DEVELOPMENT OF MONOCLONAL ANTIBODIES TO POLYHEDRIN OF MONODON BACULOVIRUS

CHUMPHOL BOONSANONGCHOKYING

A THESIS SUBMITTED IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR
THE DEGREE OF MASTER OF SCIENCE (BIOTECHNOLOGY)
FACULTY OF GRADUATE STUDIES
MAHIDOL UNIVERSITY
2005

ISBN 974-04-6211-1

COPYRIGHT OF MAHIDOL UNIVERSITY

Copyright by Mahidol University

Thesis

Entitled

DEVELOPMENT OF MONOCLONAL ANTIBODIES TO POLYHEDRIN OF MONODON BACULOVIRUS

Chumphol Boonsanongchokying

Mr. Chumphol Boonsanongchokying
Candidate

Prof. Timothy W. Flegel,
Ph.D. (Fungal Physiology)
Major-Advisor

Paisarn Sithigorngul

Assoc. Prof. Paisarn Sithigorngul,
Ph.D. (Zoology)
Co-Advisor

Saengchan Senapin

Ms. Saengchan Senapin,
Ph.D. (Molecular Biology and
Biochemistry)
Co-Advisor

Assoc. Prof. Rassmidara Hoonsawat,
Ph.D.
Dean
Faculty of Graduate Studies

Manop Suphantharika

Assoc. Prof. Manop Suphantharika,
Ph.D. (Biochemical Engineering)
Chair
Master of Science Programme in
Biothecnology
Faculty of Science

Copyright by Mahidol University

Thesis

Entitled

DEVELOPMENT OF MONOCLONAL ANTIBODIES TO POLYHEDRIN OF MONODON BACULOVIRUS

was submitted to the Faculty of Graduate Studies, Mahidol University
for the degree of Master of Science (Biotechnology)
on
25 May, 2005

Chumphol Boonsanongchokying
.....
Mr. Chumphol Boonsanongchokying
Candidate

.....
Prof. Timothy W. Flegel,
Ph.D. (Fungal Physiology)
Chair

Paisarn Sithigorngul
.....
Assoc. Prof. Paisarn Sithigorngul,
Ph.D. (Zoology)
Member

Siwaporn Longyant
.....
Mr. Siwaporn Longyant,
Ph.D. (Marine Science)
Member

Saengchan Senapin
.....
Ms. Saengchan Senapin,
Ph.D. (Molecular Biology and Biochemistry)
Member

.....
Assoc. Prof. Rassmidara Hoonsawat,
Ph.D.
Dean
Faculty of Graduate Studies
Mahidol University

.....
Prof. Amaret Bhumiratana,
Ph.D. (Microbiology)
Dean
Faculty of Science
Mahidol University

Copyright by Mahidol University

ACKNOWLEDGEMENTS

I would like to express my deepest sincere gratitude to the support of many without whom this thesis would not have happened. First, I am extremely appreciative to Prof. Dr. Timothy W Flegel, for his creative guidance, meaningful supervision, continuous discussion, and kindness throughout the course of this study which enable me to carry out my study successfully.

I am gratefully indebted to Assoc. Prof. Dr. Paisarn Sithigorngul, my co-advisor, for his esteemed advice, guidance, supervision, correcting my thesis, and giving me opportunities to do research throughout this study.

I am deeply thankful to Dr. Saengchan Senapin, my co-advisor, for correcting my thesis and beautiful smile.

In addition, I would like to give my thanks to Dr.Jeerapun Worapong for teaching me lots of valuable molecular biology techniques.

I would like to express my great thanks to Dr. Siwaporn Longyant, Mr. Sombat Rukpratarnporn, Mr.Attaphon Satidkanidkul, and Mr.Wiwat Sungoum for their valuable advice and supports on my thesis work.

I would like to thank Department of Biology, Faculty of Science, Srinakharinwirot University for providing the facilities and all instruments for my research.

I cordially thank P' BEE, P' NOOK, and P'PEN who always gave me valuable suggestion.

I would also thank Note, every body in CENTEX SHRIMP for their help, friendship, and hospitality which make me happy for being the member of CENTEX SHRIMP.

Finally, I would like to express my deepest gratitude to my beloved mother, father, grandmother, my brother and both my sister for their infinite love, supporting without conditions and understanding throughout my study.

Chumphol Boonsanongchokying

Copyright by Mahidol University

DEVELOPMENT OF MONOCLONAL ANTIBODIES TO POLYHEDRIN OF MONODON BACULOVIRUS

CHUMPHOL BOONSANONGCHOKYING 4636282 SCBT/M

M.Sc.(BIOTECHNOLOGY)

THESIS ADVISORS: TIMOTHY W. FLEGEL, Ph.D., PAISARN SITHIGORNGUL, Ph.D., SAENGCHAN SENAPIN, Ph.D.

ABSTRACT

Monodon baculovirus (MBV) is one member of the nucleopolyhedrosis virus (NPV) group that produces occlusion bodies that contain virions in epithelial cells of the hepatopancreas of the peinaeid shrimp *Penaeus monodon*. The major constituent protein of occlusion bodies is polyhedrin. Polyhedrin protein of MBV was extracted from infected hepatopancreas of *P. monodon* post larvae using Urografin gradient ultracentrifugation. A fraction at 50% Urografin was examined by electronmicroscopy that contained purified-polyhedrin protein particles of 20-23 nm diameters. This fraction was also analyzed by SDS-PAGE and yielded a single band of purified-polyhedrin at a molecular weight of 58 kDa. Therefore, purified polyhedrin was used to produce monoclonal antibodies in order to provide an immunological tool for detection of the virus. Seventeen monoclonal antibodies were obtained and reacted with 58 kDa purified-polyhedrin MBV. All of the monoclonal antibodies were specific to MBV without cross-reactivity to other viruses (HPV, YHV, and WSSV) based on immunohistochemistry. The antibodies could be used to detect purified-polyhedrin with high sensitivity up to 0.2 µg/ml by dot blot assay. Moreover, antibodies could be used to detect MBV infection in tissue by immunohistochemistry. Therefore, this study provides an immunological tool for specific detection of MBV in infected shrimp. Further development of highly sensitive assays and convenient kits using these monoclonal antibodies for detection of MBV are in progress.

KEY WORDS: IMMUNOHISTOCHEMISTRY/ MONODON BACULOVIRUS/ MONOCLONAL ANTIBODIES/ *PENAEUS MONODON*/ POLYHEDRIN

81 P. ISBN 974-04-6211-1

Copyright by Mahidol University

การพัฒนาโมโนโคลนอลแอนติบอดีต่อโพลีฮีดรินของเชื้อโมโนดอนแบคคูโลไวรัสในกุ้งกุลาดำ
(DEVELOPMENT OF MONOCLONAL ANTIBODIES TO POLYHEDRIN OF MONODON BACULOVIRUS)

จุมพล บุญสนองโชคยิ่ง 4636282 SCBT/M

วท.ม. (เทคโนโลยีชีวภาพ)

คณะกรรมการควบคุมวิทยานิพนธ์ : ทิมโมที วิลเลียม เฟลเกล, Ph.D., ไพศาล สิทธิกรกุล, Ph.D., แสงจันทร์ เสนาปิน, Ph.D.

บทคัดย่อ

การสกัดโปรตีนโพลีฮีดริน (polyhedrin) ของเชื้อโมโนดอนแบคคูโลไวรัสในกุ้งกุลาดำ (MBV) จากตับกุ้งกุลาดำในระยะตัวอ่อน (post larvae, PL) โดยการปั่นตกตะกอนอย่างหนักด้วย 20-60% Urografin สามารถแยกแถบโปรตีนได้ที่ 50% Urografin เมื่อนำไปตรวจด้วยกล้องจุลทรรศน์อิเล็กตรอนพบว่าสามารถสกัดโปรตีนโพลีฮีดรินบริสุทธิ์ที่มีอนุภาคขนาดเส้นผ่านศูนย์กลางประมาณ 20-23 นาโนเมตร จากการวิเคราะห์หาน้ำหนักของโปรตีนด้วยวิธี SDS-PAGE พบแถบโปรตีนหลักที่มีน้ำหนักประมาณ 58 กิโลดาลตัน ในงานวิจัยนี้จึงใช้โปรตีนโพลีฮีดรินบริสุทธิ์ที่สกัดได้ในการผลิตโมโนโคลนอลแอนติบอดี เพื่อใช้วินิจฉัยไวรัสบนพื้นฐานของอิมมูโนวิทยา โมโนโคลนอลแอนติบอดีที่ผลิตได้มี 17 ชนิด พบว่าทั้งหมดทำปฏิกิริยาจำเพาะต่อโปรตีนที่น้ำหนักโมเลกุล 58 กิโลดาลตัน ของ MBV แต่ไม่ทำปฏิกิริยาข้ามกับไวรัสชนิดอื่น (HPV, YHV, และ WSSV) นอกจากนี้โมโนโคลนอลแอนติบอดีบางชนิดสามารถนำมาตรวจหาโพลีฮีดรินของเชื้อโมโนดอนแบคคูโลไวรัสด้วยความไวสูงในระดับ 0.2 ไมโครกรัมต่อมิลลิลิตรโดยวิธี dot blot และสามารถตรวจติดตามการติดเชื้อโมโนดอนแบคคูโลไวรัสในเนื้อเยื่อกุ้งกุลาดำด้วยวิธี immunohistochemistry ดังนั้นการศึกษานี้ทำให้ได้วิธีในการวินิจฉัยกุ้งที่ติดเชื้อโมโนดอนแบคคูโลไวรัสด้วยวิธีทางอิมมูโนวิทยา และคาดว่าจะสามารถนำโมโนโคลนอลที่ผลิตได้ไปเตรียมชุดตรวจสำเร็จรูปที่มีความไวเพื่อใช้ในการติดตามเชื้อโมโนดอนแบคคูโลไวรัสต่อไป

81 หน้า ISBN 974-04-6211-1

Copyright by Mahidol University

CONTENTS

		Page
ACKNOWLEDGEMENTS		iii
ABSTRACT		iv
LIST OF TABLES		x
LIST OF FIGURES		xi
LIST OF ABBREVIATIONS		xiv
CHAPTER		
I	**INTRODUCTION**	1
II	**LITERATURE REVIEW**	3
	1. Effect of MBV on shrimp culture	3
	2. Baculovirus	4
	2.1 Biology of Baculovirus	4
	2.2 Monodon baculovirus	5
	2.3 Polyhedrin	7
	3. Diagnostic procedures	10
	3.1 Direct microscopic methods	10
	3.2 Histological method	10
	3.3 Polymerase chain reaction procedure	11
	3.3.1 MBV PCR method – segment of the polyhedrin gene	11
	3.3.2 MBV PCR method – segment	

Copyright by Mahidol University

CONTENTS (CONT.)

		Page
	of the polymerase gene	11
	3.3.3 MBV nested PCR method	11
	3.4 Transmission Electron Microscopy (TEM) method	12
	4. Methods of control	12
	5. Monoclonal antibodies	14
III	**MATERIALS AND METHODS**	12
	1. Animals	17
	2. Antigen preparation and purification	17
	2.1 Screening of infected shrimp	17
	2.2 Purification of MBV polyhedrin	18
	2.3 Transmission electron microscopy	19
	2.4 PCR for Hepatopancreatic parvovirus (HPV)	19
	3. Protein Analysis	19
	3.1 Determination of Protein Concentration	19
	3.2 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)	20
	3.2.1 Preparation of SDS-polyacrylamide gel	20
	3.2.2 Sample Preparation	20
	3.2.3 Coomassie Brilliant Blue Staining	20
	3.3 Molecular Weight Determination	21
	4. Immunization of Mice	22
	5. Myeloma Cell Lines	22

Copyright by Mahidol University

CONTENTS (CONT.)

		Page
	6. Fusion Procedure	22
	6.1 Serum Collection	22
	6.2 Fusion	23
	7. First Screen of Hybridomas for anti-MBV Antibodies by Dot ELISA	24
	8. Cloning of Hybrid Cells by Limiting Dilution Method	26
	9. Second Screen of Hybridomas for anti-MBV Antibodies by Western blot and immunohistochemistry	26
	9.1 Western blot	26
	9.2 Immunohistochemistry	29
	9.2.1 Preparation of tissue Specimens	29
	9.2.2 The Immunoperoxidase Assays	31
	10. Cryopreservation	31
	11. Determination of Antibody Isotypes and Subisotypes Using Sandwich ELISA	33
	12. Determination of Antibody Epitope Using Indirect ELISA	35
IV	**RESULTS**	37
	1. Screening for MBV infection	37
	2. Purification of MBV polyhedrin	37
	3. Protein analysis	44
	3.1 Determination of protein concentration	44
	3.2 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)	44
	4. Immunization of mice	47

Copyright by Mahidol University

CONTENTS (CONT.)

		Page
	4.1 Dot ELISA	47
	4.2 Western blot analysis	47
	4.3 Immunohistochemistry	47
	5. Production of monoclonal antibodies	51
	6. Characterization of monoclonal antibodies	51
	6.1 Specificity of MAbs	51
	6.2 Isotype and subisotype determination	51
	6.3 Sensitivity of MAbs for purified polyhedrin detection by dot blot	52
	7. Epitope of monoclonal antibodies determination	52
V	**DISCUSSION**	60
	1. Purification of MBV polyhedrin	60
	2. Production of monoclonal antibodies	61
VI	**CONCLUSION**	63
REFERENCES		64
APPENDIX		70
BIOGRAPHY		81

Copyright by Mahidol University

LIST OF TABLES

Table		Page
1.	Main characteristics of the two types recognized in the two groups of crustacean BP-type and MBV-type SNPVs	8
2.	Surveillance, detection and diagnostic methods for the nuclear polyhedrosis virus MBV	13
3.	MAbs mixture for determination of epitope of MAbs	36
4.	The concentration and volume of purified polyhedrin from Roong-Aroon farm, Manusfarm, and Khajee farm	45
5.	Specificities of monoclonal antibodies tested by dot blot, western blot and immunohistochemistry	53
6.	ELISA OD values of various MAbs to determine epitope of MAbs	59

Copyright by Mahidol University

LIST OF FIGURES

Figure		Page
1.	A diagram showing the baculovirus taxonomy. MBV is a single nucleocapsid polyhedrosis virus (SNPV) and it is a member of Nucleopolyhedrovirus (NPV) group.	6
2.	TEM of purified PvSNPV (a) and PmSNPV (b) polyhedra subunits (=SuOBs) negatively strained with 2% PTA	9
3.	Monoclonal antibodies production by somatic hybridization method	16
4.	A typical standard curve used for molecular weight determination of the protein components separated by SDS-PAGE	21
5.	Diagram of monoclonal antibodies production	25
6.	Dot blotting and western blot analysis of the purified-polyhedrin of MBV	28
7.	A flow chart of tissue preparation for immunohistochemistry	30
8.	A flow chart of immunohistochemistry preparation	32
9.	A flow chart of determination of antibody isotypes and subisotypes	34
10.	A. Light micrograph of a hepatopancreatic tissue squash stained with 0.05% malachite green and showing the present of MBV occlusion bodies. B. Light micrograph of the homogenate showing free MBV occlusion bodies amongst fragments of muscle tissue.	39
11.	Agarose gel electrophoresis of MBV Nested-PCR using DNA template extracted from supernatant.	40
12.	Purification of MBV polyhedrin using Urografin ultracentrifugation. The homogenate of hepatopancreatic from PL of *P. monodon* were separated in an Urografin gradient (20-60%).	41
13.	Transmission electron microscopy (TEM) of purified polyhedrin protein negatively stained with 2% PTA.	42

Copyright by Mahidol University

LIST OF FIGURES (CONT.)

Figure **Page**

14. Agarose gel electrophoresis of HPV Nested-PCR using DNA template extracted from the 50% Urografin fraction. 43
15. A typical standard curve used for protein concentration determination of the purified-polyhedrin. 45
16. SDS-PAGE gel of purified-polyhedrin from 50% Urografin fraction, Lane M contains molecular weight marker, the number denoted kDa; lane 1, 2, and 3 contains purified-polyhedrin from Roong-Aroon farm, Manus farm and Khajee farm respectively. 46
17. Dot blot analysis showing immunoreactivity of all mice antiserum at 1: 5,000 dilutions with different dilution of purified-polyhedrin of MBV. 48
18. SDS-PAGE and Western blot of purified-polyhedrin of MBV. The purified-polyhedrin (0.4µl/lane) was electrophoresed and stained with Coomassie blue or transferred to a nitrocellulose membrane and then treated with PAbs from 4 mice. 49
19. Immunohistochemistry of *P. monodon* hepatopancreas. Four sections from *P. monodon* infected with MBV were treated with PAbs from 4 mice (A; mouse no.1, B; mouse no.2, C; mouse no.3, D; mouse no.4) at dilution 1:2,500 50
20. Sensitivity of MAbs for polyhedrin of MBV as detected by dot blot. Dilution of purified-polyhedrin ($8.0 \times 10^{-3} - 2.0 \times 10^{-4}$ µg/µl) was spot on a nitrocellulose membrane (1µl/spot) and treated with 17 MAbs 54
21. SDS-PAGE and Western blot of purified polyhedrin of MBV. The purified polyhedrin was electrophoresed and stained with Coomassie blue or transferred to a nitrocellulose membrane and then treated with MAbs. 55
22. Immunohistochemistry of *P. monodon* hepatopancreas in stage post larvae (A, D), juvenile (B), and adult (C). 56

Copyright by Mahidol University

LIST OF FIGURES (CONT.)

Figure **Page**

23. Immunohistochemistry of *P. monodon* hepatopancreas infected with HPV (A, B) infected with MBV and HPV (C) and normal hepatopancreas (D). 57
24. Immunohistochemistry of *P. monodon* gills (A, B) and hepatopancreas (C, D) infected with YHV and heart (E, F) infected with WSSV. 58

Copyright by Mahidol University

LIST OF ABBREVIATION

%	=	Percent
bp	=	Base pair
°C	=	Degree Celsius
DAB	=	Diaminobenzidine
DMSO	=	Dimethylsulfoxide
ELISA	=	Enzyme-linked immunosorbent assay
FCS	=	Fetal calf serum
g	=	Grum
x g	=	Gravity force
GAM-HRP	=	goat anti mouse-horseradish peroxidase conjugated
GAR-HRP	=	goat anti rabbit-horseradish peroxidase conjugated
h	=	Hour
Ig	=	Immunoglobulin
kDa	=	Kilo Dalton
MAb	=	Monoclonal antibody
MBV	=	Monodon baculovirus
mg	=	Milligram
min	=	Minute
ml	=	Millilitre
mm	=	Millimetre

Copyright by Mahidol University

LIST OF ABBREVIATION (CONT.)

µl	=	Microlitre
µm	=	Micrometre
nm	=	Nanometre
OD	=	Optical density
OPD	=	O-phenylenediamine dihydrochloride
PAGE	=	Polyacrylamide gel electrophoresis
PAb	=	Polyclonal antibody
PBS	=	Phosphate buffer saline
PCR	=	Polymerase chain reaction
RAM-IgG	=	Rabbit anti mouse-IgG
s	=	Second
SDS	=	Sodium dodecyl sulphate
Rf	=	Running front
V	=	Voltage

Copyright by Mahidol University

CHAPTER I
INTRODUCTION

Penaeus monodon-type baculovirus (MBV) is an occluded baculo-like virus that contains double-stranded DNA as its nucleic acid type (1, 2, 3). MBV is one of the earliest found shrimp viruses, but its impact on shrimp farming has yet to be further studied. MBV is considered to be a potentially serious pathogen in the larvae, post larvae (PL), and early juvenile stages of cultivated shrimp species. Lightner and Redman (1981) first reported MBV in Taiwanese *P. monodon*. MBV has a wide geographic distribution from Southeast Asian countries, Australia (4, 5) and India (6). Its infections are characterized by the presence of prominent, spherical intranuclear occlusion bodies in affected epithelial cells of the hepatopancreas and midgut, or free within lysed cell debris in the feces (2, 7). Hepatopancreatic cells infected with MBV exhibit a 300% volume increase in the hypertrophied nuclei (8). Visible indications of MBV infected *P. monodon* include, lethargy with reduced feeding, preening activities, grey-blue to bluish-black coloration, shell disease and microbial epibiotic fouling. Acute MBV causes loss of hepatopancreatic tubule and midgut epithelia and consequently, dysfunction of these organs, often followed by secondary bacterial infections. Crowding or environmental stress may enhance the pathogenicity and increase the prevalence of MBV in its hosts (9, 10, 11). Transmission and infection by MBV is exclusively by the oral route in which cannibalism and faecal-oral contamination are the principal mechanisms of transmission (12, 13).

Polyhedrin is a crystalline protein and a characteristic feature of viruses of the genus nuclear polyhedrosis virus (NPV) belonging to the family Baculoviridae (14). Occlusion bodies (OB) consist of polyhedrin protein matrixes that appear to stabilize virions and maintain their viability over prolonged damaging conditions (8). In the shrimp, infection probably occurs through a process similar to that of NVP of insects (15). These occlusion bodies are released in to inter, or intracellular spaces, or into the

Copyright by Mahidol University

lumen of the hepatopancreatic tubules and shed in the shrimp feces. For MBV, the molecular weight of polyhedrin has been estimated at 58 kDa (16).

Diagnosis of MBV can be made by several methods. The simplest method is based on the microscopic description of the characteristic occlusion bodies produced by the virus. With direct microscopy, characteristic occlusion bodies (clusters of refractive spheres for MBV) are demonstrated in stained squashes for post larvae or by histological H&E staining in tissue sections or smears of hepatopancreatic tissue (2). Molecular methods based on gene probes applicable to in-situ hybridization assay, PCR, and nested-PCR method for MBV are also available and provide a satisfactory method for surveillance applications (5). An antibody-based enzyme-linked immunosorbent assay (ELISA) for diagnosis of MBV has been reported (17), but the method used polyclonal antibodies and the antibodies are not commercially available. Nowadays, monoclonal antibodies against *Penaeus monodon*-type baculovirus (MBV) are not produced. Serological diagnosis using monoclonal antibodies is one of the most rapid and accurate methods, in addition to being simple and low cost. The use of monoclonal antibodies results in a higher specificity to the virus than the use of polyclonal antibodies, due to the fact that monoclonal antibodies react very specifically against a particular component of the virus or occlusion bodies. An attempt in the production of polyclonal antibody against synthetic MBV polyhedrin has been made (63). However, the sensitivity was low and, therefore, it was not suitable for diagnosis. Therefore, this study used method for purify polyhedrin as described by Satidkanidkul (63).

In the present study, the aims were to purify MBV polyhedrin protein from infected shrimp and produce monoclonal antibodies (MAbs) against polyhedrin of *Penaeus monodon*-type baculovirus (MBV). These monoclonal antibodies would thereafter be used in the diagnosis of MBV.

A benefit from this study would be the use of MAbs specific to MBV polyhedrin to identify MBV infected shrimp. Also the MAbs could be utilized to develop a strip test; an easy, accurate and rapid technique for detection of MBV in hatcheries and farm, where *P. monodon* is cultivated.

Copyright by Mahidol University

CHAPTER II
LITERATURE REVIEW

1. Effect of MBV on shrimp culture

In the 1970s, shrimp aquacultures started as an industrial activity and developed rapidly with an immense increase in the number of hatcheries and farms in tropical countries in Southeast Asia, Central and South America (18). Shrimp farming provides roughly 30% of the shrimp supplied to the world market. The black tiger shrimp (*Penaeus monodon*) is an important agricultural product of Thailand. Disease is an influential in reducing shrimp number in natural populations. Pathogens, such as viruses, fungi, and bacteria along with environmental stresses and pollution can cause reduced production of cultivated shrimp (17). However, viral diseases are a major menace to the shrimp aquaculture industry because many viral infections remain undetected until they result in heavy disease outbreak (19). In Thailand, there are four important viral pathogens of *P. monodon*. These are white spot syndrome virus (WSSV), yellow head virus (YHV), hepatopancreatic parvovirus, and monodon baculovirus (MBV). Disease outbreaks cause significant losses in aquaculture production and trade and are affecting economic development of Thailand and other countries.

MBV is considered to be a potentially serious pathogen of *Penaeus monodon*. It has been reported that the disease is latent until the shrimp are faced with stress, leading to the development of disease. MBV has been observed in all life stages except eggs and nauplii of *P. monodon*. Shrimp postlarvae seem to be more susceptible to MBV than adults. MBV infections may significantly reduce growth, crop value, and over-all health status of the shrimp. MBV has been linked with high mortalities (over 90%) in late postlarvae and juvenile shrimp in many culture facilities. It has caused heavy mortalities (70% of all stages of *P. monodon* in the Philippines

Copyright by Mahidol University

and 90% of postlarvae *P. monodon* in Madras, India), and is considered partially responsible for the collapse of the shrimp culture industry in Taiwan in the late 1980s (11). Usually juvenile and adult *P. monodon* are more resistant to MBV than larval shrimp. Although good culture practices may enhance survival of MBV infected stocks, growth, crop value and performance may be significantly reduced (2). MBV, however, may pre-dispose infected shrimp to other pathogens resulting in the increase of the mortality rates. Susceptible species include *Penaeus monodon*, *Fenneropenaeus merguiensis*, *Melicertus plegejus*, *Metapenaeus ensis*, *F. penicillatus*, and *Melicertus kerathurus* (2).

Gross signs accompanying MBV infection may include reduced feeding and growth rates and increased gill and surface fouling organisms. Whitish midgut line through the abdomen has been found in severely affected larvae and post larvae. The principal clinical signs of MBV infection are the presence of single or multiple spherical occlusion bodies in the hepatopancreas and midgut epithelial cells. Histological confirmation of MBV infections is dependent upon the demonstration of prominent, single or multiple eosinophilic spherical bodies within hypertrophied nuclei of hepatopancreatic tubule or midgut epithelial cells.

MBV has been found in wild and cultured populations of shrimp in China, Taiwan, Philippines, Malaysia, Singapore, Thailand, Sri Lanka, India, Indonesia, and Australia (2). It has also been found in Kuwait, Oman, Israel, Italy, Kenya, and Gambia. Imported penaeids in Tahiti, Hawaii, Mexico, Ecuador, Brazil, Puerto Rico, and in the states of South Carolina and Texas in the United States have also been found to have MBV (2). It has not been found in the local populations of penaeids in the United States.

2. Baculoviruses

2.1 Biology of Baculoviruses

Baculoviruses, the most prominent viruses found to affect invertebrate species, are in the family Baculviridae which is a large family of occluded viruses.

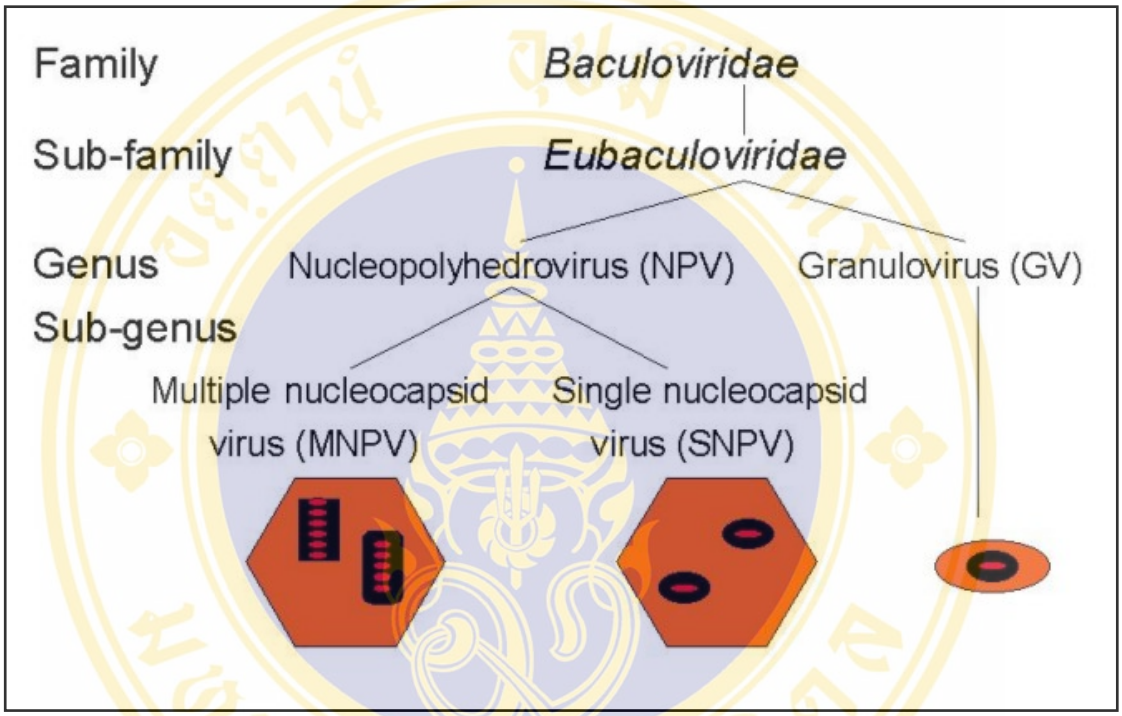
Copyright by Mahidol University

They are characterized by a large rod-shaped, enveloped nucleocapsid and double stranded circular supercoiled DNA molecules. The length of baculoviral DNA from between 80-200 kb. Virions that obtain their envelope within the nucleus are usually also occluded within a crystalline protein matrix within the nucleus. Such virions may thus be referred to as occluded virions (20, 21). Baculoviruses are divided into two distinct genera that are the nuclear polyhedrosis viruses (NPVs) and the granulosis viruses (GVs) (Figure 1). They are differentiated by the size of the virus particles and the nature of the protein in the occlusion body (www.nerc-oxford.ac.uk/. ../bactaxon.htm**).**

2.2 Monodon baculovirus

In shrimp, there are two well-known SNPV that are considered to be potentially serious pathogens in larvae, postlarvae, and early juveniles. One is *Baculovirus penaei* (BP) named by Couch (22) and the other is the monodon baculovirus (MBV) named by Lightner and Redman (3). The geographical distribution of BP is limited to the Western Hemisphere where it has a widespread distribution in cultured and wild penaeid shrimps (*Penaeus vannamei*, *Penaeus aztecus*, *Penaeus duorarum*) in North and South America. By contrast, MBV is widely distributed in cultured and wild penaeid shrimp and prawns (*Penaeus monodon Fenneropenaeus merguiensis*, *Melicertus plegejus*, *Metapenaeus ensis*, *F. penicillatus*, and *Melicertus kerathurus*) in the Eastern Hemisphere and has been observed or reported from Australia, East Africa, the Middle East, from many of the Indo-Pacific countries, and from South and East Asia. MBV is sometimes called a type-A baculoviruses indicating that they produce polyhedrin occlusion bodies that contain various with double stranded DNA as their nucleic acid (2). MBV from *P. monodon* was designated as PmSNPV (for single enveloped nuclear polyhedrosis virus from *P. monodon*) by Mari et al. (23). The nucleocapsid of *Penaeus monodon*-type baculovirus (MBV) from *P. monodon* in the Indo-Pacific measures 42 ± 3 by 246 ± 15 nm. The enveloped stage of the virus measures 75 ± 4 by 324 ± 633 nm. *Penaeus plebejus* baculovirus (PBV) is similar in size to MBV measuring 45 to 52nm by 260 to 300 nm for the nucleocapsid and 60 nm by up to 420 nm for enveloped virion (2). MBV occlusion bodies are polyhedral while those of BP are tetrahedral (16).

Copyright by Mahidol University



(From http://www.nerc-oxford.ac.uk/cehoxford/groups/virusecology/bacfam.jpg)

Figure 1. A diagram of the baculovirus taxonomy. MBV is a single nucleocapsid polyhedrosis virus (SNPV) and it is a member of Nucleopolyhedrovirus (NPV) group.

Copyright by Mahidol University

2.3 Polyhedrin

NPV virion particles were embedded in a crystalline protein matrix. The protein making up the crystalline matrix of an NPV occlusion body is known as polyhedrin. Since the embedded viral particles are covered by polyhedrin they are said to be occluded in the proteinaceous crystal of NPV (http://www.ias.ac.in). Occlusion body formation is crucial for viral infection of invertebrates in the wild, since they shield the delicate virions from harsh environmental stress. Further, occlusion bodies act as useful carriers of the virus particles; the crystalline polyhedrin matrix is solubilized in the mid-gut of the invertebrate, thus releasing the virus particles that infect the mid-gut cells. Polyhedrin is thus an extremely important protein for virus survival and propagation in its native environment (25).

There is some evidence indicating that crustacean SNPV (I.e., BP-type and MBV-type) produce polyhedrin with a much higher molecular weight than that of insect-type NPV (23, 27, 28). The molecular weight of MBV-type polyhedrin is 58 kDa and while that of BP-type is 52 kDa (Table 1). In addition, they apparently share some common epitopes since an antibody to BP-type polyhedrin can cross-react with MBV-type (16). By contrast, polyhedrin of insect baculoviruses range from about 25-30 kDa (29, 30, 31, 32). The ultrastructure of MBV-type polyhedrin is shown in Figure 2.

Copyright by Mahidol University

Table 1. Main characteristics of the two types recognized in the two groups of crustacean BP-type and MBV-type SNPVs.

characteristics	PvSNPV (BP type)	PmSNPV (MBV type)
OB shape	Tetrahedral	Rounded
Rosette	No	Yes
Polyhedra subunits (SuOBs)	Arranged in triplets	
Diameter (PTA staining)	17-19 nm	22-23nm
Diameter (in section)	15-18.5 nm	16.5-18.8 nm
M_r	52 kDa (50 kDa)[a]	58 kDa
Number of bonds/SuOB	12	8

[a]From PdSNPV (Summers, 1997)

(From Bonami et al, 1997 (16))

Copyright by Mahidol University

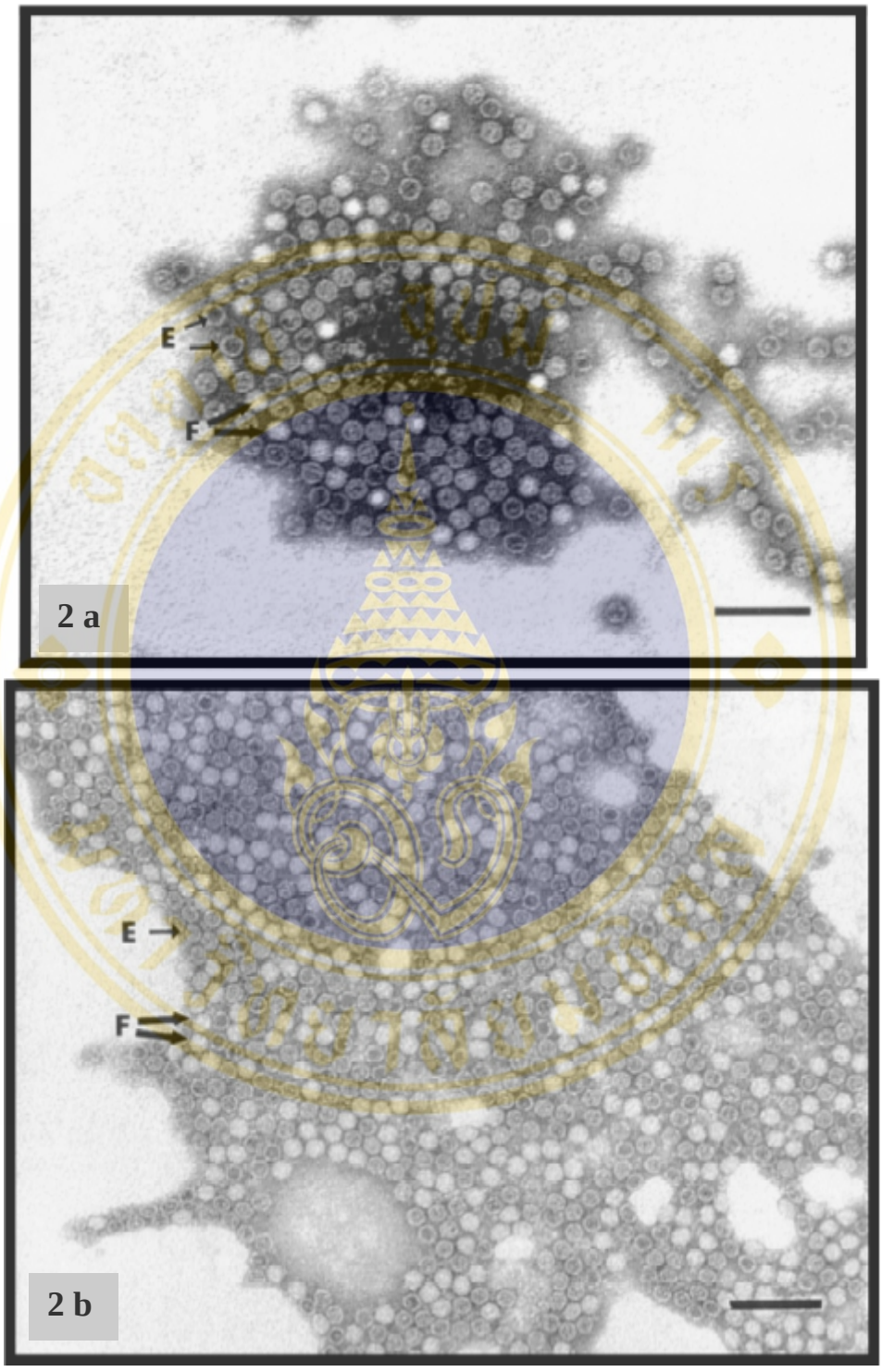


Figure 2. TEM of purified PvSNPV (a) and PmSNPV (b) polyhedra subunits (=SuOBs) negatively strained with 2% PTA (16). Full (F) and empty appearing (E) subunits are shown, some of which appear hexagonal in profiled bars are 0.1 μm.

Copyright by Mahidol University

3. Diagnostic procedures

Infection of the hepatopancreas by MBV is among the most readily diagnosable diseases of the penaeid shrimp and prawns. The methods currently available for surveillance, detection, and diagnosis of MBV are listed in Table 2.

3.1 Direct microscopic methods

MBV infection is made by demonstration of single or multiple generally spherical intranuclear occlusion bodies that range is diameter from less than 0.1 μm to nearly 20 μm in the epithelial cells of the hepatopancreas and midgut. Occlusion bodies may also be found in fresh feces examined under phase or bright filed microscopy. Staining the tissue squash with a staining such as 0.05% malachite green aids in demonstration of occlusion bodies by stain them more intensely than other similar sized spherical objects, such as normal host cell nuclei, nucleoli, secretory granules, phagolysosomes, and lipid droplets (1). A rapid and specific diagnostic tool for the identification of MBV involves epifluorescent light microscopy of tissue squashes stained with 0.001% aqueous phloxine (33).

3.2 Histological method

Histology may be used to provide a definitive diagnosis of MBV infection. MBV occlusion bodies appear as prominent eosinophilic (with haematoxylin and eosin stain) multiple polyhedral bodies within the hypertrophied nuclei of hepatopancreatic tubule or midgut epithelial cells. Early in the infection process (or in light infections) occlusion bodies may not be easily demonstrated. Routine histological stains such as Mayer-Bennett's or Harris H&E, Giemsa stains, and Gram tissue-staining methods may be used for demonstration of MBV (2, 34, 35). Another diagnostic tool for the identification of MBV involves epifluorescent light microscopy of histological sections stained with 0.005% phloxine as a component of the eosin part of normal haematoxylin and eosin staining (33).

Copyright by Mahidol University

3.3 Polymerase chain reaction procedure

PCR methods have been developed for MBV. Primer sets designed from certain segments of the MBV genome are unique and provide positive test results only in samples infected with particular stains of this virus.

3.3.1 MBV PCR method – segment of the polyhedrin gene

A PCR method with primer sequences was published by Lu et al. (36). Two primers, p35 and p36, were designed using insect baculovirus polyhedrin gene sequences as a reference, and they amplify a 674 bp segment of the MBV polyhedrin gene. The primer sequences are

p35: 5'-AC(CT)-TA(CT)-GTG-TCA-GAC-AAC-AAA-TA(CT)-TAC-AAA-3'

p36: 5'-GG(CT)-GCG-TC(GT)-GG(CT)-GCA-AA(CT)-TT(CT)-TT(AT)
AC(CT)-TT(AG)-AA-3'

However, use of this method was unable to distinguish between MBV found in Taiwan and AcMNPV (17).

3.3.2 MBV PCR method – segment of the polymerase gene

A PCR method with primer sequences was published by Hsu et al. (17). Two primers, pA1 and pA2, were designed using a conserved sequence from the insect baculovirus polymerase gene as a reference, and they amplify a 511 bp segment of the MBV polymerase gene. The primer sequences are

pA1: 5'-GTG-CTG-CAA-CGA-CTG-GAC-TGT-CC-3'

pA2: 5'-TGA-TCC-TAG-GCG-TGC-CTG-TAT-TGG-G-3'

As with the polyhedrin PCR method described in 3.3.1, this PCR method may used as a species specific assay for MBV found in Taiwan.

3.3.3 MBV nested PCR method

Belcher & Young (5) published a nested PCR method capable of detecting low concentrations of MBV (down to eight viral genome equivalents). Two external and two internal primers were designed using a DNA sequence derived from the plasmid p4Ec196, which was constructed from a 7.4 kb *Eco*RI fragment of an Australian isolate of MBV (5). The primer sequences are:

Copyright by Mahidol University

Primer	Sequence	Temperature
MBV1.4F	5'-CGA-TTC-CAT-ATC-GGC-CGA-ATA-3'	62°C (68.9°C)
MBV1.4R	5'-TTG-GCA-TGC-ACT-CCC-TGA-GAT-3'	64°C (70.8°C)
MBV1.4NF	5'-TCC-AAT-CGC-GTC-TGC-GAT-ACT-3'	64°C (70.8°C)
MBV1.4NR	5'-CGC-TAA-TGG-GGC-ACA-AGT-CTC-3'	66°C (72.8°C)

This method has been tested with MBV from Australia, Thailand, and Taiwan and shown to be an effective detection.

3.4 Transmission Electron Microscopy (TEM) method

Transmission electron microscopy (TEM) is another method used to confirm MBV infected hepatopancreas of *P. monodon*. MBV virions are easily demonstrated as enveloped virions measure about 74 x 270 nm both free in the nuclei and within the occlusion bodies (2).

4. Methods of control

Nowadays, MBV infections have no method to treatment. MBV epizootics are basically hatchery and pond management problems that are not limited to certain places or seasons. Because MBV is transmitted from adults to their offspring by faecal contamination of the spawned eggs, prevention of infection in hatcheries may be achieved by taking steps to eliminate faecal contamination of spawned eggs and larvae by thoroughly washing nauplii or eggs with formalin, iodophores, and clean sea water (2).

Copyright by Mahidol University

Table 2. Surveillance, detection and diagnostic methods for the nuclear polyhedrosis virus MBV

Method	**Screening**				**Presumptive**	**Confirmatory**
	Larvae	PLs	Juveniles	Adults		
Gross signs	-	-	-	-	+	-
Direct BF/LM	+++	+++	+	++	+++	+++
Histopathology	+++	+++	++	++	+++	+++
Bioassay	-	-	-	-	+	+
Transmission EM	-	-	-	-	+	+
Antibody-based methods	-	-	-	-	+	+
DNA probes - dot-blot	-	-	-	-	-	-
DNA probes - *in situ*	-	-	-	-	+++	+++
PCR	+++	+++	+++	+++	+++	+++

PLs = postlarvae; BF = bright field; LM = light microscopy; EM = electron microscopy; PCR = polymerase chain reaction

(From Summary spherical baculovirus 2004 (37))

Copyright by Mahidol University

5. Monoclonal antibodies

Antibodies (also known as immunoglobulin) are a class of protein molecules produced by B lymphocytes of the adaptive immune system which act as flexible adapters between the infectious agents and phagocytes (38). They are produced and present in the serum when an animal encounters an antigen either by infection or by deliberate injection. Antibodies as used in immunological test systems are powerful tools for the specific detection and identification of virus. Monoclonal antibodies (MAbs) are important reagents used in biomedical research, in diagnosis of diseases and in treatment of such diseases as infections and cancer. In 1975, Köhler and Milstein (39) developed the technique for producing hybridoma cells that could secrete antibodies with defined specificity. In this technique, an antibody-secreting cell isolated from an immunized animal is fused with a myeloma cell, to produce a type of malignant immunoglobulin secreting cell (Figure 3).

The fusion between an antibody secreting cell and myeloma cell can be facilitated by fusogens. Fusogens can be lysolecithin, or sendai virus or polyethyleneglycol (PEG). PEG is commonly used for hybridoma fusion (40,41). When cells are treated with PEG, their membranes fuse and then multinucleated cells called heterokaryons are formed. At the next cell division, the nuclei of heterokaryons fuse, and the daughter cells possess a more or less equal share of the genetic material. The resulting hybrid cells are not genetically stable and there is a strong tendency for loss of chromosomes. If one of the chromosomes that carry the rearranged immunoglobulin heavy or light chain genes in lost, production of the antibodies will end.

In the most efficient fusion, 1% of starting cells are fused and 1 to 10^5 form viable hybrids. The cells from the immunized animals do not continue to grow in tissue culture and the unfused myeloma cells must be subsequently killed by the selection procedure. The most common selection procedure, divised by Littlefield (42), depends on the fact that when the *de novo* pathway or main biosynthetic pathway for guanosine is blocked by the folic acid antagonist, aminopterine. There is an alternative, salvage pathway, in which the nucleotide metabolites hypoxanthine or guanine are converted to guanosine monophosphate via the enzyme hypoxanthine

Copyright by Mahidol University

guanine phosphoribosyl transferase (HGPRT) or thymidine kinase (TK). Myeloma cells lacking HGPRT die in a medium containing hypoxanthine, aminopterine and thymidine (HAT medium) because both the main and salvage pathway is blocked. However, an $HGPRT^-$ cell can be made to grow in HAT medium if it is provided with the missing enzyme by fusion with an $HGPRT^+$ cell. So hybrids between myeloma cells that are defective in HGPRT and an antibody secreting cell with functional HGPRT will be able to grow. With their 3 distinct characteristics, specificity of binding, homogenicity and ability to be produced in unlimited quantities, monoclonal antibodies become effective immunochemical tools. No previous or recent reports were concerned with the production of monoclonal antibodies against MBV.

The advantages of monoclonal antibodies are that each cloned B cell produces only one type of antibody of predefined specificity so that impure antigens can be used to generate monoclonal antibodies. Moreover, MAbs can be generated against rare and weakly immunogenic antigens. They have a predefined isotype and thus have variable effector functions. Finally, reproducible antibody can be made in unlimited amounts (44). Nonoclonal antibodies provide many advantages in the study of viral disease, especially as applied in immunodiagnosis, immunotherapy, and vaccine development (43). Because of their specificity, monoclonal antibodies can be used to screen foe viruses in shrimp.

Copyright by Mahidol University

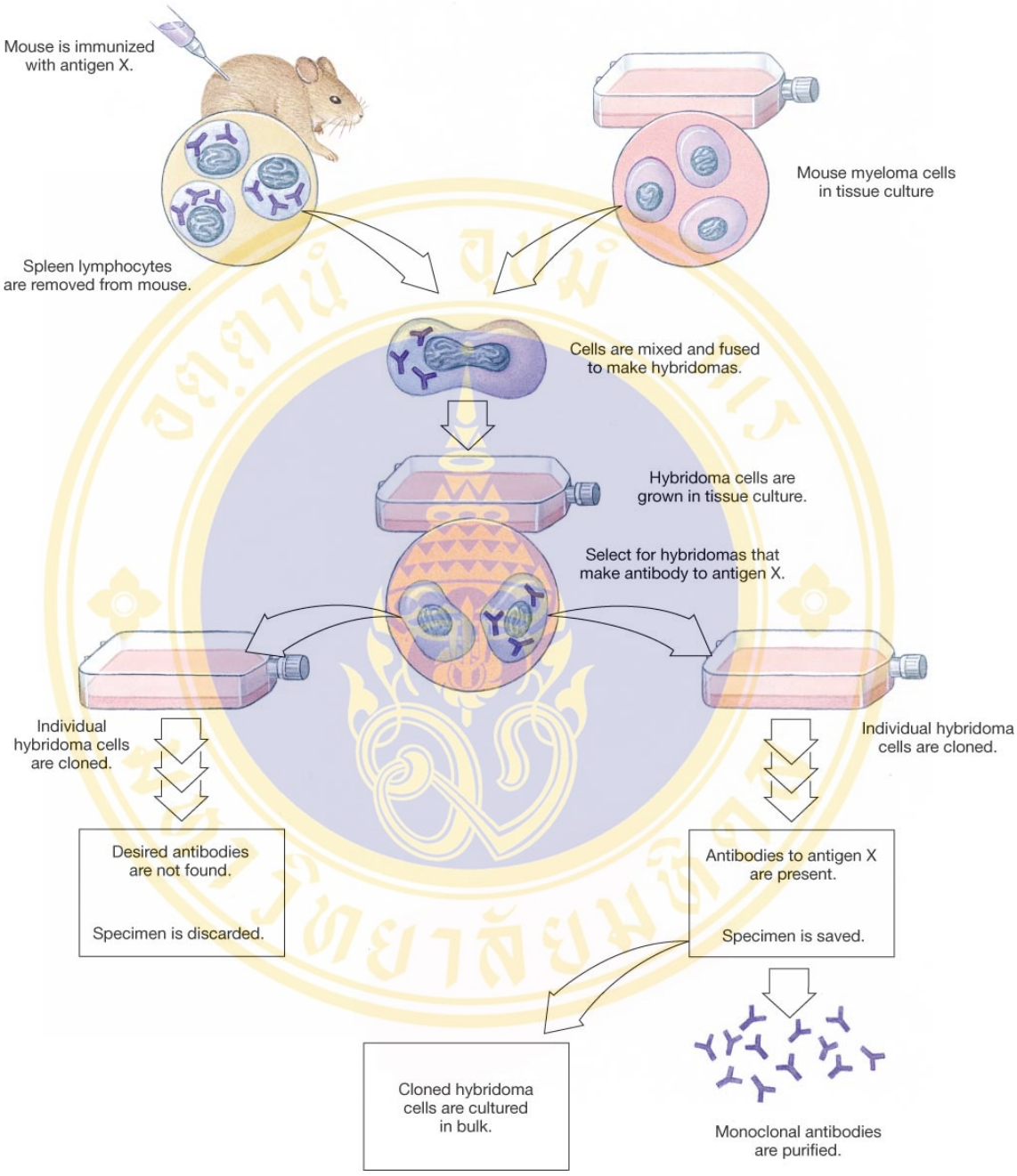


Figure 3. Monoclonal antibody production by the somatic hybridization method (From www.uccs.edu/.../ Chapter%2017/ch17.htm).

Copyright by Mahidol University

CHAPTER III
MATHERIALS AND METHODS

1. Animals

Post larvae at stage 10-15 of *Penaeus monodon* were obtained from Roong-Aroon farm, Manus farm and Khajee farm in Chachoengsao province, Thailand. Swiss albino mice used for antibody production were obtained and maintained under the auspices of National Laboratory Animal Center, Mahidol University, Thailand.

2. Antigen preparation and purification

2.1 Screening of infected shrimp

Wet-mounts of fresh tissue were used to screen for infected shrimp. Post larvae (PL) at stages PL10-15 approximately 5,000-6,000 PL were obtained and 100 randomly selected PL were subjected to microscopic checks for occlusion bodies in hepatopancreas using tissue squashes stained with 0.05% malachite green (2). Infection of MBV was confirmed by PCR and DNA from the hepatopancreas was extracted using QIAamp® DNA mini kit following the protocol from the QIAamp® DNA mini kit handbook (QIAGEN, Hilden, Germany).

Nested-PCR was performed using MBV533/361-IC NESTED-PCR READY TO USE KIT and following the protocol from the product information (Ezee Gene®, SBSL, Thailand). The first step primer was MBV533 F and R to generate a 533 bp fragment of MBV DNA and a 175 bp internal control fragment. The reaction mixtures were pre-denature at 96° C for 5 min and then performing PCR amplification for 40 cycles as following condition; denature at 94° C for 30s, annealing at 65° C for 30s and extension at 72° C for 45s. The final extension was carried out at 72° C for 7 min. PCR negative control containing no template DNA was performed for each PCR reaction. After finish the first amplification, 1 µl of the first PCR product was used as template to the second step Nested-PCR. The secondary primers are MBV 361-IC F

Copyright by Mahidol University

and R to generate a 361 bp fragment of MBV DNA and a 175 bp internal control fragment. The reaction profile consisted of pre-denature at 96° C for 5 min and then performing PCR amplification for 35 cycles under the following conditions; denaturation at 94°C for 30s, annealing at 60° C for 30s and extension at 72° C for 30s. A final extension was carried out at 72° C for 7 min. PCR products were analyzed by 1.5% agarose gel electrophoresis at 80 V for 1 h followed by staining with 1 µg/ml ethidium bromide (EtBr) solution for 3-5 min and de-staining for 30 min with water. The PCR amplicons were visualized on an UV light box. The presence of expected PCR products of 533/361 bp were considered positive for MBV. PCR positive batches were used for preparation of polyhedrin protein.

2.2 Purification of MBV polyhedrin

After confirmation MBV infection, approximately 5,000 PL MBV-infected *P. monodon* postlarvae were stunned in ice water and then place in a beaker containing 50 ml of distilled water. The plunger was removed from a 50 ml plastic syringe with no needle and a thoroughly mixed portion of the PL water mixture was poured into the syringe with the syringe opening held closed. Then the plunger was added and mixture was forced through the syringe opening into a receiving beaker on ice. This disruption procedure was repeated 10 times in rapid succession. The homogenate was then filtered 2 times through a 100 µm Nitex-mesh. The filtrate was centrifuged at 4,000g for 20 minutes at 4° C. The pellet was resuspended in TN buffer (0.01M Tris-HCl, 0.1M NaCl, pH 7.4) for layering onto the top of an Urografin^{TM} gradient (20% to 60%). Then the Urografin^{TM} gradients were ultracentrifuged at 120,000 x g for 16 h at 4° C. After ultracentrifugation, sample bands were removed and repelleted by centrifugation twice at 120,000 x g for 2 h at 4º C. The pellets were resuspended in a minimum amount of TN buffer and stored at -20º C for further experiments. The purity of the MBV polyhedrin was checked by a transmission electron microscopy (Hitachi H-600) by the negative staining technique.

Copyright by Mahidol University

2.3 Transmission electron microscopy

For transmission electron microscopy (TEM), a droplet the purified MBV polyhedrin was placed on a copper grid coated with Formvar carbon (Electron Microscopy Sciences, USA). After about 10 min for suspension settlement, excess liquid was removed by a filter paper touched to the edge of the grid. After drying, a droplet of staining solution (2% phosphotungstic acid solution in distilled water, pH 7.4) was placed on the grid for about 1 min before removal of excess liquid also by touching a filter paper to the edge of the grid (45). After air drying overnight, the grids were examined for the presence of MBV polyhedrin protein by transmission electron microscopy (Hitachi H-600).

2.4 PCR for Hepatopancreatic parvovirus (HPV)

Since Hepatopancreatic parvovirus (HPV) particles are similar in size and morphology to MBV polyhedrin, the purified polyhedrin fraction was confirmed to be free of HPV particles using nested-PCR for HPV. The fraction was subjected to DNA extraction following the protocol in the QIAamp® DNA mini kit handbook (QIAGEN, Hilden, Germany). Nested-PCR was performed using HPV465/183-IC NESTED-PCR MINI KIT following the protocol from the product information (Ezee Gene®, SBSL, Thailand). PCR products were analyzed by 1.5% agarose gel electrophoresis at 80 V for 1 h followed by staining with 1 µg/ml ethidium bromide (EtBr) solution for 3-5 min and de-staining for 30 min with water. The amplicons were visualized on an UV light box. The absence of an expected 183 bp HPV amplicon was considered to indicate freedom from HPV.

3. Protein Analysis

3.1 Determination of Protein Concentration

The concentration of proteins was determined by Bradford's method (BioRad protein assay kit). Standard BSA (Protein Standard II, BioRad) was diluted with distilled water to 5 dilutions in 200 µl of the 1:4 diluted dye reagent. The dye mixtures were incubated at room temperature for at least 5 min before measuring absorbance at 595 nm using a microplate reader (Thermo Labsystems). The

Copyright by Mahidol University

concentration of protein in the sample was calculated by referring to the concentration of this protein standard.

3.2 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

3.2.1 Preparation of SDS-polyacrylamide gel

The SDS-polyacrylamide gel contained a lower separating gel with 10% (w/v) acrylamide and 0.1% (w/v) SDS in 0.375 M Tris-HCl (pH 8.8) and an upper stacking gel with 4% (w/v) acrylamide and 0.1% (w/v) SDS in 0.062 M Tris-HCl (pH6.8). The stacking gel and separating gel were prepared to the desired concentration from a stock 30% (w/v) acrylamide solution with acrylamide to N,N'-methylene-bis-acrylamide ratio of 29:1. The mixture of separating gel was first filled into a casting mold and overlaid with small volume of distilled water and allowed to polymerize for 20 min. After polymerization, water was removed and the stacking gel solution was poured into the mold containing a comb to produce wells to hold the samples. The stacking gel was left to polymerize.

3.2.2 Sample Preparation

Protein samples were mixed with 4x protein sample buffer (0.2 M Tris-HCl pH 6.8, 8% SDS, 0.2% Bromphenol blue and 40% glycerol). The sample mixtures were heated at 100º C for 10 min before loading on the gel slot. The electrophoresis was carried out with Mini-Protein II® (BioRad) at constant current of 80 V using 25mM Tris-193 mM glycine (pH8.3) containing 0.1% SDS as buffer. The electrophoresis was carried out until the dye marker (Bromphenol blue) reached the bottom of the gel. The standard molecular weight marker consisted of a was broad range molecular weight standard mixture from BioRad.

3.2.3 Coomassie Brilliant Blue Staining

To immobilize separated proteins, the gel was removed from the mold and fixed in fixing solution (10% (v/v) acetic acid and 50% (v/v) ethanol) for 30 min at room temperature. The fixing solution was then removed and the gel was stained at room temperature for at least 1 hour in staining solution (0.3% (w/v) Coomassie

Copyright by Mahidol University

brilliant blue R-250). The stained gel was then destained with several changes of destaining solution (10% (v/v) acetic acid) until a clear background was obtained. The destained gel was then soaked in the preserve solution (5% glycerol and 30% ethanol) for at least 1 hour and dried at room temperature between two sheets of cellophane over a glass plate.

3.3 Molecular Weight Determination

A procedure used for molecular weight (MW) determination was modified according to the methods described by Weber and Osborn (46) and by Davies and Stark (47). The MW of sample could be determined by comparing its electrophoretic mobility with those of protein markers of known molecular weights. A linear relationship was obtained when the relative mobility of the standard markers was plotted against the logarithmic values of their molecular weights (Figure 4). The unstained molecular weight markers used in the present study were Myosin (212 kDa), MBP-β-galactosidase (158 kDa), β-galactosidase (116 kDa), Phosphorylase b (97.2 kDa), Serum albumin (66.4 kDa), Glutamic dehydrogenase (55.6 kDa), and MBP2 (42.7 kDa). The relative mobility of a sample was calculated according to the following formula:

$$\text{Relative mobility (Rm)} = \frac{\text{Distance of protein migration}}{\text{Distance of dry migration}}$$

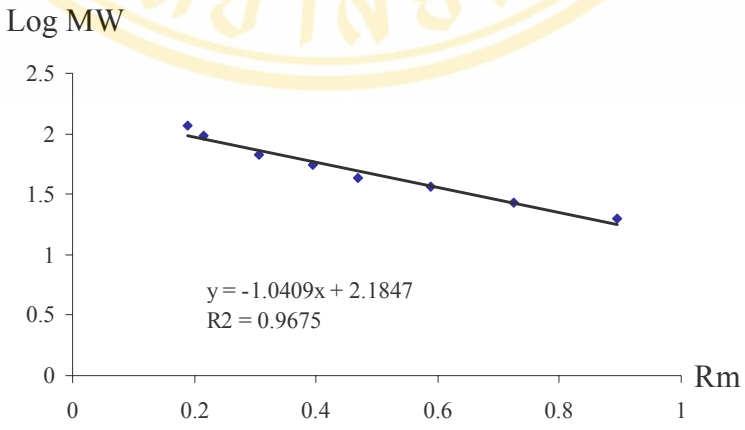


Figure 4. A typical standard curve used for molecular weight determination of the protein components separated by SDS-PAGE

Copyright by Mahidol University

4. Immunization of Mice

Four 6-week old Swiss albino mice were first immunized intraperitoneally with 0.8 mg/ml of fractions containing putative polyhedrin mixed with complete Freund's adjuvant (Sigma®) for a total volume of 100 µl per mouse (antigen: adjuvant 1:1 v/v; 40µg/mouse). The mice were boosted 2 times, first 2 weeks after the first immunization and second 1 month after the first boost with the same preparation mixed with incomplete Freund's adjuvant (Sigma®). One week after the third injection, antisera were collected, absorbed with normal hepatopancreas homogenate in PBS overnight at 4º C and tested for immunoreactivity by dot blot, western blot, and immunohistochemistry. The best performing mouse was boosted 3 days before hybridoma production.

5. Myeloma Cell Lines

Myeloma partners used for fusion were P3X. They were cultured in RPMI 1640 media supplemented with 20% calf bovine serum (CBS) in a humidified incubator in the presence of 5% CO_2 at 37° C. The myelomas were maintained in log-phased growth by means of subculture everyday for one week prior to fusion. The media volume in the petri dishes was maintained at approximately 15 ml.

6. Fusion Procedure

Hybridomas were produced according to Köhler and Milstein (39) with slight modification (48). Non-secreting murine myeloma P3X cells (49) were fused with spleen cells of the spleen donor.

6.1 Serum Collection

The donor was anethetized with diethyl ether for 20 sec before withdrawal via the orbital sinus with a Pasture pipette coated with Sigmacote®. To obtain pre-fusion immune serum, the blood was allowed to clot for 1 h at RT after collection and incubated for 30 min on ice. The serum supernatant was removed to a clean tube and spun down by centrifugation at 10,000 g for 5 min. The serum was transferred to a clean tube and stored at -20° C.

Copyright by Mahidol University

The mouse was then euthanized by cervical dislocation. It was sprayed liberally with 70% alcohol, and the spleen was removed aseptically. Three separated sets of sterile surgical utensils were prepared. The first set was for opening the skin ventrally and making the abdominal wall exposed, the second set making a small incision in the abdominal wall, the third set pulling out the spleen. Carefully the skin and loose hair was kept well away from the work surface. The spleen was placed and soaked in two sterile Petri dishes containing PBS. To minimize the chance of contamination, the spleen was taken an extensive soak in a sterile Petri dish containing RPMI 1640 media. Each soak was for a minimum of 5 min. During the extensive soak as much the connective tissues was carefully removed as possible. Prior to performing the fusion, the Petri dishes containing PBS, and containing RPMI 1640 media were placed overnight in a humidified incubator.

6.2 Fusion

To obtain a single cell suspension and to tease spleens apart, spleens were clipped off and minced using a 5 ml syringe plunger through a sterile grid. The suspension was filtered through the grid several times by means of pipeting. Ten ml of spleen suspension was added to a 50 ml polypropylene tube containing approximately 10^8 cells of P3X myeloma. It was centrifuged for 5 min at 200 x g and the supernatant was discarded as completely as possible. To facilitate fusion, one ml of pre-warmed (37ºC) 40% (w/v) polyethylene glycol (PEG 1500, Sigma®) was added to the cell suspension using a pipettor (1000 µl), and simultaneously the tube was slightly agitated (50). This step was performed for exactly 1 min. Thirty-nine ml of HT media (Appendix B) was added into the suspension, and it was then incubated in a humidified incubator for 2 hrs. The cell suspension was centrifuged for 5 min at 200 g and the supernatant was discarded. The cell pellet was suspended in HAT medium (Appendix B). One hundred µl of this mixed cell suspension was dispensed into each well of the thirty 96-well culture plates, and they were kept in the humidified incubator (Figure 5).

Six days after fusion, macroscopic growths were examined; if visible, cell supernatants were screened for anti MBV MAbs activity as described below. Selected positive hybridomas were cloned in new 96-well culture plates by the limiting dilution

Copyright by Mahidol University

method to obtain single clones per well. Wells that contained a single colony were further propagated in 20% FCS RPMI 1640 medium and cell supernatants were tested by dot ELISA and Western blots (Figure 6).

7. First Screen of Hybridomas for anti-MBV Antibodies by Dot ELISA

To determine positive/negative cell lines after the fusion, hybridoma supernatants were screened using a dot ELISA (51, 52). The nitrocellulose membrane of 0.45µm average pore size (Schleicher and Schull®, Dassel, Germany) was used as a solid phase in the dot ELISA (Figure 6). The putative polyhedrin band derived from Urografin gradient (0.8 mg/ml) diluted 1:20 in PBS, pH 7.2, was applied into the nitrocellulose membrane as 1 µl/spots by means of an adjustable Eppendorf research pipette (10 µl). The nitrocellulose membrane was allowed to air dry about 10 min or until no visible wet spot remains at RT and then placed in a shallow plastic box containing 30 ml of 5% skim milk in PBS, pH 7.2 for 30 min. After transferred to a PBS buffer without skim milk, the blots were stored at 4° C.

The membranes were cut into pieces and placed into microculture plates. Conditioned culture media (1:4 dilutions) samples were added into each well and incubated for 5 hr at RT, then washed thoroughly for 4 times with PBS, pH 7.2. Subsequently, the nitrocellulose membrane disks were incubated were for 3 hr at RT with 1:1500 diluted GAM-HRP (Bio-Rad®) in PBS. Then the nitrocellulose membrane sheets were washed 4 times with PBS and treated with DAB (0.01%3,3-Diaminobenzidine tetrahydrochloride, Sigma®) in PBS containing 0.1 % H_2O_2 and 0.05% $CoCl_2$ at RT for 5 min, and washed with distilled water. The results were scored as positive or negative after visual observations of a color reaction (dark gray) in the nitrocellulose membrane disks.

Copyright by Mahidol University

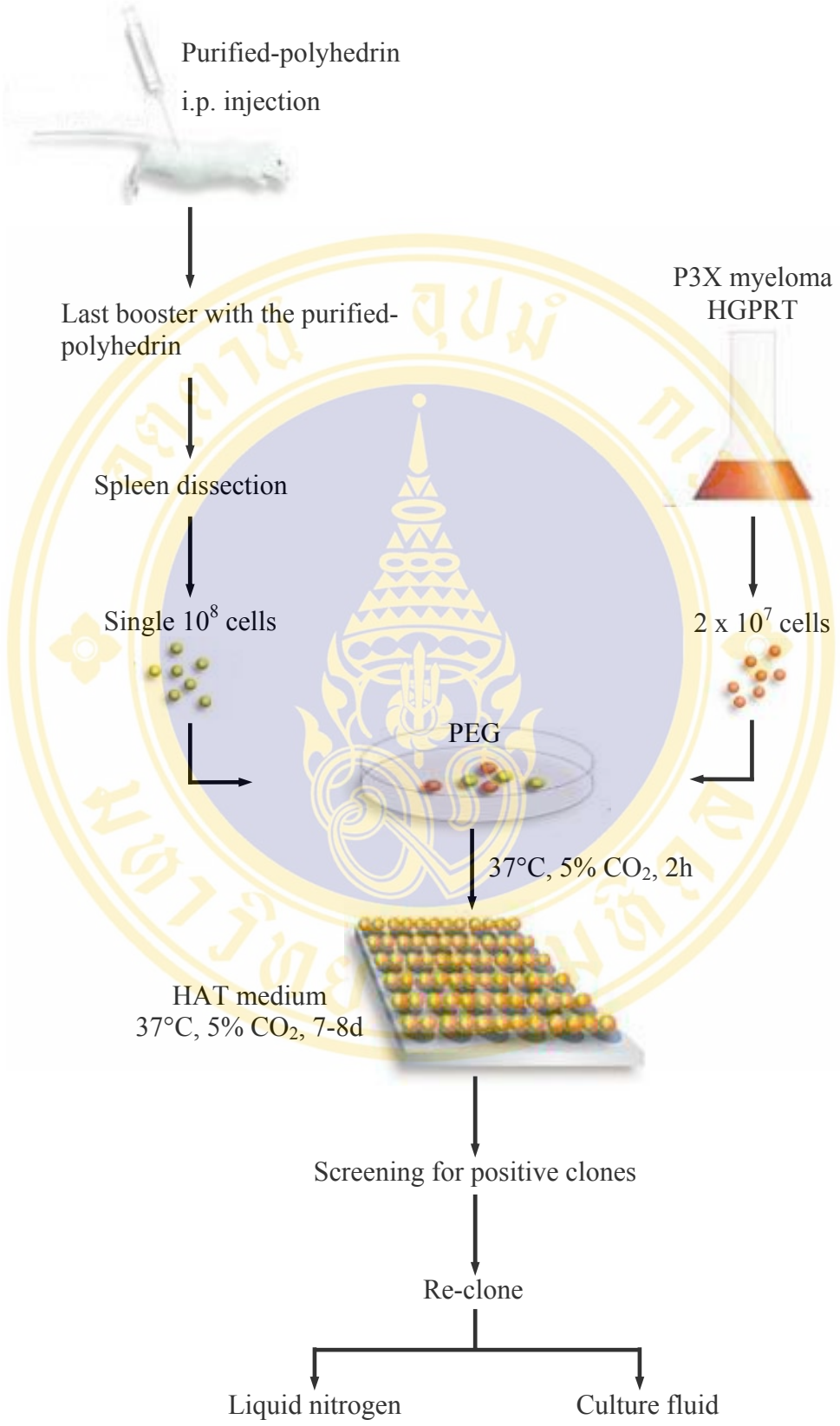


Figure 5. Diagram of monoclonal antibodies production

(From http://www.jpt.com/content/pep/immuno_tools/customized.htm)

Copyright by Mahidol University

8. Cloning of Hybrid Cells by the Limiting Dilution Method

After identification of desirable clones, all the cells from each positive well were transferred into a separate well of 96 well plates (NUNC®) 100 µl of HT media per well followed by serial dilutioning (i.e. by the limiting dilution method) (53). Approximately 24 hr prior to seeding the wells with the isolated hybridoma cells, HT media was prepared and placed into the humidified incubator. From each positive well, 10 µl of the culture suspension containing approximately 100 viable cells were drawn off and thoroughly blended with 7.5 ml of HT media. One hundred µl of the mixture was dispensed into 48 wells of the 96- well plate. More 2.5 ml of the HT media was added into the remaining mixture to lower the concentration of hybridoma cells. One hundreds of the lower the concentration was dispensed into the remaining 48 wells. The plates were incubated in a humidified incubator for 7 days. Cell growth in the bottom of the wells was monitored using an inverted microscope. Wells that showed a single confluent clone were screened for MAbs.

9. Second Screen of Hybridomas for anti-MBV Antibodies by Western blot and immunohistochemistry

9.1 Western blot

The proper size of a sheet of nitrocellulose membrane (Schleicher and Schull®, Dassel, Germany) and 6 sheets of Whatman® 3 MM were cut and one edge of the nitrocellulose membrane was marked. The cut membrane and papers were then soaked in transfer buffer (20% (v/v) methanol in 1x protein running buffer) for 5 min. The Semi Phor™ Semi-Dry Transfer Unit apparatus (Amersham Phamacia Biotec) was set by rinsing the positive and negative electrode with distilled water and placing the Mylar™ mask at the base of the unit. A sandwich of the components was assembled as follows. Three sheets of saturated blotting paper were placed one by one and the pre-wet membrane was subsequently placed onto the stack of blotting papers. The gel containing protein components, sparated by SDS-PAGE as described above that had been soaked in transfer buffer was then placed on the membrane and three sheets of blotting papers were placed on top of the gel. All air bubbles between layers were excluded by rolling a test tube from the center toward the edges of each layer.

Copyright by Mahidol University

The blot was set for a constant current depending on the size of the gel. The current was calculated by measuring the gel surface area (cm^2) and multiplying by 0.8 mA/cm^2. After the transfer was performed for 1 hour, the gel was stained with 0.3% (w/v) Coomassie brilliant blue to check the efficiency of the transfer. The membrane was lifted with a blunt forceps and air-dried. The electrophoretic blots were left 30 min at RT in 5% skim milk in PBS, pH 7.2 to saturate additional protein binding sites. Then, the nitrocellulose membranes were added with culture fluid of hybridoma (MAbs) for 5 h at RT with continuous rocking. After washing with PBS for 4 times, 5 min each, the nitrocellulose membranes were treated with 1:1500 diluted GAM-HRP (Bio-RAD®) in PBS, pH 7.2 for 3 h at RT with continuous rocking. Once completing the washing step as previously described, the nitrocellulose membranes were developed by adding freshly prepared substrate solution (0.03% DAB and 0.006% H_2O_2, and 0.05% $CoCl_2$), for 5 min before washing with distilled water several times to stop the enzymatic reaction (Figure 6).

Copyright by Mahidol University

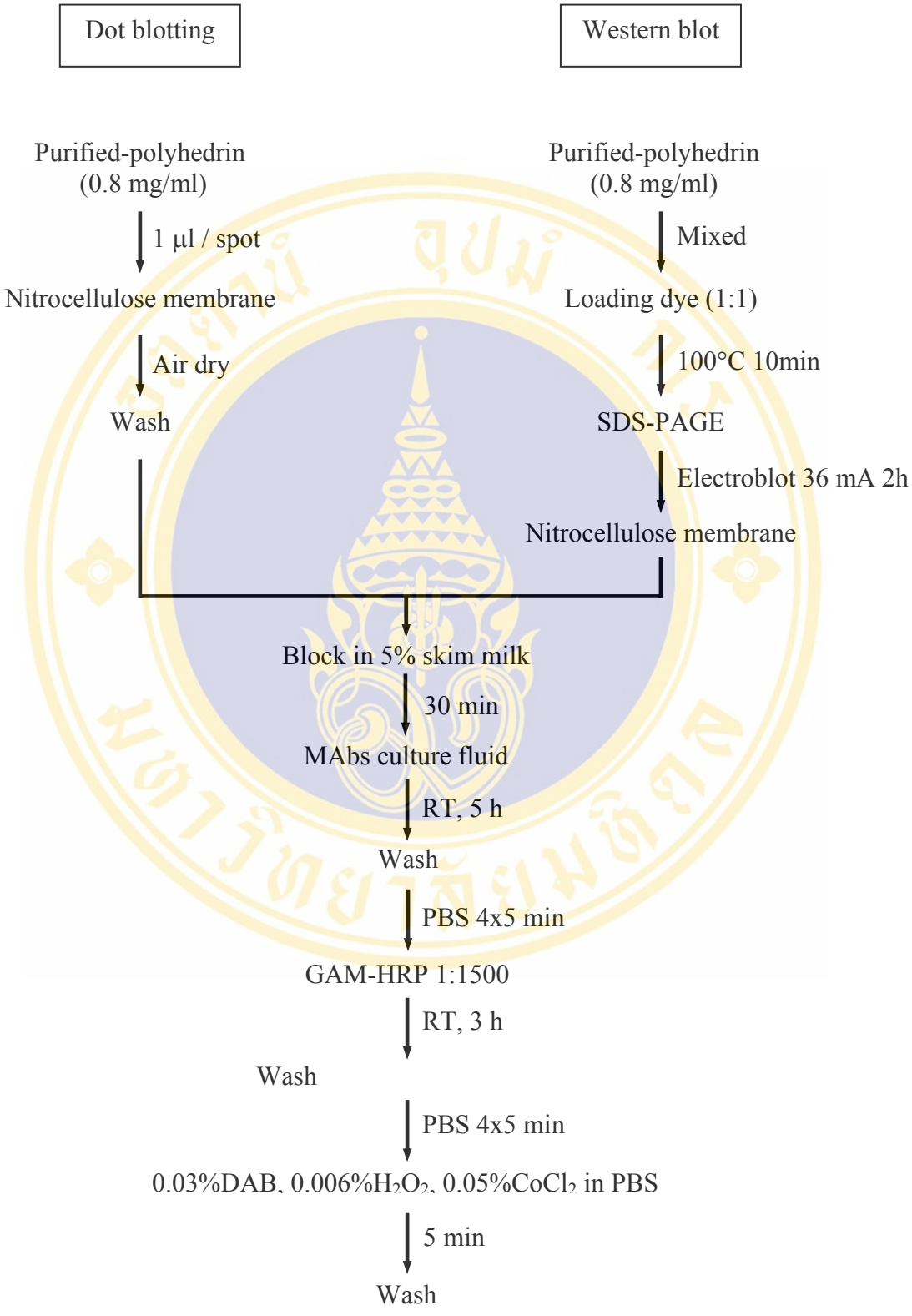


Figure 6. Dot blotting and western blot analysis of the purified-polyhedrin of MBV (54).

Copyright by Mahidol University

9.2 Immunohistochemistry

9.2.1 Preparation of tissue Specimens

Cephalothoraces from MBV-infected PL were fixed in Davidson's fixative solution (Appendix C) for 24 h at RT before processing for paraffin sectioning (55). Then, continuously rinsed in running tap water for 2 h, and dehydrated with graded series of ethyl alcohol: 70%, 90%, and 95% concentration for 3 h each; 95% ethyl alcohol in N-butyl alcohol for 1 h: and N-butyl alcohol in xylene for 1 h prior to embedding in paraffin. The tissue was completely infiltrated with xylene (a clearing agent) to make them translucent. In paraffin embedding, the tissue was infiltrated with paraffin and placed in a mold containing molten paraffin. The paraffin block was then allowed to cool at 20°C before sectioning. The tissue was then cut into 7 μm thick sections with a rotary microtome. As sections were cut, they were floated on a warm water bath to smooth out any wrinkles. They were then picked up on an albumin-coated glass microscopic slide pre-warmed and ready to use on a hot plate. To help sections adhere to slides, the slides were heated and completely dried in a warm oven (50°C) overnight. The adhered sections were then ready for further processing (Figure 7).

Copyright by Mahidol University

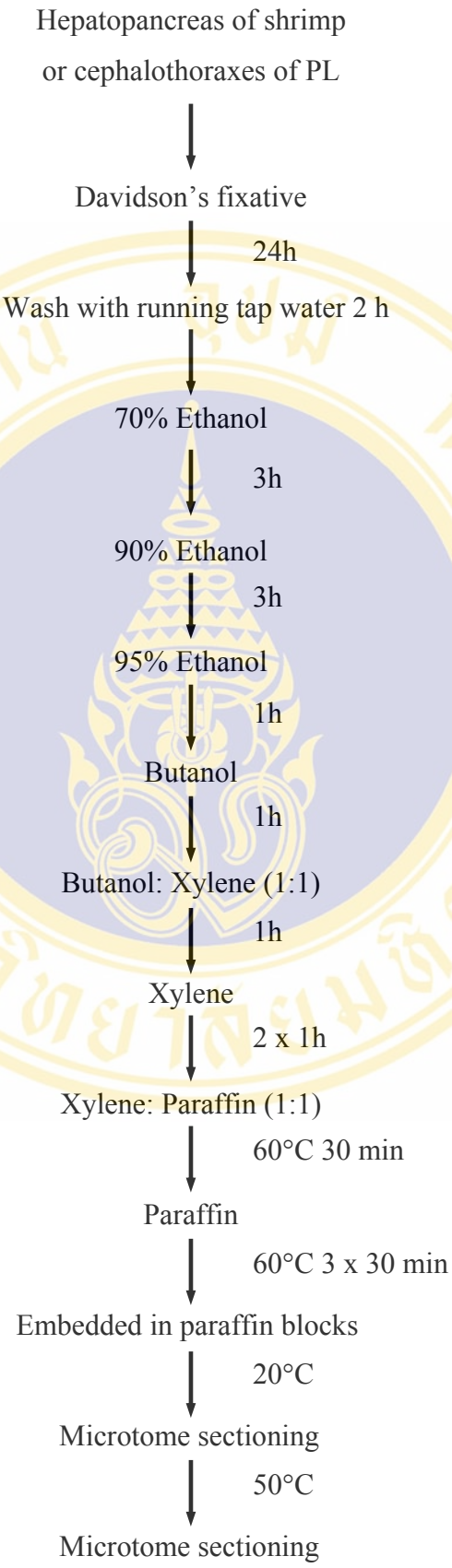


Figure 7. A flow chart of tissue preparation for immunohistochemistry (56).

Copyright by Mahidol University

9.2.2 The Immunoperoxidase Assays

The sections were deparaffinized in xylene three times, 5 min each, and then rehydrated with a graded ethanol series: N-butyl alcohol, 95%, 90%, 80%, and 70% concentration of ethyl alcohol, 5 min each, respectively. After washed with distilled water for 5 min, refixed with 10% formalin for 10 min, and washed with PBS, pH 7.2, 3 times, 15 min each, the sections were blocked with P_1^+ (10% calf serum in PBS, pH 7.2) for 30 min, incubated with MAbs specific to MBV for 5 h at 37°C. The MAbs were washed out thoroughly by dipping the slide in PBS, pH 7.2, 3 times for 10 each. Next, 2 drops of 1:1000 dilutions GAM-HRP were added into the sections and incubated at 37°C for 3 h before washing thoroughly by dipping the slide in PBS, pH 7.2, 3 times for 10 min each. Next, the slides were incubated in substrate-chromogen mixture working solution (0.03% DAB and 0.006% H_2O_2) for 5 min. The slides were rinsed well with distilled water. The slides were stained with hematoxylin, washed 10 min or until blue in solution (0.1% NaOH), and counterstained with eosin Y followed by dehydration. The slides were mounted with mounting solution and covered with cover slips (Figure 8). Positive immunoreactions were visualized as brown coloration at the site of MBV infection against purple blue of hepatopancreatic nuclei.

10. Cryopreservation

The mid-log phase proliferating hybridomas were harvested and centrifuged at 200 x g for 5 min at RT. The supernatants were kept at –20°c for any future assay; the hybridoma pellets were thoroughly mixed with 1 ml of freezing media (Appendix B), which was kept cold at 4°C. About 0.5 ml of the suspension was transferred into 2 cryopreservation tubes (NUNC®). After the caps were closed rapidly and tightly, the tubes were left in a deep freeze (-80°C) overnight and then transferred into a container of liquid N_2 (-196°C), where they could be kept for a long time. To thaw the hybridomas, the vial was agitated briefly at 37°C in a water bath with care being taken avoid contamination with water. Immediately when thawed, the cells were mixed with 10 ml RPMI 1640 medium and centrifuged at 200 x g for 5 min. After the washed media was discarded, the pellet was re-suspended in a new sterile Petri dish with 15 ml of 20% CBS RPMI 1640 media and placed in a humidified incubator.

Copyright by Mahidol University

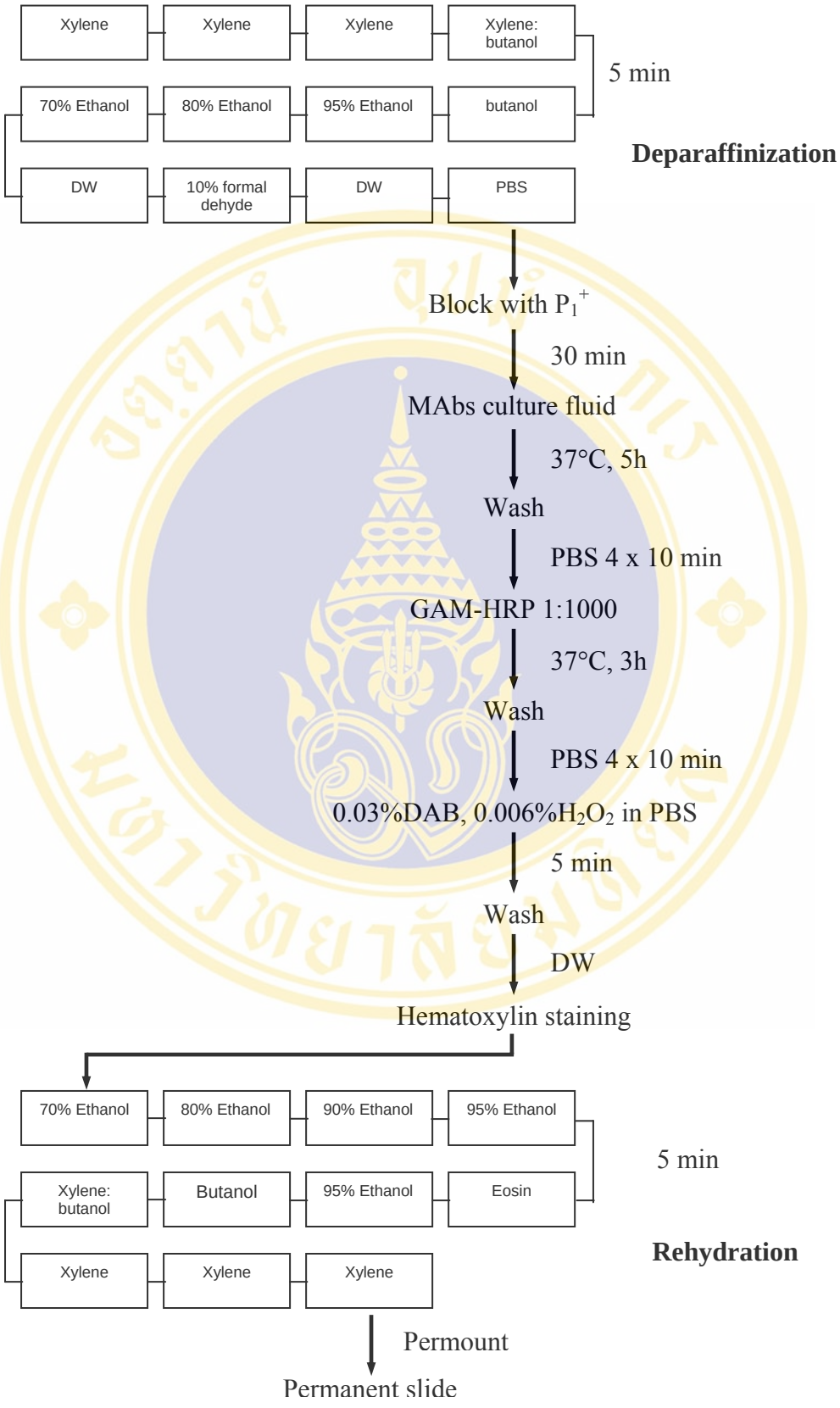


Figure 8. A flow chart of immunohistochemistry preparation (56).

Copyright by Mahidol University

11. Determination of Antibody Isotypes and Subisotypes Using Sandwich ELISA

The heavy chain classes and light chain types of MAbs were determined using Mouse Monoclonal-ID Kit (Zymed Lab, San Francisco,CA). The assay is based on an indirected sandwich ELISA principle. Rabbit anti-mouse class and subclass specific antibodies were employed in isotyping the mouse immunoglobulins secreted into the culture supernatant. Peroxidase-labelled goat anti-mouse IgG serves as the signal generating reagent. The procedure was conducted following the manufacturer's recommendations with slight modification (Figure 9). Briefly, 50 µl of goat anti-mouse IgG (GAM) were added to the microtiter wells. After the plates were incubated in a humidified box at 4˚C overnight, the solution were decanted and the plates were slapped onto paper towel until dry, and immediately all wells were washed with 150 µl of 0.5% skim milk and left at RT for 5 min. This step was repeated for 4 times. Then, 100 µl of diluted MAbs were added into the wells of each row and left with rocking at RT for 5 h. Washing steps were repeated 4 times, as previously described. Fifty µl of subclass specific rabbit anti- mouse immunoglobulins (IgG1, IgG2a, IgG2b, IgG3, IgA, IgM, κ, and λ) were added into each well of the row, respectively. The plates were left at 4°C in the humidified box overnight. Next, the washing step was repeated 4 times, 100 µl of diluted HRP-Gar IgG was added into each well, and the plates were left at RT for 3 h with rocking. To develop the reaction, 80 µl of OPD (Sigma®) working substrate solution (Appendix E) was added each well and left for 5 min before the reaction was stopped by adding 80 µl of 1N H_2SO_4 per well. The OD was measured at 490 nm using an ELISA reader (Titertek Multiskan® PLUS, Finland).

Copyright by Mahidol University

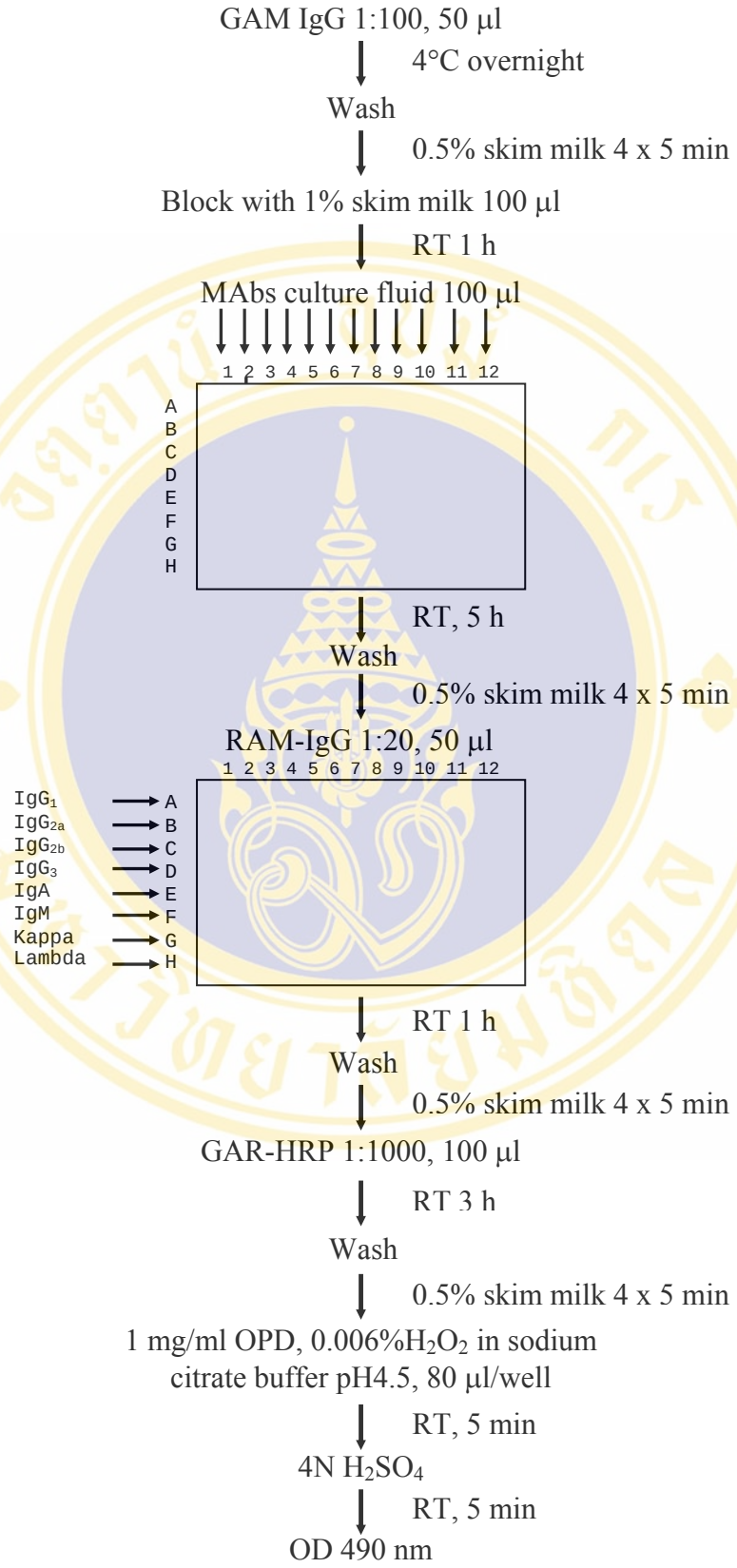


Figure 9. A flow chart of determination of antibody isotypes and subisotypes (56).

Copyright by Mahidol University

12. Determination of Antibody Epitope Using Indirect ELISA

The epitope of MAbs were determined using indirected ELISA. Fifty µl of putative polyhedrin derived from Urografin gradient (0.8 mg/ml) was diluted 1:300 in PBS, pH 7.2 and added to the microtiter wells. After the plates were incubated in a humidified box at 4˚C overnight, the solution was decanted and the plates were slapped onto paper tower until dry. Immediately all wells were washed 2 times at RT for 10 min with 150 µl of 0.5% skim milk. Then blocking wells with 5% skim milk at RT for 30 min. Then, 100 µl of diluted MAbs were added into the wells of each row and column and left with rocking at 4˚C overnight (Table 3). After that the solutions were decanted and the plates were slapped onto paper towel until dry. Immediately all wells were washed with 150 µl of 0.5% skim milk and left at RT for 10 min. This step was repeated 4 times. Next, 100 µl of diluted HRP-GAM was added to each well and the plates were left at RT with rocking for 7 h. Washing steps were repeated 4 times, as previously described but in the last wash PBS replaced 0.5% skim milk. To develop the reaction, 80 µl of OPD (Sigma®) working substrate solution (Appendix E) was added each well and left for 5 min before the reaction was stopped by adding 80 µl of 1N H_2SO_4 per well. The OD was measured at 490 nm by an ELISA reader (Titertek Multiskan® PLUS, Finland).

Copyright by Mahidol University

Table 3. MAb mixtures for determination of MAb epitope

MAbs	1 MBV 5-3H	2 MBV 5-6F	3 MBV 8-5G	4 MBV 10-5F	5 MBV 10-11H	6 MBV 13-10E	7 MBV 14-1G	8 MBV 16-3G	9 MBV 21-4B	10 MBV 21-8A	11 MBV 21-11B	12 MBV 27-8F
1 MBV 5-3H	1+1	1+2	1+3	1+4	1+5	1+6	1+7	1+8	1+9	1+10	1+11	1+12
2 MBV 5-6F		2+2	2+3	2+4	2+5	2+6	2+7	2+8	2+9	2+10	2+11	2+12
3 MBV 8-5G			3+3	3+4	3+5	3+6	3+7	3+8	3+9	3+10	3+11	3+12
4 MBV 10-5F				4+4	4+5	4+6	4+7	4+8	4+9	4+10	4+11	4+12
5 MBV 10-11H					5+5	5+6	5+7	5+8	5+9	5+10	5+11	5+12
6 MBV 13-10E						6+6	6+7	6+8	6+9	6+10	6+11	6+12
7 MBV 14-1G							7+7	7+8	7+9	7+10	7+11	7+12
8 MBV 16-3G								8+8	8+9	8+10	8+11	8+12
9 MBV 21-4B									9+9	9+10	9+11	9+12
10 MBV 21-8A										10+10	10+11	10+12
11 MBV 21-11B											11+11	11+12
12 MBV 27-8F												12+12

Copyright by Mahidol University

CHAPTER IV
RESULTS

1. Screening for MBV Infection

MBV infection was shown using the squash mount technique stained with 0.05% malachite green. A batch of *P. monodon* post larvae (PL) was selected that contained more than 95% infection in 100 randomly selected PL (Figure 10). In carefully prepared preparations, the infected hepatopancreatic cells showed spherical intranuclear occlusion bodies in hypertrophied nuclei. The nuclear membrane was persistent and held multiple occlusion bodies. MBV occlusion bodies also stained more intensely with 0.05% malachite green than similar sized normal host cell nuclei and lipid droplets (Figure 10). There was much variation in the shape and size of the occlusion bodies ranging from roughly spherical to various polygons.

For the nested PCR, primers used in this study for MBV amplified a 533-bp fragment in the non-nested reaction and a 361-bp fragment in the nested reaction. The PCR was carried out on all the 3 farms positive for MBV infected PL and all samples were positive for MBV in both amplification steps (Figure 11). PCR for MBV that were positive by II step amplification were also positive for MBV bydirect microscopic examination. Negative lanes showed the 175-bp internal control fragment confirming the validity of the PCR reaction.

2. Purification of MBV Polyhedrin

MBV-infected hepatopancreases were collected for polyhedrin purification. After Urografin gradient centrifugation at 120,000 x g for 16 h at 4°C, a white band was observed at about 50% Urografin gradient (Figure 12). A band was collected by syringe and transferred to a microcentrifuge tube. Urografin was removed from purified polyhedrin by resuspension in TN buffer and repelleting by centrifugation 2

Copyright by Mahidol University

times at 120,000 x g for 2 h at 4°C. The present of polyhedrin was determined by transmission electron microscopy (TEM) using negative staining. Ultrastructural observation indicated that the 50% Urografin fraction contained 20-23 nm diameter particles that gave “empty” and “full” appearance (Figure 13).

To confirm the purification of polyhedrin from the 50% Urografin fraction not contaminated with HPV, the DNA was extracted from this band and used as a template for PCR detection reactions. Electrophoresis of PCR products showed negative for the presence of HPV (Figure 14).

Copyright by Mahidol University

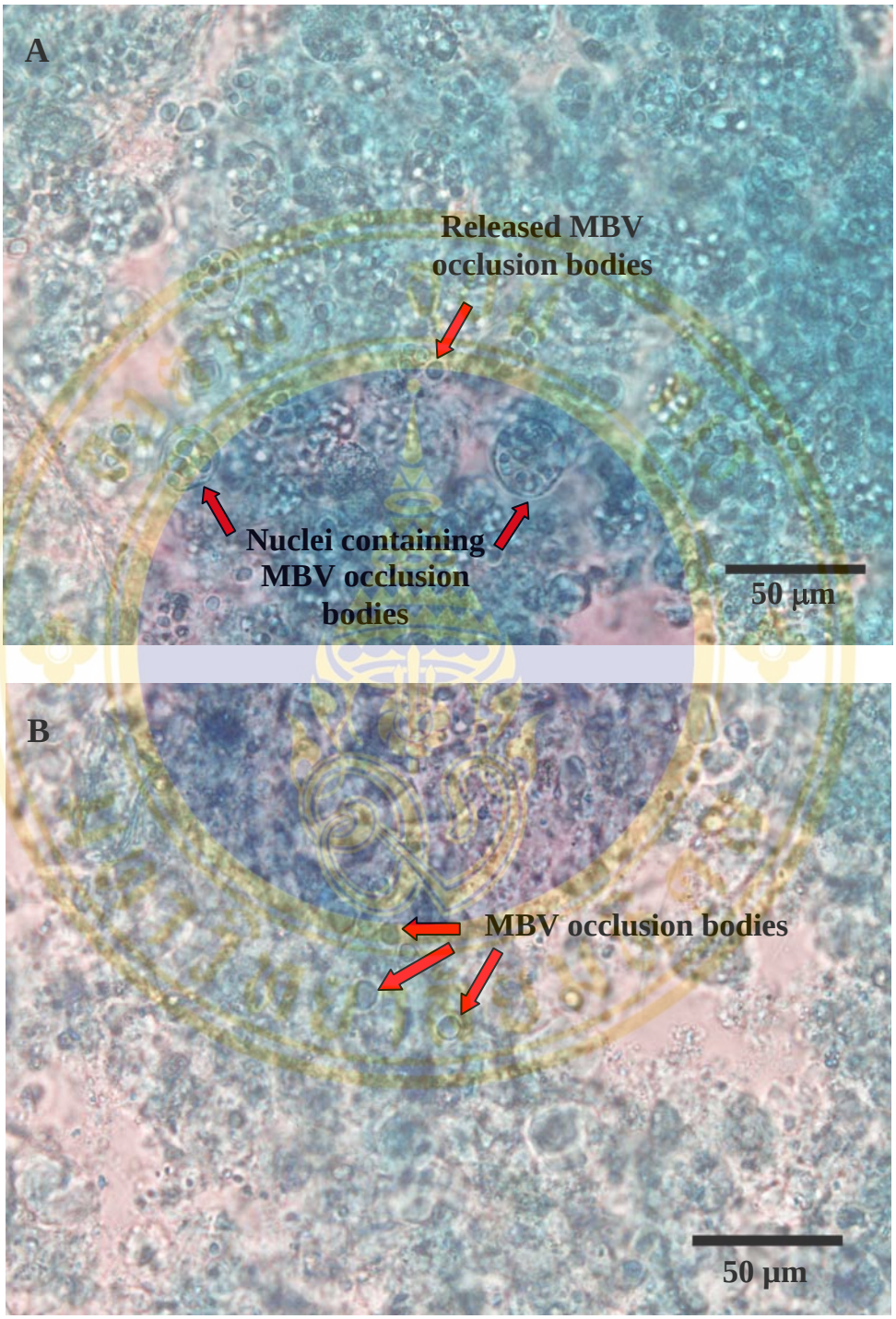


Figure 10. A. Light micrograph of a hepatopancreatic tissue squash mounts stained with 0.05% malachite green and showing ijithe presence of MBV occlusion bodies. B. Light micrograph of a homogenate showing free MBV occlusion bodies amongst fragments of muscle tissue.

Copyright by Mahidol University

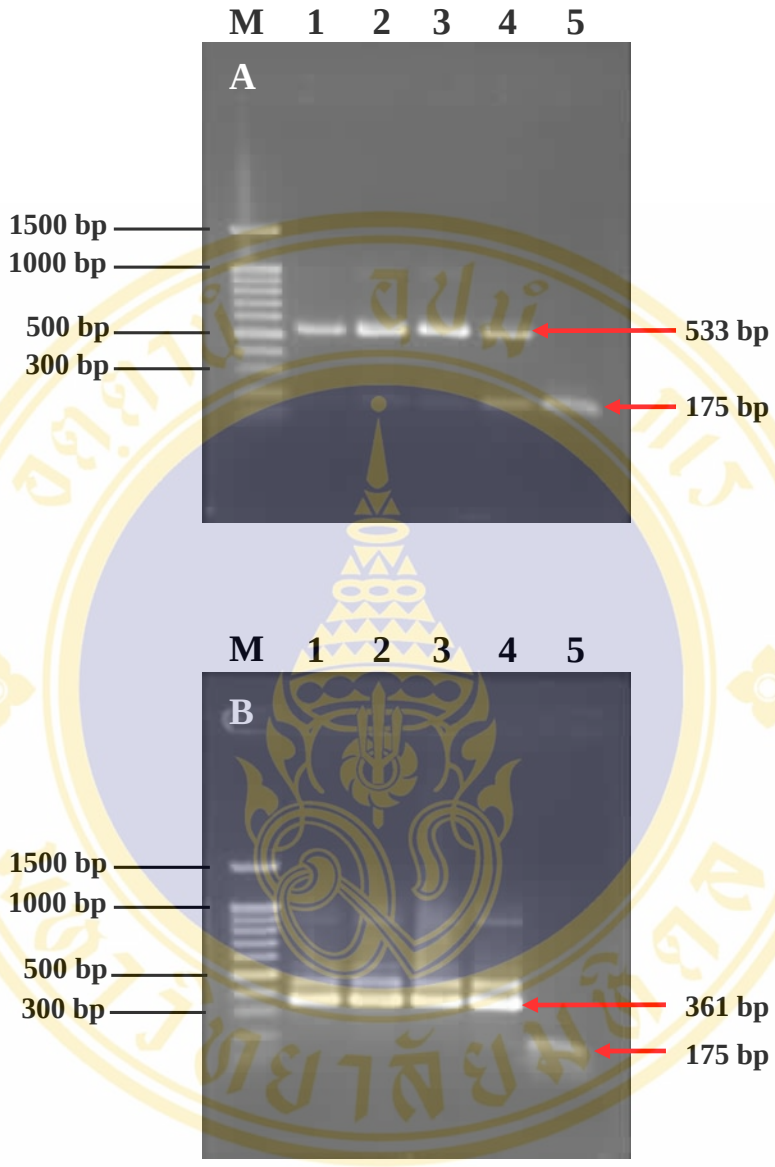


Figure 11. Agarose gel electrophoresis of MBV Nested-PCR using DNA template extracted from homogenate supernatant. (A) Primary PCR of DNA extracted supernatant as template DNA (lane 1; Roong-Aroon farm, lane 2; Manus farm and lane 3; Khajee farm) and (B) nested PCR of a 1.0 µl of primary PCR product (lane 1, 2, 3). A nested-PCR was carried out on a sample without any added DNA as a negative control with 175 bp internal control fragment (lane 5). Positive control (lane 4) and Molecular weight markers are 100 bp +1,500 bp DNA ladder (lane M). In the present of MBV DNA, an MBV-specific amplicon band would occur at 361 bp.

Copyright by Mahidol University

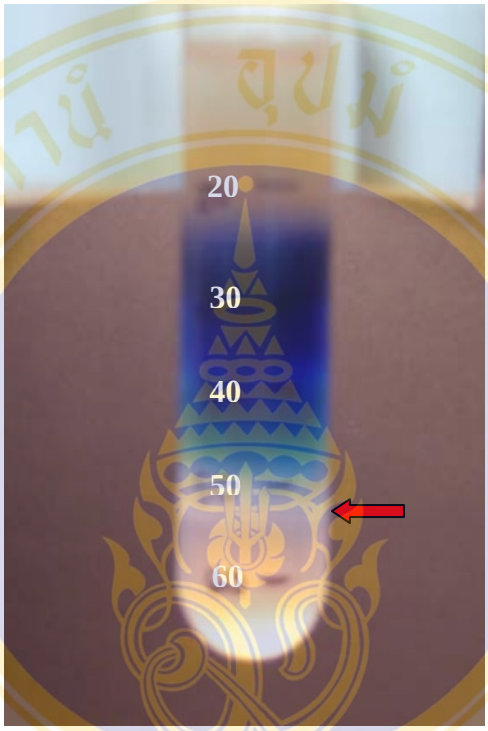


Figure 12. Purification of MBV polyhedrin using Urografin ultracentrifugation. The homogenate of hepatopancreatic tissue from PL of *P. monodon* was separated in an Urografin gradient (20-60%). A white band at about 50% Urografin (arrow) contained purified polyhedrin protein.

Copyright by Mahidol University

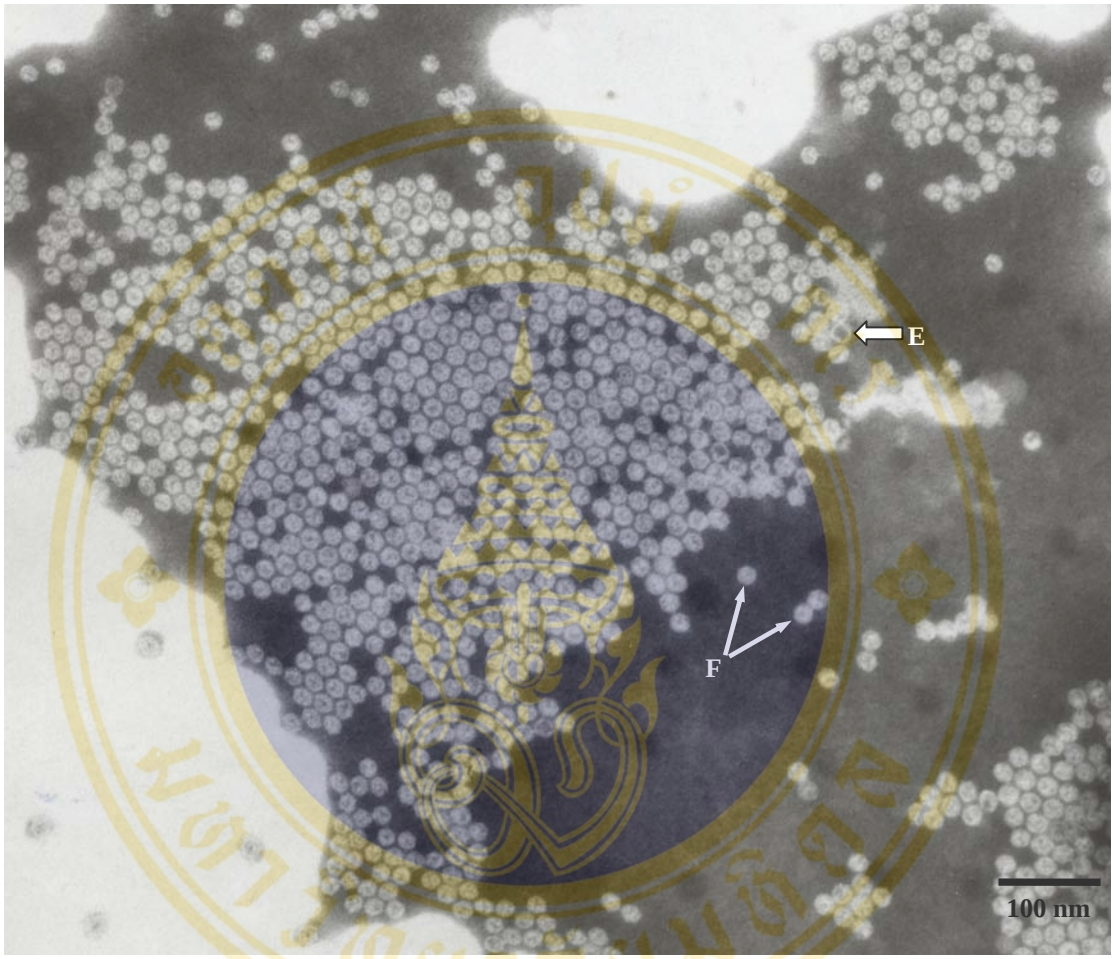


Figure 13. Transmission electron microscopy (TEM) of purified polyhedrin protein negatively stained with 2% PTA. They appear in two forms, full (F) appearing and empty (E) appearing subunits.

Copyright by Mahidol University

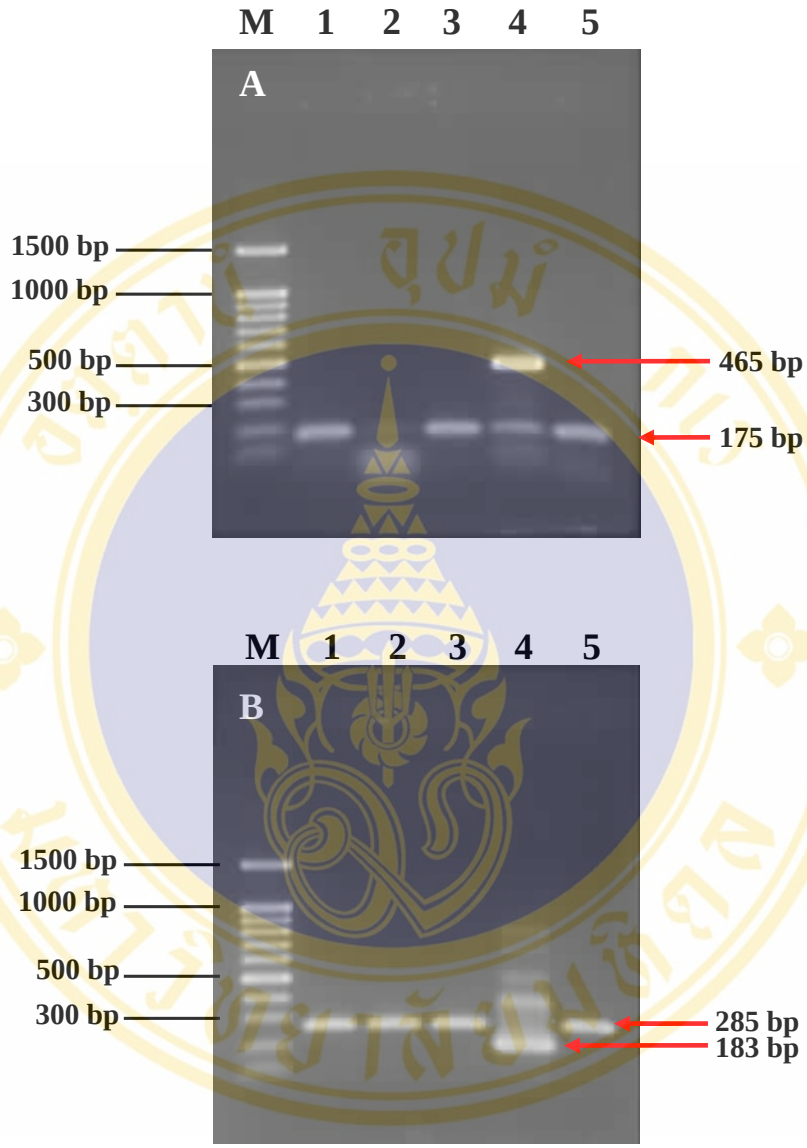


Figure 14. Agarose gel electrophoresis of HPV Nested-PCR using DNA template extracted from the 50% Urografin fraction. (A) Primary PCR of DNA extracted from the 50% Urografin fraction as template DNA (lane 1; Roong-Aroon farm, lane 2; Manus farm and lane 3; Khajee farm) and (B) nested PCR of a 1.0 µl of primary PCR product (lane 1, 2, and3). A nested-PCR was carried out on a sample without any added DNA as a negative control with 175 bp and 285 bp internal control fragment for first step and second step PCR respectively (lane 5). Positive control (lane 4) and Molecular weight markers are 100 bp +1,500 bp DNA ladder (lane M). In the present of HPV DNA, an HPV-specific amplicon band would occur at 183bp.

Copyright by Mahidol University

3. Protein Analysis

3.1 Determination of Protein Concentration

Purified-polyhedrin concentration was determined using Bradford's method. A standard curve was produced using 7 various BSA standards (Figure 15). The purified-polyhedrin from each farm was measured OD_{595} and the concentration was calculated using the equation y = 0.0809x. The concentration of purified-polyhedrin from Roong-Aroon farm, Manus farm and Khajee farm were 0.3, 0.85 and 0.82 mg/ml respectively (Table 4).

3.2 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The purified polyhedrin protein from the 50% Urografin fraction was characterized using 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) stained with Coomassie blue. It showed a major protein band (Figure 16). The molecular weight of the protein band in the gel was estimated by comparing with standard molecular weight protein markers (Figure 1). The size of a major protein band was approximately 58 kDa.

Copyright by Mahidol University

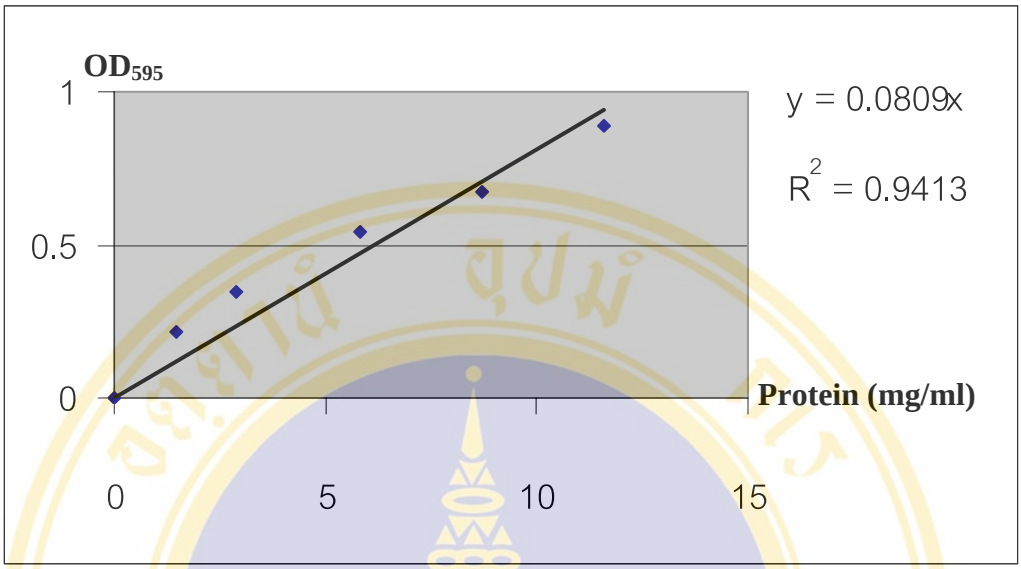


Figure 15. A typical standard curve used for protein concentration determination of the purified-polyhedrin.

Well	1	2	3	4	5	6	7	8	9
OD_{595}	0	0.215	0.345	0.540	0.671	0.888	0.051	0.334	0.321
Concentration of protein (mg/ml)	0	1.45	2.9	5.8	8.7	11.8	0.3	0.85	0.82
Total volume of sample (µl)	-	-	-	-	-	-	400	1000	1000

Table 4. The concentration and volume of purified-polyhedrin from Roong-Aroon farm, Manus farm and Khajee farm (Well 7, 8, and 9 respectively).

Copyright by Mahidol University

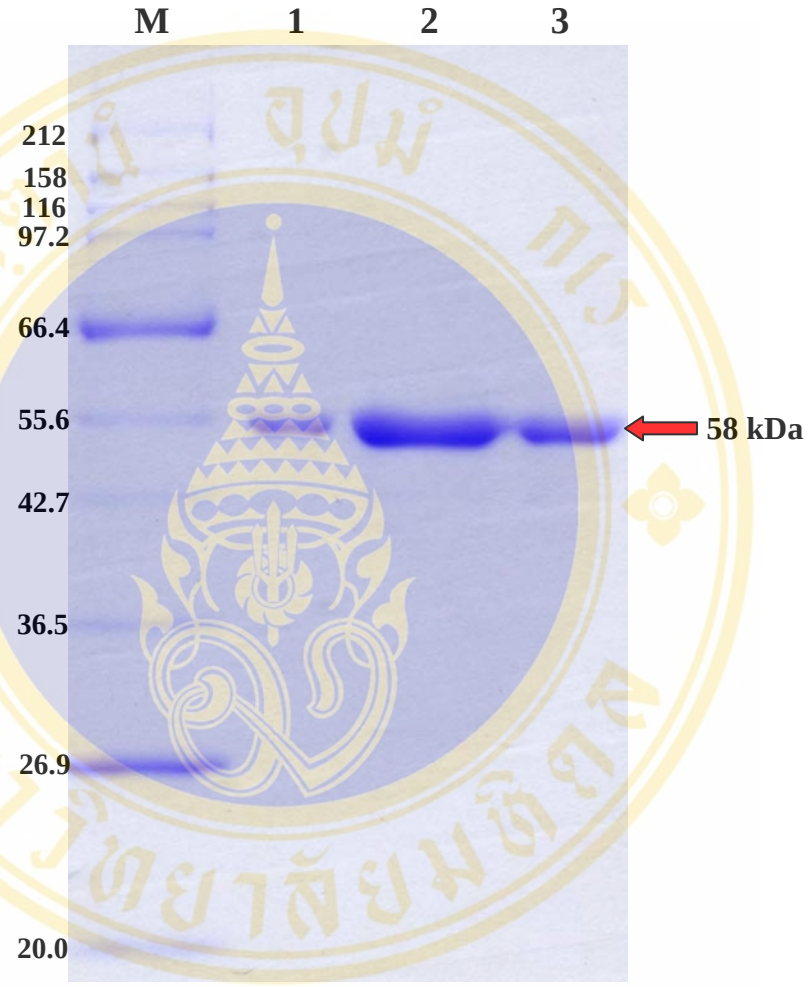


Figure 16. SDS-PAGE gel of purified-polyhedrin from a 50% Urografin fraction, Lane M contains the molecular weight marker. The number denotes kDa. Lanes 1, 2, and 3 contains purified-polyhedrin from Roong-Aroon farm, Manus farm and Khajee farm respectively. The size of the major band is indicated on the right side of the photo. The gel was stained with Coomassie blue.

Copyright by Mahidol University

4. Immunization of Mice

One week after completion of the second booster of purified-polyhedrin, sera from 4 female Swiss mice (mice number 1-4) produced antibodies against purified-polyhedrin of MBV. The specificity of the antiserum from 4 mice was determined by Dot ELIZA, western blot analysis and immunohistochemistry. Mouse number 4 was chosen as the donor for hybridoma production.

4.1 Dot ELISA

Each antiserum was tested at a dilution of 1:5000. Of these, the antiserum from mice number 2, 3, and 4 demonstrated strong immunoreactivity to the purified-polyhedrin at a concentration 1.6 ng (Figure 17).

4.2 Western blot analysis

Each antiserum was tested at a dilution of 1:5000. The antiserum from mice number 2, 3, and 4 exhibited strong immunoreactivity to the 58 kDa protein and another band (Figure 18).

4.3 Immunohistochemistry

By means of immunohistochemistry, each antiserum was tested at different dilutions (1:500, 1:1,000, and 1:2,500). The antiserum from mice number 2 and 4 at dilution 1:2,500 showed strong specific binding to eosinophilic MBV intra-nuclear occlusion bodies in hepatopancreatic tubule epithelial cells of *P. monodon* infected with MBV (Figure 19), and no cross reactivity was observed with uninfected tissues.

Copyright by Mahidol University

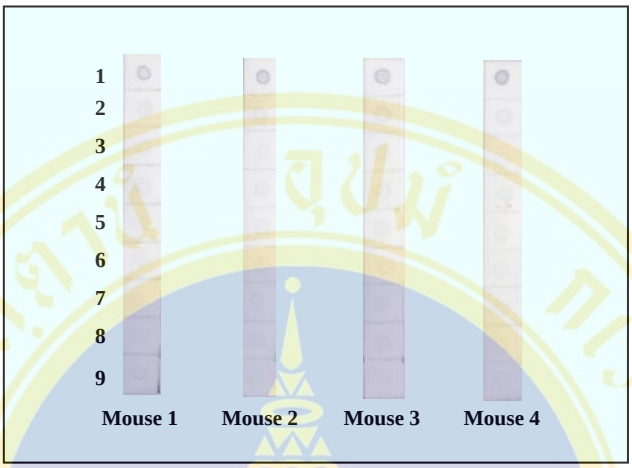


Figure 17. Dot blot analysis showing immunoreactivity of all mice antiserum at 1: 5,000 dilutions with a serial dilutions of purified-polyhedrin of MBV (0.8 mg/ml). Dilution of purified-polyhedrin (0.2 – 8.0 ng/spot) was spotted on a nitrocellulose membrane (1µl/spot) and treated with PAbs from 4 mice. Each dilution was spotted onto each block of nine rows as follows:

Row 1: dilution 1: 100
Row 2: dilution 1: 500
Row 3: dilution 1: 1,000
Row 4: dilution 1: 1,500
Row 5: dilution 1: 2,000
Row 6: dilution 1: 2,500
Row 7: dilution 1: 3,000
Row 8: dilution 1: 3,500
Row 9: dilution 1: 4,000

Copyright by Mahidol University

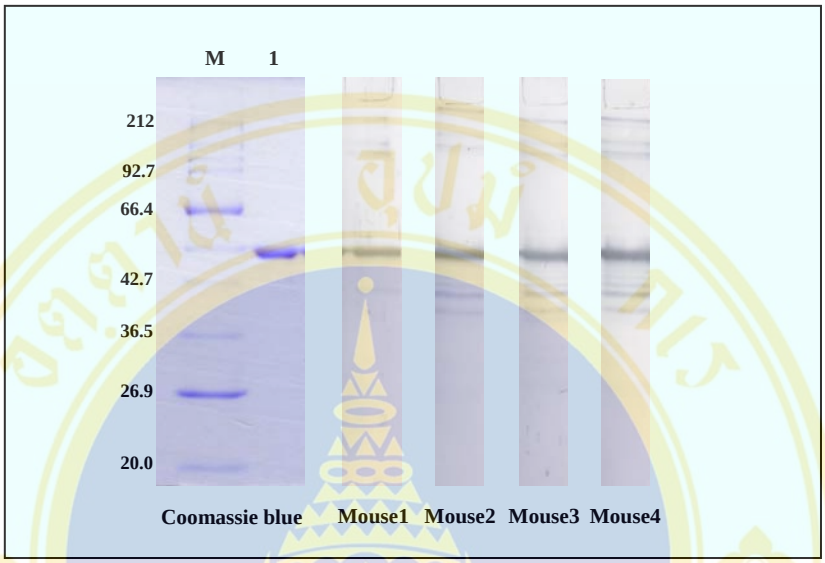


Figure 18. SDS-PAGE and Western blot of purified-polyhedrin of MBV. The purified-polyhedrin (0.4 µg/lane) was electrophoresed and stained with Coomassie blue or transferred to a nitrocellulose membrane and then treated with PAbs from 4 mice. M = Molecular weight standard protein, the number denoted kDa.

Copyright by Mahidol University

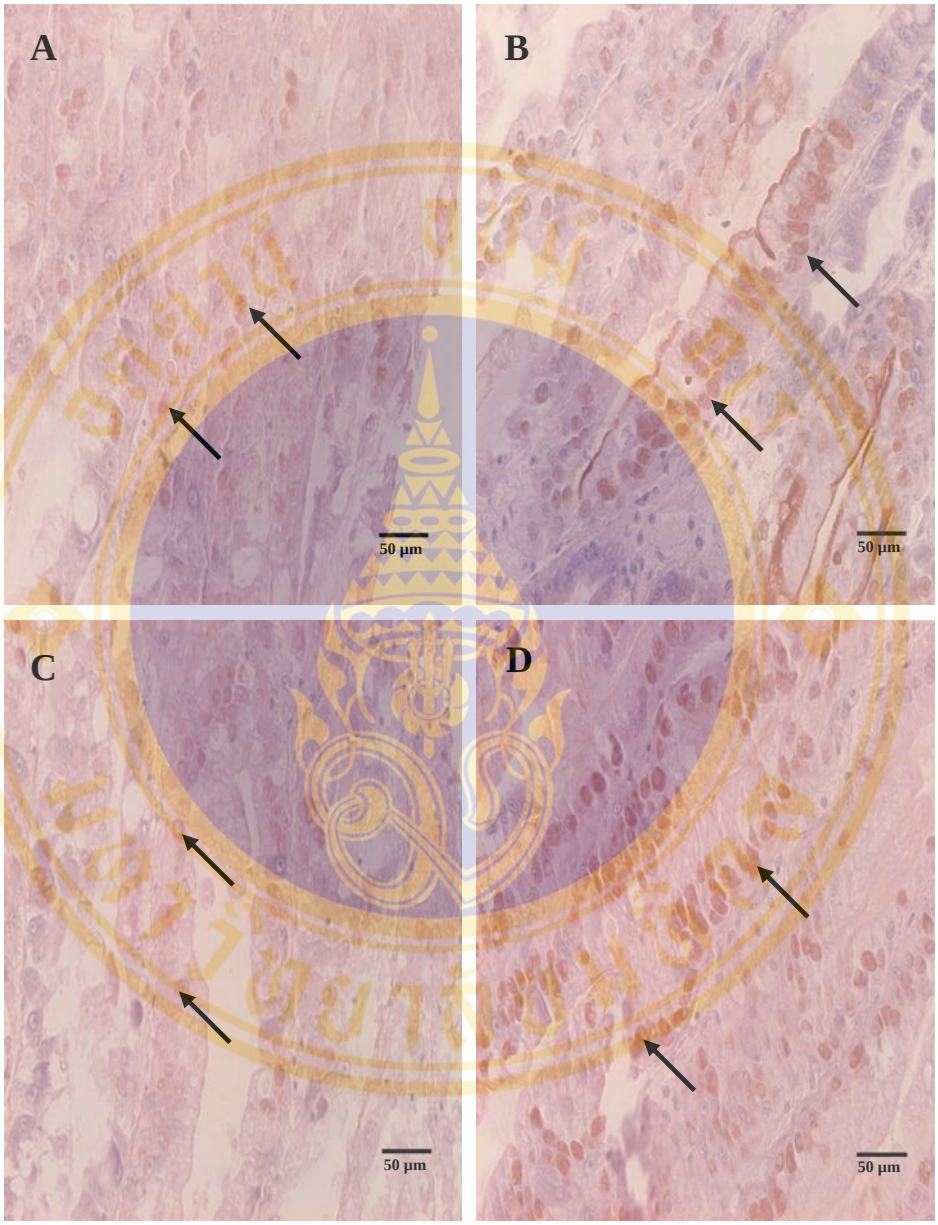


Figure 19. Immunohistochemistry of *P. monodon* hepatopancreas. Four sections from *P. monodon* infected with MBV were treated with PAbs from 4 mice (A; mouse no.1, B; mouse no.2, C; mouse no.3, D; mouse no.4) at dilution 1:2,500 and then counter stained with hematoxylin and eosin. Immunoreactivity was observed specific to occlusion bodies (arrows) in hepatopancreas cells.

Copyright by Mahidol University

5. Production of Monoclonal Antibodies

One fusion trial with 30 microculture plates yielded approximately 2000 culture wells, each giving 1 to 5 colonies of hybridoma cells. The first screening by dot-blot revealed 144 hybridoma clones that gave strong reactions with purified-polyhedrin of MBV (Figure 20). The positive culture fluids were further selected by western blot and immunohistochemistry. Supernatants from 39 clones showed specific and intense bands and specific binding to eosinophilic MBV intra-nuclear occlusion bodies in hepatopancreatic tubule epithelial cells of *P. monodon* infected with MBV (Figure 21, Figure 22). Only 17 clones showed the same pattern that strong immunoreactivity by both western blot and immunohistochemistry. Before expanding and establishing permanent cell lines, these clones were then re-cloned by the limiting dilution method in order that each clone was propagated from a single cell. Culture fluids of these hybridomas were tested against MBV occlusion bodies and other virus (HPV, YHY, WSSV) using immunohistochemistry. No cross-reactivity to other viruses and normal hepatopancreas was seen (Figure 23, Figure 24).

6. Characterization of the Monoclonal Antibodies

6.1 Specificity of MAbs

The 17 MAbs recognized a 58 kDa protein and showed strong immunoreactivity. By immunohistochemistry, these antibodies could be used to detect MBV occlusion bodies in hepatopancreatic tubule epithelial cells of *P. monodon* infected with MBV (Figure 22). The MAbs showed no cross-reactivity to other virus, normal hepatopancreas and other tissues of shrimp by means of immunohistochemistry. These antibodies detected purified-polyhedrin in dot blot assays with different sensitivities ranging from 0.2 – 0.4 µg/ml (Table 5).

6.2 Isotype and Subisotype Determination

Isotype and subisotype of MAbs obtained were IgG_1, IgG_{2a}, and IgG_{2b} (Table 5).

Copyright by Mahidol University

6.3 Sensitivity of MAbs for purified-polyhedrin detection by dot blot

These antibodies can be used to detect polyhedrin MBV by means of dot blot assay with different sensitivity ranging from 0.2 – 0.4 µg/ml (Table 5, Figure 20).

7. Epitope of Monoclonal antibodies determination

The culture fluids of the high sensitivity MAbs were determined whether they bind to overlapping epitope by indirect ELISA using mixture of 2 MAbs at excess amount of antibodies in each well coated with minimal amount of purified polyhedrin (~100 ng/well). The averages OD of the selected MAbs from 2 replicates were shown in the middle row of Table 6. The MAb MBV5-3H revealed highest sensitivity with purified polyhedrin when compare with OD value of other MAbs.

The combination of MAb MBV5-3H with the other 9 MAbs exhibit in increasing OD values at various degree except in the case of MBV14-1G and MBV16-3G the OD values decrease which indicated that the MAbs MBV5-3H and MBV14-1G, MBV16-3G[(1)] bound the overlapping position in the antigen. Combination of 2 MAbs among MAbs MBV5-6F, MBV8-5G, MBV10-5F, MBV10-11H, MBV13-10E, MBV21-8A, MBV27-8F[(2)] and among MBV16-3G, MBV21-4B, MBV21-11B[(3)] did not show improvement in OD values indicating that these MAbs bound to close nearby epitopes.

Copyright by Mahidol University

Table 5. Specificities of monoclonal antibodies tested by dot blot, western blot and immunohistochemistry. The underlined clone is the representative MAb used in other tests.

MAbs	Dot blot (ng/µl)	Western blot (kDa)	Immunohistochemistry	Subtype
MBV9-1D, MBV9-2F, MBV21-4B, MBV25-4G	0.4	58	+++	IgG_1
MBV6-9G, MBV14-1G, MBV16-3G, MBV21-11B, MBV24-5E	0.2			
MBV10-5F	0.4	58	+++	IgG_{2a}
MBV10-11H, MBV21-8A, MBV27-8F	0.4	58	+++	IgG_{2b}
MBV5-3H, MBV5-6F, MBV8-5G, MBV13-10E	0.2			

Copyright by Mahidol University

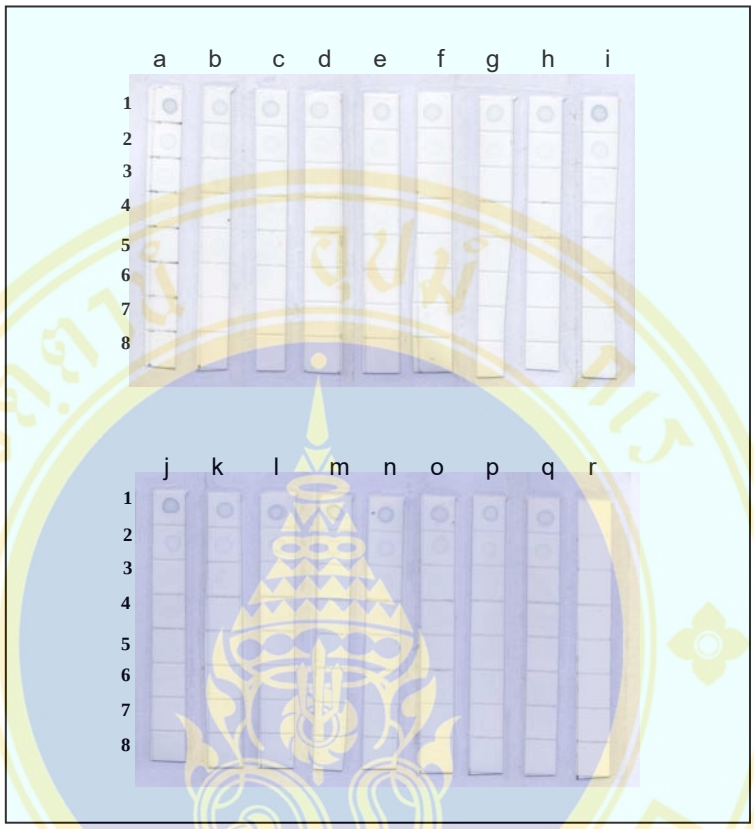


Figure 20. Sensitivity of MAbs to polyhedrin of MBV as detected by dot blot with different dilution of purified-polyhedrin of MBV (0.8 mg/ml). Dilution of purified-polyhedrin was spot on a nitrocellulose membrane (1µl/spot) and treated with 17 MAbs (a; 5-3H, b; 5-6F, c; 6-9G, d; 8-5G, e; 9-1D, f; 9-2F, g; 10-5F, h;10-11H, I; 13-10E, j; 14-1G, k; 16-3G, l; 21-4B, m; 21-8A, n; 21-11B, o; 24-5E, p; 25-4G, q; 27-8F, r; negative control). Each dilution was spotted onto each block of nine rows as follows:

Row 1: dilution 1: 100 (8 µg/ml)
Row 2: dilution 1: 500 (4 µg/ml)
Row 3: dilution 1: 1,000 (800 ng/ml)
Row 4: dilution 1: 2,000 (400 ng/ml)
Row 5: dilution 1: 4,000 (200 ng/ml)
Row 6: dilution 1: 8,000 (100 ng/ml)
Row 7: dilution 1: 16,000 (50 ng/ml)
Row 8: dilution 1: 32,000 (25 ng/ml)

Copyright by Mahidol University

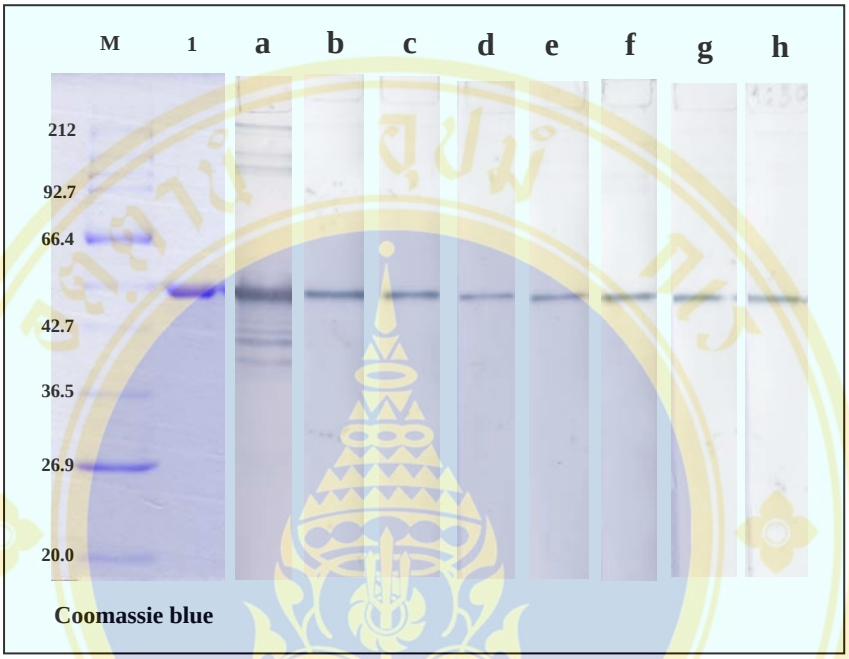


Figure 21. SDS-PAGE and Western blot of purified polyhedrin of MBV. The purified polyhedrin (9.33 ng/lane) was electrophoresed and stained with Coomassie blue or transferred to a nitrocellulose membrane and then treated with PAb (a) and representative MAbs (b; 5-3H, c; 5-6F, d; 9-2F, e; 10-5F, f; 14-1G, g; 16-3G, h; 27-8F). M = Molecular weight standard protein, the number denoted kDa.

Copyright by Mahidol University

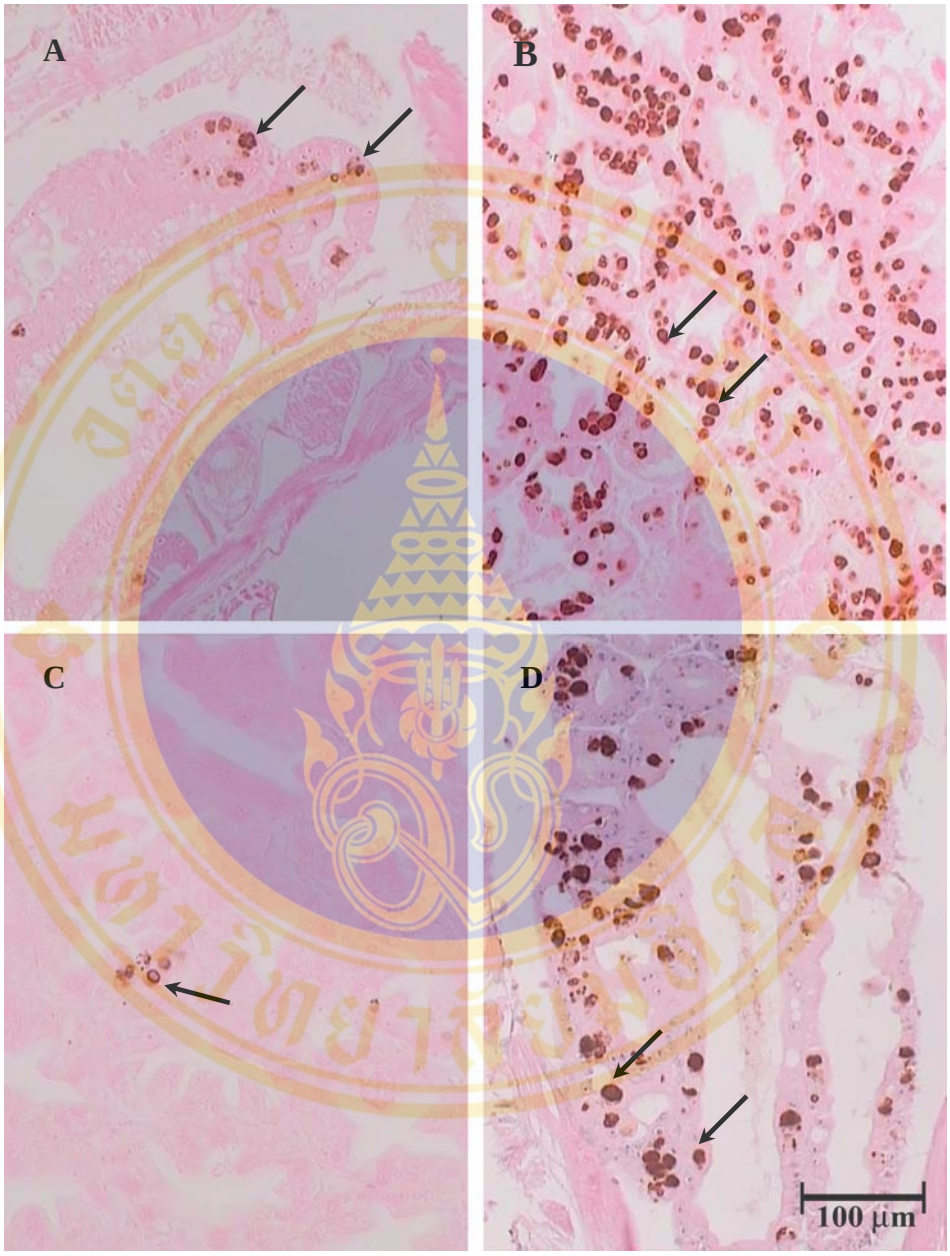


Figure 22. Immunohistochemistry of *P. monodon* hepatopancreas in stages of post larvae (A, D), juvenile (B), and adult (C). Sections from *P. monodon* infected with MBV were treated with MAb MBV5-3H and counter stained with only eosin Y (A, B, and C) or hematoxylin and eosin Y (D). Immunoreactivity showed strong specific to MBV (arrows) in all stage of infected *P. monodon*.

Copyright by Mahidol University

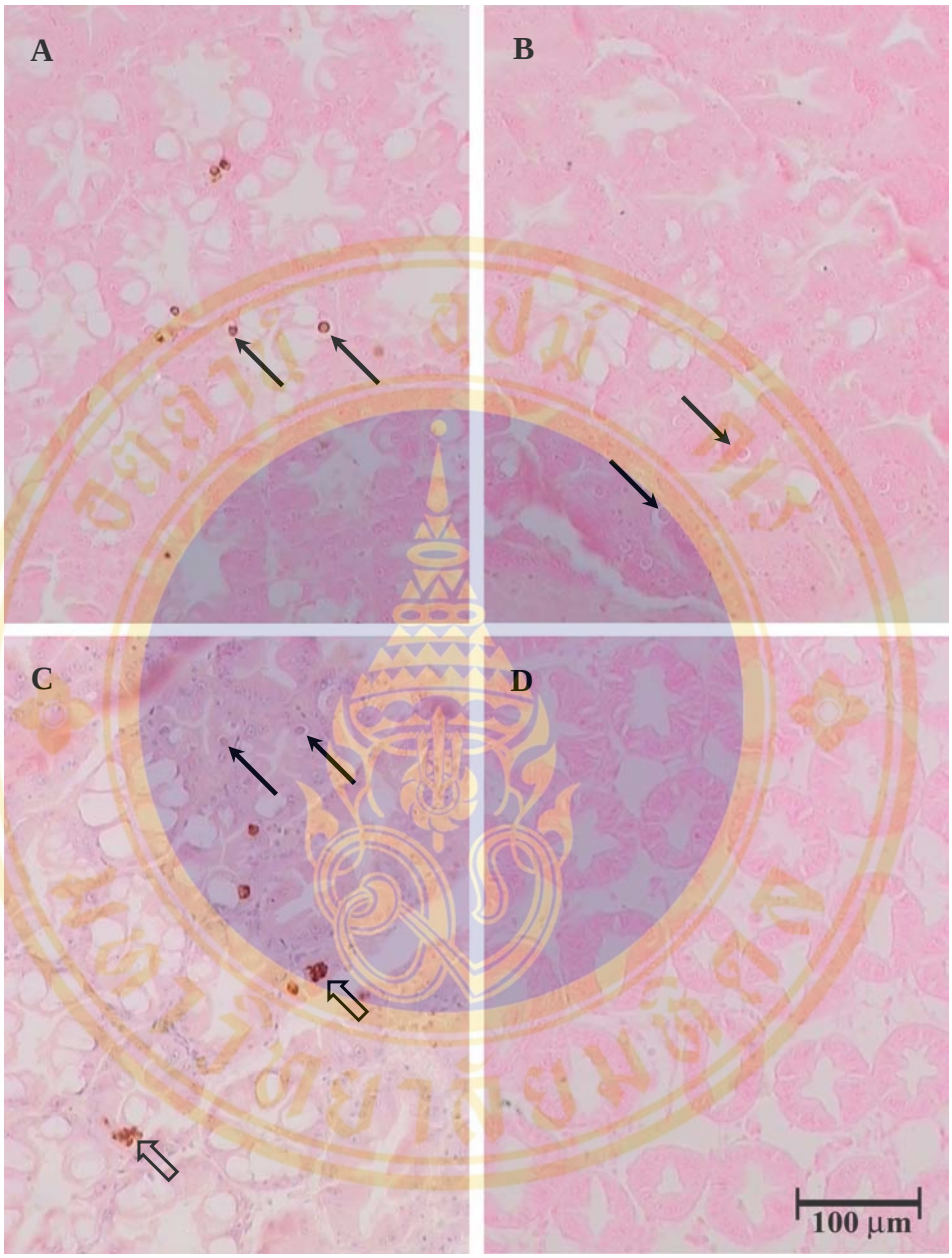


Figure 23. Immunohistochemistry of *P. monodon* hepatopancreas infected with HPV (A, B) infected with MBV and HPV (C) and normal hepatopancreas (D). Sections from *P. monodon* were treated with MAb HPV16-9C as positive control (A) treated with MAb MBV5-3H (B, C, and D) and counter stained with only eosin Y (A, B, and D) or hematoxylin and eosin Y (C). The MAb MBV5-3H showed specific immunoreactivity only to occlusion bodies of MBV (hollow arrows) and no cross-reactivity to HPV inclusion bodies (solid arrows) or normal tubule epithelial cell nuclei (B, C, and D).

Copyright by Mahidol University

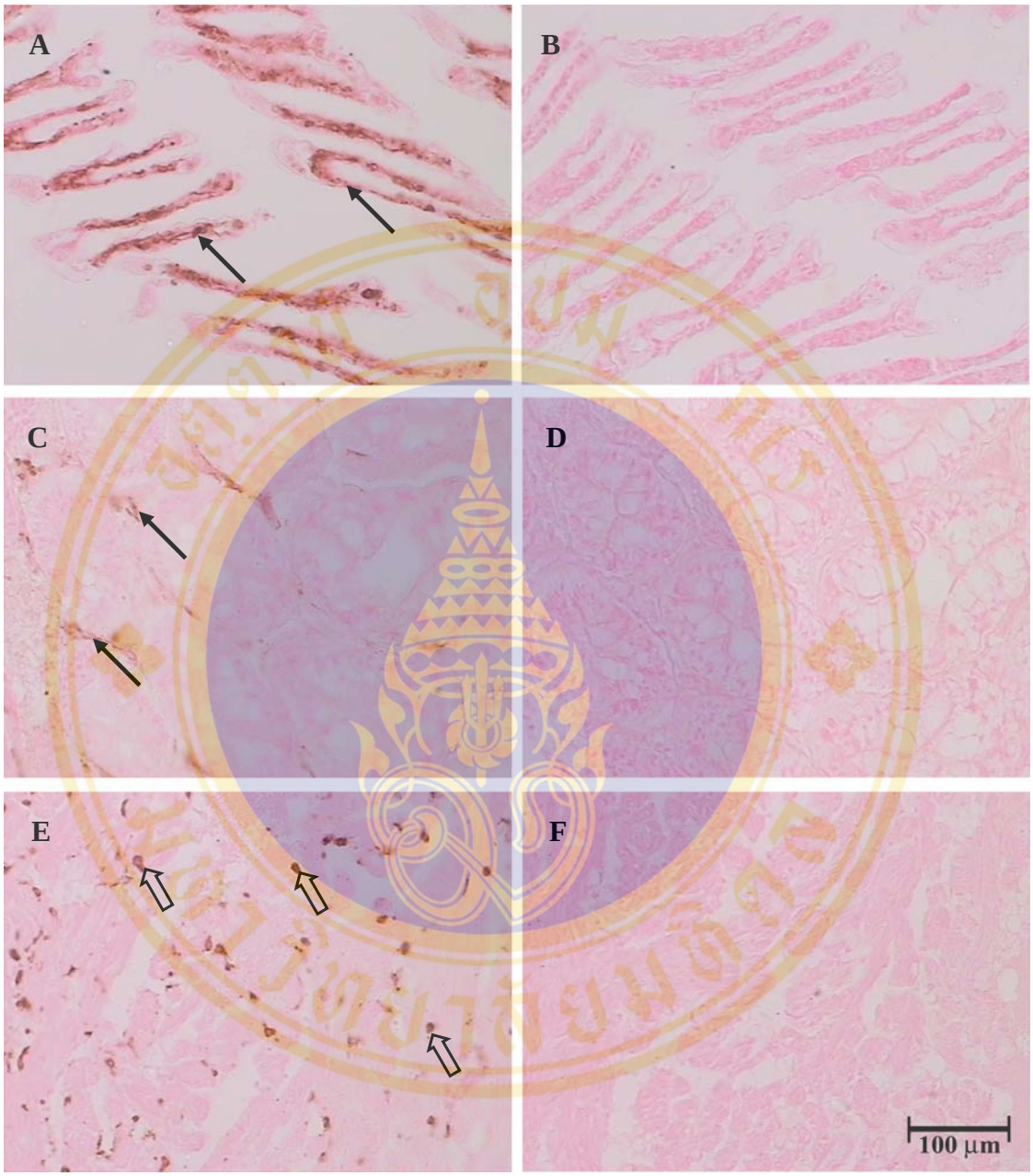


Figure 24. Immunohistochemistry of *P. monodon* gills (A, B) and hepatopancreas (C, D) infected with YHV and heart (E, F) infected with WSSV. Sections from *P. monodon* were treated with MAb Y19 as positive control (A, C; solid arrows) for YHV infection treated with MAb W28 as positive control (E; hollow arrows) for WSSV infection and treated with MAb MBV5-3H (B, D, and F) and counter stained with only eosin Y. The MAb MBV5-3H showed no cross-reactivity to YHV, WSSV and other organs (gills and heart) of *P. monodon* (B, D, and F).

Copyright by Mahidol University

Table 6. ELISA OD values of various MAbs to determine epitope of MAbs. The numbers (1, 2, 3) above the OD values in the middle row indicate the combination that did not show improvement in OD values.

MAbs	1 MBV 5-3H	2 MBV 5-6F	3 MBV 8-5G	4 MBV 10-5F	5 MBV 10-11H	6 MBV 13-10E	7 MBV 14-1G	8 MBV 16-3G	9 MBV 21-4B	10 MBV 21-8A	11 MBV 21-11B	12 MBV 27-8F
1 MBV 5-3H	1.343[1]	1.447	1.506	1.489	1.501	1.484	1.141[1]	1.166[1]	1.402	1.444	1.444	1.530
2 MBV 5-6F		0.594[2]	0.588	0.476	0.588	0.547	0.757	0.625	0.850	0.568	0.603	0.580
3 MBV 8-5G			0.526[2]	0.408	0.535	0.524	0.740	0.829	0.792	0.541	0.602	0.541
4 MBV 10-5F				0.309[2]	0.310	0.399	0.611	0.699	0.612	0.448	0.488	0.458
5 MBV 10-11H					0.364[2]	0.383	0.583	0.633	0.645	0.458	0.564	0.430
6 MBV 13-10E						0.408[2]	0.615	0.663	0.672	0.456	0.579	0.455
7 MBV 14-1G							0.333[1,3]	0.497	0.498	0.637	0.391	0.627
8 MBV 16-3G								0.384[1,3]	0.397	0.544	0.360	0.658
9 MBV 21-4B									0.370[3]	0.668	0.309	0.634
10 MBV 21-8A										0.463[2]	0.548	0.418
11 MBV 21-11B											0.257[3]	0.572
12 MBV 27-8F												0.402[2]

Copyright by Mahidol University

CHAPTER V
DISCUSSION

1. Purification of MBV polyhedrin

MBV polyhedrin protein was successfully extracted at very high purity. Our method was based on mass isolation by hydroforce and very simpler and more rapid, allowing the preparation of relatively large quantities of semi-purified polyhedrin by an Urografin gradient ultracentrifugation.

Gradient ultracentrifugation is the usual method used for purification of viral particles and their components. Cesium chloride gradient centrifugation has been used in the current purification of MBV polyhedrin (45, 46). Sucrose gradient centrifugation is another method that has been successfully to isolate occlusion bodies including complete virions (17, 47).

In the present study, purified polyhedrin proteins were successfully isolated at 50% Urografin gradient. This protein was confirmed using Transmission Electron Microscopy (TEM), which revealed 20-23 nm diameter particles that were full and empty subunits as similar to the MBV polyhedrin particles purified by Bonami *et al*. (16). Moreover, the complete virions and their component (nucleocapsid and envelope) were not found in this purified polyhedrin. These may be occurred from during hydroforce of infected hepatopancreas, proteinases present in the shrimp digestion system would be released and it unavoidably leads to rapid disruption of virions. However, it is more likely that the complete virions were separated with the supernatant solution in the first step of centrifugation before ultracentrifugation.

The molecular weight of purified polyhedrin was characterized by comparing its relative mobility on SDS-PAGE with standard protein marker (Figure 4.). It was found

Copyright by Mahidol University

that molecular weight of purified polyhedrin was 58 kDa as previously described for MBV polyhedrin (16, 23).

2. Production of monoclonal antibodies

In the past, many investigators produced monoclonal antibodies (MAbs) against specific antigens of shrimp viral pathogens for purposes. Poulos *et al*. (48), produced and identified monoclonal antibodies against viral proteins of Taura syndrome virus (TSV). The MAbs were used to compare specificities and 3 were directed at structural proteins of the virus. The production of MAbs to the white spot syndrome virus (WSSV; at MW 28 kDa) was reported by Poulos *et al*.(49) and its use in immunodiagnosis had been attempted. Sithigorngul *et al*. (50) reported the production of monoclonal antibodies specific to 22, 64, and 135 kDa proteins of yellow-head virus (YHV).

The purpose of this present study was to develop MAbs to polyhedrin of MBV. A strategy was chosen for immunization of mice to obtain MAbs that would react with both native and denatured forms of polyhedrin. The hybridomas were screened initially using a dot-immunoblot assay method that has been described previously (50). The MAbs chosen for the dot-immunoblot method were then second screened by western blot and immunohistochemistry and could also detect polyhedrin proteins in both native and denatured forms.

Seventeen MAbs were obtained from a mouse immunized with purified polyhedrin. All of MAbs bound specifically to native and denature forms of purified polyhedrin based on dot-immunoblot and western blot respectively. The western blot showed that all of MAbs reacted strongly with the 58 kDa purified polyhedrin of MBV. Moreover, all of MAbs could be used to detect natural infections of MBV in the hepatopancreas of *P. monodon* by means of immunohistochemistry. Furthermore, the MAbs showed no cross-reactivity to other viruses (HPV, YHV, and WSSV), normal hepatopancreas and other shrimp tissues (heart and gill) by means of immunohistochemistry.

Copyright by Mahidol University

Twelve MAbs with strong binding to MBV by means of dot blot were chosen to determine for sensitivity and epitope by indirect ELISA using a mixture of 2 MAbs/well in 96 well plates coated with purified-polyhedrin (0.133 µg/well). MAb MBV5-3H showed highest sensitivity with purified polyhedrin. For epitopes, results indicated that MBV5-3H, MBV14-1G, and MBV16-3G may bind to the same or overlapping epitopes because MAbs MBV14-1G and MBV16-3G showed lower sensitivity than MBV5-3H competitively bound to the same epitope with MBV5-3H. Thus, the OD value decreased when compared of MBV5-3H alone. Moreover, MAb MBV5-3H may bind to different epitope with MAbs MBV5-6F, MBV8-5G, MBV10-5F, MBV10-11H, MBV13-10E, MBV21-4B, MBV21-8A, MBV21-11B, and MBV27-8F because when mixed with MAb MBV5-3H with these MAbs increased the OD value. Results indicated that mixtures of MAb MBV5-3H with these MAbs could be used to promote high sensitivity in detection of purified polyhedrin.

The data presented here demonstrate the specificity of MAbs produced to the penaeid shrimp virus MBV. Furthermore, the results indicate that MAb MBV5-3H will be useful in the development of other serological test formats such as strip test that will permit the rapid and accurate technique for detection of MBV in hatcheries and farms of cultured *P. monodon*.

Copyright by Mahidol University

CHAPTER VI
CONCLUSIONS

1. MBV polyhedrin protein was successfully isolated at 50% Urografin fraction by means of Urografin gradient ultracentrifugation and its molecular weight was 58 kDa as evaluated by SDS-PAGE.
2. Monoclonal antibodies (MAbs) could be produced against purified polyhedrin of MBV. All of these MAbs have been found to react with 58 kDa of purified polyhedrin by means of western blot.
3. All of MAbs could be used to detect the natural infection of MBV in hepatopancreas of *P. monodon* and showed no cross-reactivity to other virus (HPV, YHV, and WSSV), normal hepatopancreas and other tissues (heart and gill) of shrimp by means of immunohistochemistry.
4. MAb MBV5-3H revealed highest sensitivity by means of indirect ELISA and was selected to be further development of strip test.

Copyright by Mahidol University

REFERENCES

1. Lightner DV, editor. A handbook of pathology and diagnostic procedures for diseases of penaeid shrimp. Baton Rough (LA): The World of Aquaculture Society; 1996
2. Lightner DV and Redman RM. A baculovirus-caused of the penaeid shrimp, *Penaeus monodon*. J. Invertebrate Pathol 1981; 38: 299-302
3. Lightner DV and Redman RM. “Shrimp diseases and current diagnostic menhods.” Aquaculture 1998; 164: 201-220
4. Owens L. Special topic review: the history of the emergence of viruses in Australian prawn aquaculture. World J. Microbiol. Biotechnol 1997; 13: 427-431
5. Belcher CR and Young PR. Colourimetric PCR-based detedtion of monodon baculovirus in world *Penaeus monodon* postlarvae. J Virol Methods 1998; 74: 21-29
6. Manivannan S, Subhendu K Otta, Karunasagar I, Karunasagar I. Mutiple viral ifection in *Penaeus monodon* shrimp postlarvae in an Indian hatchery. Dis Aquat Org 2002; 48: 233-236
7. Bondad-Reantaso M.G., McGladdery S.E., East I. & Subasinghe R.P. (eds). Asia Diagnostic Guide to Aquatic Animal Diseases. FAO Fisheries Technical Paper 402, Supplement 2. Rome, Italy 2001; 240 pp.
8. Ramasamy, P., Rajan, PR, Purushothaman, V., Brennan, GP., Ultrastructure and pathogenesis of Monodon baculovirus (Pm SNPV) in cultural larvae and natural brooders of *Penaeus monodon*. Aquaculture 2000; 184: 45-66
9. Baticados M.C.L., Pitogo, C.L., Paner M.G., de la Peza L.D., Tendencia E.A., Occurrence and pathology of *Penaeus monodon* baculovirus infection in hatcheries and ponds in the Philippines. Israeli J. Aquaculture Bamidgeh 1991; 43: 35-41.

Copyright by Mahidol University

10. Fegan D.F., Flegel T.W., Sriurairatana S. & Waiyakruttha M., The occurrence, development and histopathology of monodon baculovirus in Southern Thailand. Aquaculture 1991; 96: 205-217.
11. Natividad J.M. & Lightner D.V., Prevalence and geographic distribution of MBV and other diseases in cultured giant tiger prawns (*Penaeus monodon*) in the Philippines. *In:* Diseases of Cultured Penaeid Shrimp in Asia and the United States, Fulks W. & Main K., eds. The Oceanic Institute, Makapuu Point, Honolulu, Hawaii, USA 1992; 139-160.
12. Johnson P.T. & Lightner D.V., Rod-shaped nuclear viruses of crustaceans: gut-infecting species. Dis. Aquat. Org. 1988; 5: 123-141.
13. Paynter J.L., Vickers J.E. & Lester R.J.G., Experimental transmission of *Penaeus monodon-type baculovirus* (MBV). *In:* Diseases in Asian Aquaculture I, Shariff M., Subasinghe R. & Arthur J.R., eds. Fish Health Section, Asian Fisheries Society, Manila, Philippines 1992; 97-110.
14. Francki, R.I.B., Fauquet, C.M., Kmidson, D.L., Brown, F., Classification and nomenclature of viruses. Arch. Virol 1991; 2: 1–450
15. Rohrmann, G.F. Baculovirus structural proteins. J. Gen. Virol 1992; 73: 749–761.
16. Bonami JR, Aubert H, Mari J, Poulos BT, Vightner DV. The polyhedra of the occluded baculoviruses of marine decapod crustacea: A unique structure, crystal organization, and proposed model. J Struct Biol 1997; 120: 134-45.
17. Hsu Y.L., Wang K.H., Yang Y.H., Tung M.C., Hu C.H., Lo C.F., Wang C.H. & Hsu T. Diagnosis of *Penaeus monodon*-type baculovirus by PCR and by ELISA of occlusion bodies. Dis. Aquat. Org. 2000; 40: 93-99.
18. Rosenberry B. World shrimps farming. In : Rosenbery, R. (ed.), Shrimp News International 1996; 167.
19. Nadala E.C.B., Tapay L.M., Cai S., Loh P.C. Detection of yellow head virus and chinese baculovirus in penaeid shrimp by western blot technique. J. Virol. Methods 1997; 69: 39-44
20. O'Reilly D.R., Miller L.K., and Luckow V.A. Baculovirus expression vectors: a laboratory manual. Nw York: W.H. Freeman and Company 1992; 3-50.

Copyright by Mahidol University

21. Miller L.K. Baculovirus as gene expression vectors. Ann Rev. Microbiol 1988; 42: 177-199
22. Couch JA. An enzootic nuclear polyhedrosis virus of pink shrimp: Ultrastructure, prevalence, and enhancement. J Invertebr Pathol 1974a; 24: 311-331.
23. Mari J., Bonami J.R., Poulos B. & Lightner D.V. Preliminary characterization and partial cloning of the genome of a baculovirus from *Penaeus monodon* (PmSNPV = MBV). Dis. Aquat. Org. 1993; 16: 207-215
24. Wang S.Y., Hong C. & Lotz J.M. Development of a PCR procedure for the detection of Baculovirus penaei in shrimp. Dis. Aquat. Org.1996, 25, 123-131.
25. Ramachandran A, Bashyan MD, et al. the boundiful and blffing baculovirst: The story of polyhehrin transcription. Currnt Science 2001; 81: 339-1010
26. Doubrovsky A., Paynter J.L., Sambhi S.K., Atherton J.G. & Lester R.J.G. (1988). Observations on the ultrastructure of baculovirus in Australian *Penaeus monodon* and *Penaeus merguiensis.* Aust. J. Mar. Freshwater Res. 1988*;* 39: 743-749
27. Symmer MD. Characterization of Shrimp Baculovirus, Ecological Research Series 1977, EPA-600/3-77-130.
28. Bonami JR, Mari J, et al. Characterization of hepatopancreatic parvo-like virus, a second usual parvovirus pathogenic for penaeid shrimps. J Gen Virol 1995b; 76: 813-817.
29. Vlak JM and Rohrmann GF. The nature of polyhedrin. Viral Insecticides for Biological Control. IV: Physical, Biological, and Chemical Charaxteristics. K. Maramorosch and K. E. Sherman, Academic Press, New York 1985; 489-542
30. Maruniak JE. Baculovirus structural proteins and protein synthesis. The Biology of Baculoviruses. R.R. Granados and B.A. Federici, CRC Press, Boca Raton. 1986; 1: 129-146.
31. Adams JR and McClintock JT. Baculoviridae. Nuclear polyhedrosis viruses. Part1. Nuclear polyhedrosis viruses of insects. Atlas of Invertebrate Viruses. J. R. Adam and J. R. Bonami, CRC Press, Boca Raton 1991.

Copyright by Mahidol University

32. Tanada Y and Hess RT. Baculoviridae. Granulosis viruses. Atlas of Invertebrate Viruses. J. R. Adam and J. R. Bonami, CRC Press, Boca Raton 1991; 227-257

33. Thurman RB, Bell TA, Lightner DV, and Hazanow S. Unique physicochemical properties of the occluded penaeid shrimp baculoviruses and their use in diagnosis of infections. Journal of Aquatic Animal Health 1990; 2: 128-131.

34. Chen SN, Chang PS, and Kou GH. Observation on pathogenicity and epizootiology of *Penaeus monodon* baculovirus (MBV) in cultured shrimps in Taiwan. Fish Pathol. 1989; 24: 189-195.

35. Luna LG.(ed.). Manual of Histologic Staining Methods of the Armed Forces Institute of Pathology. McGraw-Hill Book Company, New York.

36. Lu CC, Tang F.J.K., and Chen S.N. Morphogenesis of the membranous labyrinth in penaeid shrimp cells infected with *Penaeus monodon* baculovirus (MBV). J. Fish Dis. 1996; 19: 357-364.

37. Summary of Spherical Baculovirosis (*Penaeus monodon*-type baculovirus). 2003.

38. Roitt I, Brostoff J, and Male D. Immunology. London : Gower Medical Publishing, 1985.

39. Köhler G and Milstein C. Continuous cultures of fused cells secreting antibody of predefined specificity. Nature. 1975; 256: 495-497.

40. Pontecorvo G. production of indefinitely multiplying mammalian somatic cell hybrids by polyethylene glycol (PEG) treatment. Somatic Cell Genet 1976; 1: 397-400.

41. Ringertz NR and Savage RE. Cell hybrids. New York: Academic Press 1976.

42. Littlefield JW. Selection of hybrids from matings of fibroblasts in vitro and their presumed recombinants. Sci 1964; 145: 709-710.

43. Sanyal PK and Gupta SC. The efficacy and phamacokinetics of long-term low-level intraruminal administration of triclabendazole in buffalo with induced fasciolosis. Vet Res 1996; 20: 461-468.

44. Vetterlein D. Monoclonal antibodies: production, purification and technology. Advances in clinical chemistry. 1989; 27: 303-349.

Copyright by Mahidol University

45. Haschemeyer RH and Meyer RJ. Negative staining Princ. Tech. Elec. Microsc. (M.A. Hayat, ed.) 1970; 2: 99-147.
46. Weber K. and Osborn M. the reality of molecular weight determinations of dodecyl sulfate polyacrylamide gel electrophoresis. J. Biol Chem 1969; 244: 4406-4412.
47. Davies GE. And Stark GR. Use of dimethyl suberimidate, a cross-linkingreagent, in studying the subunit struvture of oligomeric proteins. Proc Natl Acad Sci USA 1970; 66(3): 651-656.
48. Mosmann T, Bauman R, and Williamson A. Mutations affecting immunoglobulin light chain secretion by myeloma cells I. Eur. J Immunol 1979; 9: 171-172.
49. Kearney JF, Radbruch A, Liesegang B, and Rajewsky K. A new mouse myeloma cell line that has lost immunoglobulin expression but permits the construction of antibody-secreting hybrid cell lines. J Immunol 1979; 123(4): 1548-1550.
50. Adams A, Richards R, and Marin De Mateo M. Development of monoclonal antibodies to PK'X', the causative agent of proliferative kidney disease. J Fish Dis 1992; 15: 515-521.
51. Kumar S, Band AH, Samantaray JC, Dang N, and Talwar GP. A dot enzyme-linked immunosorbent assay for detection of antibodies against *Entamoeba histolytica*. J Immunol Methods 1985; 83(1): 125-133.
52. Hawkes R, Niday E, and Gordon J. A dot-immunobinding assay for monoclonal and other antibodies. Anal Biochem 1982; 119: 142-147.
53. Eshhar Z. Monoclonal antibody strategy and technique. New York: Plenum Press; 1985.
54. Winotaphan P, Sithigorngul P, Muenpol O, Longyant S, Rukpratanporn S, Chaivisuthangkura P, et al. Monoclonal antibodies specific to haemocytes of black tiger prawn *Penaeus monodon*. Fish & Shellfish Immunology 2005; 18: 189-198.
55. Bell TA and Lightner DV. A handbook of normal shrimp histology. Baton Rouge, LA: World Aquaculture Society; 1988.

Copyright by Mahidol University

56. Sithigorngul P, Panchan N, Vilaivan T, Sithigorngul W, Petsom A. Immunochemical analysis and immunocytochemical localization of crustacean hyperglycemic hormone from the eyestalk of Macrobrachium rosenbergii. Comparative biochemistry and physiology. Part B, Biochemistry & molecular biology 1999; 124(1): 73-80.
57. Bruce LD, Trumper BB, et al. “Methods for viral isolation and DNA extraction for a penaeid shrimp baculovirus” Journal of Virological Methods 1991; 34: 245-254.
58. Chang PS et al. Purification and amplification of DNA from Penaeus monodon-type baculovirus (MBV). Journal of Invertebrate Pathology 1993; 62: 116-120.
59. Lu CC, Tang F.J.K., Kou GH, and Chen S.N. Development of a Penaeus monodon-type baculovirus (MBV) DNA probe by polymerase chain reaction and sequence analysis. Journal of Fish Diseases 1993; 16: 551-559.
60. Poulos BT, Kibler R, Bradley-Dunlop D, Mohney LL, and Lightner DV. Production and use of antibodies for the detection of Taura syndrome virus in penaeid shrimp. Dis. Aquat. Org. 1999; 37: 99-106.
61. Poulos BT, Pantoja CR, Bradley-Dunlop D, Aguilar J, and Lightner DV. Development and application of monoclonal antibodies for the detection of white spot syndrome virus of penaeid shrimp. Dis. Aquat. Org. 2001; 47: 13-23.
62. Sithigorngul P, Rukpratanporn S, Longyant S, Chaivisuthangkura P, Sithigorngul W, Menasveta P. Monoclonal antibodies specific to yellow-head virus (YHV) of *Penaeus monodon*. Dis. Aquat. Org. 2002; 49: 71-76.
63. Satidkanitkul A, Sithigorngul P, Sang-oum W, Rukpratanporn S, Sriurairatana S, Withayachumnankul B, Flegel T. Synthethic peptide used to develop antibodies for detection of polyhedrin from monodon baculovirus (MBV (NOTE)). Dis. Aquat. Org. 2005; (Forthcoming publications)

Copyright by Mahidol University

APPENDIX

Copyright by Mahidol University

APPENDIX A
BUFFER AND REAGENT PREPARATION

1. Phosphate buffered saline (PBS) 0.15 M pH 7.2

NaCl	8.0	g
KCl	0.2	g
KH_2PO_4	0.2	g
Na_2HPO_4	1.15	g
Or $Na_2HPO_4.7H_2O$	2.15	g
H_2O (distilled water) adjust volume to	1000.0	ml

2. 5% Blotto solution

Skimmed milk	5.0	g
PBS 0.15 M pH 7.2	100.0	ml
1% Merthiolate (Sigma)	1.0	ml
Triton X-100	0.1	ml

3. 1% Merthiolate

Thimerosal (Sigma)	1.0	g
H_2O (distilled water) adjust volume to	100.0	ml

Copyright by Mahidol University

APPENDIX B
REAGENT PREPARATION FOR HYBRIDOMA PRODUCTION

1. RPMI medium

The medium consisted of the following ingredients:

RPMI 1640 (Roswell Park Memorial Institute- Gibco BRL, USA	10.4	g
D-glucose (Sigma)	3.6	g
L-glutamine (Sigma)	0.2923	g
Sodium pyruvate ($C_3H_3O_3Na$; Sigma)	1.1005	g
$NaHCO_3$	2.0160	g
HEPES (N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid; Sigma)	5.9525	g
H_2O	1000.0	ml

The solution of penicillin G, streptomycin and kanamycin were added to the final concentrationof 20,000 unitd, 200 mg, 200 mg per liter, repectively. The medium was sterilized by millipore membrane (pore size 0.22 μm) filtration and stored at 4ºC.

2. RPMI medium with serum

The medium consisted of following ingredients:

RPMI medium (1)	80.0	ml
Fetal calf serum (FCS, Starrate, Australia)	20.0	ml
Or bovine calf serum (BCS, Starrate, Australia)		
100 x HT supplement (Gibco BRL, USA)	1.0	ml

- 10 mM Sodiumhypoxanthine
- 1.6 mM Thymidine

3. Hybridoma selective medium (HAT medium)

The medium consisted of following ingredient

Copyright by Mahidol University

RPMI medium (1)	80.0	ml
Fetal calf serum (FCS, Starrate, Australia)	20.0	ml
100 x HT supplement (Gibco BRL, USA)	1.0	ml
50 X Aminopterin (Sigma)	2.0	ml
1 % Mouse red blood cell		

4. Fusion solution (40% polyethylene glycol)

Polyethylene glycol (PEG)	2.0	g

The solution was prepared by dissolving 2.0 g of polyethylene glycol in 3.0 ml of RPMI medium (1). The solution was incubated at 37ºC in CO_2 incubator before use.

5. Freezing medium (12% DMSO)

Dimethylsulfoxide (DMSO, Sigma)	12.0	ml
RPMI medium (1)	88.0	ml

The medium was stored in cold (4ºC) before use.

Copyright by Mahidol University

APPENDIX C
REAGENT AND SOLUTION FOR IMMUNOHISTOCHEMISTRY (IHC)

1. Coated slide solution

Gelatin	1.0	g
Clone alum (chromium potassium sulphate)	0.05	g
H_2O (distilled water) adjust volume to	100.0	ml

2. Davidson's fixative

95 % Ethanol	30.0	ml
100 % Formalin	20.0	ml
Glacial acetic acid	10.0	ml
H_2O (distilled water)	30.0	ml

3. Phosphate buffered saline (PBS) 0.15 M, pH 7.2

NaCl	8.0	g
KCl	0.20	g
KH_2PO_4	0.20	g
$Na_2HPO_4.7H_2O$	1.15	g
H_2O (distilled water) adjust volume to	1000.0	ml

4. Calf serum 10% (P_1^+)

Calf serum	10.0	ml
PBS	100.0	ml

5. Enrilich's acid hematoxylin

Hematoxylin	8.0	g
95% ethanol	400.0	ml

Copyright by Mahidol University

Aluminium potassium sulphate	8.0	g
Distilled water	400.0	ml
Glycerine	400.0	ml
Glacial acetic acid	400.0	ml

6. 0.2 % Eosin Y in 95% ethanol

Eosin Y	0.2	g
95% ethanol	100.0	ml

Copyright by Mahidol University

APPENDIX D
REAGENT FOR DETERMINATION OF ISOTYPE AND SUBISOTYPE OF MONOCLONAL ABTIBODIES USING ANTIBODY CAPTURE ON ANTI-Ig ANTIBODIES

Hybridoma sub-isotyping kit, mouse (Zymed) contents:

A) Rabbit anti-Mouse IgG1 (γ1 chain specific)
B) Rabbit anti-Mouse IgG2a (γ2a chain specific)
C) Rabbit anti-Mouse IgG2b (γ2b chain specific)
D) Rabbit anti-Mouse IgG3 (γ3 chain specific)
E) Rabbit anti-Mouse IgA (α chain specific)
F) Rabbit anti-Mouse IgM (μ chain specific)
G) Rabbit anti-Mouse kappa light chain
H) Rabbit anti-Mouse lambda light chain
I) Normal Rabbit Serum, (Negative Control)
J) Positive Control, Monoclonal Mouse IgG1
(Mouse IgG1 is in RPMI-1460 with 10% FBS)
K) Substrate Buffer, Concentration (10X)
(1 M citrate, pH 4.2, containing 0.03% H_2O_2)
L) ABTS Substrate, Concentration (50X)
(2,2-azino-di [3-ethylbenzthiazoline sulfonic acid])
M) Blocking Solution, Concentration (50X)
(25% BSA in PBS and 0.5% NaN_3)
N) HRP-Goat anti –Rabbit IgG (H+L), Concentration (50X)
O) Goat anti-Mouse IgGAM, Concentration (50X)
(0.5% mg/ml in PBS containing 10% glycerol and 0.05% NaN_3)
P) 50% Tween 20

Copyright by Mahidol University

APPENDIX E
BUFFERS AND SOLUTION FOR ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

1. 5% Blotto solution

Skimmed milk	5.0	g
PBS 0.15 M pH 7.2	100.0	ml
1% Merthiolate (Sigma)	1.0	ml
Triton X-100	0.1	ml

2. Washing solution (0.5% Blotto)

5% Blotto solution (1)	50.0	ml
PBS 0.15 M pH 7.2	950.0	ml

3. 0.1 M Citrate Buffer pH 4.5

Sodium citrate	29.4	g
1 % Merthiolate	10.0	ml
H_2O (distilled water) adjust volume to	1000.0	ml

4. 1 N H_2SO_4

Concentrated H_2SO_4	27.0	ml
H_2O (distilled water) adjust volume to	1000.0	ml

5. O-Phenylenediamine dihydrochloride (OPD)

Copyright by Mahidol University

APPENDIX F
REAGENT AND SOLUTION FOR SODIUM DODECYL SULFATE POLYACRYLAMIDE GELS

1. 10% SDS

Electrophoretic-grade SDS (Bio-Rad)	50.0	g
H_2O (distilled water) adjust volume to	500.0	ml

2. 1.5 M Tris-Cl

Tris base	90.75	g
1 N HCl	100.0	ml
H_2O (distilled water) adjust volume to	500.0	ml

3. 0.5 M Tris-Cl

Tris base	6.0	g
1 N HCl	40.0	ml
H_2O (distilled water) adjust volume to	100.0	ml

4. 1% Bromophenal blue

Bromophenol blue	0.1	g
H_2O (distilled water) adjust volume to	10.0	ml

5. 1x Running buffer

Tris base	3.0	g
Glycine	14.4	g
10% SDS	10.0	ml
H_2O (distilled water) adjust volume to	1000.0	ml

Copyright by Mahidol University

6. Fixing solution

Glacial acetic acid	50.0	ml
95% Ethanol	250.0	ml
H_2O (distilled water) adjust volume to	500.0	ml

7. Staining solution

Coomassie brilliant blue R250	1.0	g
Methanol	250.0	ml
Glacial acetic acid	50.0	ml
H_2O (distilled water) adjust volume to	500.0	ml

8. Destaining solution

Methanol	600.0	ml
Glacial acetic acid	200.0	ml
H_2O (distilled water) adjust volume to	2000.0	ml

9. Preserved solution

Glycerol	25.0	ml
95% Ethanol	142.5	ml
H_2O (distilled water) adjust volume to	500.0	ml

Copyright by Mahidol University

Components (ml) to prepare SDS-PAGE

Separating Gel					
	Total volume				
	6 ml	12 ml	24 ml	36 ml	48 ml
	30 % acrylamide gel: H_2O				
5.0% gel	1: 3.4	2: 6.8	4:13.5	6:20.3	8:27
7.5% gel	1: 2.9	3: 5.8	6: 11.5	9: 17.3	12: 23
10.0% gel	2: 2.4	4: 4.8	8: 9.5	12: 14.3	16: 19
12.5% gel	2.5: 1.9	5: 3.8	10: 7.5	15: 11.3	20: 15
15.0% gel	3: 1.4	6: 2.8	12: 5.5	18: 8.3	24: 11
1.5 M Tris-Cl pH 8.8	1.5	3	6	9	12
10% SDS	0.06	0.12	0.24	0.36	0.48
10% ammonium persulphate	0.06	0.12	0.24	0.36	0.48
TEMED	0.006	0.012	0.024	0.036	0.048

4% Polyacrylamide Stacking Gel		
	6 ml	12 ml
30% acrylamide	0.8	1.6
H_2O	4.3	8.6
0.5 M Tris-Cl pH 6.8	0.75	1.5
10% SDS	0.06	0.12
10% ammonium persulphate	0.06	0.12
TEMED	0.006	0.012

Copyright by Mahidol University

BIOGRAPHY

NAME Mr. Chumphol Boonsanongchokying

DATE OF BIRTH 13 April 1980

PLACE OF BIRTH Bangkok, Thailand

INSTITUTIONS ATTENDED Srinakharinwirot University, 2003 :
Bachelor of Science
(2^{nd} Hons, Biology)
Mahidol University, 2005 :
Bachelor of Science
(Biotechnology)

HOME ADDRESS 1134 Onnut Road Suanluang, Bangkok 10250, Thailand

Copyright by Mahidol University