Development and Evaluation of a Four-Color Multiplex Real-time PCR Assay for *Salmonella* Detection in Tomatoes

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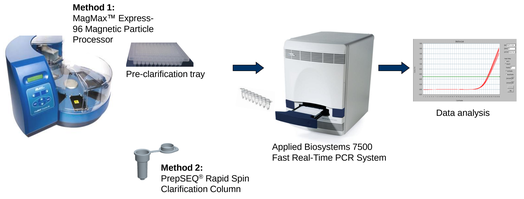
Abstract

Introduction: *Salmonella* is the leading cause of foodborne illness in the United States with an increasing number of reported *Salmonella* outbreaks originating from produce in recent years. Real-time PCR is an important rapid molecular detection tool that has great potential to minimize the risk of *Salmonella* outbreaks while also reducing time-to-result.
Purpose: We have developed a four-color real-time PCR assay that detects *Salmonella* species. The simultaneous combination of signal from *invA*, *apeE*, and *gapA* provides a unique signature for *Salmonella* detection.
Method: The real-time PCR assay was designed against three *Salmonella* gene targets (*invA*, *apeE*, and *gapA*) as well as an internal positive control to monitor inhibition. The multiplex assay was developed using *Salmonella* reference collections (SAR-A, n=72; SAR-B, n=72; SAR-C, n=16; and SAFE, n=101). The assay was also evaluated along with sample preparation methods whereby 120 g tomato samples spiked with 1 to 10 cfu *Salmonella* Typhimurium were enriched 20 hr in mBPW and then prepared using (1) a pre-clarification tray and an automated magnetic bead-based method or (2) a manual clarification column method.
Results: The assay detected 261 out of 261 of the inclusivity *Salmonella* species isolates. All 20 non-*Salmonella* exclusion strains were not detected. The assay consistently detected 100 copies of *Salmonella* genomic DNA. When used in conjunction with sample preparation in a controlled tomato-spiking study, the assay detected 1 to 10 CFU *Salmonella* post-enrichment.
Significance: Our early evaluation demonstrates the high specificity and sensitivity of the assay when testing pure cultures and spiked tomato samples. We are in the process of evaluating the assay on environmental samples from the field. The results thus far show promise that this assay and a sample preparation workflow can be applied to *Salmonella* surveillance of field samples.

Methods

Numerous TaqMan® real-time PCR assays were designed against the gene targets apeE, invA, and gapA. The assays were screened using *Salmonella* reference panels. Highly specific candidate assays were identified in the process. The performance of the multiplex was evaluated by determining assay efficiency, limit of detection of *Salmonella* genomic DNA, and limit of detection of the target organism in the background of a tomato matrix. Tomatoes were also spiked with low levels of *Salmonella* to evaluate sample preparation and detection workflow.

Sample preparation and detection:



Assay configuration

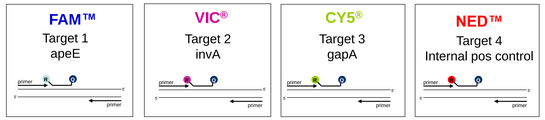


Figure 1. The assay is a four-color assay targeting apeE, invA, gapA, and an internal positive control plasmid. Degenerate probes were included to detect all *Salmonella* species. *Salmonella* should be positive for apeE, invA, and gapA. The gapA assay targets includes *Salmonella* and other Enterobacteriacea.

Inclusion and exclusion

Inclusion panel	Serotype
SAR A (72 isolates)	*S.* Heidelberg (11) *S.* Muenchen (10) *S.* Paratyphi B (22) *S.* Saintpaul (8) *S.* Typhimurium (21)
SAR B (72 isolates)	*S.* Agona (1) *S.* Anatum (1) *S.* Brandenberg (1) *S.* Choleraesuis (4) *S.* Decatur (1) *S.* Derby (3) *S.* Dublin (3) *S.* Duisburg (1) *S.* Emek (1) *S.* Enteritidis (4) *S.* Gallinarum (1) *S.* Haifa (1) *S.* Heidelberg (2) *S.* Indiana (1) *S.* Infantis (2) *S.* Miami (2) *S.* Montevideo (2) *S.* Muenchen (4) *S.* Newport (3) *S.* Panama (3) *S.* Paratyphi A (1) *S.* Paratyphi B (5) *S.* Paratyphi C (3) *S.* Pullorum (2) *S.* Reading (1) *S.* Rubislaw (1) *S.* Saintpaul (2) *S.* Schwarzengund (1) *S.* Sendai (1) *S.* Seftenberg (1) *S.* Stanley (1) *S.* Stanleyville (1) *S.* Thompson (1) *S.* Typhi (2) *S.* Typhimurium (4) *S.* Typhisuis (2) *S.* Wien (2)
SAR C (16 isolates)	*S.* Typhimurium *S.* Typhi 58:d:z6 42:f,g,t:- *S.* Arizonae (2) 501,2,3:k:z 38[k]:z35:- 45a,b:g,z51:- 16:z4,z32:- *S.* bongori (2) 45:a:e,n,x 11:b:e,n,x 1,40:g,z51:- 40:z4,z24:-

Inclusion panel	Serotype	
SAFE (101 isolates)	*S.* Newport *S.* Heidelberg *S.* Typhi 4,5,12:b:- *S.* Hadar *S.* Virchow *S.* Brandenburg II 58:l,z13,z28:z6 II 47:d:z39 II 48:d:z6 II 50:b:z6 II 53:l,z28:z39 II 39:l,z28:enx II 13,22:z29:enx II 4,12:b:- II 18:z4,z23:- IIIa 41:z4,z23:- IIIa 40:z4,z23:- IIIa 48:g,z51:- IIIa 21:g,z51:- IIIa 51:g,z51:- IIIa 62:g,z51:- IIIa 48:z4,z23,z32:- IIIa 48:z4,z23:- IIIb 60:r:e,n,x,z15 IIIb 48:i:z IIIb 61:k:1,5,(7) IIIb 61:l,v:1,5,7 IIIb 48: z10: e,n,x,z15 IIIb 38:z10:z53 IIIb 60:r:z IIIb 50:i:z IV 50:g,z51:- IV 48:g,z51:- IV 44:z4,z23: IV 45:g,z51:- IV 16:z4,z32:- IV 1:z4,z23:- IV 6,7:z36:- IV 16:z4,z32:-	IV 40:g,z51:- IV 40:z4,z24:- V 48:i:- V 40:z35:- V 44:z39:- V 60:z41:- V 66:z41:- V 48:z35:- VI 6,14,25:z10:1,(2),7 VI 11:b:1,7 VI 6,7:z41:1,7 VI 11:a:1,5 VI 6,14,25:a:e,n,x *S.* Typhimurium / DT104b *S.* Typhimurium (14) *S.* Typhimurium / DT104 (3) I 4,[5],12:i:- (6 isolates) *S.* Enteritidis (4) *S.* Agona *S.* Alachua *S.* Brisbane *S.* Cerro *S.* Cubana *S.* Fresno *S.* Gera *S.* Inverness *S.* Javiana *S.* Johannesburg *S.* Michigan *S.* Montevideo *S.* Muenchen *S.* Muenster *S.* Rubinslaw *S.* Saphra *S.* Seftenberg *S.* Tornow *S.* Urbana *S.* Vietnam

Exclusion panel	Species	
SAFE (20 isolates)	*Vibrio cholerae* *Vibrio metschnikovii* *V. parahaemolyticus* *Vibrio vulnificus* *Escherichia coli (2)* *Shigella sonnei* *Shigella flexneri* *Shigella dysenteriae* *Shigella boydii* *Proteus vulgaris*	*Klebsiella pneumoniae* *Bacillus cereus* *Bacillus subtilis* *Citrobacter freundii* *Erwinia mallotivora* *Brenneria nigrifluens* *Cronobacter sakazaki* *Cronobacter malonaticus* *Cronobacter dublinensis*

Table 1. The *Salmonella* multiplex was evaluated against *Salmonella* reference panels SAR A, B, C, and the SAFE panel. The assay detected all inclusion strains in the *Salmonella* reference panels (SAR and SAFE). It did not detection organisms in the SAFE exclusion panel. Parentheses in the table indicates the number of isolates within the panel.

Assay efficiency

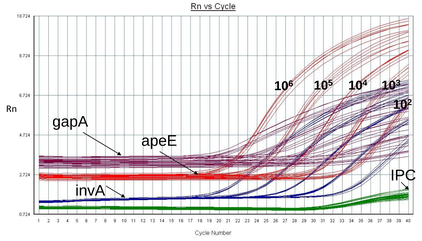


Figure 2. The *Salmonella* multiplex assay was tested against a dilution series of *Salmonella* Typhimurium purified genomic DNA ranging from 10^6 to 10^2 copies per reaction. The multiplex assay met the requirements for efficient amplification.

Sensitivity of detection of genomic DNA

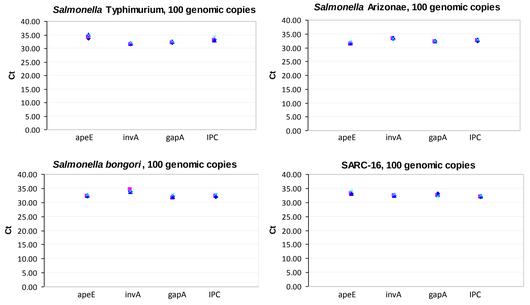


Figure 3. Genomic DNA from four serotypes of *Salmonella* were used to determine the limit of detection of the *Salmonella* multiplex assay. The four *Salmonella* strains are detected by different sets of degenerate oligos in the multiplex. The *Salmonella* multiplex assay detected 100 copies of S. Typhimurium, S. Arizonae, S. *bongori*, and SARC-16. n=16 per strain.

Limit of detection of *Salmonella* from tomato matrix

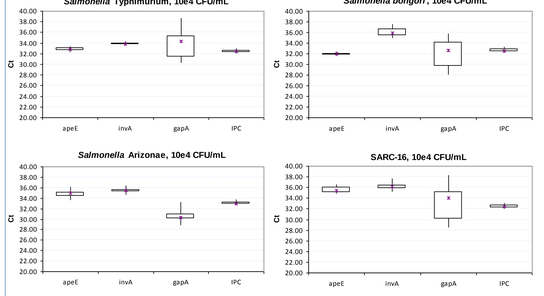


Figure 4. Specific concentrations of *Salmonella* were spiked into a tomato enrichment to determine LOD of the sample preparation and detection workflow. A tomato was quartered and enriched in mBPW for 20 hr at 37 °C in a 1:1 weight-to-volume ratio. A 100 g tomato was used for the enrichment. One gram of dirt was added to the enrichment to simulate tomatoes picked in the field. Overnight cultures of *Salmonella* grown to saturation were diluted, and each was added to individual portions of the enrichment such that the final concentrations were 10^5, 10^4, and 10^3 CFU/mL. 300 µL samples (n=16 per strain) were pre-clarified using a custom tray to remove inhibitors presented by the dirt. The samples were processed using the MagMax™ Express and the nucleic acid sample tested with the *Salmonella* multiplex assay. LOD was determined to be 10^4 CFU/mL.

Inoculation of tomatoes with *Salmonella*

	Sample prep by Rapid Spin Clarification					Sample prep by preclarification/MagMax™ Express					
Sample	apeE	invA Ct	gapA Ct	IPC Ct	Call	apeE	invA Ct	gapA Ct	IPC Ct	Call	BAM
Uninoculated											
1	-	-	+	+	Negative	-	-	+	+	Negative	Negative
2	-	-	+	+	Negative	-	-	-	+	Negative	Negative
3	-	-	+	+	Negative	-	-	+	+	Negative	Negative
4	-	-	+	+	Negative	-	-	+	+	Negative	Negative
5	-	-	+	+	Negative	-	-	+	+	Negative	Negative
Inoculated (~2 CFU)											
1	+	+	+	+	Positive	+	+	+	+	Positive	Positive
2	+	+	+	+	Positive	+	+	+	+	Positive	Positive
3	+	+	+	+	Positive	+	+	+	+	Positive	Positive
4	+	+	+	+	Positive	+	+	+	+	Positive	Positive
5	+	+	+	+	Positive	+	+	+	+	Positive	Positive
6	+	+	+	+	Positive	+	+	+	+	Positive	Positive
7	+	+	+	+	Positive	+	+	+	+	Positive	Positive
8	-	-	+	+	Negative	-	-	+	+	Negative	Negative
9	-	-	+	+	Negative	-	-	+	+	Negative	Positive
10	+	+	+	+	Positive	+	+	+	+	Positive	Positive
11	+	+	+	+	Positive	+	+	+	+	Positive	Positive
12	+	+	+	+	Positive	+	+	+	+	Positive	Positive
13	-	-	+	+	Negative	-	-	+	+	Negative	Negative
14	+	+	+	+	Positive	+	+	+	+	Positive	Positive
15	+	+	+	+	Positive	+	+	+	+	Positive	Negative
16	+	+	+	+	Positive	+	+	+	+	Positive	Positive
17	-	-	+	+	Negative	-	-	+	+	Negative	Negative
18	+	+	+	+	Positive	+	+	+	+	Positive	Positive
19	+	+	+	+	Positive	+	+	+	+	Positive	Positive
20	+	+	+	+	Positive	+	+	+	+	Positive	Positive

Table 2. Tomatoes were spiked with low level of *Salmonella* to evaluate the enrichment, sample preparation, and detection workflow. Twenty Roma tomatoes were each quartered and placed in individual stomacher bags. One gram of dirt was added to each sample and inoculated with low level of S. Typhimurium (~2 CFU per tomato). Five tomatoes with dirt were uninoculated. The samples were homogenized by squeezing and then enriched in mBPW for 20 hr at 37 °C in a 1:1 weight-to-volume ratio. The enrichments were processed by two different sample prep methods: (1) Rapid Spin Clarification Column and (2) pre-clarification followed by automated processing using the MagMax™ Express. The nucleic acid samples from both preps were tested with the *Salmonella* multiplex assay. The samples were also confirmed with the BAM for *Salmonella*. Samples 9 and 15 differed from the BAM (false negative and false positive, respectively). The two discrepancies may be due to handling error especially given that both sample methods correlated in results.

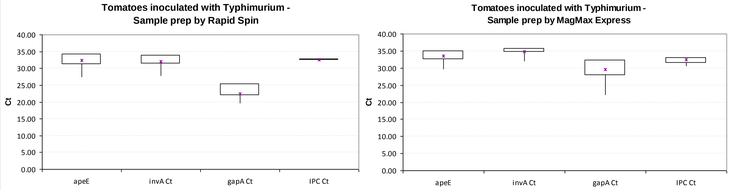


Figure 5. The Ct ranges from the 16 PCR presumptive positive samples prepared using Rapid Spin and the MagMax™ Express workflows.

Conclusions

- Through a collaboration between Life Technologies and FDA-CFSAN, a highly specific and sensitive custom *Salmonella* multiplex assay was developed for the potential use in surveillance of *Salmonella* in tomatoes and other produce.
- The assay can detect 100 copies genomic DNA and 10^4 CFU/mL culture prior to sample preparation.
- The sample preparation and detection workflows effectively address inhibitors and can detect low levels of *Salmonella* spiked onto tomatoes.

Acknowledgments

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