การพัฒนาอุปกรณ์ฐานกระดาษสำหรับการตรวจหาดีเอ็นเอของ *Vibrio parahaemolyticus* จากลูปเมดิเอเตดไอโซเทอร์มอลแอมพลิฟิเคชัน

นางสาวจุฑาภรณ์ ทิพโชติ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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DEVELOPMENT OF PAPER-BASED DEVICES FOR DETECTION OF *Vibrio parahaemolyticus* DNA FROM LOOP-MEDIATED ISOTHERMAL AMPLIFICATION

Miss Jutaporn Tipchote

A Thesis Submitted in Partial Fulfillment of the Requirements
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Thesis Title	DEVELOPMENT OF PAPER-BASED DEVICES FOR DETECTION OF *Vibrio parahaemolyticus* DNA FROM LOOP-MEDIATED ISOTHERMAL AMPLIFICATION
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จุฑาภรณ์ ทิพโชติ : การพัฒนาอุปกรณ์ฐานกระดาษสำหรับการตรวจหาดีเอ็นเอของ *Vibrio parahaemolyticus*จากลูปเมดิเอเตดไอโซเทอร์มอลแอมพลิฟิเคชัน (DEVELOPMENT OF PAPER-BASED DEVICES FOR DETECTION OF *Vibrio parahaemolyticus* DNA FROM LOOP-MEDIATED ISOTHERMAL AMPLIFICATION) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ศ. ดร. อรวรรณ ชัยลภากุลPh.D., อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ. ดร. นาตยา งามโรจนวณิชย์Ph.D., นาง วรรณสิกา เกียรติปฐมชัยM.Sc, หน้า.

Vibrio parahaemolyticus (*V. parahaemolyticus*) เป็นแบคทีเรียแกรมลบ ลักษณะรูปร่างเป็นแท่ง สามารถเจริญเติบโตได้ในสภาวะที่เป็นเกลือ เป็นเชื้อที่พบได้ในอาหารทะเล หากบริโภคอาหารทะเลดิบหรือปรุงไม่สุก และมีการปนเปื้อนของ *V. parahaemolyticus* จะทำให้เกิดโรคในทางเดินอาหาร เช่น ลำไส้อักเสบ ท้องเสีย เป็นต้น งานวิจัยนี้ได้ใช้เทคนิคการตรวจวัดด้วยสีบนอุปกรณ์ฐานกระดาษในการตรวจวัดดีเอ็นเอของ *V. parahaemolyticus* ที่เพิ่มปริมาณดีเอ็นเอโดยเทคนิคลูปเมดิเอทเตดไอโซเทอร์มอลแอมพลิฟิเคชั่น การเพิ่มปริมาณดีเอ็นเอของ *V. parahaemolyticus* ด้วยเทคนิคลูปเมดิเอทเตดไอโซเทอร์มอลแอมพลิฟิเคชั่น มีการใช้ไพรเมอร์ 6 ชุด ที่จำเพาะต่อดีเอ็นเอของ *V. parahaemolyticus* เพิ่มปริมาณดีเอ็นเอโดยใช้อุณหภูมิ 65 องศาเซลเซียส ใช้เวลา 45 นาที จากนั้นตรวจวัดดีเอ็นเอของ *V. parahaemolyticus* ที่เพิ่มปริมาณขึ้นโดยเทคนิคการตรวจวัดด้วยสีด้วยอนุภาคเงินขนาดนาโน (สีเหลือง) บนอุปกรณ์ฐานกระดาษ พบว่าเมื่อมีดีเอ็นเอ สีของอนุภาคเงินขนาดนาโนไม่เกิดการเปลี่ยนแปลงสี แต่เมื่อไม่มีดีเอ็นเอ สีของอนุภาคเงินขนาดนาโนเปลี่ยนจากสีเหลืองไปเป็นสีแดง ซึ่งผลการทดลองสามารถตรวจสอบได้ด้วยตาเปล่า ขีดจำกัดของการตรวจวัดด้วยเทคนิคการตรวจวัดด้วยสีบนอุปกรณ์ฐานกระดาษอยู่ที่ 11.15 จำนวนโคโลนีต่อมิลลิลิตร เทคนิคการตรวจวัดด้วยสีบนอุปกรณ์ฐานกระดาษสามารถตรวจวัด *V. parahaemolyticus* ในตัวอย่างอาหารทะเล (กุ้ง หอยนางรม) ได้ เมื่อเปรียบเทียบผลการตรวจวัด *V. parahaemolyticus* โดยเทคนิคการตรวจวัดด้วยสีบนอุปกรณ์ฐานกระดาษ กับเทคนิคการเลี้ยงเชื้อ *V. parahaemolyticus* บนอาหารเลี้ยงเชื้อ และเทคนิคเจลอิเล็คโตรโฟเรซิส ซึ่งเป็นวิธีมาตรฐาน ให้ผลที่ถูกต้องสอดคล้องกัน ดังนั้นเทคนิคการตรวจวัดด้วยสีบนอุปกรณ์ฐานกระดาษเป็นเทคนิคการตรวจวัดที่รวดเร็ว สามารถทำได้ง่าย ตรวจวัดได้รวดเร็ว และเป็นเทคนิคที่มีราคาถูกสำหรับการตรวจวัด *V. parahaemolyticus*

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JUTAPORN TIPCHOTE: DEVELOPMENT OF PAPER-BASED DEVICES FOR DETECTION OF *Vibrio parahaemolyticus* DNA FROM LOOP-MEDIATED ISOTHERMAL AMPLIFICATION. ADVISOR: PROF. DR. ORAWON CHAILAPAKUL, Ph.D., CO-ADVISOR: ASSOC. PROF. NATTAYA NGAMROJANAVANICH, Ph.D., MRS. WANSIKA KIATPATHOMCHAI, M.Sc, pp.

Vibrio parahaemolyticus (*V. parahaemolyticus*) is a gram-negative, halophilic marine bacterium and marine seafood-borne pathogen causing gastrointestinal disorders to humans. In this work, colorimetric paper-based device was employed for the determination of *V. parahaemolyticus* DNA amplified by loop-mediated isothermal amplification (LAMP). LAMP was performed using a set of six specially designed primers which is specific to *V. parahaemolyticus* DNA. The optimal isothermal amplification was at 65°C for 45 min. After DNA amplifyed by LAMP, colorimetric assay was carried out on paper-based device for DNA determination using silver nanoparticles (AgNPs) as colorimetric agent. It was found that the color of AgNPs did not change in the presence of amplified DNA, whereas color of AgNPs changed from yellow to red in the presence of unamplified DNA. The results of the assay can be easily evaluated by naked eyes. The limit of detection (LOD) was found to be 11.15 CFU mL^{-1}. Therefore, the developed method was used for the determination of *V. parahaemolyticus* DNA in seafood (shrimps and oysters). Comparison of the results obtained from this proposed method to those obtained by the plate count method (TCBS) and LAMP-gel electrophoresis proved that this developed method has good agreement with the conventional method in terms of accuracy for practical applications. Hence, LAMP-AgNPs on PADs serves as a rapid, inexpensive and simple assay without the need for complicated instrumentation.

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

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LIST OF ABBREVIATIONS

°C	degree Celsius
µL	Microlitre
Ag^+	Silver ion
$AgNO_3$	Silver nitrate
AgNPs	Silver nanoparticles
AuNPs	Gold nanoparticles
B3	Backward outer primer
BIP	Backward internal primer
BLP	Backward loop primer
CFU	Colony-forming unit
DNA	Deoxyribonucleic acid
dNTP	Deoxy nucleotide
EtBr	Ethidium bromide
F3	Forward outer primer
FIP	Forward internal primer
FLP	Forward loop primer
gPOCT	Gene point-of-care testing
H_2O_2	Hydrogen peroxide
HNB	Hydroxy naphthol blue
LAMP	Loop-mediated isothermal amplification
LB	Loop backward primer
LDR	Linear dynamic range
LF primer	Loop forward primer
LFD	Lateral flow dipstick
LOD	Limit of detection
LOQ	Limit of quantification

Mg^{2+}	Magnesium ion
$Mg_2P_2O_7$	Magnesium Pyrophosphate
$MgSo_4$	Magnesium sulfate
NAAT	Nucleic acid amplification test
$NaBH_4$	Sodium borohydride
NaOH	Sodium chloride
NAT	Nucleic acid test
NIH	National Institution of Health
nm	Nanometers
$P_2O_7^{4-}$	Pyrophosphate ion
PADs	Paper-based devices
PCR	Polymerase chain reaction
PEI	Polyethylene imine
RNA	Ribonucleic acid
TCBS	Thiosulfate-citrate-bile salts-sucrose agar
TDH	Thermostable direct hemolysin
TEM	Transmission electron microscopy
TLH	Thermolabile hemolysin
TRH	TDH-related hemolysin
Tris-HCL	Tris hydrochloride
UV	Ultraviolet

CHAPTER I
INTRODUCTION

1.1 Introduction

Vibrio parahaemolyticus or *V. parahaemolyticus* is a halophilic gram-negative bacterium that widely distributed in the marine environment. It is a violent foodborne pathogen that can cause gastrointestinal disorders in humans [1]. The virulent strains infect through the intake of contaminated seafood. In tropical and subtropical areas, *V. parahaemolyticus* can be isolated from seawater and seafood throughout the year [2]. Therefore, it has been considered a major cause of infectious diarrhea particularly in southern and eastern Asian countries. From above, determination of *V. parahaemolyticus* must be greatly concerned. Classical methods for detection this bacterium include biochemical identification [3] and polymerase chain reaction (PCR) [4]; however, these methods have drawbacks such as time-consuming, high operation cost and required complicated instruments and readout processing units. For that reason, the development of analytical method for rapid, cost-effective, and simple measurement of *V. parahaemolyticus* level in the marine products is extremely important.

Recently, nucleic acid test (NAT) or nucleic acid amplification test (NAAT) has been universally used in clinical diagnosis and food controlling for pathogens detection. The amplification increases the amount of nucleic acids by duplicating the specific microorganisms; as a consequence the sensitivity is enhanced. Polymerase chain reaction (PCR) is an example of the most popular techniques for the amplification of nucleic acid, both deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). However, PCR requires expensive instruments and long analysis time (due to the use of thermal cycling) in which limits the techniques in some applications.

There are plenty of methods attempt to overcome these problems. For example, Notomi and co-workers reported the use of loop-mediated isothermal amplification or LAMP for amplification of DNA [5]. LAMP is a novel nucleic acid amplification method for replicating polynucleotide under isothermal conditions. The technique has high specificity, sensitivity, and rapidity. Moreover, this technique needs basic equipment. It can be achieved simply using a water bath or an inexpensive heating block.

Such benefits of LAMP, they are interested in development for various applications. There are several detection methods used for LAMP products. Generally, it has been stained with ethidium bromide and visualized in agarose gel electrophoresis [6]. On the other hands, it can be measured indirectly using the by-product of the LAMP reaction (white precipitates of magnesium pyrophosphate) [4]. Lateral flow dipstick (LFD) format is another example of detection method used to monitor the LAMP products [7]. Currently, colorimetric assay with nanoparticles as colorimetric probe has proved to be an attractive and effective method because the results are visually with naked eyes detection. However, not many applications in nanoparticles for LAMP detection have been developed.

Nowadays, metal nanoparticles-based colorimetric assay has been attached much interest because it is highly sensitive, selective and practical. Particularly, silver nanoparticles or AgNPs, which have high extinction coefficients (higher than gold nanopaticles, AuNPs)[8], have been reported for colorimetric sensors of abundance analytical applications such as determination of heavy metals and quantification of biomarkers. Moreover, they are successfully used as detection method of nucleic acids, proteins, and small molecules. The nanoparticles-colorimetric method needs merely several tenth microliters of both reagents and samples. Therefore, the reaction can be carried out in a small tube or a miniaturized device. In addition, the

cost of vast nanoparticles synthesis per a batch is inexpensive. Together their advantages, nanoparticles have been expanded in large numbers of effort for sensing proposes, particularly for on-site assays [9].

Paper-based devices or PADs are one of miniaturized devices which have been largely applied to food, environmental and clinical applications. It processes several advantages such as ease of use, low sample and reagents consumption, rapidity, disposability, and portability [10]. Most PADs use simple visible colorimetric methods for both qualitative and quantitative detection. PADs can be fabricated from various fabrication techniques such as photolithography, plasma treatment plotting, and cutting [11]. However, some of these techniques have disadvantages, for example complicated procedures, high cost of reagents and instrumentation. Therefore, wax printing is frequently selected as the fabrication method of PADs because it is not only a simple and inexpensive, but also a high throughput method [12]. Patterns of PADs contain two parts; the hydrophilic channel and hydrophobic barriers. The reagents are diffused in the hydrophilic area in which used for separation, pretreatment, and detection. On the other hands, these reagents move to controlled area which limited by hydrophobic barriers. In colorimetric assay, the amount of targets is quantified by measurement of color intensity in a specific part of hydrophilic area that set to be test zone. The modesty of this method remarkably spread PADs into many analytical fields. In this work, colorimetric PADs were developed for LAMP amplifying for *V. parahaemolyticus*. PADs were fabricated using cheap wax printing method. On this device, LAMP products were interacted with AgNPs and produced color change that can be detected by naked-eyes. The quantification was extended by measuring grey intensity of AgNPs after the reaction. The proposed method was compared to other DNA detection methods and finally applied to real seafood samples.

1.2 Objective of the research

There are two main goals for this work including:

1. To amplify *V. parahaemolyticus* DNA using loop-mediated isothermal amplification

2. To develop paper-based devices for detection of amplified *V. parahaemolyticus* DNA

1.3 Scope of research

V. parahaemolyticus DNA was amplified and followed by detection using the aggregation principle of silver nanoparticles. The amount of analytes was amplified by LAMP method in order to increase the sensitivity for detection. Moreover, the reaction was performed on PADs which demonstrating the cost-effective, simple, and uncomplicated platform.

CHAPTER II
THEORY AND LITERATURE REVIEW

2.1 *Vibrio parahaemolyticus*

Vibrio parahaemolyticus or *V. parahaemolyticus* is a small rod gram-negative halophilic marine bacterium that has a single curve to its shape. It exists as either a swimmer cell with a single polar flagellum, or a swarmer cell covered in lateral flagella (Fig. 2.1) [13]. The halophilic nature of this bacterium was revealed in active cultivation media containing sodium chloride. *V. parahaemolyticus* is seafood-borne pathogen causing gastrointestinal disorders in humans. It infects by a consumption of contaminated raw or undercooked seafood [14].

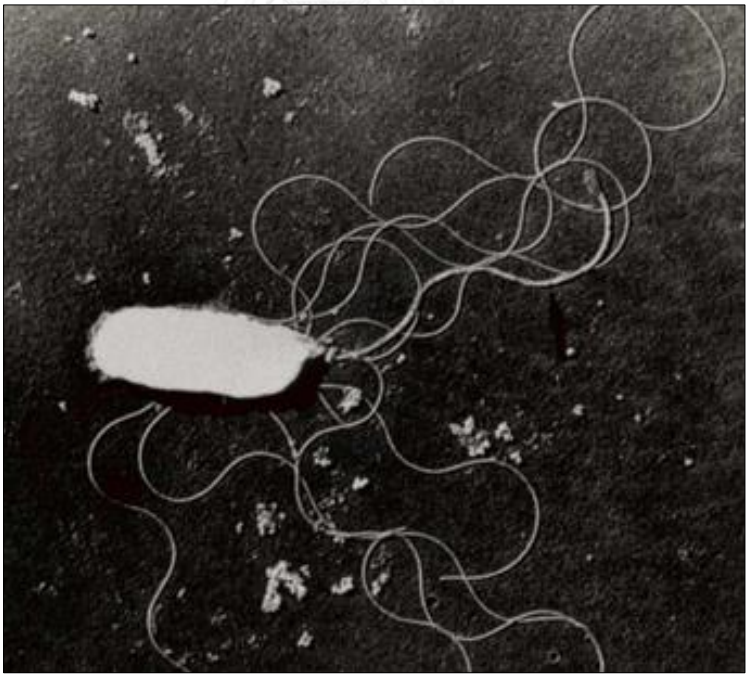

Figure 2.1 Structure of flagella of *V. parahaemolyticus* [15].

Strains of *V. parahaemolyticus* isolated from the environment or seafood are both pathogenic and non-pathogenic bacteria [16]. There are mainly three important strains including thermolabile hemolysin (TLH), thermostable direct hemolysin (TDH), and TDH-related hemolysin (TRH). TDH and TRH are concerned as pathogenic strains that cause stomach and small intestine illness in animals. Especially, TDH is most closely linked to the onset of diseases; therefore it has been extensively studied throughout the time [1]. Accordingly, TDH and TRH have been extensively considered as the major virulence factor of *V. parahaemolyticus* in clinical analysis.

2.1.1 Detection of *V. parahaemolyticus*

Detection of *V. parahaemolyticus* uses conventional culture and biochemical-based assays, which are time-consuming (requiring more than three days) and laborious. The most probable number (MPN) method described in the US Food and Drug Administration Bacterial Analytical Manual (FDA) is commonly used for the detection of *V. parahaemolyticus* in foods [17]. However, the MPN method is time-consuming (4–5 days) and cannot differentiate *V. parahaemolyticus* from some strains. To overcome the disadvantage of MPN method for detecting *V. parahaemolyticus*, Okuda et al. developed polymerase chain reaction (PCR) for detecting virulent strains of *V. parahaemolyticus* using DNA primers targeting TDH and TRH genes [18]. In addition to PCR assays, DNA–DNA hybridization methods were also developed for specific detection of *V. parahaemolyticus* [16].

Lee *et al*. also reported an enzyme-labeled oligonucleotide for detecting both TDH and TRH genes by hybridization [19]. Although PCR and DNA probe methods are available for specific detection of total or virulent *V. parahaemolyticus*, they require special instruments and skilled technicians. Chromogenic medium (Bio-

Chrome Vibrio medium, BCVM) was developed to allow differentiation of *V. parahaemolyticus* from other *Vibrio* species based on formation of unique purple colonies on the medium. Growth of *V. parahaemolyticus* on BCVM can easily be distinguished from *V. vulnificus, V. cholerae*, and *V. mimicus* which presented as blue-green colonies on the growing medium [20].

In 2008 Yamazaki *et al*. reported the use of loop-mediated isothermal amplification (LAMP) method for the identification of pathogenic organisms. It has been developed for the detection of *V. parahaemolyticus* [21]. It was found that LAMP assay is faster and easier to perform than conventional PCR assays, as well as being more specificity [5]. Because the LAMP assay synthesizes a large amount of DNA, the products can be detected by the production of precipitate or turbidity of the reaction [22]. The turbidity of the reaction mixture correlates with the increasing amount of DNA. On that account, expensive equipment is not necessary to give a high level of precision compared to PCR assays. In addition, the preparation steps of the LAMP assay are fewer than conventional PCR and real-time PCR assays, accomplishing shorter analysis time. These features allow simple, rapid, and cost-effective detection of polynucleotide [23].

2.2 Loop-mediated isothermal amplification

Loop-mediated isothermal amplification or LAMP is an alternative technique of rapid DNA amplification within one reaction tube under isothermal conditions. LAMP uses a DNA polymerase which has unique property of specially designed primer set along with the usual polymerization process. This strand displacement which recognizes at least six independent regions of the target gene which increases the specificity of the LAMP. It requires a simple incubator such as heat block to

provide a constant temperature which is a simple assay when compared with polymerase chain reaction (PCR). Apart from that, it has short analysis time, high sensitivity, and can be performed under mild condition [5]. Also, LAMP allows one-step detection of gene amplification without specialized equipment [24]. These reasons endorse LAMP as another compelling DNA amplification technique. Comparisons of PCR and LAMP are given in Table 2.1.

Table 2.1 Comparative analysis of PCR and LAMP [23]

Properties	PCR	LAMP
Denaturation	Required for separation of strands, enabling primer binding	Denaturation step is not a mandate, as the enzyme displaces the strand and take of it
Annealing extension	Usually employs 3 steps as denaturation, annealing and extension, working at the different temperature and timing	Works under a constant temperature usually between 60 - 65 $^{\circ}$C
Time required	Take 2 - 3 hours	15 - 60 minutes
Post amplification process	Need s agarose gel electrophoresis for knowing the result	By incorporating DNA binding dyes like SYBR green or any metal indicator like calcein or other dyes like hydroxy naphthol blue the result can be interpreted visually [30, 38]

Properties	PCR	LAMP
Sensitivity	Can detect up to nanogram level of DNA	Can detect up to femtogram level of DNA in the sample
Instruments	Need expensive instruments.	No need expensive instruments.
DNA template preparation	Requires template DNA preparation which should be pure and impurities can hinder the PCR reaction	Robust technique no need for process of DNA. Sample as such can be integrated to the test [39]

The amplification of DNA by LAMP is performed using a DNA polymerase and a set of six specially designed primers. Six primers are forward outer primer (F3), backward outer primer (B3), forward internal primer (FIP), and backward internal primer (BIP). Moreover, an additional of two loop primers, named forward loop primer (FLP) and backward loop primer (BLP), accelerate the remaining sites that not reacted by FIP and BIP [24]. Primers (F3, B3, FIP, and BIP) can recognize eight distinct sequences of the target regions (F3c, F2c, F1c, FLP, B1, B2, B3, and BLP) as shown in Figure 2.2. F3 and B3 primers consist of the F3 complementary to the F3c and B3 complementary to the B3c regions, respectively. FIP composes of the F2 region (at the 3' end) and F1c regions (at the 5' end). BIP has similar patterns of target regions to FIP. Sequences of FLP primers are complemented to the F1 and F2, while BLP's are complemented to B1 and B2 [25]. LAMP are operated in the temperatures between 60 and 65 degree Celsius ($^{\circ}$C) and can be finished within 15 to 60 minutes [5].

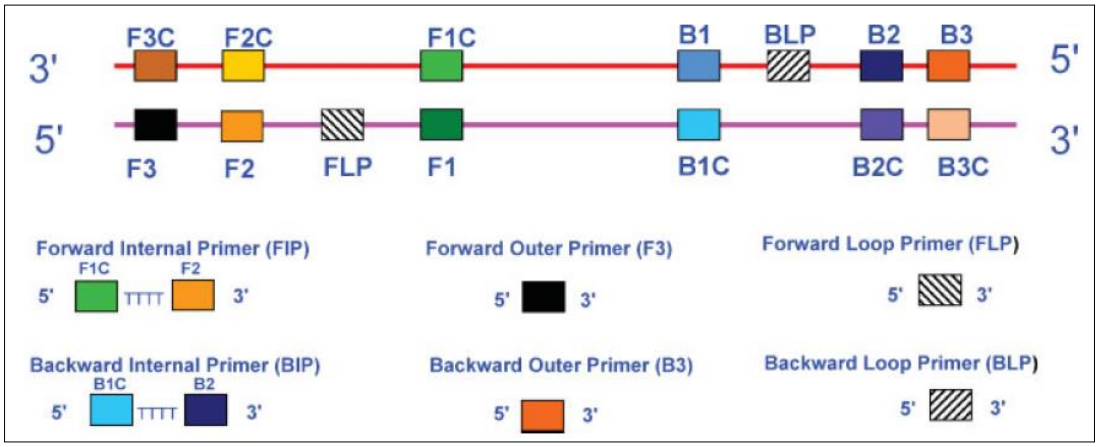

Figure 2.2 Schematic represented of primer design for LAMP method [25].

2.2.1 Principle of the LAMP method

The mechanism of LAMP is described by Maruyama F. and co-workers. The schematic is shown in Fig. 2.3. Briefly, primer FIP hybridizes to F2c in the target DNA initiating synthesis of complementary strand. Next, formed Dumbbell DNA as illustrated in structure 2, rapidly converts into stem-loop DNA (structure 3). This stem-loop DNA is the starting material for LAMP cycling. FIP repeated the process in structure 4 producing an intermediate one-gapped stem-loop DNA with an inverted replica of the target sequence formed at the opposite end by BIP sequence. Finally, the LAMP products (structure 5) are obtained. The cauliflower shaped amplicons are mixture of DNA with different length composed of many loops annealing together in the same strand [26].

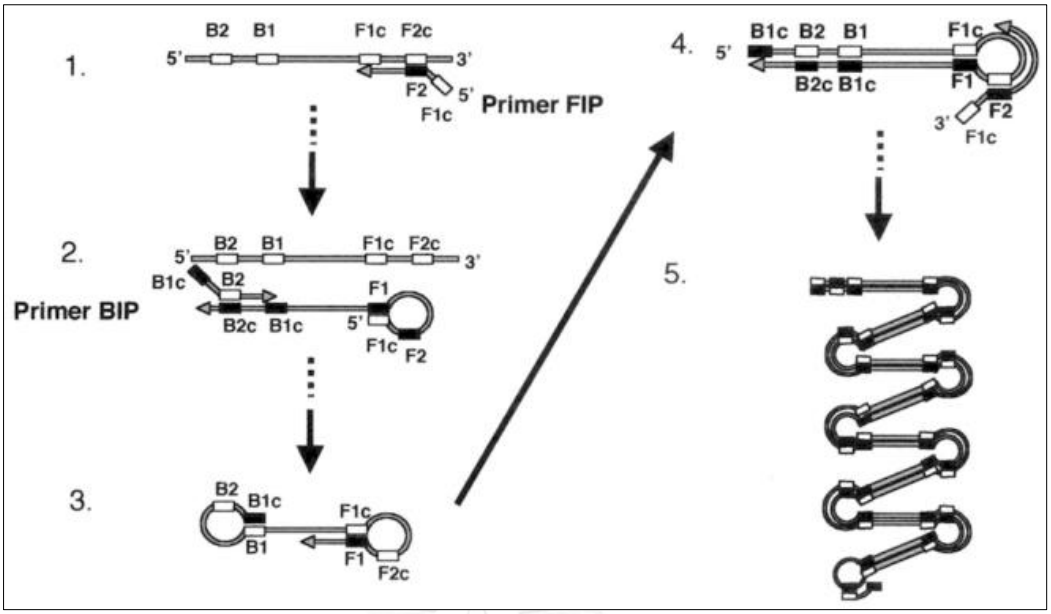

Figure 2.3 The mechanism of LAMP [26].

2.2.2 Detection of LAMP product

Copied number of the amplification genes can be quantified by agarose gel electrophoresis. Each visual bands with various sizes are observed in the gel for determining the amount of DNA. Intensity of each band is varied due to the turbidity and is used as an indicator for positive reaction. Nucleic acids amplified in LAMP reaction release large numbers of pyrophosphate ions ($P_2O_7^{4-}$). Later, this anion will combine with magnesium ions (Mg^{2+}) resulting in production of white precipitate of magnesium pyrophosphate ($Mg_2P_2O_7$). This is responsible for turbidity in case of positive reaction [22]. Polyethylene imine (PEI) is added to reaction tube after the amplification for the detection of the products. Visually detectable clear colored precipitate is formed on addition of PEI. [27]. Moreover, LAMP products can be better visualized in the presence of fluorescent intercalating dye such as ethidium bromide [28], SYBR Green [29], Hydroxy naphthol blue (HNB) [30] and calcein [31] by illuminating under a UV light (wavelength is approximately 365 nanometers). Usually,

SYBR Green is used for the visual inspection for amplification. It is performed through observation of color change after addition of the dye into the tube. In case of positive amplification, the original orange color of the dye will change into green that can be seen under natural light as well as under UV light. In case of there is no amplification, the original orange color of the dye is remained [25].

2.2.3 Applications of LAMP assay

A variety of characteristics LAMP introduces the technique in plenty of applications in a wide range of clinical and analytical fields [32]. LAMP has been proposed as kits for detecting food borne pathogens such as *Salmonella*, *Legionella*, *Listeria*, *Escherichia coli*, and *Campylobacter* [33]. Moreover, the LAMP was developed as a clinical detection kit for an acute respiratory syndrome, named corona virus (SARS CoV) [34]. Also, describing by Imai et al in 2007, throat swab specimens collected from wild birds were amplified by the LAMP assay prior to the successful detection of H5 avian influenza virus [35]. Other common severe diseases such as Malaria and Tuberculosis (TB) have also been detected by LAMP assay [36, 37]. Thus, LAMP is considered to be an effective as a gene amplification method for use in gene point-of-care testing (gPOCT) devices [25].

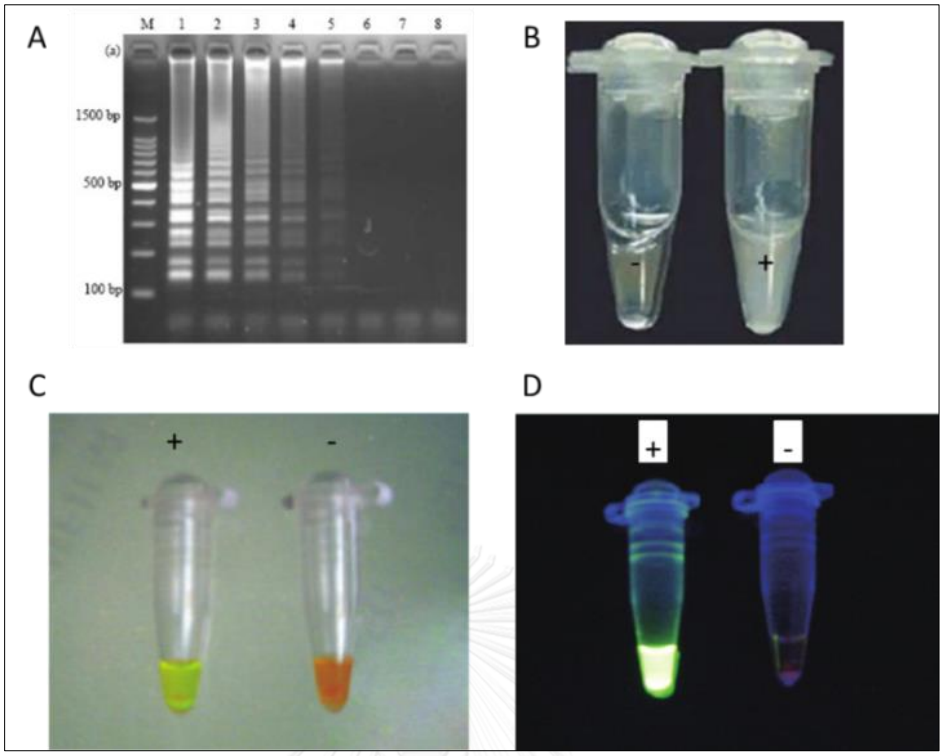

Figure 2.4 Detection of the LAMP reaction. (A) analysis of LAMP reaction products using agarose gel electrophoresis, (B) detection of LAMP products by turbidity. (C) and (D) detection of LAMP reaction using Fluorescent metal indicator. (C) Irradiating the tube using a handheld-UV lamp from the bottom. (D) Under daylight. Plus sign denotes positive reaction (with target DNA), minus sign denotes negative reaction (without target DNA).

2.3 Paper-based analysis devices

Paper-based analysis devices or PADs are a small paper platform in which entirely integrates laboratory procedures into a single device. PADs consist of hydrophobic and hydrophilic area. They have various advantages such as simplicity, low cost, and portability. Moreover, PADs offer a possibility to on-site analysis.

Addressing their advantages, a lot of research groups have been developed various methods for the fabrication of PADs. The first fabrication of PADs was introduced in 2007 by Whiteside and co-workers [10]. The developed PADs were fabricated by photolithography using SU-8 for multiple assays of glucose and protein simultaneously (Figure 2.5). Inkjet printing method was used to fabricate PADs as described by Abe *et al*. in 2008 [40]. Polystyrene was used to create hydrophobic area on the PADs, and toluene was printed for creating hydrophilic channel (Figure 2.6). However, both methods need expensive chemicals and instruments such as SU-8 and modified inkjet printer. Moreover, organic solvents are required for paper fabrication which increases the risk of toxicity exposure to human.

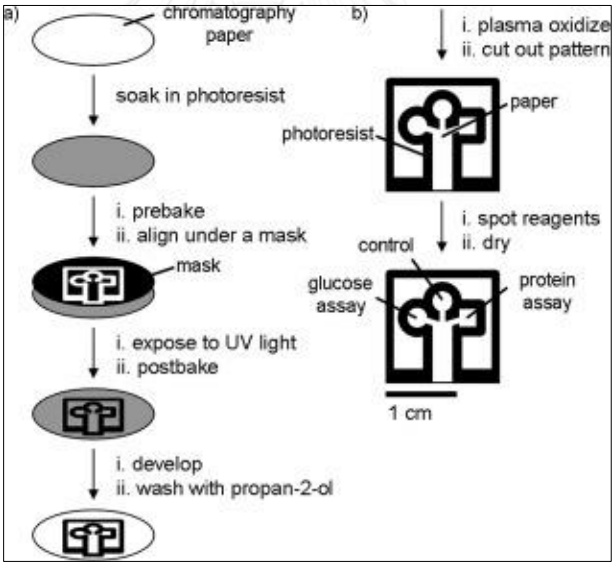

Figure 2.5 Schematic diagram depicting the method for patterning paper into millimeter-sized channels: (a) Photolithography was used to pattern SU-8 photoresist embedded into paper; (b) The patterned paper was modified for bioassays.

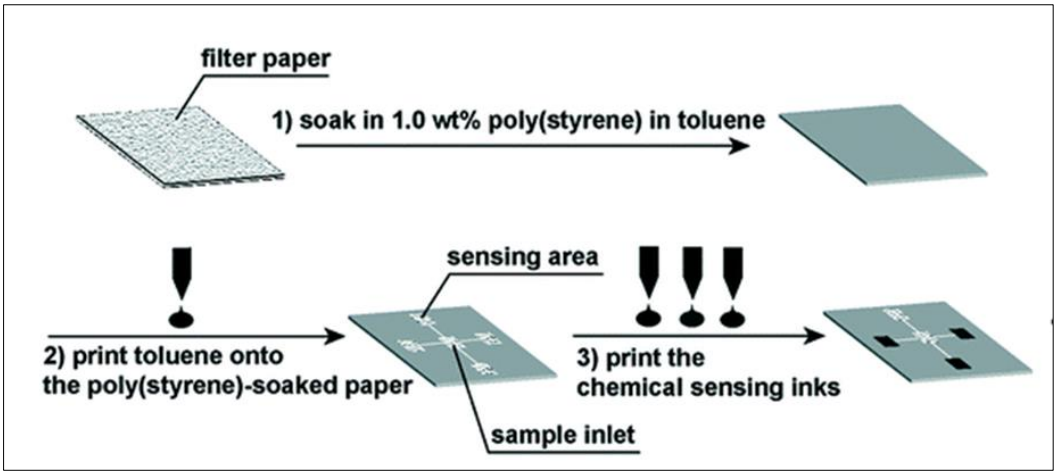

Figure 2.6 Schematic representation of the fabrication process of the inkjet-printed microfluidic multianalyte chemical sensing paper featuring microfluidic channels connecting a central sample inlet area with three different sensing areas and a reference area. Steps 2 (patterning) and 3 (chemical sensing reagent application) are performed on the same inkjet printing apparatus (the pen symbol indicates the use of the inkjet printer).

In 2009, Carrilho *et al*. [12] developed wax printing method for fabricating PADs. This novel method is simpler, faster, and safer than previous fabrication methods. To fabricate the PADs, computer designed pattern of PADs was printed onto the paper by wax printer and heated to create channel in PADs as shown in Figure 2.7. PADs can be prototyped in less than 5 minutes.

Figure 2.7 Patterning hydrophobic barriers in paper by wax printing. (1) Designed Pattern of PADs using computer software, (2) printing pattern by wax printer and (3) melting wax by hot plate for create hydrophilic channel.

Because of paper-based device methodologies are easy and readily implemented for sensing proposed. Lately, there has been considered as an interesting platform in environmental and clinical analysis. Mostly, PADs use a colorimetric assay as detection method. Specific reagents for analyte(s) on PADs were carried out for the detection zone of hydrophilic channel in which not only detection, but also pretreatment and separation are occurs. The color intensity on PADs after the reaction was finally measured in order to qualify and quantify the analytical target [41].

2.4 Optical detection

Optical detection based on colorimetric method, is a method for determining the concentration of chemical compound in a solution based on the reaction between the analytes and coloring reagents. The advantages of colorimetric method are rapidity and visualization. A requirement of expensive instruments for detection and complicated readout system can be eliminated. Thus, colorimetric detection is an attractive approach in many applications [42].

A lot of PADs use visible color changes as a detection method for qualitative and quantitative purpose. Color changes obtained from the chemical reaction between target analytes and colorimetric reagents. The results are visually detected by naked-eyes. Furthermore, it can be enhanced the detection efficiency by measurement of color intensity on computer program using simple equipment such as a digital camera, camera phone, and scanner. The accessible computer software, ImageJ, that uses for intensity measurement is developed by National Institution of Health (NIH), USA (Figure 2.8). Advantages of ImageJ are it can discard the complementary color and measure the intensity in terms of average grey intensity.

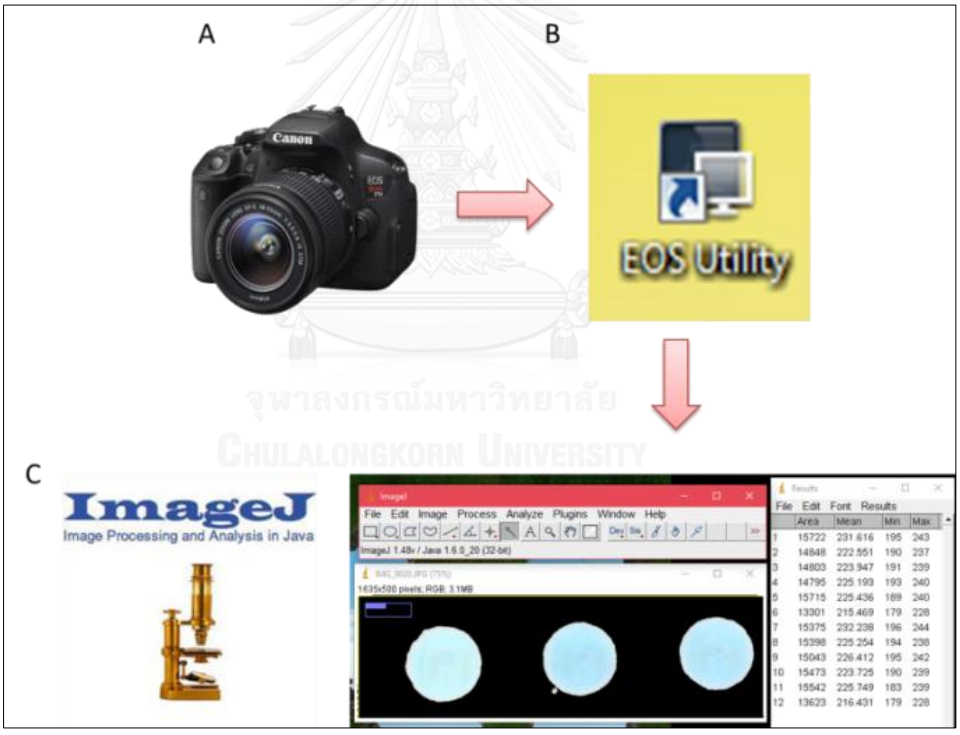

Figure 2.8 Procedure for quantifying using the Image J program.

2.5 Silver nanoparticles

Nanotechnology is most widely used in sensing, biomaterials and catalysis. They provide a wide range of applications that can be used to increase stability, selectivity, sensitivity of sensors and also analytical measurement. Most common nanotechnology-based sensing approaches utilize metal nanoparticles such as gold and silver. Such applications are enabled by the useful optical properties of these nanoparticles which can be tuned by changing the size, shape, local environment, and the synthesis method [43].

Silver nanoparticles (AgNPs) have some advantages over gold nanoparticles (AuNPs). AgNPs have higher extinction coefficients and have lower cost for synthesis due to the cheaper staring materials and stabilizing agents. However, less focus has been placed on AgNPs based sensing because of the following limitations; the chemical degradation of AgNPs to silver ions (Ag^{+}) is presented during surface functionalization and the surface of AgNPs can be easily oxidized compared with AuNPs [42]. Nevertheless, sensing systems based on the optical properties of AgNPs have been continually reported. The color change from yellow to brown of dispersed and aggregated AgNPs is distinctive and directly associated with the changing concentration of analytes. This observation promotes the accessibility of AgNPs in the colorimetric detection [43]. For example, AgNPs have been used for a colorimetric sensor of various substances including metal ions [44], proteins [45], melamine [46] and DNA [47].

2.6 Literature Review

In 2000, Natomi et al. [5] reported on the developed method for amplifying DNA by LAMP. The amplification of DNA by LAMP is performed using a DNA polymerase and a set of fore specially designed primers consisting of FIP, F3 primer, BIP and B3 primer that recognized a total of six distinct sequences (F1, F2, F3, B1, B2 and B3) of the target DNA.

In 2001, Mori *et al*. [22] detected LAMP product by visual turbidity is indicator of positive reaction. Nucleic acids are amplified in large amount in LAMP reaction. This results yielded the production of large excess of pyrophosphate ions ($P_2O_7^{4-}$), which will combine with magnesium ions (Mg^{2+}) resulting in production of white precipitate of magnesium pyrophosphate ($Mg_2P_2O_7$). This is responsible for turbidity in case of positive reaction.

In 2002, Nagami *et al.* [24] developed a method that accelerates the LAMP reaction by addition of loop primers including loop forward primer (LF primer) and loop backward primer (LB primer). The LAMP method using loop primer achieves in less reaction time than the original LAMP method without loop primer.

In 2003, Tomotada *et al.* [29] reported the used of SYBR Green I for LAMP method for detection of *Mycobacterium tuberculosis* complex, *Mycobacterium avium*, and *Mycobacterium intracellulare*. The resulting amplicons are visualized by adding SYBR Green I to the reaction tube for increasing the rate of recognition by the naked eye. The detection limit is apparently inferior to that of electrophoresis.

In 2006, Dukes *et al*. [28] developed the LAMP assay for rapid detection of foot-and-mouth disease virus. Amplification products were detected by visual

inspection, agarose gel electrophoresis, or addition of a fluorescent dye (Picogreen®) for detection by naked eye.

In 2008, Parida *et al*. [25] reported the detection of LAMP products by calcein. Calcein is fluorescent detection reagent that can be used in the reaction mixture before amplification DNA. Visual detection can be achieved without opening the tube, thus preventing carry-over contamination with post-amplification products.

In 2009, Goto *et al.* [30] reported on the colorimetric detection of LAMP by using hydroxylnaphthol blue (HNB), a metal indicator for calcium and a colorimetric reagent for alkaline earth metal ions. It was used as a new colorimetric assay of the LAMP reaction.

In 2013, Suebsing *et al*. [48] developed LAMP combined with colorimetric gold nanoparticles (AuNPs) for detection of the microsporidian *Enterocytzoon hepatopenaei (E. hepatopenaei)* in shrimp. A set of six primers was designed to successfully detect *E. hepatopenaei.* Visual detection can be observed using nanogold probe followed by salt-induced AuNPs aggregation.

CHAPTER III

EXPERIMENTAL

This chapter provided the information of apparatus, chemicals and reagents, paper-based devices preparation, LAMP assay, colorimetric assay for detection of the LAMP products.

3.1 Chemicals and apparatus

3.1.1 Loop-mediated isothermal amplification assay

The chemicals and apparatus used for LAMP assay are listed in Table 3.1

Table 3.1 List of chemicals and apparatus used for LAMP assay

Chemicals and apparatus	Suppliers
Magnesium sulfate ($MgSO_4$)	Sigma-Aldrich, MO, USA
Betaine	USA Corporation, OH, USA
Thermopol-supplied reaction buffer	New England Biolabs, USA
Deoxynucleotide (dNTP) Solution Mix	Promega, Madison, WI, USA
Bst DNA polymerase	New England Biolabs, MA, USA
Favorgen agarose	Prima Scientific, Thailand
Heating dry bath incubator	Major science, CA, USA
Micropipette and tips	Eppendorf, Hamburg, Germany
Vortex mixer	LMS, Tokyo, Japan
Electrophoresis system	Takara Bio, CA, USA

3.1.2 Preparation of paper-based devices

The chemicals and apparatus used for preparation of paper-based devices are listed in Table 3.2

Table 3.2 List of chemicals and apparatus used for preparation of PADs

Chemicals and apparatus	Suppliers
Whatman filter paper No. 1	Whatman, UK
Wax printer, Xerox Color Qube 8570	Xerox Corporation, Japan
Hot plate, HL HS-115	Harikul Science, Thailand

3.1.3 Synthesis of silver nanoparticles

3.1.3.1 Preparation of silver nanoparticles

Table 3.3 List of chemicals and apparatus used for preparation of AgNPs

Chemicals and apparatus	Suppliers
$AgNO_3$	Sigma-aldrich, Germany
$NaBH_4$	Carlo Erba, USA
H_2O_2 solution	Sigma-aldrich, Germany

3.1.3.2 Apparatus used for the characterization

For the characterization of AgNPs, the apparatus used are shown in Table 3.4

Table 3.4 List of apparatus used for characterization of AgNPs

Chemicals and apparatus	Suppliers
Microplate Spectrophotometer	Biotek, VT, USA
Transmission electron microscopy (TEM)	JEOL, CA, USA

3.2 Amplification of *V. parahaemolyticus* DNA using LAMP assay

LAMP primers of *V. parahaemolyticus* were designed according to the published sequence of the *toxR* gene (hemolysin gene, TDH and TRH). Primers were designed using Primer Explorer version 4 software (Eiken Chemical, Tokyo, Japan). The details of the primers are listed in Table 3.5.

Table 3.5 Oligonucleotide primers used for LAMP in this work

Primers	Sequence (5′–3′)
Vp-F3	GCCAGCTTCTGATAACAATGA
Vp-B3	ATCGGTAGTAATAGTGCCAA
Vp-FIP	ATTGCGTCAGAAGTCGTCGCTTTTCGCCTCTGCTAATGAGGTA
Vp-BIP	TGAACCAGAAGCGCCAGTAGTTTTTAACGCGTGGAATCCAAG
Vp-LF	AAGACGGCTCTACGATTGTTTC
Vp-LB	TACCTGAAAAAGCACCTGTGG

LAMP reactions were performed in 25 µL of total reaction mixture containing reagents listed in the table 3.6.

Table 3.6 Reagents use in LAMP for positive control

Reagents	concentration
FIP	2 µmol L^{-1}
BIP	2 µmol L^{-1}
LF	0.2 µmol L^{-1}
LB	0.2 µmol L^{-1}
F3	2 µmol L^{-1}

Reagents	concentration
B3	2 µmol L^{-1}
$MgSO_4$	6 µmol L^{-1}
Betaine	0.4 mmol L^{-1}
Thermopol-supplied reaction buffer	10 µmol L^{-1}
dNTPs mix	1.2 mmol L-1
Bst DNA polymerase	8 Units
DNA template	2 µL

A reaction mixture with DNA template was set as a positive control, whilst a mixture absence of DNA template is a negative control. Temperature and time of LAMP reaction were 65 ^{o}C and 45 minutes, respectively. The products were analyzed in 2% agarose gel electrophoresis to confirm the successful LAMP assay.

3.2.1 Gel electrophoresis

Gel electrophoresis is a universal technique for the analysis of nucleic acids and proteins. Every molecular biology research laboratory routinely uses agarose gel electrophoresis for the preparation and analysis of DNA. This work used agarose gel electrophoresis to determine the presence of LAMP products.

1. Electrophoresis is a method of separating substances based on the rate of movement under the influence of an electrical field. Agarose is a polysaccharide purified from seaweed. An agarose gel is created by suspending dry agarose 2 grams in a 100 mL of buffer solution.

2. Boiling until the solution becomes clear.

3. Pouring it into a casting tray and allowing it to cool down. During electrophoresis the gel is submersed in a chamber containing a buffer solution, and a positive and a negative electrode.

4. The DNA to be analyzed is forced through the pores of the gel by the electrical current.

5. Under an electrical field, DNA will be moved to the positive electrode and away from the negative electrode.

6. The potential of approximately 120 volts are supplied by power source and used for starting the electrodes for 30 minutes.

7. Gloves are used to remove the gel from the casting tray and place into the staining dish, followed by 15 minutes.

8. Gel and staining tray will be rinsed with water to remove residual stain. The gel is recorded under UV light while the gel is fresh.

3.2.2 Sensitivity of LAMP

The sensitivity of the LAMP assay was conducted using 10-fold dilutions DNA of *V. parahaemolyticus*. The amplification DNA by LAMP assay is heated at 65 $^{\circ}$C for 45 minutes. The products were detected in 2% agarose gel electrophoresis as described above.

3.2.3 Specificity of LAMP

The specificity of the LAMP assay was demonstrated in 2% agarose gel electrophoresis among other microorganisms listed in Table 3.7. The positive results

were obtained with LAMP primers and *V. parahaemolyticus*. Therefore, the amplified primers were specific for this analyte and used for further experiments.

Table 3.7 Other strains used in Specificity of LAMP

Microorganisms	Strains
Vibrio parahaemolyticus	ATCC 17802
Vibrio parahaemolyticus	Laboratory strain 086
Vibrio parahaemolyticu	Laboratory strain 087
Bacillus sp.	ATCC 49342
Bacillus cereus	BCC 6386
Bacillus subtilis	BCC 6327
Enterobacter cloacae	Laboratory strain
Escherichia coli O157:H7	ATCC 35150
Enterobacter aerogenes	DMST 1333
Listeria monocytogenes	DMST 1783
Staphylococcus epidermidis	TISTR 518
Salmonella typhi	Laboratory strain
Vibrio cholerae O1, El Tor, Inaba	DMST 22115
Vibrio cholerae O1, El Tor, Inaba	DMST 22116
Vibrio cholerae O1, El Tor, Inaba	DMST 22117
Vibrio cholerae O1, El Tor, Inaba	DMST 22118
Vibrio cholerae O1, El Tor, Ogawa	DMST 22125
Vibrio cholerae O1, El Tor, Ogawa	DMST 22126
Vibrio cholerae O1, El Tor, Ogawa	DMST 22127
Vibrio cholerae O1, El Tor, Ogawa	DMST 22128
Vibrio cholerae O139	DMST 22135
Vibrio cholerae O139	DMST 22136
Vibrio cholerae O139	DMST 22137
Vibrio cholerae O139	DMST 22138
Vibrio cholerae non-O1, non-O139	DMST 22140

Microorganisms	Strains
Vibrio cholerae non-O1, non-O139	DMST 22141
Vibrio cholerae non-O1, non-O139	DMST 22142
Vibrio cholerae non-O1, non-O139	DMST 22143

3.3 Preparation of paper-based devices

PADs were fabricated by using wax printing method. Briefly, device was designed by computer software, and it was printed onto the Whatman filter paper (No. 1) using commercial wax printer (Xerox Color Qube 8570, Xerox Corporation, Japan). The blue color with RGB value of (0/153/255) was selected as hydrophobic wax barrier so that it complements with the colorimetric reaction. Then, wax-printed paper was heated on hot plate at 175 °C for 50 s in order to melt the wax through the cellulose fiber. A sticky tape was used to cover one side of wax-patterned paper to protect the solution leakage through the bottom of the PADs. Schematic of PADs is shown in Figure. 3.1

Figure 3.1 Pattern of paper-based devices

3.4 Preparation of silver nanoparticles

Silver nanoparticles used in this work were obtained from Sensors Research Unit, Chemistry, Science, Chulalongkorn University. The nanoparticles were synthesized according to Parnklang, *et al*. [49]. In short, 0.63 grams of silver nitrate were dissolving in starch stabilizers. This solution was gradually added into $NaBH_4$ that used as reducing agent under vigorous stirring. The yellow silver nanoparticles was boiled and left overnight to remove the excess reducing agent. The prepared silver nanoparticles were used without further shape transformation.

3.5 Colorimetric assay detection of the *V. parahaemolyticus*

The colorimetric assays were performed on PADs. Firstly, 5 µL AgNPs and 5 µL LAMP products were subsequently added onto detection zone of PADs. The color change of AgNPs was observed after 1 min by naked eyes. After that, PADs were captured with digital camera (Cannon EOS 1000 D1, Japan). Finally, the image was imported to ImageJ software (National Institute of Health, USA) for measurement of color intensity.

3.5.1 Characterization of AgNPs-LAMP

3.5.1.1 UV-Visible spectrophotometry

To investigate spectroscopic behavior, the UV-Visible spectra of AgNPs-LAMP products were recorded in the wavelength ranging from 300 to 800 nm using microplate spectrophotometer. The spectra were compared for absorption maxima.

3.5.1.2 Transmission Electron Microscopy

The morphology and particle size of AgNPs were analyzed using Transmission Electron Microscopy (TEM). The sample for TEM measurement was prepared by dropping of AgNPs solution onto a copper grid and allowed to dry at room temperature.

3.5.2 Reagent application

5 µL of silver nanoparticles was dropped on the detection zone of PADs, followed by an equivalent volume of LAMP product. After 4 minutes the images of PADs were recorded using digital camera through EOS utility.

3.5.3 Image processing

The color change on PADs was measured for mean intensity and correlated these data with the concentration of analytes. There were 3 steps in image processing; first, the PADs were captured using digital camera (Canon EOS 1000D) at focus, ISO, and speed shutter equal to 9, 200, and 1/25, respectively. Second, the image was uploaded on the ImageJ software (developed by NIH, USA). Color threshold of the image was adjusted to remove the color of wax barrier. The parameters were selected at (100, 200), (0, 255), and (70, 200) for hue, saturation, and brightness, consecutively. Then, the image was changed to 8 bits type and inverted its color. Wand tool was chosen to fix the area for measurement of grey intensity. The obtained intensities were further used for data analysis. The schematic for image processing is shown in Figure 3.2

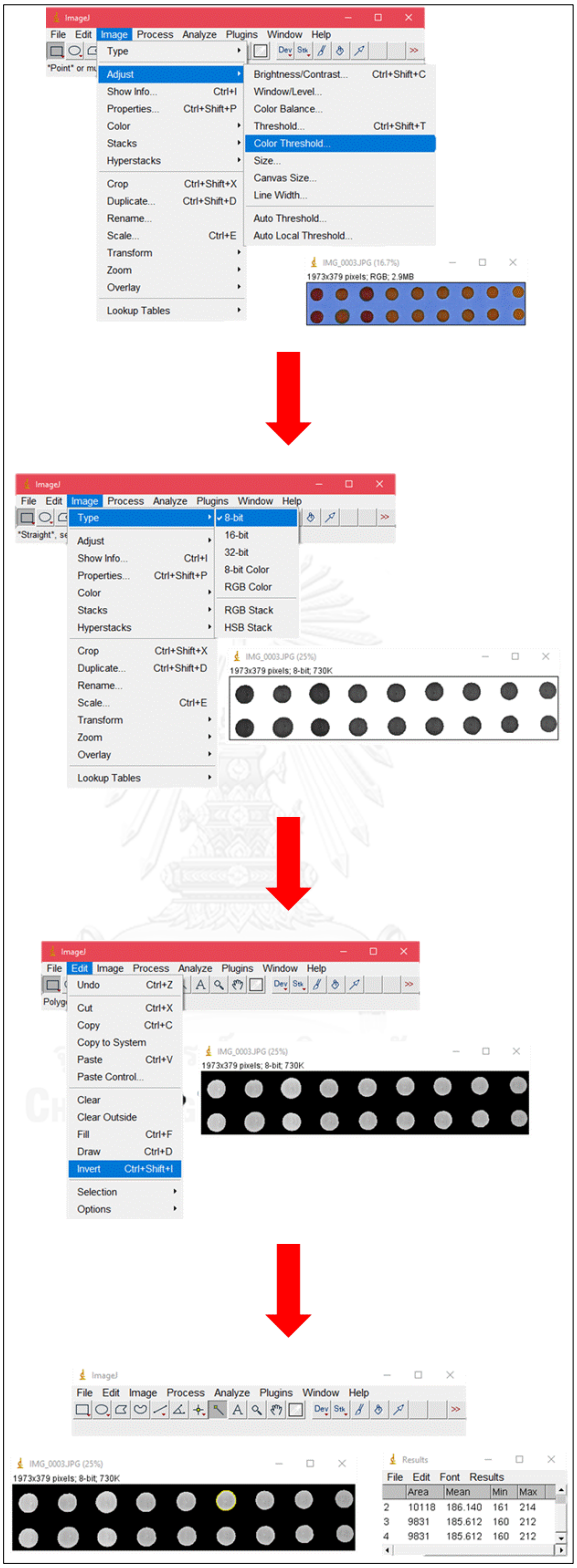

Figure 3.2 The schematic of image processing

3.5.4 Optimization of reaction time

The mixture between AgNPs and LAMP DNA was left for the completeness of the reaction. In order to obtain the rapidest time and the highest sensitivity of detection for measurement, the color change on PADs was captured using camera for 15 minutes with interval time of 1 minute. Then, the intensity of the color at each minute was plotted against time.

3.5.5 Analytical performance

3.5.5.1 Linear dynamic range

LAMP products were prepared in the range of 10 – 10^7 cfu mL^{-1} and tested on PADs. The color change was measured using grey intensity according to the steps previously described. The relationship between color intensity and concentration was plotted using scatter mode.

3.5.5.2 Limit of detection

Limit of detection (LOD) was determined using equation 1 below;

$$LOD = \frac{3SD}{Slope}$$

SD is standard deviation of blank (silver nanoparticles).

Slope is obtained from the linear regression.

3.5.6 Comparison of LAMP component with color change

3.5.6.1 Gel electrophoresis

Amplification *V. parahaemolyticus* DNA was demonstrated in 2% agarose gel electrophoresis. 5 µl of sample was loaded per well of a 2% agarose gel pre-stained with ethidium bromide (EtBr), then electrophoresed for 30 minutes at 120 V. Results were recorded under UV light using camera. In negative control (LAMP without DNA), the band will be absent, while bright band will be clearly show with DNA template (positive control).

3.5.6.2 UV-Visible spectrophotometry

Colorimetric assay using AgNPs as color reagent for LAMP products in the concentration ranged of 10 – 10^7 cfu mL^{-1} were tested and recorded on microplate spectrophotometer (data in Appendix) and UV-Visible spectra in the wavelength ranging from 300 to 800 nm. In negative control (no DNA template), there are mixture of 10 µL AgNPs and 10 µL DI water which added into 96 well plates. Positive control (DNA range 10 – 10^7 cfu mL^{-1}) consists of 10 µL AgNPs and 10 µL DNA solutions which added into 96 well plates as well.

3.6 Real sample and validation method

3.6.1 Preparation of real sample

There are three steps in the preparation of real shrimp and oyster samples. The samples are firstly collected with non-infected *V. parahaemolyticus*, including 40 samples of shrimps (n = 20) and oysters (n = 20). They were grown on selective medium (TCBS) at 30 $^{\circ}C$ for 18 hours. After that, *V. parahaemolyticus* were spiked in

real samples and pre-enrichment with alkaline peptone water for 2, 4, 6, 8 and 24 hours, consecutively. Then, DNA was extracted by 25 mM NaOH at 95 $^{\circ}$C for 5 minutes with the addition of 1 M Tris-HCL for pretreatment DNA [21]. Comparison between three detection techniques (colorimetric LAMP-AgNPs assays, LAMP-gel electrophoresis, and plate count method (TCBC)) for determination of *V. parahaemolyticus* in seafood samples was next investigated.

CHAPTER IV
RESULTS AND DISCUSSION

4.1 LAMP assay

4.1.1 Sensitivity of LAMP

V. parahaemolyticus DNA, previously amplified using LAMP assay at 65 $^{\circ}$C for 45 minutes, in the concentration ranged of 0-10^{7} CFU mL^{-1} with ten-fold serial dilutions were run on 2% agarose gel electrophoresis. The pattern in Figure 4.1 showed that LAMP assay was able to enhance *V. parahaemolyticus* detection as low as 10 CFU mL^{-1}. Therefore, this assay showed high sensitivity for the detection of target analyte.

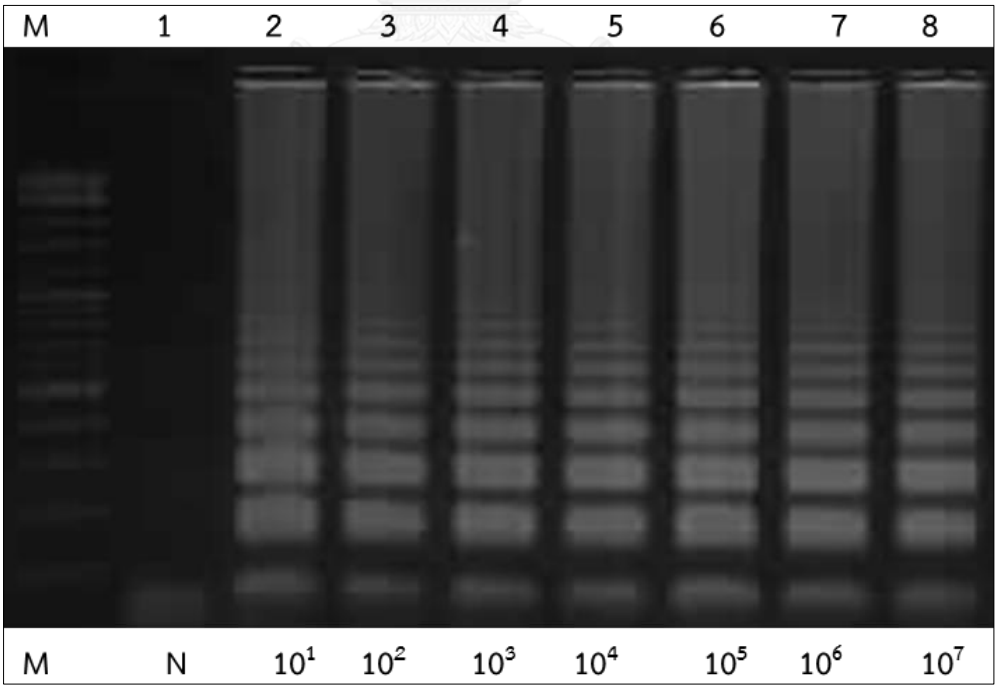

Figure 4.1 Result of amplified DNA of *V.parahaemolyticus* by LAMP assay. Lanes 1-8 represent rang *of V.parahaemolyticus* DNA from 0-10^{7} CFU mL^{-1}

4.1.2 Specificity of LAMP

V. parahaemolyticus was also studied by electrophoresis for their specificity. In the presence of other microorganisms (Table 3.7), merely positive results of *V. parahaemolyticus* were obtained as shown in Figure 4.2. In addition, specificity of *V. parahaemolyticus* and others strains of *Vibrio cholerae* (Table 3.7) were displayed in Figure 4.3. The positive results were yielded with *V. parahaemolyticus* indicating that the primers were specific to this microorganism.

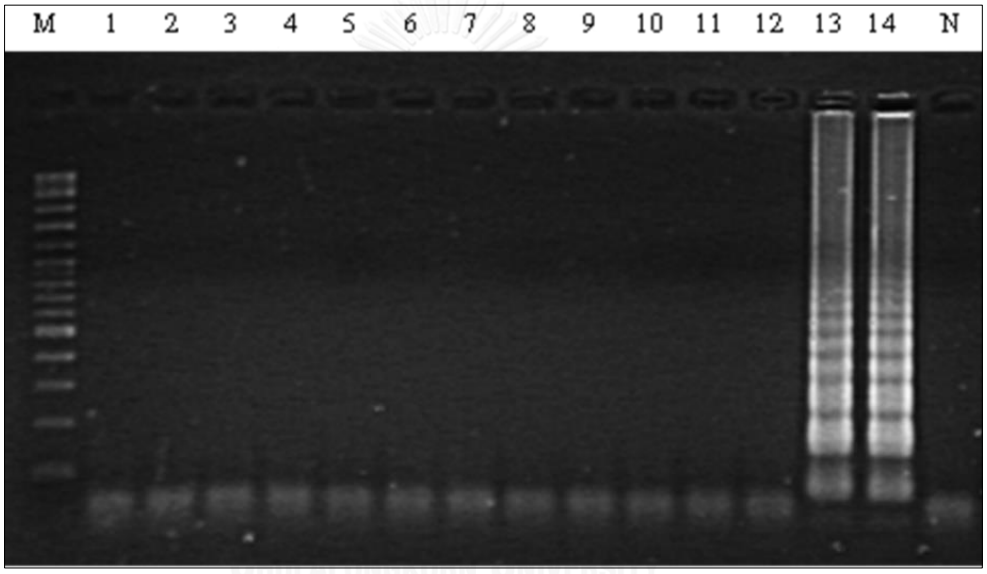

Figure 4.2 Specificity test results for *V. parahaemolyticus* detection. Agarose gel electrophoresis of the LAMP products Lane M: 2log DNA marker; lane N: no template control (negative control); lane 1: *Vibrio cholera*; lane 2: *Pseudomonas aeruginosa*; lane 3: *Salmonella enteritidis*; lane 4: *Salmonella typhimurium*; lane 5: *Enterobacter cloacae*; lane 6: *Enterobacteraerogenes*; lane 7: *Bacillus cereus*; lane 8: *Bacillus subtilis*; lane 9: *Listeria monocytogenes*; lane 10: *Clostridium perfringens*; lane 11: *Staphylococcus epidermidis*; lane 12: *Enterococcus faecium*; lane 13-14: *V. parahaemolyticus*.

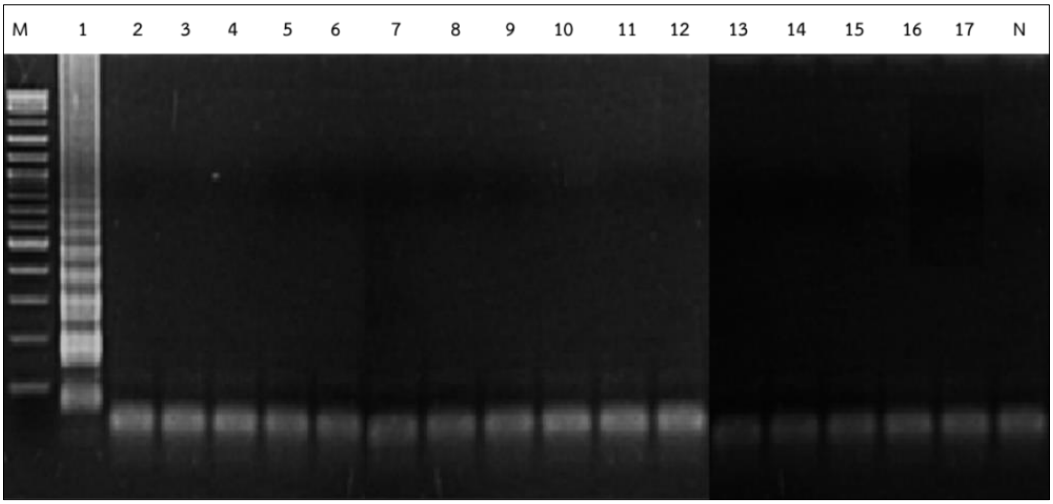

Figure 4.3 Specificity test results for *V. parahaemolyticus* detection. Agarose gel electrophoresis of the LAMP products Lane M: 2log DNA marker; lane N: no template control (negative control); lane 1: *V. parahaemolyticus*.; lane 2-17: Others strains of *Vibrio cholera* (Table. 3.7)

4.2 Colorimetric assay of LAMP product of *V. parahaemolyticus* on paper-based devices

Colorimetric assay was developed on paper-based devices for DNA determination using AgNPs as colorimetric agent. The results showed that color of AgNPs changed from yellow to red in the presence of unamplified DNA (negative control) as shown in the Fig. 4.4. In contrast, the color of AgNPs did not change after adding the LAPM product. It means that the proposed detection assay was successfully used to monitor and/or detect the product from LAMP.

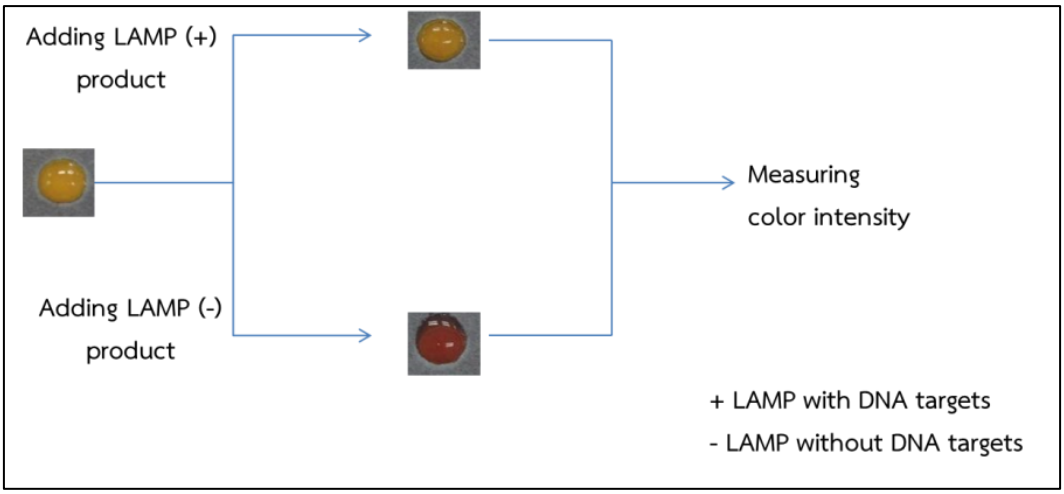

Figure 4.4 Colorimetric result of LAMP product of *V. parahaemolyticus* on paper-based devices using AgNPs.

4.2.1 Characterization of AgNPs-LAMP

4.2.1.1 UV-Visible spectrophotometry

The characterization of AgNPs-LAMP with and without DNA targets was performed using UV-Vis (Fig. 4.5). The maximum absorbance of AgNPs-LAMP with DNA targets was observed at wavelength of 400 nm while the AgNPs-LAMP without DNA was observed at 420 nm. The red shift of the UV spectra was probably due to the size increment or aggregation of AgNPs.

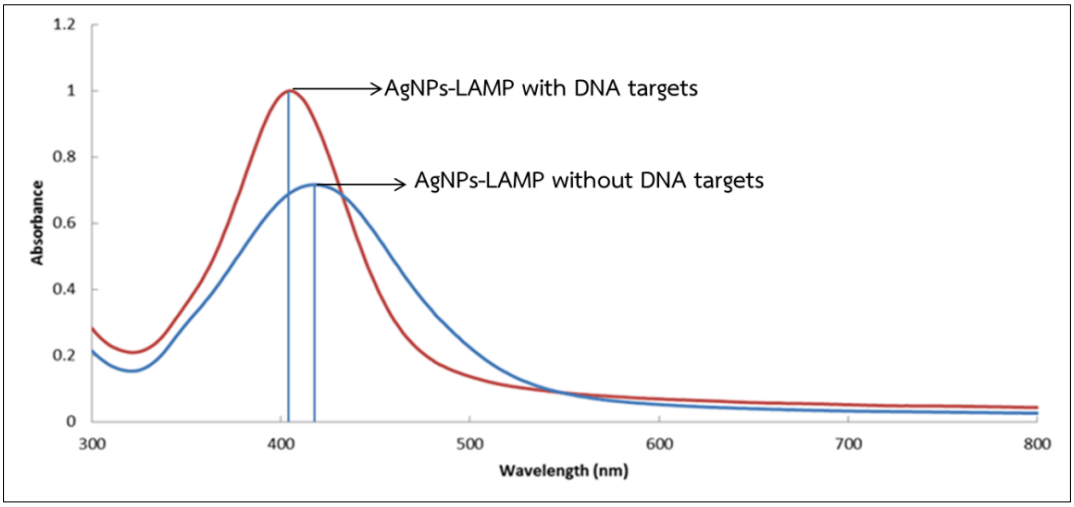

Figure 4.5 The characterization of AgNPs using UV-Visible. UV–Vis spectra of AgNPs-LAMP with DNA targets (red line) and AgNPs-LAMP without DNA targets (blue line).

4.2.1.2 TEM

In order to confirm that size of AgNPs-LAMP was enlarged, they were characterized by TEM. In Fig. 4.6, it is clearly showed that the large aggregation of AgNPs-LAMP without DNA targets (c) was observed, while AgNPs were dispersive in the solution with DNA (d). In addition, AgNPs dissolved in $MgSO_4$ solution (b) displayed more aggregation behavior comparing with AgNPs alone (a) as seen from TEM images. It indicated that the size changing of AgNPs is owing to the Mg^{2+} ion which is a component in the LAMP buffer solution. Therefore, it can be confirmed that the color changing of the AgNPs occurring by aggregation mechanism between AgNPs and Mg^{2+}.

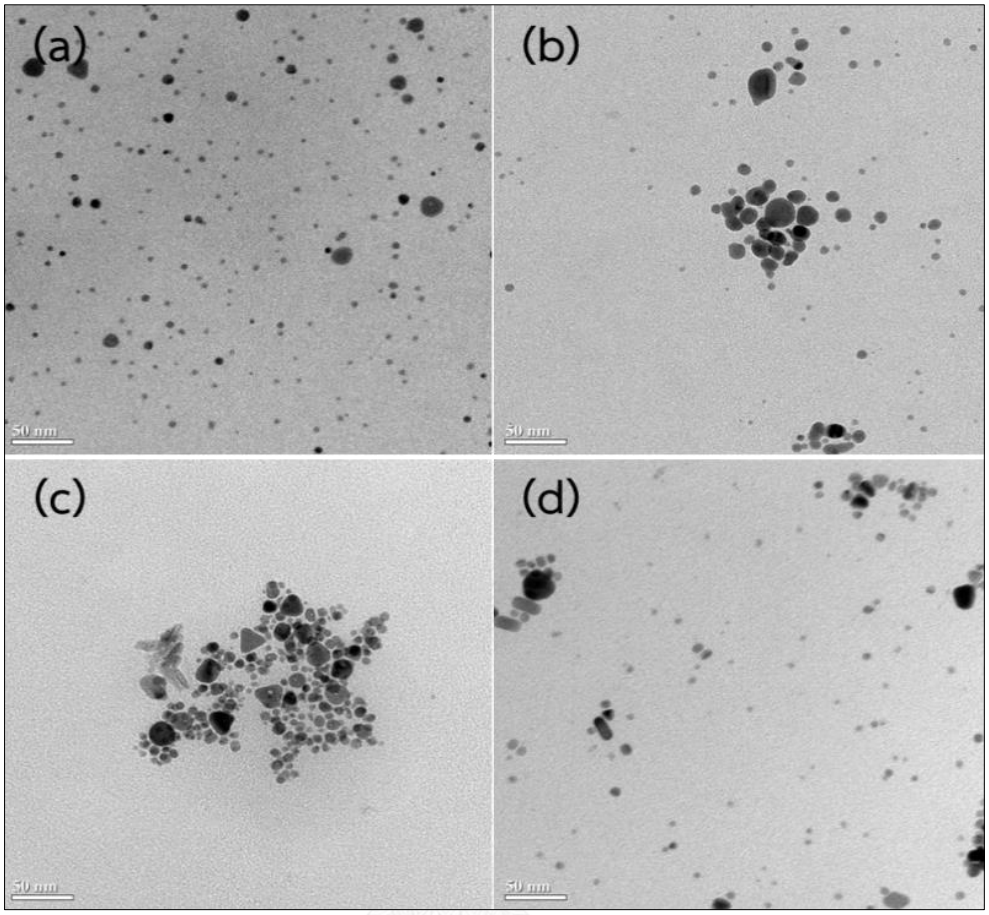

Figure 4.6 The characterization of AgNPs by TEM. (a): TEM image of AgNPs, (b): AgNPs-$MgSO_4$, (c): AgNPs-LAMP without DNA targets and (d): AgNPs-LAMP with DNA targets.

4.2.2 Mechanism of AgNPs-LAMP

The color change of the AgNPs was attributed to the aggregation between AgNPs and magnesium ion (Mg^{2+}) as previously described. During LAMP reaction, one of the by-products was pyrophosphate ion ($P_2O_7^{4-}$). This ion can combine with Mg^{2+} in solution to produce insoluble magnesium pyrophosphate ($P_2O_7 \cdot 2Mg$) [22] and to prevent AgNPs from aggregation with Mg^{2+}. The amount of pyrophosphate ion was proportional to that of those DNA. Magnesium pyrophosphate ($P_2O_7 \cdot 2Mg$) product

was enhance at the higher concentration of analytes resulting in decreasing of Mg^{2+} ion concentration. Thus, the consequence silver nanoparticles color at high level of DNA targets were close to yellow compared to the opposite situation which had red color. The schematic detection for mechanism between AgNPs and Mg^{2+} ion is illustrated in fig. 4.7.

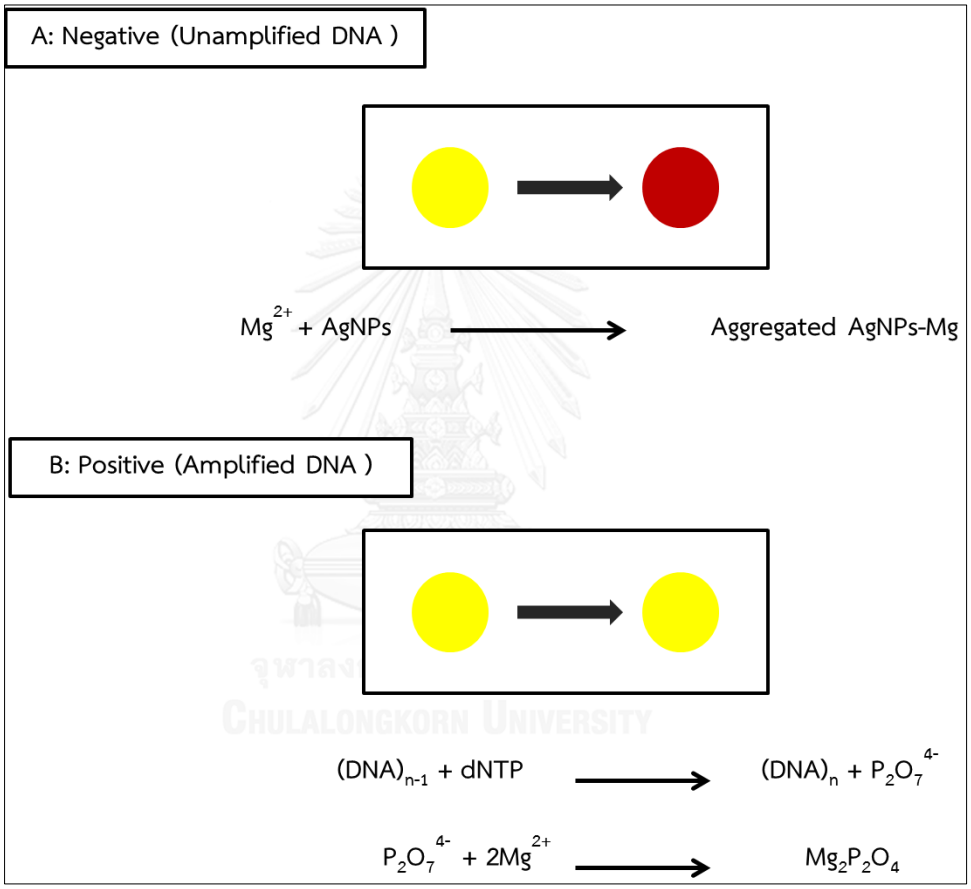

Figure 4.7 Schematic representation of the colorimetric assay addition of AgNPs to detect LAMP products. The results show A: Negative control color of AgNPs changed from yellow to red in the presence of unamplified DNA, B: Positive control color of AgNPs not changed.

4.2.3 Optimization of reaction time

The reaction time is one of parameter that concerned the sensitivity of detection. It was studied between 0 and 15 minutes using unamplified DNA or negative control as model to obtain ultimately clear colorimetric result. The relationship between color intensity and reaction time was observed as shown in Fig. 4.8. Color intensity continually increased and reached plateau after 4 minutes. Therefore, the detection time was selected at 4 minute to shorten the analysis time while maintaining the best sensitivity. The results further confirmed that not only detection LAMP products using optical assay was faster than gel electrophoresis, but also the cost of analysis was lower.

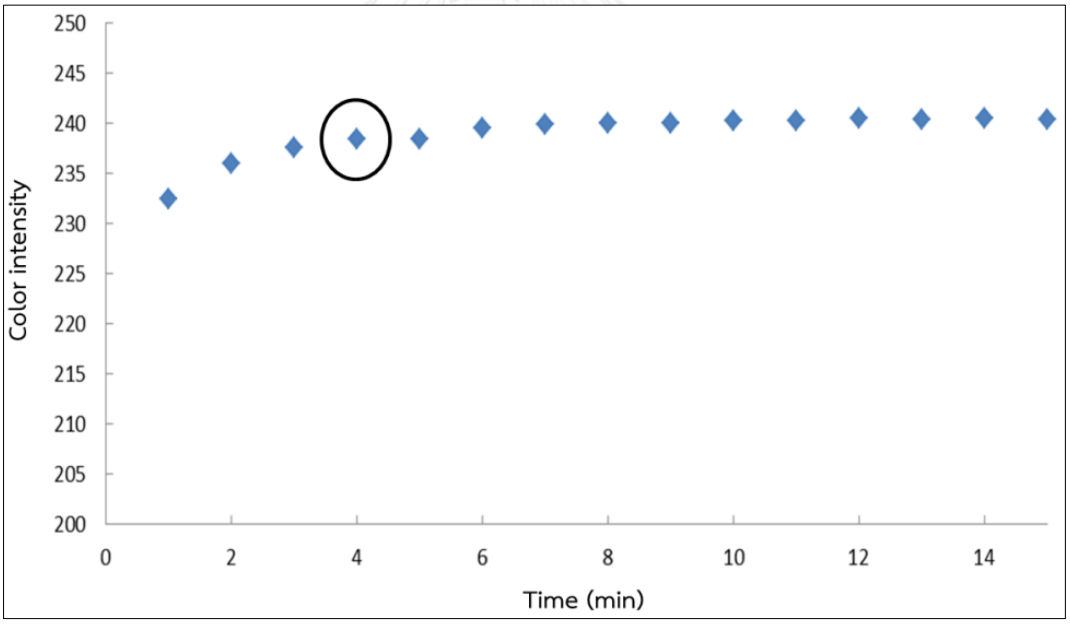

Figure 4.8 Relationship between color intensity and time of LAMP-AgNPs

4.2.4 Analytical performance

4.2.4.1 Linear dynamic range

Linear dynamic range (LDR) was determined for quantitative analysis of DNA. The color change of silver nanoparticles in the presence of analyte concentration ranging from 10 to 10^7 CFU mL^{-1} is shown in Fig. 4.9. Calibration curve between mean intensity and the concentration of *V. parahaemolyticus* was plotted in figure 4.10. Linear region was observed with a coefficient of 0.982, intercept of 24.462 and slope of 0.9637 unit/CFU mL^{-1} as shown in Figure 4.11.

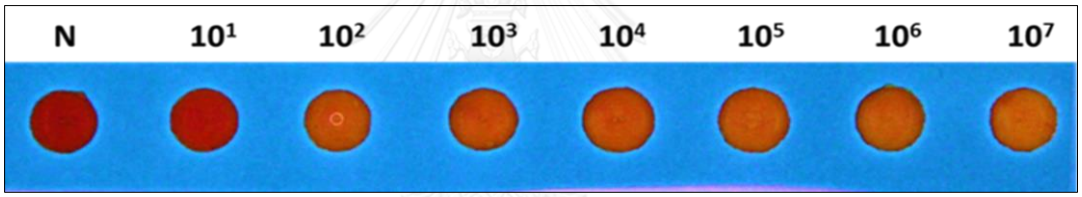

Figure 4.9 Sensitivity of assay. Detection DNA of *V. parahaemolyticus* by colorimetric assay on paper-based devices. Rang of LAMP products of *V. parahaemolyticus* DNA from 0-10^7 CFU mL^{-1}.

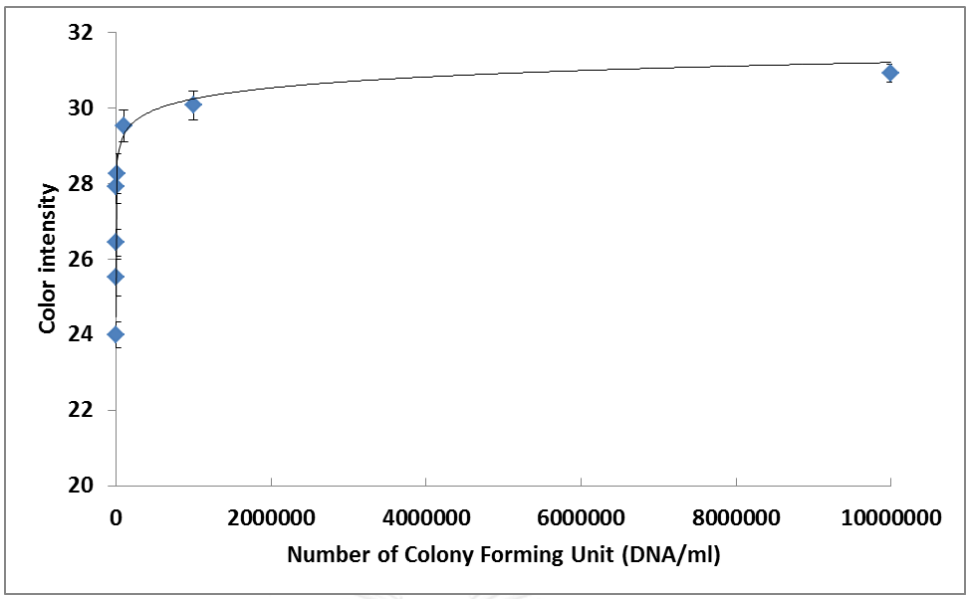

Figure 4.10 A plot of raw data between color intensity and *V. parahaemolyticus* at the concentration from 0-10^{7} CFU mL^{-1} (n=10).

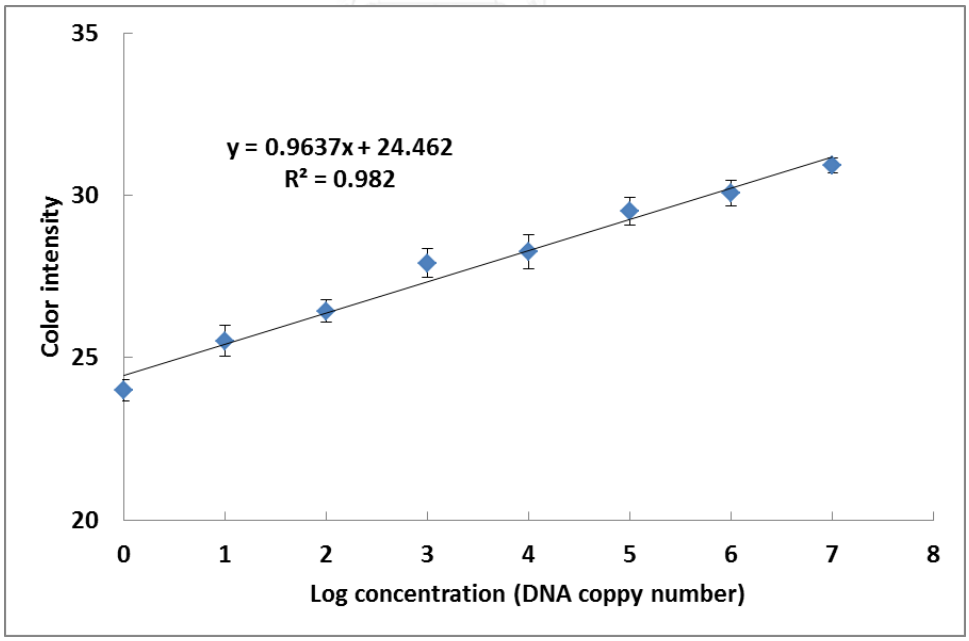

Figure 4.11 Calibration curve between color intensity and log concentration of *V. parahaemolyticus* at the concentration from 0-10^{7} CFU mL^{-1} (n=10).

4.2.4.2 Limit of detection

The same volume of silver nanoparticles and deionized water were mixed in three different detection zones. The grey intensity was averaged for their standard deviation, and then LOD was calculated using equation 1. From the calculation the detection limit of *V. parahaemolyticus* was as low as 11.15 CFU mL^{-1}.

4.2.5 Comparison of LAMP component with color change

The sensitivity of AgNPs-LAMP assay was tested using ten-fold serial dilutions of *V. parahaemolyticus* DNA in the range of 0-10^{7} CFU mL^{-1}. This result showed identical sensitivity to LAMP followed by colorimetric assay on paper based devices (Figure 4.9), followed by gel electrophoresis (Figure 4.13), and followed by UV visible spectra (Figure 4.14).

4.2.5.1 PADs

The AgNPs-LAMP was able to detect the *V. parahaemolyticus*-LAMP products at 10 CFU mL^{-1}, corresponding to the observation of color change by naked eyes (Figure 4.9). Detection method proposed in this work demonstrated a superior advantages of low cost and simplicity over those expensive and complicated techniques, while maintaining identical sensitivity for DNA sensing.

4.2.5.1 Gel electrophoresis

The same concentration range of analyte was also tested using gel electrophoresis. The results showed in Figure 4.12. It is clearly indicated that the gel technique can detect LAMP DNA similarly to this developed method. This emphasizes the efficacy of this developed colorimetric AgNPs method.

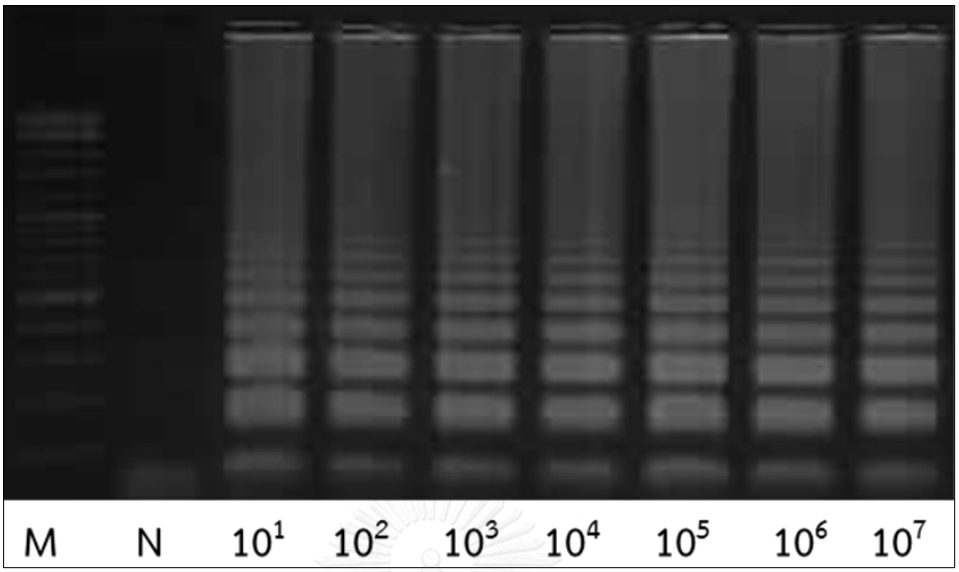

Figure 4.12 Sensitivity of assay for 0-10^7 CFU mL^{-1} *V. parahaemolyticus* detection by agarose gel electrophoresis.

4.2.5.2 UV-Visible spectroscopy

The color product on paper-based testing device was changed from red to dark yellow when concentration of DNA targets increased in the range of 0-10^7 CFU mL^{-1}. A maximum absorption in UV-Vis spectra (λ_{max}) of AgNPs-LAMP (10^7 CFU mL^{-1}) was at wavelength of 400 nanometers (nm), while the λ_{max} of AgNPs-LAMP negative control (none DNA target) increased and red shifted to 420 nm as shown in Figure 4.13. The absorbance was consecutively increased and relocated as the concentration of DNA targets was increased. The behavior was manifested visually using this method. AgNPs were less aggregated together with increasing concentration of *V. parahaemolyticus* because the products of magnesium pyrophosphate were much produced.

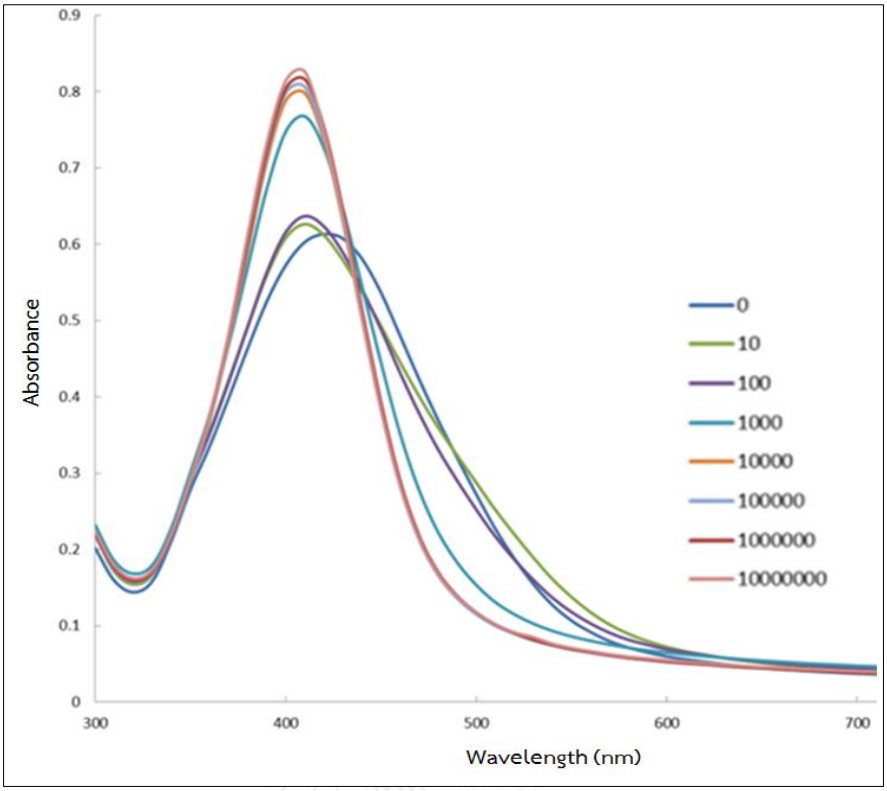

Figure 4.13 Sensitivity of assay followed by UV-visible spectra. Rang of LAMP products of *V. parahaemolyticus* DNA from 0-10^{7} CFU mL^{-1}.

The colorimetric results from LAMP-AgNPs on PADs were proved to be valid for detection of *V. parahaemolyticus* after compare with other detection methods (gel electrophoresis and UV-Visible spectrophotometry). Moreover, the proposed technique requires less instrumentation leading to both shorten analysis time and simple experimental procedures.

4.3 Real sample and validation method

The LAMP-AgNPs assay developed in this study was shown to have practical application in colorimetric detection of *V. parahaemolyticus* in real samples. The technique gave comparable sensitivity to the plate count method (TCBS). The results

between LAMP-gel electrophoresis and LAMP-AgNPs are compared in Table 4.1. The plate count method (TCBS) offers the total time of 6 hrs, LAMP-gel electrophoresis takes 1 hr and 30 min, while LAMP-AgNPs required only 50 min for detection of *V. parahaemolyticus*. Thus, the LAMP-AgNPs offers the most rapid protocol among others for the detection of *V. parahaemolyticus*.

Forty seafood samples consisting of an equal number of shrimp and oyster were collected from local markets. The results between the plate count method (TCBS) and detection methods after LAMP (LAMP-gel electrophoresis and LAMP-AgNPs) were compared in Appendix A (table A1). 35 samples (87.5%) were *V. parahaemolyticus* positive with the plate count method (TCBS), including 15 shrimps and 20 oysters. By LAMP-gel electrophoresis, 38 samples (95%), including samples of 18 shrimps and 20 oysters showed positive results. On the other hands using LAMP-AgNPs, 38 samples (95%), including 19 shrimps and 19 oysters, displayed *V.parahaemolyticus* positive. Comparing 2 LAMP detection methods, LAMP-AgNPs showed as same number of positive results as LAMP-gel electrophoresis. But the *V.parahaemolyticus* detection by LAMP-AgNPs used shorter analysis time than LAMP-gel electrophoresis, and much faster than the conventional plate count method (TCBS). Moreover, LAMP-AgNPs was safer than LAMP-gel electrophoresis because ethidium bromide (EtBr) were used in LAMP-gel techniques to make DNA fluoresce in gels running. Toxicity of EtBr preferentially induces frame shift mutations in living cells [50]. Therefore, this proposed method demonstrated the advantages of shorter analysis time, lower toxicity, and more cost efficiency than conventional methods. In addition, using LAMP-AgNPs offers more practical of on-site DNA analysis in the future.

Table 4.1 Comparison of detection of *V.parahaemolyticus* samples using plate count method (TCBS), LAMP-gel electrophoresis and LAMP-AgNPs assays.

Type of samples		**Number and % positive results**		
Samples	**No.**	**TCBS**	**LAMP-Gel**	**LAMP-AgNPs**
Shrimps	20	15	18	19
Oysters	20	20	20	19
Total	40	35 (87.5%)	38 (95%)	38 (95%)
Time		6 h	1 h 30 min	50 min

CHAPTER V
CONCLUSION

Paper-based analytical devices (PADs) using colorimetric assays of silver nanoparticles (AgNPs) coupled with loop-mediated isothermal amplification (LAMP) for detection of *V. parahaemolyticus* was successfully developed. The DNA target was firstly amplified using LAMP method which provides advantages such as simple and short time protocol over the conventional PCR method. PADs fabricated using facile wax-printing method was used to perform the reaction between AgNPs and DNA. Magnesium ions (Mg^{2+}) that normally present in the process of DNA amplification causes the yellowish of AgNPs changing into red color in the reaction of negative control, which can be directly observed by the naked eyes. The aggregation behavior was mitigated with the increase concentration of DNA because the presence of magnesium pyrophosphate (MgP_2O_7) which hinders the Mg^{2+} ions to inhibit the aggregation of AgNPs. LAMP-AgNPs on PADs exhibits the good relationship during the measurement of color intensity using ImageJ software for detection of *V. parahaemolyticus* DNA in the concentration ranged of 0-10^7 CFU mL^{-1}. The excellent sensitivity with highly selective method was acquired with the limit of detection (LOD) and limit of quantitation (LOQ) of 11.15 CFU mL^{-1} and 3.2×10^3 CFU mL^{-1} respectively. LAMP-AgNPs showed consistent results compared with other methods (gel electrophoresis and UV-visible spectroscopy). Finally, the proposed assay was applied to real seafood samples. The good results were obtained when comparing this method with conventional plate count method (TCBS) and LAMP-gel electrophoresis. Therefore, LAMP-AgNPs on PADs serves as a rapid, inexpensive and simple assay without the need for complicated instrumentation. In addition, this device shows a high potential to apply for DNA on-site analysis in the future.

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APPENDIX

APPENDIX A

Table A1 Detection of *V. parahaemolyticus* samples using plate count method (TCBS), LAMP-gel electrophoresis and LAMP-AgNPs assays.

Samples	No.	TCBS (cfu/mL)	LAMP-Gel	LAMP-AgNPs
Shrimp	1	-	+	-
	2	+	-	+
	3	+	+	+
	4	+	+	+
	5	+	+	+
	6	+	+	+
	7	-	+	+
	8	-	+	+
	9	-	+	+
	10	+	+	+
	11	+	+	+
	12	+	+	+
	13	+	+	+
	14	+	+	+
	15	+	+	+
	16	+	+	+
	17	+	+	+
	18	+	+	+
	19	+	+	+
	20	-	-	+

Samples	No.	TCBS (cfu/mL)	LAMP-Gel	LAMP-AgNPs
Oysters	1	+	+	+
	2	+	+	+
	3	+	+	+
	4	+	+	+
	5	+	+	+
	6	+	+	-
	7	+	+	+
	8	+	+	+
	9	+	+	+
	10	+	+	+
	11	+	+	+
	12	+	+	+
	13	+	+	+
	14	+	+	+
	15	+	+	+
	16	+	+	+
	17	+	+	+
	18	+	+	+
	19	+	+	+
	20	+	+	+

Table A2 Detection *V. parahaemolyticus* DNA using colorimetric assay on PADs.

Con. DNA	Intensity							
	0	10^1	10^2	10^3	10^4	10^5	10^6	10^7
1	22.555	26.364	26.757	26.778	27.781	29.117	29.326	31.112
2	22.231	26.196	26.574	26.554	27.606	28.873	29.173	31.008
3	22.156	26.161	26.531	26.565	27.447	28.806	28.959	30.882
4	23.091	26.074	26.710	26.493	27.514	29.005	29.322	31.3205
5	22.71	26.506	26.670	26.908	27.859	29.046	29.220	30.805
6	22.812	25.754	26.438	26.193	27.040	28.694	29.019	31.151
7	22.321	25.492	26.018	26.014	26.774	28.248	28.602	30.682
8	22.91	25.442	26.124	25.903	26.723	28.298	28.656	31.017
9	23.01	25.254	25.927	25.714	26.603	28.058	28.387	30.655
10	22.919	25.170	25.815	25.624	26.441	27.985	28.313	30.628
SD	0.337	0.4809	0.351	0.4490	0.5227	0.4270	0.3815	0.2343
AVG.	22.671	25.841	26.35	26.275	27.179	28.613	28.897	30.926

Table A3 Detection of *V. parahaemolyticus* DNA using UV-visible assays.

Wavelength	Absorbance	
	AgNPs-LAMP (-)	AgNPs-LAMP (+)
310	0.259	0.27525
320	0.24375	0.25875
330	0.258	0.27025
340	0.311	0.31825
350	0.378	0.3925
360	0.4345	0.474
370	0.49775	0.5795
380	0.563	0.701
390	0.6245	0.8215
400	0.67225	0.904
410	0.70275	0.91625
420	0.71325	0.854
430	0.7075	0.74525
440	0.681	0.61475
450	0.63675	0.493
460	0.58025	0.39075
470	0.52325	0.318
480	0.4695	0.27
490	0.42	0.239
500	0.37225	0.21725
510	0.327	0.2015
520	0.288	0.19025
530	0.2545	0.1815

Wavelength	Absorbance	
	AgNPs-LAMP (-)	AgNPs-LAMP (+)
540	0.227	0.1745
550	0.2065	0.169
570	0.1795	0.161
580	0.171	0.1575
590	0.16475	0.155
600	0.1595	0.1525
610	0.15575	0.1505
620	0.1525	0.149
630	0.14925	0.147
640	0.14675	0.14525
650	0.14475	0.144
660	0.14275	0.1425
670	0.141	0.1415
680	0.1395	0.1405
690	0.13825	0.13925
700	0.137	0.138
710	0.13625	0.1375
720	0.1355	0.137
730	0.135	0.1365
740	0.1345	0.136
750	0.13375	0.1355
760	0.133	0.1345
770	0.1325	0.1345
780	0.13175	0.1335

Wavelength	Absorbance	
	AgNPs-LAMP (-)	AgNPs-LAMP (+)
790	0.131	0.133
800	0.1305	0.132

Table A4 Detection of *V. parahaemolyticus* DNA using UV-visible assays.

Wave length	Absorbance							
	0	10^1	10^2	10^3	10^4	10^5	10^6	10^7
300	0.2018	0.2188	0.2198	0.2320	0.2198	0.2180	0.2203	0.2240
310	0.1590	0.1708	0.1740	0.1850	0.1753	0.1735	0.1753	0.1788
320	0.1438	0.1540	0.1573	0.1680	0.1590	0.1575	0.1588	0.1620
330	0.1580	0.1668	0.1693	0.1793	0.1698	0.1685	0.1703	0.1730
340	0.2110	0.2208	0.2208	0.2280	0.2168	0.2165	0.2183	0.2205
350	0.2780	0.2923	0.2898	0.3010	0.2883	0.2890	0.2925	0.2933
360	0.3345	0.3538	0.3508	0.3760	0.3675	0.3693	0.3740	0.3745
370	0.3978	0.4210	0.4188	0.4670	0.4700	0.4735	0.4795	0.4813
380	0.4630	0.4913	0.4913	0.5690	0.5893	0.5953	0.6010	0.6110
390	0.5245	0.5588	0.5625	0.6718	0.7075	0.7163	0.7215	0.7315
400	0.5723	0.6080	0.6155	0.7475	0.7880	0.7978	0.8040	0.8140
410	0.6028	0.6263	0.6368	0.7678	0.7983	0.8065	0.8163	0.8263
420	0.6133	0.6120	0.6235	0.7265	0.7363	0.7418	0.7540	0.7498
430	0.6075	0.5793	0.5893	0.6465	0.6300	0.6333	0.6453	0.6368
440	0.5810	0.5375	0.5415	0.5455	0.5028	0.5043	0.5148	0.5053
450	0.5368	0.4928	0.4885	0.4453	0.3840	0.3843	0.3930	0.3845
460	0.4803	0.4455	0.4315	0.3520	0.2843	0.2840	0.2908	0.2843
470	0.4233	0.4008	0.3785	0.2778	0.2135	0.2128	0.2180	0.2140
480	0.3695	0.3598	0.3310	0.2225	0.1670	0.1660	0.1700	0.1675
490	0.3200	0.3230	0.2900	0.1828	0.1370	0.1358	0.1390	0.1378
500	0.2723	0.2875	0.2523	0.1533	0.1163	0.1150	0.1173	0.1173
510	0.2270	0.2520	0.2170	0.1308	0.1008	0.0998	0.1015	0.1018
520	0.1880	0.2198	0.1868	0.1148	0.0900	0.0893	0.0903	0.0910

Wave length	Absorbance							
	0	10^1	10^2	10^3	10^4	10^5	10^6	10^7
530	0.1545	0.1893	0.1600	0.1025	0.0815	0.0805	0.0815	0.0848
540	0.1270	0.1610	0.1365	0.0930	0.0748	0.0740	0.0745	0.0760
550	0.1065	0.1375	0.1178	0.0858	0.0695	0.0685	0.0690	0.0705
560	0.0915	0.1175	0.1028	0.0800	0.0653	0.0645	0.0650	0.0663
570	0.0795	0.1013	0.0905	0.0750	0.0615	0.0605	0.0610	0.0623
580	0.0710	0.0890	0.0813	0.0708	0.0580	0.0575	0.0575	0.0590
590	0.0648	0.0795	0.0748	0.0675	0.0555	0.0550	0.0550	0.0568
600	0.0595	0.0720	0.0688	0.0645	0.0533	0.0525	0.0525	0.0543
610	0.0558	0.0660	0.0648	0.0620	0.0513	0.0508	0.0505	0.0525
620	0.0525	0.0618	0.0610	0.0598	0.0498	0.0493	0.0490	0.0508
630	0.0493	0.0573	0.0578	0.0578	0.0480	0.0473	0.0470	0.0488
640	0.0468	0.0540	0.0548	0.0558	0.0463	0.0458	0.0453	0.0470
650	0.0448	0.0510	0.0525	0.0540	0.0448	0.0443	0.0440	0.0455
660	0.0428	0.0485	0.0503	0.0525	0.0435	0.0430	0.0425	0.0445
670	0.0410	0.0463	0.0483	0.0510	0.0423	0.0418	0.0415	0.0430
680	0.0395	0.0443	0.0468	0.0495	0.0413	0.0408	0.0405	0.0420
690	0.0383	0.0428	0.0453	0.0485	0.0403	0.0398	0.0393	0.0410
700	0.0370	0.0413	0.0440	0.0473	0.0393	0.0390	0.0380	0.0400
710	0.0363	0.0398	0.0425	0.0463	0.0383	0.0383	0.0375	0.0393
720	0.0355	0.0388	0.0418	0.0455	0.0378	0.0378	0.0370	0.0388
730	0.0350	0.0383	0.0410	0.0450	0.0375	0.0373	0.0365	0.0385
740	0.0345	0.0375	0.0405	0.0445	0.0373	0.0373	0.0360	0.0380
750	0.0338	0.0365	0.0395	0.0438	0.0365	0.0365	0.0355	0.0375
760	0.0330	0.0355	0.0385	0.0428	0.0358	0.0358	0.0345	0.0365

Wave length	Absorbance							
	0	10^{1}	10^{2}	10^{3}	10^{4}	10^{5}	10^{6}	10^{7}
770	0.0325	0.0348	0.0380	0.0423	0.0353	0.0353	0.0345	0.0360
780	0.0318	0.0338	0.0370	0.0413	0.0348	0.0345	0.0335	0.0353
790	0.0310	0.0328	0.0363	0.0405	0.0338	0.0340	0.0330	0.0348
800	0.0305	0.0320	0.0355	0.0398	0.0333	0.0333	0.0320	0.0340

VITA

Miss Jutaporn Tipchote was born on January 19, 1988 in Kalasin, Thailand. She graduated from Princess Chulabhorn's College Mukdahan School in Mukdahan with high school degree in 2006. She received her Bachelor's degree of Science, majoring in Biotechnology from Kasetsart University (2007-2010). After that, she has become a graduate student in the Biotechnology, Faculty of Science, Chulalongkorn University. Furthermore, she has joined the Electrochemistry and Optical Spectroscopy Research Unit (EOSRU) under the direction of Professor Dr. Orawon Chailapakul. She graduated with Master's degree in Biotechnology of academic year 2016 from Chulalongkorn University.

Miss Jutaporn Tipchote has attended the following conferences for poster presentations.

- The 40th Congress on Science and Technology of Thailand (STT 40) held in Khon Kaen, Thailand, 2nd to 4th December 2014.
- The 27th Annual Meeting of the Thai Society for Biotechnology and International Conference "Innovative Biotechnology" (TSB 2015) held in Bangkok, Thailand, 17th to 19th November 2015.

Proceeding:

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