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# Detect live salmonella cells in produce by coupling propidium monoazide with loop-mediated isothermal amplification (PMA-LAMP)

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# DETECT LIVE *SALMONELLA* CELLS IN PRODUCE BY COUPLING PROPIDIUM MONOAZIDE WITH LOOP-MEDIATED ISOTHERMAL AMPLIFICATION (PMA-LAMP)

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# Abstract

*Salmonella* is a leading cause of foodborne illnesses worldwide. In recent years, an increasing number of *Salmonella*-related outbreaks in produce has been reported. It is therefore important that the produce industry be equipped with rapid, sensitive, specific detection methods for live *Salmonella* cells in produce to better ensure the produce safety. In this study, we first designed and optimized a loop-mediated isothermal amplification (LAMP) assay for *Salmonella* detection by targeting the invasion gene (*invA*). Then we incorporated a chemical reagent, propidium monoazide (PMA) into the sample preparation step to prevent LAMP amplification of dead *Salmonella* cells. To our knowledge, this is the first study that combined these two novel technologies for live bacterial detection. The PMA-LAMP was evaluated for false positive exclusivity, sensitivity, and quantitative capability. Finally, the PMA-LAMP assay was applied to detect live *Salmonella* cells in the presence of dead cells in several produce items (cantaloupe, spinach, and tomato). The *invA*-based PMA-LAMP could avoid detecting heat-killed dead *Salmonella* cells up to $7.5 \times 10^5$ CFU per reaction and could detect down to 3.4 - 34 live *Salmonella* cells in the presence of $7.5 \times 10^3$ heat-killed dead *Salmonella* cells per reaction in pure culture with good quantitative capability ($r^2 = 0.983$). When applied to produce testing, the assay could avoid detecting heat-killed dead *Salmonella* cells up to $3.75 \times 10^8$ CFU/g and could successfully detect down to $5.5 \times 10^3$ - $5.5 \times 10^4$ CFU/g of live *Salmonella* cells in the presence of $3.75 \times 10^6$ CFU/g of heat-killed *Salmonella* cells with good quantitative capability ($r^2 = 0.993$ - 0.949). The total assay time was 3 hours. When compared with PMA-PCR, the PMA-LAMP assay was 10 to 100-fold more sensitive, 2-hour shorter, and technically simpler. In conclusion, the *invA*-based PMA-LAMP assay developed in this study was an effective tool to specifically detect live *Salmonella* cells in produce with high sensitivity and quantitative capability.

# Chapter 1 - Introduction

Members of the genus *Salmonella* are Gram-negative, rod-shaped, facultatively anaerobic, and non-spore-forming enteric bacteria that cause typhoid fever, paratyphoid fever, and foodborne diseases in humans (Jay *et al.*, 2005). Those causing foodborne illnesses are collectively termed nontyphoidal *Salmonella*. *Salmonella* is widely distributed in the environment such as water, soil, and animal feces (CIDRP, 2009). Food products such as meat, eggs, poultry, and produce are primary vehicles of transmitting *Salmonella* infections to humans (Dolye & Beuchat, 2007).

Nontyphoidal *Salmonella* is the leading cause of foodborne illnesses in the United States and worldwide. In the U.S., it is estimated that about 1.4 million cases of foodborne *Salmonella* infections occur each year (Mead *et al.*, 1999). According to the Centers for Disease Control and Prevention (CDC)'s FoodNet report, in 2009, *Salmonella* was responsible for 7,039 cases of laboratory-confirmed foodborne infections in 10 states under FoodNet surveillance, accounting for more than 40% of the total laboratory-confirmed infections (CDC, 2010a). Furthermore, in recent years, an increasing number of *Salmonella*-related outbreaks linked to fresh produce has been observed in part due to the increasing consumption of produce (Harris *et al.*, 2003). A variety of produce items including melons, tomatoes, sprouts, spinaches, and peppers have been implicated in multiple *Salmonella* outbreaks (Hanning *et al.*, 2009). Particularly, in a large outbreak occurred between April and August 2008 in the U.S. and Canada, CDC reported that *Salmonella enterica* Serotype Saintpaul caused 1,442 cases and 2 deaths (CDC, 2008). Multiple raw produce items including fresh jalapeño peppers, serrano peppers, and raw tomatoes were implicated in this outbreak. Besides, four multistate *Salmonella* outbreaks due to raw consumption of tomatoes were reported in the U.S. between 2005 and 2006, resulting in 459

illnesses (CDC, 2007). Additionally, multistate outbreaks of *Salmonella* serotype Poona infections occurred in the spring of consecutive 2000 - 2002, associated with the consumption of Mexico-imported cantaloupes (CDC, 2002).

To identify potential contamination problems during the production, processing, and distribution of produce, it is critical for the industry to have rapid, reliable, and user-friendly techniques that can be used to better control produce safety. For detecting *Salmonella*, traditional culture-based methods are reliable but time-consuming and labor-intensive, demanding several days even weeks for definitive results (Andrews & Hammack, 2007). Besides culture-based methods, many immunological-based methods such as enzyme-linked immunosorbent assay (ELISA) and immunomagnetic separation (IMS) have also been developed to detect *Salmonella* (Barrow *et al.*, 1989; Favrin *et al.*, 2001; Mansfield & Forsythe, 2000; Prusak-Sochaczewski & Luong, 1989; Skjerve & Olsvik, 1991). However, low specificity of the immunological-based methods has limited their use. Recently, rapid molecular-based methods such as polymerase chain reaction (PCR) and real-time PCR have been widely applied in *Salmonella* detection, and demonstrated to be efficient and sensitive (Botteldoorn *et al.*, 2006; Eriksson & Aspan, 2007; Krascsenicsova *et al.*, 2008; Malorny *et al.*, 2004). However, both PCR and real-time PCR demand a dedicated thermal cycler, which are expensive, especially for real-time PCR. In 2000, a novel DNA amplification technique - loop-mediated isothermal amplification (LAMP) was developed by a group of Japanese investigators (Notomi *et al.*, 2000). Since then, LAMP has been adopted to detect multiple bacterial and viral agents including foodborne pathogens and was shown to be specific, sensitive, and rapid (Hara-Kudo *et al.*, 2005; Notomi *et al.*, 2000; Ohtsuka *et al.*, 2005; Okamura *et al.*, 2008; Ren *et al.*, 2009; Yamazaki *et al.*, 2008b; Yamazaki *et al.*, 2008c). The LAMP assay is technically simple and doesn't require a thermal cycler,

making it easy to be implemented. However, a major drawback associated with all of the molecular-based detection assays is the inability to differentiate live cells from dead ones. Recently, progress had been made in this front to circumvent the problem of false positive from dead cell amplification. First, because bacterial mRNA degrades rapidly after cell death, it can serve as a cell viability indicator and mRNA-based detection method such as reverse-transcriptase PCR (RT-PCR) and real-time RT-PCR (qRT-PCR) have been used to discriminate viable cells (Bej *et al.*, 1996; Burtscher & Wuertz, 2003; Gonzalez-Escalona *et al.*, 2009; Klein & Juneja, 1997). However, the disadvantages of these mRNA-based techniques include low amplification efficiency when compared with DNA-based methods and limited sensitivity and specificity. Very recently, chemicals such as ethidium monoazide (EMA) and propidium monoazide (PMA) have been found to be promising agents to differentiate live cells from dead ones. These compounds penetrate the membrane of dead cells (not live ones) and covalently crosslink with DNA during photolysis (Nocker *et al.*, 2006; Nogva *et al.*, 2003). Therefore, EMA and PMA have been incorporated into PCR and real-time PCR assays to distinguish live cells by inhibiting DNA amplification from the intercalated dead cell DNA (Cawthorn & Witthuhn, 2008; Lee & Levin, 2007; Nocker *et al.*, 2009; Rawsthorne & Phister, 2009; Rudi *et al.*, 2005a). A study comparing the efficiencies of EMA and PMA by Nocker, et al. (Nocker *et al.*, 2006) revealed that PMA was effectively excluded from membrane-intact live cells while EMA would somehow penetrate the membrane of live cells for some bacterial species, therefore, PMA was regarded as advantageous over EMA to be used in live cell detection .

This thesis research aimed to develop a rapid, sensitive, specific, and quantitative real-time LAMP assay for live *Salmonella* detection in produce. The specific objectives included: 1) To design and optimize a LAMP assay based on the *Salmonella* invasion gene (*invA*); 2) To

evaluate the specificity, sensitivity, and quantitative capability of PMA-LAMP to detect live *Salmonella*; and 3) To apply the PMA-LAMP to detect live *Salmonella* cells in the presence of dead cells in artificially contaminated produce items including cantaloupe, spinach, and tomato. Upon completion of the study, the developed PMA-LAMP assay would bring significant benefits to the produce producers, processors, retailers, and consumers by providing a better safety control tool, therefore potentially reducing the number of illnesses and deaths associated with the consumption of fresh produce.

# Chapter 2 - Literature Review

## 1) General information on *Salmonella*

### a. Microbiology

Members of the genus *Salmonella* are Gram-negative, rod-shaped (0.7 - 1.5 × 2.0 - 5.0 μm), facultatively anaerobic, and non-spore-forming bacteria belonging to the family of Enterobacteriaceae (Blackburn & McClure', 2004). Most of them are motile via peritrichous flagella with a few exceptions, such as *Salmonella* Gallinarum and *Salmonella* Pullorum (CIDRP, 2009). *Salmonella* ferments glucose and other monosaccharides to produce acid and gas. It grows on citrate as a sole carbon source, and is oxidase- and catalase-negative. *Salmonella* is mesophilic with an optimum growth temperature of 37$^{o}$C. However, some *Salmonella* can grow under extreme environmental conditions, such as elevated temperature (54$^{o}$C) or refrigerator temperature (2 to 4$^{o}$C) (Dolye & Beuchat, 2007).

*Salmonella* is widely distributed in natural environment, such as polluted water, soil, animal feces, equipment surfaces, and so on. It mainly dwells in the intestinal tracts of animals, with poultry, eggs, livestock, pets, and reptiles being their primary reservoirs (CIDRP, 2009). The widespread prevalence of *Salmonella* in natural environment and food animals favors the occurrence of *Salmonella* in food chains and leads to potential risk of food products contamination (Dolye & Beuchat, 2007).

### b. Taxonomy

*Salmonella* is divided into two main species: *Salmonella enterica* and *Salmonella bongori*. *S. enterica* is subdivided into six groups: *S. enterica* subsp. *enterica* (I), *S. enterica* subsp. *salamae* (II), *S. enterica* subsp. *arizonae* (IIIa), *S. enterica* subsp. *diarizonae* (IIIb), *S. enterica* subsp. *houtenae* (IV), and *S. enterica* subsp. *indica* (VI). *S. bongori,* which was

formerly classified as group V, has become a separate species of *Salmonella*. Based on the Kaufmann-White scheme, *Salmonella* species have been further grouped into more than 2,500 *Salmonella* serovars according to their somatic (O), flagellar (H) and additional surface (Vi) antigens. Among them, 1,454 serotypes are grouped into the subspecies *enterica* which contain almost all of the pathogenic serotypes to humans (Jay *et al.*, 2005).

**c. Clinical syndromes**

Foodborne diseases caused by *Salmonella* are termed salmonellosis, accounting for over 95% of the total *Salmonella* infections in the U.S. (Mead *et al.*, 1999). Salmonellosis are acute gastrointestinal infections with sudden onset (6 - 72 h) of diarrhea, nausea, vomiting, abdominal pains, headache, chills, and fever (Listorti & Doumani, 2001) and might last for 3 to 7 days. Most patients can recover without treatment. However, approximately 5% of patients, mainly immuno-compromised individuals, with gastrointestinal *Salmonella* infections might further develop bacteremia. These patients are also more likely to develop other extra-intestinal focal infections, including meningitis, septic arthritis, osteomyelitis, cholangitis and pneumonia (Hohmann, 2001). These are severe diseases that occur when the bacteria spread from the intestine to the blood stream and other body sites of the patient, and in those cases, antibiotic treatment can be life-saving.

For decades, it had been believed that the infective dose for *Salmonella* was more than $10^6$ cells. However, very low infective dose such as 15 to 20 *Salmonella* cells has also been reported to cause quite a few outbreaks implicating food products with a high fat content, such as chocolate, cheese, and salami (Blackburn & McClure', 2004). Infective dose might depend on the species of *Salmonella,* age and health condition of the host, and also the implicated food product.

### d. Virulence properties

All *Salmonella* strains possess an invasion gene (*invA*), which encodes proteins for adherence and invasion. Therefore, *Salmonella* can penetrate the gut lumen into the epithelium cells of host small intestine (Galan *et al.*, 1992). Upon internalization, *Salmonella* enters enterocytes, M cells, and dendritic cells in the intestinal epithelium and subsequently reaches to the submucosa by resident macrophages. Immediately, *Salmonella* spreads through the blood stream and accumulates in mesenteric lymph nodes and spleen, causing inflammation which leads to salmonellosis (Salcedo *et al.*, 2001). *Salmonella* can also produce enterotoxins and cytotoxins in the host intestinal tracts. But these toxins seem only have minor effects on the infection (Jay *et al.*, 2005). Therefore, *Salmonella* causes typical foodborne infection rather than intoxication.

## 2) Foodborne *Salmonella* illnesses and outbreaks

### a. Overview

*Salmonella* is the leading cause of foodborne illnesses in the United States, accounting for the largest number of foodborne outbreaks. In the U.S., it is estimated that about 1.4 million cases of foodborne *Salmonella* infections occur each year (Mead *et al.*, 1999). According to CDC's FoodNet report, in 2009 *Salmonella* was responsible for 7,039 cases of laboratory-confirmed foodborne infections in 10 states, accounting for more than 40% of the total laboratory-confirmed infections (CDC, 2010a). Furthermore, in recent years, an increasing number of *Salmonella*-related outbreaks linked to fresh produce has been observed in part due to the increasing consumption of produce (Harris *et al.*, 2003). A variety of produce items including melons, tomatoes, sprouts, spinaches, and peppers have been implicated in multiple *Salmonella* outbreaks (Hanning *et al.*, 2009). Particularly, in a large outbreak occurred between April and

August 2008 in the U.S. and Canada, CDC reported that *S. enterica* Serotype Saintpaul caused 1,442 cases and 2 deaths (CDC, 2008). Multiple raw produce items including fresh jalapeño peppers, serrano peppers, and raw tomatoes were implicated in this outbreak. Besides, multiple *Salmonella* outbreaks due to raw consumption of Roma tomatoes were reported in the U.S. and Canada in the summer of 2004, resulting in 561 illnesses with 30% of hospitalization (CDC, 2005). Additionally, U.S. Department of Agriculture, Economic Research Service (USDA-ERS) estimated that 1.4 million cases of salmonellosis occurred in 2008, costing 2.6 billion dollars in terms of medical costs and productivity lost (USDA-ERS, 2009b). Beside, a very recent report estimated the average cost per case of nontyphoidal salmonellosis at $9,146 while $1,851 for foodborne illness (Scharff, 2010).

Food-related salmonellosis is mostly associated with the consumption of poultry, undercooked meat or ground beef, dairy products, eggs, and fresh produce. Although poultry is historically regarded as the major culprit of *Salmonella*-implicated outbreaks, in recent years, fresh produce is emerging to become the main source of *Salmonella* infections. According to the Outbreak Alert! Database from the Center for Science in the Public Interest (CSPI), among 121 produce-linked outbreaks occurred between 1999 and 2001, 80 was due to *Salmonella*; and between 2002 and 2003, there were 31 produce-linked *Salmonella* outbreaks while 29 poultry-linked *Salmonella* outbreaks (CSPI, 2005). Produce-related *Salmonella* outbreaks are discussed in further detail in section 2b.

In recent years, a growing number of ingredient-oriented *Salmonella* infections has been observed, which results in large-scale *Salmonella* outbreaks throughout U.S. and even spread to other countries, resulting in great economic loss. Between September 2008 and January 2009, products containing *Salmonella* serotype Typhimurium-contaminated peanut butter and peanut

paste had sickened 529 persons from 43 states in the U.S. and one from Canada, of whom 116 were hospitalized and 8 died (CDC, 2009a). Further investigation revealed that the source of contamination might be the leaking roof of the processing plant (Schnirring, 2007). Another ingredient-driven *Salmonella* outbreak occurred between July 2009 and March 2010, in which 272 persons who had consumed salami products got infected by *Salmonella* Montevideo in 44 states and the District of Columbia. Black and red peppers used as ingredients in the salami products were believed to be source of *Salmonella* contamination and led to a recall of 1.2 million pounds of sausage products (CDC, 2010b).

Not all 2,500 *Salmonella* serotypes are created equal with regards to causing human infections. According to CDC's FoodNet report (CDC, 2010a), Enterditis, Typhimurium, Newport, and Javiana are the top 4 most common serotypes to cause *Salmonella* foodborne diseases in 10 states of the U.S. in 2009, accounting for 55.9% of the total *Salmonella* infections. Other serotypes including Heidelberg, Montevideo, I 4,[5],12:i:-, Muenchen, Saintpaul, Oranienburg, et al. (Table 1) have also been associated with food-linked infections in human.

**Table 1. Most common *Salmonella* serotypes to cause salmonellosis in 2009**

| Rank | *Salmonella* serotype | # of infections reported |
|---|---|---|
| 1 | Enteritidis | 1,226 |
| 2 | Typhimurium | 1,024 |
| 3 | Javiana | 772 |
| 4 | Newport | 544 |
| 5 | Heidelberg | 230 |
| 6 | Montevideo | 206 |
| 7 | I 4, [5], 12:i:- | 197 |
| 8 | Muenchen | 170 |
| 9 | Saintpaul | 157 |
| 10 | Oranienburg | 154 |

Source: CDC's FoodNet report (CDC, 2010a)

**b. *Salmonella* outbreaks in produce**

**i. Produce production and consumption**

In the U.S., about 130 billion pounds of vegetables (USDA-ERS, 2009a) and 60 billion pounds of fruits (USDA-ERS, 2009c) are produced by the produce industry annually. Imported fresh vegetables from foreign countries including Mexico, Canada, Peru, China, etc. share about 16% (by weight) of fresh vegetable supply in the U. S., while imported fresh fruits, mainly from Latin American countries, account for around 40% (by weight) of fresh fruit supply (USDA-FAS, 2010).

In recent decades, produce consumption in the U.S. has increased significantly. According to USDA-ERS, the consumer sales of fruits and vegetables have risen from $14.3 billion in 1980 to $20.7 billion (by 44.8%) in 1990, $27.9 billion (by 95.8%) in 2000 and $37.8 billion in 2007 (by 164.3%) (Table 2) (USDA-ERS, 2009a; USDA-ERS, 2009c). Besides, as shown in Figure 1, the U.S. annual consumption of fruits and vegetables per capita has increased by 14.6% from 614.6 pounds in 1976 to 705.4 pounds in 2007. The peak value was in 2000, with a consumption of 744.6 pounds per capita. Additionally, the organic produce market is also expanding rapidly. According to the Nutrition Business Journal, the organic produce sales in the U.S. were $4.3 billion in 2003, and were estimated to reach $8.5 billion in 2010 (NBJ, 2004).

**Table 2. Annual consumer sales (in billion dollars) of fruits and vegetables**

| Produce sales | Year | | | |
|---|---|---|---|---|
| | **1980** | **1990** | **2000** | **2007** |
| Fruits | 6.6 | 9.4 | 12.4 | 17.8 |
| Vegetables | 7.7 | 11.3 | 15.5 | 20.0 |
| Total | 14.3 | 20.7 | 27.9 | 37.8 |
| % of total increase since 1980 | - | 44.80 | 95.18 | 164.3 |

Source: USDA-ERS fruit and tree nuts yearbook and vegetables and melons yearbook (USDA-ERS, 2009a; USDA-ERS, 2009c)

There are multiple factors contributing to the increased consumption of produce in the U.S. First of all, government campaigns and federal promotions on the importance of healthy diet, such as the Food Guide Pyramid, have elevated consumers' awareness of the health benefits of fruits and vegetables, hence, the changing national diet trend follows (Eileen *et al.*, 1999). Moreover, a variety of produce items with higher quality and enhanced convenience available on the market year-round have also boosted the sales of fruits and vegetables. For example, there are more fresh-cut, prepackaged produce available on the market to meet the increasing consumer demand for convenient food products (Progressive Grocer, 1998).

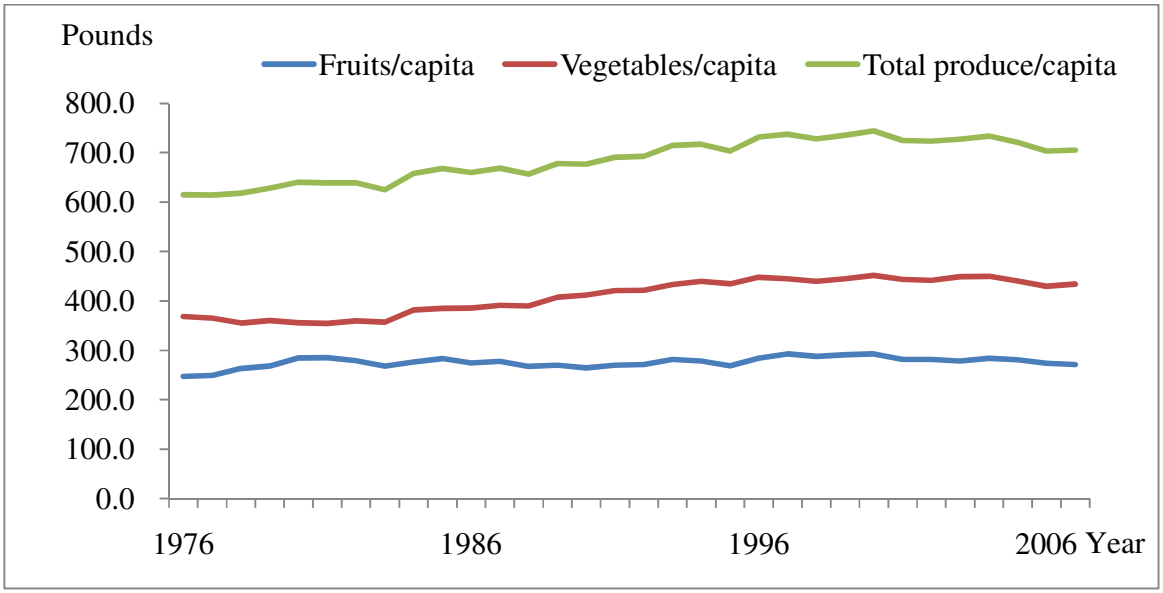


**Figure 1. Produce consumption per capita in the U.S. between 1976 and 2007**

Source: USDA-ERS fruit and tree nuts yearbook, and vegetables and melons yearbook (USDA-ERS, 2009a; USDA-ERS, 2009c)

### ii. Produce outbreaks

Ironically, the increased produce consumption since the 1970s coincides with the surge of produce-linked foodborne outbreaks. Based on data collected in the Foodborne Outbreak Surveillance System by CDC (Sivapalasingam *et al.*, 2004) and the Outbreak Alert! Database by

CSPI (Dewaal *et al.*, 2006; Dewaal & Bhuiya, 2007), produce-linked outbreaks increased from only 0.7% (13/1857) of the total foodborne outbreaks in the 1970s, to 6.0% (114/1788) in the 1990s. Between 1990 and 2003, produce items was responsible for 12.3% (554/4486) of the foodborne outbreaks and ranked as the second most frequently identified food category linked to outbreaks after seafood. Two years later, the number had increased to 13.4% as more produce-linked outbreaks had occurred (Table 3) (Dewaal & Bhuiya, 2009). Furthermore, Figure 2 shows the increased annual produce outbreaks between 1990 and 2005, which resulted in 34,049 foodborne illnesses and accounted for 21.6% of the total foodborne illnesses in that time period. This was a dramatic rise compared to only 1% in the 1970s (Hanning *et al.*, 2009; Olsen *et al.*, 2000).

**Table 3. Produce outbreaks and infection cases in the U.S. since the 1970s**

| Years | # of outbreaks | | | # of infections | | |
|---|---|---|---|---|---|---|
| | Produce-linked | Total # of cases | % | Produce-linked | Total # of cases | % |
| **1970s** | 13 | 1,857 | 0.7 | 708 | 74,592 | 1 |
| **1990s** | 114 | 1,788 | 6 | 8,245 | 68,712 | 12 |
| **1990 - 2003** | 554 | 4,486 | 12.3 | 28,315 | 138,622 | 20.4 |
| **1990 - 2005** | 713 | 5,316 | 13.4 | 34,049 | 157,830 | 21.6 |

Source: CDC Foodborne Outbreak Surveillance System (CDC, 2009b) and CSPI Outbreak Alert! Database (CSPI, 2007)

Besides, data from 1990 and 2005 has shown that the average infection cases per produce-related outbreaks were 47.9, much greater compared to 30.4, 27.4 and 9.9 cases per outbreak for other food categories, i.e., poultry, beef, and seafood, respectively (Dewaal & Bhuiya, 2007). Furthermore, according to Scharff (Scharff, 2010), it was estimated that 39 billion dollars of economic cost was attributed to produce, accounting for more than 25% of the total cost (~$152 billion) of foodborne illnesses. And it was also reported that the cost per case of produce-attributable foodborne illness was higher than that of other vehicles.

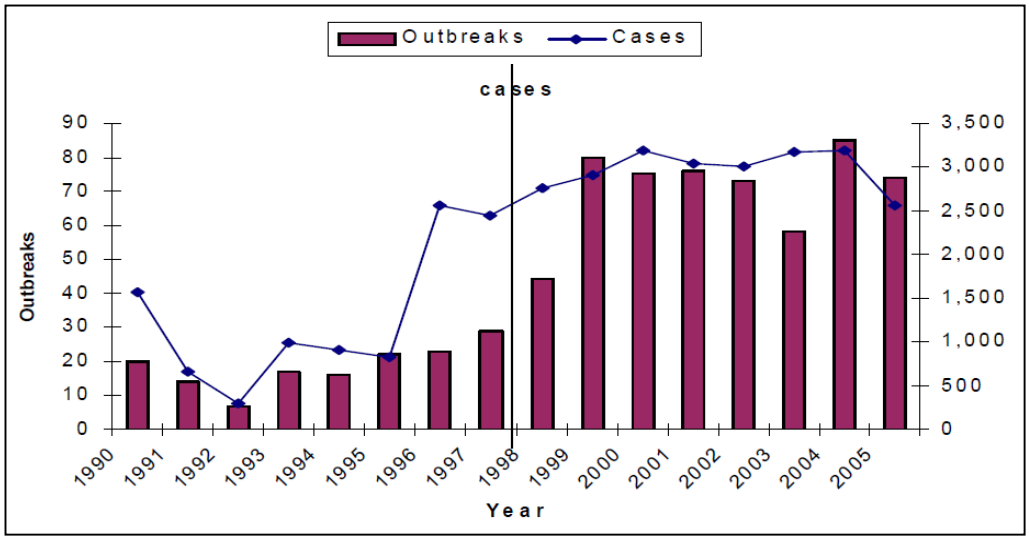


**Figure 2. Annual produce outbreaks and infection cases in the U.S. between 1990 and 2005**

Source: CSPI Outbreak Alert! Report (Dewaal & Bhuiya, 2009)

The increasing number of produce outbreaks is believed due in part to the efforts of CDC and other government agencies to improve the outbreak reporting and surveillance systems. For instance, since 1998, changes made on reporting forms and procedures for reporting allowed government agencies to react more efficiently so as to minimize the impact of outbreaks as well as to keep better track of outbreaks occurred (Dewaal & Bhuiya, 2009). Apart from the improved surveillance, other factors including the surge in produce consumption, a trend toward consumption of more convenient, but potentially more risky fresh-cut prepackaged products, increased global export of produce and so on may also play a role. Assuming that produce contamination level maintained constant, the increased produce consumption would understandably induce more produce-linked infections and outbreaks. Moreover, a trend toward more consumption of fresh-cut produce, i.e., sliced, chopped, and prepared fruits and vegetables such as ready-to-eat salad mix and prepackaged spinach, further elevates the likelihood of produce-linked outbreaks as these products are more likely to be contaminated by foodborne

pathogens during processing (Harris *et al.*, 2003). Last but not the least, the increased import of fresh fruits and vegetables from other nations, especially those with tropical or subtropical climates, further enhances the risk of produce contamination due to poor hygienic and temperature control to meet the U.S. safety standards during produce production, harvesting, and distribution.

### iii. Produce-linked *Salmonella* outbreaks

During last few decades, an increasing trend of *Salmonella*-related outbreaks linked to produce items has been observed, again due to reasons discussed above (Harris *et al.*, 2003). A closer examination of the produce-linked outbreaks between 1990 and 2005 revealed that *Salmonella* was the second leading cause after Norovirus, accounting for 18% of the total cases (Doyle & Erickson, 2008). A variety of produce items including melons, sprouts, tomatoes, spinaches and peppers have been implicated in multiple *Salmonella* outbreaks (Hanning *et al.*, 2009). Table 4 presents the top 5 produce items in terms of the number of infections and outbreaks according to reports from the Foodborne Outbreak Surveillance System by CDC (CDC, 2009b) and Outbreak Alert! Database by CSPI (Dewaal & Bhuiya, 2009) between 1990 and 2008. Particularly, in a large outbreak occurred between April and August 2008 in 43 states of the U.S., DC, and Canada, CDC reported that *S. enterica* serovar Saintpaul caused 1,442 cases and 2 deaths (CDC, 2008). Multiple raw produce items including fresh jalapeño peppers, serrano peppers and raw tomatoes were implicated in this outbreak. Besides, four multistate *Salmonella* outbreaks due to raw consumption of tomatoes were reported in the U.S. between 2005 and 2006, resulting in 459 illnesses (CDC, 2007). Additionally, multistate outbreaks of *Salmonella* serotype Poona infections occurred in the spring of consecutive 2000 - 2002, associated with the consumption of Mexico-imported cantaloupes (CDC, 2002).

**Table 4. Top 5 produce types causing *Salmonella* infections and outbreaks between 1990 and 2008**

| Produce | # of infections | # of outbreaks |
|---|---|---|
| **Sprout** | 2057 | 24 |
| **Tomato** | 1932 | 16 |
| **Pepper** | 1725 | 5 |
| **Melon** | 1169 | 19 |
| **Juice** | 710 | 6 |

Source: CDC Foodborne Outbreak Surveillance System (CDC, 2009b) and CSPI Outbreak Alert! Database (CSPI, 2007)

*Salmonella* contamination of produce might occur at any point throughout the production, harvesting, processing, and distribution, as *Salmonella* is widely distributed in the natural environment and animals are its common reservoirs (Hanning *et al.*, 2009; Harris *et al.*, 2003). Contamination during production occurs when *Salmonella*-contaminated irrigation water and animal manure as fertilizer are utilized in fields (Doyle & Erickson, 2008). Besides, over-head birds and nearby livestock harboring *Salmonella*, when come into contact with fruits and vegetables in fields, might induce contamination of the produce. During harvesting, processing, and distribution of produce, poor hygiene of handlers, unclean equipment surfaces, and ineffective disinfection methods are risk factors of *Salmonella* contamination. Therefore, to prevent contamination in the field, good agricultural practices should be implemented, including sanitation control of irrigation water and fertilizers, as well as elimination of animal contamination. While at post-harvest settings, training of employees for improved hygiene together with effective HACCP plans and disinfection treatments of produces should be implemented to monitor and control the contamination (Hanning *et al.*, 2009). For examples, FDA drafted commodity-specific guidance in 2009 for leafy greens, tomatoes, and melons to help identify and implement measures to minimize the risk of microbial contamination throughout the supply chain (FDA, 2009a; FDA, 2009b; FDA, 2009c). For that purpose, it is

therefore important that the produce industry be equipped with rapid, sensitive, specific detection methods for live *Salmonella* cells in produce to better ensure the produce safety.

## 3) Detection methods for foodborne pathogens

### a. Challenges with microbiological analysis of foods

Although remarkable progress on food microbiological analysis has been made in the past decades, inherent limitations of food analysis still pose great challenges to detecting foodborne pathogens in foods (Dolye & Beuchat, 2007). First of all, food products are very diverse. There are liquid or solid foods, homogenous or heterogeneous foods, raw or ready-to-eat foods, and so on. The great variety of food samples makes it difficult to develop efficient food sampling, sample preparation, and analytical methods. Besides, great complexity of the food matrices and compositions undermines the efficiency of microbiological analysis. A variety of compounds present in the food matrices might interfere with the functional activity of key reagents in pathogen detection, leading to false positive or false negative results. For instance, PCR enzymes such as *Taq* polymerase are particularly vulnerable to inhibitors in the food samples, rendering the limited sensitivity and false negative results of PCR in many foods (Wiedbrauk *et al.*, 1995), whereas intrinsic peroxidase in fruits and vegetables might cause false positive reaction of ELISA as it uses peroxidase conjugates (Dolye & Beuchat, 2007). In addition, there are high levels of background flora naturally present in food samples, while the target microorganism, on the other hand, is likely to account for only a small portion of the total microorganisms in foods. Therefore, the background flora may also hinder the detection of target organism. Moreover, heterogeneous distribution of target agents in foods and injuries of cells due to food processing further compromise the effective detection of pathogens in foods (Ge & Meng, 2009).

To date, various detection methods such as traditional culture-based methods, convenience-based methods, immunological-based methods, and molecular-based methods have been developed and widely used for microbiological analysis of foodborne pathogens in foods. The following section will briefly review these methods.

**b. Traditional culture-based methods**

Traditional culture-based methods are foundational and basic testing methods for microbiological analysis and are used intensively. In these methods, the target microorganisms are reproduced in selective or differential culture media under controlled laboratory conditions. To obtain isolated pure microorganism cultures, streaking on media plates is usually applied; while for enumerating the total number of viable microorganisms, spiral plating of serially diluted samples is commonly used (Gilchrist *et al.*, 1973). Determination of the type of organism and its abundance in the sample are done by observation of the colony and colony counts, as well as biochemical confirmation methods (Merker, 1998).

The U.S. Food and Drug Administration's Bacteriological Analytical Manual (BAM) details the detection of various foodborne pathogens by traditional culture methods which generally include pre-enrichment, selective enrichment, selective plating, and identification (Merker, 1998). Although regarded as the gold standard of microorganism diagnostics, the whole process usually takes several days or even weeks, which renders these time-consuming and labor-intensive (Eriksson & Aspan, 2007; Kumar *et al.*, 2008a). Therefore, quicker and simpler detection methods have been developed at a fast pace during the past several decades.

**c. Convenience-based methods**

Convenience-based methods such as 3M™ Petrifilm™ Plates are developed to allow for convenient and rapid detection of microorganisms in foods. Petrifilm plate is an all-in-one

plating system developed by the 3M corporation (St Paul, MN). It is in principle a dry media generally containing a cold water soluble gelling agent, nutrients, and an indicator for activity and enumeration. The nutrients used vary plate by plate depending on the type of microorganism to be detected. Diluted foodstuffs with presumptive microorganisms can be incubated within the circle unit of a Petrifilm plate and results are normally available within 24 h, though a few types of Petrifilm plates might need longer time. There are various types of Petrifilm™ Plates available for total aerobic plate counts, *Escherichai coli*, *Listeria*, *Staphylococcus*, yeast and mold, and so on. They are now widely applied in the food industry as a simple, convenient, and cost-effective method with enumerative results comparable to conventional plating methods. For example, a study compared the Petrifilm plate count method with conventional most probable number (MPN) for the enumeration of spiked *E. coli* from frozen shrimps, and found that Petrifilm plate counting results were in 95.7% agreement with that of conventional MPN (Suwansonthichai & Rengpipat, 2003).

**d. Immunological-based methods**

Immunological-based methods rely on the interaction between antibody and antigen for testing and have been used for many years to identify, serotype, and quantify bacteria. There are various types of immunological-based assays that have been developed for the rapid microbiological detection in foods, including enzyme-linked immunosorbent assay (ELISA) and immunomagnetic separation (IMS).

**i. ELISA**

ELISA, known as a “sandwich” assay, is an immunological method widely used to detect and quantify microorganisms and toxins in foods and is usually carried out in a 96-well microtiter plate. When loaded into the microtiter plate, target pathogen binds to the specific

antibody which has been pre-coated onto the wells of the microtiter plate. Following the binding, a secondary antibody linked to an enzyme is incubated together to again bind to the target pathogen, forming a sandwich structure. After washing off non-specific bindings, a colorless substance for the enzyme is added that reacts with the bound enzyme and generates detectable color signals (Crowther, 2000). ELISA technique is widely used due to its simplicity and quickness and has been used for detecting *Salmonella* since 1970s (Carlsson *et al.*, 1975). However, common drawbacks with ELISA include limited sensitivity and low specificity. The detection limit of ELISA is between $10^4$ and $10^5$ CFU/ml, hence enrichment is generally needed for improved sensitivity (Dolye & Beuchat, 2007). A study by Kumar et al. (Kumar *et al.*, 2008b) found that after enrichment of a variety of *Salmonella* Typhi-spiked food rinses and milk products in buffered peptone water (BPW) for about 10 h, ELISA could obtain a detection limit of $10^2$ CFU/ml, up to $10^3$ fold more sensitive than that of a ELISA without culture enrichment; when overnight enriched, it could detect as few as 2 *Salmonella* Typhi cells. For another thing, the poor binding affinity between antibody and antigen renders ELISA the characteristic of lower specificity. For example, a study in Sweden compared culture, ELISA and PCR methods for detection of *Salmonella* in fecal samples, and reported that ELISA performed worse in sensitivity and specificity compared with the standard culture methods and PCR assays, with poor ability to detect *Salmonella* Livingstone and *Salmonella* Worthington due to poor binding specificity of the antibodies (Eriksson & Aspan, 2007).

### ii. IMS

IMS method can be used to separate as well as concentrate target foodborne pathogens from food samples, greatly reducing the concentration of inhibitors from the complex composition of food matrix and at the same time eliminating the enrichment steps (Luttmann *et*

*al*, 2006). In this method, surface-activated paramagnetic beads are bound by antibodies when incubated in the refrigerator for up to 20 hours. After washing, food sample containing the antigens of target pathogen is added and incubated for minutes to hours so that antigens can be captured by the beads-coupled antibodies. After a magnetic field is applied, the target pathogens are retrieved and concentrated. In a 1993 study (Mansfield & Forsythe, 1993), IMS was used as an alternative to selective broth enrichment of *Salmonella* to shorten the detection time and it showed the potential to recover sublethelly injured *Salmonella* cells. Besides, this method can either be directly used for identification and quantification by using fluorescent antibody, or coupled with other rapid methods such as ELISA, conductance microbiology, PCR and so on (Cudjoe *et al.*, 1995; Mansfield & Forsythe, 2000; Taban *et al.*, 2009; Parmar *et al.*, 1992). For example, in a 2007 study detecting *E. coli* O157 by Kalnauwakul et al. (Kalnauwakul *et al.*, 2007), culture method followed by immunomagnetic separation could detect *E. coli* O157 in artificially contaminated stool samples with a lower level of detection of $10^2$ to $10^3$ CFU/g. Another study detected *Salmonella* in milk by combining IMS with PCR, which indicated that IMS-PCR could successfully detect 1 - 10 CFU/ml of *Salmonella* in 12 h pre-enriched milk, and the total assay was rapid, taking 16 hours (Taban *et al.*, 2009).

**e. Molecular-based methods**

Since the 1980s, advances in basic DNA research have stimulated the great surge of DNA technology, which contributed to the emergence and evolvement of molecular-based pathogen detection assays (Jay *et al.*, 2005). Methods such as PCR and real-time PCR are recognized to be rapid (requiring only several hours or even less than one hour), sensitive, and with high specificity and reproducibility. These desirable features of molecular-based assays result in their wide usage in microbiological analysis. Recently, a novel molecular-based assay -

loop-mediated isothermal amplification (LAMP) has been developed and applied in pathogen detection. In this section, PCR, real-time PCR, and LAMP will be described.

### i. PCR

PCR is a powerful molecular-based DNA amplification technique that has been widely used for foodborne pathogen detection. During PCR, a highly efficient DNA polymerase such as *Taq* polymerase is employed and within a few hours, the target DNA sequence can be exponentially amplified by $10^6$ fold (Mullis *et al.*, 1986). A gel electrophoresis is then followed to examine the amplified PCR products under UV light. PCR assays are widely regarded to be rapid and sensitive. A study for the detection of *Salmonella* in seafood samples by Kumar et al. (Kumar *et al.*, 2008a) found that PCR assay which targeted at *Salmonella*-specific *invA* gene showed 31.6% positive results in a total of 214 seafood samples, while positive rates of 23.7% and 21.3% for ELISA and culture method, respectively. The greater sensitivity of PCR assay contributed to the higher detection rate of *Salmonella* in seafood samples.

In addition, multiplex PCR in which several genes are targeted at one run is quite useful and convenient for rapid identification and characterization of the microorganism. For example, a multiplex PCR assay was developed by Panicker et al. (Panicker *et al.*, 2004) to detect potential virulent *Vibrio vulnificus* by targeting at the *viuB* gene and *vvh* gene with a detection limit of 10 pg of purified DNA. Moreover, multiplex PCR can also be designed to simultaneously detect multiple target microorganisms in food samples, which significantly reduces the time and labor needed for identification. Li et al. in 2004 (Li & Mustapha, 2004) established a multiplex PCR, in which three pairs of primers were used to identify *E. coli* O157:H7, *Salmonella*, and *Shigella*. This method could successfully detect the three bacteria in apple cider and detect down to $8 \times 10^{-1}$ CFU/g after overnight enrichment.

However, when applied to food sample testing, the sensitivity of PCR assay is likely to be dramatically reduced due to inhibitors from the complex composition of the food matrix. To solve this problem, an enrichment step is generally included during food sample processing which inevitably increases the whole processing time (Kida *et al.*, 1995; Kobayashi *et al.*, 1994; Oberst *et al.*, 2003).

### ii. Real-time PCR

Real-time PCR, also termed quantitative PCR (Q-PCR or qPCR), is an improved PCR assay which allows for both detection and quantification of the target gene simultaneously. Quantification is achieved using fluorescent dyes or fluorescence-labeled DNA probes. A fluorescent dye emits fluorescence once it is bound to double-stranded DNA (dsDNA) in PCR, allowing it to track the amplification level of the target gene after each thermal cycle. The most economically used dye is SYBR Green I. However, since the fluorescent dyes will bind to all the dsDNA, the main drawback of using fluorescent dyes in real-time PCR is the potential inaccurate quantification due to nonspecific binding of PCR products. To enhance the specificity of the assay, melting-curve analysis is usually conducted after amplification to get the melting temperature, which varies depending on sequence of the amplified product (Pryor & Wittwer, 2006).Using the fluorescence-labeled DNA probes in real-time PCR can improve specificity since the probes are designed to be specifically targeting the target sequence. However, DAN probes are rather expensive and can be difficult to design.

Real-time PCR has been reported to be more rapid, sensitive, and specific than conventional PCR. The amplification cycle times of real-time PCR are usually shorter than that of conventional PCR, and it also eliminates the necessity of running gel, which is time-consuming and does not allow precise quantification. A specific probe was designed and used in

a q-PCR to target *invA* gene of *Salmonella*, and the detection limit was 2 CFU per reaction, which was 100 fold more sensitive than conventional PCR reported previously (Cheng, 2005; Cheng *et al.*, 2008; Rahn *et al.*, 1992). Besides, q-PCR can also be used to simultaneously detect multiple targets. For example, a real-time multiplex PCR assay was developed that targeted at the *tlh*, *tdh,* and *trh* genes of *Vibrio parahaemolyticus* in order to differentiate pathogenic and nonpathogenic *V. parahaemolyticus* strains (Nordstrom *et al.*, 2007).

### iii. Loop-mediated isothermal amplification (LAMP)

LAMP was developed by a group of Japanese scientists, and firstly published in 2000 (Notomi *et al.*, 2000). This novel molecular-based assay uses a set of four specific primers, two inner and two outer, to recognize six distinct regions of the target DNA sequence (Figure 3A). It is termed loop-mediated isothermal amplification because highly specific amplification of the target gene can be achieved under isothermal conditions (at 60 - 65$^{o}$C), and a dumbbell-like structure of the DNA is formed during initial steps to facilitate subsequent amplification (Figure 3B). To facilitate the amplification, one or two loop primers targeting the dumbbell-like region of the stem-loop structure are added into the reagents mix (Nagamine *et al.*, 2002) (Figure 3C). The addition of loop primer(s) accelerates DNA amplification by increasing the number of starting points for DNA synthesis and the results can be detected within 30 min.

LAMP is found to be a simple, rapid, and cost-effective technique for DNA amplification and yielded highly specific and sensitive results. Since it is isothermal, simple equipment such as water bath or heating block that can maintain the temperature at around 60 - 65$^{o}$C is sufficient. LAMP is also highly efficient in that within an hour, a few copies of DNA can be amplified to $10^{9}$ copies (Notomi *et al.*, 2000). The addition of loop primers further speeds up the assay and reduces the reaction time to within 30 min (Nagamine *et al.*, 2002). For example, a recent study

reported that to get detectable signal for *V. parahaemolyticus* from a single colony on TCBS agar and spiked shrimp samples, LAMP assays only required 13 to 22 min and less than 35 min, respectively (Yamazaki *et al.*, 2008a). Furthermore, the large amount of DNA synthesized by LAMP can result in turbidity change which can be observed by naked eyes or quantified by a real-time turbidimeter (Mori *et al.*, 2001). When coupled with fluorescent dyes or the real-time turbidimeter, LAMP can be conducted real-time which allows quantitative analysis of DNA amplification by correlating the amplification signals with the cell numbers (Mori *et al.*, 2004).

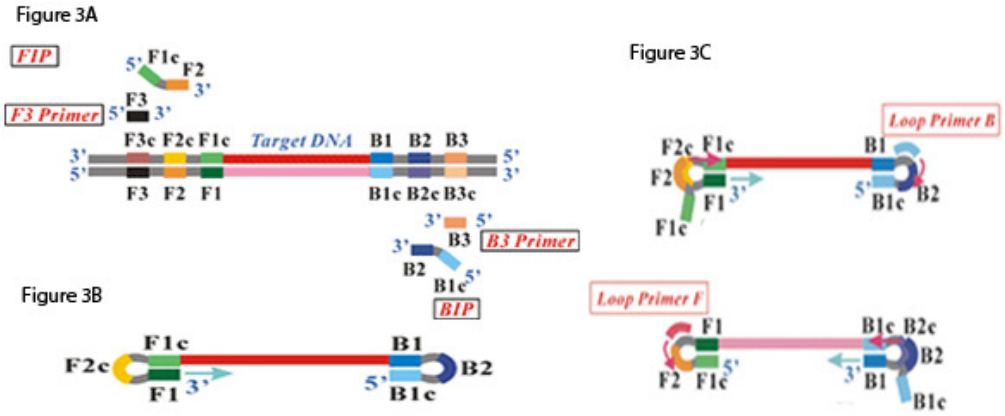


**Figure 3. LAMP primers and a dumbbell-like structure formed during LAMP**

Source: Figures are adopted from Eiken Genome Site (Eiken Genome Site, 2005); A: Inner primers (FIP and BIP) and outer primers (F3 and B3) designed for LAMP assay; B: Dumbbell-like structure formed during DNA amplification of LAMP assay; C: Loop primers designed to accelerating LAMP assay

LAMP has been applied for the detection of many foodborne pathogens such as *Vibrio cholerae* (Yamazaki *et al.*, 2008b), *V. vulnificus* (Han & Ge, 2008; Ren *et al.*, 2009), *V. parahaemolyticus* (Chen & Ge, 2010; Nemoto *et al.*, 2009), *E. coli* (Kouguchi *et al.*, 2010), *Campylobacter* (Yamazaki *et al.*, 2008c; Yamazaki *et al.*, 2009) and yielded promising results in food samples with less inhibition effect. In a study by Han et al. (Han & Ge, 2008), LAMP assay for detecting *V. vulnificus* in pure culture and raw oyster samples was found to be 10-fold and 1,000-fold more sensitive than the conventional PCR. In another study, PCR assay failed to

detect *Salmonella* in 10% of 110 raw egg samples, while LAMP assay successfully identified *Salmonella* in all samples (Ohtsuka *et al.*, 2005). In addition, LAMP has also been developed to detect specific serovars of *Salmonella*, such as the O4 and O9 group of *S. enterica* in food samples (Okamura *et al.*, 2008; Okamura *et al.*, 2009).

### f. Live detection methods

Although molecular-based assays significantly reduce assay time, simplify detection procedure, and lower the detection limit, there remains one major drawback to circumvent - the inability to differentiate live bacteria from dead ones since both dead and live cells could be amplified by DNA-based assays. Live bacteria are the primary target for food microbiological analysis rather than dead cells since they are the ones capable of causing foodborne infections in human. Therefore, these assays give potential false positive results. To achieve more reliable and accurate results, research on various live detection techniques such as mRNA-based PCR, EMA and PMA techniques, have been undertaken and yielded promising results.

#### i. mRNA-based

One of these techniques is called reverse-transcriptase PCR (RT-PCR), utilizing mRNA as a cell viability marker. mRNA has a short half life (0.5 to 50 min) and degrades rapidly upon cell death, hence can be a good candidate for live bacteria detection (Takayama & Kjelleberg, 2000). Similarly to PCR, RT-PCR can also become real-time (qRT-PCR) by employing fluorescent dyes or probes. So far, there are RT-PCR and qRT-PCR assays developed for various foodborne pathogens, including *E. coli* (de Wet *et al.*, 2008; Tsai *et al.*, 2006), *L. monocytogenes* (Klein & Juneja, 1997), *Salmonella* (Gonzalez-Escalona *et al.*, 2009; Jacobsen & Holben, 2007) and *V. cholerae* (Bej *et al.*, 1996). However, live detection relied on mRNA is unreliable as mRNA stability and quantity is heterogeneous, depending on environmental conditions and

intrinsic factors of the target gene. Besides, RT-PCR and qRT-PCR are generally of poor efficiency, specificity, and sensitivity when compared with DNA-based PCR. For instance, an investigation of RT-PCR for the detection of viable *E. coli* O157:H7 from environmental or food samples revealed that among several genes studied, only mRNA from the *rfbE* gene was reliable for live detection, however, a cell level up to $10^7$ CFU was necessary (Yaron & Matthews, 2002).

### ii. EMA and PMA

Besides the utilization of mRNA, other live detection techniques involve the use of chemicals such as EMA and PMA (Figure 4) as promising agents to discriminate live cells from dead ones. These techniques are based on the membrane integrity of cells, i.e., PMA and EMA can enter only the membrane-compromised dead cells, upon photolysis by strong visible light, the azide group of EMA or PMA converts into a highly active nitrene which then covalently binds with DNA in dead cells (Figure 5 and Figure 6), while the remaining free EMA or PMA is simultaneously degraded by reacting with water molecules. Cross-linking of EMA or PMA with DNA is reported to strongly inhibit PCR amplification of modified DNA, thus PCR or qPCR analysis coupled with EMA or PMA treatment could successfully eliminate false positive results by selectively excluding DNA from dead cells.

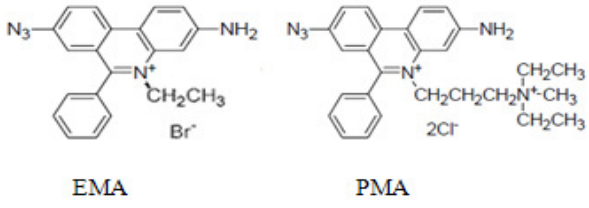


**Figure 4. Chemical structures of EMA and PMA**

Source: Product information from Biotium, Inc. (Biotium, 2009a; Biotium, 2009b)

In 2003, EMA was initially incorporated into PCR as an effective live detection assay by Nogva (Nogva *et al.*, 2003). Since then, EMA-PCR and EMA-qPCR have been developed for

detecting a variety of viable bacteria, including *Campylobacter jejuni* (Rudi *et al.*, 2005a), *L. monocytogenes* (Rudi *et al.*, 2005b), *E. coli* O157:H7 (Nocker & Camper, 2006; Wang *et al.*, 2009) , *Salmonella* Typhimurium (Nocker & Camper, 2006), *V. vulnificus* (Wang & Levin, 2006) and so on. However, further studies revealed one major drawback of EMA - potential penetration into viable cells, depending on the bacterial species (Cawthorn & Witthuhn, 2008; Flekna *et al.*, 2007; Nocker & Camper, 2006). For example, EMA was reported to enter viable *E. coli* and cause 60% genomic DNA loss of log-phase viable cells (Nocker & Camper, 2006).

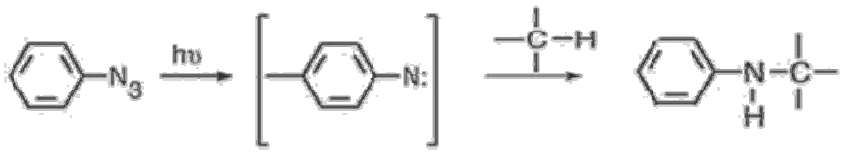


**Figure 5. Photoactive cross-linking reaction of a simple azide**

Source: modification of Molecular Probe: The Handbook (Invitrogen, 2010)

Comparative studies of PMA and EMA (Cawthorn & Witthuhn, 2008; Nocker *et al.*, 2006) suggested that PMA was a more effective agent, as it displaced higher intact-membrane impermeability and at the same time, could selectively remove nonviable cells of a wide range of bacteria. The superior properties of PMA are the results of its chemical structure and higher positive charges (Nocker *et al.*, 2006). The high selectivity and efficiency of PMA favor its application in detecting viable bacteria in environmental and food samples over EMA. For example, PMA-qPCR was developed for the enumeration of viable *L. monocytogenes* (Pan & Breidt, 2007) and for the detection of live probiotic bacteria in lyophilize products (Kramer *et al.*, 2009). Besides, PMA-qPCR was used to monitor the killing efficacy of different disinfection methods, including hypochlorite disinfection, benzalkonium disinfection, UV disinfection and heat disinfection (Nocker *et al.*, 2007).

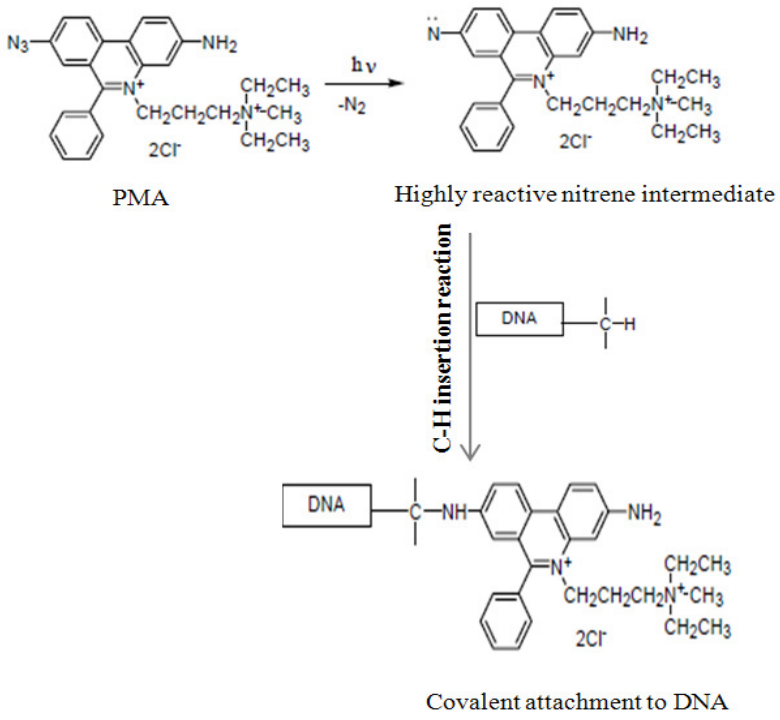


**Figure 6. Nucleic acids modification by PMA during photolysis**

Source: Product information from Biotium, Inc. (Biotium, 2009b)

## 4) Current detection methods for *Salmonella*

### a. Traditional culture-based methods

According to FDA BAM, traditional culture methods for *Salmonella* detection include pre-enrichment by BPW, selective enrichment using Rappaport-Vassiliadis (RV) broth, or tetrathionate (TT) broth, solid medium isolation by streaking on Hektoen Enteric agar, Xylose Lysine Desoxycholate agar or Bismuth Sulfite agar and identification via biochemical tests such as urease test, indole test, etc. (Andrews & Hammack, 2007). It is both time-consuming and labor-intensive, requiring several days or even a week for a definitive result. To overcome these challenges, many rapid methods such as ELISA (Kumar *et al.*, 2008b; Mansfield & Forsythe, 1993), IMS (Cudjoe *et al.*, 1995; Kumar *et al.*, 2008b), PCR (Eriksson & Aspan, 2007; Kumar *et al.*, 2008a), real-time PCR (Cheng *et al.*, 2008; Krascsenicsova *et al.*, 2008; Malorny *et al.*,

2008) and LAMP (Hara-Kudo *et al.*, 2005; Ohtsuka *et al.*, 2005; Okamura *et al.*, 2009) have been developed with the aims of reducing assay time and simplifying detection steps.

**b. Immunological-based methods**

Immunological-based methods such as ELISA and IMS have been developed for *Salmonella* detection as is mentioned in section 3d. Although these rapid methods can significantly reduce the assay time, the drawbacks of low specificity, poor sensitivity, and lack of quantitative capability still greatly limit the application of these assays. For instance, for the detection of *Salmonella* in food samples, normally ELISA can yield results within 48 h; however, a study by Rigby (Rigby, 1984) found that ELISA failed to detect 5 of 111 culture-positive poultry specimens contaminated with *Salmonella* serogroups B or C2 and also failed to detect 7 of 9 culture-positive water samples contaminated with other *Salmonella* serogroups.

**c. Molecular-based methods**

Molecular-based techniques, especially PCR and real-time PCR have been widely applied for the diagnostics of *Salmonella* in food samples due to their high specificity, superior sensitivity and rapidity as mentioned in section 3e. A variety of target genes have been studied for the detection and characterization of *Salmonella* spp., including *invA* (Galan & Curtiss III, 1991; Rahn *et al.*, 1992; Swamy *et al.*, 1996b), *spvC* (Swamy *et al.*, 1996a), *fimA* (Cohen, 1996), *himA* (Chen *et al.*, 2000), *hilA* (Pathmanathan *et al.*, 2003), *stn* (Moore & Feist, 2007) and *phoP* (Miller, 1989) genes. However, as genetic markers, all these genes except *invA* lack of species specificity (inclusivity) for *Salmonella* spp. The *Salmonella* invasive gene, *invA,* of which the DNA sequence is found to be highly conserved among the *Salmonella* population, can serve as a specific and reliable genetic indicator in PCR-based methods for detecting *Salmonella* spp. According to a study by Cheng, et al. in 2005 (Cheng, 2005), *invA*-PCR could successfully

discriminate *Salmonella* spp. from non-*Salmonella* among a total of 222 strains. Later in 2008, Cheng, et al. (Cheng *et al.*, 2008) developed an *invA*-based real-time PCR assay, which could successfully differentiate 328 *Salmonella* strains (representing 32 serogroups and 145 serotypes) from 56 non-*Salmonella* strains, and could detect *Salmonella* as low as 0.04 CFU/g in chili and shrimp samples. However, all PCR-based techniques require expensive instruments - the thermal cyclers, at the cost of more than ten thousand dollars, restricting the application of the assays in food industry and field laboratories. Furthermore, PCR-based techniques lack the ability to discriminate viable cells from dead ones, causing potential false positive results in the sample analysis.

**d. Live detection methods**

As mentioned in section 3f, live detection methods include the utilization of mRNA as viability marker, or the incorporation of EMA or PMA as a dead DNA eliminating agent into molecular-based methods. For detecting live *Salmonella* spp. in produce, *invA*-based qRT-PCR was developed with a detection limit of 40 copies of mRNA (Gonzalez-Escalona *et al.*, 2009). However, conversion factors of mRNA into viable cells depend on the growth phase of *Salmonella,* which made it difficult for the enumeration of live *Salmonella* in field tests of food samples as we have no idea at what phase the live *Salmonella* might be. In 2008, suitability of PMA and EMA incorporated with qPCR was tested by examining dead cells of *Clostridium perfringens*, *L. monocytogenes*, and *S. enterica* from environmental mix, and significant reduction of DNA was observed (Wagner *et al.*, 2008). Furthermore, Nocker et al. in 2009 (Nocker *et al.*, 2009) employed PMA treatment in combination with diagnostic microarray and qPCR to differentiate live *Salmonella* from a mixture of several isopropanol-killed bacteria and observed significant reduction of signal from dead cells.

Therefore, the LAMP assay, notable for its inexpensive, rapid, specific, sensitive and quantitative characteristics, is a desirable diagnostic tool that can be coupled with PMA to detect live *Salmonella* in food samples. In this study, we aimed to develop and optimize an *invA*-based PMA-LAMP assay to detect live *Salmonella* and evaluate the assay in terms of false positive exclusivity, live detection sensitivity, and quantitative capability in live *Salmonella* detection in produce samples.

# Chapter 3 - Materials and Methods

## 1) General methods

### a. Culture preparation

*Salmonella* strains used in this study were obtained from the American Type Culture Collection (ATCC, Manassas, VA) or our strain collection at the Department of Food Science, Louisiana State University. *S. enterica* serovar Typhimurium LT2 was used for assay optimization, sensitivity tests, and produce spiking. Additionally, 52 bacterial strains were used for the specificity test, which included 27 *Salmonella* strains with 9 serovars, and 25 other related or unrelated bacteria.

*Salmonella* serovars was streaked from the -80$^{o}$C stock on trypticase soy agar (TSA; BD Diagnostic Systems, Sparks, MD) and grown at 37$^{o}$C for 24 h. *Citrobacter, Esherichia,* and *Shigella* were incubated on TSA or blood agar at 35$^{o}$C for 24 h. For *Campylobacter* strains, microaerophilic conditions (85% $N_2$, 10% $CO_2$ and 5% $O_2$) at 42$^{o}$C were used for growth.

### b. DNA template preparation

DNA templates for specificity test were prepared by suspending colonies of *Salmonella* or non-*Salmonella* strains in 0.5 ml of TE buffer. The cell suspensions were directly boiled at 95$^{o}$C for 10 min in a dry heating block for DNA templates.

For sensitivity test of LAMP and PMA-LAMP, fresh *Salmonella* LT2 culture was prepared as follows: A loopful of *Salmonella* LT2 colonies grown on TSA at 37$^{o}$C for 24 h were inoculated into 100 ml of trypticase soy broth (TSB; BD Diagnostic Systems) for overnight growth at 37$^{o}$C with shaking at 125 rpm. After overnight incubation, the culture was diluted 100-fold and allowed to grow for 8 h to achieve mid-log culture. Cell density of the culture was adjusted to an $OD_{600}$ of 1 (~$10^9$ cells/ml) by TSB. Ten-fold serial dilutions were made using TSB

and exact cell counts were determined by standard plate counting. For sensitivity test of LAMP, aliquots (1 ml) of each dilution ($10^8$ to $10^2$ CFU/ml, equivalent to $10^5$ to $10^{-1}$ CFU/rxn) was directly boiled for templates as described above; for sensitivity of PMA-LAMP, aliquots (0.5 ml) of each dilution was distributed into 1.5 ml micro-centrifuge tubes with or without 0.5 ml of heat-killed dead *Salmonella* cells ($10^5$ CFU/ml, equivalent to $10^3$ CFU/rxn) and subjected to PMA treatment as indicated below.

### c. Data analysis

In LAMP assay, time threshold value (*Tt*; min) was obtained when a turbidity (at 650 nm) increase threshold of 0.1 was reached. *Tt* values shown in this article were all calculated as average *Tt* values ± standard deviations for corresponding *Salmonella* templates using Microsoft Excel software (Microsoft, Seattle, WA). The limit of detection was determined as the lowest detectable cell level (CFU/rxn in pure culture or CFU/g in spiked produce). Standard curve for the developed LAMP in pure culture was generated by plotting average *Tt* values against log CFU/rxn of *Salmonella* cells; similarly, standard curves for the developed PMA-LAMP assay in pure culture and spiked produce were generated by plotting average *Tt* values against log CFU/rxn or log CFU/g of live *Salmonella* cells, respectively and linear regression was calculated by using Microsoft Excel. Quantitative capabilities of the LAMP assay and the PMA-LAMP assay were evaluated based on the correlation coefficient ($r^2$) values from the standard curves.

## 2) LAMP assay development

### a. Optimization of LAMP assay

**Prototype LAMP conditions.** The prototype LAMP reaction mix in a total volume of 25 µl was based on the commercial Loopamp® DNA Amplification Kit (using Eiken company address), which consisted of 1 × thermal buffer, 6 mM of $MgSO_4$, 0.8 M of betaine, 1.6 mM of

deoxyribonucleotide triphosphate (dNTP), 0.2 µM of each outer primer, 1.6 µM of each inner primer, 0.8 µM of each loop primer, 8 U of *Bst* DNA polymerase (New England Biolabs, Ipswich, MA) and 2 µl of DNA templates. The LAMP assay was carried out in a loopamp real-time turbidimeter LA-320C (Eiken Chemical Company, Kyoto, Japan) at 65$^{o}$C for 1 h and terminated at 80$^{o}$C for 5 min. Turbidity readings at 650 nm were performed real-time and time threshold value (*Tt*; min) was obtained when a turbidity increase threshold of 0.1 was reached.

**LAMP optimization.** The prototype LAMP conditions were optimized for 9 parameters, including the concentrations of $MgSO_4$ (2 to 10 mM), betaine (0 to 1 M), dNTP (0.4 to 2 mM), enzyme (2 to 10 U), outer primers (0.05 to 0.4 µM), and inner primers (1.2 to 2.0 µM), loop primers (0.2 to 1.0 µM), assay temperature (61, 63, or 65$^{o}$C), incubation time (40, 50, or 60 min). *Salmonella* LAMP primers previously published by Hara Kudo et al. (Hara-Kudo *et al.*, 2005) (designated as Sal-HK in this study) were used for optimization testing. Eight parameters (except incubation time) were optimized one at a time and each optimization experiment was repeated five times by testing *S. enterica* serovar Typhimurium LT2 DNA template at cell level of $10^6$ CFU/rxn. A negative control was included for each LAMP run. After optimization of 8 parameters, 10-fold serial dilutions of *S. enterica* serovar Typhimurium LT2 DNA templates ranging between $10^5$ and $10^{-2}$ CFU/rxn were tested using optimized LAMP conditions. Incubation time was adjusted based on *Tt* values of lowest detection limit using optimized LAMP assay.

**LAMP primer design and comparison.** *Salmonella* invasion gene (*invA*, GenBank accession number M90846) was used as the target gene to design LAMP primers. The PrimerExplorer 4 software (Fujitsu Limited, Japan; http://primerexplorer.jp/e) was used to design six sets of primers (five to six primers per set) that each set recognized seven to eight distinct

regions of the target gene. The efficiency of the primers designed was compared with previous published Sal-HK primer set (Hara-Kudo *et al.*, 2005) listed in Table 5 for three repeats in terms of speed, sensitivity and quantitative capability.

**Table 5. Primers used for LAMP and PCR assays for *Salmonella* detection**

| Primer name | Sequence (5′- 3′) | Position * | Amplicon size (bp) | Reference |
|---|---|---|---|---|
| **Hara-Kudo's primers (Sal-HK)** | | | | |
| F3 | GGCGATATTGGTGTTTATGGGG | 225-246 | Ladder-like bands for LAMP; 244 bp for F3/B3 PCR | (Hara-Kudo *et al.*, 2005) |
| B3 | AACGATAAACTGGACCACGG | 457-483 | | |
| FIP | GACGACTGGTACTGATCGAT-AGTTTTTCAACGTTTCCTGCGG | 327-346 (F1c) 271-292 (F2) | | |
| BIP | CCGGTGAAATTATCGCCAC-ACAAAACCCACCGCCAGG | 368-386(B1) 414-434(B2c) | | |
| Loop-F | GACGAAAGAGCGTGGTAATTAAC | 297-324 | | |
| Loop-B | GGGCAATTCGTTATTGGCGATAG | 414-434 | | |
| **Our designed primers (Sal-8)** | | | | |
| F3 | CGGCCCGATTTTCTCTGG | 503-520 | Ladder-like bands for LAMP; 180 bp for F3/B3 PCR | This study |
| B3 | CGGCAATACGCGTCACCTT | 665-682 | | |
| FIP | GCGCGGCATCCGCATCAATA-TGCCCGGTAAACAGATGAGT | 573-592 (F1c) 527-546 (F2) | | |
| BIP | GCGAACGGCGAAGCGTACTG-TCGCACCGTCAAAGGAAC | 593-612 (B1c) 635-652 (B2) | | |
| Loop-F | GGCCTTCAAATCGGCATCAAT | 547-567 | | |
| Loop-B | GAAAGGGAAAGCCAGCTTTACG | 613-634 | | |
| **PCR primers** | | | | |
| invA-139 | GTGAAATTATCGCCACGTTCGGGCAA | 371-396 | 288 | (Rahn *et al.*, 1992) |
| invA-141 | TCATCGCACCGTCAAAGGAACC | 634-655 | | |

* The positions are numbered based on the coding sequence of *Salmonella* strain Typhimurium *invA* gene (GenBank accession number M98046).

**b. LAMP specificity and sensitivity.**

**LAMP specificity.** Specificity of the optimized LAMP assay, namely, the probability of LAMP assay to get positive results when testing *Salmonella* strains (inclusivity) as well as to get negative results when testing non-*Salmonella* strains (exclusivity), was determined by testing a panel of bacteria ($n$ = 53, Table 9 and Table 10) including 28 *Salmonella* strains and 25 other related or unrelated bacterial genera including *Campylobacter, Citrobacter, Escherichia,*

*Listeria, Vibrio,* and others. False positive and false negative rates, if any, were calculated. The specificity test was repeated twice.

**LAMP sensitivity.** Sensitivity of the optimized LAMP assay, namely, the lowest detectable *Salmonella* cell level by LAMP assay, was determined by testing 10-fold serial-diluted DNA templates of *Salmonella* LT2 under optimized LAMP conditions. Sensitivity test was repeated four times and the detection limit was presented as the lowest number of cells that could be detected by the optimized LAMP assay. *Tt* values were collected for the generation of standard curves by plotting the *Tt* values against the corresponding log CFU/rxn.

**PCR conditions.** To compare with LAMP assay, specificity and sensitivity tests of a PCR assay were performed using the same templates as described above. The PCR mix with a total volume of 25 µl contained 1 × PCR buffer, 0.2 mM of each dNTP, 1.5 mM of $MgCl_2$, 0.4 µM of each forward or reverse primer from Rahn et al. (Rahn *et al.*, 1992) (Table 5), 0.625 U of GoTaq Hot Start Polymerase (Promega, Madison, WI), and 2 µl of DNA templates. The PCR reactions were conducted using initial denaturation at 95$^{o}$C for 10 min followed by 30 cycles of denaturation at 95$^{o}$C for 30 s, primer annealing at 64$^{o}$C for 30 s, extension at 72$^{o}$C for 30 s and a final extension at 72$^{o}$C for 7 min in a Bio-Rad C1000 Thermal Cycler (Hercules, CA). Aliquots (10 µl) of PCR products were analyzed by electrophoresis on 1.5% agarose gel containing ethidium bromide, and visualized under UV light. Gel images were documented by a Gel Doc XR system (Bio-Rad).

## 3) Detect live *Salmonella* using PMA-LAMP

**Heat inactivation.** Fresh mid-log live *Salmonella* culture prepared as described above was incubated at 95$^{o}$C in water bath for 10 min to obtain dead cells and 0.1 ml each culture level was spread-plated to ensure the non-viability of the treated cells.

**PMA treatment.** Propidium monoazide (PMA, Biotium, Hayward, CA) was dissolved in 20% of DMSO to obtain a stock of 20 mM and stored at -20°C in the dark. Aliquots (1.0 ml) of fresh or heat-killed dead cells in a 1.5 ml translucent microcentrifuge tube were mixed with 5 µl of PMA (100 µM) and incubated in the dark for 5 min. After dark incubation, the tube was placed on ice horizontally and exposed to strong light (650W halogen light, FCW 120V, GE lighting, General Electric Co., Cleveland, OH) with a distance of 20 cm for 2 min.

**DNA purification.** The PMA treated cells were then subjected to DNA purification using the UltraClean™ Microbial DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA). The extraction procedures were followed by instructions from the manufacture except that purified DNA was finally suspended in 100 µl of elution solution.

**Dead *Salmonella* detection.** Aliquots (1.0 ml) of dead cells at levels ranging from $10^8$ to $10^2$ CFU/ml were placed on a 1.5 ml translucent microcentrifuge tube, respectively. Each tube was subjected to PMA treatment and DNA extraction for templates as described above. Aliquots (2 µl) of purified DNA were subjected to both LAMP and PCR assays. Detection in the three assays was repeated twice and data including PCR gel and *Tt* values were collected.

**Live *Salmonella* detection in the presence of dead *Salmonella*.** Aliquots (0.5 ml) of live *Salmonella* cells at levels ranging from $10^8$ to $10^2$ CFU/ml were mixed with 0.5 ml of dead *Salmonella* cells at $10^5$ CFU/ml. Each mix was subjected to PMA treatment and DNA extraction for templates as described above. Aliquots (2 µl) of purified DNA were subjected to both LAMP and PCR assays. Detection using PCR or LAMP assay was repeated four times. Data including PCR gel and *Tt* values were collected and standard curve was generated as mentioned above.

## 4) Detect live *Salmonella* in spiked produce samples

### a. Produce sample preparation

**Produce samples.** A variety of produce items (cantaloupe, spinach, and tomato) were obtained from local supermarkets and sampled immediately. These produce commodities were selected because historically they have been frequently involved in produce-associated *Salmonella* outbreaks (CDC, 2008; CDC, 2007; CDC, 2002). Three replicate samples were obtained for each produce item.

**Produce quality testing.** The produce samples were assessed for quality properties including pH, $^{o}$Brix and color using standard methods. Three samples of each produce type were tested and data were collected and analyzed as the average value ± standard deviation for each index. Briefly, the samples were blended and titrated for pH of the slurry using 836 Tirando Automatic Titrator (Metrohm, Herisau, Switzerland). Besides, the sample (except spinach) was squeezed to produce a drop of juice to be measured $^{o}$Brix in Pocket Pal-1 pocket refractometer (Atago, Tokyo, Japan). $^{o}$Brix measurement of each sample was performed three times. Additionally, color measurements were performed on the cantaloupe cube, the skin and the cut flesh of tomato, and spinach leaf using Chroma meter CR-400 with SpectraMagic NX CM-S100w software (Konica, Tokyo, Japan). Color indexes (L*, a*, b*) of each sample were measurement and repeated four times.

***Salmonella* isolation of produce samples.** To facilitate homogenization, spinach samples were cut into 4 $cm^2$ by sanitized scalpel, and cantaloupe and tomatoes and were sliced into small pieces using a sanitized knife before put into sterilized stomacher bags. Ten grams of each sample were added with 90 ml of BPW and then homogenized by a stomacher - Lab-Blander 400 (Teledyne Tekmar, Mason, Ohio) at a high speed for 2 min. Produce homogenates

were analyzed as described in the BAM with slight modifications. Briefly, forty-five milliliter of the homogenate was pre-enriched in a 37°C water bath for 6 h, and then 10 ml of the homogenate was transferred into 100 ml of TT broth (BD Diagnostic System) for overnight incubation at 42°C. After incubation, two loops of the culture were streaked on duplicate XLT4 agar and incubated at 35°C for 24 h. After incubation, typical black or pink to red with black center colonies, if any, on XLT4 agar were transferred on MacConkey agar and incubated at 35°C for 24 h. Pure colorless colonies grown on MacConkey agar were picked and suspended in TE buffer to make templates as described above and then confirmed by *invA*-PCR.

**b. Experimental contamination with *Salmonella***

**Preparation of dead *Salmonella* inoculum.** For each *Salmonella*-negative produce type, dead *Salmonella* cell inoculums were prepared by serially diluting heat-killed dead *Salmonella* cells ranging from $10^8$ to $10^2$ CFU/ml.

**Inoculation of live *Salmonella* in the presence of dead *Salmonella*.** Additionally, for each *Salmonella*-negative produce type, inoculums of live *Salmonella* in the presence of dead *Salmonella* were prepared by serially diluting fresh live *Salmonella* at cell levels ranging from $10^8$ to $10^2$ CFU/ml and then mixing each dilution with $10^5$ CFU/ml of dead *Salmonella* cells.

**Experimental spiking of produce with *Salmonella*.** *Salmonella* cells were spiked at the homogenization step. Briefly, one hundred microliter of the culture were added to 900 µl of the homogenate, mixed thoroughly, and centrifuged at 900 *g* for 3 min to remove the produce tissues. The supernatant was subjected to PMA treatment and DNA purification as described above. Aliquots (2 µl) of the DNA templates were used for LAMP and PCR assays and repeated twice. Data including PCR gel and *Tt* values were collected and standard curve was generated as mentioned above.

# Chapter 4 - Results

## 1) Characteristics of the developed LAMP assay

### a. LAMP parameter optimization

LAMP optimization results are shown in Table 6. Using a *Salmonella* Typhimurium LT2 template of $10^6$ CFU per reaction and a previously published LAMP primer set (Hara-Kudo *et al.*, 2005), the optimized $MgSO_4$ concentration (6 mM) remained the same as that in the prototype LAMP reagent mix. Other parameters including dNTP (1.2 mM), enzyme (10 U), outer primers (0.1 µM), inner primers (1.8 µM), loop primers (1.0 µM), and temperature (63°C) were slightly altered from those used in the prototype reagent mix, although the assay time was only shortened by approximately less than one minute (Table 6). For betaine (0 M), the elimination of its application in LAMP reaction mix alone dramatically speeded up DNA amplification by more than 3 min and enhanced the turbidity intensity by 2 fold (data not shown). It was also observed that as the betaine concentration decreased, the LAMP reaction progressed faster and the signals obtained were stronger (data not shown).

**Table 6. Comparison of prototype and optimized LAMP conditions and results**

| Parameters | Unit | Test range | Prototype LAMP | | Optimized LAMP | |
|---|---|---|---|---|---|---|
| | | | Value | *Tt* [a] (min) | Value | *Tt* (min) |
| MgSO4 | mM | 2, 4, 6, 8, 10 | 6 | 15.83±0.47 | 6 | 15.83±0.47 |
| Betaine | M | 0, 0.2, 0.4, 0.6, 0.8, 1.0 | 0.8 | 16.02±1.20 | 0 | 12.90±0.55 |
| dNTP | mM | 0.4, 0.8, 1.2, 1.4, 1.6, 2.0 | 1.4 | 16.60±0.52 | 1.2 | 16.47±0.77 |
| Enzyme | U | 2, 4, 6, 8, 10 | 8 | 16.13±0.42 | 10 | 15.77±1.12 |
| Outer Primers | µM | 0.05, 0.1, 0.2, 0.3, 0.4 | 0.2 | 17.10±0.23 | 0.1 | 16.75±0.15 |
| Inner Primers | µM | 1.2, 1.4, 1.6, 1.8, 2.0 | 1.6 | 16.42±0.25 | 1.8 | 16.32±0.30 |
| Loop Primers | µM | 0.2, 0.4, 0.6, 0.8, 1.0 | 0.8 | 17.15±0.68 | 1.0 | 16.77±0.45 |
| Temperature | °C | 61, 63, 65 | 65 | 18.13±0.18 | 63 | 17.62±0.12 |
| Incubation Time | min | 40, 50, 60 | 60 | NA [b] | 40 | NA |

[a] *Tt* values were calculated as based on five independent repeats of a *Salmonella* Typhimurium LT2 template of $10^6$ CFU/rxn; [b] NA means not available

Furthermore, a synergistic effect occurred when the optimized conditions were combined for all of the parameters, reducing *Tt* values by more than 4 min when compared with those of the prototype LAMP (Figure 7). When serially diluted templates ranging from $10^5$ to $10^{-2}$ CFU per reaction were tested using both the prototype and optimized LAMP conditions, the times to positive results were shortened for all templates under optimized condition (within 25 min) and the sensitivity was increased at least by 10 fold (Table 7). Based on these data, the incubation time for the optimized LAMP assay was adjusted to 40 min instead of 1 h in the prototype assay.

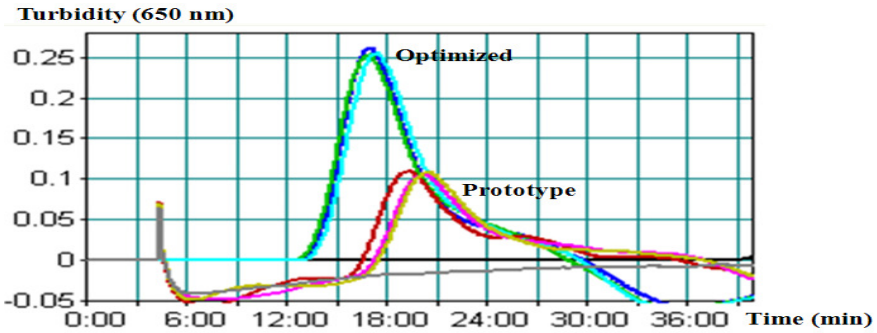


**Figure 7. Comparison of prototype and optimized LAMP results ***

* Three repeats of prototype and optimized LAMP assay comparison using a *Salmonella* Typhimurium LT2 template of $10^6$ CFU/rxn

**Table 7. Sensitivity and quantitative capability of prototype and optimized LAMP assay**

| CFU/rxn | *Tt* [a] (min) | | Reduced time [b] (min) |
|---|---|---|---|
| | Prototype LAMP | Optimized LAMP | |
| $1.3 \times 10^5$ | 18.73±0.93 | 13.80±0.00 | 4.93 |
| $1.3 \times 10^4$ | 20.43±2.23 | 14.70±0.30 | 5.73 |
| $1.3 \times 10^3$ | 21.10±2.18 | 15.15±0.70 | 5.95 |
| $1.3 \times 10^2$ | 22.20±3.12 | 16.26±0.40 | 5.94 |
| $1.3 \times 10^1$ | 24.27±1.40 | 18.50±1.48 | 5.77 |
| $1.3 \times 10^0$ | 28.50±5.65 | 19.87±1.23 | 8.63 |
| $1.3 \times 10^{-1}$ | 29.05±0.63 | 21.40±2.33 | 7.65 |
| $1.3 \times 10^{-2}$ | - | 24.33±2.97 | NA |
| **Equation** | **y = -1.16x + 23.84** | **Y= -1.30x + 20.36** | **NA** |
| **$r^2$ value** | **0.987** | **0.965** | **NA** |

[a] Tt values were calculated based on 4 independent repeats of the 10-fold serially diluted Salmonella templates; [b] Reduced time was calculated as the average Tt value of prototype LAMP minus that of optimized LAMP for the corresponding template

**b. LAMP primer comparison**

Based on the *invA* gene, five sets of LAMP primers were generated for *Salmonella* detection and designated as Sal-1, Sal-4, Sal-8, Sal-9 and Sal-13, respectively. Comparison of the speed, sensitivity, and quantitative capability of these primers with Sal-HK (Hara-Kudo *et al.*, 2005) revealed the great potential of Sal-8 primers for *Salmonella* detection (Table 8). Sal-8 primers gave positive results within 40 min for the serially diluted templates ranging from $10^5$ to $10^{-2}$ CFU per reaction, and had a detection limit of 1.3 - 13 CFU per reaction, while at the same time possessed superior quantitative capability ($r^2$ = 0.983) compared with other primer sets. Although Sal-HK primers performed faster and would yield positive result within 30 min, its detection limit of less than 1 CFU per reaction suggested its tendency to generate false positive results. In addition, Sal-HK primers had a smaller correlation coefficient value of 0.952, suggesting its weaker quantitative capability compared with the Sal-8 primer set. Therefore, Sal-8 was chosen as the LAMP primer set used for following experiments of *Salmonella* detection in this study.

**Table 8. Comparison of six *invA*-based LAMP primer sets**

| Primer set | Sal-HK | Sal-1 | Sal-4 | Sal-8 | Sal-9 | Sal-13 |
|---|---|---|---|---|---|---|
| Assay time (min) | <30 | <60 | <40 | <40 | <40 | < 60 |
| Sensitivity (CFU/rxn) | 0.013 | 1.3 - 13 | 1.3 - 13 | 1.3 - 13 | 1.3 - 13 | 13000 |
| $r^2$ value * | 0.952 | 0.960 | 0.951 | 0.983 | 0.978 | NA |

* $r^2$ value was calculated based on the linear relationship of average *Tt* values and log CFU/rxn between cell levels of $10^5$ - $10^2$ CFU/rxn

**c. LAMP specificity**

The developed *Salmonella invA*-based LAMP assay successfully detected 28 *Salmonella* strains including 9 serotypes (Table 9), while showing negative results for 25 non-*Salmonella* strains (Table 10), indicating that the *invA*-based LAMP assay was highly specific. For the 28 *Salmonella* strains, *Tt* values ranged between 15 and 17.8 min with an average of 16.26±0.40

min; for the 25 non-*Salmonella* strains, no *Tt* value was obtained. Similarly, the *invA*-based PCR could detect all *Salmonella* strains while showed negative for all non-*Salmonella* strains.

**Table 9. Specificity of *invA*-based LAMP and PCR assay for *Salmonella* strains**

| ***Salmonella* serotype** | **Strain ID** | **LAMP (*Tt* [a]; min)** | **PCR** |
|---|---|---|---|
| **Reference strains, unknown source (*n*=3)** | | | |
| Braenderup | H9812 | 15.55±0.07 | + |
| Typhimuirim | LT2 | 15.70±0.13 | + |
| | UMD373 | 15.30±0.07 | + |
| **Isolations from chicken, retail, Louisiana (*n*=25)** | | | |
| Agona | S133 | 16.65±0.07 | + |
| | S134 | 17.80±0.13 | + |
| Braenderup | S32 | 17.70±0.42 | + |
| | S33 | 16.60±0.13 | + |
| | S61 | 17.20±0.13 | + |
| | S62 | 17.60±0.00 | + |
| Enteritidis | S49 | 15.75±0.20 | + |
| | S50 | 15.15±1.20 | + |
| Hadar | S37 | 17.75±1.20 | + |
| | S38 | 17.30±0.83 | + |
| | S98 | 17.10±1.27 | + |
| | S99 | 17.10±0.83 | + |
| Kentucky | S67 [b] | 15.00±0.27 | + |
| | S68 [b] | 15.00±0.00 | + |
| | S70 | 15.55±0.77 | + |
| | S71 | 15.25±0.20 | + |
| | S127 | 15.70±0.00 | + |
| | S128 | 15.65±0.35 | + |
| Mbandaka | S16 | 15.60±0.00 | + |
| | S46 | 16.05±0.07 | + |
| | S47 | 15.05±0.07 | + |
| Montevideo | S8 | 16.70±0.00 | + |
| | S9 | 16.85±0.20 | + |
| Thompson | S25 | 15.85±0.07 | + |
| | S26 | 16.85±0.20 | + |
| | **Average:** | **16.26±0.40** | **NA** |

[a] *Tt* value was calculated as the average *Tt* values ± standard deviation based on 2 independent repeats; [b] Non-digestible Kentucky

**Table 10. Specificity of *invA*-based LAMP and PCR assay for non-*Salmonella* strains**

| Non-*Salmonella* strains | LAMP | PCR |
|---|---|---|
| **Non-*Vibrio* spp. (*n*=13)** | | |
| *Campylobacter jejuni* 81-176 | - | - |
| *Campylobacter jejuni* ATCC 33560 | - | - |
| *Citrobacter freudii* ATCC 8090 | - | - |
| *Enterobacter aerogenes* ATCC 13048 | - | - |
| *Enterococcus faecalis* ATCC 29212 | - | - |
| *Escherichia coli* ATCC 25922 | - | - |
| *Listeria monocytogenes* ATCC 13932 | - | - |
| *Listonella anguillarum* ATCC 19264 | - | - |
| *Pseudomonas aeroginosa* ATCC 27853 | - | - |
| *Shigella flexneri* ATCC 12022 | - | - |
| *Shigella sonnei* ATCC 25931 | - | - |
| *Staphylococcus aureus* ATCC 29213 | - | - |
| *Streptococcus pneumonia* ATCC 49619 | - | - |
| ***Vibrio* spp. (*n*=12)** | | |
| *V. alginolyticus* ATCC 17749 | - | - |
| *V. cholera* ATCC 14035, O:1 | - | - |
| *V. cincinnatiensis* ATCC 35912 | - | - |
| *V. harveyi* ATCC 35084 | - | - |
| *V. mimicus* ATCC 33655 | - | - |
| *V. natriegens* ATCC 14048 | - | - |
| *V. alginolyticus* ATCC 33787 | - | - |
| *V. harveyi* ATCC 14126 | - | - |
| *V. mimicus* ATCC 33653 | - | - |
| *V. Fluvicus* ATCC 33809 | - | - |
| *V. vulnificus* ATCC 27562 | - | - |
| *V. parahaemolyticus* ATCC 17802 | - | - |

**d. LAMP sensitivity and quantitative capability**

By testing the 10-fold serial dilutions of *Salmonella* Typhimurium LT2 DNA templates, sensitivity of the developed *Salmonella invA*-based LAMP assay is shown in Table 11. For templates ranging from $1.3 \times 10^5$ to $1.3 \times 10^1$ CFU per reaction, the average *Tt* values fell between 20.35 and 27.30 min. In one out of four repeats, the template of $1.3 \times 10^0$ CFU per

reaction was amplified, yielding a *Tt* value of 37.70 min. For template of $1.3 \times 10^{-1}$ CFU per reaction, no amplification occurred. Therefore, the developed LAMP assay gave the lower detection limit of 1.3 - 13 CFU per reaction. The *invA*-based PCR used to detect *Salmonella* had a sensitivity of $1.3 \times 10^{2}$ CFU per reaction, which was up to 100-fold less sensitive than that of the developed *invA*-based LAMP assay. Additionally, the correlation coefficient ($r^2$) of the *invA*-based LAMP assay, which indicated the linear relationship between *Salmonella* cell numbers and the turbidity signals, was calculated to be 0.983, which indicated excellent quantitative capability of the LAMP assay. Figure 8 presents a representative amplification graph of *invA*-based LAMP assay in detecting serially diluted *Salmonella* templates ranging from $1.3 \times 10^{5}$ to $1.3 \times 10^{-1}$ CFU per reaction (Figure 8A), the corresponding standard curve generated based on four independent LAMP repeats (Figure 8B), and the representative gel image of *invA*-based PCR assay (Figure 8C).

**Table 11. Sensitivity of *invA*-based LAMP and PCR assays in pure culture**

| **Cell level (CFU/rxn)** | **LAMP (*Tt* [a]; min)** | **PCR** |
|---|---|---|
| $1.3 \times 10^{5}$ | 20.35±1.10 | + |
| $1.3 \times 10^{4}$ | 21.50±1.27 | + |
| $1.3 \times 10^{3}$ | 22.25±1.53 | + |
| $1.3 \times 10^{2}$ | 22.93±1.92 | + |
| $1.3 \times 10^{1}$ | 27.30±3.47 | - |
| $1.3 \times 10^{0}$ | 37.70 [b] | - |
| $1.3 \times 10^{-1}$ | - | - |
| **Equation** | **y = -0.85x + 24.82** | **NA** |
| **$r^2$ [c] value** | **0.983** | **NA** |

[a] *Tt* value was calculated based on 4 independent repeats; [b] Only one out of four repeats got positive at 1.3 CFU/rxn; [c] $r^2$ value was calculated based on the linear relationship of average *Tt* values and log CFU/rxn between cell levels of $10^{5}$ - $10^{2}$ CFU/rxn

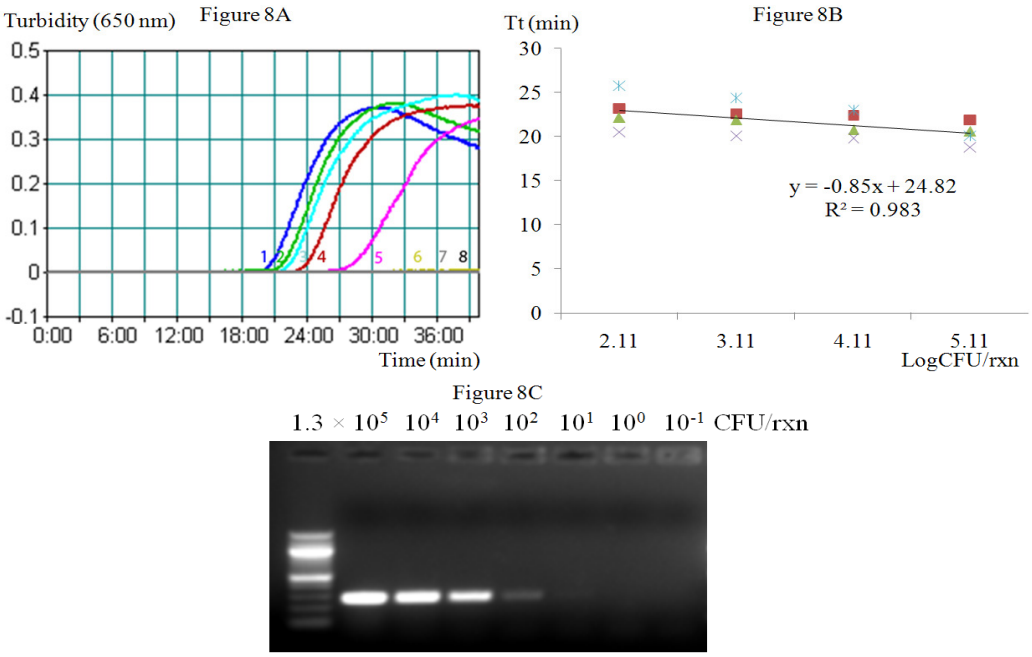


**Figure 8. Sensitivity of *invA*-based LAMP and PCR assays, and standard curve of *invA*-based LAMP in pure culture. A: A representative amplification graph generated by *invA*-based LAMP assay when detecting *Salmonella* cells in pure culture. Samples 1 - 7 correspond to templates containing *Salmonella* cells ranging from $1.3 \times 10^5$ to $1.3 \times 10^{-1}$ CFU/rxn, sample 8 is water; B: The corresponding standard curve of *invA*-based LAMP assay generated based on 4 independent repeats; C: A representative gel image generated by *invA*-based PCR assay using the same templates**

## 2) Performance of PMA-LAMP for live *Salmonella* detection

### a. False positive exclusivity of PMA-LAMP

The potential of the *invA*-based PMA-LAMP assay to specifically detect only live *Salmonella* cells were examined by testing 10-fold serial dilutions of heat-killed *Salmonella* cells. Results showed that after PMA treatment, only dead *Salmonella* of $7.5 \times 10^6$ CFU per reaction were detected by the LAMP assay at 26.70 min, while no amplification signal was observed for dead cell levels between $7.5\times 10^5$ and $7.5 \times 10^0$ CFU per reaction (data not shown), indicating that the developed *invA*-based PMA-LAMP assay could successfully avoid detecting dead *Salmonella* cells up to $7.5 \times 10^5$ CFU per reaction.

After PMA treatment of dead *Salmonella* cells, the *invA*-PCR used to detect *Salmonella* was unable to get any DNA amplification from templates ranging from $7.5\times 10^6$ to $7.5 \times 10^0$ CFU per reaction, suggesting that *invA*-based PMA-PCR could avoid detecting dead *Salmonella* cells up to $7.5 \times 10^6$ CFU per reaction.

**b. Sensitivity and quantitative capability of PMA-LAMP**

Sensitivity of the *invA*-based PMA-LAMP assay was evaluated by testing 10-fold serial dilutions of live *Salmonella* cells, in the presence of $7.5 \times 10^5$ CFU/ml, i.e., $7.5 \times 10^3$ CFU per reaction of heat-killed *Salmonella* cells. Table 12 shows that, positive results were obtained for templates ranging from $3.4 \times 10^5$ to $3.4 \times 10^1$ live *Salmonella* cells per reaction, with the average *Tt* values ranging between 19.30 and 29.55 min. In one out of four repeats, amplification occurred for the template of $3.4 \times 10^0$ live *Salmonella* cells per reaction and gave positive result at 29.70 min. No amplification took place for the template containing $3.4 \times 10^{-1}$ live *Salmonella* cells and $7.5 \times 10^3$ dead *Salmonella* cells per reaction. Therefore, the *invA*-based PMA-LAMP assay had the detection limit of 3.4 - 34 live *Salmonella* cells per reaction in the presence of $7.5 \times 10^3$ dead *Salmonella* cell. The *invA*-based PMA-PCR assay had a detection limit of 340 CFU per reaction, up to 100-fold less sensitive than the PMA-LAMP assay. Additionally, the correlation coefficient ($r^2$) was calculated to be 0.970, suggesting an excellent quantitative capability of the developed PMA-LAMP assay for live *Salmonella* detection. Figure 9 presents a representative amplification graph of *invA*-based PMA-LAMP assay in detecting *Salmonella* templates ranging from $3.4 \times 10^5$ to $3.4 \times 10^{-1}$ live *Salmonella* cells per reaction (each template containing $7.5 \times 10^3$ CFU per reaction of dead *Salmonella*) (Figure 9A), the corresponding standard curve generated based on four independent LAMP repeats (Figure 9B), and the representative gel image of *invA*-based PMA-PCR (Figure 9C).

**Table 12. Sensitivity of *invA*-based PMA-LAMP and PMA-PCR assays in pure culture [a]**

| Live cell level (CFU/rxn) | LAMP (*Tt* [b]; min) | PCR |
|---|---|---|
| $3.4 \times 10^5$ | 19.30±0.43 | + |
| $3.4 \times 10^4$ | 20.20±0.73 | + |
| $3.4 \times 10^3$ | 22.50±0.60 | + |
| $3.4 \times 10^2$ | 25.22±1.50 | + |
| $3.4 \times 10^1$ | 29.55±2.63 | - |
| $3.4 \times 10^0$ | 29.70 [c] | - |
| $3.4 \times 10^{-1}$ | - | - |
| **Equation** | **y = -2.06x + 29.58** | **NA** |
| **$r^2$ [d] value** | **0.970** | **NA** |

[a] In the presence of dead cells at $7.5 \times 10^5$CFU/ml or $7.5 \times 10^3$CFU/rxn; [b]*Tt* value was calculated based on 4 independent repeats; [c] Only one out of four repeats got positive at 3.4 CFU/rxn; [d] $r^2$ value was calculated based on the linear relationship of average *Tt* values and log CFU/rxn between cell levels of $10^5$ - $10^2$ CFU/rxn

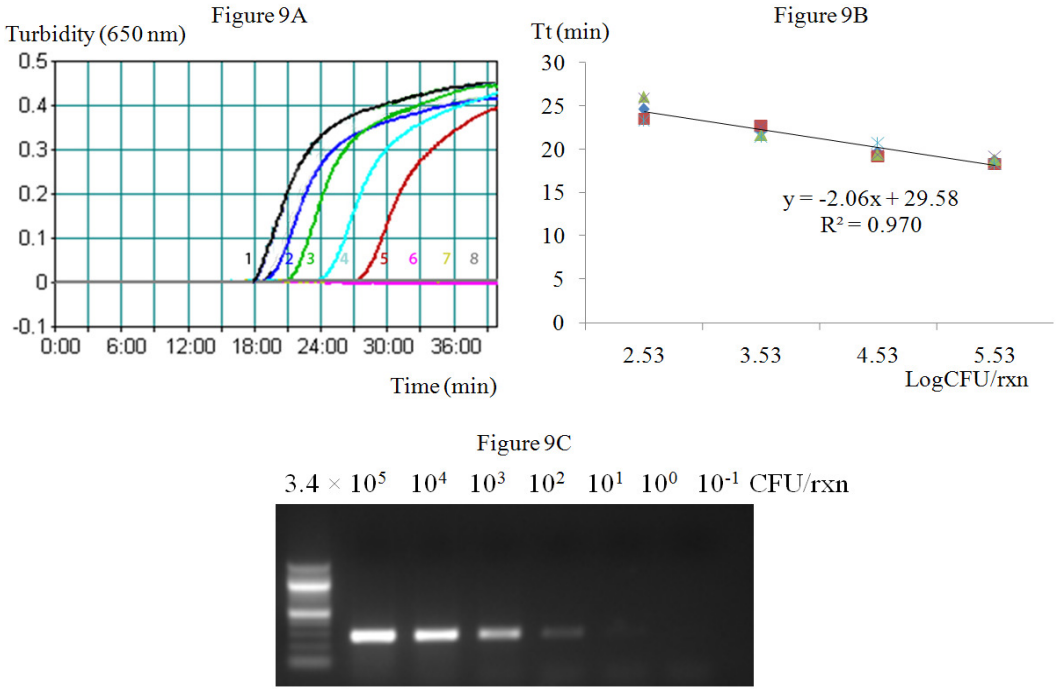


**Figure 9. Sensitivity of *invA*-based PMA-LAMP and PMA-PCR assays, and standard curve of PMA-LAMP in pure culture. A: A representative amplification graph generated by *invA*-based PMA-LAMP assay when detecting live *Salmonella* cells in pure culture. Samples 1 - 7 correspond to templates containing live *Salmonella* cells ranging from $3.4 \times 10^5$ to $3.4 \times 10^{-1}$ CFU/rxn (each template containing $7.5 \times 10^3$ CFU/rxn of dead *Salmonella*), sample 8 is water; B: The corresponding standard curve of PMA-LAMP assay generated based on 4 independent repeats; C: A representative gel image generated by *invA*-based PMA-PCR assay using the same templates**

## 3) Performance of PMA-LAMP for live *Salmonella* detection in produce

### a. Produce sample quality data

Table 13 gives the values of pH, °Brix and Color index (L*, a*, b*) of produce samples purchased from local supermarket. All the produce samples purchased were confirmed to be *Salmonella* negative.

**Table 13. pH, °Brix and color index of produce samples**

| Sample ID | | Color | | | pH | °Brix |
|---|---|---|---|---|---|---|
| | | L* | a* | b* | | |
| Cantaloupe 1 | | 57.27±6.20 | 8.01±1.30 | 31.99±1.84 | 6.17 | 17.80±0.00 |
| Cantaloupe 2 | | 59.44±2.88 | 8.32±0.84 | 34.77±1.85 | 6.37 | 15.53±0.25 |
| Cantaloupe 3 | | 66.89±3.49 | 9.45±0.87 | 37.25±1.79 | 6.21 | 12.63±0.23 |
| Spinach 1 | | 36.13±6.21 | -15.05±4.40 | 19.35±6.56 | 6.38 | NA |
| Spinach 2 | | 36.96±2.14 | -16.21±0.95 | 20.75±2.07 | 6.24 | NA |
| Spinach 3 | | 44.96±1.06 | -20.34±1.00 | 32.22±2.57 | 6.20 | NA |
| Tomato 1 | Skin | 45.25±5.71 | 9.31±2.14 | 28.71±3.81 | 4.03 | 4.30±0.17 |
| | Flesh | 42.86±6.60 | 6.64±2.59 | 21.83±2.84 | | |
| Tomato 2 | Skin | 34.77±1.85 | 21.09±1.21 | 24.20±2.13 | 4.55 | 4.2±0.1 |
| | Flesh | 38.88±4.65 | 14.92±3.78 | 24.05±2.16 | | |
| Tomato 3 | | 36.51±1.92 | 13.88±1.83 | 22.37±1.68 | 4.34 | 4.2±0.29 |
| | | 37.93±5.60 | 5.51±4.34 | 19.02±2.15 | | |

### b. False positive exclusivity of PMA-LAMP

The potential of the *invA*-based PMA-LAMP assay to specifically detect only live *Salmonella* cells in produce were examined by spiking 10-fold serial dilutions of heat-killed *Salmonella* cells into cantaloupe, spinach, and tomato samples. Results showed that produce samples containing dead *Salmonella* of $7.5 \times 10^6$ CFU per reaction yielded false positive results, with *Tt* values of 28.50, 38.80 and 25.20 min for cantaloupe, spinach and tomato, respectively; while for dead *Salmonella* cells in produce at levels between $7.5 \times 10^5$ and $7.5 \times 10^0$ CFU per reaction, no amplification was observed (data not shown). This indicated that the developed *invA*-based PMA-LAMP assay could successfully avoid false positive detection of dead

*Salmonella* presenting in produce samples up to $7.5 \times 10^5$ CFU per reaction, equivalent to $3.75 \times 10^8$ CFU/g.

The *invA*-based PMA-PCR used to detect live *Salmonella* in this study was unable to get any DNA amplification from produce containing dead *Salmonella* ranging from $7.5 \times 10^6$ to $7.5 \times 10^0$ CFU per reaction, suggesting that *invA*-based PMA-PCR could efficiently avoid false positive detection of dead *Salmonella* presenting in produce samples up to $7.5 \times 10^6$ per reaction.

**c. Sensitivity and quantitative capability of PMA-LAMP**

In the presence of $3.75 \times 10^6$ CFU/g of dead *Salmonella,* the live *Salmonella* detection limits in produce samples by the *invA*-based PMA-LAMP assay is shown in Table 14. In three independent spiking experiment, the *invA*-based PMA-LAMP consistently detected live *Salmonella* down $5.5 \times 10^3$ CFU/g in cantaloupe samples without enrichment, with the average *Tt* values ranging between 17.65 and 27.40 min; while for both spinach and tomato samples, the lowest detection limit achieved was $5.5 \times 10^4$ CFU/g, with the average *Tt* values of 19.85 - 34.15 min and 22.12 - 27.70 min, respectively. In contrast, the *invA*-based PMA-PCR consistently detected live *Salmonella* down to $5.5 \times 10^5$ CFU/g in cantaloupe, spinach and tomato samples, up to 100-fold less sensitive than the *invA*-based PMA-LAMP assay. The correlation coefficient ($r^2$) was calculated to be 0.993, 0.977 and 0.949 for cantaloupe, spinach and tomato, respectively, suggesting the excellent quantitative capabilities of the developed PMA-LAMP assay for live *Salmonella* detection in produce samples. Figure 10 shows a representative amplification graph of the *invA*-based PMA-LAMP assay in detecting *Salmonella* templates ranging from $5.5 \times 10^7$ to $5.5 \times 10^1$ live *Salmonella* cells per gram of cantaloupe sample (Figure 10A), the corresponding standard curve generated based on two independent LAMP repeats (Figure 10 B), and the representative gel image of *invA*-based PMA-PCR (Figure 10 C).

**Table 14. Sensitivity of PMA-LAMP and PMA-PCR in produce [a]**

| Live cell level (CFU/g) | Cantaloupe PMA-LAMP (*Tt* [b]; min) | Cantaloupe PMA-PCR | Spinach PMA-LAMP (*Tt*; min) | Spinach PMA-PCR | Tomato PMA-LAMP (*Tt*; min) | Tomato PMA-PCR |
|---|---|---|---|---|---|---|
| $5.5 \times 10^7$ | 20.05±4.00 | + | 19.85±1.77 | + | 22.20±2.68 | + |
| $5.5 \times 10^6$ | 21.95±3.60 | + | 24.00±0.70 | + | 25.15±2.47 | + |
| $5.5 \times 10^5$ | 25.00±1.11 | + | 27.40±3.10 | + | 26.45±2.60 | + |
| $5.5 \times 10^4$ | 27.40±2.53 | - | 34.15±0.35 | - | 27.70±0.70 | - |
| $5.5 \times 10^3$ | 30.90±0.00 | - | - | - | - | - |
| $5.5 \times 10^2$ | - | - | - | - | - | - |
| $5.5 \times 10^1$ | - | - | - | - | - | - |
| **Equation** | **y = -2.51x + 37.27** | **NA** | **y = -4.63x + 55.24** | **NA** | **y = -1.78x + 36.48** | **NA** |
| **$r^2$ [c] Value** | **0.993** | **NA** | **0.977** | **NA** | **0.949** | **NA** |

[a] In the presence of $3.75 \times 10^6$ CFU/g dead *Salmonella*; [b] *Tt* value was calculated based on 2 independent repeats; [c] $r^2$ value was calculated based on the linear relationship of average *Tt* values and log CFU/g between cell levels of $10^7$ - $10^4$ CFU/g

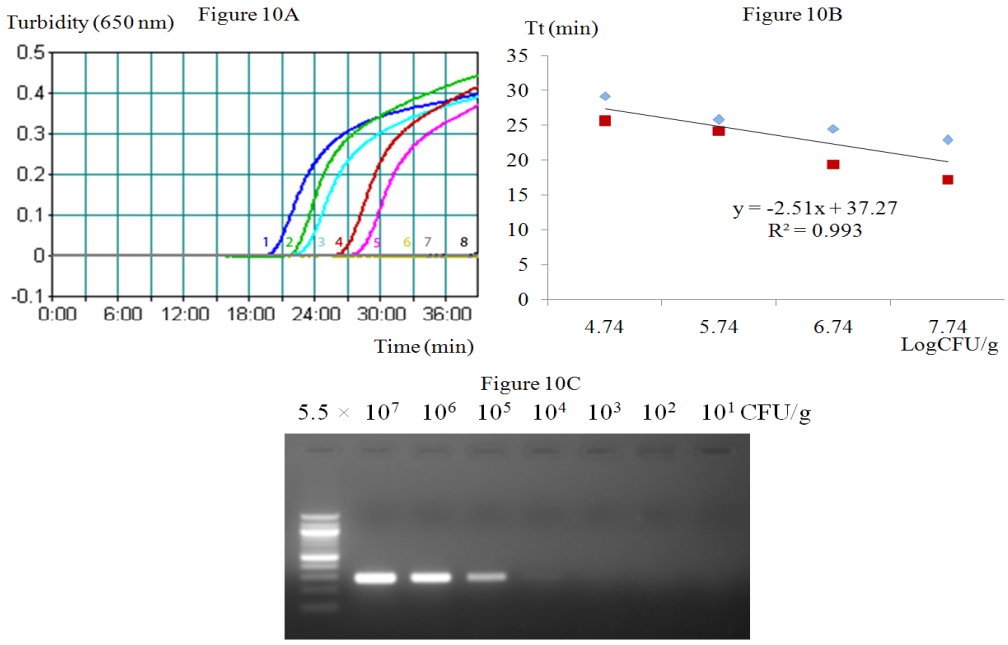


**Figure 10. Sensitivity of PMA-LAMP and PMA-PCR assays, and standard curve of PMA-LAMP in cantaloupe. A: A representative amplification graph generated by PMA-LAMP assay when detecting live *Salmonella* cells in cantaloupe sample. Samples 1 - 7 correspond to spiked samples containing live *Salmonella* cells ranging from $5.5 \times 10^7$ to $5.5 \times 10^1$ cells per gram of cantaloupe (each sample containing $3.75 \times 10^6$ CFU/g of dead *Salmonella*), sample 8 is water; B: The corresponding standard curve of PMA-LAMP assay generated based on 2 independent repeats; C: A representative gel image generated by PMA-PCR assay using the same templates**

# Chapter 5 - Discussion

## 1) The LAMP assay

### a. LAMP parameter optimization

In most LAMP studies, commercial Loopamp® DNA Amplification Kits were used regardless of the target organism or primers, though in reality the effects of different target organisms and primers on the LAMP reaction might come into play. Therefore, to develop a LAMP assay for *Salmonella* detection with high efficiency, we felt the necessity to thoroughly optimize all of the LAMP parameters and at the same time compare the optimized conditions with the prototype conditions from Loopamp® DNA Amplification Kit.

Although most parameters did not alter dramatically from the prototype conditions, surprisingly, the omission of betaine yielded an optimized assay which was 3 min faster as well as 10-fold more sensitive than using the prototype LAMP. It was observed that, with decreasing betaine concentrations, the LAMP reaction progressed faster and the turbidity signals generated were stronger. This result was in contrast to most of other studies (Haridas *et al.*, 2010; Nagamine *et al.*, 2001; Notomi *et al.*, 2000; Yeh *et al.*, 2005), in which higher concentration of Betaine could stimulate overall reaction and increase LAMP specificity. Betaine is commonly used in PCR and LAMP assays, which can isostablize DNA and prevent secondary structure formation in GC-rich region, thus promoting DNA amplification and reducing base stacking (Baskaran *et al.*, 1996; Rees *et al.*, 1993). However, we postulate that the functions of Betaine are DNA sequence-dependent and the contrast might be due to the target sequence differences.

As a cofactor for DNA polymerase, the addition of $Mg^{2+}$ enhances DNA amplification (Saiki *et al.*, 1988). In this study, the optimal $MgSO_4$ concentration for LAMP assay was 6 mM,

same as the prototype condition, corroborating finding of Yeh et al. (Yeh *et al.*, 2005), in a study using LAMP to detect *Edwardsiella ictaluri*.

Another parameter, dNTPs are substrates needed for the DNA synthesis, therefore sufficient amount of dNTP is required to faciliate DNA amplification. However, too many dNTP might result in poor specificity of reaction (Innis *et al.*, 1988). In this study, the optimal concentration of dNTP, i.e., 1.2 mM, was slightly lower than the prototype concentration. A study by Yeh et al. (Yeh *et al.*, 2005) also demonstrated the use of lower dNTP concentration, i.e., 1.0 mM to be optimal for the LAMP detection of *Edwardsiella. ictaluri*.

Concentrations of the primers are very important for the DNA amplification. In this study, optimal concentration of each outer primer (F3 and B3) was reduced to 0.1 µM while increased to 1.8 and 1.0 µM for each inner (FIP and BIP) and loop primers (Loop-F and Loop-B) respectively when compared with prototype conditions. Outer primers are primarily used in the initial stage to displace single DNA strands amplified from inner primers to form the stem-loop and dumbbell-like DNA structure. Once the dumbbell-like DNA structure is formed, outer primers are theoretically not required in the later stage - exponential stage. Therefore, the amounts of F3 and B3 required for reaction might be low. In some studies, it was reported that LAMP assay could be carried out without outer primers (Maruyama *et al.*, 2003). However, inner primers, which serve as self-primers to promote auto-cycling of DNA amplification throughout the whole process, play a vital role in the LAMP reaction. In some studies, much higher concentrations of inner primers were used to improve the efficiency of LAMP assay (Enosawa *et al.*, 2003; Ihira *et al.*, 2004; Ihira *et al.*, 2007). The addition of loop primers accelerates DNA amplification and improves sensitivity by increasing the number of starting points for DNA amplification (Nagamine *et al.*, 2002).

*Bst* DNA polymerase is the enzyme used to catalyze DNA amplification in LAMP assay. In this study, 10 U of enzyme helped to increase the reaction rate, which was not unexpected as more enzyme units existed in the reaction should catalyze more substrates per minute.

The optimal temperature for LAMP assay in this study was found to be 63$^{o}$C, which was different from a previous LAMP study for *Salmonella* detection (Hara-Kudo *et al*., 2005), in which 65$^{o}$C was used as the incubation temperature. The optimal temperature is both enzyme- and target sequence-dependent. Although the optimal temperature for *Bst* DNA polymerase is 65$^{o}$C, there are some studies that reported lower optimal temperature for LAMP assay (Chen & Ge, 2010; Li *et al.*, 2009; Varga & James, 2006). Nonetheless, LAMP reactions were generally carried out at temperatures between 60 - 65$^{o}$C.

Earlier studies of LAMP used 1 h as the standard incubation time for DNA amplification, however, in recent years, with the addition of loop primers, shorter reaction time (i.e., 30 or 45 min) was reported to be sufficient to accumulate detectable LAMP products (Ihira *et al.*, 2004; Savan *et al.*, 2004). Besides, it was reported that a shorter LAMP reaction gave more typical ladder like patterns when LAMP results were checked by gel electrophoresis (Varga & James, 2006). In our study, we shortened the LAMP incubation time to be 40 min as we noticed that in several repeats of initial experiments, all positive results were obtained within 40 min during standard 1 h incubation time.

**b. LAMP primer comparison**

In a LAMP assay development, generally several sets of primers are designed and evaluated, and the primer set with the best performed will be selected. In this study, comparisons were made for the five designed primer sets and the Sal-HK from previous study (Hara-Kudo *et al*., 2005). Among the five designed LAMP primer sets, Sal-8 primers gave the best performance

in terms of speed, sensitivity, and quantitative capability. It could detect *Salmonella* down to 1.3 CFU per reaction within 40 min, and yielded a correlation coefficient ($r^2$) of 0.983. Though Sal-HK performed faster than Sal-8, it showed inconsistent false positive results for low cell levels (<1.3 cells) and even for the negative control, and demonstrated less strong linear relationship between the *Tt* values and the bacterial cell numbers when compared with Sal-8 primer set. The unreliable amplification of Sal-HK might be due to the poor stability of the primers in the reaction. Therefore, Sal-8 was selected for further study.

**c. LAMP specificity, sensitivity and quantitative capability**

The six primers (F3, B3, FIP, BIP, and Loop-F, Loop-B) of Sal-8 targeted eight distinct regions of *Salmonella invA* gene, a gene that was previous reported to be a reliable and accurate gene marker for molecular detection of *Salmonella* (Cheng, 2005; Cheng *et al.*, 2008; Rahn *et al.*, 1992). The *invA*-based LAMP assay developed in this study was highly specific in that among a total of 28 *Salmonella* strains and 25 non-*Salmonella* strains. The assay obtained 100% inclusivity as well as 100% exclusivity. The high specificity of LAMP assay has been reported in many previous studies (Hara-Kudo *et al.*, 2005; Nemoto *et al.*, 2009; Okamura *et al.*, 2008; Ren *et al.*, 2009; Yamazaki *et al.*, 2008c).

Sensitivity of the *invA*-based LAMP in pure culture was 1.3 - 13 CFU per reaction, up to 100-fold more sensitive than the *invA*-PCR. This result was similar to many previous LAMP studies, in which detection limits of LAMP assays in pure culture were about several bacterial cells and at least10-fold more sensitive than the corresponding PCR assays (Okamura *et al.*, 2009; Yamazaki *et al.*, 2008a; Yamazaki *et al.*, 2008b; Yamazaki *et al.*, 2009). In addition, there were multiple LAMP assays developed for *Salmonella* detection targeting different serogroups or genes, all of which reported the detection limits of $10^1$ - $10^0$ CFU per reaction (Hara-Kudo *et al.*,

2005; Li *et al.*, 2009; Okamura *et al.*, 2008; Okamura *et al.*, 2009). Among them, the study by Hara-Kudo et al. (Hara-Kudo *et al.*, 2005) also developed LAMP assay based on the *Salmonella invA* gene and reported the similar sensitivity of approximately 2.2 CFU/test tube.

Few studies on the quantitative capability of LAMP assay have been reported. Stable quantitative capability of LAMP assay for monitoring ammonia-oxidizing bacteria was reported to be between $10^{10}$ to $10^{4}$ DNA copies (Aoi *et al.*, 2006). Another study by Chen et al. (Chen & Ge, 2010) also demonstrated the strong linear correlation ($r^2$ = 0.99) between the real-time LAMP signals and the bacterial cell numbers for *Vibrio parahaemolyticus* detection in spiked oysters. In this study, the quantitative capability of *invA*-based LAMP assay in pure was found to have an $r^2$ of 0.983 for cell levels between $10^{5}$ and $10^{2}$ CFU per reaction, illustrating an excellent quantitative capability of the *invA*-based LAMP assay in pure culture.

## 2) PMA-LAMP assay for live detection

To date, there has been no published study on the application of LAMP in combination with chemical agents such as EMA and PMA for detecting live bacteria. In this study, we incorporated PMA as a dead-cell DNA-eliminating agent into the *invA*-based LAMP assay to exclude dead *Salmonella* detection. PMA was chosen rather than EMA as previous studies indicated the better selectivity for dead cells and free of toxicity to live cells of PMA (Nocker *et al.*, 2006; Pan & Breidt, 2007).

The developed PMA-LAMP could avoid detecting dead *Salmonella* cells up to $7.5 \times 10^{5}$ CFU per reaction while PMA-PCR in comparison could avoid detecting dead *Salmonella* cells more than $7.5 \times 10^{6}$ CFU per reaction. The less potential of PMA-PCR to detect dead cell was likely due to the lower sensitivity of PCR compared with that of the LAMP assay. However, when compared with EMA-qPCR and PMA-qPCR assays in previous studies, it was found that

qPCR consistently gave late signals for samples containing only dead cells due to its high sensitivity, implying the great potential to get late false positive results for live bacterial detection (Kramer *et al.*, 2009; Nocker *et al.*, 2007; Nocker *et al.*, 2009; Varma *et al.*, 2009). Therefore, PMA-LAMP assay could serve as a better combination, offering high sensitivity together with less false positive potential for live *Salmonella* detection. Additionally, to improve the dead cell exclusivity of the PMA-LAMP assay, future work on the optimization of PMA treatment parameters, including PMA final concentration, PMA incubation time and light exposure time, etc., might be needed to circumvent this issue.

Sensitivity of PMA-LAMP was 3.4 - 34 live *Salmonella* cells, which was comparable to the lower detection limit of 1.3 - 13 *Salmonella* cells in LAMP assay. This indicated that the incorporation of PMA treatment before LAMP assay was not inhibitory to the LAMP assay. The *invA*-based PMA-LAMP was highly sensitive compared with a previous study by Gonzalez-Escalona et al. (Gonzalez-Escalona *et al.*, 2009), who used reserve-transcriptase qPCR assay to target *invA* mRNA of live *Salmonella* and reported the detection limit of *ca.* 120 live *Salmonella* cells at exponential growth stage. Besides, the addition of $7.5 \times 10^3$ CFU per reaction of dead *Salmonella* cells as background in each dilution of live *Salmonella* cells did not interfere with live cell detection as at lower live cell level, i.e., $3.4 \times 10^{-1}$ CFU per reaction, was not detected, illustrating the successful dead cell removal of PMA treatment. Furthermore, the PMA-LAMP assay had an $r^2$ of 0.970 for live *Salmonella* between $10^5$ and $10^2$ cells, showing comparable quantitative capability of the PMA-LAMP in detecting live *Salmonella* in pure culture.

In summary, the *invA*-based PMA-LAMP assay developed in our assay was rapid, sensitive, and quite quantitative for live *Salmonella* detection in pure culture.

## 3) PMA-LAMP assay for live detection in produce

In spiked produce, the potential of PMA-LAMP to get false positive results was similar to that in pure culture. The PMA-LAMP could avoid detecting dead *Salmonella* cells up to 3.75 × $10^8$ CFU/g, greatly reducing the false positive results for live *Salmonella* detection in produce samples.

Without enrichment, the *invA*-based PMA-LAMP had a detection limit of 5.5 × $10^3$ CFU/g for spiked cantaloupe testing and 5.5 × $10^4$ CFU/g for spiked spinach and tomato testing, up to 100-fold more sensitive than that of PMA-PCR. In addition, the time to positive results in produce testing was not or slightly delayed. These illustrated that LAMP was less affected by the inhibitory substances in produce samples than PCR. Higher tolerance of LAMP assay to biological substances such as urine, plasma, aqueous humor and vitreous than PCR assay has also been reported by Kaneko et al. (Kaneko *et al.*, 2007). On the other hand, Gonzalez-Escalona et al. (Gonzalez-Escalona *et al.*, 2009) also demonstrated the ability of *invA*-based qRT-PCR to detect 2 live *Salmonella* cells per 25 g of bagged spinach. However, this method required 24 h of pre-enrichment and lacked the dead *Salmonella* inoculation as background to interpret the potential of false positive detection. To further improve the PMA-LAMP sensitivity for detecting live *Salmonella* cells in produce, in future study we might need to introduce a short enrichment step to the spiked homogenates, i.e., 2 - 4 hours of enrichment in BPW at 37$^o$C. Besides, better sensitivity of PMA-LAMP for spiked cantaloupe samples was noticed. We speculate that one phenomenon occurred during produce homogenization might account for this result: cantaloupe samples were well blended into small particles, which were easily pelleted and removed after centrifugation; however, the tomato and spinach particles remained comparatively big after blending due to the intrinsic tissue structure and shape, making it hard to be removed by

centrifugation. Therefore, more remaining food debris of tomato and spinach gave greater inhibition during LAMP detection.

The strong linear correlation ($r^2$ = 0.993 - 0.949) between the numbers of live *Salmonella* cells ranging from $10^5$ to $10^2$ CFU per reaction in PMA-LAMP assay and the corresponding *Tt* values suggested the great quantitative capability of the *invA*-based PMA-LAMP. Again, it was found that PMA-LAMP applied better for cantaloupe analysis, while showed a lower $r^2$ of 0.949 for tomato testing. Another factor other than homogenization step that might affect the tomato testing was that, the average pH of tomato samples was 4.31, much lower than that of cantaloupe (6.25) and spinach (6.27).

In this study, we mainly targeted at the application of the *invA*-based PMA-LAMP assay in produce samples. However, in future study we will further consolidate the robustness of this developed assay by evaluating its application to a variety of foods, including poultry, seafoods, beef, eggs, etc. For that purpose, an effective sample preparation method that applies to all types of foods should be developed. Additionally, we speculate that one effect of various food matrices on PMA-LAMP assay might be the pH, which can be overcome by adjusting the pH to 8.0 to facilitate the DNA amplification during LAMP assay.

To sum up the comparison between the PMA-PCR and PMA-LAMP assays in our study, the supreme advantages of the PMA-LAMP assay were well demonstrated in terms of sensitivity, quantitative capability, rapidity, simplicity, and cost efficiency. Firstly, the PMA-LAMP assay was 10 to 100-fold more sensitive than PMA-PCR in both pure culture and produce samples. Secondly, PMA-LAMP showed an excellent quantitative capability while PMA-PCR lacked the quantitative capability due to the nature of PCR assay. Besides, the total assay time for PMA-LAMP was 3 hours, about 2 hours shorter than PMA-PCR. Moreover, the PMA-LAMP assay

was technically simple as well as cost efficient as it eliminated the gel electrophoresis and didn't require an expensive thermal cycler.

In conclusion, the *invA*-based PMA-LAMP assay developed in this study could successfully detect live *Salmonella* by dead DNA removal, and its application for live *Salmonella* detection in produce yielded highly sensitive results with strong quantitative capability.

# Chapter 6 - Summary and Conclusion

This study aimed to develop and optimize an *invA*-based PMA-LAMP assay to detect live *Salmonella* and evaluate the assay in terms of false positive exclusivity, live detection sensitivity, and quantitative capability in live *Salmonella* detection in produce samples. To our knowledge, this is the first study that combined these two novel technologies for live bacterial detection. Results of our study demonstrated that PMA, when incorporated into LAMP assay, would efficiently eliminate dead *Salmonella* detection in both pure culture and produce samples up to $7.5 \times 10^5$ CFU per reaction and $3.75 \times 10^8$ CFU/g, respectively. And in the presence of $7.5 \times 10^3$ CFU per reaction or $3.75 \times 10^6$ CFU/g dead *Salmonella,* the PMA-LAMP assay could detect down to 3.4 - 34 live *Salmonella* in pure culture or $5.5 \times 10^3$ - $5.5\times 10^4$ CFU/g in produce samples with $r^2$ values ranging between 0.99 - 0.949. The total assay time for the developed PMA-LAMP assay for detecting live *Salmonella* in produce was 3 hours. Therefore, the developed *invA*-based PMA-LAMP assay gave high false positive exclusivity, great sensitivity and excellent quantitative capability in detecting live *Salmonella* in produce samples. When compared with PMA-PCR, the PMA-LAMP assay was 10 to 100-fold more sensitive with great quantitative capability and much shorter assay time. Additionally, PMA-LAMP assay was technically simpler and more cost-efficient than PMA-PCR.

The developed PMA-LAMP assay was an effective safety control tool that would bring significant benefits to the produce producers, processors, retailers, and consumers by potentially reducing the number of *Salmonella*-linked illnesses and deaths associated with the consumption of fresh produce.

In future study, research on the optimization of the PMA treatment, incorporation of a short enrichment step into the spiked produce, development of an effective sample preparation

method for a variety of foods and so on will be performed to further improve the PMA-LAMP assay in terms of dead cell exclusivity, live cell sensitivity and assay robustness.

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# Vita

Siyi Chen was born in 1985 in Guangdong province, China. She spent her happy childhood in her hometown village which allowed her to stay close to nature and explore her interests in nature and science. Later, she was admitted to one of the best middle schools in Guangdong - Jinshan Middle School. As the school was distanced from home, she started to cultivate in herself the ability to live independently. In 2004, she entered one of the top ten universities in China, Shanghai Jiao Tong University, majoring in food science and engineering and started to build up strong and solid knowledge in science and engineering. She received her Bachelor of Science degree in 2008. After completing her undergraduate study, she came to the United States and continued her pursuit of knowledge in the Department of Food Science at Louisiana State University. At the same time, she worked as a graduate research assistant under the supervision of Dr. Beilei Ge in the area of food microbiology. Currently as a master's degree candidate, she is anticipated to graduate in summer 2010. Upon her completion of her master's degree, she will hunt for jobs in Singapore.