CERTIFICATION

AOAC® *Performance Tested*SM

Certificate No.

021108

The AOAC Research Institute hereby certifies the method known as:

MicroSEQ® *Listeria* species 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 (October 30, 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

October 30, 2021
Date

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METHOD NAME
MicroSEQ® *Listeria* species Detection Kit

CATALOG NUMBERS
4427410, 4428176, 4480466, 4426714, 4426715, 4445658, 4445659, PATHATRIXAUTO

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December 2018 Modification internal AOAC Research Institute review

APPLICABILITY OF METHOD
Target organism – *Listeria* species

Matrixes – (25 g) - Pasteurized whole cow's milk, dry infant formula, hot dogs, roast beef, Lox (smoked salmon)
(4 x 4 in surface area) - stainless steel, plastic cutting board, ceramic tile,
(1 x 1 in surface area) - rubber sheets, concrete sealed with Seal Hard®

Performance claims – Provides an effective method for the detection and identification of *Listeria* spp. In a wide variety of foods and environmental surfaces.

REFERENCE METHOD
International Organization for Standardization (1996) *Microbiology of Food and Animal Feeding Stuffs – Horizontal Method for the Detection of Listeria monocytogenes – Part I: Detection Method,* ISO 11290-1:1996(E), Geneva, Switzerland (11)

ORIGINAL CERTIFICATION DATE
February 18, 2011

CERTIFICATION RENEWAL RECORD
Renewed annually through December 2022.

METHOD MODIFICATION RECORD
1. **December 20, 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. **Edits to the 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. **Update to User Guide to include AOAC RI workflow.**
5. **Editorial/clerical changes.**

Under this AOAC® *Performance Tested*℠ License Number, 021108 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)

The MicroSEQ Pathogen Detection System is based on TaqMan(R) Real-Time PCR technology (10), providing two levels of specificity for confident pathogen detection by combining PCR amplification (10) 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.
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).
The real-time PCR assay is lyophilized to improve ease of use by reducing the number of pipetting steps and allowing for the addition of a higher volume of sample (30 µL) to the reaction mix. Two sample preparation methods were developed to meet the needs of testing both small sample numbers (PrepSEQ Rapid Spin Sample Preparation Kit) and high sample numbers (PrepSEQ Nucleic Acid Extraction Kit).
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 reactions are complete, RapidFinder Express Software interprets the individual fluorescent signals and displays the result in an intuitive, easy-to-read, color-coded presence-absence format.

DISCUSSION OF THE VALIDATION STUDY (1)

The results from validation studies presented here support the product claims of the MicroSEQ *Listeria* spp. Detection kit for detection of *Listeria* spp. in five food matrixes and five environmental surfaces, namely, hot dogs, roast beef. Lox (smoked salmon), pasteurized whole cow's milk, dry infant formula, stainless steel, plastic cutting board, ceramic tile, rubber sheets, and sealed concrete.

Table 1 (1)

Organism	Serogroup	Code	Source	MicroSEQ result
Listeria seeligeri	6B	NCTC 11289	Not available	Positive
Listeria welshimeri	6A	ATCC 43551	Human feces	Positive
Listeria welshimeri	6B	ATCC 35897	Plant	Positive
Listeria welshimeri	6B	ATCC 43549	Not available	Positive
Listeria welshimeri	1/2B	ATCC 43550	Cornfield soil	Positive

[a] ATCC = American Type Culture Collection, Manassas, VA; CWD = University of Vermont, Burlington, VT; FSL = Cornell University, Ithaca, NY; NCIMB = NCIMB Ltd, Aberdeen, Scotland, UK; NCTC = National Collection of Type Cultures, Colindale, London, UK.

Table 2. Applied Biosystems MicroSEQ *Listeria* spp. Detection Kit exclusivity[a] (1)

Organism	Code	Source	MicroSEQ result
Bacillus mycoides	ATCC 6462	Soil	Negative
Brochothrix thermosphacta	ATCC 11509	Pork sausage	Negative
Carnobacterium divergens	ATCC 35677	Minced beef	Negative
Carnobacterium gallinarum	ATCC 49517	Ice slush from around chicken carcasses	Negative
Carnobacterium maltaromaticum	ATCC 43224	Vacuum-packed beef	Negative
Citrobacter freundii	ATCC 8090	Not available	Negative
Clostridium sporogenes	ATCC 11437	Plant (cotton)	Negative
Enterobacter aerogenes	ATCC 13048	Sputum	Negative
Enterobacter sakazakii	ATCC 51329	Not available	Negative
Enterococcus faecalis	ATCC 29212	Urine	Negative
Escherichia coli	ATCC 8739	Feces	Negative
Klebsiella oxytoca	ATCC 43165	Clinical isolate	Negative
Klebsiella pneumoniae	ATCC 13883	Not available	Negative
Kurthia zopfii	ATCC 10538	Not available	Negative
Lactobacillus casei	ATCC 11578	Oral cavity	Negative
Lactobacillus fermentum	ATCC 9338	Not available	Negative
Lactobacillus lactis	ATCC 4797	Not available	Negative
Lactobacillus plantarum	ATCC 8014	Not available	Negative
Microbacterium testaceum	ATCC 15829	Paddy	Negative
Micrococcus luteus	ATCC 7468	Not available	Negative
Proteus mirabilis	ATCC 7002	Urine	Negative
Propionibacterium acnes	ATCC 11827	Not available	Negative
Rhodococcus equi	ATCC 6939	Lung abscess of foal	Negative
Salmonella enterica subsp. enterica serovar Typhimurium	ATCC 14028	Chicken hearts and livers	Negative
Salmonella enterica subsp. enterica serovar Choleraesuis	ATCC 10708	Not available	Negative
Staphylococcus aureus	ATCC 29247	Not available	Negative
Staphylococcus epidermidis	ATCC 12228	Not available	Negative
Staphylococcus haemolyticus	ATCC 29970	Human skin	Negative
Staphylococcus warneri	ATCC 29885	Not available	Negative
Streptococcus pneumoniae	ATCC 6302	Not available	Negative
Streptococcus pyogenes	ATCC 19615	Pharynx of child	Negative

[a] ATCC = American Type Culture Collection, Manassas, VA.

Table 3. Methods comparison results for the Applied Biosystems MicroSEQ *Listeria* spp. Detection Kit for food samples (1)

				MicroSEQ *Listeria* spp. method								
Inoculation level	APC count (prior to spike)	MPN/25 g	Total samples	Automated NA extraction presumptive	Automated NA extraction confirmed	Manual rapid spin presumptive	Manual rapid spin confirmed	ISO 11290-1	χ^2 (MicroSEQ vs. ISO 11290-1)	Relative sensitivity	False-negative rate	False-positive rate
Hot dogs (Inoculating organism: *Listeria ivanovii,* ATCC 19119)												
Uninoculate	6.0×10^1	<0.075	5	0	0	0	0	0	N/A	N/A	0	0
Low		0.52	20	8	8	8	8	2	4.68	4.0	0	0
High		0.90	20	19	19	19	19	11	8.32	1.7	0	0
Roast beef (Inoculating organism: *Listeria innocua*, NCTC 10528)												
Uninoculate	4.6×10^5	<0.075	5	0	0	0	0	0	N/A	N/A	0	0
Low		0.09	20	0	1	1	1	0	1.0/0.0[a]	NA	0/1.0[a]	0
High		0.72	20	8	8	8	8	9	0.10	0.89	0	0
Smoked salmon (Inoculating organism: *Listeria monocytogenes*, ATCC 49594)												
Uninoculate	1.0×10^2	<0.075	5	0	0	0	0	0	N/A	N/A	0	0
Low		0.58	20	13	13	13	13	12	0.10	1.1	0	0
High		11.5	20	16	16	16	16	18	0.76	0.89	0	0
Pasteurized whole milk (Inoculating organism: *Listeria welshimeri*, ATCC 35897)												
Uninoculate	<10	<0.075	5	0	0	0	0	0	N/A	N/A	0	0
Low		0.23	20	4	4	4	4	5	0.14	0.80	0	0
High		0.52	20	16	16	16	16	14	0.52	1.1	0	0
Dry infant formula (Inoculating organism: *Listeria seeligeri*, ATCC 35967)												
Uninoculate	1.4×10^2	<0.075	5	0	0	0	0	0	N/A	N/A	0	0
Low		1.08	20	17	17	17	17	14	1.26	1.2	0	0
High		2.32	20	20	20	20	20	19	1.00	1.1	0	0

[a] Low spike roast beef samples did not give equivalent results for Salmonella detection for samples prepared using the PrepSEQ NA Extraction Kit and the PrepSEQ RapidSpin Sample Preparation Kits. Statistical results for $\chi 2$ and false-negative rates are reported as 'NA Extraction/Rapid Spin'

DISCUSSION OF THE MODIFICATION AUGUST 2018 (13)

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.

Table 3. Results for *Listeria* 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 species 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	**6**	**9**	**8**	**4**

DISCUSSION OF THE MODIFICATION DECEMBER 2018 (14)

Samples were prepared near the limit of detection in ten replicates to compare the kit lots produced at the Austin, Texas and Vilnius, Lithuania manufacturing sites. Fractional positivity level (2-8) was reached with all kits and POD values were calculated for all targets. POD values of the kit lots from the old (Austin, Texas) and new (Vilnius, Lithuania) AmpliTaq™ UP manufacturing sites were evaluated through paired comparison. POD analysis showed that there is no statistical differences between lots produced at the Austin, Texas and Vilnius, Lithuania manufacturing sites.

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