

# MicroSEQ<sup>®</sup> *Salmonella* spp. Detection Kit Using the Pathatrix<sup>®</sup> 10-Pooling *Salmonella* spp. Kit Linked Protocol Method Modification

Performance Tested Method<sup>SM</sup> 031001

## Abstract

Real-time PCR methods for detecting foodborne pathogens offer the advantages of simplicity and quick time to results compared to traditional culture methods. The addition of a recirculating pooled immunomagnetic separation method prior to real-time PCR analysis increases processing output while reducing both cost and labor. This AOAC Research Institute method modification study validates the MicroSEQ<sup>®</sup> *Salmonella* spp. Detection Kit [AOAC Performance Tested Method (PTM) 031001] linked with the Pathatrix<sup>®</sup> 10-Pooling *Salmonella* spp. Kit (AOAC PTM 090203C) in diced tomatoes, chocolate, and deli ham. The Pathatrix 10-Pooling protocol represents a method modification of the enrichment portion of the MicroSEQ *Salmonella* spp. protocol. The results of the method modification were compared to standard cultural reference methods for diced tomatoes, chocolate, and deli ham. All three matrixes were analyzed in a paired study design. An additional set of chocolate test portions was analyzed using an alternative enrichment medium in an unpaired study design. For all matrixes tested, there were no statistically significant differences in the number of positive test portions detected by the modified candidate method compared to the appropriate reference method. The MicroSEQ *Salmonella* spp. protocol linked with the Pathatrix individual or 10-Pooling procedure demonstrated reliability as a rapid, simplified, method for the preparation of samples and subsequent detection of *Salmonella* in diced tomatoes, chocolate, and deli ham.

## Participants

### METHOD AUTHORS

JASON WALL, RICK CONRAD,<sup>1</sup> KATHY LATHAM, and ERIC LIU

Life Technologies, 2130 Woodward St, Austin, TX 78744

### CONTRACT LABORATORY

Q Laboratories, Inc., 1400 Harrison Ave, Cincinnati, OH 45214

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The method was independently tested, evaluated, and certified by the AOAC Research Institute as a Performance Tested Method<sup>SM</sup>. See <http://www.aoac.org/testkits/steps.html> for information on certification.

<sup>1</sup>Corresponding author's e-mail: rick.conrad@lifetech.com

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## REVIEWER

YI CHEN

U.S. Food and Drug Administration, Center for Food Safety and Applied Nutrition, 5100 Paint Branch Pkwy, College Park, MD 20740

## Scope of Method

(a) *Target organism.*—*Salmonella* spp.

(b) *Matrixes tested in the modification study.*—Diced tomatoes (fresh), Belgian chocolate (41% fat), sliced, cooked deli ham (2.6% fat, 1.4% sodium).

The study included the use of the Pathatrix 10-Pooling *Salmonella* spp. Kit followed by detection using the MicroSEQ *Salmonella* spp. Detection Kit on the Applied Biosystems 7500 Fast Real-Time PCR instrument.

(c) *Summary of validated performance claims.*—Performance was comparable to that of the U.S. Food and Drug Administration, *Bacteriological Analytical Manual* (FDA/BAM; 1) or the U.S. Department of Agriculture-Food Safety and Inspection Service, *Microbiology Laboratory Guidebook* (USDA-FSIS/MLG; 2) reference culture methods as determined by probability of detection (POD) analysis.

## Definitions

(a) *POD.*—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. Several POD measures can be calculated: POD<sub>R</sub> (the reference method POD); POD<sub>C</sub> (the confirmed candidate method POD); POD<sub>CP</sub> (the candidate method presumptive result POD); and POD<sub>CC</sub> (the candidate method confirmation result POD).

(b) *Difference of probabilities of detection (dPOD).*—dPOD is the difference between any two POD values. If the confidence interval of a dPOD does not contain zero, the difference is statistically significant at the 5% level (3).

## Principle

### Pathatrix 10-Pooling

The Pathatrix Auto instrument is a fully automated sample preparation system that uses paramagnetic particles with target-specific antibodies to selectively bind and concentrate target organisms from enriched media. The system utilizes a large volume recirculating immunomagnetic separation (IMS) method which enables much larger single-sample processing or the option to pool aliquots of 5 to 10 individual enrichments

for subsequent downstream analysis. Subsequent negatives for either the individual or the pooled enrichments as detected by various downstream detection methods indicate negatives for the represented samples. Positives of the pooled sample require deconvolution and running the representative samples individually.

#### *MicroSEQ Salmonella spp. Detection Kit*

The MicroSEQ *Salmonella* spp. Detection Kit is already an AOAC certified *Performance Tested Method* (PTM), and is based on TaqMan Real-Time PCR technology, providing two levels of specificity for confident pathogen detection by combining PCR amplification 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 system contains an Internal Positive Control (IPC) in the reaction mix to monitor the presence of 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 potential for contamination. A 30  $\mu$ L volume of the bead-free supernatant is transferred into MicroSEQ tubes containing lyophilized PCR reagents. The MicroSEQ assay is then run on the AB 7500 Fast Real-Time PCR instrument and results are obtained within 40 min.

#### *General Information*

The genus *Salmonella*, consisting of the two species *S. enterica* and *S. bongori*, is a member of the *Enterobacteriaceae* family. More than 2400 *Salmonella* serotypes have been reported, all of which are potentially pathogenic. *S. enterica* with its six subspecies is of clinical relevance for humans and is the causative agent of foodborne illnesses or Salmonellosis. Foodborne outbreaks due to *Salmonella* have become a major public health problem and can occur as food poisoning triggered epidemics as well as isolated cases. Outbreaks have been associated with raw meats and poultry, eggs, milk and dairy products, seafood, coconut sauces, salad dressings, cocoa, chocolate, spices, frozen products, peanuts, and vegetables such as hot peppers.

#### **Materials and Methods**

##### *Test Kit Information*

(a) *Kit names.*—MicroSEQ *Salmonella* spp. Detection Kit; Pathatrix 10-Pooling *Salmonella* spp. Kit.

(b) *Cat. Nos.*—4403930 (MicroSEQ *Salmonella* spp. Detection Kit); APS500P (Pathatrix 10-Pooling *Salmonella* spp. Kit).

(c) *Ordering information.*—Life Technologies, 5791 Van Allen Way, Carlsbad, CA 92008, Tel: 1-800-955-6288, Fax: 1-800-331-2286. Website: www.lifetechnologies.com.

(d) *Test kit components.*—MicroSEQ *Salmonella* spp. Detection Kit Part 1 of 2.—MicroSEQ *Salmonella* spp. Detection Kit (Pouch).—Contains twelve 8-tube strips placed in a 96-well

support base, a bag of optical (clear) caps, a product insert, and a desiccant pack. Each tube contains a lyophilized Real-Time PCR reaction mix and is sealed with non-optical caps. The lysosphere contains enzyme, buffers, magnesium, nucleotides, and supplements, together with primers and a FAM<sup>TM</sup>-dye labeled probe for detection of *Salmonella* species, an IPC template together with primers, and a VIC-dye labeled probe for detection of the IPC. Store at 2–8°C and protect from light and moisture.

*Part 2 of 2.*—Pathogen Detection Negative Control (box).—Contains one tube. Tube contains an aqueous buffer prepared with PCR-grade water and monitors for contamination and reagent integrity. Store at 2–8°C.

#### *Pathatrix 10-Pooling Salmonella spp. Kit*

*Part 1 of 1.*—Pathatrix 10-Pooling *Salmonella* spp. Kit Part No. APS500P.—50 tests (of 10-pooled sample). Each kit includes one vial of antibody-coated beads and sufficient numbers of sterile, single-use plastic consumables for 50 pooled tests (50 individual, enriched samples). Store beads at 5  $\pm$  3°C. Store plastics at room temperature.

#### *Additional Supplies and Reagents*

(a) *Applied Biosystems Fast Reaction Tubes (8 tubes/strip).*—125 strips, Part No. 4358293.

(b) *Applied Biosystems Optical Caps (8 caps/strip).*—300 strips, Part No. 4323032.

(c) *Aerosol-resistant pipet tips.*—Different sizes, from 0.5 to 1000  $\mu$ L. Aerosol resistant for low risk of contamination.

#### *Apparatus*

(a) *Pathatrix Auto Instrument.*—Life Technologies Part No. Pathatrix Auto. For automated/pooled IMS.

(b) *AB 7500 Fast Real-Time PCR Instrument.*—Life Technologies Part No. 4445787 (with laptop PC) or 4445785 (with desktop PC).

(c) *Incubators.*—For maintaining bacterial enrichment media at 37  $\pm$  1°C and 41  $\pm$  1°C.

(d) *Heating block.*—For sample preparation/lysis of captured bacteria at 97  $\pm$  2°C prior to real-time PCR analysis.

(e) *Food homogenizer (Stomacher or equivalent).*—For mixing food samples with culture media.

(f) *Freezer.*—For storing elution plates at –18 to –20°C.

(g) *Benchtop microcentrifuge.*—For sample concentration and sample preparation. Capable of centrifuging 1.5 mL microcentrifuge tubes at 14000–16000  $\times$  g.

(h) *Vortex.*—For sample mixing.

(i) *Pipettors.*—For transferring reagents. Set of adjustable pipets that cover volumes from 0.5 to 1000  $\mu$ L.

#### *Reference Materials*

*American Type Culture Collection (ATCC).*—Website: www.atcc.org.

#### *Standard Solutions*

(a) *Buffered peptone water (BPW).*—Mix 10 g peptone,

Result icon†	Result	Action
	Positive result	Pooled samples: proceed to "Retesting of individual samples after a positive pooled sample" on page 22. Individual samples: proceed to "Interpretation of positive results by plating and biochemical assays" on page 22.
	Negative result	Proceed to "Test result interpretation and classification" on page 23.
	Result warning	Proceed to "Result warnings and recommendations for retesting" on page 23.

† RapidFinder™ Express displays results pictorially.

Figure 1. Results from RapidFinder Express software.

5 g sodium chloride, 3.5 g disodium phosphate, and 1.5 g monosodium phosphate, with 1 L deionized water. Autoclave at 121°C for 15 min. Final pH 7.2 ± 0.2.

(b) *Ultra-high temperature milk (UHTM) or nonfat dry milk (NFDM)*.—Reconstituted (for chocolate or cocoa-based samples). Mix 100 g milk powder with 1 L distilled water. Swirl until dissolved. Autoclave 15 min at 121°C.

(c) *Brilliant Green (BG) dye solution*.—Dissolve 1 g dye in sterile water. Dilute to 100 mL. Before use, test all batches of dye for toxicity with known positive and negative test microorganisms.

### Safety Precautions

(a) *MicroSEQ Salmonella spp. Detection Kit (pouch)*.—The reagents used in the MicroSEQ *Salmonella* spp. Detection Kit pouch part pose no hazards when used as directed. Some wastes produced by operation of the system are potentially hazardous. To minimize the hazards of chemical waste, read and understand the Material Safety Data Sheet (MSDS), minimize contact with chemicals, and wear appropriate protective equipment when handling chemicals, for example, safety glasses, gloves, or protective clothing.

(b) *Pathogen Detection Negative Control (part of the MicroSEQ Salmonella spp. Detection Kit)*.—The negative control contains sodium azide at a concentration that is considered not hazardous according to U.S. Occupational Safety and Health Administration regulations. Sodium azide may react with lead and copper plumbing to form highly explosive metal azides.

(c) *Pathatrix 10-Pooling Salmonella spp. Kit*.—The reagents used in the Pathatrix 10-Pooling *Salmonella* spp. Kit pose no hazards when used as directed. Some wastes produced by operation of the system are potentially hazardous. To minimize the hazards of chemical waste, read and understand the MSDS, minimize contact with chemicals, and wear appropriate protective equipment when handling chemicals, for example, safety glasses, gloves, or protective clothing.

(d) *AB 7500 Fast Real-Time PCR System*.—Use this instrument as specified by Applied Biosystems. Using this instrument in a manner not specified by Applied Biosystems may result in personal injury or damage to the instrument. Moving parts can crush and cut. Keep hands clear of moving parts while operating the system. Disconnect power before servicing the instrument. Wear appropriate safety glasses, clothing, and gloves at all times during operation.

(e) *Enrichment*.—Biological samples such as enrichments have the potential to transmit infectious diseases. Follow all applicable local, state/provincial, and/or national regulations on disposal of biological wastes. Wear appropriate protective

equipment, which includes but is not limited to protective eyewear, face shield, clothing/lab coat, and gloves. All work should be conducted in properly equipped facilities utilizing the appropriate safety equipment (for example, physical containment devices). Individuals should be trained in accordance with applicable regulatory and company/institution requirements before working with potentially infectious materials. All enrichment broths should be autoclaved following any culture-based confirmatory steps.

### Method Overview

The high-level overview of the MicroSEQ *Salmonella* spp. Detection Kit linked with the Pathatrix 10-Pooling *Salmonella* spp. Kit consists of seven steps:

*Step 1*.—Enrich individual samples (sample matrix in enrichment media) at 37 ± 1°C for 20 ± 2 h as indicated in the directions for use depending on the sample type. Food samples, environmental contact swabs, and environmental contact sponges are supported. Alternative enrichment methods exist for chocolate/cocoa-based, dairy, and acidic/alkaline samples and should be prepared as indicated in the directions for use.

*Step 2*.—Pool aliquots (5 mL each) of the individual enriched media (10 individual samples pooled in this workflow) for a total pooled sample of 50 mL.

*Step 3*.—Load the pooled sample and reagents for the Pathatrix Auto (up to five runs simultaneously on one instrument) and start the run.

*Step 4*.—Automated Pathatrix Auto run concentrates the bacteria from the media (15 min).

*Step 5*.—DNA preparation (a brief heated lysis step performed off the instrument).

*Step 6*.—Real-Time PCR analysis via the MicroSEQ *Salmonella* spp. Detection Kit and analysis of results via the RapidFinder Express software. If the pooled sample is negative, all 10 individual samples are negative.

*Step 7*.—As needed, positive pooled samples must be deconvoluted by performing the Pathatrix Auto run and MicroSEQ *Salmonella* spp. assay run on all individual enrichments comprising the positive pooled sample(s). Retest individual samples by re-warming the individual samples to 37 ± 1°C and performing the Pathatrix Auto run on 10 mL of the enriched sample according to the instructions for use.

### Interpretation of Results

RapidFinder Express software automatically generates results from the Real-Time PCR run as shown in Figure 1.

### Confirmation

Samples identified as PCR-positive may be confirmed by plating retained unlysed Pathatrix beads onto selective agar plates, followed by biochemical and serological methods [International Organization for Standardization (ISO) 6579:2002 or FDA/BAM Chapter 5]. If individual samples are used for confirmation, using pooled samples is optional. If PCR is not used to identify *Salmonella*-positive samples, the beads from pooled samples or individual samples can be plated directly onto selective agar plates [Pub. No. MAN0006975 (10-pooled sample) or MAN0006974 (individual samples)] and confirmed

Table 1. Matrix summary table—Pathatrix/MicroSEQ method versus reference method

Matrix	Time point	Strain	MPN <sup>a</sup> /test portion	N <sup>b</sup>	Pathatrix/MicroSEQ method <sup>c</sup>				Reference method			
					x <sup>d</sup>	POD <sub>C</sub> <sup>e</sup>	95% CI <sup>f</sup>	x	POD <sub>R</sub> <sup>g</sup>	95% CI	dPOD <sub>C</sub> <sup>h</sup>	95% CI
Diced tomatoes vs FDA/BAM Chapter 5	18 h primary enrichment	<i>Salmonella enterica</i> subsp. Typhimurium ATCC 14028	0.00 (0.00, 0.18)	15	—	NA <sup>i</sup>	NA	0	0.00	0.00, 0.20	NA	NA
			0.75 (0.44, 1.20)	20	9	0.45	0.26, 0.66	9	0.45	0.26, 0.66	0.00	-0.28, 0.28
			3.00 (1.30, 6.90)	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	-0.43, 0.43
Chocolate enriched with NEDM vs FDA/BAM Chapter 5	18 h primary enrichment	<i>Salmonella enterica</i> subsp. Montevideo QL024-19	0.00 (0.00, 0.18)	15	NA	NA	NA	0	0.00	0.00, 0.20	NA	NA
			0.90 (0.54, 1.50)	20	14	0.70	0.48, 0.85	14	0.70	0.48, 0.85	0.00	-0.27, 0.27
			4.40 (1.70, 11.0)	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	-0.43, 0.43
Chocolate enriched with UHTM vs FDA/BAM Chapter 5	18 h primary enrichment	<i>Salmonella enterica</i> subsp. Montevideo QL024-19	0.00 (0.00, 0.18)	15	NA	NA	NA	0	0.00	0.00, 0.20	NA	NA
			0.90 (0.54, 1.50)	20	13	0.65	0.43, 0.82	14	0.70	0.48, 0.85	-0.05	-0.32, 0.23
			4.40 (1.70, 11.0)	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	-0.43, 0.43
Deli ham vs USDA/FSIS-MLG 4.05	18 h primary enrichment	<i>Salmonella enterica</i> subsp. Enteritidis ATCC 13076	0.00 (0.00, 0.18)	15	NA	NA	NA	0	0.00	0.00, 0.20	NA	NA
			0.43 (0.21, 0.75)	20	8	0.40	0.22, 0.61	8	0.40	0.22, 0.61	0.00	-0.28, 0.28
			2.30 (1.04, 5.02)	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	-0.43, 0.43

<sup>a</sup> MPN = MPN was calculated for each matrix using five 50 g, five 10 g, and the 20 reference method samples and the Least Cost Formulations MPN calculator.

<sup>b</sup> N = Number of test portions.

<sup>c</sup> Test method = Test method results represent both individual and 10-Pooling sample sets.

<sup>d</sup> x = Number of positive test portions.

<sup>e</sup> POD<sub>C</sub> = Candidate method confirmed positive outcomes divided by the total number of trials.

<sup>f</sup> 95% CI = If the confidence interval of a dPOD does not contain zero, the difference is statistically significant at the 5% level, calculated according to the current AOAC guidelines and using the Least Cost Formulations AOAC Binary Workbook.

<sup>g</sup> POD<sub>R</sub> = Reference method confirmed positive outcomes divided by the total number of trials.

<sup>h</sup> dPOD = Difference between the candidate method confirmed result and reference method confirmed result POD values.

<sup>i</sup> N/A = Not applicable.

by biochemical and serological methods (ISO 6579:2002 or FDA/BAM Chapter 5). See instructions for use for further details.

Once confirmed, the results are reported as:

*Salmonella* spp. Detected in 25–325 g (sample matrixes).

*Salmonella* spp. Not detected in 25–325 g (sample matrixes).

### Matrix Study

*Note:* The matrix study was conducted by Q Laboratories, Inc. on behalf of Life Technologies.

For each matrix, the study included 20 replicate test portions analyzed at the low inoculation level of 0.2–2 CFU/25 g (325 g for deli ham), five replicate test portions analyzed at the high inoculation level of 2–5 CFU/25 g (325 g for deli ham), and 15 uninoculated replicate test portions used to dilute samples for the 10-Pooling protocol. For the candidate method, individual and pooled test portions were analyzed. Most probable number (MPN) analysis was initiated on the same day as the samples.

Diced tomatoes (25 g) were inoculated with *S. enterica* subsp. Typhimurium (ATCC 14028) and enriched in BPW according to the reference method (1). Chocolate (25 g) samples were inoculated with *S. enterica* subsp. Montevideo (QL024-19) and enriched in NFDM (paired) and UHTM (unpaired) according to the reference method (1). Deli ham (325 g) was inoculated with *S. enterica* subsp. Enteritidis (ATCC 13076) and enriched in BPW according to the reference method (2).

### Sample Processing

For the Pathatrix and MicroSEQ linked protocol, an aliquot of each test portion for all three matrixes was processed using the Pathatrix instrument after 18 h of incubation. Test portions were re-incubated for an additional 6 h and confirmed according to the appropriate reference method as previously described. Test portions were processed on the Pathatrix instrument both individually and using the 10-Pooling protocol. Samples were processed individually by removing 50 mL aliquots from the primary enrichments and transferring into the Pathatrix sample vessel. For the 10-Pooling protocol, 5 mL of the primary enrichment was combined with  $9 \times 5$  mL of primary enrichment from uninoculated control samples in the sample vessel. Next, 50  $\mu$ L of the Pathatrix bead suspension was added to the spout of the lid of the sample vessel and 35 mL of phosphate buffered saline (PBS) was added to the elution vessel.

The Capture Phase Pack plastic kit was oriented over both the sample vessel and the elution vessel and firmly connected to both vessel valves. The vessels, with the plastic kit now attached, were inserted into the Pathatrix cartridge making sure that the magnetic sliders were engaged in the locked position. The cartridge was placed inside the Pathatrix apparatus, and the assay was initiated. At the end of the assay, the cartridge was removed from the Pathatrix apparatus, and the sample and elution tubes were removed from the Pathatrix cartridge. The elution vessel was placed into the tube rack for approximately 1 min to allow for the capture of the Pathatrix beads. The remaining liquid in the elution tube was removed from the tube and discarded. The elution tube was removed from the tube rack, and 100  $\mu$ L of PBS was used to resuspend the bead solution. An additional wash of the bead

solution was conducted to produce a clearer bead solution to prevent lysis and PCR inhibition.

### DNA Preparation

Following the Pathatrix procedure, 10  $\mu$ L of 1x lysis buffer was placed into 1.5 mL centrifuge tubes. The elution tubes were returned to the tube rack, and the PBS was removed from the tubes and discarded. The elution tubes were removed from the tube rack, and the Pathatrix beads were resuspended with 120  $\mu$ L of sterile Molecular Biology Grade water. For each sample, 90  $\mu$ L of this suspension was transferred into a 1.5 mL centrifuge tube containing 10  $\mu$ L of the 1x lysis buffer. The centrifuge tubes were placed into a 97°C heating block for 10 min, then removed from the heat block and allowed to cool at room temperature for approximately 1 min. The centrifuge tubes were placed into a magnetic tube rack and allowed to sit for approximately 2 min to capture the beads from the lysed suspension.

### Real-Time PCR Analysis

The MicroSEQ *Salmonella* spp Detection Kit is arranged in strip tubes of eight tubes per strip and stored frozen. The appropriate number of tubes were removed from the pouch and 30  $\mu$ L of the bead-free supernatant from the DNA preparation step was then transferred into the MicroSEQ tubes containing lyophilized PCR reagents. The MicroSEQ assay run was initiated on the AB 7500 Fast Real-Time PCR instrument, and results were obtained within 40 min.

## Results

### Diced Tomatoes

For the low inoculation level of the diced tomatoes, there were nine presumptive positives for both the individual and 10-Pooling samples. There were nine confirmed positives following the FDA/BAM reference method. For the high inoculation level, there were five presumptive positives for both the individual and 10-Pooling samples. There were five confirmed positives following the FDA/BAM reference method. All 15 uninoculated control samples, used for diluting inoculated test portions assayed by the MicroSEQ *Salmonella* test kit, were confirmed negative following the FDA/BAM reference method. For both individual and 10-Pooling samples of the low inoculation level, a dPOD<sub>C</sub> value of 0.00 was obtained with 95% confidence intervals of –0.28 and 0.28, indicating no significant difference between candidate and reference methods. For both individual and 10-Pooling samples of the high inoculation level, a dPOD<sub>C</sub> value of 0.00 was obtained with 95% confidence intervals of –0.43 and 0.43, indicating no significant difference between candidate and reference methods. Sample results are presented in Table 1.

### Chocolate-NFDM (Paired)

For the low inoculation level of the chocolate enriched in NFDM, there were 14 presumptive positives for both the individual and 10-Pooling samples. There were 14 confirmed positives following the FDA/BAM reference method. For the high inoculation level, there were five presumptive positives for both the individual and 10-Pooling samples. There were five

**Table 2. Chocolate ultra-high temperature milk (unpaired) summary table—Pathatrix/MicroSEQ Method presumptive vs. confirmed**

Matrix	Time point	Strain	MPN <sup>a</sup> /test portion	N <sup>b</sup>	Pathatrix/MicroSEQ method presumptive <sup>c</sup>			Pathatrix/MicroSEQ method confirmed				
					x <sup>d</sup>	POD <sub>c</sub> <sup>e</sup>	95% CI <sup>f</sup>	x	POD <sub>R</sub> <sup>g</sup>	95% CI	dPOD <sub>C</sub> <sup>h</sup>	95% CI
Chocolate enriched with UHTM presumptive vs confirmed	18 h primary enrichment	<i>Salmonella enterica</i> subsp. Montevideo QL024-19	0.00 (0.00, 0.18)	15	NA <sup>i</sup>	NA	NA	0	0.00	0.00, 0.20	NA	NA
			0.90 (0.54, 1.50)	20	13	0.65	0.43, 0.82	13	0.65	0.43, 0.82	0.00	-0.21, 0.21
			4.40 (1.70, 11.0)	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	-0.45, 0.45

<sup>a</sup> MPN = MPN was calculated for each matrix using five 50 g, five 10 g, and the 20 reference method samples and the Least Cost Formulations MPN calculator.

<sup>b</sup> N = Number of test portions.

<sup>c</sup> Candidate Method Presumptive = Candidate method presumptive results represent both Individual and 10-Pooling sample sets.

<sup>d</sup> x = Number of positive test portions.

<sup>e</sup> POD<sub>C</sub> = Candidate method confirmed positive outcomes divided by the total number of trials.

<sup>f</sup> 95% CI = If the confidence interval of a dPOD does not contain zero, the difference is statistically significant at the 5% level, calculated according to the current AOAC guidelines and using the Least Cost Formulations AOAC Binary Workbook.

<sup>g</sup> POD<sub>R</sub> = Reference method confirmed positive outcomes divided by the total number of trials.

<sup>h</sup> dPOD = Difference between the candidate method confirmed result and reference method confirmed result POD values.

<sup>i</sup> NA = Not applicable.

confirmed positives following the FDA/BAM reference method. All 15 uninoculated control samples, used for diluting inoculated test portions assayed by the MicroSEQ *Salmonella* test kit, were confirmed negative following the FDA/BAM reference method. For both individual and 10-Pooling samples of the low inoculation level, a dPOD<sub>C</sub> value of 0.00 was obtained with 95% confidence intervals of -0.27 and 0.27, indicating no significant difference between candidate and reference methods. For both individual and 10-Pooling samples of the high inoculation level, a dPOD<sub>C</sub> value of 0.00 was obtained with 95% confidence intervals of -0.43 and 0.43, indicating no significant difference between candidate and reference methods. Sample results are presented in Table 1.

#### *Chocolate-UHTM (Unpaired)*

For the low inoculation level of the chocolate enriched in UHTM, there were 13 presumptive and candidate confirmed positives for both the individual and 10-Pooling samples. For the high inoculation level, there were five presumptive- and candidate-confirmed positives for both the individual and 10-Pooling samples. All 15 uninoculated control samples, used for diluting inoculated test portions assayed by the MicroSEQ *Salmonella* test kit, were confirmed negative following the FDA/BAM reference method. For both the individual and 10-Pooling samples of the low inoculation level, a dPOD<sub>C</sub> value of 0.00 was obtained with 95% confidence intervals of -0.21 and 0.21, indicating no significant difference between candidate-presumptive and candidate-confirmed results. For both individual and 10-Pooling samples of the high inoculation level, a dPOD<sub>C</sub> value of 0.00 was obtained with 95% confidence intervals of -0.45 and 0.45, indicating no significant difference between candidate-presumptive and candidate-confirmed results. Sample results are presented in Table 2.

For both individual and 10-Pooling samples of the low inoculation level, a dPOD<sub>C</sub> value of -0.05 was obtained with 95% confidence intervals of -0.32 and 0.23, indicating no significant difference between candidate-confirmed and reference methods. For both individual and 10-Pooling samples of the high inoculation level, a dPOD<sub>C</sub> value of 0.00 was obtained with 95% confidence intervals of -0.43 and 0.43, indicating no significant difference between candidate-confirmed and reference methods. Sample results are presented in Table 1.

#### *Deli Ham*

For the low inoculation level of the deli ham, there were eight presumptive positives for both the individual and 10-Pooling samples. There were eight confirmed positives following the USDA/MLG reference method. For the high inoculation level, there were five presumptive positives for both the individual and 10-Pooling samples. There were five confirmed positives following the USDA/MLG reference method. All 15 uninoculated control samples, used for diluting inoculated test portions assayed by the MicroSEQ *Salmonella* test kit, were confirmed negative following the USDA/FSIS-MLG reference method. For both individual and 10-Pooling samples of the low inoculation level, a dPOD<sub>C</sub> value of 0.00 was obtained with 95% confidence intervals of -0.28 and 0.28, indicating no significant difference between candidate and reference methods. For both individual and 10-Pooling samples of the high inoculation level, a dPOD<sub>C</sub> value

of 0.00 was obtained with 95% confidence intervals of -0.43 and 0.43, indicating no significant difference between candidate and reference methods. Sample results are presented in Table 1.

### **Confirmation**

#### *FDA/BAM Method*

The diced tomatoes and chocolate were analyzed according to procedures outlined in the FDA/BAM reference method. The primary enrichment procedure of the reference method was modified by prewarming the enrichment media at 37°C. A 25 ± 1 g amount of diced tomatoes was enriched with 225 mL of pre-warmed (37°C) BPW and homogenized by a Stomacher at high speed for 2 min. The mixture was aseptically transferred into a sterile wide-mouth jar and kept at room temperature for 60 ± 5 min with the jar securely capped. Subsequently, the enrichments were incubated at 35 ± 2°C for 24 ± 2 h.

The chocolate was evaluated using two different enrichment schemes. Test portions consisting of 25 ± 0.5 g were enriched with 225 ± 22.5 mL of prewarmed (37°C), reconstituted NFDM, homogenized, and blended for approximately 2 min. The enrichment was aseptically transferred into a 500 mL wide-mouth bottle and held at room temperature for 60 ± 5 min with the jar securely capped. If necessary, the pH of the enrichment was adjusted to 6.8 ± 0.2. Next, 0.45 mL of a 1% aqueous BG dye solution was added to the enrichment. The enrichment was incubated as described above. Additionally, a set of 25 ± 0.5 g test portions was enriched in 225 ± 22.5 mL of prewarmed (37°C) UHTM, homogenized, and blended for approximately 2 min. The enrichment was aseptically transferred into a 500 mL wide-mouth bottle and held at room temperature for 60 ± 5 min with the jar securely capped. If necessary, the pH of the enrichment was adjusted to 6.8 ± 0.2. Next, 0.45 mL of a 1% aqueous BG dye solution was added to the enrichment, and the enrichment was incubated as described above. Following incubation, a 0.1 mL aliquot of primary enrichment was transferred into 10 mL of Rappaport-Vassiliadis (RV) medium and a 1.0 mL aliquot was transferred into 10 mL of Tetrathionate broth (TT). RV tubes were incubated at 42 ± 0.2°C for 24 ± 2 h; TT tubes were incubated at 35 ± 2°C for 24 ± 2 h. Following incubation, a loopful of each secondary enrichment was streaked to Bismuth Sulfite (BS) agar, Hektoen Enteric agar, and Xylose Lysine Desoxycholate agar, and incubated at 35 ± 2°C for 24 ± 2 h. If no visible colonies were present after 24 h of incubation, BS plates were reincubated for an additional 24 ± 2 h at 35 ± 2°C. Two suspect colonies from each selective agar were transferred to Triple Sugar Iron (TSI) agar and Lysine Iron Agar (LIA) and incubated at 35 ± 2°C for 24 ± 2 h. The growth from samples producing typical biochemical reactions in TSI was transferred to Brain Heart Infusion (BHI) broth and incubated at 35 ± 2°C for 5 ± 1 h. Growth from the BHI was used to conduct the flagellar H serological test. Growth from samples producing typical biochemical reactions in TSI and LIA, were streaked to Trypticase Soy Agar (TSA) slants and incubated at 35 ± 2°C for 18–24 h. Growth from the TSA slant was used to conduct the polyvalent O serological test for biochemical

confirmation and final confirmations using VITEK 2 GN according to AOAC Official Method **2011.17**.

#### USDA/FSIS-MLG 4.05 Method

The deli ham was analyzed according to the USDA/FSIS-MLG 4.05 reference method. Each test portion, consisting of  $325 \pm 6.5$  g was enriched with  $2925 \pm 58.5$  mL of prewarmed ( $37^\circ\text{C}$ ) BPW and incubated at  $35 \pm 2^\circ\text{C}$  for 18–24 h. After incubation,  $0.1 \pm 0.02$  mL of each sample was transferred to 10 mL modified RV broth, and  $0.5 \pm 0.05$  mL was transferred to 10 mL TT broth. The broths were incubated in a water bath at  $42 \pm 0.5^\circ\text{C}$  for 18–24 h. Following incubation, a loopful of each broth for each sample replicate was streaked to XL-Tergitol 4 and BG Sulfa Agar. Both selective agars were incubated at  $35 \pm 2^\circ\text{C}$  for 18–24 h. Presumptive positive *Salmonella* colonies from each selective agar were picked and transferred to TSI and LIA slants and incubated at  $35 \pm 2^\circ\text{C}$  for  $24 \pm 2$  h. Growth from samples producing typical biochemical reactions in TSI was transferred to BHI broth and incubated at  $35 \pm 2^\circ\text{C}$  for  $24 \pm 2$  h. Growth from the BHI was used to conduct the flagellar H serological test. Growth from samples producing typical biochemical reactions in TSI and LIA, were streaked to TSA slants and incubated at  $35 \pm 2^\circ\text{C}$  for 18–24 h. Growth from the TSA slant was used to conduct the polyvalent O serological test for biochemical confirmation and final confirmations using VITEK 2 GN according to AOAC Official Method **2011.17**.

#### Discussion

For both the low and high inoculation levels of each matrix, the POD was calculated as the number of positive outcomes divided by the total number of trials. POD analyses were conducted for both the individual and 10-Pooling sample sets. The POD was calculated for the  $\text{POD}_C$ , the  $\text{POD}_R$ , and the difference in the candidate presumptive and reference confirmatory results,  $\text{dPOD}_C$ . The POD values and 95% confidence intervals were calculated according to the current AOAC guidelines and using the Least Cost Formulations, Ltd, AOAC Binary Data Interlaboratory Study Workbook (4).

For the method comparison, the POD analyses between the candidate and the reference methods for all three matrices indicated no statistically significant difference between the number of positive results obtained by the two methods.

Fractionally positive results (5–15 positives out of 20 replicates) were obtained for each of the matrixes analyzed in this study. MPN results for each matrix were determined using the Least Cost Formulations MPN Calculator (5), and are presented along with an overall summary of results for each matrix in Tables 1 and 2.

#### Conclusions

The results of this study demonstrate the reliability and accuracy of the MicroSEQ *Salmonella* spp. detection assay when linked with the Pathatrix individual or 10-pooling protocols. The Pathatrix method offers a significant savings in time by processing multiple sample enrichments at once. Furthermore, the beads can be processed through the linking protocol to produce accurate presumptive results using the MicroSEQ *Salmonella* spp. Detection Kit. The MicroSEQ *Salmonella* spp. protocol linked with the Pathatrix individual or 10-pooling protocols demonstrated reliability as a rapid, simplified method for the detection of *Salmonella* in diced tomatoes, chocolate, and deli ham.

#### References

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