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# Molecular Technologies for *Salmonella* Detection

Robert S. Tebbs, Lily Y. Wong, Pius Brzoska and Olga V. Petrauskene
*Life Technologies, Foster City, CA*
*USA*

## 1. Introduction

*Salmonella* has been associated with some of the most devastating foodborne outbreaks in recent history. *Salmonella* outbreaks have been linked to a variety of foods including produce [Alfalfa Sprouts- 2009, 2010, 2011; pistachios-2009; cantaloupes-2008, 2011 etc.], processed foods [peanuts – 2009], and prepared foods [turkey burgers- 2011, Banquet Pot Pies – 2007]. The contamination of commercial shell eggs with *Salmonella* Enteriditis in 2010 led to the recall of over a half a billion eggs, and the contamination of peanut-containing products with *Salmonella* Typhimurium in 2008-2009 led to one of the largest recalls in U.S. history with over 3,900 products being recalled. The Peanut Corporation of America, responsible for the *Salmonella* outbreak in peanuts, was forced into bankruptcy. Multiple lawsuits were filed against Wright County Egg and Hillandale Farms responsible for the *Salmonella* outbreak in eggs. Despite their own internal testing which showed *Salmonella* contamination, these facilities still shipped product. The 2008 outbreak of *Salmonella* in jalapeno peppers resulted in 1442 persons infected with *Salmonella* Saintpaul across 43 states, the District of Columbia, and Canada. Unfortunately, the tomato industry was implicated early in the investigation, which resulted in economic losses to the tomato industry in hundreds of millions of dollars. Because *Salmonella* is widespread in the environment (in such places as chicken houses), vegetable plants and animals (as well as meat samples, eggs etc.), rapid, reliable, and validated pathogen detection methods are needed for use in production facilities, public health labs, as well as in the regulatory and monitoring agencies. To provide comprehensive rapid food testing solutions, all components of a pathogen detection system should be addressed: sample preparation, detection and data analysis.

Fluorescent quantitative real-time PCR is the most sensitive method for detection, monitoring and measurement of pathogen levels. The method also can be used for strain identification based on single-nucleotide polymorphism detection. A key element in designing PCR assays is an algorithm to select primers and probes because they define accuracy – specificity and inclusivity of the PCR tests. The ability to design highly specific assays becomes easier as the number of bacterial genomes added to the public domain increases.

There are a number of sample preparation methods available that are fast and easy for PCR-based pathogen detection using both low throughput (manual) and high throughput (automated) methods.

A multiplex (multi-color) real-time PCR analysis, if designed correctly, provides simultaneous and specific detection of a number of pathogens in the same reaction and can save time and money. In addition to multiplexing, other technologies improve operator experience such as lyophilized configuration and fast cycling format.

Effective data analysis software can significantly improve test workflow as well as accuracy of the presence or absence calls. Software packages can simplify analysis by displaying results graphically to make the system fail-proof even for novice users.

To further characterize detected *Salmonella* species, isolates can be sequenced using modern whole genome sequencing platforms.

## 2. Genetic methods complement biochemical and phenotypic analyses

*Salmonella* serotypes are classified by the Kauffmann-White-Schema which is maintained by the WHO and the Collaborating Center for Reference and Research at Institute Pasteur. There are only two species *Salmonella enterica,* which is associated with human infections, and *Salmonella bongori,* which is mainly found in lizards. This schema was based mainly on DNA analysis and bonified by the judiciary of bacterial nomenclature (Center for Disease Control, 2004). Under the schema, there are six subspecies. The serotypes are I: *Salmonella enterica* subsp enterica, II: *Salmonella enterica* subsp. Salamae, III: *Salmonella enterica* subsp. Arizonae, IIIb *Salmonella enteric*a subsp. Diarizonae, IV: *Salmonella enteric*a subsp. Houtenae and VI *Salmonella enterica* susp. Indica. *Salmonella* serotyping is traditionally based on immunoreactive antibodies against the O and H antigens. Different classification schemes based on phylogenetic analyses of 16S and housekeeping genes have been proposed (Boyd et al., 1996; Tindall, 2005).

Classification of bacteria is traditionally based on immunogenic and metabolic behavior. Analysis of bacterial genomes however led to reclassification and debates on the taxonomical classification. For example, Pupo et al. (2000) studied the phylogenetic relations of several housekeeping genes and the O-antigen of species of the genus *Shigella* and concluded that several species of *Shigella* are clusters of *Escherichia coli*.

The nomenclature change in *Salmonella* was subsequently supported by genomic evidence as well. McQuiston et al. (2008) showed that a set of four housekeeping genes supports the *Salmonella* classification, and microarray analysis of the gene homologues in *Salmonella* result in a similar grouping (Porwollik et al., 2002). More recently, whole genome sequencing of *Enterobacter sakazakii* isolates revealed that this group is phylogenetically different from other *Enterobacter* species and was renamed to *Cronobacter sakazakii* (Iversen et al., 2008). The breakout of *Cronobacter* was supported by biochemical and microarray analyses (Healy et al., 2009).

Since the completion of the first bacterial genome of Hemophilus influenza in 1995 (Fleischmann et al., 1995), more than 1000 bacterial genomes have been completely sequenced. Currently, 21 serovars of *Salmonella enterica* subsp. enterica have been sequenced as well as *Salmonella bongori*. Many shotgun sequencing projects are still in progress, and the number of genomes will continue to increase. New metrics for taxonomical evaluation based on complete genomes have been proposed (Kunin et al., 2005). Complete genomic information does not change the phylogeny based on 16S and

MLST substantially (Coenye & Vandamme, 2003), but it allows the study of specific genes present and absent across phylogenetic groups.

## 3. Sample preparation for real-time PCR detection of *Salmonella*

The successful detection of pathogenic organisms by genetic methods requires microbial lysis to release nucleic acids and efficient removal of inhibitors. Sample preparation can also serve to concentrate nucleic acids for improved sensitivity. Food and environmental samples create unique challenges for sample preparation due to the heterogeneous nature of the different matrices. The method used must account for the type and amount of organism to be lysed, the sample matrix, and the user's needs and limitations (cost, ease-of-use, time-to-results, sample throughput and capacity, and multi-functionality).

Samples that contains inhibitory compounds can lead to partial or complete inhibition of PCR. Food and culture media both contain components that can inhibit PCR (Rosen et al., 1992; Andersen & Omiecinski, 1992; Atmar et al., 1993; Demeke & Adams, 1992; Lofstrom et al., 2004) (for a review, see Wilson, 1997). PCR inhibitors originating from the food samples include humic acid from soil (Tsai & Olson, 1992a; Tsai & Olson, 1992b), proteins and aminoglycans from animal samples such as hemoglobin, lactoferrin and heparin (Al-Soud & Radstrom, 2001), polysaccharides from plant material (Demeke & Adams, 1992; Monteiro et al., 1997), melanin from hair and skin (Eckhart et al., 2000), etc. Media including modified Rappaport broth and phosphate buffered saline can inhibit PCR (Rossen et al., 1992). PCR can also be inhibited by contaminants from the nucleic acid extraction phase including ionic detergents (Weyant et al., 1990), phenol, ethanol, proteinase K, guanidinium, and salts (Al-Soud & Radstrom, 2001).

The control of PCR inhibition can be addressed on several fronts. Inhibitory effects can be minimized by optimizing the PCR mix. Bovine serum albumin (BSA) was shown to reduce PCR inhibition by humic acid (McGregor et al., 1996) and hemoglobin (Al-Soud & Radstrom, 2001), possibly through direct interaction with the inhibitory components such that they cannot interfere with PCR amplification. The single-stranded DNA binding protein from bacteriophage T4 (gp32) also reduced PCR inhibition caused by hemoglobin (Al-Soud & Radstrom, 2001). The addition of Tween® 20 or DMSO reversed PCR inhibition from low concentrations of the polysaccharides dextran sulfate and gum ghatti (Demeke & Adams, 1992). Inhibitors that affect polymerase activity can be partially mitigated by increasing the polymerase concentration. PCR kit manufacturers often develop proprietary formulations to optimize PCR through design of experiment (DOE) studies. For example, Environmental Master Mix version 2 (EMMv2) was developed by Applied Biosystems specifically for complex samples containing potential inhibitory components (Figure 1). PCR inhibition can be monitored using an internal positive control (IPC) (Tebbs et al., 2010). Samples that show no amplification of target and IPC either contain inhibitors or the PCR reaction was improperly prepared. Technical errors are greatly minimized with new lyophilized formulations that only require the addition of sample. Amplification of the internal control gives confidence that a negative result is not due to inhibition. A simple mitigation to inhibited samples is dilution (Tsai & Olson, 1992a; Tsai & Olson, 1992b). For samples in which the target DNA is not the limiting factor, inhibitors can be diluted below their effective threshold to allow for PCR amplification. In addition, efficient DNA extraction following bacterial enrichment removes PCR inhibitors and improves accurate detection.

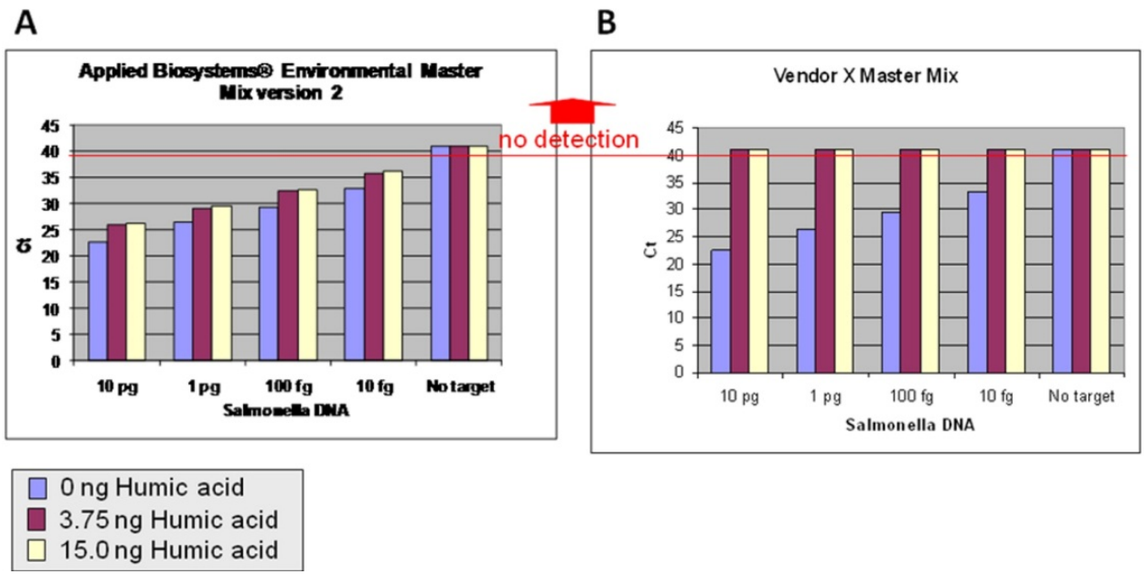


Fig. 1. Real-time PCR Master Mixes can mitigate PCR inhibition. (A) Environmental Master Mix version 2 shows detection of *Salmonella* DNA in the presence of 3.75 ng and 15.0 ng of humic acid. (B) Master mix from another source shows complete inhibition in the presence of the same amount of humic acid. Real-time PCR was performed on the 7500Fast instrument.

Food borne pathogens are usually present in small quantities in food and therefore require enrichment to detect their presence. The United States requires testing for selected pathogens in the nation's food supply. This is true for *Salmonella* species in foods such as ready-to-eat products and whole shell eggs. A standard practice for screening food for regulated pathogens is to mix 25 grams of food with 225 mL of broth (1:9 food to broth ratio). Reference methods are based on traditional culture procedures and typically use a 2-step enrichment procedure, first in non-selective broth (pre-enrichment) and second in selective broth, prior to biochemical and serological characterization. These protocols are designed to detect down to a single viable organism. The pre-enrichment step allows recovery of injured or otherwise weakened *Salmonella*, whereas selective enrichment favors growth of *Salmonella* over background flora that competes with *Salmonella* for available nutrients. The U.S. FDA *Bacteriological Analytical Manual* (BAM) for *Salmonella* pre-enriches in different broths depending on the food matrix (typically lactose broth or tryptic soy broth), followed by selective enrichment in Rappaport-Vassiliadis (RV) medium and tetrathionate (TT) broth (Andrews & Hammack, 2011). Pre-enrichment is for 24 ± 2 h at 35°C, and selective enrichment is for 24 ± 2 h at 42°C for RV and 35°C (food with low microbial load) or 43°C (food with high microbial load) for TT. The U.S. FDA protocol for sampling and detecting *Salmonella* in poultry houses pre-enriches environmental samples in buffered peptone water (24 ± 2 h at 35°C) and then selectively enriches in RV (24 ± 2 h at 42°C) and TT (24 ± 2 h at 43°C) (Food and Drug Administration, 2008). The International Organization for Standardization (ISO) reference method for the detection of *Salmonella* in food (ISO 6579:2002(E)) recommends pre-enrichment in buffered peptone water (other broths are necessary for some food types) for 18 ± 2 h at 37°C followed by selective enrichment in Rappaport-Vassiliadis medium with soya (RVS broth) for 24 ± 3 h at 41.5°C and Muller-Kauffman tetrathionate/novobiocin broth (MKTTn broth) for 24 ± 3 h at 37°C

(ISO 6579:2002 [E]). The enrichment time alone for these traditional culture methods totals 2 days. Following enrichment, the samples are plated to selective agar plates for 24-48 h growth and then transferred to slants for another 24 h growth. Presumptive detection of *Salmonella* is determined in 4 to 5 days (BS plates are left for 48 ± 2 h for BAM method).

Food producers desire faster time-to-results since it provides great cost benefits. The Food and Safety Inspection Service (FSIS) requests all meat and poultry products be held by producers until test result indicate no pathogen is present. The FSIS recently proposed new regulations requiring test results be received before meat and poultry can be shipped, the so called "Test-and Hold" policy (Department of Agriculture, 2011). To the producer, storing product is not only costly but also shortens the product shelf life.

Rapid methods can shorten time-to-results to less than 24 h for *Salmonella*. To be adopted by the food industry, new methods must undergo AOAC validation to demonstrate equivalency to reference culture methods. The AOAC developed the Performance Tested Methods℠ (PTM) program for the purpose of certifying commercial test kits (AOAC Research Institute website). Even with AOAC validation demonstrating equivalency, the FDA BAM considers positive results from rapid methods to be presumptive that must either be confirmed by culture or accepted as true positive (negative samples are accepted as true negative). Rapid methods are fast because detection is immediate, but also because enrichment requirements are usually shorter. Most PCR methods only require a single-step enrichment to demonstrate equivalency to reference standards. Well designed PCR assays can detect a single genomic copy of *Salmonella* and thus the limit of detection for PCR is largely determined by sample preparation.

Multiple methods have been used to lyse pathogens including physical, chemical and enzymatic, or combinations of the three (Table 1). Common physical methods include temperature (freeze/thaw or heat), bead-beating, and sonication. Freeze/thaw lysis is a traditional method in which the cellular suspension is transferred between freezing and warm conditions, for example between a dry ice-ethanol bath and a 37°C water bath. During the freeze cycle ice crystals cause cells to expand and rupture. Multiple freeze/thaw cycles are required for efficient lysis which makes the procedure rather lengthy and is usually only associated with "home brew" methods. Microbial cells can also be lysed by heating at 95°C to 100°C. The lysis efficiency of heat is dependent on the microorganism, but is generally poor. Heat is often combined with chemical and/or enzymatic treatment to increase the lysis efficiency which is discussed later. If enzymatic treatment is used, then the heat step serves two functions, it can break open cells and inactivate enzymatic activity. Protease is common for bacterial lysis and if used must be inactivated before adding sample to a PCR mix since proteases will destroy DNA polymerase. Sonication uses high frequency sound waves to create localized regions of low pressure resulting in micro bubbles that rapidly form and implode, ultimately breaking open cells. Bead-beating is another physical method used for breaking open cells. Typically an equal volume of silica or zirconium beads (approximately 0.1 mm diameter) are combined with a sample and mixed on a laboratory vortex. Lysis is complete in 3-5 minutes. Bead-beating has a tendency to generate foam which can be controlled by using anti-foam agents. Bead-beating and sonication can result in greatly fragmented, low molecular weight nucleic acids. However, fragmentation is of minor concern for real-time PCR since assays are designed to amplify small fragments of typically less than 100 base pairs.

| Lysis Method | Category | Product | Notes | Test Kit Name | Manufacturer |
|---|---|---|---|---|---|
| Bead-Beating/ Chemical/Filter | Manual | DNA | | IT 1-2-3™ Sample Purification Kits | Idaho Technology |
| Chemical | Automated | Lysate | DNA Hybridization | GeneQuence® *Salmonella* | Neogen |
| Chemical/ Enzyme/ Heat | Simple high throughput | Lysate | 2-step enrich (except meat and poultry) | BAX® System PCR Assay *Salmonella* | DuPont Qualicon |
| Chemical/Heat | Simple | Lysate | | PrepSEQ® Rapid Spin Sample Preparation Kit | Life Technologies |
| Chemical/Heat | Simple | Lysate | 2-step enrich | Foodproof® ShortPrep I Kit | BIOTECON Diagnostics |
| Chemical/Heat | Simple | Lysate | | iQ-Check Salmonlla II Easy Extraction I | Bio-Rad |
| Chemical/Heat | Simple high throughput | Lysate | 96-well Deepwell centrifugation | iQ-Check® *Salmonella* II Deepwell protocol | Bio-Rad |
| Chemical/Heat/ Magnetic beads | Automated | DNA | | Foodproof® Magnetic Preparation Kit I | BIOTECON Diagnostics |
| Chemical/Heat/ Magnetic beads | Semi-automated | DNA/ RNA | | PrepSEQ® Nucleic Acid Extraction Kit | Life Technologies |
| Chemical/Heat/ Filter | Manual | DNA | | Foodproof® Sample Preparation Kit | BIOTECON Diagnostics |
| Chemical/Heat/ Filter | Manual | DNA | | Biotest MMB Prep *Salmonella* | Biotest AG |
| Chemical/Heat/ Filter | Manual | DNA | | SureFood® Prep *Salmonella* | Congen Biotechnologie |
| Sonication/Heat | Automated | DNA | | GeneDisc® *Salmonella* | Pall Corp. |

Table 1. Sample preparation kits for detecting *Salmonella* by genetic methods. The kits included were chosen from the AOAC Research Institute online website of Performance Tested Methods℠ Validated for detection of *Salmonella* using genetic methods (http://www.aoac.org/testkits/testedmethods.html). Details for sample preparation were obtained from readily available information from company websites and might not be part of the AOAC approved workflow. AES and BioControl have AOAC validation for real-time PCR detection of *Salmonella,* but were omitted from the current list since details of their sample preparation methods were not available on their website.

Many chemicals have been used in cell lysis. The most common chemicals are detergents. Detergents disrupt the lipid bilayer surrounding cells. There are many types of detergents of varying strengths. Ionic detergents such as sodium dodecyl sulfate (SDS) are stronger than nonionic or zwitterionic detergents and are often employed for microbial lysis to extract DNA. In addition to disrupting lipids, SDS has the advantage of denaturing proteins, including DNAse and RNAse, and thus protecting nucleic acid during extraction. Because detergents cannot lyse cell walls, bacteria are often pre-treated with enzymes (proteases or lysozyme) before addition of detergent. Chaotropic salts are also commonly used for bacterial lysis. Guanidinium thocyanate and guanidinium chloride lyse cell membranes by denaturing proteins. Chemicals used in bacterial lysis are by nature hazardous and must be disposed of as hazardous waste.

The use of magnetic particles for sample preparation has increased in recent years. Imunomagnetic separation (IMS) uses metal beads coated with antibodies specific to the target microbe of interest. A magnet can then be used to attract the bead containing the target microbe. The beads can be washed, and the presence of the target organism can be determined by plating onto selective agar, PCR, or other detection methods. Another solution is to use solid phase capture in which antibodies are linked to a solid support. The target microbe can be captured as sample passes across the support. For example, antibodies linked to a pipette tip can capture the target organism by collecting sample with a pipetting device. In theory, the target analyte binds while unwanted material passes through. The analyte can be further purified through a series of wash steps. In practice, antibodies typically show background capture of non-target organisms (Fratamico et al., 2011), and therefore the use of IMS is often combined with a detection method such as PCR or biochemical characterization. Furthermore, the sample matrix (i.e. high fat content) can affect binding of an antibody to its antigenic substrate (Bosilevac et al., 2010; Fitzmaurice, 2006). Both false positive and false negative results have been reported for antibody-based methods. Both immunomagnetic beads and solid support systems have been used in combination with antibodies or phage-binder proteins to capture microbes. Phage-binder proteins are the proteins responsible for phage binding to host bacteria and are very host specific. Both technologies (beads and solid support) have also been used to capture nucleic acids from microbial lysates. Metal beads coated with silica and glass fiber filters are commonly used to capture nucleic acids. DNA binds under conditions of salt and high alcohol, and is eluted with aqueous solutions. Heating the elution buffer can improve recovery by increasing the ability of the nucleic acid to dissolve in solution. The advantage of these systems is increased target concentration and improved target purity without the need for centrifugation and aspiration—methods that are difficult to automate. The use of magnetic beads to capture nucleic acids eliminates the concern associated with antibody specificity since total DNA and RNA can be captured indiscriminately. The same is true for purification columns that bind nucleic acids to silica membranes. The specificity of the assay is determined by the detection method. The binding capacity for nucleic acids can be much higher for magnetic particles compared to that of standard spin columns. The disadvantage with the capture of total nucleic acids is the potential for the capture of large amounts of non-targeted DNA relative to target DNA.

There are ways to simplify sample preparation. How to simplify depends on the needs of the end-user. If obtaining results within an 8-hour work shift is critical, then simplification

becomes more challenging. Quick time-to-results requires concentrating the microbe from large sample volumes in combination with efficient microbial lysis and DNA/RNA recovery. To meet this demand sample preparation becomes more complex. Automation can simplify the process, but adds additional costs for equipment. A common practice is to use magnetic particles to capture microbes or microbial nucleic acid since magnetic particles can be easily added to and extracted from an aqueous mixture. Furthermore, procedures using magnetic particles are simple to automate. Instruments can be designed to dispense and aspirate liquids, but instruments that perform these functions are typically more complex.

Increased enrichment times have the advantage of increasing the concentration of the microbe which can greatly simplify sample preparation (Figure 2). Enrichment also dilutes dead cells that could be present in the sample matrix. A 2-step enrichment method (e.g. pre-enrichment followed by selective enrichment) will also dilute the sample matrix which can reduce the impact of inhibitory substances associated with food and environmental samples, thus creating a more consistent sample for sample preparation. Double-enrichment has not found favor in molecular methods that are expected to be rapid. Indeed, PCR is very sensitive and double-enrichment is excessive for most applications. Most food and environmental samples can be enriched overnight (16-18 h) in one broth to allow *Salmonella*, if present, to grow to concentrations that are above the detection limit of PCR even when simple sample preparation methods are used.

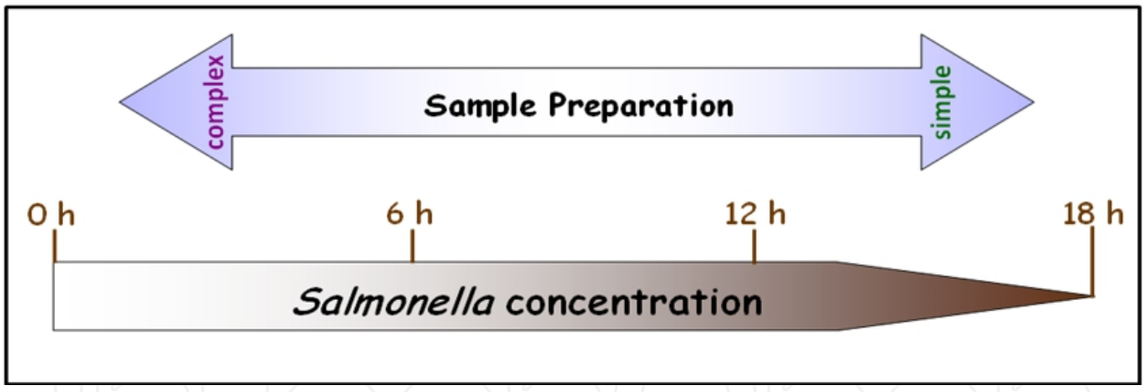


Fig. 2. Increasing *Salmonella* enrichment time can simplify sample preparation. Increased enrichment time increases *Salmonella* concentration requiring less volume and lower efficient sample preparation.

The simplest sample preparation methods dilute enriched samples into a solution that is compatible with the detection method. Indeed, many Gram-negative bacteria are lysed by boiling in water for 10 minutes. Because PCR amplification begins with a denaturation step (typically 95°C for 10 minutes) it is theoretically possible to add diluted sample directly to PCR. However, this doesn't seem to work well for many samples. Some level of lysis prior to adding to the PCR mix improves detection. For example, the addition of a simple 10 minute boiling step prior to setting up a PCR greatly improves detection. It is likely that boiling denatures enzymes and degrades substrates that disrupt the PCR reaction. A simple sample preparation method is shown in Figure 3.

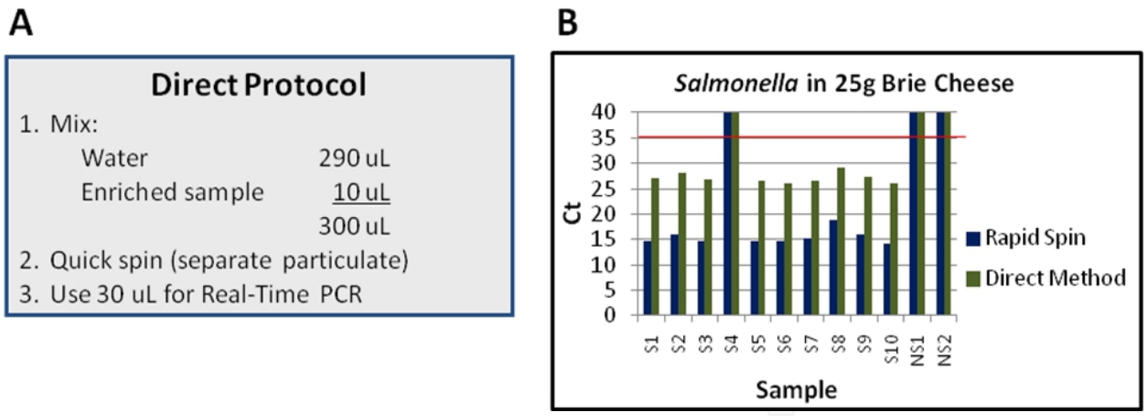


Fig. 3. Simple sample preparation for real-time PCR detection of *Salmonella*. (A) According to the direct protocol, sample is diluted in water and briefly centrifuged for 10 to 15 seconds in a table-top centrifuge. (B) Twenty-five grams of Brie cheese was spiked with 1-3 CFU *Salmonella enterica* serotype Typhimurium (strain Q210) and enriched at 37°C for 16 hours in buffered peptone water. The Direct Method showed a >10 Ct difference compared to the PrepSEQ® Rapid Spin method, but there was 100% correlation for detection of *Salmonella* between the two methods. Sample 4 (S4) which also received the spike gave negative results by both sample preparation methods, demonstrating all samples were spiked with low concentrations of *Salmonella* (i.e. fractional positive spike). Ten Cts correspond to a 1000-fold dilution in available DNA. Samples were analyzed on the 7500 Fast instrument using the MicroSEQ® *Salmonella* spp. Detection Kit.

## 4. Genetic-based methods for detection of *Salmonella* in foods

Genetic methods involve specific detection of RNA or DNA sequences to determine presence of the pathogen. There are a number of available kits in the market that apply genetic methods for the detection of *Salmonella* (Table 2). The most common genetic detection methods are PCR-based technologies. In the simplest form, conventional PCR involves amplification of a target DNA sequence using primers. The reaction is cycled between denaturing and annealing temperatures and may include a specific temperature for extension. The reaction generates an amplicon which can be detected on an agarose gel when stained with an intercalating dye such as ethidium bromide. The amplicon must be of the expected size for the sample to be called positive.

A DNA-binding dye, such as SYBR® Green, can be added to PCR and monitored by a real-time PCR instrument. SYBR® Green preferentially binds double-stranded DNA resulting in a DNA-Dye complex that shows a unique absorbance and emission spectrum. SYBR® Green dye will detect PCR products as they are amplified. Highly specific primer designs are required to avoid false positives because SYBR® Green dye will bind to all double-stranded DNA including any mis-primed products. Melt curve analysis of the PCR products of SYBR® Green dye reactions can be added to the end of a real-time PCR run to collect melting temperature (Tm) data of the PCR products amplified. This additional layer of data provides another check that the product amplified is of the expected Tm which is indicative of the fragment length. There are numerous examples of SYBR® Green PCR assays used to detect *Salmonella* in food or environmental samples (Nam et al., 2005; Techathuvanan et al., 2011).

| Method | Test Kit Name | Manufacturer |
|---|---|---|
| PCR/melt curve analysis | BAX® System for *Salmonella* | DuPont Qualicon |
| Real-time PCR | Assurance GDS® for *Salmonella* | BioControl Systems |
| | Foodproof® *Salmonella* Detection Kit | BIOTECON Diagnostics |
| | GeneDisc® *Salmonella* | Pall Corp. |
| | iQ-Check® *Salmonella* II Kit | Bio-Rad |
| | MicroSEQ® *Salmonella* spp. Detection Kit | Life Technologies |
| | *Salmonella* species LT Test Kit | Idaho Technology |
| | SureFood® *Salmonella* PLUS V | Congen |
| DNA hybridization | GeneQuence® *Salmonella* | Neogen |
| NASBA | Nuclisens EasyQ® Basic Kit* | bioMerieux |
| LAMP | LoopAmp® DNA or RNA Amplification Kit* | Eiken Chemical Company |

* General reagent kit which is not *Salmonella* specific.

Table 2. Commercial kits utilizing genetic methods to detect *Salmonella*.

Using fluorogenic probes with real-time PCR has rapidly become the standard for genetic detection because of its high specificity and sensitivity to detect low copy numbers. Many real-time instrument platforms have the ability to complete a run under one hour because of fast temperature ramping and improvements in master mix chemistries. TaqMan® assay uses target-specific primers and a probe that is labeled with a fluorescent reporter dye on the 5′ end and a quencher dye on the 3′ end (Tebbs et al., 2009; Balachandran et al., 2011). The probe anneals to the target DNA sequence between the two primer sites. As the primer extends during each cycle of the PCR, the 5′ nuclease activity of Taq polymerase displaces the probe from the DNA strand, separating the reporter dye and quencher dye in the process, and fluorescent signal is emitted. An example of a real-time assay which detects *Salmonella* Typhimurium is shown in Figure 4. Details of TaqMan data analysis will be further discussed.

An alternative fluorescent probe is the molecular beacon. The stem-and-loop structure of a molecular beacon probe consists of a target-specific sequence (which forms the loop) and non-target sequences that are complementary at the 5′ and 3′ end of the probe (forming the stem). When the probe is in a closed loop shape with the 5′ and 3′ ends hybridized to one another, the fluorescent reporter dye is quenched. When the molecular beacon probe hybridizes to the amplicon during PCR, the stem-and-loop structure opens, separating the fluorophore from the quencher releasing fluorescence. The application of molecular beacons

in *Salmonella* detection in foods has been tested in a variety of food matrices (Bhaqwat et al., 2008; Patel & Bhagwat, 2008; Liming & Bhagwat, 2004).

Another variation of real-time PCR employs Scorpions technology. Scorpions are PCR primers covalently linked to a probe (Carters, et al., 2008). The reporter dye on the probe is prevented from fluorescing by a quencher dye on a separate complementary oligo. Upon primer extension, a probe-binding sequence is created which allows the probe to bind intramolecularly and generate fluorescence.

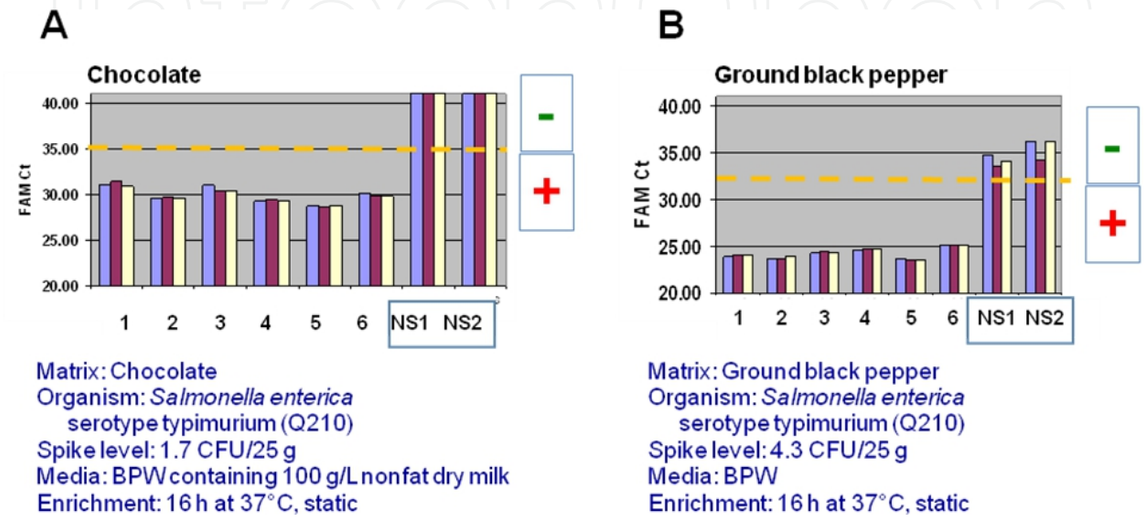


Fig. 4. Real-time PCR detection of *Salmonella.* 25 g of chocolate (A) 25 g of chocolate or (B) 25 g of black paper spiked with *Salmonella* were enriched for 16 hours. Six samples were spiked with 1-5 CFU *Salmonella* and 2 samples were not spiked with *Salmonella* (NS1 and NS2). Samples were prepared by the PrepSEQ® Rapid Spin Sample Preparation kit (in triplicate), and analyzed on the 7500Fast instrument using the MicroSEQ® *Salmonella spp.* Detection Kit.

RNA can also serve as the initial template for PCR for *Salmonella* detection. Detecting *Salmonella* RNA can serve as an indicator of viability of the bacteria (González-Escalona et al., 2009). RNA transcripts are likely present in higher copies than genomic DNA which can increase the sensitivity of the assay. In reverse transcription-PCR (RT-PCR), RNA is first converted to DNA by reverse transcriptase, and then PCR amplification occurs using the newly created DNA strands.

Alternative methods to real-time PCR have also recently emerged. The principle difference of these methods from PCR is that they use different approaches for generating new DNA or RNA with each cycle of amplification. In standard PCR, a denaturation step creates new DNA strands for DNA amplification to occur, theoretically doubling DNA template with each cycle. In contrast, loop-mediated isothermal amplification (LAMP) technology uses auto-cycling strand displacement DNA synthesis to create new DNA template. A *Bst* DNA polymerase large fragment with high strand displacement activity is added to the reaction. There are two general stages of LAMP: generation of template from the input sample and cycling amplification (Tomita et al., 2008). Typically, four primer sets are used to target six independent sequences flanking the target DNA. An inner primer hybridizes to the target DNA and elongates. This is followed by strand displacement which is primed by an outer

primer; in this step, the outer primer hybridizes to the target DNA, displacing the newly created single strand DNA. Because each inner primer consists of a 5′ overhang that becomes self-complementary to a sequence as the primer extends, the newly created single strand DNA forms a structure that has loops at each end. This DNA with stem-loop structure is the template of LAMP cycling. During LAMP cycling, the inner primer initiates auto-strand displacement with the template; also, self-priming occurs within the template. In short, the products are multiple stem-loop structures and elongated products containing the target sequence. LAMP reaction occurs at a constant temperature, typically 60-65 °C, and can be carried out in a water bath or heat block. Because a tremendous amount of DNA is formed in the reaction, the reaction by-product magnesium pyrophosphate forms a precipitate. The turbidity can be visible to the naked eye or visualized by UV after the addition of a fluorescent dye. The DNA products from LAMP can also be detected using a real-time turbidimeter. Application of LAMP in detection of *Salmonella* species in foods has previously been demonstrated (Ueda & Kuwabara, 2009). Another alternative method to real-time PCR is nucleic acid sequence-based amplification (NASBA) which amplifies RNA and creates new RNA strands by addition of a promoter site to complementary DNA. The isothermal method, which is typically run at 41 °C, first converts RNA into DNA using reverse transcriptase, then hydrolyzes RNA from the RNA-DNA hybrid using Rnase H. A target-specific primer, with a T7 promoter sequence at its 5′ end, hybridizes to the single-stranded DNA. T7 RNA polymerase binds to the promoter region of the newly created double-stranded DNA to synthesize new RNA templates. Detection of the RNA product from NASBA can be accomplished by DNA hybridization followed by electrochemiluminescence. NASBA has previously been used to detect *Salmonella* Enteritidis in foods (D'Souza & Jaykus, 2003). Although there is no AOAC certified kit specifically for *Salmonella* detection by LAMP or NASBA, there are commercially available kits to perform these types of detection.

DNA hybridization is another useful way to detect target sequences of *Salmonella*. Labeled single-stranded DNA probes are added to a sample to detect either *Salmonella* DNA or RNA sequence. In one form of DNA hybridization, a poly-dA capture probe is added to a lysed sample in a microwell coated with poly-dT to detect *Salmonella*-specific rRNA sequence; simultaneously, a detector probe with a 5′ horse-radish peroxidase (HRP) label is added to detect the same rRNA target (Mozola et al., 2007). Unhybrized probes are washed away, and after addition of a HRP substrate, the hybridization is detected by chemiluminescence. DNA hybridization can also be done using a dot blot format whereby a labeled probe is immobilized on a membrane (Iida et al., 1993).

Genetic technologies such as whole genome sequencing, microarrays, and SNP analysis are useful for identifying and typing *Salmonella*. However, these methods are not yet widely used for routine screening of food samples because they typically require more detailed workflows.

## 5. Analysis of real-time PCR data

In TaqMan® real-time PCR assay, the fluorescence released by the reporter dye during each cycle increases exponentially until the reaction reaches saturation. The cycle number at which the fluorescent signal first crosses the threshold value is the cycle threshold ($C_T$). It is dependent on the baseline which is established in the early cycles of the PCR. The threshold

value, referred to as delta Rn (normalized reporter signal), can be adjusted by the user. The threshold value should be set above baseline noise in the early cycles of PCR and within the exponential amplification phase for positive samples (Figure 5). Using PCR replicates of positive samples is a useful way to decide upon the appropriate threshold to use for data analysis. The appropriate delta Rn value will be dependent upon the assay and application and, once set, should be used for all samples for consistent analysis. Low $C_T$ values indicate high copy number of the target sequence, while high $C_T$ values indicate low copy number. The typical limit of detection is 10 to 100 copies of purified genomic DNA and $10^3$ to $10^4$ CFU/mL before sample preparation. Most assays for *Salmonella* detection are typically qualitative, producing data that can be categorized as positive or negative. However, assay runs can be designed to be quantitative. Quantitative real-time assays should be validated and shown to amplify with high efficiency. The linear range for quantitation should be based on the exponential phase of amplification. Unknown samples to be quantitated should be run alongside DNA standards during real-time PCR.

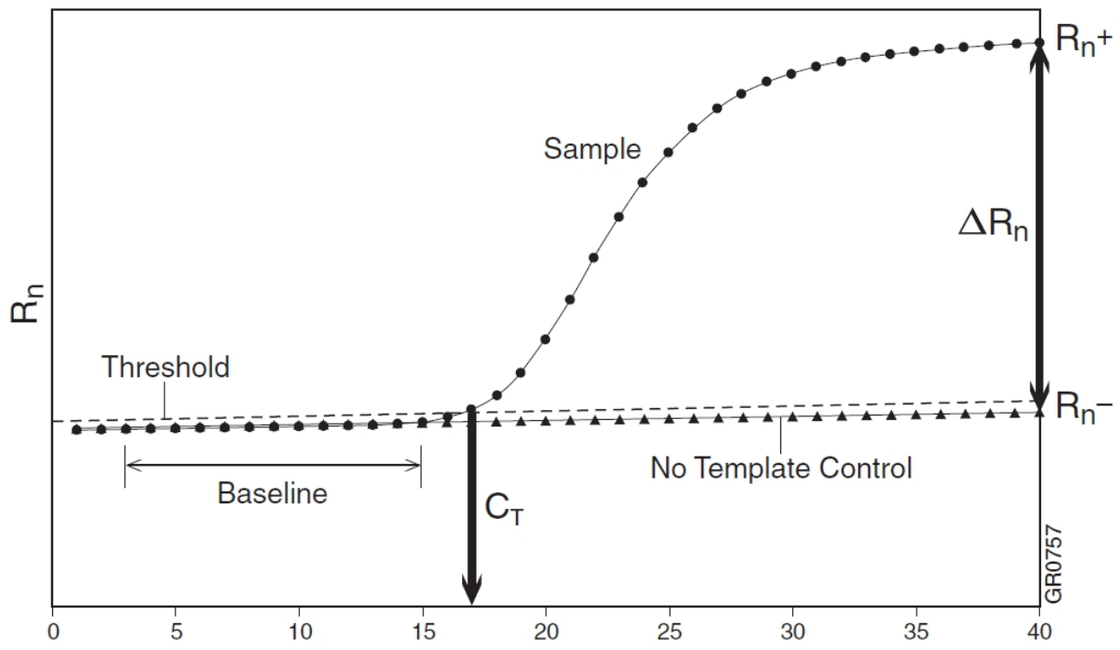


Fig. 5. Demonstration of a TaqMan® amplification curve and parameters. Amplification results in increased fluorescence (Rn). The Ct value reflects the beginning of amplification.

In PCR using SYBR® Green dye, a real-time amplification plot is generated that is similar to a TaqMan® PCR assay. Melt curve analysis (also known as a dissociation curve analysis) is typically added to the end of a SYBR® Green PCR. During the dissociation stage, the instrument increases in temperature over several minutes. For positive samples, SYBR® Green is initially bound to the amplicons. As the double-stranded amplicon dissociates, there is a drop in SYBR® Green fluorescence. The change in fluorescence is plotted against the temperature (Figure 6). Unknown samples should be compared to no-template reactions. Samples that are positive should have product of the expected Tm and, if the assay is well-designed, should not have other products such as primer-dimer or mis-primed products.

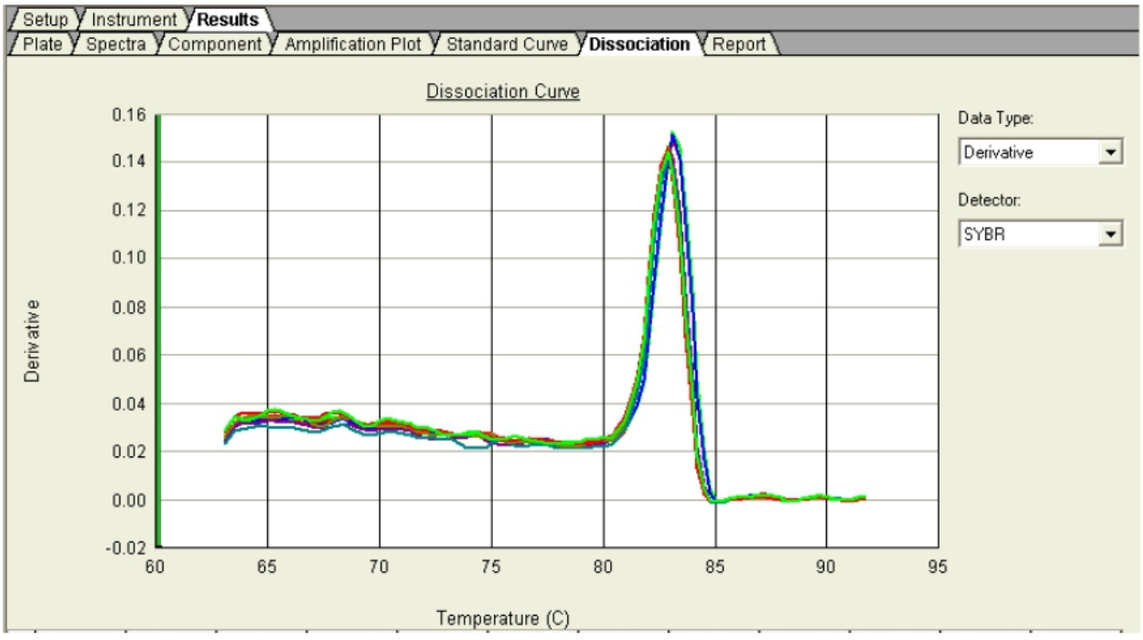


Fig. 6. Derivative of a melting curve from a reaction with SYBR® Green dye reaction. The Tm is determined by the peak of the derivative.

## 6. Software

Easy-to-use software is an important component of applying genetic methods to detect Salmonella. It is crucial to have accurate, reproducible, and unbiased data interpretation. Results should be clearly stated in convenient formats to the end user. In RapidFinder™ Express Software (Life Technologies) for food pathogen detection, algorithms were developed to interpret real-time PCR data and allow for sensitive detection while avoiding false positive results. Results are stated as positive or negative depending on cutoff values. In cases in which a positive or negative assessment cannot be made (e.g, the internal positive control failed indicating inhibition), a warning assessment is generated along with an explanation (Figure 7).

## 7. Bioinformatic tools for real-time PCR assays design

Selecting a good genomic target is critical to designing a real-time PCR assay. Appropriate genomic targets have to be sequenced from multiple strains in order to design highly specific primers and probes which cover a set of desired target species (*inclusion set*) and exclude from detecting a set of other bacterial genomes (*exclusion set*). Examples of broadly sequenced bacterial DNA targets are the 16S gene, *gapA, recA*, *rpoB*, among others. We developed and validated a standard bioinformatics assay design tool to generate primer and probe combinations for real-time PCR pathogen detection. The first step of the design is generation of a target consensus sequence based on multiple sequence alignments of all available target sequences using clustalW algorithm (Larkin et al., 2007). Sequences can be used from all available public databases. At the second step, a set of well described selection criteria (Larkin et al., 2007; Endrizzi et al., 2002; Kramer & Coen, 2000) has to be applied,

such as optimal Tm, nucleotide distribution (*e.g.* avoid high GC content and poly-N stretches), absence of cross-hybridization; and amplicon size (optimal size is 60-150 base pairs). This set of rules can be applied to select candidate primer and probe sequences that target a signature nucleic acid sequence in a microbe of interest. A set of optimal assays has to be evaluated, considering criteria of hybridization patterns of the two primers and a TaqMan® probe to the intended target sequence (Endrizzi et al., 2002). At the third step, those assays with the highest specificity are selected. The specificity is determined based on nucleic acid sequence comparisons of the binding sites for the assay primers and probes with genomic sequences from other bacterial species (exclusion set), being sure to include closely related species in the analysis. These genomic sequences have to be from multiple available databases, as well as from additional sequences that can be specifically generated for the design of the TaqMan® detection assays. Based on this sequence comparison, specific primers and probes can be selected. The best primers contain the highest number of mismatches to other non-target bacteria genomes, with mismatches preferentially located at the 3'-prime region of the primers. This minimizes the possibility that an assay will be selected that generates a false-positive signal (Furtado et al., 2004).

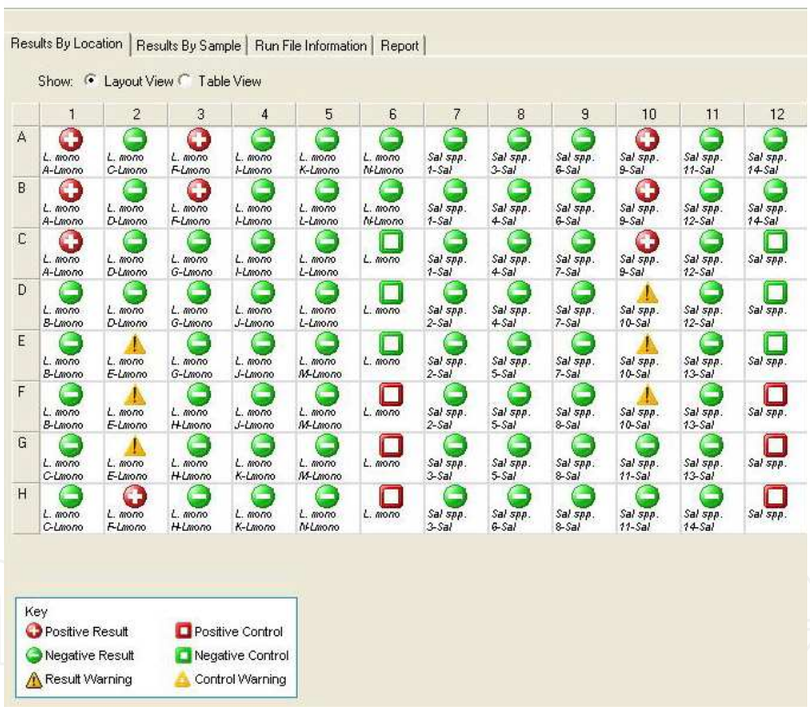


Fig. 7. RapidFinder™ Express Software for food pathogen detection displays positive, negative, and warning assessments for real-time PCR.

Additional DNA targets can be amplified and sequenced. This is often required when inclusion/exclusion testing show non-detection of inclusion strains or unwanted detection of exclusion strains. This requires the design of sequencing primers upstream and downstream of the assay target. It might also be useful to create a specific signature sequence database by sequencing additional samples, which can increase the confidence of specific assay designs. Sequence files can be analyzed using clustalW multiple sequence

alignment. The Applied Biosystems® MicroSEQ® ID validated method can be used for sequencing the 16S fragment. Using the 16S gene as a target for specific pathogen detection is challenging because it contains conservative sequences across multiple bacterial species. However, when primers and probes have to detect a broad range of species, families, genus or higher order, the 16S target is ideal or often is the only choice.

Experimental validation of the assay specificity is a critical step in the final assay selection. To test for assay specificity, a panel containing a diverse group of *bacteria* isolates (target and exclusion strains) has to be established and used for experimental validation. The panel must be well characterized by stereotyping or sequence-based bacterial identification (Tanner et al., 2006). Preferably, the panel will contain ATCC type-strains.

## 8. *Salmonella* detection background

More than 2,400 *Salmonella* serotypes have been reported, all of which are potentially pathogenic. The species *Salmonella* enterica, with its 6 main subspecies is of clinical relevance for humans and is the causative agent of food borne illnesses or salmonellosis. However, for tracking purposes it is often important to have a very specific serotype assay to measure and contain the spread of an outbreak pathogen. Food borne outbreaks due to *Salmonella* have become a major public health problem and can occur either as food poisoning triggered epidemics or as isolated cases. Outbreaks have been associated with raw meats and poultry, eggs, milk and dairy products, seafood, coconut sauces, salad dressings, cocoa, chocolate, and peanuts. The combination of efficient sample preparation protocols and reliable detection of *Salmonella* is a solid path forward for molecular detection methods.

## 9. Design options: Single-plex, multiplex, degenerate primers and probes & optimization

The most important advantages of real-time PCR are the ability for quantification of target concentration and multiplexing with different dyes per target. Since real-time PCR measures target amplification by monitoring the increase in fluorescence generated by probe degradation, the only limitation to multiplexing within a single reaction tube is the number of fluorophores that can be distinguished by the detection system (i.e. optics and software). From the chemistry side, one of the keys to maximizing the number of fluorogenic signals measured in a single reaction mix is developing dyes with well-separated and narrow emission spectra. Life Technologies identified several dye sets and real-time PCR systems that allow for detection of up to five fluorophores in a single reaction mix. It is important to note that instruments have different detection ranges for monitoring fluorescent energy. Detecting multiple targets can also be achieved through novel engineering designs; an example being the 384-well format Custom TaqMan® Array card that can be used in combination with the Applied Biosystems 7900HT Fast real-time PCR System (Tebbs et al., 2010), and the OpenArray® real-time PCR System. These Systems split a common sample amongst multiple reaction chambers prior to real-time PCR amplification and detection.

Since the 7500Fast real-time PCR system capability allows for measuring five fluorophores in a single reaction mix, it is possible to create real-time reaction applications capable of detecting 5 target organisms in a single tube. However, for high accuracy applications, two of the five channels are often used as system controls. We created a number of applications

where one channel is assigned to the internal positive control (IPC) to monitor the presence of PCR inhibitors, and another channel is used to control for well-to-well variation and normalization of fluorescence detection by using a ROX™ dye as a standard dye. Detection of IPC is indicative of a successful PCR amplification. Thus, with the inclusion of two controls, three targets can be detected in a single reaction tube. Each assay target is evaluated independently through the dye assigned to that target. Important considerations when designing multiplex real-time PCR assays include: first, development of several specific working assays for each target to have a choice in a final configuration. Each assay should be tested for quantification efficiency. Second, each assay must be tested against a large panel of microorganisms that include both inclusive and exclusive strains to identify highly specific assays. It is possible to add additional primers and probes containing variant base sequences (degenerate sequences) if some inclusive strains are not detected or weakly detected. Third, fluorogenic dye signals should be balanced by adjusting primer/probe concentrations: it is essential to perform statistics-based DOE (Design of Experiments) studies to optimize primer and probe concentrations for optimal detection of each target species. The final multiplex assay can be further optimized by standard PCR optimization techniques including adjusting magnesium and enzyme concentration, annealing temperature, probe lengths, and instrument settings.

## 10. Design and validation of existing real-time PCR *Salmonella* assays

Specific assays were designed and tested for detection of (i) *Salmonella* species in food samples, (ii) *Salmonella* Enteriditis in eggs and environmental samples, and (iii) *Salmonella* Typhimurium (Table 3). The selection of the target genes was based on specific applications: whether targeting detection of all *Salmonella* species in one reaction or targeting a specific serotype. Experimental validation is an essential part of molecular assay development. Testing and validation of the complete workflow is a critical element in the acceptance of a detection assay in food safety testing laboratories.

| ***Salmonella* target organism** | **Gene Target** |
|---|---|
| *Salmonella* species | *hilA* gene |
| *Salmonella* Enteriditis | *Prot6e* gene |
| *Salmonella* Typhimurium | *Target 1/Target 2* |

Table 3. Real-time PCR assays for *Salmonella*.

Our *Salmonella* spp. detection assay is a rapid, sensitive real-time PCR test, that, when combined with the automated PrepSEQ® Nucleic Acid extraction method or the manual PrepSEQ® Rapid Spin extraction method, allows the completion of *Salmonella* spp. detection within 18-19 hours, compared to 3-5 days required by for the traditional culture-based methods (Andrews & Hammack, 2011). As described in the previous section, each real-time PCR reaction contains an Internal Positive Control (IPC) that monitors for the presence of inhibitors for reliable negative results. The assay was designed as a complete reaction mix (no target DNA) in a lyophilized format to allow for minimal pipetting steps and addition of maximum sample volume. This workflow allows for robust *Salmonella* detection and creates the possibility of testing composite or pooled samples reliably.

The assay was validated for *Salmonella* detection in different food matrices including a variety of food matrices which were previously associated with food recalls or outbreaks (Carroll, 2009; Cahill et al., 2008; van Cauteren et al., 2009; Munnoch et al., 2009; Reiter et al., 2007): raw ground beef, raw chicken wings, chocolate, raw shrimp, Brie cheese, shell eggs, cantaloupe, black pepper, dry infant formula, and dry pet food. The detection was complemented by two sample preparation protocols for flexible detection work-flow set up: manual Rapid Spin column-based method and high-throughput automated method. The complete workflow was evaluated against the reference ISO 6579 culture confirmation method. Examples for detecting *Salmonella* in "difficult" matrices chocolate and black pepper are shown in Figure 4 (above). The sensitivity and specificity rates for *Salmonella* spp. detection assays were 100%, with no false negative or false positive samples observed with both sample preparation methods. Inclusivity panel contained 100 *Salmonella* strains based on variety of serotypes. Exclusivity panel contained 30 different bacterial isolates, including genetically close microorganisms as well as bacteria that are common organisms in the environment (Balachandran et al., 2011).

A special study was conducted to demonstrate detection of *Salmonella* in peanut butter, as a part of the Emergency Response Validation program, that followed the peanut butter outbreak in the United States in 2009 (Tebbs et al., 2009). The method was evaluated using *S. enterica* ser. Typhimurium ATCC14028 strain and the reference FDA-BAM protocol was used as culture confirmation method. There was complete agreement between the automated PrepSEQ® NA Extraction method and culture confirmation for uninoculated samples (see Table 5 in Tebbs et al., 2009). Also, Chi-square analyses indicated that the proportions of positives for the *Salmonella* spp. detection method and the reference method were not statistically different at the 5% level of significance.

A specific *Salmonella* Typhimurium assay was designed using duplex assay approach: two different assay probes carry different fluorescent dyes. This two-target approach increases confidence calling the test positive: both fluorescent signals should be positive (Figure 8). An example of positive *Salmonella* Typhimurium detection using this two-target assay is presented in Figure 9.

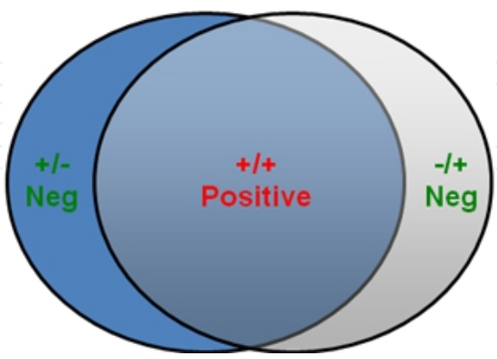


Fig. 8. Diagram representing a duplex real-time PCR assay for detection of *Salmonella* Typhimurium. Each assay detects *S.* Typhimurium plus some non-Typhimurium strains. Because each assay detects a different set of non-Typhimurium strains, only when both assays are positive is the sample positive for *S.* Typhimurium. The assays are labeled with a different fluorophore to be detected independently by real-time PCR.


Fig. 9. Example showing positive detection of *Salmonella* Typhimurium with a two-target assay. The assay for target 1 uses FAM™, the assay for target 2 uses VIC®, and the assay for IPC uses NED™. The assay was analyzed on the 7500Fast real-time PCR instrument.

Real-time PCR methods for detecting foodborne pathogens offer the advantages of simplicity and quick time to results compared to traditional culture methods. Our assays demonstrated high accuracy detection of *Salmonella* strains in inclusivity panels, and good discrimination against detection of exclusivity panels.

## 11. Conclusion

Rapid methods offer great advantages to food producers minimizing risks associated with long hold times during pathogen testing. This is clearly illustrated with requirements for testing whole shell eggs for *Salmonella enterica* serovar Enteritidis (SE). In July of 2009 the U.S. Food and Drug Administration announced The Federal Egg Safety Program, a new regulation that requires routine environmental tests of poultry houses for presence of *Salmonella* Enteritidis (Food and Drug Administration, 2009). If SE is present in the environment, there is a requirement to test eggs prior to their distribution for sale. According to the regulation, 50 egg pools consisting of 20 eggs per pool must be tested every 2 weeks for 8 weeks. The traditional method, designed and approved by the FDA, takes up to ten days to get results (Andrews & Hammack, 2011). According to the FDA approved method, egg pools sit for 4 days at room temperature to allow growth of SE, and then a 25 gram sample is pre-enriched in modified typtic soy broth with ferrous sulfate. The sample is then grown in selective media, then selective agars for presumptive detection, and then confirmed by biochemical and serological methods. The procedure is laborious and expensive. A recently developed real-time PCR assay allows detection of SE in egg pools in less than 27 hours. The TaqMan® *Salmonella* Enteritidis Detection Kit (Life Technologies) enriches egg pool samples for 24 hours, following which the samples are prepped and combined with a PCR reaction mix for detection by real-time PCR. Because sample prep is

fully automated, the total hands on time following enrichment is less than one hour. A method comparison study showed that the real-time PCR method was equivalent to the FDA reference method (Table 4). The FDA reported that the real-time PCR kit was equivalent to the FDA BAM Chapter 5, *Salmonella* method for detection of *Salmonella* Enteritidis in accuracy, precision, and sensitivity (FDA website). The Pennsylvania Layer Industry and Penn Ag approved the use of the real-time PCR method as an option for testing of egg and environmental samples without the need for culture confirmation. The trend appears to show increased acceptance of real-time PCR and other fast methods as alternatives to the more cumbersome culture methods.

| Inoculation Level | Inoculating Organism | U.S. FDA BAM | TaqMan® Salmonella Enteritidis Method | | $X^2$ | Relative Sensitivity | False Negative Rate | False Positive Rate |
|---|---|---|---|---|---|---|---|---|
| | | | Presumed | Confirmed | | | | |
| **Experiment 1** | | | | | | | | |
| **Control** | N/A | 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** | N/A | 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 4. Methods comparison showed that the TaqMan® Real-time PCR method was equivalent to the FDA BAM method for detection of *Salmonella* Enteritidis in whole shell eggs. The results from chi-square analysis on two independent experiments indicated no difference between the two methods ($\chi^2 = 0$ and 0.41 for Experiment 1 and Experiment 2, respectively). No false positive or false negative results were observed.

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