THE DEVELOPMENT OF LOOP-MEDIATED ISOTHERMAL AMPLIFICATION WITH LATERAL-FLOW DIPSTICKS (LAMP-LFD) TO DETECT INFECTION OF HEPATOPANCREATIC PARVOVIRUS (HPV)

TONGCHAI NIMITPHAK

A THESIS SUBMITTED IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR
THE DEGREE OF MASTER OF SCIENCE (BIOTECHNOLOGY)
FACULTY OF GRADUATE STUDIES
MAHIDOL UNIVERSITY
2008

Thesis
Entitled

THE DEVELOPMENT OF LOOP-MEDIATED ISOTHERMAL AMPLIFICATION WITH LATERAL-FLOW DIPSTICKS (LAMP-LFD) TO DETECT INFECTION OF HEPATOPANCREATIC PARVOVIRUS (HPV)

…….
Mr. Tongchai Nimitphak
Candidate

…….
Mrs. Wansika Kiatpathomchai, M.Sc.
Co-Advisor

…….
Prof. Timothy William Flegel, Ph.D.
Major-Advisor

…….
Assoc. Prof. Wasana Sukhumsirichart, Ph.D.
Co-Advisor

.....
Prof. Boonsirm Withyachumnarnkul, Ph.D.
Co-Advisor

…….
Prof. Banchong Mahaisavariya, M.D.
Dean
Faculty of Graduate Studies

…….
Assoc. Prof. Chuenchit Boonchird, Ph.D.
Chair
Master of Science Programme in Biotechnology
Faculty of Science

Thesis
Entitled

THE DEVELOPMENT OF LOOP-MEDIATED ISOTHERMAL AMPLIFICATION WITH LATERAL-FLOW DIPSTICKS (LAMP-LFD) TO DETECT INFECTION OF HEPATOPANCREATIC PARVOVIRUS (HPV)

was submitted to the Faculty of Graduate Studies, Mahidol University
for the degree of Master of Science (Biotechnology)
on
May 26, 2008

……
Mr. Tongchai Nimitphak
Candidate

……
Assoc. Prof. Manop Suphantharika, Ph.D.
Chair

……
Prof. Timothy William Flegel, Ph.D.
Member

……
Mrs. Wansika Kiatpathomchai, M.Sc.
Member

……
Prof. Boonsirm Withyachumnarnkul, Ph.D.
Member

……
Assoc. Prof. Wasana Sukhumsirichart, Ph.D.
Member

……
Prof. Paisarn Sithigorngul, Ph.D.
Member

……
Prof. Banchong Mahaisavariya, M.D.
Dean
Faculty of Graduate Studies
Mahidol University

……
Prof. Skorn Mongkolsuk, Ph.D.
Dean
Faculty of Science
Mahidol University

ACKNOWLEDGEMENTS

I would like to express my gratitude and appreciation to all those who gave me the possibility to complete this thesis. I wish to thank my advisor, Prof. Timothy William Flegel, for his supervision, valuable helpful consultation and kind attention which have enabled me to carry out until this thesis complete successfully. Their criticism, advice and helpfulness will be long remembered with respect.

I wish to thank Mrs. Wansika Kiatpathomchai, for her kindness consultation, encouragement the research and providing suggestions for improvement in this experiment which have enabled me to carry out until this thesis complete successfully.

I am very grateful to Assoc. Prof. Wasana Sukhumsirichart, Department of Biochemistry, Faculty of Medicine**,** Srinakharinwirot University, for her guidance, helpful suggestion, and comments which have enabled me to carry out until this thesis complete successfully. Her helpfulness will be long remembered with respect.

I would like to acknowledge Dr. Pongsopee Attasart, Institute of Molecular Biology and Genetics, Mahidol University, for her providing the HPV-infected hepatopancreas samples of *P. monodon* used in this experiment.

I am thankful to National Center for Genetic Engineering and Biotechnology, Thailand (BIOTEC), for financial support in this experiment.

Finally, I am grateful to my family for their entirely care, infinite love, understanding, financial support and encouragement in every way possible me to succeed in my education.

Tongchai Nimitphak

THE DEVELOPMENT OF LOOP-MEDIATED ISOTHERMAL AMPLIFICATION WITH LATERAL-FLOW DIPSTICKS (LAMP-LFD) TO DETECT INFECTION OF HEPATOPANCREATIC PARVOVIRUS (HPV)

TONGCHAI NIMITPHAK 4837145 SCBT/M

M.Sc. (BIOTECHNOLOGY)

THESIS ADVISORS: TIMOTHY WILLIAM FLEGEL, Ph.D., BOONSIRM WITHYACHUMNARNKUL, Ph.D., WASANA SUKHUMSIRICHART, Ph.D., WANSIKA KIATPATHOMCHAI, M.Sc.

ABSTRACT

Shrimp hepatopancreatic parvovirus (HPV) is a DNA virus that retards growth in cultivated shrimp. Present methods for its detection include normal or nested PCR followed by electophoresis, PCR-ELISA, real-time PCR, immunodetection and histological examination. These techniques entail various disadvantages such as high cost, long assay time or use of toxic substances. Loop-mediated isothermal amplification (LAMP) is a novel method for rapid, sensitive and specific pathogen detection via amplification of target nucleotide sequences under inexpensive isothermal conditions. Combining LAMP with amplicon detection by chromatographic lateral-flow dipsticks (LFD) instead of electrophoresis allows interpretation of results within 10 min. A set of four LAMP primers was designed from the capsid protein gene of HPV and LAMP conditions were optimized at 63^{o}C for 1 h. The FITC-labeled DNA probe for the LFD test was optimized at 20 pmole. With a quantified plasmid template containing the LAMP target sequence, LAMP-LFD was 100 times more sensitive than PCR-electrophoresis and equal to nested PCR-electrophoresis. With DNA extracts from HPV-infected shrimp, it was 10 times more sensitive than normal PCR-electrophoresis but 10 times less sensitive than a commercial kit for nested PCR-electroporesis. The LAMP-LFD method gave negative test results with DNA extracts from normal shrimp and from shrimp infected with other DNA viruses including *monodon baculovirus* (MBV), white spot syndrome virus (WSSV) and infectious hypodermal and hematopoietic necrosis virus (IHHNV).

KEY WORDS: HEPATOPANCREATIC PARVOVIRUS/ LOOP-MEDIATED ISOTHERMAL AMPLIFICATION/ LFD

55 pp.

การพัฒนาเทคนิค Loop-mediated isothermal amplification ร่วมกับเทคนิค Lateral-flow dipsticks (LAMP-LFD) เพื่อการศึกษาการมีอยู่ของเชื้อไวรัส Hepatopancreatic parvovirus (HPV) ที่เป็นสาเหตุของโรคกุ้งแคระ (THE DEVELOPMENT OF LOOP-MEDIATED ISOTHERMAL AMPLIFICATION WITH LATERAL-FLOW DIPSTICKS (LAMP-LFD) TO DETECT INFECTION OF HEPATOPANCREATIC PARVOVIRUS (HPV))

ธงชัย นิมิตภาคย์ 4837145 SCBT/M

วท.ม.(เทคโนโลยีชีวภาพ)

คณะกรรมการควบคุมวิทยานิพนธ์: ทิมโมที วิลเลียม เฟลเกล, Ph.D., บุญเสริม วิทยชำนาญกุล, Ph.D., วาสนา สุขุมศิริชาติ, Ph.D., วรรณสิกา เกียรติปฐมชัย, M.Sc.

บทคัดย่อ

Hepatopancreatic parvovirus หรือ HPV เป็นไวรัสที่มีสารพันธุกรรมเป็นดีเอ็นเอสายเดี่ยว ที่เป็นสาเหตุของโรคกุ้งแคระ ในปัจจุบันมีวิธีตรวจสอบไวรัสชนิดนี้หลายวิธี ได้แก่ PCR หรือ nested PCR ที่ตามด้วยการตรวจสอบผลโดยการแยกด้วยกระแสไฟฟ้าบนแผ่นเจลอะกาโรส เทคนิค PCR-ELISA เทคนิค real-time PCR เทคนิคการตรวจสอบทางภูมิคุ้มกัน และเทคนิคทางพยาธิวิทยาของเนื้อเยื่อ ซึ่งเทคนิคเหล่านี้ล้วนมีข้อเสีย เช่น ราคาแพง ใช้เวลาในการตรวจนาน หรือใช้สารก่อพิษ เทคนิค Loop-mediated isothermal amplification หรือ LAMP เป็นเทคนิคใหม่ที่รวดเร็ว ราคาถูก มีความไว และ ความจำเพาะสูงในการเพิ่มปริมาณสารพันธุกรรมภายใต้สภาวะอุณหภูมิเดียว การรวมเทคนิค LAMP กับเทคนิค lateral-flow dipsticks หรือ LAMP-LFD เป็นการตรวจสอบผลผลิต LAMP โดยอาศัยหลักการของเทคนิคโครโมโตกราฟฟีแทนการแยกด้วยกระแสไฟฟ้า ทำให้สามารถวิเคราะห์ผลได้ภายในเวลาเพียง 10 นาที การศึกษาเริ่มจากการออกแบบไพรเมอร์สำหรับปฏิกิริยา LAMP 4 ตัว จากส่วนของโปรตีน capsid ของไวรัส HPV ซึ่งสภาวะของปฏิกิริยา LAMP ที่เหมาะสมคืออุณหภูมิ 63°C เป็นเวลา 1 ชั่วโมง ผลผลิต LAMP ที่ได้ถูกตรวจสอบด้วยเทคนิค LFD โดยการเติม DNA ติดตามที่เชื่อมติดกับ FITC ปริมาณ 20 pmole เมื่อทดสอบความไวในการตรวจด้วยเทคนิค LAMP-LFD โดยใช้พลาสมิดซึ่งมีลำดับเบสเป้าหมายที่ทราบจำนวนพบว่าเทคนิคนี้มีความไวมากกว่าเทคนิค PCR 100 เท่า ซึ่งเทียบเท่ากับเทคนิค nested-PCR อย่างไรก็ตามเมื่อตรวจสอบกับ DNA ของตับกุ้งที่ติดเชื้อไวรัส HPV พบว่า LAMP-LFD มีความไวมากกว่า PCR 10 เท่า แต่น้อยกว่าชุดตรวจ nested-PCR 10 เท่า นอกจากนั้น LAMP-LFD ได้ให้ผลลบต่อ DNA ที่สกัดจากกุ้งปกติ และกุ้งที่ติดเชื้อไวรัสชนิดอื่นๆ เช่น *monodon baculovirus* หรือ MBV ไวรัสตัวแดงดวง ขาว หรือ WSSV และ ไวรัส infectious hypodermal and hematopoietic necrosis virus หรือ IHHNV

55 หน้า

CONTENTS

	Page
ACKNOWLEDGEMENT	iii
ABSTRACT (ENGLISH)	iv
ABSTRACT (THAI)	v
LIST OF TABLES	viii
LIST OF FIGURES	ix
LIST OF ABBREVIATION	xi
CHAPTER	
I INTRODUCTION	1
II LITERATURE REVIEW	4
1. Shrimp Hepatopancreatic Parvovirus (HPV)	4
1.1 Viral characteristics	4
1.2 Symptoms and Infection	5
1.3 Detection of HPV	6
1.3.1 Histological techniques	6
1.3.2 Molecular techniques	8
2. Loop-mediated isothermal amplification (LAMP)	10
3. Lateral-Flow Dipsticks (LFD)	14
III MATERIALS AND METHODS	18
1. Shrimp Samples	18
2. DNA extraction	18
3. Design of biotin-labeled primers for LAMP	18
4. Recombinant plasmid construction	19
5. Optimize the LAMP condition	19
6. LAMP sensitivity determination using a recombinant plasmid	20
7. One step sensitivity determination with recombinant plasmid	20
8. LAMP sensitivity determination with HPV-infected shrimp samples	20
9. One step PCR sensitivity determination with HPV-infected shrimp samples	21

CONTENTS

(Continued)

	Page
10. Nested PCR sensitivity determination with HPV-infected shrimp samples	21
11. Specificity of LAMP detection	23
12. Design and optimization of FITC-labeled DNA probe for LFD	23
13. LFD sensitivity determination	23
14. LFD specificity determination	24
IV RESULTS	25
1. Design primers for LAMP	25
2. Optimization LAMP conditions	25
3. Comparison in sensitivity between one-step PCR and LAMP using recombinant plasmid	25
4. Comparison of sensitivity by one-step PCR, Nested PCR and LAMP using total DNA from HPV-infected shrimp	29
5. Specificity of LAMP detection	30
6. Optimization DNA probe amount in LFD	30
7. Sensitivity determination using lateral-flow dipsticks	33
8. Specificity of LFD detection	33
V DISCUSSION	36
VI CONCLUSION	43
REFERENCES	45
APPENDIX	51
BIOGRAPHY	55

LIST OF TABLE

TABLE **Page**

1. LAMP primers and DNA probe sequences and its positions on capsid protein gene 26

LIST OF FIGURES

FIGURE		Page
1.	A comparison of HPV-infected shrimp and normal shrimp from a rearing pond.	6
2.	Histopathological features of HPV-infected hepatopancreatic tubule epithelial cells of shrimp stained with H&E.	7
3.	HPV inclusion body containing virions in the nucleus of an infected hepatopancreatic cell of shrimp.	8
4.	The amplification step and the base sequences of LAMP products and their intermediates during strand displacement DNA synthesis in the LAMP method.	13
5.	The mechanism of the lateral-flow dipstick (LFD).	17
6.	Different test results and their interpretation when using a lateral flow dipstick (LFD).	17
7.	DNA sequences of partial capsid protein of HPV Thai strain for designing LAMP primers and the positions at which LAMP primers and probe attach for amplification and hybridization.	27
8.	Temperature optimization tests for the LAMP reaction to detect HPV.	28
9.	Sensitivity of one-step PCR detection of HPV using the LAMP primers F3 and B3 with recombinant plasmid.	28
10.	Sensitivity for LAMP detection of HPV with recombinant plasmid.	29
11.	Sensitivity of two one-step PCR detection methods for HPV detection in DNA extracted from HPV-infected sample.	30
12.	Sensitivity of two nested PCR detection methods for HPV detection in DNA extracted from HPV-infected sample.	31
13.	Sensitivity of LAMP detection of HPV in DNA extracted from HPV-infected sample.	31
14.	Specificity of the LAMP method for HPV detection.	32
15.	LAMP assay for HPV detection in *M. rosenbergii* infected with an HPV-like virus.	32

LIST OF FIGURES

(Continued)

FIGURE	Page
16. FITC probe optimization for detection of HPV amplicons by LFD detection.	33
17. Sensitivity tests using the lateral flow dipstick for detection of LAMP products from a plasmid template.	34
18. Sensitivity tests using the lateral flow dipstick for detection of LAMP products from DNA of HPV-infected shrimp.	34
19. Specificity tests using the lateral flow dipstick for detection of LAMP products.	35
20. Lateral flow dipstick for detection of LAMP products using DNA extracted from *M. rosenbergii* infected with an HPV-like virus.	35
21. Alignment (5’ to 3’) of Thai HPV sequences and other geographical HPV sequences (Indian, Korean and Australian) in the target region for the LAMP F3 and B3 primers.	38

LISTS OF ABBREVIATIONS

%	=	Percent
A	=	Absorbance
bp (s)	=	Base pair (s)
^{o}C	=	Degree Celsius
DNA	=	Deoxyribonucleic acid
dNTPs	=	Deoxyribonucleotide triphosphate
E. coli	=	*Escherichia coli*
EDTA	=	Ethylenediaminetetra acetic acid
e.g.	=	Exempli gratia (Latin), for example
ELISA	=	Enzyme-linked immunosorbent assay
et al.	=	Et alii (Latin), and others
Fig.	=	Figure
g	=	Gram
HP	=	Hepatopancreas
h	=	Hour
HCl	=	Hydrochloric acid
IPTG	=	Isopropyl thio-β-D-galactoside
KCl	=	Potassium chloride
LAMP	=	Loop-mediated isothermal amplification
LB	=	Laria – Bertani
$MgCl_2$	=	Magnesium chloride
$Mg_2P_2O_7$	=	Magnesium pyrophosphate
$MgSO_4$	=	Magnesium sulphate
µg	=	Microgram
µl	=	Microliter
µM	=	Micromolar
mg	=	Milligram
ml	=	Milliliter

LISTS OF ABBREVIATIONS

(Continued)

mM	=	Millimolar
min	=	Minute
M	=	Molar
NaCl	=	Sodium chloride
ORF (s)	=	Open reading frame (s)
PCR	=	Polymerase chain reaction
PL	=	Post larvae
rpm	=	Round per minute
RT	=	Room temperature
SDS	=	Sodium dodecyl sulfate
sec	=	Second
U	=	Unit
UV	=	Ultraviolet
X-Gal	=	5-bromo-4-chloro-3-indolyl- beta-D-galactopyranoside

CHAPTER I
INTRODUCTION

In Thailand, shrimp are economically important aquatic animals that currently encounter infections by microorganisms such as bacteria, fungi, protozoa and viruses. Viruses cause the most serious losses to shrimp farmers in many countries. They can be divided into DNA and RNA viruses. Among the most important DNA viruses commonly found in Thailand are WSSV (white spot syndrome virus), MBV (monodon baculovirus) and HPV (hepatopancratic parvovirus). YHV (yellow head virus) is an important RNA virus. Some viruses such as WSSV and YHV cause losses by killing shrimp while others such as HPV and MBV cause losses by retarding growth. Since retarded growth can be caused by many factors, farmers may not know that their shrimp are infected with HPV even after they are harvested. By the time they might suspect its presence in a pond after the development of wide variation in shrimp sizes and an overall retardation in growth, it may be too late to rectify the situation.

Since shrimp viral infections generally cannot be treated in shrimp ponds, the best approach for shrimp farmers is to adopt management practices that will prevent or reduce the probability of infections occurring. One approach is to inspect shrimp post larvae prior for the presence of dangerous pathogens to the stocking of ponds. Another is to prevent wild carriers from entering ponds before stocking and after cultivation has begun. Choosing not to adopt these management practices but to rear shrimp in open systems increases the risk of pathogen transfer from the outside environment and among rearing ponds. The most common approach currently adopted in Thailand is to strictly manage water and soil sanitation prior to stocking of ponds, to use high quality post larvae and to cultivate shrimp under closed or semi-closed conditions. Under good management practices, shrimp farmers also avoid negative impacts on the environment such as accumulation of chemicals or antibiotic residues in the environment around shrimp farms and in the shrimp themselves.

In monitoring post larvae and shrimp in rearing ponds, there are many available methods for detection of viral pathogens. These include normal

histopathological techniques (Lightner, 1996b) such as tissue smears, squash mounts and hematoxylin and eosin (H&E) staining of paraffin-embedded tissue sections. Another direct detection method is transmission electron microscopy (TEM) of thin tissue sections (Pantoja and Lightner, 2001). These techniques may give false negative results in cases of low levels of infection and they are also destructive processes (i.e., require death of the sampled shrimp). To avoid these problems, new methods have been developed for viral detection using non-descructive methods. Examples include techniques such as polymerase chain reaction (PCR) assays (Pantoja and Lightner, 2000; Phromjai et al., 2002; Sukhumsirichart et al., 1999, 2002), PCR-ELISA (Sukhumsirichart et al., 2002) and dot blot hybridization (Phromjai et al., 2002) using small quantities of shrimp hemolymph, shrimp feces or small tissue fragments removed without killing the shrimp. These techniques are useful not only for screening shrimp in rearing ponds but also for rapid and easy screening of large numbers of potential hosts and their life stages as potential carriers even if lightly infected (Flegel, 2006). However these techniques have some limitations with respect to the long time required to carry them out, the high cost of required instruments, the possibility of non-specific amplification products and the need for toxic substances such as ethidium bromide (EtBr) to interpret results.

Thus, the purpose of this work was to develop a new detection technique for HPV based on loop-mediated isothermal amplification (LAMP) with high sensitivity and specificity to target DNA. Since this technique can amplify target DNA under isothermal conditions, it has the advantage that high cost instruments are not needed and only a heating block or water bath is sufficient. Another advantage over PCR is the fact that amplification is continuous and there is no need to wait temperature adjustment between amplification steps. This allows the LAMP to be completed within 1 hour. To further improve the detection method, another objective was to replace the traditional method of cDNA amplicon detection by gel electrophoresis and subsequent staining with ethidium bromide (a carcinogen) that takes approximately 30-60 min after LAMP. To this end, an alternative method was chosen for cDNA amplicon detection within 10 min by lateral flow chromatography with a lateral flow dipstick (LFD). In summary, the objectives of the experimental work were:

- To develop a LAMP technique for detection of HPV in shrimp.

- To combine this with cDNA amplicon detection using a lateral flow chromatography technique.

CHAPTER II
LITERATURE REVIEW

1. Shrimp *Hepatopancreatic Parvovirus* (HPV)

1.1 Viral characteristics

HPV was first described from farmed marine shrimp in Singapore (Chong and Loh, 1984) and has been seen in Thailand for many years (Flegel et al., 1995, 1992). It is a single-stranded DNA (ssDNA) virus classified in the family Parvoviridae (Lightner and Redman, 1985) in the densovirus group (DNV). However, it has distinctive capsid proteins and a distinctive genomic structure when compared to other densoviruses. By Transmission electron microscopy (TEM), purified viral preparations show unenveloped icosahedral viral particles of 22 to 24 nm in diameter. A recent publication described the complete genomic nucleotide sequence of Thai HPV with a size around 6.3 Kb that consisted of 3 ORF. ORF1 and ORF2 encode putative non-structural proteins-2 (NS-2) and the major nonstructural protein (NS-1) and the minor (NS-2) are similar to those of other parvovirus, and ORF3 encodes for a capsid protein (VP) (Sukhumsirichart W. et al., 2006). Three types of HPV have been described at the molecular level from penaeid shrimp. One is from *P. chinensis* from Korea (Bonami et al., 1995), one from *P. monodon* in Thailand (Sukhumsirichart et al., 2002) and one from *P. merguiensis* in Australia (La Fauce et al., 2007). These viruses differ in total genome length (approximately 4, 6.3 and 5.9 Kb, respectively). Lightner (1996) has proposed that HPV originated in the Indo-Pacific but was later reported in wild shrimp from the Americas probably due to importation of live shrimp for aquaculture. Although most shrimp farmers do not consider HPV to be a very serious problem when compared to major viruses like WSSV and YHV, this does not mean that the disease can be ignored and control abandoned. Simple measures such as

broodstock monitoring and washing of eggs and nauplii can reduce its impact substantially.

1.2 Symptoms and Infection

Although a preliminary report on HPV in Thailand suggested that it might kill shrimp larvae within the first month after stocking (Flegel et al., 1995), it was later found that this was not the case (Flegel et al. 1999). During pond rearing, most HPV-infected shrimp show no specific gross signs that can be ascribed specifically to HPV. However, in severe cases whitening and shrinkage of the hepatopancreas have been reported due to incomplete function in food digestion and to tissue resorption. Another characteristic of shrimp in ponds infected with HPV is a wide variation in shrimp sizes and an overall decreased growth rate. In one report, the mean size of HPV-infected shrimp was approximately 6 cm in length (Fig. 1) and 5 g in weight (Flegel et al., 1999, 2006) when compared to 11 cm or more for shrimp from the same pond that were not infected with HPV. Moreover, the shrimp may show a decreased appetite and low preening activity leading to fouling by external protozoa on the body and gills. This may predispose them to infection by opportunistic pathogens such as *Vibrio* bacteria. Based on histological analysis, PL stages of several species of penaeid shrimp have been reported to be susceptible to HPV infection (Lightner, 1996). Examples are *Penaeus monodon*, *P. merguiensis*, *P. indicus* and *P. chinensis* (Chong & Loh, 1984). The river prawn *Macrobrachium rosenbergii* has also been reported to be susceptible to infection with an HPV-like virus, but molecular tests indicated that it was not closely related to HPV in peaneid shrimp (Lightner et al., 1994).

With respect to infection route, Lightner (1996) has reported that F1 larvae of of *P. chinensis* were found to be infected with HPV, suggesting that vertical transmission could occur via parental broodstock. There are similar reports of HPV infection in hatchery larvae of *P. monodon* in India (Manivannan et al., 2002; Umesha et al., 2003). In contrast, in Thailand HPV was not seen in larvae in hatcheries but in PL in outdoor nurseries and in pond suggesting that there was horizontal transmission from unknown carriers (Flegel et al., 2004). Recently (Catap et al., 2003), it has been shown that PL of *P. monodon* can be successfully infected with HPV by the oral route,

indicating that horizontal transmission is possible. This work opens the way for further laboratory studies on the effects of HPV on shrimp mortality and growth and on its modes of infection.

Fig. 1. A comparison of HPV-infected shrimp (*P. monodon*) (left) and normal shrimp (right) from a rearing pond (Flegel, 2006).

1.3 Detection of HPV

In order to control HPV in shrimp production systems, it is necessary to have effective detection methods. Because high variation in shrimp size and other gross signs in HPV infection are not specific, histological and molecular methods of analysis such as PCR assay are required for its detection. The following paragraphs review the techniques currently used for HPV detection.

1.3.1 Histological techniques

Preliminary detection of HPV can be achieved squash mounts of hepatopancreatic tissue from shrimp post-larvae (PL) stained with malachite green (wet-mount technique) or in hepatopancreatic tissue smears from juvenile or adult shrimp in a drop of 10% formalin in 2.8% NaCl followed by drying and normal haematoxylin and eosin (H&E) staining (Flegel, 2006) or by Giemsa staining. It can also be detected in H&E stained tissue sections examined under the light microscope. H&E staining reveals the presence of large, usually single, basophilic intranuclear

inclusion bodies 5 to 11 µm in diameter that lead to displacement of the nucleoli in the dividing cells (E-cells) of the hepatopancreatic tubule epithelium (Fig. 2) and sometime in adjacent mid-gut cells (Lightner, 1996). Transmission electron microscopy reveals that these inclusion bodies are composed of icosahedral, electron-dense virions measuring 22-24 nm in diameter arranged either randomly or in para-crystalline arrays and (Fig. 3). These lesions are typical for all types of HPV reported from shrimp. All of these techniques are destructive, since they require sacrifice of the tested shrimp samples. Because of this, they are not suitable for broodstock testing or for monitoring large numbers of shrimp from production ponds. In addition, they may give false negative results for very lightly infected specimens.

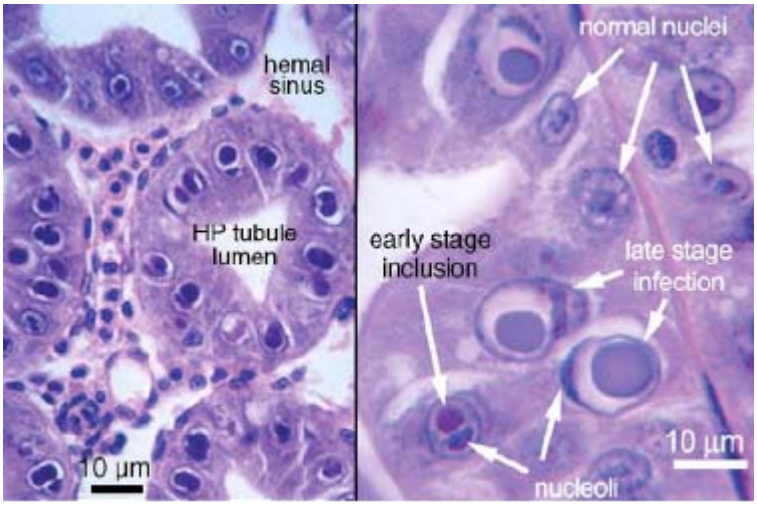

Fig. 2. Photomicrographs of histopathological features of HPV-infected hepatopancreatic tubule epithelial cells of shrimp (*P. monodon*) stained with H&E (Flegel, 2006).

1.3.2 Molecular techniques

Although all types of HPV give similar histopathlogical and ultrastructural features, DNA sequence analysis and comparison has revealed that there are significant differences HPV from different shrimp species and from different geographical regions. For example, DNA sequences of HPV from *P. monodon* in

Thailand (HPVmon, AY008257) have only 70% identity with HPV from *P. chinensis* in Korea (HPVchin, AF456476). Their genome sizes also differ by approximately 1 Kb (Sukhumsirichart et al., 1999). Therefore, the molecular techniques are needed to distinguish among the types. They are also needed for non-destructive analysis of small tissue or hemolymph samples that do not harm broodstock or pond-reared shrimp.

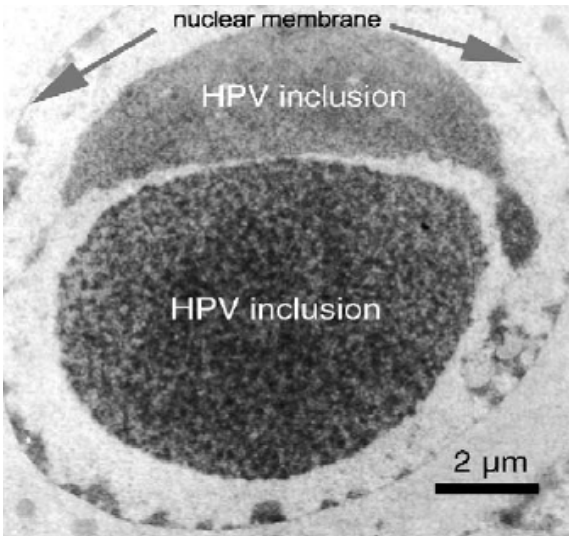

Fig. 3. Electron micrograph of an HPV inclusion body containing virions in the nucleus of an infected hepatopancreatic cell of shrimp (*P. monodon*) at low magnification (Flegel, 2006).

***In situ* hybridization, PCR-ELISA, Southern Hybridization:** Although genomic sequence comparison has shown that HPVmon and HPVchin differ by approximately 30%, both give positive *in situ* hybridization reactions using a commercial probe prepared from HPVchin (Phromjai et al., 2001). This probe also gives a positive reaction with HPV in *P. merquiensis* and *P. esculentis* from Australia (Owens, 1997). By contrast, the probe gave negative results with an HPV-like virus in *M. rosenbergii* (Lightner et al., 1994) from Malaysia. Thus, positive *in situ*

hybridization results cannot be used to conclude high genomic sequence identity (Flegel, 2006).

The first specific polymerase chain reaction (PCR) assay method for HPV detection in post larvae of the black tiger shrimp (*P. monodon*) yielded an amplicon product of 156 bp that was detected by Gel electrophoresis. In addition, both ELISA and southern blot hybridization methods were also developed for its detection from as little as 1 pg to 0.01 fg of purified HPV DNA (Sukhumsirichart et al., 2002).

One-step PCR and Nested PCR Kits: The first commercial primers designed from HPVchin (DiagXotics Co. Ltd, Nashville TN) amplifed a 350 bp product from HPVchin but gave a weak signal with a 732 bp product form HPVmon (Phromjai et al., 2002). Thus, the HPVmon specific primers were designed to yield a 441 bp product that gave a high effective amplification to HPVmon (Phromjai et al., 2002) and also with HPV in *P. monodon* from India (Manjanaik et al., 2005). On the other hand, the primers from HPVchin could not amplify fragments from HPV in India (Umesha et al., 2003). These results suggest that HPV from India is more closely related to HPV from Thailand than HPV from Korea. Since the various one-step PCR methods may not amplify fragments from lightly infected shrimp, nested PCR methods were developed in India (Manjanaik et al., 2005) and Thailand (SBBU, Pathumthani) to confirm the viral infection by re-amplification using a second pair of primers that target positions within the sequence of the amplicon from the first round of PCR amplification. For example, the HPV 465/183 nested PCR kit (Ezee Gene, SBBU, Pathumthani) gives one band of 465 bp in the first step and two bands 465 bp and 183 bp in the nested step for heavy infections. With light infections, only the 183 bp band from nested step is seen. The Indian nested PCR method (Manjanaik et al., 2005) developed from the one-step PCR method of Phromjai et al. (2002) gives the original 441 bp product from the first step and a 265 bp product from the second.

2. Loop-mediated isothermal amplification (LAMP)

Efficient, rapid and timely disease detection is of critical importance for successful aquaculture management and improvement. For field application at shrimp farms, the optimal detection procedure would be cheap, quick and easy-to-operate by relatively untrained personnel but also give satisfactory sensitivity and specificity. The current methods used widely for PCR detection are difficult to apply in a farm setting because of the highly trained personnel and sophisticated equipment required (e.g., a themocycler and electrophoresis equipment). Loop-mediated isothermal amplification (LAMP) is the novel method to amplify targeted nucleic acid sequences. It can amplify rapidly and with high specificity and sensitivity under isothermal conditions using inexpensive equipment. This technique employs a DNA polymerase called "*Bst* DNA Polymerase" with strand displacement activity and a set of 4 designed primers that hybridize specifically to 6 positions on targeted nucleotides (Notomi et al., 2000). These four primers can be divided into 2 types "outer primers" (F3 and B3) and "inner primers" (e.g., FOP = forward outer primer, BOP = backward outer primer, FIP = forward inner primer and BIP = backward inner primer). The outer primers are designed for the ends of the region targeted for amplification. In addition, the inside ends of F3 and B3 (17-21 nt.) are named F2C and B2 (23-24 nt.), and within the F2C and B2 regions (40 nt.) located F1C and B1 (23-24 nt.) regions, respectively. Thus, FIP contains sequences F1C and the complementary sequence F2C (F2), while BIP contains the sequence B2 and the complementary sequence B1 (B1C). The 2 regions of both inner primers are linked together with a TTTT spacer.

The LAMP mechanism can be described as follows (Fig. 1A). The LAMP reaction consists of 3 steps. The first is called the "LAMP initial step". It is started by inner primer FIP that contains both sense and anti-sense sequences for the targeted nucleotides. These hybridize to the F2C region of the targeted DNA and start the complementary strand synthesis. Next, the shorter outer primer F3that is added in lower concentration than the inner primer, hybridizes to its complementary sequence (F3C) to initiate strand displacement DNA synthesis. This releases a new single strand DNA that forms a loop structure at its end since it contains linked complementary sequences (Fig. 4A, No. 4). This strand now acts as the template upon which the

backward primers hybridize to initiate strand displacement DNA synthesis followed by another loop formation ending in a stem-loop DNA structure called a “dumb-bell like structure” (Fig. 4A, No. 6).

The dumb-bell like structure is the starter for the second step in the LAMP reaction called the “LAMP cycling step”. This is initiated by self-primed stand displacement DNA synthesis to yield a stem-loop DNA structure (Fig. 4A, No. 7). The FIP primer hybridizes to the dumb-bell like structure at the loop position to prime strand displacement DNA synthesis that produces an intermediate stem-loop molecule that contains an inverted copy of the target sequences and one gapped position with a loop formed at the BIP site (Fig. 4A, No.8). Subsequent self-primed stand displacement DNA synthesis yields a fragment of twice the length with a filled gap stem-loop structure (Fig. 4A, No. 9) plus another dumb-bell like structure containing complementary sequences of the original structure (Fig. 4A, No. 20). Next, both of these products hybridize with the BIP primer and self-primed strand displacement DNA synthesis starts again. Some of the LAMP products lead to displacement DNA synthesis in the third step of the LAMP reaction called the “elongation and recycling step”.

When a LAMP reaction is carried out, the whole mixture contains a complex of dumb-bell structures, stem-loop DNA structures and cauliflower-like structures with multiple loops formed by annealing between alternately inverted repeats of target sequences in the same strand (Fig. 4A, No. 16-18). The sequences of the LAMP products and their intermediates are illustrated in Fig. 4B. Because it is a continuous method independent of thermocycling, it is easy and rapid and can be completed within 1 h with high sensitivity and specificity. Recently reports have shown that LAMP reactions can proceed directly with samples collected form water, culture medium and ocular fluid suggesting that it is less susceptible to inhibitory substances than is normal PCR cycling (Enomoto et al., 2005; Kaneko et al., 2005). Since the LAMP method was first published in 2000, it has been tested for diagnosis of shrimp viruses including Taura syndrome virus (TSV) (Teng et al., 2007; Kiatpathomchai et al., 2007), yellow head virus (YHV) (Mekata et al., 2006), infectious hypodermal and hematopoietic necrosis virus (IHHNV) (Sun et al., 2006) and white spot syndrome

virus (WSSV) (Kono et al., 2004). It has also been used for detection of bacteria (Enosawa et al., 2003; Iwamoto et al., 2003; Maruyama et al., 2003; Savan et al., 2004), protozoa (Kuboki et al., 2003) and fungi (Endo et al., 2004).

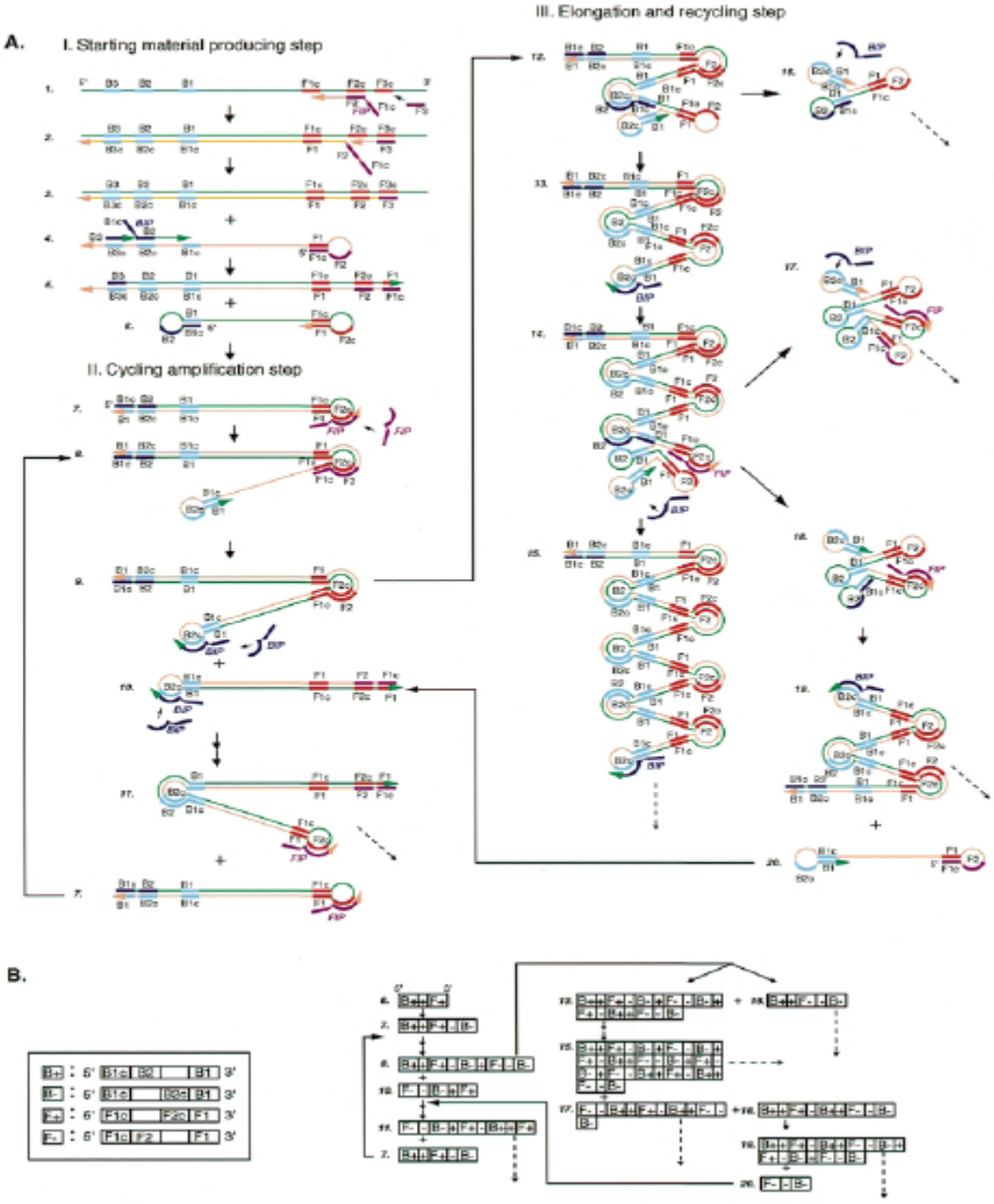

Fig. 4. Schematic diagrams showing the amplification step (A) and the base sequences of LAMP products and their intermediates (B) during strand displacement DNA synthesis by the LAMP method (Notomi et al., 2000).

3. Lateral-Flow Dipsticks (LFD)

Although there are highly sensitive and specific tools for nucleic acid amplification, the detection of the amplification products is often dependent on complex or expensive instruments such as those for electrophoresis and may require the use of harmful carcinogens such as ethidium bromide (EtBr) to reveal a signal. For LAMP products detection is also possible by the naked-eye as a white precipitate of magnesium pyrophosphate. Sensitivity and rapidity can be increased by real-time assay of a fluorescent dye (Dukes et al., 2006) or real-time assay of magnesium pyrophosphate accumulation by detection of its turbidity (Enomoto et al., 2005; Mori et al., 2004; Poon et al., 2006). However, these techniques have drawbacks due to the lack of specific amplicon detection. One method to overcome this limitation is specific-amplicon identification of LAMP products by sequence-specific probe hybridization combined with cationic polymer precipitation and followed by visualization of fluorescent dye tagged probes using a UV transilluminator (Mori et al., 2006). Some of these procedures use expensive equipment like reflectometers and involve several complex steps including, incubation and washing steps that take some time to complete. Thus, they are not easily applied to field application at shrimp farms.

Lateral-flow dipsticks (LFD) can detect target cDNA sequences rapidly based on the principles of nucleic acid hybridization and chromatography in membrane strips that allow visual detection. This technique is simple and cost-effective when compared to complex and instrument-dependent commercial methods or classical enzyme immunoassays. No electronic devices other than a simple heating apparatus are needed and detection can be accomplished within 10-15 min after completion of the LAMP reaction whereas gel electrophoresis would take 45-60 min. Moreover, LFD is visual and no equipment is needed to read the results. This makes it more suitable for shrimp farm applications. However, their limitation is that sensitivity is to low for direct detection of nucleic acids from pathogens (Dineva M.A. et al., 2005). Thus, LFD must be combined with a highly sensitivity nucleic acid amplification step for practical applications using shrimp specimens. An example of disease detection in this format is LFD detection of PCR amplicons from white spot syndrome virus (WSSV)

(AMODIA, 2006). Another recent report describes the combination of RT-LAMP with LFD for detection of TSV in infected shrimp (Kiatpathomchai et al., 2008).

The LFD strip consistes of three main parts. The first part is called the “sample application area”. It contains a purple-red gold-conjugated primary antibody (1^o Ab) that binds specifically with a fluorescein isothiocyanate (FITC) label on a DNA probe. The second part consists of the “test line” that contains a biotin binding molecule such as streptavidin and the third part consists of the “control line” that contains a secondary antibody (2^o Ab) that binds specifically with the gold-labeled 1^o Ab contained in the first part of the strip.

The mechanism of the LFD strip is as follows. First, the LAMP reaction must be carried out using either FIP or BIP labeled with biotin, so that amplicons will become biotin-labeled during LAMP amplification. After amplification, an FITC-lobeled cDNA probe specific to the inner region of the WSSV target region is added to the amplification mixture and allowed to hybridize with any WSSV amplicons that have arisen during the LAMP reaction. If there are any WSSV amplicons present, this reaction results in formation of a DNA duplex labeled with FITC. If not, the FITC probe will remain free. Next, the LFD is dipped into a tube containing some of the LAMP reaction mixture in assay buffer. By capillary force, the reaction mixture migrates along the membrane and through the area containing the gold-labeled 1^o Ab specific for FITC. After attachment of the 1^o Ab to any FITC-labeled cDNA duplexes, a “primary antibody-probe complex” (complex) is formed (i.e., containing biotin-labeled cDNA amplicons duplexed with FITC-labeled DNA probe attached to the gold-labeled 1^o Ab). Present also in the migrating solution is free FITC probe, free gold-labeled 1^o Ab and FITC probe attached to gold-labeled 1^o Ab. Moving up the membrane the test line contains immobilized strepavidin that traps the biotin labeled complex to produce a reddish line. Uncomplexed biotin-labeled primers are also trapped here but have no color. All other free components and the 1^o Ab complexed with excess FITC probe move through the test line to the control line that contains a 2^o Ab to trap the gold-labeled 1^o antibody with or without attached FITC-probe and to produce a second reddish line. Thus, after incubation for 10 min, a reddish mark at the

test line and at the control line constitutes a positive test result for WSSV amplicons (Fig. 5 and Fig. 6, the first strip). In samples without target virus, the LAMP reaction will produce no amplified complementary cDNA with which the FITC-labeled probe can interact so no primary antibody-probe complex will be formed. Free biotin-labeled primers (no color) will be trapped at the test line while gold-labeled 1^{o} antibody with or without attached FITC-probe will be trapped at the control line. Thus, a negative test result for WSSV will yield a single reddish mark at the control line (Fig. 6, the second strip).

From this explanation, it is clear that this LFD detection method is generic and that it can be adapted for detection of any cDNA amplicon simply by changing the sequence (specificity) of the biotin labeled primers and of the FITC-probe used to match those of a different target sequence.

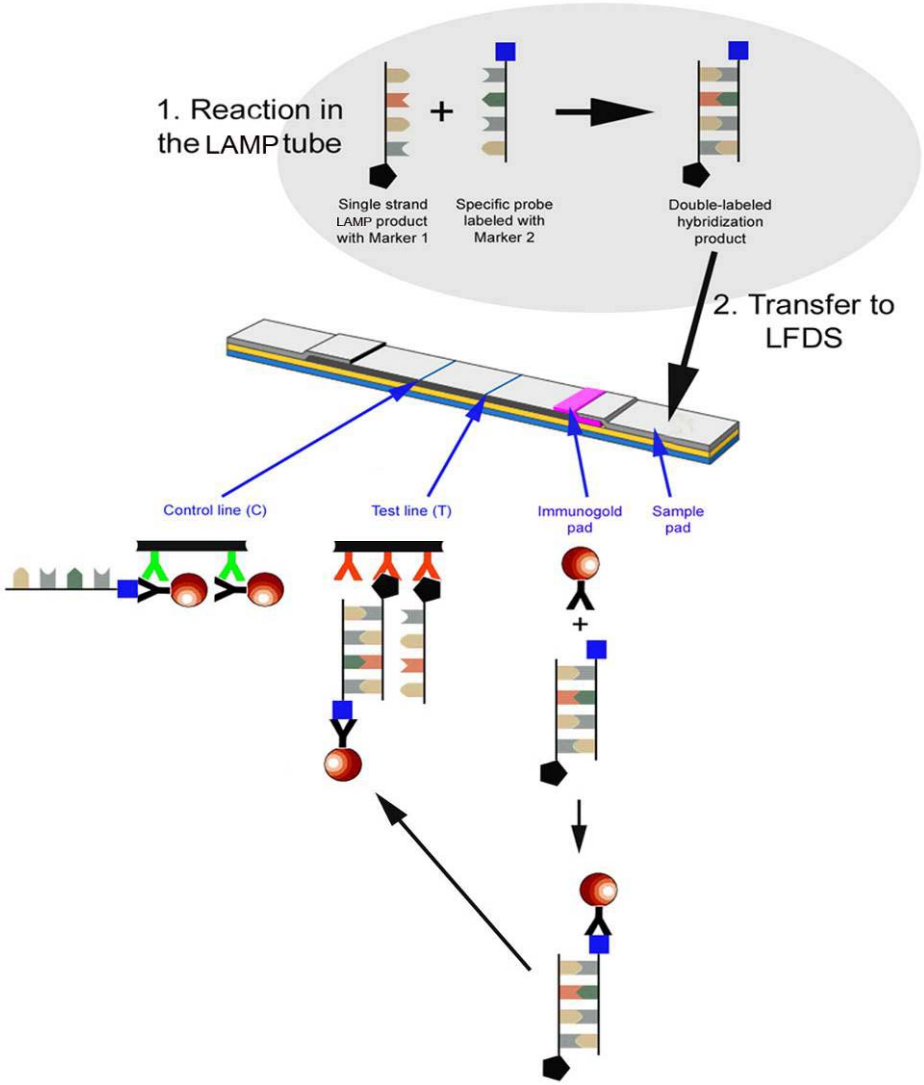

Fig. 5. Schematic diagram showing the mechanism of the lateral-flow dipstick (LFD).

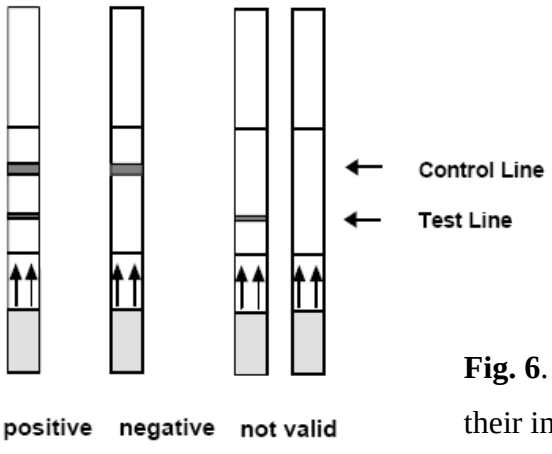

Fig. 6. Diagram of different test results and their interpretation when using a lateral flow dipstick (LFD).

CHAPTER III
MATERIALS AND METHODS

1. Shrimp Samples

Hepatopancreas samples of HPV-infected *P. monodon* were provided by the Institute of Molecular Biology and Genetics (MB), Nakhon Pathom province, Thailand. They were stored at -20 ^{o}C until DNA extraction.

2. DNA extraction

HPV-infected hepatopancreatic tissue was homogenized in 500 µl of Lysis buffer (10 mM Tris-Cl pH 8.0, 100 mM EDTA, 1% SDS). Then mercaptoethanol (1% v/v) and Proteinase K (0.1 mg/ml) was added and followed by incubation at 55 ^{o}C for 1 hr. Next, 700 µl of phenol : chroloform : isoamylalcohol (25: 24: 1) was added with mixing by inverting 5-6 times. After incubation at RT for 3 min, the mixture was centrifuged at 27,000 g for 10 min. The aqueous phase was transferred to a new tube and extracted twice more to remove impurities. Next, 3M sodium acetate (1:10 v/v) was added followed by DNA precipitation using 2 volumes of ice-cold absolute ethanol accompanied by gentle mixing inverting 2-3 times. After centrifugation at and centrifuged at 27,000 g for 5 min the DNA pellet was washed with 70% (v/v) ethanol, air dried and dissolved in 100 µl of DNase-free water (Leonard G. Davis, et al., 1994). Finally, total DNA is measured for quantity and quality using a UV spectrometer set at $A_{260/280}$.

3. Design of biotin-labeled primers for LAMP

LAMP primers for HPV detection were designed according to the capsid protein gene sequence of HPV from *P. monodon* in Thailand (GenBank Accession no. NC007218, Sukhumsirichart et al., 2006) using PrimerExplorerV3 program (Fujitsu, Tokyo, Japan) available at the website (http://loopamp.eiken.co.jp/e/lamp/primer.

html). LAMP primer selection was carried out according to the criteria described by Notomi et al. (2000) and either the FIP or BIP primers were 5´-labeled with biotin for use in the subsequent LFD step.

4. Recombinant plasmid construction

Targeted sites of the HPV genome were amplified by PCR with 100 ng of HPV-infected shrimp total DNA using F3 and B3 primers (232 bp sizes). The PCR reaction was carried out in 50 µl reaction mixture containing, 1x PCR reaction buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl) 1.5 mM $MgCl_2$, 200 µM dNTPs, 0.5 µM of each primer and 1.25 units Taq DNA polymerase (Invitrogen). Conditions for amplification were pre-denaturation at 95 ^{o}C for 5 min, followed by the 35 cycles of denaturing at 95 ^{o}C for 30 sec, annealing at 50 ^{o}C for 30 sec and extension at 72 ^{o}C for 30 sec followed by a final extension step at 72 ^{o}C for 7min. PCR products were purified using a NucleoSpin® Extract II kit (MACHEREY-NAGEL) and ligated into plasmid pGEM®-T Easy Vector (Promega) at 4 ^{o}C overnight. Next, 4 µl of recombinant plasmid was transformed into 100 µl of *E. coli* strain JM109, incubated for 20 min on ice, heat shocked for 1 min at 42 ^{o}C and incubated for 2 min on ice before addition of 600 µl of SOC medium. The mixture was then incubated at 37 ^{o}C for 1.5 h with shaking and centrifuged at 10,000 g for 2 min for cell precipitation. After that 500 µl of supernatant was removed and the remaining 200 µl was used to resuspend the transformants by gently pipetting up and down. Each of 100 µl transformed cell suspension was spread on LB-Agar contain 100 µg/ml of ampicillin and 100 µl of 20 mM IPTG and 50 µl of 50 mg/ml X-Gal and the plates were incubated at 37 ^{o}C overnight followed by blue-white colony selection. White colonies were picked for colony PCR by F3 and B3 primers set and amplified overnight in 2 ml of LB broth medium containing 100 µg/ml of ampicillin. The positive colonies from broth were selected for plasmid extraction using GeneJET™ Plasmid Miniprep Kits (Fermentas). Plasmids were kept at -20 ^{o}C until used.

5. Optimize the LAMP condition

LAMP reactions were carried out by varying temperatures between 60 ^{o}C and 65 ^{o}C for 1 h. The reaction mixture contained 2 µM each of inner primers FIP and

BIP, 0.2 μM each of outer primers F3 and B3, 1.4 mM of dNTP mix (Promega, Madison, WI, USA), 0.7 M betaine (Sigma-Aldrich, St. Louis, MO, USA), 6 mM $MgSO_4$, 8 U of *Bst* DNA polymerase (large fragment; New England Biolabs Inc., Beverly, MA, USA) along with 1X of the supplied buffer and 5 μl of a specified amount of template DNA in a final volume of 25 μl. Reaction mixture containing distilled water served as the negative control.

6. LAMP sensitivity determination using a recombinant plasmid

The recombinant plasmid stock solution was subjected to spectrophotometric analysis and the plasmid copy number was calculated. Then, the stock solution was ten-fold diluted with DNase-free water and used as template for LAMP detection (10^5, 10^4, 10^3, 10^2, 10 and 5 copies). After LAMP, 5 μl of the LAMP reaction solution was electrophoresed in a 2% agarose gel for 30-35 min and stained with ethidium bromide so that bands could be visualized with a UV transilluminator.

7. One step sensitivity determination with recombinant plasmid

The recombinant plasmid stock was 10-fold diluted and used as template in one step PCR (10^5, 10^4, 10^3, 10^2, 10 and 5 copies) using primers F3 and B3. After one step PCR, 10 μl of the PCR reaction solution was electrophoresed in a 1.5% agarose gel for 30-35 min and stained with ethidium bromide so that bands could be visualized with a UV transilluminator.

8. LAMP sensitivity determination with HPV-infected shrimp samples

Total DNA extracted from HPV infected shrimp was ten-fold diluted with DNase-free water and used as the template (100 ng, 10 ng, 1 ng, 0.1 ng, 0.01 ng and 1 pg) in sensitivity tests for optimum LAMP conditions. After LAMP, 5 μl of the LAMP reaction solution was electrophoresed in a 2% agarose gel for 30-35 min and stained with ethidium bromide so that bands could be visualized with a UV transilluminator.

9. One step PCR sensitivity determination with HPV-infected shrimp samples

Total DNA extracted from HPV infected shrimp was ten-fold diluted and used as the template (100 ng, 10 ng, 1 ng, 0.1 ng, 0.01 ng and 1 pg) in PCR sensitivity tests. PCR was carried out in 50 µl of reaction mixture containing 1x PCR reaction buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl), 1.5 mM $MgCl_2$, 200 µM dNTPs, 2 units *Taq* DNA polymerase (Invitrogen) and 0.1 µM each of primers for HPV detection (Sukhumsirichart et al. (2002). These were forward primer (5´-GCA CTT ATC ACT GTC TCT AC-3´) and reverse primer (5'-GTG AAC TTT GTA AAT ACC TTG -3´). The PCR protocol consists of pre-denaturation at 94 ^{o}C for 3 min and followed by 40 cycles of denaturation at 94 ^{o}C for 30 sec, annealing at 52 ^{o}C for 30 sec and extension at 72 ^{o}C for 30 sec followed by a final extension step at 72 ^{o}C for 5 min. This yielded a 156 bp amplicon. A second PCR test was carried out using primers F3 and B3 as described above for the plasmid template. Then 10 µl of PCR reaction solution from both methods was electrophoresised in 1.5% agarose gel for 30-35 min before staining with ethidium bromide so that bands could be visualized with a UV transilluminator.

10. Nested PCR sensitivity determination with HPV-infected shrimp samples

Total DNA extracted from HPV infected shrimp was ten-fold diluted and used as the template (100 ng, 10 ng, 1 ng, 0.1 ng, 0.01 ng and 1 pg) for amplification in nested-PCR sensitivity tests. The first PCR reaction step was carried out in 50 µl of reaction mixture containing, 1x PCR reaction buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl), 1.5 mM $MgCl_2$, 200 µM dNTPs, 1.25 unit *Taq* DNA polymerase (Invitrogen).and 0.2 µM each primer for HPV detection described by Phromjai et al. (2002). The forward primer was 5´-GCA TTA CAA GAG CCA AGC AG-3´ and the reverse primer was 5´-ACA CTC AGC CTC TAC CTT GT-3´. The PCR protocol consisted of pre-denaturation at 95^{o}C for 5 min followed by 35 cycles of denaturion at 95^{o}C for 1 min, annealing at 60^{o}C for 1 min, extension at 72 ^{o}C for 1 min and a final extension at 72 ^{o}C for 7 min. This yielded an amplicon of 441 bp. For the 2nd (nested)

PCR step, 2 µl of the first PCR reaction solution containing the 441 bp amplicon was added to 30 µl of reaction mixture containing, 1x PCR reaction buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl), 1.5 mM $MgCl_2$, 50 µM dNTPs, and 0.9 unit *Taq* DNA polymerase Recombinant (Invitrogen) and 0.16 µM each nested primer as described by Manjanaik et al. (2005). These were forward primer 5′-GGA ACT TCA AGA ACA AGA AAG-3′) and reverse (5′-AAC TCT CTG AGC CAC ATA TAC GT-3′),. The PCR protocol consisted of pre-denaturation at 94°C for 5 min followed by 30 cycles of denaturion at 94°C for 30 sec, annealing at 55°C for 30 sec, extension at 72 °C for 30 sec and final extension at 72 °C for 5 min. This yielded a 265 bp amplicon.

An additional nested PCR system tested consisted of a commercial kit (Ezee Gene, SBBU, Thailand). The first step of this nested PCR system was carried out in 50 µl of reaction mixture containing, 1x PCR reaction buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl), 1.5 mM $MgCl_2$, 200 µM dNTPs, 1.25 unit *Taq* DNA polymerase (Invitrogen) and 0.5 µM each primer. The primers were forward primer 5′-ACA AGA GCC AAG CAG TGT AGC ACA-3′) and reverse primer 5′-GAC CAC GAT AAC TTG TCC CCC ACT-3′). The PCR protocol consisted of pre-denaturion at 95°C for 5 min and followed by 35 cycles of denaturion at 95°C for 30 sec, annealing at 55°C for 30 sec extension at 72 °C for 20 sec and final extension at 72 °C for 7 min. This yielded a 465 bp amplicon. For the second nested step, 1 µl of PCR reaction solution containing the 465 bp amplicon was used in 50 µl of reaction mixture containing, 1x PCR reaction buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl), 1.5 mM $MgCl_2$, 200 µM dNTPs, 1.25 unit *Taq* DNA polymerase (Invitrogen) and 0.5 µM of each primer. These were forward primer 5′-CGA ACC AGG AAC TTC AAG AAC-3′ and reverse primer 5′-TTC CGT ACC CTG CAG ATT CTC-3′). The PCR protocol consisted of pre-denaturation at 95°C for 5 min and followed by 35 cycles of denaturation at 95°C for 30 sec, annealing at 50°C for 30 sec, extension at 72 °C for 30 sec and final extension at 72 °C for 7 min. This yielded a 183 bp amplicon. Both of nested PCR sensitivities were tested and compared to the sensitivity of the LAMP method. For amplicon detection, 10 µl of PCR reaction mixture was electrophoresised in 1.5% agarose gel for 30-35 min followed by staining with ethidium bromide for visualization using a UV transilluminator.

11. Specificity of LAMP detection

Total DNA was extracted from shrimp infected with DNA viruses commonly found in Thaiand. These included hepatopancretic parvovirus (HPV), infectious hypodermal and hematopoietic necrosis virus (IHHNV), white spot syndrome virus (WSSV) and monodon baculovirus (MBV) and used as DNA template (100 ng) for LAMP detection. In addition, 100 ng of total DNA extracted from normal shrimp *P. monodon*, *P. vannamei*, *Macrobrachium rosenbergii* and distilled water were used as the negative control. For the positive control, 10^6 copies of recombinant plasmid containing the LAMP target fragment were used. Also used was 100 ng DNA extracted from *M. resenbergii* infected with an HPV-like virus. Then, 5 µl of LAMP products were electrophoresed in a 2% agarose gel for 30-35 min followed by staining with ethidium bromide and visualization using a UV transilluminator.

12. Design and optimization of FITC-labeled DNA probe for LFD

The DNA probe for hybridization with LAMP amplicons prior to LFD detection was designed from the HPV sequences between the B1 and B2 primer regions. These are illustrated in a succeeding section of this thesis (Fig. 7). According to the test protocol, 5 µl of the LAMP reaction mixture was used for detection of amplicons by electrophoresis, while other fractions of 20 µl each were mixed with 5 µl of solution containing the the DNA probe at 3 different concentrations (200, 20 and 2 pmole). This was followed by incubation at 63^{o}C for 5 min. Then, 8 µl of the reaction mixture was added to 150 µl of the assay buffer (Milenia *Genline* HybriDetect MGHD1, Milenia) with mixing by pulse vortex. Finally, the LFD strip was dipped into the assay buffer and left for 10-15 min to for strip reactions to occur and allow interpretation of test results.

13. LFD sensitivity determination

From both of the LAMP sensitivity tests described above in (Method 6 and 8) of this thesis document. For each sample, 20 µl of LAMP reaction mixture was mixed with 5 µl of the optimum concentration of FITC-labeled DNA probe followed by incubation at 63^{o}C for 5 min. Then, 8 µl of the resulting mixture was added to 150 µl

of assay buffer with mixing by pulse vortex. Finally, the LFD strip was dipped into the assay buffer and left for 10-15 min to for strip reactions to occur and allow interpretation of test results.

14. LFD specificity determination

These tests were carried out as in the preceding paragraph except that the 20 µl of LAMP reaction mixture used came from LAMP reactions using templates from shrimp viruses other than HPV and templates from uninfected shrimp.

CHAPTER IV
RESULTS

1. Design primers for LAMP

After DNA sequences of HPV capsid protein genes were submitted for analysis using the online program software, several sets of LAMP primer sequences were presented. These included F3, B3, FIP and BIP that were used in this study. An additional 4 primers presented were not used in this study. The primer sequences are shown in Table 1 together with the sequence of the FITP DNA probe for hybridization with the LAMP amplicon. The locations of these sequences in the genome of HPV from Thailand are illustrated in Fig. 7.

2. Optimization LAMP conditions

From the isothermal variation found that after electrophoresis, LAMP products showed a ladder-like pattern in agarose gel due to, its structural feature of LAMP product (Notomi et al., 2000). From optimization temperatures of LAMP (60 ^{o}C, 63 ^{o}C and 65 ^{o}C), the LAMP products showed that no difference in amplification between at 63^{o}C and 65^{o}C, nevertheless no such typical pattern product was amplified obviously at 60^{o}C (Fig. 8).

3. Comparison in sensitivity between one-step PCR and LAMP using recombinant plasmid

To compare the sensitivity of detection limit, LAMP, one-step PCR and nested PCR were carried out using 10-fold diluted plasmid as a template at a different copy number (10^5, 10^4,10^3,10^2, 10 and 5 copies). After gel electrophoresis, it was found that one-step PCR can detect recombinant plasmid to 1,000 copies in 100 ng of

norml shrimp DNA (*P. monodon*) (Fig. 9), whereas LAMP can detect recombinant plasmid as little as 10 copies (Fig. 10).

Table 1. Names of primers and probes and their sequences for used for LAMP amplification followed by LFD detection. Each of the inner primer sequences is linked together with 4 thymidine bases (T) to form a "T-linker" (indicated in bold letters).

Primer & Probe name	**Genome position**	**Sequences (5´___3´)**
F3	1055-1073	GCAACAGTAACTCTGGAGA
B3	1264-1286	GCACTTGTATATCCCACTACAG
biotin-FIP	1075-1094/ **TTTT**/ 1115-1138	biotin-TACCCAGAGCAAGATTTCCCAAAC**TTTT**ATTAAGCACAGTTTCTACACAC
BIP	1164-1185/ **TTTT**/ 1226-1244	CAACATATCCAAGAGCGAGGAC**TTTT**ACCATCTCAAATGCCTGTA
DNA probe	1202-1223	CCACCAGGGGCAAGATGAACCA

(a)

```
                                    F3                  F2
Targeted DNA 1045 GGAATAAACG GCAACAGTAA CTCTGGAGAT ATTAAGCACA GTTTCTACAC 1094
Complement        CCTTATTTGC CGTTGTCATT GAGACCTCTA TAATTCGTGT CAAAGATGTG

                                                        F1c
                                          B1c
1095 ACAGCTATCT GGAAGTATTG GTTTGGGAAA TCTTGCTCTG GGTAACTACA 1144
     TGTCGATAGA CCTTCATAAC CAAACCCTTT AGAACGAGAC CCATTGATGT

1145 TAAACTCGTG GGGTATGGAC AACATATCCA AGAGCGAGGA CAGTTGGGCT 1194
     ATTTGAGCAC CCCATACCTG TTGTATAGGT TCTCGCTCCT GTCAACCCGA

                FITC-HPV probe
1195 ATCATAGCCA CCAGGGGCAA GATGAACCAT CTACAGGCAT TTGAGATGGT 1244
     TAGTATCGGT GGTCCCCGTT CTACTTGGTA GATGTCCGTA AACTCTACCA
                                                  B2
1245 TCCACAATAC CAAGGAGAAA CTGTAGTGGG ATATACAAGT GCACC      1289
     AGGTGTTATG GTTCCTCTTT GACATCACCC TATATGTTCA CGTGG
                                      B3
```

(B)

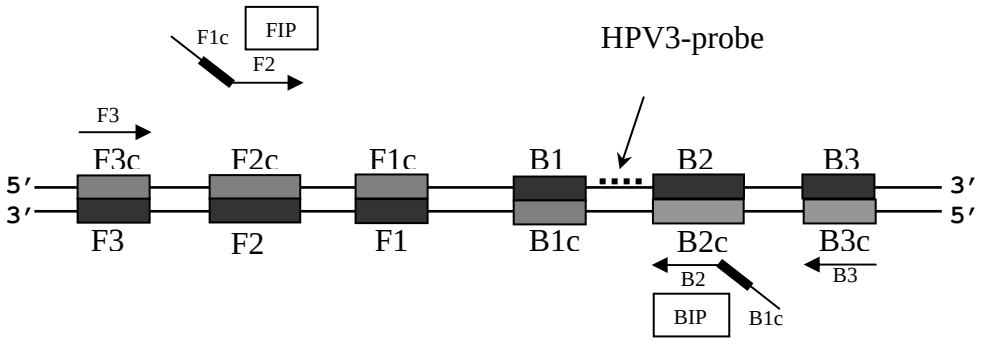

Figure 7. **(A)** DNA sequences of partial capsid protein of HPV Thai strain (Accession No.: NC007218) used for designing LAMP primers and positions (both outer and inner primers). **(B)** A schematic diagram representing the positions and map at which LAMP primers and probe attach for amplification and hybridization, respectively. DNA sequences used for design of LAMP primers are indicated by solid lines. The target for the FITC labeled probe is indicated with a dashed line.

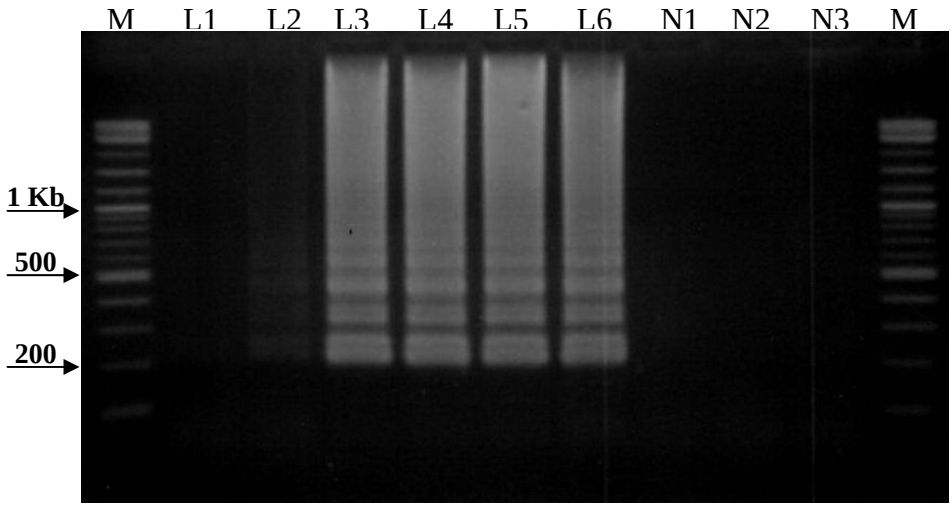

Figure 8. Electrophoresis gel showing the results from temperature optimization tests for the LAMP reaction to detect HPV. Lane L1-2: incubated at 60^{o}C, L3-4: incubated at 63^{o}C, L5-6: incubated at 65^{o}C, Lane N1: Negative control (distilled water), Lane N2-3: Negative control (100 ng and 1 ng of DNA extracted from normal *P. monodon*), lane M: 2-Log DNA ladder.

Figure 9. Example of electrophoresis gel showing sensitivity of one-step PCR detection of HPV using the LAMP primers F3 and B3. Lane L1-6: 10^5 to 5 copies of recombinant plasmid in 100 ng of normal shrimp DNA, Lane N1-2: Negative control (100 ng of DNA extracted from normal *P. monodon* and distilled water), lane M: 2-Log DNA ladder.

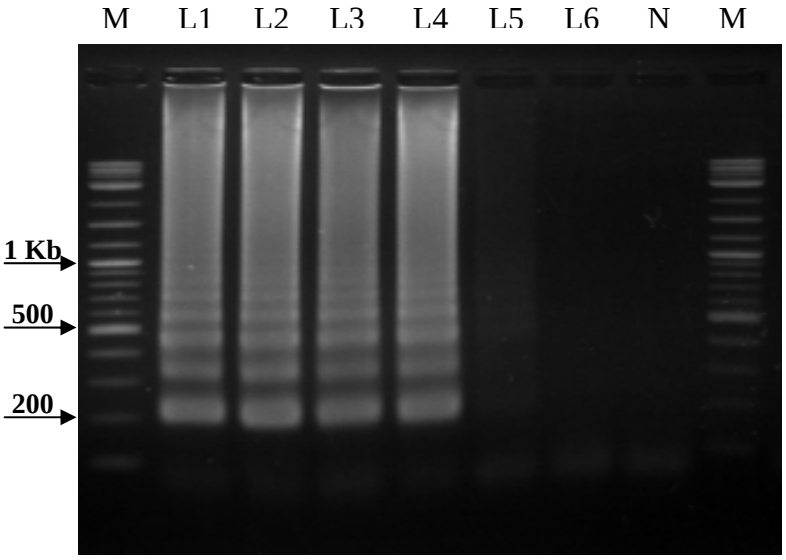

Figure 10. Agarose gel showing sensitivity for LAMP detection of HPV. Lane L1-6: 10^5 to 5 copies of recombinant plasmid, lane N: negative control (distilled water), lane M: 2-Log DNA ladder.

4. Comparison of sensitivity by one-step PCR, Nested PCR and LAMP using total DNA from HPV-infected shrimp

To compare the sensitivity of various detection methods, LAMP, one-step PCR and nested PCR were carried out using 10-fold dilutions of total DNA extracted from HPV-infected hepatopancreatic tissue as a template at various concentrations (100 ng, 10 ng, 1 ng, 0.1 ng, 0.01 ng and 1 pg). After gel electrophoresis, it was found that both one-step PCR methods [i.e., the one using LAMP primers F3 and B3 and the one using those of Sukhumsirichart et al., (2002)] could detect targeted DNA in 10 ng of total DNA (Fig. 11). By contrast, LAMP could detect target DNA in 1 ng of total DNA (Fig. 13). The latter was equivalent to the nested PCR detection method described by Manjanaik et al. (2005) (Fig. 12b). The Ezee Gene kit (SBBU, Thailand) could detect targeted DNA at 0.1 ng total DNA (Fig. 12a).

5. Specificity of LAMP detection

To determine the specificity of LAMP detection for HPV, viral DNA from shrimp infected with other viruses commonly found in Thailand was used as template DNA. Viruses tested included MBV, WSSV and IHHNV. In addition, DNA templates from various normal shrimp were also tested as a negative control. After electrophoresis, positive results were seen for only HPV-infected samples (Fig. 14) and no cross-reactions were seen with templates from normal shrimp or shrimp infected with other viruses. This indicated that the designed LAMP method had high specificity to HPV. Negative results were also was obtained when using 100 ng DNA template from *M. rosenbergii* infected with an HPV-like virus (Fig. 15). These results were further tested using the LFD detection method.

6. Optimization DNA probe amount in LFD

When varied amounts of DNA probe (200, 20 and 2 pmole) were added to the final LAMP reaction mix, it was found that 20 pmole of FITC probe gave the clearest purple-red positive test line result (Fig. 16).

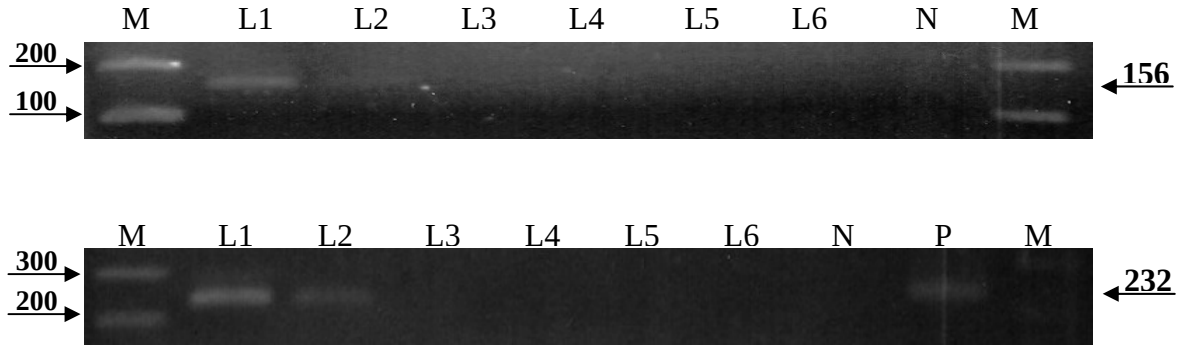

Figure 11. Agarose gels showing the sensitivity two one-step PCR detection methods for HPV. The upper result is for the Sukhumsirichart et al. (2002) method and the lower for the method employing F3 and B3 LAMP primers. Lane L1-6: 100 ng -1 pg of DNA extracted from HPV-infected sample. Lane N: Negative control, lane P: positive control, lane M: 2-Log DNA ladder.

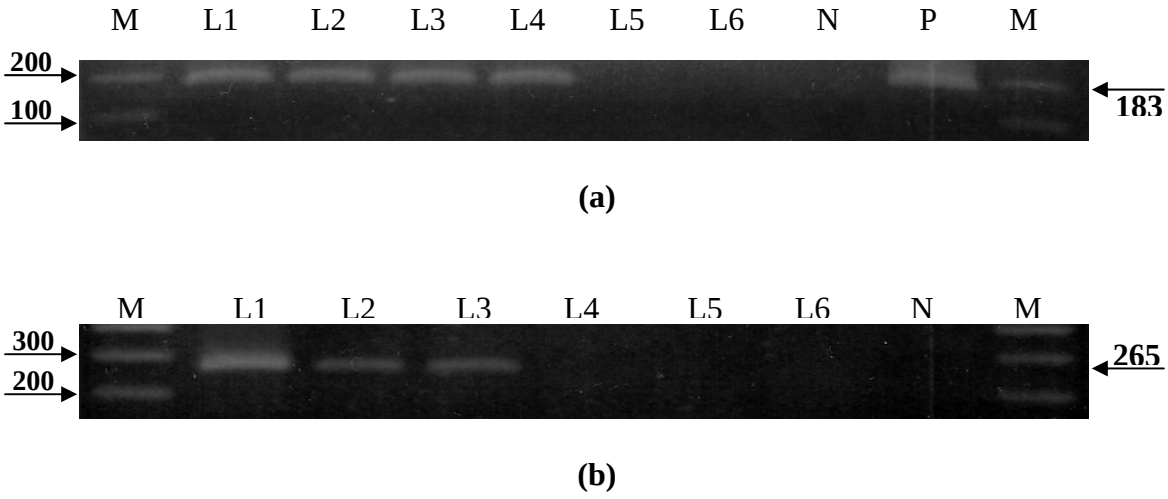

Figure 12. Agarose gels showing the sensitivity two nested PCR detection methods for HPV. The upper result is for the 465/183 nested PCR kit method and the lower for the method of Manjanaik et al. (2005). Lanes L1-6: 100 ng -1 pg of DNA extracted from an HPV-infected shrimp sample. Lane N: Negative control, lane P: positive control, lane M: 2-Log DNA ladder.

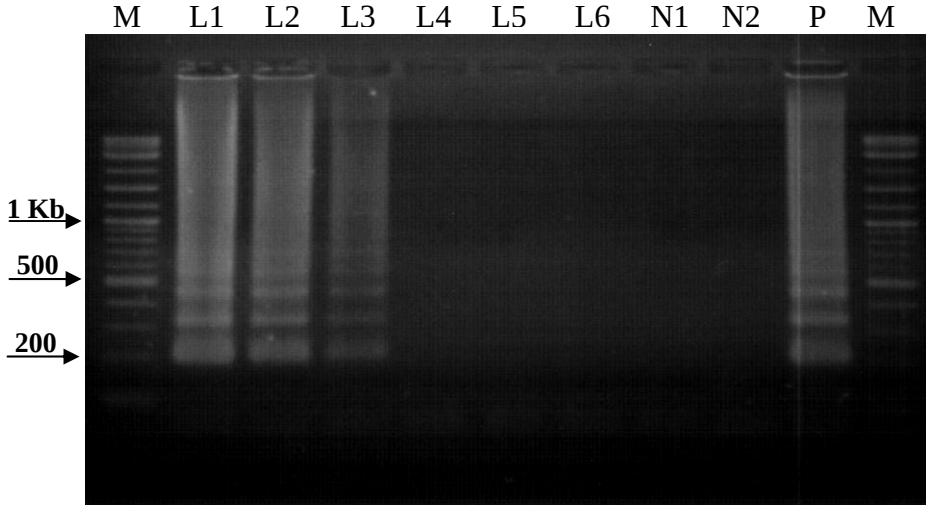

Figure 13. Agarose gels showing the sensitivity of LAMP detection of HPV. Lane L1-6: 100 ng -1 pg of DNA extracted from an HPV-infected shrimp sample, lane N1-2: negative control (100 ng of DNA extracted from normal *P. monodon* and distilled water), lane P: positive control, lane M: 2-Log DNA ladder.

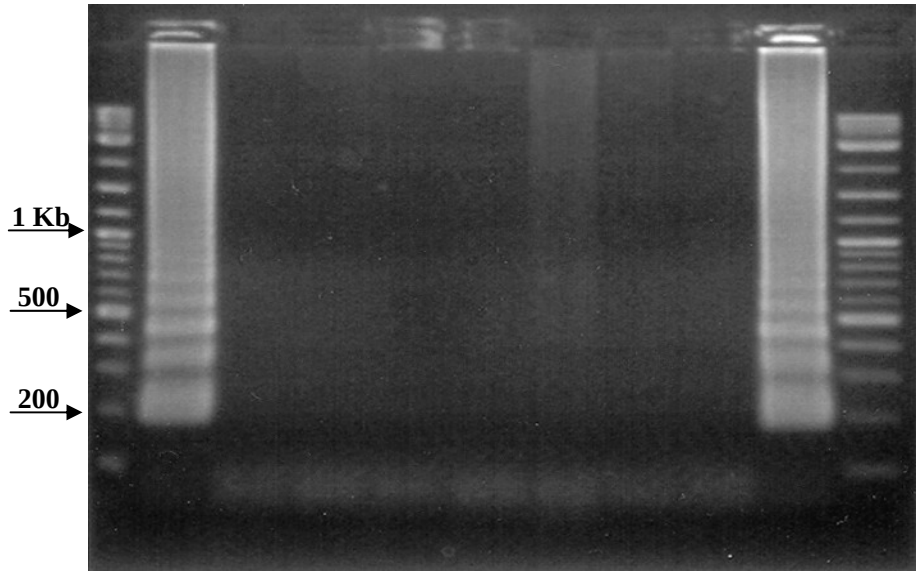

Figure 14. Agarose gel showing specificity of the LAMP method for detection of HPV. Lane L1-4: 100 ng of DNA extracted from HPV, MBV, WSSV and IHHNV-infected shrimp samples, lane L5-7 100 ng of DNA extracted from normal *P. monodon*, *M. rosenbergii* and *P. vannamei*., lane N: negative control (distilled water), lane P: positive control, lane M: 2-Log DNA Ladder.

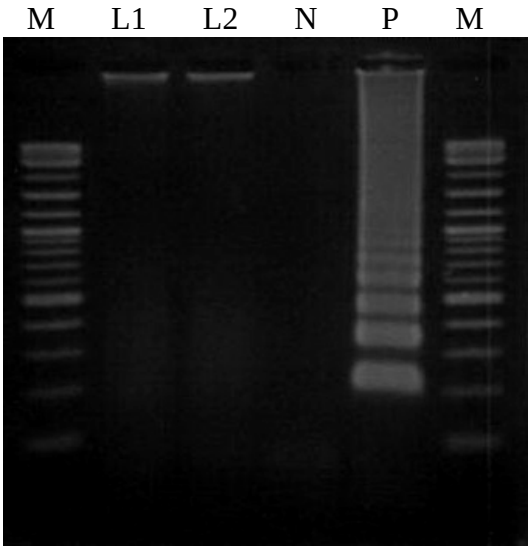

Figure 15. Agarose gel showing result of LAMP assay for HPV in *M. rosenbergii* infected with an HPV-like virus. Lane L1-2: 100 ng of infected shrimp DNA, lane N: negative control (distilled water), lane P: positive control, lane M: 2-Log DNA Ladder.

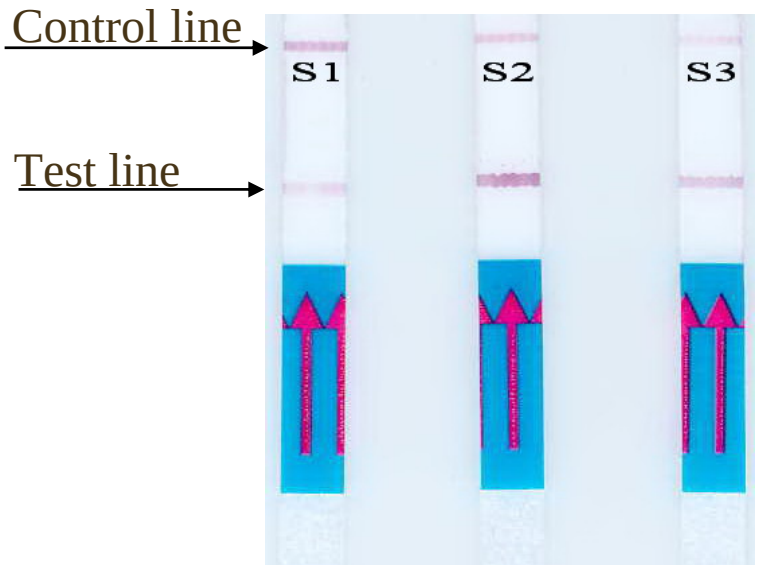

Figure 16. Photograph of results from FITC probe optimization for detection of HPV amplicons by LFD detection.. Strips S1-3: Diluted DNA probe at 200, 20 and 2 pmole, respectively. A positive test result consisted of red lines at both the test and control lines.

7. Sensitivity determination using lateral-flow dipsticks

When the sensitivity of detection was compared using various templates with LAMP-LFD, it was found that LAMP-LFD was capable of detecting LAMP product at 10 copies per reaction using recombinant plasmid template (Fig. 17) and 1 ng of total DNA extracted from HPV-infected hepatopancreas (Fig. 18).

8. Specificity of LFD detection

With specificity tests using the LAMP-LFD method for HVP detection, it was found that there were no cross-reactions with other DNA viruses commonly found in shrimp from Thailand (Fig. 19). This result indicated that the LFD method had high specificity for the DNA virus. A negative result was also observed in 100 ng of DNA template from HPV-infected *M. rosenbergii* (Fig. 20).

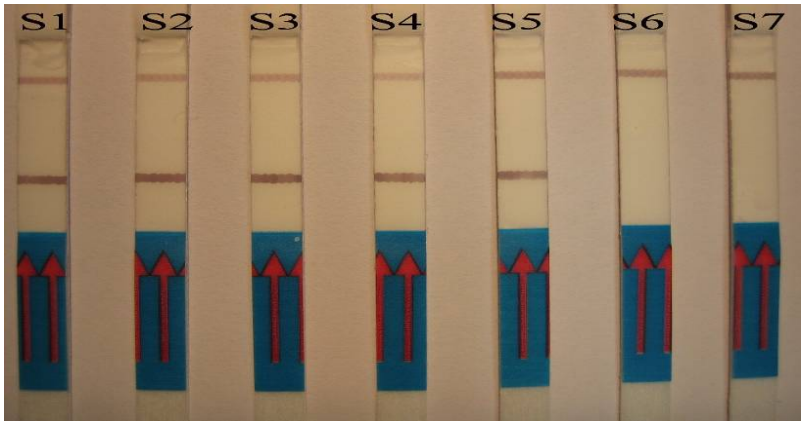

Figure 17. Photograph showing results of sensitivity tests using the lateral flow dipstick for detection of LAMP products from a plasmid template. Strip S1-6: 10^5 - 5 copies of recombinant plasmid in 100 ng of normal shrimp DNA, Strip S7: negative control (distilled water).

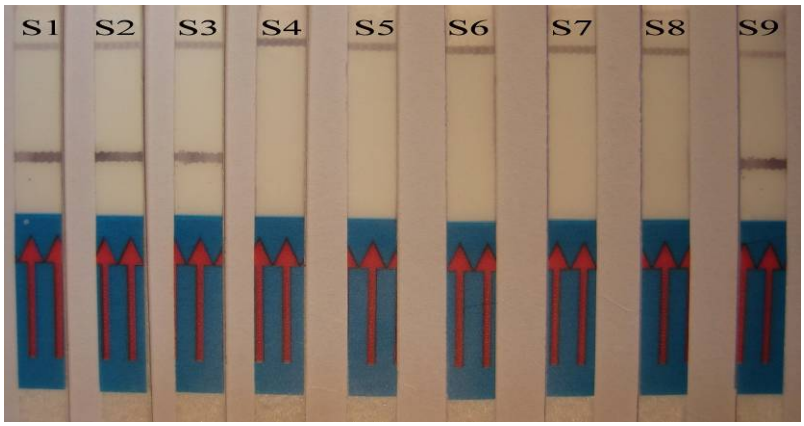

Figure 18. Photograph showing results of sensitivity tests using the lateral flow dipstick for detection of LAMP products from DNA of HPV-infected shrimp. Strip S1-6: 100 ng -1 pg of shrimp DNA template, Strip S7-8: negative control (100 ng of DNA extracted from normal *P. monodon* and distilled water), Strip S9: positive control.

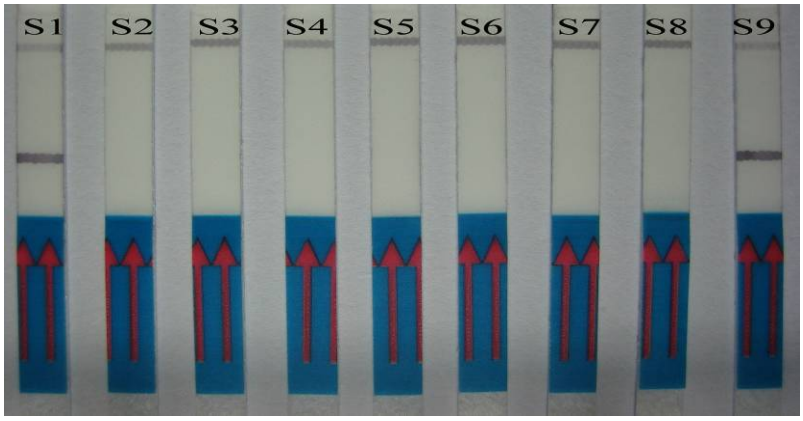

Figure 19. Photograph showing results of specificity tests using the lateral flow dipstick for detection of LAMP products using DNA of shrimp infected with various viruses. Strip S1-4: 100 ng of DNA extracted from HPV, MBV, WSSV and IHHNV-infected shrimp, Strip S5-7 100 ng of DNA extracted from normal *P. monodon*, *M. rosenbergii* and *P. vannamei*., Strip S8: negative control (distilled water), Strip S9: positive control.

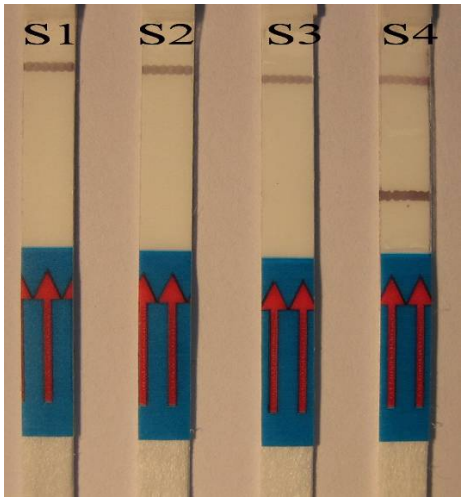

Figure 20. Photograph showing results of a LAMP-LFD test carried out using DNA template extracted from lateral flow dipstick for detection of LAMP products using DNA of *M. rosenbergii* infected with an HPV-like virus. Strip S1-2: 100 ng of DNA extracted from HPV-infected *M. rosenbergii*, Strip S3: negative control (distilled water), Strip S4: positive control.

CHAPTER V
DISCUSSION

Hepatopancreatic parvovirus (HPV) is currently considered as a member of the family Parvoviridae (Bonami et al., 1995). Its host range encompasses both wild and cultured penaeid species worldwide (Paynter et al., 1985; Lightner, 1996; Spann et al., 1997) and the freshwater prawn *Macrobrachium rosenbergii* (Anderson et al., 1990). To date, three Austral-Asian strains of HPV have been relatively well characterized at the molecular level and the results suggest that HPV isolated from different prawn species and/or different geographic regions is genetically different. These 3 types are HPV in *P. chinensis* from Korea (Bonami et al., 1995), HPV in *P. monodon* from Thailand (Sukhumsirichart et al., 2002) and HPV in in *Penaeus merguiensis* from Australia (La Fauce et al. 2007). HPV in *P. semisulcatus* from India (Manjanaik et al., 2005) has also been described partially at the molecular level.

For diagnosis of HPV infections, classical histopathology with H&E staining or transmission electron microscopy (Lightner 1996) is complicated, time consuming and requires experience for interpretation. A simple method using Giemsa-stained impression smears of the hepatopancreas (Lightner et al. 1993) is more convenient and rapid; however, this still requires experience for interpretation and may be difficult with light infections. In addition, the methods require destruction or biopsy of tested shrimp. To solve these problems, molecular techniques have been replaced for HPV detection such as conventional polymerase chain reaction (PCR) assays (Pantoja and Lightner, 2000; Phromjai et al., 2002; Sukhumsirichart et al., 1999, 2002), PCR-ELISA (Sukhumsirichart et al., 2002), nested PCR (Manjanaik et al., 2005) and dot blot hybridization (Phromjai et al., 2002) without killing shrimp. Nevertheless these techniques suffer from various disadvantages including long assay times required, high cost of required instruments, occurrence of non-specific amplification products and/or the need for toxic substances such as ethidium bromide (EtBr) to interpret results.

Loop-mediated isothermal amplification (LAMP) is the novel and rapid DNA amplification via auto-cycling strand displacement DNA synthesis by employing a DNA polymerase under isothermal conditions. Thus, only simple equipment such as a heating block or water bath is needed instead of an expensive and sophisticated PCR machine. Moreover the method has high sensitivity and specificity due to the use of 6 primers that recognize 6 distinct sequences on the sense and anti-sense strands of the targeted DNA (Notomi et al., 2000).

The LAMP primers used in this experiment were designed according to the sequence of the capsid protein gene of HPV in *P. monodon* from Thailand (Sukhumsirichart et al. 2006). Since, the capsid gene contains both conserved and variable sequences, primer sequences designed to target conserved sequences can sometimes be used for detection of several variants of a pathogen that may differ in genome sequence at more variable regions of their genomes. To examine the possibility co-detection of HPV variants other than the Thai HPV for which our LAMP method was designed, we aligned the LAMP target region for primers F3 and B3 with the homologous regions of other HPV sequences listed at the GenBank database. These included sequences from India (GenBank Accession number: EU617324), Korea (GenBank Accession number: AY008257) and Australia (GenBank Accession number: DQ458781).

According to the data from the Eiken company website (http://loopamp.eiken. co.jp/e/lamp/snps_index. html), the specificity of LAMP reaction depends not only on the six positions of the LAMP primers, but especially on the sequence at their 5' ends where differences of as little as one nucleotide will result in no DNA synthesis from any dumbbell-like structures that might arise from sequence variants generated at the start of the LAMP amplification series. As a result, no cycling (i.e., amplification) would occur. This is supported by the fact that the DNA extracts from *M. rosenbergii* infected with an HPV-like virus did not give positive results with the LAMP-LFD method.

Our alignment analysis revealed that the sequence of bases for HPV from India, Korea and Australia were identical in the target region for the 5´ end of the FIP primers. This analysis suggests that our LAMP method may be suitable for detection of HPV not only from Thailand but also from India, Korea and Australia. However,

actual tests would have to be performed to determine whether this prediction is correct.

Thai and India

```
SeqA Name    Len(nt)  SeqB Name    Len(nt)  Score
================================================
1    Thai    232      2    India   232      85
================================================
```

```
Thai         GCAACAGTAA CTCTGGAGAT ATTAAGCACA GTTTCTACAC ACAGCTATCT GGAAGTATTG
India        GCAACAGTAA CACAGGAGAT ATCAAGCACG GGTTTTCGGC GTCACTACCT GGAAGTATTG
Clustal Co   ********** * * ****** ** ******  * ** *   *     *** ** **********

Thai         GTTTGGGAAA TCTTGCTCTG GGTAACTACA TAAACTCGTG GGGTATGGAC AACATATCCA
India        GACTGGGAAA TCTAGCATTG GGTAACTACA TAAACTCATG GGGCATGGAC AACATATCGA
Clustal Co   *  ******* *** **  ** ********** ******* ** *** ****** ******** *

Thai         AGAGCGAGGA CAGTTGGGCT ATCATAGCCA CCAGGGGCAA GATGAACCAT CTACAGGCAT
India        AGAGCGAGGA CAGCTGGGCT ATCATCGCCA CAAGGGGTAA GATGAACCAC TTGCAGGCAT
Clustal Co   ********** *** ****** ***** **** * ***** ** *********   * *******

Thai         TTGAGATGGT TCCACAATAC CAAGGAGAAA CTGTAGTGGG ATATACAAGT GC
India        TTGAGATGGT GCCACAGTAC CAAGGAGAGA CCATAGTGGG ATATACAAGT GC
Clustal Co   **********  ***** *** ******** * *  ******* ********** **
```

Thai and Korea

```
SeqA Name    Len(nt)  SeqB Name    Len(nt)  Score
================================================
1    Thai    232      2    Korea   228      82
================================================
```

```
Thai         GCAACAGTAA CTCTGGAGAT ATTAAGCACA GTTTCTACAC ACAGCTATCT GGAAGTATTG
Korea        ----CAGTAA CAGTGGAGAT ATTAAGTACA GTTTTAACGC TCTACTGCAT GGAAGTATTG
Clustal Co       ****** *  ******* ****** *** ****  ** *  *  **   * **********

Thai         GTTTGGGAAA TCTTGCTCTG GGTAACTACA TAAACTCGTG GGGTATGGAC AACATATCCA
Korea        GACTTGGAAA TCTTGCGTTG AGTAACTACA TAAACGCATG GGGTATTGAC AACATGGCAA
Clustal Co   *  * ***** ******  **  ********* ***** * ** ****** *** *****  * *

Thai         AGAGCGAGGA CAGTTGGGCT ATCATAGCCA CCAGGGGCAA GATGAACCAT CTACAGGCAT
Korea        AGAGCGAGGA CAGTTGGGCT ATTATAGCTA CAAGAGGTAA GATGAACCAC TTGCAAGCAT
Clustal Co   ********** ********** ** ***** * * ** ** ** *********   * ** ****

Thai         TTGAGATGGT TCCACAATAC CAAGGAGAAA CTGTAGTGGG ATATACAAGT GC
Korea        TTGAAATGAT TCCACAAATG CAAGGAGAAA CCATAGTGGG ATACACAAGT GC
Clustal Co   **** *** * *******    ********** *  ******* *** ****** **
```

Thai and Australia

SeqA	Name	Len(nt)	SeqB	Name	Len(nt)	Score
1	Thai	232	2	Australia	232	76

```
Thai          GCAACAGTAA CTCTGGAGAT ATTAAGCACA GTTTCTACAC ACAGCTATCT GGAAGTATTG
Australia     GCGCCAGTAA CCAAGGAAAC TTCAAGTACA GCTTTAAGTC AATCCTAGGT GGAAGTGTCT
Clustal Co    **  ****** *   *** *   * *** *** * **  *  * *   ***  * ****** *

Thai          GTTTGGGAAA TCTTGCTCTG GGTAACTACA TAAACTCGTG GGGTATGGAC AACATATCCA
Australia     GTCTGGGGAA TCTTAACCTA TCCAACTACA TAAATGCATG GGGCATAGAC AACATAGCCA
Clustal Co    ** **** ** ****   **     ******* ****  * ** *** ** *** ****** ***

Thai          AGAGCGAGGA CAGTTGGGCT ATCATAGCCA CCAGGGGCAA GATGAACCAT CTACAGGCAT
Australia     AGAGCGAGGA CAGTTGGGCT ATTTTATGTA CGAGAGGCAA GATGAACCAT TTACAGGCAT
Clustal Co    ********** ********** **  **   * * ** ***** **********  *********

Thai          TTGAGATGGT TCCACAATAC CAAGGAGAAA CTGTAGTGGG ATATACAAGT GC
Australia     TTGAGATGAT TCCACAACTT CAGGGAGAAA CTGTGATAGG ATACACAAGT GC
Clustal Co    ******** * *******    ** ******* ****  * ** *** ****** **
```

= F3 primer

= B3 primer

= Forward inner primer (FIP)

= Backward inner primer (BIP)

Figure 21. Alignment (5' to 3') of Thai HPV sequences and other geographical HPV sequences (Indian, Korean and Australian) in the target region for the LAMP F3 and B3 primers.

Kono et al. (2004) have recently reported that LAMP sensitivity is higher than that of one-step PCR and almost equivalent to that of nested PCR or higher. Here, we found that our LAMP method with HPV-infected shrimp samples was 10 times higher than that of traditional, one-step PCR for HPV but 10 times lower than for nested PCR. This lower sensitivity was similar to that reported for an RT-LAMP study (Mekata et al., 2006; Kiatpathomchai et al., 2007). Our sensitivity was similar to that obtained by using a PCR-ELISA method for HPV (Sukhumsirichart et al., 2002) that was also 10 times more sensitive than one-step PCR followed by

electrophoresis. By contrast, our LAMP sensitivity using a plasmid template gave the same sensitivity as nested PCR from the Ezee Gene kit and as previously reported for LAMP (Notomi et al., 2000). It is difficult to explain the difference in sensitivity given the fact that our plasmid samples were diluted in buffer containing normal shrimp DNA. Thus, the lower sensitivity with DNA extracts from HPV-infected shrimp should not have been the result of inhibitors present in the DNA extracts from shrimp. We currently have no explanation for this difference unless it arose from random variation in target DNA quantity in each DNA dilution, despite the fact that both experiments were done in triplicate. It is also possible that the biotin labeling efficiency of the primers or the labeling efficiency of the FITC probe was not ideal. If these probes were labeled at low efficiency, it would mean that some LAMP products might not be biotin labeled and that some of the LAMP products might not be hybridized with an FITC labeled probe. Complexes lacking biotin would not be trapped at the LFD test line and those lacking FITC would not be gold labeled and these features would reduce the sensitivity of the LFD. Understanding the reason for the difference may make it possible to alter the LAMP method in a manner that would improve the sensitivity with infected shrimp extracts to be equal to that obtained using the plasmid template.

After highly sensitive and specific LAMP, the detection of LAMP products is usually carried out by electrophoresis on agarose gel followed by staining with toxic ethidium bromide and visualization by UV transilluminator. The detection protocol is time consuming and lacks a way of specifically identifying amplicons. Lateral-Flow Dipsticks (LFD) provide an alternative mode of detection that is more rapid, that does not employ toxic chemicals and that confirms the identity of the LAMP product by nucleic acid hybridization. This technique is cost-effective and simple, and does not require expert personnel that would be needed for more complex PCR and immunochemical tests that are employ sophisticated electronic devices. For these reasons, it is more suitable for disease diagnosis in field situations. The LAMP-LFD combination has been used for hepatitis B, C and human immuno deficiency virus (HIV) (Dinava et al., 2005) detection. In shrimp, PCR-LFD has been used for WSSV detection (Srisala et al., 2008 (personal communication)) and RT-LAMP-LFD for TSV detection (Kiatpathomchai et al., 2008 (submitted)). RT-LAMP has also been

combined with dot-blot hybridization for TSV detection (Teng et al., 2007), that is similar to LFD detection

For FITC probe optimization (200, 20 and 2 pmole), we found that at 20 pmole gave the best color intensity. Nevertheless, this constituted a high amount of probe (4 pmole/μl) when compared to the quantity of probe used for HPV detection by PCR-ELISA (7.5 pmole/ml by Sukhumsirichart et al., 2002). It is obvious that if the FITC probe concentration is too low, little of the LAMP product would be labeled and this would result in low combination with gold-labeled anti-FITC leading to low color intensity at the test line. On the other hand, excess FITC probe would be wasteful since it would lead to binding of the probe and the antibody alone, increasing the color intensity of the control line instead. To reduce as much as possible competition between self-hybridization of LAMP products with probe hybridization, we used a hybridization temperature of 63^{o}C.

Using a plasmid template or DNA extracted from infected shrimp, the HPV detection sensitivity demonstrated using our LAMP-LFD method was equal to that of nested PCR followed by gel electrophoresis (i.e., 10 target copies and 1 ng total DNA, respectively) while the detection of TSV using LAMP-LFD by Kiatpathomchai et al., 2008 (submitted) and the detection of WSSV by Srisala et al., 2008 (personal communication) improved these limits by 100 times and 10 times, respectively. To solve this problem, LFD may be improved more sensitive than electrophoresis by labeled biotin with FIP and BIP.

For specificity test, we found no cross-reaction with other shrimp viruses commonly found in Thailand (i.e., WSSV, MBV, IHHNV). In addition, no positive results were obtained with DNA extracts from healthy shrimp or with buffer controls. This indicates that this LAMP-LFD method has good specificity for HPV. In addition, the LAMP-LFD gave negative detection result with and HPV-like virus in *M. rosenbergii*. This is consistent with a previous report indicating that it did not react by *in situ* hybridization with a DNA probe designed for detection of HPV in *P. chinensis* (Lightner et al., 1994), although that probe does hybridize (albeit somewhat less efficiently) with HPV from *P. monodon* (Phromjai, J. et al., 2001). In any case, knowledge of the sequence of any pathogen would allow appropriate re-design of LAMP primers and the FITC probe sequence to result in a viable test method.

We have shown that LAMP-LFD methods for HPV detection in template DNA extracted from shrimp requires relatively inexpensive equipment, is highly sensitive and specific and gives results within approximately 75 minutes. This can be compared to 120 min for LAMP followed by electrophoresis and very long for traditional nested PCR followed by electrophoresis. Furthermore, the test confirms the presence of amplicons by hybridization and without using a carcinogen such as ethidium bromide. Since the cost for LAMP-LFD detection is comparable with that for standard nested PCR followed by electrophoresis, it should be a highly sensitive, safe and rapid alternative for HPV detection.

CHAPTER VI
CONCLUSIONS

We have developed a LAMP-LFD detection method that is rapid, simple and cost effective for detection of HPV in *P. monodon* in Thailand and possibly also in Australia, India and Korea. The test is more sensitive than one-step PCR (Sukhumsirichart W. et al., 2002) and equal to a nested PCR test from India (Manjanaik et al., 2005), but less sensitive than a commercial nested PCR test kit from Thailand (Ezee Gene, SBBU, Bangkok) when using extracted DNA from HPV-infected shrimp. The test gives negative results with normal shrimp and does not cross-react with other DNA viruses commonly found in shrimp in Thailand. Nor does it give positive results with an HPV-like virus found in *M. rosenbergii* from Thialand. These data indicate that the LAMP test has high sensitivity and specificity for the target virus. It has additional advantages over traditional electrophoresis detection of amplicons in confirmation of the pathogen amplicon by nucleic acid hybridization, and a total analysis time of 75 minutes (excluding DNA extraction) without the use of any carcinogenic reagents. The method is more adaptable to field conditions than traditional PCR methods based on thermocycling and electrophoresis detection of amplicons. It is suitable for rapid, simple and cost-effective monitoring for HPV detection in shrimp and carriers such as insect larvae or other crustaceans that live together with shrimp in shrimp ponds and in the surrounding environment.

Our curious result of lack of increase in sensitivity by use of LFD rather than electrophoresis after the LAMP reaction should be explored further in an attempt to increase the test sensitivity by a further 10 times. The most likely avenues of investigation should focus initially on labeling efficiency of the biotin primers and the FITC labeled probes used and how these might be further optimized. Other possibilities include increasing 2 loop primers for more rapid, sensitive and specific DNA amplification (Nagamine et al., 2002) and labeling of both inner primers with biotin.

For even more field-friendly application, it is possible that our LAMP detection method could be further simplified by development of hand-held devices for quantitative electronic translation of LAMP product detection either directly by turbidity or by fluorescence of intercalated dyes such as Cyber-green, or indirectly by hybridization with labeled probes. If possible, this should be done in a manner that would reduce the probability of LAMP product contamination upon repeated use of any hand-held, field device. For example, this might be accomplished by development of pre-packaged, disposable “capsules” designed for one time use.

Other directions for future development include, multiplex detection of various types of HPV in shrimp and carriers using universal HPV LAMP primers but various labeled DNA probes that target variable regions of strain-specific amplicons to give different test lines on a single LFD. In a similar manner, it may be possible to design multiplex LAMP-LFD methods simultaneous detection of two or more viruses. An interesting pair might be HPV and MBV since they commonly occur in the HP of *P. monodon*, sometimes in mixed infections.

REFERENCES

Anderson, I.G., Law, A.T., Shariff, M., & Nash, G. (1990). A parvo-like virus in the giant freshwater prawn, *Macrobrachium rosenbergii*. Journal of Invertebrate Pathology, 55 (3), 447-449.

Bonami, J.R., Mari, J., Poulos, B.T., & Lightner, D.V. (1995). Characterization of hepatopancreatic parvo-like virus, a second unusual parvovirus pathogenic for penaeid shrimps. Journal of General Virology; 76, 813–817.

Catap, E.S., Lavilla-Pitogo, C.R., Maeno, Y., & Traviña, R.D. (2003). Occurrence, histopathology and experimental transmission of hepatopancreatic parvovirus (HPV) infections in *Penaeus monodon* postlarvae. Diseases of Aquatic Organisms, 57, 11–17.

Chong, Y.C., & Loh, H. (1984). Hepatopancreas chlamydial and parvoviral infections of farmed marine prawns in Singapore. Singapore Veterinary Jounal, 9, 51–56.

Dineva, M.A., Candotti, D., Fletcher-Brown, F., Allain, J.P., & Lee, H. (2005). Simultaneous Visual Detection of Multiple Viral Amplicons by Dipstick Assay. Journal of Clinical Microbiology, 43, 4015-4021.

Dukes, J.P., King, D.P., & Alexandersen, S. (2006). Novel reverse transcription loop-mediated isothermal amplification for rapid detection foot-and-mouth disease virus. Archives of Virology, 151, 1093-1106.

Endo, S., Komori, T., Ricci, G., Sano, A., Yokoyama, K., Ohori, A., Kamei, K., Franco, M., Miyaji, M., & Nishimura, K. (2004). Detection of gp43 of *Paracoccidioides brasiliensis* by the loop-mediated isothermal amplification (LAMP) method. FEMS Microbiology Letters, 234 (1), 93–97.

Enomoto, Y., Yoshikawa, T., Ihira, M., Akimoto, S., Miyake, F., Usui, C., Suga, S., Suzuki, K., Kawana, T., Nishiyama, Y., & Asano, Y. (2005). Rapid diagnosis of herpes simplex virus infection by a loop-mediated isothermal amplification method. Journal of Clinical Microbiology, l43, 951–955.

Enosawa, M., Kageyama, S., Sawai, K., Watanabe, K., Notomi, T., Onoe, S., Mori, Y., & Yokomizo, Y. (2003). Use of loop-mediated isothermal amplification of the IS900 sequence for rapid detection of cultured *Mycobacterium avium* subsp. paratuberculosis. Journal of Clinical Microbiology, 41, 4359– 4365.

Flegel, T.W., Fegan, D.F., Kongsom, S., Vuthikornudomkit, S., Sriurairatana, S., Boonyaratpalin, S., Chantanachookhin, C., Vickers, J.E., Macdonald, O.D. (1992). Occurrence, diagnosis and treatment of shrimp diseases in Thailand. In: Fulks, W., Main, K.L. (Eds.). Diseases of Cultured Penaeid Shrimp in Asia and the United States. Oceanic Institute, Honolulu, HI, 57–112.

Flegel, T.W., Fegan, D.F., & Sriurairatana, S. (1995). Environmental control of infectious shrimp diseases in Thailand. In: Shariff, M., Subasinghe, R.P., Arthur, J.R. (Eds.), Diseases in Asian Aquaculture, vol. II. Fish Health Section, Asian Fisheries Society, Manila, 65–79.

Flegel, T.W., Thamavit, V., Pasharawipas, T., & Alday-Sanz, V. (1999). Statistical correlation between severity of *hepatopancreatic parvovirus* infection and stunting of farmed black tiger shrimp (*Penaeus monodon*). Aquaculture, 174 (3-4), 197-206.

Flegel, T.W., Nielsen, L., Thamavit, V., Kongtim, S., & Pasharawipas, T. (2004). Presence of multiple viruses in non-diseased, cultivated shrimp at harvest. Aquaculture, 240 (1-4), 55–68.

Flegel, T.W. (2006). Detection of major penaeid shrimp viruses in Asia, a historical perspective with emphasis on Thailand. Aquaculture, 258, 1-33.

Iwamoto, T., Sonobe, T., & Hayashi, K. (2003). Loop-mediated isothermal amplification for direct detection of *Mycobacterium tuberculosis* complex, M. avium, and M. intracellulare in sputum samples. Journal of Clinical. Microbiology, 41, 2616– 2622.

Kaneko, H., Iida, T., Aoki, K., Ohno, S., & Suzutani, T. (2005). Sensitive and rapid detection of herpes simplex virus and varicella-zoster virus DNA by loop-mediated isothermal amplification. Journal of Clinical Microbiology, 43, 3290–3296.

Kiatpathomchai, W., Jareonram, W., Jitrapakdee, S., & Flegel, T.W. (2007). Rapid and sensitive detection of Taura syndrome virus by reverse transcription loop-mediated isothermal amplification. Journal of Virological Methods, 146 (1-2), 125-128.

Kiatpathomchai, W., Jaroenram, W., Arunrut, N., Jitrapakdee, S., Flegel, T.W. (2008). Reverse transcription loop-mediated isothermal amplification combined with the lateral flow dipstick test for specific detection of the Taura syndrome virus. Journal of Virological Methods, (submitted).

Kono, T., Savan, R., Sakai, M., & Itami, T. (2004). Detection of white spot syndrome virus in shrimp by loop-mediated isothermal amplification. Journal of Virological Methods, 115 (1), 59–65.

Kuboki, N., Inoue, N., Sakurai, T., Cello F.D., Grab, D.J., Suzuki, H., Sugimoto, C., & Igarashi, I. (2003). Loop-mediated isothermal amplification for detection of African trypanosomes. Journal of Clinical Microbiology, 41 (5), 5517–5524.

La Fauce, K.A., Elliman, J., & Owens, L. (2007). Molecular characterisation of hepatopancreatic parvovirus (*Pmerg*DNV) from Australian *Penaeus merguiensis*. Virology, 362 (2), 397-403.

Lightner, D.V., & Redman, R.M. (1985). A parvo-like virus disease of penaeid shrimp. Journal of Invertebrate Pathology, 45 (1), 47–53.

Lightner, D.V., Redman, R.M., Poulos, B.T., Mari, J.L., Bonami, J.R., & Shariff, M. (1994). Distinction of HPV-type virus in *Penaeus chinensis* and *Macrobrachium rosenbergii* using a DNA probe. Asian Fisheries Society, 7, 267–272.

Lightner, D.V., A Handbook of Pathology and Diagnostic Procedures for Diseases of Penaeid Shrimp. World Aquaculture Society, Baton Rouge, LA, 1996.

Manivannan, S., Otta, S.K., Karunasagar, I., & Karunasagar, I. (2002). Multiple viral infection in *Penaeus monodon* shrimp postlarvae in an Indian hatchery. Diseases of Aquatic Organisms, 48 (3), 233–236.

Manjanaik, B., Umesha, K.R., Karunasagar, I., & Karunasagar, I. (2005). Detection of *hepatopancreatic parvovirus* (HPV) in wild shrimp from India by nested Polymerase chain reaction (PCR). Diseases of Aquatic Organisms, 63, 255-259.

Maruyama, F., Kenzaka, T., Yamaguchi, N., Tani, K., & Nasu, M. (2003). Detection of bacteria carrying the stx2 gene by in situ loop-mediated isothermal amplification. Applied and Environmental Microbiology, 69, 5023–5028.

Mekata, T., Kono, T., Savan, R., Sakai, M., Kasornchandra, J., Yoshida, T., & Itami, T. (2006). Detection of yellow head virus in shrimp by loop-mediated isothermal amplification (LAMP). Journal of Virological Methods, 135 (2), 151–156.

Mori, Y., Kitao, M., Tomita, N., & Notomi, T. (2004). Real-time turbidimetry of LAMP reaction for quantifying template DNA. Journal of Biochemical and Biophysical Methods, 59 (2), 145–157

Mori, Y., Hirano, T., & Notomi, T. (2006). Sequence specific visual detection of LAMP reactions by addition of cationic polymers. BMC Biotechnology, 6, 3.

Nagamine, K., Hase, T., & Notomi, T. (2002). Accelerated reaction by loop-mediated isothermal amplification using loop primers. Molecular and Cellular Probes, 16 (3), 223-229.

Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T.,Watanabe, K., Amino, N., & Hase, T. (2000). Loop-mediated isothermal amplification of DNA. Nucleic Acids Research, 28 (12), E63.

Owens, L. (1997). Special topic review: the history of the emergence of viruses in Australian prawn aquaculture. World Journal of Microbiology and Biotechnology. 13 (4), 427–431.

Pantoja, C.R., & Lightner, D.V. (2000). A non-destructive method based on the polymerase chain reaction for detection of hepatopancreatic parvovirus (HPV) of penaeid shrimp. Diseases of Aquatic Organisms, 39, 177–182.

Pantoja, C.R., & Lightner, D.V. (2001). Detection of hepatopancreatic parvovirus (HPV) of penaeid shrimp by in situ hybridization at the electron microscope level. Diseases of Aquatic Organisms, 44 (2), 87–96.

Paynter, J.L., Lightner, D.V., Lester, R.J.G. (1985). Prawn virus from juvenile Penaeus esculentus. In: Rothlisberg, P.C., Hill, B.J., Staples, D.J. (Eds). Second Australian National Prawn Seminar, NPS 2, Cleveland, Australia, 61-64.

Phromjai, J., Sukhumsirichart, W., Pantoja, C., Lightner, D.V., & Flegel, T.W. (2001). Different reactions obtained using the same DNA detection reagents for Thai and Korean hepatopancreatic parvovirus of penaeid shrimp. Diseases of Aquatic Organisms, 46, 153–158.

Phromjai, J., Boonsaeng, V., Withyachumnarnkul, B., & Flegel, T.W. (2002). Detection of hepatopancreatic parvovirus in Thai shrimp Penaeus monodon by *in situ* hybridization, dot blot hybridization and PCR amplification. Diseases of Aquatic Organisms, 51, 227–232

Poon, L.L., Wong, B.W., Ma, E.H., Chan, K.H., Chow, L.M., Abeyewickreme, W., Tangpukdee, N., Yuen, K.Y., Guan, Y., Looareesuwan, S., & Peiris, J.S. (2006). Sensitive and inexpensive molecular test for falciparum malaria: detecting *Plasmodium falciparum* DNA directly from heat-treated blood by loop-mediated isothermal amplification. Clinical Chemistry, 52, 303–306.

Savan, R., Igarashi, A., Matsuoka, S., & Sakai, M. (2004). Sensitive and rapid detection of edwardsiellosis in fish by a loop-mediated isothermal amplification method. Applied and Environmental Microbiology. 70 (1), 621–624.

Spann, K.M., Adlard, R.D., Hudson, D.A., Pyecroft, S.B., Jones, T.C., & Voigt, M.O.C. (1997). Hepatopancreatic parvo-like virus (HPV) of *Penaeus japonicus* cultured in Australia. Diseases of Aquatic Organisms, 31, 239-241.

Srisala, J., Tacon, P., Flegel, T.W., & Sritunyalucksana, K. (2008). Comparison of white spot syndrome virus (WSSV) PCR-detection methods that use electrophoresis or antibody-mediated lateral flow chromatographic strips (LFCS) to visualize PCR amplicons, (personal communication).

Sukhumsirichart, W., Wongteerasupaya, C., Boonsaeng, V., Panyim, S., Sriurairatana, S., Withyachumnarnkul, B., Flegel, T.W. (1999). Characterization and PCR detection of hepatopancreatic parvovirus (HPV) from Penaeus monodon in Thailand. Diseases of Aquatic Organisms, 38 (1), 1–10.

Sukhumsirichart, W., Kiatpathomchai, W., Wongteerasupaya, C., Withyachumnarnkul, B., Flegel, T.W., Boonseang, V., & Panyim, S. (2002). Detection of hepatopancreatic parvovirus (HPV) infection in Penaeus monodon using PCR-ELISA. Molecular and Cellular Probes, 16 (6), 409–413.

Sukhumsirichart, W., Attasart, P., Boonsaeng, V., Panyim, S. (2006). Complete nucleotide sequence and genomic organization of hepatopancreatic parvovirus (HPV) of Penaeus monodon. Virology, 346 (2), 266–277.

Sun, Z.F., Hu, C.Q., Ren, C.H., & Shen, Q. (2006). Sensitivity and rapid detection of infectious hypodermal and hematopoietic necrosis virus (IHHNV) in shrimps by loop-mediated isothermal amplification. Journal of Virological Methods, 131 (1), 41-46.

Teng, P.H., Chen, C.L., Sung, P.F., Lee, F.C., Ou, B.R., & Lee, P.Y. (2007). Specific detection of reverse transcription-loop-mediated isothermal amplification amplicons for Taura syndrome virus by colorimetric dot-blot hybridization. Journal Virological Methods, 146 (1-2), 317-326.

Umesha, R.K., Uma, A., Otta, S.K., Karunasagar, I., Karunasagar, I. (2003). Detection by polymerase chain reaction (PCR) of hepatopancreatic parvovirus (HPV) and other viruses in hatchery-reared Penaeus monodon postlarvae. Diseases Aquatic Organisms, 57 (1-2), 141–145.

APPENDIX

APPENDIX

Reagent for DNA Extraction

Lysis Buffer

2 M Tris-HCl, pH 8.0	2.5	ml
0.5 M Ethylenediaminetetra-acetic acid (EDTA)	20	ml
5 M NaCl	1	ml
10% SDS	20	ml
Distilled water	56.5	ml

Autoclave under 121^{o}C, 15 lb pressure for 15 min before usage.

Phenol:Chloroform:Isoamyl alcohol (25:24:1)

Saturated phenol	25	ml
Chloroform	24	ml
Isoamyl alcohol	1	ml

Overlaying solution with Tris-HCl, pH 8.0 then, mixes all solutions homogenously and allow phase separate completely before usage.

3 M Sodium Acetate, pH 5.2

Sodium Acetate	12.3	g

Add 20 ml of distilled water then, adjust pH to 5.2 by glacial acetic acid and adjust final volume to 50 ml. Autoclave under 121^{o}C, 15 lb pressure for 15 min before usage.

Reagent for Gel Electrophoresis

5X TBE Buffer, pH 8.0

Trizma® Base	108	g
Boric acid	55	g
EDTA	7.4	g

Add 500 ml of distilled water then, adjust pH to 8.0 by NaOH and adjust final volume to 1 L. Dilute Stock buffer to 1X or 0.5X final concentration before usage.

Reagent for Cell transformation

20 mM Isopropyl thio-β-D-galactoside (IPTG)

Isopropyl thio-β-D-galactoside	0.0477	g

Add 8 ml of distilled water then mixes homogenously and adjust final volume to 10 ml. Filter through 0.2 μm filter paper and keep under -20^{o}C in foiled tube.

2 % 5-bromo-4-chloro-3-indolyl- beta-D-galactopyranoside (X-Gal)

X-Gal	0.04	g

Add 2 ml of dimethylformamide then mixes homogenously. Filter through 0.2 μm filter paper and keep under -20^{o}C in foiled tube.

SOB media solution

Bactotryptone	20	g
Yeast Extract	5	g
NaCl	5	g

1 M KCl	2.5	ml

Adjust final volume to 980 ml. Autoclave under 121^{o}C, 15 lb pressure for 15 min. Followed by adding: (**Note** that these two solutions must be filtered through 0.2 μm filter paper before added).

1 M $MgCl_2$	10	ml
1 M $MgSO_4$	10	ml

Keep under 4^{o}C until usage.

SOC media solution

SOB media solution	99	ml
2 M glucose	1	ml

Keep under 4^{o}C until usage.

(**Note** that 2 M glucose solution must be filtered through 0.2 μm filter paper before added).

BIOGRAPHY

NAME	Mr. Tongchai Nimitphak
DATE OF BIRTH	December 11, 1982
PLACE OF BIRTH	Bangkok, Thailand
INSTITUTIONS ATTENED	Mahidol University, 2001-2004: Bachelor of Science (Biotechnology) Mahidol University, 2005-2008: Master of Science (Biotechnology)
SCHOLARSHIP	National Center for Genetic Engineering and Biotechnology (BIOTEC), Thailand.
ADDRESS	228/70 Sathupradit 20, Bangkloa, Bangkorlam, Bangkok, 10120, Thailand.
E-MAIL ADDRESS	benzjee115@hotmail.com benzjee115@gmail.com

PRESENTATION

1. The 6th National Symposium on Marine Shrimp. March 29-30, 2007. Pathumthani, Thailand (Poster).
2. ASIAN–PACIFIC AQUACULTURE 2007. August 5-8, 2007. Hanoi, Vietnam (Poster).