

**Helicase-dependent Isothermal Amplification for the Detection of  
*Salmonella enterica* serovar Typhimurium**

by

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## Abstract

*Salmonella enterica* serovar Typhimurium is a common foodborne pathogen, frequently causing gastroenteritis in humans. Much effort has been given recently to develop rapid detection methods for foodborne pathogens such as *Salmonella*. In this study, a real-time thermophilic helicase-dependent isothermal amplification (tHDA) was developed for simple and specific detection of *S. Typhimurium*. A set of highly specific primers was designed and synthesized to target the STM4497 gene of *S. Typhimurium*. The real-time tHDA conditions were optimized for specificity and sensitivity tests. Five *Salmonella* strains and one other pathogen were used to test the method's specificity. Tenfold serial dilution method was used to test its sensitivity. The result showed that the tHDA method is capable of performing at a constant temperature of 65 °C. The method presented positive amplification for the two *S. Typhimurium* target strains, but no specific products were amplified by non-target strains. tHDA methods showed very high sensitivity, being able to detect  $17 \times 10^{-5}$  ng of genomic DNA (per reaction mixture) from *S. Typhimurium* pure culture within 2 hours. The regression curve/standard curve based on the relationship between threshold cycle number (Ct value) and DNA concentration was generated for analyzing the performance of the real-time tHDA method. Slopes of the curve from each test were -3.594 ( $R^2=0.969$ ), -3.037 ( $R^2=0.997$ ), and -3.193 ( $R^2=0.991$ ) respectively, indicating high efficiency and reproducibility of the reaction. The capability of running at lower temperature with high sensitivity makes tHDA a potential detection platform applicable to portable microfluidic devices.

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## List of Abbreviations

|       |   |
|-------|---|
| AOAC  | Association of Official Analytical Chemists International |
| ATCC  | American Type Culture Collection                          |
| Bp    | Base pair   |
| CDC   | Centers of Disease Control                                |
| dNTPs | deoxyribonucleoside triphosphates                         |
| ELISA | Enzyme-linked immunosorbent Assay                         |
| ERS   | Economic Research Service                                 |
| FDA   | Food and Drug Administration                              |
| HDA   | Helicase-dependent Amplification                          |
| LAMP  | Loop-mediated isothermal amplification                    |
| LOC   | Lab-on-a-Chip   |
| MMWR  | Morbidity and Mortality Weekly Report                     |
| NCBI  | National Center for Biotechnology Information             |
| NTS   | Nontyphoidal <i>Salmonella</i>                            |
| PCR   | Polymerase Chain Reaction                                 |
| PDMS  | Poly(dimethylsiloxane)                                    |
| RCA   | Rolling circle Amplification                              |
| SDA   | Strand Displacement Amplification                         |
| USDA  | United States Department of Agriculture                   |

## Chapter 1 Introduction

Nontyphoidal *Salmonella* spp. (NTS) is an important pathogen that causes salmonellosis, a foodborne zoonose that threatens public health in the form of morbidity and mortality (CDC 2011). It is reported that approximately 1 million people get sick from consuming food contaminated with *Salmonella*, resulting in 19,336 hospitalizations and 378 deaths each year (Scallan and others 2011).

*Salmonella* infections spread widely and fast because they can be transmitted by many different types of food. Meats, raw or undercooked eggs, vegetables, raw milk and dairy products, seafood and even processed foods, such as peanut butter, could serve as sources of contamination (Kegode and others 2008; Camps and others 2005; Abadias and others 2008; Vasavada 2004; Pinu and others 2007). People infected by *Salmonella* often develop diarrhea, fever, and abdominal cramps from a mild-to-moderate self-limited illness to severe disease. Salmonellosis can resolve without treatment, but people may require treatment with antibiotics. Some people with compromised immune systems (such as infants, the elderly and pregnant women) are more likely to have severe infections. According to the CDC report, nontyphoidal *Salmonella* spp. infections caused more hospitalizations and deaths than any other germ, and the numbers have not been reduced, but increased by 3% instead over the past 15 years (CDC 2011).

Over 2500 different *Salmonella* serovars have been identified; however, only a few can cause human infections. In most developed countries, *Salmonella* Enteritidis and *Salmonella* Typhimurium in the NTS spp. group are the most prevalent and frequently reported to cause

human salmonellosis. In the United States, *Salmonella* serovar Typhimurium has been the most predominant since 2001 (Hendriksen and others 2011). Moreover, *Salmonella* Typhimurium presented the highest isolation rates from both human and non-human sources from 1970 to 2009 (CDC 2011). Its apparent resistance to multi-antimicrobial drugs has also been reported (CDC 2010).

Although the food supply is reasonably safe, *Salmonella* infections by directly contaminated (contamination from direct contact) or indirectly contaminated (contamination from processing steps) food still cause a huge economic burden to the food industry and society. It is estimated that salmonellosis costs approximately \$2.8 billion annually in terms of direct medical cost and the loss of productivity, which is around \$1,938 per case of salmonellosis (ERS 2011). Therefore, rapid and accurate identification and detection of *Salmonella* is urgently needed for the health of people as well as economic reasons. As a long established method, traditional culture identification based on colonies' biochemical characteristics has been used for detection due to its high accuracy and reliability. However, it is time-consuming and labor intensive because sufficient time is needed for media preparation, colony growth and strain identification. In this case, the detection and confirmation period for *Salmonella* spp. requires several days to obtain results (Andrews and others 2001).

Microfluidic technology, an integrated system which is capable of processing small amounts of fluids by using nanoliter to microliter chambers, provides a platform to perform detection with high speed and sensitivity. Various types of miniaturized analysis devices have been successfully designed and developed based on microfluidic systems (called "Lab-on-Chip") and applied in food diagnostics for rapid detection of pathogens (e.g., the micro-PCR chip or the capillary electrophoresis microchip). By using the miniaturized system, the consumption of

reagents, production of waste, and requirement for power are reduced, yet sample analysis becomes faster and more sensitive. Moreover, reduction of the size allows integrating monitoring techniques into a single device, which results in efficient measurement and analysis as well as the possibility for real-time detection.

Polydimethylsiloxane (PDMS) as a polymer has been the most widely used material for the fabrication of microchips through the molding process. PDMS is a gas permeable, optically transparent elastomer whose stiffness can be easily controlled from soft to stiff, making it a flexible material to handle biological materials, such as cells, within the micrometer dimension channels because the softness of the inside wall of the channels can prevent cellular damage. Compared to silicon (another commonly used material in microfluidic device), PDMS is a disposable, much less expensive material. Its transparency to visible and UV light make it a suitable substrate easily used in real-time fluorescent detection. A variety of microfluidic-based devices have been developed using PDMS and commercially used in gene detection. High temperature gene amplification techniques, such as PCR techniques, can be integrated into the microfluidic system for rapid and sensitive identification of pathogens. Detection for single template can be achieved by this PCR-microfluidics system (Ottesen and others 2006). However, manipulation of the microfluidic system under high temperature cycles is challenging to control, which might cause potential evaporation difficulties. In this case, processes at lower temperatures may have the advantages of easier handling and more flexible control because the microfluidic detection platform can be easily set and applied on a simple heating block or in a water bath.

In this research, an isothermal thermophilic helicase dependent amplification (tHDA) detection method was developed which mimics DNA unwinding and amplification *in vivo* at a

constant temperature of 65 °C. Specific primers were designed for targeting *Salmonella* serovar Typhimurium. EvaGreen, a double-stranded DNA binding dye, was selected and used for rapid detection of the tHDA products in real time.

## Chapter 2 Literature Review

### *Salmonella*

#### Characteristics of the organism

*Salmonella* spp. are Gram-negative rod-shaped and non-spore forming bacteria belonging to the Enterobacteriaceae family (D'Aoust 1997). They are non-fastidious organisms that can grow readily under a variety of conditions (e.g., various types of foods, water) and can even survive in some extreme conditions (e.g., low pH, desiccated state). Moreover, *Salmonella* spp., especially subspecies I of *Salmonella enterica*, can colonize and cause diseases in all warm-blooded animals (Porwollik and others 2004). Of the 2,500 different serotypes of *Salmonella* spp., only a few of them can be found in humans and described as the cause of disease. Typically, *Salmonella enterica* serovars Typhimurium and Enteritidis have been reported as the major non-restricted host serotypes frequently causing self-limited gastroenteritis in humans (Herikstad and others 2002). Among them, *Salmonella* Typhimurium has been prevalent in Europe and U.S. (Herikstad and others 2002). Infection caused by these germs is primarily thought to be transmitted by ingestion of uncooked food and inappropriate handling (Bangtrakulnonth and others 2004). The infections transmitted by those germs give rise to a variety of disease syndromes including nausea, fever and diarrhea. The severe infections could lead to death without prompt treatment (e.g., intravenous fluid injection, antibiotics). According to estimates from CDC (2011), the annual number of domestic foodborne hospitalizations caused by nontyphoidal *Salmonella* spp. is around 19,336 with 378 deaths. It is estimated that around 1

million cases of salmonellosis occur annually, which would cost approximately \$2.8 billion. Therefore, *Salmonella* has become a more severe economic burden to society in addition to threatening people's lives.

#### Pathogenesis and virulence factors

Nontyphoidal *Salmonellae* are the major cause of human enteritis with 90% of this infection being caused by *Salmonella* serovar Typhimurium and serovar Enteritidis. *Salmonella* Typhimurium has been the model germ used in the study of gastroenteritis for understanding pathogenesis of *Salmonella*. Much research has been successfully performed on *Salmonella* Typhimurium-induced enteritis in humans using a bovine model because of the similarity of the intestinal pathology and the pattern of inflammatory response in calves with that in humans. It has been determined that human *Salmonella* infection relies on the ability of the bacterium to colonize and invade the host intestinal epithelial cells (Maurer and Bailey 2001). Pathogenic *Salmonella* can invade cell membranes, survive and grow in host cells, and finally cause apoptosis. All these behaviors are induced by sophisticated collaborations of secreted virulent proteins encoded by *Salmonella* pathogenicity islands prime one (SPI1), virulence plasmid genes and *Salmonella* essential virulence genes scattered around the chromosome (Olsen 2005). The type III secretion system encoded by SPI1 was initially identified as an essential virulence factor required for *Salmonella* attachment to and invasion into the intestinal epithelial cells. The secretion system encodes at least 33 proteins, of which SipB, SipC, and SipD are secreted and released from *Salmonella* cells, localizing and binding on the membrane of host epithelial cells. They also help to translocate and deliver other invasive proteins into the cytoplasm of a host cell. SopB and SopE2 proteins, which are thought to contribute virulence, are primarily involved in cytoskeletal rearrangement on specialized sites of host cell membranes, resulting in cell

membranes ruffling and the uptake of the bacterium. The pathogenicity island is induced *in vivo* to protect *Salmonella* from attack by the host defense system (e.g., phagocyte) (Cirillo and others 1998); virulence plasmids enhance the invasive ability and most importantly allow the bacterium to multiply fast inside cells (Gulig and Curtiss, 1988; Gulig and Doyle, 1993).

## **Detection methods**

### Traditional culture-based methods

Conventional culture-based methods have the longest history for bacterial detection and remain the standard microbiological detection techniques. It identifies and characterizes microorganisms by using specific selective microbiological media to isolate and enumerate viable bacterial cells in samples. This method usually involves several basic steps: sampling, pre-enrichment, selective enrichment, selective plating, biochemical and serological identification (Andrews and others 2001).

The food analyzed is usually a composite of smaller representative aliquots taken from the food, which enables the food to be examined in one pre-enrichment and thus reduce the number of samples to be analyzed and the volume of pre-enrichment media (Sperber and others 2001). For *Salmonella* identification, 25 g of food sample is suggested as an analyzed unit followed by 10-fold dilution by pre-enrichment media/buffer for recovering *Salmonella* in foods (Andrews and others 2011). The pre-enriched sample is homogenized in a sterile bag and incubated at 35 °C for 24 hours. For recovering viable microorganisms in the tested sample, pre-enrichment is a necessary step to neutralize the inhibitors in food. Ten different pre-enrichment media are described for the recovery of *Salmonella* in various foods, of which lactose broth is the most common media used with foods (Andrews and others 2011).

Selective enrichment allows the target microorganism to proliferate to detectable levels while suppressing or inhibiting other competing microorganisms. The selective agents could be pH, temperature, antibiotics, and metals (Sperber and others 2001). For *Salmonella*, 0.1 mL pre-enriched sample is transferred in 10 mL Rappaport-Vassiliadis (RV) medium (FDA recommended selective medium for *Salmonella*) and incubated at 42 °C for 24 hours (Andrews and others 2011).

Plating of the culture on selective agar by either a pour-plate method or spread-plate method is used to identify target microorganisms and for further target cell enumeration. Target microorganisms multiply on the selective medium forming visible colonies after approximately 24 hours incubation. Microbial colonies present their biochemical characteristics on the selective medium due to specific metabolic reactions with the selective agents or differential agents (e.g., H<sub>2</sub>S indicators). For *Salmonella* spp., Xylose Lysine Desoxycholate (XLD) agar is the recommended medium to select *Salmonella* cells, which appear as black-purple colonies on the solid medium. Further biochemical and serological tests are applied to confirm the microorganisms. Some convenience identification kits (such as API-20E, Minitek, Enterotubes, and Vitek GNI systems) have been approved by the Association of Official Analytical Chemists International to characterize *Salmonella* isolates.

Although the traditional culture methods have been considered very accurate, sensitive and inexpensive, medium preparation, colony counting, biochemical characterization and serological confirmation make this method time-consuming and very labor intensive. Therefore, laboratories, clinical diagnostics, and food industries are still looking for alternative detection methods that are rapid, sensitive, and accurate.

### Traditional immunological-based methods

Immunological-based methods have been used as powerful analytical tools to detect a wide variety of targets in food, including bacterial cells, spores, viruses, and toxins (Iqbal and others 2000). Immunological methods rely on the specific binding of an antibody to an antigen which is unique to a particular microbial group. Immunoassays can be classified as homogenous assays or heterogeneous assays based on whether reagents are being separated and washed away after reaction. In homogenous assays, the bound and unbound antibodies do not need to be separated and the antigen-antibody complexes are usually visible or measurable (Boer and Beumer 1999). In contrast, in heterogeneous assays, unbound antibodies must be separated and washed away from the bound antibody (Boer and Beumer 1999). Enzyme-linked immunosorbent assay (ELISA), a heterogeneous assay, is currently the most prevalent antibody assay used for pathogen detection in food (Crowther 1995). Among several established types of ELISA assays, the “sandwich assay” is commercially designed and available. In a typical sandwich assay, an antibody is immobilized on a solid phase (such as test tubes, blotting membrane, or the well of a microtiter plate) as a support for capturing the specific antigen (Entis and others 2001). After adding the sample (contains antigen) and waiting for a short incubation period, the unbound substances are washed away. The secondary reporter antibody labeled with an enzyme is added and followed by an incubation period. The enzyme-labeled antibody is detected by adding a substrate which specifically reacts with the reporter enzyme to produce colored products and indicates the presence of the target in the original sample. The intensity of the colored product is proportional to the concentration of the targets in the sample and can be detected by either colorimetric or fluorometric technique (Entis and others 2001; Chen and Durst 2006).

ELISA has been successfully used to detect many kinds of bacteria in a variety of food. The common pathogens detected by ELISA methods include *Escherichia coli* O157:H7 (Chen and Durst 2006), *Salmonella* Typhimurium (Magliulo and others 2007; Chen and Durst 2006), *Campylobacter* spp. (Chen and Durst 2006), *Yersinia enterocolitica* (Magliulo and others 2007), and *Listeria monocytogenes* (Beumer and Brinkman 1989; Palumbo and others 2003). In the study by Palumbo and others (2003), 89 serotypes of *L. monocytogenes* were successfully identified from 101 isolates, and 100 previously uncharacterized Listerial isolates were serotyped by the ELISA method. Chen and Durst (2006) developed a new immunoassay reagent, protein G-liposomal nanovesicles, which are able to enhance the fluorescent signal and simultaneously detect *Salmonella* Typhimurium, *E. coli* O157:H7 and *L. monocytogenes* in both pure culture and mixed culture. Ng and others (1996) successfully identified *Salmonella* spp. in 26 contaminated food samples including eggs, pork, and infant formula with an ELISA method.

In recent decades, immunological-based methods have been coupled with other techniques for pathogen detection. For example, magnetic beads are able to be coupled with immunology for immunomagnetic separation of the target (Schlosser and others 2007).

However, since the limit of detection of whole bacterial cells is  $10^3$  to  $10^5$  cfu/mL by ELISA methods, direct detection of pathogens in food becomes impractical, and thus enrichment is required for at least 16-24 hours to allow cell numbers to reach the lower detection limit. Therefore, compared to molecular/nucleic acid-based detection methods ELISA is more time consuming and labor intensive for sample preparation.

## Nucleic acid-based methods

### *Nucleic acid hybridization*

Nucleic acid hybridization basically relies on a labeled DNA probe interacting and identifying a particular nucleotide sequence in the target complementary DNA (cDNA) or ribosomal RNA (rRNA) by base pairing. The probe-target complexes are signaled by the bound label and detected in the hybridization assay. The DNA probe commonly consists of short pieces of single-strand DNA (ssDNA) referred to as an oligonucleotide which usually contains 20-30 base pairs (Entis and others 2001). The DNA oligonucleotide probe can be easily prepared synthetically or biologically to detect the nucleic acid sequences from the target samples (Entis and others 2001). The DNA probes may be labeled with either radioactive isotope elements ( $^{32}\text{P}$  or  $^{125}\text{I}$ ) or non-isotopic fluorescent and enzyme labels (Keller and Manak 1989). The latter have become more appealing because they are more easily handled, readily disposed of and have very low toxic effects on the environment.

There are various formats of hybridization that are capable of being used for bacterial detection; they are generally classified into solid-phase hybridization and liquid-phase hybridization based on the medium involved for the assays (Entis and others 2001). In solid-phase hybridization, a solid support is offered to bind and immobilize the target sequence, and when the probe is added to match the target nucleic acid, hybrids form on the support. The excess free hybrids and unhybridized DNA probes are washed away, and the probe-target hybrids can then be detected with appropriate methods. Different types of solid support include membranes, nitrocellulose filters, microtiter plates or microscope slides (Keller and Manak 1989). Bacterial colony hybridization is a typical application of the solid-phase format. In this case, individual colonies are transferred to the solid support, usually a membrane or a paper filter

(Meinkoth and Wahl 1984). The surface of the solid support is then treated with reagents for cell lysis and DNA denaturation. With its porous structure, the solid support can easily catch and immobilize the ssDNA on its surface.  $^{32}\text{P}$  or enzyme-labeled DNA probes with a specific sequence are added onto the membrane filter and incubated with the target nucleic acid for hybridization via base pairing. The excess DNA probe that failed to form probe-target hybrids is removed by washing the solid support. The empirically-determined washing temperature is particularly important to the specificity of the reaction (Entis and others 2001) and the hybridization rate (Hill and others 1998). After hybridization, the probe-target complexes are detected with appropriate methods depending upon the probe labeling methods. For the radioactive probe, an X-ray film is placed over the support followed by being exposed, developed and detected by autoradiography (Hill and others 1998). Thus, black spots on the film are representative of the locations of the target sequence. If an enzyme-labeled probe is used, a chromogenic substrate is added to develop visible spots directly on the support. The colony hybridization has been successfully performed for detection of foodborne pathogens, including *E. coli*, *Listeria*, *Vibrio*, *Shigella*, *Salmonella* (Hill and others 1998). The colony hybridization methods have been coupled with advanced techniques, such as hydrophobic grid membrane filtration, to reduce the numbers of dilutions (Sharpe and others 1983) and provide more efficient identification of *E. coli* (Todd and others 1999) and *Listeria* colonies (Peterkin and others 1991).

Compared to solid-phase hybridization, the liquid-phase hybridization format offers faster hybridization rates (Britten and others 1974). In this format, both probe and target nucleic acids are suspended in solution, where the hybridization occurs. The hybrids are then separated from the solution by a capture solid support. In liquid-phase hybridization, a dual-probe system referred to as sandwich hybridization is commonly applied (Keller and Manak 1989). The

labeled probe binds to one end of the target sequence for further detection while the other end of the target sequence binds to a capture probe which is immobilized on a solid support for separating hybrids from solution. The detection process in liquid-phase method is fairly similar to that of the solid-phase method. Liquid-phase hybridization has been commercially used for the detection of various foodborne pathogens, including *Salmonella*, *E. coli*, *Listeria*, *Staphylococcus*, *Campylobacter*, and *Yersinia* (Feng 1997).

Nucleic acid hybridization methods have been used for the identification of microorganisms in various food samples (Fitts and others 1983; D'Aoust and others 1995). D'Aoust and others (1995) used a commercial GENE-TRAK<sup>®</sup> Colorimetric probe to detect target rRNA, and 110 strains of *Salmonella* in both high moisture food samples and low moisture food samples, including poultry, fish, pork, chocolate, peanut butter, and spices. Samadapour and others (1990) used colony hybridization techniques to successfully detect Shiga-Like-Toxin producing *E. coli* from 44 food samples with a detection limit of 1.3 cfu/g. Todd and others (1999) developed a method using hydrophobic grid membrane filters incorporated into the DNA hybridization method for detecting verotoxigenic *E. coli* and efficiently lowered the detection limit to 0.1 cfu/g in ground beef.

However, in practice, food ingredients may interfere with the probe's ability to anneal to the target, which could significantly lower the efficiency and accuracy of the hybridization. Although the sensitivity of this method has been improved by current techniques, it may not be reliable and stable for all types of pathogen detection, especially not suitable for the zero tolerance foodborne pathogens.

### *Polymerase chain reaction (PCR)*

Kary B. Mullis first developed the polymerase chain reaction (PCR) in 1983, which was a big breakthrough for molecular biology (Entis and others 2001). Over the past decade, PCR has become an essential tool in food microbiology (Malorny and others 2002; Prasad and Vidyarthi 2009).

By using PCR technology, a million-fold copies of the target sequence, referred to as the template, can be enzymatically amplified within 1-3 hours. The mechanism of PCR technology is similar to the basic principle of DNA hybridization; short nucleic acid sequences, called primers, are hybridized to the target sequence, enabling DNA polymerase to add nucleotides to the 3 prime end of the primer and generate a new DNA strand which is complementary to the template. A thermocycler is necessary to supply repeating cycles of alternating high and low temperature that brings about the amplification. Each cycle of the reaction basically consists of three steps: denaturation, annealing, and extension. In the first step, hydrogen bonds between double helical DNA are broken by high temperature (95 °C) and thus the double-strand DNA (dsDNA) is denatured and separated into ssDNA templates. The temperature is lowered to around 58 °C, at which primers can anneal and match over the specific region of the template. The temperature at this step is particularly essential to the specific attachment of primers to the template, and the optimum annealing temperature is usually obtained experimentally. The temperature is then increased again to 72 °C which allows the DNA polymerase to add nucleotide bases to the 3 prime end of the primer and make the extension for its complementary sequence. Theoretically, the target is amplified exponentially in PCR since the amount of product is doubled after each cycle. A single template can be detected after 20 to 30 cycles of amplification. The amplified products are commonly measured by running gel electrophoresis. In

practice, however, detection of single templates in food samples are difficult to achieve by PCR technology since some food ingredients inhibit DNA polymerase and base pairing, and thus reduce PCR sensitivity and efficiency. A preliminary enrichment step is common in PCR technology to dilute potential inhibitors in food and increase the target amount to detectable levels.

In the late 1990's, multiplex PCR was developed for the detection of multiple genes or microorganisms simultaneously within a few hours (Bej and others 1990; Khan and others 2000). Multiple primers were designed for identifying different genes of one target or for multiple targets in mixed culture or complex food samples. Thus, molecular detection became more practical and efficient for contaminated food samples (Lee and others 2007).

Later, real-time PCR technology was developed for even more sensitive, portable and recordable amplification (Heid and others 1996). In this modified real-time PCR, a fluorescent labeling technique was involved, acting as a detection marker during amplification so that target amplification could be quantitatively monitored, measured, and recorded at each cycle. Hence, the traditional end point measurement methods, such as gel electrophoresis, were not necessary. Several ready-to-use commercial assays, such as TaqMan<sup>TM</sup> fluorescent probe system (Jung and others 2005), BAX (Johnson and others 1998), and GeneDisc<sup>®</sup> (Bugarel and others 2011), have been incorporated with PCR technology and reported as efficient tools for pathogen detection. In the study by Jung and others (2005), TaqMan<sup>TM</sup> assay was applied, and primers and a double fluorescence-labeled probe were designed for the specific detection of invasion A gene (*invA*) of *Salmonella* Typhimurium in contaminated milk by real-time PCR. Similar assays were also used for *Staphylococcus aureus* (Hein and others 2001) and *Listeria* (Bassler and others 1995) detection. Shanmugasundaram and others (2009) successfully identified *Salmonella* serovar

Typhimurium from 58 *Salmonella* isolates in spiked water, chicken, and mutton samples by performing multiplex PCR. Seven genes of *Salmonella* Typhimurium were specifically targeted and differentiated from other closely related *Salmonella* serovars. In their study,  $10^2$  cfu/100 mL and 60 cfu/mg detection limits were obtained in water samples and food samples, respectively (Shanmugasundaram and others 2009).

PCR technology has been widely used for detecting other foodborne pathogens, including *Clostridium perfringens* (Fach and Popoff 1997), *Clostridium botulinum* (Aranda and others 1997), *Staphylococcus aureus* (Wilson and others 1991), *Shigella* (Lampel and others 1990), and *Campylobacter* (Waage and others 1999).

Limitations of PCR detection in food are (1) inhibitors' effects on the efficiency of DNA polymerase; (2) training required for personnel; (3) large energy use by the thermocycler.

### Biosensors

Recently, various biosensors have been designed and widely applied to solve bio-analytical problems within the food, pharmaceutical, and environmental areas (Velusamy and others 2010). Biosensors have been generally defined as an analytical device which consists of two main components: a bioreceptor element and a transducer element. The bioreceptor can be an antibody, enzyme, or nucleic acid which recognizes specific targets. The transducer converts the corresponding biological responses into equivalent electrical signals which are further processed and analyzed by signal processors and other analyzing systems. Based on the transduction methods, biosensors are usually classified into optical-based biosensors, electrochemical biosensors, and mass-based biosensors (Velusamy and others 2010; Lazcka and others 2007)

Optical biosensors have received much attention, becoming the most popular method for bacterial pathogen detection because of their high sensitivity and selectivity (Ko and Grant 2003).

Optical biosensors can be categorized into several subclasses based on their optical detection methods: reflection, absorption, refraction, infrared, Raman, or fluorescence (Velusamy and others 2010). The common principle of these methods is the recording of the photochemical properties of the analyte molecules by the corresponding spectrometers. Among these methods, surface plasmon resonance (SPR) is one of the simplest, no-labeling-needed techniques which allow analysis in real-time (Homola and others 1999). It has been successfully used to detect foodborne pathogens such as *Salmonella* (Koubova and others 2004; Oh and others 2004), *Listeria monocytogenes* (Bhunja and others 2004), *E. coli* O157:H7 (Taylor and others 2006), and *Campylobacter jejuni* (Taylor and others 2006).

In the SPR biosensor, a prism is coated by a thin layer of metal, usually gold, which is then coated with specific antibodies. Electrons in the metal are excited by the external laser light source, forming an electromagnetic wave, called a surface plasmon, which results in a reduced intensity of the reflected light. As the antibodies specifically interact with antigens in the analyte, the local refractive index (RI) on the metal surface is changed, resulting in the shift of the reflection angle. Changes in the angle of reflection can be monitored by recording the intensity of the reflected light, which can be used to identify the target. A SPR-based Spreeta<sup>TM</sup> biosensor was successfully fabricated for detection of *E. coli* O157:H7 in apple juice, pasteurized milk, and ground beef extracts within 30 minutes with limits of detection down to  $10^2$  to  $10^3$  cfu/mL (Waswa and others 2007).

#### Microfluidic system in food safety testing

Microfluidics is generally defined as the science and technology of systems which accurately process very small liquid volumes ( $10^{-9}$  to  $10^{-18}$  L) by using channels with dimensions of 10 to 100  $\mu\text{m}$  (Whitesides 2006).

Typical detection methods for food safety (such as improved culture, immunoassay, PCR) are labor intensive and expensive, usually taking 2 to 4 days (Mairhofer and others 2009). Fortunately, microfluidic systems contribute great advantages to transferring complex and tedious macro-manipulation steps into a micro/nano portable and cost-effective system where all process steps occur within a small single device (also known as lab-on-a-chip). Therefore, it has decreased processing complexity, lowered cost and labor intensity, reduced the risk of cross-contamination resulting from multi-step manipulation, and increased the fluid controllability (Berthier 2013). As a powerful tool in the food safety area, microfluidic systems have been successfully used for common foodborne pathogen detection (Varshney and others 2007; Ikeda and others 2006), antigen detection (Zhan and others 2009), toxin recognition (Hervas and others 2011; Frisk and others 2008), and antibiotic detection (Suarez and others 2009).

Traditional sample preparation for pathogen detection requires large amounts of reagent consumption and numerous tedious handling steps. The implementation of microfluidic DNA chips could solve these issues because the microfluidic system is capable of integrating all process steps (cell isolation, cell lysis, DNA isolation and DNA purification) within a single chip using nanoliter volumes. Therefore, this platform saves reagents and reduces handling steps which may cause cross-contamination (Hong and others 2004).

Due to its important characteristic of large surface-to-volume ratios of the miniaturized systems, microfluidic technology has easier and faster heat transfer and molecular diffusion (Atalay and others 2011), resulting in high sensitivity, unprecedented speed and accurate analysis in a variety of fields. As food safety and quality become more important worldwide, microfluidic technology is being used in food processing and food diagnostics areas as the result of the urgent need for rapid, accurate, cheap, portable, high throughput analytical tools (Whitesides 2006).

For wide application, microfluidic systems have been integrated into many other existing detection techniques, such as immunoassays, nanotechnologies, biosensor technology, and PCR.

#### *Microfluidic immunoassay*

Over the years, traditional immunoassay technologies have been the predominant analytical tools for detection of antigens for use in medical diagnostics, food safety and environmental testing (Sun and others 2010; Stokes and others 2001; Ekins 1999). The mechanism of conventional immunoassay was introduced previously. These methods typically required large amounts of samples and reagents, needed dedicated instruments and took between several hours to two days to obtain data due to multiple processing steps (Lin and others 2010).

Microfluidic lab-on-a-chip (LOC) technology utilized the advantages of immunoassay and enhanced the sensitivity of detection in food applications (Ikeda and others 2009; Dong and others 2006) and point-of-care diagnostics (Gervais and Delamarche 2009). It was able to save the multiple macro-manipulation steps (described previously in traditional immunoassay methods) and shorten the reaction period to a few minutes by integrating all processes into a one-step immunoassay within a single disposable chip (Gervais and Delamarche 2009). As an established and mature technique, microfluidic immunoassays made significant contributions to the food safety field including the detection of foodborne pathogens (Lee and others 2006), toxins (Sun and others 2010), allergens (Heyries and others 2008), and antibiotics (Suarez and others 2009) in food samples.

Hervas and others (2011) developed an electrokinetic magnetic bead-based immunoassay on a double-T channels microchip for the rapid detection of zearalenone (ZEA), a mycotoxin produced by fungi, in infant foods. In their design, an immunological reaction (ELISA) and enzymatic reaction were sequentially performed in different chambers. An electric field drives

reagents from sample reservoirs into the different chambers where reactions occur. In the immunological reaction chamber, ZEA was recognized and captured by its specific antibody immobilized on magnetic beads. The magnetic beads carrying the antibody-antigen hybrid were then directed to the enzymatic reaction chamber by applying an electric field. Antibody-antigen complexes were made detectable by the addition of an enzymatic substrate. The complete process took less than 15 minutes with good sensitivity of  $0.4 \mu\text{g L}^{-1}$ .

#### *Microfluidic biosensor*

Biosensor techniques coupled with a microfluidic platform provide a simpler and more portable analytical tool than conventional biosensor devices that greatly increase detection speed in food safety. Several microfluidic biosensors for bacteria on-chip identification have been developed by using magnetic beads (Zaytseva and others 2005), electrophoresis (Suehiro and others 2006), and membrane filters (Floriano and others 2005). The analyte signal in these devices was measured using fluorescence (Zaytseva and others 2005), impedance (Suehiro and others 2006), acoustics (Godber and others 2005) or electrochemistry (Ivnitski and others 2000). In recent years, impedance-based microfluidic biosensors have become popular because they provide high sensitivity, rapid responses, and ease of fabrication. Most microfluidic biosensors for pathogen detection use labeled capture-antibodies to convert antigen-antibody interaction to detectable signals.

Varshney and others (2007) recently developed a novel label-free impedance microfluidic biosensor. This device contains a microfluidic flow cell with an embedded gold interdigitated array microelectrode (IDAM) coupled with magnetic nanoparticle-antibody conjugates (MNAC) for rapid and sensitive detection of *E. coli* O157:H7 in ground beef. The MNAC were used to capture and separate target cells from the food matrix and to concentrate the cells into a detection

microchamber for the impedance measurement using a microfluidic flow cell with embedded IDAM. This device is accommodating for direct impedance measurement without probe-labeling or antibodies on the surface of electrodes. It was able to detect  $1.6 \times 10^2$  cfu/mL and  $1.2 \times 10^3$  cfu/mL of *E. coli* O157:H7 in pure culture and ground beef, respectively, within 35 minutes (Varshney and others 2007).

### *Microfluidic PCR*

As introduced previously, millions of copies of DNA can be produced within approximately 2 hours by the PCR heat cycling process. However, conventional PCR uses bulky equipment and large amounts of reagents. Moreover, the ramping rate for the PCR instrument is low (1 to 2 °C/s) due to the large thermal capacity of the PCR system (Zhang and others 2006). Hence, in conventional PCR, the efficiency of the reaction could be inhibited. The integration of microfluidic technology with PCR techniques brought about faster, sensitive detection methods with the introduction of the first microfluidic PCR chip (Northrup and others 1993, 1995). In the miniaturized PCR devices, large surface-to-volume ratios promoted better heat transfer, enhanced ramping rates, and shortened the amplification cycles required to obtain detectable DNA copies. The small dimensions of microfluidic devices, with a variety of additional functional components (such as micropumps, micromixers, and microvalves) enhance sample handling capacity and portability (Atalay and others 2011). Based on different reaction types and chip configurations, microfluidic PCR has been classified into stationary PCR, flow-through PCR, and thermal convection-driven PCR.

Stationary PCR works much the same way as conventional PCR, only with much smaller size and high automation, where the reaction solution is kept stationary within single or multiple micro reaction chambers (Northrup and others 1993; Chartier and others 2003). Heating occurs

with an external or embedded thermocycler. Stationary PCR, while simple with respect to handling, require a longer time to achieve results because the heating rate is slower.

On the other hand, PCR solutions in a flow-through platform (Nakano and others 1994) are moved by pressure sources and cycle through different temperature zones to achieve amplification. With rapid heat transfer, solutions are able to be heated up and cooled quickly, which allows for high-efficiency and fast amplification. The “one-channel” design of the flow-through method greatly reduced the possibility of cross-contamination and ensured specific amplification.

Thermal convection-driven PCR was more recently developed and was first reported by Krishnan and others (2002). This method does not require pressure to make the solutions flow. Instead, it uses buoyancy force to drive the fluid through different temperature zones. Because of its predominant advantages of simplicity in design, ease of handling, and low-cost fabrication, multiple types of this platform were subsequently developed (Krishnan and others 2002; Braun and Libchaber 2004; Wheeler and others 2003, 2004; Chen and others 2004).

Another application of microfluidic systems is for digital PCR (dPCR). dPCR, first introduced by Vogelstein and Kinzler (1999), made a great contribution to molecular genetic analysis and cancer diagnostics. Templates, usually diluted DNA samples, are introduced into multi-well plates so that there is one template molecule per two well on average. PCR is then performed, and the DNA with different alleles or mutants is able to be discriminated and detected using different fluorescent labels (Vogelstein and Kinzler 1999). Therefore, digital PCR converts the exponential data of conventional PCR to linear, digital data, allowing easier and more visualized data analysis.

The use of microfluidic technology with digital PCR optimized the application of the conventional digital PCR platform, allowing single cells to partition from a complex sample and the precise measurement of multiple templates individually. Ottesen and others (2006) developed a microfluidic digital PCR chip to identify the microbial community, which encoded a key enzyme (formyl-tetrahydrofolate synthetase (FTHFS)) involved in symbiosis with termites, by analyzing their specific 16S rRNA gene sequence. This device consisted of 12 sample panels and each panel used micro-mechanical valves to partition a single PCR mixture into 1176 independent 6.25 nL reaction chambers for efficient amplification of particular genes, as shown in Figure 2.1 (Ottesen and others 2006). The FTHFS-encoding genes and the “all-bacterial” 16S rRNA gene were identified on the chip by digital PCR (Figure 2.1 left). Simultaneously, the members of “termites cluster” of the bacterial genus *Treponema* were detected on the microfluidic chip for the identity’s confirmation (Figure 2.1 right). The microfluidic digital PCR of single cells were successfully performed using diluted termites’ gut contents that had been partitioned on the chip. Compared to traditional digital PCR, microfluidic digital PCR is able to identify large amounts and varieties of targets with much less reagent consumption and DNA dilution.

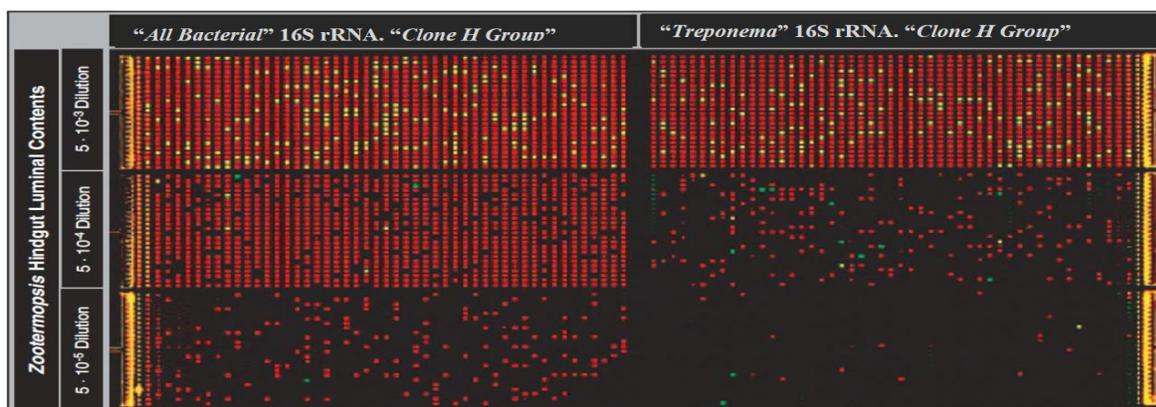


Figure 2.1 Multiplex microfluidic digital PCR chip from single cells in environmental samples. Panels showed microfluidic digital PCR on hindgut contents from termites-*Z. nevadensis*. Left: multiplex PCR using “all-bacterial” 16S rRNA gene (red) and “Clone H group” enzymatic encoding gene (green) primers and probes. Right: multiplex PCR using “all-bacterial” 16S rRNA gene (red) and *Treponema* 16S rRNA gene primers. (Genus *Treponema* is major cluster symbiotic with termites). Ottesen and others (2006).

## **Isothermal amplification and fluorescent detection methods**

Isothermal nucleic acid sequence amplification is an alternative method to PCR-based amplification for molecular analysis and point-of-care diagnosis. Unlike PCR methods, isothermal amplification methods do not require thermal cycling *in vitro*; instead, they only need a lower constant temperature (usually 64 °C to 65 °C) to denature DNA and execute the whole amplification process. Thus, isothermal amplification not only saves energy as an easy-to-use method without using bulky thermocycler, but becomes an easy-handling platform for those portable devices not suitable to carry out reactions at high temperature. Three major isothermal DNA amplification techniques have been developed. They are strand-displacement amplification (SDA), rolling circle amplification (RCA), and helicase-dependent amplification (HDA).

### Strand displacement amplification (SDA) method

One of the most popular SDA-based amplification techniques is loop-mediated isothermal amplification (LAMP) developed by Notomi and others (2000). It has been widely used in rapid pathogen detection. The principle of LAMP relies on four specially designed primers (two inner primers and two outer primers) recognizing six distinct regions on the target sequence. The inner primers annealing to the ssDNA initiate the synthesis of a new cDNA. Then the outer primers anneal to the same template, initiate strand synthesis, and displace the original newly formed ssDNA. The released ssDNAs form double-end stem-loop structures themselves that serve as a starting structure for the subsequent LAMP cycling. The final products of LAMP cycling eventually contain detectable stem-loop DNAs with several inverted repeats of the target sequences and cauliflower-like structures with multiple loops formed by interplays between alternately inverted repeats of the target in the same strand (Notomi and others 2000). The LAMP amplification is performed at a constant temperature of 65 °C. A web-based, detailed

description of LAMP as an animation is available at the website of Eiken Chemical Co., Ltd. (2005).

LAMP is a very sensitive and specific method that has been successfully used for foodborne pathogen detection because multiple specific primers are designed for precisely recognizing six distinct regions of the target sequence. For example, Hara-Kudo and others (2005) developed six pairs of primers (two inner primers, two outer primer and two loop primers) for specifically targeting the *Salmonella* invasion gene, and 220 strains of *Salmonella* were successfully identified in artificially inoculated liquid egg samples, with high sensitivity of 2.2 cfu/mL in 1 hour.

Recently, a LAMP detection system has been successfully integrated into a microfluidic device and achieved the detection limit of  $10^{-5}$  ng of *Pseudorabies* viral DNA within one hour (Fang and others 2010), which made it 100 to 1000 fold more sensitive than the conventional PCR technique for the same target. Although LAMP allows highly sensitive rapid detection and does not require special reagents and sophisticated instrument, precisely designing multiple specific primers from a highly specific gene segment remains challenging.

#### Rolling circle amplification (RCA)

In rolling circle amplification (Lizardi and others 1998), a short linear probe is designed to anneal to a specific sequence of the template forming a circular shape with a gap on the target. The gap is filled by the process of DNA polymerase, adding dNTPs (deoxyribonucleoside triphosphates, including dATP, dTTP, dCTP, and dGTP) to the 3'-OH end of the probe with the concomitant ligation by DNA ligase. The ring-shaped probe serves as a template to start rolling circle amplification led by DNA polymerase, displacing and releasing the original single-strand target sequence. The new linear single-stranded DNA sequence with multiple repeats of the

target sequence was generated and extended by DNA polymerase running along the circular template for many repetitions. A smaller circular template size caused a higher amplification rate (Kim and Easley 2011). As many as  $10^{12}$  copies of the target sequence were able to be created in a single reaction by the RCA method within an hour (Mothershed and Whiney 2005).

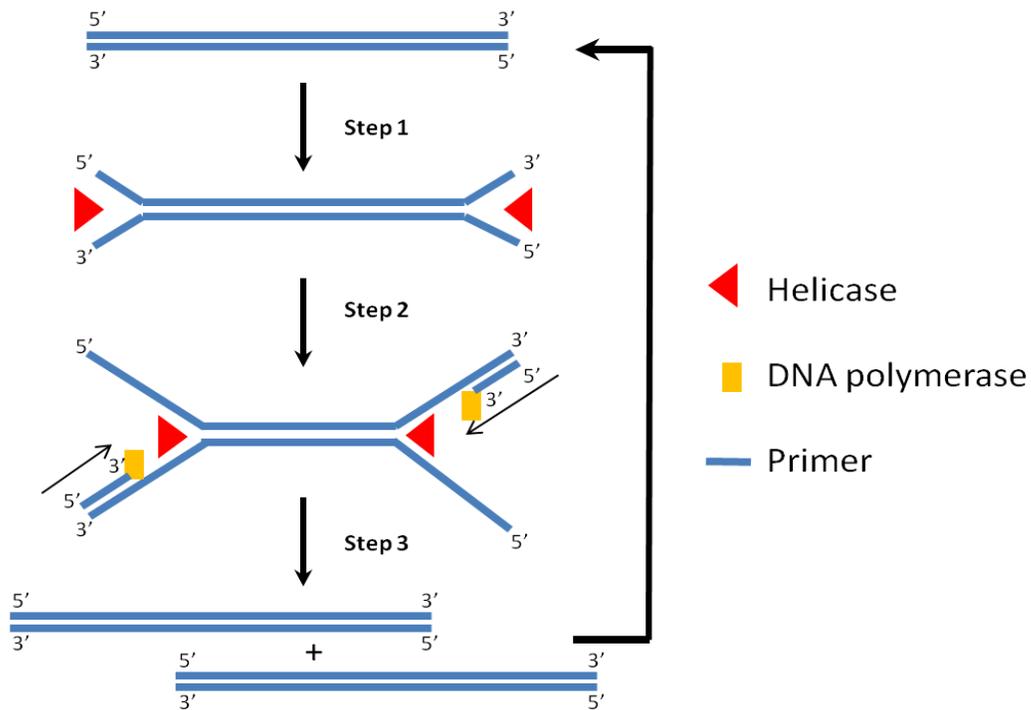
The high sensitivity of RCA is an advantage for single-molecule counting, single mutation detection (Lizardi and others 1998) and detection of foodborne pathogens. To increase the sensitivity of this technique, and consequently the detection of low amounts of pathogens in food, RCA has been coupled with magnetic bead-based electrochemiluminescence (ECL) technology for *Listeria monocytogenes* detection (Long and others 2011). With their approach, as low as 0.0002 ng/ $\mu$ L of genomic DNA from *L. monocytogenes* were successfully detected (Long and others 2011).

#### Helicase-dependent amplification (HDA) method

The technique of HDA mimics the natural DNA replication mechanism *in vivo*. It relies on *Escherichia coli* UvrD helicase to unwind and separate duplex DNA to allow hybridization of the primers to the free ssDNA templates. DNA polymerase extends the primers and produces new double-stranded DNA (dsDNA) target copies.

The original HDA method, known as mesophilic HDA (mHDA) first developed by Vincent and others (2004), was carried out at a consistent 37 °C for the entire process, and a million-fold increase of DNA targets were achieved from nanogram quantities of the template. However, two additional proteins were required in this method. A MutL protein (an accessory protein of UvrD helicase) stimulated and enhanced the unwinding activity of helicase, and a single-stranded DNA-binding protein (SSB) bonded to the ssDNA, preventing their re-association.

Later, thermophilic HDA (tHDA) was developed owing to the application of thermostable UvrD (helicase) extracted from *Thermoanaerobacter tengcongensis*, a thermophilic bacterium. The reaction occurred at 45-65 °C which not only abandoned the two accessory proteins, but



**Figure 2.2 Principle of thermophilic helicase-dependent isothermal amplification technology. Step 1, the helicase unwind DNA duplex. Step 2, the primers anneal to ssDNA. Step 3, DNA polymerase extends the 3'-OH end of primers; one DNA duplex is amplified to two DNA duplexes. The dsDNAs are separated by helicase and this chain reaction repeats itself. An and others (2005).**

greatly improved specificity, sensitivity and the DNA yield of the reaction (An and others 2005).

The simple mechanism of tHDA, as shown in Figure 2.2, made it a very attractive platform to be

used for pathogen detection (Goldmeyer and others 2007, 2008). Some foodborne pathogens

have been detected by tHDA platform within 2 hours, such as *Staphylococcus aureus*

(Goldmeyer and others 2008), toxigenic *Clostridium* (Chow and others 2008), *Bacillus* (Tong

and others 2008) and *E. coli* (Mahalanabis and others 2010). Mahalanabis and others (2010)

reported tHDA was capable of integrating into a microfluidic chip for high sensitivity of *E. coli*

detection with low detection limit of  $10^1$  cfu/mL within 50 min; all processes including DNA extraction, purification and detection can be achieved in the chip, which indicates that tHDA is amenable for use in point-of-care microfluidic devices.

### Fluorescent detection techniques

For the quantitative detection of target DNA sequences, fluorescent techniques have been commonly used as an optical detection method, allowing the measurement of amplified product in real time. As the target sequences are multiplied during the amplifying process, the fluorescence intensity of the DNA-binding dye or fluorescent-labeled probe increases to a detectable level. Several commercial fluorescent dyes, such as SYBR Green, EvaGreen, and fluorophore-containing DNA probes (e.g., TaqMan probe), have been successfully used for real time detection. The characteristics of each fluorescent dye are listed in Table 2.1. The advantages of EvaGreen dye compared to other probes are high binding specificity, good detection sensitivity, cost-effectiveness, and environmental safety. EvaGreen dye's preference of binding high adenine/thymidine (AT) DNA sequences is another attractive characteristic for the tHDA method because the amplification products from the tHDA reaction usually have high AT contents in the dsDNA sequences.

### **Objective**

Compared to other isothermal detection methods, tHDA is relatively new, yet it has been successfully applied in many areas due to its simple mechanism. However, as for *Salmonella* detection by tHDA, few reported studies exist. The objective of this study was to develop and test a tHDA method for *Salmonella* serovar Typhimurium detection in real time.

**Table 2.1 Comparison of Three Fluorescent Detection Techniques Used in Quantitative Detection of Target Sequence**

|  | <b>SYBR Green Dye (SG)</b>  | <b>EvaGreen Dye (EG)</b>  | <b>TaqMan Probe</b>  |
|--|---|---|--|
| <b>Detection mechanism</b>               | <ul style="list-style-type: none"> <li>▪ a dsDNA binding dye; detect product as it accumulates during amplification techniques</li> </ul>   | <ul style="list-style-type: none"> <li>▪ a dsDNA binding dye; detect product as it accumulates during amplification techniques</li> </ul>   | <ul style="list-style-type: none"> <li>▪ fluorescence labeled probe specific detect target sequence as it accumulates during amplification techniques</li> </ul>   |
| <b>Excitation &amp; Emission spectra</b> | <ul style="list-style-type: none"> <li>▪ Excitation: 494 nm</li> <li>▪ Emission: 521 nm</li> </ul>  | <ul style="list-style-type: none"> <li>▪ Excitation: 495 nm</li> <li>▪ Emission: 525 nm</li> </ul>  | <ul style="list-style-type: none"> <li>▪ depend on the labeled fluorescent dye</li> </ul>  |
| <b>Features</b>                          | <ul style="list-style-type: none"> <li>▪ prefer to bind GC-rich sequences</li> </ul>  | <ul style="list-style-type: none"> <li>▪ prefer to bind AT-rich sequences compared to SYBR Green</li> </ul>   | <ul style="list-style-type: none"> <li>▪ no preferences</li> </ul>   |
| <b>Advantages</b>                        | <ul style="list-style-type: none"> <li>▪ monitor the amplification of any double-stranded DNA sequence.</li> <li>▪ no probe is required, which can reduce assay setup and running costs</li> <li>▪ low affinity to very short ddDNA (10 bp) &amp; long ddDNA (500 bp)</li> </ul>                                  | <ul style="list-style-type: none"> <li>▪ monitor the amplification of any double-stranded DNA sequence.</li> <li>▪ no probe is required, which can reduce assay setup and running costs</li> <li>▪ low affinity to very short ddDNA (10 bp) and long ddDNA (500 bp)</li> <li>▪ fluorescence brighter than SG</li> <li>▪ much less likely to cause nonspecific amplification</li> <li>▪ allow high conc. for permitting a more robust signal</li> <li>▪ stable during freeze &amp; thaw and high temperature condition</li> <li>▪ serve both as a quantitative amplification dye and DNA gel stain</li> <li>▪ environmental safe: Non-mutagenic safe to handling and easy disposal down the drain</li> </ul> | <ul style="list-style-type: none"> <li>▪ high specific hybridization</li> <li>▪ high sensitivity (1-10 copies)</li> <li>▪ probes can be labeled with different, distinguishable reporter dyes</li> <li>▪ allows detection of two distinct sequences in one reaction tube</li> <li>▪ post-processing (e.g., melting curve) is eliminated</li> </ul> |
| <b>Disadvantages</b>                     | <ul style="list-style-type: none"> <li>▪ may generate false positive signal</li> <li>▪ high specific well-designed primers are required</li> <li>▪ reaction optimization is extremely important</li> <li>▪ high conc. can inhibit amplification reaction</li> <li>▪ extremely toxic; effective mutagen</li> </ul> | <ul style="list-style-type: none"> <li>▪ high specific well-designed primers are required</li> <li>▪ reaction optimization is extremely important</li> </ul>  | <ul style="list-style-type: none"> <li>▪ synthesis of different probes is required for different sequences</li> <li>▪ very expensive</li> </ul>  |

## Chapter 3 Materials and Methods

### Bacterial strains and isolates

Two *Salmonella* Typhimurium isolates, one *Salmonella* Typhi isolate, one *Salmonella* Newport isolate, one *Salmonella* Heidelberg isolate and *Escherichia coli* isolate were used in this study (Table 3.1). Isolates from American Type Culture Collection (ATCC) were revived in tryptic soy broth (Becton, Dickinson and Company, Franklin Lakes, NJ) and stored with 30% glycerol at -20 °C. Purity of each strain was then tested and confirmed using API 20E biochemical test panels (bioMérieux, Inc., Durham, NC).

**Table 3.1 Bacterial Strains and Isolates Used in the Real-time tHDA**

| <b>Species</b>         | <b>ATCC #</b>                           | <b>Other ID</b> |
|------------------------|---|-----------------|
| <i>S. Typhimurium</i>  | 19585                                   | LT2             |
| <i>S. Typhimurium</i>  | 13311                                   | NCTC 74         |
| <i>S. Typhi</i>        | 9993                                    | AMC 42-A-63     |
| <i>S. Newport</i>      | USDA lab in Athens GA, Dr. Nelson A Cox |                 |
| <i>S. Heidelberg</i>   | USDA lab in Athens GA, Dr. Nelson A Cox |                 |
| <i>E. coli</i> O157:H7 | 43895                                   | CDC EDL 933     |

### Culture and Medium conditions

To recover strains from -20 °C glycerol stock suspensions, a sterile inoculation loop was used to scrape the ice of the frozen culture, and then inoculated into 3 mL of appropriate broth mediums (Table 3.2). Frozen stocks were not thawed to prevent the loss of cell viability. Cell cultures were shaken at 160 rpm in an incubator at 37 °C overnight. A sterile inoculation loop

was then dipped into the cell culture. The bacteria on the loop were then streaked three times on each prepared medium plate for pure strain isolation. Medium types for different bacterial strains are shown in Table 3.2.

**Table 3.2 The Culture Mediums Prepared for Each Bacterial Strain**

| <b>Species</b>         | <b>ATCC #</b> | <b>Growth mediums</b>              |
|------------------------|---------------|------------------------------------|
| <i>S. Typhimurium</i>  | 19585         | Nutrient Agar/Broth with 0.5% NaCl |
| <i>S. Typhimurium</i>  | 13311         | Nutrient Agar/Broth                |
| <i>S. Typhi</i>        | 9993          | Nutrient Agar/Broth                |
| <i>S. Newport</i>      |               | Nutrient Agar/Broth                |
| <i>S. Heidelberg</i>   |               | Nutrient Agar/Broth                |
| <i>E. coli</i> O157:H7 | 43895         | Tryptic Soy Agar/Broth             |

All plates were subsequently incubated for 24 h at 37 °C under microaerobic conditions (85% nitrogen, 10% carbon oxide, 5% oxygen) using anaerobic jars. A single colony of each strain was picked using a loop and suspended into phosphate buffered saline (PBS). The turbidities of cell suspensions were adjusted to OD<sub>600</sub>=0.1 by adding PBS buffer. In order to ensure the efficiency of DNA isolation, a maximum of  $2 \times 10^9$  cells were allowed to be used in the isolation process. One mL of the suspension from each strain was used for fresh DNA extraction. Simultaneously, the suspension was serially diluted in PBS buffer, and 100 µL from each dilution were spread on media plates. Plates were incubated at 37 °C under microaerobic conditions, and colony numbers were counted after 48 hours of incubation.

#### Genomic DNA isolation

Cells were harvested in a microcentrifuge tube by centrifuging for 10 min at  $5000 \times g$ . The supernatant was discarded, and the pellet was re-suspended in 180 µL animal tissue lysis (ATL) buffer (breaks open cells and nuclear membranes) for DNA isolation. Genomic DNA (gDNA)

from bacterial standard media (*S. Typhimurium* ATCC 19585; *S. Typhimurium* 13311; *S. Typhi* 9993; *S. Newport*; *S. Heidelberg*; *E. coli* O157: H7 43895) were sequentially extracted using a QIAGEN DNeasy Blood & Tissue Kit (QIAGEN, Valencia, CA) according to the manufacturer's instructions. The final extracted genomic DNAs (gDNAs) were stored in TE buffer (10 mM Tris-Cl, pH 7.5, 1 mM EDTA) included in the DNA isolation kit. The absorbance of the DNA solution for each strain was measured at 260 nm 3 times using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, West Palm Beach, FL), and the mean values were recorded as the actual absorbance. The absorbances of DNA solutions were also measured at 280 nm and 230 nm for contaminant evaluation. Lower A<sub>260/280</sub> and/or A<sub>260/230</sub> values may indicate protein contamination and/or contamination with salts and solvents (e.g., phenol). For acceptable purity, it is recommended that A<sub>260/280</sub> ≥ 1.8 and A<sub>260/230</sub> ≥ 2.0. An aliquot (1 μL) of the diluted gDNA from each strain was used in the tHDA reaction to evaluate the specificity and the sensitivity.

#### Oligonucleotides design and screening

In this experiment, tHDA primers were designed based on the *inv* gene cluster and the STM4497 gene of *S. Typhimurium* LT2 (PrimerQuest<sup>®</sup> program 2013). The parameters in the PrimerQuest program were set as follows to fit the requirements of the tHDA reaction. Product size (the DNA segment to be amplified in the reaction) was in the range from 80 to 120 base pairs (bp), as tHDA is not efficient with larger products. The melting temperature (T<sub>m</sub>) of amplicon ranged from 68 to 75 °C with the optimal temperature being 71 °C. The primer size ranged from 24 to 33 bp with the optimum being 27 bp. Primer T<sub>m</sub> ranged from 60 to 74 °C with the optimal temperature being 68 °C. The guanine-cytosine content (G+C content) of the target sequence ranged from 35 to 60% with the optimum being 44%. However, the optimal primer T<sub>m</sub>

was set at 64 to 66 °C when the G+C content of target sequence was smaller than 37.5 % and at 70 to 72 °C when the G+C content was larger than 45%. The detailed procedures of primer design are outlined in Figure 3.1. Fifty pairs of primers for the target gene were obtained from the PrimerQuest program output. All primer sequences were analyzed using Primer-BLAST program on the National Center for Biotechnology Information website for theoretical specificity (NCBI 2011). The second structure (structures including primer hairpins, primer self-dimers, and hetero-dimers, which may form during the reaction and reduce amplification efficiency) of those primers with relatively high specificity to *S. Typhimurium* were further analyzed and measured by web-based Oligoanalyzer version 3.1 (Integrated DNA Technologies, Inc). A less negative Gibbs free energy of the primers would give a better performance during amplification.

Twenty-four primer candidates out of the 50 pairs of primers with good configurations and high specificity were selected and ordered from Life Technologies (Carlsbad, CA). The actual performance of each of the primer was tested in a two-step tHDA platform as described later. The amplified products were loaded on a 2% agarose gel stained with ethidium bromide; the electrophoresis method is also described later. One pair of primer design based on the STM4497 gene was finally selected for the amplification of the 88 bp target gene fragment because of its high specificity, stability and good sensitivity in the tHDA reaction. The target sequence, with a theoretical T<sub>m</sub> value of 79.5 °C, was located at 4751059-4751146 of the genomic DNA of *S. Typhimurium* LT2. The set of the primers used in this experiment were for the forward primer 5'-TCCTTTTCCAGATT-ACGCAACAGATACT (28 bp, T<sub>m</sub>=65.7 °C) and for the reverse primer 5'-TTGGGTTCTGGA-TTTTTGATTATCCTGC (28 bp, T<sub>m</sub>=65.5 °C), which were synthesized by Life Technologies (Carlsbad, CA).

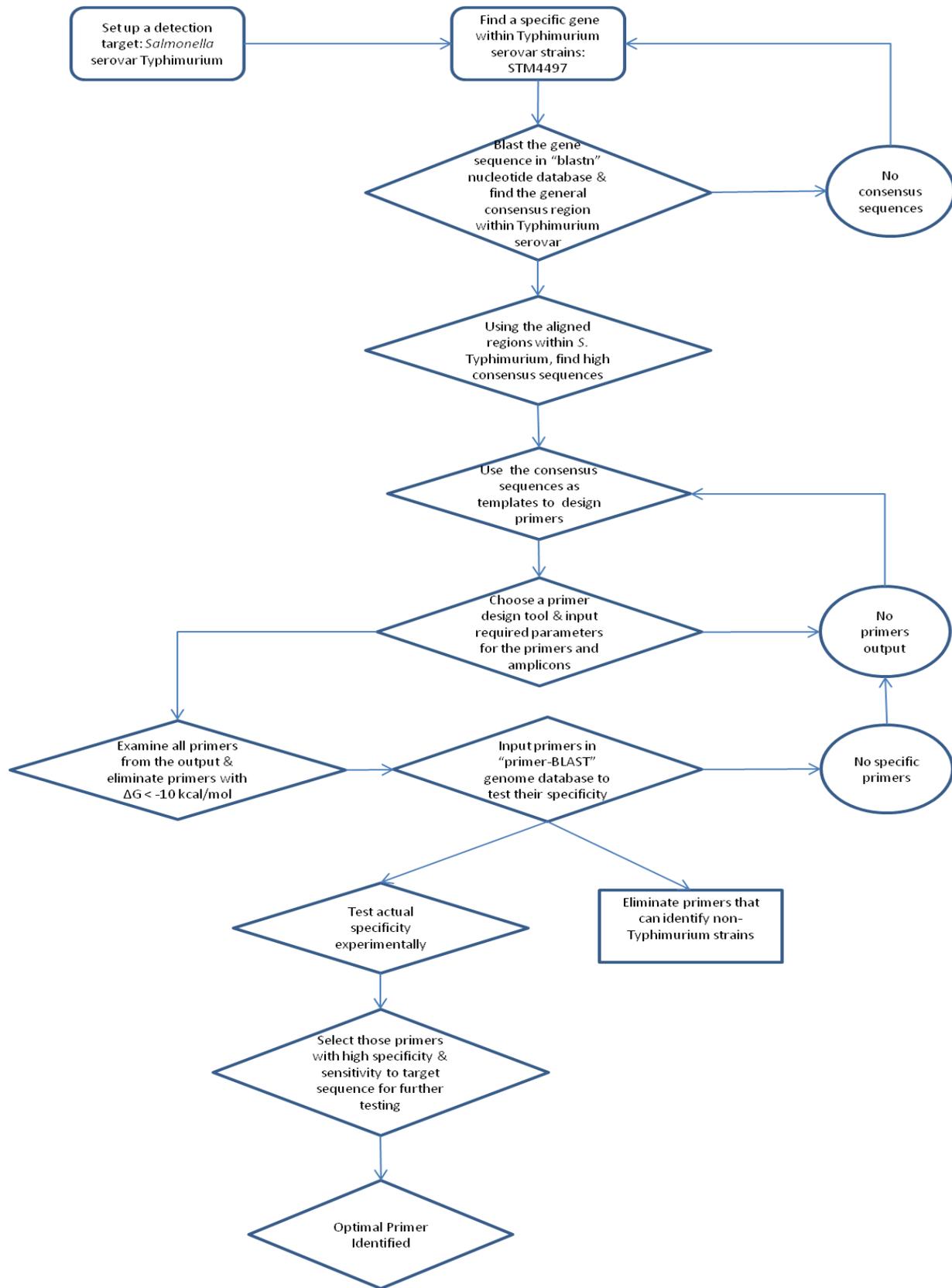


Figure 3.1 The procedures for primer design to identify *Salmonella* Typhimurium using tHDA

### tHDA amplification assay

For the real-time tHDA isothermal amplification, IsoAmp III Universal tHDA kit from Biohelix (Beverly, MA) was used. Other reagents, including EvaGreen dye and ROX reference dye, were bought from Biotium, Inc. (Hayward, CA) and Invitrogen Corporation (Carlsbad, CA), respectively. EvaGreen dye, a green fluorescent nucleic acid dye, is highly fluorescent when bound to dsDNA, and its excitation and emission spectra are very close to other commonly used fluorescent dyes (FAM and SYBR Green I), which make it compatible with most real-time monitoring methods. EvaGreen dye is nontoxic to cell membranes and nonmutagenic compared to SYBR Green I, which is well known to be a powerful mutation-enhancer. EvaGreen can be used at a higher concentration than SYBR Green I and is less likely to cause nonspecific amplification, resulting in a robust signal for specific products. In this experiment, a 0.2X concentration was used in the tHDA reaction to obtain a detectable signal. ROX (6-Carboxyl-X-Rhodamine), as a passive reference dye in the reaction, was used for normalizing noisy background signal variation and offered a baseline for the actual reading of the fluorescent intensity.

The tHDA reactions were prepared as 50  $\mu$ L volumes for amplifying the freshly isolated genomic DNA template. The tHDA reaction was dependent on the combination of two reaction mixtures, A and B (25  $\mu$ L of each), which were separately prepared. The compositions of mix A and mix B are listed in Table 3.3. The final 50  $\mu$ L mixture contained 1  $\mu$ L diluted gDNA (~1.7 ng) and 100 nM of each primer in a 1X tHDA annealing buffer II (10 mM KCl and 20 mM Tris-Cl, pH 8.8, at 25 °C), 4.5 mM MgSO<sub>4</sub>, 40 mM NaCl, 0.2 mM dNTPs, 0.4 mM dATP, 0.2X EvaGreen (100-fold dilution with water from the original solution), 1X ROX reference dye and the enzyme mix (helicase, DNA polymerase, and SSB protein). The enzyme mix was thawed on

ice, immediately added into the premixed mixture B solution, and mixed thoroughly by gentle pipetting followed by brief centrifugation. All preparations were done on ice.

**Table 3.3 Mix A (left) and Mix B (right) Solutions Composition for the Real-time tHDA Reaction**

|                                 |              |                                 |              |
|---------------------------------|--------------|---------------------------------|--------------|
|                                 |              | H <sub>2</sub> O                | 9.25 $\mu$ L |
|                                 |              | 10 $\times$ Annealing buffer II | 2.5 $\mu$ L  |
|                                 |              | MgSO <sub>4</sub> (100 mM)      | 2.25 $\mu$ L |
| H <sub>2</sub> O                | 19.5 $\mu$ L | NaCl (500 mM)                   | 4.0 $\mu$ L  |
| 10 $\times$ Annealing buffer II | 2.5 $\mu$ L  | dNTP                            | 3.5 $\mu$ L  |
| Forward Primer (5 $\mu$ M)      | 1.0 $\mu$ L  | EvaGreen (20 $\times$ )         | 0.5 $\mu$ L  |
| Reverse Primer (5 $\mu$ M)      | 1.0 $\mu$ L  | ROX dye (50 $\times$ )          | 1.0 $\mu$ L  |
| DNA template                    | 1.0 $\mu$ L  | Enzyme                          | 2.0 $\mu$ L  |
| The total volume of Mix A       | 25.0 $\mu$ L | The total volume of Mix B       | 25.0 $\mu$ L |

The tHDA amplification was performed in a two-step isothermal reaction in which mixture A was preheated at a high temperature, and then mixed with mixture B to induce the isothermal amplification at constant 65 °C. Mixture A was preheated at 95 °C for 2 min to completely denature and separate dsDNA. After immediately cooling to 4 °C, 25  $\mu$ L of mixture B was pipetted into each mixture A reaction and gently mixed by pipetting (8-tube PCR strip was used, and the volume of each tube was 0.2 mL). The negative control was composed of all of the same reagents but substituted molecular water (Dnase and Rnase free water) for the DNA template. The complete 50  $\mu$ L mixture (A and B) was incubated at 65 °C for around 2 hours using an ABI 7500 real-time PCR machine (Applied Biosystems, Grand Island, NY). To run tHDA in this machine, the cycle temperature was set up as 65 °C for 5 s and 64 °C for 1 min 55 s. The intensity of fluorescence increased as the target dsDNAs multiplied because the EvaGreen dye emits light when bound to the dsDNA, and this fluorescent intensity can be monitored during

amplification in real-time. The amplification plot and the melting curve of the product were automatically generated when the reaction finished. The molecular weight and the yield of the tHDA products were confirmed by loading 20  $\mu$ L of the product on a 2% agarose gel.

#### tHDA optimization assay

In the DNA amplification process, magnesium ions are required for DNA polymerase to polymerize. Hence, it was critical to identify the optimal concentration of  $MgSO_4$  for the most efficient activity of DNA polymerase. In this study, experiments were performed to optimize both concentrations of  $MgSO_4$  and primers to improve the overall tHDA performance and therefore achieve higher product yields. Genomic DNA from *S. Typhimurium* LT2 was used as the template. Three concentrations of  $MgSO_4$  (3.5 mM, 4.0 mM, and 4.5 mM) and three concentrations of primer (50 nM, 75 nM and 100 nM) were tested in the study. Therefore, each combination (3.5 mM/50 nM, 3.5 mM/75 nM, 3.5 mM/100 nM, 4.0 mM/50 nM, 4.0 mM/75 nM, 4.0 mM/100 nM, 4.5 mM/50 nM, 4.5 mM/75 nM, 4.5 mM/100 nM) was tested in individual tHDA reactions.

#### Specificity test

For the evaluation of the specificity, genomic DNA was isolated from two target *S. Typhimurium* strains and four other non-target strains, including *S. Typhi*, *S. Newport*, *S. Heidelberg*, and *E. coli* O157: H7. tHDA was run with the gDNA from each strain to determine the specificity of the DNA primer for *S. Typhimurium* serotype. One nanogram (representing approximately  $10^5$  genome copies) of genomic DNA of each of these six strains was added alone to each tHDA reaction and analyzed by the Real-time 7500 PCR system. One negative control containing the same reagents but replacing the DNA template with an equal amount of molecular water (Dnase and Rnase free water) was included in each test. One positive control (pCNG1,

plasmid DNA from *Naegleria gruberi*) contained in the IsoAmp III Universal tHDA kit (Biohelix Corporation, Beverly, MA) was included in the specificity test. The analytical specificity test was performed in triplicate, and the amplified products were confirmed on 2% agarose gel stained with ethidium bromide.

#### Sensitivity study and standard curve of the real-time tHDA with genomic DNA

Sensitivity of the method was assayed based on whether target sequences could be amplified by the real-time tHDA system from a specific starting amount of DNA template. The detection limit and standard curve of the real-time tHDA was determined using *S. Typhimurium* LT2. The overnight-grown culture of *S. Typhimurium* LT2 was diluted to  $OD_{600}=0.1$  for gDNA isolation, as described in the earlier DNA isolation procedure. Simultaneously, bacterial counts were obtained using 10-fold serial dilutions of the cell culture ( $10^{-1}$  to  $10^{-6}$ ) and the standard plate method with nutrient agar (Becton, Dickinson and Company, NJ) containing 0.5% NaCl for obtaining the original cell numbers. Each dilution was prepared in triplicate. A negative control consisting of broth spread on triplicate plates was also prepared for each of the dilutions. Ten-fold serial dilutions of gDNA from *S. Typhimurium* LT2 cell culture ( $OD_{600}=0.1$ ) were prepared in TE buffer (QIAEN, CA) and 1  $\mu$ L of each dilution was subjected to the real-time tHDA for the evaluation of the sensitivity of the test. A standard curve was then generated based on the relationship of the concentration of gDNA and the threshold cycle (Ct) value, which is determined by the cycle number at which the fluorescence starts to be detectable. The amplified products were further confirmed on a 2% agarose gels stained with ethidium bromide.

#### Agarose gel electrophoresis

For preparation of a 2% agarose gel, one gram pure agarose was accurately weighed and dissolved in 50 mL tris-borate-EDTA (TBE) buffer. The solution was then microwaved for 2

min. The 0.5 µg/mL working concentration of ethidium bromide (Sigma-Aldrich, Louis, MO) was used in the agarose gel for visualizing DNA products. Ten µL of DNA ladder (Promega Corporation, Madison, WI) and 20 µL of the tHDA products from different targets were loaded under the TBE buffer into each well, and the electrophoresis was run at the voltage of 80 mV for 1 hour. The molecular weights of products were compared with the standard DNA ladder. In this experiment, the sizes of tHDA products ranged from 74 to 112 bp. Therefore, the 50 bp DNA ladder was selected as a marker of molecular weight, and performed in a concentrated 2% agarose gel because the smaller sizes of molecular products need to be performed at more concentrated gel solution to prevent running too fast.

## Chapter 4 Results and Discussion

### Genomic DNA isolation

Genomic DNA (gDNA) from two target *S. Typhimurium* strains (*S. Typhimurium* ATCC 19585; *S. Typhimurium* ATCC 13311) and four non-*Salmonella* Typhimurium strains (*S. Typhi* ATCC 9993; *S. Newport*; *S. Heidelberg*; *E. coli* O157:H7 ATCC 43895) were sequentially extracted, and the gDNA concentration and purity from each strain was determined. The results are shown in Table 4.1. As previously described, the ratios of absorbance 260 nm to 280 nm (A260/280) and 260 nm to 230 nm (A260/230) are used to assess the purity of DNA. All the A260/280 ratios and A260/230 of gDNAs were above 1.8 and 2.0, respectively, indicating that the gDNAs were pure and suitable to be used in experimental tests.

**Table 4.1 Concentrations, Absorbances, and Absorbance Ratio and Information of the Isolated Genomic DNAs Solutions**

|                             | Nucleic Acid Conc. (ng/μL) | A260  | A280  | A230  | A260/A280* | A260/A230** |
|-----------------------------|----------------------------|-------|-------|-------|------------|-------------|
| <i>S. Typhimurium</i> LT2   | 170.5                      | 3.411 | 1.679 | 1.416 | 2.03       | 2.41        |
| <i>S. Typhimurium</i> 13311 | 136.4                      | 2.729 | 1.425 | 1.181 | 1.92       | 2.31        |
| <i>S. Typhi</i>             | 90.2                       | 1.842 | 0.928 | 0.837 | 1.98       | 2.20        |
| <i>S. Newport</i>           | 50.8                       | 0.978 | 0.454 | 0.496 | 2.16       | 1.97        |
| <i>S. Heidelberg</i>        | 65.3                       | 1.289 | 0.602 | 0.626 | 2.14       | 2.06        |
| <i>E. coli</i> O157:H7      | 156.4                      | 2.966 | 1.373 | 1.476 | 2.16       | 2.01        |

\*A260/A280  $\geq$  1.8 indicates good purity; \*\*A260/A230  $\geq$  2.0 indicates good purity.

### Primer test

After primer screening and selection, the properties of these primer sequences, such as G+C content and theoretical melting temperature (T<sub>m</sub>), were calculated by the PrimerQuest

program. The theoretical melting temperatures of different sizes of target sequences were also calculated and are listed in Table 4.2. The secondary structures of 24 pairs of primers were analyzed for the 3 prime end stability (hairpin), homogeneous primer self-dimer, and heterogeneous primer dimers, as shown in Table 4.2. The value of  $\Delta G$  is generally used to measure the stability of secondary structures of primer dimer which might form during amplification reaction. Usually, more positive values of  $\Delta G$  than -4 kcal/mol for primer hairpin and more positive values than -10 kcal/mol for primer self-dimer and hetero-dimer are acceptable for a good amplification reaction. The specificity of the candidate primers and target sequence was subsequently subjected to primer-BLAST check in the BLASTN database (includes all registered nucleic acid sequences). All of the 24 pairs of primers showed relatively high specificity to *S. Typhimurium*, and multiple mismatch base-pairs existed between the primers and non-*Salmonella Typhimurium* gene sequences in the BLASTN database.

#### Specificity test

To experimentally evaluate the specificity and performance of the 24 primers, extracted DNA from pure cultures of two *Salmonella Typhimurium* strains and four non-*Salmonella Typhimurium* strains were examined as templates. Only one primer pair, whose design was based on STM4497 gene from *S. Typhimurium* LT2 showed high specificity and good amplification performance in the real-time tHDA reaction. Therefore, this primer pair with forward sequence 5'-TCCTTTTCCAGATTACGCAACAGATACT and reverse sequence 5'-TTGGGTTCTGGATTTTGGATTATCCTGC (the primer pair marked with the red box in Table 4.2) was finally selected for amplifying an 88 bp target sequence, which is located from 4751059 to 4751146 in *S. Typhimurium* LT2 gDNA (Figure 4.1 and Table 4.2).

**Table 4.2 Primers Design Based on the STM4497 Gene and *inv* Gene Cluster of *Salmonella* Typhimurium LT2 and the Analysis of the Secondary Structures of Primers**

| #  | type          | sequence                         | %GC  | Tm (°C) | Amplicon (bp) | Amplicon Tm (°C) | Secondary structure analysis [kcal.mole <sup>-1</sup> ] |               |                 |
|----|---------------|----------------------------------|------|---------|---------------|------------------|---|---------------|-----------------|
|    |               |                                  |      |         |               |                  | ΔG hairpin  | ΔG self-dimer | ΔG hetero-dimer |
| 1  | STM4497 FP    | AGAATGACAAACTCTTGATTCTGA         | 33.3 | 60.4    | 90            | 78.4             | -1.71   | -6.59         | -6.57           |
|    | STM4497 RP    | AAAACAACTCGTTGGCTTATTGAATC       | 34.6 | 62.9    |               |                  | -2.1  | -5.24         |                 |
| 2  | STM4497 FP    | GAATGACAAACTCTTGATTCTGA          | 34.8 | 58.9    | 94            | 78.0             | -1.62   | -4.99         | -3.9            |
|    | STM4497 RP    | CTGAAAAACAACCTCGTTGGCTTA         | 37.5 | 62.1    |               |                  | -2.1  | -5.24         |                 |
| 3  | STM4497 FP    | AATGACAAACTCTTGATTCTGA           | 31.8 | 58.1    | 93            | 78.3             | -1.62   | -3.9          | -3.9            |
|    | STM4497 RP    | CTGAAAAACAACCTCGTTGGCTTA         | 37.5 | 62.1    |               |                  | -2.1  | -5.24         |                 |
| 4  | STM4497 FP    | AGAATGACAAACTCTTGATTCTGA         | 33.3 | 60.4    | 96            | 78.6             | -1.71   | -6.59         | -3.9            |
|    | STM4497 RP    | GCTGAAAAACAACCTCGTTGGCTTA        | 40.0 | 64.3    |               |                  | -2.1  | -5.24         |                 |
| 5  | STM4497 FP    | GAATGACAAACTCTTGATTCTGA          | 34.8 | 58.9    | 95            | 78.3             | -1.62   | -4.99         | -3.9            |
|    | STM4497 RP    | GCTGAAAAACAACCTCGTTGGCTTA        | 40.0 | 64.3    |               |                  | -2.1  | -5.24         |                 |
| 6  | STM4497 FP    | AATGACAAACTCTTGATTCTGA           | 31.8 | 58.1    | 94            | 78.5             | -1.62   | -3.9          | -3.9            |
|    | STM4497 RP    | GCTGAAAAACAACCTCGTTGGCTTA        | 40.0 | 64.3    |               |                  | -2.1  | -5.24         |                 |
| 7  | STM4497 FP    | AGAATGACAAACTCTTGATTCTGA         | 33.3 | 60.4    | 97            | 78.7             | -1.71   | -6.59         | -3.9            |
|    | STM4497 RP    | CGCTGAAAAACAACCTCGTTGGCTTA       | 42.3 | 66.0    |               |                  | -2.1  | -5.24         |                 |
| 8  | STM4497 FP    | GAATGACAAACTCTTGATTCTGA          | 34.8 | 58.9    | 96            | 78.4             | -1.62   | -4.99         | -3.9            |
|    | STM4497 RP    | CGCTGAAAAACAACCTCGTTGGCTTA       | 42.3 | 66.0    |               |                  | -2.1  | -5.24         |                 |
| 9  | STM4497 FP    | AATGACAAACTCTTGATTCTGA           | 31.8 | 58.1    | 95            | 78.7             | -1.62   | -3.9          | -3.9            |
|    | STM4497 RP    | CGCTGAAAAACAACCTCGTTGGCTTA       | 42.3 | 66.0    |               |                  | -2.1  | -5.24         |                 |
| 10 | STM4497 FP    | AGAATGACAAACTCTTGATTCTGA         | 33.3 | 60.4    | 95            | 78.4             | -1.71   | -6.59         | -3.9            |
|    | STM4497 RP    | CTGAAAAACAACCTCGTTGGCTTATTG      | 37.0 | 63.6    |               |                  | -2.1  | -5.24         |                 |
| 11 | STM4497 FP    | GAATGACAAACTCTTGATTCTGA          | 34.8 | 58.9    | 94            | 78.0             | -1.62   | -4.99         | -3.9            |
|    | STM4497 RP    | CTGAAAAACAACCTCGTTGGCTTATTG      | 37.0 | 63.6    |               |                  | -2.1  | -5.24         |                 |
| 12 | STM4497 FP    | AATGACAAACTCTTGATTCTGA           | 31.8 | 58.1    | 93            | 78.3             | -1.62   | -3.9          | -3.9            |
|    | STM4497 RP    | CTGAAAAACAACCTCGTTGGCTTATTG      | 37.0 | 63.6    |               |                  | -2.1  | -5.24         |                 |
| 13 | STM4497 FP    | AGAATGACAAACTCTTGATTCTGA         | 33.3 | 60.4    | 88            | 78.4             | -1.71   | -6.59         | -6.59           |
|    | STM4497 RP    | CTGAAAAACAACCTCGTTGGCTTA         | 37.5 | 62.1    |               |                  | -2.1  | -5.24         |                 |
| 14 | STM4497 FP    | TCCTGTCAGAATGACAAACTCTTGATTC     | 39.3 | 64.7    | 103           | 79               | -3.68   | -6.82         | -5.13           |
|    | STM4497 RP    | GCTGAAAAACAACCTCGTTGGCTTATTG     | 39.3 | 65.4    |               |                  | -2.1  | -5.24         |                 |
| 15 | STM4497 FP    | TCAAATAACCCACGTTCCAGTGAGCATG     | 44.4 | 66.6    | 112           | 80.1             | -2.6  | -6.3          | -7.07           |
|    | STM4497 RP    | TGCGTGAACACCTGAAGTATCTGTTG       | 46.2 | 66.2    |               |                  | -1.12   | -5.24         |                 |
| 16 | STM4497 FP    | TCCTTTCCAGATTACGCAACAGATACT      | 39.3 | 65.7    | 88            | 78.9             | -0.03   | -3.61         | -9.77           |
|    | STM4497 RP    | TTGGGTTCTGGATTTTTGATTATCCTGC     | 39.3 | 65.5    |               |                  | -1.88   | -6.12         |                 |
| 17 | STM4497 FP    | AGCAGGATAATCAAAAATCCAGAACCCA     | 39.3 | 66.3    | 74            | 78.7             | -2.42   | -6.12         | -5.12           |
|    | STM4497 RP    | TTAGCGAAGAGCGCTTCTCAAAAAC        | 42.3 | 65.6    |               |                  | -4.66   | -13.09        |                 |
| 18 | <i>inv</i> FP | CCAACCTGCATAGTGTGCTGATTTA        | 37.0 | 64.3    | 103           | 78.8             | -1.62   | -7.5          | -5.09           |
|    | <i>inv</i> RP | AGATGACAATATCTGAATTTTTGCTGC      | 33.3 | 62.9    |               |                  | -0.82   | -5.36         |                 |
| 19 | <i>inv</i> FP | TGAAAGTCATCCGTTCCACTACGTTCT      | 44.4 | 66.9    | 87            | 79.2             | -1.46   | -6.3          | -4.88           |
|    | <i>inv</i> RP | CAGTAGCAAGGCAGGTTGTTGAACTT       | 44.4 | 67.0    |               |                  | -1.76   | -3.9          |                 |
| 20 | <i>inv</i> FP | TGAAAGTCATCCGTTCCACTACGTTCT      | 44.4 | 66.9    | 90            | 79.3             | -1.46   | -6.3          | -4.86           |
|    | <i>inv</i> RP | GAACAGTAGCAAGGCAGGTTGTTGAA       | 44.4 | 67.0    |               |                  | -0.93   | -5.24         |                 |
| 21 | <i>inv</i> FP | TGAAAGTCATCCGTTCCACTACGTTCT      | 44.4 | 66.9    | 92            | 79.7             | -1.46   | -6.3          | -5.0            |
|    | <i>inv</i> RP | ATGAACAGTAGCAAGGCAGGTTGTTG       | 44.4 | 67.1    |               |                  | -0.93   | -5.24         |                 |
| 22 | <i>inv</i> FP | AGGTGTGGGAAATTACGGATAAAGGGT      | 44.4 | 67.1    | 101           | 79.4             | -0.46   | -5.36         | -6.61           |
|    | <i>inv</i> RP | ACGTTATTGGCGGTATTCTTCGGTAT       | 44.4 | 67.1    |               |                  | -1.9  | -6.3          |                 |
| 23 | <i>inv</i> FP | TCTTCTGTTCTTTCTGTTTGCATGC        | 44.4 | 67.0    | 90            | 79.7             | -0.28   | -3.61         | -3.61           |
|    | <i>inv</i> RP | TGGCGTCTTCTTCTTCTTCTGTC          | 44.4 | 67.0    |               |                  | 0   | -3.61         |                 |
| 24 | <i>inv</i> FP | TGATGCAATCATTATGAACTGATGCCG      | 39.3 | 65.9    | 87            | 78.5             | -1.53   | -7.05         | -6.6            |
|    | <i>inv</i> RP | GCTAATTTTCTATTTTAAATCAGCAAACACTA | 25.0 | 62.9    |               |                  | -1.25   | -8.74         |                 |

```

1  gtgatcgatcccgtgcttgaataccgctgtcacaggttcagagccgcattagcgaagag
   cactagctagggcacgaacttatggcggacagtgtccaagtctcggcgtaatcgcttctc
61  cgctttctcaaaaaacaacggctccggtaatgagatttggggttctggatTTTTgattatcct
   gcgaaagagTTTTTgTTgCCgaggccattactctaaCCcaagacctaaaaactaatagga
   RP 5' - tTggggttctggatTTTTgattatcct
                                     3' - tcatagacaacgcattagaccttttc
121 gctcagaatgagctgcaggtgCGTgaacacctgaagtatctgTTgCGtaatctggaaaag
   cgagtcttactcgacgtccacgcactTgtggacttcatagacaacgcattagaccttttc
   gc-3'
ct-5' FP
181 gaccacaagttcgcgcacctcaacatctttcagatcatcgtcgacatgctcactgaacgt
   ctgggtgttcaagcgcgtggagttgtagaaagtctagtagcagctgtacgagtgacttgca
241 gggttatttgaccgCGTctgtcagcaggaagtgaaagtcggTaccgaagcgtgaaaaaa
   cccaataaaactggcgcagacagtcgTccttcactttcagccatggcttcgcgactTTTTT
301 caactcgttggcttatttgaatcagaaaaagatcgCGgattacatagcaaaaaaagtcgat
   gttgagcaaccgaataacttagtctTTTTctagcgcctaattgtatcgTTTTTTTcagcta
361 cttcagaatcaagagtttgtcattctgacaggaatgggtaacgcctggcCGctggttcgt
   gaagtcttagttctcaaacagtaagactgtccttaccattgCGgaccggcgaccaagca
421 ggccatgagctgatgagcgccttgcaggacgtgatgggTTTTacccccctgctgatgttt
   ccggtactcgactactcgcggaacgtcctgcactacccaaaatggggggacgactacaaa
481 tatccgggaacctatagcggacacgatctctccccattggcggggcattgattcccgaaat
   ataggcccttggatatcgcctgtgctagagaggggtaaccgcccgtaactaagggcttta
541 tattatcgcgccttcaggctggtacccgaaagcgggctgCGgCGacattgaatcctcgt
   ataatagcgcggaagtccgacctgggctttcgcCCggacgCGctgtaacttaggagca
601 taa
   Att

```

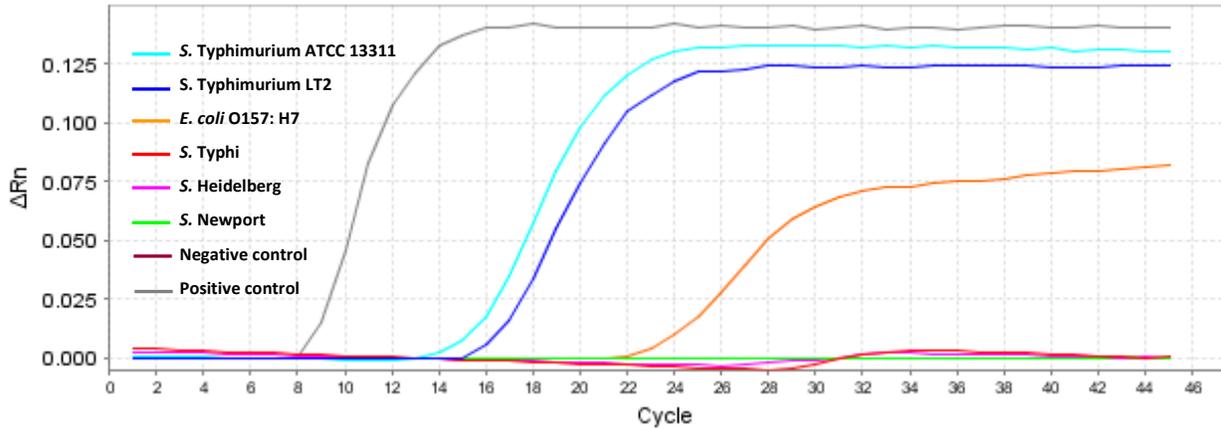
**Figure 4.1** The STM4497 gene sequence and the amplicon sequence from the gDNA of *S. Typhimurium* LT2. The yellow brightened region is the target sequence being amplified; forward and reverse primers are labeled in red.

With respect to primer specificity, the base-pair mismatches between the primer sequences and the non-target gene sequences were an essential principle for differentiating the target and non-target strains. Mismatches presented within the last three base pairs of the 3 prime end of the primer appear to be the critical region that determines the specificity of a primer. One or two base-pair mismatches in the last five base pairs of the 3 prime end of primer may result in

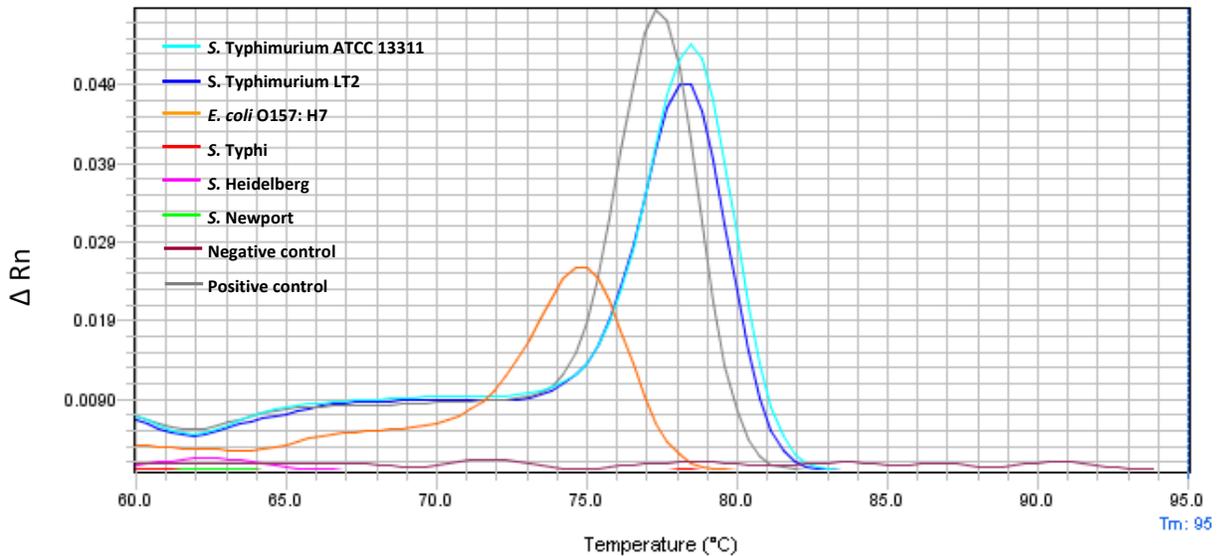
completely failed annealing. Even though more mismatches (2 or 3 mismatches) exist in the primer sequence elsewhere than the last five base pairs of the 3 prime end, DNA amplification was still able to continue. DNA polymerase can add free nucleotides only to the 3 prime end of the strand and too many mismatches at the 3 prime end could cause amplification to fail.

The *Salmonella* Typhimurium strains tested positive in the real-time tHDA assay using the primer, and showed early amplification (Figure 4.2). The DNAs from all non-*Salmonella* Typhimurium, except *E. coli* O157:H7 did not result in any amplification of tHDA products. The *E. coli* O157:H7 strain showed a late (amplification started from the 22<sup>th</sup> cycle) and low amplification trend (Figure 4.2), but not in the size range of the products generated by *Salmonella* Typhimurium. Specific tHDA products were identified by melting curve analysis; the melting points of *S. Typhimurium* LT2 (ATCC 19585) and *S. Typhimurium* (ATCC 13311) were 78.2 °C and 78.4 °C, respectively (Figure 4.3), which are close to the theoretically calculated  $T_m$  (78.9 °C) of the target amplicon. However, the melting point of the *E. coli* O157:H7 amplified products was 74.8 °C. Because  $T_m$  is 4 °C lower than the  $T_m$  value of the target amplicon, the amplification products from *E. coli* O157:H7 do not correspond to the target products. The amplified products in the *E. coli* O157:H7 reaction could be unspecific gene segment amplification or primer dimers, which could be shorter than the target amplicon (because the  $T_m$  is lower), and thus these products would be easily distinguished from the target products. Other target strains and the negative control did not show peaks in dsDNA melting curves that corresponded to 78.9 °C. A positive control obtained in the analytical kit was also run simultaneously in the tHDA reaction. Specificity of primers and absence of unspecific products or primer dimers were tested and confirmed by analyzing the reactions in 2% agarose gel stained

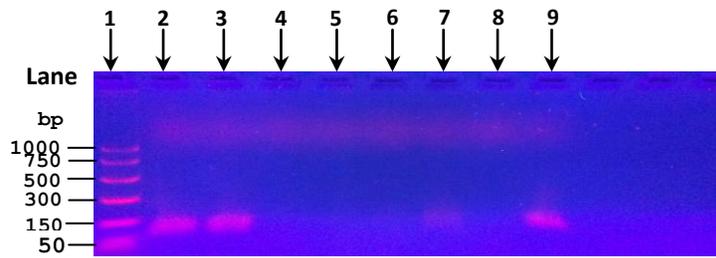
with ethidium bromide. The molecular weight of the amplified product is as expected (Figure 4.4).



**Figure 4.2** tHDA amplification curves for primer specificity test using *S. Typhimurium* target strains and non-target strains, where relative fluorescence is plotted as a function of cycle numbers.



**Figure 4.3** Melting curves for evaluating the specificity of the real-time tHDA products. The measured  $T_m=78.3$  °C of the target amplicons were close to the theoretically calculated  $T_m$  value, indicating the specific amplification. Small melting peak was found in *E. coli* O157: H7 reaction at lower  $T_m$ , indicating primer-dimer or smaller nucleic acid sequences other than the target sequence were amplified.



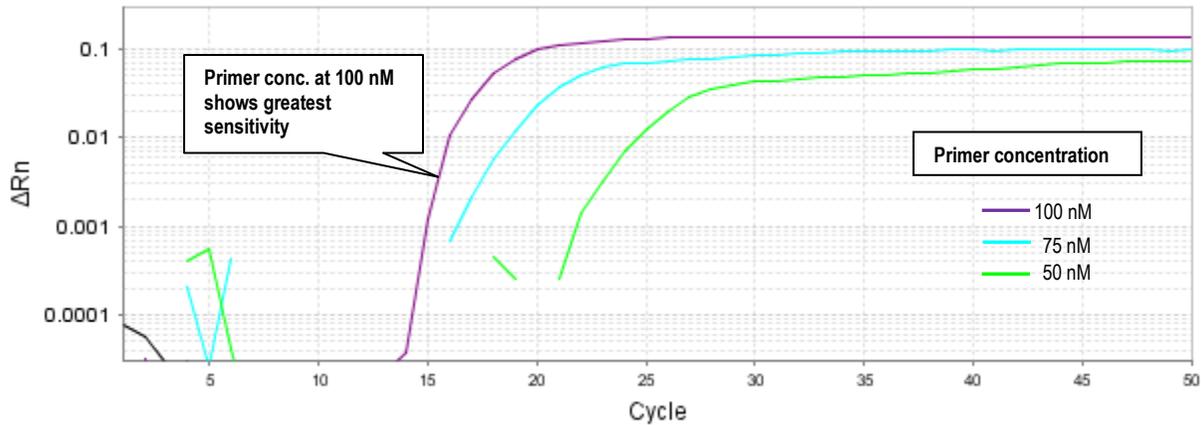
**Figure 4.4** 2% agarose gel electrophoresis for confirmation of the real-time tHDA products. Lane 1, DNA ladder; Lane 2 and 3, target strains (*S. Typhimurium* LT2 and *S. Typhimurium* 13311); Lane 4 to 7 are non-target strains (*S. Typhi*, *S. Newport*, *S. Heidelberg*, *E. coli* O157: H7); Lane 8, negative control; Lane 9, positive control.

### tHDA optimization

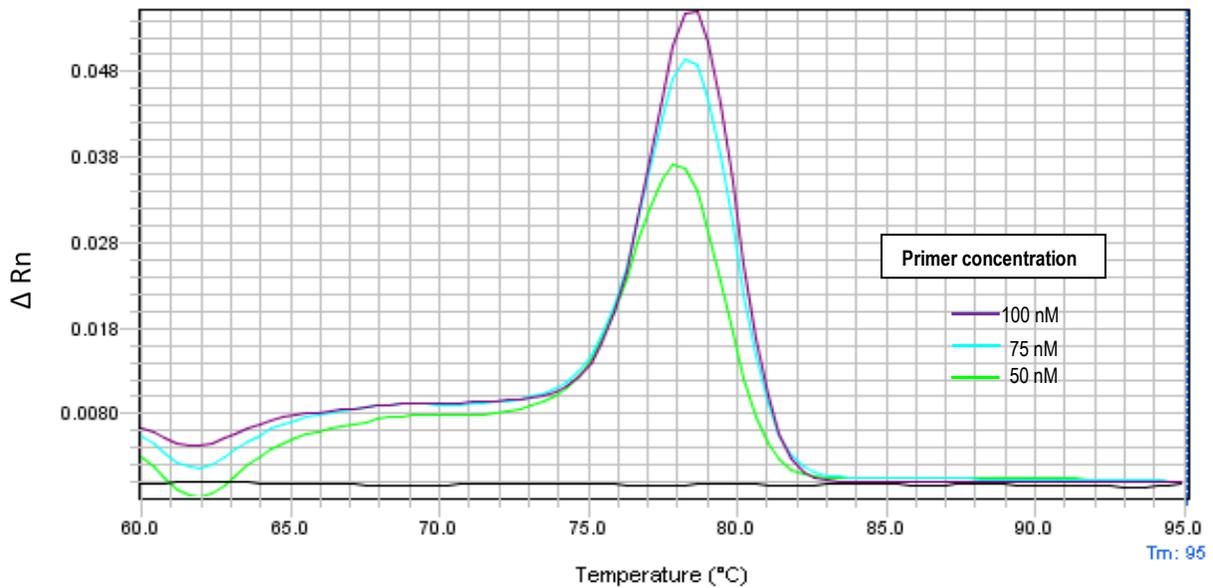
For tHDA, a reaction mix of 50  $\mu$ L was prepared, as described previously. Genomic DNA from *S. Typhimurium* LT2 was added at a concentration of around 1.7 ng/reaction (representing  $10^5$  copies of gDNA). The forward and reverse primers were systematically tested at equimolar concentrations of 50 nM, 75 nM and 100 nM in the tHDA reaction for their optimum performance (Figure 4.5). Also, the concentration of  $MgSO_4$  was titrated and standardized at each concentration of 3.5 mM, 4.0 mM, and 4.5 mM for the optimum performances in tHDA reaction (Figure 4.7). The combinations of primer/ $MgSO_4$  concentration were assayed in real-time tHDA, and the amplification products were confirmed by analyzing the melting temperatures as shown in Figure 4.6 and Figure 4.8. The amplification products were loaded into a 2% agarose gel for molecular weight confirmation (Figure 4.9).

In this study, when the concentrations of forward and reverse primers were 100 nM and the concentration of  $MgSO_4$  was 4.5 mM in the real-time tHDA reaction, the amplification started at the earliest cycle, which was cycle 13 (Figure 4.5). Specific tHDA products were identified by melting curve analysis. The melting points of the products amplified under the optimal concentration of primer and  $MgSO_4$  ranged from 77.7 to 78.4  $^{\circ}C$  (Figure 4.6) and 78.1 to 78.4  $^{\circ}C$

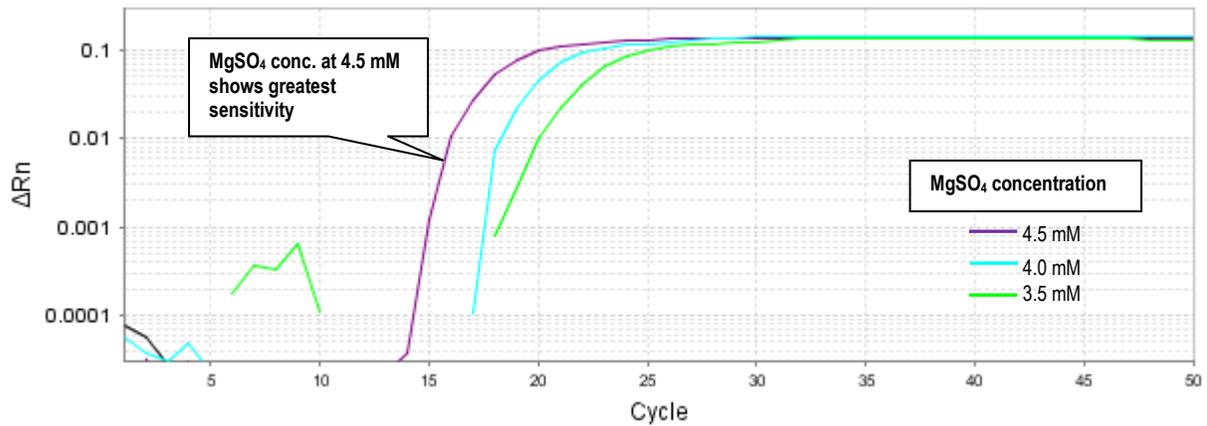
(Figure 4.8), respectively, which indicates specific amplification of the target amplicon. Products were further loaded into 2% agarose gel and the molecular weight of the amplified product was as expected (Figure 4.9).



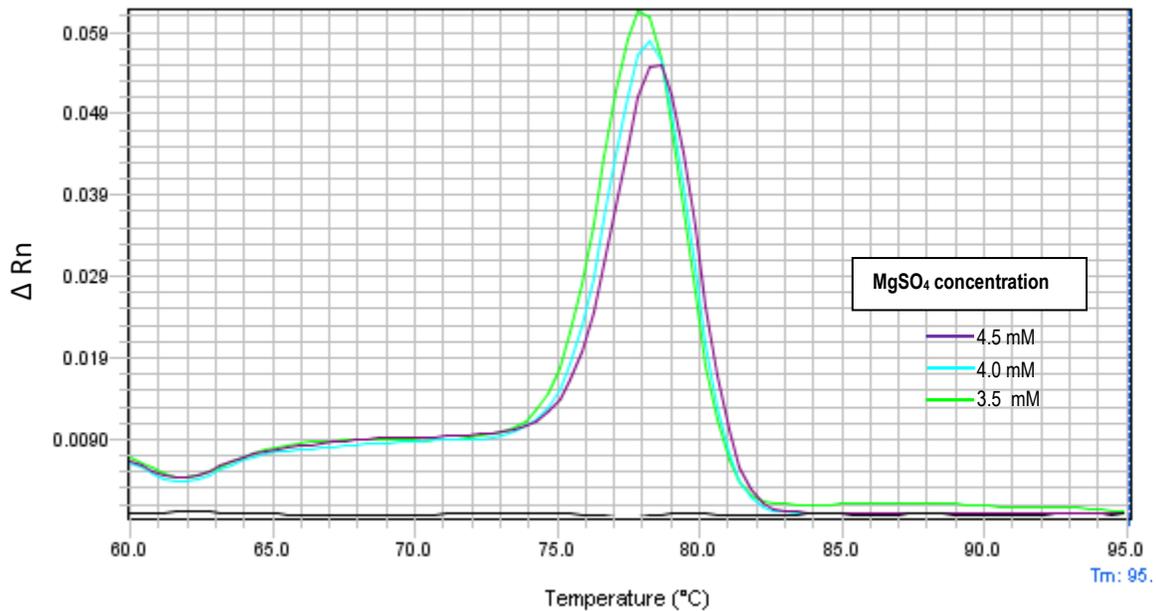
**Figure 4.5 Real-time tHDA amplification curves with different primer concentrations for optimization of the reaction conditions in the presence of 4.5 mM MgSO<sub>4</sub>. The optimum amplification condition is when the concentrations of forward and reverse primers are 100 nM. Relative fluorescence is plotted as a function of cycle numbers.**



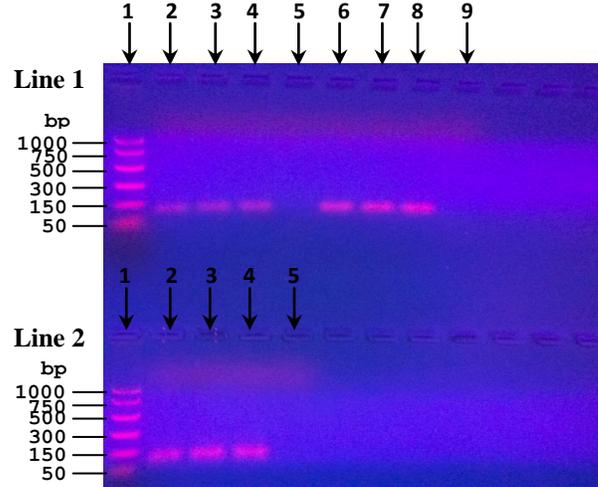
**Figure 4.6 Melting curves for verification of the specificity of real-time tHDA products under different primer concentrations in the presence of 4.5 mM MgSO<sub>4</sub>. The T<sub>m</sub> ranged from 77.7 to 78.4 °C.**



**Figure 4.7** Real-time tHDA amplification curves with different magnesium concentrations for optimization of the reaction conditions in the presence of 100 nM primers. The optimum amplification condition is when the concentration of  $MgSO_4$  is 4.5 mM. Relative fluorescence is plotted as a function of cycle numbers.



**Figure 4.8** Melting curves for verification of the specificity of real-time tHDA products under different magnesium concentrations in the presence of 100 nM primer. The  $T_m$  ranged from 78.1 to 78.4 °C.



**Figure 4.9** 2% agarose gel electrophoresis for confirmation of the real-time tHDA products, amplified in different reaction conditions. Nine groups of tHDA product, amplified in different primer/MgSO<sub>4</sub> concentrations were loaded in line 1 and Line 2. Line 1 (1-5) are 100-bp DNA ladder, 50 nM/3.5 mM, 50 nM/4.0 mM, 50 nM/4.5 mM, negative control. Line 1 (6-9) are 75 nM/3.5 mM, 75 nM/4.0 mM, 75 nM/4.5 mM, negative control. Line 2 (1-5) are 100-bp DNA ladder, 100 nM/3.5 mM, 100 nM/4.0 mM, 100 nM/4.5 mM, negative control.

*Sensitivity study and standard curve of the real-time tHDA with genomic DNA*

The fresh isolated gDNA from *S. Typhimurium* LT2 overnight culture was used in the sensitivity study. The final concentration of the gDNA was 170.5 ng/μL. The ratios of 260/280 and 260/230 were 2.03 and 2.41, respectively, which satisfied the requirements for the pure DNA products, as previously described.

DNA was serially diluted tenfold with TE buffer to give DNA concentrations of 17.0 ng/μL to  $17.0 \times 10^{-5}$  ng/μL (representing  $3.3 \times 10^6$  to  $3.3 \times 10^1$  copies of gDNA), which were subjected to the real-time tHDA for the low detection limit test (Figure 4.10, Figure 4.14 and Figure 4.18). The diluted culture (OD<sub>600</sub>=0.1) was simultaneously diluted tenfold and plated on nutrient agar with 0.5% NaCl to determine initial bacterial counts. After 24 h incubation of all inoculated plates at 37 °C, cell density in the OD<sub>600</sub>=0.1 culture was found to be originally  $5.2 \times 10^7$  cfu/mL. Examination of the sensitivity and performance of the real-time tHDA shows that

the reaction had a limit of detection of  $17.0 \times 10^{-5}$  ng of gDNA per reaction mixture, which is very sensitive for *S. Typhimurium* detection. The analysis of the melting curves of the tHDA products from each DNA diluted reaction confirmed that the amplified products are the target amplicon (Figure 4.11, Figure 4.15 and Figure 4.19). The amplification products were loaded into a 2% agarose gel for further molecular weight confirmation as shown in Figure 4.12, Figure 4.16 and Figure 4.20.

To measure the efficiency of the real-time tHDA method, a standard curve between the threshold cycle (Ct) value and DNA concentration ( $10^1$  to  $10^6$  copies per reaction mixture) was generated. The slope of this curve represents the efficiency of the real-time tHDA reaction. The threshold cycle (Ct) value, as explained, was calculated by determining the cycle number at which the fluorescence generated within a reaction crosses the fluorescence threshold line. More templates in a reaction would need less amplification cycles to cross the threshold. Triplicate experiments were conducted to ensure the reproducibility and accuracy of the real-time tHDA method. The slopes for the regression curves were of -3.594 (Figure 4.13), -3.037 (Figure 4.17), and -3.193 (Figure 4.21), which indicate a good efficiency of the real-time tHDA amplification; slopes ranging between -3.1 and -3.6 represent efficient amplification (Bustin and Nolan 2009). The correlation coefficients ( $R^2$ ) of the three standard curves were 0.969 (Figure 4.13), 0.997 (Figure 4.17), and 0.991 (Figure 4.21).

tHDA methods have been reported for the successful detection of various bacteria. Chow and others (2008) detected the genomic DNA from pure culture of toxigenic *Clostridium difficile* with the low detection limit of 20 copies of gDNA. An and others (2005) used tHDA to detect bacteria in pure culture, and as few as 10 copies of gDNA from bacterial pure culture were detected by the method. Recently, Mahalanabis and others (2010) used tHDA method coupled

with a microfluidic device to successfully detect gDNA isolated from 10 cfu *E. coli* pure culture. In this study, sensitivity of the real-time tHDA was tested, and as low as  $17 \times 10^{-5}$  ng or  $10^1$  copies of gDNA from *S. Typhimurium* LT2 were detected by a specially designed primer, which showed a high specificity to *S. Typhimurium* strains.

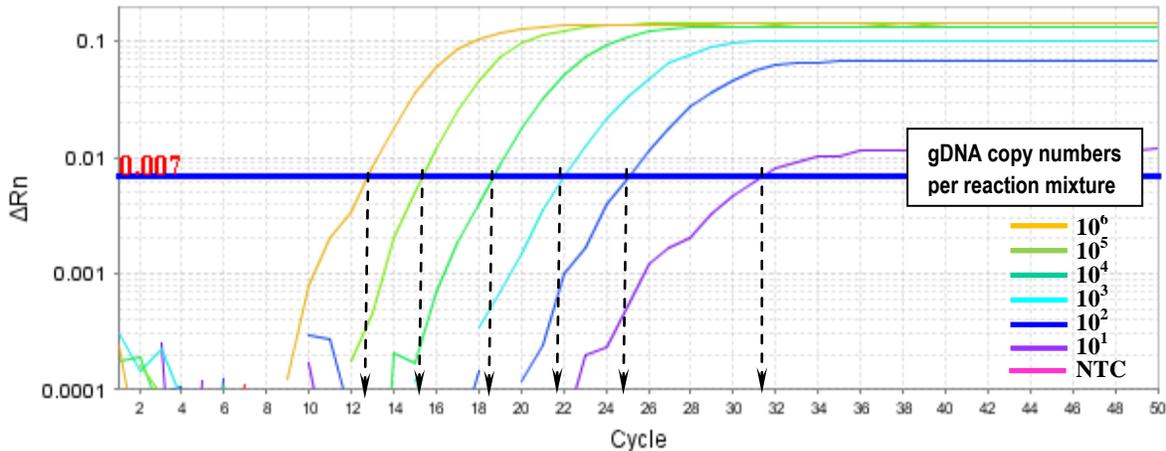
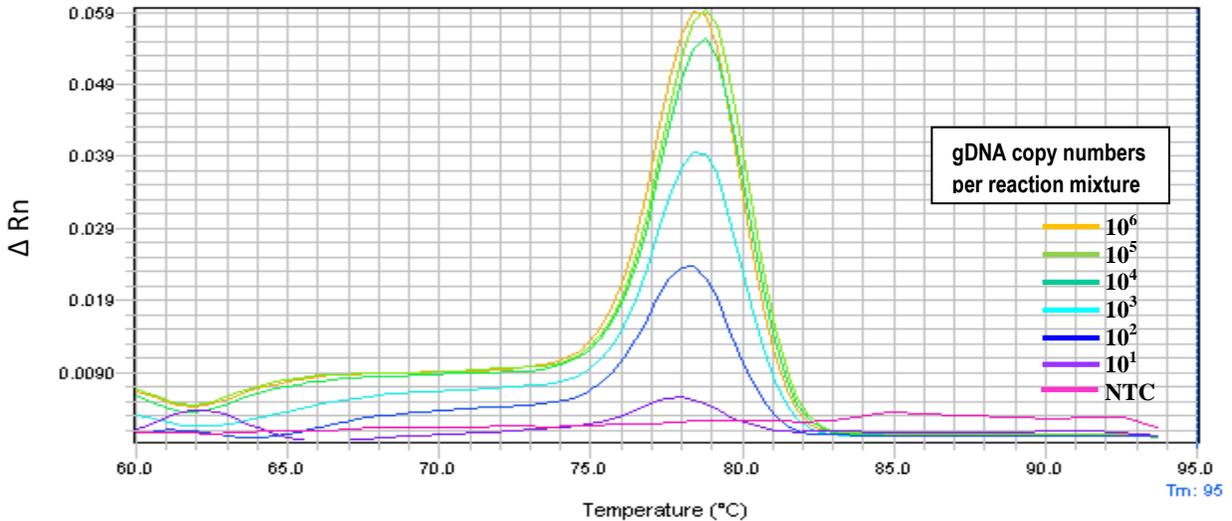


Figure 4.10 First gDNA amplification curves for the real-time tHDA sensitivity test using ten-fold diluted gDNA from *S. Typhimurium* LT2. The different colored curves represent the different amounts of gDNA from  $10^6$  to  $10^1$  copies per reaction mixture. Relative fluorescence is plotted as a function of cycle numbers. The threshold line was automatically set at the exponential amplification phase and the threshold cycle values are where the amplification curves cross the threshold line, which are used to measure the reaction efficiency later.



| Copy number | 2.6e+006 | 2.6e+005 | 2.6e+004 | 2.6e+003 | 2.6e+002 | 2.6e+001 |
|-------------|----------|----------|----------|----------|----------|----------|
| Tm (°C)     | 78.578   | 78.769   | 78.769   | 78.578   | 78.195   | 77.812   |

Figure 4.11 First melting curves for the real-time tHDA products specificity check. The table below the curves shows the Tm of the tHDA products, amplified from each initial gDNA concentration per reaction mixture.

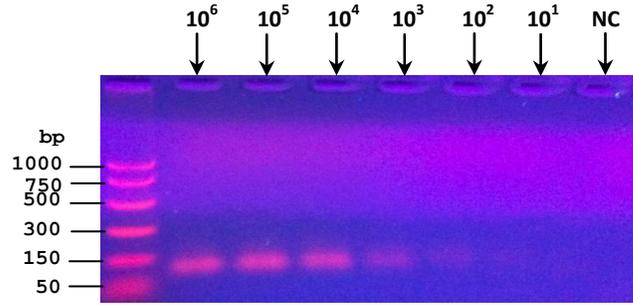
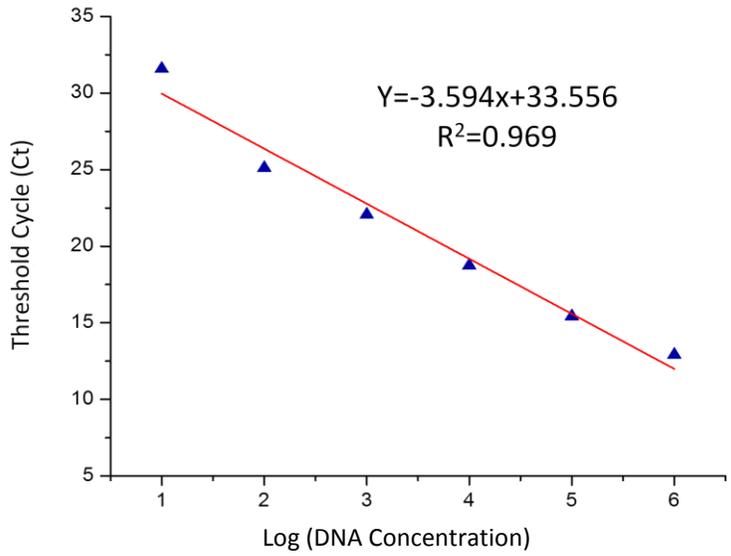


Figure 4.12 First 2% agarose gel electrophoresis for confirmation of the sensitivity of real-time tHDA reaction, amplified in different DNA concentrations (copies per reaction mixture).



|             |          |          |          |          |          |          |
|-------------|----------|----------|----------|----------|----------|----------|
| Copy number | 2.6e+006 | 2.6e+005 | 2.6e+004 | 2.6e+003 | 2.6e+002 | 2.6e+001 |
| Ct values   | 12.911   | 15.423   | 18.741   | 22.068   | 25.118   | 31.590   |

Figure 4.13 First standard curve based on the various gDNA concentrations per reaction mixture and their corresponding Ct values for evaluating the efficiency of the real-time tHDA.

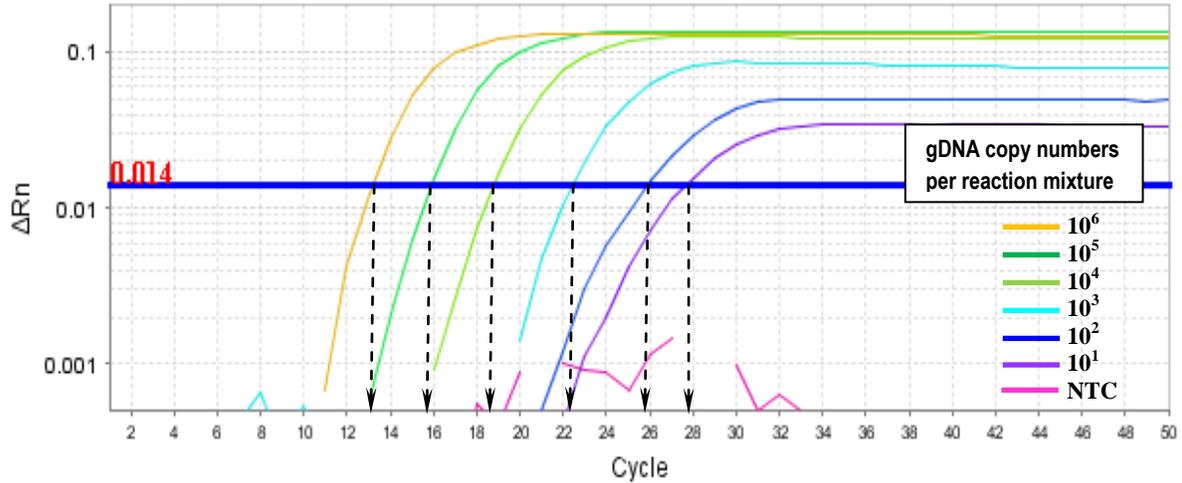
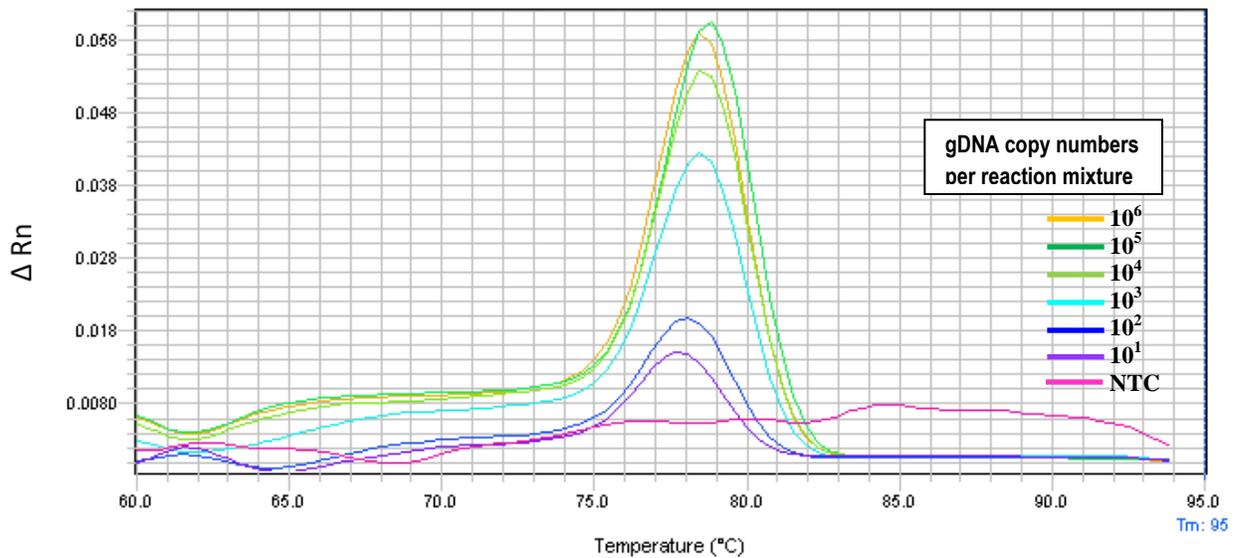
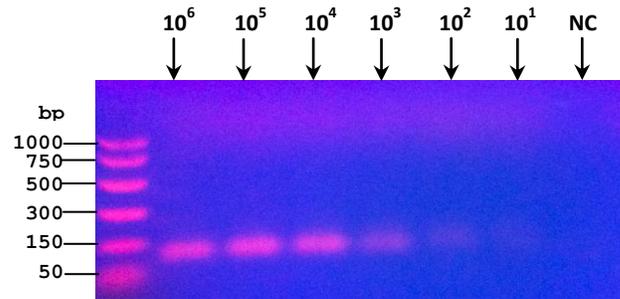


Figure 4.14 Second gDNA amplification curves for the real-time tHDA sensitivity test using ten-fold diluted gDNA from *S. Typhimurium* LT2. The different colored curves represent the different amounts of gDNA from  $10^6$  to  $10^1$  copies per reaction mixture. Relative fluorescence is plotted as a function of cycle numbers. The threshold line was automatically set at the exponential amplification phase and the threshold cycle values are where the amplification curves cross the threshold line, which are used to measure the reaction efficiency later.

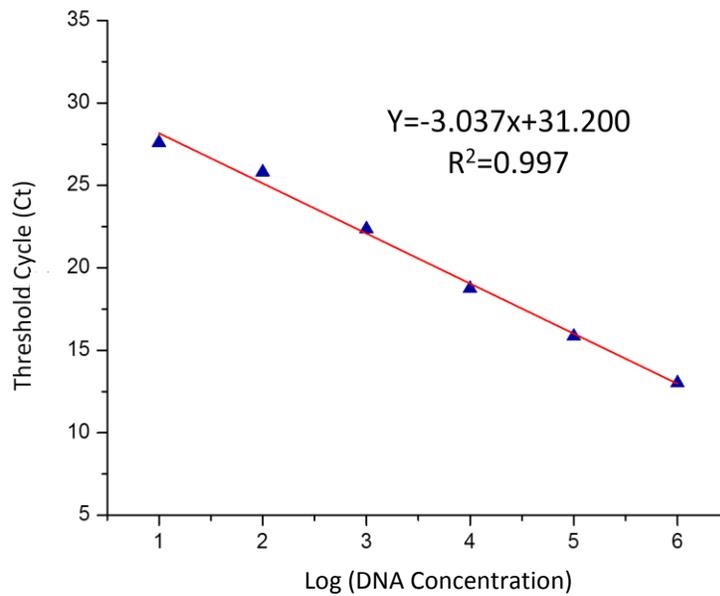


| Copy number         | 2.6e+006 | 2.6e+005 | 2.6e+004 | 2.6e+003 | 2.6e+002 | 2.6e+001 |
|---------------------|----------|----------|----------|----------|----------|----------|
| T <sub>m</sub> (°C) | 78.441   | 78.633   | 78.825   | 78.441   | 78.056   | 77.864   |

Figure 4.15 Second melting curves for the real-time tHDA products specificity check. The table below the curves shows the T<sub>m</sub> of the tHDA products, amplified from each initial gDNA concentration per reaction mixture.



**Figure 4.16** Second 2% agarose gel electrophoresis for confirmation of the sensitivity of real-time tHDA reaction, amplified in different DNA concentrations (copies per reaction mixture).



|             |          |          |          |          |          |          |
|-------------|----------|----------|----------|----------|----------|----------|
| Copy number | 2.6e+006 | 2.6e+005 | 2.6e+004 | 2.6e+003 | 2.6e+002 | 2.6e+001 |
| Ct values   | 13.030   | 15.857   | 18.753   | 22.365   | 25.815   | 27.595   |

**Figure 4.17** Second standard curve based on the various gDNA concentrations per reaction mixture and their corresponding Ct values for evaluating the efficiency of the real-time tHDA.

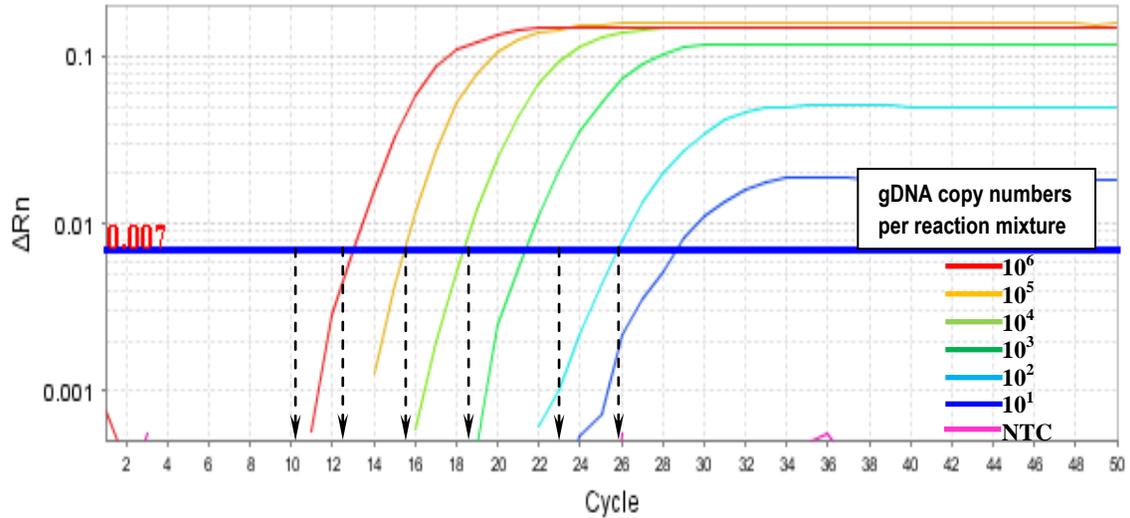
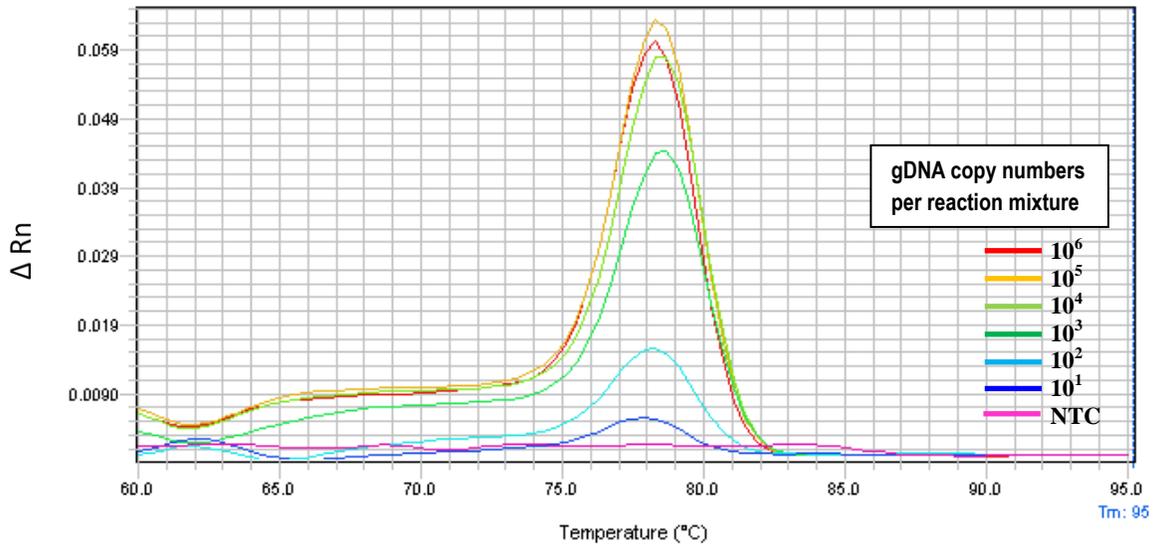


Figure 4.18 Third gDNA amplification curves for the real-time tHDA sensitivity test using ten-fold diluted gDNA from *S. Typhimurium* LT2. The different colored curves represent the different amounts of gDNA from  $10^6$  to  $10^1$  copies per reaction mixture. Relative fluorescence is plotted as a function of cycle numbers. The threshold line was automatically set at the exponential amplification phase and the threshold cycle values are where the amplification curves cross the threshold line, which are used to measure the reaction efficiency later.



|                     |          |          |          |          |          |          |
|---------------------|----------|----------|----------|----------|----------|----------|
| Copy                | 2.6e+006 | 2.6e+005 | 2.6e+004 | 2.6e+003 | 2.6e+002 | 2.6e+001 |
| T <sub>m</sub> (°C) | 78.296   | 78.296   | 78.495   | 78.495   | 78.097   | 77.898   |

Figure 4.19 Third melting curves for the real-time tHDA products specificity check. The table below the curves shows the T<sub>m</sub> of the tHDA products, amplified from each initial gDNA concentration per reaction mixture.

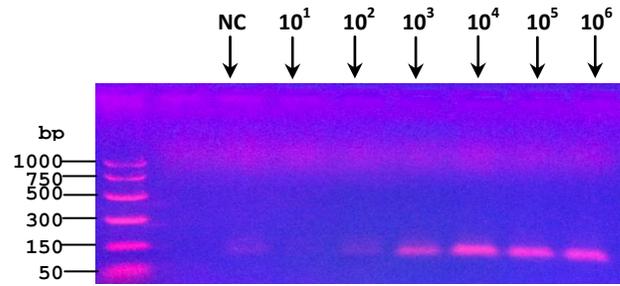
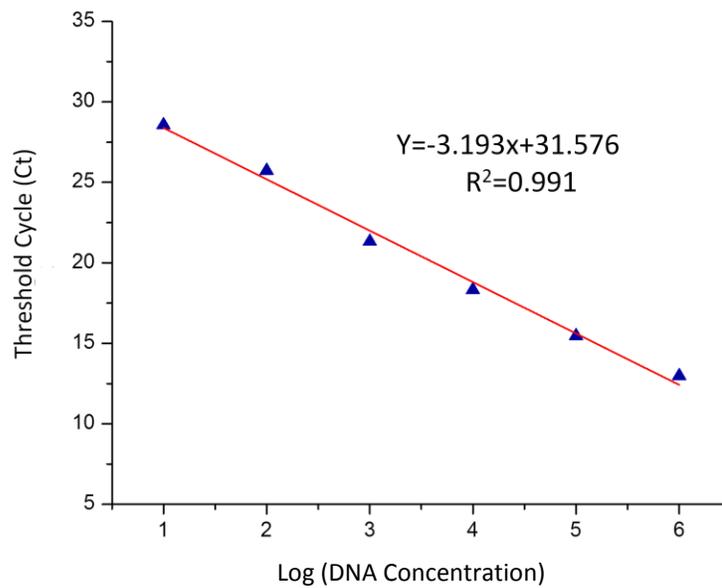


Figure 4.20 Third 2% agarose gel electrophoresis for confirmation of the sensitivity of real-time tHDA reaction, amplified in different DNA concentrations (copies per reaction mixture).



|             |          |          |          |          |          |          |
|-------------|----------|----------|----------|----------|----------|----------|
| Copy number | 2.6e+006 | 2.6e+005 | 2.6e+004 | 2.6e+003 | 2.6e+002 | 2.6e+001 |
| Ct values   | 12.985   | 15.458   | 18.320   | 21.330   | 25.735   | 28.571   |

Figure 4.21 Third standard curve based on the various gDNA concentrations per reaction mixture and their corresponding Ct values for evaluating the efficiency of the real-time tHDA.

## Chapter 5 Summary and Conclusion

The need for rapid and sensitive methods for foodborne pathogen detection is becoming increasingly urgent in food safety. In this study, the development of a tHDA method provided an isothermal platform, allowing specific detection of *Salmonella* spp. at the serovar level. Data was collected in a real-time system, so the results were obtained within 3 hours.

For the specific detection of *Salmonella* Typhimurium, 24 pairs of primers, specially designed based on the STM4497 gene (23 pairs) and the *inv* cluster genes (1 pair) of gDNA of *Salmonella* Typhimurium LT2, were tested in the real-time tHDA system. One pair of those primers with good performance and high specificity to the target sequence was selected as the candidate for the further specificity and sensitivity tests. It was found that the locations of the mismatched base pairs on a primer sequence affected the amplification process, in terms of the functionality of DNA polymerase. Mismatches presented at the 3 prime end of primer sequence provided a great benefit to differentiate the target and non-target sequences.

The efficiency and sensitivity of the tHDA can be affected by the concentration of magnesium ions and the primer concentration. With the optimal reaction configurations determined experimentally (4.5 mM MgSO<sub>4</sub> and 100 nM forward and reverse primers), the lower detection limit of  $17 \times 10^{-5}$  ng genomic DNA or  $10^1$  copies of gDNA (per reaction mixture) from a pure culture can be achieved by the real-time tHDA method.

The mechanism of the tHDA method is similar to the PCR method except it denatures and separates double DNA strands by helicase, a DNA enzyme, instead of high temperature. The fact

that the amplification is performed at a constant temperature of 65 °C eliminates the requirement for thermocycler equipment and reduces power waste. Thus, the tHDA method is a simple and cost effective alternative to real-time PCR that could be used in point-of-care diagnostics as well as for those portable devices not suitable for conducting reactions at high temperature.

Future studies will investigate incorporating the general tHDA isothermal technique into microchip devices, so a rapid, more sensitive detection method may be achieved.

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