

FOOD BIOLOGICAL CONTAMINANTS

Validation of a Microwell DNA Probe Assay for Detection of *Listeria* spp. in Foods

*Performance-Tested Method*SM 010403

Abstract

A new DNA hybridization assay in microwell format for detection of *Listeria* spp. in foods and environmental samples was developed. This assay uses *Listeria*-specific oligonucleotide probes labeled with horseradish peroxidase and a photometrically determined end point. Validation studies with 15 different food commodities and a variety of environmental sample types were conducted to compare the performance of this alternative test versus reference methods. Meats, seafood, dairy products, and vegetables comprised the categories of food tested. Food samples were inoculated at 2 levels and refrigerated or frozen for at least 72 h. Uninoculated (negative) control samples were included in each trial. Samples were enriched according to the procedure recommended by either the U.S. Food and Drug Administration (FDA) or the U.S. Department of Agriculture (USDA), Food Safety and Inspection Service (FSIS). Samples enriched for 24 h were transferred to Oxford agar plates and incubated for 24 h. The surface of the plates was then swabbed and any growth present was transferred to phosphate buffer solution for the performance of the DNA assay. A standard confirmation procedure was used to compare the number of positive samples obtained with the DNA method versus reference methods. Statistical analyses of the results indicate that the proposed alternative method performs equally to cultural reference methods. The DNA assay is able to detect as low as 1 colony-forming unit of *Listeria* in a 25 g food sample, with results available as early as 48 h after the start of sample enrichment.

1 Scope of Method

(1.1) *Target organisms.*—*Listeria* spp.

(1.2) *Matrixes.*—Raw and processed meats, raw and processed seafoods, dairy products, and vegetables.

Environmental swab or sponge samples taken from stainless steel, plastic, concrete, cast iron, ceramic, and painted wood surfaces.

(1.3) *Summary of validated performance claims.*—Based on internal validation study: sensitivity $\geq 97\%$; specificity $\geq 98\%$; agreement with FDA (*Bacteriological Analytical*

Manual; BAM) culture method $\geq 94\%$; agreement with USDA/FSIS culture method $\geq 97\%$.

2 Participants

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3 Introduction

3.1 Principle

The GeneQuenceTM *Listeria* Test is a DNA probe-based diagnostic in kit format, which permits rapid and accurate detection of *Listeria* spp. in selected foods and selected environmental surfaces. Following sample pre-enrichment and selective enrichment, target bacteria are lysed enzymatically at 37°C, and *Listeria*-specific oligonucleotide probes are added for a 60 min hybridization at 45°C. If *Listeria* ribosomal RNA (rRNA) is present in the test sample, the detector probe, labeled with horseradish peroxidase (HRP), and polydeoxyadenylic acid (poly dA)-tailed capture probe will hybridize to the target rRNA sequences. Concurrently, base pairing between the poly dA-tailed capture

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probe and polydeoxythymidylic acid (poly dT)-coated polystyrene microwells facilitates solid-phase capture of probe-target molecules. Unbound probe is removed by washing, and then substrate chromogen is added to react with HRP to yield a blue color. The reaction is stopped by the addition of sulfuric acid, which changes the color of the substrate from blue to yellow. A microwell plate or microwell strip reader measures absorbance at 450 nm. An absorbance in excess of the threshold value indicates the presence of *Listeria* in the test sample. Positive assay results must be confirmed by standard culture methods.

3.2 Summary of Results

The inclusivity and exclusivity of the test were assessed on 52 different strains of *Listeria* spp. and 33 strains of non-*Listeria* bacteria. Inclusivity and exclusivity were both 100%. Comparative testing of 17 different foods and 6 environmental surfaces demonstrated a sensitivity $\geq 97\%$ and a specificity $\geq 98\%$. The agreement between the GeneQuence microwell and FDA (BAM) methods was calculated at 94.0% while the agreement between the GeneQuence and USDA/FSIS methods was calculated at 97.1%. Based on the results of the internal food study and the independent laboratory validation studies, it is concluded that the GeneQuence *Listeria* Test is an effective method for the detection of *Listeria* spp. in a wide variety of food and environmental samples, with low rates of false-negative and false-positive reactions.

4 Materials and Method

4.1 Test Kit Information

(4.1.1) *Kit name.*—GeneQuence™ *Listeria* Test.

(4.1.2) *Cat. No.*—6700.

(4.1.3) *Ordering information.*—Inside the United States: Neogen Corp., 620 Leshar Pl, Lansing, MI 48912, Tel: 517-372-9200, Fax: 517-372-0108, Website: www.neogen.com; outside the United States: contact above for local distributor information.

(4.1.4) *Test kit reagents.*

(4.1.4.1) *96-Well microwell plate in divisible strips.*—Polystyrene microwells, high binding, 12 row by 8-well configuration, coated with poly dT.

(4.1.4.2) *Pretreatment concentrate (labeled 1a).*—Contains lyophilized mutanolysin/lysozyme. Once reconstituted, it must be stored at -20°C and is stable for 60 days.

(4.1.4.3) *Pretreatment buffer (labeled 1b, 12 mL).*—Contains Tris pH 7.4, disodium EDTA, and bromophenol blue.

(4.1.4.4) *Lysis reagent concentrate (labeled 2a).*—Contains Proteinase K. Once reconstituted, it must be stored at -20°C and is stable for 60 days.

(4.1.4.5) *Lysis reagent buffer (labeled 2b, 12 mL).*—Contains Tris pH 7.4, disodium EDTA, *n*-lauryl sarcosine, and brilliant yellow.

(4.1.4.6) *Hybridization solution (labeled 3, 18 mL).*—Contains formamide. (*Caution:* Avoid contact with skin; if contact occurs, wash skin thoroughly with water.)

(4.1.4.7) *Listeria probe solution (labeled 4, 5 mL).*—Contains *Listeria*-specific oligonucleotide labeled with HRP (detector probe) and *Listeria*-specific oligonucleotide 3' labeled with polydeoxyadenylic acid (capture probe).

(4.1.4.8) *Wash solution 20 \times concentrate (labeled 5, 50 mL).*—Contains Tris, pH 7.5, EDTA, NaCl, and Tween 20.

(4.1.4.9) *Substrate chromogen solution (labeled 6, 15 mL).*—Standard HRP detection reagent for use in enzyme-linked immunosorbent assay (ELISA) and other HRP-based colorimetric assays. Contains urea peroxide and tetramethylbenzidine.

(4.1.4.10) *Stop solution (labeled 7, 5 mL).*—Contains 4.0 N sulfuric acid. (*Caution:* Corrosive. Avoid contact with skin; if contact occurs, wash skin thoroughly with cold water.)

(4.1.4.11) *Positive control (labeled +, 5 mL).*—Phosphate buffer solution with *Listeria*-specific DNA oligos to produce absorbance ≥ 1.0 when tested in assay.

(4.1.4.12) *Negative control (labeled -, 5 mL).*—Contains formaldehyde-killed *Enterococcus faecium* in concentrations sufficient to produce absorbance >0.15 in assay when stringency conditions of assay are incorrect (e.g., low hybridization temperature). Actual cell concentration used may vary depending on strain of organism used, media, and conditions used for its preparation.

(4.1.5) *Storage requirements.*—Items 4.1.4.1–4.1.4.12 are available as GeneQuence *Listeria* Test from Neogen Corporation. Store microwell plate, positive control, negative control, and bottles labeled 1a, 2a, 3, 4, and 6 at $2-8^{\circ}\text{C}$. Other reagents may be stored at $2-25^{\circ}\text{C}$. Alternatively, the entire kit may be stored at $2-8^{\circ}\text{C}$. Once reagents 1a and 2a have been reconstituted, they must be stored at -20°C and are stable for 60 days. Optionally, the reconstituted reagents 1a and 2a can be combined into bottle 2a and stored at -20°C for up to 60 days.

4.2 Additional Supplies and Reagents Available from Neogen Corp.

(4.2.1) *Microwell reader.*—To read at 450 nm.

(4.2.2) *Incubators.*— $30 \pm 1^{\circ}\text{C}$ and $35 \pm 1^{\circ}\text{C}$.

(4.2.3) *Test tubes.*— 12×75 mm borosilicate glass, and racks.

(4.2.4) *Micropipettor and tips.*— $20-200 \mu\text{L}$.

(4.2.5) *Minute timer.*

(4.2.6) *Heater block.*— $37 \pm 1^{\circ}\text{C}$.

(4.2.7) *Heater block or incubator.*— $45 \pm 1^{\circ}\text{C}$.

(4.2.8) *Wash bottle.*—500 mL.

(4.2.9) *Sterile serological pipets.*—10 mL.

(4.2.10) *Multichannel adjustable pipettor and tips.*

(4.2.11) *UVM modified Listeria enrichment broth.*—Available from Acumedia (Lansing, MI) and other vendors.

(4.2.12) *Buffered Listeria enrichment broth base (BLEB).*—Available from Acumedia and other vendors.

(4.2.13) *Oxford Listeria agar base (OXA).*—Available from Acumedia and other vendors.

Apparatus and Equipment not Available from Neogen Corp.

- (4.3.1) *Blender or homogenizer*.—e.g., Stomacher.
 (4.3.2) *Phosphate-buffered saline (PBS)*.
 (4.3.3) *BLEB base supplements*.
 (4.3.4) *Oxford medium antimicrobics*.
 (4.3.5) *Modified Oxford AOAC (MOX)*.
 (4.3.6) *Modified Oxford medium antimicrobics*.
 (4.3.7) *Sodium pyruvate supplement*.—10% aqueous solution.
 (4.3.8) *Culture bottles*.—For sample pre-enrichment.
 (4.3.9) *Graduated cylinder*.—500 mL.
 (4.3.10) *Sterile cotton swabs and absorbent paper*.
 (4.3.11) *Apparatus for plate washing with vacuum source*.
 (4.3.12) *Disposable graduated pipets*.—2 mL.
 (4.3.13) *Repeater syringe and tips*.—To dispense 50, 100, 125, and 150 μ L volumes (optional).
 (4.3.14) *Water bath*.—37°C. (If not using heater block).
 (4.3.15) *Test tubes, sterile*.—12 \times 75 mm, with caps.
 (4.3.16) *Petri plates*.—100 \times 15 mm.
 (4.3.17) *Diagnostic reagents*.—As necessary for culture confirmation of positive DNA hybridization assays (1, 2).

4.4 Standard Reference Materials

Bacterial cultures used in inclusivity testing, exclusivity testing, and inoculated food experiments were obtained from the Centers for Disease Control and Prevention, ATCC, and other sources (Tables 1 and 2).

4.5 General Preparation

(4.5.1) *General notes*.—All pipetting transfers should be made using either a disposable pipet and pipetting bulb or micropipet with disposable tips. Do not mouth pipet. This assay should be performed in a normal laboratory environment with respect to humidity, lighting, etc. Steps requiring room temperature incubation should be performed at 18–30°C. Kit reagents that have been refrigerated should be equilibrated to room temperature before use but should not be left out unrefrigerated for long periods of time.

(4.5.2) *Prior to starting the assay*.

(4.5.2.1) Allow refrigerated reagents to equilibrate to room temperature.

(4.5.2.2) Turn on water bath or heater block and adjust to $37 \pm 1^\circ\text{C}$. Fill the water bath to a level of approximately 1.5 in., or fill heater block wells about 1/3 with deionized water.

(4.5.2.3) Prepare pretreatment reagent by adding 12 mL pretreatment buffer (bottle 1b) directly to the pretreatment concentrate (bottle 1a). Dissolve contents by gently swirling. Prepare lysis reagent by adding 12 mL lysis reagent buffer (bottle 2b) directly to the bottle of lysis reagent concentrate (bottle 2a). Dissolve contents by gently swirling. *Note*: The prepared pretreatment reagent and prepared lysis reagent may be combined in bottle 2a. Separate pretreatment and lysis reagents, or the reagents combined in bottle 2a, are stable in the reconstituted form for 60 days when stored at -20°C . To

thaw, place bottles at room temperature. When thawed, gently swirl contents. Return reconstituted reagents to storage at -20°C immediately after each use.

(4.5.2.4) For each sample to be tested, label a 12 \times 75 mm glass test tube with the appropriate sample designation and place in a rack. Include tubes for 1 positive control and 1 negative control per experimental run.

(4.5.2.5) Prepare the wash solution by mixing entire contents of wash solution concentrate (bottle 5) with 950 mL distilled or deionized water. (If washing manually with a 500 mL wash bottle, use 25 mL concentrate with 475 mL water.) Fill the buffer reservoir of the plate-washing device (*see* manufacturer's instructions for set-up and use). *Note*: Wash solution can be stored in a closed bottle at room temperature for up to 60 days.

(4.5.2.6) Prepare a 4:1 hybridization/probe mixture by mixing hybridization solution (bottle 3) and probe solution (bottle 4) in a plastic container. For mixture guidelines, refer to the mixing chart provided with this test kit or use the formula below.

$$\text{Volume hybridization solution (bottle 3)} = [(N \times 0.1) + 1.6] \text{ mL}$$

$$\text{Volume probe solution (bottle 4)} = [(N \times 0.025) + 0.4] \text{ mL}$$

where N is the number of samples to be tested including controls.

(4.5.2.7) Without touching the bottoms of the wells, place the appropriate number of microwells in the plate frame, filling the frame left to right and front to back in rows of 8. Include wells for the reagent blank, negative control, and positive control. *Note*: If the last row has fewer than 8 wells and a plate-washing device is used, fill the last row with colored wells. (Colored wells are available free of charge from Neogen.)

4.6 Sample Preparation

Note: Food samples should be obtained and handled according to standard practices appropriate to analysis for *Listeria* spp. All enrichment media should be prewarmed to room temperature before use. If needed, media formulations are available from Neogen Corp.

(4.6.1) *Sample preparation and enrichment*.

(4.6.1.1) *For red meat and poultry*.—Homogenize (Stomacher for 1–2 min) 25 g sample in 225 mL UVM broth. Incubate 24 ± 2 h at 30°C . Remove UVM culture from incubation and mix well.

(4.6.1.2) *For dairy products, seafood, and fruits and vegetables*.—Homogenize (Stomacher for 1–2 min) 25 g sample in 225 mL BLEB base. Incubate 4 h at 30°C . Add BLEB base supplements and incubate 20 ± 1 h at 30°C . Remove culture from incubation and mix well.

(4.6.1.3) *Environmental swab samples*.—Place swab in 10 mL UVM broth. Mix on a Vortex mixer or mix vigorously for 10 s. Leave swab in broth. Incubate 24 ± 4 h at $30\text{--}35^\circ\text{C}$. Remove UVM culture from incubation and mix well.

Table 1. Inclusivity testing results

Strain No.	Organism	Serotype (if known)	Source	OD ^a		Results
				1:10 ^b Dilution	1:100 ^b Dilution	
BLEB primary enrichment						
A169	<i>L. monocytogenes</i>	2	ATCC 19112	2.950	0.542	Positive
A207	<i>L. monocytogenes</i>	4b	ATCC 13932	2.950	0.154	Positive
A170	<i>L. monocytogenes</i>	4a	ATCC 19114	2.950	0.621	Positive
GT3829	<i>L. monocytogenes</i>	1a	C. Donnelly	2.950	0.713	Positive
GT1072	<i>L. monocytogenes</i>	1a	C. Donnelly	2.950	1.122	Positive
GT1880	<i>L. monocytogenes</i>	1a	J. Lovett	2.950	0.472	Positive
GT3812	<i>L. monocytogenes</i>	1a	J. Lovett	2.950	0.514	Positive
GT1021	<i>L. monocytogenes</i>	1/2a	CDC	2.406	0.355	Positive
GT3727	<i>L. monocytogenes</i>	1/2a	H. Seeliger	2.950	0.202	Positive
GT4340	<i>L. monocytogenes</i>	1/2a	CDC	2.934	0.489	Positive
GT1038	<i>L. monocytogenes</i>	1/2a	H. Seeliger	2.950	0.243	Positive
GT3635	<i>L. monocytogenes</i>	1/2b	H. Seeliger	2.769	0.261	Positive
GT3728	<i>L. monocytogenes</i>	1/2b	H. Seeliger	1.577	0.154	Positive
GT3856	<i>L. monocytogenes</i>	1/2b	H. Seeliger	2.950	0.274	Positive
GT3698	<i>L. monocytogenes</i>	1/2c	H. Seeliger	2.950	0.961	Positive
GT3648	<i>L. monocytogenes</i>	1/2c	H. Seeliger	2.950	0.410	Positive
GT3730	<i>L. monocytogenes</i>	1/2c	H. Seeliger	2.950	0.461	Positive
GT3741	<i>L. monocytogenes</i>	1/2c	H. Seeliger	2.950	1.205	Positive
GT3720	<i>L. monocytogenes</i>	3a	H. Seeliger	2.460	0.160	Positive
GT1035	<i>L. monocytogenes</i>	3a	H. Seeliger	2.950	0.266	Positive
GT1057	<i>L. monocytogenes</i>	3b	J. Lovett	2.029	0.169	Positive
GT3715	<i>L. monocytogenes</i>	3b	H. Seeliger	2.950	1.879	Positive
GT3817	<i>L. monocytogenes</i>	3b	H. Seeliger	2.022	0.125	Positive
GT3857	<i>L. monocytogenes</i>	3b	J. Lovett	2.196	0.305	Positive
GT1019	<i>L. monocytogenes</i>	4b	GENE-TRAK	2.950	0.813	Positive
GT1081	<i>L. monocytogenes</i>	4b	CDC	2.950	0.173	Positive
GT3819	<i>L. monocytogenes</i>	4c	H. Seeliger	2.950	0.317	Positive
GT4800	<i>L. grayi</i>		GENE-TRAK	1.145	0.157	Positive
GT674	<i>L. grayi</i>		Inst. Pasteur	2.944	0.846	Positive
GT3881	<i>L. grayi</i> subsp. <i>murrayi</i>		GENE-TRAK	1.908	0.201	Positive
GT3785	<i>L. innocua</i>		CDC	0.993	0.162	Positive
GT3627	<i>L. innocua</i>	6a	H. Seeliger	2.240	0.209	Positive
GT3631	<i>L. innocua</i>	6a	H. Seeliger	2.950	0.540	Positive
GT1026	<i>L. innocua</i>	6b	H. Seeliger	1.444	0.141	Positive
GT1042	<i>L. innocua</i>	6b	H. Seeliger	2.355	0.246	Positive
GT1044	<i>L. innocua</i>	6b	H. Seeliger	2.950	0.285	Positive
A102	<i>L. innocua</i>	6a	ATCC 33090	2.950	0.287	Positive
GT1050	<i>L. innocua</i>	6b	H. Seeliger	2.049	0.166	Positive
GT1052	<i>L. innocua</i>		J. Farber	2.934	0.389	Positive
A140	<i>L. ivanovii</i>		ATCC 19119	2.551	0.282	Positive
GT1028	<i>L. ivanovii</i>	5	H. Seeliger	2.950	2.348	Positive
GT1040	<i>L. ivanovii</i>	5	H. Seeliger	2.950	0.849	Positive
GT3699	<i>L. ivanovii</i>	5	H. Seeliger	2.950	0.351	Positive

Table 1. (continued)

Strain No.	Organism	Serotype (if known)	Source	OD ^a		Results
				1:10 ^b Dilution	1:100 ^b Dilution	
GT3794	<i>L. seeligeri</i>	1	J. Lovett	2.950	1.725	Positive
A201	<i>L. seeligeri</i>		ATCC 51334	2.950	2.943	Positive
GT3693	<i>L. seeligeri</i>	1/2b	H. Seeliger	2.950	2.943	Positive
GT289	<i>L. seeligeri</i>	4a	H. Seeliger	2.950	2.619	Positive
A199	<i>L. welshimeri</i>		ATCC 35897	2.045	0.945	Positive
A200	<i>L. welshimeri</i>		ATCC 43550	2.950	2.314	Positive
GT1773	<i>L. welshimeri</i>		Environmental isolate	2.950	1.479	Positive
GT1729	<i>L. welshimeri</i>		Dairy plant	2.950	2.943	Positive
GT293	<i>L. welshimeri</i>	6a	H. Seeliger	2.950	0.424	Positive
UVM primary enrichment						
A169	<i>L. monocytogenes</i>	2	ATCC 19112	2.949	2.374	Positive
A207	<i>L. monocytogenes</i>	4b	ATCC 13932	2.949	2.948	Positive
A170	<i>L. monocytogenes</i>	4a	ATCC 19114	2.949	1.543	Positive
GT3829	<i>L. monocytogenes</i>	1a	C. Donnelly	2.949	1.815	Positive
GT1072	<i>L. monocytogenes</i>	1a	C. Donnelly	2.949	2.948	Positive
GT1880	<i>L. monocytogenes</i>	1a	J. Lovett	2.949	0.485	Positive
GT3812	<i>L. monocytogenes</i>	1a	J. Lovett	2.949	1.099	Positive
GT1021	<i>L. monocytogenes</i>	1/2a	CDC	2.949	0.521	Positive
GT3727	<i>L. monocytogenes</i>	1/2a	H. Seeliger	2.949	1.286	Positive
GT4340	<i>L. monocytogenes</i>	1/2a	CDC	2.949	2.379	Positive
GT1038	<i>L. monocytogenes</i>	1/2a	H. Seeliger	2.949	2.288	Positive
GT3635	<i>L. monocytogenes</i>	1/2b	H. Seeliger	2.949	0.775	Positive
GT3728	<i>L. monocytogenes</i>	1/2b	H. Seeliger	2.949	0.763	Positive
GT3856	<i>L. monocytogenes</i>	1/2b	H. Seeliger	2.949	0.629	Positive
GT3698	<i>L. monocytogenes</i>	1/2c	H. Seeliger	2.949	2.563	Positive
GT3648	<i>L. monocytogenes</i>	1/2c	H. Seeliger	2.949	1.178	Positive
GT3730	<i>L. monocytogenes</i>	1/2c	H. Seeliger	2.949	1.391	Positive
GT3741	<i>L. monocytogenes</i>	1/2c	H. Seeliger	2.949	2.948	Positive
GT3720	<i>L. monocytogenes</i>	3a	H. Seeliger	2.949	1.564	Positive
GT1035	<i>L. monocytogenes</i>	3a	H. Seeliger	2.949	2.478	Positive
GT1057	<i>L. monocytogenes</i>	3b	J. Lovett	2.949	0.769	Positive
GT3715	<i>L. monocytogenes</i>	3b	H. Seeliger	2.949	2.948	Positive
GT3817	<i>L. monocytogenes</i>	3b	H. Seeliger	2.949	0.733	Positive
GT3857	<i>L. monocytogenes</i>	3b	J. Lovett	2.949	0.817	Positive
GT1019	<i>L. monocytogenes</i>	4b	GENE-TRAK	2.949	2.948	Positive
GT1081	<i>L. monocytogenes</i>	4b	CDC	2.949	2.948	Positive
GT3819	<i>L. monocytogenes</i>	4c	H. Seeliger	2.949	1.689	Positive
GT4800	<i>L. grayi</i>		GENE-TRAK	2.949	0.404	Positive
GT674	<i>L. grayi</i>		Inst. Pasteur	2.949	1.044	Positive
GT3881	<i>L. grayi</i> subsp. <i>murrayi</i>		GENE-TRAK	0.418	0.133	Positive
GT3785	<i>L. innocua</i>		CDC	2.949	2.058	Positive
GT3627	<i>L. innocua</i>	6a	H. Seeliger	2.949	0.759	Positive
GT3631	<i>L. innocua</i>	6a	H. Seeliger	2.949	1.752	Positive
GT1026	<i>L. innocua</i>	6b	H. Seeliger	2.949	0.878	Positive
GT1042	<i>L. innocua</i>	6b	H. Seeliger	2.949	0.600	Positive

Table 1. (continued)

Strain No.	Organism	Serotype (if known)	Source	OD ^a		Results
				1:10 ^b Dilution	1:100 ^b Dilution	
GT1044	<i>L. innocua</i>	6b	H. Seeliger	2.949	0.521	Positive
A102	<i>L. innocua</i>	6a	ATCC 33090	2.949	1.265	Positive
GT1050	<i>L. innocua</i>	6b	H. Seeliger	2.949	1.394	Positive
GT1052	<i>L. innocua</i>		J. Farber	2.949	1.189	Positive
A140	<i>L. ivanovii</i>		ATCC 19119	2.949	0.627	Positive
GT1028	<i>L. ivanovii</i>	5	H. Seeliger	2.949	0.354	Positive
GT1040	<i>L. ivanovii</i>	5	H. Seeliger	2.949	2.948	Positive
GT3699	<i>L. ivanovii</i>	5	H. Seeliger	2.949	0.712	Positive
GT3794	<i>L. seeligeri</i>	1	J. Lovett	2.949	2.363	Positive
A201	<i>L. seeligeri</i>		ATCC 51334	2.949	2.948	Positive
GT3693	<i>L. seeligeri</i>	1/2b	H. Seeliger	2.949	0.532	Positive
GT289	<i>L. seeligeri</i>	4a	H. Seeliger	2.949	2.948	Positive
A199	<i>L. welshimeri</i>		ATCC 35897	2.949	2.948	Positive
A200	<i>L. welshimeri</i>		ATCC 43550	2.949	2.948	Positive
GT1773	<i>L. welshimeri</i>		Environmental isolate	2.949	2.948	Positive
GT1729	<i>L. welshimeri</i>		Dairy plant	2.949	2.948	Positive
GT293	<i>L. welshimeri</i>	6a	H. Seeliger	2.949	2.948	Positive

^a OD = Optical density. OD values ≥ 0.10 were considered positive.

^b Dilutions (1:10 and 1:100) of a PBS resuspension of growth from an OXA plate were tested.

(4.6.1.4) Environmental sponge samples.—Place sponge in appropriate volume (e.g., 100–200 mL) UVM broth. Mix on a Vortex mixer or mix vigorously for 10 s. Leave sponge in broth. Incubate 24 ± 4 h at $30\text{--}35^\circ\text{C}$. Remove UVM culture from incubation and mix well.

(4.6.2) Dip a sterile cotton swab into culture and swab onto the entire surface of an OXA plate, expressing as much liquid from the swab as possible. Incubate plate 24 ± 2 h at 35°C .

(4.6.3) With a sterile cotton swab, swab off growth from the plate. (Swab entire surface of plate, removing as much growth as possible.) Suspend the growth in 1 mL PBS in a sterile, capped tube by swirling swab vigorously for 5 s. Express as much liquid as possible before discarding the swab.

(4.6.4) Perform GeneQuence assay on 0.2 mL aliquot of growth suspension. Save remaining growth suspension for possible culture confirmation.

4.7 Analysis

(4.7.1) Mix the test samples by gently shaking the test tubes. Shake positive and negative control solutions by inverting the bottles several times. Add 0.2 mL each control and test sample to the appropriately labeled tubes.

(4.7.2) Add 0.05 mL reconstituted pretreatment reagent (bottle 1a) and 0.05 mL reconstituted lysis reagent (bottle 2a) to each tube. Optionally, add 0.1 mL of the combined pretreatment/lysis solution (bottle 2a) to each tube. Mix by

gently shaking the rack of tubes by hand for 5 s. The resulting solution should be green. If any tubes are not green, check for proper reagent addition. Incubate the rack of tubes in the 37°C water bath or heater block for 5 min.

(4.7.3) Remove the tubes from the heat source. Transfer 0.15 mL of each lysed sample, including the controls, to designated microwells. The first well should be reserved for the reagent blank and receives no sample. The second well should be used for the negative control, and the third for the positive control.

(4.7.4) Vigorously mix the hybridization/probe solution prepared earlier by shaking or mixing on a Vortex mixer. Add 0.125 mL to each microwell, and mix each well 5 times with the pipettor. Do not add hybridization/probe solution to the reagent blank microwell.

(4.7.5) Incubate the plate at 45°C for 60 min in the incubator or covered heater block.

(4.7.5.1) Wash the wells using one of the following procedures:

(4.7.5.1.1) Plate-washing device.—Wash 5 times at room temperature. For each wash, process one 8-well strip at a time by aspirating the liquid, filling the wells, and then proceeding to the next strip. After the last wash, aspirate the liquid from the wells, then remove any residual liquid by inverting the plate and tapping it onto absorbent paper. Hold the plate by gently squeezing on the sides of the frame to keep the strips in place.

Table 2. Exclusivity testing results

Strain No.	Organism	Source	OD ^a	Results
GT803	<i>Bacillus brevis</i>	ATCC 8186	0.007	Negative
A208	<i>Bacillus cereus</i>	ATCC 25621	0.004	Negative
GT811	<i>Bacillus coagulans</i>	ATCC 7050	0.034	Negative
GT10	<i>Bacillus fragilis</i>	ATCC 23745	0.001	Negative
GT2128	<i>Bacillus megaterium</i>	ATCC 14581	0.005	Negative
GT804	<i>Bacillus subtilis</i>	ATCC 23059	0.002	Negative
GT4373	<i>Bacillus stearothermophilus</i>	ATCC 12980	0.010	Negative
GT664	<i>Brocothrix thermosphacta</i>	ATCC 11509	0.000	Negative
GT918	<i>Enterococcus durans</i>	ATCC 11576	0.019	Negative
GT406	<i>Enterococcus faecalis</i>	ATCC 19433	0.015	Negative
GT919	<i>Enterococcus faecium</i>	ATCC 6057	0.016	Negative
GT923	<i>Enterococcus hirae</i>	ATCC 35220	0.022	Negative
GT2129	<i>Kurthia gibsonii</i>	ATCC 43195	0.004	Negative
GT1941	<i>Kurthia zopfii</i>	ATCC 33403	0.002	Negative
GT256	<i>Lactobacillus acidophilus</i>	ATCC 4356	0.007	Negative
GT4082	<i>Lactobacillus buchneri</i>	ATCC 11305	0.006	Negative
GT805	<i>Lactobacillus casei</i>	ATCC 393	0.010	Negative
GT4063	<i>Lactobacillus fermentum</i>	ATCC 9338	0.019	Negative
GT3516	<i>Lactococcus lactis</i>	ATCC 11454	0.019	Negative
GT1943	<i>Micrococcus luteus</i>	ATCC 381	0.028	Negative
GT1944	<i>Micrococcus roseus</i>	ATCC 186	0.008	Negative
GT4404	<i>Micrococcus varians</i>	ATCC 15306	0.017	Negative
GT665	<i>Rodococcus equi</i>	ATCC 6939	0.029	Negative
GT3524	<i>Rodococcus fascians</i>	ATCC 12974	0.030	Negative
GT3474	<i>Rodococcus sputi</i>	ATCC 29627	0.015	Negative
A179	<i>Staphylococcus aureus</i>	ATCC 12600	0.020	Negative
A183	<i>Staphylococcus epidermidis</i>	ATCC 14990	0.016	Negative
A185	<i>Staphylococcus saprophyticus</i>	ATCC 15305	0.015	Negative
GT668	<i>Streptococcus bovis</i>	ATCC 9809	0.005	Negative
GT3596	<i>Streptococcus equi</i>	ATCC 33398	0.042	Negative
GT412	<i>Streptococcus mutans</i>	ATCC 25175	0.018	Negative
GT408	<i>Streptococcus pneumoniae</i>	ATCC 6303	0.013	Negative
GT411	<i>Streptococcus sanguis</i>	ATCC 10556	0.015	Negative

^a OD = Optical density. OD values < 0.10 were considered negative.

(4.7.5.1.2) Manual.—Thoroughly wash the microwells at least 5 times using a plastic wash bottle filled with the wash solution prepared earlier. Wash by emptying the wells into a suitable container, tapping the inverted plate on absorbent paper, completely filling the wells with wash solution, and vigorously shaking out the contents. *Note:* All air bubbles must be removed before proceeding to the next step. If air bubbles remain, repeat vigorous striking of the wells onto an absorbent paper towel on a flat surface until eliminated.

(4.7.6) Add 0.15 mL substrate chromogen solution (bottle 6) to each microwell, including the reagent blank microwell. Incubate the plate at room temperature for 20 min.

(4.7.7) Add 0.05 mL stop solution (bottle 7) to each microwell, including the blank microwell.

(4.7.8) Gently tap the side of frame a few times to ensure mixing.

(4.7.9) Read absorbance at 450 nm using a plate or strip reader according to the manufacturer's instructions. Blank

using the first microwell that contains the mixture of substrate chromogen and stop solution (do not blank with air).

4.8 Interpretation and Test Result Report

(4.8.1) Control values.—The absorbance value for the negative control must be ≤ 0.15 , and the absorbance value for the positive control must be ≥ 1.00 . If either control is outside the acceptance range, the test is invalid and should be repeated.

(4.8.2) Negative criterion.—Tests producing absorbance values < 0.10 are negative for the presence of *Listeria* spp. in the test samples.

(4.8.3) Positive criterion.—Tests producing absorbance values ≥ 0.10 are positive for the presence of *Listeria* spp. in the test samples. A positive test result should be confirmed by standard culture procedures.

(4.8.4) Recommended confirmation procedure.—Neogen recommends that positive results be confirmed culturally by swabbing a portion of the remaining PBS growth resuspension onto a *Listeria* selective/differential agar plate (MOX agar plate is recommended), and by continuing with biochemical identification of presumptive *Listeria* isolates using standard procedures, e.g., miniaturized biochemical identification strips such as API or Micro-ID (available from bioMérieux, Hazelwood, MO).

5 Safety Precautions

Use of this test should be restricted to individuals with appropriate laboratory training in microbiology. Reagents are for laboratory use only. Stop solution contains 4.0 N sulfuric acid. Hybridization solution contains formamide. Avoid contact with skin and mucous membranes. Refer to Material Safety Data Sheet available from Neogen for more information. Reagents from different kit lots should not be interchanged. GeneQuence *Listeria* Test reagents are not interchangeable with other GeneQuence test reagents. Reagents should not be used beyond their expiration dates. Enrichment cultures should be handled and disposed of as potentially infectious material. The preferred method for disposal of contaminated materials, including cultures, pipets, etc., is autoclaving. Items that cannot be autoclaved should be decontaminated by treatment with disinfectant solution and rinsing with water. This test should be performed in a normal laboratory environment with respect to humidity and lighting. Steps requiring room temperature incubation should be performed at 18–30°C.

6 Summary of Results

6.1 Internal Validation Studies

(6.1.1) Inclusivity testing.—Fifty-two different strains of *Listeria* spp. were tested with the DNA hybridization assay to assess the inclusivity of the method. Strains tested (Table 1) are from the collections of the Centers for Disease Control and Prevention, ATCC, Neogen Corp., and other sources. The microwell-format hybridization assay uses the same DNA

probe set used in the dipstick-format GeneQuence *Listeria* DLP assay, which has been previously evaluated and approved in the *Performance-Tested Method*SM program (Method No. 981201).

(6.1.2) Methodology.—A single colony of the test organism from a trypticase soy agar plate was inoculated into BLEB containing supplements and incubated static for 24 h at 30°C. A second colony was inoculated into UVM and incubated static for 24 h at 30°C. A sterile cotton swab was inserted into the BLEB culture and swabbed onto the surface of an OXA plate. A second OXA plate was swabbed from the UVM culture. The OXA plates were incubated for 24 h at 35°C. Sterile cotton swabs were then used to collect growth from the OXA plates, and the growth was resuspended in 1 mL aliquots of PBS. Serial dilutions were made in PBS, and 1:10 and 1:100 dilutions were tested with the *Listeria* microwell assay.

(6.1.3) Results.—All strains tested produced positive results with the microwell assay from both primary enrichment media (Table 1).

6.2 Exclusivity Testing

Thirty-three strains of non-*Listeria* bacteria, representing 10 genera, were tested in the DNA hybridization assay to assess the inherent specificity of the method. Strains tested are shown in Table 2.

(6.2.1) Methodology.—A single colony of the test organism was inoculated into 10 mL TSB and incubated static for 18–24 h at 35°C. These growth conditions produced final titers of approximately $1\text{--}5 \times 10^9$ CFU/mL. The cultures were tested undiluted with the microwell assay (approximately 2×10^8 CFU/assay).

(6.2.2) Results.—All non-*Listeria* strains were negative with the microwell assay (Table 2).

6.3 Test Sensitivity and Specificity for Inoculated Foods

The microwell DNA hybridization assay was compared to either the USDA/FSIS (1) or FDA (2) reference methods for the recovery of *Listeria* in 15 foods: ground beef, deli turkey, hot dog, frozen ground pork, deli ham, parmesan cheese, brie cheese, pasteurized milk, ice cream, raw shrimp, pasteurized crab meat, smoked salmon, lettuce, mixed vegetables, and alfalfa sprouts.

(6.3.1) Methodology.

(6.3.2) Preparation of inocula and inoculation of food samples.—The inoculated foods were prepared using methods intended to simulate normal food storage conditions, including subjecting the inoculum strains to sublethal stress when appropriate. A summary of tested food products, organisms, and inoculation levels is shown in Tables 3 and 4. The inoculum culture was started by transferring growth from a refrigerated (4°C) TSA slant to 9 mL TSB tubes. TSB tubes were incubated at 35°C. After 24 h incubation, 0.1–1.0 mL of this culture was transferred to 9 mL TSB and incubated at 35°C for 24 h. Serial dilutions in PBS were made from these

second TSB tubes to prepare the inoculum. Samples were inoculated to yield a fractional number of positives.

When required, food products were homogenized prior to inoculation by aseptic mechanical blending (e.g., cheeses, deli meats, seafoods, mixed vegetables) or finely chopping (e.g., lettuce). Certain foods did not require homogenization before inoculation, including milk, ground meats, and alfalfa sprouts. Three 600 g batches of homogenized food were weighed aseptically.

From one uninoculated batch, ten 25 g samples were weighed in sterile plastic bags (Whirl-Pak® Nasco, Fort Atkinson, WI). These were the control (uninoculated) samples. A second 600 g food batch was inoculated with

approximately 40 CFU, which resulted in approximately ≤ 2 CFU/25 g. The inoculum was manually mixed (e.g., sterile spoon) with the food product in a large sterile beaker under aseptic conditions to ensure uniform distribution of the inoculum. Twenty 25 g samples were weighed from this batch for the low inoculation level. The third 600 g food batch was inoculated with approximately 200 CFU, which would result in approximately 10 CFU/25 g, and mixed as described above. Twenty 25 g samples were weighed from this batch for the high inoculation level. All samples were blind-coded to assure that the personnel processing the samples did not know the inoculation level of the samples. All test samples were either refrigerated at 5°C (ground beef, deli turkey, hot dogs, deli

Table 3. Results of inoculated food experiments; comparison between the DNA method and the USDA FSIS method

Foods	Organism	No. of samples	MPN, CFU/g	Total positive ^a	Samples positive			
					Assay ^b	Confirmed ^c	USDA/FSIS	Chi square ^d
Ground beef (Trial 1)	<i>L. monocytogenes</i> , sero 4b	20	11.00	20	20	20	20	NA ^e
		20	0.04	18	18	18	18	NA
		10	0.00	2	1	1	2	0.00
Ground beef (Trial 2)	<i>L. monocytogenes</i> , sero 4b	20	4.60	20	20	20	20	NA
		20	0.28	18	13	13	18	3.20
		20	0.00	0	0	0	0	NA
Deli turkey (Trial 1)	<i>L. monocytogenes</i> , sero 1a	20	2.40	20	20	20	20	NA
		20	<0.03	5	5	5	5	NA
		10	0.00	0	0	0	0	NA
Deli turkey (Trial 2)	<i>L. monocytogenes</i> , sero 1a	20	0.38	20	20	20	20	NA
		20	0.23	14	14	14	14	NA
		10	0.00	0	0	0	0	NA
Hot dogs	<i>L. monocytogenes</i> , sero 4b	20	0.75	16	16	16	16	NA
		20	<0.03	5	5	5	5	NA
		10	0.00	0	0	0	0	NA
Ground pork (Trial 1)	<i>L. monocytogenes</i> , sero 1/2a	20	2.40	19	19	19	18	0.00
		20	<0.03	8	7	7	6	0.00
		10	0.00	1	1	1	1	NA
Ground pork (Trial 2)	Naturally contaminated, uninoculated	20	0.00	0	1	0	0	NA
		20	0.23	18	16	16	18	0.50
		20	0.00	0	0	0	0	NA
Deli ham	<i>L. monocytogenes</i> , sero 1/2b	20	0.15	19	19	19	19	NA
		20	<0.03	2	2	2	2	NA
		10	0.00	0	0	0	0	NA
Total		420		225	217	216	222	

^a Number of samples confirmed positive by one or more methods.

^b Number of samples positive by DNA hybridization assay not considering subsequent culture confirmation.

^c Number of samples positive by DNA hybridization assay and confirmed by plating from associated cultures.

^d Chi square value by McNemar's test comparing confirmed DNA hybridization assay and reference culture method. Chi square values ≥ 3.84 indicate a statistically significant difference at $P \leq 0.05$.

^e NA = Not applicable, results in perfect agreement.

Table 4. Results of inoculated food experiments, comparison of the DNA method and the FDA (BAM) method

Foods	Organism	No. of samples	MPN, CFU/g	Total positive ^a	Samples positive			Chi square ^d
					Assay ^b	Confirmed ^c	FDA method	
Parmesan	<i>L. monocytogenes</i> , sero 3b	20	0.93	20	19	19	17	0.25
		20	0.23	14	14	14	12	0.50
		10	0.00	0	0	0	0	NA ^e
Brie cheese	<i>L. monocytogenes</i> , sero 1/2a	20	0.04	20	20	20	14	4.17
		20	0.06	19	19	19	18	0.00
		10	0.00	0	0	0	0	NA
Pasteurized milk	<i>L. monocytogenes</i> , sero 1/2a	20	0.23	19	19	19	19	NA
		20	0.03	1	2	1	1	NA
		10	0.00	0	1	0	0	NA
Ice cream	<i>L. monocytogenes</i> , sero 4c	20	0.40	13	13	13	13	NA
		20	<0.03	0	2	0	0	NA
		10	0.00	0	0	0	0	NA
Raw shrimp	<i>L. monocytogenes</i> , sero 1/2c	20	0.04	9	8	8	5	0.80
		20	<0.03	0	0	0	0	NA
		10	0.00	0	0	0	0	NA
Pasteurized crab meat	<i>L. monocytogenes</i> , sero 1/2c	20	0.75	20	20	20	20	NA
		20	0.04	18	18	18	18	NA
		10	0.00	0	0	0	0	NA
Smoked salmon	<i>L. monocytogenes</i> , sero 1/2c	20	2.40	20	20	20	20	NA
		20	0.23	18	15	15	18	1.33
		10	0.00	0	0	0	0	NA
Lettuce	<i>L. ivanovii</i>	20	0.43	20	20	20	20	NA
		20	0.15	19	19	19	19	NA
		10	0.00	0	0	0	0	NA
Mixed vegetables	<i>L. seeligeri</i> , sero 1 (<i>L. monocytogenes</i> , natural contaminant)	20	0.09	20	20	20	20	NA
		20	0.75	18	18	18	15	1.33
		10	0.43	9	9	9	8	0.00
Alfalfa sprouts	<i>L. monocytogenes</i> , sero 3a	20	11.00	20	20	20	19	0.00
		20	0.38	15	14	14	11	0.80
		10	0.00	0	0	0	0	NA
Total		500		312	310	306	288	

^a Number of samples confirmed positive by one or more methods.

^b Number of samples positive by DNA hybridization assay not considering subsequent culture confirmation.

^c Number of samples positive by DNA hybridization assay and confirmed by plating from associated cultures.

^d Chi square value by McNemar's test comparing confirmed DNA hybridization assay and reference culture method. Chi square values ≥ 3.84 indicate a statistically significant difference at $P \leq 0.05$.

^e NA = Not applicable, results in perfect agreement.

ham, parmesan cheese, brie cheese, pasteurized milk, smoked salmon, lettuce, alfalfa sprouts) or frozen at -20°C (ground pork, ice cream, raw shrimp, pasteurized crab meat, mixed vegetables) for at least 72 h prior to analysis.

The number of viable *Listeria* spp. per gram of food was estimated by a most probable number (MPN) determination performed on the day of sample pre-enrichment. For the analysis, 10, 1, and 0.1 g portions (in triplicate) of the food were placed into pre-enrichment media, and then analyzed for the presence of *Listeria* by carrying the samples through the steps of pre-enrichment and selective enrichment described by either the FDA or the USDA/FSIS method. MPN values are shown in Tables 3 and 4.

(6.3.3) Analysis of test products.—For each food trial, fifty 25 g samples were prepared for analysis. Twenty samples were inoculated with *Listeria* spp. at a high level of approximately 0.4–2 CFU/g of food, 20 samples were inoculated at a low level of approximately 0.04–0.2 CFU/g of food, and 10 samples served as uninoculated controls.

The test food samples were analyzed in parallel by the DNA hybridization method and either the FDA or the USDA/FSIS reference method. The initial enrichment step used was the one indicated by the reference method. After enrichment, a sterile cotton swab was dipped into the enrichment broth and swabbed onto the entire surface of an OXA plate, expressing as much liquid from the swab as possible. Plates were incubated for 24 ± 2 h at 35°C . After incubation, the entire surface of the OXA plate was swabbed with a sterile cotton swab, removing as much growth as possible. The collected growth was suspended in 1 mL PBS in a sterile, capped tube by swirling the swab vigorously. The microwell assay was performed on a 0.2 mL aliquot of growth suspension, with the remainder stored at 4°C for possible culture confirmation.

Positive results were confirmed culturally by streaking from the growth resuspension onto MOX plates, and by confirmation of presumptive *Listeria* isolates by the use of API *Listeria* tests (bioMérieux).

(6.3.4) Results.—Summaries of the comparative data for all food samples tested by the DNA assay versus the USDA/FSIS method, and the DNA assay versus the FDA method, are presented in Tables 3 and 4, respectively. A total of 537 out of 920 samples were confirmed positive for the presence of *Listeria* spp. The DNA method detected 522 of 537 total positives for an overall sensitivity of 97.2%. There were 5 unconfirmed positive assay results by the DNA method resulting in a specificity of 98.7%.

The total number of samples tested with the USDA method was 420. The USDA method detected 222 of 225 positive samples, for a sensitivity of 98.7%. The DNA method detected 216 positive samples for a sensitivity of 96.0%. There was one unconfirmed positive result by the DNA method, for a specificity of 99.5%.

The total number of samples tested with the FDA method was 500. The FDA method detected 288 samples out of 312 samples confirmed positive, resulting in a sensitivity of 92.3%.

The DNA method detected 310, of which 306 were confirmed positive. Therefore, the DNA method gave 98.1% sensitivity, with a specificity of 97.9%.

Listeria spp. were detected in uninoculated samples for 3 raw food commodities: ground beef, ground pork, and mixed vegetables. In ground beef, *L. welshimeri* and *L. monocytogenes* were isolated from 2 control samples. In ground pork, one control sample presented *L. monocytogenes*. Because raw meats frequently contain *Listeria* spp., it is not unreasonable to assume that these positive samples represent natural contaminants. However, since it could not be proven that the isolates from these samples were distinguishable from the inoculum strains, additional trials were performed with ground beef and ground pork. In the second trial with ground beef, all uninoculated control samples were negative (Table 3). For ground pork, prescreening experiments (data not shown) indicated a high incidence of *Listeria* spp. in this commodity. Considering this, it was decided to use uninoculated material in the repeat experiment. Ground pork was obtained from 3 different sources, and 20 samples from each batch were tested. Two batches yielded no confirmed positives, while the third batch produced 18 confirmed positives out of the 20 samples (Table 3).

For the mixed vegetables, the inoculated strain was *L. seeligeri*, but the majority of the positive samples presented *L. monocytogenes*, and only *L. monocytogenes* was isolated from the control samples. This is clear evidence that the vegetables were naturally contaminated with *L. monocytogenes*.

For deli turkey, 2 trials were performed, as the first trial did not yield the required number of fractionally positive samples at a single inoculation level (5 observed versus the required 9–19). The second trial produced an acceptable number of positive samples (14) at one inoculation level. Data from both trials are included in Table 3.

(6.3.5) Agreement results.—The agreement between the reference methods and the GeneQuence *Listeria* Test was as follows: GeneQuence versus USDA/FSIS method 408/420 = 97.1%; GeneQuence versus FDA (BAM) method 470/500 = 94.0%.

6.4 Ruggedness Testing

The ability of the microwell-format assay to withstand perturbations to basic procedural specifications was examined in a series of ruggedness experiments. The following parameters were assessed: variation in the number of mixing steps of the lysed sample and the probe/hybridization solution; variation in the premixed probe/hybridization solution volume; variation in the number of wash steps; variation in the hybridization incubation temperature; and variation in the hybridization incubation time. These parameters were chosen for examination because they had been determined to be the most critical to proper performance of the microwell format assay based on results of developmental work.

(6.4.1) Methodology.—Each parameter was tested on 2 different days. In each case, performance of the assay at the specified condition was compared to performance at

2 conditions deviating from that specified. In each experiment, 6 samples were tested in triplicate. The samples included kit negative control, kit positive control, kit positive control diluted 1:10, undiluted cultures of *Enterococcus faecium*, and cultures of *L. monocytogenes* and *L. innocua* diluted 1:10. The bacterial cultures were grown from single colonies in TSB at 35°C for approximately 24 h. These conditions produced final titers of approximately $0.5\text{--}2.0 \times 10^9$ CFU/mL. Positive controls, *L. innocua* and *L. monocytogenes*, were used to assess the effects on assay sensitivity, while negative controls and *E. faecium* samples were used to measure effects on assay background or specificity.

(6.4.2) Variation in the number of mixing steps of the lysed sample and the probe/hybridization solution.—Three conditions were tested: no mixing, mixing 5 times, and mixing 10 times. In the first trial, a false positive was detected in a negative control at no mix. In addition, an *E. faecium* sample gave a false-positive signal in the set with 5 times mixing. Mixing does not seem to be an important contributor to the

Table 5. Quality control testing of 3 production lots of GENE-TRAK *Listeria* Test kits

Test sample	OD ^a			Acceptance criteria
	Lot No.			
	80001	80002	80003	
Negative control	0.070	0.039	0.029	
Negative control	0.050	0.035	0.022	≤0.07
Negative control	0.069	0.043	0.026	
Positive control	2.953	2.941	2.944	
Positive control	2.949	2.943	2.943	≥1.30
Positive control	2.949	2.942	2.942	
Positive control 1:5	2.953	2.941	2.944	
Positive control 1:5	2.949	2.943	2.943	≥0.60
Positive control 1:5	2.949	2.942	2.942	
Positive control 1:25	1.213	0.740	0.835	
Positive control 1:25	1.224	0.813	0.513	≥0.20
Positive control 1:25	1.296	0.836	0.775	
<i>Listeria monocytogenes</i>	2.953	2.941	2.944	
<i>Listeria monocytogenes</i>	2.949	2.943	2.943	
<i>Listeria monocytogenes</i>	2.949	2.942	2.942	
<i>Listeria innocua</i>	2.953	2.941	2.944	
<i>Listeria innocua</i>	2.949	2.943	2.943	
<i>Listeria innocua</i>	2.949	2.942	2.942	
<i>Enterococcus faecium</i>	0.040	0.038	0.026	
<i>Enterococcus faecium</i>	0.054	0.064	0.028	
<i>Enterococcus faecium</i>	0.049	0.050	0.025	

^a OD = Optical density; values shown are assay absorbance at 450 nm.

appearance of background. We still recommend mixing the lysed samples with the probe/hybridization solution 5 times to ensure complete distribution of formamide in the solution.

(6.4.2.1) Variation in the premixed probe/hybridization reagent volume.—Three conditions were tested: addition of 0.1, 0.125, and 0.15 mL probe/hybridization solution to the sample in the well. Results from these experiments clearly demonstrate that addition of less than the recommended amount of probe/hybridization solution may result in false-positive readings. Without the proper concentration of formamide (hybridization solution) in the hybridization reaction, the assay conditions do not have the proper stringency, and false-positive results may occur due to mismatching of the probe sequences with nontarget rRNA sequences. We recommend a volume of 0.125 mL ($\pm 5\%$) of probe/hybridization solution.

(6.4.2.2) Variation in the number of wash steps.—Three conditions were tested: washing 4 times, 5 times, and 6 times with the wash solution. In the first trial, only one negative control gave a false positive when washed 4 times. In the second trial, all results were within normal values. These results indicate that a minimum of 4 washes may be enough to achieve acceptable results in most cases. The recommendation, however, is to wash the wells 5 times before adding the substrate chromogen solution.

(6.4.2.3) Variation in the hybridization incubation temperature.—Three conditions were tested: 43, 47, and the prescribed 45°C hybridization incubation temperature. In terms of assay signal, no differences were apparent between the 3 conditions, although there was more assay background at 43°C than at the other 2 temperatures, as evidenced by somewhat higher absorbance values with the 3 negative control samples in the second trial. We recommend a hybridization temperature of $45 \pm 1^\circ\text{C}$.

(6.4.2.4) Variation in the hybridization incubation time.—The conditions tested were incubation for 45, 60, and 75 min. Results indicate that a variation of up to 15 min above or below the recommended time of 60 min does not affect the performance of the test.

6.5 Stability and Lot-to-Lot Variability

(6.5.1) Methodology.—Three production lots were tested in accordance with established quality control acceptance/rejection criteria. Test samples included kit positive and negative controls, dilutions of positive controls, and one strain each of *L. monocytogenes*, *L. innocua*, and *E. faecium*. Quality control testing results of 3 production lots and the acceptance criteria are shown in Table 5.

(6.5.2) Results.—Reagent stability was assessed in a real-time study in which kits were kept under the selected conditions of storage (4°C). Data were collected up to 26 weeks post-manufacture. Coated microwell plates were kept in resealable foil pouches with desiccant. Kit positive control diluted 1:5 and 1:25 in PBS and kit negative control were used as the test samples. The positive control dilutions typically produce assay absorbance values in the linear range in which differences will be most readily apparent. Results of

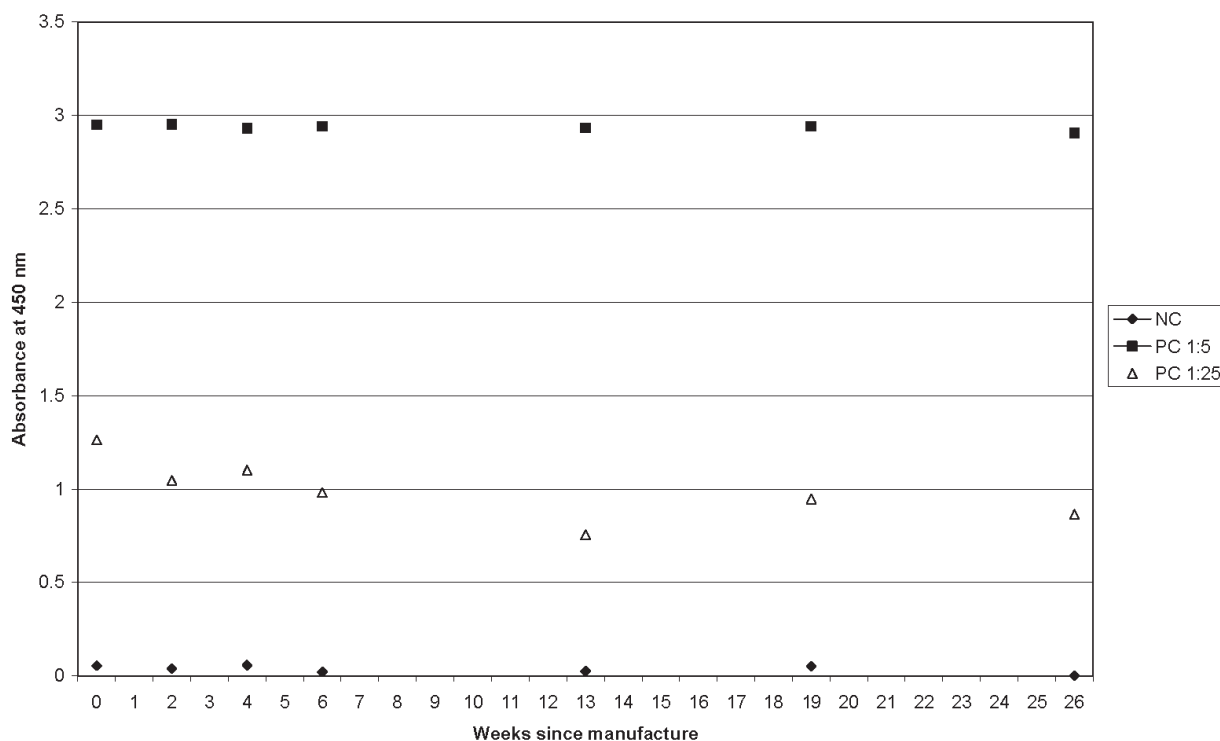


Figure 1. Stability study results for the GeneSequence *Listeria* Test. Tests were conducted with kit reagents at several time points post-manufacture, up to 26 weeks. Assay results in A_{450} are shown. PC 1:5 and PC 1:25 = kit positive control diluted 1:5 and 1:25 in PBS, respectively; NC = kit negative control.

stability testing (Figure 1) show that the positive control 1:5 and the negative control values remained constant throughout the study. The signal for the positive control 1:25 declined slightly during the testing storage time. However, the average value for the positive control 1:25 was always within the normal expected range (0.35–1.5 A_{450} units). Based on the results of this study, a 6 month shelf life was established for the test kit.

6.6 Independent Laboratory Validation Study

(6.6.1) Methodology.

(6.6.1.1) Food testing.—Two foods were chosen for analysis: cottage cheese and mayonnaise. Foods were inoculated with *L. innocua* (cottage cheese) or *L. monocytogenes* (mayonnaise) at 2 levels to yield at least one level containing fractional positives (Table 6). For each trial, 20 samples were prepared at each level, plus 5 uninoculated controls. Samples were analyzed by the microwell DNA hybridization method and the FDA culture method. See section 6.3 above for procedural details concerning sample preparation and analysis. Inoculation levels were determined on the day of analysis by MPN procedures.

(6.6.1.2) Environmental samples: food plant.—Environmental samples (sponge or swab) were collected from food manufacturing facilities over the course of 3 weeks and analyzed by the microwell DNA hybridization and USDA

culture methods. (See section 6.3 for details of the enrichment methods.) A common UVM broth primary enrichment was used for both methods, thus avoiding sample-splitting issues. Environmental surfaces sampled included stainless steel, plastic (polypropylene), glass, concrete, wood, rubber, painted surfaces, cast iron, and ceramic.

(6.6.1.3) Environmental samples: inoculated.—This study involved 6 environmental surface types (stainless steel, ceramic tile, cast iron, plastic, concrete, and painted wood) inoculated with *Listeria* spp. to produce sample sets yielding fractional positive results. Analyses were conducted in parallel by the microwell DNA hybridization and USDA culture methods (see section 6.3).

(6.6.1.3.1) Inoculated surface preparation.—For each surface type, 20 replicate inoculated surfaces and 5 uninoculated controls were prepared. A different strain of *Listeria* spp. was used for each surface type. For stainless steel, in addition to inoculation with *L. monocytogenes*, the surface was also inoculated with a cocktail of 3 competitor organisms (*Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212, and *Bacillus licheniformis* ATCC 12759) in a 1:1 ratio of competitors to *L. monocytogenes*. Test strains and inoculum levels are shown in Table 7. Test organisms were grown on trypticase soy agar with yeast extract (TSA-YE) and incubated for 20 ± 2 h at $35 \pm 1^\circ\text{C}$. Following incubation, a McFarland colorimeter was used to make a suspension of approximately 1×10^8 CFU/mL

Table 6. Results of independent laboratory inoculated food experiments; comparison of the DNA method and the FDA culture method

Foods	Organism	No. of samples	MPN, CFU/g	Total positive ^a	Samples positive			Chi square ^d
					Assay ^b	Confirmed ^c	FDA	
Cottage cheese	<i>L. innocua</i> ATCC 33090	20	0.075	14	14	14	14	NA ^e
		20	0.64	20	20	20	20	NA
		5	0.00	0	0	0	0	NA
Mayonnaise	<i>L. monocytogenes</i> , sero 4b ATCC 19115	20	0.46	12	12	12	12	NA
		5	0.00	0	0	0	0	NA

^a Number of samples confirmed positive by one or more methods.

^b Number of samples positive by DNA hybridization assay not considering subsequent culture confirmation.

^c Number of samples positive by DNA hybridization assay and confirmed by plating from associated cultures.

^d Chi square value by McNemar's test comparing confirmed DNA hybridization assay and reference culture method. Chi square values ≥ 3.84 indicate a statistically significant difference at $P \leq 0.05$.

^e NA = Not applicable, results in perfect agreement.

in 0.1% peptone water. The organism suspension was diluted to the appropriate titer in 0.1% peptone water, and inoculated onto the surface. Each surface type was pretested to determine an inoculum that would yield fractional positive results. The inoculation titer was verified by serial dilution and plating on TSA-YE. The exception to this preparation was painted wood. After the 1×10^8 CFU/mL suspension was made, further dilutions were made in Dey-Engley neutralizing broth (D/E broth) and the dilutions were used to inoculate the painted wood surface. Stainless steel, plastic, and painted wood surfaces were inoculated using 0.1 mL of the appropriate dilution and spreading with a pipet tip over a 1×1 in. area. Ceramic tile, cast iron, and concrete surfaces were inoculated by spreading 0.25 mL of the appropriate dilution over a 4×4 in. area using a sterile "hockey stick" spreader. The inoculated surfaces were allowed to dry for 18 ± 2 h before testing. For each surface type, 20 replicate inoculated surfaces were prepared.

(6.6.1.3.2) Surface sampling.—Three of the surfaces (stainless steel, plastic, and painted wood) were sampled with polyester swabs in a 1×1 in. test area, and 3 surfaces (ceramic tile, cast iron, and concrete) were sampled with sponges in a 4×4 in. test area. Before testing the surface, the swabs were moistened with D/E broth by dipping the swab into the D/E broth and expressing the excess. Sponges were moistened with 10 mL D/E broth before testing the surface.

(6.6.1.3.3) Enrichment and analysis.—UVM broth was used as the primary enrichment medium for both the DNA hybridization and USDA/FSIS analyses. Swab samples were enriched in 10 mL UVM, and sponges in 200 mL UVM. All enrichments were incubated at $30 \pm 1^\circ\text{C}$ for 24 ± 2 h. Secondary enrichments and all subsequent steps in the analyses were conducted as previously described (6.3.3).

(6.6.1.4) Results.

(6.6.1.4.1) Food samples.—Results of the cottage cheese trial are summarized in Table 6. One level yielded fractional positive results; the same 14 of 20 samples were positive by both the DNA hybridization and FDA methods. All DNA hybridization assay positive samples were confirmed by culture. The second level yielded 20 positive results by both methods. Again, all positive DNA hybridization assay samples were confirmed. All 5 uninoculated control samples were negative by both methods.

Results of the mayonnaise trials are also summarized in Table 6. In total, 5 trials were performed due to a high level of die-off of the inoculum in the highly acidic matrix. Four trials were invalid as they did not yield the minimum acceptable number of positive results (10 of 20). One trial yielded fractional positive results and met the requirement for the minimum number of positives. In this trial, the same 12 of 20 samples were positive by both the DNA hybridization and FDA culture methods. All DNA hybridization positive samples were confirmed by culture. All 5 uninoculated control samples were negative by both methods.

(6.6.1.4.2) Environmental samples: naturally contaminated.—A total of 104 environmental samples were analyzed. Results are shown in Table 7. There were a total of 17 samples positive by at least one of the methods. There were 14 samples positive (and confirmed) by the DNA hybridization method and 15 samples positive by the USDA culture method. There were 3 false-negative results by the DNA hybridization method and 2 false-negative results by the USDA culture method. The McNemar's χ^2 value for this comparison is 0.00, indicating that the difference in performance between the methods is not statistically significant at $P < 0.05$. Of 87 negative samples, there were no false-positive results by the DNA hybridization method. A variety of *Listeria* spp. were isolated from the individual positive swabs, including *L. monocytogenes*, *L. seeligeri*,

Table 7. Results of independent laboratory testing of naturally contaminated environmental samples; comparison of the DNA hybridization method and the USDA culture method

Swab No.	Swab type	Surface type	USDA method result	DNA hybridization method result
200	Sponge	Plastic	Suspect–conf. negative	Negative
201	Sponge	Plastic	Negative	Negative
202	Sponge	Stainless steel	Negative	Negative
203	Sponge	Stainless steel	Negative	Negative
204	Sponge	Stainless steel	Negative	Negative
205	Sponge	Stainless steel	Negative	Negative
206	Sponge	Stainless steel	Negative	Negative
207	Sponge	Stainless steel	Negative	Negative
208	Sponge	Stainless steel	Negative	Negative
209	Sponge	Stainless steel	Negative	Negative
210	Sponge	Stainless steel	Negative	Negative
211	Sponge	Stainless steel	Suspect–conf. negative	Negative
212	Sponge	Stainless steel	Negative	Negative
213	Sponge	Stainless steel	Positive– <i>L. monocytogenes</i>	Positive– <i>L. monocytogenes</i>
214	Sponge	Stainless steel	Negative	Negative
215	Sponge	Stainless steel	Negative	Negative
216	Sponge	Glass	Negative	Negative
217	Sponge	Plastic	Positive– <i>L. seeligeri</i>	Positive– <i>L. seeligeri</i>
218	Sponge	Plastic	Negative	Negative
219	Sponge	Plastic	Negative	Negative
220	Sponge	Plastic	Suspect–conf. negative	Negative
221	Sponge	Plastic	Negative	Negative
222	Sponge	Concrete	Negative	Negative
223	Sponge	Concrete	Negative	Negative
224	Sponge	Concrete	Negative	Negative
225	Sponge	Concrete	Negative	Negative
226	Sponge	Concrete	Negative	Negative
227	Sponge	Wood	Suspect–conf. negative	Negative
228	Sponge	Wood	Negative	Negative
229	Sponge	Wood	Positive– <i>L. monocytogenes</i>	Positive– <i>L. monocytogenes</i>
230	Sponge	Wood	Suspect–conf. negative	Negative
231	Sponge	Wood	Negative	Negative
232	Sponge	Wood	Negative	Negative
233	Sponge	Wood	Negative	Negative
234	Sponge	Wood	Negative	Negative
235	Sponge	Wood	Suspect–conf. negative	Positive– <i>L. welshimeri</i>
236	Sponge	Wood	Suspect–conf. negative	Negative
237	Sponge	Wood	Negative	Negative
238	Sponge	Wood	Negative	Negative
239	Sponge	Wood	Negative	Negative
240	Sponge	Wood	Negative	Negative
241	Sponge	Wood	Negative	Negative
242	Sponge	Wood	Positive– <i>L. welshimeri</i>	Positive– <i>L. innocua</i>
243	Sponge	Wood	Negative	Negative
244	Sponge	Wood	Negative	Negative

Table 7. (continued)

Swab No.	Swab type	Surface type	USDA method result	DNA hybridization method result
245	Sponge	Wood	Negative	Negative
246	Sponge	Wood	Negative	Negative
247	Sponge	Wood	Negative	Negative
248	Sponge	Wood	Negative	Negative
249	Sponge	Wood	Negative	Negative
250	Sponge	Wood	Positive— <i>L. welshimeri</i>	Positive— <i>L. welshimeri</i>
251	Sponge	Wood	Negative	Negative
252	Sponge	Wood	Negative	Negative
253	Sponge	Wood	Negative	Negative
254	Sponge	Wood	Negative	Negative
255	Sponge	Wood	Negative	Negative
256	Sponge	Plastic	Negative	Negative
257	Sponge	Plastic	Negative	Negative
258	Sponge	Plastic	Positive— <i>L. innocua</i>	Positive— <i>L. innocua</i>
259	Sponge	Plastic	Negative	Negative
260	Sponge	Plastic	Negative	Negative
261	Sponge	Plastic	Negative	Negative
262	Sponge	Plastic	Negative	Negative
263	Sponge	Plastic	Negative	Negative
264	Sponge	Plastic	Positive— <i>L. monocytogenes</i>	Positive— <i>L. monocytogenes</i>
265	Sponge	Plastic	Positive— <i>L. monocytogenes</i>	Positive— <i>L. monocytogenes</i>
266	Sponge	Stainless steel	Negative	Negative
267	Sponge	Stainless steel	Negative	Negative
268	Sponge	Stainless steel	Negative	Negative
269	Sponge	Stainless steel	Negative	Negative
270	Sponge	Stainless steel	Negative	Negative
271	Sponge	Stainless steel	Positive— <i>L. monocytogenes</i>	Positive— <i>L. monocytogenes</i>
272	Sponge	Rubber	Negative	Negative
273	Sponge	Rubber	Negative	Negative
274	Sponge	Rubber	Negative	Negative
275	Sponge	Concrete	Negative	Positive— <i>L. innocua</i>
276	Sponge	Concrete	Positive— <i>L. innocua</i>	Positive— <i>L. innocua</i>
277	Sponge	Concrete	Positive— <i>L. monocytogenes</i>	Positive— <i>L. monocytogenes</i>
278	Sponge	Concrete	Positive— <i>L. welshimeri</i>	Negative
279	Sponge	Concrete	Negative	Negative
280	Sponge	Concrete	Negative	Negative
281	Sponge	Concrete	Negative	Negative
282	Sponge	Concrete	Positive— <i>L. innocua</i>	Negative
283	Sponge	Concrete	Positive— <i>L. innocua</i>	Negative
284	Sponge	Concrete	Negative	Negative
285	Sponge	Concrete	Negative	Negative
286	Sponge	Concrete	Negative	Negative
287	Sponge	Concrete	Negative	Negative
288	Sponge	Concrete	Negative	Negative
289	Sponge	Concrete	Positive— <i>L. monocytogenes</i>	Positive— <i>L. monocytogenes</i>
290	Tip swab	Air vent	Negative	Negative

Table 7. (continued)

Swab No.	Swab type	Surface type	USDA method result	DNA hybridization method result
291	Tip swab	Plastic	Negative	Negative
292	Tip swab	Stainless steel	Negative	Negative
293	Tip swab	Painted surface	Negative	Negative
294	Tip swab	Steel grate	Negative	Negative
295	Tip swab	Plastic	Negative	Negative
296	Tip swab	Cast iron	Negative	Negative
297	Tip swab	Cast iron	Negative	Negative
298	Tip swab	Ceramic	Negative	Negative
299	Tip swab	Cast iron	Negative	Negative
300	Tip swab	Plastic	Negative	Negative
301	Tip swab	Painted surface	Negative	Negative
302	Tip swab	Cast iron	Negative	Negative
303	Tip swab	Drain-cast iron	Negative	Negative
Total			15	14

L. welshimeri, and *L. innocua*. Positive swabs were obtained from the following surfaces: stainless steel, plastic, concrete, and wood.

(6.6.1.4.3) Environmental samples—inoculated.—Results are summarized in Table 8. Of 120 inoculated samples, 85 were confirmed positive by the DNA hybridization method and 87 were positive by the USDA/FSIS culture procedure. There were 2 false-negative results by the DNA hybridization method (both on plastic). Therefore, sensitivity rates were 97.7% (85/87) for the DNA hybridization method and 100% for the reference method. There were 5 false-positive results by the DNA hybridization method (all on ceramic, 4 on uninoculated control samples, and one on an inoculated surface) for an overall specificity of 83.3% (25/30). Overall agreement between the methods (confirmed results) was 98.7% (148/150).

The situation regarding false-positive results from the ceramic surface was further investigated in a subsequent experiment. Twenty uninoculated ceramic surface samples were prepared and tested as described in 6.6.1.3. All 20 samples tested negative by both the DNA hybridization and reference culture procedures. It is concluded that the false-positive results experienced in the initial experiment are not matrix related but are simply aberrant results.

(6.6.1.4.4) Comments from independent laboratories.—One laboratory commented that the DNA hybridization assay was easy to use and comparable to other microwell assay kits in technician time and expertise requirements. They also commented that, in the case of environmental samples, there were many instances of suspect USDA culture method samples requiring extended days of work that eventually confirmed as negative. In contrast, there were no cases of presumptive DNA hybridization assay results that did not confirm positive. The second laboratory commented that the DNA hybridization test was a user

friendly assay that can yield presumptive positive results up to 3 days sooner than the reference method. This laboratory initially experienced some invalid assay runs due to high negative control values. These problems were solved by using a 45°C heater block rather than an air incubator and using a multichannel pipettor. After these procedural adjustments, no further difficulties were encountered.

7 Discussion

Results of the 15 food trial clearly show that the microwell format assay is capable of detecting low levels of *Listeria* spp. in a variety of foods. The assay also has proven to be very specific, with no cross-reactions observed in exclusivity testing.

The DNA method has a high rate of agreement with both the FDA and USDA culture methods. In internal trials, the false-negative rate for all food commodities was 2.8%. In testing of meat and poultry samples, the DNA assay had a false-positive rate of 0.5%, and a false-negative rate of 4.0%. In comparison, the USDA method had a false-negative rate of 1.3%. In testing of dairy products, seafood, and fruits and vegetables, the DNA assay had a false-positive rate of 2.1%, and a false-negative rate of 1.9%. In comparison, the FDA method showed a false negative rate of 7.7%.

In comparing the DNA method and the reference culture method for the ability to detect *Listeria* spp. in foods, there was no significant difference in detection for any food tested, with the single exception of brie cheese for which the DNA method showed a statistically significant advantage over the FDA method for the high level samples ($\chi^2 = 4.17$; Table 4).

Results of the independent laboratory validation studies confirm the results from the internal trials. No false-negative or false-positive results were obtained in the testing of cottage cheese and mayonnaise samples. Results of these studies

Table 8. Results of independent laboratory testing of inoculated environmental samples; comparison between the DNA hybridization method and the USDA culture method

Surface	Inoculum strain	Inoculation level ^a	No. of samples	Total positive ^b	DNA assay positive ^c	DNA assay confirmed ^d	USDA/FSIS positive	Chi square ^e
Stainless steel	<i>L. monocytogenes</i> ATCC 19112 + competitors	5.0×10^5	20	15	15	15	15	NA ^f
		0	5	0	0	0	0	NA
Ceramic	<i>L. monocytogenes</i> ATCC 19118	2.5×10^3	20	15	16	15	15	NA
		0	5	0	4	0	0	NA
Cast iron	<i>L. monocytogenes</i> ATCC 19116	2.7×10^4	20	10	10	10	10	NA
		0	5	0	0	0	0	NA
Plastic	<i>L. welshimeri</i> ATCC 43551	1.0×10^7	20	10	8	8	10	0.50
		0	5	0	0	0	0	NA
Concrete	<i>L. innocua</i> ATCC 51742	1.7×10^8	20	18	18	18	18	NA
		0	5	0	0	0	0	NA
Painted wood	<i>L. ivanovii</i> ATCC 49954	5.0×10^2	20	19	19	19	19	NA
		0	5	0	0	0	0	NA
Total				87	90	85	87	

^a Inoculation level in CFU per surface.

^b Number of samples confirmed positive by one or more methods.

^c Number of samples positive by DNA hybridization assay not considering subsequent culture confirmation.

^d Number of samples positive by DNA hybridization assay and confirmed by plating from associated cultures.

^e Chi square value by McNemar's test comparing confirmed DNA hybridization assay and reference culture method. Chi square values ≥ 3.84 indicate a statistically significant difference at $P \leq 0.05$.

^f NA = Not applicable, results in perfect agreement.

further showed that the DNA hybridization method is applicable to the detection of *Listeria* spp. in environmental samples, with a sensitivity equivalent to that of the USDA culture method.

8 Conclusions

Based on the results of the 15 food internal study and the independent laboratory validation studies, it is concluded that the GeneQuence *Listeria* Test is an effective method for the detection of *Listeria* spp. in a wide variety of foods and

environmental samples, with low rates of false negative and false positive reactions.

9 References

- (1) U.S. Department of Agriculture, Food Safety and Inspection Service (1998) *Microbiology Laboratory Guidebook*, 3rd Ed., Rev. No. 3, 4/29/02, Ch. 8, Washington, DC
- (2) U.S. Food and Drug Administration (2003) *Bacteriological Analytical Manual*, Ch. 10, <http://www.cfsan.fda.gov/~ebam/bam-10.html>