APPLICATION NOTE: Pathogen testing and typing kits

Improve your poultry testing by going molecular

Detection of pathogens for food safety and environmental monitoring in the poultry industry—switching to molecular methods

Employing the most advanced scientific technology and the best practices in breeding, housing, feeding and medical interventions, poultry producers now have the choice to employ molecular pathogen testing methods to reduce hold times for faster, fresher product release (Table 1).

Microbial identification using traditional culture methods requires growth and isolation of individual colonies that can then be characterized through a series of biochemical and serological methods. While proven to be reliable and accurate, waiting up to several days for a result can have a real financial impact on a business. PCR identifies genetic sequences that are specific for the organism(s) of interest, thus removing the subjectivity that can sometimes be an issue when using culture methods. In particular, samples from primary production stages (PPS) can create a real challenge when using culture methods and other techniques due to the presence of very high levels of competing flora. PCR only requires growth of the microbe to a concentration necessary for detection (typically about 1,000 CFU/mL). The presence of background microbial flora has little impact on PCR detection (Figure 1).

The technology and capability of real-time PCR

The genome is the blueprint of life that is made up of DNA sequences. Some DNA sequences within an organism are highly similar to other organisms because they code for common “housekeeping” functions, whereas other sequences are highly specific to a serovar (a distinct variant) within a

species. These highly specific sequences are the regions of the blueprint that provide a microbe with its unique identity. In between these two extremes are genomic sequences of varying degrees of similarity between organisms. This range of genomic diversity works to the advantage of PCR because it allows a skilled bioinformatician to design PCR assays that can, for example, detect either multiple species within a genus or a specific serovar within a single species. For example, one PCR assay can detect whether any *Salmonella* species are within a sample, while another PCR assay can detect *Salmonella enterica* subspecies *enterica* serovar Enteritidis (e.g. Applied Biosystems™ MicroSEQ™ *Salmonella* spp. Detection Kit versus TaqMan™ *Salmonella* Enteritidis Detection Kit).

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Table 1. Time to actionable result comparison between real-time PCR and traditional poultry pathogen identification methods.

Description	Traditional method*	Real-time PCR method*	Improvement
Salmonella Enteritidis/shell eggs	8 – 9 days† (FDA BAM)	1 day	7 – 8 days
Salmonella species/raw poultry	5 – 6 days (USDA MLG)	1 day	4 – 5 days
Salmonella species/environmental swabs	5 – 6 days‡ (FDA Env)	1 day	4 – 5 days
Salmonella species/primary production	3 days (ISO 6579, D)	1 day	2 days
Campylobacter/chicken rinse	3 – 4 days (ISO 10272-1)	2 days	1 – 2 days
Listeria species/environment	3 – 5 days (ISO 11290)	1 day	2 – 4 days
Salmonella serotyping	≥15 days	NA	NA

* The times reported in Table 1 for Traditional methods and the Real-Time PCR methods are time to actionable result. Confirmation may require up to several additional days depending on the required confirmatory steps.

† Method as referenced in 21 CFR Parts 16 and 118: Federal Register Final Rule (July 9, 2009, 74 FR 33030): Prevention of *Salmonella* Enteritidis in shell eggs during production, storage, and transportation [FDA BAM Chapter 5 *Salmonella* (2007)].

‡ Method as described in FDA environmental sampling and detection of *Salmonella* in poultry houses (2008).
NA = Not applicable.

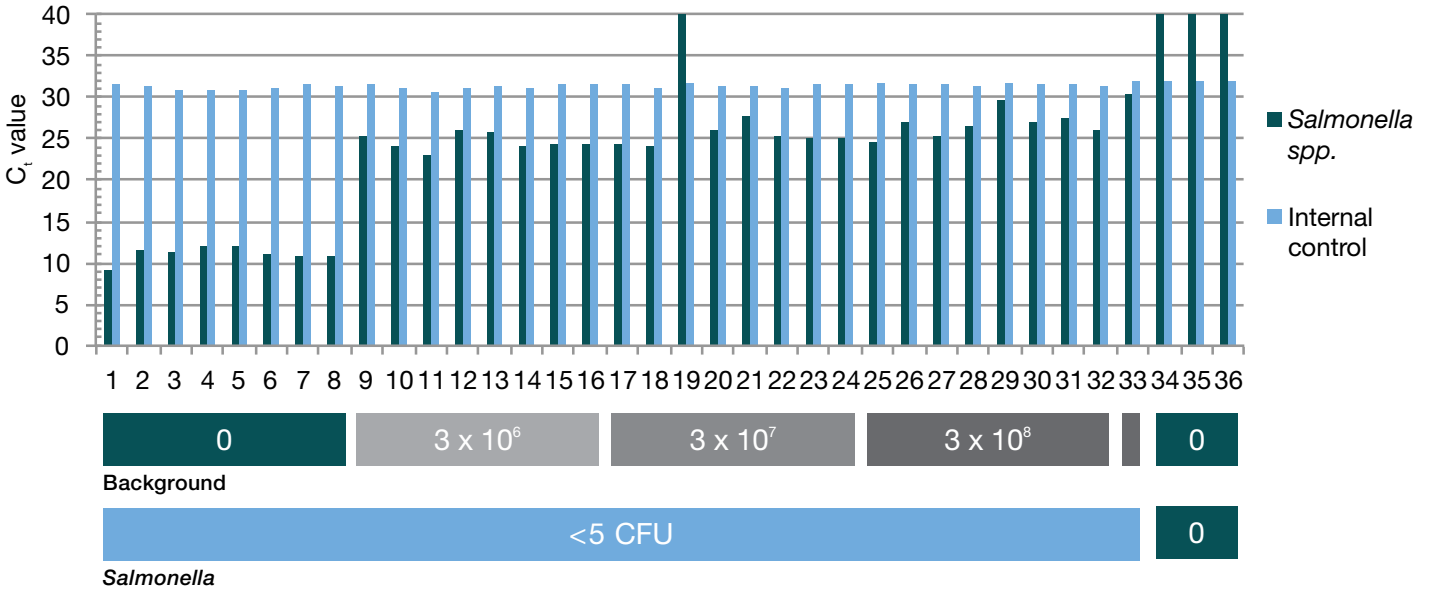

Figure 1. Detection of *Salmonella* in the presence of high concentrations of non-*Salmonella* bacteria.

Summary

Samples 1 – 33 were spiked with <5 CFU of *Salmonella* Poona, and samples 34 – 36 are the negative "no-spike" controls. The presence of *Salmonella* in all 36 samples was determined by both the MicroSEQ *Salmonella* spp. method and the US FDA BAM reference method. Sample 19 was negative for *Salmonella* by both the MicroSEQ and the FDA BAM method (a C_t value of 40 means no detection of *Salmonella* after 40 PCR cycles). The remaining 32 spiked samples were positive for *Salmonella* by both the MicroSEQ method and the FDA BAM method, and the negative control samples were negative by both methods. Thus, there was a perfect correlation between the MicroSEQ method and the FDA BAM reference method. For the real-time PCR method, lower C_t values correspond to higher concentrations of *Salmonella* target DNA, because fewer PCR cycles are needed for detection. Microbes common in the environment were added to samples 9 – 33, in the form of a mixture of *Enterobacter cloacae, Klebsiella pneumonia,* and *Citrobacter fruendii*. Samples 1 – 8 and 34 – 36 were not spiked with background microbial flora. Samples 9 – 16 were spiked with 3 x 10^6 CFU of background microbial flora, samples 17 – 24 were spiked with 3 x 10^7 CFU of background microbial flora, samples 25 – 32 were spiked with 3 x 10^8 CFU of background microbial flora, and sample 33 was spiked with 3 x 10^9 CFU of background microbial flora. The light blue bars show results from the internal positive control, which is consistent between all samples, demonstrating no inhibition of the PCR reaction.

The benefits of using real-time TaqMan PCR

PCR can detect trace amounts of microbes because PCR amplifies a genomic target exponentially prior to detection. In the case of real-time PCR the genetic target is detected coincident with amplification. The PCR reaction mix contains enzymes and reagents that double the concentration of a small genetic region of the microbe with each PCR cycle. A single PCR cycle consists of at least two temperatures, a lower temperature used to double the genetic target and the higher temperature used to separate the newly made DNA duplex so the two new strands are available for the next cycle. One PCR cycle is fast, typically 60°C for 30 seconds, followed by 95°C for three seconds. Within 45 minutes, a single genomic copy can be amplified with 40 PCR cycles to create more than 100 billion copies, assuming there were enough reagents available and the doubling reaction was 100% efficient.

Real-time PCR is an improvement over standard PCR for microbial detection. Real-time PCR doesn't require post-PCR manipulation of the sample and is therefore easier to perform and enables faster results. Furthermore, real-time PCR methods that utilize a TaqMan probe, such as the MicroSEQ and TaqMan assays, show increased specificity over standard PCR methods because real-time PCR requires hybridization of three oligonucleotides to the target DNA instead of the two oligonucleotides that are required for standard PCR. The forward and reverse oligonucleotides are required for both standard PCR and real-time PCR to amplify the target sequence. For real-time PCR a third oligo, the TaqMan probe, is needed to detect amplification and binds to a unique sequence between the forward and reverse oligos. The TaqMan probe is labeled with a fluorescent dye and quencher molecule that prevents fluorescent signal detection when no target is present. During PCR when the target is present, the quencher molecule and fluorescent dye are separated as part of the chemistry of the reaction, resulting in an increase in fluorescence. In the presence of the target microbe, the TaqMan probe releases light from fluorescence with each PCR cycle during amplification. Thus, the fluorescence increases exponentially together with amplification of the genomic target. It is fluorescence that is measured in real-time PCR.

Many real-time PCR instruments can detect multiple dyes individually as well as simultaneously in the same PCR reaction mix. This feature of real-time PCR allows detection of multiple microbial targets in a single reaction. The Applied Biosystems™ 7500 Fast Real-Time PCR System (Thermo Fisher Scientific) can detect up to five dyes in a single reaction. Our products incorporate an internal positive control to monitor for PCR inhibition and a normalization standard to correct for sample-to-sample variation. Thus, three dyes are available for detecting up to three separate microbial targets. For example, the TaqMan™ *Campylobacter* Multiplex Assay Beads detect and differentiate the three *Campylobacter* species, *C. jejuni*, *C. coli*, and *C. lari*, in a single reaction.

Helping you respond to a dynamic market

Thermo Fisher Scientific helps your lab adapt and respond to a dynamic and competitive market by providing end-to-end solutions for today and the future (Figure 2). We have designed and validated a number of different workflows for detecting *Salmonella* species in animal feed, raw poultry meat, and primary production samples, and for testing eggs and environmental samples for *Salmonella* Enteritidis (Table 2). These validated products are largely used by poultry producers for routine microbial testing required to meet regulatory requirements. In addition, we have

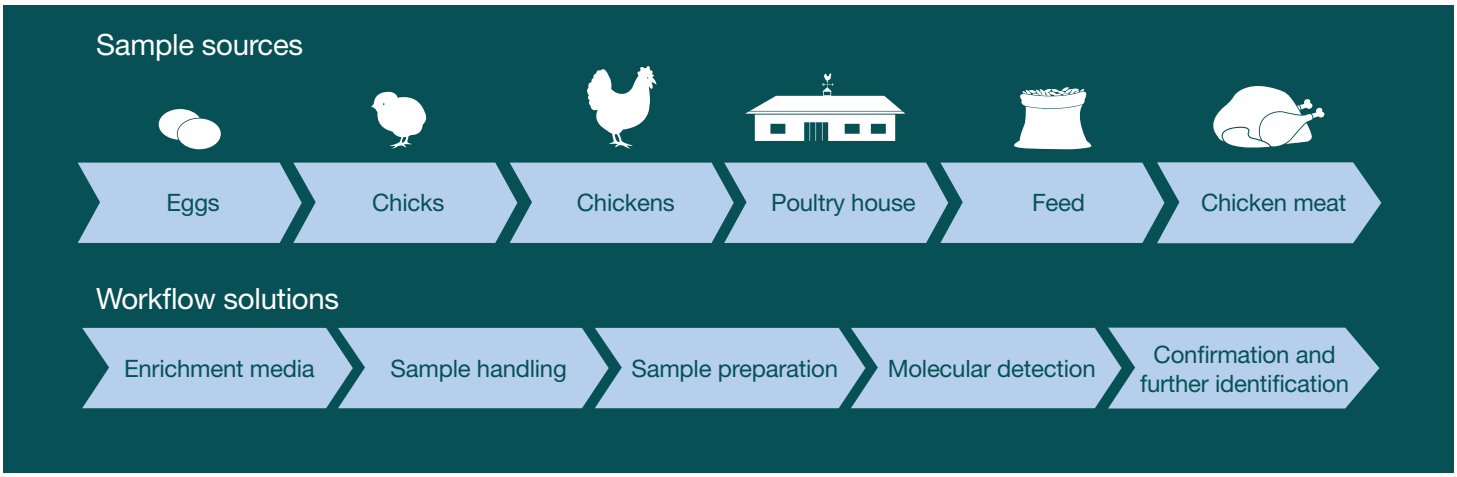

Figure 2. Thermo Fisher Scientific supports end-to-end testing solutions for the poultry industry.

Table 2. Validated poultry pathogen identification tests.
AFNOR = Association Française de Normalisation, AOAC = Association of Analytical Communities, DAFF = Department of Agriculture, Fisheries and Forestry, FDA = US Food and Drug Administration, NPIP = National Poultry Improvement Plan.

Detection kit	Sample sources	Validations	Cat. No.
TaqMan™ *Salmonella* Enteritidis	Egg pools, drag swabs	FDA, NPIP	4457030
MicroSEQ™ *Salmonella* species	Eggs, dry pet food, raw chicken wings, environmental surfaces (stainless steel, sealed concrete, plastic, ceramic tile, and rubber)	AOAC, DAFF	4403930
MicroSEQ™ *Salmonella* species	Meat products, egg products, feed products	AFNOR, NPIP	4403930
MicroSEQ™ *Salmonella* species	Primary production samples	AFNOR, NPIP	4403930
MicroSEQ™ *Listeria* species	Environmental surfaces (stainless steel, sealed concrete, plastic, ceramic tile, and rubber)	AOAC, DAFF	4427410

also developed detection assays against individual *Salmonella* serovars found in poultry and poultry environments that have been used to identify and track the presence of local and/or persistent contamination within a manufacturing facility (Table 3). Our solutions have shown high success rates even with very complex matrices such as poultry rinses, eggs, chick paper, feces, booties, swabs, etc.

We can also assist with onsite validation studies, and offer custom support if new assays or methods are needed to identify and track recurrent outbreaks within a facility. Our products and services include:

- Growth media
- Scientific instruments (real-time PCR, DNA and RNA sequencing, mass spectroscopy)
- Detection kits (real-time PCR kits optimized for pathogen detection)
- Sample preparation kits (multiple kits and automated high-throughput systems)
- Data analysis software (simplifies data analysis)
- Validated workflows (AOAC, AFNOR, DAFF, FDA, and NPIP approved Pathogen Detection Kits)
- Consumables (plastics, reagents, oligonucleotide synthesis, fluorescent dyes, etc.)
- Validation support services
- Custom sequencing including comparative genomics for *Salmonella* fingerprint and trace-back analysis
- Custom real-time PCR assay development
- Dedicated Technical Support

We have a long-established relationship with government regulators, including the USDA and the US FDA, as well as many academic leaders. The TaqMan™ *Salmonella* spp. Ultimate Assay (Cat. No. 4485049) was developed as part of a joint venture with the FDA. This multiplex assay was designed against two gene targets recommended by the FDA, namely apeE and invA, both of which are highly specific for *Salmonella*. The apeE target and the invA target are detected independently, each linked

Table 3. Other poultry pathogen identification tests.

Assay beads	Cat. No.
TaqMan™ *Salmonella* Typhimurium	4485053
TaqMan™ *Salmonella* Hadar	4485051
TaqMan™ *Salmonella* Heidelberg	4485050
TaqMan™ *Salmonella* Seftenberg	4485052
TaqMan™ *Salmonella* Newport	4485048
TaqMan™ *Salmonella* species and Enteritidis multiplex	4485086
TaqMan™ *Campylobacter* Multiplex (detects *C. jujeni*, *C. coli*, and *C. lari*)	4485027

Table 4. The TaqMan *Salmonella* spp. Ultimate Assay 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 detect any organisms in the SAFE exclusion panel. Parentheses in the table indicate the number of isolates within the panel.

Inclusion panel	*Salmonella* serotype		
SAR A (72 isolates)	Heidelberg (11) Muenchen (10)	Paratyphi B (22) Saintpaul (8)	Typhimurium (21)
SAR B (72 isolates)	Agona (1) Anatum (1) Brandenberg (1) Choleraesuis (4) Decatur (1) Derby (3) Dublin (3) Duisburg (1) Emek (1) Enteritidis (4) Gallinarum (1) Haifa (1) Heidelberg (2)	Indiana (1) Infantis (2) Miami (2) Montevideo (2) Muenchen (4) Newport (3) Panama (3) Paratyphi A (1) Paratyphi B (5) Paratyphi C (3) Pullorum (2) Reading (1) Rubislaw (1)	Saintpaul (2) Schwarzengund (1) Sendai (1) Seftenberg (1) Stanley (1) Stanleyville (1) Thompson (1) Typhi (2) Typhimurium (4) Typhisuis (2) Wien (2)
SAR C (16 isolates)	Typhimurium Typhi 58:d:z6 42:f:g,t:- *Salmonella arizonae* (2)	501,2,3:k:z 38[k]:z35 45a,b:g,z51:- 16:z4,z32:- *Salmonella bongori* (2)	45:a:e,n,x 11:b:e,n,x 1,40:g,z51:- 40:z4,z24:-
SAFE (101 isolates)	Newport Heidelberg Typhi 4,5,12:b:- Hadar Virchow Brandenburg II 58:l,z13,z28:z6 II 47:d:z39 II 48:d:z6 II 50:b:z6 II 53:lz28:z39 II 39:lz28: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:gz51:- 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 11: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 Typhimurium / DT104b Typhimurium / DT104 (3) I 4,[5],12:i:- (6 isolates) Enteritidis (4) Agona Alachua Brisbane Cerro Cubana Fresno Gera Inverness Javiana Johannesburg Michigan Montevideo Muenchen Muenster Rubinslaw Saphra Seftenberg Tornow Urbana Vietnam

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

to a different fluorescent dye. Inclusion/exclusion testing of the TaqMan Salmonella spp. Ultimate Assay demonstrated 100% detection of 261 Salmonella isolates tested with both gene targets, and no detection of 20 non-Salmonella isolates tested with either gene target (Table 4).

Our expertise in bioinformatics and next-generation sequencing were used for the recent development of a TaqMan™ *Salmonella* Seftenberg Assay (Cat. No. 4485052). Twenty poultry-derived *Salmonella* isolates were received from a collaborator and sequenced using the Ion Torrent™ PGM™ System. Draft genomes were assembled and several real-time PCR assays were designed and tested for sensitivity and specificity of the assays to *Salmonella* Seftenberg. One real-time PCR assay was selected and found to detect 100% of all 16 *Salmonella* Seftenberg isolates tested, with no detection of an exclusion panel consisting of 11 non-Seftenberg *Salmonella* isolates and other related pathogens.

Shortly after the US Food and Drug Administration announced new mandatory requirements for environmental testing of poultry houses for the presence of *Salmonella* Enteritidis in 2010, we worked with an academic collaborator to develop the TaqMan™ *Salmonella* Enteritidis Assay (Cat. No. 4457030). The real-time PCR workflow for detecting *Salmonella* Enteritidis in egg pools allowed next-day results comparable to the FDA BAM protocol, which requires 8–9 days for confirmed detection of *Salmonella* Enteritidis in egg pools (6 – 7 days for presumptive detection of *Salmonella* Enteritidis) (Figure 3). For *Salmonella* Enteritidis, the time-to-result for real-time PCR detection was 12.5% of the time required using traditional methods (see Table 1). The TaqMan *Salmonella* Enteritidis real-time PCR detection method workflow was certified by the US FDA and NPIP for detecting *Salmonella* Enteritidis in egg pools and environmental drag swabs. Tables 5 and 6 show the results from validation testing of the TaqMan *Salmonella* Enteritidis assay in egg pools and environmental drag swabs, respectively. The results from chi-square analysis on two independent experiments of each matrix demonstrated no outcome difference between the FDA BAM reference method and the TaqMan™ real-time PCR method. X^2 in Tables 5 and 6 is the result of a chi-square statistical comparison of the reference method with the MicroSEQ™ real-time PCR detection method. AOAC considers a chi-square result under 3.84 to indicate the two methods being compared are not statistically different.

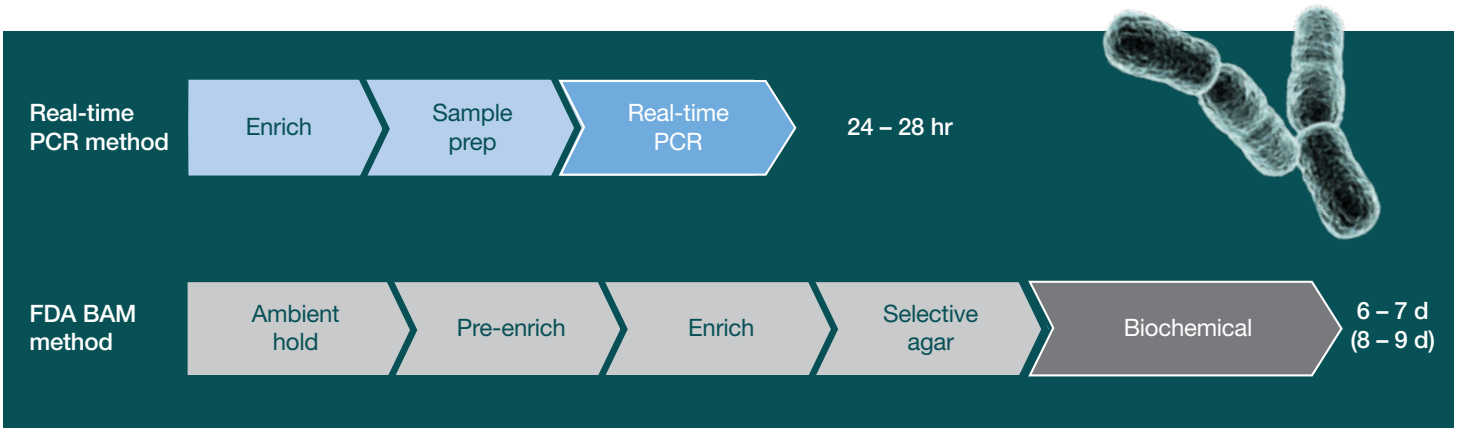

Figure 3. Comparison of the Thermo Fisher Scientific real-time PCR protocol for detection of *Salmonella* Enteritidis in whole-shell eggs and the FDA BAM protocol. The FDA BAM protocol is referenced in 21 CFR Parts 16 and 118: Federal Register Final Rule (July 9, 2009, 74 FR 33030): Prevention of *Salmonella* Enteritidis in shell eggs during production, storage, and transportation.

Table 5. Validation results for detecting *Salmonella* Enteritidis in whole-shell eggs. The TaqMan *Salmonella* Enteritidis real-time PCR detection method shows results equivalent to the US FDA BAM reference method for the detection of *Salmonella* Enteritidis in egg-pool samples.

TaqMan® *Salmonella* Enteritidis detection method versus US FDA BAM method								
Inoculation level	Inoculating organism	US FDA BAM	TaqMan *Salmonella* Enteritidis method		X^2	Relative sensitivity	False-negative rate	False-positive rate
			Presumed	Confirmed				
Experiment 1								
Control	NA	0/5	0/5	0/5	—	—	0%	0%
Spike	*S. enterica ser.* Enteritidis ATCC™ 13076	16/20	16/20	16/20	0	100%	0%	0%
Experiment 2								
Control	NA	0/5	0/5	0/5	—	—	0%	0%
Spike	*S. enterica ser.* Enteritidis ATCC™ 13076	11/20	13/20	13/20	0.41	118%	0%	0%

Table 6. Validation results for detecting *Salmonella* Enteritidis in environmental drag swabs from poultry houses. The TaqMan *Salmonella* Enteritidis real-time PCR detection method shows equivalence to the US FDA BAM reference method for the detection of *Salmonella* Enteritidis in environmental drag swab samples.

TaqMan® *Salmonella* Enteritidis detection method versus US FDA BAM method								
Inoculation level	Inoculating organism	US FDA BAM	TaqMan *Salmonella* Enteritidis method		X^2	Relative sensitivity	False-negative rate	False-positive rate
			Presumed	Confirmed				
Experiment 1								
Control	NA	0/5	0/5	0/5	—	—	0%	0%
Spike	*S. enterica ser.* ARS-12	9/20	12/20	12/20	0.88	133%	0%	0%
Experiment 2								
Control	NA	0/5	0/5	0/5	—	—	0%	0%
Spike	*S. enterica ser.* Enteritidis ATCC™ 13076	7/20	9/20	9/20	0.41	128%	0%	0%

A single platform for multiple tests

Traditional culture methods for microbial identification are complex workflows with significant differences between the methods, depending on the pathogens being analyzed. Each pathogen requires a different set of supplies for enrichment, selective plating, serotyping, and biochemical evaluations. Depending on the number of pathogens being screened and the level of screening being done, maintaining supplies for traditional testing can be a logistical challenge. Real-time PCR, on the other hand, is a single technology. The main differences between real-time PCR workflows for detecting different pathogens are the primary enrichment conditions and the choice for sample preparation (several choices are available). For real-time PCR, the pathogen detected is determined by the real-time PCR detection kit used (a single reagent).

Notably, different pathogen detection kits can be used during the same real-time PCR run. The analyst chooses which samples to load onto the instrument and is not limited to a specific pathogen type. In other words, different samples can be screened for the presence of *Salmonella* species, *Listeria monocytogenes*, and *E. coli* O157:H7 on the same instrument run.

The 7500 Fast Real-Time PCR System is an open system. This means an analyst can run real-time PCR on the 7500 Fast PCR instrument with our test kits, or with a custom real-time PCR detection kit or homebrew assays.

Conclusion

In an industry of ever-changing regulations and complex poultry samples, you need a partner that can adapt to your needs and empower your operations. With proven solutions for every step of the test workflow, Thermo Fisher Scientific brings together the best from sample preparation to identification for pathogens, and more. Choose from manual or automated solutions designed to deliver accurate results and maximize productivity.

Find out more at **thermoscientific.com/poultry**

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