EXPRESSION OF RECOMBINANT NS1 PROTEIN IN BACULOVIRUS SYSTEM AND DEVELOPMENT OF MONOCLONAL ANTIBODY AGAINST NS1 PROTEIN OF AVIAN INFLUENZA A VIRUS (H5N1)

PATUMPORN JIEAN-UMPUNKUL

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……..
Miss.Patumporn Jiean-umpunkul
Candidate

……..
Prof. Tararaj Dharakul,
M.D., Ph.D.
Major advisor

……..
Assist. Prof. Kanokwan poomputsa,
Ph.D.
Co-advisor

……..
Asst. Prof. Auemphorn Mutchimwong,
Ph.D.
Acting Dean
Faculty of Graduate Studies
Mahidol University

……..
Prof. Kovit Pattanapanyasat,
Ph.D.
Program Director
Master of Science Program in
Immunology
Faculty of Medicine Siriraj Hospital
Mahidol University

Thesis
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EXPRESSION OF RECOMBINANT NS1 PROTEIN IN BACULOVIRUS SYSTEM AND DEVELOPMENT OF MONOCLONAL ANTIBODY AGAINST NS1 PROTEIN OF AVIAN INFLUENZA A VIRUS (H5N1)

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for the degree of Master of Science (Immunology)
on
July 21, 2010

…….....
Miss.Patumporn Jiean-umpunkul
Candidate

……..
Assoc. Prof. Porntippa Lekcharoensuk, Ph.D.
Chair

……..
Assist. Prof. Kanokwan poomputsa, Ph.D.
Member

……..
Prof. Tararaj Dharakul, M.D., Ph.D.
Member

……..
Asst. Prof. Auemphorn Mutchimwong, Ph.D.
Acting Dean
Faculty of Graduate Studies
Mahidol University

……..
Clin.Prof. Teerawat Kulthanan Ph.D. Ph.D. M.D., F.I.M.S., F.R.C.S.T., F.I.C.S., Certificate in Orthopedics (Essen)
Dean
Faculty of Medicine Siriraj Hospital
Mahidol University

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PATUMPORN JIEAN-UMPUNKUL 4937153 SIIM/M

M.Sc. (IMMUNOLOGY)

THESIS ADVISORY COMMITTEE: TARARAJ DHARAKUL, M.D.,Ph.D., KANOKWAN POOMPUTSA, Ph.D.

ABSTRACT

Influenza A viruses infect a variety of avian and mammalian species, including humans; and have a significant impact on global health. The nonstructural protein, NS1 is an important non-structural protein that is abundantly expressed in influenza virus infected cells, but it is not incorporated into mature virions. In this study, the 6xHis-tag recombinant NS1 protein (rNS1) was produced from full length NS1 cDNA from (A/chicken/Nakorn-Pathom/Thailand/cu-k2/04), and expressed by Baculovirus Expression System using Sf9 cells and purified by metal affinity chromatography on a Co^{2+} column. The identity of the purified rNS1 protein was confirmed by Western blot analysis. Furthermore, our results showed that Sf9 insect cells can produce biologically active recombinant NS1, utilizing it as antigen for immunization for monoclonal antibody (MAb) production. The hybridoma producing MAb to NS1 protein was screened by ELISA and Western blot analysis. Two MAbs were shown to be the best MAb pair to develop the NS1 sandwich ELISA to identify NS1 antigen. The developed MAb-based NS1 sandwich ELISA was shown to react specifically with NS1 protein of several isolates of H1N1 and H3N2 human influenza A virus infected MDCK cell culture. This NS1 sandwich ELISA can be used to identify NS1 protein of influenza A virus not only in infected MDCK cell culture, but also influenza A infected clinical specimens in the future.

KEY WORDS: INFLUENZA A VIRUS/ NS1/NON-STRUCTURAL PROTEIN 1/ BACULOVIRUS EXPRESSION SYSTEM/ MONOCLONAL ANTIBODY

112 pages

การผลิตโปรตีน NS1 ด้วยระบบ Baculovirus และการพัฒนาโมโนโคลนัล แอนติบอดี ต่อโปรตีน NS1 ของไวรัสไข้หวัดนก ชนิด H5N1

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ปทุมพร เจียรอำพันกุล 4937153 SIIM/M

วท.ม. (วิทยาภูมิคุ้มกัน)

คณะกรรมการที่ปรึกษาวิทยานิพนธ์ : ธารารัชต์ ธารากุล M.D.,Ph.D., กนกวรรณ พุ่มพุทรา,Ph.D.

บทคัดย่อ

ไวรัสไข้หวัดใหญ่ชนิดเอเป็นสาเหตุสำคัญของการติดเชื้อในสิ่งมีชีวิตหลายหลายชนิดรวมถึงมนุษย์ด้วย เมื่อมีการติดเชื้อเกิดขึ้น ไวรัสจะสร้างโปรตีนออกมามากมาย หนึ่งในนั้นก็คือโปรตีน NS1 ซึ่งเป็นโปรตีนที่ถูกสร้างขึ้นในเซลล์ที่ติดเชื้อในขณะที่ไวรัสมีการขยายจำนวน นอกจากนี้โปรตีน NS1 จะพบเป็นจำนวนมากในเซลล์ที่ติดเชื้อแต่จะไม่พบในอนุภาคไวรัส ในการศึกษานี้ได้ทำการสร้างโปรตีนลูกผสม NS1 ที่มีการต่อของ 6xHis จากไวรัสสายพันธุ์ H5N1 (A/chicken/Nakorn-Pathom/Thailand/cu-k2/04) ด้วย Baculovirus Expression System โดยใช้เซลล์แมลงชนิด Sf9 โปรตีน NS1 ที่ถูกสร้างขึ้นถูกนำไปทำให้บริสุทธิ์โดยวิธี affinity chromatography ด้วยไอออนโลหะชนิด Co^{2+} และนำมาทดสอบเพื่อยืนยันด้วยวิธี Western Blot analysis ซึ่งผลการทดสอบบ่งบอกว่า เซลล์แมลงชนิด Sf9 สามารถผลิต recombinant โปรตีน NS1ที่มี biological activity และสามารถนำไปใช้ต่อเพื่อเป็น antigen ในการฉีดหนูทดลองเพื่อการสร้างโมโนโคลนอลแอนติบอดี เซลล์ hybridoma ที่สร้างโมโนโคลนอลแอนติบอดีที่มีความจำเพาะต่อโปรตีน NS1 ได้นั้นผ่านการคัดเลือกด้วยวิธี ELISA และ Western Blot analysis โมโนโคลนอลแอนติบอดีจำนวนสองโคลนที่มี activity ดีต่อโปรตีน NS1 สามารถนำมาจับคู่กันเพื่อพัฒนาวิธีการตรวจสอบโปรตีน NS1 ด้วยวิธี sandwich ELISA ซึ่งวิธีการตรวจสอบนี้ได้แสดงให้เห็นว่าโมโนโคลนอลแอนติบอดีที่เลือกสามารถทำปฏิกิริยาอย่างจำเพาะกับโปรตีน NS1 ในเซลล์ MDCK ที่ถูกติดเชื้อด้วยไวรัสไข้หวัดใหญ่ชนิดเอ ที่ติดเชื้อในมนุษย์สายพันธุ์ H1N1และ H3N2 ผลการทดลองสามารถสรุปได้ว่าวิธีการตรวจหาโปรตีน NS1 ด้วยวิธีการ sandwich ELISA นั้นนอกจากจะสามารถตรวจสอบโปรตีน NS1 ได้ในเซลล์ทดลองที่ถูกติดเชื้อแล้ว ยังสามารถนำไปพัฒนาต่อเพื่อใช้ในการตรวจหาโปรตีน NS1 ในสิ่งส่งตรวจที่มีเซลล์ที่ติดเชื้อไข้หวัดใหญ่ชนิดเอด้วย

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LIST OF ABBREVIATIONS

Abbreviations	Term
°C	Degree Celsius
AIV	Avian influenza virus
RNA	Ribonucleic acid
DNA	Deoxyribonucleic acid
APS	Ammonium persulfate
BSA	Bovine serum albumin
ELISA	Enzyme linked immunosorbent assay
EDTA	Ethylenediamine tetra acetic acid
FBS	Fetal bovine serum
g	Gram
HRP	Horseradish peroxidase
Ig	Immunoglobulin
kDa	Kilodalton
LB	Luria-Bertani broth
M	Molar
MAb	Monoclonal antibody
min	Minutes
mg	Milligram
ml	Milliliter
mM	Millimolar
MOI	Multiplicity of inflection
2-ME	2-Mercaptoethanol
NS1	Nonstructural protein 1
NP	Nucleoprotein
N	Normal

LIST OF ABBREVIATIONS (cont.)

Abbreviation	Term
nm	Nanometer
OD	Optimal density
PBS	Phosphate-buffered saline
PBST	Phosphate-buffered saline containing 0.05% Tween20
rpm	Revolutions per minute
rNS1	Recombinant Nonstructural protein 1
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TMB	3,3´,5,5´-tetramethylbenzidine
µg	Microgram
µl	Microliter
w/v	Weight per volume

CHAPTER I
INTRODUCTION

Avian influenza (AI) infection is a significant cause of morbidity and mortality in both human and animal population, especially H5N1 strain of avian influenza A virus. The H5N1 infections are of increasing public health concern because of their occasional transmission to human and also causing highly contagious respiratory disease (1, 2). During an outbreak of bird flu in Hong Kong in 1997, H5N1 viruses have crossed the species barrier from bird to human and killed six of eighteen infected persons (3). The virus was seriously pathogenic to human and confirmed cases of influenza A (H5N1) virus infection have globally increased. WHO reported the high mortality rate for infected humans (currently 62.7%) and it has been proposed that the HPAI-H5N1 viruses are strong candidate for causing the next pandemic if viruses acquire an efficient ability for human-to-human transmission (4, 5). Furthermore, the highly pathogenic avian influenza virus (HPAI) of the H5N1 subtype caused outbreak in poultry population in several countries in Southeast and East Asia during late 2003 and early 2004. Over 100 million birds died from bird flu or were killed by countries authority in effort to control the outbreak (6).

The control of AI infections in poultry appears to be crucial in order to reduce the pandemic risk, as actively circulating virus in domestic poultry populations represents the main source of infectious virus for humans (7). Traditional control measures for HPAI have centered on stamping out, which entails the large scale culling of infected flocks and contact flocks. This policy has in the past proven effective however the high concentration of poultry found in certain areas leads to the culling of millions of animals. Vaccination against AI has proven to be a successful additional control measure implemented alongside with control culling.

The expected advantages of a vaccination are twofold. Firstly, vaccination reduced susceptibility to infection, a higher dose of virus is necessary for establishing an infection in vaccinated birds. Secondly, there is a significant reduction in the amount of virus shed by infected birds, thus less virus to contaminate the environment reducing the risk of spread to other avian species and reducing the occupational risk faced by poultry workers (8). Although AI vaccines have been successful at providing protection against clinical signs and death among poultry (9), vaccination alone will not achieve eradication and if not used appropriately it may result in the infection becoming endemic (10). After vaccination, the antibody level monitoring constitutes an important step of serologic surveillance to identify the antibodies to AI vaccines in individual vaccinated. The tradition method of AI vaccination includes the use of inactivated whole-virus vaccines (11) but one limitation of the inactivated vaccines is that vaccinated birds cannot be differentiated serologically from naturally infected birds using the commonly available diagnostic tests (9, 11, 12)

As it is very crucial to control and surveillance of influenza, the more rapid and accurate detection of influenza tests is required. The use of MAb increases the specificity, accuracy and efficiency of diagnosis and provides an unlimited quality and consistent quality of reagents (13). The aim of this study is to demonstrate the production of MAb against AI NS1 protein subtype H5N1. For induction of the MAb against AI NS1 protein, mice were immunized with the recombinant NS1 protein that was generated by using Baculovirus expression system. The MAb against AI NS1 protein and recombinant NS1 protein can be used to develop various tests for differentiating infected and vaccinated with inactivated virus both in human and animal. In addition, this generated MAbs could be used to develop various tests for NS1 antigen detection for Influenza A diagnostic test.

CHAPTER II
OBJECTIVES

This study is aimed to produce monoclonal antibody specific to nonstructural protein 1 (NS1) of influenza A virus H5N1. These antibodies will be useful for further study of the NS1 protein of Influenza A virus. The objectives of this study are included:

1. To generate recombinant nonstructural protein 1 (NS1) from avian influenza A virus subtype H5N1.
2. To obtain purified recombinant NS1 protein by purification under Immobilized metal affinity chromatography (IMAC).
3. To produce monoclonal antibodies against nonstructural protein 1 (NS1) protein of avian influenza A virus subtype H5N1.
4. To characterize the produced monoclonal antibodies in term of antigen specificity.

CHAPTER III
LITERATURE REVIEW

1. Epidemiology of Influenza A virus infection

Flu season is a regularly re-occurring time period characterized by the prevalence of outbreaks of influenza. As influenza is caused by a variety of species and strains of viruses, in any given year some strains can die out while others create epidemics, while yet another strain can cause a pandemic. Typically, in a year's normal two flu seasons, there are between three and five million cases of severe illness and up to 500,000 deaths worldwide, which by some definitions is a yearly influenza epidemic (14). New influenza viruses are constantly evolving by mutation or by reassortment (15). Mutations can cause small changes in the hemagglutinin and neuraminidase antigens on the surface of the virus. This is called antigenic drift, which slowly creates an increasing variety of strains until one evolves that can infect people who are immune to the pre-existing strains. This new variant then replaces the older strains as it rapidly sweeps through the human population often causing an epidemic (16). However, since the strains produced by drift will still be reasonably similar to the older strains, some people will still be immune to them. In contrast, when influenza viruses reassort, they acquire completely new antigens for example by reassortment between avian strains and human strains; this is called antigenic shift. If a human influenza virus is produced that has entirely new antigens, the novel influenza will spread uncontrollably, causing a pandemic (17).

In contrast to this model of pandemics based on antigenic drift and shift, an alternative approach has been proposed where the periodic pandemics are produced by interactions of a fixed set of viral strains with a human population with a constantly changing set of immunities to different viral strains (18). In addition to these annual epidemics, Pandemic as well as seasonal outbreaks of influenza A virus represent

major threats to global public health. In the last century three major pandemics have occurred, in 1918, 1957, and 1968, caused by H1N1 (Spanish flu), H2N2 (Asian flu), and H3N2 (Hong Kong flu) viruses, respectively. Of these, the Spanish flu was the most severe and is estimated to have caused over 40 million deaths worldwide (19). These pandemics were caused by strains of Influenza A virus that had undergone major genetic changes and for which the population did not possess significant immunity (14, 20). Current pandemic is the 2009 flu pandemic which is an A (H1N1) pandemic, a global outbreak of a new strain of influenza A virus subtype H1N1, identified in April 2009 and transmitted between humans. The overall effects of these pandemics and epidemics are summarized in Table 1 below.

Recent human infections by highly pathogenic H5N1 avian influenza A viruses have increased the concern that another global pandemic may occur. One strain of HPAI A (H5N1) is spreading globally after first appearing in Asia. It is epizootic (an epidemic in nonhumans) and panzootic (affecting animals of many species, especially over a wide area), killing tens of millions of birds and spurring the culling of hundreds of millions of others to stem its spread. Most references to "bird flu" and H5N1 in the popular media refer to this strain (21). According to the FAO Avian Influenza Disease Emergency Situation Update, H5N1 pathogenicity is continuing to gradually rise in endemic areas but the avian influenza disease situation in farmed birds is being held in check by vaccination. Eleven outbreaks of H5N1 were reported worldwide in June 2008 in five countries (China, Egypt, Indonesia, Pakistan and Vietnam) compared to 65 outbreaks in June 2006 and 55 in June 2007. The global HPAI situation can be said to have improved markedly in the first half of 2008, cases of HPAI are still underestimated and underreported in many countries because of limitations in country disease surveillance systems (22).

Table 1 Known flu pandemic

Name of pandemic	Year	Deaths (est.)	Influenza A virus Subtype	Case fatality rate
Asiatic (Russian) Flu	1889–1890	1 million	possibly H2N2	?
1918 flu pandemic (Spanish flu)	1918–1920	20 to 100 million	H1N1	>2.5%
Asian Flu	1957–1958	1 to 1.5 million	H2N2	<0.1%
Hong Kong Flu	1968–1969	0.75 to 1 million	H3N2	<0.1%
2009 flu pandemic	2009–Present	?	H1N1	?

Hilleman MR. Realities and enigmas of human viral influenza: pathogenesis, epidemiology and control. Vaccine. 2002 Aug 19;20(25-26):3068-87.(23)

Potter CW. A history of influenza. J Appl Microbiol. 2001 Oct;91(4):572-9.(24)

Taubenberger JK, Morens DM. 1918 Influenza: the mother of all pandemics. Emerg Infect Dis. 2006 Jan;12(1):15-22.(25)

2. Molecular biology of Influenza A virus

Influenza A virus is a genus of the Orthomyxoviridae family of viruses. Influenza virus A includes only one species: *Influenza A virus*. Influenza A viruses are not only primarily avian viruses which wild aquatic birds are the natural hosts for a large variety of viruses but also infect a wild variety of animals including pigs, horses and seals. Only a few antigenic subtypes of influenza A virus are known to infect humans. Strains of all subtypes of influenza A virus have been isolated from wild birds, although disease is uncommon. Some isolates of influenza A virus cause severe disease both in domestic poultry and, rarely, in humans. Occasionally viruses are transmitted from wild aquatic birds to domestic poultry and this may cause an outbreak or give rise to human influenza pandemics. Because of the human epidemic and pandemic have been caused by influenza A viruses, The type A viruses are the most virulent human pathogens among the three influenza types causing the most severe disease.

2.1. Genetic structure

The physical structure of all influenza A viruses is similar. The virions or virus particles are enveloped and can be either spherical or filamentous in form. Its genome is organized into eight single stranded, negative-sense RNA segments that code for 11 identified viral protein (HA, NA, NP, M1, M2, NS1, NEP, PA, PB1, PB1-F2, PB2). The total genome size is 13,588 bases. The segmented nature of the genome allows for the exchange of entire genes between different viral strains during cellular cohabitation. The eight RNA segments are:

2.1.1. HA encodes hemagglutinin, it is an integral membrane protein as an antigenic glycoprotein found on surface of influenza A virus. The extent of infection into host organism is determined by HA. There are at least 16 different HA antigens. These subtypes are labeled H1 through H16. The primary function of HA are recognize of target vertebrate cells, accomplished through the binding of these

cells' sialic acid-containing receptors, and entry of the viral genome into the target cells by causing the fusion of host endosomal membrane with the viral membrane.

2.1.2. NA encodes neuraminidase, it is an integral membrane glycoprotein as an antigenic determinants found on the surface of the Influenza virus. There are 9 subtypes of NA antigen. The function is to free virus particles from host cell receptor by catalyze the hydrolysis of terminal sialic acid residues (26). And include assistance in the mobility of virus particles through the respiratory tract mucus and in the elution of virion progeny from the infected cell (27).

2.1.3. NP encodes nucleoprotein, it is a basic protein rich in arginine with a net positive charge at neutral pH. Influenza virus NP is a major structural protein in virus particles and has multiple functions in the viral infectious cycle. The primary function of NP is to encapsidate the virus genome for the purposes of RNA transcription, replication and packaging (28). Beside structural RNA-binding protein, NP is able to interact with a variety of other macromolecules, of both viral and cellular origins. As well as binding ssRNA, NP is able to self-associate to form large oligomeric complexes. It also binds the PB1 and PB2 subunits of the polymerase and the matrix protein M1. NP has also been shown to interact with cellular polypeptides, including actin, components of the nuclear import and export apparatus and a nuclear RNA helicase.

2.1.4. M encodes two matrix proteins (the M1 and the M2) by using different reading frames from the same RNA segment. Matrix proteins are structural proteins linking the viral envelope with the virus core (29).

The M1 protein is a matrix protein of the influenza virus. It forms a coat inside the viral envelope, showing affinity to the glycoproteins of the host cell membrane on one side of its molecule and nonspecific affinity for the RNA molecules on its other end, which allows it to form a layer under the membrane, to which the assembled complexes of viral ribonucleoprotein and viral RNA bind and are enveloped and bud out of the cell as new mature viruses. It also has multiple regulatory functions, performed by interaction with the components of the host cell.

The M2 protein is a proton-selective ion channel protein, integral in the viral envelope of the influenza A virus. It is activated by low pH. The M2 protein has an important role in the life cycle of the influenza A virus (30). It is located in the viral envelope. It enables hydrogen ions to enter the viral particle (virion) from the endosome, thus lowering pH of the inside of the virus, which causes dissociation of the viral matrix protein M1 from the ribonucleoprotein RNP. This is a crucial step in uncoating of the virus and exposing its content to the cytoplasm of the host cell.

2.1.5 NS encodes two distinct non-structural proteins (NS1 and NEP) by using different reading frames from the same RNA segment.

The NS1 protein is designated as non-structural because it is synthesized in infected cells, but is not incorporated into virions (31). More detail about NS1 protein will be described later.

The nuclear export protein or NEP formerly referred to as the NS2 protein, which mediates the export of vRNPs complexes (32, 33).

2.1.6 PA encodes an RNA polymerase, The PA subunit is a phosphoprotein and it is a member of the RNA-dependent RNA polymerase complex along with PB1and PB2. Genetic evidence suggests a role for the PA subunit in vRNA synthesis (34). For a role in genome transcription and replication by the polymerase complex; PA protein and a functional PA binding α domain on PB1 requires for efficiently transcription of viral reporter minigenome into translatable mRNA. The PB2-PB1-PA interaction is essential for maximum influenza virus PB1 transcriptase activity. It is possible that PA is mediating this enhancement by way of its ability to induce proteolysis of many cellular proteins (35).

2.1.7 PB1 encodes an RNA polymerase and PB1-F2 protein (induces apoptosis) by using different reading frames from the same RNA segment.

PB1 is the core of the complex and accounts for the polymerase activity(34). The PB1 subunit is involved in the elongation of the growing mRNA chain. It contains motifs typical of an RNA-dependent RNA polymerase that are essential for its biological activity (36). A map of intersubunit contact regions has been

established. Thus, the N-terminal sequences of PB1 interact with the C-terminal region of the PA subunit, while sequences next to the C-terminus of PB1 interact with the N-terminal region of the PB2 subunit.

The PB1-F2 protein has an apoptotic induction effect on macrophages, thus reducing their ability to contribute to an immune response. It has been previously suggested that this PB1-F2 protein contributes to viral pathogenicity solely because of it causes an inhibition of viral clearance, thus increasing cytoxicity (37).

2.1.8 PB2 encodes an RNA polymerase, The PB2 protein is one member of polymerase complex providing viral RNA-dependent RNA polymerase activity. The PB2 protein is an essential constituent of the transcriptase complex and has been shown to interact with the type 1 cap structure of host cell heterogeneous nuclear RNA, utilizing it as a primer for mRNA synthesis (38). The PB2 protein recognizes the cap structures on host cell mRNAs, and its possible involvement in the endonucleolytic cleavage of the host cellular mRNA precursors (36). The PB2 subunit has cap-binding and cap-dependent endonuclease activities. Nevertheless, both transcription and cap-dependent endonuclease activity require the presence of the three subunits of the polymerase and the RNA template (34).

The genome segments have common terminal sequences, and the ends of the RNA strands are partially complementary, allowing them to bond to each other by hydrogen bonds. After transcription from negative-sense to positive-sense RNA the +RNA strands get the cellular 5' cap added by cap snatching, which involves the viral protein NS1 binding to the cellular pre-mRNAs. The cap is then cleaved from the cellular pre-mRNA using a second viral protein, PB2. The short oligo cap is then added to the influenza +RNA strands, allowing its processing as messenger RNA by ribosomes. The +RNA strands also serve for synthesis of -RNA strands for new virions. The RNA synthesis and its assembly with the nucleoprotein takes place in the cell nucleus, the synthesis of proteins takes place in the cytoplasm. The assembled virion cores leave the nucleus and migrate towards the cell membrane, with patches of viral transmembrane proteins (hemagglutinin, neuraminidase and M2 proteins) and an

underlying layer of the M1 protein, and bud through these patches, releasing finished enveloped viruses into the extracellular fluid.

3. H5N1

Influenza A virus subtype H5N1, also known as "bird flu," A (H5N1) or simply H5N1, is a subtype of the Influenza A virus which can cause illness in humans and many other animal species. A bird-adapted strain of H5N1, called HPAI A (H5N1) for "highly pathogenic avian influenza virus of type A of subtype H5N1", is an emerging avian influenza virus that has been causing global concern as a potential pandemic threat. HPAI A (H5N1) is considered an avian disease, although there is some evidence of limited human-to-human transmission of the virus (39). A risk factor for contracting the virus is handling of infected poultry, but transmission of the virus from infected birds to humans is inefficient (40). Still, around 60% of humans known to have been infected with the current Asian strain of HPAI A (H5N1) have died from it, and H5N1 may mutate or reassort into a strain capable of efficient human-to-human transmission.

H5N1 has killed millions of poultry in a growing number of countries throughout Asia, Europe and Africa. Health experts are concerned that the co-existence of human flu viruses and avian flu viruses (especially H5N1) will provide an opportunity for genetic material to be exchanged between species-specific viruses, possibly creating a new virulent influenza strain that is easily transmissible and lethal to humans. Since the first H5N1 outbreak occurred in 1997, there have been an increasing number of HPAI H5N1 bird-to-human transmissions leading to clinically severe and fatal human infections. However, because there is a significant species barrier that exists between birds and humans, the virus does not easily cross over to humans, though some cases of infection are being researched to discern whether human to human transmission is occurring. More research is necessary to understand the pathogenesis and epidemiology of the H5N1 virus in humans. Exposure routes and other disease transmission characteristics such as genetic and immunological factors, that may increase the likelihood of infection, are not clearly understood.

The Avian Flu claimed at least 200 humans in Indonesia, Vietnam, Laos, Romania, China, Turkey and Russia. Epidemiologists are afraid that the next time such a virus mutates, it could pass from human to human. If this form of transmission occurs, another pandemic could result. Thus disease-control centers around the world are making avian flu a top priority. These organizations encourage poultry-related operations to develop a preemptive plan to prevent the spread of H5N1 and its potentially pandemic strains. The recommended plans center on providing protective clothing for workers and isolating flocks to prevent the spread of the virus (41).

4. DIVA strategies

For surveillance of avian influenza purpose, it is important to differentiate between naturally infection and vaccination. However, there are several potential strategies to differentiate infected from vaccinated animals (DIVA) (11, 42). The most common is the use of unvaccinated sentinel birds in flocks containing vaccinated birds which the sentinel may become infected with H5N1 which increase the risk of spread to human.

A second approach is the use of subunit vaccines targeted to the HA protein that allows serologic surveillance to the internal proteins (11). Nevertheless, these infected birds may not have enough virus replication to stimulate an antibody response to the novel viral proteins, and therefore may not seroconvert.

A third strategy is to vaccinate with a homologous HA subtype to the circulating field strain and a heterologous NA subtype allowing serological evidence of natural infection using a test for the homologous NA subtype. However, in certain countries, both homologous H5N1 vaccines and heterologous H5N2 vaccines are used in vaccination programs, which complicate validation of such NA DIVA serology testing (43).

The fourth strategy is to measure the serologic response to the nonstructural protein 1 (NS1). The NS1 protein is produced in large quantities in infected cells, but it is not packaged in the virion. Since killed vaccines for influenza are primarily made with whole virions, a differential antibody response can be seen between naturally infected and vaccinated animals. Therefore both homologous and

heterologous vaccination would allow the use of anti-NS1 antibodies to be used as a 'DIVA' tool. The ability to detect antibodies to this protein has been demonstrated experimentally with equine influenza (44) indicated that the NS1 protein could be used for serological diagnosis to distinguish horses infected with equine influenza viruses from those immunized with inactivated vaccines and recently, antibodies to the NS1 protein could possibly be used as part of a DIVA strategy as seroconversion to antibodies against the NS1 protein was achieved in chickens and turkeys experimentally infected with different subtypes of influenza A virus. A similar reaction was not detected in birds inoculated with inactivated vaccines (9).

5. NS1 protein

The NS1 protein of influenza A is translated from the collinear transcript of segment 8, which also encodes nuclear export protein (NS2 protein) from a spliced mRNA. The NS1 protein is a 26 kDa protein which is designated as a nonstructural protein because it is synthesized in large amounts in virus-infected cells, but it is not incorporated into the mature virions (31). NS1 protein of influenza virus is only produced during active replication of the virus thereby the detection of antibodies to this protein could be used as a 'marker' of infection (45). NS1 protein could represent the ideal candidate to elicit a specific immune response only in the presence of active viral replication. The NS1 protein is a multifunctional protein that participates in both protein-protein and protein-RNA interactions. It binds non-specifically to double-stranded RNA (dsRNA) and to specific protein targets. Folding of proteins into highly ordered structures is especially critical for carrying out their multiple functions. In addition, multifunctional proteins usually show a modular organization, with different domains responsible for different functions.

5.1. Intracellular localization of NS1 protein

A number of studies have reported various intra-cellular localization patterns for NS1. In infected cells, the distribution of NS1 may be dependent on several factors, including, virus strain, expression level of NS1, cell fixation procedure, cell type used, cell polarity and time post-infection. Nevertheless, in virus-

infected cells NS1 predominantly localizes to the nucleus, but a significant proportion can also be found in the cytoplasm (46).

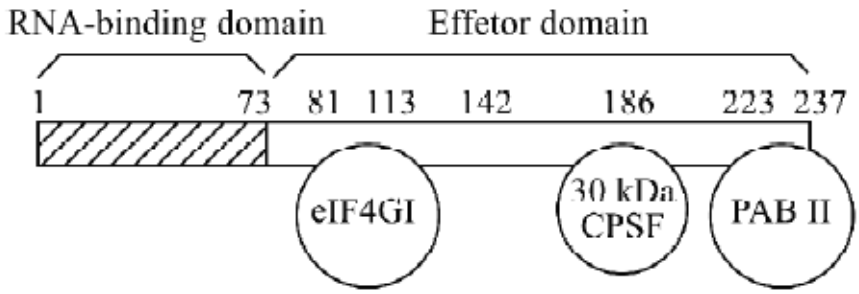
5.2. Phylogenetic analysis of NS1 protein

Based on their amino acid sequence the NS1 of influenza A viruses, NS1 proteins can be divided into two major groups, referred to as alleles A and B (47). A number of NS1 proteins from avian influenza viruses together with those of all human, swine and equine influenza viruses are described as allele A NS1 proteins, whereas those of allele B are exclusively from avian viruses. The level of homology within each allele is 93–100 %; however, between the two alleles it can be as low as 62%. There are no reports of functional differences associated with the different alleles to date (48). When a recombinant human virus containing an allele B NS1 protein was used to infect squirrel monkeys, there was a decrease in the ability of the virus to replicate in the respiratory tract compared with wild-type virus (47). This suggested that allele A NS1 proteins have a replicative advantage in mammalian hosts. Further analysis revealed that allele A NS1 proteins are under continual selection pressure to mutate, whereas those of allele B are not (49). It is possible that allele B NS1 proteins represent the archaic version of this protein and that, after entering the human influenza virus population via reassortment events, NS1 has been under a strong selection pressure to mutate, giving rise to the allele A NS1 proteins. The large degree of evolutionary divergence between the two alleles may indicate that there are significant functional constraints on NS1 proteins between host species. The significance of NS1 alleles for the virulence and pathogenicity of certain influenza viruses is not clear (46); however, the majority of highly pathogenic avian influenza viruses isolated from humans have contained an allele A NS1 protein (50).

5.3. Structure of NS1 protein

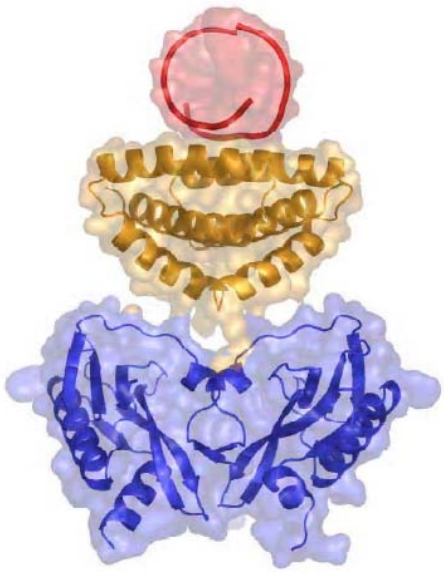
The NS1 protein normally consists of 230 amino acid residues. It can be divided into two major domains (Figure 1), the N-terminal RNA-binding domain (amino acids 1 to 73), which protects the virus against the antiviral state induced by IFN-α/β, primarily by blocking the activation of the 2'-5'-oligo (A) synthetase/RNase L pathway and the C-terminal effector domain (amino acids 74 to 230) which inhibits

the maturation and exportation of the host cellular antiviral mRNAs by binding cleavage and polyadenylation specificity factor (CPSF) and inhibiting poly (A)-binding protein (PAB II) function. The effector domain is crucial for the function of the RBD according to its functions. It was revealed that dimerization of these two domains was essential for NS1 protein to interact with RNA or cellular proteins (Figure 2).



Lin D, Lan J, Zhang Z. Structure and function of the NS1 protein of influenza A virus. Acta Biochim Biophys Sin (Shanghai). 2007 Mar;39(3):155-62. (31)

Figure 1 Binding sites of cellular proteins on the domains of the NS1 protein.
(CPSF, cleavage and polyadenylation specificity factor; eIF4GI, eukaryotic initiation factor 4GI; NES, nuclear export signal; PAB II, poly (A)-binding protein)



Bornholdt ZA, Prasad BV. X-ray structure of influenza virus NS1 effector domain. Nat Struct Mol Biol. 2006 Jun;13(6):559-60. (51)

Figure 2 Model for the dimer of the NS1 protein. The top area represents RNA, the middle area represents the RNA-binding domain dimer, and the lower area represents the effector domain dimer.

5.4. Function of NS1 protein

The function of the dsRNA-binding activity of the NS1 protein during influenza A virus infection has not been elucidated yet. Genetic analyses of NS1 have shown that viral replication, spread, and pathogenesis are very dependent on the function of this protein. Several interesting functions for NS1 have been described (52). NS1 is an RNA-binding protein that can interact with a variety of RNA species, including double stranded RNA (dsRNA). NS1 dsRNA-binding activity provides the protection of influenza A virus against the antiviral state primarily by inhibiting the IFN-α/β-induced 2'-5'-oligo (A) synthetase/RNase L pathway (53) for degradation of viral RNAs, blocks the activity of transcription factor pathways that depend on dsRNA, and inhibits activation of protein kinase RNA-regulated (PKR), by the direct binding of NS1 protein and the N-terminal 230 amino acid region of PKR (54).

Besides type I IFN, the NS1 protein is also involved in the inhibition of other pro-inflammatory cytokines, such as tumor necrosis factor-α, interleukin (IL) 6, chemokine (CC motif) ligand 3 (CCL3), macrophage inflammatory protein-1 alpha (MIP-1α), IL1β and IL18 (review in reference (31). NS1 protein is to inhibit the splicing of pre-mRNA in virus-infected cells by binding to a specific stem bulge in one of the spliceosomal small nuclear (sn) RNAs, U6 snRNA, a key component of the catalytic core within the spliceosome. NS1 also inhibits the induction of RNA interference through its ability to sequester small interfering RNAs (55).

Interactions of the NS1 with eIF4GI and PABP1, as well as with viral mRNAs, could promote the specific recruitment of the viral mRNA translation initiation complexes, thus enhancing the translation of the viral mRNA. NS1 protein binds and inhibits the function of two cellular proteins, CPSF30 and PABP II by way of its effector domain that are essential for the 3′-end processing of cellular pre-mRNAs, thereby inhibiting the production of mature cellular mRNAs, including IFN-β mRNA. Binding of NS1 and PABPII, which facilitates the elongation of oligo (A) tails during the generation of the 3′ poly (A) ends of mRNAs, prevents PABP II from properly extending the poly-A tail of pre-mRNA within the host cell nucleus, and blocks these pre-mRNAs exporting from the nucleus (56). It was also reported that another role of the C-terminal of the NS1 protein *in vivo* is to stabilize and/or facilitate formation of NS1 dimers and multimers and, therefore, to promote the RNA binding function of the NS1 N-terminal domain (31).

Some of the functions of NS1 serve to inhibit the host antiviral response that is mediated by interferon (IFN) which the NS1 protein encoded virally IFN antagonists. It has been proposed that NS1 protein plays a significant role in resisting the cellular immune response during the viral life cycle and is essential for a viable infection by multiple mechanisms. Other function of NS1 is to inhibit cellular gene expression so as to favor viral gene expression, such as effects on cellular RNA metabolism and export (52).

5.5. Contribution of NS1 protein to the pathogenicity and virulence of influenza A viruses.

The NS1 protein as a molecular determinant that contributes to the pathogenicity and virulence of influenza A viruses has been extensively studied in recent years. In the background of a human influenza virus, the NS1 protein of an H5N1 virus was able to reduce levels of pro-inflammatory cytokine induction. A recombinant WSN virus containing the 1918 pandemic NS1 gene was more efficient at blocking the expression of IFN-regulated genes than its parental influenza A/WSN/33 virus (57). Large-scale sequence analysis of avian influenza viruses indicated that the C-terminal four residues of the NS1 protein have the consensus sequence of a PDZ domain ligand (PL) and it was speculated that it may represent a virulence determinant. The effects of avian NS1 PL sequences on the virulence of a human influenza virus were recently reported (58) that, Viruses containing NS1 sequences from the 1918 H1N1 and H5N1 highly pathogenic avian influenza (HPAI) viruses demonstrated increased virulence in infected mice compared with wt A/WSN/33 virus. Infection with viruses containing the avian-like PL in NS1 was characterized by a severe loss of body weight, decreased survival, decreased MLD_{50}, severe alveolitis and increased viral spread in the infected lung.

NS1 is a functionally complex protein and is a central player in the virus's response to host defense mechanisms and the establishment of efficient viral gene expression (52). The multifunctional nature of NS1 is in many ways surprising given its relatively low molecular mass (~26 kDa). It may be that intra-cellular concentration and/or localization of NS1 contributes towards any possible hierarchy of binding, or NS1 may intrinsically have different affinities towards its binding partners. Unfortunately, such studies are complicated by that fact that some functions of NS1 are strain- or cell-type specific, and with no doubt influence virus pathogenicity and host range (46).

6. Baculovirus

The baculoviruses are classified in Family Baculoviridae, a family of large rod-shaped viruses that is specific to Arthropods. Their genomes are circular double-stranded DNA ranging from 80-180 kbp. The baculoviruses can be divided into two genera: nucleopolyhedroviruses (NPV) and granuloviruses (GV). While GVs contain only one nucleocapsid per envelope, NPVs contain either single (SNPV) or multiple (MNPV) nucleocapsids per envelope. The enveloped virions are further occluded in granulin matrix in GVs and polyhedrin for NPVs. Moreover, GV have only single virion per granulin occlusion body while polyhedra contain multiple embedded virions. Baculoviruses have very species-specific tropisms among the invertebrates with over 600 host species having been described. Immature (larval) forms of moth species are the most common hosts, but these viruses have also been found infecting sawflies, mosquitoes, and shrimp. They are not known to replicate in mammalian or other vertebrate animal cells.

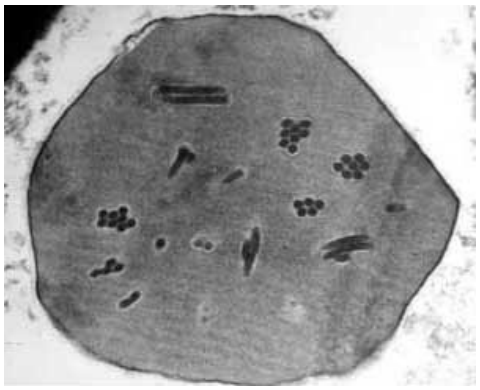


Figure 3 Electron micrograph of nucleopolyhedrovirus by J.R.Adams. From Wikipedia, the free encyclopedia (http://en.wikipedia.org/wiki/File:Baculovirus.jpg)

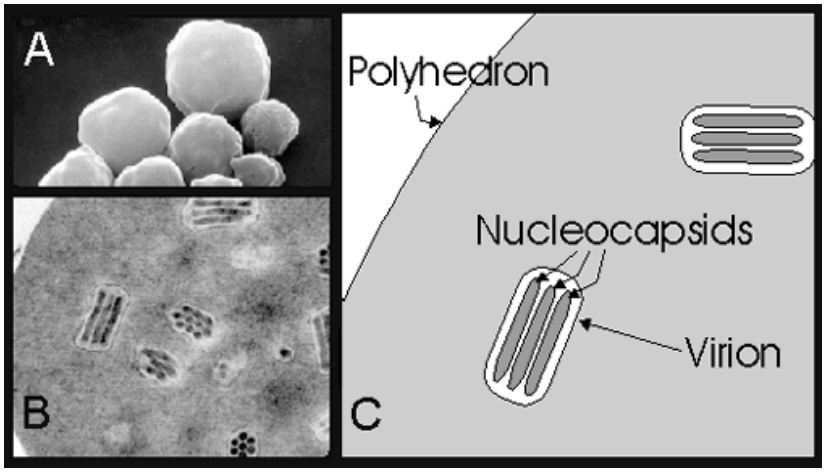


Figure 4 Electron micrographs A) Baculovirus particles, or polyhedra; B) Cross-section of a polyhedron; C) Diagram of polyhedron cross-section. Electron micrographs (A&B) by Jean Adams, graphic © by V. D'Amico. (http://www.nysaes.cornell.edu/ent/biocontrol/pathogens/baculoviruses.html)

6.1. Baculovirus life cycle

Currently, the most widely used Baculovirus expression system utilizes a lytic virus known as Autographa californica nuclear polyhedrosis virus (AcMNPV; hereafter called Baculovirus). This virus is the prototype of the family Baculoviridae. It is a large, enveloped, double-stranded DNA virus that infects arthropods. The Baculovirus expression system takes advantage of some unique features of the viral life cycle (59). The Baculovirus life cycle involves two distinct forms of virus. Occlusion derived virus (ODV) is present in a protein matrix (polyhedrin or granulin) and is responsible for the primary infection of the host while the Extracellular virus (ECV) is released from the infected host cells later during the secondary infection.

Typically, the initial infection occurs when a susceptible host insect feeds on plants that are contaminated with the occluded form of the virus. The protein matrix dissolves in the alkaline environment of the host midgut (stomach), releasing

ODV that then enter cells by adsorptive endocytosis, virus infects the gut epithelial cells by fuse to the columnar epithelial cell membrane of the host intestine and are taken into the cell in endosomes and move to the nucleus where their DNA is released. Nucleocapsids escape from the endosomes and are transported to nucleus. This step is possibly mediated by actin filaments. Both DNA replication and viral assembly take place in the nuclei of infected cells to generate two types of viral progeny. These include extracellular (nonoccluded) virus particles and polyhedra-derived (occluded) virus particles. The new ECV particles are budded out from the basolateral side to spread the infection systemically. During budding, ECV acquires loosely fitting host cell membrane with expressed and displayed viral glycoproteins.

Baculovirus infection can be divided to three distinct phases, early (0-6 hr post-infection), late (6-24 hr postinfection) and very late phase (18-24 to 72 hr postinfection). DNA replication begins ~6 hr after infection and is followed by viral assembly in the nucleus of the infected cell. Two types of viral progeny are produced during the life cycle of the virus: extracellular virus particles (nonoccluded viruses) during the late phase and polyhedra-derived virus particles (occluded viruses) during the very late phase of infection. ECV is released from the cell by budding, beginning at ~12 hr postinfection, and is produced at a logarithmic rate until 20 hr postinfection, after which production drops off. Polyhedra-derived virus, on the other hand, appears in the nucleus at ~18 hr postinfection and continues to accumulate as late as 72 hr postinfection, or until the cells lyse. Occluded viral particles are embedded in proteinaceous viral occlusions called polyhedral within the nucleus of infected cells. The polyhedron protein (29 kDa) is the major protein component of the occlusion bodies. When cells lyse, these occlusion bodies are released to further spread Baculovirus infection to next host. To adapt survival in the wild, ODV-polyhedrin particles are resistant to heat and light inactivation, whereas ECV is more sensitive to environment (60).

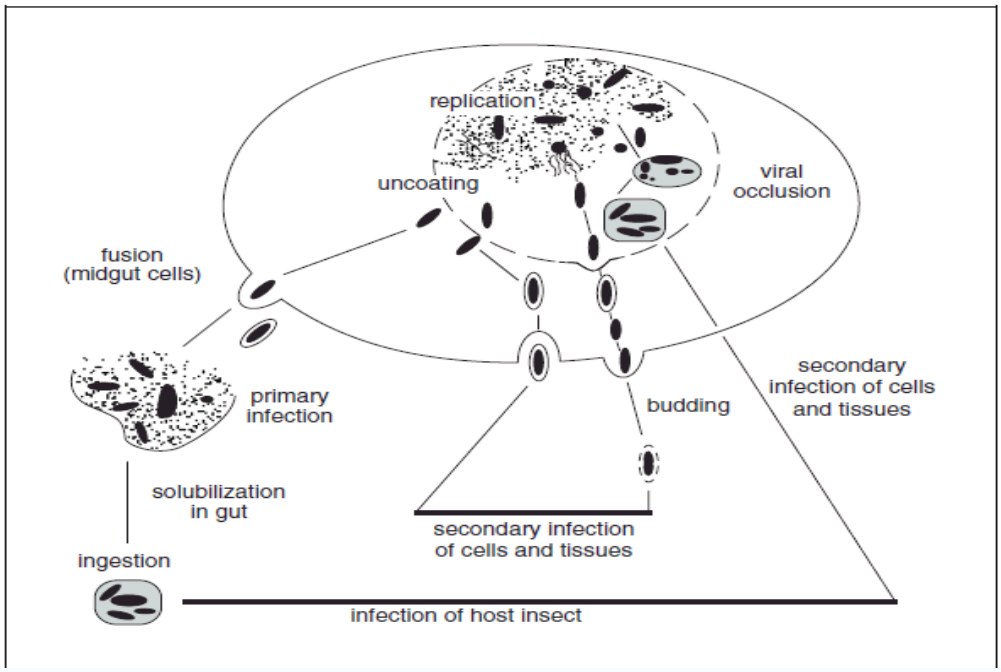


Figure 5 Baculovirus life cycle. The protein matrix of the occluded form of the virus dissolves in the alkaline environment of the host midgut (stomach), releasing ODV that then enter cells by adsorptive endocytosis, virus infects the gut epithelial cells by fuse to the columnar epithelial cell membrane of the host intestine and are taken into the cell in endosomes and move to the nucleus where their DNA is released. Both DNA replication and viral assembly take place in the nuclei of infected cells to generate two types of viral progeny. These include extracellular (nonoccluded) virus particles and polyhedra-derived (occluded) virus particles. ECV is released from the cell by budding, starting at ~12 hr postinfection and ending ~36 hr postinfection. Polyhedra-derived virus, on the other hand, appears later (~18 hr postinfection) and accumulates in the nuclei of infected cells ≤72 hr postinfection or until cellular lysis. Secondary infection of cells and tissues occurs by two pathways. In the first, the extracellular virus, once budded from the site of primary infection, is free to infect neighboring cells. Alternatively, polyhedra-derived virus is released from occlusion bodies after an infected food source is ingested by a new host. Reproduced from Summers and Smith (1987) with permission from the Texas Agricultural Experiment Station.

6.2. Baculovirus expression system

Baculovirus have emerged as a popular system for overproducing recombinant proteins in eukaryotic cells and used in research and scientific industrial communities for the production of high levels of properly post-translationally modified (folding, disulfide bond formation, oligomerization, glycosylation, acylation, proteolytic cleavage), biologically active and functional recombinant proteins (61). A number of unique features distinguish the Baculovirus expression system from other expression systems. First, unlike bacterial expression systems, the Baculovirus-based system is a eukaryotic expression system and thus uses many of the protein modification, processing, and transport systems present in higher eukaryotic cells. In addition, the Baculovirus expression system uses a helper-independent virus that can be propagated to high titers in insect cells adapted for growth in suspension cultures, making it possible to obtain large amounts of recombinant protein with relative ease. The majority of this overproduced protein remains soluble in insect cells, in contrast to the insoluble proteins often obtained from bacteria. Furthermore, the viral genome is large (~130 kbp) and thus can accommodate large segments of foreign DNA. Finally, baculoviruses are noninfectious to vertebrates, and their promoters have been shown to be inactive in most mammalian cells, which gives them a possible advantage over other systems when expressing oncogenes or potentially toxic proteins (59).

6.3. Bac-to-bac baculovirus system

The Bac-to-Bac baculovirus system (Invitrogen) provides a rapid way for the generation of recombinant baculoviruses (Figure 6). It is based on site-specific transposition of an expression cassette into a baculovirus shuttle vector (bacmid). The interest gene is inserted into the N-terminus of the lacZα gene, is a short segment containing the attachment site for the bacterial transposon Tn7 (mini-attTn7) that does not disrupt the reading frame of the lacZα peptide. The bacmid DNA can be propagated in E. coli DH10Bac™ as a large plasmid that confers resistance to kanamycin and can complement a lacZ deletion present on the chromosome to form colonies that are blue (Lac+) in the presence of a chromogenic substrate such as Bluo-gal or X-gal and the inducer IPTG. Transposition of the gene of interest from the

pFastBac donor plasmid into the bacmid disrupts the lacZ sequence causing recombinant bacmids to appear as white colonies. Transfection of the recombinant bacmid DNA into insect cells leads to the generation of recombinant baculovirus particles that can be amplified in successive rounds of infection and eventually used for protein expression studies (see Invitrogen manual for further details).

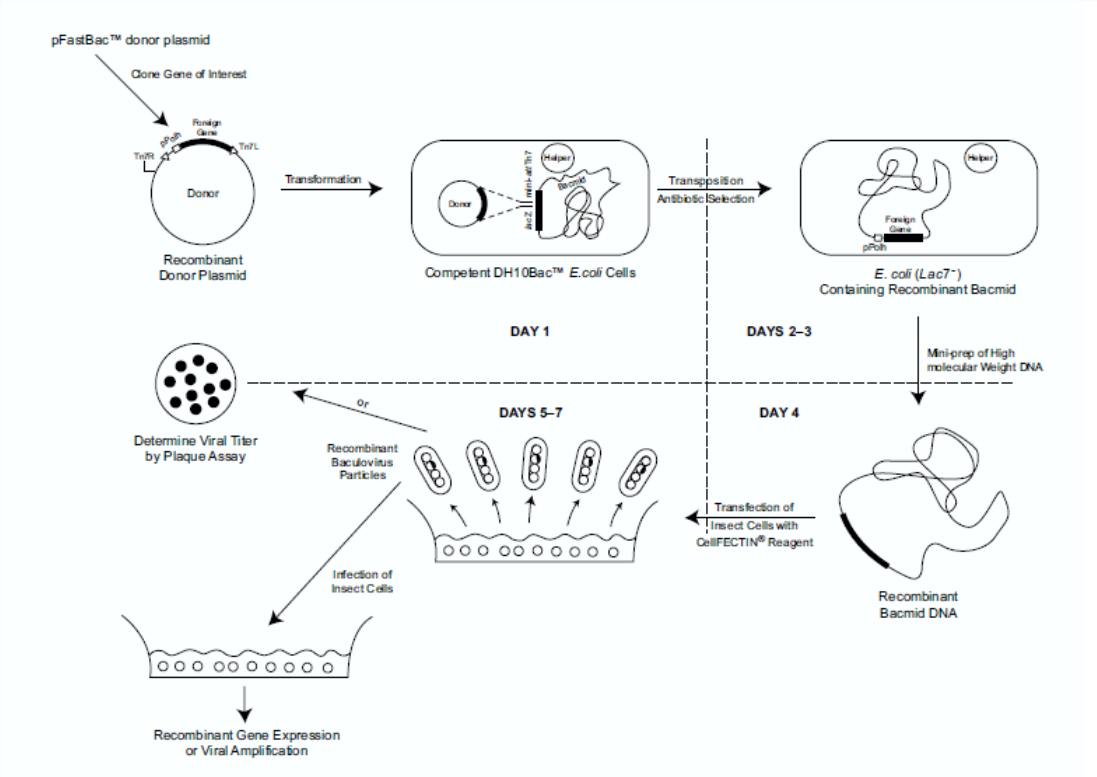


Figure 6 Generation of recombinant baculoviruses and gene expression with the Bac-to-Bac® Expression System. The gene of interest is cloned into a pFastBac™ donor plasmid and the recombinant plasmid is transformed into DH10Bac™ competent cells which contain the bacmid with a mini-*att*Tn7 target site and the helper plasmid. The mini-Tn7 element on the pFastBac™ donor plasmid can transpose to the mini-*att*Tn7 target site on the bacmid in the presence of transposition proteins provided by the helper plasmid. Colonies containing recombinant bacmids are identified by disruption of the *lacZα* gene. High molecular weight mini-prep DNA is prepared from selected *E. coli* clones containing the recombinant bacmid, and this DNA is then used to transfect insect cells.

7. Laboratory diagnostic of influenza A virus

Influenza is unique among viral infections because of its propensity for seasonal epidemics and occasional pandemics, and because of the morbidity and mortality that result from its pulmonary complications. In contrast to the majority of viruses, effective well-tolerated influenza vaccines, antiviral and chemoprophylaxis are available. The need for a timely diagnosis allows for optimal use of these treatments. However, during influenza season, clinical diagnosis (based on cough and high fever of acute onset) can be highly predictive of influenza. Thus diagnostic tests are not required for all patients with suspected influenza but may be of value if the clinical diagnosis is unclear and if antiviral or antibiotic treatment is a consideration. The laboratory diagnostic tests for influenza A virus infection are of four main types; virus isolation (conventional), detection by serology, detection of viral antigen and detection of virus nucleic acid.

7.1. Virus culture

Conventional classic laboratory diagnosis of influenza is based on virus isolation and serologic testing. Virus culture using primary rhesus monkey (PMK) or Madin-Darby canine kidney (MDCK) cells is the currently accepted ‘gold-standard’ for the laboratory diagnosis of influenza virus. Cell cultures are examined for cytopathic effect and screened by hemadsorption with confirmation by immunofluorescent monoclonal antibody against influenza A or B. However, traditional virus isolation and identification takes time. Because virus culture results are generally available in four to five days (range, two to 14 days).

7.2. Detection of virus antigen

For rapid diagnosis of influenza, the direct detection of viral antigens in clinical specimens is considered the method of choice. Diagnostic tests for detection of influenza virus antigen are of two main types (i) direct fluorescent antibody tests and (ii) Rapid enzyme/optical immunoassays. The technique most commonly used is the immunofluorescence test (IFT) (62). The nasopharyngeal specimens fixed on slides

can be sent and turnaround time required for a result is within 2–3 h. As solid-phase assays, enzyme-linked immunosorbent assays (ELISA) are the preferred option.

Using antigen tests, an influenza subtype-specific diagnosis is not possible. However, the detection of viral antigens does not require viable virus, therefore, less prone to delayed receipt of specimens by the laboratory. More recently, bed-side test kits have been developed. In these tests, viral antigen is separated chromatographically and detected immunologically by a color reaction. Such assays often require as little as 15 min. Despite their hitherto mostly low sensitivity and specificity, these tests will gain importance in future.

7.3. Molecular diagnosis

The extreme genetic variability of Influenza virus is a challenge for design of molecular-based diagnostic tests. However, various molecular based techniques have been developed. Reverse transcriptase polymerase chain reaction (RT-PCR) assays, these techniques are still rather expensive. Turnaround time of RT-PCR for influenza is one to two days. In addition, molecular testing requires considerable skill and expertise to perform and must be integrated into laboratory workflow. Several RT-PCR, which use nested primers to detect and sub-type influenza virus, have demonstrated greater sensitivity than other rapid diagnostic tests and conventional cell culture (63). Although the complexity and turnaround time of conventional RT-PCR may exclude its use for rapid influenza diagnosis, real-time RT-PCR, in which amplification and detection occur in the same reaction tube, can provide truly rapid results within four to five hours.

However, in the event of large antigenic drift or major antigenic shift in influenza, novel strains may not be detected by existing primer sets, and diagnosis may depend on traditional virus culture. This was highlighted by the detection of the first case of human influenza A H5N1 in Hong Kong, in May, 1997 (64). This strain, which was initially identified by culture and DFA as influenza A, was not detected by pre-existing PCR primers specific for H1 and H3. Thus, rather than replacing traditional virus isolation, molecular methods will likely complement cell culture, which will continue to play a major role in global epidemiologic influenza surveillance and vaccine strain selection.

7.4. Serological diagnosis

Serologic diagnosis of influenza infection is based on demonstration of a four-fold or greater rise in specific antibody titer between acute and convalescent serum samples. The need for paired serum samples, the first collected as soon after the onset of illness as possible and the second collected about two to four weeks later. Due to about 1 week after onset of clinical symptoms, a specific immune reaction becomes demonstrable. After the virus has been eliminated, antibodies can be detected in serum or in nasal secretions. Conventional methods for antibody detection are the complement fixation test, the hemagglutination inhibition test, EIA or neutralization test in which viral nucleoprotein (NP) or hemagglutinin (HA) serve as antigens. The conserved NP antigen allowed the detection of type-specific influenza antibodies, subtype-specific antibodies can be determined by means of the HI test. In this way, serological findings may complement the results of virus detection. Although serology may occasionally establish a diagnosis when all other means have failed, its main value lies in epidemiology or as a research tool.

CHAPTER IV
MATERIALS AND METHODS

1. Cell culture

1.1. Insect cell lines

Sf9 cells, a cell line from *Spodoptera frugiperda* were cultured in suspension cell culture at 27°C ± 0.5°C at a constant stirring rate of 100 rpm in Grace's insect cell culture medium (Invitrogen) containing 10% Fetal Bovine Serum (FBS) (Hyclone), 8% L-glutamine 200 mM (Invitrogen), 1% yeastolate (Invitrogen) and 1% lactabumin hydrolysate (GibcoBRL).

The insect cells were routinely subcultured using a hemocytometer chamber to determine cell density and trypan blue to determine the cell number and viability.

Spodoptera frugiperda (Sf9) cells insect cells were maintained in the animal cell culture laboratory of School of Bioresources and Technology, King Mongkut's University of Technology Thonburi. Sf9 cells grow in Grace's complete insect cell culture medium using formula by Dr. Porntippa Lekcharoensuk, Department of Microbiology and Immunology, Faculty of Veterinary medicine, Kasetsart University.

1.2. Mammalian cell lines

Mouse myeloma cell line myeloma cells (P3-X63-Ag 8.653) was cultured in RPMI-1640 (Invitrogen) supplemented with 20% FBS (Hyclone) at 37°C with 80% humidity in a 5% CO_2 incubator.

2. Antibodies

2.1. Rabbit polyclonal to Influenza A virus NS1 protein (H5N1) (Abcam)

2.2. Mouse polyclonal to Influenza A virus NS1 protein (H5N1)

2.3. Goat anti-mouse IgG conjugated horse-radish peroxidase (HRP) (KPL)

2.4. Goat anti-mouse IgG, IgA, IgM conjugated alkaline phosphatase (ALP) (KPL)

2.5. Goat anti-human IgG, IgA, IgM (H+L) conjugated alkaline phosphatase (ALP) (KPL)

2.6. Goat anti-mouse IgM conjugated horse-radish peroxidase (HRP) (KPL)

2.7. Goat anti-mouse IgA conjugated horse-radish peroxidase (HRP) (KPL)

3. Viruses

3.1. Avian influenza virus, A/chicken/Nakorn-Pathom/Thailand/cu-k2/04 (H5N1).

3.2. rgH5N1_3P, a reverse genetic derived virus containing HA, NP, NA, M and NS genes of avian influenza virus subtype H5N1.

3.3. Swine influenza virus, A/swine/Thailand/KU21/06.

3.4. H1N1, 4 isolates; 14/04, 20/07, 04/08 and 09/08.

3.5. H3N2, 4 isolates; 04/04, 02/07, 03/07 and 08/08.

3.6. Baculovirus *Autographa califonica* Nucleopolyhedrovirus (AcMNPV) is a recombinant baculovirus (bacmid) from *E.coli* DH10Bac^{TM} (Invitrogen).

4. Bacteria

The bacterial cells; *E.coli* DH5α and *E.coli* DH10Bac^{TM} used in this study were from Invitrogen Corporation.

E.*coli* DH5α^{TM} was used as a host strain of pFastBac HTb Donor plasmid. The chromosomal genotype of this strain is fhuA2 Δ (argF-lacZ) U169 phoA glnV44 Φ80 Δ (lacZ) M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17.

E.coli DH10Bac™ was used as the host strain for propagate a baculovirus shuttle vector (bacmid). The bacmid (bMON14272) contains the low-copy-number mini-F replicon, a kanamycin resistance marker, and a segment of DNA encoding the *lacZ*α peptide from a pUC-based cloning vector. The bacmid was propagated in *E*.coli DH10Bac™ as a large plasmid that confers resistance to kanamycin and can complement a *lacZ* deletion present on the chromosome to form colonies that are blue (Lac+) in the presence of a chromogenic substrate such as Bluo-gal or X-gal and the inducer IPTG.

4.1. Culture *E.coli* cells

4.1.1. *E.coli* DH5α ™ was cultured in LB medium, 5 ml at 37°C, 250 rpm overnight. However, in case that *E.coli* DH5α containing pFastBac HTb Donor plasmid, antibiotic ampicillin 100 μg/ml was adding into LB medium.

4.1.2. *E.coli* DH10Bac™ was cultured in LB medium containing antibiotics, kanamycin 50 μg/ml and tetracycline 10 μg/ml, 5 ml at 37°C, 250 rpm overnight.

4.2. Freezing bacterial cell

Culture the *E.coli* cells in appropriate condition at 37°C, 250 rpm overnight. Add sterile glycerol into culture cells medium for final concentration at 15% glycerol and mix before aliquot in 1.5 ml tube and store at -80°C until use.

4.3. Preparation of competent *E.coli* cells for transformation

The *E.coli* cells; DH5α™ and DH10Bac™ were made competent by using $CaCl_2$ treatment cells. A single bacterium colony was inoculated into 5 ml of LB medium. In addition *E.coli* DH10Bac™ cells containing bacmid, the antibiotic must be added into LB medium and incubated overnight at 37°C with shaking at 250 rpm. The 1 ml culture was transferred to 100 ml appropriate LB medium and incubated at 37°C with shaking at 250 rpm for 2-3 hr. to early log phase. The culture was transferred to 50 ml centrifuge tube for 4 tubes and stayed on ice for 5 min. The tubes were centrifuged at 4,000 rpm at 4°C for 15 min. then, supernatant was removed. The each pellet was resuspended with 4 ml of cold Transformation buffer I (TFB I) and centrifuged at 4,000 rpm at 4°C for 15 min and, supernatant was removed. The pellet

was pooled to 1 tube and resuspended with 8 ml of cold Transformation buffer II (TFB II) and stayed on ice for 15 min. The 200 µl of mixture was transferred to microcentrifuge tubes and immediately cooled with liquid nitrogen and stored at -80°C until use.

5. Primers

The two primers NS-F5-*Bam*HI and NS-R6-*Eco*RI were designed from coding region part of NS1 gene starts at ATG and stops at TGA. Primer sequences were based on the consensus sequence of the NS1 gene of influenza A virus obtain from CLUSTAL W (1.83) multiple sequence alignment. The primers were designed subject to the PCR product contain *Bam*HI restriction endoneuclease recognition sequence upstream of the start codon and *Eco*RI restriction endoneuclease recognition sequence downstream of the stop codon of NS1 gene.

The sequence of the PCR forward primer and reverse primer used to generate the recombinant NS1 gene with adding the restriction site to both 3' and 5' ends of the constructed gene were from Operon Biotechnology, Germany. The sequences of primers are shown in Table 2.

The sequence of the PCR forward primer and reverse primer used to verify the presence of the NS1 gene in the recombinant bacmid were synthesized from Bioservice unit (BSU), National Center for Genetic Engineering and Biotechnology (BIOTEC), Thailand. The sequences of primers are shown in Table 2.

Table 2 Oligonucleotide primers using in this study

Name	Gene*	Strand	Sequence 5'to3'**
NS-F5-*Bam*HI	NS1	+	CGG GAT CCA TGG ATT CCA ACA CTG TGT CAA GC
NS-R6-*Eco*RI	NS1	-	TTG AAT TCC TCA AAC TTC TGA CTC AAT TGT TCT CG
M13F	*lacZ*	+	GTA AAA CGA CGG CCA GT
M13R	*lacZ*	-	AAC AGC TAT GAC CAT G

*NS1; NS1 gene of influenza A virus, lac*Z*; β-galactosidase gene

**The sequences corresponding to restriction endoneuclease recognition site are underlines.

6. Generation of recombinant Donor plasmid.

6.1. Preparation of NS1 gene

6.1.1. Extract viral RNA

Viral RNA was extracted from whole genome of influenza A virus using QIAamp viral RNA kit (QIAGEN) according to the manufacturer's instructions. The final extracted RNA was used directly for RT-PCR.

6.1.2. RT-PCR

The extracted viral RNA was yielded to NS1cDNA by reverse transcribed and polymerase chain reaction (RT-PCR) on a thermal cycle using QIAGEN one step RT-PCR kit (QIAGEN) according to the manufacturer's instructions with specific designed primers; NS-F5-*Bam*HI and NS-F6-*Eco*RI to converted the NS1 segmented RNA to full-length NS1 gene.

6.1.3. Preparation of amplified DNA fragment

Approximately 50 µl of amplified products were purified using QIA quick PCR purification kit (QIAGEN), according to the manufacturer's instructions. The RT-PCR product (NS1cDNA) amplified using NS1 specific primers; NS-F5-*Bam*HI and NS-R6-*Eco*RI was digested with *Eco*RI and *Bam*HI restriction endonucleases

enzyme (Promega) at 37°C for overnight. The fragmented DNA was purified using QIA quick Gel Extraction Kit (QIAGEN), according to the manufacturer's instructions. The final DNA product was eluted in 30 µl of DI water and used directly for ligation.

6.2. Preparation of Donor plasmid

6.2.1. Small-scale preparation of pFastBac HTb plasmid

The plasmid DNA was prepared using GeneJET™ Plasmid Miniprep Kit (Fermentus), according to the manufacturer's instructions. A single bacteria colony (DH5α^{TM} containing pFastBac HTb) was inoculated in 5 ml of LB medium containing 100µg/ml ampicillin and incubated at 37°C for overnight with shaking at 250 rpm. The 5 ml culture was transferred to microcentrifuge tube and centrifuged at 14,000 for 1 min. Then the supernatant was removed and pellet was collected. The pellet was resuspended in 250 µl of the resuspension Solution by pipetting up and down until no cell clumps remain. The 250 µl of Lysis Solution was added and mixed thoroughly by inverting until the solution became viscous and slightly clear. The 350 µl of the Neutralization Solution was added and mixed thoroughly by inverting. The mixture was centrifuged at 14,000×g for 5 min to pellet cell debris and chromosomal DNA. The supernatant was transferred to the supplied GeneJET™ spin column and centrifuged for 1 min and the flow-through was discarded. The 500 µl of Wash Solution was added to the GeneJET™ spin column and centrifuged for 1 min and the flow-through was discarded again. The 50 µl of Elution Buffer was added at the center of GeneJET™ spin column membrane in order to elute the plasmid DNA and then incubated for 2 min at room temperature and centrifuged for 2 min. The purified plasmid DNA was collected and stored at -20°C.

6.2.2. Preparation of pFastBac HTb donor plasmid

The plasmid DNA was digested with *Eco*RI and *Bam*HI restriction endonucleases enzymes (Promega) at 37°C for overnight. The plasmid DNA was purified using QIA quick Gel Extraction Kit (QIAGEN), according to the manufacturer's instructions. The final plasmid DNA product was eluted in DI water and used directly for ligation.

6.3. Agarose gel electrophoresis DNA

Agarose gel 1% was prepared by solubilizing agarose in 1x TAE buffer. The gel was casted by pouring the melting agarose onto gel tray taped at both ends, and the comb was inserted to form the wells. After the gel was become solidified, the comb and the tabbed was removed and the gel tray was placed in the electrophoresis tank containing 1x TAE buffer. The DNA samples were mixed with 6x loading buffer (0.25% bromophenol blue, 40% sucrose) at a volume ratio of 5:1 and loaded into each wells. Electrophoresis was carried out from cathode to anode using constant voltage at 100V for 30 min. When electrophoresis was completed, the gel was stained in ethidium bromide solution (0.5μg/ml) at room temperature for 15 min and destained with water. The fragmented DNA in the gel was visualized under UV transilluminator.

6.4. Ligation of DNA fragment

The prepared plasmid DNA and NS1 gene insert were calculated for appropriate ligation condition by following formula:

$$\text{ng of insert gene} = \frac{\text{ng of plasmid} \times \text{size of insert (kb)} \times 3}{\text{size of plasmid (kb)}}$$

Moreover, the T4 ligase buffer and T4 DNA ligase enzyme (Promega) were added into the reaction to 1 unit of enzyme and final concentration of glycerol is not more than 5% in the reaction. The sterile distilled water was added to a final volume to 20 μl and the reaction was incubated at 4°C for overnight. The reaction was analyzed for ligation by electrophoresis in 1% agarose gel and the fragments were detected by ethidium bromide staining and visualized under UV light.

6.5. Transformation of plasmid DNA to *E.coli* cells

The ligated plasmid DNA was introduced into the *E.coli* cells; DH5α™ competent cells by heat shock. The 5 μg of ligated plasmid DNA was mixed to 200 μl of competent cells in cold polypropylene tube and then stayed on ice for 30 min. The mixture was heat shock by transferring to 42°C water bath for 45 s, followed by chilling the mixture on ice for 2 min. 800 μl of LB medium was added to the mixture

and placed in a shaking incubator at 37°C with shaking at 250 rpm for 1 hr. The mixture was centrifuged at 4,000 rpm for 1 min and then 900 µl of supernatant was removed. The transformed cells were mixed by pipetting up and down, and plated the transformation mix onto LB agar plates containing 100µg/ml ampicillin. The plate was incubated at 37°C for overnight so as to selected transformant.

6.6. Analysis of recombinant plasmid

6.6.1. Analysis by PCR

The single colony from transformed agar plate was selected in order to analysis for recombinant plasmid DNA. For analysis of a directional cloning experiment, 10 colonies were selected to screen by PCR analysis with specific designed primers; NS-F5-*Bam*HI and NS-F6-*Eco*RI. The PCR reaction was carried out in 25 µl reaction mixture in the presence of 1 single colony, 2.5 µl of 10x Taq buffer, 0.75 µl of 50 mM $MgCl_2$, 2 µl of 2.5mM each of dNTP mix, 0.25 µl of 50 pmoles/µl of each primers, 0.2 µl of 5 U/µl of Taq DNA polymerase, The distilled water was added to a final volume to 25 µl. The PCR was amplified by following thermal cycle; after incubation at 95°C for 3 min, 95°C for 1 min, 55°C for 1 min, 72°C for 1 min for 30 cycles and final extension for 10 min at 72°C. The PCR products were analyzed by electrophoresis in 1% agarose gel and detected by ethidium bromide staining and visualized under UV light.

6.6.2. Analysis by restriction enzyme digestion

The recombinant plasmid DNA was prepared from overnight cultures of single colony and using a mini-preparation procedure and verified correct insertion of the NS1 gene by restriction endonuclease digestion with *Eco*RI and *Bam*HI and/or *Hin*dIII restriction endonucleases enzymes (Promega) at 37°C for overnight. The fragmented products were analysis by electrophoresis in 1% agarose gel and detected by ethidium bromide staining and visualized under UV light.

7. Generation of recombinant bacmid

After the recombinant pFastBac HTb-NS1 plasmid has been determined to be correct, the recombinant pFastBac HTb-NS1 plasmid DNA was transformed into DH10Bac™ competent cells which contained the bacmid with a mini-attTn7 target site and the helper plasmid. The mini-Tn7 element on the pFastBac HTb plasmid could transpose to the mini-attTn7 target site on the bacmid in the presence of transposition proteins provided by the helper plasmid. Colonies containing recombinant bacmid was identified by disruption of *lacZ* gene and incubated at 37 °C for at least 24 h. Recombinant bacmid containing NS1cDNA was isolated.

7.1. Transposition

The DH10Bac™ competent cells were thawed on ice and dispense 200 µl of the cells into 15-ml round-bottom cold polypropylene tubes. Approximately 1 ng recombinant donor plasmid (in 5 µl) was added and gently mixes the DNA into the cells by tapping the side of the tube. Then, the mixture was incubated on ice for 30 min. The mixture was heat shock by transferring to 42°C water bath for 45 s, followed by chilling the mixture on ice for 2 min. The reaction was added with 800 µl of LB medium to the mixture. The mixture was placed in a shaking incubator at 37°C with shaking at 250 rpm for 4 hr. The mixture was centrifuged at 4,000 rpm for 1 min and then removed 900 µl of supernatant. The transformed cells were mixed by pipetting up and down, and plated 100 µl of the transformation mix onto LB agar plates containing 50µg/ml kanamycin, 7µg/ml gentamicin, 10µg/ml tetracycline, 100µg/ml Bluo-gal, and 40µg/ml IPTG and spread evenly over the surface. The plates were incubated for 24 to 48 h at 37°C (colonies are very small and blue colonies may not be discernible prior to 24 h).

7.2. Verify the phenotype

The white colonies containing the recombinant bacmid were selected for isolation of recombinant bacmid DNA. The white candidate colonies were streaked to fresh LB plate agar containing Bluo-gal and IPTG and antibiotics (kanamycin, gentamicin, and tetracycline) to verify the phenotype and the plates were incubated at

37°C overnight. From a single colony confirmed as having a white phenotype on LB agar plates.

7.3. PCR analysis of recombinant bacmid DNA

The presence of NS1 gene in the recombinant bacmid was verified by PCR analysis with M13 Forward and M13 Reverse primers. The PCR reaction was carried out in 25 µl reaction mixture in the presence of 1 µl of recombinant bacmid DNA, 2.5 µl of 10x Taq buffer, 0.75 µl of 50 mM $MgCl_2$, 2 µl of 2.5mM each of dNTP mix, 0.25 µl of 50 pmoles/µl of each primers, 0.2 µl of 5 U/µl of Taq DNA polymerase, The distilled water was added to a final volume to 25 µl. The reaction tubes were then place in thermal cycling. After incubation at 95°C for 3 min, the PCR was performed for 30 cycles of PCR as followed 95°C for 1 min, 55°C for 2 min, 72°C for 1 min and final extension for 10 min. at 72°C. The PCR products were analyzed by electrophoresis in 1% agarose gel and the fragments were detected by ethidium bromide staining and visualized under UV light.

7.4. Isolation of recombinant bacmid DNA

The isolated single colony was picked by using a sterile toothpick and inoculated into 5 ml LB medium supplemented with 50µg/ml kanamycin, 7µg/ml gentamicin, and 10µg/ml tetracycline. The innoculum was placed in a shaking incubator at 37°C with shaking at 250 rpm to stationary phase (up to 24 h). The culture was transferred to 1.5 ml microcentrifuge tube and centrifuge at 14,000 × g for 1 min. Then the supernatant was removed and pellet was collected. Each pellet was resuspended in 0.3 ml of Solution I (15 mM Tris-HCl (pH 8.0), 10 mM EDTA, 100 µg/ml RNase A) by pipetting up and down. 0.3 ml of Solution II (0.2 N NaOH, 1% SDS) was added and gently mixed by inverting, then incubated at room temperature for 5 min. (The appearance of the suspension should change from very turbid to almost translucent.) 0.3 ml of 3 M potassium acetate (pH 5.5) was slowly addedand then mixed gently by inverting during addition. A thick white precipitate of protein and *E. coli* genomic DNA was formed. The sample was placed on ice for 5 to 10 min and centrifuged for 10 min at 14,000×g. During the centrifugation, another microcentrifuge tube was labeled and 0.8 ml absolute isopropanol was added to this

tube. After that, the supernatant was gently transferred to the tube containing isopropanol and mixed gently by inverting tube a few times and place on ice for 5 to 10 min. The sample was centrifuged for 15 min at 14,000×g at room temperature. The supernatant was removed and added 0.5 ml 70% ethanol to each tube. The tube was inverted several times to wash the pellet and centrifuged for 5 min at 14,000×g at room temperature. The supernatant was removed as much of as possible. The pellet was air dried for 5 to 10 min at room temperature and the DNA was dissolved in 40 µl DI water. The solution was allowed to sit in the tube with occasional gentle tapping of the bottom of the tube. The DNA is generally ready to use within 10 min. The DNA was stored at –20°C.

8. Generation of recombinant baculovirus

8.1. Transfection of Sf9 insect cells

The 1×10^6 cells of Sf9 insect cells growing in mid-log phase with a viability of >97% were seeded into 35 mm petridish. The cells were allowed to attach at 27°C for at least 1 hr. The solutions were prepared as the following formula:

Solution A: For each transfection, 5 µl of recombinant AcMNPV bacmid DNA was diluted into 100 µl insect cell media without antibiotics.

Solution B: For each transfection, 6 µl CellFECTIN® reagent (Invitrogen) was diluted into 100 µl insect cell media without antibiotics.

Then, the two solutions were combined and incubated for 15 to 45 min at room temperature and 0.8 ml of insect cell media was added to the mixture solution. The cells once washed with 2 ml of insect cell media and aspirated wash media from cells and overlaid the mixture solution (diluted lipid-DNA complexes) onto the cells. The cells were incubated for 5 hr in a 27°C incubator. After that, the transfection mixtures were removed and add 2 ml of insect cell medium containing FBS and incubated cells in a 27°C incubator for 72 hr. The extracellular baculovirus was harvested from cell culture medium at 72 h post-transfection and store at 4 °C protected from light as a baculovirus stock.

8.2. Amplification of Viral stock

For amplification of viral stocks, infect a suspension culture at a multiplicity of infection (MOI) of 0.01-0.1 according to the following formula:

$$\text{Inoculum required (ml)}: \frac{\text{desired MOI (pfu/ml)} \times \text{(total number of cells)}}{\text{Titer of viral inoculums (pfu/ml)}}$$

For an MOI of 1, infect a 50 ml culture at 1×10^6 cells/ml each viral stock. The culture supernatant containing baculovirus was harvested 48 hr post-infection and clarified by centrifugation at 10,000 rpm at 4°C for 10 min usually results in 2-log amplification. The clarified supernatant was determined for virus titer and stored as virus stock at -70°C for long time.

8.3. Viral Titer

8.3.1. Viral plaque assay

The infectious potency of a stock of Baculovirus is determined by examining and counting plaque formations in an immobilized monolayer culture. Under sterile conditions, the 1×10^6 cells of cell suspension were seeded into 35 mm petridish and cells were allowed to attach at 27°C for at least 1 hr. After the 1 hr incubation, the cells were observed monolayer under the inverted microscope to confirm cell attachment and 50% confluence. The virus stock was prepared at 10^{-1} to 10^{-8} serial dilution in Grace's Insect Cell Culture Medium, supplemented, without FBS. The supernatant from each dish was removed and discarded and immediately replaced with 1 ml of the respective virus dilution then, Incubated for 4 hr at room temperature. The plaquing overlays were prepared as the following formula:

Solution 1: 80% Grace's Insect Cell Culture Medium, Supplemented, without FBS, 20% FBS and antibiotic

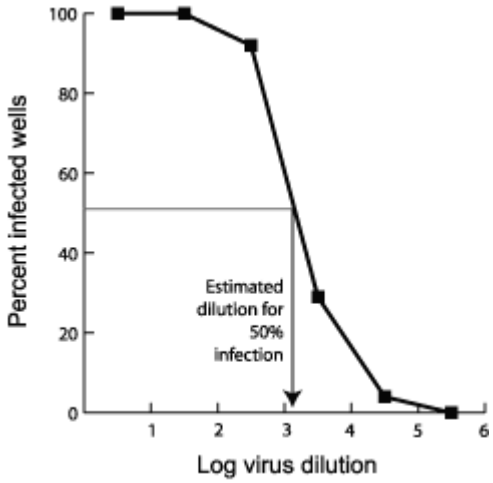
Solution 2: 1.5% Sea plaque agarose in Grace's Insect Cell Culture Medium, Supplemented, without FBS, this bottle was melted before move to a sterile hood.

Then, the two solutions were combined by transferring solution 1 to solution 2 and gently mixing and the plaquing overlay was warmed the 45°C water

bath until use. After the 4 hr incubation with virus, the virus was sequentially (from high to low dilution) removed inoculum from the dish and replaced with 4 ml of the plaquing overlays agarose. The gel was let harden for 10 to 20 min before moving the plates and the plates were incubated at 27°C in a humidified incubator for 4 to 10 days. The plates were monitored daily until the number of plaques counted was not change for 2 consecutive days.

8.3.2. End-point dilution assay

The log-phase Sf9 cells(with greater than 98% viability) were diluted to $1x10^5$ cells/ml with fresh Grace's Insect Cell Culture Medium, Supplemented with 10% FBS and seeded 100 µl of Sf9 cells to each well of 96 wells plate. The cells were allowed to attach firmly about 10 min. The virus stock was prepared at 10^{-1} to 10^{-8} serial dilution in Grace's Insect Cell Culture Medium, Supplemented, without FBS and 20 µl of each virus dilution was sequentially (from high to low dilution) added to each well. The cells were incubated at 27°C for 4 to 7 days and examined for signs of infection. Endpoint dilution assays provide a basis for the calculation of virus titers in which this is done is shown below:



The 50% infectious dose ($TCID_{50}$ or tissue culture 50% infectious dose) is calculated using a mathematical analysis of the data. The $TCID_{50}$ /ml are converted to pfu/ml (plaque forming unit/ml) by mathematical program of Reed and Munch (1938).

9. Expression of recombinant NS1 protein

9.1. Optimizing protein expression

To express the recombinant NS1 protein, insect cells were infected with the recombinant Baculovirus. Cultures should be infected while in the mid-logarithmic phase of growth; infect the suspension culture at a cell density of 1×10^6 cells/ml with a Multiplicity of Infection (MOI) of 0.1, 1 and 5. Infect duplicated flasks at each of the following MOIs: 0.1, 1 and 5. Maintain one set of flasks as uninfected growth controls. Samples were taken from at 24, 48, 72, and 96 h post-infection were collected to compare morphologies and cell densities of infected cultures against non-infected controls to confirm progress of infection. Total and viable cells were counted. Cell pellets and supernatants were determined for the recombinant NS1 proteins expression from the recombinant Baculovirus. The optimal MOI and the harvest time that produced the highest product yield and quality/homogeneity were selected and these infection parameters were used for large scale production.

9.2. Preparation of infected cell lysate

The 50 ml suspension culture of Sf9 insect cells was infected at a cell density of 1×10^6 cells/ml with MOI of 1. The suspension was cultured at 27°C at a constant stirring rate of 120 rpm for 72 hr. The infected cells were collected by centrifugation at 10,000 rpm at 4°C for 10 min and then culture supernatant was removed and discarded. The infected cells pellet was washed twice in cold PBS and lysed by RIPA buffer (1% Triton X-100, 0.5% sodium deoxyclolate, 0.1% SDS in PBS pH 7.2) for 2 hr with continuously mixed by vertex. After 2 hr, the pellet was sonicated for 10 seconds with 30% pulse for 3 cycles. The mixture was clarified by centrifugation at 10,000 rpm at 4°C for 10 min to pellet the cells debris. The supernatant was transferred to further analysis by SDS-PAGE analysis and western blot analysis and use directly for purification under Immobilized metal affinity chromatography (IMAC).

9.3. Sodium Dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed to separate and determine the molecular weight of the recombinant NS1 protein. The protein samples were separated by SDS-PAGE in slab gel apparatus containing 5% stacking gel and 15% resolving gel in reducing condition. The samples were denatured by mixing with 2X loading buffer (50 mM Tris-Cl pH 6.8, 4% SDS, 20% glycerol, and 0.02% bromophenol blue) and heating at 95 °C for 5 min. Electrophoresis was performed in vertical direction in electrode buffer (25 mM Tris, 192 mM glycine, and 0.1% SDS) with constant voltage of 100 volts from cathode to anode until dry front reached the bottom of the gel. The separated proteins were stained with Coomassie Brilliant Blue, allowing visualization of the separated proteins.

9.4. Western blot anlysis

The Sf9 infected cells lysate were electrophoretically separated in SDS-PAGE and transferred onto nitrocellulose membranes (Satorious, Goettingen, Germany) in Towbin's buffer (25 mM Tris, 192 mM glycine, 20% v/v methanol) for 1 hr. After blotting, membranes were blocking with 3% BSA in PBST for 1 hr at room temperature and washed 3 times with PBST for 5 min each. The membrane was further reacted to rabbit polyclonal to influenza A virus NS1 (H5N1) (abcam) diluted at 1:200 in 1% BSA in PBST for 2 hr. After washing, the membrane was incubated with 1:2,000 diluted goat anti-rabbit conjugated alkaline phosphatase (ALP) in 1% BSA in PBST, as the secondary antibody, for 1 hr and washed again with PBST. Finally, the reaction was visualized by adding chromogenic substrate solution containing 2 ml of 4CN (4-cholro-1-Naphthol) (Sigma-aldich) substrate in methanol, 10 ml of 4CN diluents (10mM Tris-Cl pH 7.5, 150 mM NaCl) and 51.5 µl of 3% H_2O_2 for approximately 1 hr in dark. The antigen-antibody complexes were seen as dark brown band on the membrane.

10. Purification of recombinant NS1 protein

10.1. Talon affinity resin

The purification was based on the TALON® metal affinity resin (Clontech). The TALON Resin was thoroughly resuspended and transferred the 2 ml of resin suspension to a sterile tube. The resin was pellet by centrifugation at 700 x g for 2 min and removed and discarded the supernatant. The Co^{2+} resin was pre-equilibrated with 10 bed volumes of 50 mM sodium phosphate buffer, 300 mM NaCl at pH 7.5 for twice. The prepared lysate Sf9 cells were transferred to pre-equilibrated Co^{2+} resin and incubated with gentle agitation at 4°C for 1 hr on a platform shaker to allow the polyhistidine-tagged protein to bind the resin then centrifuged at 700 x g for 5 min and carefully removed as much supernatant as possible without disturbing the resin pellet. The bound resin was washed 5 times with 10-20 bed volumes of wash buffer consisting of 50 mM sodium phosphate buffer, 300 mM NaCl at pH 7.5 containing 5 mM imidazole with gently agitate the suspension at 4°C for 10 min on a platform shaker to promote thorough washing. The recombinant NS1 protein was eluted by the addition of 2 ml of elution buffer consisting of 50 mM sodium phosphate buffer, 300mM NaCl at pH 7.5 containing 150 mM imidazole and incubated for 10 min each for 4 times. Purified recombinant NS1 protein-His was dialyzed overnight in phosphate-buffered saline (PBS) and concentrated by ultrafiltration at 4 °C with YM-10 filter (MW cut-off at 10,000; Millipore). The concentration was calculated using the Bio-Rad protein assay (Bio-rad) with BSA as a standard.

11. Mouse immunization

Two female BALB/C mice were first immunized with 20 μg of purified recombinant NS1 protein with complete Freund's adjuvant by intraperitoneal injection. Three boosters of 10 μg of purified recombinant NS1 protein with incomplete Freund's adjuvant were given at 4 weeks interval. The blood samples were collected to test bleed for identify and monitoring of anti-NS1 protein antibody level. When an increasing antibody titer was detected in mouse sera either by indirect ELISA or western blot analysis, mice were last boosted with purified recombinant

NS1 protein in phosphate buffer saline (PBS) by intravenous injection three days before fusion.

12. Production of hybridoma clones producing anti-NS1 monoclonal antibodies

12.1. Preparation of the mouse spleen cells

Three days after the last boost, the individual mouse was sacrificed. The spleen was removed aseptically and ground to separate spleen cells in serum free RPMI-1640. The spleen cells suspension were prepared in 50 ml sterile centrifuged tube. The spleen cells were washed with serum free RPMI-1640 3 times at 300 x g for 5 min at 4°C and the viable spleen cells concentration was determined by trypan blue staining and counting in a heamatocytometer.

12.2. Preparation of Myeloma cells

Approximately 2 weeks before fusion remove one vial of myeloma cells Ag 8.653 from the liquid nitrogen freezer, thaw rapidly at 37 °C, and plate myeloma cells in a flask containing complete-media. Myeloma cells were cultured in RPMI-1640 supplemented with 20% FBS at 37°C with 80% humidity and 5% CO_2. On fusion day, the healthy myeloma cells from culture flasks were pooled and harvested in 50 ml sterile centrifuged tube. The myeloma cells were washed with serum free RPMI-1640 3 times at 300 x g for 5 min at 4°C and the viable myeloma cells concentration was determined by trypan blue staining and counting in a heamatocytometer.

12.3. Fusion procedure

The myeloma cells were mixed with spleen cells at the ratio of: Spleen: Ag 8.653 = 2:1 and the mixed cells were washed with serum free RPMI-1640 at 300 x g for 5 min at room temperature. The supernatant was removed as completely as possible. The dropwise 1.0-1.5 ml of PEG-3000-3700 pre-warm at 37°C was slowly added into the pellet while gently resuspended the cells by stirring the tube by hand within 1 min then, the cells were stand for 30 s. The 5 ml of pre-warm serum free RPMI-1640 was slowly add dropwisely over the first 5 min followed by 15-20 ml. The

tube was centrifuged at room temperature at 300 x g for 5 min and the supernatant was removed and discarded. The cells were resuspended in pre-warm HAT medium at 37°C to a concentration of 2-4.5×10^5 cells/ml. The cells suspension were gently mixed and subsequently plated 150 µl/well into 96 well culture plates using multi-channel pipette. The plates were incubated in a tissue culture incubator at 37°C with 80% humidity and 5% CO_2. After fusion 7-10 days, the 75 µl of supernatant was removed from each well and discarded, followed by added 75 µl of fresh complete media (HT) containing feeder cells and further incubated at 37°C with 80% humidity and 5% CO_2. The supernatant was removed and replaced with complete media after 2-3 days and complete media was changed every 2-3 days. The growth of hybridoma cells were observed under microscope when hybridoma cells grow about 2-3 mm, three days after last feeding the supernatant those culture supernatants were collect to screening for specific antibody to NS1 protein by Indirect ELISA.

12.4. Single cell cloning

Once the positive culture has been identified, then the cells from the selected wells were clone by modified limiting dilution method in 96 well tissue culture plate. The cells were determine growth in well and the 1-5 µl of cell suspension from the well was diluted in 10 ml complete media containing feeder cells and plated 1 drop into each well of the former six columns and 2 drops into the later six columns (using 10 ml seropipette, 1 drop from 10 ml pipette is 75 µl). The 1 drop of feeder cells were plated into each well of the plates and the plates were leaved in incubator at 37°C with 80% humidity and 5% CO_2 undisturbed for 7 days, and then the plates were screened for single colony growth.

13. Immunodetection for determination of anti-NS1 antibody

13.1. Western blot analysis for determination of anti-NS1 in mouse sera or hybridoma culture supernatants

The mouse sera and/or hybridoma culture supernatants were analyzed their specific binding to NS1 protein by Western blot analysis as mentioned above. In order to assess the ability of the MAb to react with NS1 antigen, lysate obtained from MDCK cells infected with influenza A viruses were used as antigen. Both of lysate

MDCK infected with influenza A and purified rNS1 protein were separated in SDS-PAGE and blotted onto nitrocellulose membrane. The membranes were cut into strips and reacted with mouse sera diluted at 1:1,000 and 1:4,000 and/or hybridoma culture supernatants diluted at 1:10 in diluents (1% BSA in PBST) and/or MAbs. Therefore, RNA binding domain (RBD) was kindly provided by Dr. Porntippa Lekcharoensuk, Department of Microbiology and Immunology, Faculty of Veterinary medicine, Kasetsart University, also used as antigen for Western blot analysis. The RBD was reacted with both selected MAbs. Then, the membrans were incubated with 1:2,000 diluted goat anti-mouse IgG, IgA, IgM conjugated alkaline phosphatase (ALP), as the secondary antibody. The antigen-antibody complexes were seen as dark brown band on the strips by adding substrates BCIP (KPL) and incubated in dark for 15 min.

13.2. ELISA for determination of anti-NS1 in mouse sera or hybridoma culture supernatants

13.2.1. NS1 ELISA

Microtitre plates (Nunc Maxisorb) were coated with 50 µl of 1 µg/ml of purified rNS1 protein in 50 mM carbonate buffer (pH 9.6) followed by incubating at 4°C overnight. After blocking with 3% BSA in PBST at 37°C for 1 hr, mouse sera diluted at 1:1,000, 1:5,000 and 1:10,000 or hybridoma culture supernatants diluted at 1:5 in 1% BSA in PBST was added to each well and further incubated at 37°C for 30 min. Each well was 5 times washed with PBST (0.05% Tween 20 in PBS). The 50 µl of 1: 10,000 goat anti-mouse IgG conjugated horse-radish peroxidase (HRP) diluted in 1% BSA in PBST was added to each well and incubated at 37°C for 30 min. Each well was 5 times washed with PBST again and then TMB (Sureblue^{TM} TMB Microwell peroxidase substrate) (KPL) was added and incubated at room temperature for 30 min in dark. After 30 min, an equal volume of 0.6N sulfuric acid was added to stop color reaction. The optical absorbance (OD) was measured at 450/630 nm using an automated plate reader.

13.2.2. Antibody captured ELISA

The Antibody captured ELISA was used to screen the hybridoma clones secreting MAb to NS1 protein in order to select the clones that containing different

properties from clones that positive by the NS1 ELISA. Microtitre plates (Nunc Maxisorb) were coated with 50 µl of 0.5 µg/ml of anti-mouse IgG, IgA, IgM in 50 mM carbonate buffer (pH 9.6) followed by incubating at 4°C overnight. After blocking with 3% BSA in PBST at 37°C for 1 hr, hybridoma culture supernatants diluted at 1:5 in 1% BSA in PBST was added to each well and further incubated at 37°C for 30 min. Each well was 5 times washed with PBST. The 50 µl of 1:500 NS1 conjugated biotin diluted in 1% BSA in PBST was added to each well and incubated at 37°C for 30 min. Each well was 5 times washed with PBST. The 50 µl of 1: 2,000 streptavidin conjugated horse-radish peroxidase (HRP) was added to each well and further incubated at 37°C for 30 min. Each well was 5 times washed with PBST again and then TMB (KPL) was added and incubated at room temperature for 30 min in dark. After 30 min, an equal volume of 0.6N sulfuric acid was added to stop color reaction. The optical absorbance (OD) was measured at 450/630 nm using an automated plate reader.

14. Determination of immunoglobulin isotype

The isotype of each Mab was determined by ELISA. Microtitre plates (Nunc Maxisorb) were coated with 50 µl of 0.5 µg/ml of anti-mouse IgG, IgA, IgM in 50 mM carbonate buffer (pH 9.6) followed by incubating at 4 °C overnight. The plate was 5 times wash with PBST and blocking with 3% BSA in PBST at 37°C for 1 hr and then hybridoma culture supernatants diluted at 1:10 in 1% BSA in PBST was added to each well and further incubated at 37°C for 30 min. After washing, 50 µl of each dilution of rabbit anti-mouse immunoglobulin isotype IgG1, IgG2a, IgG2b, IgG3, Kappa and Lambda was added to each well and further incubated at 37°C for 30 min. The plate was washed and 1:2,000 anti-rabbit conjugated-HRP was added into each wells and incubated at 37°C. For IgM and IgA isotype, goat anti-mouse IgM conjugate-HRP and goat anti-mouse IgA conjugate-HRP dilution 1:10,000 were added to each well. After 30 min incubation and washing, the complexes were visualized by adding TMB (KPL) and incubated at room temperature for 30 min in dark. The color reaction was stopped by 0.6N sulfuric acid. The optical absorbance (OD) was measured at 450/630 nm using an automated plate reader.

15. Purification of monoclonal antibody

The monoclonal antibodies were purified using two steps, precipitation and affinity chromatography.

15.1. Precipitated by ammonium sulfate precipitation

First, the hybridoma culture supernatants were centrifuged at 3,000g at 4°C for 30 min then transfer to clean container and add a stirring bar and place on a magnatic stirrer. The protein in solution were precipitated by slowly add saturated ammonium sulfate solution in an equal volume and transfer to 4°C for overnight. The solutions were centrifuged at 10,000g at 4°C for 20 min. The pellets were collected and resuspended in 0.1 volumes of the starting volume in PBS and dialyze versus three changes of PBS overnight. The monoclonal antibodies were collected and centrifuged at 10,000g at 4°C for 10 min and store at -20°C.

15.2. Purification of monoclonal antibody

Protein G Sepharose is designed for purification and isolation of monoclonal and polyclonal IgG from ascites, serum and cell culture supernatants. Therefore, the IgG monoclonal antibodies were purified by Affinity chromatography on a HiTrap Protein G HP (GE Healthcare) according to the manufacturer's instructions. The sample monoclonal antibodies were adjusted to the composition of the binding buffer by diluting the sample with binding buffer (20 mM sodium phosphate, pH 7.0) and then filtration immediately before loading on the column to remove particulate material (0.45 μm filter). The binding buffers were filtered by passing them through a 0.45 μm filter before use them to fill the system then the column was connected to the syringe (with the provided adaptor) by "drop to drop" to avoid introducing air into the column. The column was washed with 10 column volumes of binding buffer at flow rate 1 ml/min. The sample was then applied to column at flow rate 1 ml/min and collected flow through at 6 min after. The column was washed with 5–10 column volumes of binding buffer or until no material appears in the effluent. The IgG monoclonal antibodies were eluted with 2–5 column volumes of elution buffer (0.1 M glycine-HCl, pH 3) at flow rate 0.5 ml/min. The 500 μl

fractions were collected into collection tubes prepared by adding 50 µl 1 M Tris-HCl, pH 9.0.

16. Epitope comparison

16.1. Antibody biotinylation (biotin-conjugated MAb)

The purified monoclonal antibody was conjugated with EZ-Link NHS-PEO Solid Phase Biotinylation Kit (Pierce Biotechnology) according to the manufacturer's instructions. Briefly, 0.2 mg of purified MAb was coupled to the appropriate volume of PBS and NHS-PEO_4 Biotin for 30 min at room temperature. The labeled MAB was eluted with 200 µl of elution buffer by centrifugation for 2 times. The labeled MAB was dialyzed against PBS for overnight at 4°C and stored at 4°C for short or at -70°C for long-term storage.

16.2. Competitive analysis of monoclonal antibodies

Antigenic specificity of monoclonal antibody was compared in competitive ELISA (cELISA). Microtitre plates (Nunc Maxisorb) were coated with 50 µl of 1 µg/ml of purified recombinant NS1 protein in 50 mM carbonate buffer (pH 9.6) followed by incubating at 4°C overnight. The nonspecific binding sites were blocked with 3% BSA in PBST at 37°C for 1 hr. The unlabeled antibodies in a concentration 2 µg/ml was added into each well together with biotin-conjugated MAb in dilution giving OD 1.0-1.5 at 450 nm and further incubated at 37°C for 30 min. The results were showed as inhibition degree of the binding of the biotin-conjugated MAb with recombinant NS1 protein coated plate at the present of unlabeled monoclonal antibodies. Each well was 5 times washed with PBST and The 50 µl of 1: 2,000 streptavidin conjugated horse-radish peroxidase (HRP) was added to each well and further incubated at 37°C for 30 min. Each well was 5 times washed with PBST again and then TMB (KPL) was added and incubated at room temperature for 30 min in dark. After 30 min, an equal volume of 0.6N sulfuric acid was added to stop color reaction. The optical absorbance (OD) was measured at 450/630 nm using an automated plate reader.

17. NS1 sandwich ELISA

The MAb were applied to the development of sandwich ELISA for detection of NS1 antigen in approach that differentiated between infected animal from vaccinated animal or NS1 as infection marker. Microtitre plates (Nunc Maxisorb) were coated with 50 µl of optimal dilution of anti-NS1 as capture MAb in 50 mM carbonate buffer (pH 9.6) at 4°C overnight. All washes between incubation steps were performed with PBST for 5 times and all dilutions were done in 1%BSA in PBST. After washing, the plates were blocked by incubation with 200 µl of 3% BSA in PBST at 37°C for 1 h, washed, and incubated with 50 µl of purified rNS1 or lysate of MDCK cells infected with 4 isolates each of H1N1 or H3N2 influenza A viruses at 37°C for 30 min. The plates were then washed and 50 µl of anti-NS1 as detector MAb was added and the mixture was incubated at 37°C for 30 min, washed, and further incubated with 50 µl of 1: 2,000 streptavidin conjugated-HRP or 1:2,000 anti-mouse IgA conjugated-HRP at 37°C for 30 min. After final wash, the plates were incubated with TMB (KPL) at room temperature for 30 min in dark. Reactions were stopped by the addition of 50 µl of 0.6N sulfuric acid and the optical absorbance (OD) was measured at 450/630 nm using ELISA reader.

18. Immunoperoxidase staining

The cells were infected with the swine influenza virus (strain no. SIV.21.1) was kindly provided by Dr. Porntippa Lekcharoensuk, Department of Microbiology and Immunology, Faculty of Veterinary medicine, Kasetsart University. The infected cells were fixed onto the slide, and store at-20°C until use. The slide was chilled at room temperature for 5 min, All washes between incubation steps were performed with PBS for 5 times and all dilutions were done in 1%BSA in PBS. After washing, the slide was blocked with 1% BSA in PBS and incubated at room temperature in moist chamber for 30 min. Then wash, and incubated with MAb at concentration 2 µg/ml diluted in PBS for 30 min, wash and incubated with 1:500 anti-mouse IgG, IgA, IgM conjugated alkaline phosphatase (ALP) diluted in PBS for 30 min. The antigen-antibody complexes were seen as dark brown as observed under microscope by adding substrates BCIP (KPL) and incubated in dark for 15 min.

CHAPTER V
RESULTS

1. Preparation of nonstructural protein 1(NS1) gene

To generate the nonstructural protein 1 (NS1) gene, the viral RNA was extracted from whole genome of influenza A virus strain H5N1. The extracted viral RNA was used as a template for reverse transcribed in order to generate NS1cDNA and then amplified by PCR on a thermal cycle with specific designed primers; NS-F5-*Bam*HI and NS-R6-*Eco*RI to convert the NS1 segmented RNA into full-length NS1 gene. The primers were designed to contain the *Bam*HI restriction site at the 5' end of forward primer; NS-F5-*Bam*HI and *Eco*RI restriction site at the 5' end of reverse primer; NS-R6-*Eco*RI. The PCR product of NS1 gene was 690 bp which 5' end and 3' end of the NS1 gene compose of *Bam*HI restriction site and *Eco*RI restriction site, respectively. The PCR product was purified by using purification Kit and cut with restriction endonuclease enzyme *Bam*HI and *Eco*RI. Then, the cut fragmented DNA was purified by using QIA quick Gel Extraction Kit. Finally, the NS1 gene had sticky end at 5' end and 3' end with restriction endonuclease enzyme *Bam*HI and *Eco*RI site, respectively.

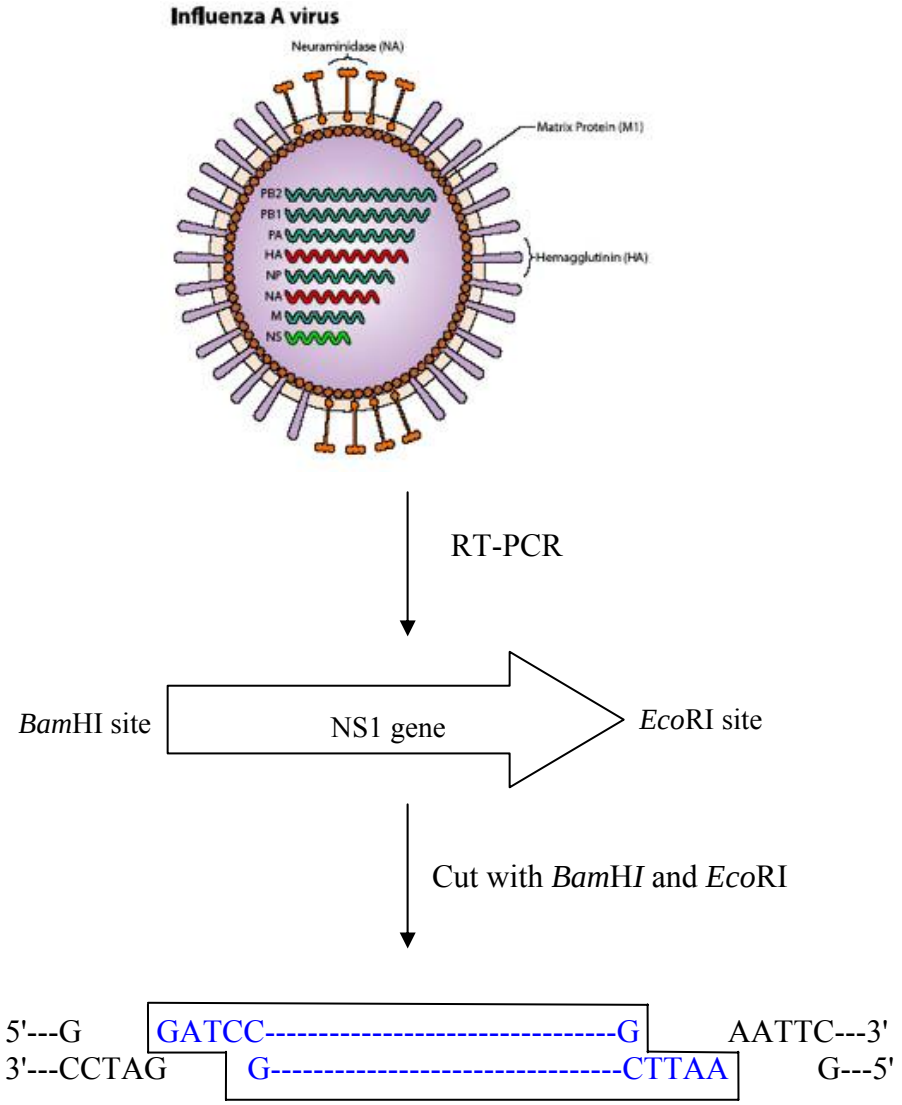


Figure 7 Generation of the NS1 gene with the sticky end. NS1 gene insert cut with *Bam*HI and *Eco*RI restriction endonuclease enzymes from whole genome of H5N1 influenza A virus by RT-PCR.

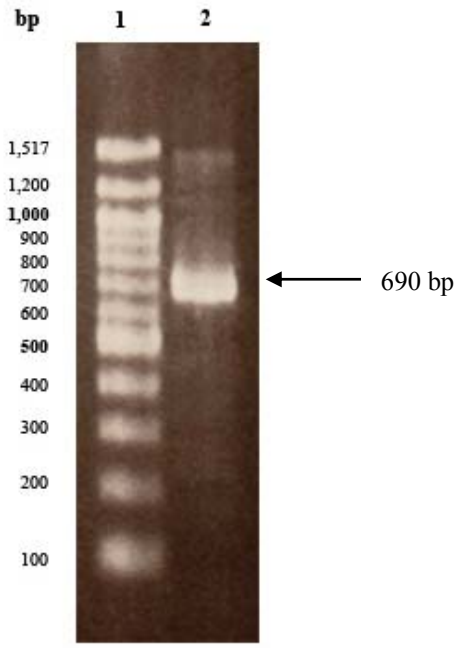


Figure 8 The PCR products from RT-PCR showed the nucleotide 690 base pairs identify to full-length NS1 gene. Lane 1: 100 bp DNA Ladder marker, lane 2: NS1 gene PCR product, 690 bp.

2. Preparation of recombinant pFastBac HTb plasmid

The pFastBac HTb plasmid was transformed into the *E.coli* strain DH5α cells in order to propagate plasmid in bacterial cells. The *E.coli* strain DH5α containing pFastBac HTb Donor plasmid was grown overnight and then the plasmid DNA was isolated by plasmid miniprep. Kit. The plasmid DNA was cut with the restriction endonuclease enzyme; *Bam*HI and *Eco*RI enzyme and then purified using QIA quick Gel Extraction Kit. Finally, the plasmid DNA had sticky end at 5' end and 3' end with restriction endonuclease enzyme *Bam*HI and *Eco*RI site, respectively. The NS1 gene DNA was cloned into pFastBac HTb plasmid in order to generate recombinant plasmid containing a polyhistidine-tag (6xHis) at the N-terminal. Consequence that, the NS1 gene and the pFastBac HTb plasmid already containing sticky end with restriction sites of *Bam*HI and *Eco*RI at 5' end and 3' end, respectively were ligated with T4 DNA ligase enzyme. Then, the ligation reaction was transformed into the competent *E.coli* DH5α cells and cultured onto LB agar plate containing ampicillin at 37°c overnight. Colonies which grown on LB agar plate were selected and cultured again into LB broth containing ampicillin and recombinant plasmid DNA was extracted with plasmid miniprep Kit.

The extracted recombinant plasmid (recombinant pFastBac HTb-NS1) was checked for the inserted NS1 gene by PCR with specific primers; NS-F5-*Bam*HI and NS-R6-*Eco*RI. Both primers are specific to NS1 gene insert of recombinant pFastBac HTb plasmid. The PCR product was 690 bp (Figure 10) which identical to the size of NS1 gene as shown on the agarose gel electrophoresis. This indicated that the NS1 gene was inserted into the recombinant pFastBac HTb-NS1 plasmid. Then, the restriction analysis was performed to confirm the NS1 gene inserted of the recombinant pFastBac HTb-NS1 plasmid. The cutting site of each enzyme was shown in Figure 11 and the expected size of DNA after cut with each enzyme was shown in the Table 3. The recombinant pFastBac HTb-NS1 plasmid was cut with restriction endonuclease enzyme, *Bam*HI, *Eco*RI and *Hin*dIII enzymes (Figure 12).

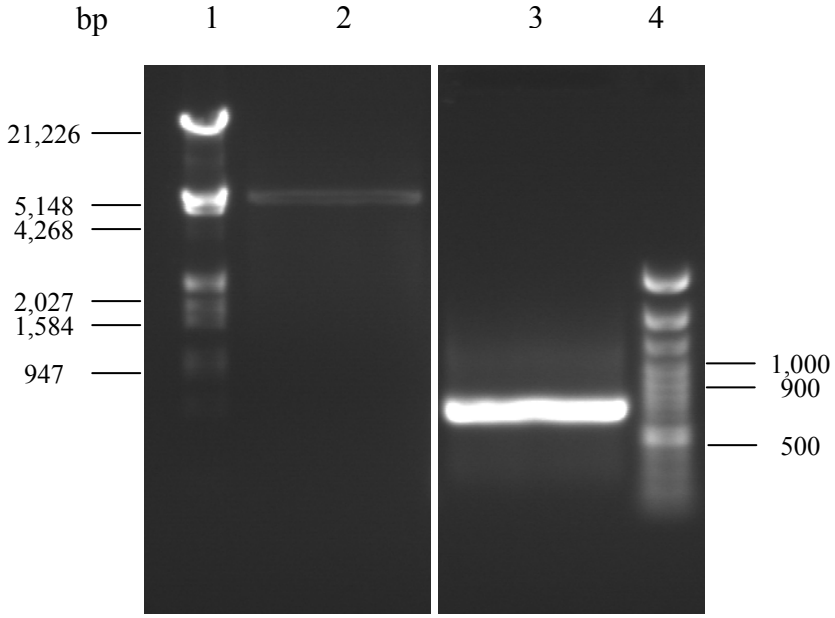


Figure 9 The gel electrophoresis of pFastBac HTb and NS1 gene insert cut with *Eco*RI and *Bam*HI enzymes. Lane 1: standard DNA marker (λDNA cut with *Eco*RI/*Hin*dIII), lane 2: pFastBac HTb plasmid DNA cut (4856 bp), lane 3: NS1 gene insert cut (690 bp), lane 4: 100 bp DNA Ladder marker.

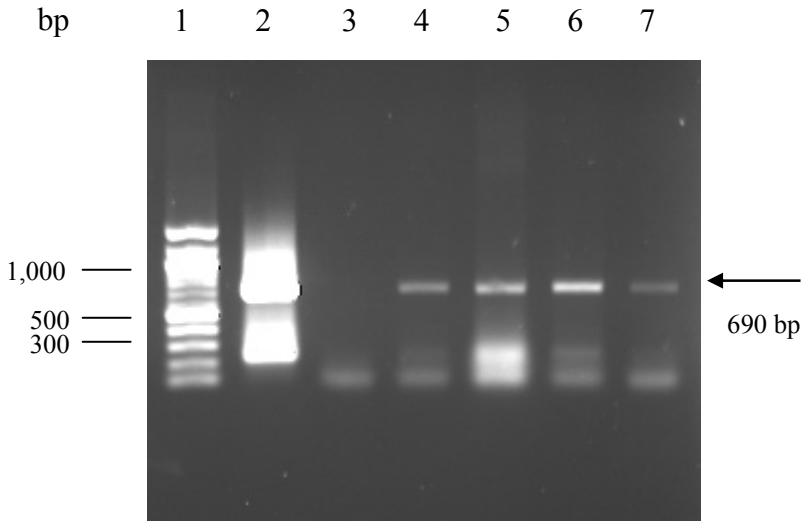


Figure 10 A agarose gel electrophoresis of PCR products from recombinant pFastBac HTb-NS1 plasmid. Lane 1: 100 bp DNA Ladder marker, lane 2: positive control NS1 gene as templates, lane 3: negative control, lane 4-7: PCR products from the bacterial colonies showed nucleotides band size 690 identical to NS1 gene.

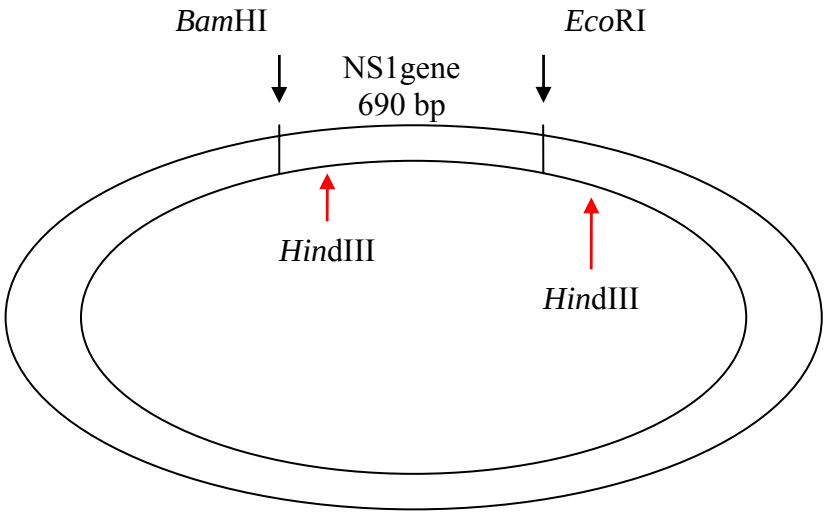


Figure 11 The restriction sites of recombinant pFastBac HTb-NS1 plasmid.

Table 3 The site of DNA after cut the recombinant pFastBac HTb-NS1 plasmid with restriction endonuclease enzymes

Restriction endonuclease enzymes	Size of DNA after cut with enzyme (bp)	
	Recombinant pFastBac HTb-NS1 plasmid	pFastBac HTb plasmid (negative control)
*Bam*HI and *Eco*RI	4,856 and 690	4,856
*Hin*dIII	4,780 and 740	4,856

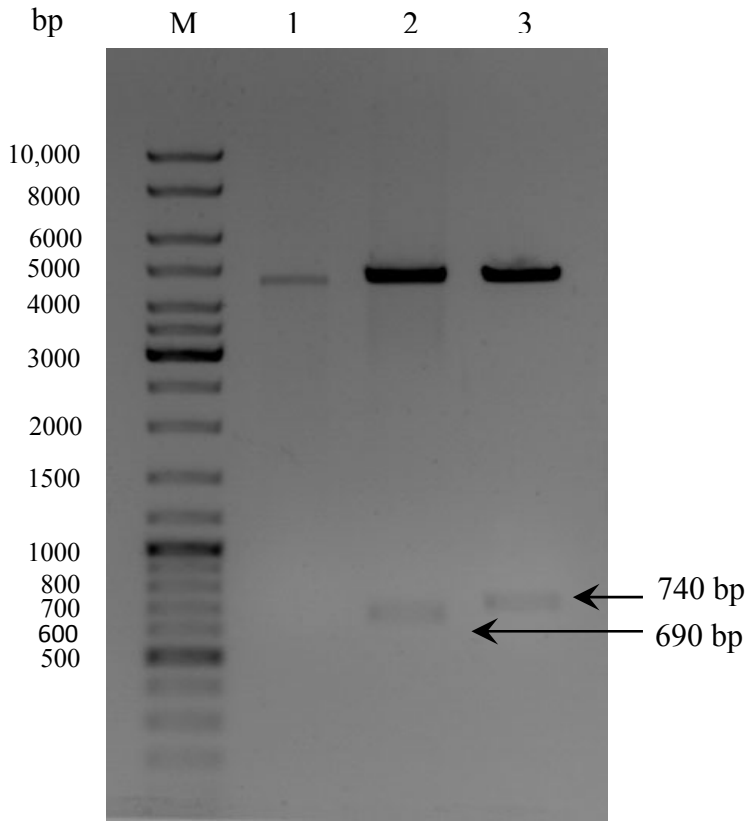
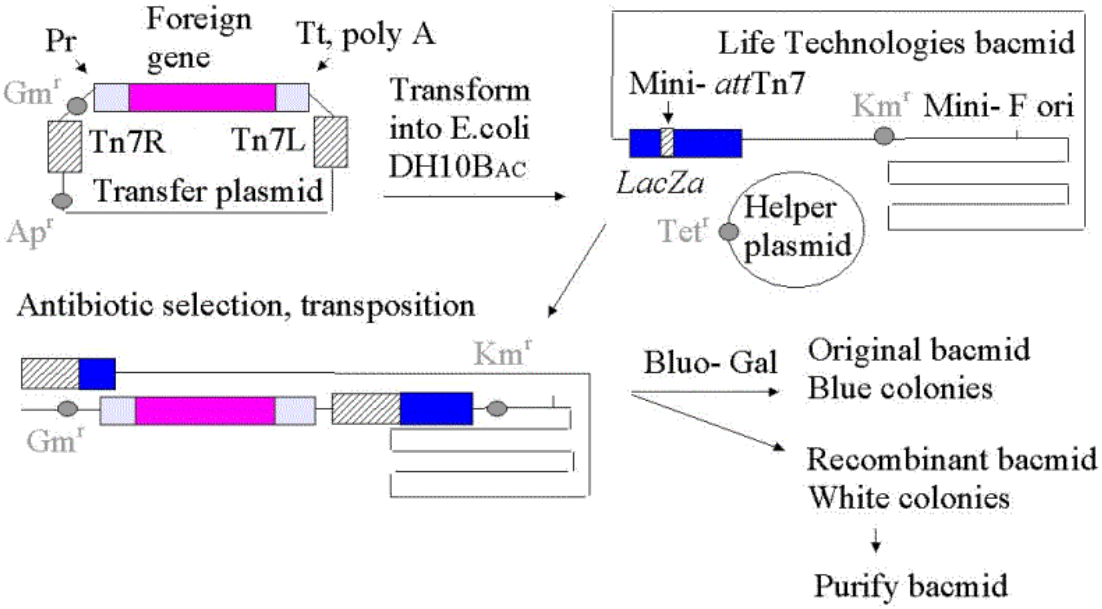


Figure 12 The recombinant pFastBac HTb-NS1 plasmids were cut with different restriction endonuclease enzymes *Bam*HI / *Eco*RI and/or *Hin*dIII. Lane M: GeneRuler™ DNA Ladder Mix (Fermentus), lane 1: negative control (pFastBac HTb) showed 4,856 bp, lane 2: recombinant pFastBac HTb-NS1 plasmid cut with *Bam*HI and *Eco*RI showed 4,856 and 690 bp, lane 3: recombinant pFastBac HTb-NS1 plasmid cut with *Hin*dIII showed 4,780 and 740 bp.

After the restriction endonuclease enzyme analysis, the recombinant pFastBac HTb-NS1 plasmid showed DNA size corresponding to expected DNA size in Table3 by agarose gel electrophoresis. Together with PCR, these indicated that the NS1 gene was successfully cloned into the recombinant pFastBac HTb-NS1 plasmid which containing a polyhistidine-tag (6xHis) at the N-terminal.

3. Generation of recombinant AcMNPV bacmid DNA

It is based on site-specific transposition of an expression cassette into a baculovirus shuttle vector (bacmid) propagated in *E. coli*. The bacmid (bMON14272) contains the low-copy-number mini-F replicon, a kanamycin resistance marker, and a segment of DNA encoding the *lacZ*α peptide from a pUC-based cloning vector. Inserted into the N-terminus of the *lacZ*α gene is a short segment containing the attachment site for the bacterial transposon Tn7 (mini-*att*Tn7) that does not disrupt the reading frame of the *lacZ*α peptide. The bacmid propagates in *Escherichia coli* DH10Bac™ as a large plasmid that confers resistance to kanamycin and can complement a *lacZ* deletion present on the chromosome to form colonies that are blue (Lac+) in the presence of a chromogenic substrate such as Bluo-gal or X-gal and the inducer IPTG (Figure 13).



http://strubiol.icr.ac.uk/extra/baculovirus/introduction.html

Figure 13 Generation of recombinant AcMNPV bacmid DNA. The recombinant plasmid is transformed into DH10Bac™ competent cells which contain the bacmid with a mini-*att*Tn7 target site and the helper plasmid. The mini-Tn7 element on the pFastBac™ donor plasmid can transpose to the mini-*att*Tn7 target site on the bacmid in the presence of transposition proteins provided by the helper plasmid. Colonies containing recombinant bacmids are identified by disruption of the *lacZ*α gene.

The recombinant pFastBac HTb-NS1 plasmids confers resistance to gentamicin were transformed into competent DH10Bac™ *E.coli* Cells containing a baculovirus shuttle vector (bacmid) confers resistance to kanamycin with a mini-*att*Tn7 target site and a helper plasmid, pMON7124 (13.2 kb). Recombinant bacmids were constructed by transposing a mini-Tn7 element from a recombinant pFastBac HTb-NS1 plasmid to the mini-*att*Tn7 attachment site on the bacmid. The Tn7 transposition functions were provided *in trans* by a helper plasmid which encodes the transposase and confers resistance to tetracycline. Therefore, the transformation mix was plated onto LB agar plates containing gentamicin, kanamycin, tetracycline, Bluo-gal and IPTG and nncubated for 24 to 48 h at 37°C. Insertions of the mini-Tn7 into the mini-*att*Tn7 attachment site on the bacmid disrupted the expression of the *lacZ*α peptide, so colonies containing the recombinant bacmid were been white in a background of blue colonies that harbor the unaltered bacmid (Figure 14). White colonies were selected for analysis.

The phenotypes of white colonies were verified by selecting and restreaked them on fresh LB agar plates containing kanamycin, gentamicin, tetracycline, Bluo-gal, and IPTG. The plates were incubated overnight at 37°C. Then a single white colony on restreaked plates containing Bluo-gal and IPTG was inoculated in a liquid culture containing kanamycin, gentamicin, and tetracycline. The recombinant bacmid DNA was isolated using minipreparation method and analyze by PCR analysis to veryify the presence of NS1 gene in the recombinant bacmid. Since recombinant bacmid DNA is greater than 135 kb in size, restriction analysis is difficult to perform. The bacmid contains M13 sequence flanking mini-*att*Tn7 site. The presence of NS1 gene at attachment site in the recombinant bacmid was verified by PCR using *Taq* polymerase, the M13 Forward (-40) and M13 Reverse primers and a combination of the M13 Forward (-40) or M13 Reverse primers and a primer specific for NS1 gene insert were used as primer pairs to produce amplification product whether transposition has occurred. If transposition has occurred, the expected PCR product shown in Table 4 should be observed on the agarose gel. As a result, the amplification of the recombinant AcMNPV bacmid using each primer pair was shown corresponding to expected size by agarose gel analysis (Figure 16).

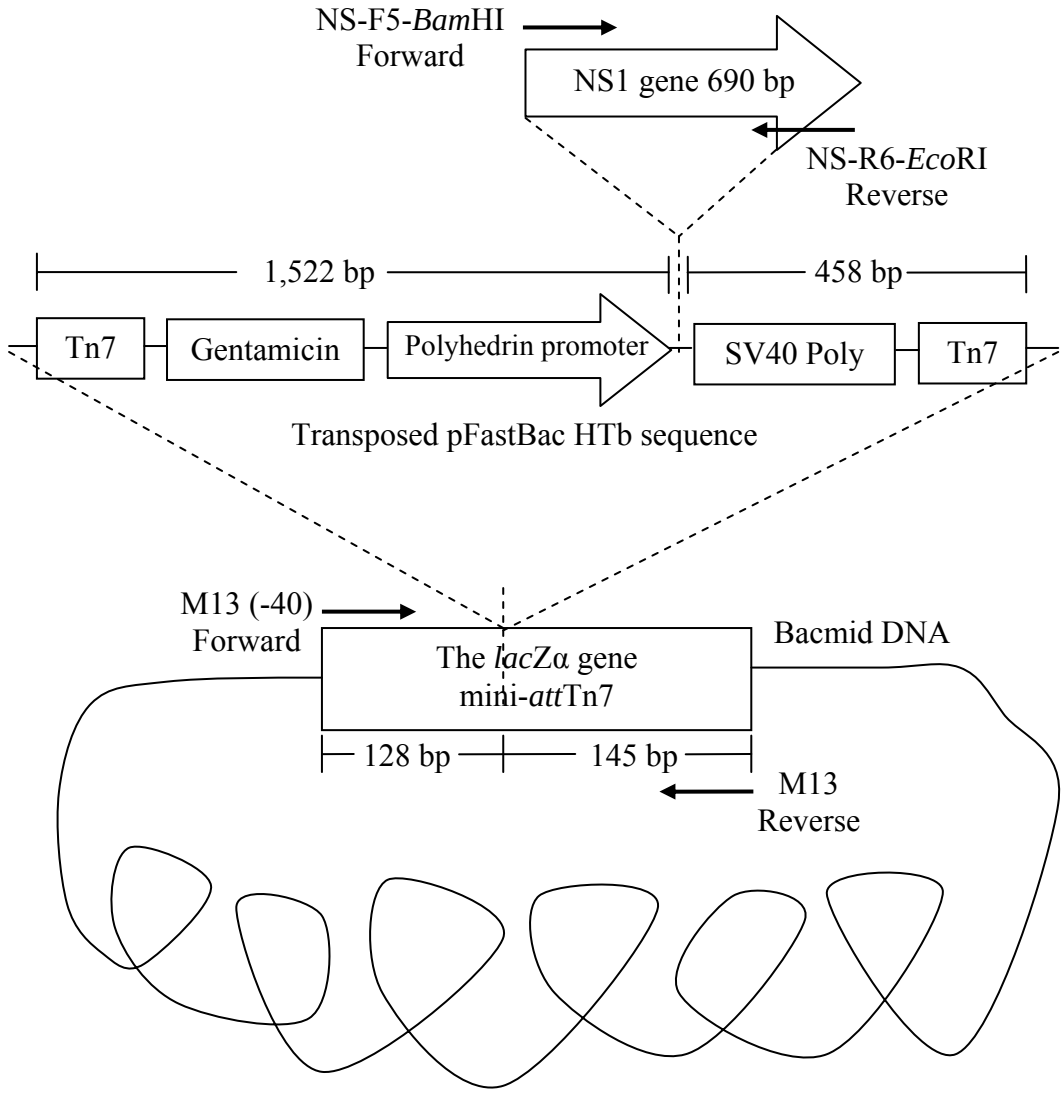


Figure 14 Transposition Regions. The bacmid contains M13 Forward (-40) and M13 Reverse priming sites flanking the mini-*att*Tn7 site within the *lacZ*α-complementation region to facilitate PCR analysis.

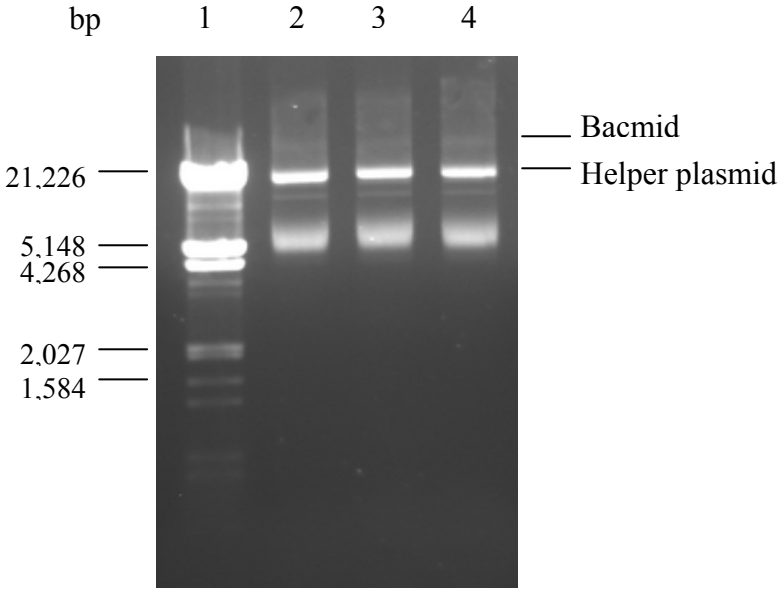


Figure 15 Agarose gel analysis of mini-prep recombinant AcMNPV bacmid DNA. Lane 1: standard DNA marker (λ DNA cut with *Eco*RI/*Hin*d III, lane 2-4: Mini-prep of high-molecular-weight (bacmid) DNA.

Table 4 The expected results from PCR to verify the presence of NS1 gene in the recombinant bacmid when use the recombinant AcMNPV bacmid and AcMNPV bacmid as template.

The primer using in PCR reaction	The PCR product size from recombinant AcMNPV bacmid as template (bp)	The PCR product size from AcMNPV bacmid as template (bp)
NS-F5-BamHI and NS-R6-EcoRI	690	-
NS-F5-BamHI and M13R	1293	-
M13F and NS-R6-EcoRI	2340	-
M13F and M13R	2943	273

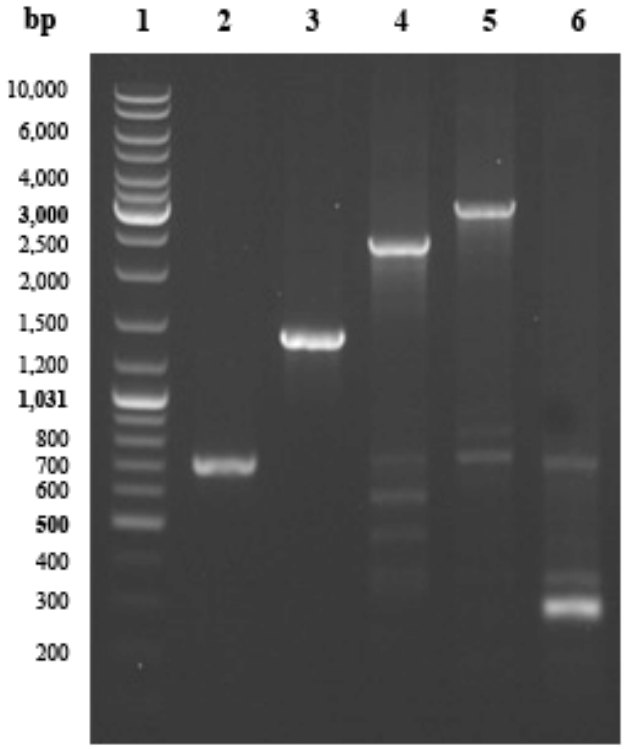


Figure 16 Agarose gel analysis of PCR products that used for verification of the presence of NS1 gene in the recombinant AcMNPV bacmid. Lane 1: 1 Kb ladder DNA marker, lane 2: NS-F5-BamHI and NS-R6-EcoRI *690 bp*, lane 3: NS-F5-BamHI and M13R *1293 bp*, lane 4: M13F and NS-R6-EcoRI *2340 bp*, lane 5: M13F and M13R *2943 bp*, lane 6: negative control (AcMNPV bacmid) M13F and M13R *273 bp*.

4. Generation of recombinant AcMNPV baculovirus

A baculovirus expression system (Bac-to-Bac; Life Technologies) was used to express recombinant AcMNPV baculovirus.

4.1 Generation of low-titer viral stock

To generate low-titer viral stock, transfection of Sf9 insect cells with the recombinant bacmid DNA was performed. The recombinant AcMNPV bacmid containing NS1 gene was introduced into Sf9 insect cells by transfection with CellFECTIN, the cationic-lipid formulated reagent designed for optimal transfection of insect cells. The hydrophobic region of CellFECTIN is not a large lipid, therefore it should not get in the way as much when complexing with nucleic acids. Because of this, it binds DNA more tightly and not as toxic to the cell.

The reagent forms particle sizes structures leading to interact spontaneously with DNA to form lipid-DNA complexes. This lipid DNA complex fuses with the plasma membrane of tissue culture cells, resulting in efficient uptake and expression of the DNA. CellFECTIN entraps the DNA, the DNA/ CellFECTIN complex allows the DNA to enter the cells and into the nucleus via a direct route through the cell cytosol as opposed to endocytosis. The extracellular virus progeny of recombinant AcMNPV baculovirus released from the cell by budding. Polyhedra-derived virus appears at 18 hr post-infection and accumulates in the nuclei of infected cells 72 hr post-infection or until cellular lysis. Therefore, virus was harvested from cell culture medium at 72 hr post-transfection.

4.2 Amplification of recombinant AcMNPV baculovirus and generation of a high-titer viral stock

To determine and confirm the viral titer, viral plaque assay and/or end-point dilution assay were used. The virus stock was then amplified before the analyzed protein expression. The culture supernatant containing baculovirus was harvested at 72 hr post-infection usually results in 2-log amplification of baculovirus stock and clarified by centrifugation at 10,000 rpm at 4°C for 10 min. The clarified supernatant was determined for virus titer by viral plaque assay and End point dilution assay. The gold viral stock was 1.95×10^{8} pfu/ml.

5. Production of recombinant NS1 protein

The recombinant NS1 protein was expressed in a fusion form with 6xHistidine tag in baculovirus expression system. The predicted size of fusion protein recombinant NS1 protein was 31 kDa consisting of the 26 kDa native NS1 proteins and 5 kDa of 6xHistidine tag. The recombinant NS1 protein was confirmed the identity by Western blot using commercial rabbit polyclonal to influenza A virus NS1 (H5N1), a band size 31 kDa corresponding to the size of recombinant NS1 protein was observed (Figure 17).

5.1. Expression of recombinant NS1 protein

The optimal infection conditions to express recombinant NS1 protein was that a Sf9 insect cells suspension culture was infected with recombinant baculovirus at a Multiplicity of Infection (MOI) of 1 and the protein expression was assayed upon harvesting the infected cells at 72 hr post-infection (Figure 18 A and B). The SDS-PAGE of Sf9 infected cells showed a protein band of 31 kDa resolved by 15% polyacrylamide gel with reducing condition corresponding to the calculated fusion protein recombinant NS1 size. The recombinant NS1 protein band 31 kDa was presented in the ACMNPV-NS1 infected Sf9 cells lysate but not in the mock-infected cells lysate. Moreover, the intensity of protein band also indicated a good level of expression of the recombinant NS1 protein (Figure 19).

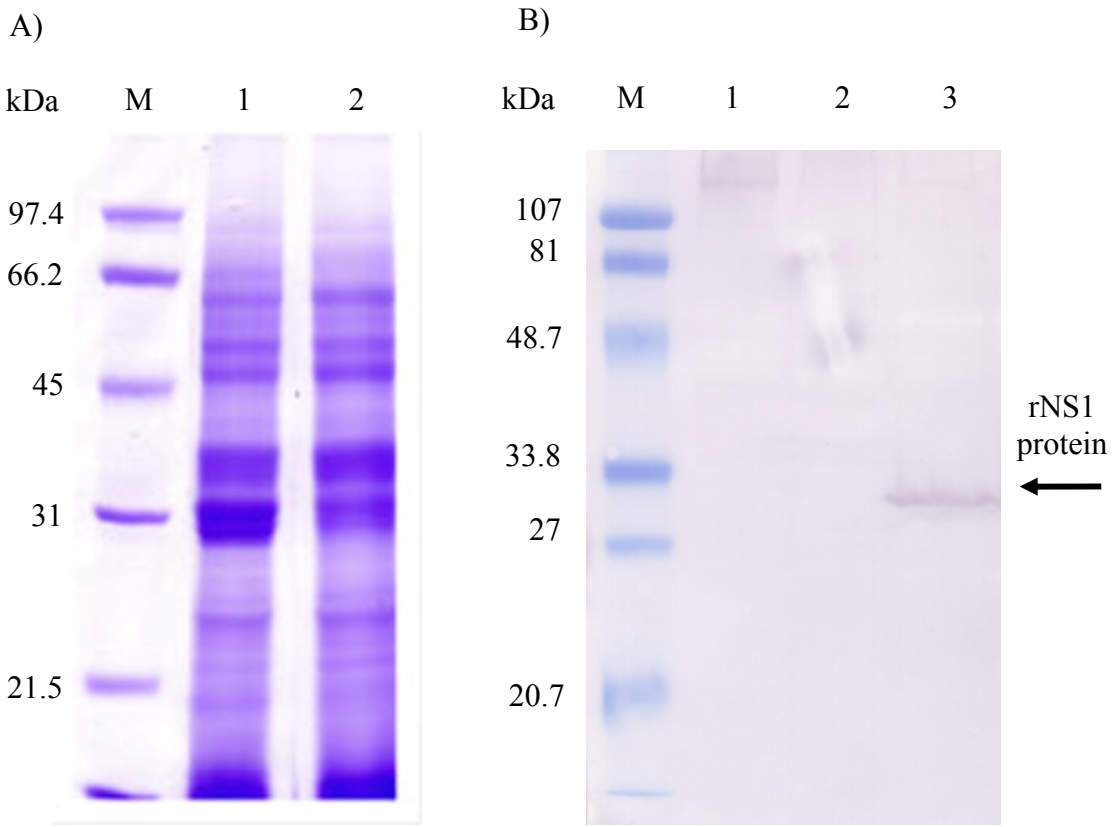


Figure 17 A) SDS-PAGE of the ACMNPV-NS1 and mock infected Sf9cell lysate. Crude lysate from ACMNPV-NS1 infected (lane 1) and mock- infected cells (lane 2), stained with Coomassie brilliant blue R250, protein molecular weight markers (lane M). B) Western blot analysis of recombinant NS1 protein and mock-infected and uninfected Sf9 cells control. Crude lysates from uninfected Sf9 cells control (lane 1), and mock-infected (lane 2), and ACMNPV-NS1 infected (lane 3). Crude lysates were separated by SDS-PAGE and then transferred onto nitrocellulose membrane and Western bloting with comercial rabbit polyclonal antibody to influenza A virus NS1 (H5N1) dilution 1: 1000. Note that 31 kDa protein was detected only in the ACMNPV-NS1 infected Sf9 cells lysate but not other lysates.

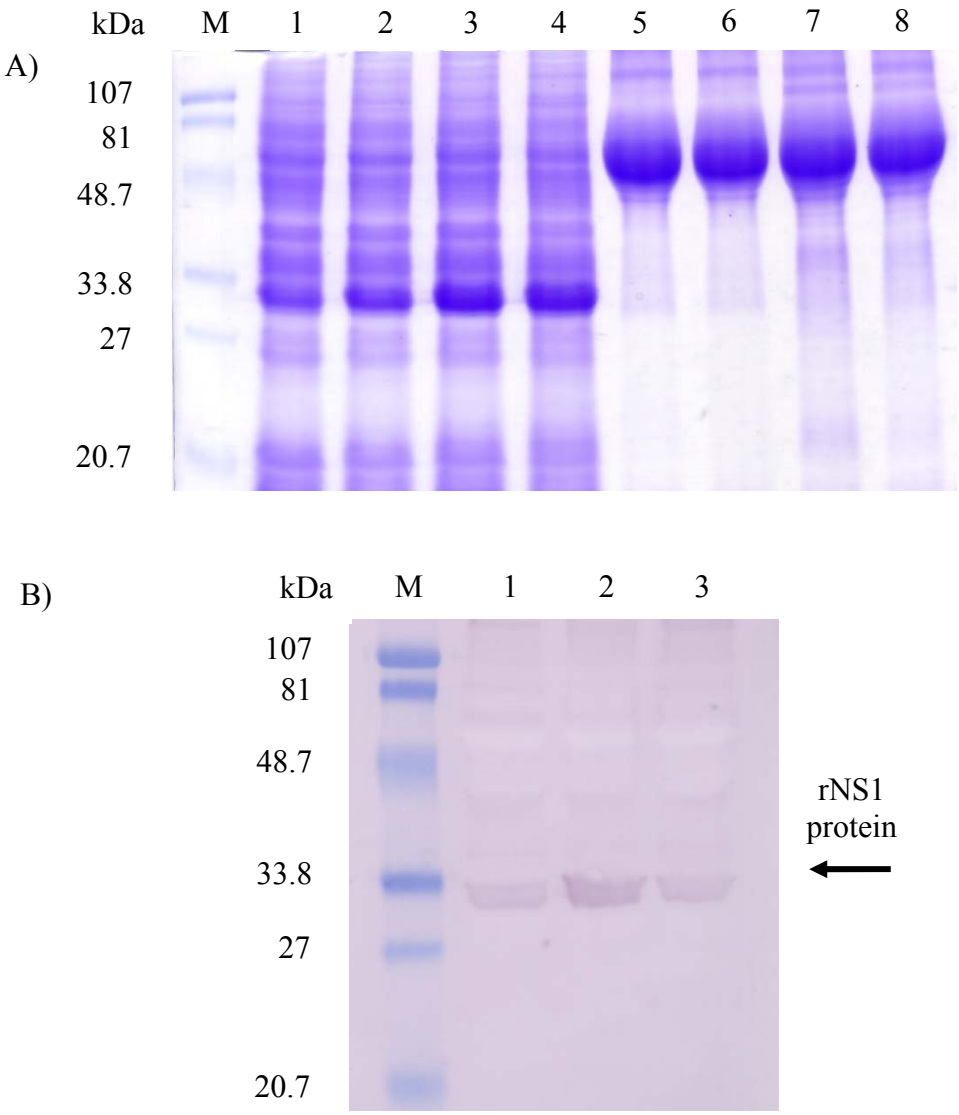


Figure 18 MOI optimization and time course. Infect a population of cells at MOIs 0.1, 1 and 5 and assay protein expression upon harvesting the infected cells. A) Harvest cells and culture media at the following time intervals: 48 h, 72 h. and subjected to SDS-PAGE (Coomassie brilliant blue staining). Crude extract in RIPA buffer show in lane 1-4 and culture media show in lane 5-8. MOI of 0.1, harvest time at 48 hr.(1,5) MOI of 1, harvest time at 48 hr (2,6) MOI of 0.1, harvest time at 72 hr.(3,7) MOI of 1, harvest time at 72 hr.(4,8). B) Western bloting with comercial rabbit polyclonal antibody to influenza A virus NS1 (H5N1). Lane M: standard molecular weight, lane 1: MOI of 0.1, lane 2: MOI of 1, lane 3: MOI of 5. Note that at MOI of 1, harvest time at 72 hr expressed recombinant NS1 protein more than other MOIs.

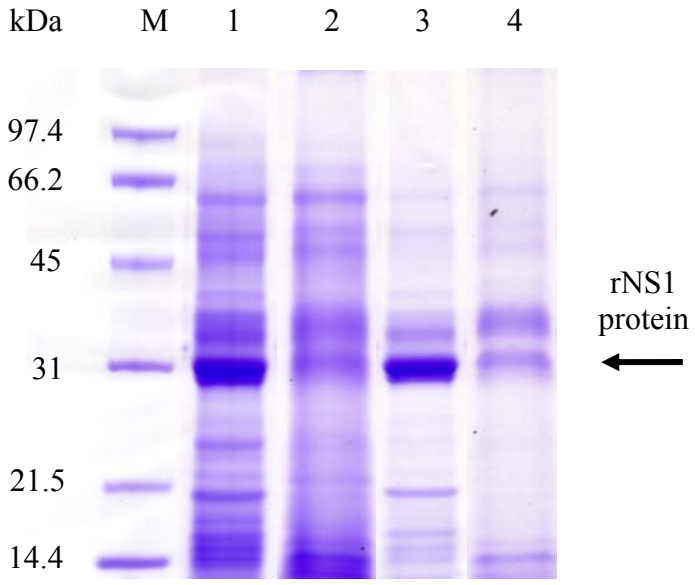


Figure 19 SDS-PAGE of the ACMNPV-NS1. Crude lysate from ACMNPV-NS1 infected Sf9 cells and mock infected Sf9 cells lysate after treated with RIPA buffer (lane 1and 2) and after sonication (lane 3 and 4), respectively. The recombinant NS1 protein band 31 kDa was presented in the ACMNPV-NS1 infected Sf9 cells lysate. Sf9 cells were subjected to SDS-PAGE under reducing conditions. The gel was subsequently stained with Coomassie brilliant blue R-250 to visible the bands. The standard Low- range molecular weight marker (lane M).

6. Purification of recombinant NS1 protein using immobilized metal affinity chromatography

IMAC is a popular affinity-purification method to purify recombinant protein. The principle is based on the reversible interaction between various amino acid side chains such as cysteine, and tryptophan and especially, histidine and immobilized transition metal ions (Ni^{2+}, Co^{2+}, and Zn^{2+}). The recombinant protein is exposed to immobilized metal ions ligated to Sepharose beads. After binding, non-specific adsorption of protein is washed and the target protein is eluted with imidazole. The bound polyhistidine-tagged protein is competitively eluted by simply adding imidazole to the elution buffer, because imidazole is identical to the histidine side chain.

The recombinant NS1 protein containing 6xHistidine tags on the N-terminus was engineered to give advantage in purification under Immobilized metal affinity chromatography (IMAC). Purification of NS1 protein was initially performed using Co^{2+}-based IMAC techniques. The recombinant NS1 protein was eluted with 150 mM imidazole and then validated the authenticity with SDS-PAGE analysis (Figure 20). The purity and authenticity of the purified proteins was confirmed with western blot analysis. The purified NS1 protein appeared as a single band with an apparent molecular weight of 31 kDa recognized by the mouse polyclonal anti-NS1 protein antibody. The yields of recombinant NS1 proteins expressed and purified by this method was approximately 1 mg per liter of culture and no impurities were co-eluted with the NS1 protein.

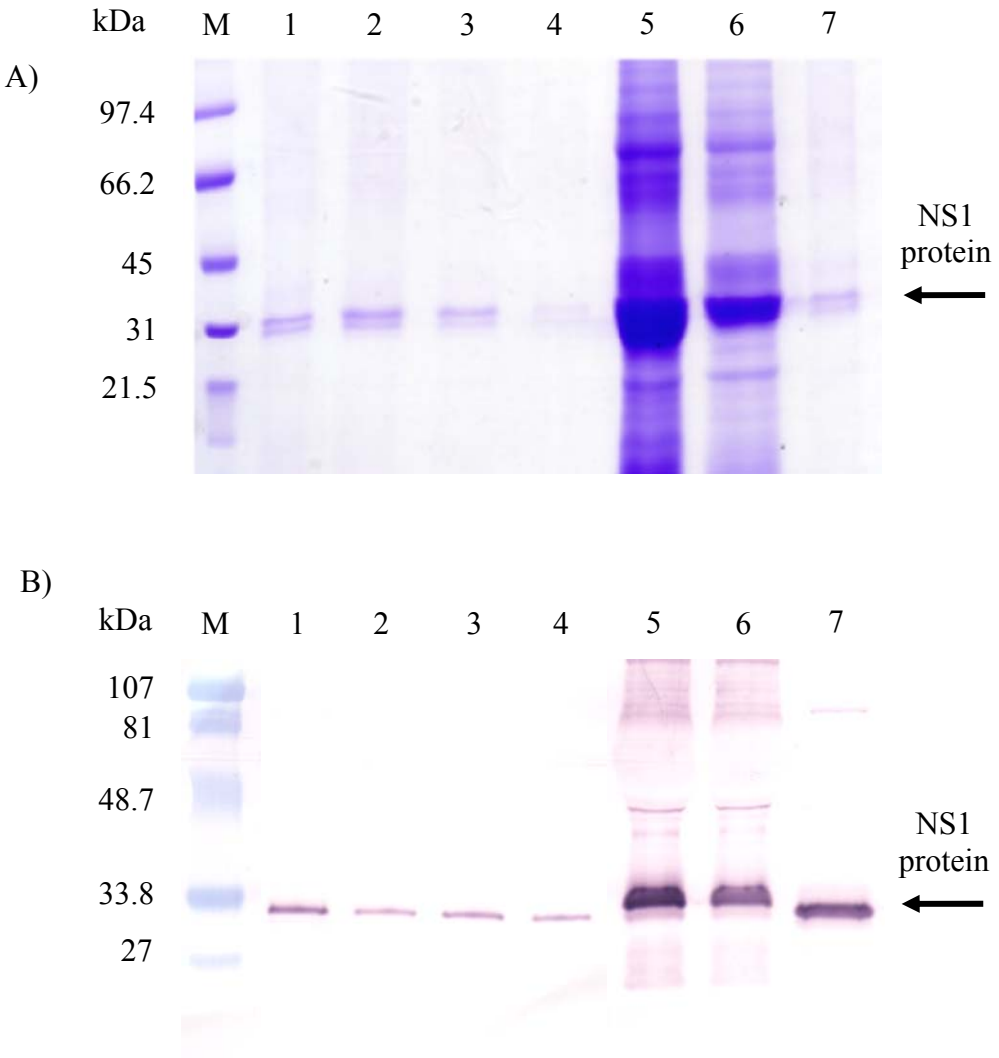


Figure 20 SDS-PAGE (A) and Western blot analysis (B) of recombinant NS1 protein purified by Immobilised metal affinity purification of recombinant NS1 protein with TALON Co^{2+} beads. Molecular weight markers (lane M), elution with 150mM imidazole lanes 1–4, crude lysate of ACMNPV-NS1 infected Sf-9 cell following incubation with TALON resin (lane 5), unbound other protein from TALON resin (lane 6), bound protein on the beads (lane 7). The eluted of protein with an expected size of 31 kDa, indicated that no other proteins were observed after elution from the beads.

7. Production of hybridoma clones producing anti-NS1 protein MAb

7.1. Mice immunization and detection of anti-NS1 protein antibody in immunized mouse sera

To produce anti-NS1 protein monoclonal antibody in this study, the purified recombinant NS1 protein (rNS1) was used as immunogen to immunize BALB/c mice. Following the immunization procedure (see section 11 in Materials and Methods), Mice were immunized with 20 ug of immunogen. The test bleed was performed to monitor the antibody level that specified to NS1 protein by purified rNS1-based indirect ELISA. The result of ELISA in mouse sera at each dilution indicated that a specific anti-NS1 antibody titers were significantly increased in the bleed 2nd (which was taken 2 weeks after the 2nd immunization), compared to the bleed 1st (which was taken 2 weeks after the 1st immunization) and pre-immune serum (which was taken before immunization) in mouse sera as show in Figure 21 (Both mice showed the same results of rNS1-based indirect ELISA), reflecting the immune responses to NS1 protein in both mice after boosting.

In addition, immunized mouse sera were further analyzed for NS1 protein specificity by Western blot analysis using ACMNPV-NS1 Sf9 infected cell lysate as antigen as shown in Figure 22. Pre-immune sera of mice gave no reaction band (data not shown). While, the bleed 2nd showed strong reactivity to both ACMNPV-NS1 Sf9 infected cell lysate and purify rNS1 protein. When mice were demonstrated antibody specific to NS1 protein, the fusion was then designed. Both mice were finally boosted with purified rNS1 protein, mouse that showed high anti-NS1 antibody titer was therefore sacrificed and collected the spleen cells for fusion with myeloma cells.

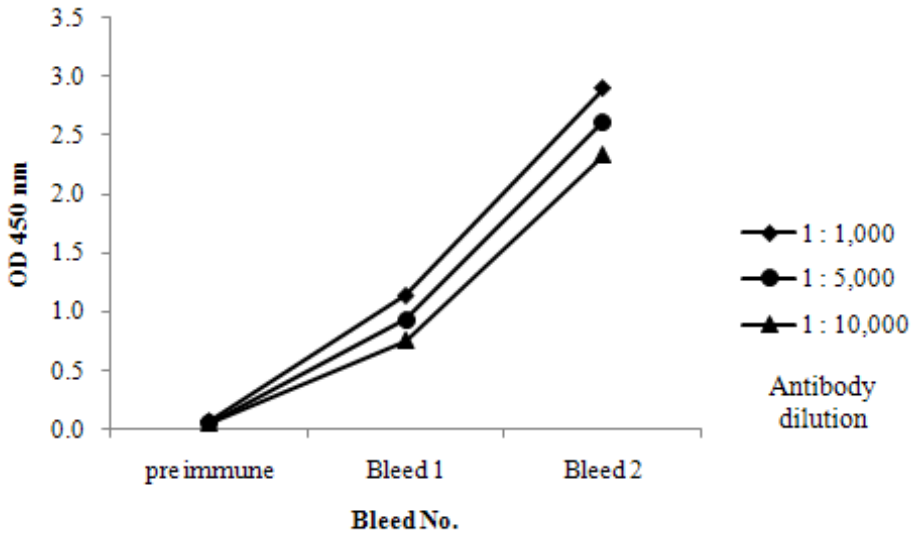


Figure 21 Detection of anti-NS1 antibody responses in immunized mouse sera by indirect ELISA. Immunized mouse sera from various bleeding (pre-immune, bleed^{1st}, bleed^{2nd}) were diluted to 1:1,000, 1:5,000 and 1:10,000 , then analyzed by using purified recombinant NS1 protein coated-indirect ELISA.

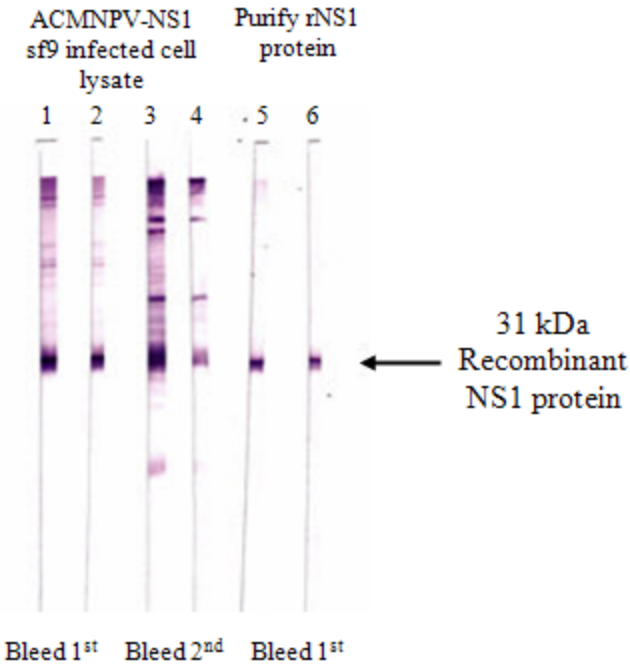


Figure 22 Detection of anti-NS1 antibody responses in immunized mouse sera by Western blot analysis. The Sf9 infected cell lysate and purify recombinant NS1 protein were electrophoretically separated, blotted onto nitrocellulose membrane, and then cut into strips. Each strip was reacted with immunized mouse sera at dilution 1:1,000. Lane 1 and lane 5: Bleed 1st from mouse No.1, lane 2 and lane 6: Bleed 1st from mouse No.2, lane 3: Bleed 2nd from mouse No.1, lane 4: Bleed 2nd from mouse No.2, respectively

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7.2. Selection of hybridoma cells producing anti-NS1 protein antibodies.

Three days after the final boosted, fusion of mouse spleen cells and mouse myeloma cells was then accomplished to achieve hybridoma clones in 96 wells tissue culture plates. The hybridoma clones producing anti-NS1 antibody were screened by indirect ELISA which was performed in two formats of indirect ELISA in order to select different properties of positive hybridoma clones. More than 700 hybridoma clones were obtained, and the fusion efficiency was calculated as 40% of total culture wells. We selected the wells that containing only single or two hybridoma clone for initial screening. Total 564 hybridoma supernatants were collected for both indirect ELISA assays. The results showed positive for 46 hybridoma clones that were reactive to NS1 antigen; 7 for NS1 ELISA, 18 for antibody captured ELISA and 21 for both assays.

The hybridoma clones in each positive well were further subcultured for single cell cloning by modified limiting dilution method. After first limiting dilution, 26 hybridoma clones were expanded and containing their NS1-specific antibody activity which tested by two assays of indirect ELISA. From 26 hybridoma clones, we collected 8 representative supernatants of each hybridoma clone for indirect ELISA and found that 14 hybridoma clones with 8 out of 8 supernatants (100%) were positive by ELISA, indicated that the 14 hybridoma clones were single cell hybridoma clone. Consequently, those 12 hybridoma clones which were not single cell clone (positive percentage was lower than 100%) were subcultured by modified limiting dilution method again. After second limit dilution, 1 hybridoma clone died. The others, 11 clones were tested for their specificity and single cell cloning by ELISA. It found that all 11 clones were positive by ELISA (Table 5). The results demonstrated that all the hybridoma clones were single cell clone.

During expanded hybridoma cells, 5 single cell hybridoma clones died. Therefore, all 20 single cells hybridoma clones were further tested for their affinity to rNS1 protein by using two assays of indirect ELISA; NS1 ELISA and antibody captured ELISA. The affinity ratio was calculated from OD_{450} nm of rNS1 at 0.2μg/ml divided by OD_{450} nm of rNS1 at 1μg/ml for NS1 ELISA and from OD_{450} nm of NS1 conjugated biotin at dilution 1:1,000 divided by OD_{450} nm of NS1 conjugated biotin at

dilution 1:200 for Antibody captured ELISA. The NS1 ELISA affinity ratio showed 3 hybridoma clones with the highest ratio namely 4F12H8H3, 2E2B1 and 18B1E9. These three clones were selected to expand and conjugate with biotin for further experiments. Surprisingly, the Antibody captured ELISA affinity ratio gave a lower ratio (in between 0.2 to 0.5) in all hybridoma clones (Table 5). However, they all 20 hybridoma clones were picked up for storage and expansion for further experiments.

8. Characterization of the anti-NS1 MAbs obtained from the selected hybridoma clones

All 20 hybridoma clones secreting MAb to NS1 antigen were selected and then culture the hybridoma clones to produce the MAb of each clone in midi-scale (50-100 ml) for the following characterization.

8.1 Immunoglobulin isotype

The MAbs from all 20 hybridoma clones were determining their isotype by ELISA isotype kit. The results demonstrated that the generated MAbs composed of various types of immunoglobulin; IgG1, IgG2a, IgG2b, IgA and IgM as shown in Table5.

8.2. Epitope comparison

Since the generated MAbs were selected by rNS1 antigen by two assays of indirect ELISA, therefore we further investigated whether they could bind to different epitopes. The MAb binding to different epitopes could be used as MAb pair for development of sandwich ELISA assay. The biotin were used to conjugate with 3 selected MAbs from previous experiment; 4F12H8H3, 2E2B1 and 18B1E9. The three MAbs were selected because they were highest positive in the NS1 ELISA could act as detector antibody for the further experiment.

After three MAbs were purified and conjugated with biotin, they were analyzed by competitive ELISA (cELISA) with all 20 MAbs. The results showed that only MAb 2E2B1 (NS1.1) was able to inhibit itself; biotin-conjugated MAb 2E2B1 (B-NS1.1) from binding to rNS1 coated plate and no cross-blocking of B-NS1.1 with other MAbs, indicating its unique binding epitope of MAb NS1.1. Whereas the other

two biotin-conjugated MAb 4F12H8H3 and 18B1E9 were not inhibited the binding by all MAbs even if themselves, suggesting that they may binding to NS1 antigen in not different epitopes.

8.3. Specific binding activity

As the positive result by indirect ELISA did not give the information about NS1 protein in the influenza virus-infected cells of monoclonal antibody secreting from selected hybridoma clones, therefore, All 20 generated MAbs were determined by Western blot analysis with lysate MDCK cells infected with influenza A virus and purified rNS1 protein. The results showed that 18G11H5, 3B11G6C11 and 2E2B1 reacted with both lysate MDCK cells infected with influenza A virus (H1N1 and H3N2) and purify rNS1 protein. Whereas 5B2A1 reacted with lysate MDCK cells infected with influenza A virus (H1N1 and H3N2). The 18H8H3E3 reacted only with purify rNS1 protein. The 5C1H11B8 reacted with only lysate MDCK cells infected with H1N1 but no H3N2 influenza A virus. The other 14 MAbs were no reactive band with both lysate MDCK cells infected with influenza A virus (H1N1 and H3N2) and purify rNS1 protein by Western blot analysis (Table 5).

The specific binding reactivity of monoclonal antibodies was examined whether the MAbs NS1.1 and MAb NS1.2 can recognize RNA binding domain of NS1 protein from avian influenza virus (RBD) by Western blot analysis. Both MAb NS.1 and MAb NS1.2 gave a specific reactivity with recombinant RBD by Western blot analysis (Figure 23B). This result indicated that both selected MAbs have specific reactivity not only full length NS1 protein but also RNA binding domain of avian influenza A virus. However, both MAbs would react against a linear epitope conformed by Western blot analysis using full length NS1 protein and RBD of NS1 protein.

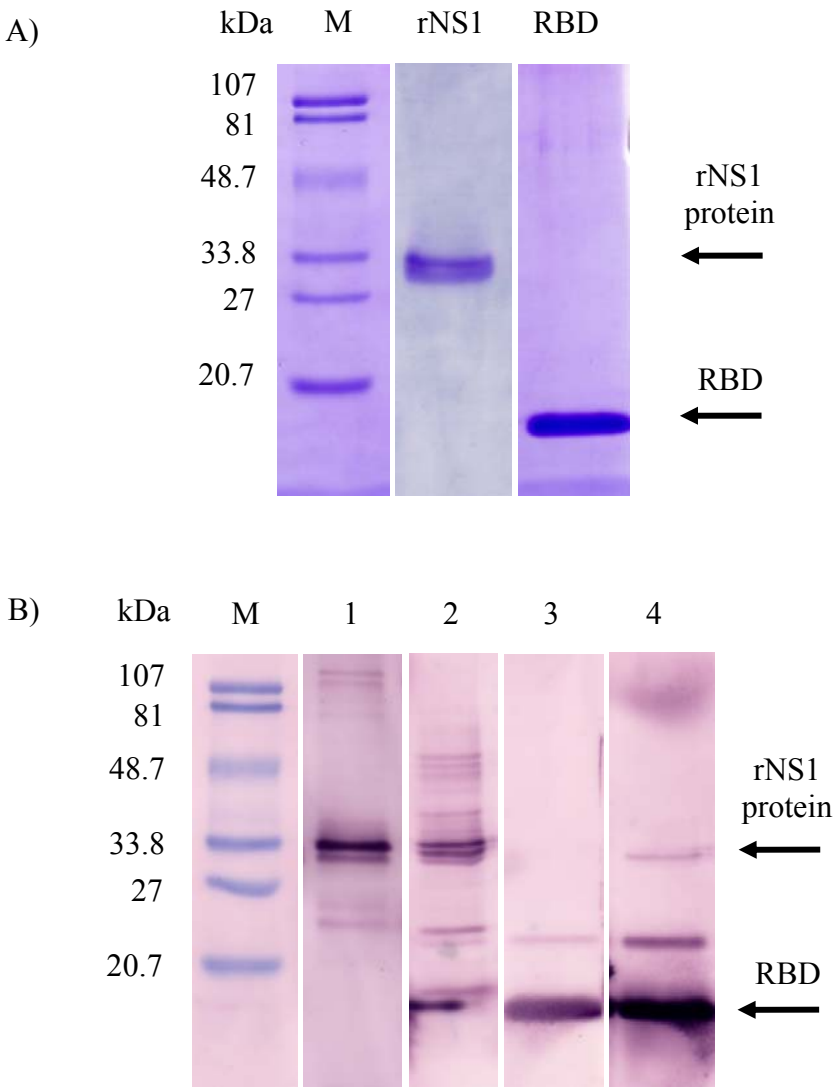

Figure 23 SDS-PAGE and Western blot analysis showed specific binding reactivity of monoclonal antibodies. A) SDS-PAGE of purified rNS1 and RBD. B) Western blot analysis, the purified rNS1 (lane 1and 2) and RBD (lane 3 and 4) was used as antigen to react with MAb NS1.1 (lane 1 and 3) and MAb NS1.2 (lane 2 and 4). Lane M, molecular weight markers.

Table 5 Screening and characterization of hybridoma clones producing MAb to NS1 protein of influenza A virus.

MAbs	Screening assays		Single cell cloning (% positive*)		Isotype	Affinity ratio		Western blot analysis			Reactivity after expansion	
	NS1 ELISA (ΔOD)	Ab capture ELISA (ΔOD)	First limit dilution	Second limit dilution		NS1 ELISA (ΔOD)	Ab capture ELISA (ΔOD)	rNS1	H3N2	H1N1	NS1 ELISA (ΔOD)	Ab capture ELISA (ΔOD)
2E2B1(NS1.1)	1.087	0.097	100%		IgG1 Kappa	0.70	0.31	+	+	+	2.730	0.696
18G11H5	0.343	0.302	100%		IgG1 Kappa	0.22	0.26	+	+	+	1.303	0.341
5B2A1	0.422	0.091	100%		IgG1 Lambda	0.18			+	+	2.986	0.042
1G7H5	0.452	0.147	100%		IgG1 Kappa	0.31	0.25				1.346	0.476
6D8E7	0.77	0.244	100%		IgG1 Kappa	0.21	0.49				0.487	1.501
18B1E9	0.272	0.159	100%		IgG2a kappa	0.49	0.31				0.825	0.210
17G1C11	0.931	0.455	100%		IgG2a kappa	0.23	0.56				1.388	0.258
13E 12	1.179	0.399	100%		IgG2a Kappa	0.24	0.55				0.500	1.561
18G12C2	0.53	0.16	100%		IgG2b Kappa		0.28				0.004	0.149
3B11G6C11(NS1.2)	0.08	0.208	87.5%	100%	IgA kappa		0.28	+	+	+		1.087
18H8H3E3	0.309	0.278	87.5%	100%	IgG1 Kappa	0.25	0.27	+			0.223	0.619
5C1H11B8	0.356	0.205	87.5%	100%	IgG1 Kappa	0.17	0.25			+	0.293	0.561
4F12H8H3	0.69	0.09	87.5%	100%	IgG1 Kappa	0.96					1.313	
3F8E1H6 (NS1.3)	0.067	0.19	87.5%	100%	IgG1 Kappa		0.25					0.367
1E10B2D10	0.637	0.34	87.5%	100%	IgG1 Kappa	0.22	0.36				0.222	0.906
15F8E6E11	0.145	0.151	87.5%	100%	IgG1 Kappa		0.25				0.065	0.257
4D2D3G2	1.492	0.458	87.5%	100%	IgG2b Kappa	0.25	0.52				0.816	0.775
17G7E2B11	0.816	0.106	75%	100%	IgG1 Kappa	0.25	0.26				0.669	0.561
4C1F1B8	0.059	0.179	75%	100%	IgG2a kappa		0.28					0.384
1B2C4C2	0.045	0.339	75%	100%	IgM Kappa		0.27					0.579

*% positive of single cell cloning calculated from (No. of ELISA positive / total supernatants) ×100

9. Development of MAb-based NS1 sandwich enzyme-linked immunosorbent assay (sandwich ELISA) for detection of NS1 antigen

The MAbs were purified by affinity chromatography in mini-scale for using in the following experiment. After the mini-scale purification, the MAbs were tested for the binding activity to NS1 antigen by ELISA and its concentration determined by Bio-rad protein assay. There are six MAbs that lost their specific binding activity to NS1 and contained a lower concentration for further experiment.

9.1. Selection of MAb pair for NS1 protein detection

A sandwich ELISA was performed to select the optimal pairs by using each monoclonal antibody interchangeably as capture and/or detector antibodies. Antibody pairs specific to NS1 were examined for their reactivity with both rNS1 and NS1 antigen in MDCK cell lysate infected with influenza A virus. Fourteen MAbs clones with specific activity to NS1 antigen were used to select the antibody pairs. From previous experiment, the MAb NS1.1 consisting of highest activity in indirect ELISA using rNS1 as antigen and containing unique binding epitope showed the best activity compared to other MAbs as the detector antibody in the sandwich ELISA. Based on MAb NS1.1, there are two MAbs 3B11G6C11 (MAb NS1.2) and 3F8E1H6 (MAb NS1.3) of different MAbs selected from the panel matched pairs with MAb NS1.1. This two matched pair were compared their reactivity based on rNS1 by ELISA, but pairing between MAb NS1.1 and NS1.2 showed the highest activity with rNS1 in a sandwich ELISA. Initially, the sandwich ELISA for NS1 antigen was performed by using MAb NS1.2 as capture antibody and B-NS1.1 as detector antibody. The further experiment was done using unlabelled MAb NS1.1 and NS1.2 interchangeably as capture and detector antibodies to optimize the capable sandwich ELISA using both rNS1 and NS1 antigen in lysate MDCK cells as antigen. According to these two formats, MAb NS1.1 as capture antibody and MAb NS1.2 as detector antibody and detected with anti-mouse IgA conjugated-HRP showed a stronger reactivity with both rNS1 and NS1 antigen in lysate MDCK cells than another initial format; MAb NS1.2 as capture antibody and B-NS1.1 as detector antibody (Figure 24A). The best anti-NS1 antibody pair was MAb NS1.1 as capture antibody and MAb NS1.2 as detector antibody, with anti-mouse IgA conjugated-HRP.

9.2. Optimization of MAb pair in sandwich ELISA

The sandwich ELISA condition of the best performance MAb pair was first optimized by checkerboard titration method using the selected MAbs in different concentrations. The optimal concentration of capture MAbs NS1.1 was selected at 2μg/ml, while detector antibody MAb NS1.2 was chosen at 1μg/ml. To evaluate the sensitivity of the assay, a twofold serial dilutions of rNS1 was performed by the sandwich ELISA assay ranging from 800 ng to 6.25 ng of rNS1 per well. Bovine serum albumin (BSA) was used to establish the baseline of the assay. The cut-off value was calculated as mean OD_{450} nm plus 3 standard deviations. These criteria revealed that the sandwich ELISA can detect as little as 6.25 ng of purified rNS1 (Figure 24B).

9.3. Detection of NS1 antigen in infected cells by sandwich ELISA

The sandwich ELISA for NS1 antigen detection test was performed by using lysate of MDCK cells infected with 4 isolates each of H1N1 or H3N2 influenza A and rgH5N1_3P, a reverse genetic derived virus containing NS genes of avian influenza virus subtype H5N1, after treatment with the extraction buffer. The NS1 antigen detection was clearly obtained in all samples of MDCK cell lysate infected with H1N1, H3N2 influenza A virus and avian influenza virus H5N1 with MAb pair specific to NS1 antigen by NS1 sandwich ELISA (Figure 25). Moreover, the NS1 antigen was also consistently detected in reconstituted samples. The results suggested type A NS1 could be detected in specimens from H1N1 and H3N2 human influenza viruses and also H5N1 avian influenza virus.

When the NS1 sandwich ELISA was tested with NS1 antigen in MDCK cell lysate infected with influenza A virus, a clearly signal was seen, as compared to uninfected MDCK cells. In addition, the NS1 sandwich ELISA compared with those MAbs recognizing the nucleoprotein (NP) or matrix (M), a lower absorbance value was seen. The lower level of signal seen with NS1 protein detection than NP and M may indicated that in the samples contained lower level of NS1 antigen than that of NP and M antigen.

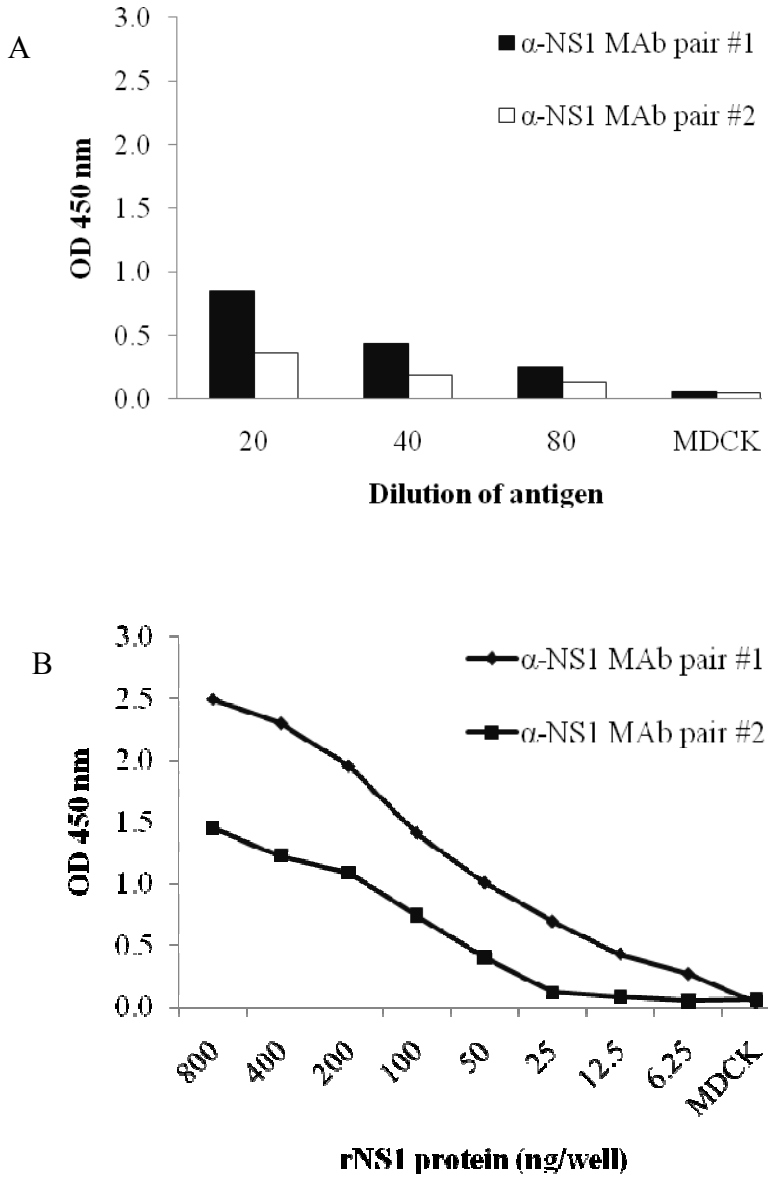

Figure 24 Selection of optimal monoclonal antibody pairs for detection of NS1 protein by sandwich ELISA. (A) The NS1 antigen in MDCK cell lysate infected with influenza A virus was used as antigen to examined the reactivity of each MAb pair. (B) The rNS1 was used to determine the sensitivity of sandwich ELISA of each MAb pair. (α-NS1 MAb pair #1, NS1.1/ NS1.2; α-NS1 MAb pair #2, NS1.2/ B-NS1.1).

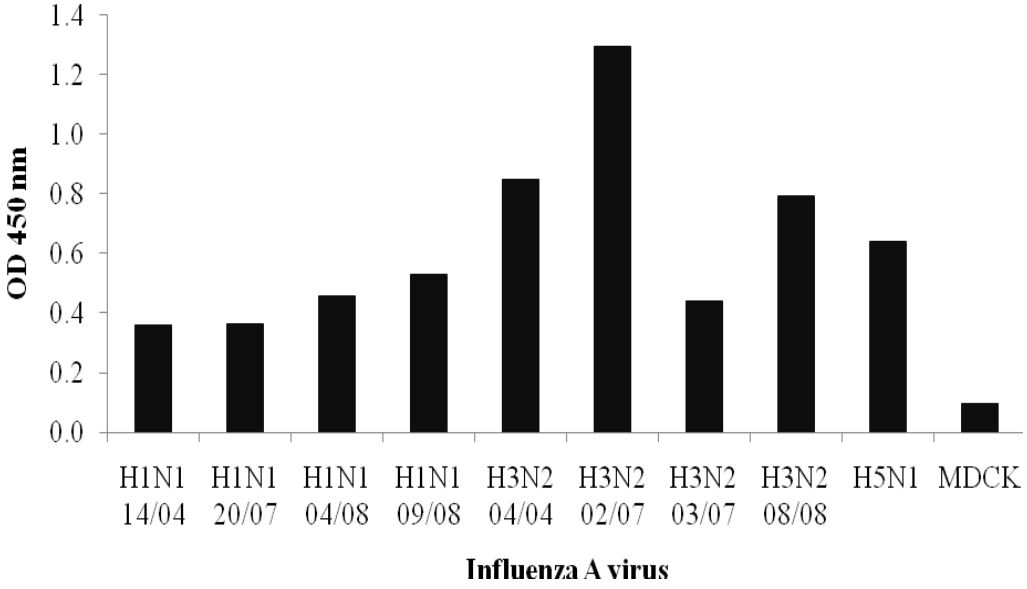

Figure 25 The sandwich ELISA for NS1 antigen detection test. Equal amount of NS1 antigen in MDCK cell lysate infected with influenza A viruses total 9 isolates, 4 each of H1N1 and H3N2 influenza A virus and rgH5N1_3P, a reverse genetic derived virus containing HA, NP, NA, M and NS genes of avian influenza virus subtype H5N1 and uninfected MDCK cells were tested by sandwich ELISA. Numbers represent the mean OD_{450} nm from triplicate wells.

10. Immunoperoxidase staining

In order to examine whether the MAbs NS1.1 and MAb NS1.2 can identify NS1 protein allele A of other source, the swine flu was used. Monoclonal antibodies were tested for their ability to recognized NS1 of swine influenza virus in infected cells using immunoperoxidase staining. The MAb specific to NP of influenza A virus was used as positive control whereas the MAb specific to influenza B virus was used as the negative control. Two MAbs; MAb NS1.1 and MAb NS1.2 were tested. Both MAbs gave specific reactivity with swine influenza (Figure 26). This result indicated that both selected MAbs still maintained some binding epitopes which were a conserved region of allele A NS1 antigen.

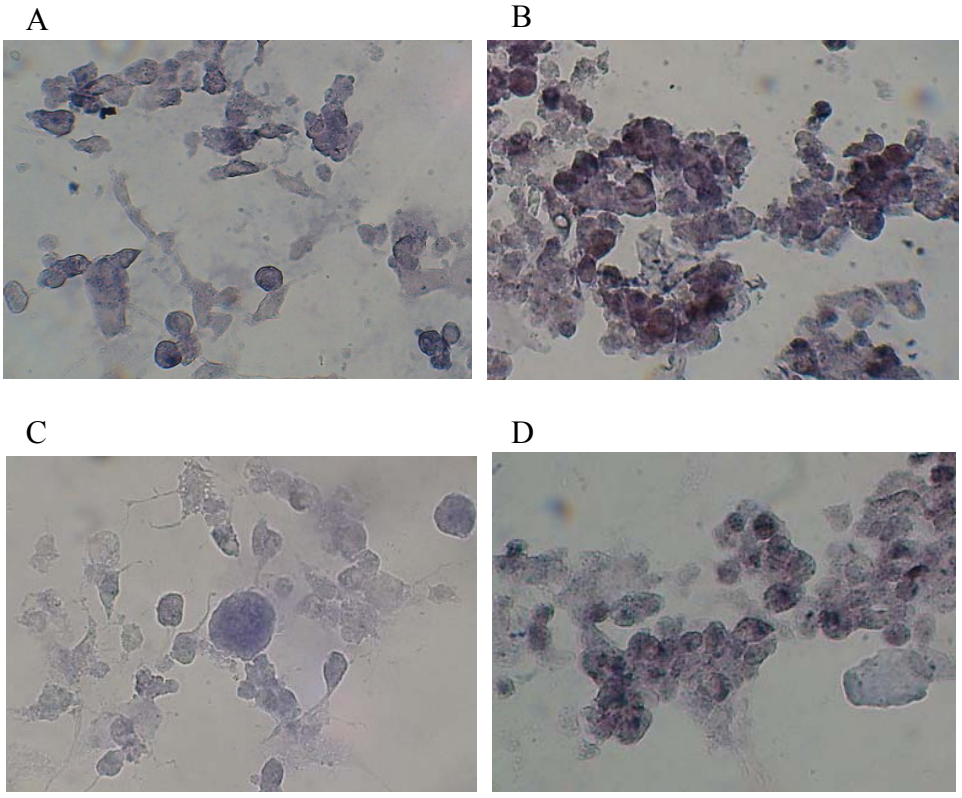

Figure 26 Immunoperoxidase staining using monoclonal antibodies. The swine flu infected cells were incubated with MAbs and then goat anti-mouse antidody conjugated-ALP; infected cells detected with MAb specific to NP (A) and MAb NS1.1(B) and MAb specific to influenza B virus (C) and MAb NS1.2.

CHAPTER VI
DISCUSSION

Influenza A virus infects a variety of avian and mammalian species including humans and it has a significant impact on the global health. During the infection, various viral proteins were produced within the infected cells. Nucleoprotein (NP) and Matrix (M), as well as NS1 non-structural proteins are among the highly conserved and highly expressed viral proteins present in infected epithelium. In this present study we described the diagnostic test emphasized on the use of a conserved NS1 protein.

The NS1 protein has been previously demonstrated to be a good candidate for differentiation between vaccinated and infected animals (DIVA) (7, 9, 65, 66). Antibodies to NS1 could be detected in sera of equine infected with equine influenza virus, but not in vaccinated animals (44). Antibodies to the NS1 were also detected in sera of mouse, chickens and turkeys experimentally infected with different subtypes of influenza A virus (9, 67, 68). These findings led to a general conclusion that antibody to NS1 protein or NS1 protein in infected cells could be useful as a marker of virus replication.

To achieve the recombinantly expressed NS1 protein which resembled native NS1 protein, the rNS1 protein used in this study was expressed in Sf9 insect cells. The expression of eukaryotic proteins in baculovirus/insect cell system served a purpose to circumvent problems associated with the incorrect folding, lack of disulfide bond formation, and lack of post-translational modifications found in prokaryotic systems (59). The NS1 is known to be subjected to post-translational modifications by phosphorylation (46, 51).

The rNS1 protein was designed to have a 6xHis-tag in order to facilitate purification by affinity under immobilized metal affinity chromatography (IMAC)

principle. In this study, the His-tagged protein was purified utilizing cobalt (II) (Co^{2+}) resin. The Co^{2+} can bind the recombinant His-tagged protein more efficiently and have less non-specific binding of non-His tagged protein resulting in the highly pure yield (69, 70).

In this study, the purified rNS1 protein was used as the immunogen for immunization. The rNS1 exhibited high antigenic activity demonstrated by the reactivity of polyclonal sera obtained after immunization, reacting with Sf9 infected cells lysate, as well as with purified rNS1 and MDCK cells infected with H3N2 and H1N1 influenza A virus by ELISA and Western blot analysis. The immunogenic properties of rNS1 were confirmed by a large number of hybridoma clones specific to NS1 protein (46 out of 564). Finally, two MAbs specific to NS1 protein with highest reactivity were selected. The selected 2 MAbs; MAb NS1.1 and MAb NS1.2 reacted specifically with NS1 protein in the lysate MDCK cells infected cells with influenza virus and with purified rNS1 protein by ELISA and Western blot analysis.

The hybridoma screening step was designed to obtain antibody with different binding epitopes, to facilitate subsequent selection of MAb pairs. Two formats of ELISA were used; an NS1 ELISA employed rNS1 coated wells to select NS1 binding antibody, and antibody-captured ELISA using anti-mouse antibody coated well to capture MAb and tested for the ability to bind biotin conjugated rNS1. The MAbs specific to NS1 protein obtained from two different ELISA formats recognized different epitope on NS1. The results from these two ELISA assays were independent. The selected MAb NS1.1 showed strong positive reactivity by both assay. MAb NS1.2 showed strong positive reactivity only by the latter assay. If only NS1 ELISA format was performed for screening hybridoma clones, the best one, MAb NS1.2, might not be selected.

During the selection process to identify the one suitable pair for sandwich ELISA, all MAbs were subjected to epitope mapping. The epitope comparison was investigated whether MAbs bind to different epitopes by cELISA utilizing biotin-conjugated MAbs. The MAbs NS1.1 exhibited the unique binding epitope different from the other clones. Therefore MAb NS1.1 could be paired with all other MAbs for developing sandwich ELISA. The selection of MAb pair was obtained by two MAbs were used interchangeably as capture and/or detector antibodies in sandwich ELISA

for detecting NS1 antigen. The MAb pair with best performance was MAb NS1.1 (IgG) as capture antibody while MAb NS1.2 (IgA) as detector antibody and detected with anti-mouse IgA conjugated-HRP. The detection limit of this sandwich ELISA was also determined using purified rNS1. As little as 0.125 μg/ml (6.25 ng/well) of NS1 antigen can be detected.

NS1 gene can be divided into two groups on the basis of nucleotide sequence homology, referred to as allele A and B (47). The alignment sequences analysis of the NS1 gene of avian influenza A virus H5N1 used in the present study revealed a high degree of homology to human influenza A (H1N1 and H3N2) previously reported to have NS1 gene of the allele A (47). Similar finding reported that the majority of highly pathogenic avian influenza viruses isolated from humans had an allele A NS1 gene (50). However, MDCK cells lysate infected with a reverse genetic H5N1 avian influenza virus was tested in the present study. It implied that our NS1 sandwich ELISA may also be a useful diagnostic tool for the detection of the homotypic avian influenza virus. Although the obtained Mab pair was generated with rNS1 of H5N1 influenza, it was expected to cross-react with other influenza strains with allele A NS1. The developed NS1 sandwich ELISA was tested with MDCK cells infected with 4 isolates each of H1N1 or H3N2 human influenza A, also a representative of NS1 protein allele A. The NS1 sandwich ELISA could detect NS1 antigen from infected cells of all H1N1 and H3N2 human influenza virus tested, suggesting that this assay was able to use for viral NS1 antigen detection for all strains with NS1 that were belonged to allele A. Therefore, our two MAbs, MAb NS1.1 and MAb NS1.2, also demonstrated the reactivity with swine flu by immunoperoxidase staining, this result indicated that our generated MAbs still maintained some binding epitopes which were a conserved region of allele A NS1 antigen.

In this study, the anti-NS1 MAbs specific to NS1 protein of influenza A virus were generated and a sandwich ELISA for detection of NS1 protein in cell-associated antigen was successfully developed. The assay could be further applied to detect NS1 protein in influenza patient specimens. This developed sandwich ELISA assay could also be further combined with other influenza A viral conserved protein detection in order to detect multiple antigens, an approach that should improve the sensitivity of the rapid influenza A antigen detection test.

CHAPTER VII
CONCLUSION

Influenza NS1 protein is an important non-structural protein that is abundantly expressed in influenza virus infected cells and only produced during active replication of the virus. This study involved generation of rNS1 in Baculovirus expression system and production of MAbs specific to NS1 protein of influenza A virus H5N1. The rNS1 was expressed with 6xHis-tagged to facilitate purification by metal affinity chromatography. The rNS1 was purified by utilizing Co^{2+} which exhibited specific binding efficiency and specificity and also yield a highly purified rNS1 protein. The purified rNS1 was successfully used as immunogen to immunize BALB/c mice for the production of MAbs against NS1 protein of influenza A virus.

The spleen cells of immunized mouse giving the highest titer to NS1 were fused with myeloma cells. Following hybridoma fusions and subsequent single cell cloning by limiting dilution, 20 positive clones were obtained. The best hybridoma clones were selected by various immunological methods to identify immunoglobulin isotype, their affinity binding to rNS1 by ELISA. In term of antigen binding specific reactivity to NS1 antigen, when both purified rNS1 and native NS1 antigen in MDCK cells infected with H1N1 and H3N2 influenza A virus were used as antigen in Western blot analysis and ELISA. Finally, two MAbs clones namely MAb NS1.1 and MAb NS1.2 were shown to be the best MAb pair for the NS1 sandwich ELISA.

The developed MAb-based NS1 sandwich ELISA was shown to react specifically with NS1 protein of several isolates of H1N1 and H3N2 human influenza A virus and also H5N1 avian influenza virus infected MDCK cell culture. This assay could be used to detect NS1 antigen in cell-associated form. This NS1 sandwich ELISA can be used to identify NS1 protein of influenza A virus not only in infected MDCK cell culture but also influenza A infected clinical specimens in the future.

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APPENDIX

1. Cell culture and Hybridoma

Reagent:

Fetal bovine serum (FBS)

Heat inactivated at 56 °C for 30 minutes.

L-glutamine 200 mM

L-glutamine	2.92	g
DW to	100	ml

The solution was sterilized by filtration through a 0.2 μm disposable filter and stored at 4°C.

50X lactabumin hydrolysate

Lactabunin hydrolysate (sigma)	16.65	g
DW to	100	ml

The solution was sterilized by filtration through a 0.2 μm disposable filter and stored at 4°C.

0.1 M IPTG (Isopropylthio-β-D-galactoside)

Isopropylthio-β-D-galactoside	0.24	g
DW to	10	ml

The solution was sterilized by filtration through a 0.2 μm disposable filter and dispensed the solution into 1 ml aliquot stored in dark at -20°C.

Antibiotic:

Ampicillin (100 mg/ml)

Ampicillin	1	g
Sterile DW to	10	ml

The solution was sterilized by filtration through a 0.2 μm disposable filter and dispensed the solution into 1 ml aliquot stored at -20°C.

Kanamycin (50 mg/ml)

Kanamycin	0.5	g
Sterile DW to	10	ml

The solution was sterilized by filtration through a 0.2 μm disposable filter and dispensed the solution into 1 ml aliquot stored at -20°C.

Gentamicin (7 mg/ml)

Gentamicin	70	mg
Sterile DW to	10	ml

The solution was sterilized by filtration through a 0.2 μm disposable filter and dispensed the solution into 1 ml aliquot stored at -20°C.

Tetracycline (10 mg/ml)

Tetracycline	0.1	g
Ethanol	10	ml

The solution was sterilized by filtration through a 0.2 μm disposable filter and dispensed the solution into 1 ml aliquot stored at -20°C.

Penicillin (100,000u/ml)-Streptomycin (100,000μg/ml)

Penicillin G	5×10^6	unit
Streptomycin sulfate	5	g

Dissolved in DW and adjusted the volume to 50 ml. The solution was sterilized by filtration through a 0.2 μm disposable filter and stored at -20°C.

Media for cell culture and hybridoma:

Stock Grace's insect cell culture medium

Grace's insect cell culture medium (Invitrogen)	1	pack
$NaHCO_3$	0.35	g

Dissolved in DI, adjusted to pH 6.2 with 2 N NaOH and adjusted the volume to 1,000 ml. The media was sterilized by filtration through a 0.2 μm disposable filter and stored at 4°

Maintenance media for sf9 insect cells

Stock Grace's insect cell culture medium	78	ml
FBS (Hyclone)	10	ml
L-glutamine 200 mM	8	ml
50X Yeastolate (Invitrogen)	2	ml
50X lactabumin hydrolysate	2	ml

Mix them together and stored at 4°C.

Stock RPMI-1640 media

RPMI-1640 (Invitrogen)	1	pack
$NaHCO_3$	2	g

Dissolved in DI, adjusted to pH 7.2 and adjusted the volume to 1,000 ml. The media was sterilized by filtration through a 0.2 μm disposable filter and stored at 4°C.

Maintenance media for hybridoma cells (Complete media)

Stock RPMI-1640	400	ml
FBS (Hyclone)	100	ml
L-glutamine 200 mM	5	ml
2-Mercaptoethanol (stock)	2.5	ml
Penicillin-Streptomycin	0.5	ml

Mix them together and stored at 4°C.

Media for bacteria culture:

LB (Luria-Bertani) broth medium

Bacto-Tryptone	1	g
NaCl	0.5	g
Bacto-yeast extract	0.5	g

Dissolved in DW and adjusted the volume to 100 ml.

The solution was sterilized by autoclaving at 121°C at 15 lb/in^2 for 15 minutes.

LB (Luria-Bertani) agar plates

Bacto-Tryptone	1	g
NaCl	0.5	g
Bacto-yeast extract	0.5	g
Agarose	2	g

Dissolved in DW and adjusted the volume to 100 ml.

The solution was sterilized by autoclaving at 121°C at 15 lb/in^2 for 15 minutes and left the media cool down to 50°C, poured the media on petri dish.

Reagent for fusion:

15% DMSO in PBS

DMSO		4.5	ml
Sterile PBS	25.5	ml	

41.3% PEG

Dissolved 5 g PEG 3000-3700 in 7 ml of 15% DMSO in PBS, mix thoroughly in 50°C water bath.

2-Mercaptoethanol stock (2-ME)

2-Mercaptoethanol (14.34 M solution)	0.07	ml
Sterile PBS	100	ml

Mix them together and stored at 4°C for only 2 weeks.

Aminopterin (A) stock 100X

Aminopterin	1.76	g
DW	90	ml

Add 1 N NaOH to dissolved, adjusted to pH 7.2 with 1 N HCl and adjusted the volume to 100 ml. The solution was sterilized by filtration through a 0.2 μm disposable filter and dispenses the solution into 1 ml aliquot stored in dark vials at -20°C.

Hypoxanthine and Thymidine (HT) stock 100X

Hypoxanthine	136.1	mg
Thymidine	38.75	mg
DW	90	ml

Warm and add 1 N NaOH to dissolved, adjusted to pH 8.0 with 1 N NaOH and adjusted the volume to 100 ml. The solution was sterilized by filtration through a 0.2 μm disposable filter and dispenses the solution into 1 ml aliquot stored at -20°C.

HAT working media (freshly prepared)

Complete media	100	ml
HT stock 100X	1	ml
A stock 100X	1	ml

Mix them together and stored at 4°C.

HT working media (freshly prepared)

Complete media	100	ml
HT stock 100X	1	ml

Mix them together and stored at 4°C.

Freezing media

Sterile complete media (50% FBS)	90	ml
Dimethysulfoxide (DMSO)	10	ml

Mix them together and stored at 4°C.

(Do not filter sterile or autoclave the DMSO because the filters are destroyed by DMSO and the DMSO is destroyed by autoclaving. The DMSO is already sterile by itself)

Phosphate buffer saline (PBS) pH 7.2

NaCl	8.5	g
$Na_2HPO_4 \cdot 2H_2O$	1.34	g
$NaH_2PO_4 \cdot 2H_2O$	0.39	g

Dissolved in DI, adjusted to pH 7.2 with 2 N NaOH and adjusted the volume to 1,000 ml. The solution was sterilized by filtration through a 0.2 μm disposable filter and stored at room temperature.

2. Reagent for PCR and Agarose gel electrophoresis

0.1% DEPC-treated water

Diethylpyrocarbonate	1	ml
DI to	1,000	ml

Incubated overnight at room temperature and then sterilized by autoclaving at 121°C at 15 lb/in^2 for 15 minutes.

5X TAE buffer

Tris base	24.2	g
Glacial acetic acid	5.71	ml
0.5 M EDTA pH 8.0	10	ml

Dissolved in DW and adjusted the volume to 1,000 ml

Ethidium bromide solution (10 mg/ml)

Ethidium bromide	1	g

Dissolved in DW and adjusted the volume to 100 ml.

6x loading buffer

Bromophenol blue	25	mg
Sucrose	4	g

Dissolved in DW and adjusted the volume to 10 ml.

3. Reagent for preparation of plasmid or bacmid DNA

1.0 M Tris-Cl pH 8.0

Tris base	12.11	g

Dissolved in DW, adjusted to pH 8.0 with concentrated HCl and adjusted the volume to 100 ml.

2 N NaOH

NaOH	80	g
DW	100	g

10% Sodium dodecyl sulfate (SDS) (w/v)

Sodium dodecyl sulfate	10	g
DW	90	ml

70% Ethanol

Ethanol	70	ml
DW	30	ml

0.5 M EDTA (Ethylenediamine tetraacetic acid, disodium salt) pH 8.0

EDTA	18.61	g

Dissolved in DW, adjusted to pH 8.0 with 2 N NaOH and adjusted the volume to 100 ml.

Ribonuclease A (RNase A)

Ribonuclease A was dissolved in 10 mM Tris-Cl (pH 7.5) and 15 mM NaCl to a concentration 10 mg/ml. The solution was boiled at 100°C for 15 minutes, then left the solution cool down at room temperature and stored at -20°C.

Solution I

1.0 M Tris-Cl pH 8.0	1.5	ml
0.5 M EDTA	2.0	ml
10 mg/ml RNase A	1.0	ml
DW	95.5	ml

Mix them together, sterilized by filtration through a 0.2 μm disposable filter and stored at 4°C.

Solution II

2 N NaOH	10	ml
10% SDS (w/v)	10	ml
DW	80	ml

Mix them together and sterilized by filtration through a 0.2 μm disposable filter and stored at room temperature.

Solution III (3 M potassium acetate pH 5.5)

Potassium acetate	29.46	g

Dissolved in DW, adjusted to pH 5.5 with glacial acetic acid and adjusted the volume to 100 ml and stored at 4°C.

4. Reagent for preparing competent cells

Transformation buffer I (TFB I)

1 M CH_3COOK	0.3	ml
1 M RbCl	1	ml
1 M $CaCl_2$	1	ml
1 M $MnCl_2$	0.5	ml
0.2 M Acetic acid	0.044	ml
Glycerol	1.5	ml
DW	5.656	ml

Transformation buffer (TFB II)

1 M MOPS	0.1	ml
1 M RbCl	0.1	ml
1 M $CaCl_2$	0.75	ml
1 M KOH	0.028	ml
Glycerol	1.5	ml
DW	7.522	ml

5. Reagent for polyacrylamide gel electrophoresis

Stock 2x sample buffer

0.5 M Tris-Cl pH 6.8	1.2	ml
0.1 % Bromophenol blue (w/v)	0.5	ml
10% SDS (w/v)	2	ml
Glycerol	1	ml
DW	4.8	ml

Mix them together and stored at room temperature.

SDS Reducing sample buffer (prepare immediately before use)

Stock 2X sample buffer	0.05	ml
2- Mercaptoethanol (2-ME)	0.95	ml

1.5 M Tris-Cl pH 8.8 (resolving gel buffer)

Tris base	18.16	g

Dissolved in DW, adjusted to pH 8.8 with concentrated HCl and adjusted the volume to 100 ml.

1.0 M Tris-Cl pH 6.8 (Stacking gel buffer)

Tris base	12.11	g

Dissolved in DW, adjusted to pH 6.8 with concentrated HCl and adjusted the volume to 100 ml.

30% Acrylamide-bisacrylamide solution

Acrylamide (Bio-rad)	29	g
N, N-bismethylene acrylamide (Sigma)	1	g
DW	100	ml

The solution was stored in dark bottle at room temperature.

10% Ammonium persulfate (APS) (w/v)

Ammonium persulfate	1	g

Dissolve in DW 10 ml, the solution was stored in dark bottle at 4 °C.

12% separating gel

30% Acrylamide-bisacrylamide solution	2	ml
DW	1.6	ml
1.5 M Tris-Cl pH 8.8	1.3	ml
10% SDS (w/v)	0.05	ml
10% APS (w/v)	0.05	ml
TEMED	0.002	ml

After TEMED was added, the gel solution was immediately loaded on the vertical gel electrophoresis (Mini-protein II Electrophoresis cell, Bio-Rad) and then overlay with water. The gel solution was allowed to polymerize for 30 min.

15% separating gel

30% Acrylamide-bisacrylamide solution	2.5	ml
DW	1.1	ml
1.5 M Tris-Cl pH 8.8	1.3	ml
10% SDS (w/v)	0.05	ml
10% APS (w/v)	0.05	ml
TEMED	0.002	ml

After TEMED was added, the gel solution was immediately loaded on the vertical gel electrophoresis (Mini-protein II Electrophoresis cell, Bio-Rad) and then overlay with water. The gel solution was allowed to polymerize for 30 min.

5% stacking gel

30% Acrylamide-bisacrylamide solution	0.5	ml
DW	2.1	ml
1.0 M Tris-Cl pH 6.8	0.38	ml
10% SDS (w/v)	0.03	ml
10% APS (w/v)	0.03	ml
TEMED	0.003	ml

After TEMED was added, the gel solution was immediately loaded on the vertical gel electrophoresis. The comb was gently inserted on the top of the gel to make well for sample application. The gel solution was allowed to polymerize for 10 min.

Electrode buffer

Tris base	3.02	g
SDS	1	g
Glycine	14.24	g

Dissolved in DW, adjusted to pH 8.3 with 1.0 N HCl and adjusted the volume to 1,000 ml.

Staining solution

Coomassie brilliant blue R250	0.25	g
Methanol	45	ml
Glacial acetic acid	10	ml

Dissolved in DW and adjusted the volume to 100 ml.

Destaining solution

Methanol	300	ml
Glacial acetic acid	100	ml

Dissolved in DW and adjusted the volume to 1,000 ml.

6. Reagent for Western blot analysis

Towbin's buffer

Tris base	3.04	g
Glycine	14.41	g
Methanol	200	ml

Dissolved in DW and adjusted the volume to 1,000 ml. The solution was stored at 4°C.

10x PBS

NaCl	80	g
KCl	2	g
Na_2HPO_4	14.4	g
KH_2PO_4	2.4	g

Dissolved in DW, adjusted to pH 7.2 with 2 N NaOH and adjusted the volume to 1,000 ml.

Washing buffer (PBST)

10X PBS	100	ml
Tween 20	0.5	ml

Dissolved in DW and adjusted the volume to 1,000 ml. The solution was stored at 4°C.

Blocking buffer (3% BSA (w/v) in PBST)

BSA	3	g
PBST	100	ml

Mix them together and stored at 4°C.

Diluent buffer (1% BSA (w/v) in PBST)

3% BSA (w/v) in PBST	15	ml
PBST	30	ml

Mix them together and stored at 4°C.

Chromogenic substrate stock (4CN)

4-Chloro-1-Naphthol tablet	30	mg

Dissolved in ice cold methanol 10 ml and stored in dark bottle at -20°C.

Working substrate (Freshly prepared)

Triethanolamine buffer saline (TBS) pH 7.5	10	ml
4CN stock	2	ml
3% H_2O_2	51.5	µl

7. Reagent for cells dissociation solution

RIPA buffer

Triton X-100	1	ml
SDS	0.1	g
Sodium deoxycholate	0.5	g

Dissolved in 1X PBS adjusted the volume to 100 ml and stored at 4°C.

8. Reagent for protein purification

0.2 M Na_2HPO_4 (dibasic sodium phosphate)

Na_2HPO_4	28.4	g

Dissolved in DI and adjusted the volume to 1,000 ml

0.2 M NaH_2PO_4 (monobasic sodium phosphate)

NaH_2PO_4	27.4	g

Dissolved in DI and adjusted the volume to 1,000 ml

50 mM sodium phosphate buffer, pH 7.5

0.2 M Na_2HPO_4	42	ml
0.2 M NaH_2PO_4	8	ml

Dissolved in DI and adjusted the volume to 200 ml.

20 mM sodium phosphate buffer, pH 7.0

0.2 M Na_2HPO_4	30.5	ml
0.2 M NaH_2PO_4	19.5	ml

Dissolved in DI and adjusted the volume to 500 ml.

0.1 M Glycine, pH 3.0

Glycine	3.75	g

Dissolved in DI and adjusted the volume to 1,000 ml

Saturated ammonium sulfate

$(NH_4)_2SO_4$	1,000	g

Dissolved in DI at 50°C, stirred until it was dissolved and let stand overnight at room temperature. The solution was filtered through a N0.1 filter paper, adjusted to neutral pH with NaOH and kept in a closed container.

9. Reagent for ELISA

Coating buffer (Carbonate-bicarbonate, pH 9.6)

Na_2CO_3	1.7	g
$NaHCO_3$	2.86	g

Dissolved in DW, adjusted to pH 9.6 and adjusted the volume to 1,000 ml.

4 N H_2SO_4

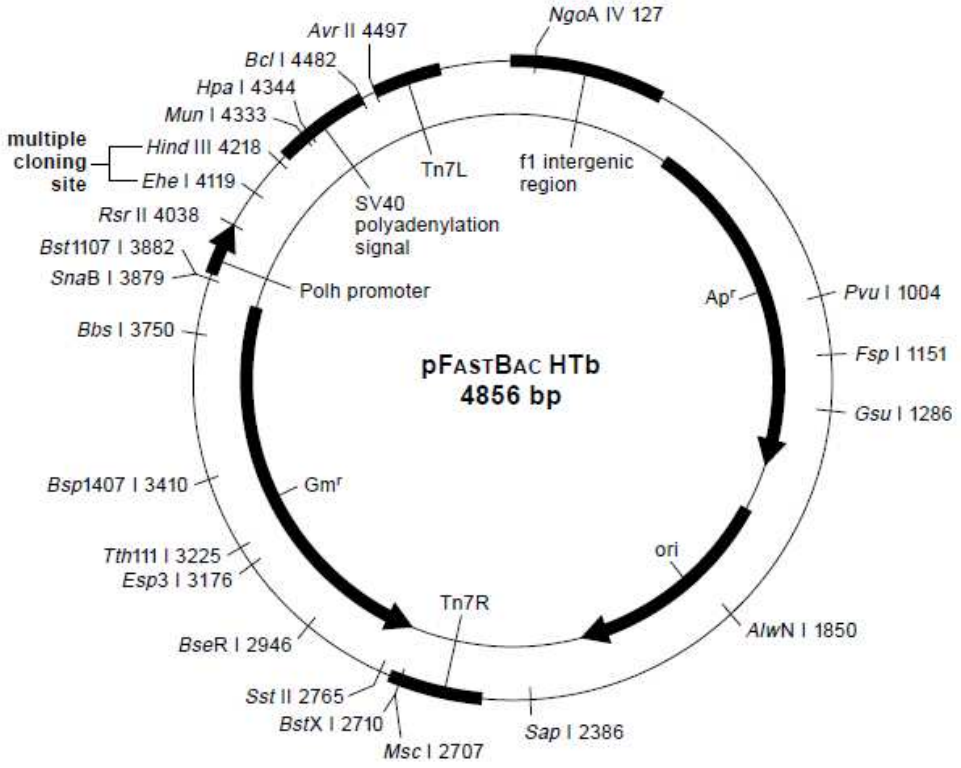
H_2SO_4	10	ml
DW	90	ml

Stop solution (0.6 N Sulfuric acid)

4 N H_2SO_4	15	ml
DW	85	ml

Mix them together and stored at room temperature.

10. Maps and Restriction Endonuclease Sites for pFastBac HTb Expression Vectors



Restriction endonucleases that do not cleave pFastBac HT DNAs:

Aat II	*Bpu*1102 I	*Bst*E II	*Eco*72 I	*Nhe* I	*Pfl*M I	*Pvu* II	*Sma* I	*Sun* II
Afl II	*Bsg* I	*Cla* I	*Eco*O109 I	*Nru* I	*Pin*A I	*Sex*A I	*Srf* I	*Swa* I
Apa I	*Bsp*M I	*Cvn* I	*Mlu* I	*Nsi* I	*Pme* I	*Sfi* I	*Sse* I	*Xcm* I
Asc I	*Bss*H II	*Eco*47 III	*Nde* I	*Pac* I	*Psp*5 II	*Sgr*A I	*Sse*8387 I	

Restriction endonucleases that cleave pFastBac HTb DNA twice:

Acc I	3882	4151	*Bsa*H I	835	4119	*Rca* I	536	1544
Afl III	2264	3246	*Bsm* I	4328	4427	*Sca* I	893	4231
Ban II	157	4157	*Bsm*F I	4015	4856	*Tfi* I	2290	4182
Bgl II	2547	3017	*Dra* III	230	3578	*Xmn* I	772	3797
Bsa I	1304	3661	*Eam*1105 I	1371	4732			

Figure 27 Map and restriction endonuclease sites for pFastBac HTb expression vectors. The circle map of pFastBac HTb (4856 bp) is presented. Restriction endonucleases that cleave pFastBac HTb once are shown on the outer circle. The nucleotide position refers to the 5´ base of the recognition sequence.

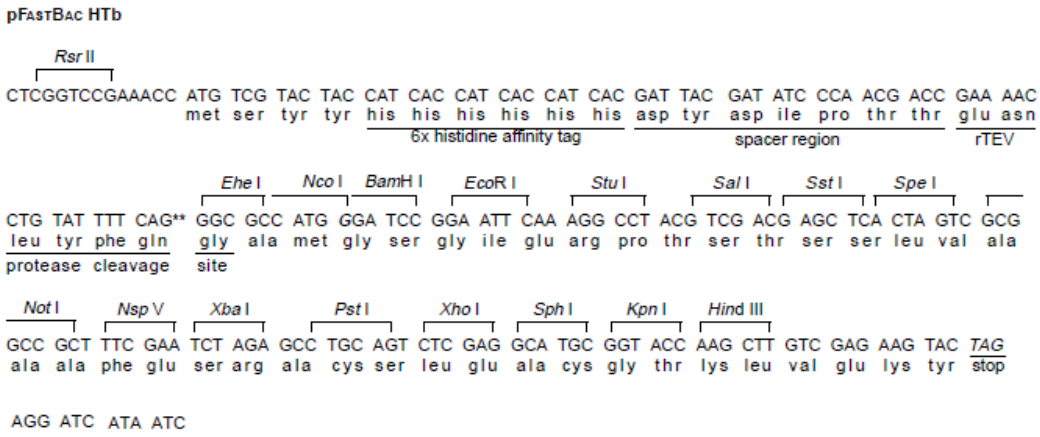


Figure 28 Multiple cloning site sequences of pFastBac HT expression vectors. The multiple cloning sites (MCS) for the vector are shown above. The sequence for the 6x Histidine affinity tag, spacer region and rTEV protease cleavage site are underlined. The cleavage with rTEV protease occurs between the gln and gly and is signified by **. The stop codon is underlined.

BIOGRAPHY

NAME	Miss Patumporn Jiean-umpunkul
DATE OF BIRTH	6 December 1984
PLACE OF BIRTH	Samutsongkhram, Thailand
INSTITUTIONS ATTENDED	Mahidol University, 2002-2005: Bachelor of Science (Medical Technology) Mahidol University, 2006-2010: Master of Science (Immunology)
RESEARCH GRANT	Siriraj Research Grant for Graduate studies, Faculty of Medicine Siriraj Hospital, Mahidol University, Thailand

POSTER PRESENTATION

A part of result from this thesis was presented as a poster entitled "Improved sensitivity of Influenza A antigen test by Multiple antigen detection" by **Patumporn Jiean-umpunkul**, Charin Thepthai, Nisachon Apiwat, Kanokwan Poomputsa, and Tararaj Dharakul at EID 2010- Thailand Conference on Emerging Infectious and Neglected Diseases, 3-4 June 2010 at Amari Orchid Resort and Tower, Pattaya, Chonburi, Thailand,.