Technical Bulletin: Detection of *Salmonella* and *Listeria monocytogenes* from Anhydrous Milk Fat Using the BAX® System Real-Time PCR Assays

An unpaired matrix study was conducted at an independent laboratory to measure the performance of the BAX® System Real-Time PCR Assay for *Salmonella* and Real-Time PCR Assay for *Listeria monocytogenes* against the United States Food and Drug Administration's reference methods to detect each target organism in Anhydrous Milk Fat (AMF). Samples tested in this study were artificially inoculated at levels expected to produce low (0.2-2 CFU/test portion) or high (5 CFU/test portion) spike levels after a 2 week equilibration at room temperature. The results obtained for each method were compared using the probability of detection (POD). For both *Salmonella* and *L. monocytogenes,* the BAX® System method demonstrated equivalent performance to the reference method.

Introduction

Anhydrous milk fat (AMF) is essentially a pure form of milk fat obtained from fresh cream or butter. It is widely used as an ingredient to intensify the taste and creaminess of many food products including ice cream, cake, chocolate and more. AMF is the preferred dairy-based fat source to use in these food types because its consistency makes it easy to work with and it has a long shelf life (1). However, like most milk products, microbial contamination can be a problem.

Sample Preparation and Enrichment

Salmonella Kentucky ATCC 9263 and *Listeria monocytogenes* ATCC 19111 were used to inoculate samples of AMF independently. For each target organism, AMF was inoculated with a broth culture in bulk to create a low fractional spike level or a high spike level. Each master sample was thoroughly mixed to achieve equal distribution of the inoculum and stored at room temperature for 2 weeks. The master samples were then separated into 25 g test portions to create 20 low-spike and 5 high-spike samples per method. An additional 5 samples per method were left uninoculated to serve as negative controls.

For the BAX® System Real-Time *Salmonella* method, 375 g test portions were analyzed by combining a 25 g sample of inoculated AMF with 350 g of uninoculated AMF. Samples were homogenized with 1500 mL pre-warmed (35°C) Buffered peptone water (BPW) and incubated at 35°C for 22-26 hours. Samples were tested by the BAX® System method directly from the primary enrichment and after a BHI regrowth. The regrowth was performed by transferring 10 µL of the primary enrichment to 500 µL pre-warmed (37°C) BHI broth and incubating at 37°C for 3 hours before proceeding to the lysis procedure described below.

For the BAX® System Real-Time *Listeria* methods, 25 g test portions of AMF were analyzed. Samples were homogenized with 225 mL with pre-warmed (20-35°C) 24 LEB Complete media and incubated at 35°C for 26-48 hours. Samples were tested by the BAX® System method at 26 and 48 hours.

For the FDA BAM reference methods, 25 g samples were analyzed for both *Salmonella* and *Listeria*. *Salmonella* samples were homogenized with 225 mL of Lactose Broth (LB) and incubated at 35°C for 22-26 hours.

Listeria samples were homogenized with 225 mL of BLEB containing pyruvate and incubated at 30°C for 4 hours. After 4 hours, solutions of three selective agents (acriflavine, cycloheximide and nalidixic acid) were added to the enrichment, mixed and incubated at 30°C for an additional 44 hours.

Method

BAX® System Method – For *Salmonella* samples, 5 µL of enrichment was added to 200 µL prepared lysis reagent (150 µL of protease to one 12 mL bottle of lysis buffer) in cluster tubes. Lysis was performed by heating tubes for 20 minutes at 37°C and 10 minutes at 95°C, and then cooling tubes at 4°C. Real-Time *Salmonella* PCR tubes were hydrated with 30 µL of lysate and held for 10 minutes on a chilled (2-8°C) cold block. All PCR tubes were then loaded into the BAX® System Q7 instrument, and a full process was run according to the procedure described in the BAX® System User Guide.

For *Listeria* samples, 5 µL of enrichment was added to 200 µL prepared lysis reagent (150 µL of protease and 200 µL of Lysing Agent 2 to one 12 mL bottle of lysis buffer) in cluster tubes. Lysis was performed by heating tubes for 30 minutes at 55°C and 10 minutes at 95°C, and then cooling tubes at 4°C. Real-Time Genus *Listeria* and Real-Time *L. monocytogenes* PCR tubes were hydrated with 30 µL of lysate. All PCR tubes were loaded into the BAX® System Q7 instrument, and a full process was run according to the procedure described in the BAX® System User Guide.

Reference Method – All samples were culture confirmed regardless of presumptive BAX® System results following the FDA BAM Chapter 5 for *Salmonella* or the FDA BAM Chapter 10 for *Listeria monocytogenes*.

Results and Discussion

Statistical analysis using the probability of detection (POD) and the difference in POD (dPOD) values were calculated with 95% confidence intervals to compare the results between the BAX® System method presumptive and confirmed results (Table 1) and between the BAX® System method and reference method (Table 2).

For *Salmonella* samples enriched in BPW, the BAX® System method returned positive results for 14/20 low spiked and 5/5 high spiked samples with and without a BHI regrowth. All results were identical to culture. The corresponding samples enriched using the FDA BAM method returned culture positive results for 18/20 low spiked and 5/5 high spiked samples. The statistical analyses between the BAX® System presumptive and confirmed results as well as the BAX® System method and reference method demonstrated no significant difference.

Table 1. BAX® System Presumptive vs. Confimed Results

Sample Type	Target Organism	MPN/25 g	Test Portions	BAX® System Presumptive			BAX® System Confirmed			$dPOD_{CP}$	95% CI
				X	POD_{CP}	95% CI	X	POD_{CC}	95% CI		
AMF (375 g)	*Salmonella* Kentucky ATCC 9263	Control	5	0	0.00	0.00, 0.45	0	0.00	0.00, 0.45	0.00	-0.43, 0.43
		2.34	20	14	0.70	0.48, 0.85	14	0.70	0.48, 0.85	0.00	-0.27, 0.27
		2.43	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	-0.43, 0.43
AMF (25 g)	*L. monocytogenes* ATCC 19111	Control	5	0	0.00	0.00, 0.45	0	0.00	0.00, 0.45	0.00	-0.43, 0.43
		0.53	20	8	0.40	0.22,0.61	8	0.40	0.22,0.61	0.00	-0.28, 0.28
		9.26	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	-0.43, 0.43

MPN/25 g = Most Probable Number is based on the POD of reference method test portions

X = Number of positive test portions

POD_{CP} = BAX® method presumptive positive results divided by the total number of test portions

POD_{CC} = BAX® method confirmed positive results divided by the total number of test portions

$dPOD_{CP}$ = Difference between the BAX® method presumptive result and BAX® method confirmed result POD values

95% CI = If the confidence interval of dPOD does not contain zero, then the difference is statistically significant at the 5%

Table 2. BAX® System Results vs. Reference Method Results

Sample Type	Target Organism	MPN/25 g	Test Portions	BAX® System Method			Reference Method			$dPOD_C$	95% CI
				X	POD_C	95% CI	X	POD_R	95% CI		
AMF	*Salmonella* Kentucky ATCC 9263	Control	5	0	0.00	0.00, 0.45	0	0.00	0.00, 0.45	0.00	-0.43, 0.43
		2.34	20	14	0.70	0.48, 0.85	18	0.90	0.70, 0.97	-0.20	-0.43, 0.05
		2.43	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	-0.43, 0.43
	L. monocytogenes ATCC 19111	Control	5	0	0.00	0.00, 0.45	0	0.00	0.00, 0.45	0.00	-0.43, 0.43
		0.53	20	8	0.40	0.22,0.61	9	0.45	0.26, 0.65	-0.05	-0.32, 0.23
		9.26	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	-0.43, 0.43

MPN/25 g = Most Probable Number is based on the POD of reference method test portions

X = Number of positive test portions

POD_C = Confirmed BAX® method positive results divided by the total number of test portions

POD_R = Confirmed reference method positive results divided by the total number of test portions

$dPOD_C$ = Difference between the BAX® method and reference method POD values

95% CI = If the confidence interval of dPOD does not contain zero, then the difference is statistically significant at the 5% level

For *Listeria* samples enriched in 24 LEB Complete media, the BAX® System method returned positive results for 8/20 low spiked and 5/5 high spiked samples at both 26 and 48 hours. All results were identical to culture. The corresponding samples enriched using the FDA BAM method returned culture positive results for 9/20 low spiked and 5/5 high spiked samples. The statistical analyses between the BAX® System presumptive and confirmed results as well as the BAX® System method and reference method demonstrated no significant difference.

Conclusions

Overall, the results of this study demonstrate the ability of the BAX® System Real-Time PCR Assay for *Salmonella* and Real-Time PCR Assay for *L. monocytogenes* to accurately detect *Salmonella* species and *L. monocytogenes,* respectively in AMF equivalent to the reference methods using the following enrichment protocols:

- For *Salmonella*, homogenize 375 g samples with 1500 mL pre-warmed (35°C) BPW and incubate at 35°C for 24 hours.
- For *Listeria*, homogenize 25 g samples with 225 mL pre-warmed (20-35°C) 24 LEB Complete media and incubate at 35°C for 26-48 hours.

References

1. Tetra Pak. Dairy Processing Handbook. Chapter 13: Anhydrous Milk Fat (AMF) and Butteroil. https://dairyprocessinghandbook.com/chapter/anhydrous-milk-fat-amf-and-butteroil