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Idaho Technology Inc.
Innovation Amplified

Performance Tested Method℠ Evaluation and Approval of Idaho Technology's R.A.P.I.D.® LT *Salmonella* Food Security System for Dry Pet Food and Stainless Steel Environmental Surfaces

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ABSTRACT

INTRODUCTION

The *Salmonella* LT Food Security System (FSS) is a PCR-based detection method that rapidly and specifically identifies *Salmonella* species in foods with previous AOAC Performance Tested Method approval. The method includes: a single 16 or 20 hour enrichment of samples, mechanical lysis of bacteria to release DNA, amplification and melting of target DNA in Idaho Technology, Inc.'s R.A.P.I.D. LT instrument in under an hour, internal amplification controls, and automatic interpretation of results by the system software which provides users with a positive or negative call.

PURPOSE

The *Salmonella* LT FSS was evaluated for sensitivity in additional matrices: dry pet food and sponges from stainless steel environmental surfaces in an AOAC Research Institute PTM study.

RESULTS

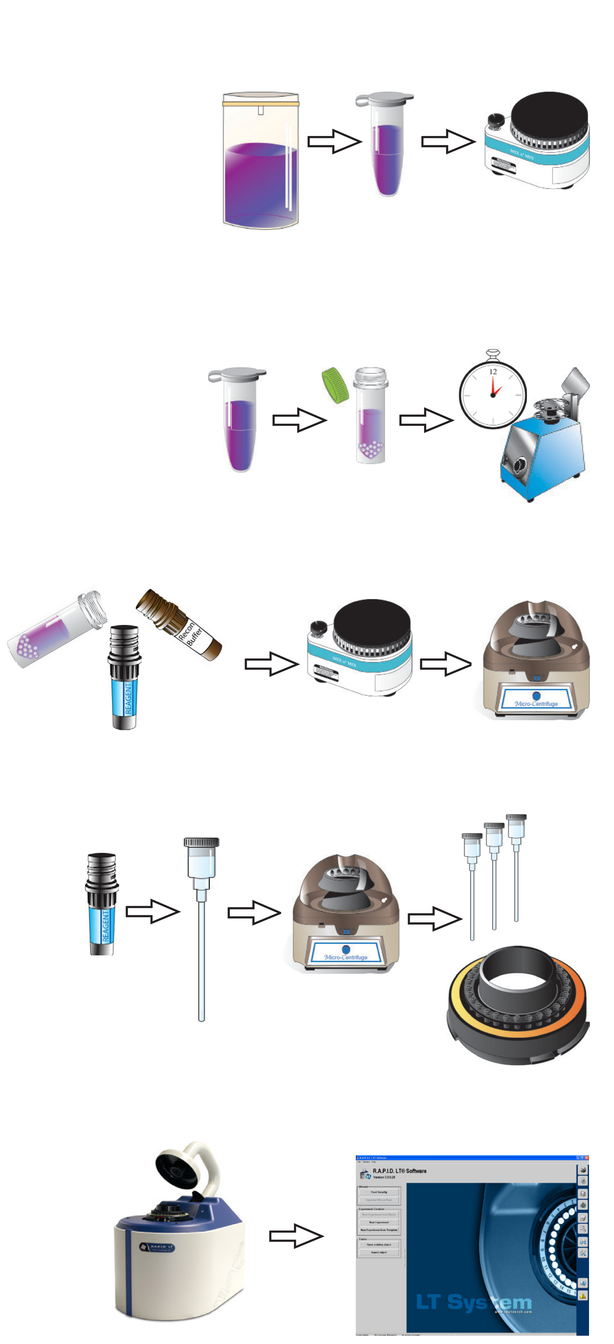
The *Salmonella* LT FSS is equivalent to the reference method for dry pet food and stainless steel environmental surfaces with background organism. The system detects all 121 *Salmonella* strains tested and did not detect 31 closely related strains. The system is robust and reproducible as demonstrated by ruggedness and stability studies. The *Salmonella* LT FSS obtains results for dry pet food and stainless steel in less than 21 h while the reference method can take 3–4 days.

BACKGROUND

The R.A.P.I.D. LT Food Security System (FSS) is a PCR-based pathogen detection method used to detect pathogens from enriched food or environmental surface sponge samples. The method involves enriching a sample for a specified amount of time in commercially available media, performing mechanical cell lysis to release the DNA, rehydration of freeze-dried PCR reagents, DNA amplification and melting peak analysis in the R.A.P.I.D. LT instrument, and automated data and results interpretation by the R.A.P.I.D. LT software.

The *Salmonella* LT FSS has previously been evaluated for detection of *Salmonella* in cooked ham, chicken, chocolate, liquid whole egg, lettuce, and raw ground beef (AOAC PTM Certificate 030803). ITI has completed a method comparison evaluation for two additional matrices: dry pet food and stainless steel environmental surface sponges. A matrix extension has been granted to include these two matrices.

Figure 1: *Salmonella* LT FSS Protocol



Step 1: Enrich Individual Samples and Prepare
- Add sample and media to a filtered blender bag and mix.
- Enrich sample for specified amount of time.
- From an enrichment broth sample, transfer **1 mL** of sample into micro-centrifuge tube and vortex for 10 seconds.

Step 2: Lyse Bacteria and Isolate DNA
- Transfer **5 µL** of sample into bead tube
- Bead beat on Disruptor Genie for 5 minutes

Step 3: Reconstitute Reagent
- Add **10 µL** of reconstitution buffer into a reagent tube.
- Transfer **10 µL** of sample into tube.
- Mix the reagent tube by pipetting the sample up and down or by vortexing the tube for 3 seconds.
- Spin the reagent tube.

Step 4: Add Sample to Capillary Tube
- Pipette **18 µL** into a capillary tube.
- Cap capillary tube.
- Centrifuge at low speed in a micro-centrifuge for 3-5 seconds.
- Load the capillary tubes into the carousel.

Step 5: Run Protocol and Automatic Software Results
- Place the carousel into the thermal cycler.
- Start the run using FSS R.A.P.I.D. LT software. The software automatically calls the results in less than an hour.

METHODS

METHOD COMPARISON

Food

The *Salmonella* LT FSS was evaluated with dry pet food and compared to the reference method. Dry pet food was divided into two portions. One portion was not inoculated; the second portion was inoculated in a large batch to provide enough samples for testing by the *Salmonella* LT FSS, MPN analysis, and the reference method. Both inoculated and uninoculated batches were handled in the same manner. The inoculum concentrations were selected to result in approximately 1–5 CFU of *Salmonella* per 25 g sample (fractional positive levels, 5–15 positives out of 20 replicates) after equilibration. Samples were inoculated with lyophilized culture (*Salmonella* NTCC 6017) and allowed to equilibrate at room temperature for 2 weeks by at least one method (see figure 2).

Environmental

Forty inoculated food-grade stainless steel surfaces and 10 uninoculated surfaces were prepared. Inoculated surfaces were prepared using a co-inoculation of *Salmonella* (ATCC 14028) and *Escherichia coli* (ATCC 25922). *Salmonella* inoculation levels were targeted to result in fractionally positive results (5–15 positives out of 20 replicates tested) by at least one of the methods (FSS or reference method). *E. coli* inoculation levels were 10-fold higher than the analyte. A 50 µL aliquot of each organism was applied directly to the stainless steel surface. Surface samples were allowed to dry at room temperature for 16–24 hours before sampling with a sterile sponge (see figure 3).

For each matrix, 25 samples (20 inoculated and 5 uninoculated) were prepared, enriched, plated, and evaluated according to the reference method—FDA BAM 8th edition, Chapter 5 (1).

Twenty-five samples (20 inoculated and 5 uninoculated) were prepared, enriched, and tested according to the *Salmonella* LT FSS method. After the required 1 mL aliquot for PCR was removed, the samples were returned to the incubator to incubate for a total of 24 hours at 37°C. Aliquots from the enrichments were transferred to tetrathionate broth and Rapport Vassiliadis broth and FDA BAM procedures were followed for the remainder of the confirmation.

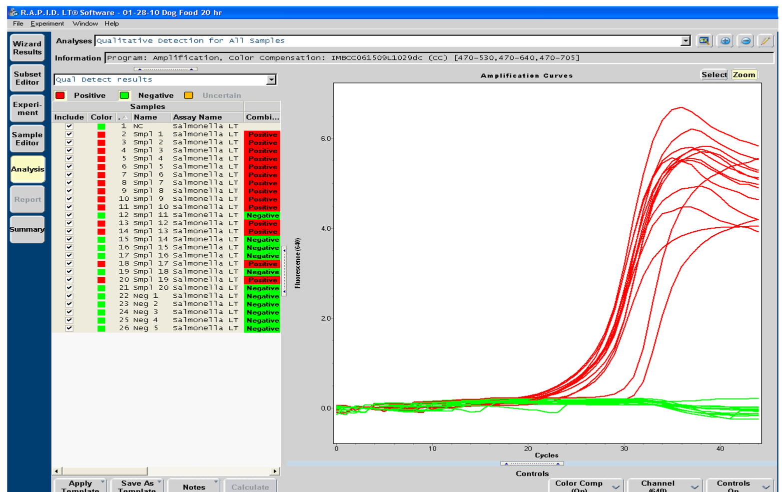
Most Probable Number (MPN) quantification was conducted on the day that sample testing was initiated for the dry pet food (there is no MPN method for environmental samples). The MPN was calculated according to the FDA BAM 8th edition, Appendix 2 (1).

Table I. Method Comparison Results

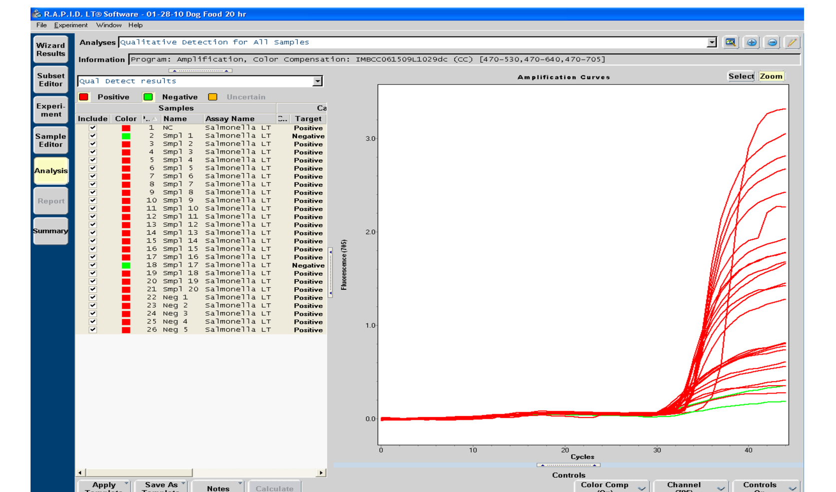
					Reference Method	Test Kit			Test Kit Performance	
Matrix	Inoculating Organism	Inoculation Level	MPN /25g[a]	No. test portions for each method	Positive	Presumptive Positive	Confirmed Positive	$\chi^{2\,b}$	False Negative (%)	False Positive (%)
Dry Pet Food	*Salmonella* Abony NCTC 6017	Inoculated	<0.75	20	13	15	15	0.46	0	0
		Control	0	5	0	0	0	-	-	-
Stainless Steel Surface Sponge	*Salmonella* Typhimurium ATCC 14028 and *E. coli* ATCC 25922	Inoculated	N/A	20	14	16	16	0.5	0	0
		Control	N/A	5	0	0	0	-	-	-

[a]Most Probable Number: Colony forming units in a 25 g sample. MPN is not performed on environmental surface samples.
[b]Mantel-Haenszel chi square test for significant difference between two methods that use different primary enrichments. Chi square (χ^2) <3.84 indicates that the proportions positive for the test method and reference methods are not statistically different at the 5% level of significance.

Figure 2.

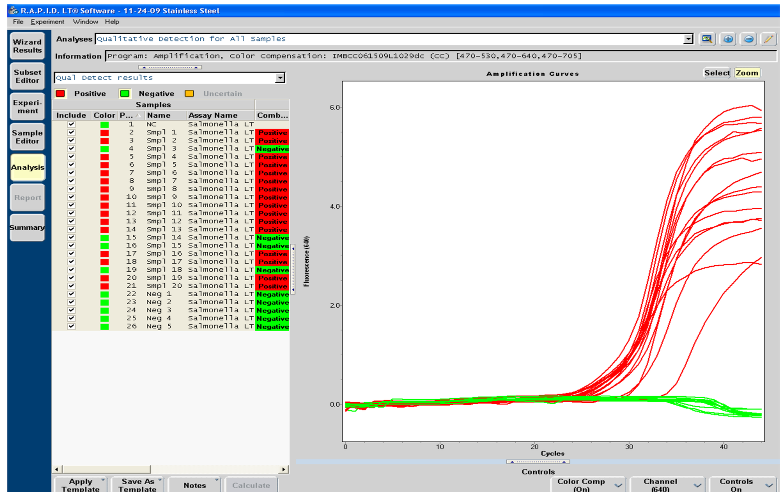


2A: Dry pet food samples target channel (640). Negative control (position 1), 15/20 fractionally spiked positive samples confirmed by culture (positions 2–21), and 5 uninoculated (positions 22–26).

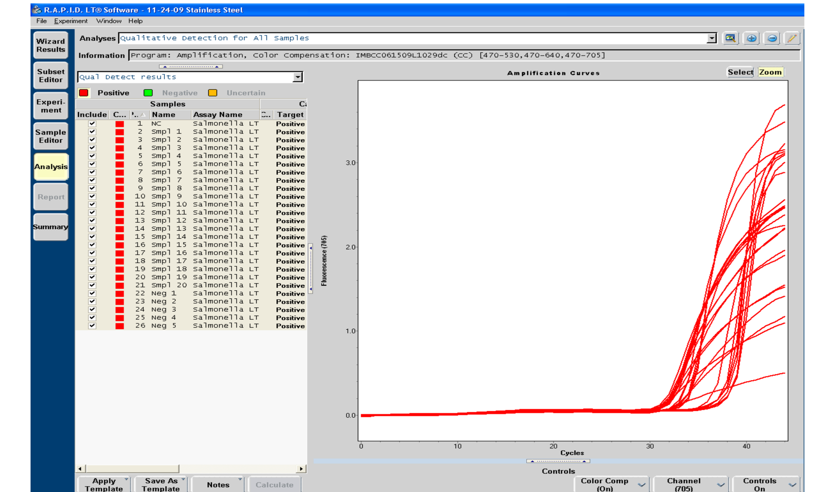


2B: Dry pet food samples AC channel (705), internal positive control (included in each reagent vial). Negative control (position 1), 15/20 fractionally spiked positive samples confirmed by culture (positions 2–21), and 5 uninoculated (positions 22–26).

Figure 3.



3A: Stainless steel environmental sponge samples target channel (640). Negative control (position 1), 16/20 fractionally spiked positive samples confirmed by culture (positions 2–21), and 5 uninoculated (positions 22–26).



3B: Stainless steel environmental sponge samples AC channel (705), internal positive control (included in each reagent vial). Negative control (position 1), 16/20 fractionally spiked positive samples confirmed by culture (positions 2–21), and 5 uninoculated (positions 22–26).

RESULTS

METHOD COMPARISON

The results obtained for dry pet food and stainless steel environmental surface samples show that the *Salmonella* LT FSS is statistically as effective as the reference method at detecting *Salmonella* in these matrices. Results are summarized in Table I. The *Salmonella* LT FSS uses different enrichment media than the reference method for both dry pet food samples and environmental surface samples; therefore, the study was conducted using unpaired samples. Statistical analysis using the Mantel-Haenszel Chi square calculation (χ^2) determined that the *Salmonella* LT FSS is statistically equivalent to the reference method (χ^2 is less than 3.84) for both dry pet food and environmental surface sample methods.

SPECIFICITY

A total of 121 strains of *Salmonella* were evaluated. At least 50 of these strains were isolated from food sources. All *Salmonella* strains were detected by the R.A.P.I.D. LT (Results not shown). None of the 31 non-*Salmonella* bacteria strains were detected (Table II).

Table II. Strains Not Detected in the AOAC Exclusivity Study.

Organism	Strain/Source	Organism	Strain/Source	Organism	Strain/Source	Organism	Strain/Source
Citrobacter amalonaticus	Gene-Trak	*Enterobacter cloacae*	ATCC 7256	*Klebsiella pneumoniae*	ATCC 9591	*Shigella flexineri*	MicroBioLogics
Citrobacter freundii	ATCC 6879	*Cronobacter sakazakii*	ATCC 51329	*Morganella morganii spp. morganii*	ATCC 29853	*Shigella sonneii*	U. Chicago Hosp.
Citrobacter braakii	ATCC 29063	*Enterococcus faecalis*	ATCC 33186	*Proteus mirabilis*	MicroBioLogics	*Staphyloccus aureus*	ATCC 13565
Citrobacter farmerii	ATCC 51112	*Escherichia blattae*	ATCC 29907	*Proteus vulgaris*	ATCC 8427	*Yersinia enterocolitica*	ATCC 29913
Citrobacter youngae	ATCC 29935	*Escherichia coli*	ATCC 11229	*Providencia stuartii*	Org-Tek, clinical	*Yersinia frederiksenii*	Envir. Dairy
Enterobacter aerogenes	ATCC 35028	*Escherichia coli* O157:H7	ATCC 35150	*Serratia liquefaciens*	MicroBioLogics	*Yersinia*	Org-Tek, clinical
Enterobacter agglomerans	ATCC 29917	*Hafnia alvei*	ATCC 25927	*Serratia marcescens*	ATCC 14041	*Enterococcus faecalis*	ATCC 49452
Enterobacter cloacae	ATCC 35030	*Klebsiella oxytoca*	Gene-Trak	*Shigella boydii*	MicroBioLogics		

RUGGEDNESS AND REAGENT VARIATION

None of the parameters tested led to a negative result; all of the reagent lot performed equivalently (results not shown).

Discussion

The *Salmonella* LT FSS results were statistically equivalent to results for the reference method at low inoculum levels (1–5 CFU/25 g food or 100 cm² surface samples). All positive and negative samples were confirmed. No false positive or false negative results were observed; 0% false positive and 0% false negative. Enrichment of food and environmental sponges in buffered peptone water performed equivalently to the reference method enrichment. Buffered peptone water is well suited for most food types and environmental surface samples coinoculated with a background organism. The system specifically identified 121 *Salmonella* strains and did not identify 31 non-*Salmonella* species. The system is robust and reproducible as demonstrated by ruggedness, lot-to-lot and shelf life studies.

COMPARISON TO REFERENCE METHODS

Organism die-off can be a problem when using a minimal diluent for *Salmonella* spiking. The selection of diluent is critical when spiking and equilibrating low level *Salmonella* on surfaces for fractional recovery. *Salmonella* appears to be less stable in minimal buffers than in BPW when spiking and equilibrating on surfaces. The use of Butterfield's phosphate buffer (BPB) resulted in discontinuity between presumptive and confirmed results. Investigation revealed the detection of DNA from dead cells contributed to these discordant results.

A high inoculum level was required to achieve fractional recovery when BPB was used (10,000 CFU/100 µL). Fractional recovery of *Salmonella* was achieved at a low inoculation level (300 CFU/100 µL) when BPW was used as the diluent. *Salmonella* die-off during the spiking and equilibration (drying at room temperature for 16–24 h before sampling with a sterile sponge) was significantly greater in BPB and required a higher inoculum level. When an elevated spike level was required due to die-off, DNA from dead cells may be detected leading to false positive results.

Table III. Organism Die-Off – Stainless Steel Surface Spiking Trials

			Salmonella	Background Organism	No. Test Portions	Confirmation	Reference Method	Test Kit	
Trial	Inoculating Organisms	Diluent	Spike Level CFU	Spike Level CFU		FDA BAM 5.D.2	Positive	Presumptive Positive	Confirmed Positive
A	***Salmonella* Typhimurium and *Citrobacter freundii***	BPB	10,000/100 µL	100,000/100 µL	20	High	11	15	10
B	***Salmonella* Typhimurium and *Citrobacter freundii***	BPB	10,000/100 µL	100,000/100 µL	20	Low	4	10	2
C	***Salmonella* Tennessee and *E. coli***	BPB	10,000/50 µL	100,000/50 µL	20	High	NC	17	9
D	***Salmonella* Tennessee and *E. coli***	BPW	300/50 µL	3,000/50 µL	20	High	14	16	16

NC = confirmation not completed
High = FDA BAM protocol for foods with a high microbial load
Low = FDA BAM protocol for foods with a low microbial load

SPECIFICITY

The *Salmonella* LT FSS is highly specific and was able to detect 121 strains tested in the inclusivity panel. It did not detect the 31 non-*Salmonella* species in the exclusivity panel.

RUGGEDNESS AND REAGENT VARIATION

The *Salmonella* LT FSS is robust and reproducible as demonstrated by the ruggedness, lot-to-lot, and shelf life studies. The ruggedness study demonstrated that the system produced consistent results even with variability in reagent preparation time and sample volume pipette. The lot-to-lot and shelf life study demonstrated that the *Salmonella* LT FSS gave consistent results with several lots of reagents produced at different times.

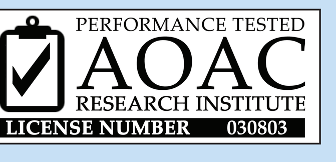
CONCLUSION

The R.A.P.I.D. LT can reliably detect low level *Salmonella* contamination in dry pet food or on stainless steel environmental surfaces in about 21 hours as opposed to 3–4 days for the FDA BAM method. The data presented demonstrate that the *Salmonella* LT FSS is equivalent to the current FDA BAM official methods used to detect low levels of *Salmonella* in dry pet food and on environmental surfaces.

The *Salmonella* LT FSS is Easy, Accurate, and Timely™ and represents a significant improvement over standard methods in a number of ways:

- Single enrichment step and reduced enrichment times
- Minimal sample handling
- Easy to use freeze-dried PCR reagents, including an internal amplification control
- Easy to interpret results (software gives a "Positive" or "Negative" result)
- Accuracy of real-time PCR
- Results in hours, not days

The *Salmonella* LT FSS is AOAC Research Institute *Performance Tested Method*℠ validated for 8 matrices: cooked ham, raw chicken, raw ground beef, lettuce, liquid whole egg, chocolate, and stainless steel environmental surface sponges.



References

1. U.S. Food and Drug Administration, *Bacteriological Analytical Manual*, http://www.fda.gov/Food/ScienceResearch/LaboratoryMethods/BacteriologicalAnalyticalManualBAM/default.htm