STUDY REPORT

Thermo Scientific RapidFinder Salmonella species, Typhimurium, and Enteritidis PCR Kit AOAC-RI PTM Matrix Extension: Method Comparison Study

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Summary

As part of the method modification study for AOAC Research Institute (RI) Performance Tested Method℠ certification (PTM 081701), method developer and independent laboratory studies were conducted to extend the claim for use of the Thermo Scientific™ RapidFinder™ Salmonella species, Typhimurium and Enteritidis PCR Kit (candidate method) with chicken carcass rinse and shell egg matrices (lysates prepared using the Applied Biosystems™ SimpliAmp™ Thermal Cycler) and 375 g ground turkey matrices (lysates prepared using the Thermo Scientific™ KingFisher™ Flex Purification System). For testing using the KingFisher Flex instrument, the use of Applied Biosystems™ Dynabeads™ anti-*Salmonella* is required. The Dynabeads anti-*Salmonella* have been added as a kit component of the Thermo Scientific™ RapidFinder™ Salmonella Multiplex Flex PCR Kit. PCR analysis was performed using the Applied Biosystems™ 7500 Fast Food Safety Real-Time PCR Instrument and associated Applied Biosystems™ RapidFinder™ Express Software (version 2.0 or greater), and also the Applied Biosystems™ QuantStudio™ 5 Food Safety Real-Time PCR Instrument and associated Thermo Scientific RapidFinder Analysis Software (version 1.0 or greater), for the detection and differentiation of *Salmonella* species, *Salmonella* Typhimurium and *Salmonella* Enteritidis. Results showed that the candidate method showed comparable or better performance than the corresponding reference method. The following is a summary of the method comparison study.

Methodology

The performance of the candidate method was assessed as an unpaired study in comparison to the U.S. Department of Agriculture (USDA) Food Safety and Inspection Service (FSIS) Microbiology Laboratory Guidebook (MLG) 4.09 for chicken carcass rinse and ground turkey samples. The performance of the candidate method was evaluated in comparison to the U.S. Food and Drug Administration (FDA) Bacteriological Analytical Manual (BAM) Chapter 5 for shell egg samples.

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Method developer studies were conducted by Thermo Fisher Scientific on chicken carcass rinse, shell egg and 375 g ground turkey matrices. The independent laboratory study was conducted by Q Laboratories, Inc., Ohio, USA with the ground turkey matrix.

Sample Preparation

For the shell egg matrix, before use, the exterior surface of the eggs were disinfected using 1 part 70% alcohol to 1 part iodine/potassium iodide solution. To prepare the sample size of 20 eggs (approximately 4 L), the eggs were cracked aseptically into a sterile beaker and mixed thoroughly with a sterile tool.

For the chicken carcass rinse matrix, following spiking, the carcass sample were held for 48–72 hours at 2–8 °C prior to analysis to allow microorganisms to stabilize in the food environment. To perform the carcass rinse, 400 mL of collection medium were poured into the cavity of the carcass inside the bag and rinsed thoroughly assuring that all surfaces were rinsed.

For the ground turkey matrix, the appropriate number of 25 g portions for each spike level were added into a sterile sample bag for the FDA BAM Ch. 5 reference method samples. For the candidate method samples, to each 25 g portion, 350 g of unspiked ground turkey were added to give a total of 375 g. The ground turkey samples were held for 48–72 hours at 2–8 °C prior to analysis to allow microorganisms to stabilize in the food environment.

RapidFinder Salmonella species, Typhimurium, and Enteritidis Multiplex PCR Kit Method for Shell Egg and Carcass Rinse Samples

Day: 0

Shell egg samples: Add 20 eggs to 2 L of Buffered Peptone Water (BPW) (ISO) broth (CM1211B) prewarmed to 37 ±1°C

Carcass rinse samples: Add 30 mL of sample to 90 mL of Buffered Peptone Water (ISO) (CM1211B) with novobiocin (12 mg/L) prewarmed to 41.5 ±1°C

Day: 1

Add 1 mL enrichment to 10 mL BPW. This regrowth is used for confirmation testing

Remove ~1.5 mL of enrichment to a sterile sealable tube

First add 10 µL of Proteinase K to each required Lysis Tube

Second add 10 µL of enrichment to the Lysis Tube

Incubate Lysis Tubes at 37 ±1°C for 10 min followed by 95 ±1°C for 5 min

Allow lysates to cool at room temperature for at least 2 min, then transfer 20 µl to RapidFinder PCR Tubes

QuantStudio 5 PCR Instrument

Load into the QuantStudio 5 PCR Instrument and start PCR, review results at end of run (approx. 50 min)

7500 Fast PCR Instrument

Load into the 7500 Fast PCR Instrument, with the addition of a negative control tube containing nuclease free sterile water and start PCR, review results at end of run (approx. 45 min)

Confirm PCR results by culturing onto Thermo Scientific™ *Brilliance*™ Salmonella Agar and Tryptone Soya Agar (TSA)

Confirm colonies grown on the *Brilliance* Agar and TSA using serological and biochemical procedures

RapidFinder Salmonella species, Typhimurium, and Enteritidis PCR Kit Method for Ground Turkey (375 g) Samples

Day: 0

Ground turkey samples: Add 375 g of food sample to 1,125 mL of Buffered Peptone Water (ISO) (CM1211B) with 12 mg/L novobiocin prewarmed to 41.5 ±1°C

Incubate at 41.5 ±1°C for 20-24 hours

Day: 1

Remove ~1.5 mL of enrichment to a sterile sealable tube

Set up the KingFisher Flex processing plates:

- Tip Comb: place a 96-well Deep Well Tip Comb in the plate
- Elution plate: add 180 µL Lysis reagent 1 and 10 µL Proteinase K to each sample and control well
- Wash plate 1: Add 1000 µL of Wash Buffer to each sample and control well

Set up the KingFisher Flex processing plates:

- Add 500 µL of Wash Buffer to each sample and control well
- Add 25 µL Applied Biosystems Dynabeads anti-*Salmonella* to each sample and control well
- Add 500 µL of enriched sample to each sample and control well
- In addition, Add 500 µL of sterile water to one or more wells to prepare a mock-purified (negative extraction control)

Select the A33227KF_RF_Sal program on the KingFisher Flex instrument and press start

Load the prepared plates when prompted onto the instrument and allow the run to complete

When the run is complete, remove the elution plate from the instrument. The elution plate contains the sample lysate. Proceed directly to PCR. (The sealed elution plate can be stored 2-8°C for up to 24 hours).

QuantStudio 5 PCR Instrument

Load into the QuantStudio 5 PCR Instrument and start PCR, review results at end of run (approx. 50 min)

7500 Fast PCR Instrument

Load into the 7500 Fast PCR Instrument, with the addition of a negative control tube containing nuclease free sterile water and start PCR, review results at end of run (approx. 45 min)

Confirm PCR results by culturing onto Thermo Scientific™ *Brilliance*™ Salmonella Agar and Tryptone Soya Agar (TSA)

Confirm colonies grown on the *Brilliance* Agar and TSA using serological and biochemical procedures

USDA FSIS MLG 4.09 Reference Method Protocol for Ground Turkey and Carcass Rinse Samples

Day: 0

Carcass rinse samples: Add 30 ±0.6 mL of sample to 30 ±0.6 mL of BPW

Incubate at 35 ±2°C for 20–24 hours

Ground turkey samples: Add 25 g of sample to a 1 in 10 ratio of Buffered Peptone Water

Incubate at 35 ±2°C for 20–24 hours

Day: 1

Transfer 0.5 ±0.05 mL of each sample into 10 mL TT broth (Hajna) and 0.1 ±0.02 mL into 10 mL modified Rappaport Vassiliadis (mRV) broth

Incubate at 42 ±0.5°C for 22–24 hours

Day: 2

Streak onto Brilliant Green Sulfa Agar (BGS) and either Double Modified Lysine Iron Agar (DMLIA) or Xylose Lysine Tergitol 4 Agar (XLT4) plates using a 10 µL loopful of inoculum for each plate. Streak the entire agar plate with a single sample enrichment

Incubate at 35 ±2°C for 18–24 hours

Day: 3

Examine plates for colonies that meet the description for suspect *Salmonella* colonies. Pick at least 1 or more typical or atypical colonies from each plate and inoculate Triple sugar iron (TSI) and Lysing iron agar (LIA) agar slants

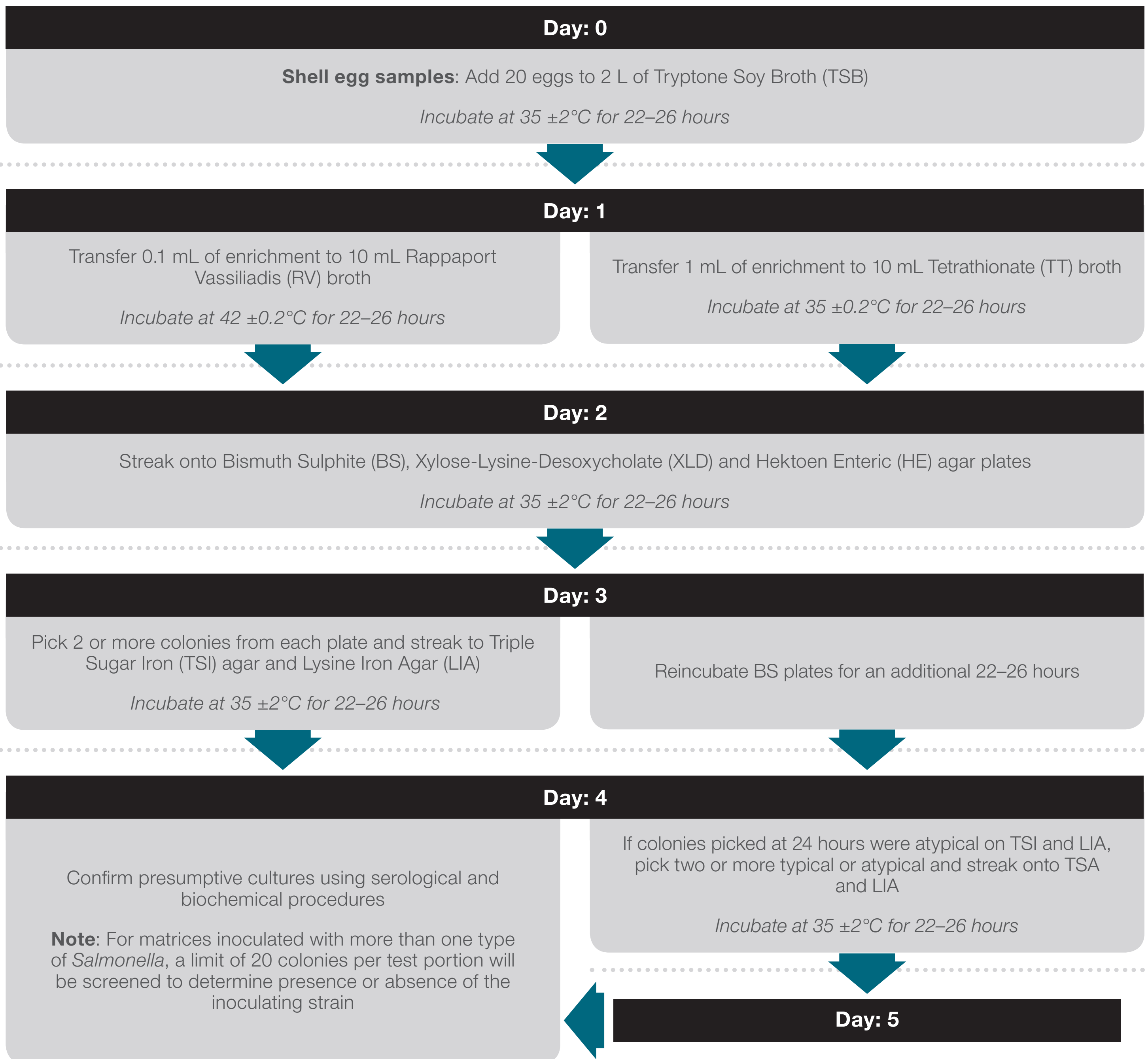
Incubate at 35 ±2°C for 22–26 hours

Day: 4

Confirm presumptive cultures using serological and biochemical procedures

Note: For matrices inoculated with more than one type of *Salmonella*, a limit of 20 colonies per test portion is screened to determine presence or absence of the inoculating strain

FDA BAM Chapter 5 Reference Method Protocol for Shell Egg Samples



Results

All test results were analyzed by probability of detection (POD) statistical analysis to 95% confidence intervals. POD analysis of the study data were conducted as detailed in the AOAC INTERNATIONAL validation guidelines. The POD analysis of the candidate method and MLG/BAM reference methods are detailed in Appendix 1.

Throughout the matrix study at the method developer laboratory, dual colony morphology and difficulty in O group serological confirmations were observed whilst testing *Salmonella* Enteritidis and *Salmonella* Bareilly isolates. It was confirmed by gene sequencing that cells from both colony morphologies were from the same isolate, cells from one morphology could be confirmed by O group presence and cells from the other morphology had a mutation (causing the O antigen to not be expressed). All *Salmonella* Enteritidis and *Salmonella* Bareilly isolates were confirmed by H antigen presence and the data for both (O-) and (O+) colonies have been combined to create one data set.

For all matrices tested, when using both the 7500 Fast PCR Instrument with RapidFinder Express software and the QuantStudio 5 PCR Instrument with RapidFinder Analysis software, there was no statistical difference by POD analysis between the candidate method presumptive PCR results compared to the candidate method confirmed PCR results using both candidate and reference method confirmation protocols. There was no POD statistical difference between the candidate method confirmation protocol and reference method confirmation protocols.

For all matrices tested, the candidate presumptive PCR results were not statistically different by POD analysis compared to the candidate confirmed results (via *Brilliance* Salmonella Agar) when tested using the 7500 Fast Real-Time PCR instrument and interpreted by the RapidFinder Express software and the QuantStudio 5 Real-Time PCR instrument and interpreted by the RapidFinder Analysis software.

The POD analysis of the shell egg data has shown statistically significant differences for the detection of *Salmonella* Enteritidis between the candidate method presumptive and candidate method confirmed via FDA BAM Ch. 5, between the candidate method (confirmed via *Brilliance* Salmonella Agar) and both the FDA BAM Ch. 5 method and candidate method confirmed (via FDA BAM Ch. 5), and also between the candidate method confirmed (via FDA BAM Ch.5) and the FDA BAM Ch.5 method. The statistically significant differences are in favor of the candidate method; it is likely that during the FDA BAM Ch.5 enrichment the *Salmonella* Enteritidis and *Salmonella* Heidelberg were competing for growth during dual inoculation, reducing the likelihood of *Salmonella* Enteritidis confirmation via the FDA BAM Ch. 5 method.

There were statistical differences observed in the POD analysis for the detection of *Salmonella* Heidelberg in shell egg samples in favor of the candidate method. The statistically significant differences were between both the candidate method confirmed (via *Brilliance* Salmonella Agar and FDA BAM Ch. 5) and the FDA BAM Ch. 5 reference method.

The POD analysis of the carcass rinse data has shown statistically significant differences for the detection of *Salmonella* Typhimurium between the candidate method confirmed (via *Brilliance* Salmonella Agar, and confirmed via MLG 4.09) and the MLG 4.09 reference method. The statistically significant differences were in favor of the candidate method; it is likely that during the MLG 4.09 enrichment the *Salmonella* Enteritidis and *Salmonella* Typhimurium were competing for growth during the dual inoculation, reducing the likelihood of *Salmonella* Typhimurium confirmation. There were no statistically significant differences in the detection of *Salmonella* Enteritidis between all methods tested.

The original testing of the ground turkey matrix at the method developer laboratory returned non-fractional results for *Salmonella* Bareilly (<25% positivity) when tested as dual inoculation with *Salmonella* Typhimurium. The *Salmonella* Bareilly was later confirmed with acceptable fractional positivity using the dilute-and-spread troubleshooting method described in the manufacturer's guidelines. It is likely that the candidate confirmed method (via *Brilliance* Salmonella Agar) failed to confirm *Salmonella* Bareilly presence due to strain competition during dual inoculation.

The original testing of the ground turkey matrix returned non-fractional results for *Salmonella* Typhimurium (>75% positivity), therefore the *Salmonella* Typhimurium low spike level was repeated as single inoculation in the ground turkey matrix. The original test low spike data (MPN of 2.26) represents the high spike for the repeat test.

During the repeat testing of the ground turkey matrix, the MLG 4.09 reference method and candidate method confirmed (via MLG 4.09 method) failed to detect a natural contaminant that was successfully identified by the candidate method presumptive and candidate method confirmed (via the extended confirmation method detailed in the manufacturer guidelines for high background samples; includes RVS and step prior to *Brilliance* Salmonella Agar). This is likely due to the larger sample size of the candidate test portion (375 g) compared to the reference method test portion (25 g) increasing the likelihood of natural contaminant presence. This has led to a statistically significant difference by POD analysis in favor of the candidate method. For the detection of *Salmonella* Typhimurium in ground turkey samples there were no statistically significant differences between all methods tested.

Conclusions

The data collected demonstrates that the candidate method showed equivalent or improved sensitivity performance to the USDA FSIS MLG 4.09 reference method for the detection of Salmonella spp., *Salmonella* Enteritidis, and Salmonella Typhimurium from chicken carcass rinse and raw ground turkey, and the FDA BAM Ch. 5 reference method for shell eggs. POD analysis conducted during the validation study demonstrated statistically significant differences in favor of the RapidFinder Salmonella species, Typhimurium and Enteritidis Multiplex PCR Kit. The AOAC-RI PTM validation certificate (License number: 081707) is available from either **www.thermofisher.com** or the AOAC Research Institute at **www.aoac.org**.

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Appendix 1. RapidFinder Salmonella species, Typhimurium and Enteritidis Multiplex PCR Kit POD summary of: Candidate method confirmed result (using candidate method confirmation protocol) vs. USDA FSIS MLG 4.09/FDA BAM Ch.5 reference method result

Matrix[a]	Inoculating strain(s)	MPN[b]/ test portion	N[c]	RapidFinder *Salmonella* Multiplex PCR Kit *Brilliance* Salmonella Agar confirmed[f]			Reference method[f]			$dPOD_{cc}$[h]	95% CI[i]
				x[d]	POD_{cc}[e]	95% CI	x	POD_R[g]	95% CI		
Shell eggs	*Salmonella* spp. total[j] (*S.* Heidelberg & *S.* Enteritidis)	N/A[k]	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	-0.43, 0.43
		0.77	20	19	0.95	0.76, 1.00	12	0.60	0.39, 0.78	0.35	0.09, 0.57
		1.78	5	5	1.00	0.57, 1.00	4	0.80	0.38, 1.00	0.20	-0.28, 0.62
	Salmonella Heidelberg	N/A	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	-0.43, 0.43
		0.53	20	16	0.80	0.58, 0.92	9	0.45	0.26, 0.66	0.35	0.05, 0.58
		0.40	5	4	0.80	0.38, 1.00	2	0.40	0.12, 0.77	0.40	-0.16, 0.75
	Salmonella Enteritidis	N/A	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	-0.43, 0.43
		0.18	20	16	0.80	0.58, 0.92	4	0.20	0.08, 0.42	0.60	0.29, 0.77
		1.08	5	4	0.80	0.38, 1.00	2	0.40	0.12, 0.77	0.40	-0.16, 0.75
Ground turkey[l] (Original test)	*Salmonella* spp. total[j] (S. Bareilly & S. Typhimurium)	N/A	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	-0.43, 0.43
		3.18	20	19	0.95	0.76, 1.00	20	1.00	0.84, 1.00	-0.05	-0.24, 0.12
		N/A	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	-0.43, 0.43
	Salmonella Bareilly	N/A	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	-0.43, 0.43
		0.65	20	10	0.50	0.30, 0.70	11	0.55	0.34. 0.74	-0.05	-0.33, 0.24
		0.52	5	4[q]	0.80	0.38, 1.00	3	0.60	0.23, 0.88	0.20	-0.31, .062
	Salmonella Typhimurium[n]	N/A	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	-0.43, 0.43
		2.26	20	17	0.85	0.76, 1.00	19	0.95	0.76, 1.00	-0.10	-0.32, 0.11
		N/A	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	-0.43, 0.43
Ground turkey[m] (Repeat)	*Salmonella* spp. total[j] (natural contaminant & *S. Typhimurium*)	N/A	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	-0.43, 0.43
		0.46	20	14	0.70	0.48, 0.85	6	0.30	0.15, 0.52	0.40	0.09, 0.62
		N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Salmonella natural contaminant	N/A	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	-0.43, 0.43
		N/A	20	3	0.15	0.05, 0.36	0	0.00	0.00, 0.16	0.15	-0.04, 0.36
		N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A

	Salmonella Typhimurium	N/A	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	-0.43, 0.43
		0.46	20	11	0.55	0.34, 0.74	6	0.30	0.15, 0.52	0.25	-0.05, 0.50
		N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Ground turkey[o]	*Salmonella* spp. total[j] (S. Bareilly & S. Typhimurium	N/A	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	-0.43, 0.43
		0.89	20	14	0.70	0.48, 0.85	13	0.65	0.43, 0.82	0.05	-0.23, 0.32
		1.97	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	-0.43, 0.43
	Salmonella Bareilly	N/A	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	-0.43, 0.43
		0.89	20	14	0.70	0.48, 0.85	13	0.65	0.43, 0.82	0.05	-0.23, 0.32
		1.97	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	-0.43, 0.43
	Salmonella Typhimurium	N/A	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	-0.43, 0.43
		0.81	20	12	0.60	0.39, 0.78	12	0.60	0.39, 0.78	0.00	-0.28, 0.28
		1.97	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	-0.43, 0.43
Chicken carcass rinse	*Salmonella spp. total*[j] (S. Typhimurium & S. Enteritidis)	N/A	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	-0.43, 0.43
		2.85	20	20	1.00	0.84, 1.00	20	1.00	0.84, 1.00	0.00	-0.16, 0.16
		2.75	5	5	1.00	0.57, 1.00	4	0.80	0.38, 1.00	0.20	-0.28, 0.62
	Salmonella Enteritidis	N/A	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	-0.43, 0.43
		1.34	20	15	0.75	0.53, 0.89	16	0.80	0.58, 0.92	-0.05	-0.30, 0.21
		0.72	5	5	1.00	0.57, 1.00	3	0.60	0.23, 0.88	0.40	-0.12, 0.77
	Salmonella Typhimurium	N/A	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	-0.43, 0.43
		0.75	20	20	1.00	0.84, 1.00	11	0.55	0.34, 0.74	0.45	0.20, 0.66
		0.28	5	5	1.00	0.57, 1.00	1	0.20	0.00, 0.62	0.80	0.19, 1.00

[a] Matrix = for each matrix the data is shown combined for both 7500 Fast and QuantStudio 5 PCR instruments unless otherwise specified

[b] MPN = Most Probable Number is based on the POD of reference method test portions using the Least Cost Formulations MPN calculator, with 95% confidence interval

[c] N = Number of test portions

[d] x = Number of positive test portions

[e] PODCC = Candidate method presumptive positives (confirmed via *Brilliance* Salmonella Agar) divided by the total number of trials

[f] All strains were confirmed by serotyping

[g] PODR = Reference method positive outcomes divided by the total number of trials

[h] dPODCC = Difference between the candidate method confirmed (via *Brilliance* Salmonella Agar) and reference method POD values

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

[j] RapidFinder presumptive PCR result for *Salmonella* species reflects all species present and therefore does not discriminate between the species

[k] N/A = Not applicable

[l] Ground turkey original test (failed on fractional positivity for *Salmonella* Typhimurium)

[m] Ground turkey repeat test data

[n] Data from the *Salmonella* Typhimurium ground turkey low spike original test represents the high level spike for the ground turkey repeat test

[o] Matrix tested at the independent laboratory

[q] 1 out of 4 positives was not confirmed via O:7 latex testing. This isolate exhibited H: 1,5 antigens and therefore is reported as a *Salmonella* Bareilly (it is likely the isolated colony had lost the O antigens due to a mutation).