การพัฒนาชุดตรวจอีไลซ่าเพื่อตรวจแอนติบอดีต่อนิวคลีโอโปรตีนของเชื้อไข้หวัดใหญ่ชนิดเอในสุกร

นางสาวคณนา หรั่งประเสริฐ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาพยาธิชีววิทยาทางสัตวแพทย์ ภาควิชาพยาธิวิทยา
คณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2555

DEVELOPMENT OF ELISA FOR DETECTING ANTIBODY AGAINST INFLUENZA A NUCLEOPROTEIN IN PIGS

Miss Kanana Rungprasert

A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Master of Science Program in Veterinary Pathobiology

Department of Veterinary Pathology

Faculty of Veterinary Science

Chulalongkorn University

Academic Year 2012

Thesis Title	DEVELOPMENT OF ELISA FOR DETECTING ANTIBODY AGAINST INFLUENZA A NUCLEOPROTEIN IN PIGS
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คณนา หรั่งประเสริฐ : การพัฒนาชุดตรวจอีไลซ่าเพื่อตรวจแอนติบอดีต่อนิวคลีโอโปรตีนของเชื้อไข้หวัดใหญ่ชนิดเอในสุกร . (DEVELOPMENT OF ELISA FOR DETECTING ANTIBODY AGAINST INFLUENZA A NUCLEOPROTEIN IN PIGS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก : รศ.น.สพ.ดร.คณิศักดิ์ อรวีระกุล , อ.ที่ปรึกษาวิทยานิพนธ์ร่วม : รศ.สพ.ญ.ดร.สันนิภา สุรทัตต์, 90 หน้า.

การตรวจวินิจฉัยโรคทางซีรั่มวิทยามีความสำคัญยิ่งในการสำรวจการติดเชื้อไข้หวัดใหญ่ชนิดเอในฝูงสัตว์ร่วมถึงฝูงสุกร ปัจจุบันมีการผลิตชุดตรวจอีไลซ่าเพื่อตรวจหาแอนติบอดีต่อเชื้อไข้หวัดใหญ่ชนิดเอออกวางขายตามท้องตลาดเป็นจำนวนมากซึ่งเป็นวิธีที่สะดวก ให้ผลรวดเร็ว แต่อย่างไรก็ตามมักมีราคาแพง จึงเป็นข้อจำกัดที่จะนำมาใช้งานในประเทศกำลังพัฒนาอย่างเช่นประเทศไทย วัตถุประสงค์หลักของงานวิจัยนี้เพื่อผลิตรีคอมบิแนนท์นิวคลีโอโปรตีนของเชื้อไข้หวัดใหญ่ชนิดเอในสุกร เพื่อนำมาใช้ในการพัฒนาเป็นชุดตรวจอีไลซ่าเพื่อตรวจแอนติบอดีต่อนิวคลีโอโปรตีนของเชื้อไข้หวัดใหญ่ชนิดเอในสุกรโดยการเพิ่มจำนวนนิวคลีโอโปรตีนยีนขนาดเต็มเส้น (1,500 คู่เบส) และขนาดสั้น (1,000 คู่เบส) ของเชื้อไข้หวัดสุก รสายพันธุ์ H1N1 (A/swine/Thailand/ CU-CBP18/2009) ด้วยวิธีปฏิกิริยาลูกโซ่โพลีเมอเรสแบบ RT-PCR และยีนเหล่านั้นถูกนำไปตัดต่อเข้าสู่ pThioHisA expression vector เพื่อสร้ างรีคอมบิแนนท์ พลาสมิด Np-pThioHisA และ NPt-pThioHisA หลังจากนั้นรีคอมบิแนนท์พลาสมิดเหล่านี้ถูกนำเข้าสู่เซลล์ของแบคทีเรีย *E. coli* สายพันธุ์ TOP10 เพื่อทำการกระตุ้นการสร้างรีคอมบิแนนท์นิวคลีโอโปรตีนขนาดเต็มเส้นและขนาดสั้นด้วย IPTG ความเข้มข้น 1 mM โดยสามารถตรวจพบรีคอมบิแนนท์นิวคลีโอโปรตีนขนาดเต็มเส้นและขนาดสั้นด้วยวิธี SDS-PAGE (ขนาด 56 kDa และ 48 kDa ตามลำดับ) ซึ่งโปรตีนดังกล่าวให้ผลบวกกับชุดทดสอบอีไลซ่าสำเร็จรูปที่ใช้ตรวจหานิวคลีโปรตีนของเชื้อไข้หวัดใหญ่ชนิดเอ อย่างไรก็ตามจากการศึกษาครั้งนี้พบว่าโปรตีนที่ผลิตออกมาอยู่ในรูปแบบของโปรตีนที่ไม่ละลายน้ำ ดังนั้นจึงมีความพยายามที่จะปรับสภาวะ ระยะเวลาและความเข้ม ข้นของสารต่างๆ เพื่อที่จะเพิ่มความสามารถในการละลายน้ำของโปรตีนดังกล่าว ได้แก่ การเพิ่มระยะเวลาในบ่มโปรตีนในตัวทำละลายโปรตีน (6 M guanidine hydrochloride) ให้มีระยะเวลานานขึ้นและปรับเปลี่ยนสภาวะในการผลิตโปรตีนทั้งอุณหภูมิในช่วงกระตุ้นโปรตีน ความเข้มข้นของ IPTG และสายพันธุ์ของแบคทีเรียที่ใช้ จากผลการทดลองพบว่ายังไม่สามารถที่จะทำให้โปรตีนละลายน้ำได้ ซึ่งมีผลให้โปรตีนดังกล่าวไม่มีความบริสุทธิ์ ทำให้ไม่สามารถนำโปรตีนนี้ไปผลิตชุดตรวจสอบอีไลซ่าต่อไปได้ อย่างไรก็ตามงานวิจัยนี้มีสามารถใช้เป็นข้อมูลพื้นฐานสำหรับงานวิจัยที่ต้องการผลิตรีคอมบิแนนท์นิวคลีโอโปรตีนเพื่อที่จะนำไปพัฒนาเป็นชุดตรวจอีไลซ่าเพื่อตรวจแอนติบอดีต่อนิวคลีโอโปรตีนของเชื้อไข้หวัดใหญ่ชนิดเอต่อไปในอนาคต

ภาควิชา พยาธิวิทยา ลายมือชื่อนิสิต .....

สาขาวิชา พยาธิชีววิทยาทางสัตวแพทย์ ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

ปีการศึกษา 255 5 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม .....

5275554331: MAJOR VETERINARY PATHOBIOLOGY
KEYWORDS: INFLUENZA / SEROLOGY / NUCLEOPROTEIN

KANANA RUNGPRASERT: DEVELOPMENT OF ELISA FOR DETECTING ANTIBODY AGAINST INFLUENZA A NUCLEOPROTEIN.
ADVISOR: ASSOC. PROF. KANISAK ORAVEERAKUL, D.V.M., Ph.D.,
CO-ADVISOR: ASSOC. PROF. SANIPA SURADHAT, D.V.M., Ph.D.,
90 pp.

Serological diagnosis is the major role in the surveillance of influenza A virus occurrence in animal herds including pig herd. Commercial ELISA is a convenient and practical method, but it is expensive for routine work in Thailand. The objective of this study is to produce a recombinant nucleoprotein of swine influenza virus for developing an ELISA-based test to detect antibody against nucleoprotein (NP) in order to use it as influenza A seroscreening test in Thai pig herds. Viral RNA was extracted from swine influenza H1N1virus (A/swine/Thailand/CU-CBP18/2009). The full-length NP gene (1,500 bp) and truncated NP gene (1,000 bp) were amplified by RT-PCR. These RT-PCR products were ligated into pThioHisA *E.coli* expression vector. The recombinant NP-pThioHisA and NPt-pThioHisA *E. coli* expression vectors were constructed and transformed into host cells (*E. coli* strain TOP10). The recombinant full-length NP and truncated NP proteins were expressed by using 1 mM of IPTG. Our result showed that the expressed full-length NP and truncated NP proteins were detected by SDS-PAGE (56 kDa and 48 kDa, respectively). Moreover, these proteins could be detected by commercial ELISA using for detection of IAV NP antigen. However, these NP proteins were expressed in insoluble forms, so the proteins could not be purified in native condition. In order to solve this problem, various optimizing conditions were used for enhancing the solubility of the expressed proteins, including the increasing of incubation period in denaturant (6 M guanidide hydrochloride) and using variation of protein expression conditions, including induction temperature, concentration of IPTG, and bacterial strains. Our results demonstrated that, the expressed NP proteins were not purifiable, leading to the unsuccessful establishment of the ELISA-based method. However, this research can be used as the basic information for the future researches that need to produce the recombinant NP proteins for developing an ELISA test kit for antibody detection against NP protein of influenza A virus.

Department: Veterinary Pathology Student's Signature .....
Field of Study: Veterinary Pathobiology Advisor's Signature .....
Academic Year: 2012 Co-advisor's Signature .....

ACKNOWLEDGEMENTS

I would like to express my deepest appreciation to my advisors, Associate Professor Dr. Kanisak Oraveerakul and Associate Professor Dr.Sanipa Suradhat. I am grateful for their patience and forbearance. Both of them have not only given me scientific thinking process but also have guided my thought in social-life living by their straightforward advices and valuable encouragement from their truly spirit. I have always touched with their kindness and sincere support. I really have learned so much from them. I don't have any sufficiently valuable thing to give back to them, just only the words "Thank you".

My gratitude is also extended to Associate Professor Dr. Anudep Rungsipipat, Associate Professor Dr. Alongkorn Amonsin, and Dr. Sudarat Damrongwatanapokin for serving as the member of my research committee and their advices and useful comments.

My special thanks to Assistant Professor Dr. Pravina Kitikoon and Dr. Juthatip Keawcharoen for their kindness and valuable suggestions. I have known them and learned from them. Even though, the time that we spent together is not much but I love it. It's so precious and meaningful to me. Moreover, I would like to thank to a number of people in Chulalongkorn University in particularly Dr. Piya Wongyanin who provided me guidance and helping in all processes of my laboratory work during study time. My special thanks to all virology unit staffs and colleagues, Dr. Aunyaratana Thontiravong, Dr. Suwarak Wannaratana, Dr. Pachariporn Ninvilai, Ms. Ranida Tuanudom, Ms. Duangduean Praguyrungnamthip and Ms. Wikanda Tunterak, all of them have given me helping in many different way and have made my graduate student life are memorable and delightful.

I would like to express my deepest gratitude to my parents, Mr. Chalok and Mrs. Sumalee Rungprasert, and my special aunt, Mrs. Suwimon Rukchon. I could never been succeeded today without your edification, education and evocation. Thank all of them for their unconditional and unlimited love. Special thanks for my lovely sister, Ms. Akaul Rukchon, for her comprehensive suggestion and attention. She has been immediately connected with my emotion and cheers me up when I was feeling tired and depressed. I thank my little sister, Ms. Kattiya Rungpasert, for her responsibility over than mine that make me have more comfortable life. Both of them always accept whatever I am. Finally, I could not have completed this thesis without unexpected love and emotional support of my truly best friends, Ms. Chanida Chalermpolyothin, Ms. Kanokporn Kitvorapong, Ms. Pimsuthee Sangsuwan, and Ms. Siriratchanee Sirisawat. All of them have been devoted to make me happy and laugh when I got weak. They have consoled me to recognize my capability and to appreciate myself. I will always be pleasurable and thankful for having them to share all thing together not also happy and funny stuffs but also the pretty messy or crazy things together.

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LIST OF ABBREVIATIONS

AMV	Avian Myeloblastosis virus
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
$CaCl_2$	Calcium chloride
cDNA	Comlementary deoxyribonucleic acid
DNase	Deoxyribonuclease
ELISA	Enzyme-linked immunosorbent assay
E. coli	*Escherichia coli*
IgG	Gamma immunoglobulin
IPTG	Isopropyl β-D-1-thiogalactopyranoside
LB	Luria-Bertani
OD	Optical density
OD_{280}	Optical density at 280 nm wave length
OD_{600}	Optical density at 600 nm wave length
Rpm	Revolution per minute
RT-PCR	Reverse transcription-PCR
NaCl	Sodium Chloride
NaOH	Sodium Hydroxide
SDS	Sodium dodecyl sulfate
TEMED	N, N, N', N',-tetramethylenediamine
Tris-HCl	Tris-(hydroxymethyl)-aminoethane
WHO	World Health Organization
X-Gal	5-bromo-4-chloro-3-indolyl-ß-D-galactoside

CHAPTER I

INTRODUCTION

Background and rationale

Influenza A is a significant viral respiratory disease of human and animal worldwide. This disease is not only affect on clinical health but also can cause economic loss in swine and poultry industry (Kay et al., 1994; Tiensin et al., 2005). Influenza A virus (IAV) is a member of the *Orthomyxoviridae* family. IAVs are further characterized into different subtypes according to the antigenicity of two surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA). At present there are 17 hemagglutinin (H1 to H17) and 9 neuraminidase (N1-N9) antigens (Lamb and Krug, 2001; Tong et al., 2012). A recent new IAV HA subtype has been identified from bat and then classified as H17 (Tong et al., 2012). Although, the majority of IAV subtypes can infect avian species, only few IAV subtypes can infect mammals such as pig, horse and human (Pringle, 1996).

Swine influenza virus (SIV) can cause respiratory disease in all ages of pig population worldwide and can also cause abortion in pregnant pigs due to high fever (Vannier, 1999). In addition, SIV is one of the primary causes of porcine respiratory disease complex (PRDC) (Yazawa et al., 2004). Although, mortality rate of swine influenza (SI) infection is less than 1% but this disease can cause high economic loss in swine industry of many countries including Thailand due to the mobility rate that can be increased nearly 100% (Kay et al., 1994). In Thailand, SI subtype H1N1, H1N2 and H3N2 have been detected in pig population (Chutinimitkul et al., 2008; Sreta et al., 2009). The genetic variation of IAV is the result from an antigenic drift and antigenic shift mechanism. Interspecies transmission of IAV is rare, however it can occur occasionally. There are many evidences in the literature demonstrating avian and SI viruses as the cause of respiratory disease in humans (Reeth, 2007). There were many evidences

showed that pigs were an important “mixing vessel” or “intermediate host”. The genetic reassortment of avian, swine or human influenza can occur in pigs. Therefore, pig population is considered to be an important host for generating new IAV lead to pandemic occurrence (Zhou et al., 1999; Reeth, 2007; Ma et al., 2009). Recently, a novel influenza A (H1N1-2009) virus infected human in Mexico and the United States in early April 2009. Since the emergence, the virus has been spread worldwide through many countries by human-to-human transmission establishing the 1st human pandemic in this century (Smith et al., 2009). Genetic characteristic of the novel IAV known as “pandemic H1N1” contains a unique combination of gene segments from both North American and Eurasian swine lineages (Garten et al., 2009). Based on the genetic data, the World Health Organization (WHO) supported surveillance studies to detect IAVs not only in human but in many animal species, especially in pigs and vaccines have been used for prevention of the disease in human population worldwide (Galwankar and Clem, 2009).

To diagnose IAV infection, both virus detection and serological evaluation are important tools for monitoring the disease in various species. Reverse transcription-polymerase chain reaction (RT-PCR), DNA sequencing and virus isolation are used to detect viral genome, genetic characterization of the virus. Live virus that can be found in nasal secretion but the direct detection is limited due to the short shedding time of the virus. Therefore, methods to detect influenza-specific antibody can be useful for diagnosis (Kim et al., 2006). Many serological methods are available for influenza A virus diagnosis but among all of those serological methods, hemagglutination-inhibition test (HI test) is considered as the gold standard. This method is used to detect specific antibody against the HA surface glycoprotein of IAVs. HI test has high specificity and is easy to perform. However, many factors including pH of buffer and types of red blood cells, that are selected for the test, can affect the result of the test (Skibbe et al., 2004). Other difficulties of HI test are variation of HA in different strains of virus and the serum of some animal species that contain a variety of biological substances known as nonspecific inhibitors of HA (Ryan-Poirier and Kawaoka, 1991). Thus, procedures to eliminate nonspecific inhibitors of

HA in serum by appropriate treatment such as red blood cell absorption, kaolin treatment are required prior to the beginning of HI test (Boliar et al., 2006). Enzyme-linked immunosorbent assay (ELISA) is a promising method since it is practical to use and has been developed for detection of antibodies to many viral pathogens (Khan et al., 1982; Callow, 1983). To date, there are various commercial ELISAs available for detection of specific antibodies against swine H1N1 or swine H3N2 in pig herds (Lee et al., 1993). Moreover, a commercial ELISA has been available to detect specific antibody against multi-subtype avian influenza but it is recommended for using only in avian species. One drawback is that these available commercial ELISAs must be imported and are considered too expensive for routine work in Thailand. In addition, the tests are limited for using in certain species only.

Many studies reported that not only HA and NA glycoproteins of IAV could induce antibody responses during infection but also nucleoprotein (NP), matrix protein (M1) or non-structural protein (NS1 and NS2). Although, NP and M are highly conserved among IAVs but M1 was shown to be less immunogenic than NP (Hensley and Yewdell, 2009). From the experiment in swine, the characterization of antibody response to NP was similar to HA. The antibodies against HA and NP could be detected at 7 day post-inoculation by western blot analysis and prolonged until 28 day post-inoculation. In contrast, antibodies against M1 protein is not detected until 14 day post-inoculation (Kim et al., 2006). All over, anti-NP antibody may be a good choice for use as a target in multi-subtype serological test.

This study is proposed to generate a recombinant nucleoprotein of swine influenza virus for developing an ELISA-based test for antibody detection against NP for influenza A seroscreening test. Moreover, this ELISA-based will be useful for monitoring antibody response to IAV vaccination or infection in pig herds.

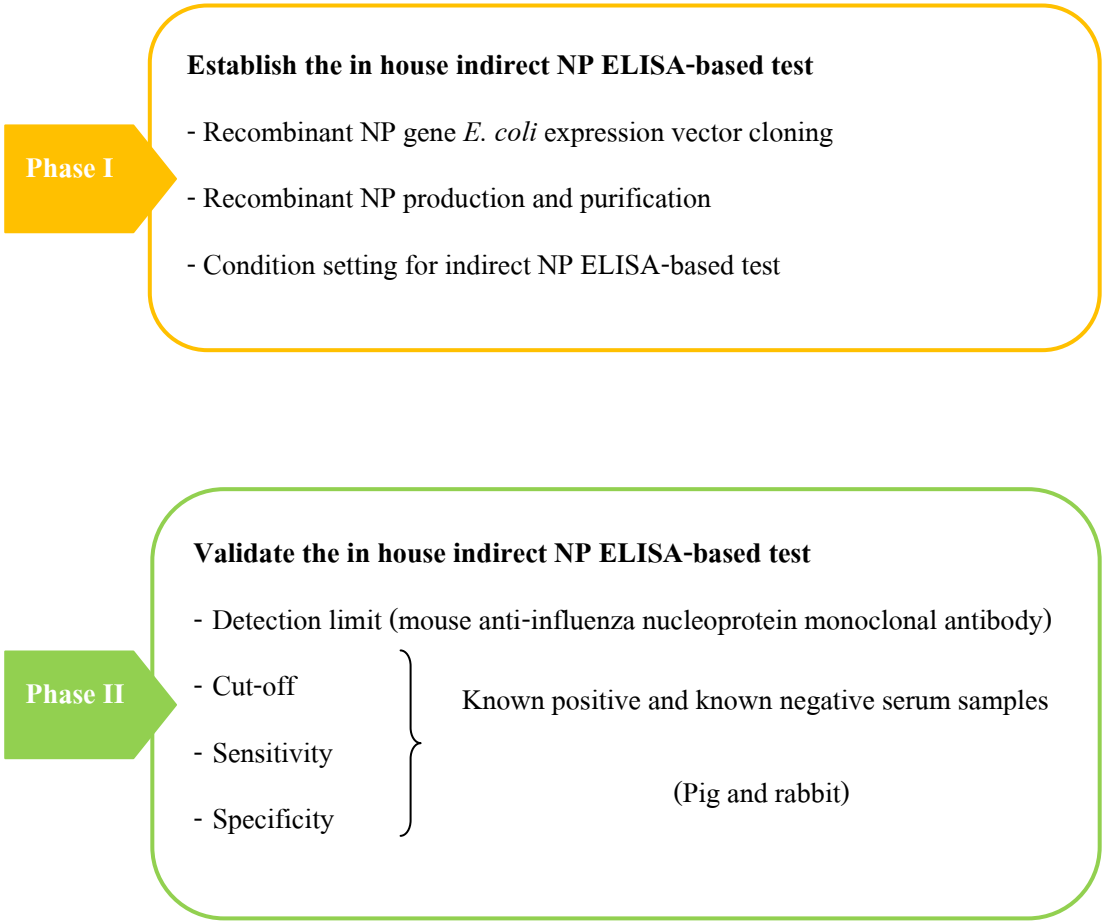
Objective of this study

To produce a recombinant nucleoprotein of swine influenza virus for developing an ELISA to detect antibody against influenza A nucleoprotein (NP) in pigs.

Expected advantage of the study

Establish a practical ELISA for influenza A serological surveillance in Thai pig herd.

Conceptual framework



CHAPTER II

LITERATURE REVIEW

Influenza A

Influenza or flu is respiratory disease caused by virus. The disease emerges not only in occasionally pandemic respiratory disease outbreak but also affect worldwide by annual epidemic outbreak in every winter. For the last century, influenza A virus (IAV) appeared as main causative agent that introduced unpredictable pandemic and most of all annual seasonal epidemic of influenza outbreak worldwide (Hampson and Mackenzie, 2006).

Annually, human worldwide have been hospitalized with severe respiratory symptoms by seasonal influenza about 3-5 millions cases and more than 250,000 cases have been died (Suzuki, 2005). Many data have shown that seasonal IAV infection can increase hospitalization rate, prolong illness and increase risk of death in young and old people, pregnant women and patients infected with chronic cardiopulmonary disease or human immunodeficiency virus (HIV) (Safrin et al., 1990; Lin and Nichol, 2001). However, pandemic influenza could attack humankind with higher number of death (mortality rate) than seasonal epidemic influenza. The novel IAV subtype was introduced into new human population which complete absence or adequate preexisting immunity to limit the infection of the virus (Cowling et al., 2010). Three major pandemics in human population have been occurred in 20^{th} century. In 1918, the pandemic Spanish flu (H1N1) occurred and killed 50 million human lives around the world. The death is higher than the number of people who died in World war I. Pandemic of Asian flu (H2N2) in 1957 and Hong Kong flu (H3N2) in 1968 cause death of approximate 2 million (Forbes, 2004; Viboud et al., 2005; Taubenberger and Morens, 2006).

Apart from its importance in avian and swine species, influenza A disease is an important zoonosis that affects both human and animal health (Vincent et al., 2008). Generally, IAV

cross-transmission among animal species is rare. However, avian influenza or swine influenza could be introduced into human population. Novel influenza virus can be adapted in animal species and cause influenza pandemic. For instance, the outbreak of avian influenza H5N1 virus (Hong Kong flu) in 1997 presented the direct transmission of avian influenza to human and in 2004 to 2005. The outbreaks of avian influenza (H5N1) in Asia, including Thailand were reported not only in poultry industry but also in human with mortality rate of approximately 50% (Hampson and Mackenzie, 2006). First influenza pandemic outbreak in 21th century occurred in April 2009 (H1N1-2009 virus). The swine-origin H1N1 IAV circulated in human population and then spread worldwide by human-to-human transmission (Smith et al., 2009).

Influenza A virus classification and virion

The *Orthomyxoviridae* is a family of enveloped virus containing single-stranded negative sense RNA segments. This family is composed of five genera: influenza A, influenza B, Influenza C, Thogotovirus and Isavirus (Webster et al., 1992). Thogotovirus is a tick-borne viral disease in hamster (Wang and Nuttall, 2001). Isavirus is identified as causative agent of infectious salmon anemia (Raynard et al., 2001). Since 1960s, Influenza viruses have been given name because of their ability to bind mucus membrane. Influenza A, B, C viruses could be distinguished based on (1) the antigenic differences between nucleoprotein (NP) and matrix protein (M), (2) morphology, (3) the genome organization, (4) the variability of surface glycoproteins and (5) the host range even though all these viruses could infect human (Lamb and Krug, 2001).

Morphology of IAVs is usually spherical but elongated shape can occur with the measuring size of 80-120 nm in diameter. The IAVs virion is composed of 8 viral structural proteins. The outer lipid bilayer-envelope of IAVs is derived from plasma membrane of host cell that contains three types of viral transmembrane protein; (1) hemagglutinin (HA) glycoprotein, (2) neuraminidase (NA) glycoprotein and (3) matrix protein 2 (M2) ion channel. The matrix

protein 1 (M1) underlines the envelope protein and is considered as the major structural component of the virion. Inside the shell of M1 lie, there are the helical ribonucleoprotein (RNP) particles: these consist of the viral RNA genome segments, trimeric RNA polymerase proteins (polymerase basic 1 (PB1), polymerase basic 2 (PB2) and polymerase acid (PA)) and nucleoprotein (NP). There are eight separated RNPs in a virion that relate with genome feature of the virus (Lamb and Krug, 2001). Each viral RNA segment is wrapped by multiple copies of NP, predominant protein of viral capsid and each NP subunit interacts with approximately 24 nucleotides of RNA, and some amount of NS2/NEP. The trimeric polymerase is located at the end of RNP complex (Ortega et al., 2000). The structure diagram of IAV is shown in Figure 2.1.

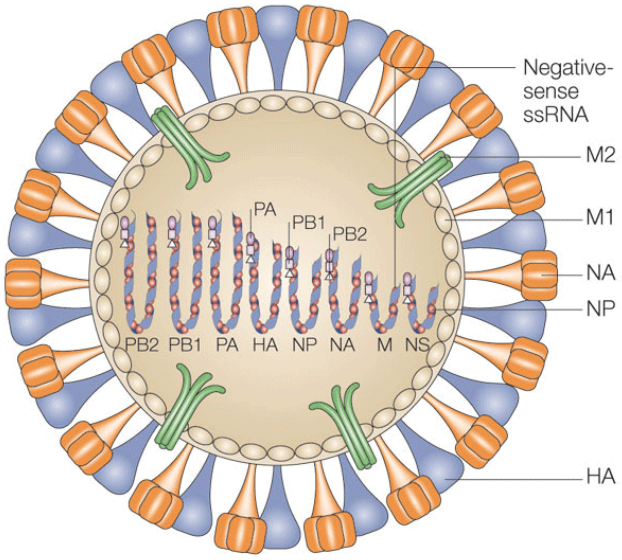


Figure 2.1 Virion diagram of influenza A virus (Horimoto and Kawaoka, 2005)

The arrangement feature of HA and NA glycoprotein are spikes radiating outward from virus envelope. Traditionally, IAVs are classified into subtypes base on antigenic characteristics of these two surface glycoproteins (HA and NA). At present, 17 HA (H1-H17) and 9NA (N1-N9) subtypes have been identified. All subtypes of IAVs have been isolated from avian species and the natural reservoir of IAVs is wild aquatic birds, except the recent novel subtype of IAV (H17)

has been identified from bat (Fouchier et al., 2005; Tong et al., 2012). Occasionally, some combination subtypes of IAVs which circulate and are non-pathogenic in avian species can change and directly transmit from avian to mammal species, including human, which may introduce the pandemics of IVAs to human and mammal species (Guo, 1992; Peiris et al., 2007; Ma et al., 2009). Even nowadays, only combination subtypes of H1, H3 subtypes and N1, N2 subtypes are circulated in human and pig population (Ma et al., 2009). However, there were many evidences reported about occasionally transmission of avian influenza H5N1, H7N7, H7N9 and H9N2 to human (Lin et al., 2000; Fouchier et al., 2004; Peiris et al., 2004; Wen and Klenk, 2013).

Influenza A virus genome and encoded protein function

Genome of IAVs consists of 8 negative-sense-single-stranded RNA segments encoding 10 proteins or more in some cases for different function as description in Table 2.1 and 2.2. Totally, IAV genome is completely about 13,600 nucleotides, separates into 8 different gene segments, and the nucleotides length of each segments are from 890 to 2,341 nucleotides (Lamb and Krug, 2001).

Table 2.1 RNA segments and their encoded protein of IAVs (Lamb and Krug, 2001)

Segment (no. of nucleotides)	Gene product (no. of amino acids)	Molecule per virion
1 (2,341)	Polymerase basic 2 (759)	30-60
2 (2,341)	Polymerase basic 1 (757)	30-60
3 (2,233)	Polymerase acid (716)	30-60
4 (1,778)	Hemagglutinin (566)	500
5 (1,565)	Nucleoprotein (498)	1,000
6 (1,413)	Neuraminidase (454)	100
7 (1,027)	Matrix protein 1 (252)	3,000
	Matrix protein 2 (97)	20-60
8 (890)	Non-structural protein 1 (230)	-
	Non-structural protein 2 (121)	130-200

Both HA and NA surface glycoproteins are inserted into the lipid envelope and are the major antigenic determinant of IAVs. HA can bind with sialic acid receptor of epithelial cell initiating virus attachment. NA, has enzymatic activity which can cleave the terminal sialic acid and thus release the newly produced virus from the infected cells (Su et al., 2009). Balance between HA receptor binding activity and NA receptor cleavage activity is important for efficiency of virus replication (Ma et al., 2009). Antibodies to the HA and NA proteins can therefore neutralize the virus as well as inhibit virus spread, respectively (Johansson et al., 1989; Cox et al., 2004). Another inserted envelope protein is M2 which acts as an ion channel involving viral uncoating and HA maturation. In the viral core, each RNA segment interacts with NP and viral RNA polymerase complex (PB2, PB1 and PA) to form a RNP complex. The RNPs play the major role for virus replication. All RNPs are surrounded by M1 (Lamb and Krug, 2001). NP contacts with a variety of cellular and other viral molecules so the function of NP is for virus genome encapsulation and, RNA transcription, replication, and packaging (Portela and Digard, 2002). Function of M1 is associated with virus assembly and budding. Over these viral structural proteins, there are non-structural proteins (NS1 and NS2) which encoded from the 8^{th} RNA segment with overlapping reading frame. NS1 have mainly function to inhibit interferon-mediated antivirus response of host cells including virus replication. NS1 has been found only in the infected cell. NS2 or NEP is the important protein which interacts with M1 to mediate the exportation of RNPs from nucleus to cytoplasm of host cells (Lamb and Krug, 2001). PB1-F2 and N40, the recently novel proteins of IAV are encoded by an alternate open reading frame in 2^{nd} gene segment of IAV. PB1-F2 has been shown as a pro-apoptotic protein mediating death of alveolar macrophages, thus it plays as a virulence factor of IAVs (Coleman, 2007). Lacking of N40 has been shown reducing of viral polymerase activity (Wise et al., 2009). Moreover, PA-X is encoded by second open reading frame in 3^{rd} gene segment of IAV. The previous study showed its ability to modulate IAV virulence by modulating host response to infection (Jagger et al., 2012).

Table 2.2 Function of encoded protein of IAVs genome (Sreta, 2010; Jagger et al., 2012)

Segment	Encoded protein	Function of viral protein
1 (PB2)	PB2	Initiation of viral mRNA transcription through recognition and binding of the 5'cap-1 structures if host pre-mRNAs used to genetic primers for viral transcription.
2 (PB1)	PB1	Responsible for elongation of the primed nascent viral mRNA and elongation in template RNA and vRNA synthesis. It contains the conserved motifs characteristic of RNA-dependent RNA polymerase. It also contains site for sequence-specific binding to conserved 5'– and 3'-terminal sequences of vRNA and cRNA molecules.
	PB1-F2	May have a role in modulating the host response to influenza A virus by hastening the death of immune cells.
	N40	Binding with PB2 and shifts polymerase activity toward replication
3 (PA)	PA	Thought to be involved in viral RNA replication. Strong suggestion has been made that PA is involved in the assembly of functional viral RNA polymerase complexes from their inactive intermediates.
	PA-X	Modulates host response to infection
4 (HA)	HA	Binding of virion to host cell receptor and fusion between the virion envelope and the membrane of the endosome.
5 (NP)	NP	Binds to and encapsidates viral RNA to from coiled ribonucleoprotein complex to which the three polymerase protein associate.
6 (NA)	NA	Cleave terminal sialic acid from glycoprotein or glycolipids of free progeny virions from host cell receptors.
7 (M)	M1	Forms a shell surrounding the virion nucleocapsids underneath the virion envelope. Play an important role in progeny virus assembly initiation.
	M2	The membrane-spanning domain serves as a signal for transport to the cell surface. Act as a portion channel to control the pH of the cell Golgi during HA synthesis and to allow acidification of the interior of the virion during virus coating
8 (NS1)	NS1	Regulates nuclear export of mRNA and inhibits pre-mRNA splicing. Probably inhibits IFN-mediated antivirus responses of the host
	NS2/NEP	Provides M1 with a nuclear export signal that mediates the nuclear export of vRNA from the nucleus to the cytoplasm

Influenza A virus replication

IAV replication cycle can be described into following steps by beginning with (1) IAV receptor binding and host cell entry by receptor mediated endocytosis, (2) virus uncoating in cytoplasm and transporting of viral RNPs into nucleus, (3) mRNA transcription and viral RNA replication in nucleus of infected cell, (4) viral internal protein translation in cytoplasm and viral membrane protein translation in endoplasmic reticulum (ER) of host cell, and finally (5) virus assembly and releasing of progeny virus at plasma membrane by budding (Samji, 2009). The life cycle of IAV has been shown in Figure 2.2.

In human, target cell of IAV is epithelial cell in respiratory tract. IAV can bind to the sialic acid residual (SA) of the receptor on target cell surface by HA. HA precursor (HA0) comprises two subunits, HA1 and HA2, which is linked by disulphide bond. HA1, a globular domain at the distal end of the spike, plays as a cellular sialic acid receptor binding site and contains major antigenic epitopes. HA2, stem of the spike, contains "fusion peptide" at N-terminal (Skehel and Wiley, 2000). Binding between HA and host cell receptor initiate the host cell entry through receptor mediated endocytosis. The virus is engulfed by plasma cell and formed the endosome. The endosome that containing virus in the lumen are delivered into cytoplasm of the host cell (Lakadamyali et al., 2004). Under a low pH (5-6) condition in the endosome, conformational change of HA0 is induced that results in maintenance of HA1 receptor binding site but exposing fusion peptide of HA2 to the distal part of the spike. Subsequently, fusion peptides insert into endosomal membrane and bring virus envelope into contact with the endosomal membrane. Merging of the two membranes leads to the forming of fusion pore (Huang et al., 2003). Low pH condition in the endosome is not the only a key for inducing HA conformational change but also initiates opening of M2 ion channel in viral envelope (Pinto et al., 1992). When M2 proton channel open up, protons influx from lumen of endosome into viral core. This acidification brings the weakening interaction between M1 underneath viral

envelope and viral RNPs, thus, the viral RNPs are free and can directly move through fusion pore from viral core into cytoplasm of infected cell (Pinto and Lamb, 2006). After that, RNPs are transported into the nucleus of infected cell where viral messenger RNAs (mRNA) are synthesized and viral RNAs are replicated. mRNA transcription and viral RNA replication are carried out by viral RNA polymerase complex (PB2, PB1 and PA) and NP. mRNAs are exported into cytoplasm for translation of viral proteins. Viral proteins which are necessary for transcription and replication are moved back into nucleus (Neumann, 2009). Synthesis of viral envelope proteins (HA, NA and M2) occurs in the cytoplasm prior to transporting into ER for glycosylation and folding. Subsequently, those are transported into Golgi apparatus and then are moved to insert into plasma membrane of the host cell (Lamb and Krug, 2001). Synthesized viral RNPs are localized from nucleus to cytoplasm of the host cell by interaction with nuclear export signal on M1 and NS2/NEP (Samji, 2009). M1 interacts with HA and NA on plasma membrane and then condenses HA and NA forming patch that M1 lining underneath plasma membrane with high density of HA and NA. After the synthesized viral RNPs attached with M1 at the patch, the packaging of viral RNPs occurs and then budding of progeny virus appears at plasma membrane (Nayak et al., 2004). After budding, progeny virus is still attached with plasma membrane of host cell by binding between HA and SA. NA, which has an enzymatic activity, can cleave SA resulting in releasing of progeny virus from the infected cell (Su et al., 2009).

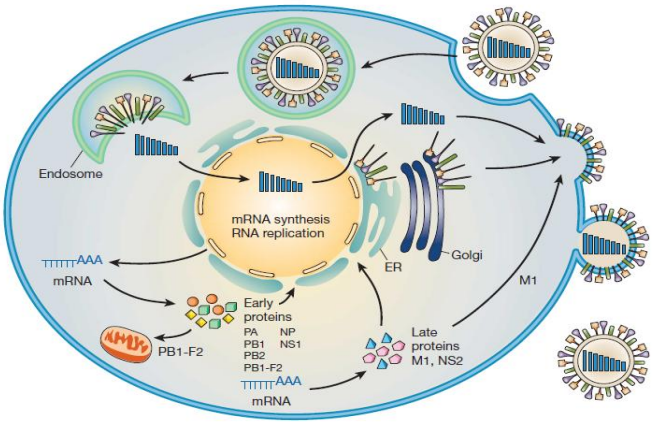


Figure 2.2 Schematic diagram of influenza A virus's life cycle (Neumann, 2009)

Antigenic variation of influenza A virus

The most importance characteristic of IAV is antigenic variation. There are many subtypes and strains of IAV that circulate in different host species as the results of antigenic evolution of the virus (Nelson and Holmes, 2007). Moreover, occurrence of yearly-seasonal influenza epidemic and influenza pandemic are based on antigenic evolution of the virus that allow virus to evade host immunity previously induced by influenza subtype (Suzuki, 2005). There are two major evolution mechanism of IAV for changing its antigen, "antigenic drift" and "antigenic shift" (Lamb and Krug, 2001).

Evolution of IAV for escaping from host immunity that induced by other strain is the accumulation of point mutation of viral genome resulting in antigenic changing. This antigenic adaption process is referred to "antigenic drift" (Nelson and Holmes, 2007). Mutation of influenza A genome is continuous occurrence due to lack of fidelity of viral RNA polymerase (Ma et al., 2009). Mutation rate of influenza A genome is approximately 1 in 10^5 nucleotides per infectious cycle (Lamb and Krug, 2001). Each viral RNA replication cycle, the mixed population of IAV are produced under a natural selective condition or pre-existing immunity of host, some viruses that have advantage mutation can be a dominant and established in host population (Suzuki, 2005). Evolution rates of influenza A genes are depended on host immune selective pressure or adaptation to novel host so the evolutions of viral surface protein genome segments, such as HA or NA, are more rapid than the viral internal proteins (NP, M, PA, PB1, PB2) (Webster et al., 1992). Antigenic drift is a continuous mechanism so the new strain virus which arised by this process is still partially recognized by pre-existing immunity (Nelson and Holmes, 2007). Antigenic drift plays an important role in supporting the annual seasonal influenza epidemic occurrence by the same virus subtype circulating in the same host population. Antigenic drift of influenza virus is necessary for vaccine efficacy and annually vaccine

formulation adjustment (Francis et al., 1947; Hampson and Mackenzie, 2006; Taubenberger and Morens, 2006).

Antigenic shift, another important antigenic evolution of IAV, is a dramatic antigenic changing of IAV. This process leads to new subtype of IAV into the population. Antigenic shift is driven by occasionally interspecies transmission of virus into non-native host or through the process of genetic reassortment between the viruses circulating in the host population and the new virus (Olsen, 2002). The genome characteristic of IAV is segmented by nature. Co-infection of two different origin IAVs in the same cell during a process of virus progeny packaging can induce the exchange of RNA segments of both parental viruses to produce the genetic variety of viral progeny. The new progeny virus contains 8 selective RNA segments from both parental viruses. The process is known as “reassortment” (Ma et al., 2009). Thereby, a new subtype virus may be introduced into immunologically naïve population and lead to the high morbidity and mortality rate. Therefore, the genetic reassortment of IAV is a major role in emergence of new strain virus pandemic occurrence (Scholtissek, 1998). Two important IAV pandemic in human, Asian flu (H2N2) in 1957 and Hong Kong flu (H3N2) in 1968, were the result of antigenic shift. These both cases, the previously circulating human influenza virus (H1N1) imported their HA and NA genes from Eurasian avian influenza viruses (Taubenberger and Morens, 2006; Taubenberger and Morens, 2008; Ma et al., 2009). The genetic reassortment between avian influenza and human influenza viruses may occur in susceptible host so called “intermediate host” or “mixing vessel”. There were many evidences indicating that pigs were an important mixing vessel (Zhou et al., 1999; Brown, 2001; Reeth, 2007; Ma et al., 2009; Trebbien et al., 2011).

Swine influenza

Swine influenza virus (SIV) can cause respiratory disease in pigs of all ages and abortion in pregnant pigs due to high fever (Vannier, 1999). Clinical signs of SIV- infected pigs are similar to those observed in humans such as losing appetite, sneezing, nasal discharge, conjunctivitis and

fever (Alexander and Brown, 2000). Mortality rate of swine influenza (SI) is less than 1% but this disease can cause severe economic loss due to high morbility rate which can increase up to 100% (Kay et al., 1994). In addition, SIV is one of the primary causes of porcine respiratory disease complex (PRDC) (Kay et al., 1994; Yazawa et al., 2004). In the British swine industry, the amount of money loss per year due to SI was approximately £60 million with £7 per pig affected with PRDC (Kay et al., 1994). SI was first reported after the Spanish flu outbreak in human in 1918 but swine influenza virus (SIV) could be isolated from pigs in 1930 (Shope, 1931). SIV H1N1, H1N2 and H3N2 subtypes are commonly detected in swine population worldwide but the origin and characteristic of all subtypes are different depending on geographic locations. SIV H1N1 subtype in the U.S. is well-known as "classical H1N1 swine virus" and "triple-reassorted H1N1 SIV". The gene constellation of SIV circulating in European pig population is called "avian-like" H1N1 as it has an avian origin. After pandemic of human Hong Kong flu (H3N2) in 1968, SIV H1N2 and H3N2 subtypes have been generated due to genetic reassortment between human influenza (H3N2) and swine influenza (H1N1) which circulated in each area (Vincent et al., 2008). In Asia, there were several reports about SIV in China that SIV H1N1 was direct transmission from bird to pig population whereas HA genes of SIV H3N2 in China related with avian and human H3 (Kida et al., 1988; Guan et al., 1996). In Thailand, all these subtypes have been isolated from pigs (Kupradinun et al., 1991; Chutinimitkul et al., 2008; Sreta et al., 2009). As mention earlier, all of three subtypes, H1N1, H1N2 and H3N2 have been found in Thai swine herds. Recently, HA and NA genes of swine influenza H1N1 in Thailand were characterized. The HA gene of Thai swine influenza is subordinate to the classical swine lineage while the NA gene belongs to avian-like lineage. HA and NA genes of SIV H3N2 isolated in Thailand were characterized and those results showed the circulation of American, European, Asian SIV H3N2 and also Human IAV H3N2 in Thai pigs. SIV H1N2 isolated in Thailand, characteristic of HA is closely related to American SIV and Asian SIV H1N2 but NA gene is related to European SIV (Chutinimitkul et al., 2008; Sreta et al., 2009).

Since early 2009, there was an emergence of novel influenza A (H1N1) virus which could infect human in Mexico and the United States. Genetic characteristic of the novel influenza known as "pandemic H1N1" contained a unique combination of gene segments from both North American and Eurasian swine lineages (Garten et al., 2009). During the outbreak, there was a report of the co-circulation of pandemic H1N1 and Thai swine H1N1 in Thai commercial pig herd (Sreta et al., 2010).

Pig population is considered to be an important host for IAV pandemic occurrence because pigs are known as mixing vessel or intermediate host of IAV (Ma et al., 2009). The previous study showed that pigs are susceptible host for swine, avian, and human influenza viruses due to the presence of α2,3 and α2,6-galactose sialic acid receptor in upper respiratory tract (Trebbien et al., 2011). Thereby, the genetic reassortment of avian, swine, or human influenza viruses can occur in pigs (Ma et al., 2009). Recently, there was a report of the emergence of novel reasserted pandemic H1N1 (rH1N1) virus in Thailand. From the genetic analysis of the virus is demonstrating that this virus was generated from genetic reassortment of swine origin human pandemic H1N1 and Thai swine H1N1 virus (Kitikoon et al., 2011). Moreover, the serological study revealed an evidence of pig to human transmission of novel influenza A (H1N1) in Thai swine-expose workers (Kitikoon et al., 2011).

The World Health Organization (WHO) supported surveillance studies to detect influenza viruses not only in human but in all animal species, especially in pigs and vaccines have been used for prevention of the disease in human population worldwide (Galwankar and Clem, 2009). Based on the data of Thai SIV, influenza A surveillance in Thai pig population should be continuously monitored.

Influenza A virus diagnosis

Influenza A virus diagnosis is difficult to performed only by clinical signs only because clinical signs of the disease are similar to those symptoms caused by other respiratory infection

agents. Influenza A virus diagnosis can be performed by virus isolation and culture, direct and indirect methods (Webster et al., 2002). Influenza A virus can be isolated and cultured in embryonated eggs or cultured cells. Because many influenza viruses may not present the appearance of cytopathic effect (CPE) on cell culture so the supernatant of virus culture have to test with other virus replication screening test such as immunochemistry assay, hemagglutination test and viral nucleic acid testing. The virus detection using these method required at least 4-5 days so many rapid antigen detection assay have been developed (Dwyer, 2006). The direct method involves the detection of viral nucleic acid or antigen. Direct virus detection is limited by the short shedding period of IAV infected animals (Kim et al., 2006). Indirect method to detect influenza A-specific antibody response is an alternate way for IAV diagnosis (Webster et al., 2002). Antibody response against HA and NA of IAV are associated with protection from infection while antibodies to internal conserved proteins such as M and NP are not. Major immune response against M and NP is cytotoxic T-cell, although cytotoxic T-cells do not provide protective immunity to these protein but they play an important role for viral clearance (Cox et al., 2004). Hemagglutination inhibition test (HI test) is the gold standard for diagnosis influenza A infection by measurement of HA-specific antibodies (Webster et al., 2002). However, many factors may contribute to the accurate HI test result such as variations of HA in different strains of IAV and the source of erythrocytes used in the test (Cunningham, 1973). More importantly, HI test can be interfered by many nonspecific inhibitors found in different animal sera that can cause inaccurate results (Ryan-Poirier and Kawaoka, 1991). Most of the time, serum samples must be treated first by using different substances (Subbarao et al., 1992) and enzymes to eliminate nonspecific inhibitors and thus contribute to the cost and turnaround time of the test (Boliar et al., 2006).

ELISA based test is an interesting optional method to evaluate the serological status in different species. ELISA based test may be suitable because it is simple and less labor intensiveness. Nowadays, many commercial ELISAs have been developed for practically use in

the field such as ELISA for the detection of antibody against HA of SIV H1N1 or SIV H3N2 in pigs and ELISA for the detection of antibody against avian influenza (for avian species only) (Erickson, 2005; Wu et al., 2007; Pérez-Ramírez, 2010; Toennessen et al., 2010). Moreover, ELISA for detection of antibody against influenza A virus nucleoprotein in multi-species have been developed and can be used in avian flock and swine herds (Ciacci-Zanella et al., 2010). The disadvantage is that most commercial ELISAs must be imported. As a result, these kits become expensive as well as certain kits may not be applied due to the difference of virus test antigen and field virus strains circulating in Thailand. In summary ELISA that can be used for the detection of antibody response to multi-IAV subtypes with the reasonable price is needed for the developing countries including Thailand.

Development of ELISA for antibody detection against influenza A virus

To develop an ELISA, the target protein used to detect influenza A specific antibody is considered to be the most essential component. For seroscreening test development, the choice protein should be the highly conserved protein among all IAVs. Nucleoprotein (NP) of IAV is encoded from gene segment 5th of virus genome. NP is an abundant protein found in the virion of IAV. The ratio of NP:HA is about 2:1. The NP antigen is used for differentiation of influenza A, B and C viruses (Lamb and Krug, 2001).

Although, the phylogenetic analysis of NP genes showed that NP had evolved into 5 lineages, including (1) Equine/Prague/56, (2) recent equine strain, (3) classical swine and human, (4) gull H13 and (5) avian strain, many previous studies revealed the conservative nature of NP gene and protein (Gorman et al., 1990; Thippamom et al., 2010). NP is well conserved among all IAVs with less than 11% of a maximum amino acid difference (van Wyke et al., 1980; de Boer et al., 1990; Thippamom et al., 2010). To date, the complete antigenic structure of NP is still unknown (Prokudina et al., 2008) but the antigenic determinants of NP is preserved among different strains of the viruses (van Wyke et al., 1980). Some studies reported that NP specific

antibody was consistent across host species (de Boer et al., 1990; Wu et al., 2007; Ciacci-Zanella et al., 2010). Moreover, there was a report of high sensitivity of a competitive ELISA developed by using recombinant NP of avian IAV for detecting antibody against IAV (Zhou et al., 1998). The previous study had characterized the humoral immunity to SIV in pigs and found that antibodies against NP could be detected at 7 day post-inoculation and prolonged until 14 day post-inoculation. In contrast, antibodies against another conserved protein of IAVs (M1 protein) could not be detected until 14 day post-inoculation (Kim et al., 2006). Therefore, NP could be an excellent choice to use as antigen in multi-subtypes influenza A serological test. Many researchers reported that NP expressed from *Escherichia coli* (*E. coli*) expression system could be used for detection of antibody against NP in many animal species (de Boer et al., 1990; Jin et al., 2004; Ciacci-Zanella et al., 2010). Prokaryotic expression system, particularly *E. coli* expression system, is the most widely used for protein productions (Baneyx, 1999). There are several characters of this system that make it suitable for using as a recombinant protein expression system. *E. coli* is easily manipulated and it has rapid growth rate in inexpensive media. Protein yield produced in *E. coli* expression system is generally higher than other expression systems such as yeast, mammalian cell, insect cell (Ghosh et al., 2004). Moreover, the fusion protein in recombinant *E. coli* expression system is reliable for quality improvement of protein expression and purification (Esposito and Chatterjee, 2006).

CHAPTER III

MATERIALS AND METHODS

All steps involving the usage of commercial kits in the following methods were performed according to the manufacturer's protocol.

Viral RNA extraction

Viral RNA was extracted from swine influenza (A/swine/Thailand/CU-CBP18/2009 (H1N1) nucleoprotein gene, accession number HM142754) stock virus by using NucleoSpin Extract Viral RNA Kit (Macherey-Nagel, Düren, Germany). The extracted viral RNA was kept at -80°C until used.

Primer design

In this study, primers were designed for amplifying the NP gene of swine influenza virus H1N1 virus. Published NP sequences of the virus were retrieved from GenBank. These NP sequences were aligned by CLUSTAL X multiple alignment program (version 1.8) and used as reference sequences of NP gene. Start (ATG) and stop codon (ATT) of NP gene were identified and used for primer design. All primers in this study were designed and analyzed by OLIGO primer design program (version 9.1).

NP_F61 and NP_R1557_*Sal*I were designed for amplifying of full-length NP gene, (coding region). RT-PCR product from these primers contained start codon of NP gene and *Sal*I restriction endonuclease recognition site at downstream of stop codon of NP gene. Amplicon size was about 1,500 base pairs (bp). The sequences of both primers were shown in Table 3.1.

NP49_*Xho*I and NP377_*Xba*I were designed for amplifying of truncated NP gene at amino acid residual 49-337 of NP protein with the amplicon size of about 1,000 bp. The forward primer (NP49_*Xho*I) began with 5' *Xho*I restriction endonuclease recognition site and followed

by specific the 5' coding region of NP gene (nucleotide 145-167). The reverse primer (NP377_*Xba*I) coded for 3' amplification (nucleotide 1114-1131) started by *Xba*I restriction endonuclease recognition site. The sequences of primers for amplifying truncated NP gene were indicated in Table 3.2.

Table 3.1 Primers for amplifying full-length NP gene of influenza A virus

Primer	**Code name**	**Primer sequence (5'-3')**
Forward primer	NP_F61	ATGGCGTCTCAAGGCACCAAA Start
Reverse primer	NP_R1557_*Sal*I	GGCGGCGTCGACTTAATTGTCATACTCCTCTGCAT *Sal*I Stop

Table 3.2 Primers for amplifying truncated NP gene of influenza A virus

Primer	**Code name**	**Primer sequence (5'-3')**
Forward primer	NP49_*Xho*I	GCCGCGCTCGAGAAAAGACTCAGTGACTATGAAGG *Xho*I
Reverse primer	NP377_*Xba*I	GGCGGCTCTAGAAACGGAATCCATTGTTTC *Xba*I

Reverse transcriptase-polymerase chain reaction (RT-PCR) and RT-PCR product purification

Full-length and truncated cDNA of NP gene were produced and amplified by using One-step RT-PCR (Promega, WI, USA). Each mixture of reactions was combined with 25 µl of 2x AccessQuick™ Master Mix, 1 µl (5 U) of AMV reverse transcriptase, 1 µl (20 µmol) of each primers (forward and reverse primer), 3 µl of extracted RNA and Nuclease-free water to a final volume of 50 µl. RT-PCR conditions were shown in Table 3.3. The RT-PCR products were

analyzed with 1.5% agarose gel electrophoresis, stained with ethidium bromide and visualized by UV transilluminator. The RT-PCR products were purified from agarose gel using NucleoSpin® Extract II (Macherey-Nagel, Düren, Germany).

Table 3.3 RT-PCR condition for amplifying full-length and truncated NP genes

RT-PCR step	**Condition**
Reverse transcription reaction	49 °C for 45 minutes
PCR amplification	
Initial denaturation	94 °C for 3 minutes
Denaturation	94 °C for 30 seconds } 40 cycles
Primer annealing	55 °C for 30 seconds } 40 cycles
Extension	72 °C for 1.5 minutes } 40 cycles
Final extension	72 °C for 10 minutes

Bacterial strains and plasmids

Escherichia coli (*E. coli)* strain JM109 (Madison, WI, USA) was used as the host of pGEM®-T easy vector (Promaga, WI, USA) and *E.coli* strain TOP10 (Invitrogen™, CA, USA) was used as the host of pThioHisA vector (Invitrogen™, CA, USA). In addition, *E.coli* strain BL21 (GE Healthcare, Dornstadt, Germany) and JM109 were used as the host of expression vector. The genotype of these bacterial strains and vector map demonstrating the promoter and multiple cloning sequences were shown in Appendix A.

Preparation of *E. coli* competent cells

E. coli strain JM109, TOP10, and BL21 were used as host cells. Bacterial stocks were recovered in Luria-Bertani (LB) agar media and incubated overnight at 37°C until single colony was visible. A single colony was incubated in 5 ml LB broth overnight at 37°C with shaker (250 rpm). Subsequently, 1 ml of overnight culture was incubated into 50 ml of LB broth at 37°C

with shaking until OD_{600} was 0.5 (approximately 3-4 hours). Bacteria in the culture were harvested by centrifugation at 2,000 rpm, at 4°C for 15 minutes. Bacterial pellets were resuspended in 10 ml of 0.1 M sterile $CaCl_2$, incubated on ice for 30 minutes, and centrifuged at 2,000 rpm, 4°C for 15 minutes. The bacterial pellets were resuspended in 3 ml of cold sterile 0.1 M $CaCl_2$ with 10% glycerol. The competent *E. coli* suspension was aliquoted 200 µl per each microcentrifuge tube and stored at -80°C for further transformation.

Transformation of *E. coli* by heat shock protocol

The frozen competent cells were thawed on ice. The ligation mixtures were added to the competent cells and incubated on ice for 30 minutes. The cells were heat shocked in a water bath at 42°C for 60 seconds without shaking, followed by immediately chilling on ice for 2 minutes. The transformed cells were added with 800 µl of LB broth then incubated at 37°C with shaking for 1 hour. The transformation cultures were centrifuged at 8,000 rpm for 1 minute. Supernatants were removed and the pellets were resuspended with 100 µl residual media. The resuspensions were spread on selective media agar plate and incubated at 37°C overnight until *E.coli* colonies could be observed. Selective media for pGEM®-T easy vector was LB agar plate with ampicillin/IPTG/X-Gal while selective media for pThioHisA vector is LB agar plate with ampicillin. The compositions and preparations of the media were shown in Appendix B.

Construction of the *E. coli* recombinant full-length NP expression vector (NP-pThioHisA)

RT-PCR product of full-length NP gene was amplified using PCR cloning pGEM®-T easy vector system for enhancing gene concentration before using for construction of NP-pThioHisA. Concentration of RT-PCR product of NP gene was measured by spectrophotometer at 280 nm wave length to calculate the appropriate amount of PCR product for ligation mixture. Suitable molar ratio of inserted DNA to vector is 3:1. The ligation mixture contained 20 µl of mixture as followed: 10 µl of 2x T4 DNA Ligase Rapid Ligation Buffer, 2 µl of pGEM®-T easy vector (50ng/µl), 6 µl of diluted RT-PCR product (using sterile distilled water)

and 2 µl of T4 DNA Ligase (3 Weiss units/µl). The ligation mixture was incubated overnight at 4°C before being transformed into *E. coli* competent cell strain JM109. Bacterial colonies indicating positive NP gene insertion were propagated and verified for the presence of NP gene and the direction of NP gene by PCR and DNA sequencing. The NP positive clones were propagated and the plasmids were extracted using NucleoSpin® Plasmid (Macherey-Nagel, Düren, Germany). The extracted DNAs were verified for NP gene orientation by DNA sequencing and designated as NP-pGEM.

The extracted NP-pGEM and pThioHisA vector were digested by *Not*I and *Sal*I restriction endonuclease (NEB, MA, USA). Each reaction mixture was composed of 5 µl of 10x NEBuffer3, 5 µl (50 U) of *Not*I, 5 µl of (100 U) *Sal*I, 0.5 µl (5 µg) bovine serum albumin (BSA) and extracted plasmid to adjust the final volume to 50 µl. The reactions were spun down and incubated at 37ºC for 3 hours. Then, the digested plasmids were run on 1.5% agarose gel electrophoresis, stained with ethidium bromide, and visualized under UV light to check the completion of digestion. The completely digested samples were purified from 1.5% agarose gel using NucleoSpin® Extract II (Macherey-Nagel, Düren, Germany).

The purified digested pThioHisA vector and NP inserted gene concentrations were measured by spectrophotometer at 280 nm wave length and diluted with distilled water to the appropriate concentration for ligation at molar ratio of 1:3 (vector : inserted gene). Total 20 µl of ligation mixture consisted of 100 ng of pThioHisA vector, 100 ng of NP inserted gene, 2 µl of T4 DNA Ligase (3 Weiss units/µl) and 2 µl of 10x T4 DNA Ligase Buffer (Promaga, WI, USA). Before being transformed into *E. coli* competent cell strain TOP10, the ligation mixture was incubated overnight at 4°C.

Construction of the *E. coli* recombinant truncated NP expression vector (NPt-pThioHisA)

The RT-PCR product of truncated NP and pThioHisA vector were digested by FastDigest® *Xho*I and FastDigest® *Xba*I restriction enzyme (Fermentas, MD, USA). Each reaction

mixtures consisted of 5 µl of 10x FastDigest® Buffer, 5 µl of FastDigest® *Xho*I, 5 µl of FastDigest® *Xba*I and 35 µl of DNA samples, to final volume of 50 µl. The reactions were spun down and incubated at 37ºC for 1 hour. The completed digestion were analyzed and purified before ligation at molar ratio of 1:3 using T4 DNA Ligase (Promaga, WI, USA) and transformation into *E. coli* competent cell strain TOP10. All steps after digestion until transformation into *E. coli* were performed as in NP-pThioHisA construction.

Analysis of inserted gene in recombinant plasmids

All single colonies that could be found on LB agar plate with ampicillin after transformation into *E. coli* were suspected to contain the recombinant plasmid. The colonies were incubated in 10 ml of LB broth with ampicillin at 37°C overnight with shaking (250 rpm) for analysis of inserted gene in recombinant plasmids and then stored in 25% glycerol stock at -80ºC.

1. **Polymerase chain reaction (PCR)**

Aliquots of 50 µl of each bacterial culture were spun down at 8,000 rpm for 1 minute to remove the supernatants. The bacterial pellets were resuspended in 50 µl DNase free water and boiled at 100ºC for breaking cells. The boiled suspensions were spun down at 8,000 rpm for 1 minute and supernatants were collected and used for PCR.

All conditions of PCR for screening inserted gene in plasmid were similar to PCR condition for NP gene amplification, using different primers depending on the types of plasmid. T7 promoter primer and NP_R1557_*Sal*I were used as forward and reverse primers for screening inserted full-length NP gene in NP-pGEM. Whereas, the primers for PCR screening of the inserted full-length NP gene in NP-pThioHisA are NP_F61 and Trx reverse. Furthermore, Trx forward and Trx reverse primers were used for PCR screening truncated NP gene in NPt-pThioHisA. The sequences of primers for screening test described as above (Table 3.1) were

shown in Table 3.4. The PCR products were analyzed with 1.5% agarose gel electrophoresis, stained with ethidium bromide, and visualized by UV transilluminator.

2. Analysis of inserted gene by restriction enzyme

Samples for screening of inserted gene by restriction enzyme were prepared as PCR sample preparation for inserted gene screening. Recombinant plasmids were detected by digestion with the same enzyme used for ligation. Each reaction mixture consisted of 2 µl of 10x FastDigest® Buffer, 1 µl of FastDigest® *Xho*I, 1 µl of FastDigest® *Xba*I and 16 µl of DNA samples, to final volume of 20 µl. The reactions were spun down and incubated at 37ºC for 1 hour. The products of the digestion were analyzed with 1.5% agarose gel electrophoresis, stained with ethidium bromide, and visualized under UV light.

3. DNA sequencing

For positive samples by PCR screening or restriction enzyme screening test, the suspected recombinant plasmids were extracted from 5 ml of overnight bacterial cultures using NucleoSpin® Plasmid. Each sample was sequenced with inserted gene amplification primers (both directions) and plasmid sequencing primers (both directions) by 1st BASE company using Big dye terminator version 3.0 cycle sequencing ready reaction (ABI, Foster City, CA). T7 promoter and SP6 promoter primers were used as plasmid sequencing primers for DNA sequencing of inserted gene in pGEM®-T easy vector, while Trx forward and Trx reverse primers were used for sequencing inserted gene in pThioHisA plasmids. The sequences of plasmid sequencing primers were shown in Table 3.4.

The sequences of inserted gene in recombinant plasmids were aligned by CLUSTAL X multiple alignment program (version 1.8) and analyzed for sequence and orientation accuracy of inserted gene prior to perform protein expression.

Table 3.4 Primers for analysis the insertions of gene in plasmid

Code name	Primer	Primer sequence (5'-3')
T7 promoter*	Forward primer	TAATACGACTCACTATAGGG
SP6 promoter*	Reverse primer	ATTTAGGTGACACTATAGAA
Trx forward**	Forward primer	TTCCTCGACGCTAACCTG
Trx reverse**	Reverse primer	TGTAAAACGACGGCCAGTGC

* sequencing primer of recombinant pGEM®-T easy vector

** sequencing primer of recombinant pThioHisA vector

4. B-cell epitope prediction

The 3D structures of full-length NP were predictably constructed from the protein amino acid sequences using HHPred server (http://toolkit.tuebingen.mpg.de/hhpred). The predicted structures were saved as PDB-file. The B-cell epitopes of NP were predicted from the PBD-file using DiscoTope server (http://www.cbs.dtu.dk/services/DiscoTope/) and Ellipro server (http://tools.iedb.org/tools/ElliPro/iedb_input). The positions of mutated amino acid residuals of inserted gene in recombinant plasmids were analyzed for determining whether they are B-cell epitope regions by comparing with the predicted B-cell epitopes of the protein.

Protein expression and purification

1. Performing of pilot expression

E. coli strain TOP10 containing NP-pThioHisA or NPt-pThioHisA was incubated in 1 ml of LB broth with ampicillin at 37°C overnight with shaking (250 rpm). Subsequently, 200 µl of bacterial culture was added into 10 ml of LB broth with ampicillin at the dilution of 1:50 and incubated at 37°C with shaking. When OD_{600} of bacteria cultures was reached to 0.6 (approximately 3-4 hours), 1 ml of the culture was collected and spun down to pellet the cell at 8,000 rpm for 1 minute. The supernatant was removed and the pellet was stored at -20ºC

as “zero-time sample” (non-induced sample) until analysis. IPTG (Fermentas, MD, USA) was added into bacterial culture at a final concentration of 1 mM and incubated at 37ºC with shaking. At 1 hour, 4 hours and overnight after adding IPTG, 1 ml of bacterial cultures were collected and spun down to pellet the cells at 8,000 rpm for 1 minute, The supernatant were removed and the cell pellets were stored at-20ºC as induced sample at 1 indicated time points for protein expression analysis using sodium dedocyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

2. Protein purification

E. coli strain TOP10 that contained NP-pThioHisA or NPt-pThioHisA was further grown into large scale with same culture condition of pilot expression. Bacterial culture was divided into non-induced and induced bacterial culture for protein expression by incubation with 1mM IPTG for overnight. Harvested cells from 50 ml of each bacterial culture were used for protein purification by Probond™ Purification system (Invitrogen™, CA, USA) under native and denaturing conditions performed according to the manufacturer’s protocol. Aliquots of purified protein were store at -20 ºC. Eluted proteins were verified for the correct molecular weight and protein specificity by SDS-PAGE and immunoblot analysis, respectively.

Protein expression and purification verification

1. Sodium dedocyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-polyacrylamide gel was prepared according to the formulations as shown in Appendix B. The protein samples were denatured by mixing with 3x sample buffer at the ratio 1:2 and heated at 100ºC for 5 minutes. Electrophoresis was performed at constant voltage 100 V in Tris-glycine running buffer. The gel was stained with Coomassie brilliant blue or SimplyBlue™ SafeStain (Invitrogen™, CA, USA). For the Coomassie brilliant blue staining, the protein bands were visualized when no background was observed by destaining solution. Formulation of

SDS-polyacrylamide gel, 3x sample buffer, running buffer, Coomassie brilliant blue staining and destaining solution were shown in Appendix B.

2. Enzyme-linked immunosorbent assay (ELISA)

Both induced and non-induced groups of bacterial cultures were verified for NP protein expression by using IVtA EIA KIT, the commercial sandwich-ELISA for IAV-NP detection in tissue culture and swab material (European Veterinary Laboratory, Woerden, The Netherlands).

3. Immunoblot analysis

The protein samples were denatured and separated on SDS-polyacrylamide gel by electrophoresis without Coomassie brilliant blue staining. Then, these protein samples were electrophoretically transferred from gel to a nitrocellulose membrane using a semi-dry blot apparatus. The electric current was performed at 60 V (constant voltage) for 3 hours. The transferred membrane was incubated in blocking solution (5% BSA in PBS buffer) at room temperature for 1 hour. The membrane was probed with mouse anti-influenza nucleoprotein monoclonal antibody clone EVS 238 (HB65-like) (B.V.EUROPEAN VETERINARY LABORATORY, Woerden, The Netherlands) as a primary antibody. The primary antibody was diluted in 1% BSA in PBS buffer with dilution of 1:500 and incubated with membrane at room temperature on rocking plate for 2 hours. The membrane was washed 3 times with PBS 15 minutes each. The membrane was subsequently incubated with rabbit anti-mouse IgG conjugated horseradish peroxidase (Dako Cytomation, CA, USA) in 1% BSA in PBS buffer at the dilution of 1:250, 1:500, and 1:1000 for 1 hour at room temperature with rocking. After 3 time washings with PBS, the color was developed using a chromogen 3-amino-9-ethylcarbazole substrate (Sigma-Aldrich, MO, USA) with H_2O_2.

Native protein purification verification

E. coli strain TOP10 containing NP-pThioHisA was further grown and induced for protein expression with the same condition as described above. Generally, for protein purification under native condition, the harvested cells from 50 ml of each bacterial culture were resuspended in native binding buffer (phosphate-based buffer) and lysed by sonication and freeze-thaw cycle. The lysates were then centrifuged at 3,000 rpm for 15 minutes to remove cell debris. The Probond resins were suspended in the prepared supernatant and incubated at 4 ºC with gently inverting for 60 minutes to allow the binding of recombinant protein with the resin. After washing with native washing buffer to remove contaminated proteins, the recombinant protein-bound Probond resins were allowed to settle in the column. The recombinant proteins were eluted from resin using 3 ml of elution buffer containing imidazole. One ml eluted fractions were collected for analysis by SDS-PAGE.

In order to find the limitation of the native protein purification, the prepared supernatant, through-flow of supernatant after binding, washing buffer and eluted fraction of induced and non-induced protein expression group were verified by SDS-PAGE.

Variation of imidazole concentration in native elution buffer

The recommended concentration of imidazole in elution buffer according to the manufacturer's protocol was 250 mM imidazole in native binding buffer. In this study, the concentration of imidazole in elution buffer was varied as following concentration: 125, 250, 500 and 1,000 mM imidazole in native binding buffer. The lysate preparation of this trial was performed as the manufacturer's protocol of Probond™ Purification system. One ml of the lysate was collected for protein expression analysis by SDS-PAGE. The recombinant proteins were eluted from resin using 2 ml of each imidazole concentration elution buffer. All 1 ml eluted fractions were collected to verify the appearance of purified recombinant protein by SDS-PAGE.

Protein solubility determination

In order to determine the solubility of expressed recombinant protein, *E. coli* strain TOP10 containing NP-pThioHisA plasmid was grown and induced protein expression with the same protocol as described above. The harvested pellet of induced group was resuspended in 50 ml of PBS and lysed by sonication and freeze-thaw cycles as lysate preparation protocol of Probond™ Purification system. The lysate was centrifuged at 3,000 rpm for 15 minutes. After centrifugation, both pellet and supernatant were collected for protein profiles analysis by SDS-PAGE. The supernatant was centrifuged at 12,000 rpm for 15 minutes then the pellet and supernatant from second centrifugation were collected again for protein profiles analysis by SDS-PAGE. The pellet from second centrifugation was resuspended in 50 ml of 0.5% Triton-X and incubated at 4 °C with gently inverting for 4 hours. Finally, this suspension was centrifuged at 12,000 rpm for 15 minutes. The final pellet and supernatant were collected to verify protein profiles by SDS-PAGE.

Performing for enhancing solubility of expressed recombinant protein

1. **Variation of incubation period for denaturant of denaturing protein purification**

For protein purification under denaturing condition, the harvested pellets of bacterial culture were induced for full-length NP expression. The protein was lysed and unfolded by incubation in 8 ml of 6 M guanidine hydrochloride lysis buffer with gently inverting for 10 minutes before adding the resin into the lysate for binding with recombinant protein. Protein purification under this condition, the binding buffer, washing buffer and elution buffer were included 8 M urea and the purification process was performed according to the manufacturer's protocol of Probond™ Purification system. Denaturing protein purification verification was performed as previously described for native protein purification verification.

In this study, incubation period of harvested pellet in lysis buffer containing denaturant (6 M guanidine hydrochloride) was increased from 10 minutes to 12 hours. Subsequently, the lysate was used for protein purification under denaturing condition with same protocol as described above. The eluted fractions were detected for eluted protein by SDS-PAGE.

2. Variation of expression conditions

This study attempted to enhance solubility of recombinant protein by reducing induction temperature from 37 °C to room temperature. Moreover, the final concentrations of IPTG that used for inducing protein expression were varied as following concentrations: 0.01, 0.1 and 0.5 mM IPTG. The expressions of full-length NP under these conditions were detected by SDS-PAGE. Subsequently, the expressed full-length NPs were purified under denaturing condition with protocol as described above. The eluted fractions of each group were verified purified protein by SDS-PAGE.

3. Variation of bacterial strains

Full-length NP expression in other *E. coli* strains was performed to improve the solubility of expressed recombinant protein. The purified NP-pThioHisA expression vectors were transformed into *E. coli* strain JM109 and BL21 and were verified the containing this expression vector by PCR with same protocol and condition as described for inserted gene analysis in recombinant plasmids. The positive colonies were selected and protein expression induction was conducted at room temperature for overnight by adding 0.01 mM IPTG (final concentration). Before using the sample in protein purification, the full-length NP expressions of the induced and non-induced groups of both *E. coli* strains were verified by SDS-PAGE. The pellets harvested from expressed groups were used for protein purification under modified denaturing condition. The eluted proteins were analyzed by SDS-PAGE.

CHAPTER IV

RESULTS

Construction of the *E. coli* recombinant full-length NP expression vector (NP-pThioHisA)

1. RNA extraction and RT-PCR

Viral RNA was extracted from swine influenza H1N1 using NucleoSpin Extract Viral RNA Kit. Viral RNA was used as a template for the RT-PCR according to the optimized protocol and condition of One-step RT-PCR. The RT-PCR conditions were in Table 3.3. The RT-PCR product of full length NP gene could be generated using NP_F61 and NP_R1557_*Sal*I primer (Table 3.1). The RT-PCR amplicon size of 1,500 bp was analyzed with 1.5% agarose gel electrophoresis and visualized under ultraviolet (UV) light as shown in Figure 4.1. The RT-PCR product was purified from agarose gel using NucleoSpin® Extract II. Before using for DNA cloning, the purified RT-PCR product was checked by running 1 µl of purified product on 1.5% agarose gel as shown in Figure 4.1.

2. Cloning and analysis of inserted full-length NP gene in recombinant NP-pGEM

Blue and white colonies of bacteria were observed on the selective media after ligation of full-length NP gene (1,500 bp) into pGEM®-T easy vector and transformation the ligation mixture into *E. coli* competent cell strain JM109 (data not shown). Twenty two white colonies were randomly selected and prepared for inserted gene analysis. All of these colonies were screened for the corrected insertion of full length NP gene in pGEM®-T easy vector by PCR and analyzed by 1.5% agarose gel electrophoresis with 1,500 bp of product size as shown in Figure 4.2. In this case, fifteen out of twenty two colonies were confirmed with the insertion of full length NP gene and were analyzed for orientation accuracy by DNA sequencing. These fifteen colonies were named as NP-pGEM (1-15). The sequence of full length NP gene of selected NP-pGEM was

shown in Figure 4.3. The nucleotide sequence of NP gene in NP-pGEM was similar to NP gene of swine influenza H1N1 virus (A/swine/Thailand/ CU-CBP18/2009) with 99.66 % identity by pair-wise sequence comparison. The comparative result of the deduced amino acid sequence between NP gene in NP-pGEM and NP gene of the stock virus showed a similarity with 99.59 % identity and showed amino acid mutations from phenylalanine to leucine at position 346 (F to L) and glycine to arginine at position 394 (G to R) (Figure 4.4). However, the comparative result between the mutated amino acid residues and B-cell epitope regions of NP showed that the positions of mutations were not in B-cell epitope region of the NP (Figure 4.5).

3. Cloning and analysis of inserted full-length NP gene in recombinant NP-pThioHisA

The extracted NP-pGEM and pThioHisA vectors were digested by *Not*I and *Sal*I restriction endonuclease (NEB, MA, USA). The success of the digestion was verified by running the digests on 1.5% agarose gel electrophoresis and visualized under UV light. NP gene digested from recombinant NP-pGEM was observed with product size of 1,500 bp and the product size of digested pThioHisA vector was 4,400 bp. These digested DNAs were purified from agarose gel using NucleoSpin® Extract II (Macherey-Nagel, Düren, Germany) and the purified products were checked before ligation (Figure 4.6).

After the purified digested pThioHisA vector and NP inserted gene were ligated with DNA ligation condition as described above, the ligation mixture was transformed in *E. coli* competent cell strain TOP10. Three colonies of the bacteria were observed on selective media. All of these colonies were prepared for inserted gene analtsis. The result of PCR for insertion analysis of full-length NP gene in pThioHisA expression vector was in Figure 4.7. The inserted clone was grown and the recombinant plasmid was extracted for DNA sequencing. The recombinant plasmid was named as “NP-pThioHisA”. The sequence of inserted NP gene in pThioHisA expression vector was in Figure 4.3. The sequence of the inserted NP gene in NP-pThioHisA was similar to inserted NP gene in pGEM®-T easy vector easy with 100%

identity. Moreover, analysis of the sequencing results revealed that this clone contained the proper start and stop codons for the open reading frame of the vector.

Protein expression and purification of full-length NP (NP-pThioHisA)

1. Protein expression verification of full-length NP (NP-pThioHisA)

Pellet samples prepared from bacterial culture were used for verification of protein expression by using SDS-PAGE and commercial ELISA for influenza A antigen detection. The SDS-PAGE protein profile of 4 hours and overnight sample showed the expected recombinant full-length NP band at approximately 68 kDa (56 kDa of full-length NP with 12 kDa of thioredoxin fusion protein) (Figure 4.8).

Samples collected at 0 hour and overnight were verified for protein expression using IVtA EIA KIT, the commercial ELISA for NP of IAV detection in tissue culture. These samples were performed according to manufacturer's protocol. For the result interpretation, the sample considered to be positive when OD at 450 nm was at least 2 times higher than those of negative control. And the ELISA has been validated. The test is valid when the positive control is $\geq$ 1.5 time of OD negative control. The ELISA results from checking of full-length NP expression were shown in Table 4.1.

Table 4.1 ELISA results for verification of full-length NP expression (NP-pThioHisA)

Sample name	OD at 450 nm (mean $\pm$ SD)	Interpreted result
Zero-time sample NP-pThioHisA	0.259 $\pm$ 0.031	Negative
Overnight sample NP-pThioHisA	2.111 $\pm$ 0.143	Positive
Positive control	0.399 $\pm$ 0.022*	-
Negative control	0.232 $\pm$ 0.025*	-

* Positive control $\geq$ 1.5 times of OD negative control

** Cut off = 2 times of OD negative control = 0.464

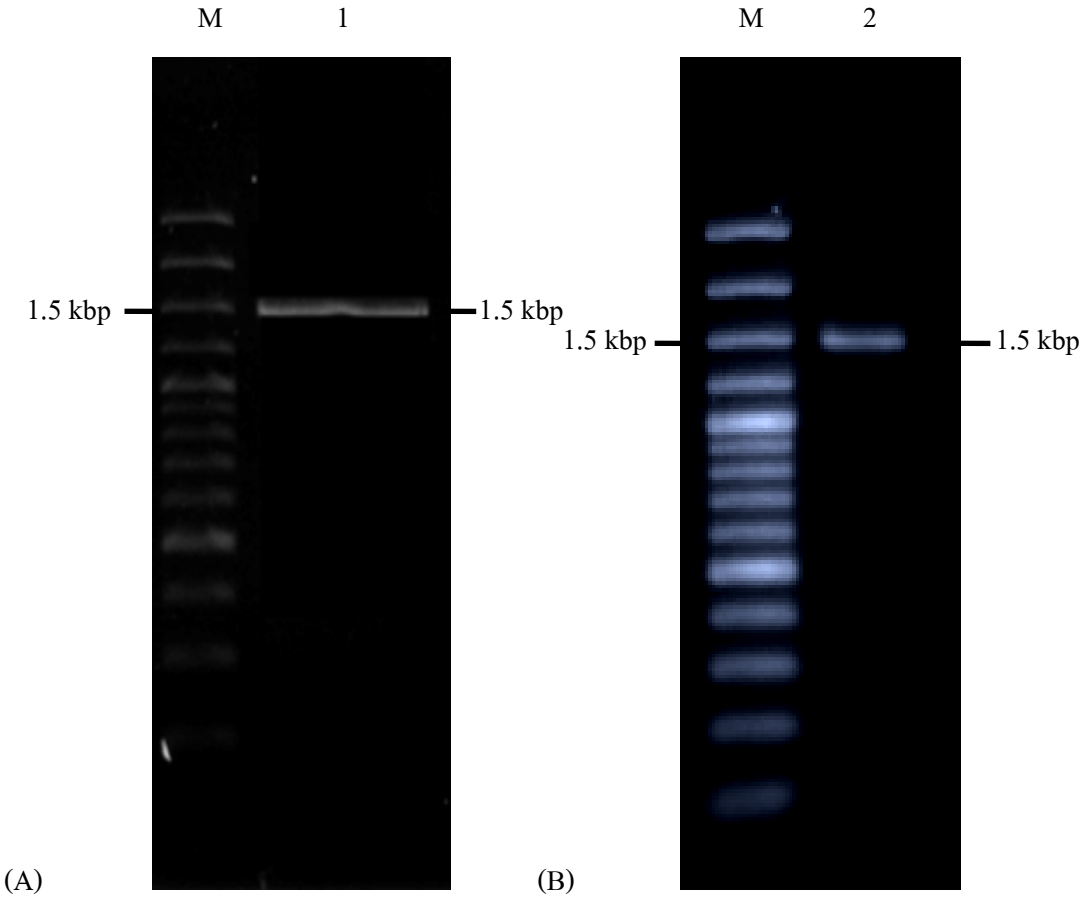


Figure 4.1 Ethidium bromide stainning agarose gel electrophoresis pattern of RT-PCR product of full-length NP gene (A) and purified RT-PCR product (B)

Lane M = GeneRuler™ 100 bp plus DNA ladder (Fermentas, MD, USA)

Lane 1 = The RT-PCR product of full-length NP gene

Lane 2 = The purified RT-PCR product of full-length NP gene

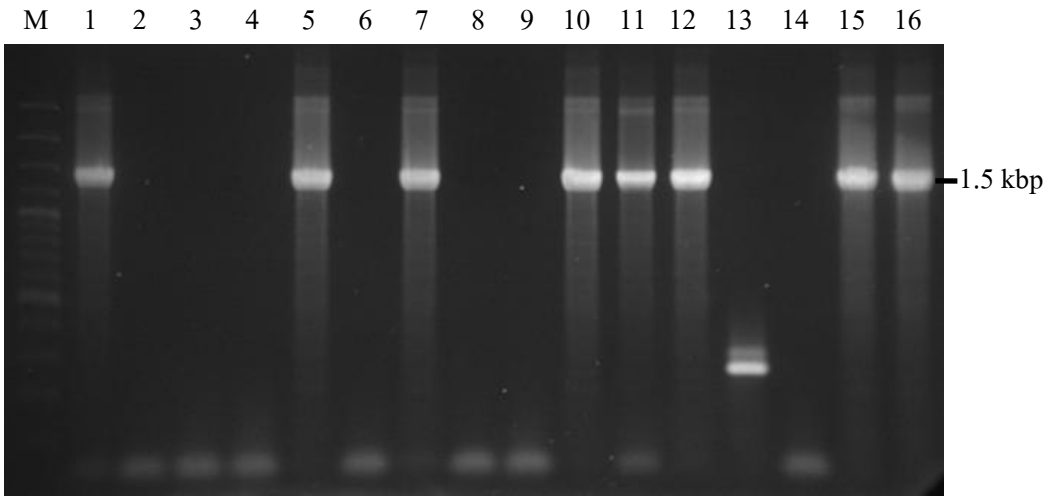


Figure 4.2 Ethidium bromide stainning agarose gel electrophoresis pattern of PCR products of full-length NP gene for screening inserted NP gene in pGEM®-T easy vector by using samples prepared from white colonies bacteria as templates

Lane M = GeneRuler™ 100 bp plus DNA ladder (Fermentas, MD, USA)

Lane 1, 5, 7, 10, 11, 12, 15, 16 = The PCR product of full-length NP gene from positive clones

Lane 2, 3, 4, 6, 8, 9, 13, 14 = The PCR product of full-length NP gene from negative clones

```
                        10         20         30         40         50         60         70         80         90         100
                   ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
NP CU-CBP18        ATGGCGTCTC AAGGCACCAA ACGATCTTAT GAGCAGATGG AAACTGGTGG GGAACGCCAG AATGCTACTG AAATCAGAGC ATCTGTTGGG AGAATGGTTG
NP-pGEM            ATGGCGTCTC AAGGCACCAA ACGATCTTAT GAGCAGATGG AAACTGGTGG GGAACGCCAG AATGCTACTG AAATCAGAGC ATCTGTTGGG AGAATGGTTG
NP-pThioHisA       ATGGCGTCTC AAGGCACCAA ACGATCTTAT GAGCAGATGG AAACTGGTGG GGAACGCCAG AATGCTACTG AAATCAGAGC ATCTGTTGGG AGAATGGTTG
Clustal Consensus  ********** ********** ********** ********** ********** ********** ********** ********** ********** **********

                       110        120        130        140        150        160        170        180        190        200
                   ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
NP CU-CBP18        GAGGAATTGG AAGATTTTAC ATACAAATGT GCACTGAACT TCAACTCAGT GACTATGAAG GGAGGCTGAT CCAGAACAGC ATAACAATAG AGAGAATGGT
NP-pGEM            GAGGAATTGG AAGATTTTAC ATACAAATGT GCACTGAACT TCAACTCAGT GACTATGAAG GGAGGCTGAT CCAGAACAGC ATAACAATAG AGAGAATGGT
NP-pThioHisA       GAGGAATTGG AAGATTTTAC ATACAAATGT GCACTGAACT TCAACTCAGT GACTATGAAG GGAGGCTGAT CCAGAACAGC ATAACAATAG AGAGAATGGT
Clustal Consensus  ********** ********** ********** ********** ********** ********** ********** ********** ********** **********

                       210        220        230        240        250        260        270        280        290        300
                   ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
NP CU-CBP18        TCTCTCGGCG TTTGACGAGA GAAGAAACAG ATACCTGGAG GAACATCCCA GTGCGGGGAA AGACCCGAAG AAAACTGGTG GTCCAATCTA CAAAAAGAGA
NP-pGEM            TCTCTCGGCG TTTGACGAGA GGAGAAACAG ATACCTGGAG GAACATCCCA GTGCGGGGAA AGACCCGAAG AAAACTGGTG GTCCAATCTA CAAAAAGAGA
NP-pThioHisA       TCTCTCGGCG TTTGACGAGA GGAGAAACAG ATACCTGGAG GAACATCCCA GTGCGGGGAA AGACCCGAAG AAAACTGGTG GTCCAATCTA CAAAAAGAGA
Clustal Consensus  ********** ********** * ********* ********** ********** ********** ********** ********** **********

                       310        320        330        340        350        360        370        380        390        400
                   ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
NP CU-CBP18        GACGGAAAAT GGATGAGGGA GCTGGTTCTG TATGATAAAG ATGAGATCAG GAGAATTTGG CGCCAAGCAA ACAATGGTGA AGATGCTACC GCTGGTCTCA
NP-pGEM            GACGGAAAAT GGATGAGGGA GCTGGTTCTG TATGATAAAG ATGAGATCAG GAGAATTTGG CGCCAAGCAA ACAATGGTGA AGATGCTACC GCTGGTCTCA
NP-pThioHisA       GACGGAAAAT GGATGAGGGA GCTGGTTCTG TATGATAAAG ATGAGATCAG GAGAATTTGG CGCCAAGCAA ACAATGGTGA AGATGCTACC GCTGGTCTCA
Clustal Consensus  ********** ********** ********** ********** ********** ********** ********** ********** ********** **********

                       410        420        430        440        450        460        470        480        490        500
                   ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
NP CU-CBP18        CCCACTTGAT GATTTGGCAT TCCAATCTGA ATGATGCCAC ATATCAAAGA ACGAGGGCTC TTGTGCGTAC TGGGATGGAT CCCAGAATGT GCTCTCTAAT
NP-pGEM            CCCACTTGAT GATTTGGCAT TCCAATCTGA ATGATGCCAC ATATCAAAGA ACGAGGGCTC TTGTGCGTAC TGGGATGGAT CCCAGAATGT GCTCTCTAAT
NP-pThioHisA       CCCACTTGAT GATTTGGCAT TCCAATCTGA ATGATGCCAC ATATCAAAGA ACGAGGGCTC TTGTGCGTAC TGGGATGGAT CCCAGAATGT GCTCTCTAAT
Clustal Consensus  ********** ********** ********** ********** ********** ********** ********** ********** ********** **********

                       510        520        530        540        550        560        570        580        590        600
                   ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
NP CU-CBP18        GCAAGGCTCA ACTCTCCCGA GGAGATCTGG AGCTGCTGGG GCAGCAGTAA AGGGAGTTGG GACAATGGTA ATGGAACTGA TTCGGATGAT AAAGCGAGGG
NP-pGEM            GCAGGGCTCA ACTCTCCCGA GGAGATCTGG AGCTGCTGGG GCAGCAGTAA AGGGAGTTGG GACAATGGTA ATGGAACTGA TTCGGATGAT AAAGCGAGGG
NP-pThioHisA       GCCAGGGCTCA ACTCTCCCGA GGAGATCTGG AGCTGCTGGG GCAGCAGTAA AGGGAGTTGG GACAATGGTA ATGGAACTGA TTCGGATGAT AAAGCGAGGG
Clustal Consensus  *** ****** ********** ********** ********** ********** ********** ********** ********** **********
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610 620 630 640 650 660 670 680 690 700
NP CU-CBP18 ATCAATGACC GGAACTTCTG GAGAGGCGAA AATGGACGAA GAACAAGAAT TGCATATGAG AGAATGTGCA ACATCCTCAA AGGGAAATTT CAAACAGCAG
NP-pGEM ATCAATGACC GGAACTTCTG GAGAGGCGAA AATGGACGAA GGACAAGAAT TGCATATGAG AGAATGTGCA ACATCCTCAA AGGGAAATTT CAAACAGCAG
NP-pThioHisA ATCAATGACC GGAACTTCTG GAGAGGCGAA AATGGACGAA GGACAAGAAT TGCATATGAG AGAATGTGCA ACATCCTCAA AGGGAAATTT CAAACAGCAG
Clustal Consensus
710 720 730 740 750 760 770 780 790 800
NP CU-CBP18 CGCAACGAGC AATGATGGAC CAGGTGCGAG AAAGCAGAAA TCCTGGGAAT GCTGAGATTG AAGATCTTAT CTTTCTAGCA CGATCTGCAC TCATTCTGAG
NP-pGEM CGCAACGAGC AATGATGGAC CAGGTGCGAG AAAGCAGAAA TCCTGGGAAT GCTGAGATTG AAGATCTTAT CTTTCTAGCA CGATCTGCAC TCATTCTGAG
NP-pThioHisA CGCAACGAGC AATGATGGAC CAGGTGCGAG AAAGCAGAAA TCCTGGGAAT GCTGAGATTG AAGATCTTAT CTTTCTAGCA CGATCTGCAC TCATTCTGAG
Clustal Consensus
810 820 830 840 850 860 870 880 890 900
NP CU-CBP18 AGGATCAGTA GCTCACAAAT CCTGTCTACC TGCTTGTGTA TACGGACTTG TTGTGGCAAG TGGATATGAC TTTGAAAGAG AAGGGTACTC TCTCGTCGGA
NP-pGEM AGGATCAGTA GCTCACAAAT CCTGTCTACC TGCTTGTGTA TACGGACTTG TTGTGGCAAG TGGATATGAC TTTGAAAGAG AAGGGTACTC TCTCGTCGGA
NP-pThioHisA AGGATCAGTA GCTCACAAAT CCTGTCTACC TGCTTGTGTA TACGGACTTG TTGTGGCAAG TGGATATGAC TTTGAAAGAG AAGGGTACTC TCTCGTCGGA
Clustal Consensus
910 920 930 940 950 960 970 980 990 1000
NP CU-CBP18 ATAGATCCTT TTCGTCTGCT TCAGAACAGC CAGGTGTTCA GCCTCATTAG ACCGAATGAG AACCCAGTAC ATAAGAGTCA GCTTATGTGG ATGGCATGCC
NP-pGEM ATAGATCCTT TTCGTCTGCT TCAGAACAGC CAGGTGTTCA GCCTCATTAG ACCGAATGAG AACCCAGTAC ATAAGAGTCA GCTTATGTGG ATGGCATGCC
NP-pThioHisA ATAGATCCTT TTCGTCTGCT TCAGAACAGC CAGGTGTTCA GCCTCATTAG ACCGAATGAG AACCCAGTAC ATAAGAGTCA GCTTATGTGG ATGGCATGCC
Clustal Consensus
1010 1020 1030 1040 1050 1060 1070 1080 1090 1100
NP CU-CBP18 ATTCTGCGGC ATTTGAAGAT CTGAGAGTGT CAAGTTTCAT CAGAGGAACC AAAGTGATCC CAAGAGGGCA ACTGTCCACC AGAGGAATTC AAATTGCTTC
NP-pGEM ATTCTGCGGC ATTTGAAGAT CTGAGAGTGT CAAGTCTCAT CAGAGGAACC AAAGTGATCC CAAGAGGGCA ACTGTCCACC AGAGGAATTC AAATTGCTTC
NP-pThioHisA ATTCTGCGGC ATTTGAAGAT CTGAGAGTGT CAAGTCTCAT CAGAGGAACC AAAGTGATCC CAAGAGGGCA ACTGTCCACC AGAGGAATTC AAATTGCTTC
Clustal Consensus
1110 1120 1130 1140 1150 1160 1170 1180 1190 1200
NP CU-CBP18 AAATGAAAAC ATGGAAACAA TGGATTCCGT TACTCTTGAA TTGAGGAGCA AATACTGGGC TATAAGAACC AGGAGCGGAG GAAACACTAA CCAACAGAGA
NP-pGEM AAATGAAAAC ATGGAAACAA TGGATTCCGT TACTCTTGAA TTGAGGAGCA AATACTGGGC TATAAGAACC AGGAGCGGAA GAAACACTAA CCAACAGAGA
NP-pThioHisA AAATGAAAAC ATGGAAACAA TGGATTCCGT TACTCTTGAA TTGAGGAGCA AATACTGGGC TATAAGAACC AGGAGCGGAA GAAACACTAA CCAACAGAGA
Clustal Consensus

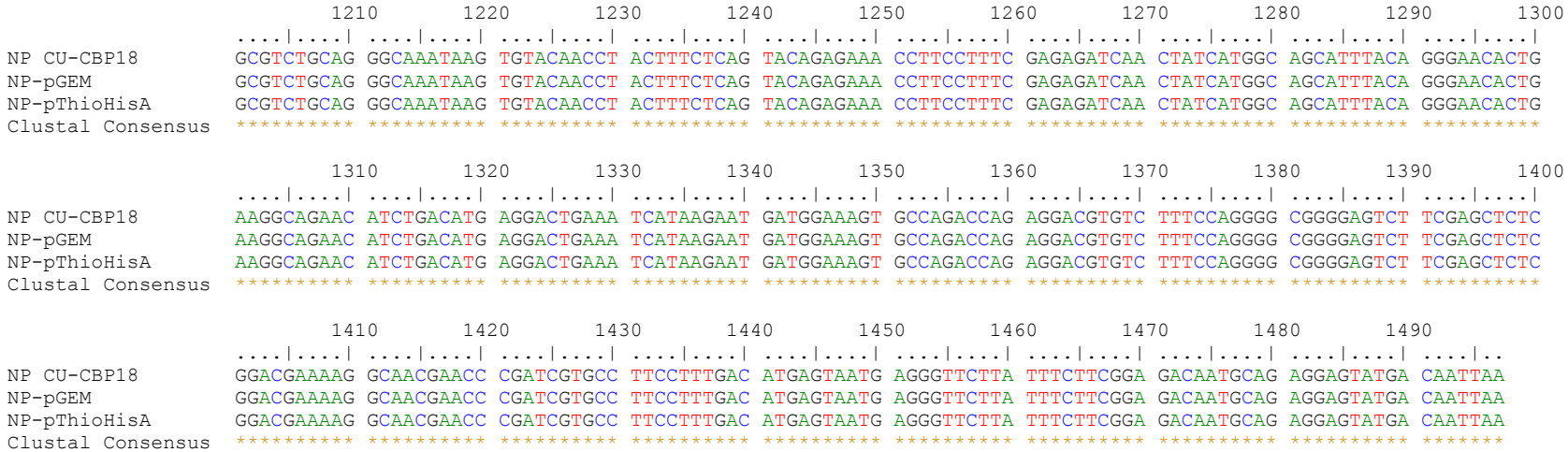


Figure 4.3 Nucleotide sequence alignment of NP gene in NP-pGEM and NP-pThioHisA expression vector. The nucleotide sequences of NP gene in both plasmids were compared with NP gene of swine influenza H1N1 virus (A/swine/Thailand/CU-CBP18/2009)

* = Homology

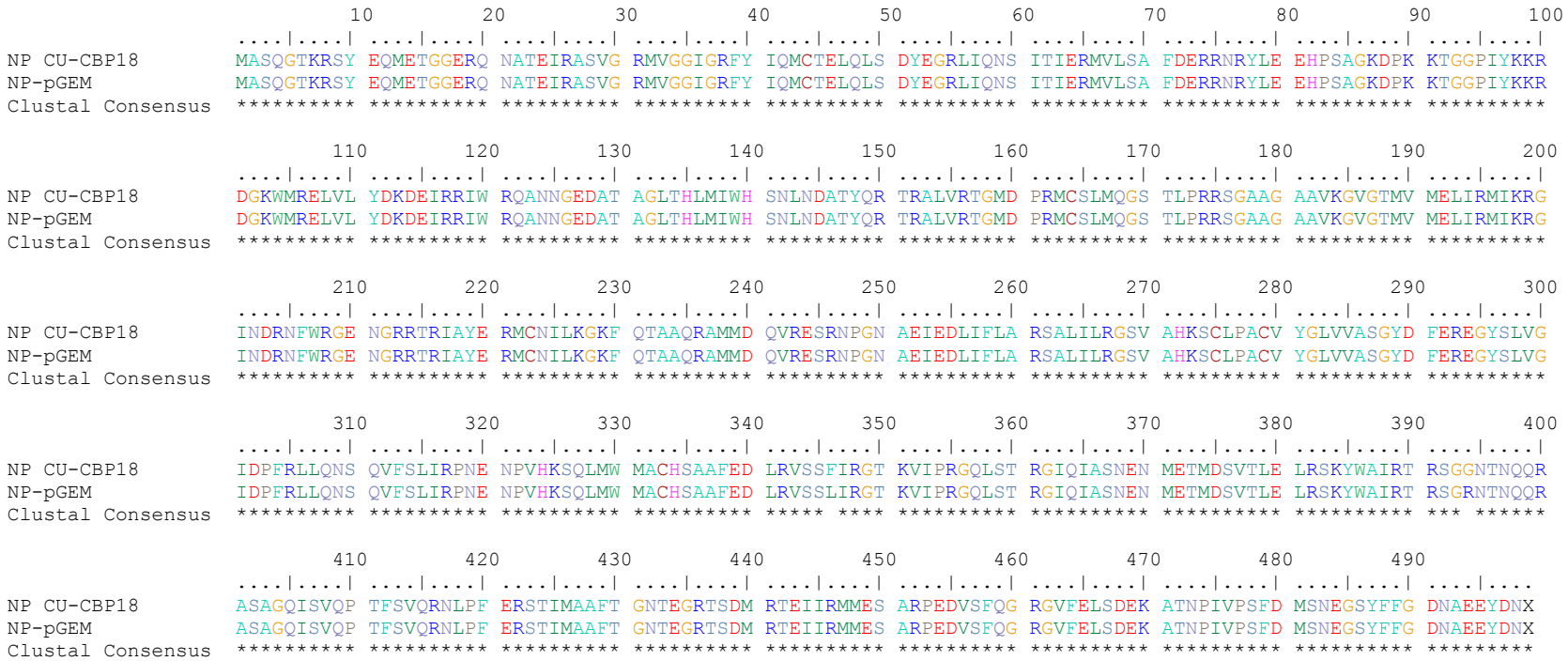


Figure 4.4 Amino acid sequence alignment of NP in NP-pGEM was compared with NP of swine influenza H1N1 virus (A/swine/Thailand/CU-CBP18/2009)

* = Homology

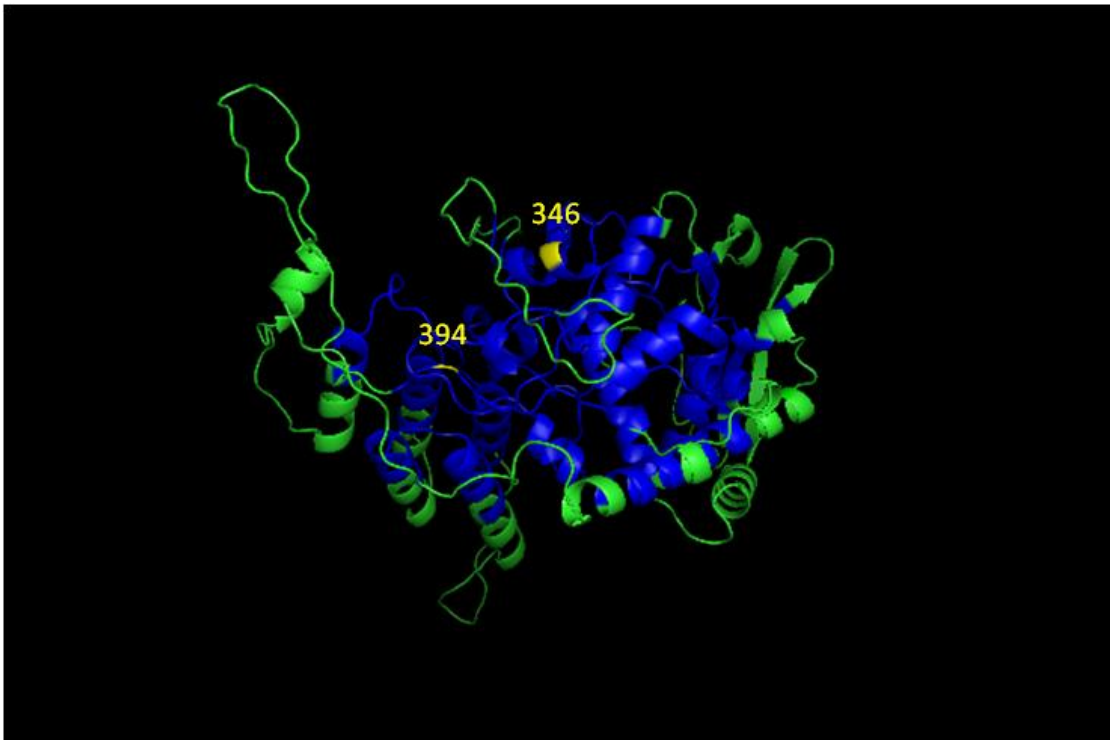


Figure 4.5 Predicted B-cell epitopes and mutated regions of the recombinant full-length NP

Green region = Predicted B-cell epitopes of the NP

Yellow region = Mutated regions

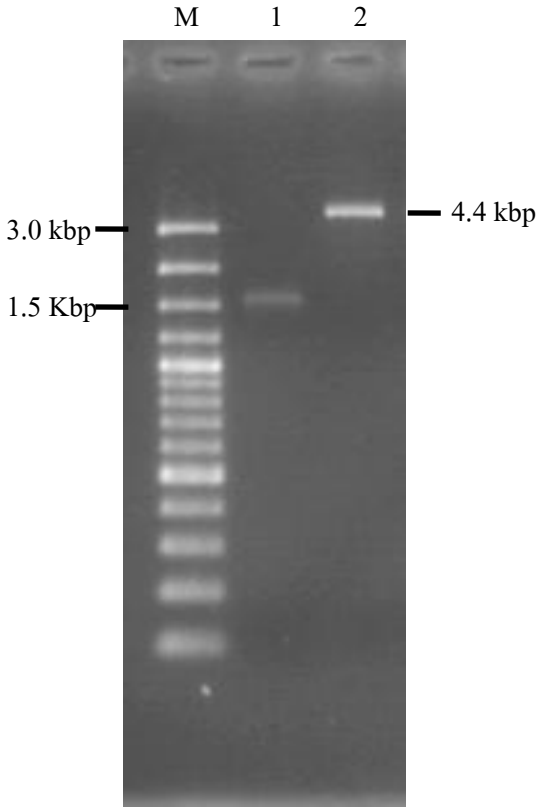


Figure 4.6 Ethidium bromide stainning agarose gel electrophoresis pattern of purified digested full-length NP gene from pGEM®-T easy vector and digested pThioHisA expression vector by *Not*I and *Sal*I restriction endonuclease (NEB, MA, USA)

Lane M = GeneRuler™ 100 bp plus DNA ladder (Fermentas, MD, USA)

Lane 1 = *Not*I and *Sal*I digested NP-pGEM

Lane 2 = *Not*I and *Sal*I digested pThioHisA expression vector

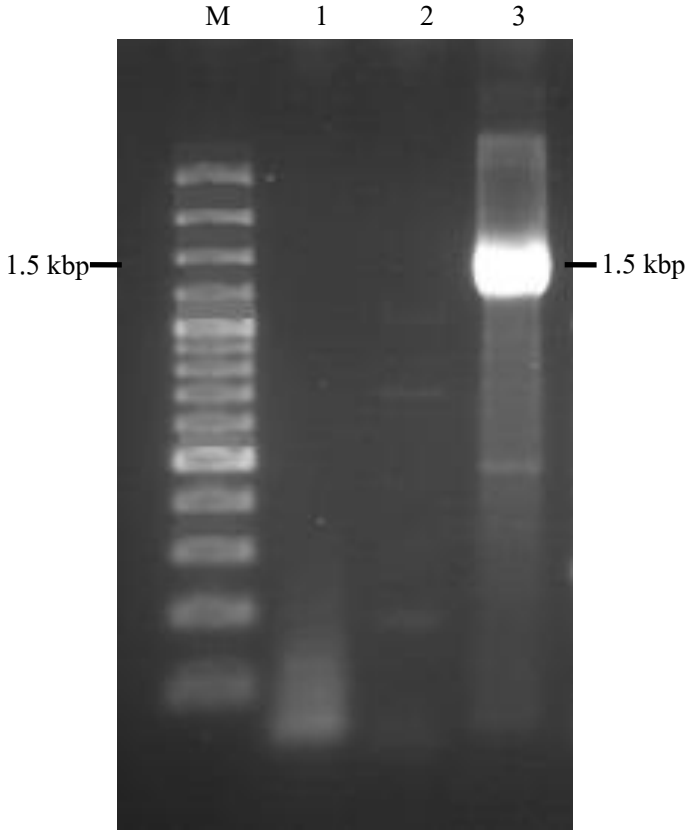


Figure 4.7 Ethidium bromide stainning agarose gel electrophoresis pattern of PCR products for screening inserted full-length NP gene in pThioHisA expression vector

Lane M = GeneRuler™ 100 bp plus DNA ladder (Fermentas, MD, USA)

Lane 1, 2 = The PCR products from non-NP gene inserted clones (negative clone)

Lane 3 = The PCR product from full-length NP gene inserted clones (positive clone)

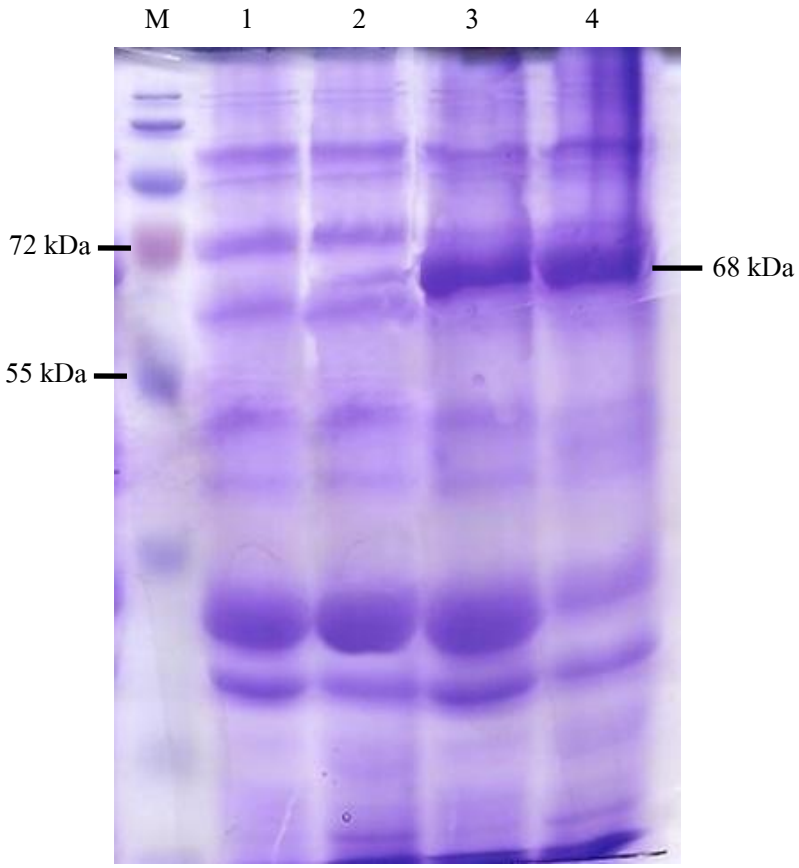


Figure 4.8 Coomassie brilliant blue staining SDS-PAGE profile of total protein from NP-pThioHisA transformed *E.coli* strain TOP10

Lane M = PageRuler™ Prestained Protein ladder (Fermentas, MD, USA)

Lane 1 = NP-pThioHisA expression at 0 hour of induction (Non-induced group)

Lane 2 = NP-pThioHisA expression at 1 hour of induction

Lane 3 = NP-pThioHisA expression at 4 hours of induction

Lane 4 = NP-pThioHisA expression at overnight of induction

2. Protein purification verification of full-length NP (NP-pThioHisA)

E. coli strain TOP10 containing NP-pThoHisA was grown in large scale. Bacterial culture was divided into (1) non-induced culture and (2) induced culture. The recombinant full-length NP was expressed in induced culture after induction overnight and could be observed from SDS-PAGE protein profile with the expected band as observed from pilot scale induction. The recombinant protein of both culture were purified by Probond™ Purification system under native condition. The purified protein samples were verified by SDS-PAGE and immunoblot analysis. No purified full-length NP bands were observed with Coomassie brilliant blue straining SDS-PAGE (Figure 4.9) and immunoblot analysis from both native and hybrid purification condition of induced culture group (data not shown).

Our approaches used to produce recombinant NP

1. Native protein purification verification

Since no purified full-length NP bands were observed with Coomassie brilliant blue straining SDS-PAGE, the following trial was performed to find the missing recombinant protein in the native protein purification. The prepared supernatant, through-flow of supernatant after binding, washing buffer and eluted fraction of induced and non-induced protein expression group were verified by SDS-PAGE. From SDS-PAGE result, the expressed full-length NP band (68 kDa) was shown only in the supernatant sample of induced group but no expected bands were observed in other samples. Moreover, the expressed full-length NP was not lost during binding and washing processes as no expected band were detected in the through-flow of supernatant (Lane 3) and the washing buffer (Lane 5). Those results indicated that the recombinant protein bound with the resin and could not be eluted by the native elution buffer (Figure 4.10).

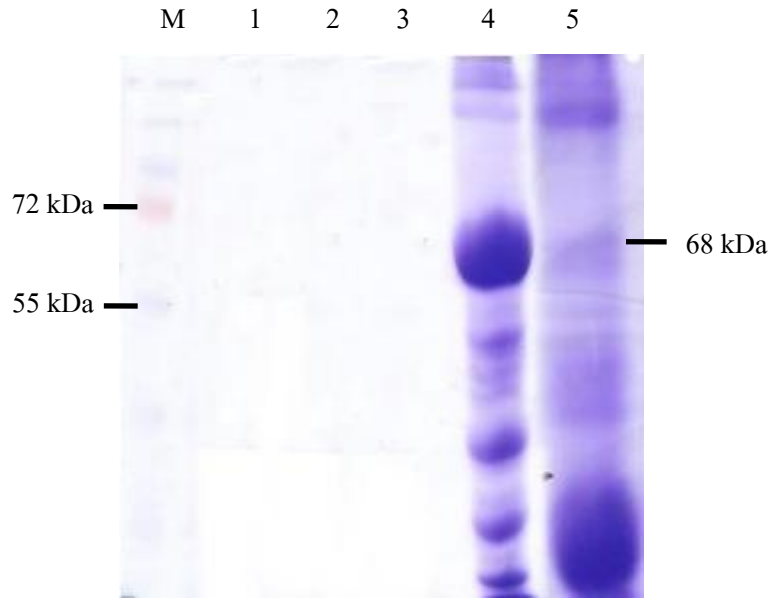


Figure 4.9 Coomassie brilliant blue staining SDS-PAGE profile of purified full-length NP from NP-pThioHisA contained *E.coli* strain TOP10 using native protein purification system

Lane M = PageRuler™ Prestained Protein ladder (Fermentas, MD, USA)

Lane 1, 2, 3 = No expected purified protein from 1st fraction, 2nd fraction and 3rd fraction of eluted samples, respectively (induced group)

Lane 4 = Lysate of induced NP-pThioHisA expression

Lane 5 = Lysate of non-induced NP-pThioHisA expression (No recombinant protein expression)

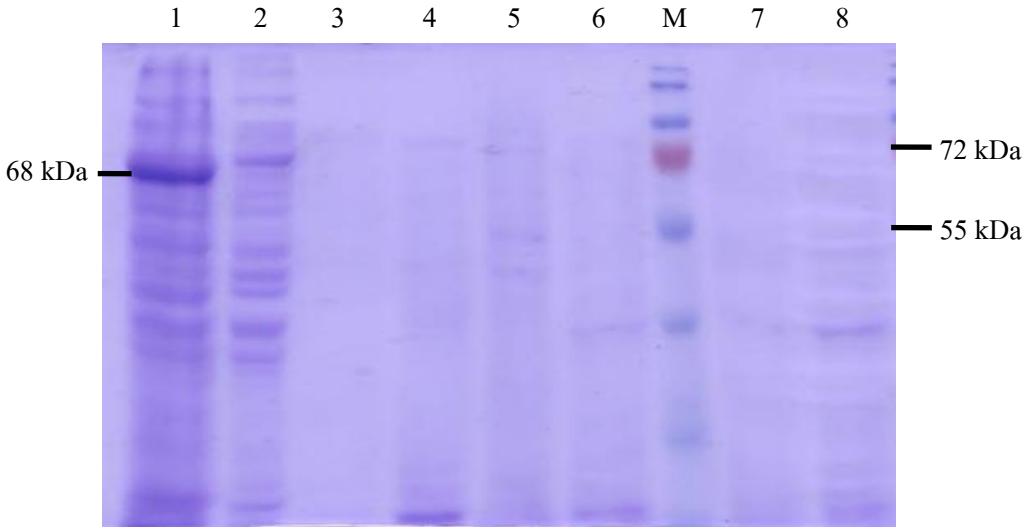


Figure 4.10 Coomassie brilliant blue staining SDS-PAGE profile of expressed and purified full-length NP from NP-pThioHisA contained *E.coli* strain TOP10 for verifying native protein purification system

Lane M = PageRuler™ Prestained Protein ladder (Fermentas, MD, USA)

Lane 1 = Supernatant of induced group (68 kDA expected band)

Lane 2 = Supernatant of non-induced group

Lane 3 = Through-flow supernatant after binding of induced group

Lane 4 = Through-flow supernatant after binding of non- induced group

Lane 5 = Washing buffer of induced group

Lane 6 = Washing buffer of non- induced group

Lane 7 = Eluted fraction of induced group

Lane 8 = Eluted fraction of non- induced group

2. Variation of imidazole concentration in native elution buffer

The higher concentration of imidazole in elution buffer was suspected to increase the efficiency of elution. Another attempt to purify full-length NP in native form was performed using the different concentration of imidazole in elution as the following concentrations: 125, 250, 500 and 1,000 mM in native binding buffer. The lysates of induced and non-induced bacterial culture were collected for protein expression analysis by SDS-PAGE. The result of SDS-PAGE showed expressed full-length NP band (68 kDa) in lysate of induced group (Figure 4.11). The lysate was used as a sample for this modified native protein purification. After washing process, the recombinant protein was eluted from resin using 2 ml of each imidazole concentration elution buffer. All 1 ml eluted fractions were collected to verify purified recombinant protein by SDS-PAGE. No purified full-length NP bands were observed from SDS-PAGE protein profiles of all eluted fractions (Figure 4.11).

3. Protein solubility determination

The protein solubility could affect on the protein purification. To determine solubility of expressed recombinant protein, *E. coli* strain TOP10 containing NP-pThioHisA plasmid was induced for protein expression. The lysate of this harvested pellet was centrifuged at 3,000 rpm. After this centrifugation, both pellet and supernatant were collected for protein profiles analysis by SDS-PAGE. As seen from this SDS-PAGE protein profiles, most of expressed proteins were found in supernatant after the 1st centrifugation (Figure 4.12). The supernatant from the 1st centrifugation was centrifuged again at 12,000 rpm, then the pellet and supernatant were collected for protein profiles analysis by SDS-PAGE. Most of expressed proteins were settled in the pellet from the 2nd centrifugation as shown in Figure 4.12 (Lane 4). Subsequently, the pellet from the 2nd centrifugation was incubated with detergent (50 ml of 0.5% Triton-X) for 4 hours before centrifugation at 12,000 rpm. These final pellet and supernatant were collected for protein profile verification by SDS-PAGE. The SDS-PAGE protein profiles of supernatant after Triton-X

incubation showed most of expressed proteins were in pellet. No expressed full-length NP band was observed in supernatant of detergent (Figure 4.12). The results indicated that our expressed recombinant NP was an insoluble protein and was not a plasma membrane which should be solubilized by detergent.

4. Denaturing protein purification for enhancing solubility of expressed protein

After induction, the harvested pellet of NP-pThioHisA contained E. coli strain TOP10 expression were used for protein purification under denaturing condition performed according to the manufacturer's protocol. The verification of denaturing protein purification was performed by SDS-PAGE as that of native protein purification. The results were similar to that from native protein purification. No purified full-length NP bands were observed with Coomassie brilliant blue straining SDS-PAGE and the expressed full-length NP band (68 kDa) was shown only in the prepared supernatant sample of induced group but none were observed in through-flow of supernatant after binding, washing buffer and eluted fraction of the group (data not shown).

5. Variation of incubation period for denaturant of denaturing protein purification

Moreover, the eluted proteins were purified under modified denaturing condition (incubation course in denaturant was increased to be 12 hours) and were verified by SDS-PAGE. The results were similar to those of denaturing protein purification performed according to the manufacturer's protocol. No purified NPs were observed in all eluted fractions (data not shown).

6. Variation of expression conditions

The lower temperature and longer time induction were important to increase the expression of soluble protein (Huang et al., 2011). This study attempted to enhance solubility of recombinant protein by inducing protein expression at room temperature and using the different concentrations of IPTG. The concentration was varied as following: 0.01, 0.1, and 0.5 mM IPTG. The expressions of full-length NP (68 kDa) under these conditions were observed by SDS-PAGE

(Figure 4.13). These expressed full-length NP groups were used as samples for protein purification under modified denaturing conditions. However, the results of SDS-PAGE were similar to those of denaturing protein purification. No purified bands were observed in all eluted fractions (data not shown).

7. Variation of bacterial strains

Changing of bacterial strains was used to improve soluble expression (Sorensen and Mortensen, 2005). In the study, the purified NP-pThioHisA contained *E. coli* strain JM109 and BL21 were induced for protein expression by adding 0.01, 0.1, and 0.5 mM IPTG (final concentration) and incubated at room temperature overnight. Before using as sample in protein purification, the expressions of full-length NP (68 kDa) in induced group of bacterial strains were observed by SDS-PAGE (Figure 4.14). Then, the harvested pellets from induced group of both strains were used for protein purification under modified denaturing condition. The eluted fractions were analyzed by SDS-PAGE and the proteins were purified. The results of SDS-PAGE were similar to those of denaturing protein purification. No purified bands were observed in all eluted fractions of both groups (data not shown).

Construction of the *E. coli* recombinant truncated NP expression vector (NPt-pThioHisA)

Another attempt was to produce truncated NP due to the failure in generation of the recombinant full-length NP. The truncated NP that contained immunogenic activity was decided to be the target in the expression attempt according to previous study describing about the successful of truncated influenza A NP expression and purification by other *E. coli* expression vectors (Jin et al., 2004; Boonmoh, 2006).

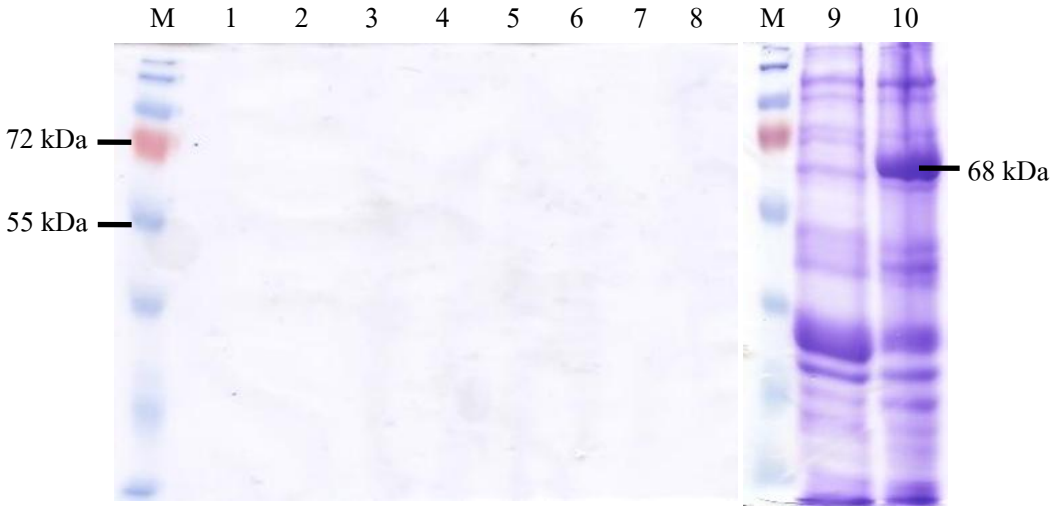


Figure 4.11 Coomassie brilliant blue staining SDS-PAGE profile of purified full-length NP from NP-pThioHisA contained *E.coli* strain TOP10 for using different imidazole concentration in elution buffer

Lane M = PageRuler™ Prestained Protein ladder (Fermentas, MD, USA)

Lane 1,2 = 1st fraction and 2nd fraction by using 125 mM imidazole elution buffer

Lane 3,4 = 1st fraction and 2nd fraction by using 250 mM imidazole elution buffer

Lane 5,6 = 1st fraction and 2nd fraction by using 500 mM imidazole elution buffer

Lane 7,8 = 1st fraction and 2nd fraction by using 1,000 mM imidazole elution buffer

Lane 9 = Lysate of non-induced NP-pThioHisA expression

Lane 10 = Lysate of induced NP-pThioHisA expresssion (68 kDa expected band)

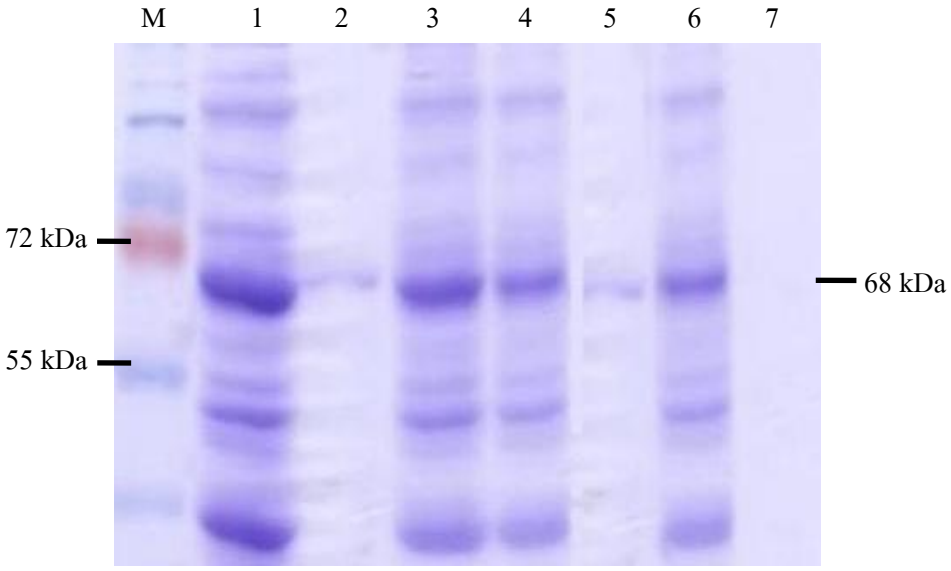


Figure 4.12 Coomassie brilliant blue staining SDS-PAGE profile of expressed protein from NP-pThioHisA contained *E.coli* strain TOP10 for solubility determination

Lane M = PageRuler™ Prestained Protein ladder (Fermentas, MD, USA)

Lane 1 = Lysate of non-induced NP-pThioHisA expression

Lane 2 = Pellet (after centrifuged at 3,000 rpm)

Lane 3 = Supernatant (after centrifuged at 3,000 rpm)

Lane 4 = Pellet (after centrifuged at 12,000 rpm)

Lane 5 = Supernatant (after centrifuged at 12,000 rpm)

Lane 6 = Pellet (after incubated in 0.5% Triton-X and centrifuged at 12,000 rpm)

Lane 7 = Supernatant (after incubated in 0.5% Triton-X and centrifuged at 12,000 rpm)

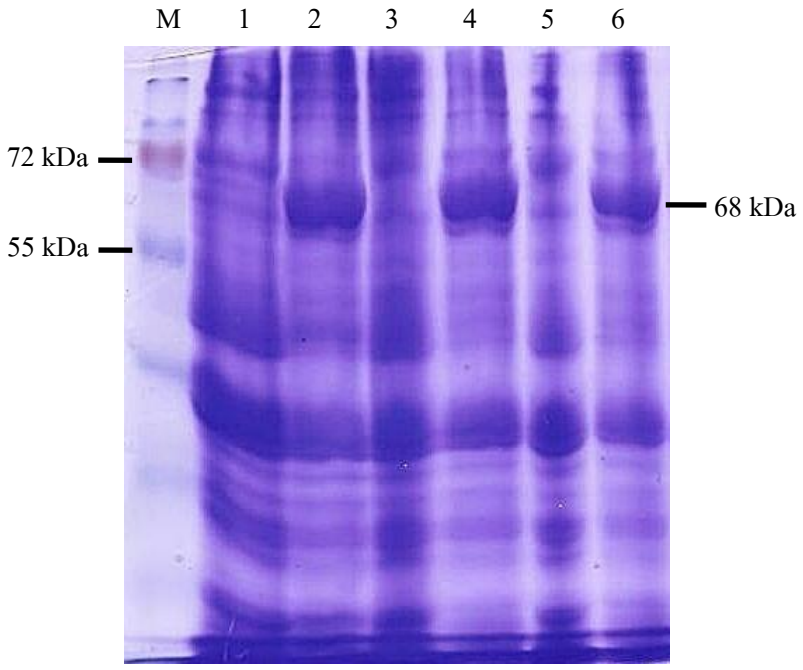


Figure 4.13 Coomassie brilliant blue staining SDS-PAGE profile of total protein from NP-pThioHisA contained *E.coli* strain TOP10 after inducing protein expression at room tempurature

Lane M = PageRuler™ Prestained Protein ladder (Fermentas, MD, USA)

Lane 1, 3, 5 = Non-induced NP-pThioHisA expression

Lane 2 = Induced NP-pThioHisA expression by 0.01 mM IPTG (68 kDa expected band)

Lane 4 = Induced NP-pThioHisA expression by 0.1 mM IPTG (68 kDa expected band)

Lane 6 = Induced NP-pThioHisA expression by 0.5 mM IPTG (68 kDa expected band)

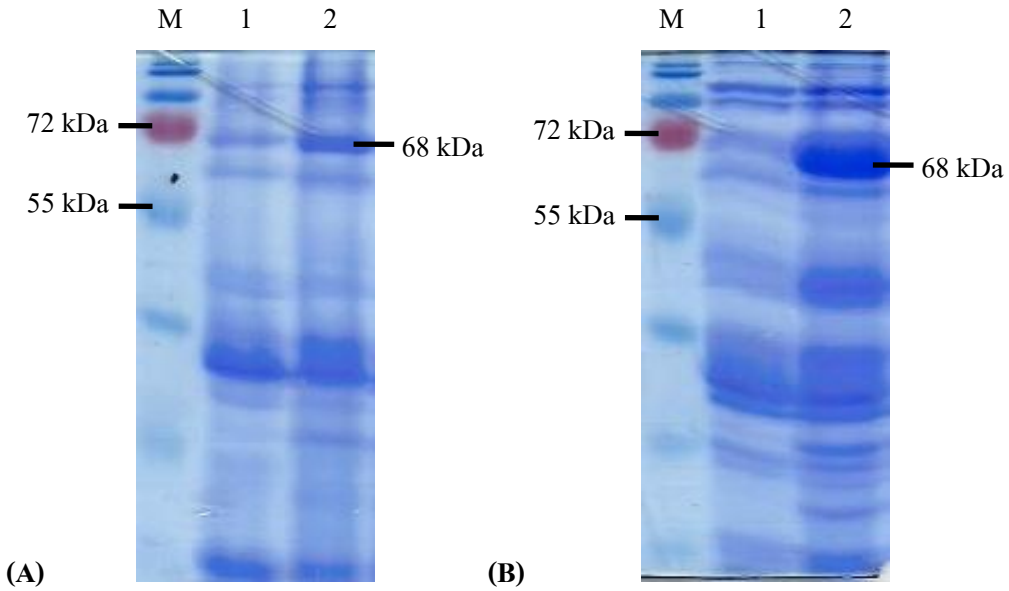


Figure 4.14 SimplyBlue™ SafeStain staining SDS-PAGE profile of total proteins from NP-pThioHisA contained *E.coli* strain JM109 (A) and BL21 (B) after inducing protein expression at room tempurature

Lane M = PageRuler™ Prestained Protein ladder (Fermentas, MD, USA)

Lane 1 = Non-induced NP-pThioHisA expression

Lane 2 = Induced NP-pThioHisA expression by 0.01 mM IPTG (68 kDa expected band)

1. RNA extraction and RT-PCR

The RT-PCR for truncated NP gene amplification was performed according to the optimized protocol and condition as described for full-length NP gene amplification. The RT-PCR amplicons size of 1,000 bp were analyzed with agarose gel electrophoresis and visualized under ultraviolet (UV) light as shown in Figure 4.15.

2. Cloning and analysis of inserted truncated NP gene in recombinant NPt-pThioHisA

The success of digestion was verified by running the digests on 1.5% agarose gel electrophoresis and visualized under UV light (Figure 4.16). The digested truncated NP gene was observed with the product size of 1,000 bp. The product size of digested pThioHisA vector was 4,400 bp. These digested DNA were purified and checked before ligation as described for cloning full-length NP gene.

Twenty-three single colonies of bacteria were observed on selective media after ligation and transformation. The result of PCR for insertion analysis of truncated NP gene in pThioHisA expression vector was shown in Figure 4.17. Moreover, the observed colonies were screened for insertion by restriction enzymes. The result was shown in Figure 4.18. According to application of PCR screening and restriction enzymes, there were 22 colonies of bacteria containing truncated NP gene plasmid. Ten positive colonies were extracted and prepared for DNA sequencing. These recombinant plasmids were named as “NPt-pThioHisA” (1-10). The nucleotide and deduced amino acid sequence of truncated NP gene in the selected NPt-pThioHisA clone was shown in Figure 4.19 and 4.20, respectively. Only one amino acid sequence of inserted gene in the plasmid was mutated. The mutation of phenylalanine to valine at position 298 (F to L) was not in B-cell epitopes of NP protein (Figure 4.21). The analysis of the sequencing results revealed that this clone was appropriated for protein expression study.

Protein expression and purification of truncated NP (NPt-pThioHisA)

The pellet samples from the pilot expression were collected and used for verification of protein expression by SDS-PAGE and commercial ELISA for NP of IAV detection in tissue culture. The recombinant truncated NP bands of approximately 49 kDa (37 kDa of truncated NP with 12 kDa of thioredoxin fusion protein) were observed in the SDS-PAGE protein profile at 1 hour, 4 hours and overnight samples (Figure 4.21).

Zero-time and overnight samples were tested for protein expression using IVtA EIA KIT for IAV-NP detection in tissue culture. The ELISA results for truncated NP expression were shown in Table 4.2.

Table 4.2 ELISA results for verification of truncated NP expression (NPt-pThioHisA)

Sample name	**OD at 450 nm (mean ± SD)**	**Interpreted result**
Zero-time sample NPt-pThioHisA	0.198 ± 0.009	Negative
Overnight sample NPt-pThioHisA	1.508 ± 0.373	Positive
Positive control	0.424 ± 0.018*	-
Negative control	0.192 ± 0.001*	-

*Positive control ≥ 1.5 times of OD negative control

** Cut off = 2 times of OD negative control = 0.383

Purification of truncated NP was performed and verification by SDS-PAGE and immunoblot analysis as previously was described for full-length NP. The results were similar to the those of the purification of full-length NP. The purified truncated NP band was not observed with Coomassie brilliant blue straining SDS-PAGE and immunoblot analysis (data not shown).

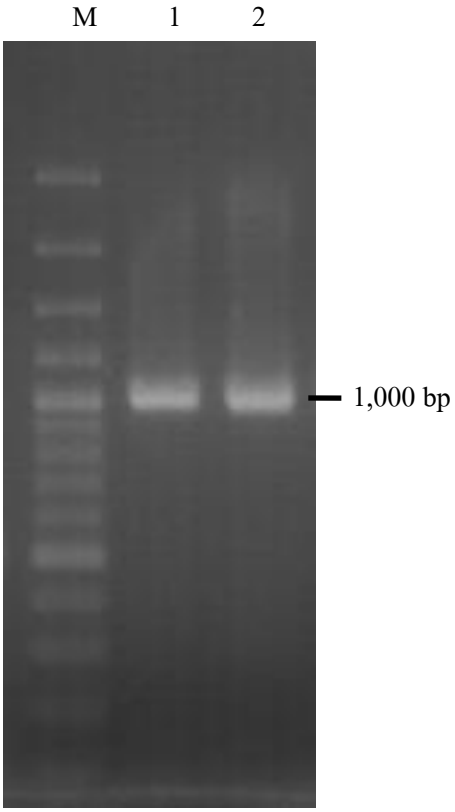


Figure 4.15 Ethidium bromide stainning agarose gel electrophoresis pattern of RT-PCR products of truncated NP gene

Lane M = GeneRuler™ 100 bp plus DNA ladder (Fermentas, MD, USA)

Lane 1,2 = The RT-PCR product of truncated NP gene

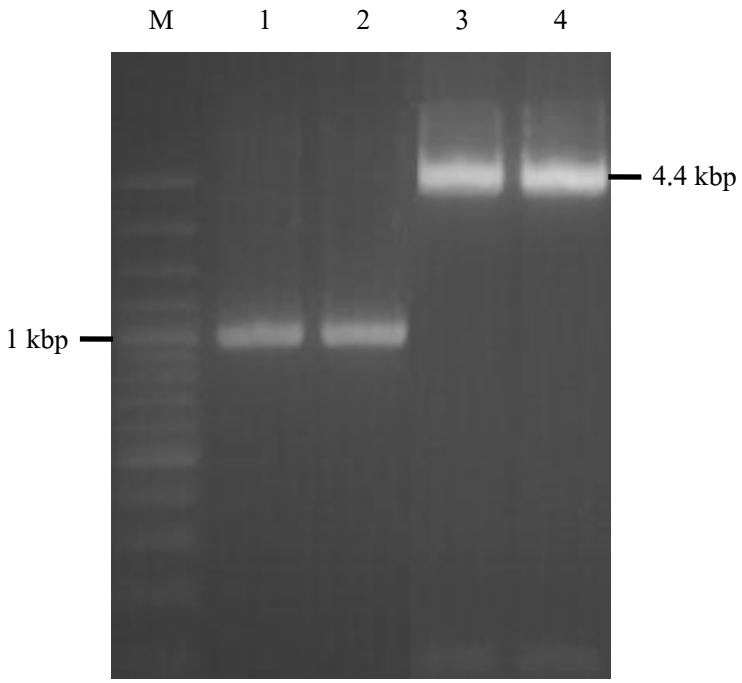


Figure 4.16 Ethidium bromide staining agarose gel electrophoresis pattern of digested truncated NP gene RT-PCR product and digested pThioHisA expression vector by *Xho*I and *Xba*I restriction endonuclease (NEB, MA, USA)

Lane M = GeneRuler™ 100 bp plus DNA ladder (Fermentas, MD, USA)

Lane 1, 2 = *Xho*I and *Xba*I digested truncated NP

Lane 3, 4 = *Xho*I and *Xba*I digested pThioHisA expression vector

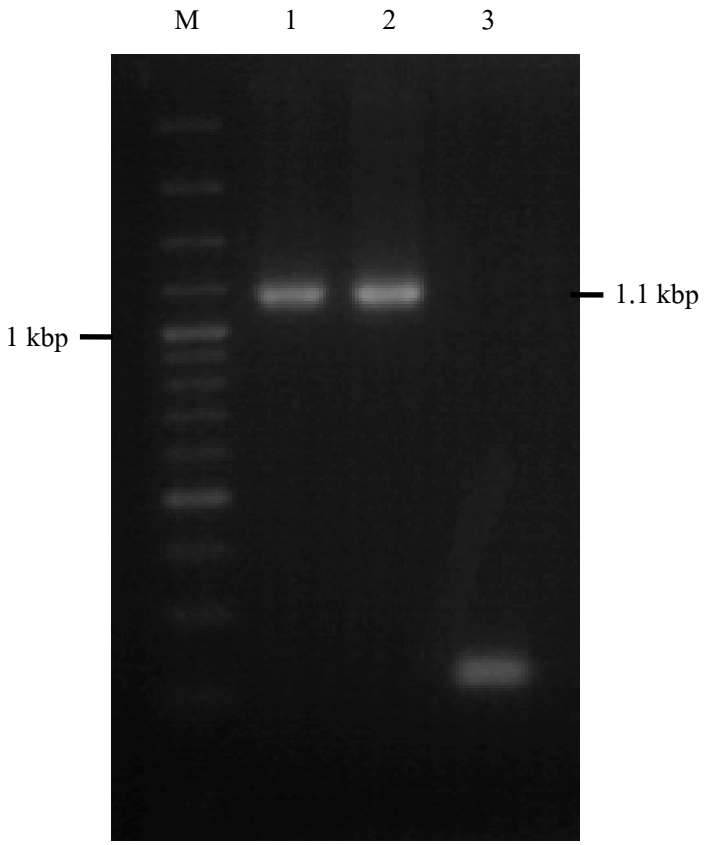


Figure 4.17 Ethidium bromide staining agarose gel electrophoresis pattern of PCR products for screening inserted truncated NP gene in pThioHisA expression vector

Lane M = GeneRuler™ 100 bp plus DNA ladder (Fermentas, MD, USA)

Lane 1, 2 = The PCR products form truncated NP gene inserted clones (positive clone)

Lane 3 = The PCR product from non-truncated NP gene inserted clones (negative clone)

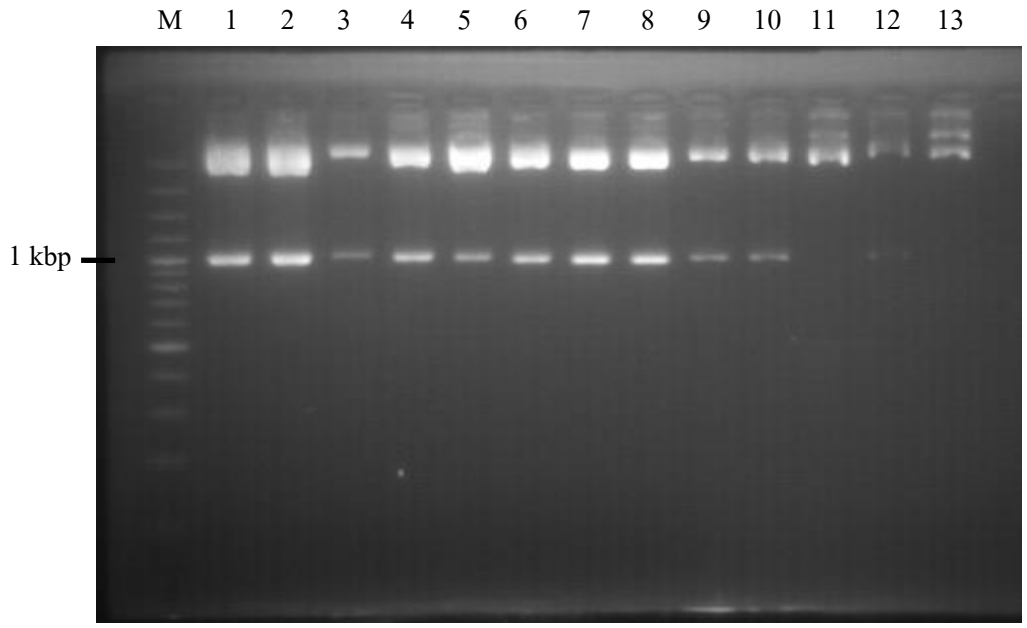


Figure 4.18 Ethidium bromide staining agarose gel electrophoresis pattern of Xho*I* and Xba*I* digested recombinant plasmid for screening truncated NP gene insertion in pThioHisA expression vector using samples prepared from bacterial colonies as templates

Lane M = GeneRuler™ 100 bp plus DNA ladder (Fermentas, MD, USA)

Lane 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12 = Truncated NP genes were cut from recombinant plasmid (Positive truncated NP inserted clones)

Lane 11, 13 = No expected digested DNA (Negative truncated NP inserted clones)


10 20 30 40 50 60 70 80 90 100
Truncated-NP CU-CBP18 CTCAGTGACT ATGAAGGGAG GCTGATCCAG AACAGCATAA CAATAGAGAG AATGGTTCTC TCGGCGTTTG ACGAGAGAAG AAACAGATAC CTGGAGGAAC
NPt-pThioHisA CTCAGTGACT ATGAAGGGAG GCTGATCCAG AACAGCATAA CAATAGAGAG AATGGTTCTC TCGGCGTTTG ACGAGAGGAG AAACAGATAC CTGGAGGAAC
Clustal Consensus
110 120 130 140 150 160 170 180 190 200
Truncated-NP CU-CBP18 ATCCCAGTGC GGGGAAAGAC CCGAAGAAAA CTGGTGGTCC AATCTACAAA AAGAGAGACG GAAAATGGAT GAGGGAGCTG GTTCTGTATG ATAAAGATGA
NPt-pThioHisA ATCCCAGTGC GGGGAAAGAC CCGAAGAAAA CTGGTGGTCC AATCTACAAA AAGAGAGACG GAAAATGGAT GAGGGAGCTG GTTCTGTATG ATAAAGATGA
Clustal Consensus
210 220 230 240 250 260 270 280 290 300
Truncated-NP CU-CBP18 GATCAGGAGA ATTTGGCGCC AAGCAAACAA TGGTGAAGAT GCTACCGCTG GTCTCACCCA CTTGATGATT TGGCATTCCA ATCTGAATGA TGCCACATAT
NPt-pThioHisA GATCAGGAGA ATTTGGCGCC AAGCAAACAA TGGTGAAGAT GCTACCGCTG GTCTCACCCA CTTGATGATT TGGCATTCCA ATCTGAATGA TGCCACATAT
Clustal Consensus
310 320 330 340 350 360 370 380 390 400
Truncated-NP CU-CBP18 CAAAGAACGA GGGCTCTTGT GCGTACTGGG ATGGATCCCA GAATGTGCTC TCTAATGCAA GGCTCAACTC TCCCGAGGAG ATCTGGAGCT GCTGGGGCAG
NPt-pThioHisA CAAAGAACGA GGGCTCTTGT GCGTACTGGG ATGGATCCCA GAATGTGCTC TCTAATGCAG GGCTCAACTC TCCCGAGGAG ATCTGGAGCT GCTGGGGCAG
Clustal Consensus
410 420 430 440 450 460 470 480 490 500
Truncated-NP CU-CBP18 CAGTAAAGGG AGTTGGGACA ATGGTAATGG AACTGATTCG GATGATAAAG CGAGGGATCA ATGACCGGAA CTTCTGGAGA GGCGAAAATG GACGAAGAAC
NPt-pThioHisA CAGTAAAGGG AGTTGGGACA ATGGTAATGG AACTGATTCG GATGATAAAG CGAGGGATCA ATGACCGGAA CTTCTGGAGA GGCGAAAATG GACGAAGGAC
Clustal Consensus
510 520 530 540 550 560 570 580 590 600
Truncated-NP CU-CBP18 AAGAATTGCA TATGAGAGAA TGTGCAACAT CCTCAAAGGG AAATTTCAAA CAGCAGCGCA ACGAGCAATG ATGGACCAGG TGCGAGAAAG CAGAAATCCT
NPt-pThioHisA AAGAATTGCA TATGAGAGAA TGTGCAACAT CCTCAAAGGG AAATTTCAAA CAGCAGCGCA ACGAGCAATG ATGGACCAGG TGCGAGAAAG CAGAAATCCT
Clustal Consensus
610 620 630 640 650 660 670 680 690 700
Truncated-NP CU-CBP18 GGGAATGCTG AGATTGAAGA TCTTATCTTT CTAGCACGAT CTGCACTCAT TCTGAGAGGA TCAGTAGCTC ACAAATCCTG TCTACCTGCT TGTGTATACG
NPt-pThioHisA GGGAATGCTG AGATTGAAGA TCTTATCTTT CTAGCACGAT CTGCACTCAT TCTGAGAGGA TCAGTAGCTC ACAAATCCTG TCTACCTGCT TGTGTATACG
Clustal Consensus

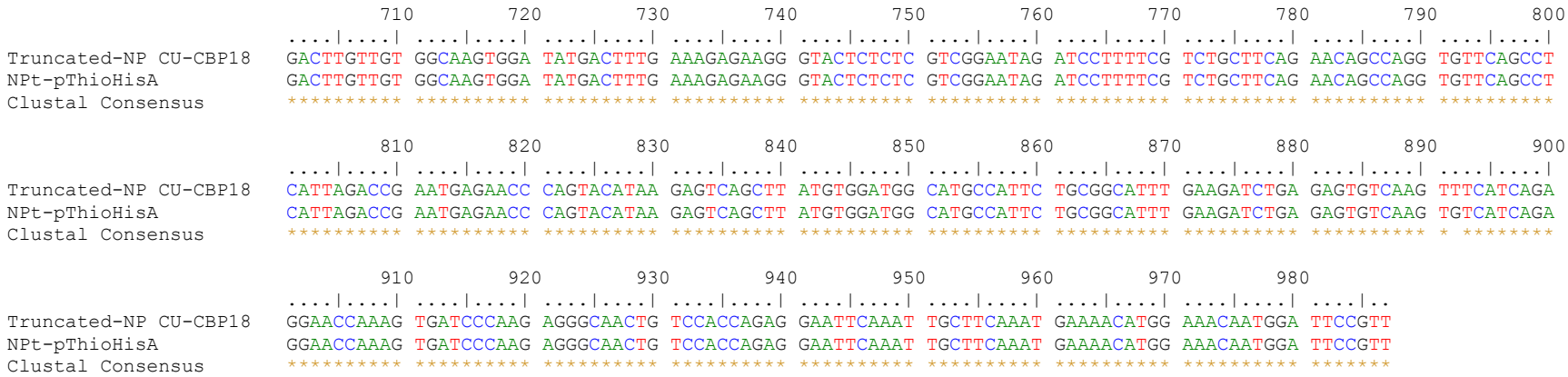


Figure 4.19 Nucleotide sequence alignment of truncated NP gene in NPt-pThioHisA expression was compared with truncated NP gene of swine influenza H1N1 virus (A/swine/Thailand/CU-CBP18/2009)

* = Homology

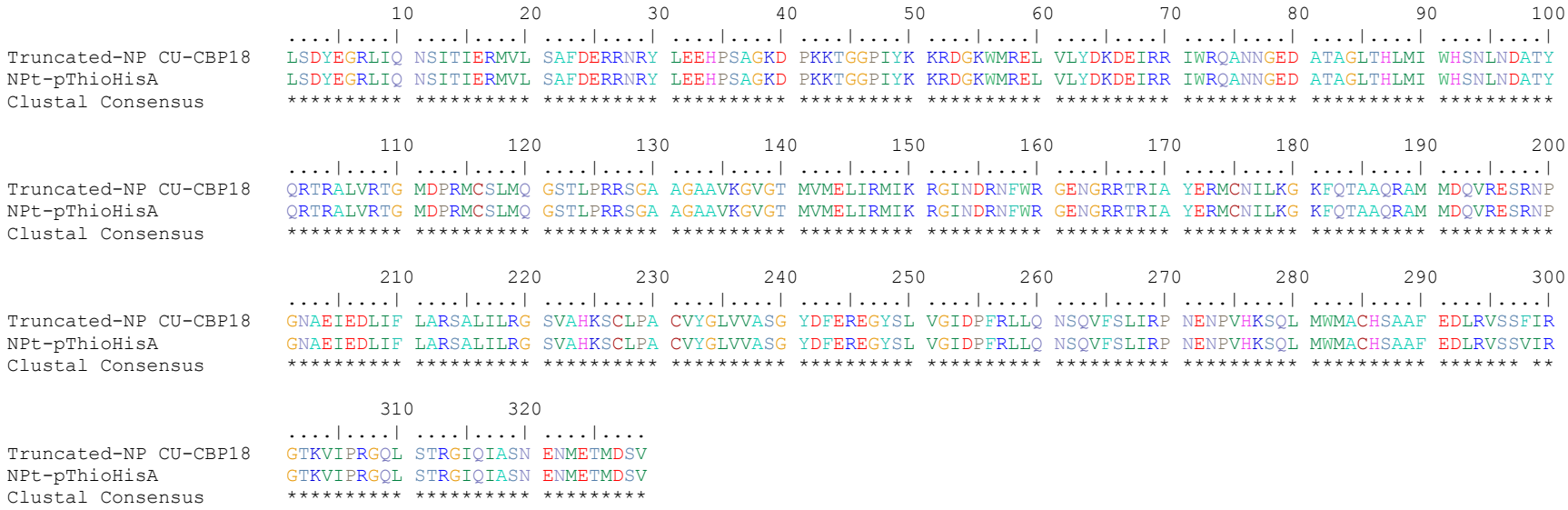


Figure 4.20 Amino acid sequence alignment of truncated NP in NPt-pThioHisA was compared with truncated NP of swine influenza H1N1 virus (A/swine/Thailand/CU-CBP18/2009)

* = Homology

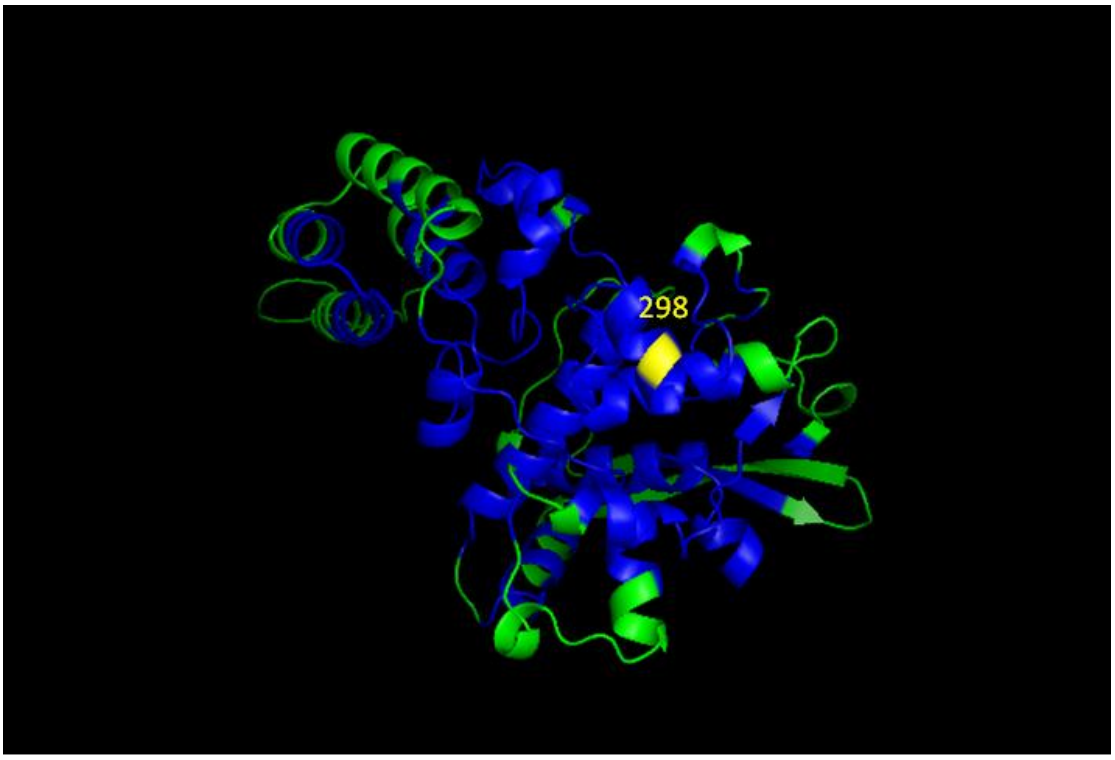


Figure 4.21 Predicted B-cell epitopes and mutated region of the recombinant truncated NP

Green region = Predicted B-cell epitopes of the NP

Yellow region = Mutated region

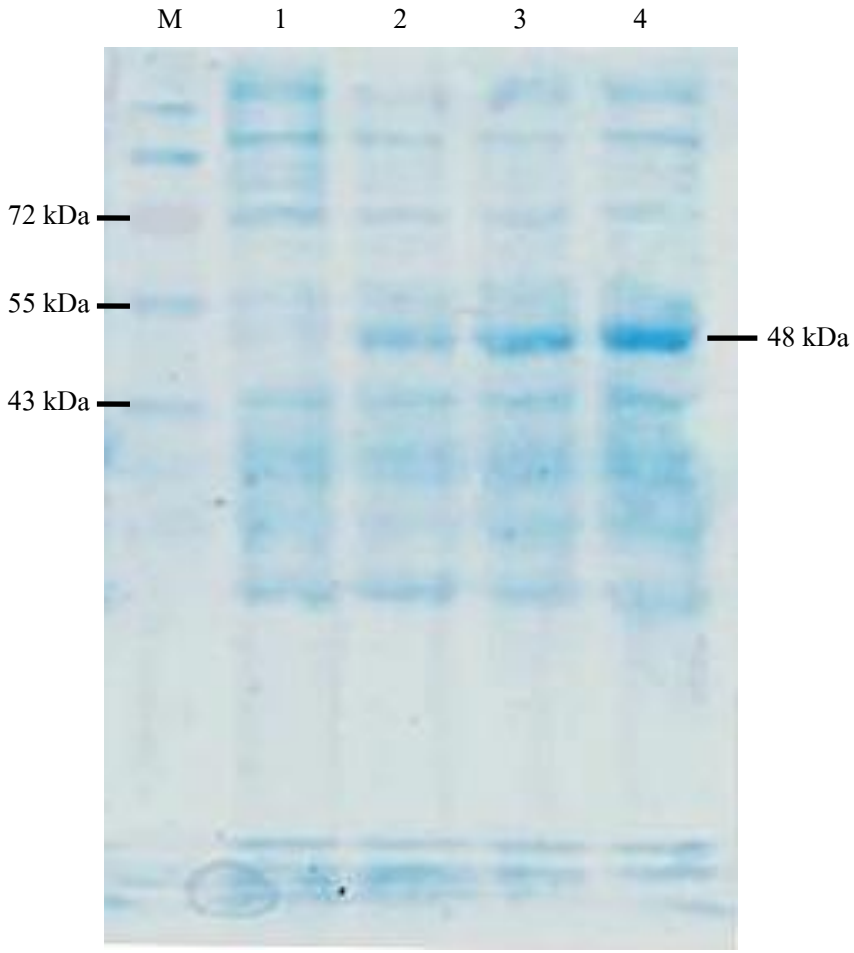


Figure 4.22 SimplyBlue™ SafeStain staining SDS-PAGE profile of total protein from NPt-pThioHisA transformed *E.coli* strain TOP10

Lane M = PageRuler™ Prestained Protein ladder (Fermentas, MD, USA)

Lane 1 = NPt-pThioHisA expression at 0 hour of induction (Non-induced group)

Lane 2 = NPt-pThioHisA expression at 1 hour of induction

Lane 3 = NPt-pThioHisA expression at 4 hour of induction

Lane 4 = NPt-pThioHisA expression at overnight of induction

CHAPTER V

DISCUSSIONS AND CONCLUSION

Discussions

Serological diagnosis is the major role in surveillance of IAV occurrence in animal herds including pig herd because direct diagnosis of the virus is limited caused by short shedding period (Kim et al., 2006). Commercial ELISA, a practical method, has been developed for detection of antibodies against multi-subtypes of IAV (Ciacci-Zanella et al., 2010; Pérez-Ramírez, 2010; Toennessen et al., 2010). The disadvantage of these commercial ELISAs is that they are imported and very costly for routine use in Thailand. The objective goal of this study is to produce a recombinant NP of SIV for ELISA-based test development for NP antibody detection for influenza A surveillance in Thai swine farms. Influenza A nucleoprotein (NP) is a conserved protein among influenza subtypes, so it is an interesting choice for using as an antigen to develop ELISA-base test for serological investigation in animal herds. This research attempted to generate full-length NP of IAV and use for the development of an ELISA-based test.

In this study, full-length NP gene of influenza A virus could be successfully cloned into pThioHisA expression vector (NP-pThioHisA). Even though, the nucleotide sequence of NP gene in NP-pGEM showed some point mutations in comparison with that of the stock virus, the deduced amino acid sequence of the NP gene had the proper start and stop codons in the open reading frame. In addition, the changing of two amino acids in the NP was not significant in amino acid composition of the protein because the position of mutated amino acids were not in predicted B-cell epitope regions of the protein. Thus the NP gene in NP-pGEM was used for NP-pThioHisA construction. The point mutations of NP gene in NP-pGEM might occur during RT-PCR amplification. The lacks of repairing system of reverse transcriptase and DNA polymerase have been mentioned for the reason of this error (Yu and Goodman, 1992; Cottam et

al., 2009). In this study, RT-PCR was performed following One-step RT-PCR (Promega, WI, USA). This RT-PCR kit contained T*fl* (*Thermus flavus*) polymerase. Using T*fl* polymerase is similar with using Taq polymerase which is well-known for no-proofreading activity enzyme. The previous study showed that percentage of mutated PCR products was 16% by using T*fl* polymerase while using high fidelity polymerase such as P*fu* (*Pyrococcus furiosus*) revealed only 2% of mutated PCR products (Jozwiakowski and Connolly, 2009; Sharifian, 2010). Thus, using high-fidelity polymerase should be an appropriated choice to decrease the mutation in cloning works.

The recombinant full-length NP of influenza A virus was expressed from NP-pThioHisA in *E. coli* expression vector. The protein expression could be verified by SDS-PAGE. Moreover, the interpretation result of commercial ELISA for influenza A NP antigen detection could be distinguished between induced and non-induced protein expression group supporting the recombinant full-length NP expression. However, during the protein purification and verification process, the result of SDS-PAGE profile could not demonstrate the purified protein by Probond™ Purification system under native condition.

Generally, thioredoxin recombinant fusion protein containing 6 tandem histidine residues (HP-thioredoxin) showed high affinity with divalent cations thus the recombinant HP-thiredoxins could be purified by Nickel-Chelating resin (Probond resin). The recombinant protein-bound resin could be eluted by competition with imidazole. In this study, the expressed recombinant NP had high affinity with the resin but cloud not be eluted from the resin even using the high concentration of imidazole elution buffer.

The attempts to purify the expressed full-length NP under native condition were not successful suggesting the insolubility of the expressed protein. In order to prove the insolubility of the expressed proteins, the protein solubility determination trial was performed. The result

supported that the recombinant protein was insoluble protein and might not be a membrane protein which can be solubilized by detergent.

Although protein yield that produced in *E. coli* expression system is quite high, the over expression can lead to a production of insoluble protein lacking of folding mediator (Chaperone) during rapid protein expression in cell and the aggregation of misfolded protein which is often lack of biological activity (Choi and Lee, 2004). Accumulation of misfolded proteins into insoluble aggregates have been known as inclusion proteins (Sorensen and Mortensen, 2005). Generally, solubilization of inclusion proteins can be achieved by using solubilization buffer containing denaturing agents to unfold the aggregated protein. The common denaturing agents are 6 M of guanidine hydrochloride and 8 M of urea buffers (Tsumoto et al., 2003). In this study, although protein purification was performed under denaturing condition, the recombinant full-length NP cloud not be solubilized and purified.

Previous study showed that the NP has more solubility when optimized the NP expression condition by reducing induction temperature from 37ºC to 25ºC (Boonmoh, 2006; Huang et al., 2011). Some studies supported this finding and reported that the production of chaperone in *E. coli* was increased at temperature about 30ºC (Mogk et al., 2002; Sorensen and Mortensen, 2005). Furthermore, each strain of *E. coli* is used for protein expression with differential potency (Sorensen and Mortensen, 2005). Various protein expression conditions were performed in this study, we were not able to purify the expressed protein.

In our study, the recombinant truncated NP protein was successfully expressed, but could not be purified. The reason being unable to purify the recombinant full-length and truncated NP was caused by the insolubility of the protein products.

Changing *E. coli* expression cassette could improve solubility of recombinant NP protein (Boonmoh, 2006; Yan et al., 2009). However, previous studies on expression of recombinant influenza A NP in *E. coli* expression system reported the same limitation (Sianglum et al., 2010;

Pongpeng et al., 2011). Alternatively, eukaryotic expression system, yeast and mammalian expression system should be used as the alternate option to produce the soluble protein (Demain and Vaishnav, 2009).

Conclusion

In conclusion, this project was successful on the amplification and cloning of full-length and truncated NP of IAV into *E.coli* expression vector. The recombinant NP-pThioHisA and NPt-pThioHisA *E. coli* expression vector were constructed. These constructed expression vectors were able to express full-length and truncated NP of IAV in prokaryotic expression system. Unfortunately, these expressed proteins could not be purified leading to unsuccessfully development of an ELISA-based test for antibody detection against influenza A nucleoprotein (NP) in pigs. The improvement of certain technical aspects of recombinant influenza A nucleoprotein expression system and the purification method to produce the recombinant protein are necessary for the future studies. Further improvements from this study might be useful for others to produce recombinant influenza A NP for development a practical and cost effective serological diagnostic test which can provide the great benefit for the disease surveillance in Thai pig herds as well as in other developing countries.

REFERENCES

Alexander, D.J. and Brown, I.H. 2000. Recent zoonoses caused by influenza A viruses. Rev Sci Tech. 19(1): 197-225.

Baneyx, F. 1999. Recombinant protein expression in *Escherichia coli*. Curr Opin Biotechnol. 10(5): 411-421.

Boliar, S., Stanislawek, W. and Chambers, T.M. 2006. Inability of Kaolin Treatment to Remove Nonspecific Inhibitors from Equine Serum for the Hemagglutination Inhibition Test Against Equine H7N7 Influenza Virus. J Vet Diagn Invest. 18(3): 264-270.

Boonmoh, S. 2006. Molecular cloning and expression. Thesis of M.S. Thailand: Mahidol university.

Brown, I. 2001. The pig as an intermediate host for influenza A viruses between birds and humans. Proceedings of the international congress series of virology department, veterinary laboratories agency-Weybridge. United Kingdom, p. 173-178.

Callow, K.A. 1983. Measurement of antibodies to influenza virus neuraminidase by an enzyme-linked immunosorbent assay. Infect Immun. 41(2): 650-656.

Choi, J.H. and Lee, S.Y. 2004. Secretory and extracellular production of recombinant proteins using *Escherichia coli*. Appl Microbiol Biotechnol. 64(5): 625-635.

Chutinimitkul, S., Thippamom, N., Damrongwatanapokin, S., Payungporn, S., Thanawongnuwech, R., Amonsin, A., Boonsuk, P., Sreta, D., Bunpong, N. and Tantilertcharoen, R. 2008. Genetic characterization of H1N1, H1N2 and H3N2 swine influenza virus in Thailand. Arch Virol. 153(6): 1049 - 1056.

Ciacci-Zanella, J.R., Vincent, A.L., Prickett, J.R., Zimmerman, S.M. and Zimmerman, J.J. 2010. Detection of anti-influenza A nucleoprotein antibodies in pigs using a commercial influenza epitope-blocking enzyme-linked immunosorbent assay developed for avian species. J Vet Diagn Invest. 22(1): 3-9.

Coleman, J.R. 2007. The PB1-F2 protein of Influenza A virus: increasing pathogenicity by disrupting alveolar macrophages. Virol J. 4(1): 9.

Cottam, E.M., King, D.P., Wilson, A., Paton, D.J. and Haydon, D.T. 2009. Analysis of Foot-and-mouth disease virus nucleotide sequence variation within naturally infected epithelium. Virus Res. 140(1-2): 199-204.

Cowling, B.J., Chan, K.H., Fang, V.J., Lau, L.L.H., So, H.C., Fung, R.O.P., Ma, E.S.K., Kwong, A.S.K., Chan, C.-W., Tsui, W.W.S., Ngai, H.-Y., Chu, D.W.S., Lee, P.W.Y., Chiu, M.-C., Leung, G.M. and Peiris, J.S.M. 2010. Comparative Epidemiology of Pandemic and Seasonal Influenza A in Households. N Engl J Med. 362(23): 2175-2184.

Cox, R.J., Brokstad, K.A. and Ogra, P. 2004. Influenza Virus: Immunity and Vaccination Strategies. Comparison of the Immune Response to Inactivated and Live, Attenuated Influenza Vaccines. Scand J Immunol. 59(1): 1-15.

Cunningham, C.H. 1973. Hemagglutination, Hemagglution-inhibition and Hemadsorption. A Laboratory Guide in virology. 7th ed. Minnesota: Burgess Publishing Company. 6 pp.

de Boer, G.F., Back, W. and Osterhaus, A.D.M.E. 1990. An ELISA for detection of antibodies against influenza A nucleoprotein in humans and various animal species. Arch Virol. 115(1): 47-61.

Demain, A.L. and Vaishnav, P. 2009. Production of recombinant proteins by microbes and higher organisms. Biotech Adv. 27(3): 297-306.

Dwyer, D.E., Smith, D.W., Catton, M.G. and Barr, I.G. 2006. Laboratory diagnosis of human seasonal and pandemic inflenza virus infection. Med J Aust. 185(10): 48-53

Erickson, G.A., Gramer, M.R. and Webby, R.J. 2005. How to evaluate herds using swine influenza serology. Swine Health Prod. 13(4): 222-224.

Esposito, D. and Chatterjee, D.K. 2006. Enhancement of soluble protein expression through the use of fusion tags. Curr Opin Biotechnol. 17(4): 353-358.

Forbes, K.J. 2004. The Asian flu and Russian virus: the international transmission of crises in firm-level data. J Intern Econ. 63(1): 59-92.

Fouchier, R.A., Schneeberger, P.M., Rozendaal, F.W., Broekman, J.M., Kemink, S.A., Munster, V., Kuiken, T., Rimmelzwaan, G.F., Schutten, M., Van Doornum, G.J., Koch, G., Bosman, A., Koopmans, M. and Osterhaus, A.D. 2004. Avian influenza A virus (H7N7) associated with human conjunctivitis and a fatal case of acute respiratory distress syndrome. Proc Natl Acad Sci U S A. 101(5): 1356-1361.

Fouchier, R.A.M., Munster, V., Wallensten, A., Bestebroer, T.M., Herfst, S., Smith, D., Rimmelzwaan, G.F., Olsen, B. and Osterhaus, A.D.M.E. 2005. Characterization of a Novel Influenza A Virus Hemagglutinin Subtype (H16) Obtained from Black-Headed Gulls. J Virol. 79(5): 2814-2822.

Francis, T.J., Salk, J.E. and Guilligan, J.J. 1947. Experience with Vaccination Against Influenza in the Spring of 1947. Am J Public Health Nation Health. 37(8): 1013-1016.

Galwankar, S. and Clem, A. 2009. Swine influenza A (H1N1) strikes a potential for global disaster. J Emerg Trauma Shock. 2(2): 99-105.

Garten, R.J., Davis, C.T., Russell, C.A., Shu, B., Lindstrom, S., Balish, A., Sessions, W.M., Xu, X., Skepner, E., Deyde, V., Okomo-Adhiambo, M., Gubareva, L., Barnes, J., Smith, C.B., Emery, S.L., Hillman, M.J., Rivailler, P., Smagala, J., de Graaf, M., Burke, D.F., Fouchier, R.A., Pappas, C., Alpuche-Aranda, C.M., Lopez-Gatell, H., Olivera, H., Lopez, I., Myers, C.A., Faix, D., Blair, P.J., Yu, C., Keene, K.M., Dotson, P.D., Jr., Boxrud, D., Sambol, A.R., Abid, S.H., St George, K., Bannerman, T., Moore, A.L., Stringer, D.J., Blevins, P., Demmler-Harrison, G.J., Ginsberg, M., Kriner, P., Waterman, S., Smole, S., Guevara, H.F., Belongia, E.A., Clark, P.A., Beatrice, S.T., Donis, R., Katz, J., Finelli, L., Bridges, C.B., Shaw, M., Jernigan, D.B., Uyeki, T.M., Smith, D.J., Klimov, A.I. and Cox, N.J. 2009. Antigenic and genetic characteristics of swine-origin 2009 A(H1N1) influenza viruses circulating in humans. Science. 325(5937): 197-201.

Ghosh, S., Rasheedi, S., Rahim, S.S., Banerjee, S., Choudhary, R.K., Chakhaiyar, P., Ehtesham, N.Z., Mukhopadhyay, S. and Hasnain, S.E. 2004. Method for enhancing solubility of the expressed recombinant proteins in *Escherichia coli*. Biotechniques. 37: 418-423.

Gorman, O.T., Bean, W.J., Kawaoka, Y. and Webster, R.G. 1990. Evolution of the nucleoprotein gene of influenza A virus. J. Virol. 64(4): 1487-1497.

Guan, Y., Shortridge, K.F., Krauss, S., Li, P.H., Kawaoka, Y. and Webster, R.G. 1996. Emergence of avian H1N1 influenza viruses in pigs in China. J Virol. 70(11): 8041-8046.

Guo, Y., Wang, M., Kawaoka, Y., Gorman, O., Ito, T., Saito, T., Webster, R.G. 1992. Characterization of a new avian-like influenza A virus from horse in China. Virology. 188(1): 245-255.

Hampson, A. and Mackenzie, J. 2006. The influenza virus. Med J Aust. 185(10): 39-43.

Hensley, S.E. and Yewdell, J.W. 2009. Que sera, sera: evolution of the swine H1N1 influenza A virus. Expert Rev Anti Infect Ther. 7(7): 763-768.

Horimoto, T. and Kawaoka, Y. 2005. Influenza: lessons from past pandemics, warnings from current incidents. Nat Rev Microbiol. 3(8): 591-600.

Huang, B.Y., Wang, W.L., Wang, X.P., Jiang, T., Tan, W.J. and Ruan, L. 2011. Efficient soluble expression and purification of influenza A nucleoprotein in *Escherichia coli*. Bing Du Xue Bao. 27(1): 50-57

Huang, Q., Sivaramakrishna, R.P., Ludwig, K., Korte, T., Böttcher, C. and Herrmann, A. 2003. Early steps of the conformational change of influenza virus hemagglutinin to a fusion active state: Stability and energetics of the hemagglutinin. BBA-Rev Biomembranes. 1614(1): 3-13.

Jagger, B.W., Wise, H.M., Kash, J.C., Walters, K.-A., Wills, N.M., Xiao, Y.-L., Dunfee, R.L., Schwartzman, L.M., Ozinsky, A., Bell, G.L., Dalton, R.M., Lo, A., Efstathiou, S., Atkins, J.F., Firth, A.E., Taubenberger, J.K. and Digard, P. 2012. An overlapping protein-coding region in influenza A virus segment 3 modulates the host response. Science. 337(6091): 199-204.

Jin, M., Wang, G., Zhang, R., Zhao, S., Li, H., Tan, Y. and Chen, H. 2004. Development of enzyme-linked immunosorbent assay with nucleoprotein as antigen for detection of antibodies to avian influenza virus. Avian Dis. 48(4): 870-878.

Johansson, B.E., Bucher, D.J. and Kilbourne, E.D. 1989. Purified influenza virus hemagglutinin and neuraminidase are equivalent in stimulation of antibody response but induce contrasting types of immunity to infection. J Virol. 63(3): 1239-1246.

Jozwiakowski, S.K. and Connolly, B.A. 2009. Plasmid-based lacZα assay for DNA polymerase fidelity: application to archaeal family-B DNA polymerase. Nucl Acids Res. 37(15): 102.

Kay, R., Done, S. and Paton, D. 1994. Effect of sequential porcine reproductive and respiratory syndrome and swine influenza on the growth and performance of finishing pigs. Vet Rec. 135(9): 199-204.

Khan, M.W., Gallagher, M., Bucher, D., Cerini, C.P. and Kilbourne, E.D. 1982. Detection of influenza virus neuraminidase-specific antibodies by an enzyme-linked immunosorbent assay. J. Clin. Microbiol. 16(1): 115-122.

Kida, H., Shortridge, K.F. and Webster, R.G. 1988. Origin of the hemagglutinin gene of H3N2 influenza viruses from pigs in China. Virology. 162(1): 160-166.

Kim, W.I., Wu, W.H., Janke, B. and Yoon, K.J. 2006. Characterization of the humoral immune response of experimentally infected and vaccinated pigs to swine influenza viral proteins. Arch Virol. 151(1): 23-36.

Kitikoon, P., Sreta, D., Nuntawan Na Ayudhya, S., Wongphatcharachai, M., Lapkuntod, J., Prakairungnamthip, D., Bunpapong, N., Suradhat, S., Thanawongnuwech, R. and Amonsin, A. 2011. Brief report: molecular characterization of a novel reassorted pandemic H1N1 2009 in Thai pigs. Virus Genes. 43(1): 1-5.

Kitikoon, P., Sreta, D., Tuanudom, R., Amonsin, A., Suradhat, S., Oraveerakul, K., Poovorawan, Y. and Thanawongnuwech, R. 2011. Serological evidence of pig-to-human influenza virus transmission on Thai swine farms. Vet microbiol. 148(2-4): 413-418.

Kupradinun, S., Peanpijit, P., Bhodhikosoom, C., Yoshioka, Y., Endo, A. and Nerome, K. 1991. The first isolation of swine H1N1 influenza viruses from pigs in Thailand. Arch Virol. 118: 289 - 297.

Lakadamyali, M., Rust, M.J. and Zhuang, X. 2004. Endocytosis of influenza viruses. Microbes Infect. 6(10): 929-936.

Lamb, R.A. and Krug, R.M. 2001. Orthomixoviridae: The Viruses and Their Replication. In: Fields virology. 4th ed. A.M. Knipe and P.M. Howley (ed). Philadelphia, PA: Lippincott Williams & Wilkins. 1487-1531.

Lee, B.W., Bey, R.F., Baarsch, M.J., Morrison, R.B. and Freese, W. 1993. Determination of hemagglutination-inhibition titers to influenza A virus in porcine sera by use of an enzyme-linked immunosorbent assay. Am J Vet Res. 54(8): 1270-1276.

Lin, J.C. and Nichol, K.L. 2001. Excess mortality due to pneumonia or influenza during influenza seasons among persons with acquired immunodeficiency syndrome. Arch Intern Med. 161(3): 441-446.

Lin, Y.P., Shaw, M., Gregory, V., Cameron, K., Lim, W., Klimov, A., Subbarao, K., Guan, Y., Krauss, S., Shortridge, K., Webster, R., Cox, N. and Hay, A. 2000. Avian-to-human transmission of H9N2 subtype influenza A viruses: relationship between H9N2 and H5N1 human isolates. Proc Natl Acad Sci U S A. 97(17): 9654-9658.

Ma, W., Kahn, R.E. and Richt, J.A. 2009. The pig as a mixing vessel for influenza viruses: Human and veterinary implication. J Mol Genet Med. 3(1): 158-166.

Ma, W., Lager, K.M., Vincent, A.L., Janke, B.H., Gramer, M.R. and Richt, J.A. 2009. The role of swine in the generation of novel influenza viruses. Zoonoses Public Health. 56(6-7): 326-337.

Mogk, A., Mayer, M.P. and Deuerling, E. 2002. Mechanisms of Protein Folding: Molecular Chaperones and Their Application in Biotechnology. Chembiochem. 3(9): 807-814.

Nayak, D.P., Hui, E.K.-W. and Barman, S. 2004. Assembly and budding of influenza virus. Virus Res. 106(2): 147-165.

Nelson, M.I. and Holmes, E.C. 2007. The evolution of epidemic influenza. Nature rev genet. 8: 196-205.

Neumann, G., Noda, T., and Kawaoka, Y. 2009. Emergence and pandemic potential of swine-origin H1N1 influenza virus. Nature. 459: 931-939.

Olsen, C.W. 2002. The emergence of novel swine influenza viruses in North America. Virus Res. 85(2): 199-210.

Ortega, J., Martin-Benito, J., Zurcher, T., Valpuesta, J.M., Carrascosa, J.L. and Ortin, J. 2000. Ultrastructural and functional analyses of recombinant influenza virus ribonucleoproteins suggest dimerization of nucleoprotein during virus amplification. J Virol. 74(1): 156-163.

Peiris, J.S., de Jong, M.D. and Guan, Y. 2007. Avian Influenza Virus (H5N1): a Threat to Human Health. Clin Microbiol Rev. 20(2): 243-267.

Peiris, J.S., Yu, W.C., Leung, C.W., Cheung, C.Y., Ng, W.F., Nicholls, J.M., Ng, T.K., Chan, K.H., Lai, S.T., Lim, W.L., Yuen, K.Y. and Guan, Y. 2004. Re-emergence of fatal human influenza A subtype H5N1 disease. Lancet. 363(9409): 617-619.

Pérez-Ramírez, E., Rodríguez, V., Sommer, D., Blanco, J.M., Acevedo, P., Heffels-Redmann, U. and Höfle, U. 2010. Serologic testing for avian influenza viruses in wild birds: comparison of two commercial competition enzyme-linked immunosorbent assays. Avian Dis. 54(1): 729-733.

Pinto, L.H., Holsinger, L.J. and Lamb, R.A. 1992. Influenza virus M2 protein has ion channel activity. Cell. 69(3): 517-528.

Pinto, L.H. and Lamb, R.A. 2006. The M2 proton channels of influenza A and B viruses. J Biol Chem. 281(14): 8997-9000.

Pongpeng, A., Phecharat, N. and Lekcharoensuk, P. 2011. Cloning and production of nucleoprotein of swine influenza virus in *E.coli*. Proceedings of the the 49th Kasetsart University Annual Conference: Animals, Veterinary Medicine. Thailand, p. 57-64.

Portela, A. and Digard, P. 2002. The influenza virus nucleoprotein: a multifunctional RNA-binding protein pivotal to virus replication. J Gen Virol. 83(4): 723-734.

Pringle, C.R. 1996. Virus taxonomy 1996 — A Bulletin from the Xth International Congress of Virology in Jerusalem. Arch Virol. 141(11): 2251-2256.

Prokudina, E., Semenova, N., Chumakov, V. and Stitz, L. 2008. An antigenic epitope of influenza virus nucleoprotein (NP) associated with polymeric forms of NP. Virology Journal. 5(37): 1-5.

Raynard, R.S., Murray, A.G. and Gregory, A. 2001. Infectious salmon anaemia virus in wild fish from Scotland. Dis Aquat Organ. 46(2): 93-100.

Reeth, K.V. 2007. Avian and swine influenza viruses: our current understanding of the zoonotic risk. Vet. Res. 38(2): 243-260.

Ryan-Poirier, K.A. and Kawaoka, Y. 1991. Distinct glycoprotein inhibitors of influenza A virus in different animal sera. J Virol. 65(1): 389-395.

Safrin, S., Rush, J.D. and Mills, J. 1990. Influenza in patients with human immunodeficiency virus infection. Chest. 98(1): 33-37.

Samji, T. 2009. Influenza A: understanding the viral life cycle. Yale J Biol Med. 82(4): 153-159.

Scholtissek, C. 1998. Genetic reassortment of human influenza viruses in nature. In: Textbook of influenza. 1st ed. K.G. Nicholson, Webster, R.G. and Hay. A.J. (ed). Malden, MA: Blackwell Science, Ltd. 120-125.

Sharifian, H. 2010. Errors induced during PCR amplication. Thesis of M.S. Zürich, Switzerland: Swiss Federal Institute of Technology.

Shope, R.E. 1931. Swine influenza: III - filtration experiments and etiology. J Exp Med. 54(3): 373-385.

Sianglum, A., Namwat, W., Chreonsudjai, S. and Lulitanond, V. 2010. Cloning and expression of nucleoprotein of influenza virus in *Escherichia coli*. Proceedings of the 11th graduate research conference, Khon Kaen University. Thailand, p. 967-973.

Skehel, J.J. and Wiley, D.C. 2000. Receptor binding and membrane fusion in virus entry: the influenza hemagglutinin. Annu Rev Biochem. 69(1): 531-569.

Skibbe, D., Zhou, E. and Janke, B. 2004. Comparison of a commercial enzyme-linked immunosorbent assay with hemagglutination inhibition assay for serodiagnosis of swine influenza virus (H1N1) infection. J Vet Diagn Invest. 16(1): 86-89.

Smith, G.J., Vijaykrishna, D., Bahl, J., Lycett, S.J., Worobey, M., Pybus, O.G., Ma, S.K., Cheung, C.L., Raghwani, J., Bhatt, S., Peiris, J.S., Guan, Y. and Rambaut, A. 2009. Origins and evolutionary genomics of the 2009 swine-origin H1N1 influenza A epidemic. Nature. 459(7250): 1122-1125.

Sorensen, H. and Mortensen, K. 2005. Soluble expression of recombinant proteins in the cytoplasm of *Escherichia coli*. Microb Cell Fact. 4(1): 1-8.

Sreta, D. 2010. Serological study and genetic characterization of swine influenza A virus in four high pig density provinces in Thailand between 2006-2009. Thesis of Ph.D. Thailand: Chulalongkorn University.

Sreta, D., Kedkovid, R., Tuamsang, S., Kitikoon, P. and Thanawongnuwech, R. 2009. Pathogenesis of swine influenza virus (Thai isolates) in weanling pigs: an experimental trial. Virol J. 6(1): 34.

Sreta, D., Tantawet, S., Nuntawan Na Ayudhya, S., Thontiravong, A., Wongphatcharachai, M., Lapkuntod, J., Bunpapong, N., Tuanudom, R., Suradhat, S., Vimolket, L., Poovorawan, Y., Thanawongnuwech, R., Amonsin, A. and Kitikoon, P. 2010. Pandemic (H1N1) 2009 virus on commercial swine farm, Thailand. Emerg Infect Dis. 16(10): 1587-1590.

Su, B., Wurtzer, S., Rameix-Welti, M.A., Dwyer, D., van der Werf, S., Naffakh, N., Clavel, F. and Labrosse, B. 2009. Enhancement of the influenza A hemagglutinin (HA)-mediated cell-cell fusion and virus entry by the viral neuraminidase (NA). PLoS One. 4(12): 8495.

Subbarao, E.K., Kawaoka, Y., Ryan-Poirier, K., Clements, M.L. and Murphy, B.R. 1992. Comparison of different approaches to measuring influenza A virus-specific hemagglutination inhibition antibodies in the presence of serum inhibitors. J Clin Microbiol. 30(4): 996-999.

Suzuki, Y. 2005. Sialobiology of influenza: molecular mechanism of host range variation of influenza viruses. Biol Pharm Bull. 28(3): 399-408.

Taubenberger, J. and Morens, D. 2006. 1918 influenza: the mother of all pandemics. Emerg Infect Dis. 12(1): 15-22.

Taubenberger, J. and Morens, D. 2008. The pathology of influenza virus infections. Annu Rev Pathol Mech Dis. 3: 499-522.

Thippamom, N., Sreta, D., Kitikoon, P., Thanawongnuwech, R., Poovorawan, Y., Theamboonlers, A., Suwannakarn, K., Parchariyanon, S., Damrongwatanapokin, S. and Amonsin, A. 2010. Genetic variations of nucleoprotein gene of influenza A viruses isolated from swine in Thailand. Virol J. 7(1): 185.

Tiensin, T., Chaitaweesub, P., Songserm, T., Chaisingh, A., Hoonsuwan, W., Buranathai, C., Parakamawongsa, T., Premashthira, S., Amonsin, A., Gilbert, M., Nielen, M. and Stegeman, A. 2005. Highly pathogenic avian influenza H5N1, Thailand, 2004. Emerg Infect Dis. 11(11): 1664-1672.

Toennessen, R., Germundsson, A., Jonassen, C., Haugen, I., Berg, K., Barrett, R. and Rimstad, E. 2010. Virological and serological surveillance for type A influenza in the black-legged kittiwake (Rissa tridactyla). Virol J. 8(1): 21.

Tong, S., Li, Y., Rivailler, P., Conrardy, C., Castillo, D.A.A., Chen, L.-M., Recuenco, S., Ellison, J.A., Davis, C.T., York, I.A., Turmelle, A.S., Moran, D., Rogers, S., Shi, M., Tao, Y., Weil, M.R., Tang, K., Rowe, L.A., Sammons, S., Xu, X., Frace, M., Lindblade, K.A., Cox, N.J., Anderson, L.J., Rupprecht, C.E. and Donis, R.O. 2012. A distinct lineage of influenza A virus from bats. Proceedings of the national academy of sciences of the united state of america. USA. February 27, 2012, p. 1-6.

Trebbien, R., Larsen, L. and Viuff, B. 2011. Distribution of sialic acid receptors and influenza A virus of avian and swine origin in experimentally infected pigs. Virol J. 8(1): 434.

Tsumoto, K., Ejima, D., Kumagai, I. and Arakawa, T. 2003. Practical considerations in refolding proteins from inclusion bodies. Protein Express Purif 28(1): 1-8.

van Wyke, K.L., Hinshaw, V.S., Bean, W.J. and Webster, R.G. 1980. Antigenic variation of influenza A virus nucleoprotein detected with monoclonal antibodies. J Virol. 35(1): 24-30.

Vannier, P. 1999. Infectious causes of abortion in swine. Reprod Domestic Anim. 34(3-4): 367-376.

Viboud, C., Grais, R.F., Lafont, B.A.P., Miller, M.A. and Simonsen, L. 2005. Multinational impact of the 1968 Hong Kong influenza pandemic: evidence for a smoldering pandemic. J Infect Dis. 192(2): 233-248.

Vincent, A.L., Ma, W., Lager, K.M., Janke, B.H., Richt, J.A., Karl Maramorosch, A.J.S. and Frederick, A.M. 2008. Swine influenza viruses: a North American perspective. Adv Virus Res. 72: 127-154.

Wang, H. and Nuttall, P.A. 2001. Intra-stadial tick-borne Thogoto virus (Orthomyxoviridae) transmission: accelerated arbovirus transmission triggered by host death. Parasitology. 122(4): 439-446.

Webster, R., Bean, W., Gorman, O., Chambers, T. and Kawaoka, Y. 1992. Evolution and ecology of influenza A viruses. Microbiol Rev. 56: 152 - 179.

Webster, R., Cox, N. and Stohi, K. 2002. WHO Manual on animal influenza diagnosis and surveillance: World Health Organization. 7-64 pp.

Webster, R.G., Bean, W.J., Gorman, O.T., Chambers, T.M. and Kawaoka, Y. 1992. Evolution and ecology of influenza A viruses. Microbiol Rev. 56(1): 152-179.

Wen, Y. and Klenk, H. 2013. "H7N9 avian influenza virus - search and re-search " [Online]. Available: http://www.nature.com/emi/journal/v2/n4/full/emi201318a.html.

Wise, H.M., Foeglein, A., Sun, J., Dalton, R.M., Patel, S., Howard, W., Anderson, E.C., Barclay, W.S. and Digard, P. 2009. A Complicated message: identification of a novel PB1-related protein translated from influenza A virus segment 2 mRNA. J Virol. 83(16): 8021-8031.

Wu, R., Hu, S., Xiao, Y., Li, Z., Shi, D. and Bi, D. 2007. Development of indirect enzyme-linked immunosorbent assay with nucleoprotein as antigen for detection and quantification of antibodies against avian influenza virus. Vet Res Commun. 31(5): 631-641.

Yan, Y., Orcutt, S.J. and Strickler, J.E. 2009. The use of SUMO as a fusion system for protein expression and purification. Chem Today. 27(6): 42-47.

Yazawa, S., Okada, M., Ono, M., Fujii, S., Okuda, Y., Shibata, I. and Kida, H. 2004. Experimental dual infection of pigs with an H1N1 swine influenza virus (A/Sw/Hok/2/81) and *Mycoplasma hyopneumoniae*. Vet Microbiol. 98(3-4): 221-228.

Yu, H. and Goodman, M.F. 1992. Comparison of HIV-1 and avian myeloblastosis virus reverse transcriptase fidelity on RNA and DNA templates. J Biol Chem. 267(15): 10888-10896.

Zhou, E.M., Chan, M., Heckert, R.A., Riva, J. and Cantin, M.F. 1998. Evaluation of a competitive ELISA for detection of antibodies against avian influenza virus nucleoprotein. Avian Dis. 42(3): 517-522.

Zhou, N.N., Senne, D.A., Landgraf, J.S., Swenson, S.L., Erickson, G., Rossow, K., Liu, L., Yoon, K.-j., Krauss, S. and Webster, R.G. 1999. Genetic reassortment of avian, swine, and human influenza A viruses in American pigs. J Virol. 73(10): 8851-8856.

APPENDICES

APPENDIX A

Genetic characteristic of bacteria and physical map of plasmid

Genotype of *E.coli* strain JM109

*rec*A1, *end*A1, *gyr*A96, *thi*, *hsd*R17 (rK–,mK+), *rel*A1, *sup*E44, Δ(*lac-pro*AB), [F′, *tra*D36, *pro*AB, *lac*IqZΔM15]

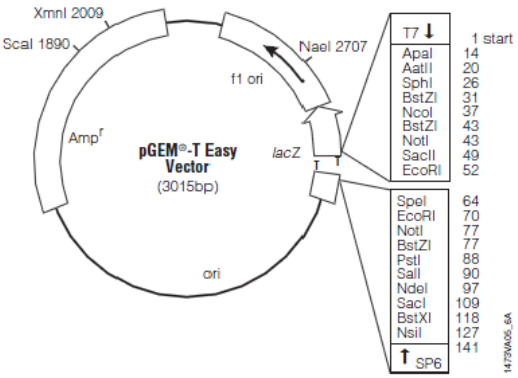
Genotype of *E.coli* strain TOP10

F-*mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) ϕ80*lac*Z ΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L *end*A1 *nup*G

Genotype of *E.coli* strain BL21

F-, ompT, hsdS (rB-, mB -), gal, dcm

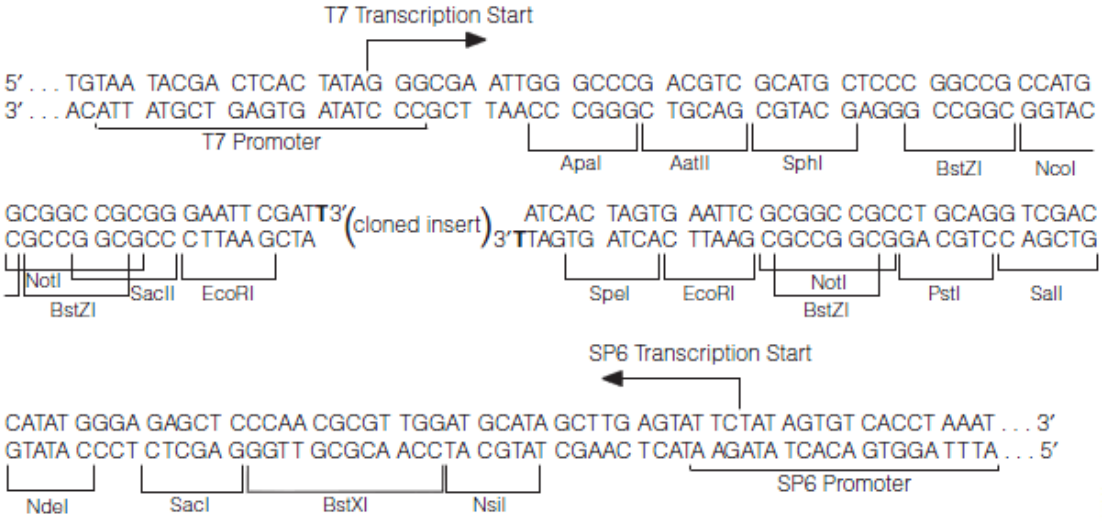
Physical mapping and sequence reference point of pGEM®-T easy vector (Promaga, WI, USA)



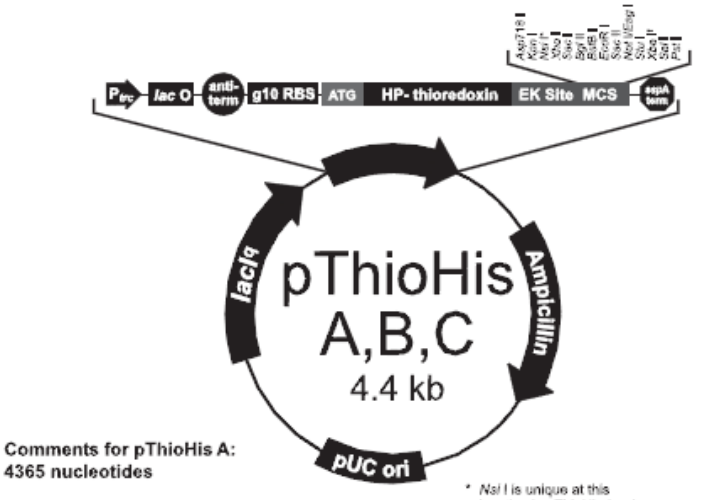
pGEM®-T Easy Vector sequence reference points:

T7 RNA polymerase transcription initiation site	1
multiple cloning region	10–128
SP6 RNA polymerase promoter (–17 to +3)	139–158
SP6 RNA polymerase transcription initiation site	141
pUC/M13 Reverse Sequencing Primer binding site	176–197
lacZ start codon	180
lac operator	200–216
β-lactamase coding region	1337–2197
phage f1 region	2380–2835
lac operon sequences	2836–2996, 166–395
pUC/M13 Forward Sequencing Primer binding site	2949–2972
T7 RNA polymerase promoter (–17 to +3)	2999–3

Multiple cloning site of pGEM®-T easy vector



Physical mapping and sequence reference point of pThioHisA vector (Invitrogen™, CA, USA)



Comments for pThioHis A:
4365 nucleotides

Ampicillin resistance ORF: bases 201-1061
pUC origin: bases 1206-1879
Lac Repressor (*lacI*^{q}) ORF: bases 1967-3049
Trc Promotor Region: bases 3261-3470
-35 region: bases 3281-3292
-10 region: bases 3304-3309
lac operator (*lac*O): bases 3316-3336
*rrn*B antitermination: bases 3352-3421
gene 10 region: bases 3434-3442
Ribosome binding site: bases 3457-3461
Thioredoxin ATG: bases 3471-3473
Trx forward priming site: bases 3777-3794
Enterokinase site: bases 3813-3827
Multiple cloning site: bases 3827-3892
*asp*A Termination: bases 3893-3959
Trx reverse priming site: bases 3967-3986

* *Nsi* I is unique at this location in pThioHis A only.
Nco I is unique at this location in pThioHis B only.
Sph I is unique at this location in pThioHis C only.

† There is a stop codon following the *Xba* I site in pThioHis C

Multiple cloning site of pThioHisA vector

```
3708  CTG CTG TTC AAA AAC GGT GAA GTG GCG GCA ACC AAA GTG GGT GCA CTG
      Leu Leu Phe Lys Asn Gly Glu Val Ala Ala Thr Lys Val Gly Ala Leu
                                  Trx forward sequencing priming site
3756  TCT AAA GGT CAG TTG AAA GAG TTC CTC GAC GCT AAC CTG GCC GGC TCT
      Ser Lys Gly Gln Leu Lys Glu Phe Leu Asp Ala Asn Leu Ala Gly Ser
                        Enterokinase recognition sequence  Asp718 I  Kpn I    Nsi I      Xho I Sac I Bgl II
3804  GGA TCC GGT GAT GAC GAT GAC AAG GTA CCT ATG CAT GAG CTC GAG ATC
      Gly Ser Gly Asp Asp Asp Asp Lys Val Pro Met His Glu Leu Glu Ile
                                      ▲ Enterokinase cleavage site
      BstB I EcoR I     Sac II Not I        Stu I  Xba I      Sal I              Pst I
3852  TTC GAA TTC CGC GGC CGC AGG CCT CTA GAG TCG ACC TGC AGT AAT CGT
      Phe Glu Phe Arg Gly Arg Arg Pro Leu Glu Ser Thr Cys Ser Asn Arg
                                  aspA transcriptional terminator
3900  ACA GGG TAG TACAAATA AAAAAGGCAC GTCAGATGAC GTGCCTTTTT TCTTGTGAGC
      Thr Gly ***
                        Trx reverse sequencing priming site
3957  AGTAAGCTTG GCACTGGCCGT CGTTTTACAA CGTCGTGACT GGGAAAA
```

APPENDIX B

Reagent preparations

Reagents for bacteria culture media

1. Luria-Bertani (LB) broth

Tryptone	10 g
Yeast extract	5 g
Sodium chloride (NaCl)	10 g
Add distilled water to	1000 ml

Adjust pH to 7.0 with sodium hydroxide (NaOH) and sterilize by autoclave before use.

2. Luria-Bertani (LB) broth with ampicillin

Prepare LB broth as above. Allow to cool down to 37 ºC before adding ampicillin to the final concentration of 100 µg/ml.

3. Luria-Bertani (LB) agar media

Tryptone	10 g
Yeast extract	5 g
Sodium chloride (NaCl)	10 g
Agar	15 G
Add distilled water to	1000 ml

Adjust pH to 7.0 with sodium hydroxide (NaOH), sterilize by autoclave. Pour 20-25 ml of medium into Petri-dishes and let agar set. Store at 4 ºC (should use within 1 month).

4. Luria-Bertani (LB) agar media with ampicillin

Tryptone	10 g
Yeast extract	5 g
Sodium chloride (NaCl)	10 g
Agar	15 g
Add distilled water to	1000 ml

Adjust pH to 7.0 with sodium hydroxide (NaOH), sterilized by autoclave and allow to cool down to 37 ºC before adding ampicillin to the final concentration of 100 µg/ml. Pour 20-25 ml of medium into Petri-dishes and let agar set. Store at 4 ºC (should use within 1 month).

5. Luria-Bertani (LB) agar media with ampicillin/IPTG/X-Gal

Prepare LB agar with ampicillin plate as above. Spread with 50 µl of 200 mM IPTG and 50 µl of 20 mg/ml X-Gal (Fermentas, MD, USA) over the surface of agar plate and let it absorb for 30 minutes at 37 ºC before use.

Reagents for SDS-PAGE

1. 3x Sample buffer

Pure β-mercaptoethanol	1.6 ml
1 M Tris-HCl pH 6.8	2.4 ml
Sterile pure glycerol	3 ml
20% SDS (w/v)	3 ml
Bromphenol blue	0.006 g

2. SDS-PAGE gel

2.1. Components of 10% separating gel (for 2 gel)

Distilled water	4.5 ml
40% Acrylamide	2.4 ml
1.5 M Tris-HCl, pH 8.8	2.5 ml
10% SDS (w/v)	100 µl
10% Ammonium persulphate (w/v)	60 µl
TEMED	40 µl

2.2. Component of 5% stacking gel (for 2 gel)

Distilled water	3 ml
40% Acrylamide	550 ml
0.5 M Tris-HCl, pH 6.8	1.25 ml
10% SDS (w/v)	50 µl
10% Ammonium persulphate (w/v)	40 µl
TEMED	20 µl

3. 10x Tris-glycine running buffer (500 ml)

Tris-HCl	15.15	g
Glycine	72	g
SDS	5	g
Add distilled water to	1000	ml

4. Coomassie brilliant blue staining (100 ml)

Distilled water	45	ml
Methanol	45	ml
Acetic acid	10	ml
Coomassie brilliant blue R 250	0.25	g

5. Destaining solution (400 ml)

Distilled water	180	ml
Methanol	180	ml
Acetic acid	40	ml

Reagent for immunoblot

1. Western blot transfer buffer

Tris-HCl	6	g
Glycine	28.8	g
SDS	2	g
Add distilled water to	1600	ml

Add 400 ml of Cold Methanol into prepared buffer before using to transfer protein

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