Feasibility Evaluation for the Real-Time Analyzers *Listeria* Detection Assay for the Detection of *Listeria* species from Environmental Samples

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Performing Laboratory

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Project ID Number

QL # 17077-14A

Study Sponsor

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STUDY DESIGN

Purpose:

The purpose of this feasibility study was to conduct a method comparison evaluation for the detection of *Listeria* species from stainless steel environmental surfaces and an inclusivity and exclusivity evaluation for the Real-Time Analyzers, Inc. (Study Sponsor) *Listeria* detection assay. A modified AOAC validation design was conducted on one environmental surface, stainless steel, to verify the detection level and the enrichment time point of the assay (7 or 8 hours) that will produce acceptable data for an AOAC validation (fractional positive results by the RTA or reference method, along with passing POD statistical analysis).

Materials and Methods:

For the method comparison evaluation, the candidate method was analyzed by testing stainless steel environmental surfaces at three target inoculation levels: 10 replicates at a low inoculum level ~50 CFU/4" x 4" test area, 3 replicates at a high inoculum level ~150 CFU/test area, and 3 replicates at an uninoculated control level of 0 CFU/test area. All test candidate method samples were analyzed at 7 and 8 hours post-enrichment by the RTA *Listeria* detection assay, and all 8 hours enrichment samples, regardless of presumptive results, were confirmed by the USDA/MLG 8.10 reference method. In addition, an unpaired set of test samples was analyzed by the USDA/MLG 8.10 [1]. A summary of the method comparison feasibility evaluation is presented in Table A.

Table A: Study Summary

Matrix	Inoculating Organisms[1]	Target Inoculum Level # of Replicates	Equilibrium Protocol	Reference Method
Stainless Steel Environmental Surface (4" x 4" area)	*Listeria monocytogenes* 4B ATCC 19115 & *Staphylococcus aureus* ATCC 25923 (10x Level of *Listeria*)	0 CFU/test area – 3 ~50 CFU/ test area - 10 ~150 CFU/ test area - 3	16-24 hours at 20-25°C	USDA MLG 8.10

[1]Strains obtained from the American Type Culture Collection

A total of 15 inclusivity isolates, as well as 5 exclusivity isolates were evaluated by the RTA *Listeria* detection assay. Species and sources of each inclusivity isolate are listed in Table B while species and sources for each exclusivity isolate are listed in Table C. Inclusivity isolates were cultured according to the assay protocol and diluted to 100 x the LOD_{50}. Exclusivity isolates were cultured in non-selective media in accordance with current AOAC Appendix J guidelines, [2] and additionally in the prescribed candidate method media, and tested undiluted.

Table B: Inclusivity Organisms

No.	Organism	Reference Number	Source
1	*Listeria monocytogenes* (4B)	19115	ATCC
2	*Listeria monocytogenes* (1/2C)	7644	ATCC
3	*Listeria maarthi*	BAA-1595	ATCC
4	*Listeria innocua* (6A)	33090	ATCC
5	*Listeria innocua* (6B)	33091	ATCC
6	*Listeria ivanovii*	19119	ATCC
7	*Listeria ivanovii*	49954	ATCC
8	*Listeria grayi*	19120	ATCC
9	*Listeria grayi*	25401	ATCC
10	*Listeria seeligeri* (6B)	11289	ATCC
11	*Listeria seeligeri* (1/2B)	35967	ATCC
12	*Listeria welshimeri* (6B)	35897	ATCC
13	*Listeria welshimeri* (1/2B)	43550	ATCC
14	*Listeria weihenstephanensis*	FSL R9-0317	Cornell
15	*Listeria rocourtiae*	FSL F6-0920	Cornell

Table C: Exclusivity Organisms

No.	Organism	Reference Number	Source
1	*Enterococcus faecalis*	29212	ATCC
2	*Lactobacillus casei*	11578	ATCC
3	*Rhodococcus equi*	6939	NCTC
4	*Staphylococcus aureus*	25923	ATCC
5	*Streptococcus salivarius*	19258	ATCC

METHOD COMPARISON STUDY

Listeria monocytogenes ATCC 19115 and *Staphylococcus aureus* ATCC 25923 were retrieved from frozen stock stored at -70° C, transferred to Trypticase Soy Agar with 5% sheep blood (SBA) and incubated at 35 ± 1° C for 24 ± 2h. An isolated colony was transferred from SBA to Brain Heart Infusion (BHI) broth and incubated at 35 ± 1°C for 18-24 hours. 4" x 4" test areas for both the low and high inoculation levels were inoculated with 250 µL of culture prepared in pre-warmed (37 ± 1°C, for 30 minutes) BHI broth. The test areas were randomized and blind coded so that the analyst performing the assay was unaware of the level of bacterial culture present. Uninoculated BHI was used for the uninoculated control test areas. The inoculated test areas were allowed to dry for 16-24 hours at 20-25° C.

For the RTA candidate method assay and for the USDA/MLG 8.10 reference method, 16 total samples were evaluated. Three (3) test areas were uninoculated for each method, 10 test areas were inoculated with low level target inoculum (~ 50 CFU/test area), and 3 test areas were inoculated with high inoculum (~ 150 CFU/test area) for each method. For each test area, the target organism, *Listeria monocytogenes* ATCC 19115, was co-inoculated with the competitor organism, *Staphylococcus aureus* (10x the level of the target strain). To determine the

inoculation level for the environmental surfaces, aliquots of each inoculum were plated onto Tryptic Soy agar (TSA) and modified Oxford agar (MOX) in duplicate.

Modified USDA/FSIS MLG 8.10 Confirmation Method

Sampling sponges pre-moistened with 10 mL of Dey-Engley (D/E) Neutralizing Broth were used in horizontal and vertical sweeping motions to sample the area. All sampling sponges were held at ambient temperature (24 ± 2 °C) for a minimum of 2 hours prior to enrichment. After the hold time, each individual sponge was enriched with 225 ± 5 mL of modified University of Vermont broth (UVM) and incubated at 30 ± 2°C for 20 – 26 hours.

After incubation, 0.1 ± 0.02 mL of the enrichment for the 8 hour enrichment RTA samples and the USDA/FSIS MLG 8.10 samples were transferred to 10 ± 0.5 mL of Fraser Broth (FB) containing 0.1 mL of 5% ferric ammonium citrate and incubated at 35 ± 2°C for 26 ± 2 hours. A loopful of each sample enrichment containing Demi-Fraser or UVM was also streaked to a MOX plate and incubated at 35 ± 2°C for 26 ± 2 hours. After 26 ± 2 hours, FB was examined for any degree of darkening due to esculin hydrolysis. Any FB tubes that displayed darkening were streaked to a MOX plate. If no darkening occurred, FB was re-incubated at 35 ± 2°C for a total of 48 ± 2 hours and re-examined for evidence of darkening. All FB tubes were streaked to MOX plates and incubated at 35 ± 2°C for 26 ± 2 hours. MOX agar plates streaked from the primary enrichment or the FB secondary enrichment were examined after 26 ± 2 hours for typical *Listeria* colonies. If no suspect colonies were present; the MOX agar plate was re-incubated for an additional 26 ± 2 hours at 35 ± 2°C ± 2°C for a total of 48 ± 2 hours. MOX agar plates having typical *Listeria* colonies were considered positive; no additional reference method biochemical confirmation was conducted per the design of the study.

Real-Time Analyzer (RTA) Assay for the Detection of Listeria spp.

Using World Bioproducts PUR-Blue polyurethane DUO Swabs (pre-moistened with Neutralizing Broth), excess neutralizing buffer was removed by expressing the swab against the test tube walls and each area sampled using horizontal and vertical sweeping motions. The DUO swabs were separated from the enrichment broth component of the sampling device allowing the swabs to be held at room temperature (24 ± 2 °C) for a minimum of 2 hours while the tubes containing 5 mL of Demi-Fraser enrichment broth were pre-warmed to 37°C. Once the hold time was complete, the polyurethane tip swabs were added to the tube containing Demi-Fraser Broth and incubated for up to 8 hours at 37 ± 1°C. After 7 and 8 hours of incubation, a 1.5 mL subsample was removed and processed according to the RTA protocol described in Appendix 2.

All samples, regardless of presumptive results were confirmed as described in the USDA method section from the 8 hour enrichment time subsample.

INCLUSIVITY/EXCLUSIVITY

The inclusivity isolates were culture in Demi-Fraser broth for 8 hours at 37 ± 1°C. After incubation, the cultures were serially diluted using DF broth to 100 x the LOD_{50} of the assay. The exclusivity isolates were cultured in BHI Broth at the appropriate temperature, time and environmental conditions for optimal growth. Exclusivity isolates were evaluated undiluted. Additionally, exclusivity strains were grown in DF broth for 8 hours at 37±1°C. All isolates were randomized, blind coded and processed according to the RTA protocol described in Appendix 2. To confirm the inoculation level for each isolate, aliquots of each inoculum were plated onto TSA and MOX in duplicate.

RESULTS

The probability of detection (POD) was calculated as the number of positive outcomes divided by the total number of trials [3]. The POD was calculated for the candidate presumptive results, POD_{CP}, the candidate confirmatory results, POD_{CC}, the difference in the candidate presumptive and confirmatory results, $dPOD_{CP}$, presumptive candidate results that confirmed positive, POD_C, the reference method, POD_R, and the difference in the confirmed candidate and reference methods, $dPOD_C$ [4]. The POD analysis between the Real-Time Analyzer (RTA) Platform for the Detection of *Listeria* spp. and the reference method indicated that there was no significant difference at the 5% level between the number of positive results by each enrichment time point. The POD analysis between the RTA Platform for the Detection of *Listeria* spp. presumptive and confirmed results for each enrichment time point indicated that there was no significant difference at the 5% level.

The inoculum results are presented in Table 1 of Appendix 1. A detailed summary of results are presented in Table 2 of Appendix 1. A summary of POD analyses are presented in Tables 3 - 5 of Appendix 1.

Method Comparison Evaluation

For the 7 hour enrichment of the RTA *Listeria* Detection Assay, there were 3 presumptive positives for the low inoculation level, 3 presumptive positives for the high inoculation level and 0 presumptive positives for the uninoculated level.

For the 8 hour enrichment of the RTA *Listeria* Detection Assay, there were 6 presumptive positives and 6 confirmed positives for the low inoculation level following the modified USDA/MLG 8.10 reference method confirmation procedure. For the high inoculation level, there were 3 presumptive positives and 3 confirmed positives following the modified USDA/MLG 8.10 reference method confirmation procedure. For the uninoculated level, there were 0 presumptive positives and 0 confirmed positives following the modified USDA/MLG 8.10 reference method confirmation procedure.

For the low inoculation level of the 8 hour enrichment, a $dPOD_C$ value of 0.20 was obtained with a 95% confidence interval of (-0.21, 0.53), indicating no statistically significant difference between the candidate and reference method. A $dPOD_{CP}$ value of 0.00 was obtained with a 95% confidence interval of (-0.37, 0.37), indicating no statistically significant difference between the candidate presumptive and confirmed results.

For the high inoculation level of the 8 hour enrichment, a $dPOD_C$ value of 0.00 was obtained with a 95% confidence interval of (-0.56, 0.56), indicating no statistically significant difference between the candidate and reference method. A $dPOD_{CP}$ value of 0.00 was obtained with a 95% confidence interval of (-0.56, 0.56), indicating no statistically significant difference between the candidate presumptive and confirmed results. Detailed results of the POD analyses are presented in Tables 3 and 4 of Appendix 1.

Inclusivity/Exclusivity

The inclusivity and exclusivity analysis was performed on using a different Raman Analyzer instrument than used in the method comparison study. Out of the 15 inclusivity isolates evaluated, 10 were correctly detected by the Raman Analyzer. The 5 inclusivity cultures that were not detected were re-inoculated to Demi Fraser Broth and reanalyzed. Out of the 5 inclusivity isolates that were reanalyzed, 3 were correctly detected by the Raman Analyzer. Table 6 of Appendix 1 presents the detailed results of the inclusivity isolates.

Of the 5 exclusivity isolates evaluated, one (1) was correctly excluded. The 4 isolates that were detected were re-analyzed using Demi Fraser Broth that was incubated for 8 hours at 37 ± 1°C. Out of the 4 exclusivity isolates that were re-analyzed, 2 were correctly excluded by the Raman Analyzer. Table 7 of Appendix 1 presents the detail results of the exclusivity isolates.

REFERENCES

[1] USDA/FSIS-MLG 8.10 *Isolation and Identification of Listeria monocytogenes from Red Meat, Poultry, Ready-To-Eat Siluriformes (Fish) and Egg Products, and Environmental Samples,* January 2, 2017. (Accessed January 2017)

[2] AOAC International Methods Committee Appendix J: *Guidelines for Validation of Microbial Methods for Food and Environmental Surfaces*. 20th Ed. 2016. (Accessed January 2017) http://www.eoma.aoac.org/app_j.pdf

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

[4] Least Cost Formulations, Ltd., AOAC Binary Data Interlaboratory Study Workbook Version 2.3 (2014) -http://lcfltd.com/aoac/aoac-binary-v2-2.xls (Accessed March 2015)

APPENDIX 1

Table 1: Inoculum Summary Table

Matrix	Stainless Steel	
Inoculating Organism	*Listeria monocytogenes* 4B ATCC 19115	*Staphylococcus aureus* ATCC 25923
Low-Inoculum Level CFU[a]/Test Area[b]	170	1400
High-Inoculum Level CFU[a]/Test Area[b]	310	19000

[a]CFU: aliquots of the inocula were plated in triplicate onto TSA and averaged
[b]Test Area: 4" x 4" Surface Area

Table 2: Detailed Results for the Real-Time Analyzers *Listeria* Detection Assay for Stainless Steel (4" x 4" Test Area)

Stainless Steel (4" x 4" Test Area) *Listeria monocytogenes* ATCC 19115				
Low Level 170 CFU/Test Area				
Sample #	**RTA *Listeria* Detection Assay**		**8 Hour Confirmed**	**USDA/FSIS MLG 8.10**
	7 Hour	**8 Hour**		
1	+	+	+	+
2	+	+	+	-
3	-	+	+	-
4	+	+	+	+
5	-	+	-	-
6	-	-	-	+
7	-	-	+	+
8	-	-	-	-
9	-	+	+	-
10	-	-	-	-
Total	**3/10**	**6/10**	**6/10**	**4/10**
High Level 310 CFU/Test Area				
1	+	+	+	+
2	+	+	+	+
3	+	+	+	+
Total	**3/3**	**3/3**	**3/3**	**3/3**
Uninoculated 0 CFU/Test Area				
1	-	-	-	-
2	-	-	-	-
3	-	-	-	-
Total	**0/3**	**0/3**	**0/3**	**0/3**

Table 3: RTA *Listeria* Detection Assay, Candidate vs. Reference – POD Results

Matrix:	Enrichment Time Point	Inoculum Level (*Listeria monocytogenes* ATCC 19115)	N[a]	Candidate			Reference			$dPOD_C$[e]	95% CI[f]
				X[b]	POD_C[c]	95% CI	x[c]	POD_R[d]	95% CI		
Stainless Steel (4" x 4" Test Area)	8 Hour	Uninoculated	3	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	-0.43, 0.43
		Low	10	6	0.60	0.31, 0.83	4	0.40	0.17, 0.69	0.20	-0.21, 0.53
		High	3	3	1.00	0.57, 1.00	3	1.00	0.57, 1.00	0.00	-0.56, 0.56

[a]N = Number of test portions
[b]x = Number of positive test portions
[c]POD_C = Candidate method confirmed positive outcomes divided by the total number of trials
[d]POD_R = Reference method confirmed positive outcomes divided by the total number of trials
[e]$dPOD_C$= Difference between the confirmed candidate method result and reference method confirmed result POD values
[f]95% CI = If the confidence interval of a dPOD does not contain zero, then the difference is statistically significant at the 5% level

Table 4: RTA *Listeria* Detection Assay, 8 Hour Presumptive vs. Confirmed – POD Results

Matrix:	Enrichment Time Point	Inoculum Level (*Listeria monocytogenes* 4B ATCC 19115)	N[a]	Presumptive			Confirmed			$dPOD_{CP}$[e]	95% CI[f]
				X[b]	POD_{CP}[c]	95% CI	x[c]	POD_{CC}[d]	95% CI		
Stainless Steel (4" x 4" Test Area)	8 Hour	Uninoculated	3	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	-0.43, 0.43
		Low	10	6	0.60	0.31, 0.83	6	0.60	0.31, 0.83	0.00	-0.37, 0.37
		High	3	3	1.00	0.57, 1.00	3	1.00	0.57, 1.00	0.00	-0.43, 0.43

[a]N = Number of test portions
[b]x = Number of positive test portions
[c]POD_{CP} = Candidate method presumptive positive outcomes divided by the total number of trials
[d]POD_{CC} = Candidate method confirmed positive outcomes divided by the total number of trials
[e]$dPOD_{CP}$= Difference between the candidate method presumptive result and candidate method confirmed result POD values
[f]95% CI = If the confidence interval of a dPOD does not contain zero, then the difference is statistically significant at the 5% level

Table 5: RTA *Listeria* Detection Assay, 7 Hour Presumptive vs. 8 Hour Confirmed – POD Results

Matrix:	Inoculum Level (*Listeria monocytogenes* 4B ATCC 19115)	N[a]	7 Hour Presumptive			8 Hour Confirmed			$dPOD_{CP}$[e]	95% CI[f]
			X[b]	POD_{CP}[c]	95% CI	x[c]	POD_{CC}[d]	95% CI		
Stainless Steel (4" x 4" Test Area)	Uninoculated	3	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	-0.43, 0.43
	Low	10	3	0.30	0.11, 0.60	6	0.60	0.31, 0.83	-0.30	-0.60, 0.12
	High	3	3	1.00	0.57, 1.00	3	1.00	0.57, 1.00	0.00	-0.43, 0.43

[a]N = Number of test portions
[b]x = Number of positive test portions
[c]POD_{CP} = Candidate method presumptive positive outcomes divided by the total number of trials
[d]POD_{CC} = Candidate method confirmed positive outcomes divided by the total number of trials
[e]$dPOD_{CP}$= Difference between the candidate method presumptive result and candidate method confirmed result POD values
[f]95% CI = If the confidence interval of a dPOD does not contain zero, then the difference is statistically significant at the 5% level

Table 6: Detailed Results for the Inclusivity Evaluation

Organism	Source	Reference Number	Inoculum Level	Real-Time Analyzers Result	Reanalysis Result[1]
Listeria monocytogenes (4B)	ATCC	19115	6.6×10^8	-	+
Listeria monocytogenes (1/2C)	ATCC	7644	2.2×10^8	+	
Listeria maarthi	ATCC	BAA-1595	5.1×10^8	+	
Listeria innocua (6A)	ATCC	33090	8.7×10^8	+	
Listeria innocua (6B)	ATCC	33091	7.4×10^8	+	
Listeria ivanovii	ATCC	19119	5.9×10^8	+	
Listeria ivanovii	ATCC	49954	2.1×10^8	+	
Listeria grayi	ATCC	19120	7.6×10^5	+	
Listeria grayi	ATCC	25401	6.4×10^6	-	-
Listeria seeligeri (6B)	ATCC	11289	1.0×10^9	+	
Listeria seeligeri (1/2B)	ATCC	35967	7.6×10^8	-	+
Listeria welshimeri (6B)	ATCC	35897	5.8×10^8	+	
Listeria welshimeri (1/2B)	ATCC	43550	9.0×10^8	+	
Listeria weihenstephanensis	Cornell	FSL R9-0317	2.6×10^7	-	-
Listeria rocourtiae	Cornell	FSL F6-0920	1.7×10^8	-	+

[1]Inclusivity organisms originally producing a negative result were re-inoculated in Demi Fraser and reanalyzed.

Table 6: Detailed Results for the Exclusivity Evaluation

Organism	Source	Reference Number	Inoculum Level	Real-Time Analyzers Result	Demi Fraser Inoculum Level	Demi Fraser Result[1]
Enterococcus faecalis	ATCC	29212	5.6×10^8	-		
Lactobacillus casei	ATCC	11578	5.3×10^7	+	<10	-
Rhodococcus equi	NCTC	6939	4.3×10^7	+	6.1×10^6	+
Staphylococcus aureus	ATCC	25923	8.6×10^7	+	1.2×10^4	-
Streptococcus salivarius	ATCC	19258	2.6×10^8	+	3.1×10^7	+

[1]Exclusivity organisms that produced a positive result were reanalyzed after being cultured in Demi Fraser Broth.

APPENDIX 2

Feasibility Evaluation for the Real-Time Analyzers *Listeria* Detection Assay for the Detection of *Listeria* species from Environmental Samples

Protocol # MRD17011-14B

Prepared for:

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Prepared by:

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1. Purpose

The purpose of this study is to conduct a feasibility evaluation for the detection of *Listeria* species from environmental samples for Real-Time Analyzers, Inc. (Study Sponsor).
A modified AOAC validation design will be conducted on one environmental surface, stainless steel, to verify the detection level of the assay that will produce acceptable data for an AOAC validation (fractional positive results by the RTA or reference method, along with passing POD statistical analysis). Additionally, an inclusivity and exclusivity evaluation for 15 isolates of *Listeria* species and 5 exclusivity strains will be evaluated by the RTA platform.

2. Quality Assurance

2.1. The bacterial strains specified for use in this validation study will be obtained directly from American Type Culture Collection (ATCC) or another internationally recognizable reference source. Regardless of the source of the strains, confirmation documentation (biochemical and serological) is on file for each organism (Table 1, 2, and 3).

2.2. One or more technicians will prepare the 4 x 4" test areas and different technicians will perform the assays on the "blind" coded test regions.

2.3. Test areas will be fully randomized and labeled with a code so that the analyst performing the assay is unaware of the level or type of bacterial culture present.

3. Microbiological Media/Reagents:

3.1. Trypticase soy agar with 5% sheep blood (SBA)
3.2. Brain Heart Infusion (BHI) broth
3.3. UVM, Modified Listeria Enrichment Broth
3.4. Fraser Broth, Fraser broth base supplement
3.5. Oxford Listeria Agar Base
3.6. Modified Oxford Listeria Supplement
3.7. Dey-Engley Neutralizing broth

4. Equipment/Supplies:

4.1. Sterile Whirl-Pak® Speci-Sponge® environmental surface sampling bags
4.2. Calibrated micropipettors, capable of delivering ≥ 500 µL
4.3. Sterile micropipette tips, capable of delivering ≥ 500 µL
4.4. Sterile serological pipettes
4.5. Sterile inoculation loops
4.6. Vortex mixer
4.7. Incubator capable of maintaining 35 ± 1°C, 37 ± 1°C, and 30 ± 1°C
4.8. Refrigerator capable of maintaining 2 - 8°C
4.9. World Bioproducts, PUR-Blue™ DUO Swab HiCap™ Neutralizing Broth/Demi-Fraser Broth, catalog number DUO-HC9DFB, with 5 mL removed for a total of 5 mL Neutralizing Broth/Demi-Fraser Broth.
4.10. 1.5 mL microcentrifuge tubes
4.11. RTA magnetic *Listeria* beads
4.12. 6 tube magnetic separation rack, catalog #S1506S
4.13. RTA magnetic stand
4.14. VWR Tube rotator 120V, Part number: 10136-084
4.15. Glass vial 5/8 dram clear 15 x 26 vial with 13-425 black polycone cap, part # W1526C-CONE
4.16. RTA Wash buffer (20x)
4.17. RTA *Listeria* probes
4.18. RTA positive *Listeria* control

5. Method Comparison

5.1. 3 levels will be tested

5.1.1. **Control** – Uninoculated level 0 CFU/test area (4 x 4")

5.1.1.1. 3 replicates for the reference and candidate method

5.1.2. **Fractional/low** - Low level contamination ~50 CFU/ test area (4 x 4")

5.1.2.1. 10 replicates for the reference and candidate method

5.1.3. **High** - High level of contamination ~150 CFU/ test area (4 x 4")

5.1.3.1. 3 replicates for the reference and candidate method

5.2. Inoculation and Matrix Preparation Summary

5.2.1. Matrix Evaluation:

5.2.1.1. Stainless Steel Environmental Surface (Table 1)
Target organism: *Listeria monocytogenes* 4B ATCC 19115
Competitive organism: *Staphylococcus aureus* ATCC 25923

Table 1: Matrix and Inoculation Organisms

Matrix	Inoculating Organism	Target Inoculum Level # of Replicates	Equilibrium Protocol	Reference Method
Stainless Steel Environmental Surface	*Listeria monocytogenes* 4B ATCC 19115 *Staphlyococcus aureus* ATCC 25923 10x Level	0 CFU/test area – 3 ~50 CFU/ test area - 10 ~150 CFU/ test area - 3	Unstressed 16-24 hours at 20-25°C	USDA MLG 8.10

5.2.2. Appropriate cultures are retrieved from a frozen stock stored at -70° C, transferred to Trypticase Soy Agar with 5% sheep blood (SBA) and incubated at 35 ± 1° C for 24 ± 2h.

5.2.3. Propagate the culture from SBA in Brain Heart Infusion (BHI) broth at 35°C for 18-24 hours.

5.2.3.1. Prepare dilutions for quantification and for inoculation of the low and high contamination levels by inoculating pre-warmed (37 ± 1°C, for 30 minutes) BHI broth and immediately pipetting 250 µL of sample dropwise (approximately 10 drops) onto test area (4 x 4").

5.2.3.2. Let dry for 16-24 hours at 20-25° C

5.3. Real-Time Analyzer (RTA) Platform for the Detection of *Listeria* spp.

5.3.1. Sample test area

5.3.1.1. Pre-warm World Bioproducts DUO swabs to 37 ± 1°C immediately before use

5.3.1.2. Wring out swab of excess neutralizing buffer by pressing swab against tube walls.

5.3.1.3. Swab the stainless steel surface thoroughly over the spike area.

5.3.1.4. Store swab samples at ambient temperature (20-25° C) for a minimum of two hours.
Note: The sampling swab portion of the sampling device will remain capped at 20-25° C. The Demi-Fraser Broth portion of the sampling device will be capped with a new sterile cap and pre-warmed at 37 ± 1°C.

5.3.1.5. After the minimum two hour hold, add swab to the pre-warmed Demi-Fraser Broth (5 mL) that is pre-warmed to 37 ± 1°C.

5.3.1.6. Shake and vortex the tube for 5 seconds.

5.3.1.7. Incubate candidate samples at 37 ± 1°C and analyze at 7 and 8 hours of incubation by vortexing for 30 seconds and removing a 1.5 mL subsample at each time point.

5.3.2. Real-Time Analyzer testing procedures

5.3.2.1. Add 1.0 mL from each subsample into a clean microcentrifuge tube.

5.3.2.2. Add 1.0 mL of RTA positive *Listeria* control into clean microcentrifuge tube and 1.0 mL of sterile enrichment broth (blank) into clean microcentrifuge tubes.

5.3.2.3. To each 1.0 mL sample, 1.0 mL RTA positive *Listeria* control sample, and 1.0 mL blank enrichment broth sample add 7.5 µL of room temperature magnetic beads.

5.3.2.4. Shake tubes and place on rotator for 10 minutes at room temperature.

5.3.2.5. Place tubes in magnetic separation rack and tilt back and forth for 3 minutes or until beads form a visible pellet.

5.3.2.6. Carefully remove and discard supernatant making sure not to disrupt the bead pellet.

5.3.2.7. Add 1 mL of wash buffer to each tube, remove from magnetic rack and gently shake the tubes.

5.3.2.8. Place tubes in magnetic separation rack and tilt back and forth for 3 minutes or until beads form a visible pellet.

5.3.2.9. Carefully remove and discard supernatant making sure not to disrupt the bead pellet.

5.3.2.10. Add 250 µL of probe solution to each tube.

5.3.2.11. Shake tubes and place on rotator for 10 minutes at room temperature.

5.3.2.12. Add 1 mL of wash buffer to each tube and shake.

5.3.2.13. Place tubes in magnetic separation rack and tilt back and forth for 3 minutes or until beads form a visible pellet.

5.3.2.14. Carefully remove and discard supernatant making sure not to disrupt the bead pellet.

5.3.2.15. Add 1 mL of wash buffer to each tube, remove the sample from the magnetic separation rack, and gently shake sample to mix.

5.3.2.16. Transfer the contents of the micro centrifuge tube to a glass vial.

5.3.2.17. Place glass vial on magnetic separation stand for 3 minutes.

5.3.2.18. Carefully remove and discard supernatant making sure not to disrupt the bead pellet.

5.3.2.19. Leave the glass vial on the magnetic separation stand for 5 additional minutes.

5.3.2.20. Turn on the lap-top computer and open the RTA software application (SERS Pathogen Detection icon), connect the Raman Analyzer and insure that the computer and instrument are synced.

Note: Syncing is indicated by a Computer Desktop image appearing on the Roman Analyzer.

5.3.2.21. Carefully place blank glass vial in the Raman Analyzer

5.3.2.22. Close the lid

5.3.2.23. Acquire spectrum 1 by following software prompts.

5.3.2.24. Open lid, rotate vial approximately 90 degrees and repeat steps 5.3.2.21. – 5.3.2.23. three additional times to acquire spectra 2, 3, and 4.

5.3.2.25. Remove vial and place positive RTA *Listeria* control glass vial in the Raman Analyzer and continue with steps 5.3.2.21. – 5.3.2.24.

5.3.2.26. Once the blank and positive controls have been run, analyze all samples by placing the glass vials in the Raman Analyzer and performing steps 5.3.2.21.- 5.3.2.24.

5.3.2.27. RTA SERS Pathogen Detection software averages the four spectra readings and compares them to the blank and positive controls. It then provides a "Presumptive Positive," or "None Detected" or "Invalid" result.

Note: In the case of an "Invalid" result, rerun the sample and document both the invalid and retested result.

Note: To further identify invalid or unexpected results open the Raman VISTA software to view the graph of the spectra.

5.3.2.28. All samples at the 8 hour time point, regardless of presumptive results, will be confirmed for the candidate method by a modification of the procedures in the MLG 8.10 reference method outlined below. The modification confirms *Listeria* through agar plate level as colonies exhibiting typical *Listeria* morphology. Any additional confirmations will be conducted at the request of the study sponsor.

5.4. Modified MLG 8.10 Reference Method

5.4.1. Add 10 mL of D/E neutralizing broth to sampling sponge and squeeze several times to allow the sponge to become fully immerse.

5.4.2. Sample the stainless steel surface, and hold sponges at ambient temperature (20-25°C) for 2 hours.

5.4.3. Enrich sample with 225 mL of UVM and incubate for 20-26 hours at 30 ± 1°C.

5.4.4. After incubation, transfer 0.1 ± 0.02 ml of the enriched sample to 10 ± 0.5 ml of Fraser Broth (FB). As per media preparation instructions, be sure that appropriate supplements have been added to the FB prior to inoculation.

5.4.5. Mix by vortex and incubate the inoculated FB tubes at 35 ± 2°C for 26 ± 2 h.

5.4.6. Using the enriched sample, streak a loopful or a drop approximating 0.1 ml of the sample over the surface of a Modified Oxford Listeria Agar (MOX) plate. Incubate the plates at 35 ± 2°C for 26 ±2 h.

5.4.7. Examine the MOX plates for colonies with morphology typical of *Listeria* spp. At 26 ± 2 h, suspect colonies are typically small (ca. 1 mm) and are surrounded by a zone of darkening due to esculin hydrolysis.

5.4.8. If suspect colonies are present on MOX, sample is considered positive for *Listeria* spp.

5.4.9. If no suspect colonies are evident, re-incubate the MOX plate for an additional 26 ± 2 hour.

5.4.10. After 26 ± 2 h of incubation, examine the FB for the potential presence of Listeria spp., by visual examination of the broth for darkening due to esculin hydrolysis.

5.4.11. If any degree of FB darkening is evident, aseptically dispense a drop approximating 0.1 ± 0.02 ml of FB onto a MOX plate. Swab or streak 25-40% of the surface of the MOX plate with the FB inoculum. Use a loop to streak for isolation from the initial swab/streak quadrant onto the remainder of the plate. Incubate the MOX plate at 35 ± 2°C for 26 ± 2 h.

5.4.12. If no FB darkening is evident, re-incubate the FB at 35 ± 2°C until a total incubation time of 48 ± 2 h has been achieved.

5.4.13. Re-examine the FB for evidence of darkening after 48 ± 2 h of total incubation. If any degree of darkening is evident, swab, streak and incubate a MOX plate.

5.4.14. If no darkening of FB is evident and no suspect MOX colonies have been demonstrated, the sample is considered negative for Listeria spp.

5.4.15. If suspect colonies are present on MOX, sample is considered positive for *Listeria spp.*

5.4.16. Any additional confirmations will be conducted at the request of the study sponsor.

6. Inclusivity/Exclusivity Test Methodology

6.1. Fifteen *Listeria* species (Table 2) will be cultured from a frozen stock stored at -70° C, transferred to Trypticase Soy Agar with 5% sheep blood (SBA) and incubated at 35 ± 1° C for 24 ± 2h.

6.1.1. The isolates will then be cultured in Demi-Fraser broth for 8 hours at 37 ± 1°C. After incubation, the cultures will be serially diluted to 100 x the LOD_{50} of the assay and processed according to the RTA method protocol.

6.2. Five exclusivity strains (Table 3) will be cultured from a frozen stock stored at -70° C, transferred to Trypticase Soy Agar with 5% sheep blood (SBA) and incubated at 35 ± 1° C for 24 ± 2h.

6.2.1. All exclusivity organisms will be grown in non-selective media for 18 – 24 hours and tested undiluted.

6.3. All cultures will be randomized, blind coded and analyzed by the RTA Raman Analyzer. Results will be reported by strain as Presumptive Positive or None Detected.

Table 2- Inclusivity Organisms

No.	Organism	Reference Number	Source
1	*Listeria monocytogenes* (4B)	19115	ATCC
2	*Listeria monocytogenes* (1/2C)	7644	ATCC
3	*Listeria maarthi*	BAA-1595	ATCC
4	*Listeria innocua* (6A)	33090	ATCC
5	*Listeria innocua* (6B)	33091	ATCC
6	*Listeria ivanovii*	19119	ATCC
7	*Listeria ivanovii*	49954	ATCC
8	*Listeria grayi*	19120	ATCC
9	*Listeria grayi*	25401	ATCC
10	*Listeria seeligeri* (6B)	11289	ATCC
11	*Listeria seeligeri* (1/2B)	35967	ATCC
12	*Listeria welshimeri* (6B)	35897	ATCC
13	*Listeria welshimeri* (1/2B)	43550	ATCC
14	*Listeria weihenstephanensis*	FSL R9-0317	Cornell
15	*Listeria rocourtiae*	FSL F6-0920	Cornell

Table 3-Exclusivity Organisms

Organism	Source	Origin
Enterococcus faecalis	ATCC 29212	Urine
Lactobacillus casei	ATCC 11578	Salad Dressing
Rhodococcus equi	NCTC 6939	Not available
Staphylococcus aureus	ATCC 25923	Clinical Isolate
Streptococcus salivarius	ATCC 19258	Pasteurized Milk

7. Statistical Analysis

7.1. Probability of Detection (POD)

7.1.1. The proportion of positive analytical outcomes for a qualitative method for a given matrix at a given analyte level or concentration. POD is concentration dependent. For this study POD measurements include: POD_R (reference method POD), POD_C (Confirmed candidate method POD), POD_{CP} (candidate method presumptive result POD) and POD_{CC} (candidate method confirmation result POD)

7.1.2. The POD estimate is calculated as the number of positive outcomes divided by the total number of trials. Estimate the POD with a 95% confidence interval for the candidate method, the reference method and the presumptive and confirmed results.

7.1.3. Difference of Probabilities of Detection (dPOD).—Difference of probabilities of detection is the difference between any two POD values.

7.1.4. Estimate the $dPOD_{CP}$ as the difference between the candidate presumptive result POD (POD_{CP}) and the candidate confirmed result POD (POD_{CC}) values. Calculate the 95% confidence interval on the $dPOD_{CP}$. $dPOD_{CP} = POD_{CP} - POD_{CC}$

7.1.5. Estimate the $dPOD_C$ as the difference between the candidate method and reference method POD values. Calculate the 95% confidence interval on the $dPOD_C$. $dPOD_C = POD_C - POD_R$

7.1.6. If the confidence interval of a dPOD does not contain zero, then the difference is statistically significant at the 5% level.

8. Report Format

A validation manuscript including a tabularized summary of results will be submitted at the completion of the independent laboratory testing.

APPROVALS:

Q Laboratories, Inc. Approval

MRD Supervisor/Designee _____

Real-Time Analyzers Designee _____