BAX® System Real-Time PCR Assay for *Salmonella*: Detecting *Salmonella* from Difficult Food and Environmental Samples Using Actero™ *Salmonella* Enrichment Media

Hygiena™ has collaborated with FoodChek™ Systems Inc. to develop a new combined testing method for detecting *Salmonella* from food and environmental samples. This protocol includes a single-stage enrichment in FoodChek's proprietary Actero™ *Salmonella* Enrichment Media followed by automated processing with the BAX® System real-time PCR assay.

This new protocol for the BAX® System Real-Time PCR Assay for *Salmonella* was evaluated in a series of validation-level studies by FoodChek™ Laboratories on dry pet food, milk chocolate and chocolate liquor. Stainless steel and plastic surfaces were also tested in the presence of high levels of competing background flora. Independent laboratory validation studies were performed for dry pet food and stainless-steel samples. The results of these studies have been submitted to the AOAC Research Institute (AOAC-RI) for validation as a *Performance Testing Method*℠.

The results of these studies demonstrate that the performance of the BAX® System method using Actero™ *Salmonella* Enrichment Media is statistically equivalent or superior to the reference culture methods for detecting *Salmonella* in the evaluated sample types.

Equipment, Reagents and Supplies

- BAX® System Real-Time PCR Assay for *Salmonella* (KIT2006)
- BAX® System Q7 instrument, equipment and supplies
- Actero™ *Salmonella* Enrichment Media (FoodChek™ MediaBox #MED2024 or equivalent)
- Lactose Broth
- 10% non-fat skim milk
- Phosphate Buffered Saline (PBS)
- Tetrathionate (TT) Broth
- Rappaport Vassiliadis (RV) Broth
- Selective agar plates

Sample Preparation and Enrichment

The strains of *Salmonella* were selected from FoodChek Laboratories Inc. Collection to artificially contaminate food matrices and environmental surfaces. *Citrobacter freundii* was selected for inoculation of stainless steel surfaces to represent competing flora (see Table 1).

TABLE 1. BACTERIAL STRAINS SELECTED FOR ARTIFICIAL INOCULATION

SAMPLE TYPE	INOCULATING ORGANISM	STRAIN ID	STRAIN SOURCE
Dry pet food	*S. Anatum E1*	MSR0070	Chicken feed
Milk chocolate	*S. Senftenberg E4*	MSR0112	ATCC 8400
Chocolate liquor	*S. Virchow C1*	MSR0048	N/A
Stainless steel	*S. Braenderup C1* *C. freundii*	MSR0027 MSR0325	Environment ATCC 8090
Plastic	*S. Oranienburg C1*	MSR0030	Environment

Note: The same Salmonella strains were used for internal and independent laboratory validation studies

For dry pet food, lyophilized *S. Anatum* was used to inoculate samples. For milk chocolate and chocolate liquor, a pure culture of each *Salmonella* strain was grown overnight in TSB at 35°C then diluted in 40% glycerol to levels expected to produce fractional positive results. After inoculation, all food samples were held at room temperature for at least two weeks prior to testing.

For environmental surfaces, a pure culture of each *Salmonella* strain was grown overnight in TSB at 35°C then diluted in 10% non-fat dry milk to levels expected to produce fractional positive results. *C. freundii* was diluted to a level approximately 10-fold higher than the *Salmonella* strain dilution for inoculating stainless steel samples. Decontaminated surfaces of 100 cm2 were spread evenly with 250 µL of the appropriate inoculation strain(s) and allowed to dry at room temperature for 18-20 hours. Each surface was then swabbed with a sponge pre-moistened with 10 mL D/E neutralizing broth and held at room temperature for at least 2 hours before testing.

For the BAX® System method, samples were enriched according to the protocols described in Table 2. For the FDA-BAM reference method, samples were blended for 2 minutes in 225 mL of lactose broth (dry pet food), 10% non-fat skim milk (milk chocolate and chocolate liquor) or lactose broth (environmental sponges). All the samples were allowed to stand for 60 minutes at room temperature, then pH was adjusted to 6.8±0.2 if necessary. For milk chocolate and chocolate liquor, 0.45 mL 1% aqueous brilliant green dye solution was added to each sample. All the samples were incubated at 35°C for 22-26 hours before processing with the reference method as described below.

TABLE 2. BAX® SYSTEM ENRICHMENT PROTOCOLS				
SAMPLE TYPE	SAMPLE SIZE	ACTERO™ MEDIA VOLUME	INCUBATION TEMPERATURE	INCUBATION TIME
Dry pet food	25 g	225 mL	35°C	18-22 hours
Dry pet food	375 g	2625 mL	35°C	18-22 hours
Milk chocolate	25 g	175 mL	35°C	22-26 hours
Chocolate liquor	25 g	225 mL	35°C	26-30 hours
Stainless steel	sponge	90 mL	35°C	14-18 hours
Plastic	sponge	90 mL	35°C	14-18 hours

Test Methods

For the BAX® System method, 40 µL enrichment (for food samples) or 80 µL enrichment (for environmental samples) was transferred to 2 mL of PBS and mixed by pipetting. BAX® System lysis reagent was prepared by adding 150 µL protease to 12 mL of lysis buffer. For each sample, 5 µL sample diluted with PBS was added to 200 µL prepared lysis reagent in cluster tubes. Tubes were heated for 20 minutes at 37°C and 10 minutes at 95°C, and then cooled for at least 5 minutes at 2-8°C. PCR tablets were hydrated with 30 µL lysate and cooled for 10-30 minutes before loading into the BAX® System Q7 instrument. All results obtained with the BAX® System method were confirmed using the appropriate reference method.

For the FDA-BAM reference method, 1.0 mL of each primary enrichment was transferred into 10 mL TT broth and incubated at 35°C in a circulating, thermostatically controlled water bath for 24 hours. An additional 0.1 mL primary enrichment was transferred into 10 mL RV broth and incubated at 42°C in a circulating, thermostatically controlled water bath for 24 hours. All secondary enrichments were streaked onto selective agar plates, and typical *Salmonella* colonies were confirmed using the appropriate biochemical and serological procedures.

Results and Discussion

Probability of detection (POD) statistical model was used to evaluate the differences between presumptive and confirmed results as well as between the alternative and the reference methods. All the data are presented in summary tables of POD values, dPOD values, and confidence intervals by matrix and concentration.

The results for food and environmental samples tested are summarized in Tables 3-6. For dry pet food, milk chocolate, stainless steel and plastic samples, POD analysis showed no statistically significant differences between the performance of the BAX® System method and the reference methods (the 95% confidence interval of the dPODs contains zero). For chocolate liquor samples, POD analysis showed a statistically superior performance for the BAX® System method compared to the appropriate reference method.

TABLE 3. BAX® SYSTEM METHOD PRESUMPTIVE VS CONFIRMED RESULTS (FOOD SAMPLES)

SAMPLE TYPE	MPN, CFU/SAMPLE	N	X	PRESUMPTIVE		X	CONFIRMED		dPOD$_{CP}$	95%CI
				POD$_{CP}$	95%CI		POD$_{CC}$	95%CI		
Dry pet food (25 g)	<0.075	5	0	0.00	(0.00; 0.43)	0	0.00	(0.00; 0.43)	0.00	(-0.43; 0.43)
	1.1	20	13	0.65	(0.43; 0.82)	13	0.65	(0.43; 0.82)	0.00	(-0.28; 0.28)
	3.7	5	5	1.00	(0.57; 1.00)	5	1.00	(0.57; 1.00)	0.00	(-0.43; 0.43)
Dry pet food (375 g) Ind. Lab	<0.075	5	0	0.00	(0.00; 0.43)	0	0.00	(0.00; 0.43)	0.00	(-0.43; 0.43)
	1.5	20	14	0.70	(0.48; 0.86)	14	0.70	(0.48; 0.86)	0.00	(-0.28; 0.28)
	8.7	5	5	1.00	(0.57; 1.00)	5	1.00	(0.57; 1.00)	0.00	(-0.43; 0.43)
Milk chocolate	<0.075	5	0	0.00	(0.00; 0.43)	0	0.00	(0.00; 0.43)	0.00	(-0.43; 0.43)
	0.5	20	10	0.50	(0.30; 0.70)	10	0.50	(0.30; 0.70)	0.00	(-0.28; 0.28)
	8.7	5	5	1.00	(0.57; 1.00)	5	1.00	(0.57; 1.00)	0.00	(-0.43; 0.43)
Chocolate liquor	<0.075	5	0	0.00	(0.00; 0.43)	0	0.00	(0.00; 0.43)	0.00	(-0.43; 0.43)
	0.1	20	9	0.45	(0.26; 0.67)	9	0.45	(0.26; 0.67)	0.00	(-0.28; 0.28)
	2.7	5	5	1.00	(0.57; 1.00)	5	1.00	(0.57; 1.00)	0.00	(-0.43; 0.43)

TABLE 4. BAX® SYSTEM METHOD VS REFERENCE METHOD RESULTS (FOOD SAMPLES)

SAMPLE TYPE	MPN, CFU/SAMPLE	N	X	BAX® SYSTEM METHOD		X	FDA-BAM METHOD		dPOD$_{CP}$	95%CI
				POD$_{CP}$	95%CI		POD$_{CC}$	95%CI		
Dry pet food (25 g)	<0.075	5	0	0.00	(0.00; 0.43)	0	0.00	(0.00; 0.43)	0.00	(-0.43; 0.43)
	1.1	20	13	0.65	(0.43; 0.82)	16	0.80	(0.58; 0.92)	-0.15	(-0.28; 0.28)
	3.7	5	5	1.00	(0.57; 1.00)	5	1.00	(0.57; 1.00)	0.00	(-0.43; 0.43)
Dry pet food (375 g) Ind. Lab	<0.075	5	0	0.00	(0.00; 0.43)	0	0.00	(0.00; 0.43)	0.00	(-0.43; 0.43)
	1.5	20	14	0.70	(0.48; 0.86)	15	0.75	(0.53; 0.89)	-0.05	(-0.28; 0.28)
	8.7	5	5	1.00	(0.57; 1.00)	5	1.00	(0.57; 1.00)	0.00	(-0.43; 0.43)
Milk chocolate	<0.075	5	0	0.00	(0.00; 0.43)	0	0.00	(0.00; 0.43)	0.00	(-0.43; 0.43)
	0.5	20	10	0.50	(0.30; 0.70)	9	0.45	(0.26; 0.66)	0.05	(-0.28; 0.28)
	8.7	5	5	1.00	(0.57; 1.00)	5	1.00	(0.57; 1.00)	0.00	(-0.43; 0.43)
Chocolate liquor	<0.075	5	0	0.00	(0.00; 0.43)	0	0.00	(0.00; 0.43)	0.00	(-0.43; 0.43)
	0.1	20	9	0.45	(0.26; 0.67)	2	0.10	(0.03; 0.30)	0.35	(-0.28; 0.28)
	2.7	5	5	1.00	(0.57; 1.00)	5	1.00	(0.57; 1.00)	0.00	(-0.43; 0.43)

TABLE 6. BAX® SYSTEM METHOD VS REFERENCE METHOD RESULTS (ENVIRONMENTAL SAMPLES)

SAMPLE TYPE	CFU / 100 CM^2	N	X	BAX® SYSTEM METHOD		X	FDA-BAM METHOD		dPOD$_C$	95%CI
				POD$_C$	95%CI		POD$_R$	95%CI		
Stainless steel (14 hours)	0.0	5	0	0.00	(0.00; 0.43)	0	0.00	(0.00; 0.43)	0.00	(-0.43; 0.43)
	50.0	20	12	0.60	(0.39; 0.78)	12	0.60	(0.39; 0.78)	0.00	(-0.28; 0.28)
	437.0	5	5	1.00	(0.57; 1.00)	5	1.00	(0.57; 1.00)	0.00	(-0.43; 0.43)
Stainless steel (16 hours)	0.0	5	0	0.00	(0.00, 0.43)	0	0.00	(0.00, 0.43)	0.00	(-0.43, 0.43)
	47.0	20	10	0.50	(0.30, 0.70)	9	0.45	(0.26, 0.66)	0.05	(-0.24, 0.33)
	313.0	5	5	1.00	(0.57, 1.00)	5	1.00	(0.57, 1.00)	0.00	(-0.43, 0.43)
Stainless steel (18 hours)	0.0	5	0	0.00	(0.00; 0.43)	0	0.00	(0.00; 0.43)	0.00	(-0.43; 0.43)
	50.0	20	10	0.50	(0.30; 0.70)	9	0.45	(0.26; 0.66)	0.05	(-0.24; 0.33)
	437.0	5	5	1.00	(0.57; 1.00)	5	1.00	(0.57; 1.00)	0.00	(-0.43; 0.43)
Plastic (14 hours)	0.0	5	0	0.00	(0.00; 0.43)	0	0.00	(0.00; 0.43)	0.00	(-0.43; 0.43)
	59.4	20	10	0.50	(0.30; 0.70)	14	0.70	(0.48; 0.85)	-0.20	(-0.45; 0.10)
	400.0	5	5	1.00	(0.57; 1.00)	5	1.00	(0.57; 1.00)	0.00	(-0.43; 0.43)
Plastic (18 hours)	0.0	5	0	0.00	(0.00; 0.43)	0	0.00	(0.00; 0.43)	0.00	(-0.43; 0.43)
	53.0	20	11	0.55	(0.34; 0.74)	12	0.60	(0.39; 0.78)	-0.05	(-0.33; 0.24)
	120.0	5	5	1.00	(0.57; 1.00)	5	1.00	(0.57; 1.00)	0.00	(-0.43; 0.43)

Conclusions

The results of these studies demonstrate that the performance of the BAX® System method using Actero™ *Salmonella* Enrichment Media is statistically equivalent or superior to the reference culture methods for detecting *Salmonella* in the evaluated sample types.