---|-----------------|--------|-----------------|------------------|
| Stainless steel (4”x 4”)| *L. monocytogenes* 4a (CWD 1554)/E. faecalis ATCC 29212 | 48 & 510          | 20           | 8            | 0.40            | 0.22, 0.61| 0           | 0 | 0.00            | 0.00, 0.43 | 0.00            | -0.28, 0.28  |
|                         |                               | 160 & 1800        | 5            | 5            | 1.00            | 0.57, 1.00 | 0           | 0 | 0.00            | 0.00, 0.43 | 0.00            | -0.43, 0.43  |
| Ceramic (4”x 4”)        | *L. innocua* (ATCC 43547 6b) | 60                | 20           | 9            | 0.45            | 0.26, 0.66| 0           | 0 | 0.00            | 0.00, 0.43 | 0.00            | -0.43, 0.43  |
|                         |                               | 200               | 5            | 5            | 1.00            | 0.57, 1.00 | 0           | 5 | 1.00            | 0.57, 1.00 | 0.00            | -0.43, 0.43  |

<sup>a</sup>MPN = Most Probable Number is based on the POD of reference method test portions using the Least cost Formulations MPN calculator, with 95% confidence interval.

<sup>b</sup>N = Number of test portions.

<sup>c</sup>x = Number of positive test portions.

<sup>d</sup>POD<sub>C</sub> = Candidate method presumptive positive outcomes confirmed positive using the reference method procedure.

<sup>e</sup>POD<sub>R</sub> = Reference method confirmed positive outcomes divided by the total number of trials.

<sup>f</sup>dPOD<sub>C</sub> = Difference between the candidate method and reference method POD values.

<sup>g</sup>95% CI = If the confidence interval of a dPOD does not contain zero, then the difference is statistically significant at the 5% level.

<sup>h</sup>CWD = University of Vermont, Burlington, VT.

<sup>i</sup>ATCC = American Type Culture Collection, Manassas, VA.

<sup>j</sup>N/A = Not applicable.
Table 7. PhageDx™ Listeria Results: Presumptive vs. Confirmed using the PhageDx™ Listeria confirmation procedure

| Matrix               | Strain                          | CFU\(^a\)/test area | N\(^b\) | x\(^c\) | POD\(_{CP}^d\) | 95% CI     | x | POD\(_{CC}^e\) | 95% CI     | dPOD\(_{CP}^f\) | 95% CI     |
|----------------------|--------------------------------|----------------------|---------|---------|---------------|------------|---|---------------|------------|---------------|------------|
| Stainless steel (4“x 4”) | *L. monocytogenes* 4a (CWD\(^h\) 1554)/*E. faecalis* ATCC 29212 | N/A\(^j\)            | 5       | 0       | 0.00         | 0.00, 0.43 | 0 | 0.00         | 0.00, 0.43 | 0.00         | -0.47, 0.47 |
|                      |                                | 48 & 510             | 20      | 8       | 0.40         | 0.22, 0.61 | 8 | 0.40         | 0.22, 0.61 | 0.00         | -0.13, 0.13 |
|                      |                                | 160 & 1800           | 5       | 5       | 1.00         | 0.57, 1.00 | 5 | 1.00         | 0.57, 1.00 | 0.00         | -0.47, 0.47 |
| Ceramic (4“x 4”)     | *L. innocua* (ATCC 43547 6b)  | N/A\(^j\)            | 5       | 0       | 0.00         | 0.00, 0.43 | 0 | 0.00         | 0.00, 0.43 | 0.00         | -0.47, 0.47 |
|                      |                                | 60                   | 20      | 9       | 0.45         | 0.26, 0.66 | 9 | 0.45         | 0.26, 0.66 | 0.00         | -0.13, 0.13 |
|                      |                                | 200                  | 5       | 5       | 1.00         | 0.57, 1.00 | 5 | 1.00         | 0.57, 1.00 | 0.00         | -0.47, 0.47 |

\(^a\) CFU = Colony forming units applied per test area.
\(^b\) N = Number of test potions.
\(^c\) x = Number of positive test portions.
\(^d\) POD\(_{CP}^\) = Candidate method presumptive positive outcomes divided by the total number of trials.
\(^e\) POD\(_{CC}^\) = Candidate method confirmed positive outcomes (confirmed using the PhageDx Listeria recommended procedure) 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.
\(^h\) CWD = University of Vermont, Burlington, VT.
\(^i\) ATCC = American Type Culture Collection, Manassas, VA.
\(^j\) N/A = Not applicable.
Table 8. Method Comparison Results: PhageDx™ *Listeria* vs. BAM Ch. 10

| Matrix          | Strain                        | CFU\(^a\)/test area | \(\text{N}\)^b | \(\times\)^c | \(\text{POD}_{\text{CP}}\)^d | 95% CI  | BAM Ch. 10                  | \(\text{x}\) | \(\text{POD}_{\text{CP}}\)^e | 95% CI  | \(\text{dPOD}_{\text{CP}}\)^f | 95% CI  |
|-----------------|-------------------------------|----------------------|---------------|--------------|-----------------|--------|-----------------------------|----------|-----------------|--------|-----------------|--------|
| Stainless steel | *L. monocytogenes* 4a (CWD\(^h\) 1554)/E. faecalis ATCC 29212 | N/A\(^j\)           | 5             | 0            | 0.00            | 0.00, 0.43 |           | 0           | 0.00            | 0.00, 0.43 | 0.00            | -0.47, 0.47 |
|                 |                               | 48 & 510             | 20            | 8            | 0.40            | 0.22, 0.61 |           | 8           | 0.40            | 0.22, 0.61 | 0.00            | -0.13, 0.13 |
|                 |                               | 160 & 1800           | 5             | 5            | 1.00            | 0.57, 1.00 |           | 5           | 1.00            | 0.57, 1.00 | 0.00            | -0.47, 0.47 |
| Ceramic         | *L. innocua* (ATCC 43547 6b) | N/A                  | 5             | 0            | 0.00            | 0.00, 0.43 |           | 0           | 0.00            | 0.00, 0.43 | 0.00            | -0.43, 0.43 |
|                 |                               | 60                   | 20            | 9            | 0.45            | 0.26, 0.66 |           | 7           | 0.35            | 0.18, 0.57 | 0.10            | -0.19, 0.37 |
|                 |                               | 200                  | 5             | 5            | 1.00            | 0.57, 1.00 |           | 5           | 1.00            | 0.57, 1.00 | 0.00            | -0.43, 0.43 |

\(^a\)MPN = Most Probable Number is based on the POD of reference method test portions using the Least cost Formulations MPN calculator, with 95% confidence interval.

\(^b\)N = Number of test portions.

\(^c\)\(\times\) = Number of positive test portions.

\(^d\)\(\text{POD}_{\text{CP}}\) = Candidate method presumptive positive outcomes confirmed positive using the PhageDx™ *Listeria* recommended procedure.

\(^e\)\(\text{POD}_{\text{R}}\) = Reference method confirmed positive outcomes divided by the total number of trials.

\(^f\)\(\text{dPOD}_{\text{CP}}\) = Difference between the candidate method and reference method POD values.

\(^g\)95% CI = If the confidence interval of a \(\text{dPOD}\) does not contain zero, then the difference is statistically significant at the 5% level.

\(^h\)CWD = University of Vermont, Burlington, VT.

\(^i\)ATCC = American Type Culture Collection, Manassas, VA.

\(^j\)N/A = Not applicable.