การพัฒนาจีโนเซ็นเซอร์บนพื้นฐานของการเพิ่มปริมาณดีเอ็นเอที่ขึ้นอยู่กับเฮลิเคสและการตรวจการเรืองแสงเพื่อการตรวจหา *Listeria monocytogenes* ในอาหารทะเล

นางสาวกัลย์กนิต พิสมยรมย์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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DEVELOPMENT OF GENOSENSOR BASED ON HELICASE-DEPENDENT DNA AMPLIFICATION AND FLUORESCENCE ASSAY FOR DETECTION OF *Listeria monocytogenes* IN SEAFOOD

Miss Kankanit Pisamayarom

A Dissertation Submitted in Partial Fulfillment of the Requirements
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Thesis Title	DEVELOPMENT OF GENOSENSOR BASED ON HELICASE-DEPENDENT DNA AMPLIFICATION AND FLUORESCENCE ASSAY FOR DETECTION OF *Listeria monocytogenes* IN SEAFOOD
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Field of Study	Biotechnology
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กัลย์กนิต พิสมยรมย์ : การพัฒนาจีโนเซ็นเซอร์บนพื้นฐานของการเพิ่มปริมาณดีเอ็นเอที่ขึ้นอยู่กับเฮลิเคสและการตรวจการเรืองแสงเพื่อการตรวจหา *Listeria monocytogenes* ในอาหารทะเล (DEVELOPMENT OF GENOSENSOR BASED ON HELICASE-DEPENDENT DNA AMPLIFICATION AND FLUORESCENCE ASSAY FOR DETECTION OF *Listeria monocytogenes* IN SEAFOOD) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. ดร.ปิยะศักดิ์ ชอุ่มพฤกษ์, 98 หน้า.

โรคลิสเทอริโอซิส (Listeriosis) เป็นโรคที่เกิดจากการบริโภคอาหารที่มีการปนเปื้อน *Listeria monocytogenes* ซึ่งเป็นแบคทีเรียที่มีความรุนแรงของโรคสูงโดยเฉพาะในกลุ่มมารดาที่ตั้งครรภ์ ทารก ผู้สูงอายุ หรือกลุ่มผู้ป่วยที่มีความบกพร่องทางภูมิคุ้มกัน อีกทั้งก่อให้เกิดปัญหาในด้านความปลอดภัยในการบริโภคอาหาร ในหลายประเทศมีกฎหมายที่นำมาควบคุมการเกิดโรคลิสเทอริโอซิส เช่น สหรัฐอเมริกากำหนดให้ไม่มีการปนเปื้อนในอาหารพร้อมบริโภค (RTE) สหภาพยุโรปมีการตั้งค่ายอมรับการปนเปื้อนในระดับต่ำ ด้วยเหตุนี้ทำให้มีความต้องการวิธีการตรวจสอบที่รวดเร็ว การศึกษาในครั้งนี้ได้คิดค้นการตรวจสอบยีน hly ของ *L. monocytogenes* โดยอาศัยการทำงานร่วมกันของการเพิ่มปริมาณดีเอ็นเอที่ขึ้นอยู่กับเฮลิเคส และการตรวจสัญญานดีเอ็นเอด้วยปฏิกิริยาการเรืองแสงและการจับของดีเอ็นเออานาล็อกโพรบในอาหารทะเล ตัวอย่างแบคทีเรียจะถูกนำมาเลี้ยงด้วยเทคนิคการนำก้อนสำลีเช็ดบริเวณผิวของตัวอย่างและนำไปเลี้ยงในอาหารเหลว terrific broth จากนั้นทำการเพิ่มปริมาณยีนเป้าหมายด้วยวิธีการเพิ่มปริมาณดีเอ็นเอที่ขึ้นอยู่กับเฮลิเคสที่อุณหภูมิ 65 องศาเซลเซียส ตรวจผลิตภัณฑ์ดีเอ็นเอที่ได้ด้วยปฏิกิริยาการเรืองแสงและการไฮบริไดซ์ดีเอ็นเออานาล็อกโพรบ ตัวอย่างที่เป็นบวกจะเกิดปฏิกิริการเรืองแสงภายใต้แสงยูวี ตัวอย่างที่ให้ผลลบจะไม่ปรากฏการเปลี่ยนแปลงของปฏิกิริยาการเรืองแสง ปริมาณดีเอ็นเอต่ำสุดที่สามารถตรวจสอบได้ที่ 100 ก็อปปี้ของดีเอ็นเอของ *L. monocytogenes* ต่อปริมาณตัวอย่างอาหารทะเล 50 กรัม การตรวจสอบในครั้งนี้เป็นครั้งแรกที่มีการนำการเพิ่มปริมาณดีเอ็นเอที่ขึ้นอยู่กับเฮลิเคส, ปฏิกิริยาเรืองแสงของ molecular beacon probe และการไฮบริไดเซชั่นระหว่างดีเอ็นเอและดีเอ็นเออานาล็อกโพรบในการตรวจสอบยีน hly ของ *L. monocytogenes* นอกจากนี้ยังสามารถใช้ตรวจสอบตำแหน่งนิวคลีโอไทด์ที่ผิดปกติบนยีนเพื่อตรวจจำแนกชนิดที่มีการกลายพันธุ์และชนิดที่ปกติ การศึกษาพบว่าเทคนิคที่ใช้ในการตรวจสอบมีความรวดเร็ว ง่าย และสามารถใช้ตรวจสอบการปนเปื้อนในอาหารทะเลในพื้นที่ได้ โดยไม่ต้องพึ่งพาห้องปฏิบัติการ

สาขาวิชา เทคโนโลยีชีวภาพ ลายมือชื่อนิสิต .....

ปีการศึกษา 2558 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

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KEYWORDS: HELICASE DEPENDENT AMPLIFICATION , HDA, HLY GENE, LISTERIA MONOCYTOGENS, FLUORESCENCE BINDER, CLAMP LOCK

KANKANIT PISAMAYAROM: DEVELOPMENT OF GENOSENSOR BASED ON HELICASE-DEPENDENT DNA AMPLIFICATION AND FLUORESCENCE ASSAY FOR DETECTION OF *Listeria monocytogenes* IN SEAFOOD. ADVISOR: ASST. PROF. PIYASAK CHAUMPLUK, Ph.D., 98 pp.

Listeriosis, a foodborne disease caused by ingesting food contaminated with *Listeria monocytogenes.* This serious illness posed hazard especially to pregnant women, newborn, elderly as well as people with weakened immune systems (immunocompromised). The introduced legislation to control the incidence of listeriosis in several countries including a zero-tolerance in ready-to-eat (RTE) foods in the United Stated and a low acceptable level setting in the EU made a requirement for a rapid monitoring method. In this study, a rapid assay based on a combination of both helicase dependent amplification (HDA) and DNA signal detection via fluorescence DNA-DNA hybridization and analog probe as clamp lock was established to detect *hly* gene of *Listeria monocytogens* in seafood. Assay processes did include a short period of enrichment in terrific broth using cotton ball swabbing technique on seafood surface. HDA amplification of *hly* gene at 65°C allowed DNA signals to be increased, whereas the rendered DNA products were detected via fluorescence visualization based on FRET and analog DNA probe via DNA hybridization. The positive specimens induced fluorescence signals from a reporter at 5’ (FAM) while the negative specimens did not due to a resonance transfer of energy from 5’ (FAM) to 3’ (BHQ-1). The method had a detection limit at 100 copies or equivalence to 100 CFU of *L. monocytogenes* per 50 g of sample. This method was the first report of the application of molecular beacon and analog probe hybridization with Helicase Dependent Amplification to detection of *hly* gene. Furthermore these techniques could be used to discriminate the single nucleotide polymorphic mutant of *hly* determinant in food. This developed method is rapid, simple, and relied less on laboratory facilities which is suitable for monitoring of safety in frozen seafood in the field.

Field of Study: Biotechnology Student's Signature .....

Academic Year: 2015 Advisor's Signature .....

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CHAPTER I

INTRODUCTION

Foodborne Disease is a disease caused by consumption of the food or beverages contaminated with pathogen and/or their toxins such as *Listeria monocytogenes* and *Salmonella enterica* which cause a severe infectious disease (Kim et al., 1995). Most foodborne diseases are the result of microorganisms. The majority of pathogens involved in the infection are bacteria, viruses and parasites (Table 1.1).

Table 1.1 List of foodborne pathogens including bacteria, virus and human parasites.

Microorganisms	Name
Bacteria	*Aeromonas hydrophila*
	Bacillus cereus
	Campylobacter jejuni
	Clostridium botulinum
	Clostridium perfringens
	Escherichinia coli O157:H7
	Listeria monocytogenes
	Salmonella enteritidis
	Shigella sp.
	Staphylococcus aureus
	Vibrio cholerae,
	Vibrio parahaemolyticus
	Vibrio vulnificus
	Yersinia enterocolitica
Virus	Hepatitis A
Parasites	*Giardia lamblia*
	Trichinella spiralis

There are three major types of foodborne diseases; foodborne intoxication, foodborne toxin-mediated infection and foodborne infection.

Foodborne intoxication is caused by consuming food containing toxins formed by microorganism as a result of the bacterial growth in the food. The pathogens related with this intoxication are *Staphylococcus aureus, Clostridium botulinum, C. perfringens*

and *Bacillus cereus*. The symptoms are rapidly occurred after food consumption with less than 7 hours of incubation period.

Foodborne toxin-mediated infection is caused by consuming food contaminated with toxin producing pathogenic bacteria. The causal bacteria strain having foodborne toxin-mediated pathway are *Shigella* spp. and *Escherichia coli* O157:H7.

Foodborne infection is caused by the ingestion of food containing live bacteria grown and established themselves in the human intestinal tract. The examples of these foodborne bacteria are *Salmonella* spp., *Campylobacter jejuni, Vibrio parahaemolyticus, V. vulnificus*, *Yersinia enterocolitica* and *Listeria monocytogenes*. The incubation period of these types is longer (more than 7 hours). Some species of these groups are highly pathogenic and infection through the gastrointestinal tract to blood system may cause in a septicemia. Nausea, vomiting, abdominal cramps and diarrhea are often common symptoms. Long term studies suggested that up to 10% of human gastrointestinal tracts may be caused by *L. monocytogenes* (Ramaswamy et al., 2007). Nevertheless, clinical diseases due to *L. monocytogenes* are more frequently recognized.

The genus of *Listeria* consists of eight species including *L. monocytogenes, L. innocua, L. welshimeri, L. seeligeri, L. ivanovii, L. grayi, L. marthii* (Graves et al., 2010) and *L. rocourtiae* (Leclercq et al., 2010). *L. monocytogenes* is both a human and animal pathogen, while *L. ivanovii* is pathogenic for animals, mainly cattle and sheep. Infrequently occasions, *L. ivanovii* and *L. seeligeri* were founded to be connected with human infections (Rocourt and Cossart, 1997; Schmid et al., 2005). *L. ivanovii* mighty causes infections in ruminants, but has been associated with rare infections in humans (Cummins et al., 1994; Lessing et al., 1994).

Although the several strains in the genus were involved, the listeriosis was caused by a *L. monocytogenes* bacterial infection. Once ingested, bacteria might damage the central nervous system including meningitis, meningoencephalitis (meninges and encephalitis), brain abscess, cerebritis and bacteremia. Pregnant women, newborn babies, people with weakened immune systems (immunocompromised people) and the elderly were among much higher risk of bacterial infection, specifically.

Symptoms of listerioisis are high fever, muscle aches, stiff neck, confusion, weakness, vomiting, nausea, diarrhea or gastrointestinal symptoms. The incubation period is around 3-70 days. And, almost everyone who has *L. monocytogenes* spreaded to the gastrointestinal tract can show different symptoms. These include headache, stiff neck, confusion, loss of balance, and convulsions in addition to fever and muscle aches. Furthermore, listeriosis in pregnant women and newborns lead also to septicemia, abortions, stillbirth, meningitis and meningoencephalitis (Low and Donachie, 1997).

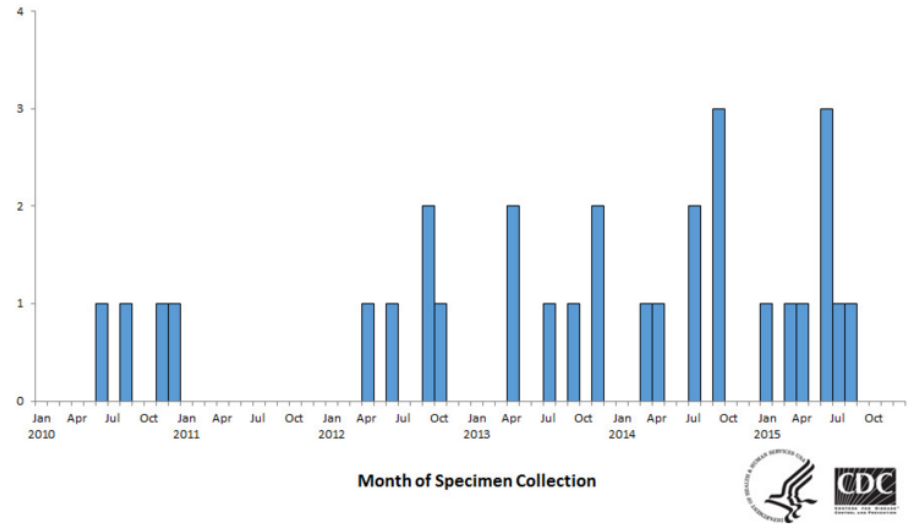
L. monocytogenes is a gram-positive intracellular bacterium, rod shape with rounded ends, distributed in the environments (Figure 1.1). This bacteria is wildly distributed in the environment (Farber and Peterkin, 1991), and is commonly isolated from soil and silage and foods. It can grow under a wide range of pH, osmolality and temperature conditions, which underline many foods manufacturing process. Several food matrices including lettuce, carrots, commercially prepared coleslaw, pasteurized 2% milk (contaminated after pasteurization), pâté, pork tongue in jelly, soft cheese made from raw milk, chocolate milk hotdogs and seafood products were reported to have incidences of the bacteria.

L. monocytogenes has emerged as a significant foodborne pathogen in recent decades, along with a trend toward increasing consumption of ready-to-eat (RTE), convenience food products such as deli meat, milk, cheese and seafood via fish and shrimp.

In raw foods, raw meats and vegetables, for example, have been found contamination with the bacteria. In processed foods, such as soft cheeses and cold cuts, unpasteurized milk or foods which made from unpasteurized milk may contain the bacterium. As *L. monocytogenes* infection often produces non-specific initial manifestation, without prompt antibiotic therapy, it can have severe clinical consequences.

Figure 1.1 Rod shape with gram stain of *L. monocytogenes* (Weinstein, 2015)

In the United States and Canada, the outbreaks of listeriosis were reported during 1979-1999. In 1998, the outbreak of listeria infections in hot dogs and deli meats killed at least 21 people. To this the International Commission on Microbiology specification on Foods (ICMSF) had announced the threshold for bacterial contamination. Based on report, food samples should not contain more than 100 colony forming unit (CFU) per gram of *L. monocytogenes* (Nogva et al., 2000). Since then, *L. monocytogenes* has been recognized as an important public health problem with listeiosis in the United States. The Centers for Disease Control and Prevention (CDC) issued several reports concerning Listeria outbreak. In 2011, at least 33 people (Anonymous, 2012) are dead amid 72 sickened in 18 states in an outbreak of listeria food poisoning, tied to contaminated cantaloupes in eight states, including four in New Mexico, two in Colorado, two in Texas and one each in Kansas, Maryland, Missouri, Nebraska and Oklahoma. In July 2002, sliced turkey and deli meat contaminated with listeria led to 54 illnesses and eight deaths, including three stillbirths from Pilgrim's Pride Corp., Franconia, Pennsylvania (Aleccia, 2011). In 2015, There was a reports of ten patients from four states; Arizona (1), Kansas (5), Oklahoma (1), and Texas (3), infected with *L. monocytogenes*. These included the Ice cream products from Blue Bell Creameries from January 2010 till January 2015. (Administration, 2015A). People were infected with the outbreak strains of *L. monocytogenes*, during month of specimen collection. Though this outbreak investigation was over, however, there was a continuing growth of incidence of *L. monocytogenes*. Data on this might be enlarged because the recalled cheeses may still be in homes, restaurants, or retail locations. And, consumers, restaurants, and retailers were unaware of the recall, the eat, serve, or sell them (Anonymous, 2015).



*n=30 for whom information was reported as of October 20, 2015

Figure 1.2 People infected with the outbreak strains of *L. monocytogenes*, by month of specimen collection

Contamination of Listeria in seafood was also important. In seafood, Listeria contamination cases divided the incidences to 3 main seafood products; 1) fresh seafood including raw fish, raw mollusk and raw crustacean, 2) ready to eat (RTE) seafood including cold smoked fish, hot smoked fish and 3) gravid and semi process seafood. The predominance of the foodborne pathogen *L. monocytogenes* in raw fish is quite low level, ranging from 0 to 1% (Autio et al., 1999) to 10% (Jemmi and Keusch, 1994). An overall prevalence 3% of *L. monocytogenes* was observed in European fish (Davies et al., 2001). *L. monocytogenes* was isolated from 8% fish samples (Swetha et al., 2012). Normally Listeria contaminated problem occurs after or during processing, handling and storing. Furthermore, seafood contamination with *L. monocytogenes* had also led to product recalls (Wan Norhana et al., 2010; Zhu et al., 2005). The Food Standards Agency (FSA) reported that the Fine Seafood Company recalled all date codes of its whiskey-cured, oak-smoked salmon line via a total of 444 individual packs and five products of its kind via 150 g and 240 g packs of plain and peppered smoked mackerel after high levels of *L. monocytogenes* contamination was detected (White, 2014).

RTE food products are ready to eat without cooking including seafood salads, deli salads, soft cheeses, and pre-packed fresh vegetables and fruits. Example of RTE seafood which contaminated with foodborne pathogen is shrimp, smoked salmon,

smoked New Zealand green-lipped mussel. In Thailand, seafood products are important. Country's export statistics showed the value of export of seafood products more than 4838.28 million US dollars (169339.8 million Baht) in 2013 and 1161.09 million US dollars (40638.15 million Baht) in 2014 (January-March) with several export products such as were among the world top list (Figure 1.3) . Export trend in 2012 also increased over the same period a year ago.

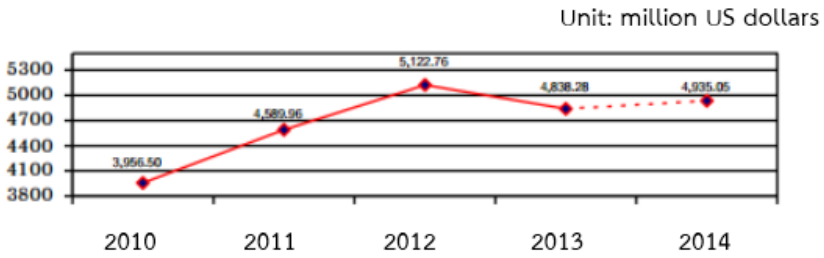


Figure 1.3 The value of export of seafood products

While the importing countries are stringent on the import product's standards and its food safety measure, e.g. Food Safety Modernization Act (FSMA) in USA, the European Food Safety Authority (EFSA) in Europe, and strict regulations concerning the safety and quality control and assurances were implemented, Thai industries and government needed to well prepare accordingly.

Among the reports concerning common contaminating agents, *L. monocytogenes* was found as a major pathogen associated with fish and fishery product. Monitoring of foodborne pathogen contamination in seafood had been established for food safety and creating for standard product (Administration, 2015B).

So far, conventional methods for foodborne pathogenic microbial detection were based on standard culture method and biochemical tests. The detail included an enrichment of bacteria in culture media, a biological test for their metabolisms to confirm the involved species in a number of isolated colonies (Ryser and Marth, 1999) in term of standard plate count. The use of the selective and differential media for bacterial isolation which contain the growth factor for bacteria was very reliable. The detection method as well as biochemical systems for identification of specific food isolates had been proceed routinely.

Although the standard culture method did simplify the diagnosis of the bacterial infection (Chernesky et al., 1994; Nelson and Helfand, 2001), some bacteria were still difficult to be isolated, some grew slowly in the culture due to stringent growth requirements, or some might not grow because of prior empirical treatment of patients with antimicrobial agents (Cosgrove et al., 2005).

For the *L. monocytogenes* detection, the demerits of the standard culture method were its laborious and time-consuming, requiring 10 days to identity and nonselective pre-enrichment, selective enrichment, plating on differential agar media, and its complexity on presumptive biochemical identification and serological confirmation. Immunoassays and nucleic acid based assays such as polymerase chain reaction (PCR) method, have been elaborated to accelerate *L. monocytogenes* detection. Although PCR-based methods are the most widely used and several PCR systems have been demonstrated (Bessesen et al., 1990; Bickley et al., 1996; Border et al., 1990). The PCR method had a limitation due to its need a thermocyclic temperature to separate double-stranded DNA and amplify nucleic acids. It is, therefore not suitable for field monitoring application or use as a point-of-care test. The time consuming and laborious are also major concerns. Moreover, the reaction of PCR can be inhibited by the presences of compound from food matric.

Biosensor was developed to fulfill the addressed problems by improving the relevance and extent of the tests. The inherent specificity, selectivity, and adaptability of the technique make it ideal candidate for food industries' use. In principle, biosensor is an analytical device or tool that combines a biological component with a physicochemical detector (Turner et al., 1987). It combines the bio-recognition component and transducer interplay.

Specific recognition for targeting nucleic acid can be obtained through specific hybridization between target nucleic acid sequence and synthetic oligonucleotide. So far target sequence of gene was distinguished either by selective capturing, through nucleic acid hybridization with and selectively duplicating (amplifying) using oligonucleotide primers. Since DNA sensing in real specimen in this case involves with extremely small amount of target DNA in the total background with corresponded to few hybridization event, thus, there is no choice but select a recognition platform based on a selectively amplifying system.

Isothermal amplification methods have recently been developed as an alternative to PCR method (Andras et al., 2001). The isothermal amplification methods which do not require heating for separating of double strands DNA and synthesized DNA product was focused as the alternative. This provides merit suit for onsite monitoring application. So far, several platforms such as nucleic acid sequence-based amplification (NASBA), strand displacement amplification (SDA), rolling circle amplification (RCA), loop-mediated isothermal amplification of DNA (Schmid et al., 2005) and helicase-dependent amplification (HDA) are addressed (Notomi et al., 2000; Vincent et al., 2004; Walker et al., 1992; Walker et al., 1996).

NASBA utilizes the function of an enzyme DNA dependent RNA polymerase (T7 RNA polymerase) to transcribe RNA from a T7 promoter previously incorporated at the 5' of primer region, and a reverse transcriptase, to produce DNA from the RNA templates. At the beginning, the first primer (with T7 promoter at 5' plus specific primer sequence at 3') hybridizes to the target RNA. Reverse transcriptase generates cDNA by extension from the 3'end of the promoter primer. T7 RNA polymerase recognizes the promoter sequence in the cDNA and initiates transcription. Next, the second primer binds to the RNA copy and reinitiates synthesis of double strand DNA having a complete of T7 promoter structure by reverse transcriptase. All steps are repeated. Each of the newly synthesized RNA amplicons reenters the amplification process and serves as a template for a new round of replication.

SDA combines the ability of a *Hinc*II restriction endonuclease to nick the unmodified strand of its target DNA and the action of an *E. coli* exo$^-$ Klenow (exonuclease-deficient DNA polymerase) to extend the 3' end at the nick and displace the downstream DNA strand. The product of displaced strand then serves as a template for an antisense reaction and vice versa, resulting in amplification of the target DNA exponentially. Reaction requires four specific primers (B1, B2, S1, and S2). Primers S1 and S2 have *Hinc*II recognition sequences (5'-GTTGAC-3') located 5' to the target sequences and target binding region at 3' end. Primer B1 and B2 are only target specific primers located at 5' to S1 and S2.

RCA is also another isothermal technique. It generates multiple copies of sequence through the rolling circle DNA replication. The RCA reaction involves infinite

rounds of isothermal enzymatic synthesis of DNA using DNA polymerase from bacteriophage ø29 (Fire and Xu, 1995).

The extension of hybridized primer on circle to replicate its sequence again and again by moving around the circular DNA results in a mass production of high molecular weight DNA products of wide length distribution, seen as smear band upon gel electrophoresis separation. Moreover, further adaptation of RCA by combining the unique properties of ø29 DNA polymerase and random primers renders a new technique called multiply-primed rolling circle amplification (multiply-primed RCA) which can help to achieve a 10,000-fold amplification. The process can be applied for amplification of circular DNA directly from cells or plaques, generating, or cloning (Dean et al., 2001).

Another isothermal DNA amplification techniques is called loop mediated isothermal DNA amplification. It relies on set of primers, 2 to 3 pairs, recognized to 6 areas within target gene, and a strand displacement thermophillic *Bst* DNA polymerase. Position of each primer related to the target template and reaction mechanism are as illustrated in Figure 1.4. The reaction starts from binding of F2 and B2 from inner primers to target template at condition preferred for single strand DNA binding to the primers, and then further extending their synthesis along each strand of the templates. Once extension proceeded, F3 and B3 primers (the outer primers) perform next initiation of newly strand synthesis via strand displacement activity, allowing self-binding of F1 to its F1c in the displaced strand and B1 to B1c vice versa. These create dumbbell shape structures which are key structures for a mass accelerated amplification of target DNA products. By repeatedly performing DNA synthesis from inner primer (F2 and B2) and outer primer (F3 and B3) at the dumbbell shape, then a large amount of complicated stem loop DNA products in zigzag form are obtained.

In nucleic acid replication, not only an enzyme DNA dependent DNA polymerase but also various accessory proteins are involved. One of these is DNA helicase enzyme. Helicase is used in living organisms during DNA replication to unwind two complementary DNA strands during template preparation (Kornberg and Baker, 1992). By mimicking nature of the helicase, a special thermophillic helicase is applied to generate single stranded templates prior to primer hybridization and subsequent extension (Vincent et al., 2004).

The technique is called helicase-dependent amplification (HDA). Using thermophillic helicase allows simple DNA amplification to be performed without the use of initial heat denaturation as being required by PCR. The process of HDA, similar to that of PCR, starts from sequence-specific primers bind to target DNA template and then extends using *Bst* DNA polymerases to amplify the target sequence. This process repeats itself so that exponential amplification can be achieved at an isothermal temperature (60-65°C). The process allows multiple cycles of replication to be carried out at a single incubation temperature, completely omitting the need for denaturation of DNA template by thermocycler device. So far several demonstration of HDA including the detection via gel electrophoresis, real-time format, and enzyme-linked immuno sorbent assay can be reviewed. Although HDA system is inefficient at amplifying long target sequences, its simplicity and its true isothermal nature of the HDA platform offer a great opportunity for the development of hand-held DNA device that could be used to do nucleic acid detection at POC or in the field.

Of these, although LAMP offers better and more specific DNA amplification due to its utilize four specific primers that hybridize to six different region of the target DNA sequence (Figure 1.4), providing with greater specificity at isothermal temperature condition, the set of multi primers in LAMP reaction were difficult to design. This difficulty hinders the application of LAMP to be applied in some of genetic situation whose element consensus was limit in both length and similarity.

To this, the HDA, is a candidate choice. Its principle was based on the action of thermophillic helicase enzyme to unwind double-stranded DNA to single-stranded DNA (ssDNA) instead of heating prior to primer hybridization and subsequent extension using DNA polymerase. Mechanism for HDA reaction was shown in figure 1.5. Amplification was started as the following. Double-strand DNA was shown as two lines: the top strand and the bottom strand. Step 1, helicase (black triangle) enzyme separates the double strand DNAs, which were bound by SSB (small DNA binding protein; grey circles). Step 2, two specific primers (lines with arrow heads) hybridized to the target region on the single strand DNA template. Step 3, DNA polymerase (squares with mosaic patterns) extended the primers hybridized on the template DNA. Step 4, Amplified products (double-strand DNA) entered the next round of amplification.

Among them HDA provided much of advantages in term of primer and amplification conditions which were closed to that of PCR, in term of reaction sensitivity, specificity and productivity. Since DNA helicase can unwind the double-stranded DNA without the need for heating, this system provides a very useful tool to amplify DNA under isothermal conditions. HDA provided several advantages over PCR such as its rapidity, its equal sensitivity, specificity and its condition involved with the use of isothermal temperature conditions enable to perform DNA amplification without the need of thermocycler. These advantages were suitable for the point-of-care application in field. Several HDA studies had focused on the DNA signal detection via the analysis of DNA products using agarose gel electrophoresis prior to detect after nucleic acid staining. However, these complicated processes were very laborious and time consuming.

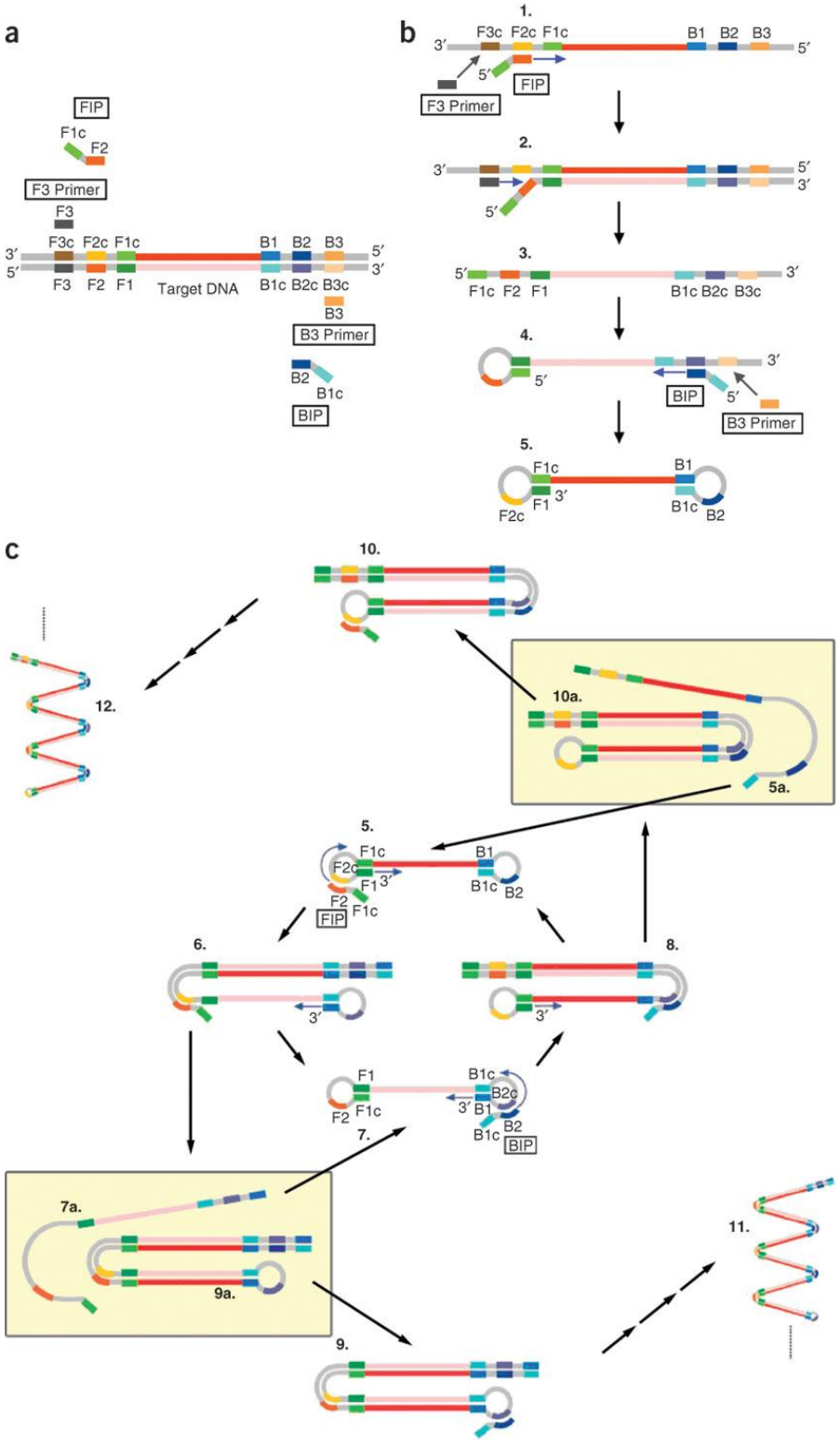


Figure 1.4 Principle of loop-mediated isothermal amplification method (Tomita et al., 2008)

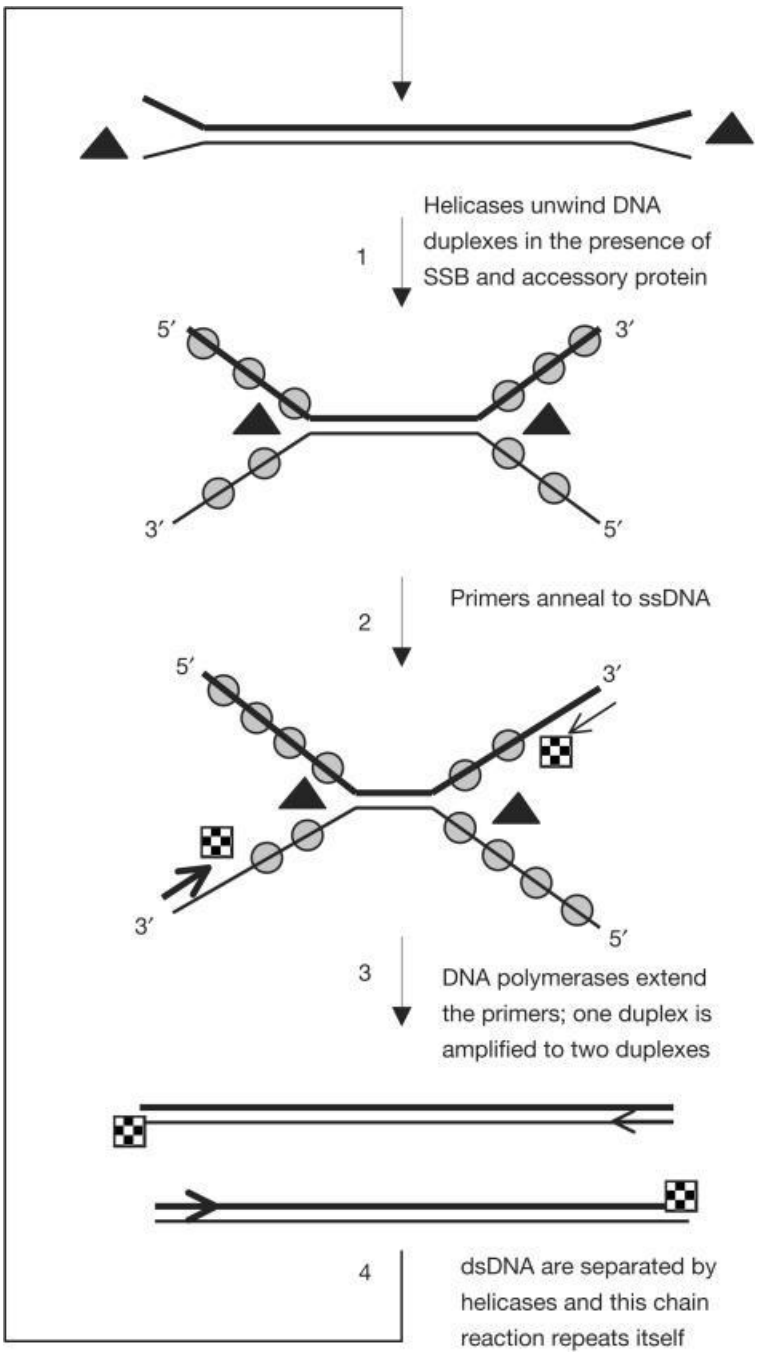


Figure 1.5 Helicase Dependent Amplification (HDA) (Vincent et al., 2004)

Further step of signals detection is also important. In this study, the combination of oligonucleotide probe hybridization along with fluorescent detection method based on FRET technology made the DNA signal detection system was employed.

In practical, the principle of a rapid detection of DNA signal is based on the enrichment of bacteria taken from surface area of seafood and later *Listeria* detection using isothermal specific amplification of the target gene in combination with fluorescence detection via oligonucleotide probe hybridization.

Although DNA amplification had been used for detection of several pathogenic bacteria, it did not provide absolute sequence confirmation result unless one needed to perform further Southern hybridization. The Southern hybridization technique is a sequence-specific detection of nucleic acid. It had been chosen as gold standard (Sambrook et al., 1989). So far, several hybridization platforms had been developed using target probes with either radioactive, or fluorescent, or chemiluminescent, or other types of labeled probes by oligonucleotide or fragment of DNA (Osborn, 2000). None could be performed in a short period of time, and the hybridization results could not be observed with naked eye.

Detection of DNA hybridization at room temperature with high specificity in short period of time could be performed via the application of oligonucleotide. Oligonucleotide probe corresponding to a target gene was synthesized to have, a reporter, 5'Flu-O-group (Nielson and Haaima, 1997). In parallel the Quencher molecule was also synthesized to have 3' BHQ-1 at 3'terminus. The strategy for oligonucleotide quencher and target DNA interaction was depicted in figure 1.6. Positions of both primers and probes were matched to have a structure fit for fluorescence resonance energy transfer (FRET). When this structure was locked, the transfer of energy from reporter to quencher allowed the release of energy not reaching excitation step. However when target DNA was presence, the competitive molecule of quencher did bind to target DNA allowing incomplete transfer of energy and a resulting in an illumination of fluorescence light. This help detection be carried out by visual inspection of fluorescence signals after hybridization with oligonucleotide probe. Observation of fluorescence emission could be done by UV or black light source.

Fluorescence was visualized with naked eye. After UV illumination, no fluorescent signals was detected in non target sample (negative sample, non target DNA or non template control sample on the left) due to no competitive hybridization occurred between quencher and oligonucleotide probe signals, leaving

oligonucleotide quencher complex in quenching mode, however, there was a clear fluorescent signals detectable on target sample on right.

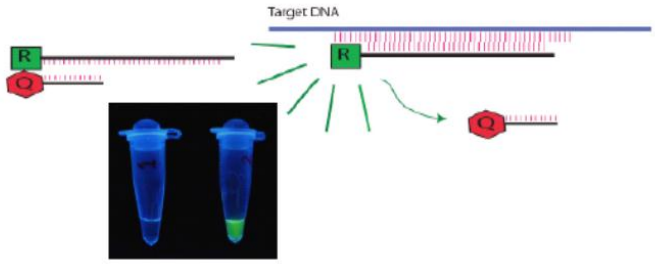


Figure 1.6 R is a reporter fluorophore (Fam) labeled oligonucleotide and Q is a quencher competitive probe. Detection of the hybridization signals were based on fluorescence resonance energy transfer (FRET).

In this study, oligonucleotide probe were designed to match with *hly* gene of *L. monocytogenes* because this candidate was commonly employed in nucleic acid discrimination. The system consists of two components which were fluorescent reported dye and quencher dye for labeling oligonucleotide. The quencher molecule inhibits the natural fluorescence emission of the reporter by fluorescence resonance energy transfer (FRET). The fluorescent reported dye as 6-fluorescein amidite (6-FAM) at 5'-end and the fluorescence quencher as Black Hole Quencher (BHQ) dyes (BHQ-1) at the 3'-end were designed to serve as Molecular beacon (Figure 1.7).

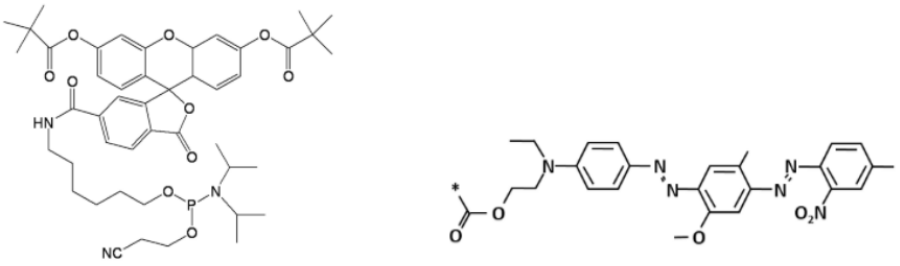


Figure 1.7 Structure of 6-FAM™ Fluorescein and BHQ-1

Molecular beacon structure in form "stem-loop" is consist of the complementary sequences at the 5' and 3' ends, usually four-five bases in length as stem and a specific target region in the center as probe, which forms the loop (Figure

1.8). The probe sequence in the loop portion of the molecule is designed to complement a target DNA sequence.

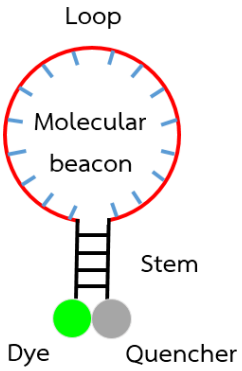


Figure 1.8 Molecular beacon structure (Wile et al., 2014)

This structure brings the 5’ reporter (6-FAM) and 3’ BHQ-1 into close proximity so that fluorescence is quenched. The presence of the “stem-loop” increase the probe’s stringency for the target. Their fluorescence changes with temperature in the presence and in the absence of their targets. At lower temperatures molecular beacons exist in a closed state, the fluorophore and the quencher are held in close proximity to each other by the hairpin stem, and there is no fluorescence. When the target DNA was presence, the binding between loop structure and target labeling the 5’ reporter and 3’ BHQ to be independently separated and no more FRET phenomenon and occurred. This result in term of bright illuminescent of the signal visible with naked eye.

Recently, for DNA signal detection, an option of colorimetric assay method using non-functionalized blue silver nanoplates (AgNPls) was employed. The blue AgNPls with an average bisector length of 180 nm were synthesized by our developed wet chemical technique using hydrogen-peroxide-induced shape conversion of silver nanospheres under an ambient condition (Figure 1.9). DNA signals could be read by mixing the DNA oligoprobe and LAMP products and performing a DNA hybridization assay to the surface of the nanoparticles and adding salt which triggered nanoparticle aggregation.

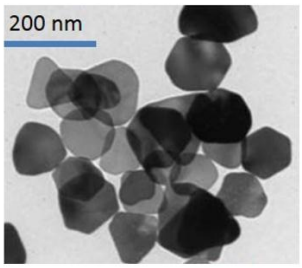


Figure 1.9 TEM image blue silver nanoplates (AgNPls) by hydrogen-peroxide-induced shape conversion of silver nanospheres.

Recently Vilaivan et al (2006) had introduced a novel conformationally rigid pyrrolidinyl PNA (pPNA) which can hybridize to DNA with exceptionally high affinity and specificity. With a new conformationally rigid pPNA, a derivative of oligonucleotide analogues, in which the sugar phosphate backbone had been replaced by a pseudopeptide chain of *N*-aminoethylglycine monomers, the recognition of target DNA via pPNA/DNA hybridization could be carried out with exceptionally high affinity and specificity (Vilaivan and Srisuwannaket, 2006).

Peptide Nucleic Acid (PNA) is an artificially synthesized polymer having nitrogenous base similar to natural DNA or RNA. It was invented by Peter E. Nielsen (Nielson and Haaima, 1997) (Figure 1.9). The backbone of PNA is composed of repeating N-(2-aminoethyl)-glycine units linked by peptide bonds of protein structure. The various purine and pyrimidine bases are linked to the backbone by a methylene bridge (-CH2-) and a carbonyl group (-(C=O)-). PNAs are acted like peptides, with the N-terminus at the first (left) position (Figure 1.10) and the C-terminus at the last (right) position while forming a complementary binding with target DNA sequence following Watson and Click's rule. Due to the backbone of PNA contains no charge of phosphate groups, the binding between PNA/DNA is then stronger than between natural DNA/DNA form due to the lack of electrostatic repulsion (Nielson and Haaima, 1997).

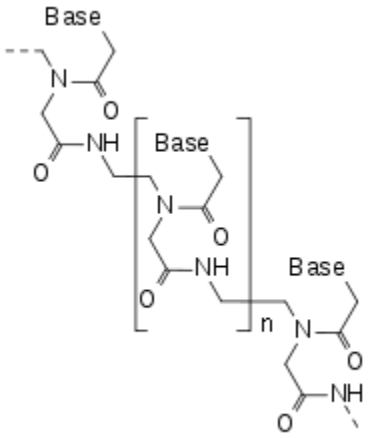


Figure 1.10 structure of Peptide Nucleic Acid (PNA)

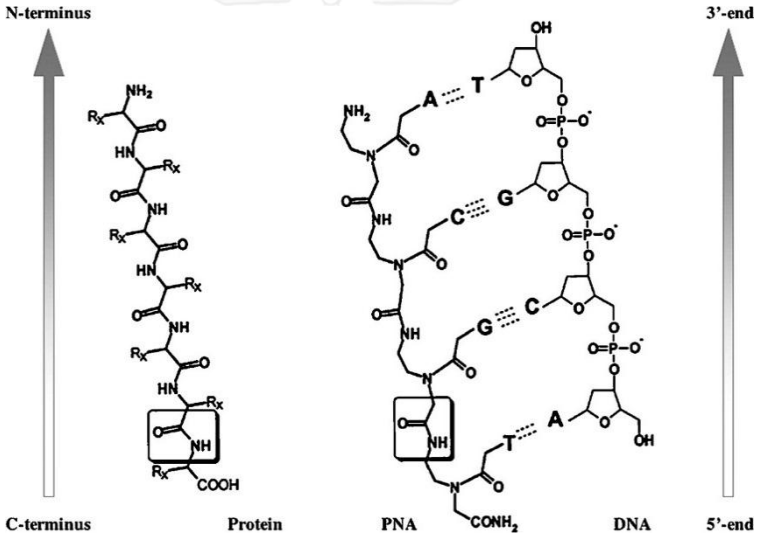


Figure 1.11 Structure of DNA and PNA molecule (Nielsen and Egholm, 1999)

The use of pPNA not only allowed complementary DNAs to be hybridized according to the Watson–Crick hydrogen-bonding rules, it also offered several advantages over a classical DNA hybridization since its neutral-charged backbone increases the binding strength (Nielson and Haaima, 1997). This result allowed a rapid sequence-specific detection in a short period of time at room temperature. Moreover, the pPNA probe usually showed higher selectivity against mismatches compared to DNA probes thus improving discrimination of DNA to a perfect level.

Nucleic acid hybridization can also be operated in different manner using analog DNA as a clamp to block nucleic acid amplification. Because analog DNA holds chemical structure differed from natural DNA and it has stronger affinity than DNA/DNA binding, both provide unique structure resisted to the 5'-3' exonuclease activity of polymerase while kept strong binding to the template. These properties allow nucleic acid amplification to be tampered when analog DNA presented in the amplification reaction. By comparing the capability of amplification especially at common sequence having single nucleotide polymorphic marker amplified with and without clamp, one can differentiate target *L. monocytogenes*'s DNA from the rest directly.

The aim of this study is to develop a novel method for *Listeria monocytogenes* detection by combining Helicase-Dependent Amplification platform with DNA signal detection using fluorescence binder and clamp lock amplification and applied for *L. monocytogenes*. This will help avoiding the previous limitation of the PCR method that depends heavily on lab facility.

CHAPTER II

LITERATURE REVIEW

Listeria monocytogenes is a Gram-positive, rod shape with round end, non-spore forming intracellular bacterium that causes serious invasive disease or listeriosis in humans and animals (Gasanov et al., 2005; Perrin et al., 2003). This bacterium is able to distribute in the diverged environments and has a record of the highest rate of fatality among all foodborne pathogens. Due to its ability to survive at high and low temperature as well as at low pH, it could resist various food processing technologies and grow at storage temperature.

Seafood especially the one with fish and frozen has been one of the list for high risk of *L. monocytogenes* contamination. Major Foodborne diseases listeriosis outbreaks have been reported since 1983 in Europe and North America (Allerberger and Guggenbichler, 1989; Salamina et al., 1996; Tham et al., 2000) mainly due to ready-to-eat foods. Some review was reported that found the proportion of *Listeria* spp and *L. monocytogene* isolation from food in Japan is similar to reported from other countries (Okutani et al., 2004).

Ready-to-eat sea foods are prepared food for easy of consumption that can be sold as hot, ready-to-eat dishes; as room-temperature, shelf-stable products; or as refrigerated or frozen products that require minimal preparation (typically just heating). These ready-to-eat foods have a high risk of causing foodborne illness. Some examples of ready-to-eat foods include canned products, refrigerated smoked seafood products, deli-type salads such as tuna, etc. (Anonymous, 2015).

Seafood products are important export items of Thailand as the one of the world's export of frozen shrimp and the biggest exporter of canned tuna in the world. Trend of export fresh and frozen seafood products in 2012 increased over the same period a year ago. Recent stringent of product standards and food safety measures in several importing countries, eg. Food Safety Modernization Act (FSMA) in USA, the European Food Safety Autherity (EFSA) in Europe (TFFA, 2012) has been addressed. Among foodborne pathogens found in frozen seafood products, *L. monocytogenes* is the most common contaminant. In some countries, food agencies have established a zero-tolerance in ready-to-eat food products such as gravid or cold-smoked fish, as

other countries have established an upper limit of 100 cells *L. monocytogenes* per gram (Jorgensen and Huss, 1998).

The most at risk of infection of *L. monocytogenes* are pregnant women, neonates, newborns, immunocompromized persons and the elderly (Schuchat et al., 1991). The pregnant women infected with *L. monocytogenes* may have spontaneous abortions, stillborn fetuses or newborns with meningitis (Fayol et al., 2009; McClure and Goldenberg, 2009; Rappaport et al., 1960). In the United States, The frequency of *L. monocytogenes* infection is 9.7 cases per million population. Annually, 2500 cases are reported, with higher incidence rates of listeriosis during the summer months (Pappas et al., 2006). Twenty-seven percentages of all case were pregnant women and most occur during the third trimester. Seventy percent of all non-perinatal infections occur in immunocompromised patients. Corticosteroid therapy is the most important predisposing association in patients who are not pregnant. Other risk factors include advanced age and recent chemotherapy. Clinical manifestations of listeriosis range from gastrointestinal disturbances as non-bloody diarrhea, nausea and vomiting, to influenza-like illness with high fever, headache and myalgia, and to serious septicemia, meningitis, encephalitis and death (Begley et al., 2003; Bortolussi, 2008; Kalorey et al., 2008). *L. monocytogenes* infections are not frequent, but with a mean mortality rate of 20-30%, they are among the most deadly foodborne infection in industrial countries (Vázquez-Boland et al., 2001). In the United State, the food-related death of bacterial origin is nearly 28% (Anonymous, 2010).

Classical assay for *L. monocytogenes* was by standard culture method that involves bacteriological tests starting with enrichment in a suitable culture medium, colony isolation on selective agar plate, biochemical and serological confirmation of presumption colonies, and these steps took several days to be completed. Immunoassay and Polymerase Chain Reaction (PCR) have been elaborated to accelerate *L. monocytogenes* detection. PCR has been used in molecular biological research, biomedical research and disease diagnostic for a long time (Saiki et al., 1988). Especially, the clinical microbiology have been used the development of PCR method for detection and identification of pathogens (Bernet et al., 1989; Brisson-Noël et al., 1989; Duggan et al., 1988; Holland et al., 1990; Holodniy et al., 1991; Hoshina et al., 1990; Lisby and Dessau, 1994; Victor et al., 1991) and *L. monocytogenes* (Bassler et al., 1995; Batt, 1997; Makino et al., 1995; Nogva et al., 2000; Rodriguez-Lazaro et al., 2004).

BAX-PCR and iQ-Check PCR were examined for *L. monocytogenes* detection in fresh produce (Liming et al., 2004). The detection via PCR in general was by separating the PCR products on agarose (2.0%) gel electrophoresis following by visual observation by ethedium staining (Bhagwat et al., 2001; Liming et al., 2004; Shearer et al., 2001). A Polymerase Chain Reaction (PCR) based detection system, BAX was also used to evaluate for the detection of *Salmonella enteritidis*, *E. coli* O157:H7, *Listeria* spp. and *Listeria monocytogenes* on fresh fruits and vegetables (Shearer et al., 2001). Although PCR is the most widely used method for DNA amplification, it requires a thermocycler to separate double strands DNA and amplify nucleic acid (Jeong et al., 2009; Vincent et al., 2004). Moreover, the platform with cycling temperature restricted the PCR applications in term of time consuming, laborious, not fit for field monitoring or point of care test.

Biosensors offer an opportunity to fulfill this limitation by enhancing the relevance and extent of the tests being carried out through measuring specific target analyses that are directly related to produce safety. The inherent specificity, selectivity, and adaptability of the technique make it ideal candidate for use in food industries. The developed DNA sensor, in principle, depends on the detection of sequence specific signals of target nucleic acid among the rest of signals available in each specimen and signals detection in point of care manner. Specific recognition for targeting nucleic acid can be obtained through specific hybridization between target nucleic acid sequence and synthetic oligonucleotide. So far target sequence of gene was distinguished either by selective capturing, usually through nucleic acid hybridization with complementary DNA or RNA or synthetic analog probe, or selectively duplicating (amplifying) using oligonucleotide primers as found in every platforms of nucleic acid amplification. Since DNA sensing in real specimen in this case involves with extremely small amount of target DNA in the total background with corresponding to few hybridization events, thus, there is no choice but choosing a recognition platform based on a selectively duplicating (amplifying) system.

For signal amplification, isothermal amplification methods have recently been developed as alternative choice to PCR (Andras et al., 2001). It does not require heating for the separation of double strands DNA and the synthesized DNA products. So far several isothermal nucleic acid amplification methods have been developed such as the nucleic acid sequence-based amplification (NASBA) (Compton, 1991), the strand

displacement amplification (SDA) (Walker et al., 1992), the rolling circle amplification (RCA) (Demidov, 2002), the loop-mediated isothermal amplification of DNA (Notomi et al., 2000; Schmid et al., 2005), and the helicase-dependent amplification (HDA) (Vincent et al., 2004). Although each method provided its own unique criteria, the HDA is only a novel isothermal DNA amplification method run based on a similar principle with that of PCR (Vincent et al., 2004). In the reaction, the DNA helicase unwinds the double strands DNA into single strand DNA without the need of heat treatment, allowing DNA polymerase to synthesize new strand. The HDA process can then be performed at single temperature (Vincent et al., 2004). The advantages of HDA are not only the increasing of reaction rapidity, specificity and sensitivity over that of other nucleic acid amplification methods while still using only one pair of primers, but also its simplicity and its true isothermal nature of the HDA platform offering a great opportunity for the development of genosensor that could be used to do nucleic acid detection at point-of–care (POC) or in the field.

HDA had been applied and demonstrated for rapid detection of *Mycobacterium tuberculosis* (MTB). In this case, HDA reduced the amplification time from an input of 2-copy MTB genomic from 60 min to less than 30 min (Motre et al., 2011). In 2014, detection of high-risk HPV16, HPV18 and HPV45 using isothermal helicase-dependent amplification were analyzed (Barbieri et al., 2014). Limit detection for HPV16-E7 was one copy/reaction. This method could be used as cost-effective diagnostic method for highest incidence cervical cancer in low-income countries laboratories.

Furthermore LAMP method that have been used to detect *L. monocytogenes* was stills increased the specificity and sensitivity but its required a set of 4 primers to amplify target DNA. A real-time quantitative loop-mediated isothermal amplification (Schmid et al., 2005) method was developed to amplified *hly* gene for detection of *L. monocytogenes* in food (Shan et al., 2012; Wang et al., 2012; Wang et al., 2011) developed LAMP method for detection *L. monocytogenes* and the detection limit of the method was 100 CFU/reaction. Detection of *L. ivanovii* using LAMP assay based on a special identifiable target *smcL* gene were established and compared with qPCR an PCR assays (Wang et al., 2014). Loop-Mediated Isothermal Amplification were developed and compared with PCR for identification of *Staphylococcus aureus* (*arcC* gene) (Lim et al., 2013). HDA, so far, has not yet been applied for *L. monocytogenes*

based biosensor detection. This application have been allow the amplification of *L. monocytogenes* signal in shorter period of time.

To detect *L. monocytogenes*, the specific virulence element must be selected as a target sequence for signal recognition so that once recognized and transduced the resulting signals can be used to represent result on bacterial incidence. So far pathogenic determinants including hemolysin gene, listeriolysin 0 gene (Cossart et al., 1989), phosphatidylinositol-specific phospholipase C gene (Leimeister-Wachter et al., 1990), lecithinase gene (Vazquez-Boland et al., 1992), metalloproteases gene (Domann et al., 1991), and a membrane anchor *act* gene (Kocks et al., 1992) had been investigated (Portnoy et al., 1988). Selection of candidate target is based on sequence variations and similarity among target group and non-target group of bacteria per determinant.

Apart from the selectively duplicating of DNA signals, the amplified signals in term of target products are normally analyzed by agarose gel electrophoresis and ethidium bromide staining and observed under an ultraviolet (UV) trans-illuminator. However, processes of agraose gel electrophoresis technique take a long-time and rely on laboratory facilities. Moreover the use of ethidium bromide during staining also poses risk of cancer. Although the amplified products detection has been further developed in real-time PCR by using oligonucleotide probe with fluorescence reporters, the use of optical instrument in the real-time PCR device made this detection relied heavily on laboratory facilities.

The integration of a biological recognition element having nucleic acid sequence as a target with a transducer to generate a measurable signal related to the amount of that target nucleic acid sequence can be obtained through specific hybridization between target nucleic acid sequence and synthetic oligonucleotide. This can be carried out using fluorophore based on FRET (Fluorescence Resonance Energy Transfer) hybridization assay. First established the molecular structure called molecular beacons (MBs) technology, which produce fluorescence without having to separate probe-target hybrids from excess probes in hybridization assays (Tyagi and Kramer, 1996). By selecting a wavelength-shifting among fluorophores in MBs structure, two independent groups at each fluorophore absorbed excitation energy and emitted fluorescence. This study is the first report of the application of the MB probe being

used for Helicase Dependent Amplification (HDA) detection of *L. monocytogenes* in seafood. A faster method based on utilization of a hybridization probe with real-time PCR was developed and applied for detection of *E. coli* O157:H7 and *L. monocytogenes* in contaminated raw ground beef and fully cooked beef hotdogs (Nguyen et al., 2004). The 6-carboxy-2', 4, 7, 7'-tetrachlorofluorescein (TET) and 6-carboxy-fluorecein (FAM) were used as fluorescent reporter dyes and conjugated to 5' ends of probe. And the quencher dye 6-carboxytetramethyl-rhodamine (TEMRA) was attached to 3' ends to detect *hlyA* gene that encode for the virulence factor listeriolysin O for *L. monocytogenes*. Molecular Beacon probe PCR (MB probe PCR) has been developed that uses an internal fluorogenic probe that is specific to the target gene (Chen et al., 2000; Hoorfar et al., 2000; Liming et al., 2004). PCR product was recognized and visualized by the fluorescent probe moiety (Tyagi and Kramer, 1996).

The reaction was practically involved with two functional groups, a reporter and a quencher. When there were no target DNA both groups come at enclosure, quencher will absorb energy excited from reporter group resulting in fluorescence energy transferring crossing no fluorescence illumination. In contrast, when there was target DNA in the system, DNA probe binding with target result in reporter and quencher separation and no FRET taking place. With this condition, the DNA had been produced strong fluorescence signals upon UV illumination. So far several nucleic acid probing systems and wavelength-shifting fluorophore combinations had been addressed (Jung et al., 2007; Petersen et al., 2004; Tyagi et al., 2000).

Normal signal detection was performed in real-time PCR device using oligonucleotide at dual high and low temperature following a melting nature of template DNA/probe during hybridization. Although this routine reaction allows nucleic acid hybridization is carried out stably, its platform does not meet demand of sensor condition requiring less on laboratory facilities. Since detection of double helix DNA structure using conventional oligonucleotide at room temperature is difficult to be arranged, modified structure in term of analog DNA is used instead. The DNA analog probe designed based on similar FRET technology had been introduced for the detection of *E. coli* since 2010 (Chaumpluk and Chaiprasart, 2012). The process involved with prolyl-aminocyclopentane carboxylic acid dipeptide DNA analog as a probe to bind with target DNA at very high affinity. This binding occurred at lower Tm of 40-45 °C lower compared with that of classical hybridization (Suparpprom et al.,

2005). Application of analog DNA then allows detection to be carried out at room temperature while also still compromise the biosensor requirement. By designing the analog DNA to have right wavelength-shifting fluorophore combination and to have a sequence matching with *L. monocytogenes*'s target determinant, DNA signals can then be measured directly.

Recently, DNA signal detection based on a colorimetric assay using gold nanoparticles has been reported (Fu et al., 2013); however, there are limits to gold nanoparticle stability and functionalization that make this method of detection complicated and confine its use to specific conditions, i.e., tests must be performed rapidly using fresh gold nanoparticles, and nanoparticle functionalization requires specialized experience (Kreibig and Genzel, 1985). These limitations reduce the utility of this method for real-world applications. The use of non-functionalized AgNPls instead of gold simplified the signal detection steps solved the stability problem of nanoparticle and reduced the cost of the assay. These advantages enabled the colorimetric test to be applied to point-of-care testing.

Nucleic acid hybridization can also be operated in different manner using analog DNA as a clamp to block nucleic acid amplification (Ugozzoli et al., 2004). Because analog DNA holds chemical structure differed from that of natural DNA and it has stronger affinity than DNA/DNA binding, both reasons provide this unique structure resisted to the 5'-3' exonuclease activity of polymerase while kept strong binding to the template. These properties allow nucleic acid amplification to be tampered when analog DNA presented in the amplification reaction. By comparing the capability of amplification especially at common sequence having single nucleotide polymorphic marker amplified with and without clamp, one can differentiate target *L. monocytogenes*'s DNA from the rest directly.

The purpose of this study was developed a genosensor for the detection of *Listeria monocytogenes* by applying the Helicase-Dependent Amplification (HDA) to target genetic determinant in signal recognition step and applied a detection technique selected after a comparison of two fluorescence assays, the fluorophore based on FRET probe hybridization and a probe locked clamp hybridization for a rapid detection of *L. monocytogenes* in seafood in a signal detection step.

CHAPTER III

METHODOLOGY

3.1 Materials

3.1.1 Samples

- Seafood samples

Seafood samples including salmon, tuna and shrimp were obtained from supermarkets and local markets in Bangkok.

- Bacterial strains

Type strains of bacteria were obtained either from The Department of Medical Science, Ministry of Public Health, Thailand (DMSc) or from The Food Research and Testing Laboratory, Faculty of Science, Chulalongkorn University, Bangkok (FRTL), Thailand (see Table 3.1, for the detail).

3.1.2 Instruments

- UV Transilluminator (UV Electronic Dual Light™ Transilluminator, USA)

- Gel electrophoresis machine (Mupid Advance Co., Ltd., Japan)

- Thermo cycler (Biorad Gene Cycler™, USA)

- Vertex mixer (Vortex Geni, USA)

- Evaporator (Eyela, Japan)

- Centrifugal machine (MIKRO 12-24 Hettich Zentrifugen, Germany)

- Balance model ME1002 (METTLER TOLEDO, Thailand)

- Augusta safety cabinet (Lio Lab Co., Thailand)

- Microwave (Sharp Co., Ltd., Japan)

- Heat block ThermoE incubator (Bioer Technology, Co., Ltd, china)

- Freezer -20°C (Panasonic, Thailand)

- Micropipette (Gibson, France)

- Microcentrifuge tube (Axygen, India)

- Pipette tips (Axygen, India)

3.1.3 Chemicals

- StrataClone PCR Cloning Kit (Agilent Technologies, USA)

- IsoAmp® III Universal tHDA Kit (Biohelix)

- Wizard® resin column (Promega, Madison, WI USA)

- Master mix

- Agarose (Promega, USA)

- Ethidium bromide (Gibco BRL, USA)

- 100 base pair DNA ladder (New England Biolabs, USA)

- Lauria broth (Sambrook et al., 1989)

- Lauria medium (Sambrook et al., 1989)

- SOC medium (Sambrook et al., 1989)

- Alcohol 99.5% (Merck, Germany)

- Primer (Wardmedic Co. Ltd.)

3.2 Methods

3.2.1 Collect and culture of the bacteria

A total of 120 seafood samples including salmon, tuna and shrimps were collected from supermarkets and local markets in Bangkok, Thailand. All samples were analyzed after collection in a day. *Listeria monocytogenes*, standard strain, and other pathogenic strains of bacteria including 7 strains *of L. monocytogenes* and 17 strains of *non-L. monocytogenes* including the same genus as *L. innocua, L. ivanovii, L. welshimeri*, *Vibrio cholera, V. parahaemolyticus, Salmonella enteritidis, Escherichia coli* O157:H7*,* Enterotoxigenic *E. coli* (ETEC), Enteropathogenic *E. coli* (EPEC)*, Pseudomonas putida, Shigella flexneri, Campylobacter jejuni* and *Staphylococcus aureus* were obtained from Department of Medical Sciences, Ministry of Public Health, Bangkok (DMSc) and Food Research and Testing Laboratory, Faculty of Science,

Chulalongkorn University, Bangkok (FRTL) (Table 3.1). For stock maintenance, the bacteria were cultured separately in 3 ml of Luria Broth (LB) in 30 ml glass bottles and incubated at 37°C for 16-18 hours in shaking incubator. For long term maintenance, the culture were stored in 20% glycerol and kept at -20°C.

Table 3.1 The strains of *L. monocytogenes* and non-*L. monocytogenes* and sources

No.	Bacteria Strain	Source
1	*Listeria monocytogenes*	DMST* 17303
2	*L. monocytogenes*	DMST* 1327
3	*L. monocytogenes*	DMST* 20093
4	*L. monocytogenes*	DMST* 23145
5	*L. monocytogenes*	DMST* 31802
6	*L. monocytogenes*	FRTL** 1299
7	*L. monocytogenes*	FRTL** 1401
8	*L. innocua*	DMST* 9011
9	*L. innocua*	FRTL** 1265
10	*L. innocua*	FRTL** 1445
11	*L. innocua*	FRTL** 1446
12	*L. ivanovii*	FRTL** 1243
13	*L. ivanovii*	DMST* 9012
14	*L. welshimeri*	DMST* 20559
15	*Vibrio cholera*	FRTL** 1322
16	*V. parahaemolyticus*	FRTL** 0886
17	*Salmonella enteritidis*	DMST* 15676
18	*Escherichia coli* O157:H7	DMST* 12743
19	*E. coli* (ETEC)	DMST* 30543
20	*E. coli* (EPEC)	DMST* 30546
21	*Pseudomonas putida*	DMST* 16074
22	*Shigella flexneri*	DMST* 4423
23	*Campylobacter jejuni* ATCC 33291	DMST* 15190
24	*Staphylococcus aureus* ATCC 25923	DMST* 8840

* DMST = Department of Medical Sciences, Ministry of Public Health, Thailand

** FRTL = Food Research and Testing Laboratory, Faculty of Science, Chulalongkorn University, Thailand

3.2.2 Primer and probe design

The candidate virulence genes of *L. monocytogenes* were *hly, inlA, inlB, iap, prfA, plcA, plcB*, and *actA* (Vázquez-Boland et al., 2001), however, the most frequently chosen target was the *hly* gene, encoding for listeriolysin O (Bohnert et al., 1992; Border et al., 1990; Furrer et al., 1991). Then the *hly* gene was chosen as a target gene here

for *L. monocytogenes* detection in this study. The specific oligonucleotide sequences for *hly* gene (GenBank, Accession No. AF253320.1) were employed as references and primers were designed using Primer3 (version 0.4.0) (Table 3.2). Once selected, the specific primers for LAMP technic were designed based on the program Primer Explorer Version 4 (Fujitsu, Japan). In order to confirm their binding specificity, target sequences in FASTA format were aligned using ClustalW alignment program (Higgins, 1994) and compared against databank via the Basic Local Alignment Search Tool (BLASTn) to search for the similarity among sequences (Altschul et al., 1990). For comparative study between HDA assay and classical PCR, standard PCR primers as described were used (Furrer et al., 1991). For nucleic acid hybridization, oligonucleotide probe (DNA analog) specific to target *hly* gene was also designed using program Primer3 (version 0.4.0) and resulting primer at the position 272 to 281 (Table 3.2) was obtained.

Table 3.2 Oligonucleotide sequence for amplification assay of *L. monocytogenes*

Primer name	Primer sequence (5'-3')	Size of product (bp)	Position*
hlyF **	5'-CGG AGG TTC CGC AAA AGA TG-3'	234	1044-1063
hlyR **	5'-CCT CCA GAG TGA TCG ATG TT-3'		1258-1277
HDAhlyF	5'-TAC CAC GGA GAT GCA GTG AC-3'	91	232-251
HDAhlyR	5'-TGG ATT TCT TCT TTT TCT CCA CA-3'		300-322
F3	5'-ACA ATG TAT TAG TAT ACC ACG GA-3'	211	218-240
B3	5'-TCT GGT TGA TTT TCT ACT AAT TCC-3'		405-428
F1c	5'-GGA TTT CTC TTT TTC TCC ACA ACG 3'		297-321
F2	5' GA TGC AGT GAC AAA TGT GC-3'		241-259
B1c	5'-GCA GAC ATC CAA GTT GTA AAT GC 3'		337-359
B2	5' CGC TTT TAC GAG AGC ACC-3'		382-399
Analog Probe	5'-CAA TGT TTC TAC C-3'	13	272-281
FRET Probe	5'-6-FAM-CGC GAT CGT GCC GCC AAG AAA AGA TCG CG-3'	29	256-270

* With reference to sequence Accession Number AF253320.1 (GenBank)

** (Furrer et al., 1991)

3.2.3 DNA extraction

Genomic DNA was extracted from bacterial overnight at 37°C for 16-18 hours in LB. After collecting the cells by centrifugation at 6000 rpm for 2 min, only bacterial pellet was extracted for DNA via standard phenol extraction method (Sambrook et al., 1989). The genomic DNA was dissolved in TE-RNase and purified on the Wizard® resin

column (Promega, Madison, WI USA) as described in manufacturer protocol and used in experiments. For seafood samples, the bacteria was obtained using cotton ball swab technique from surface of each seafood sample and enrich-cultured in 2.5 ml Terrific broth at 37°C for 3.5 hours in shaking incubator as described (Chaumpluk and Chaiprasart, 2013; Sambrook et al., 1989). The resulting culture was collected in a 1.5 ml microfuge tube, centrifuged to collect the cells at 6,000 rpm for 2 min and the supernatant was discarded. Only the obtained pellet was used for amplification after DNA extraction.

3.2.4 PCR amplification

Genomic DNA from positive control was amplified using Polymerase Chain Reaction (PCR) method with the specific designed primers, hlyF and hlyR (Table 3.2) as described by Furrer (1991). PCR products were analyzed by 2.5% agarose gel electrophoresis and visualized under UV illumination after stained with ethidium bromide (Sambrook et al., 1989). The expected product size was 234 base pairs.

3.2.5 Cloning of PCR products and nucleotide sequencing

DNA products were cloned into StrataClone PCR cloning vector using StrataClone PCR Cloning Technology (STRATAGENE, USA) as described by manufacturer and transferred into StrataClone SoloPack competent cells (*E. coli* strain SG13009). Plasmid screening was carried out according to Sambrook et al. (1989), and confirmed for insert target DNA by PCR technique. DNA fragments were confirmed using nucleotide sequencing.

3.2.6 Isothermal DNA amplification of *hly* gene

Isothermal DNA amplification based on Helicase dependent amplification (HDA) and Loop-mediated isothermal DNA amplification or LAMP were employed to amplify the DNA. Genomic DNA from samples and positive control DNA were amplified using IsoAmp® II Universal tHDA kit (BioHelix, Beverly, MA, USA) with two specific HDA primers; HDAhlyF and HDAhlyR (Table 3.2), and two-step tHDA (thermophilic HDA) as described.

In detail, HDA reaction have been performed by generating two mixtures. Mixture A contained with the DNA template, 10 pmol of specific HDA primers, 10x Annealing buffer II and DW. In addition, mixture B was prepared in a separate PCR tube

in sterile hood. It contained 100 mM $MgSO_4$, 500 mM NaCl, 1.75 mM dNTP solution, 2.0 µl enzyme mix, 10x Annealing buffer II and DW, gently mixed the solution by pipetting followed by brief centrifugation. Mixture A was coated with mineral oil and placed the tube on ice. Then, mixture A was heated at 95°C for 2 min and places promptly on ice. Once finished, mixture B was added into mixture A under the oil layer, gently mixes the reaction by pipetting and places the tube on ice. The obtaining mixture were then incubated at 65°C for 90 min using a heat block incubator, a water bath or thermo cycler or equivalence. Finally 10 µl of the HDA products were separated on 2% agarose gel and visualized under UV light and ethidium bromide staining (Sambrook et al., 1989). All strains of *L. monocytogenes* and non-*L. monocytogene*s DNA were amplified using HDA technique and compared with a classical PCR method (Furrer et al., 1991).

For LAMP method, the amplification reaction was performed in ThermoPol™ reaction buffer (20 mM Tris-HCl, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Triton X-100, pH 8.8) with 3 mM $MgSO_4$, 1.6 M Betaine, 5 mM dNTP, 8U *Bst* DNA polymerase large fragment (New England Biolabs, UK), 5 pmol each of F3 and B3 primer, and 40 pmol each of FIP and BIP primer and incubated at 63°C for 40 min using a heat block ThermoE incubator (Bioer, China). The LAMP DNA products were separated on a 2.5% agarose gel and visualized under UV light after ethidium bromide staining, as described elsewhere (Yano et al., 2007). *L. monocytogenes* and non-*L. monocytogenes* DNA was amplified using the LAMP method and compared with the products of PCR, as described elsewhere (Furrer et al., 1991). The sensitivity and specificity of the LAMP technique was also evaluated based on DNA copy number, as described elsewhere (Fawcett, 2006), and by spiking *L. monocytogenes* (10-fold dilutions from 10^7 to 1 CFU) directly onto 50 g of frozen salmon, followed by detection after 3.5 hours of enrichment**.**

3.2.7 Establish the DNA signals detection

DNA signals detection was performed via both agarose gel analysis and a visual observation of fluorescence illumination based on oligonucleotide probe (Table 3.2) hybridizations on UV illuminator and colorimetric detection using blue silver nanoplates by nake-eyed. The signal detection system was carried out via the use of FRET probe hybridization (Tyagi and Kramer, 1996). FRET probe was designed based on

the target amplified sequence using Primer3 program (Table 3.2). The specificity of probe was confirmed based on basic local alignment search. Once determined, probe was synthesized as described earlier and the used fluorophores were FAM and BHQ-1 at 5' and 3' respectively. Hybridization assay was customized under room temperature by at least 3x3x3 probes templates and salt concentration conditions. For an analog probe locked clamp hybridization (Ugozzoli et al., 2004), the design of probe was focused at single nucleotide polymorphic determinant, and at least 5 levels of probe concentration will be tested for its locking suitability. Analog probe was synthesized as described earlier (Suparpprom et al., 2005). The DNA signal detection with blue AgNPls was performed. The plate shape structure of the AgNPls was confirmed by TEM image as well as surface plasmon resonance spectrum with a unique out-of-plane quadrupole plasmon resonance at 340 nm [29]. DNA signals could be read by mixing the DNA oligoprobe and LAMP products that performed a DNA hybridization assay to the surface of the nanoparticles and adding salt which triggered nanoparticle aggregation. Briefly, 10 pmol of DNA probe was mixed with 1 µl of LAMP products, heated to allow DNA hybridization, and 15 µl of nanoparticle solution was then added to the reaction. For color detection, 1.5 µl of 50 mM PBS, pH 7.0, was added, and the color of the blue silver nanoplates was observed both by the naked eye and by UV-visible spectroscopy (Ocean Optics® USB2000, USA) at λ=380-800 nm.

3.2.8 The specificity and sensitivity of primer for HDA and LAMP amplification

The specificity of the primers of HDA method was performed using genomic DNA of *L. monocytogenes* and non-*L. monocytogenes* (Table 3.1) as a template and a specific set of *hly* primers using IsoAmp® II Universal tHDA kit (BioHelix, Beverly, MA, USA). DNA products were then assayed by both nake-eyed fluorescence visualization on UV illuminator based on analog probe hybridizations and agarose gel electrophoresis. Results were compared between HDA method and PCR (Furrer et al., 1991). The sensitivity of HDA method was tested using the serial dilution of the plasmid harboring *hly* gene of *L. monocytogenes* from 0 to 10^7 copies as described as described (Kuribara et al., 2002). The DNA products were visualized by ethidium bromide staining after agarose gel electrophoresis and by nake-eyed fluorescence visualization.

For LAMP amplification, the strains of *L. monocytogenes* and non-*L. monocytogenes* in the table 3.1 were amplified using the specific designed LAMP

primers, and the results were detected as described above. The results from LAMP were compared with those of PCR with gel electrophoresis (Furrer et al., 1991). In parallel, the sensitivity of the reaction (i.e., the detection limit) was determined using both the 10-fold dilutions of cloned *L. monocytogenes* target DNA, as described elsewhere (Kuribara et al., 2002).

The sensitivity of HDA and LAMP method was examined using 100 blind samples containing 30 real samples, 40 samples spiked with *L. monocytogenes* (500 CFU), and 30 samples without *L. monocytogenes*, and the results were compared with those of the PCR assay. The specificity and sensitivity of the methods were evaluated as described (Fawcett, 2006). The specific detection of this method was described as the number of true negatives x 100 divided by the sum of the number of false positives and true negatives. The sensitivity was calculated from the number of true positives x 100 divided by the sum of the number of true positives and false negatives (Yano et al., 2007).

3.2.9 Strain differentiation

In addition, occurring signals from locked clamp hybridization with and without a clamp probe will also be determined to established wild type and mutant of *L. monocytogenes*.

3.2.10 Field trial

One hundred and twenty samples of seafood such as fish and shrimp products were collected for analysis from supermarkets and local markets in Bangkok. DNA extraction and Listeria assay are as described earlier using specific primer for HDA, and LAMP method and results were compared with that of PCR, reported earlier as a gold reference (Furrer et al., 1991) (Table 3.2). Result of the test came from comparative fluorescence signals produce under UV light.

For the spiking trial, a strain of *L. monocytogenes* (DMST* 17303) was cultured in LB and incubated at 37°C for 16-18 hours in shaking incubator. The serial dilutions of bacteria were determined from 10^{10} to 0 CFU. Each of diluted bacteria was inoculated on 50 g of seafood product and incubated at 37°C for 15 minutes. Then bacteria was obtains by swapping with a sterile cotton ball and enriching in Terrific broth for 3.5 hours at 37°C in a shaking incubator. The bacteria culture was harvested

as previously described, and to measure the amount of bacteria count, 50 µl of the bacterial serial dilution were plated onto LM media and incubated at 37ºC for 16-18 hours. The numbers of colonies were counted as a population from each serial dilution. The inoculums, transferred to the plate, contained a known proportion of the original sample as it was the product of a serial dilution.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Primers and Probe design

Traditional methods for detection for *Listeria* spp. not only requires 5 official days to obtain the result but relies heavily on laboratory facilities (de Boer and Beumer, 1999).

For this reason a number of alternative rapid screening tests have been proposed. PCR based screening platform and immunochromatographic platform were among the popular models employed recently (Nogva et al., 2000; Rodriguez-Lazaro et al., 2004; Saiki et al., 1988), but both had drawn backs on time consuming, depending heavily on laboratory facilities, and the latter had some problem with test specificity.

In this study, HDA platform were combined with a novel colorimetric DNA signal detection based on fluorescence binder and clamp lock amplification. To establish DNA amplification, several candidate genes of *L. monocytogenes*, including *hly, iap*, *inl*A, *inl*B, *prf*A and *act*A, were investigated. Among them, the *hly* gene was selected as the target sequence because it was highly conserved among different strains. The *hly* gene encodes a hemolysin called listeriolysin O (Cossart et al., 1989) that is essential for the lysis of the phagosomal membrane during infection. This gene was also the most commonly employed in several molecular analysis (Churchill et al., 2006; Furrer et al., 1991; Jallewar et al., 2007; Portnoy et al., 1988). The specific oligonucleotide sequence for *hly* gene were determined as references and designed primer for *hly* gene using Primer3 (version 0.4.0) and using the program Primer Explorer Version 4 (Fujitsu, Japan) for a set of LAMP primer. To this, specific set of primers for *L. monocytogenes* (*hly* gene) detection was designed (Table 3.2). The primers were screened based on BLASTn program on 8 June 2015 (Reference number RGUEX4PY014 and RGUVTT4Y014) (Table 4.1). The position of each primer on *hly* gene were shown; HDAhlyF/R for HDA in the Figure 4.1; F3 and B3 for LAMP in the Figure 4.2; hlyF/R for PCR in the Figure 4.3 and HDAhlyF/R, PNA probe and FRET probe in the Figure 4.4.

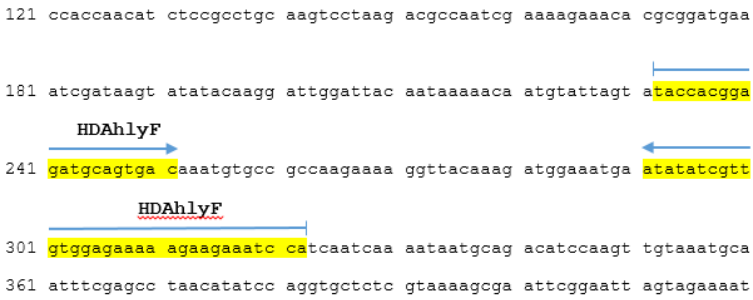


Figure 4.1 The position of HDAhlyF/R designed Primer on *hly* gene (accession No. AF253320)

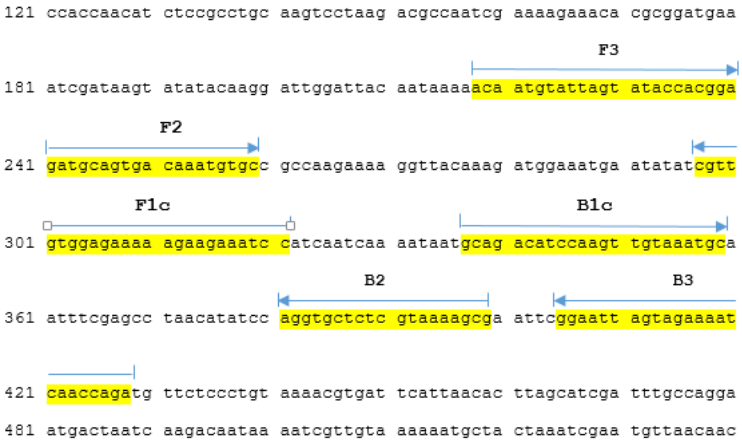


Figure 4.2 The position of LAMP (F3, B3) designed Primer on *hly* gene (accession No. AF253320)

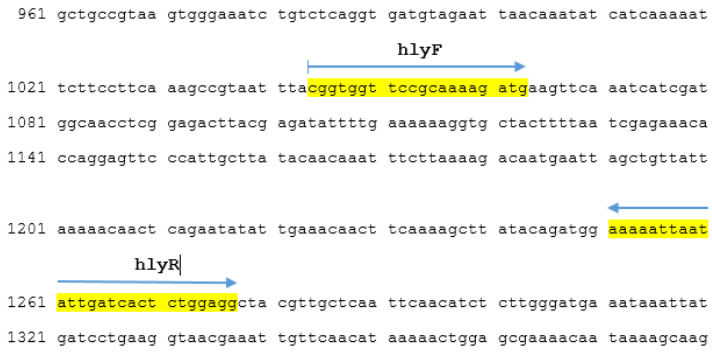


Figure 4.3 The position of hlyF/R designed Primer as reference primer on *hly* gene (accession No. AF253320)

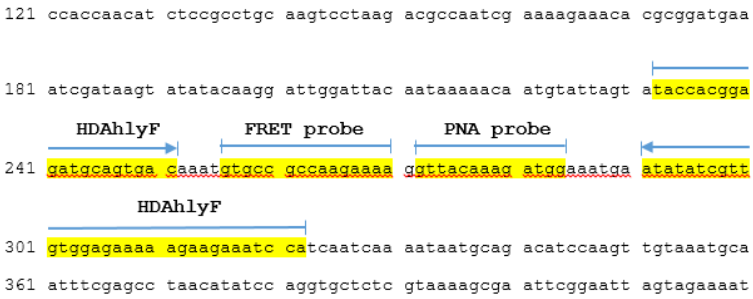


Figure 4.4 The position of HDAhlyF/R designed Primer, analog probe and FRET probe on *hly* gene (accession No. AF253320)

All designed HDAhlyF/R primers had a Query cover 100%, Maximum score 40.1 and 46.1 and Expected value low to 0.027 and 0.001. Moreover, the local alignments revealed the matching of the primers with very specific to *L. monocytogenes* (Table 4.1). Both primers located within the genetic determinant of *hly* gene at nucleotide 41247-41266t to 41176-41198 with related to sequence of known *Listeria* sp. (accession number NZ_CP007600). The lower of Expected value indicated the higher in the specificity of the primer to target gene. When this was tested further via Blastn analysis, results of each primers then confirmed the appropriate of each for the use in amplification detection.

For LAMP primers, the F3 and B3 primers had a Query cover 100%, Maximum score 46.1 and 48.1 with Expected value low to 0.004 and 0.001 respectively, while the inner primer of both forward and reverse had a Query cover 91-100%, Maximum score 38.2-40.1 and Expected value low to 0.063-0.083 (Table 4.1). When these primers were aligned to the DNA database, there were the high specific relationships to genetic determinant of *hly* gene of known *Listeria monocytogenes* at nucleotide 41258-41280 to 490073-490096 with related to sequence accession number NZ_CP007600 and NZ_HG813249.

Table 4.1 Specificity of Primer using the Basic Local Alignment Search Tool (BLASTn)

Primer	Definition	Max Score	Ident.	E Value	Accession No.
hlyF (5FWAV5TM015)	*Listeria monocytogenes* strain Giza7 listeriolysin O (*hlyA*) gene, partial cds	40.1	100%	0.18	KR812477
	Listeria monocytogenes strain Giza6 listeriolysin O (*hlyA*) gene, partial cds	40.1	100%	0.18	KR812476
	Listeria monocytogenes strain Giza5 listeriolysin O (*hlyA*) gene, partial cds	40.1	100%	0.18	KR812475
	Listeria monocytogenes strain Giza3 listeriolysin O (*hlyA*) gene, partial cds	40.1	100%	0.18	KR812474
hlyR (5FWFVFAF01R)	*Listeria monocytogenes* listeriolysin O (*hlyA*) gene, partial cds	40.1	100%	0.18	KR534875
	Listeria monocytogenes strainJP2008 listeiolysin O precursor (*hly*) gene, complete cds	40.1	100%	0.18	KR185738
	Listeria monocytogenes J1-220, complete genome	40.1	100%	0.18	CP006047
	Listeria monocytogenes J1816, complete genome	40.1	100%	0.18	CP006046
HDAhlyF (5AA18YMK014)	*Listeria monocytogenes* strain CFSAN006122, complete genome	40.1	100%	0.027	NZ_CP007600
	Listeria monocytogenes 6179 chromosome, complete genome	40.1	100%	0.027	NZ_HG813249
	Listeria monocytogenes R479a chromosome, complete genome	40.1	100%	0.027	NZ_HG813247
	Listeria monocytogenes WSLC1042, complete genome	40.1	100%	0.027	NZ_CP007210
HDAhlyR (5AA5WNXY015)	*Listeria monocytogenes* strain CFSAN006122, complete genome	46.1	100%	0.001	NZ_CP007600
	Listeria monocytogenes strain Lm60, complete genome	46.1	100%	0.001	NZ_CP009258
	Listeria monocytogenes strain J1926, complete genome	46.1	100%	0.001	NC_021840
	Listeria monocytogenes strain J2-031, complete genome	46.1	100%	0.001	NC_021837
F3 (5FV4R9UV01R)	*Listeria monocytogenes* strainJP2008 listeiolysin O precursor (*hly*) gene, complete cds	46.1	100%	0.004	KR185738
	Listeria monocytogenes J1-220, complete genome	46.1	100%	0.004	CP006047
	Listeria monocytogenes J1816, complete genome	46.1	100%	0.004	CP006046
	Listeria monocytogenes strain LM850658, complete genome	46.1	100%	0.004	CP009242
B3 (5FU0WFTE014)	*Listeria monocytogenes* strain LM850658, complete genome	48.1	100%	0.001	CP009242
	Listeria monocytogenes strain L2074, complete genome	48.1	100%	0.001	CP007689
	Listeria monocytogenes strain L1846, complete genome	48.1	100%	0.001	CP007688
	Listeria monocytogenes strain L2625, complete genome	48.1	100%	0.001	CP007687

4.2 Amplification of target gene, specificity and sensitivity of primers

The amplification of *hly* gene with the primer set using HDA delivered DNA products at 91 nucleotides of expected size. Similarly, amplification of *hly* gene with a set of 4 primers using LAMP provided DNA products of multiple of 211 nucleotides size ladder. The results of DNA amplification with HDA did not provide any non-specific DNA products nor primer dimers (Figure 4.5). The result of DNA amplification was also in agreement with those obtained by classical PCR having 234 nucleotide size (Furrer et al., 1991).

For primers specificity study, attempts to amplify different DNA templates from 7 strains of *L. monocytogenes* and 17 strains of non-*L. moncytogenes* including *L. innocua, L. ivanovii, L. welshimeri* of the same genus, *Vibrio cholera, V. parahaemolyticus, Salmonella enteritidis, Escherichia coli* O157:H7, Enterotoxigenic *E. coli* (ETEC), Enteropathogenic *E. coli* (EPEC)*, Pseudomonas putida, Shigella flexneri,*

Campylobacter jejuni and *Staphylococcus aureus,* using HDA were made. Only the target DNAs from *L. monocytogenes* were amplified compared with that of PCR. DNA products were amplified using gel electrophoresis. The results revealed that only the strains of *L. monocytogenes* were amplified (Figure 4.6). When non *L. monocytogenes* strains were used as a template, either non-specific amplification nor a primer-dimer was observed (Figure 4.6). The amplification of *hly* gene using specific primer thus occurred only when *L. monocytogenes* was presented. Results also confirmed primer specificity with *L. monocytogenes* but not *L. innocua*, *L. ivanovi* and *L. welshimeri,* another three species in the same Listeria genus. The results of primer specificity was also in consistent with those study using PCR technique (Furrer et al., 1991), indicating the reliability of primer for Listeria amplification. All these results confirmed the specificity of the HDA primer set for *L. monocytogenes* DNA amplification.

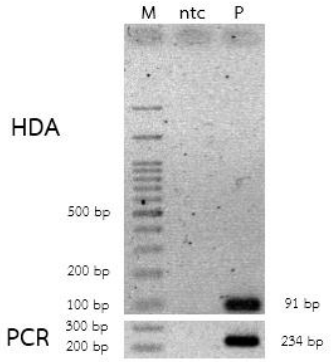


Figure 4.5 The DNA products of *hly* gene using HDA method compared the products from the amplification of *hly* gene by PCR method (Furrer et al., 1991) with specific primers and detected based on agarose gel electrophoresis. Lane M, 100 base pair DNA ladder; Lane ntc, non-template control; Lane P, Positive DNA from *L. monocytogenes*.

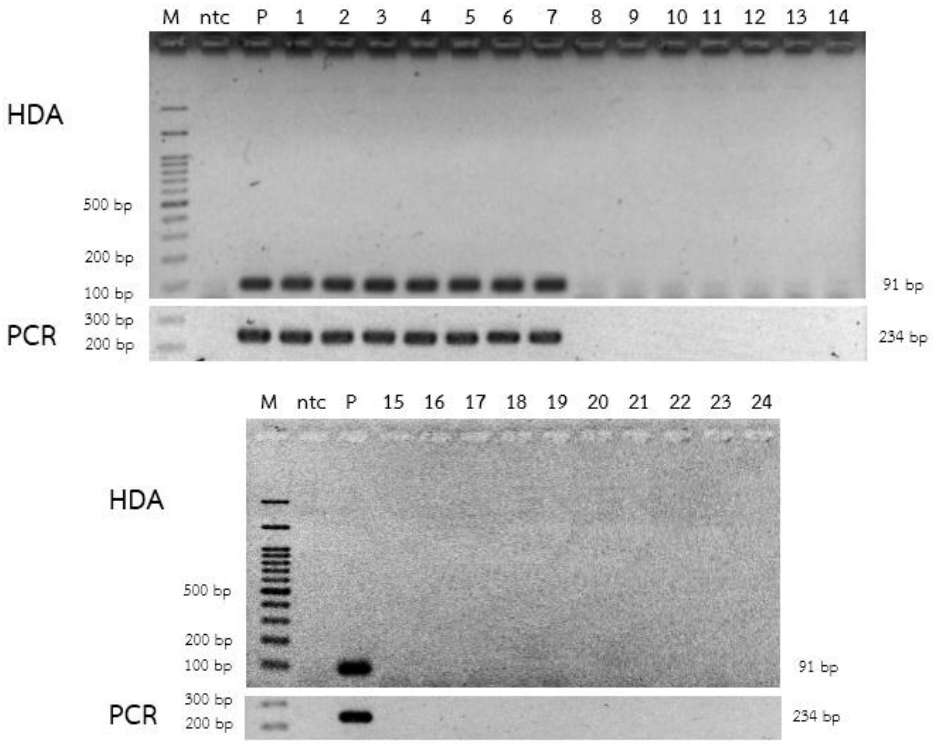


Figure 4.6 The specificity of HDA method for detection of *L. monocytogenes* compared with PCR (Furrer et al., 1991). Lane M, 100 base pair DNA ladder; Lane ntc, non-template control; Lane P, positive DNA from *L. monocytogenes*, Lane 1-7; *L. monocytogenes* from difference source, Lane 8-11, four strains of *L. innocua*; Lane 12-13, two strains of *L. ivanovii*; Lane 14-24, *L. welshimeri*; *Vibrio cholera*; *V. parahaemolyticus*; *Salmonella enteritidis*; *Escherichia coli* O157:H7; *E. coli* (ETEC); *E. coli* (EPEC); *Pseudomonas putida*; *Shigella flexneri*; *Campylobacter jejuni* and *Staphylococcus aureus* respectively.

The sensitivity of HDA method was carried out using DNA plasmid with ten-fold dilution as described (Kuribara et al., 2002). The amplified with HDA method gave *hly* gene products at size of 91 base pairs even when template was diluted to 100 copies (Figure 4.7). In our attempt to compare limit of detection using PCR (Furrer et al., 1991), it was found that the DNA products size 234 nucleotides of PCR method were found only when the plasmid templates were diluted to 1000 copies. Since the study was based on purified plasmid template, results then indicated the possibility of better sensitivity for HDA primer than the PCR.

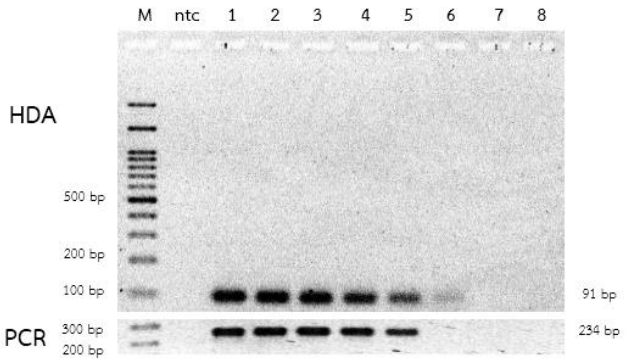


Figure 4.7 The sensitivity of HDA method for detection of *L. monocytogenes* compared with PCR (Furrer et al., 1991). Lane M, 100 base pair DNA ladder; Lane ntc, non-template control; Lanes 1-8, ten fold serial dilutions of *L. monocytogenes* template DNA, ranging from Lane 1, 10^7 copies; Lane 2, 10^6 copies; Lane 3, 10^5 copies; Lane 4, 10^4 copies; Lane 5, 10^3 copies; Lane 6, 10^2 copies; Lane 7, 10 copies and Lane 8, 0 copies.

Sensitivity of the primer was also investigated based on the spiking of tenfold serially diluted bacterial cultures on 50 g of frozen seafood together with colony forming plate count evaluation, results revealed that the limit of detection was approximate 100 CFU in contaminated frozen seafood, which was in agreement with the results obtained from DNA test (Figure 4.8). Based on the above, the limit of detection of the primer for Listeria detection was as low as 100 copies of *hly* template.

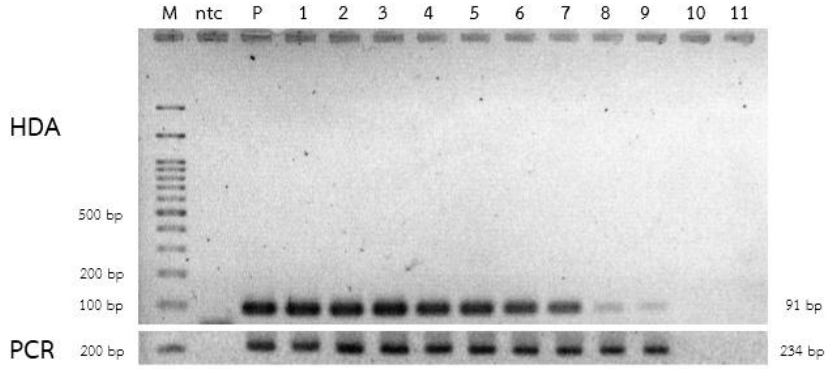


Figure 4.8 The detection limit by spiking of the seafood sample with *L. monocytogenes* diluents using HDA method compared with PCR (Furrer et al., 1991); Lane M, 100 base pair DNA ladder; Lane ntc, non-template control; Lane P, *L. monocytogenes* plasmid, Lane 1-5, from 10^{10} to 10^6 CFU, Lane 6, 1.02 x 10^5 CFU, Lane 7, 10250 CFU, Lane 8, 1033 CFU, Lane 9, 100 CFU, Lane 10, 7 CFU and Lane 11, 0 CFU, respectively.

For the amplification with LAMP technique, successful amplification of *hly* set of primers provided the DNA products of a multiple ladder of DNA, related to a unique pattern of LAMP products (Figure 4.9). The results of DNA amplification were also in agreement with those obtain by classical PCR having 234 nucleotides size (Furrer et al., 1991).

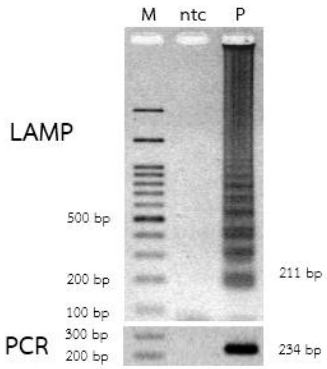


Figure 4.9 DNA products from the amplification of the hly gene by LAMP compared with the products of PCR (Furrer et al., 1991). Lane M, 100 base pair DNA ladder; Lane ntc, non-template control; Lane P, positive DNA from *L. monocytogenes*.

For the specificity assay, similar DNA from 22 strains of bacteria, including seven strains of *L. monocytogenes*; four strains of *L. innocua*; two strains of *L. ivanovii*; *L. welshimeri*; *Vibrio parahaemolyticus*; *V. cholera*; *Salmonella enteritidis*; *E. coli* O157:H7; *E. coli* (ETEC); *E. coli* (EPEC); *Pseudomonad putida and Shigella flexneri* were used as the templates for LAMP and were compared with the results of the same PCR previously used in HDA study (Furrer et al., 1991). Target *hly* gene amplification occurred only in the presence of *L. monocytogenes* (Figure 4.10). Neither non-specific amplification products nor primer dimers were observed when using non-*L. monocytogenes* strains as templates. Thus, the amplification of the *hly* gene via LAMP undoubtedly occurred only when *L. monocytogenes* was present.

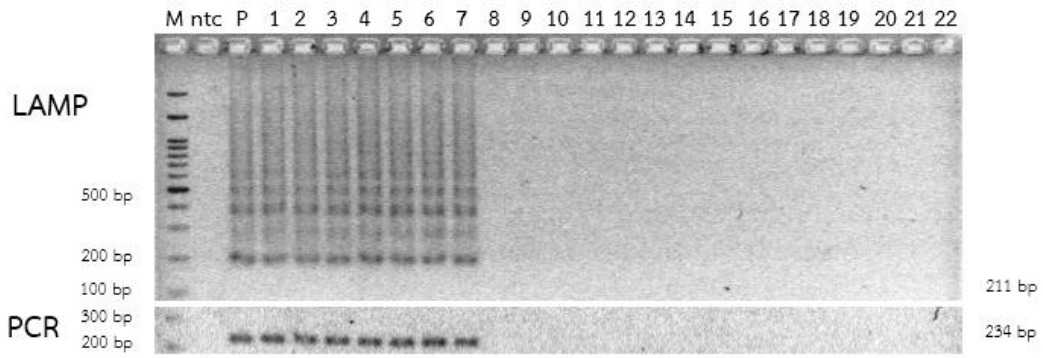


Figure 4.10 The specificity of the LAMP reaction for *L. monocytogenes* compared with that of PCR (Furrer et al., 1991). Lane M, 100 base pair DNA ladder; Lane ntc, Non-template control; Lane P, *L. monocytogenes* plasmid; Lane 1-7, seven strains of *L. monocytogenes* DNA; Lane 8-11, four strains of *L. innocua*; Lane 12-13; two strains of *L. ivanovii*; Lane 14-22, DNA from *L. welshimeri*; *Vibrio cholera*; *V. parahaemolyticus*; *Salmonella enteritidis*; *Escherichia coli* O157:H7; *E. coli* ETEC; *E. coli* EPEC; *Pseudomonas putida* and *Shigella flexneri.*

Similarly, serial dilutions of a plasmid containing the *hly* gene were employed to determine the sensitivity of the primers. Following amplification, the limit of detection was as low as 100 copies of the *hly* template (Figure 4.11). Because the LAMP technique is based on DNA detection, one copy of DNA corresponds to the presence of one bacterial cell in the test system. Thus, the sensitivity in this case was 100 cells. This value was also similar to the previous studies showing the DNA based methods had a detection limit of DNA range between 10 to 100 copies or equivalence to colony forming units/mL of sample (D'Urso et al., 2009; Poltronieri et al., 2009).

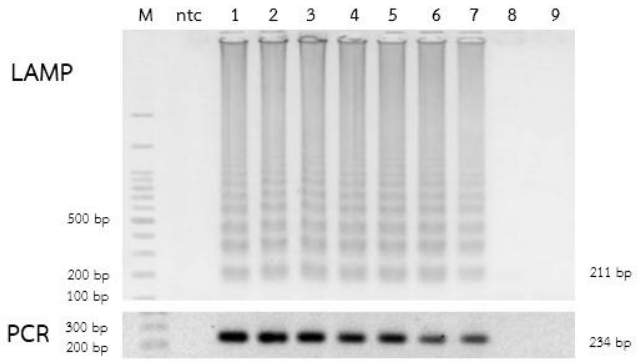


Figure 4.11 The detection limit of the LAMP reaction using DNA as the template compared with that of PCR (Furrer et al., 1991). Lane M, 100 base pair DNA ladder;

Lane ntc, non-template control; Lanes 1-9, 10-fold serial dilutions of *L. monocytogenes* template DNA, ranging from 10^8 copies to 0 copies.

Sensitivity assays based on the spiking of 10-fold serially diluted bacterial cultures on 50 g of frozen salmon were also carried out. Results revealed that the limit of detection was 100 CFU in contaminated frozen seafood, which is similar to the results from PCR test (Figure 4.12).

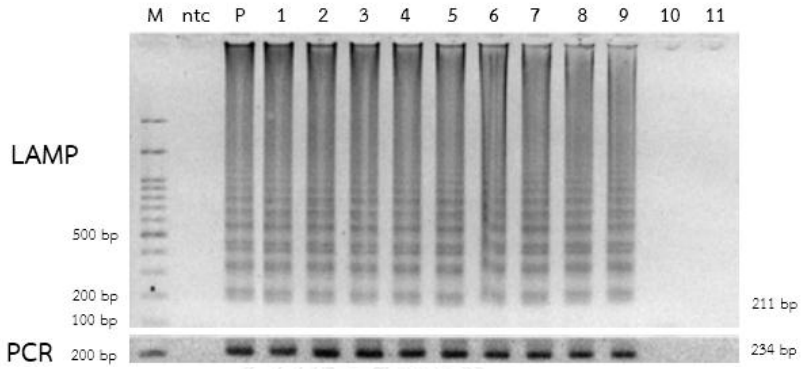


Figure 4.12 The detection limit by spiking of the seafood sample with *L. monocytogenes* diluents with Lane M, 100 base pair DNA ladder; Lane ntc, non-template control; Lane P, *L. monocytogenes* plasmid; Lane 1-5, from 10^{10} CFU to 10^6 CFU, Lane 6, 1.02 x 10^5 CFU, Lane 7, 10250 CFU, Lane 8, 1033 CFU, Lane 9, 100 CFU, Lane 10, 7 CFU and Lane 11, 0 CFU, respectively.

4.3 Detection of target gene via fluorescence signal

The DNA signal detection was carried out via both agarose gel elelctrophoresis and fluorescence visualization based on nucleic acid hybridization. The latter system relied on DNA/DNA hybridization via fluorescent biosensing using molecular beacon as a probe. The combination of target DNA-DNA molecular beacon probe hybridization along with fluorescent detection method based on FRET technology made the DNA signal detection system reliable and more effective. In the beacon structure, the 5' fluorophore group of FAM was in the proximity with a stem loop and 3'BHQ-1. And once the stem loop was formed, the proximity structure would force both fluorophore groups to come to close side by side and induced FRET phenomenon. This resulted in no fluorescence illumination. In contrast, when target *hly* gene was presented, target *hly* gene binded with beacon probe and separated the 5' FAM and 3' BHQ-1 fluorophores, allowing a bright illumination of the fluorescence signal.

In the study, the absence of target sequence, the fluorophore and quencher was stayed in close proximity causing the resonance energy transfer of fluorophore to quencher thus, fluorophore was unable to emit fluorescent light. On the other hands, when the target sequence was presented, the fluorescent DNA probe was complemented to target *hly* sequence which forced the quencher to be far away from fluorophore. Therefore, the emission of fluorescent light was produced when illuminated under UV light. Results of fluorescence illumination could be observed for both HDA based and LAMP amplification. There was no emission occurred in the tube containing non-template control while a green fluorescent light was emitted in the tube containing positive control (Figure 4.13 to 4.19).

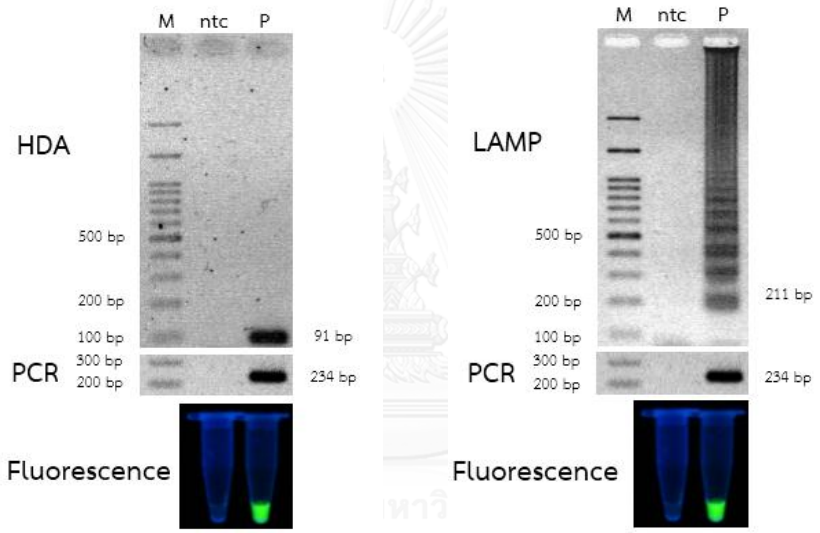


Figure 4.13 The DNA products of *hly* gene using HDA and LAMP method compared the products from the amplification of *hly* gene by PCR method (Furrer et al., 1991) with specific primers and detected based on agarose gel electrophoresis and fluorescent detection. Lane M, 100 base pair DNA ladder; Lane ntc, non-template control; Lane P, Positive DNA from *L. monocytogenes*.

In comparison, though both HDA and LAMP platform did provide fluorescence signals, the intensity of signal on LAMP was better. One possible reason behind this was the amount of target DNA that produced more in LAMP platform than the other. The more DNA signals was available, that better chance of hybridization and the bright of signal intensity. The format using fluorescence signal detection presented here was the first demonstration of a kind in detection of food borne bacteria. The fluorescence signals here agreed well with the results observed via electrophoresis and gel staining.

This allowed the test to be assayed without relying on the gel electrophoresis process at all and this reduced the test retention time at least for 1 hour.

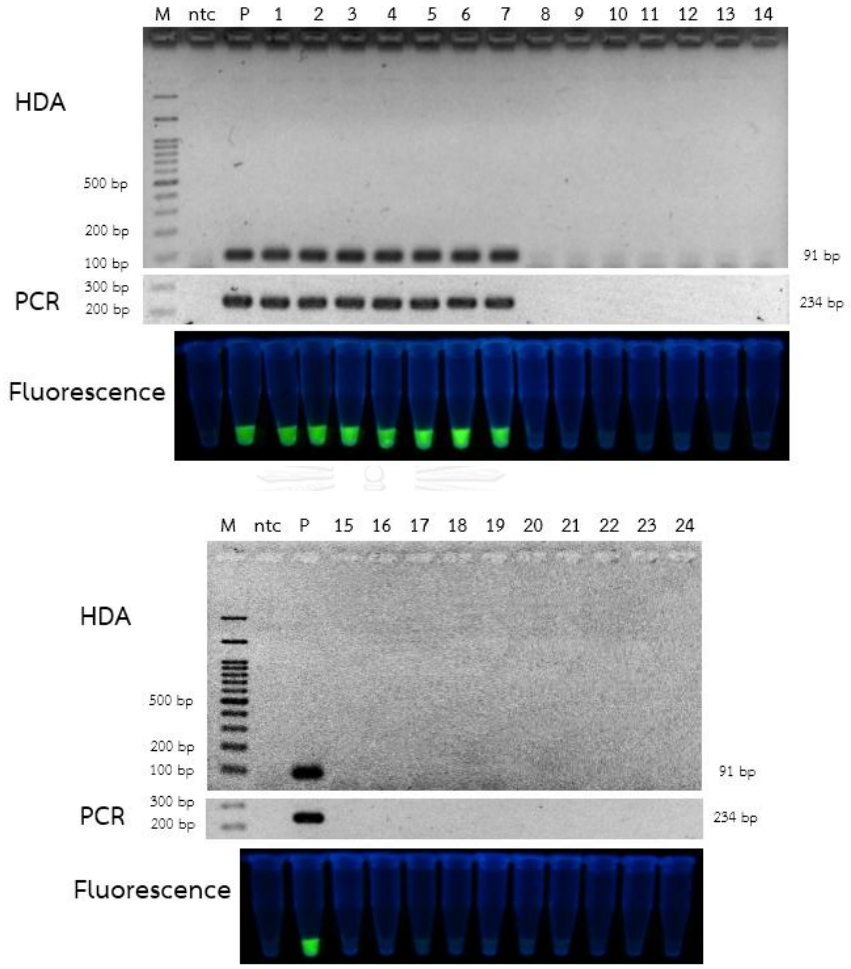


Figure 4.14 The specificity of HDA method for detection of *L. monocytogenes* compared with PCR method (Furrer et al., 1991) with specific primers and detected based on agarose gel electrophoresis and fluorescent detection. Lane M, 100 base pair DNA ladder; Lane ntc, non-template control; Lane P, positive DNA from *L. monocytogenes*, Lane 1-7; *L. monocytogenes* from difference source, Lane 8-11, four strains of *L. innocua*; Lane 12-13, two strains of *L. ivanovii*; Lane 14-24, *L. welshimeri*; *Vibrio cholera*; *V. parahaemolyticus*; *Salmonella enteritidis*; *Escherichia coli* O157:H7; *E. coli* (ETEC); *E. coli* (EPEC); *Pseudomonas putida*; *Shigella flexneri*; *Campylobacter jejuni* and *Staphylococcus aureus* respectively.

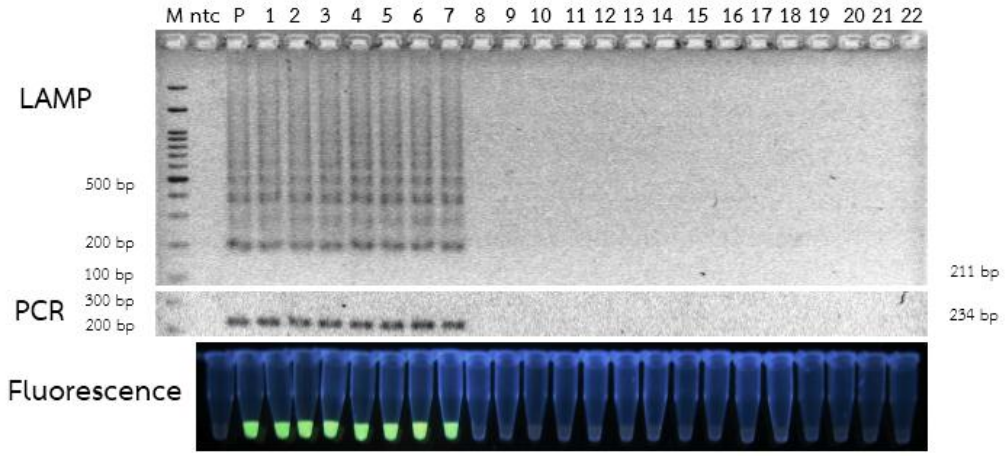


Figure 4.15 The specificity of the LAMP reaction for *L. monocytogenes* compared with that of PCR method (Furrer et al., 1991) with specific primers and detected based on agarose gel electrophoresis and fluorescent detection. Lane M, 100 base pair DNA ladder; Lane ntc, Non-template control; Lane P, *L. monocytogenes* plasmid; Lane 1-7, seven strains of *L. monocytogenes* DNA; Lane 8-11, four strains of *L. innocua*; Lane 12-13; two strains of *L. ivanovii*; Lane 14-22, DNA from *L. welshimeri*; *Vibrio cholera; V. parahaemolyticus; Salmonella enteritidis; Escherichia coli O157:H7; E. coli ETEC; E. coli EPEC; Pseudomonas putida* and *Shigella flexneri*.

Application of fluorescence detections were further carried out for specificity and sensitivity detection in both HDA and LAMP platform. In all aspects, results were in corresponding with that of gel electrophoresis and the classical PCR.

In signal strength comparison, it should be noted that the platform using molecular hybridization was not worked well for HDA sensitivity test especially at the near limit of detection (Figure 4.16). This was due to the lower in products amplification efficiency with in turn affected the product yields. This phenomenon did affect the hybridization efficiency directly and resulted in only faint fluorescence signals availably. However LAMP was provided with a contrast results which plenty of DNA products were delivered even at limit of detection (Figure 4.17). These increased hybridization phenomena which in turn created the strong signal upon illumination with UV.

In the past, the designed molecular beacon was routinely employed in real-time PCR even though the reaction limit was presented without any restrictions. This was because the detector device with high sensitivity used in real-time PCR allowing

even small trace of signal could be measured effectively. In contrast, when the detection was set aside via visualization with naked eye, one must concern with a minimum threshold setting in order to bring signal to the level of the observable. Thus, the comparison of both amplification cases provided a tendency of better signal detection on LAMP platform than of HDA.

The detection using fluorescence beacon costs about 2 US$ (30 baht/US$ rate) per test not included the operational costs. This was still lower than the Southern hybridization performing cost. And result could be obtained instantly once the DNA amplification was accomplished.

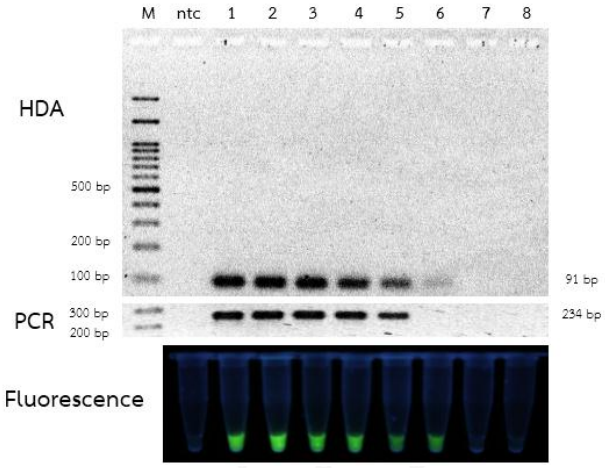


Figure 4.16 The sensitivity of HDA method for detection of *L. monocytogenes* compared with PCR (Furrer et al., 1991) with specific primers and detected based on agarose gel electrophoresis and fluorescent detection. Lane M, 100 base pair DNA ladder; Lane ntc, non-template control; Lanes 1-8, ten fold serial dilutions of *L. monocytogenes* template DNA, ranging from Lane 1, 10^7 copies; Lane 2, 10^6 copies; Lane 3, 10^5 copies; Lane 4, 10^4 copies; Lane 5, 10^3 copies; Lane 6, 10^2 copies; Lane 7, 10 copies and Lane 8, 0 copies.

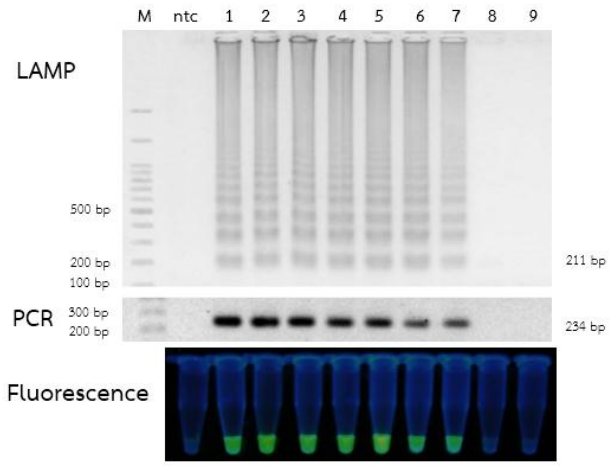


Figure 4.17 The detection limit of the LAMP reaction using DNA as the template compared with that of PCR (Furrer et al., 1991) with specific primers and detected based on agarose gel electrophoresis and fluorescent detection. Lane M, 100 base pair DNA ladder; Lane ntc, non-template control; Lanes 1-9, 10-fold serial dilutions of *L. monocytogenes* template DNA, ranging from 10^8 copies to 0 copies.

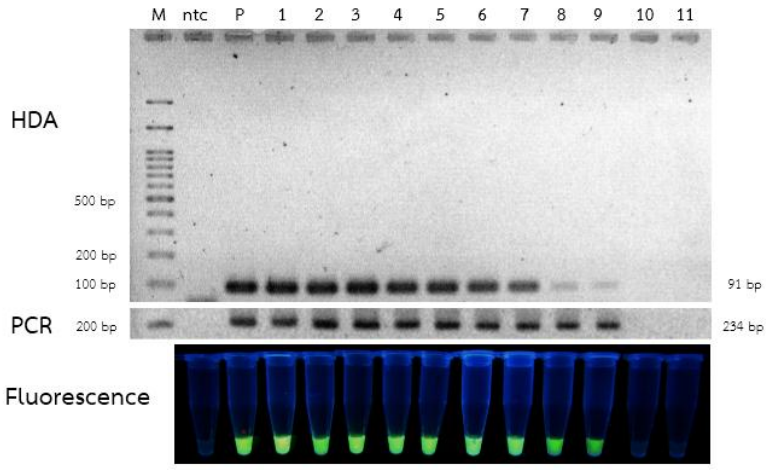


Figure 4.18 The detection limit by spiking of the seafood sample with *L. monocytogenes* diluents using HDA method compared with PCR (Furrer et al., 1991) with specific primers and detected based on agarose gel electrophoresis and fluorescent detection. Lane M, 100 base pair DNA ladder; Lane ntc, non-template control; Lane P, *L. monocytogenes* plasmid, Lane 1-5, from 10^{10} to 10^6 CFU, Lane 6, 1.02×10^5 CFU, Lane 7, 10250 CFU, Lane 8, 1033 CFU, Lane 9, 100 CFU, Lane 10, 7 CFU and Lane 11, 0 CFU, respectively.

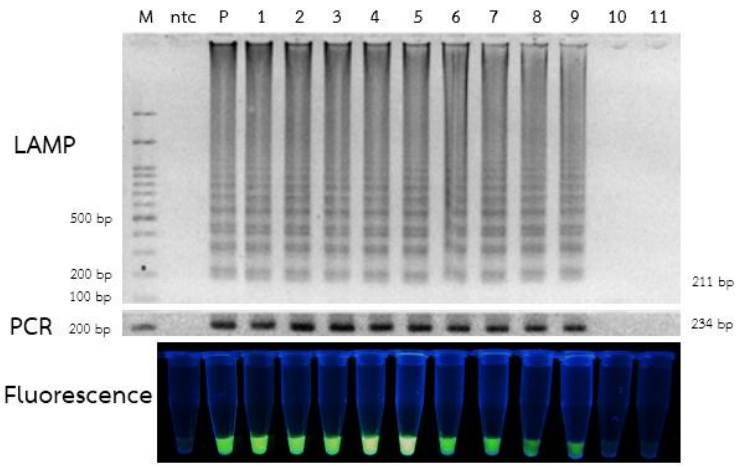


Figure 4.19 The detection limit by spiking of the seafood sample with *L. monocytogenes* diluents using LAMP method compared with PCR (Furrer et al., 1991) with specific primers and detected based on agarose gel electrophoresis and fluorescent detection. Lane 1-5, from 1010 CFU to 106 CFU, Lane 6, 1.02 x 105 CFU, Lane 7, 10250 CFU, Lane 8, 1033 CFU, Lane 9, 100 CFU, Lane 10, 7 CFU and Lane 11, 0 CFU, respectively. Lane M, 100 base pair DNA ladder; Lane ntc, non-template control and Lane P, *L. monocytogenes* plasmid.

Thus, the colorimetric method using non-functionalized blue silver nanoplates (AgNPls) was developed to facilitate the detection of DNA products from HDA and LAMP method. The results of DNA signal detection were also in agreement with those obtain by agarose gel electrophoresis and fluorescent detection (Figure 4.20 to 4.29).

In practical, the DNA signal detection was based on colorimetric assay using non functionalized metal nanoparticles due to its advantages on speed and simplicity of the test (Li and Rothberg, 2004). Moreover colorimetric assay this way rendered the results observable without instrumentation. The original platform depended on the electrostatic property different between ssDNA (primers without PCR products) and dsDNA (eg the PCR products). The structure of ssDNA allows its configuration to uncoil and expose its bases, whereas that of dsDNA has a stable double-helix geometry, always presenting the negatively charged phosphate backbone (Bloomfield et al., 1999). The stabilized Gold tended to adsorb negative ions whose repulsion prevents the strong van der Waals attraction between the particles from causing them to aggregate (Hunter, 2001). Repulsion between the charged phosphate backbone of dsDNA and the adsorbed citrate ions dominates the electrostatic interaction resulting

in unabsorbing on the gold surface. Adsorption of ssDNA stabilizes the gold nanoparticles against salt induced aggregation and the gold colloid remained ruby red but was not the case for dsDNA, where salt induced aggregation occurred and the gold colloid turned purple.

Recently, Fu et al. attempted to combine DNA signals detection using modified gold nanoparticles strategy via the thiolized primer molecule. When the 5' thiol labeling of oligonucleotide primer was used for *hly* gene amplification in the PCR platform, the result of amplified DNA products yielded the DNA with thiol groups at 5' terminus. The obtaining molecules then attached to the particle via the strong interaction between thiol groups and gold creating created thick barriers containing a chain of negative charges that protected the gold nanoparticles from salt-induced aggregation.

However, the above functionalization of the gold with thiol group was complicated laborious and had problem with stability. Moreover, the functionalization efficiency was partially dependent on the reaction conditions, i.e., the pH, electrolyte concentration, heat and magnetic field (Zhou et al., 2009). These obstacles limited the applicability for on-site testing.

This study, short oligonucleotides (30 nucleotides) were used as oligoprobes and performed a colorimetric assay based on non-functionalized, blue-colored silver nanoplates (AgNPls). The use of non-functionalized nanoparticles avoided the problems associated with thiol-group labeling, while blue AgNPls were used instead of gold nanoparticles because they provided long-term stability during a year of storage, unlike the 1-2 month stability of gold nanoparticles. These particles also had a 100 times greater molar extinction coefficient than gold, thus increasing the sensitivity of the assay (Kreibig and Genzel, 1985). The blue AgNPls also provided more clearly visible signals upon aggregation of them, transitioning from blue to colorless, than did the common yellow AgNPls used by Thomson's group, which switched from pale yellow to pale pink (Thompson et al., 2008). The colorimetric change in the blue AgNPls could be observed by UV–vis spectroscopy at wavelengths from 400-800 nm (Figure 4.20). Color changes in the AgNPl solution were observed in *L. monocytogenes*-positive samples but not in samples lacking *L. monocytogenes.*

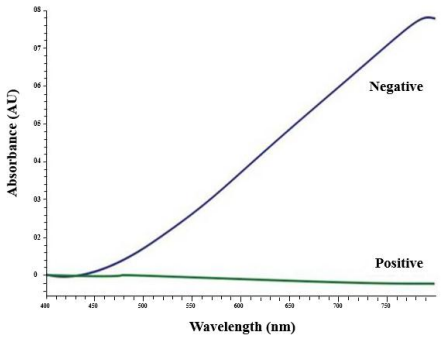


Figure 4.20 Absorption spectra of the aggregated blue AgNPls (positive) and dispersed blue AgNPls (negative) by UV-vis spectroscopy at wavelengths from 400-800 nm.

The effect of signal detection was based on the control of particle dispersion and aggregation using target DNA products and oligonucleotide probe. When oligonucleotide probes was added at the first step to the diluted HDA products and performed denaturation by heat incubation at 65°C for 5 min, the binding of HDA products with probes would result in two different conditions.

For positive HDA sample, a large amount of target double stranded DNAs of *hly* gene, produced by HDA technique had opened up a lot of target binding domains which attracted the affinity and consumed large amount of probes. Shorter oligonucleotide had tendency to bind to target DNA before the longer DNA counterparts. These hybridizations affected the available amount of single strand oligoprobes in the system to less molar level. In general, single strand oligoprobes tended to bind dispersedly on surface of AgNPls via nitrogenous base/silver van der Waals forces and protected AgNPls from salt induced aggregation.[44] When the probes were totally consumed through the nucleic acid hybridization as that found in *hly* gene of HDA positive case, there were less available probes to protect nanoplates' aggregation in the second step, thus, resulting in the stacking of plates after adding of salt which turning color of colloid AgNPls from blue to pale gray to colorless at the end. These color changes could be visualized by naked eye. In negative HDA sample, there was no DNA amplification and no related products available at all. Then, the adding of the diluted HDA products in the second step did not interfered with the molar amount of single strand oligoprobes in the system, leading to a complete protection of AgNPls from aggregation and inducing no such a color change in fugure 4.21. In the test, with 1 µl of diluted HDA products, a suitable amount of probe was at

10 pmole, the amount of blue AgNPls (200 ppm) was at 15 µl and the amount of salt (100 mM PBS pH 7.0) solutions was 1.5 µl. These rendered particles aggregation which in turn provided a colorimetric change of AgNPls' colloid from blue to pale gray to colorless in positive, while no such a change was observed in non-aggregated negative one. Thus, the positive DNA of *hly* gene were observed in aggregated (pale gray) of the Blue AgNPls and the negative DNA were still observed in blue color of non-aggregation blue AgNPls because of single strand of oligonucleotide probes prevented blue AgNPls from aggregation in high saline solutions.

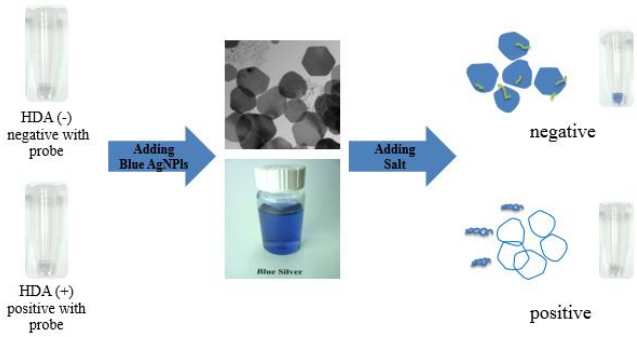


Figure 4.21 DNA signal detection based on the control of particle dispersion and aggregation using HDA products and oligonucleotide probe for differentiating between single stranded and double stranded oligonucleotides.

Similarly, when oligonucleotide probes was added at the first step to the diluted LAMP products and performed denaturation by heat incubation at 65°C for 5 min, the binding of LAMP products with probes would result in two different conditions.

In positive LAMP sample, a large amount of several inverted repeats of target double stranded DNAs of *hly* gene, produced by LAMP had opened up a lot of target binding domains which attracted the affinity and consumed large amount of probes. Shorter oligonucleotide had tendency to bind to target DNA before the longer DNA counterparts. These hybridizations affected the available amount of single strand oligoprobes in the system to less molar level. In general, single strand oligoprobes tended to bind dispersedly on surface of AgNPls via nitrogenous base/silver van der Waals forces and protected AgNPls from salt induced aggregation. When the probes were totally consumed through the nucleic acid hybridization as that found in hly LAMP positive case, there were less available probes to protect nanoplates'

aggregation in the second step, thus, resulting in the stacking of plates after adding of salt which turning color of colloid AgNPls from blue to pale gray to colorless at the end. These color changes could be visualized by naked eye. In negative LAMP sample, there was no DNA amplification and no related products available at all. Then, the adding of the diluted LAMP products in the second step did not interfered with the molar amount of single strand oligoprobes in the system, leading to a complete protection of AgNPls from aggregation and inducing no such a color change in figure 4.22. In the test, with 1 µl of diluted LAMP products, a suitable amount of probe was at 10 pmole, and the amount of blue AgNPls (200 ppm) was at 15 µl and the amount of salt (100 mM PBS pH 7.0) solutions was 1.5 µl These rendered particles aggregation which in turn provided a colorimetric change of AgNPls' colloid from blue to pale gray to colorless in positive, while no such a change was observed in non aggregated negative one.

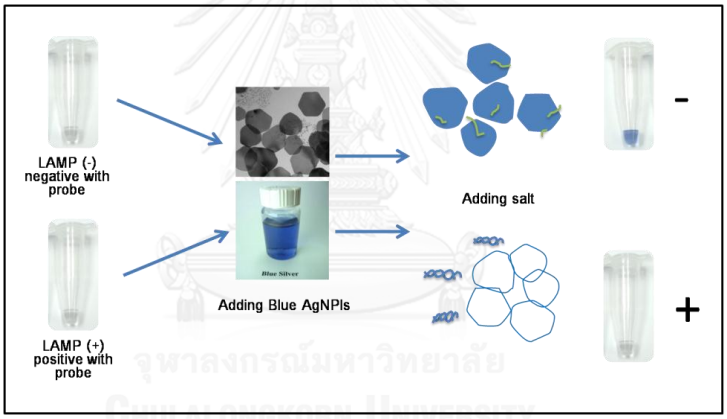


Figure 4.22 DNA signal detection based on the control of particle dispersion and aggregation using LAMP products and oligonucleotide probe for differentiating between single stranded and double stranded oligonucleotides.

The specificity and sensitivity of the assay via colorimetric assay using AgNPls also agreed with those obtained by gel electrophoresis. However, it should be noted that in the case of lower level of DNA template (diluted DNA template less than 100 copies), the test provided less amplification, which produced insufficient DNA products to induce nanoplate aggregation. The sensitivity test was also carried out using living bacteria as the template. Result on limit of detection via both nanoplate aggregation and gel electrophoresis was also observed when living bacteria were diluted to 100 CFU.

To evaluate the liability of the method, HDA and LAMP method with colorimetric detection was employed for the 120 samples of seafood product test. All bacteria were directly detected using HDA and LAMP method with specific designed primer and colorimetric detection base on blue AgNPls. All samples of seafood product were negative for *hly* gene of *L. monocytogenes*. The detection step of colorimetric detection exhibited blue color in of blue AgNPls. All the sample were assayed for *hly* gene, 40 samples were obtained with spiking of known *L. monocytogenes* concentration and 60 samples were obtained without *L. monocytogenes* contamination. PCR method was used as standard reference. The spiking concentration of *L. monocytogenes* was near the limit detection. The assay using PCR and HDA with AgNPls were shown in the table 4.2. The specificity and sensitivity detection were calculated as 100%, thus this assay is a good candidate for screening and monitoring bacterial contamination in the field (Chaumpluk and Chaiprasart, 2013).

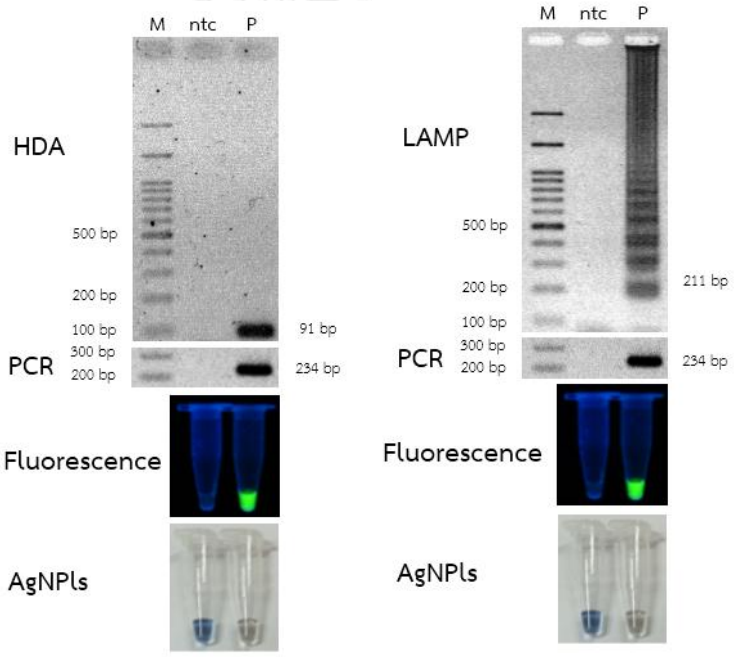


Figure 4.23 The DNA products of *hly* gene using HDA and LAMP method compared the products from the amplification of *hly* gene by PCR method (Furrer et al., 1991) with specific primers and detected based on agarose gel electrophoresis, fluorescent detection and blue silver nanoplates. Lane M, 100 base pair DNA ladder; Lane ntc, non-template control; Lane P, Positive DNA from *L. monocytogenes*.

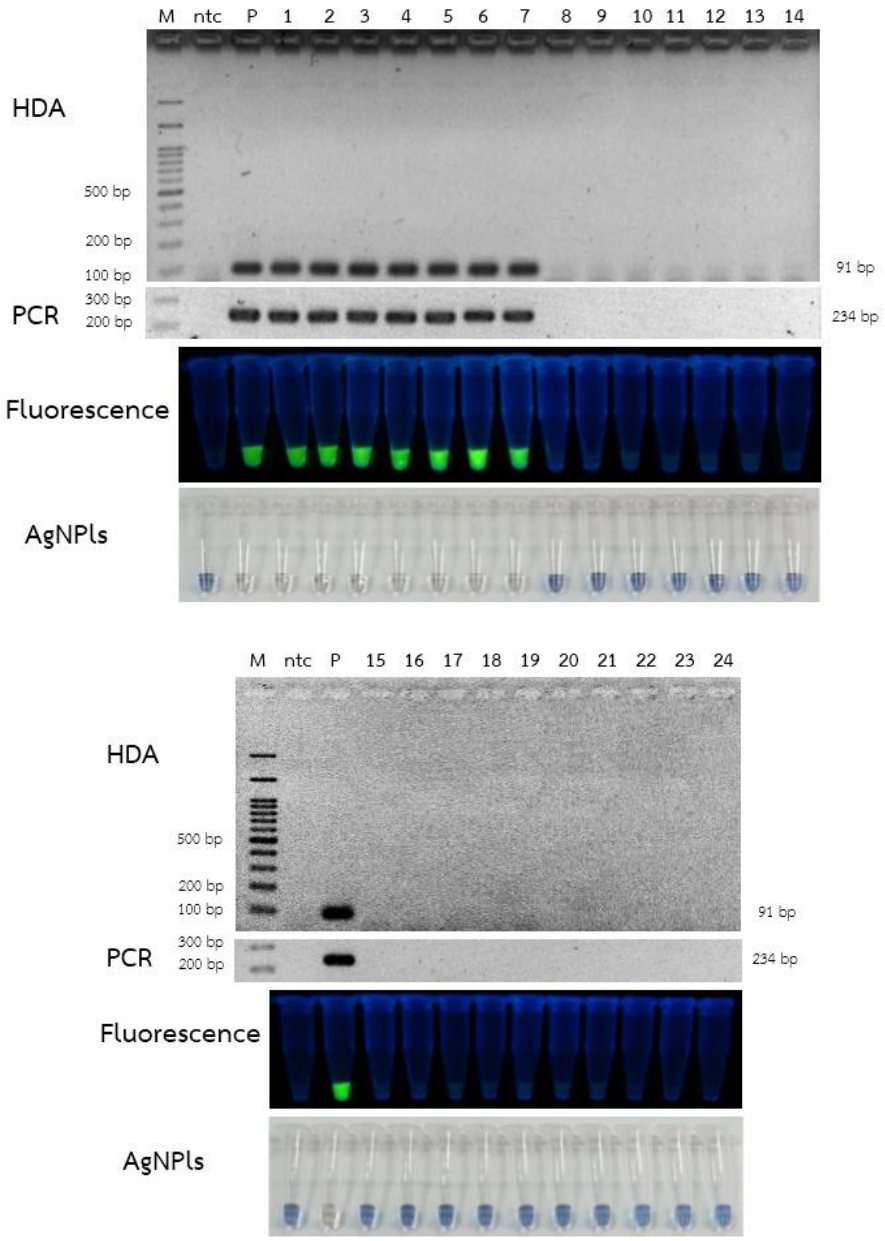


Figure 4.24 The specificity of HDA method for detection of *L. monocytogenes* compared with PCR method (Furrer et al., 1991) with specific primers and detected based on agarose gel electrophoresis, fluorescent detection and blue silver nanoplates. Lane M, 100 base pair DNA ladder; Lane ntc, non-template control; Lane P, positive DNA from *L. monocytogenes*, Lane 1-7; *L. monocytogenes* from difference source, Lane 8-11, four strains of *L. innocua*; Lane 12-13, two strains of *L. ivanovii*; Lane 14-24, *L. welshimeri*; Vibrio cholera; *V. parahaemolyticus*; *Salmonella enteritidis*; *Escherichia coli* O157:H7; *E. coli* (ETEC); *E. coli* (EPEC); *Pseudomonas putida*; *Shigella flexneri*; *Campylobacter jejuni* and *Staphylococcus aureus* respectively.

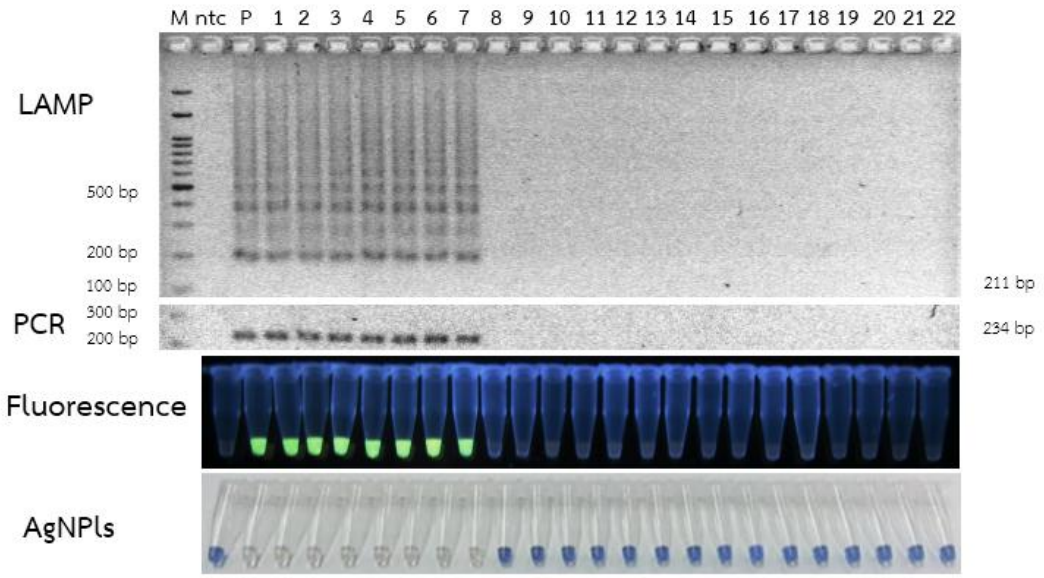


Figure 4.25 The specificity of the LAMP reaction for *L. monocytogenes* compared with that of PCR method (Furrer et al., 1991) with specific primers and detected based on agarose gel electrophoresis, fluorescent detection and blue silver nanoplates. Lane M, 100 base pair DNA ladder; Lane ntc, Non-template control; Lane P, *L. monocytogenes* plasmid; Lane 1-7, seven strains of *L. monocytogenes* DNA; Lane 8-11, four strains of *L. innocua*; Lane 12-13; two strains of *L. ivanovii*; Lane 14-22, DNA from *L. welshimeri*; *Vibrio cholera; V. parahaemolyticus; Salmonella enteritidis; Escherichia coli O157:H7; E. coli ETEC; E. coli EPEC; Pseudomonas putida* and *Shigella flexneri*.

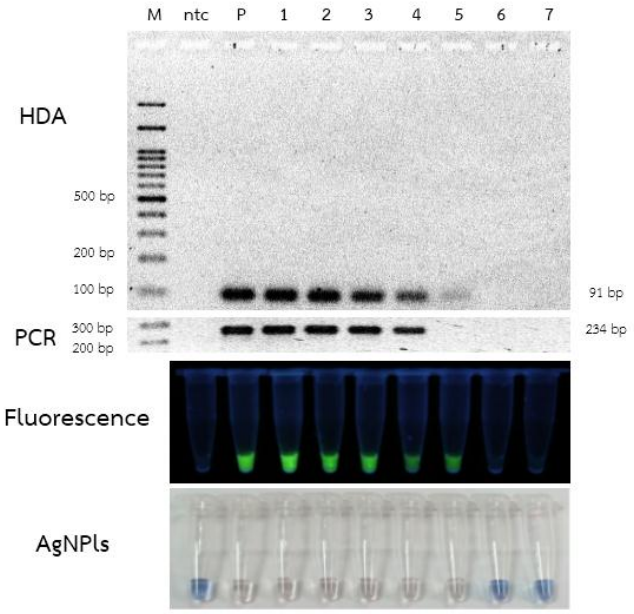


Figure 4.26 The sensitivity of HDA method for detection of *L. monocytogenes* compared with PCR (Furrer et al., 1991) with specific primers and detected based on agarose gel electrophoresis, fluorescent detection and blue silver nanoplates. Lane M, 100 base pair DNA ladder; Lane ntc, non-template control; Lanes 1-8, ten fold serial dilutions of *L. monocytogenes* template DNA, ranging from Lane 1, 10^7 copies; Lane 2, 10^6 copies; Lane 3, 10^5 copies; Lane 4, 10^4 copies; Lane 5, 10^3 copies; Lane 6, 10^2 copies; Lane 7, 10 copies and Lane 8, 0 copies.

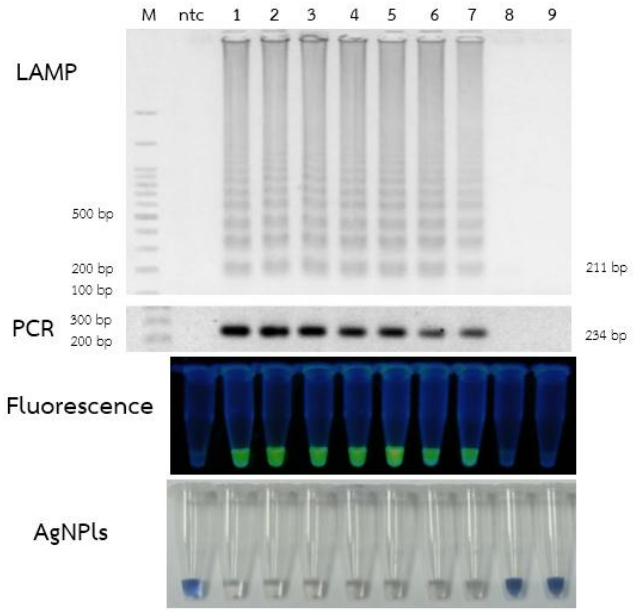


Figure 4.27 The detection limit of the LAMP reaction using DNA as the template compared with that of PCR (Furrer et al., 1991) with specific primers and detected based on agarose gel electrophoresis, fluorescent detection and blue silver nanoplates. Lane M, 100 base pair DNA ladder; Lane ntc, non-template control; Lanes 1-9, 10-fold serial dilutions of *L. monocytogenes* template DNA, ranging from 10^8 copies to 0 copies.

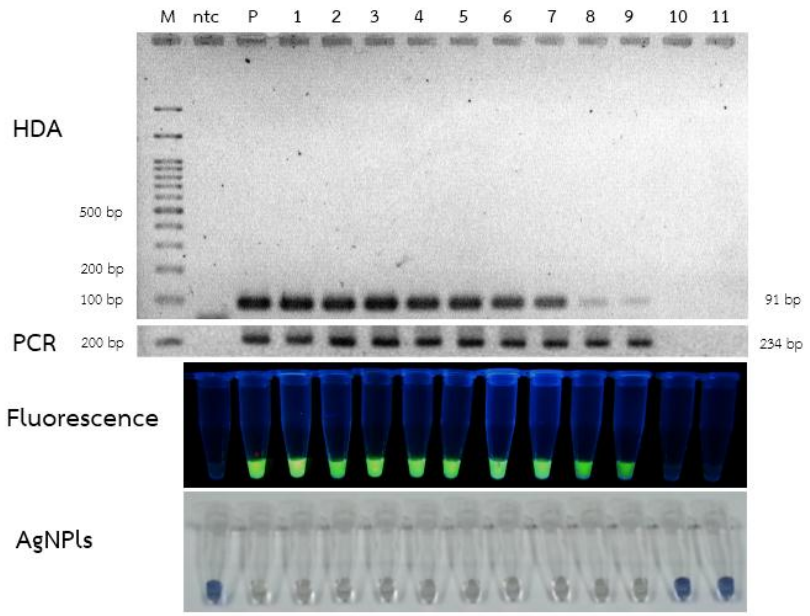


Figure 4.28 The detection limit by spiking of the seafood sample with *L. monocytogenes* diluents using HDA method compared with PCR (Furrer et al., 1991) with specific primers and detected based on agarose gel electrophoresis, fluorescent detection and blue silver nanoplates. Lane M, 100 base pair DNA ladder; Lane ntc, non-template control; Lane P, *L. monocytogenes* plasmid, Lane 1-5, from 10^{10} to 10^{6} CFU, Lane 6, 1.02 x 10^{5} CFU, Lane 7, 10250 CFU, Lane 8, 1033 CFU, Lane 9, 100 CFU, Lane 10, 7 CFU and Lane 11, 0 CFU, respectively.

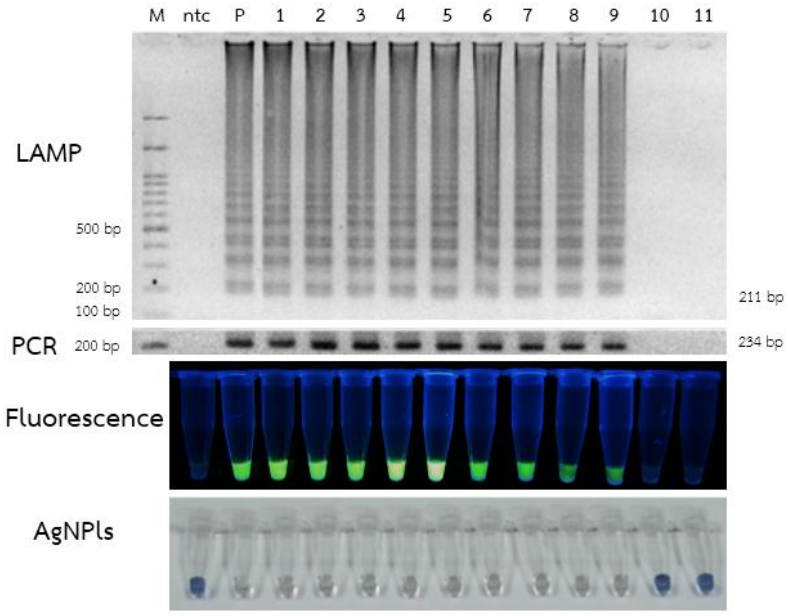


Figure 4.29 The detection limit by spiking of the seafood sample with *L. monocytogenes* diluents using LAMP method compared with PCR (Furrer et al., 1991) with specific primers and detected based on agarose gel electrophoresis, fluorescent detection and blue silver nanoplates. Lane 1-5, from 1010 CFU to 106 CFU, Lane 6, 1.02 x 105 CFU, Lane 7, 10250 CFU, Lane 8, 1033 CFU, Lane 9, 100 CFU, Lane 10, 7 CFU and Lane 11, 0 CFU, respectively. Lane M, 100 base pair DNA ladder; Lane ntc, non-template control and Lane P, *L. monocytogenes* plasmid.

4.4 Strain differentiation via point mutation detection

In the past PNA had been used to block initiative of prime amplification. The process was called PNA based PCR clamping. Its principle relied on a amplification only at the mutated target DNA sequence as a minor portion in the mixture with the major wild type DNA sequences. It takes full advantages of the nature of PNA probes that has strong binding affinity and specificity to its target DNA and not being recognized by DNA polymerase as primer.

In the study, DNA amplification with HDA could be performed to both wild type and mutant strain L312. Both delivered DNA products at 91 nucleotides in size as well as the fluorescence signals when observed under UV light. The use of LAMP primer also could not discriminate both strains from each other. Thus, LAMP was still provided with a unique ladder of DNA products as well as the fluorescence signals when observed under UV light similar to those observed via HDA.

However, when PNA clamp specific with wild type target was used, mutant strain could be amplified by both HDA and LAMP platform while the wild type one could not. When DNA signals were identified via fluorescence platform developed in this study, only mutant strain could rendered a fluorescence signal observable with naked eye (Figure 4.30 and 4.31).

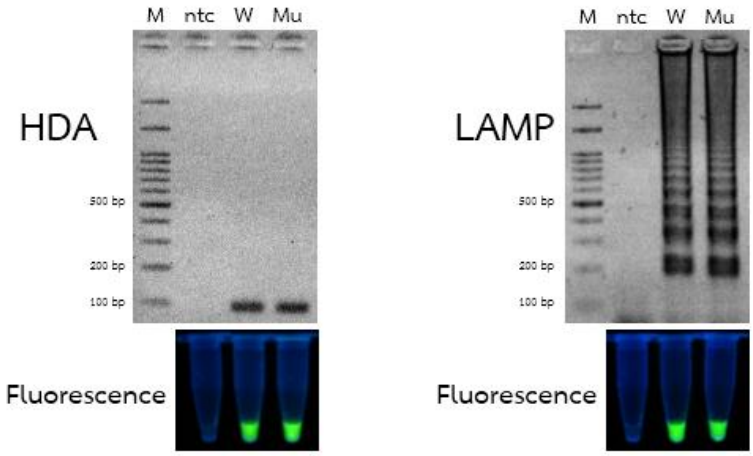


Figure 4.30 DNA amplification without PNA clamp specific with wild type platform

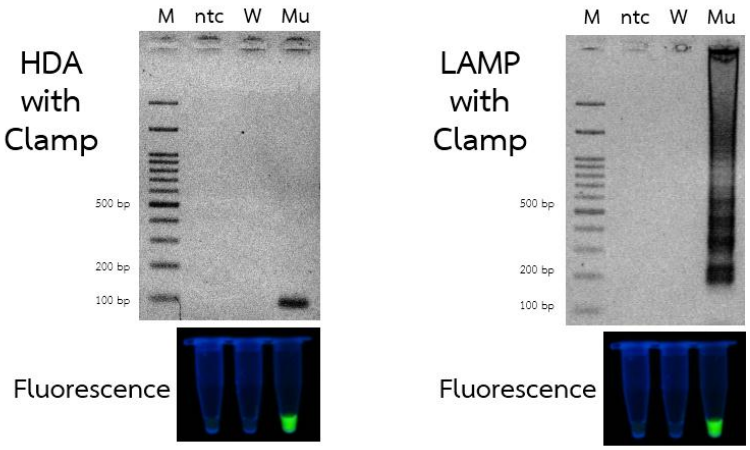


Figure 4.31 DNA amplification with PNA clamp specific with wild type platform

This was the first time that PNA clamp was used to detect mutant in Listeria detection using HDA platform. The merits of employing PNA clamp in this study were a rapidity of the test in short period of time with simple protocol, a requirement of only small amount of DNA sample needed for detection and its high sensitivity.

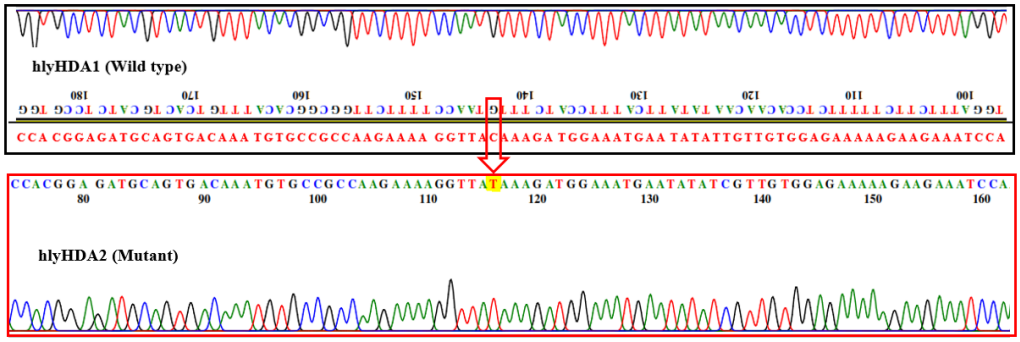


Figure 4.32 Nucleotide sequence of *hly* gene via wild type and mutant type by DNA sequencing

4.5 Validation of the test

To evaluate the liability of the method, HDA and LAMP were employed for the 100 samples of seafood product test. All bacteria were directly detected using HDA and LAMP method with specific designed primer. All samples of seafood product were negative for *hly* gene of *L. monocytogenes*. All the sample were assayed for *hly* gene, 40 samples were obtained with spiking of known *L. monocytogenes* concentration and 60 samples were obtained without *L. monocytogenes* contamination. PCR method was used as standard reference. The spiking concentration of *L. monocytogenes* was near the limit detection. The assay using PCR, HDA and LAMP with fluorescence were shown in the table 4.2. The specificity and sensitivity detection were calculated as 97.6 and 96.8%, respectively. Therefore, this assay is a good candidate for screening and monitoring bacterial contamination in the field (Chaumpluk and Chaiprasart, 2013). Furthermore, the advantages of PCR, HDA and LAMP method were compared (Table 4.3). In term of primer and amplification condition, HDA method is very similar to that of PCR and required only 2 primers for DNA amplification. But LAMP method required 4 to 6 primers and complex design.

Table 4.2 The comparison of PCR with HDA and LAMP detection

Specimens	PCR	HDA	LAMP
Positive	40	39	39
Negative	60	58	59
False positive	-	2	1
False negative	-	1	1
Sensitivity [A]		97.6	97.5
Specificity [B]		96.8	98.3

A; Sensitivity = 100x true positive/(true positive + false negative)

B; Specificity = 100x true negative/(false positive + true negative)

Table 4.3 Merits of HDA method compared with LAMP and PCR

	PCR	HDA	LAMP
Temperature	thermocycler	isothermal	isothermal
Time	3-4 hours	90 min	40 min
Cost	Reagent + equipment	Reagent w/o equipment	Reagent w/o equipment
Product	10^7	10^8-10^9	10^{10}-10^{15}
Primer	2 primers	2 primers	4-6 primers
Sensitivity	1000 CFU	100 CFU	100 CFU
Design primer	simple	simple	complex

Finally, one hundred and twenty seafood samples from supermarket and local market (including frozen tuna, salmon, and shrimp) were also tested using both PCR and our assay. The results confirmed that *L. monocytogenes* contamination was present in 3 samples of seafood from the local market (data not shown). This test provided a visual readout based on fluorescence visualization and analog probe hybridization. Although this assay is qualitative, the total cost was as low as approximately US$ 5 per test, and the entire procedure required less than 4.5 hours to complete (3.5 hours for enrichment and 40 min for the *L. monocytogenes* assay). This test provides an alternative for monitoring this pathogen in the field, especially for food quality and safety purposes.

CHAPTER V

CONCLUSION

The isothermal DNA amplification via Helicase Dependent Amplification (HDA) and loop-mediated isothermal DNA amplification (Schmid et al.) and DNA signal detection base on fluorescence visualization, blue silver nanoplates (AgNPls) and analog probe hybridization were successfully established for the rapid detection of *hly* gene from *Listeria monocytogenes* in seafood. In this study DNA amplification using LAMP was compared with HDA, in order to prove the efficiency of amplification and detection system. Result revealed that HDA technique that required less primers (only two primers) and run on the same principle with PCR had high efficiency for Listeria assay with its sensitivity at 97.6%, comparable with that of LAMP technique (97.5%).

The specific designed primer of *hly* gene provided a merit of detection of only *L. monocytogenes* but not the rest of others in the same genus. The amplification using HDA was better sensitivity than PCR but equally to LAMP with the limit of detection of *hly* gene at 100 copies. DNA products when assayed via fluorescent detection on FRET technology and analog DNA probe provided a way to discriminate mutant from wild type. This enabled the method to be applied to detect SNPs of *hly* gene between wild type and mutant from *L. monocytogenes* with naked-eyed. Surveillance test revealed all samples of seafood from supermarkets and local markets were contaminated with foodborne pathogen as *L. monocytogenes.* The three samples from the local market were contaminated.

The HDA assay provided 96.8% specificity and 97.6% sensitivity. And it could be checked simply via fluorescence illumination under UV light. The total cost of the developed assay was about 5US$ per test and the process required 4.5 hours to complete the whole assay including enrichment process.

APPENDIX

Luria Broth

1. Add the following to 1,000 ml of distilled H_2O

   Yeast extract 5.0 g

   Tryptone 10.0 g

   NaCl 5.0 g

2. Adjust to pH 7.5

LM

1. Add the following to 1,000 ml of distilled H_2O

   Yeast extract 5.0 g

   Tryptone 10.0 g

   NaCl 0.5 g

   Agar 20.0 g

2. Adjust to pH 7.5

   Adding $MgSO_4$ 1.5 ml/150 ml of LM and antibiotic 300 µl before used

SOC medium

1. Add the following to 900 ml of distilled H_2O

   Bacto Tryptone 20.0 g

   Bacto Yeast Extract 5.0 g

   5M NaCl 2.0 ml

   1M KCl 2.5 ml

   1M $MgCl_2$ 10.0 ml

   1M $MgSO_4$ 10.0 ml

   1M glucose 20.0 ml

2. Adjust to 1L with distilled H_2O

3. Sterilize by autoclaving

Terrific broth

1. Add the following to 800 ml distilled H_2O

   Tryptone 12.0 g

   Yeast extract 24.0 g

   Glycerol 4.0 ml

2. Adjust to 900 ml with distilled H_2O
3. Sterilize by autoclaving
4. Allow to cool to room temperature
5. Adjust volume to 1,000 ml with 100 ml of a filter sterilized solution of 0.17 M KH_2PO_4 and 0.72 M K_2HPO_4

Potassium solution is made by dissolving 2.31 g of KH_2PO_4 and 12.54 g of K_2HPO_4 in 90 ml of H_2O. After the salts have dissolved, adjust the volume of the solution to 100 ml with H_2O and sterilize by autoclaving

DNA Transformation

1. Add 60 µl of competent cell into microfuge tube containing 5 µl of DNA. Keep on ice for 15 minutes.
2. Heat shock at 42°C in water bath and soak a DNA for 45 seconds and immediately keep on ice for 15 minutes.
3. Add 800 µl of SOC medium and homogenize the mixture. Incubate at 37°C for 2-3 hours in incubator shaker.
4. Pipette the mixture and spread it onto medium containing Ampicillin and $MgSO_4$.
5. Incubate the bacterial culture plate at 37°C for 16-18 hours.
6. Observe the growth of bacterial colony and record it.

Plasmid DNA extraction

1. Culture the bacteria in tube containing 3.0 ml of LB medium. Incubate at 37°C for 16-18 hours.
2. Pipette all of bacteria culture into microfuge tube and centrifuge at 6,000 rpm for 2 minutes and discard the supernatant.
3. Add 100 µl of Lysozyme solution and vortex.
4. Add 200 µl of Alkaline solution (2N NaOH: 10%SDS: DW) and keep on ice for 5 minutes.
5. Add 150 µl of KOAC and invert the tube 2-3 times and keep on ice for 15 minutes.
6. Centrifuge at 12,000 rpm for 5 minutes and pipette the supernatant in new tube.
7. Add Phenol:Chloroform (v/v) and vortex.
8. Centrifuge at 12,000 rpm for 5 minutes and pipette the supernatant in new tube.
9. Add propanal (v/v), keep it at RT 15 minutes. Centrifuge at 12,000 rpm for 10 minutes.
10. Discard the propanol solution and wash the pellet with 1 ml of 70% Ethanol. Centrifuge at 12,000 rpm for 10 minutes and discard the Ethanol solution.
11. Dry the pellet using Lyophilizer machine and 30 µl of TE-RNase buffer.
12. Incubate at 37°C for 5 minutes.
13. Keep it at -20°C for long-term maintenance. Check the plasmid by loading on (1%) agarose gel electrophoresis.

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