CERTIFICATION

AOAC® *Performance Tested*SM

Certificate No.

011002

The AOAC Research Institute hereby certifies the method known as:

MicroSEQ® *Listeria monocytogenes* Detection Kit

manufactured by

Life Technologies, part of Thermo Fisher Scientific
Wade Road
Basingstoke, Hampshire
RG24 8PW, United Kingdom

This method has been evaluated in the AOAC® *Performance Tested Methods*SM Program and found to perform as stated by the manufacturer contingent to the comments contained in the manuscript. This certificate means that an AOAC® Certification Mark License Agreement has been executed which authorizes the manufacturer to display the AOAC *Performance Tested*SM certification mark along with the statement - "THIS METHOD'S PERFORMANCE WAS REVIEWED BY AOAC RESEARCH INSTITUTE AND WAS FOUND TO PERFORM TO THE MANUFACTURER'S SPECIFICATIONS" - on the above-mentioned method for a period of one calendar year from the date of this certificate (November 2, 2021 – December 31, 2022). Renewal may be granted at the end of one year under the rules stated in the licensing agreement.

Scott Coates

Scott Coates, Senior Director
Signature for AOAC Research Institute

November 2, 2021
Date

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MODIFICATION AUGUST 2018: Life Technologies, part of Thermo Fisher Scientific
MODIFICATION DECEMBER 2018: Tiina Karla

SUBMITTING COMPANY
Applied Biosystems, Inc.
850 Lincoln Centre Drive
Foster City, CA 94404

CURRENT SPONSOR
Life Technologies, part of Thermo Fisher Scientific
Wade Road
Basingstoke, Hampshire, RG24 8PW
United Kingdom

METHOD NAME
MicroSEQ® *Listeria monocytogenes* Detection Kit

CATALOG NUMBERS
4403874, 4426714, 4426715, 4428176, 4480466, 4415045, 4412637

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December 2018 modification internal AOAC Research Institute review

APPLICABILITY OF METHOD
Target organism – *Listeria monocytogenes*

Matrixes – (25 g) - Pasteurized whole milk, dry infant formula, ice cream, roast beef, cured bacon, Lox (cold-smoked salmon), lettuce, salad dressing, mayonnaise

Performance claims - Sensitivity was statistically similar to the reference ISO culture-based method and specificity > 99%.

REFERENCE METHOD
ISO 11290-1: Microbiology of Food and Animal Feeding Stuffs: Horizontal Method for the Detection and Enumeration of *Listeria monocytogenes* –Part I: Detection Method (6)

ORIGINAL CERTIFICATION DATE
January 05, 2010

CERTIFICATION RENEWAL RECORD
Renewed annually through December 2022.

METHOD MODIFICATION RECORD

1. **December 2017 Level 1**
2. **August 2018 Level 2**
3. **December 2018 Level 2**
4. **December 2018 Level 1**
5. **November 2019 Level 1**

SUMMARY OF MODIFICATION

1. **Editorial changes including company name update on insert and labels.**
2. **Equivalency study for KingFisher™ Flex-96 Deep Well Magnetic Particle Processor.**
3. **Location change of critical raw materials from Austin, Texas to Vilnius, Lithuania.**
4. **Edits to User Guide to update AOAC RI Workflow.**
5. **Clerical changes to decommission negative control labels.**

Under this AOAC® *Performance Tested*℠ License Number, 011002 this method is distributed by:
NONE

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NONE

Thermocyclers characteristics to run the Real-Time PCR:
Applied Biosystems™ 7500 Fast Real-Time PCR Instrument and equivalents manufactured by Thermo Fisher Scientific and/or subsidiaries with the following characteristics:

Characteristics	7500 Fast Real-Time PCR Instrument
Optics	12v 75w halogen bulb
Filters	5 excitation and 5 emission filters
Sample ramp rate	Standard mode: ± 1.6°C/sec Fast mode: ± 3.5°C/sec
Thermal range	4-100°C
Thermal accuracy	± 0.5°C
Thermal uniformity	± 1°C
Format	96-well, 0.1-mL block

PRINCIPLE OF THE METHOD (1)

Real-Time PCR Amplification

The MicroSEQ® Pathogen Detection System is based on TaqMan® Real-Time PCR technology [4], providing two levels of specificity for confident pathogen detection by combining Polymerase Chain Reaction (PCR) amplification [5] and signal detection in a single reaction. The first level of specificity is provided by target specific PCR primers that identify the DNA sequence of the organism in the sample. The identification of the organism is confirmed simultaneously by TaqMan probes, which represent the second level of specificity. As a result, a fluorescent signal is emitted only if the unique genetic signature of the pathogen has been recognized. By addressing the unique genetic signature of the target organism, the Real-Time PCR system delivers results independent of environmental factors such as temperature or pH.

In addition, the MicroSEQ Pathogen Detection System contains an Internal Positive Control (IPC) in the reaction mix to monitor the presence of PCR inhibitors. Amplification of IPC demonstrates the absence of PCR inhibition, providing more confident negative results (reducing false negative calls). After PCR amplification and detection, reaction tubes remain sealed, thus significantly reducing the potential for contamination (false positives).

Data Analysis

The RapidFinder™ Express software simplifies Real-Time PCR setup and processing by providing a software-guided workflow and automated analysis of results. Designed specifically for pathogen detection in food and environmental samples, RapidFinder Express software guides the user through each step of the assay and performs all required calculations. During PCR, real-time fluorescence data is collected cycle by cycle for each individual reaction. Positive signals result in an increase of the target-specific fluorescent signal while the fluorescence of negative signals remains below the threshold applied by RapidFinder Express software. When the assay is complete, RapidFinder™ Express software transforms the individual fluorescent signals and displays them in an intuitive, easy-to-read, color-coded format.

DISCUSSION OF THE VALIDATION STUDY (1)

The procedure for detecting *L. monocytogenes* in food matrixes validated in this report included the use of two sample preparation kits (the PrepSEQ™ Nucleic Acid Extraction Kit and the PrepSEQ Rapid Spin Sample Preparation Kit), the MicroSEQ *Listeria monocytogenes* Detection Kit, the 7500 Fast Real-Time PCR system, and RapidFinder Express v1.0 software for data analysis (all from Applied Biosystems, Inc.), which in this report is referred to as the MicroSEQ *Listeria monocytogenes* Detection Method or the MicroSEQ® method. In this study, no difference in detection was observed between the two DNA extraction procedures (Rapid Spin and NA Extraction). The MicroSEQ *Listeria monocytogenes* Detection Method successfully detected low numbers of *L. monocytogenes* in roast beef, cured bacon, lox, lettuce, whole cow's milk, dry infant formula, ice cream, salad dressing and mayonnaise. In the method comparison study no discrepancies were observed between the MicroSEQ method and the confirmation method when the two tests were performed on the same enriched sample. The method comparison study between the MicroSEQ® method and the ISO 11290-1 reference method was an unpaired design, therefore the two methods were analyzed by the Mantel-Haenszel formula for χ^2 analysis to determine statistical differences. The acceptance criteria for the two methods to be "not significantly different" requires χ^2 to be ≤ 3.84. Based on chi-square analysis there is no statistical difference between the two methods. In all cases (internal and independent validation), chi-square was ≤ 1.00 for fractional positive spike levels (5 to 15 out of 20 samples were confirmed positive).

The MicroSEQ® *Listeria monocytogenes* Detection method was able to detect all of the *L. monocytogenes* strains tested (50/50), and none of the non-target strains gave rise to false-positive results (0/30). Lot-to-lot and stability studies showed consistent performance. The ruggedness study demonstrated that the MicroSEQ® *Listeria monocytogenes* Detection Method was not sensitive to changes in factors most likely to adversely impact assay performance including the incubation time for sample pre-enrichment, and small changes in buffer volumes and incubation times for the two sample preparation methods.

The MicroSEQ *Listeria monocytogenes* Detection Method is a rapid and simple procedure, giving results within approximately 28 hr (including 24 hr enrichment of sample). The MicroSEQ method offers a complete solution following sample enrichment that includes a choice of two sample preparation kits, a complete Real-Time PCR reaction mix that is lyophilized for ease of use and is re-hydrated with sample, Real-Time instrumentation that processes samples with a ~40 minutes run time, and RapidFinder Express software for automated analysis of data such that results are displayed as either positive or negative.

Results obtained with the *Listeria* Inclusivity strains and the MicroSEQ *Listeria monocytogenes* Kit by two DNA extraction procedures (1)					
				MicroSEQ® *Listeria monocytogenes* Detection Kit result	
Number	Organism	Campden code	CFU/mL	Rapid Spin Kit	NA Extraction Kit
1.	*Listeria monocytogenes*	6600	5.4E +05	Positive	Positive
2.	*Listeria monocytogenes*	6601	4.7E +05	Positive	Positive
3.	*Listeria monocytogenes*	1100	5.1E +05	Positive	Positive
4.	*Listeria monocytogenes*	1101	5.1E +05	Positive	Positive
5.	*Listeria monocytogenes*	1102	6.9E +05	Positive	Positive
6.	*Listeria monocytogenes*	1103	6.3E +05	Positive	Positive
7.	*Listeria monocytogenes*	1104	7.5E +05	Positive	Positive
8.	*Listeria monocytogenes*	1105	6.9E +05	Positive	Positive
9.	*Listeria monocytogenes*	1108	6.7E +05	Positive	Positive
10.	*Listeria monocytogenes*	1109	6.3E +05	Positive	Positive
11.	*Listeria monocytogenes*	1149	1.2E +06	Positive	Positive
12.	*Listeria monocytogenes*	1150	1.6E +05	Positive	Positive
13.	*Listeria monocytogenes*	1151	6.0E +05	Positive	Positive
14.	*Listeria monocytogenes*	1152	6.1E +05	Positive	Positive
15.	*Listeria monocytogenes*	1153	7.7E +05	Positive	Positive
16.	*Listeria monocytogenes*	1154	1.1E +06	Positive	Positive
17.	*Listeria monocytogenes*	1155	8.7E +05	Positive	Positive
18.	*Listeria monocytogenes*	1156	1.1E +06	Positive	Positive
19.	*Listeria monocytogenes*	1157	6.5E +05	Positive	Positive
20.	*Listeria monocytogenes*	1158	5.9E +05	Positive	Positive
21.	*Listeria monocytogenes*	1160	5.4E +05	Positive	Positive
22.	*Listeria monocytogenes*	1161	9.5E +05	Positive	Positive
23.	*Listeria monocytogenes*	1162	6.5E +05	Positive	Positive
24.	*Listeria monocytogenes*	1163	6.8E +05	Positive	Positive
25.	*Listeria monocytogenes*	1164	7.4E +05	Positive	Positive
26.	*Listeria monocytogenes*	1165	9.5E +05	Positive	Positive
27.	*Listeria monocytogenes*	1166	7.4E +05	Positive	Positive
28.	*Listeria monocytogenes*	1168	5.9E +05	Positive	Positive
29.	*Listeria monocytogenes*	1169	8.6E +05	Positive	Positive
30.	*Listeria monocytogenes*	1170	9.5E +05	Positive	Positive
31.	*Listeria monocytogenes*	1171	1.1E +06	Positive	Positive
32.	*Listeria monocytogenes*	1172	8.9E +05	Positive	Positive
33.	*Listeria monocytogenes*	1173	5.7E +05	Positive	Positive
34.	*Listeria monocytogenes*	1174	4.6E +05	Positive	Positive
35.	*Listeria monocytogenes*	1175	7.5E +05	Positive	Positive
36.	*Listeria monocytogenes*	1176	5.5E +05	Positive	Positive
37.	*Listeria monocytogenes*	1177	6.7E +05	Positive	Positive
38.	*Listeria monocytogenes*	1178	2.8E +05	Positive	Positive
39.	*Listeria monocytogenes*	1179	3.3E +05	Positive	Positive
40.	*Listeria monocytogenes*	1180	2.4E +05	Positive	Positive
41.	*Listeria monocytogenes*	1181	6.9E +05	Positive	Positive
42.	*Listeria monocytogenes*	1182	9.4E +05	Positive	Positive
43.	*Listeria monocytogenes*	1183	6.7E +05	Positive	Positive
44.	*Listeria monocytogenes*	1184	5.5E +05	Positive	Positive
45.	*Listeria monocytogenes*	1186	6.5E +05	Positive	Positive
46.	*Listeria monocytogenes*	1187	5.3E +05	Positive	Positive
47.	*Listeria monocytogenes*	1980	6.8E +05	Positive	Positive
48.	*Listeria monocytogenes*	3630	6.0E +05	Positive	Positive
49.	*Listeria monocytogenes*	3650	7.0E +05	Positive	Positive
50.	*Listeria monocytogenes*	2080	9.5E +05	Positive	Positive

Results obtained with the *Listeria* Exclusivity strains and the MicroSEQ *Listeria monocytogenes* Kit by two DNA extraction procedures (1)					
				MicroSEQ® *Listeria monocytogenes* Detection Kit result	
Number	Organism	Campden code	CFU/mL	Rapid Spin Kit	NA Extraction Kit
1.	*Listeria innocua*	1110	4.1E +08	Negative	Negative
2.	*Listeria innocua*	1111	7.0E +08	Negative	Negative
3.	*Listeria innocua*	1112	1.2E +09	Negative	Negative
4.	*Listeria innocua*	1117	6.0E +08	Negative	Negative
5.	*Listeria innocua*	6602	1.2E +09	Negative	Negative
6.	*Listeria welshimeri*	1130	3.8E +08	Negative	Negative
7.	*Listeria welshimeri*	1132	4.5E +08	Negative	Negative
8.	*Listeria welshimeri*	1134	1.2E +08	Negative	Negative
9.	*Listeria welshimeri*	1135	7.3E +08	Negative	Negative
10.	*Listeria seeligeri*	1139	1.1E +08	Negative	Negative
11.	*Listeria seeligeri*	1146	5.0E +07	Negative	Negative
12.	*Listeria seeligeri*	6603	2.4E +08	Negative	Negative
13.	*Listeria ivanovii*	1123	8.9E +08	Negative	Negative

14.	*Listeria ivanovii*	6599	9.7E +08	Negative	Negative
15.	*Listeria grayi*	9298	5.6E +08	Negative	Negative
16.	*Listeria grayi*	12524 A	4.5E +06	Negative	Negative
17.	*Listeria grayi*	12526 A	2.4E +07	Negative	Negative
18.	*Bacillus cereus*	4110	8.0E +06	Negative	Negative
19.	*Bacillus licheniformis*	8478	2.3E +07	Negative	Negative
20.	*Bacillus pumilus*	16384	6.5E +06	Negative	Negative
21.	*Bacillus subtilis*	4112	6.8E +06	Negative	Negative
22.	*Brochothrix thermosphacta*	16019	3.7E +07	Negative	Negative
23.	*Enterococcus durans*	16464	6.2E +07	Negative	Negative
24.	*Enterococcus faecalis*	16049	1.3E +08	Negative	Negative
25.	*Lactobacillus gasseri*	6804	2.3E +07	Negative	Negative
26.	*Lactobacillus plantarum*	166	8.5E +08	Negative	Negative
27.	*Micrococcus luteus*	16258	5.9E +07	Negative	Negative
28.	*Pediococcus pentosaceus*	16030	6.6E +07	Negative	Negative
29.	*Rhodococcus equi*	4055	2.8E +07	Negative	Negative
30.	*Staphylococcus aureus*	1216	9.0E +06	Negative	Negative

Table C: Summary of Method Comparison Results* (1)

Roast Beef				**Cured Bacon**			**Lox**		
Level	ISO	MicroSEQ®		ISO	MicroSEQ®		ISO	MicroSEQ®	
		Rapid Spin	NA		Rapid Spin	NA		Rapid Spin	NA
Low	1/20	2/20	2/20	11/20	10/20	10/20	0/20	0/20	0/20
High	8/20	5/20	5/20	19/20	17/20	17/20	5/20	8/20	8/20
Control	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
	Lettuce			**Pasteurized Whole Milk**			**Dry Infant Formula**		
Level	ISO	MicroSEQ®		ISO	MicroSEQ®		ISO	MicroSEQ®	
		Rapid Spin	NA		Rapid Spin	NA		Rapid Spin	NA
Low	10/20	11/20	11/20	7/20	6/20	6/20	9/20	10/20	10/20
High	18/20	19/20	19/20	18/20	17/20	17/20	18/20	20/20	20/20
Control	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
	Ice Cream			**Salad Dressing**			**Mayonnaise**		
Level	ISO	MicroSEQ®		ISO	MicroSEQ®		ISO	MicroSEQ®	
		Rapid Spin	NA		Rapid Spin	NA		Rapid Spin	NA
Low	5/20	4/20	4/20	15/20	13/20	13/20	11/20	13/20	13/20
High	17/20	20/20	20/20	20/20	20/20	20/20	19/20	20/20	20/20
Control	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5

*Results are confirmed positives.

DISCUSSION OF THE MODIFICATION AUGUST 2018 (8)

The purpose of the additional study was to generate more performance data for MagMAX Express-96 Deep Well and KingFisher Flex-96 Deep Well magnetic particle processors and to supplement the existing data set generated to facilitate transfer of the MagMAX Express-96 protocols to KingFisher Flex-96 and assure AOAC-RI on the comparable performance of these instruments.

Both instrument types produced similar results in terms of number of positive calls returned from the sample set. In this study, the number of positive calls generated with MagMAX Express-96 instruments was at the high end of desired amount of positive results (2-8 from 10 test replicates), staying at the fractional level (8 and 9 positives generated). When the same sample set was analysed with KingFisher Flex-96 instruments, not only did the amount of positive results stay almost identical with little difference but also variance within instrument type remained similar. Although nine positive reactions were recorded for one MagMAX and one KingFisher instrument, the other instruments' fractional results indicate that the spiking level had been at the correct fractional level.

Also, when evaluating the total amount of positives within instrument type the results are very similar; 17 positives with MagMAX Express-96 instrument from MicroSEQ Listeria monocytogenes Detection Kit compared to 16 positives with KingFisher Flex-96 instrument using the same assay and nucleic acid extraction kits. The average Ct values recorded from the samples are within 0,3 cycles across instruments indicating comparable DNA yield from the instrument in this challenging study setup. Internal Positive Controls performed as expected and no sign of inhibition was observed.

In the 10x LoD study setup all samples received positive interpretation. Ct value comparison further confirmed the results generated in the LoD study and previous studies, as the Ct values generated from the instruments were almost identical (Ct values were within 0,5 cycles across the instruments). Ct values this close between the instruments indicate that the sample preparation kits perform almost identically between the instruments and are able to collect, concentrate and purify considerable amounts of target DNA from the sample matrixes even in the presence of saturating background flora DNA.

(Additional Study June 2018) Table 1. Presence / absence results for *L. monocytogenes* LoD spiked samples ran with two MagMAX Express-96 and two KingFisher Flex-96 instruments and interpreted by RapidFinder Express 2.0 software using the MicroSEQ Listeria monocytogenes Detection Kit. (8)

Test replicate	*MagMAX Express-96*		*KingFisher Flex-96*	
	Instrument 1	*Instrument 2*	*Instrument 1*	*Instrument 2*
1	-	+	+	+
2	+	-	+	+
3	+	+	-	+
4	+	+	+	+
5	+	+	+	-
6	+	+	+	+
7	+	+	-	+
8	-	+	-	+
9	+	+	+	+
10	+	+	+	+
Σ Positive results	**8**	**9**	**7**	**9**

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