GENETIC DETERMINANTS OF TEMPERATURE SENSITIVITY OF H5N1 AVIAN INFLUENZA VIRUS

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ABSTRACT

The ability of H5N1 virus to grow at high temperatures is an important feature for viral pathogenesis in human. It has previously been reported that this ability is associated with the viral polymerase function. Mutations occurring in the viral genome, which are a result of the error prone nature of RNA polymerase, also play an important role in determining the viral phenotype and pathogenesis.

This study aimed to identify the genetic determinants of temperature sensitivity of H5N1 avian influenza virus. The temperature sensitive (*ts*) mutant virus (SP83ts20) was generated from the wild type A/Thailand/3(SP-83)/2004 virus (SP83wt) by serial adaptation at low temperature. After 20 passages, it was confirmed that the virus showed temperature sensitive phenotype, and did not grow at 40ºC. Making use of a reverse genetic system, ten reassortant viruses were constructed. Each virus contained one mutated gene from SP83ts20 or wild type gene from SP83wt in a backbone derived from mouse adapted influenza virus, A/Puerto Rico/8/34 (H1N1). These viruses were determined for their *ts* phenotype by kinetic growth curve at 33 and 40ºC. The NP gene derived from SP83ts20 exhibited temperature sensitive phenotype in mammalian cells, but not in avian cells. It was found that NP of *ts* mutant virus contained only one point mutation: serine to asparagine at position 314 (S314N).

The NP protein plays a role in many steps of the viral life cycle, including nuclear transport mechanism. To test whether NP S314N mutation affected the nuclear transporting system, an indirect immunofluorescence assay was conducted to detect the NP subcellular localization. NP possessing S314N mutation exhibited a defect in nuclear uptake when compared with wild type NP protein at high temperature. These results suggest that NP S314N in SP83ts20 virus was responsible for temperature sensitive phenotype, and the lack of ability to replicate at high temperature involved a defect in interaction with cellular factors for its importation into the nucleus of infected cells.

KEY WORDS: H5N1 VIRUS/ TEMPERATURE SENSITIVE/ NP PROTEIN/ REVERSE GENETICS/ NUCLEAR TRANSPORT

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ตัวกำหนดทางพันธุกรรมของเชื้อไวรัสไข้หวัดนก H5N1 ที่ตอบสนองต่อความไวต่ออุณหภูมิสูง
GENETIC DETERMINANTS OF TEMPERATURE SENSITIVITY OF H5N1 AVIAN INFLUENZA VIRUS

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บทคัดย่อ

ความชอบต่ออุณหภูมิสูงของเชื้อไวรัสไข้หวัดนกสายพันธุ์ H5N1 นั้น เป็นปัจจัยสำคัญต่อการก่อพยาธิสภาพในคน เนื่องจากคุณสมบัติของเอนไซม์ RNA polymerase ในการสังเคราะห์รหัสพันธุกรรมมีความผิดพลาดสูง ดังนั้นจึงมีการกลายพันธุ์เกิดขึ้นได้ง่าย ซึ่งมีบทบาทสำคัญในการกำหนดลักษณะของการแสดงออกของไวรัส

จุดประสงค์ในการศึกษาครั้งนี้ก็เพื่อหาตัวกำหนดทางพันธุกรรมที่มีผลต่อความไวต่ออุณหภูมิสูงของไวรัสไข้หวัดนก SP83ts20 ซึ่งเป็นไวรัสที่แสดงออกถึงความไวต่ออุณหภูมิสูง โดยถูกสร้างจากไวรัสไข้หวัดนก H5N1 สายพันธุ์ A/Thailand/3 (SP-83)/2004 virus (SP83wt) ซึ่งเลี้ยงที่อุณหภูมิต่ำเป็นเวลา 20 passages จนได้ไวรัส SP83ts20 ที่ไม่สามารถเจริญเติบโตได้ที่อุณหภูมิ 40ºC งานวิจัยนี้สามารถสร้างไวรัสลูกผสมได้ 10 แบบโดยวิธี reverse genetics ภายในตัวไวรัสนั้นประกอบด้วย หนึ่งยีนจากตัวไวรัส SP83ts20 ที่พบว่ามีการกลายพันธ์เกิดขึ้นในลำดับเบส หรือยีนเดียวกันจากไวรัส SP83wt และยีนที่เหลือมาจากไข้หวัดใหญ่สายพันธุ์ A/Puerto Rico/8/34 (H1N1) ไวรัสที่ได้จะถูกนำไปทดสอบดูว่ายีนใดที่เป็นตัวกำหนดลักษณะความไวต่ออุณหภูมิ ซึ่งศึกษาโดยดูการเจริญเติบโตของไวรัสแต่ละตัวที่อุณหภูมิ 33 และ 40ºC ผลการศึกษาพบว่า ยีน NP ของไวรัส SP83ts20 เป็นตัวที่แสดงออกของลักษณะดังกล่าวเฉพาะในเซลล์จากสัตว์เลี้ยงลูกด้วยนม แต่ไม่พบการแสดงออกนี้ในเซลล์จากสัตว์ปีก นอกจากนี้ตำแหน่งของการกลายพันธุ์ที่เกิดขึ้นนั้นมีเพียงตำแหน่งเดียวคือที่ 314 โดยเปลี่ยนจาก serine (S) ไปเป็น asparagines (N)

โปรตีน NP นั้นมีความเกี่ยวข้องในหลายขั้นตอนของวงจรชีวิตของไวรัสซึ่งรวมถึง กลไกในการขนส่ง vRNPs เข้านิวเคลียสด้วย เพื่อเป็นการทดสอบดูผลจากกลายพันธุ์ที่ตำแหน่ง S314N ว่ามีผลต่อกลไกดังกล่าวหรือไม่ ผู้ทำการวิจัยจึงใช้วิธี indirect immunofluorescence เพื่อหาตำแหน่งที่อยู่ภายในเซลล์ของโปรตีน NP ผลปรากฏว่า เมื่อทดสอบที่อุณหภูมิสูง โปรตีน NP ที่มีการกลายพันธุ์ของตำแหน่ง 314 นั้น ทำให้กลไกในการเข้าสู่นิวเคลียสมีความบกพร่องเมื่อเปรียบเทียบกับโปรตีนที่ไม่มีการกลายพันธุ์ และจากผลการศึกษาเหล่านี้แสดงให้เห็นถึงความสำคัญของ NP S314N ต่อการเป็นตัวกำหนดลักษณะความไวต่ออุณหภูมิสูง และการที่ไวรัสไม่สามารถเจริญเติบโตได้ในที่มีอุณหภูมิสูงเกี่ยวข้องกับความบกพร่องต่อการกระทำระหว่างโปรตีน NP กับโปรตีนภายในเซลล์ที่ใช้ในกระบวนการนำโปรตีนเข้าสู่นิวเคลียส

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LIST OF ABBREVIATIONS

Abbreviation	Term
ºC	Degree Celcius
bp	Base pair
BSA	Bovine serum albumin
BSL-3	Biosafety Level 3
ca	Cold-adapted
cDNA	Complementary Deoxyribonucleic acid
CEF	Chicken embryonic fibroblast
CNS	Central nervous system
Crm1	Chromosome maintenance region 1
DNA	Deoxyribonucleic acid
DMEM	Dulbecco's Modified Eagle Media
dNTPs	Deoxyribonucleotide triphosphate
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmic reticulum
FITC	Fluorescein isothiocyanate
Gal	Galactose
HA	Hemagglutinin
HEK	Human embryonic kidney
IFN	Interferon
IgG	Immunoglobulin G
IL-6	Interlukin-6
IPTG	Isopropyl-β-D-galactoside
Kb	Kilobase
LB	Lauria Bertani

LIST OF ABBREVIATIONS (cont.)

Abbreviation	Term
M	Matrix
MDCK	Mardin-Darby canine kidney
MDV	Master donor virus
MEM	Minimum essential media
min	minute
ml	Milliliter
mM	Millimolar
mRNA	Messenger ribonucleic acid
NA	Neuraminidase
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NCR	Noncoding region
NLS	Nuclear localization signal
NP	Nucleoprotein
NPC	Nuclear pore complex
NS	Nonstructural protein
OE-PCR	Overlap extension polymerase chain reaction
PA	Polymerase acidic
PB	Polymerase basic
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PFU	Plaque-forming units
RBC	Red blood cell
RBS	Receptor binding site
RNA	Ribonucleic acid
RNP	Ribonucleoprotein

LIST OF ABBREVIATIONS (cont.)

Abbreviation	Term
rpm	Round per minute
RT-PCR	Reverse transcriptase polymerase chain reaction
SA	Sialic acid
SAα2,3Gal	Sialic acid linked to galactose by α2,3 linkages
SAα2,6Gal	Sialic acid linked to galactose by α2,6 linkages
ssRNA	Single stranded RNA
TCID50	50% tissue culture infectious dose
TNF-α	Tumor necrosis factor alpha
ts	Temperature sensitive
U	Unit
WHO	World Health Organization
X-Gal	5-Bromo-4-chloro-3-indolyl- β-D-galactoside
µg	Microgram
µ1	Microliter
A/ Ala	Alanine
D/Asp	Aspartic acid
E/ Glu	Glutamic acic
H/ His	Histidine
K/ Lys	Lysine
Q/ Gln	Glutamine
R/ Arg	Arginine
S/ Ser	Serine
Y/ Tyr	Tyrosine

CHAPTER I
INTRODUCTION

Highly pathogenic avian influenza (HPAI) H5N1 virus is member of Influenza A viruses which are classified in the family *Orthomyxoviridae*. H5N1 virus is enveloped virus carrying negative sense single stranded RNA genome, which comprises eight segments (PB2, PB1, PA, HA, NP, NA, M and NS) encoding 11 proteins [1-3]. Normally, H5N1 and all known subtypes of influenza A viruses circulate in their natural reservoir host, aquatic birds, and host range barrier can prevent some interspecies transmission [1, 2]. However, emergences of H5N1 broke the host range barrier to infect and cause severe respiratory disease with high fatality in several mammalian species, including humans [2]. In 1997, the first outbreak of human infection with H5N1 virus was documented in Hong Kong. The outbreak caused lethal infection 6 of 18 confirmed cases [4, 5]. In Southeast Asia in late 2003, occurrence of human cases has paralleled with a wide spread of H5N1 viruses in poultry. The epidemic has moved to many countries in Africa and Europe [6]. Currently, highly pathogenic H5N1 avian influenza virus is a worldwide concern because of its ability to infect humans with over 60% mortality. The total cumulative numbers of confirmed cases up to April 2010 are more than 400 with more than 250 deaths [7].

There are many factors involved in the complex process of viral replication and survival in its hosts. One factor that can differentiate ability in human infection between human and avian influenza viruses is growth temperature. Human influenza A viruses have a natural temperature sensitive (*ts*) phenotype, which shows restricted growth at non-permissive temperature. Therefore, viruses are able to replicate efficiently in the upper respiratory tract of human at a temperature of about 33ºC, while avian influenza A viruses have an ability to replicate efficiently at the body temperature of bird, which is close to 41ºC [8, 9].

Occasional transmission across the species barrier from avian to human can have a huge impact. The ability of H5N1 to grow at high temperature plays a crucial role to threaten human life. It acts as important factor for the viral pathogenesis and high fatality. Because high temperature is optimal for H5N1 replication, it can replicate well in the human lower respiratory tract. Previous studies performed on victims of H5N1 infection have shown that these viruses cause primary viral pneumonia that can lead to acute respiratory distress syndrome (ARDS) and multi-organ failure [6, 10-12]. Several viral components, such as ssRNA, dsRNA and virosomes, activate the host defense mechanism, resulting in production of many cytokines during viral infection. Many important cytokines, such as IL-1 α/β, TNF α/β, Il-6, serve as endogenous pyrogens and are involved in the pathogenesis of fever [13]. However, the mechanism of fever that increases body temperature to suppress viral replication is not effective for inhibiting the replication of H5N1 virus. This agrees with the study of de Jong et al., 2006, which show that fatal outcome of avian influenza H5N1 infection was associated with high level of viral replication and hypercytokinemia [11].

It has previously been reported that the ability of H5N1 to grow at high temperature is associated with the viral polymerase function. Mutations occurring in the viral genome, which is a result of the error prone nature of RNA polymerase, also play an important role in determining the viral phenotype and pathogenesis. This thesis had the aim to identify genetic determinants of temperature sensitivity of H5N1 avian influenza virus. A variant of H5N1 virus strain A/Thailand/3(SP-83)/2004; abbreviated hereafter as SP83wt, which exhibited the temperature sensitive (*ts*) phenotype, was studied in this thesis. The *ts* mutant virus was kindly provided by Dr. Ornpreya Suptawiwat and generated by a temperature shift experiment. Briefly, SP83wt virus was serially cultured in MDCK cells at 30ºC for 5 passages, then shifted to 27ºC for 9 passages and finally 25ºC for 6 passages. After 20 passages the virus is called SP83ts20. The phenotype of mutant virus was compared with wild type virus by evaluating on kinetic growth curve. Kinetic growth curve was done at 37 and 40ºC, and viral output was determined by using hemagglutination (HA) and plaque assays as shown in Figure 1.1. It demonstrated that this virus did not grow at 40ºC. Therefore,

aim of this thesis was to identify the genetic determinant responsible for temperature sensitive phenotype of H5N1 avian influenza virus.

A *ts* mutant usually has low virulence. The knowledge of *ts* determinant may apply in production of live attenuated vaccine. Live-attenuated vaccine is an effective tool for prevention of influenza pandemic. The *ts* phenotype is a prerequisite for successful and safe live-attenuated vaccine. This phenotype restricts the replication of vaccine virus to the cooler region of the upper respiratory tract and, therefore, contributes to the overall attenuation and safety of vaccine [14].

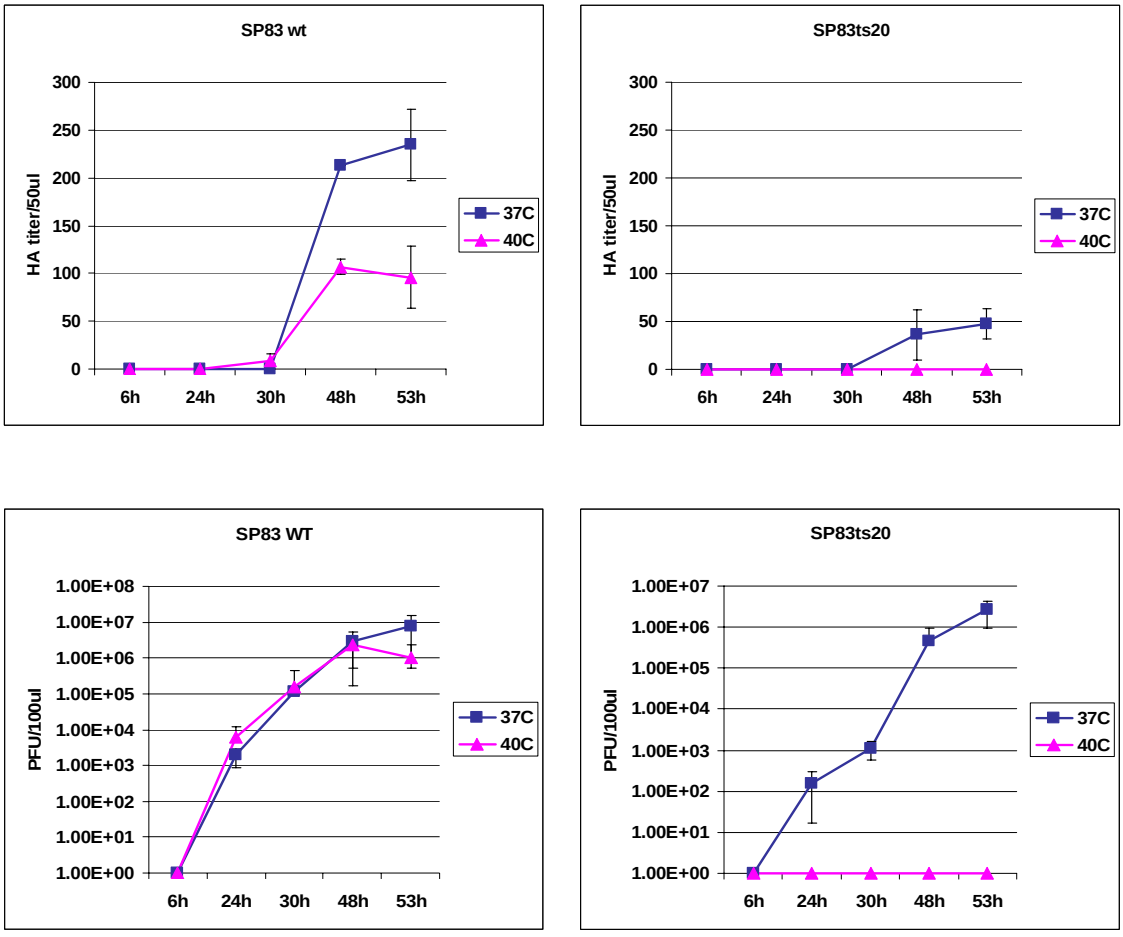

Figure 1.1 Comparison of kinetic growth curve between SP83wt and SP83ts20 at 37 and 40ºC. Viral titer at each time point was determined by hemagglutination and plaque assay. The values were means of three independent determinations.

CHAPTER II
OBJECTIVES

1. To generate reassortant viruses carrying a single mutated gene from SP83ts20 or wild type gene from SP83wt in the backbone of a mouse adapted influenza virus, A/Puerto Rico/8/34 (H1N1), and determine for their phenotypes using kinetic growth curve assay
2. To identify the genetic determinant of temperature sensitive (*ts*) phenotype in SP83ts20
3. To study the mechanism of temperature sensitive phenotype of SP83ts20

CHAPTER III
LITERATURE REVIEWS

The highly pathogenic avian influenza A H5N1 virus in Asia is a threat to domestic poultry, human live and public health because of the potential for pandemic spread of this virus [2, 15]. H5N1 virus was first documented in diseased geese in Guangdong Province, People's Republic of China in 1996. The first incidence of direct interspecies transmission from avian to human was reported in Hong Kong in 1997. The outbreak caused lethal infection in 6 of 18 confirmed cases, and 1.5 million of all poultry in farms and markets of Hong Kong were slaughtered in order to abort this outbreak. However, the re-emergence of H5N1 virus in Southeast Asia in 2003 and the expansion into three continents by migratory birds and shipment of poultry have led to huge economic losses and high fatality in human [6, 16]. Nowadays, H5N1 is still circulating in wild birds and transmitting to other bird species, particularly domestic poultry, and human population. Cumulative confirmed cases of human infection with H5N1 virus have reached more than 400 infected cases and over 250 deaths [7].

3.1 Classification and biology of highly pathogenic avian influenza virus H5N1

Avian influenza H5N1 virus belongs to one subtype of type A influenza virus of the family *Orthomyxoviridae*. The classification of subtypes is based on major surface proteins of virus, Hemagglutinin (HA) and Neuraminidase (NA). The morphology of virus particle can exhibit a variety of shapes and sizes, such as spherical or elongated filamentous form with diameter of about 100 nm or more than 300 nm, respectively (Figure 3.1). The complex virion structure of type A influenza viruses is enveloped virus. The viral envelope is derived from the host plasma membrane. It contains both cholesterol-enriched lipid raft and non raft lipid [17]. On

the viral surface are HA, NA and the M2 proteins, and matrix protein (M1) is underlying the envelope surface. The core of viral particle is consisted of the viral ribonucleoprotein (vRNP) containing the viral polymerase gene complex: the polymerase basic 1 (PB1), polymerase basic 2 (PB2), and polymerase acid (PA) and the nucleoprotein (NP), which is tight associated with each viral RNA (vRNA) segment (Figure 3.2). The viral genome of influenza A virus comprises eight segmented negative sense single stranded RNA genome (HA, NA, M, NP, PA, PB1, PB2, and NS) encoding 11 proteins (Table 3.1 and Figure 3.3) [1, 18, 19]. An additional, structural NEP/NS2 (nuclear export protein/nonstructural protein 2) is present in small amount in the viral particle.

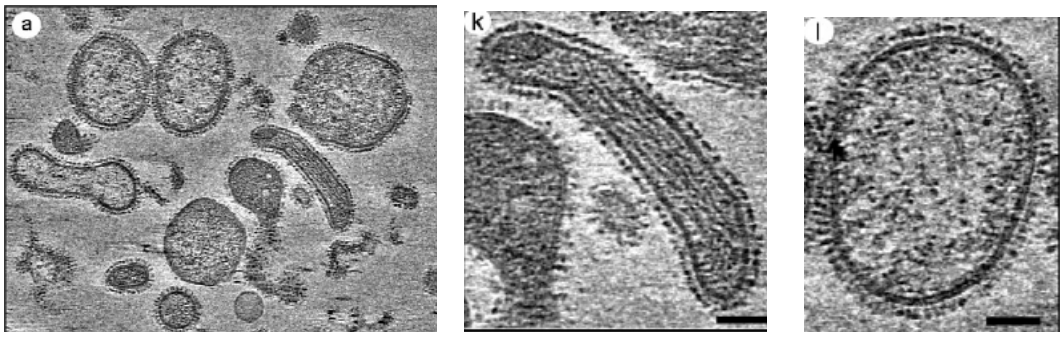

Figure 3.1 Electron micrograph of a negatively stained influenza virus particle with HA and NA spikes visible on the surface. Scale bar 50 nm [20].

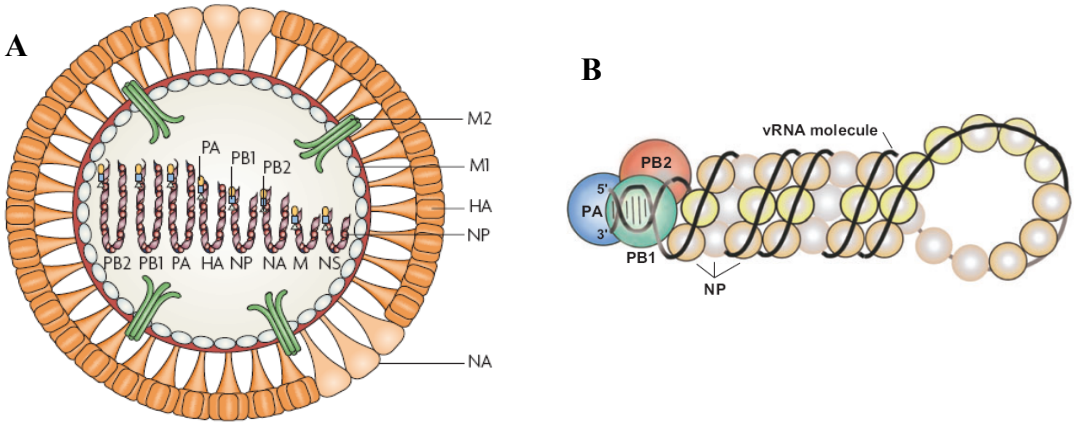

Figure 3.2 Schematic of influenza virus A virus and structural organization of influenza virus ribonucleoproteins (RNPs). (A) The influenza A virus particle has three major surface proteins consisted of HA, NA and M2 protein are embedded in the lipid bilayer of the viral envelope [2]. (B) Schematic model of RNP organization. The viral RNA molecule (*black line*) is coiled into a hairpin structure tight associated with nucleoprotein (NP) (*yellow circles*). At the 5' and 3'end of partially complementary vRNA constitutes the binging site for bound to the heterotrimeric polymerase complex (PB1,PB2,PA)[1].

Table 3.1 Influenza virus genes and proteins [21].

Table 1. Influenza virus genes and proteins

Seg	Protein	Localisation & features	Function
1	PB2, 96K	Virion interior, infected cell nuclei	Heterotrimeric P complex associated with NP & virion RNA, role in transcription & replication of viral RNA
2	PB1, 87K	Virion interior, infected cell nuclei	
3	PA, 85.5K	Virion interior, infected cell nuclei	
4	Haemagglutinin (HA), 220K homotrimer	Virion envelope, infected cell surface Globular head bears antigenic sites & receptor binding site; stem; transmembrane span; cytoplasmic tail	Virus binding to sialic acid-containing receptors on host cell; penetration of virus genome into host cell cytoplasm by fusion of virus & host cell membranes; major antigenic determinant
5	Nucleoprotein (NP), 55K	Virion interior, associated with P complex & viral RNA	Interacts with RNAs & polymerase proteins as major component of nucleocapsid; role in vRNA replication; role in virus maturation & packaging.
6	Neuraminidase (NA), 240K homotetramer	Virion envelope, infected cell surface Cytoplasmic tail; transmembrane span; extracellular stalk; globular head bears antigenic sites & enzyme active site	Sialidase enzyme catalysing cleavage of terminal sialic acid residues from glycoconjugates thereby: digesting mucin to enable virus to reach target epithelium & facilitating release of infectious progeny virus; antigenic molecule
7	Matrix protein (M1), 28K	Beneath lipid bilayer of virion envelope; associates with vRNPs in mature virion to form nucleocapsid	Central role in replication & virus assembly, modulating nuclear transport of viral RNP Early infection: bound to viral RNP prior to RNP transport to nucleus Late infection: binds RNP, signaling RNP-transport from nucleus to cell surface Mediates association of RNP with HA & NA at cell membrane, promoting virion formation and budding
	M2, 15K homotetramer	Virion envelope, infected cell surface (abundant) Extracellular region; transmembrane span; cytoplasmic tail; tetramers form cation-selective channel	Membrane cation channel activity Infected cell: specifically raises pH of golgi to protect pH-sensitive HA Virion: may permit acidification of virus interior during passage through endosomal pathway, to dissociate viral RNP from M1
8	Non-structural protein 1 (NS1), 25K dimer	Infected cell nuclei	Binds RNA thereby: inhibiting host mRNA translation, regulating viral pre-mRNA splicing and translation & viral polymerase activity; down-regulating dsRNA-induced anti-viral responses
	Non-structural protein 2 (NS2), 14K	Associated with core components of virion; cytoplasm of infected cells	Mediates nuclear export of viral RNPs

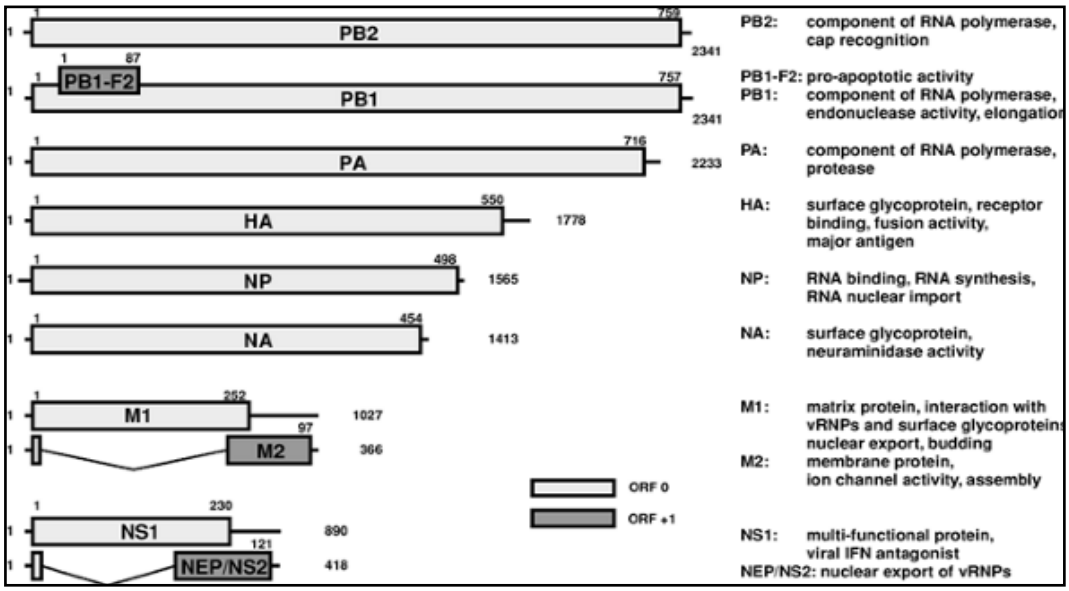

Figure 3.3 Genome structure of influenza A virus. Viral RNA segments encode 11 proteins. The 5' and 3' end represent the noncoding regions. The PB1 gene contains second ORF in the +1 frame for encoding PB1-F2 protein. M and NS mRNA were spliced into M2 and NS2 proteins [22].

The natural reservoir host of highly pathogenic H5N1 virus and all known subtypes of influenza A virus are wild aquatic birds. The infection of avian influenza H5N1 in natural host does not usually cause illness [1, 2]. Moreover, the expansion of the host range can be found in this virus by disrupting species barrier to cross transmission to other animal species, particularly domestic poultry, and humans (Figure 3.4). The interspecies crossing of H5N1 virus can cause severe disease and high fatality in animals and humans. Although, the transmission of avian H5N1 virus to human may be restricted because of the species barrier including receptor specificity and optimal temperature for viral polymerase enzyme function, the increasing incidence of worldwide spread of H5N1 infection in humans is a serious pandemic threat. Because the property of viral RNA polymerase has error-prone nature, it can promote viral adaptation and fitness in new hosts by positive selection. In the previous study, the victim of H5N1 virus had high viral load, suggesting a high efficiency of viral replication in human [16].

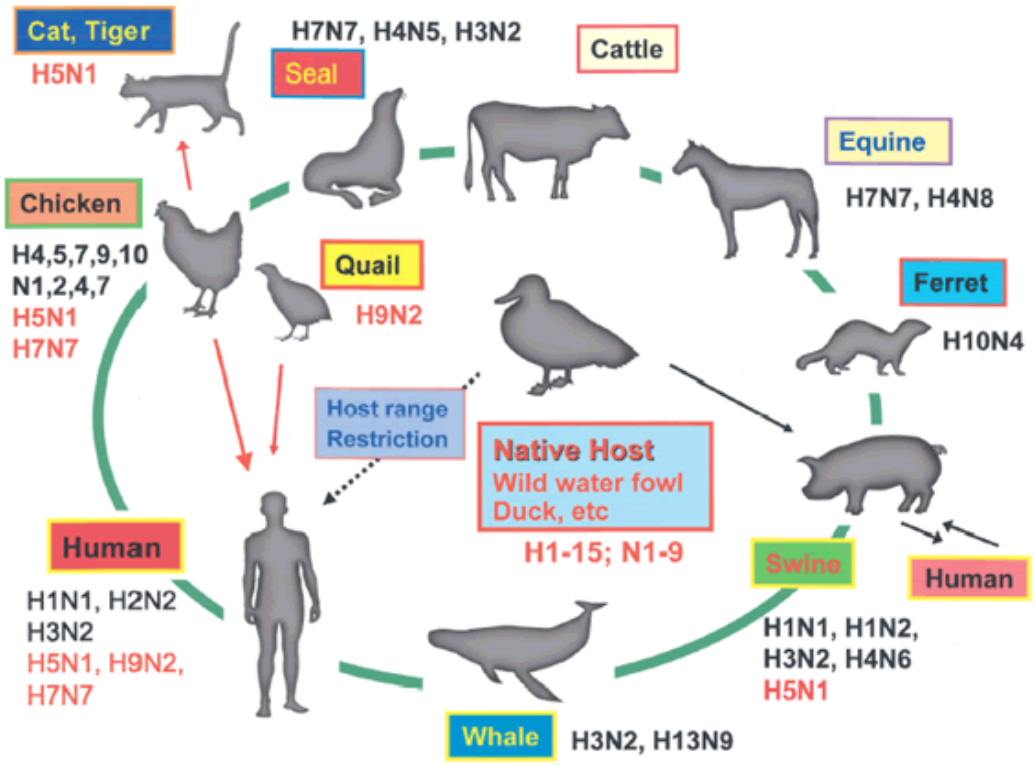

Figure 3.4 The ecology of influenza A viruses and crossing species transmission. (Available from URL: http:// www.medicalecology.org/ diseases/influenza/ print_ influenza. html)

3.2 The replication cycle of virus

Both processes of replication of the viral genome and transcription of the viral mRNA occur in the nucleus of infected cells, which can be divided into main five steps, as illustrated in Figure 3.9.

3.2.1 Viral entry into the host cell

The process of viral replication is carried out by initiating with a major surface protein, HA, for binding with specific sialic acid (SA) receptor on the host cell's surface. HA of virus is in precursor form (HA0) containing the cleavage site, which is recognized by cellular protease. Thus, HA0 will be cleaved into two subunits of active form: HA1, which contains the receptor-binding site (RBS), and HA2, which contains the fusion peptide. After the binding of HA to host receptor, the process of receptor-mediated endocytosis occurs, and consequently initializes the virus entry into infected cells in an endosome [6, 17, 18, 23, 24]. The fusion of viral membrane and an endosomal membrane is triggered by low pH of around 5 to 6 resulting in conformational change of HA2 fusion peptide. The fusion of two membranes is carried out by inserting of fusion peptide into endosomal membrane. Then, protons in the acidic endosome are pumped into the inside of virion through proton-selective ion channel, M2 protein, and viral RNP also dissociates from M1, resulting in the releasing of viral RNPs into the cytoplasm. The vRNPs are imported into the nucleus in an ATP-dependent manner [17, 24, 25].

3.2.2 Entry of viral RNPs into the nucleus

The free vRNPs must enter the nucleus for transcription and replication by using cellular nuclear import mechanism. Although known nuclear localization signals (NLSs) on viral RNP protein (PB2, PB1, PA, and NP) can interact with cellular import protein, the NLS of NP is shown to be sufficient to mediate the nuclear import [26, 27].

The vRNPs crossing of nuclear membrane by NP is a key event for influenza virus life cycle. Influenza A virus RNA segment 5 encodes NP (a polypeptide of 498 amino acids in length), which is a 56 kDa and rich in arginine, glycine and serine residues. The NP structure contains several functional domains (Figure 3.5) that are able to interact with a variety of macromolecules of both viral and

cellular origins as summarized in Table 3.2 .The presence of three NLSs on the NP of influenza A viruses as shown in Figure 3.6. An unconventional NLS (M_1ASQGTKRSYEQM_{13}) is at the very N-terminus. The second NLS residue (K_{198}RGINDRNFWRGFNGRRTR$_{216}$) in the central part of NP is the bipartite signal appears to be weaker than the unconventional NLS [76]. The third NLS proposed to be located between amino acid 320 and 400 [77, 78]. The Crm1 dependent pathway has been well characterized for importing protein into the nucleus by binding to karyopherins, which are involved in nuclear import, for example, importin α and β as shown in Figure 3.7 [17].

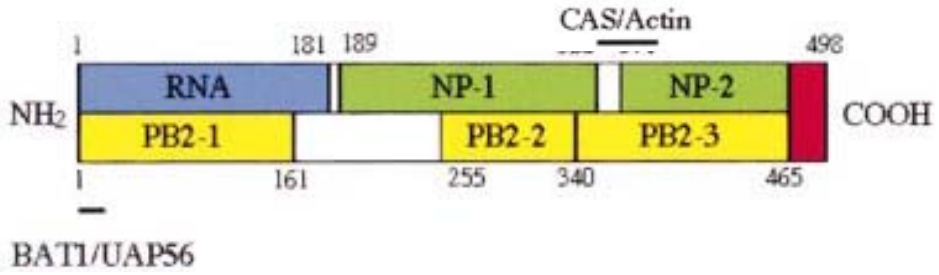

Figure 3.5 Functional domains of NP. Sub-fragments of NP identified as capable of binding RNA (blue), NP (green) or PB2 (yellow) are indicated on a linear representation of the NP molecule. Numbers refer to the amino acid co-ordinates. Black bars indicate regions shown to be important for binding the cellular polypeptide origin [73].

Table 3.2 Macromolecules bound by influenza virus nucleoprotein [73]

Macromolecule	Function	Reference
Viral		
ssRNA	RNP structure	Pons *et al.* (1969) Baudin *et al.* (1994)
NP	RNP structure	Pons *et al.* (1969) Ruigrok & Baudin (1995) Elton *et al.* (1999a)
PB1	Transcription?	Biswas *et al.* (1998) Medcalf *et al.* (1999)
PB2	Transcription?	Biswas *et al.* (1998) Medcalf *et al.* (1999)
M1	Transcription inhibition Nuclear export Assembly?	Zvonarjev & Ghendon (1980) Martin & Helenius (1991)
Cellular		
Importin α	Nuclear import	O'Neill & Palese (1995) O'Neill *et al.* (1995)
F-actin	Cytoplasmic retention?	Digard *et al.* (1999)
CRM1/exportin-1	Nuclear export?	Elton *et al.* (2001)
BAT1/UAP56	Transcription?	Momose *et al.* (2001)

Figure 3.6 Schematic representation the three nuclear localization signals (NLSs) in red hatch on the NP genome [26].

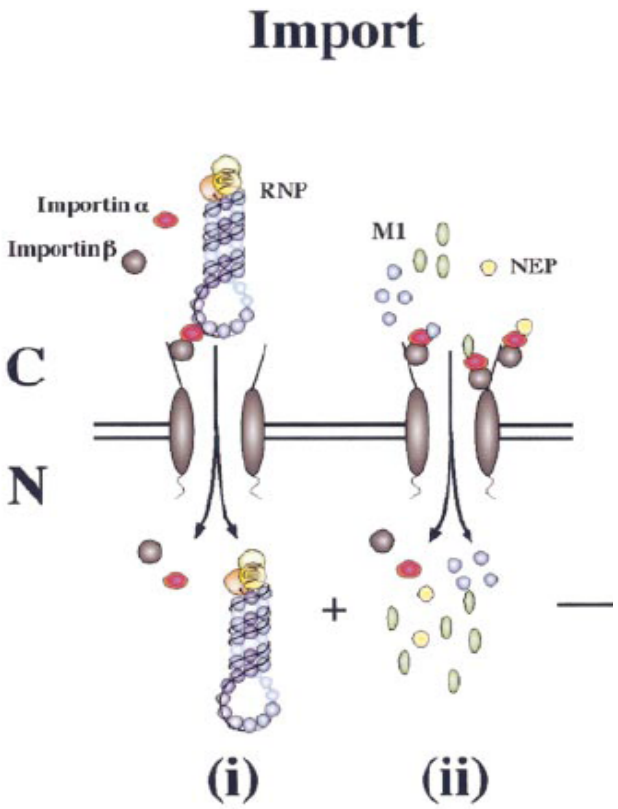

Figure 3.7 Schematic representation of nuclear import mechanism. (i) RNP and (ii) monomeric NP, M1 and NEP polypeptides are imported from cytoplasm (C) to the nucleus (N) through NLS-mediated interaction with importin α1 followed by importin β docking with the nuclear pore complex (gray ovals) [73].

3.2.3 Transcription and replication of the viral genome

The structure of viral mRNA containing both of 5'methylated cap and a poly (A) tail at 3'end differs from viral genome. The initiation of viral mRNA synthesis requires primers from cellular mRNA by using "cap-snatching" mechanism. The PB2 protein is used to bind with capped cellular premessenger RNA, a m^7GpppXm cap, which is cleaved by endonuclease activity of PB1 [1]. The negative sense strand of RNA acts as a template for primer binding and extension, which start from a G residue complementary to the C residue at position 2 of the vRNA 3'end [28]. Termination of mRNA synthesis occurs through the polyadenylation process. The polyadenylation takes place at viral polyadenylation signal, which is at five to seven U residues approximately 17 nucleotides from the 5'end of vRNA [1, 28]. The viral

mRNA is translated into viral protein using the host's translation machinery. During this process, M2 and NEP (NS2) are generated by splicing mRNA.

In addition, the synthesis of viral genome does not require primer, resulting in the generation of co-linear complementary copies of the vRNAs, or cRNAs (Figure 3.8). The cRNAs may serve as a template for new rounds of transcription and replication, and newly synthesized vRNPs may exist the nucleus for assembly process [1].

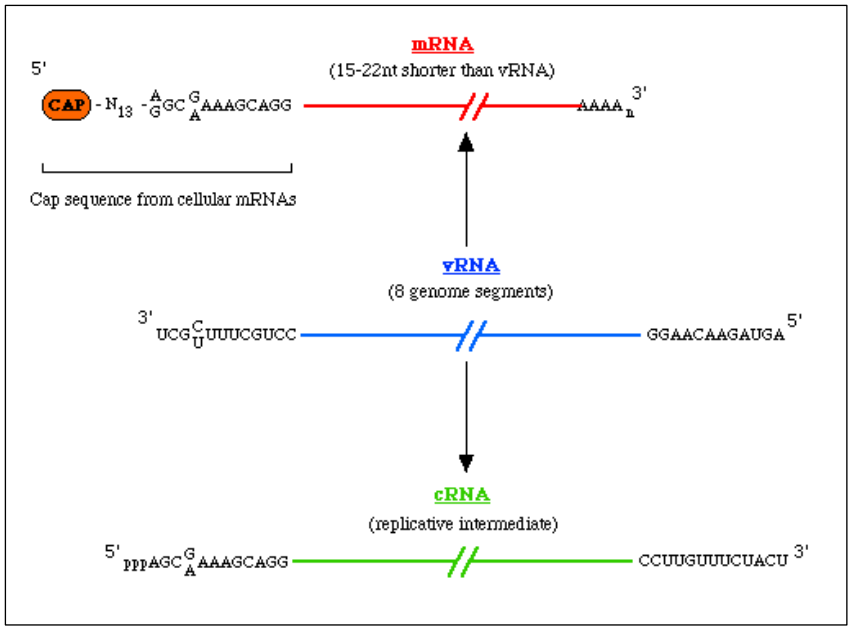

Figure 3.8 Influenza vRNA synthesis. (Top) Schematic of mRNA showing cap and 10 to 13 nucleotides derived from cellular mRNAs, and poly(A) stretch. (Middle) Schematic of (-) strand genomic RNA showing conserved 3'- and 5'-terminal nucleotide sequences. (Bottom) Schematic of full-length (+) strand RNA made by unprimed end-to-end copying of (-) strand genomic RNA.
(Available from URL: http://www.microbiologybytes.com/virology/3035pics/ Orthomyxo2 .gif)

3.2.4 Export of vRNPs from the nucleus

During vRNA replication, vRNPs are tightly bound with the chromatin and nuclear matrix. The dissociation of vRNPs occurs through the expression of late protein encoding the M1, which is proposes that promote the vRNP formation [26]. The C-terminal of M1 binds with NS2/NEP, and it interacts with Crm1 (Chromosome maintenance region 1), leading to the complex of RNP-M1-NEP/NS2. This complex is responsible for export its from the nucleus to the cytoplasm via the Crm1 dependent manner. Another viral protein, NP, which is proposed that can bind to Crm1 that suggest to play a role in both import and export vRNPs [22, 26].

3.2.5 Assembly and budding at the host cell's plasma membrane

The next step is viral assembly and budding from the host cell via the apical surface. Three integral membrane proteins, HA, NA, and M2 synthesize on membrane-bound ribosome, enter to the endoplasmic reticulum (ER) and transport to the Golgi apparatus. The apical sorting signals, which reside in transmembrane protein for HA and NA, but M2 has not been characterized, promote the viral assembly at the apical plasma membrane [22]. For the packaging of viral genomic segments into virion, there are two models for explanation: the random incorporation model and the selective incorporation model. The first model proposes that viral genomics are randomly packaged, while the second model assumes that each vRNA segment contains an unique packaging signal, which has been identified in the 5' and 3'end, non-coding or coding region [17, 22]. For the viral budding, the important protein that involves in this step is M1, which stimulates the bud formation by accumulation itself at the inner leaflet of the lipid bilayer [17]. M1 contains a late domain, which has been identified in the YRKL motif at position 100 to 103. This domain is responsible for recruiting host factors (VPS28 and Cdc42), which require for the late stages of viral budding [22].The budding process carries out when the membrane fuse at the base of the bud and the NA, which is function as sialidase enzyme, will cleave the sialic acid residue from host receptors to release new virions.

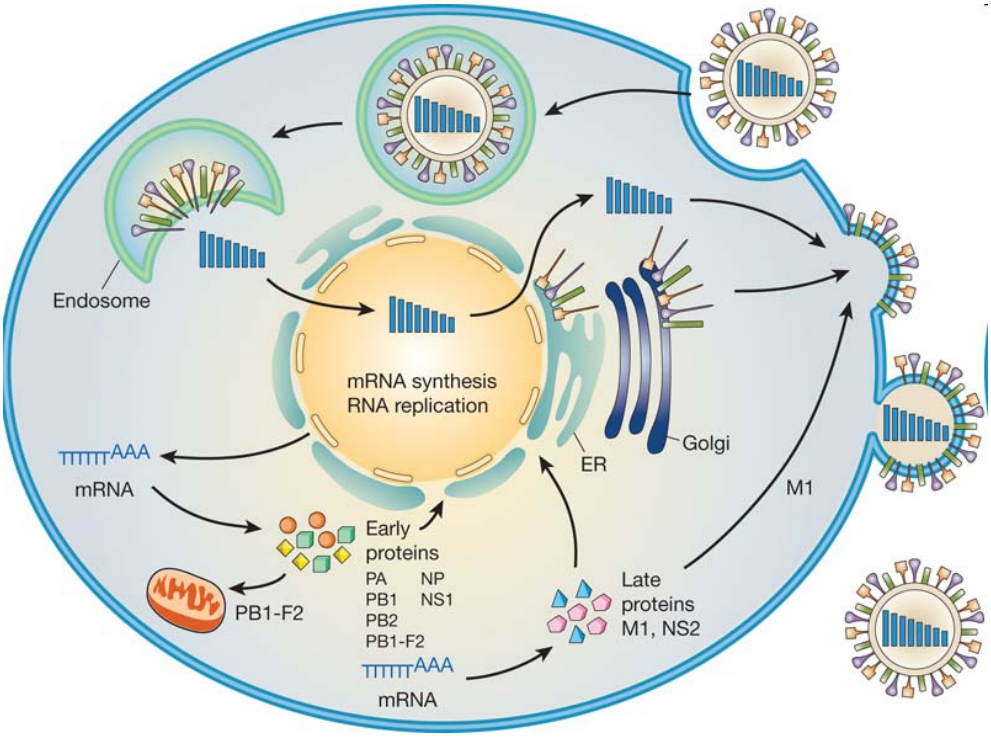

Figure 3.9 Illustration of the influenza virus replication cycle [29].

3.3 The history of crossing species from avian to human of H5N1 virus

In 1997 Hong Kong, the first evidence of H5N1 virus, which broke the host range barrier to infect in human, was found in a 3 years old child [5, 10]. The increasing incidence of human infection was documented with 18 cases and six deaths [16]. Although, a million of domestic poultry were slaughtered to abort this outbreak, the re-emergence of H5N1 was found again in 2003 in Southeast Asia. In Hong Kong in February, a father and son, who had just come back from Fujian Province, People's Republic of China, were infected by a genotype Z virus [30], and in November, another case of H5N1 was found in Beijing [31]. Subsequently, the spreading alarm of H5N1 virus was expanded into another human population in Vietnam, Thailand, Cambodia, Indonesia etc., and it had been moved to circulate in Africa and Europe due to the bird migration. To date, WHO reports the cumulative number of confirmed human cases of H5N1 as shown in Table 3.3.

3.3.1 Host range barrier

Since H5N1 has seemingly overcome species barrier, but the effectiveness in human to human transmission is still low. This phenomenon reflects insufficient adaptation of virus in human host. From the previous study, highly pathogenic avian influenza virus H5N1 has low efficiency to infect among human population or nonhuman primates [32, 33], and also human virus has low efficiency to replicate in ducks [34]. Host range barriers between avian and human virus comprise host receptor specificity and the ability of viral polymerase function at different temperature. The molecule of HA and NA are considered as the possible genetic determinant of host specificity, tissue tropism and virulence [21]. Human and avian influenza viruses differ in ability to recognize host receptor. There are two major linkages of sialic acid receptor, which are found in human respiratory tract for viral entry: SA α 2,3 Gal and SA α 2,6 Gal (Figure 3.10). HA of Human virus preferentially binds to SA α 2,6 Gal receptor, whereas most avian viruses preferentially bind to SA α 2,3 Gal receptor [6, 35]. In the human airway, SA α 2,6 Gal receptor is highly expressed on epithelial cells in the human nasal mucosa, paranasal sinuses, the pharynx trachea and bronchi, while SA α 2,3 Gal receptor is hardly detected in these cells, but it predominates in the non-ciliated cuboidal bronchiolar cells at the junction between the respiratory bronchiole, alveolus and alveolar type II cells [6, 36].

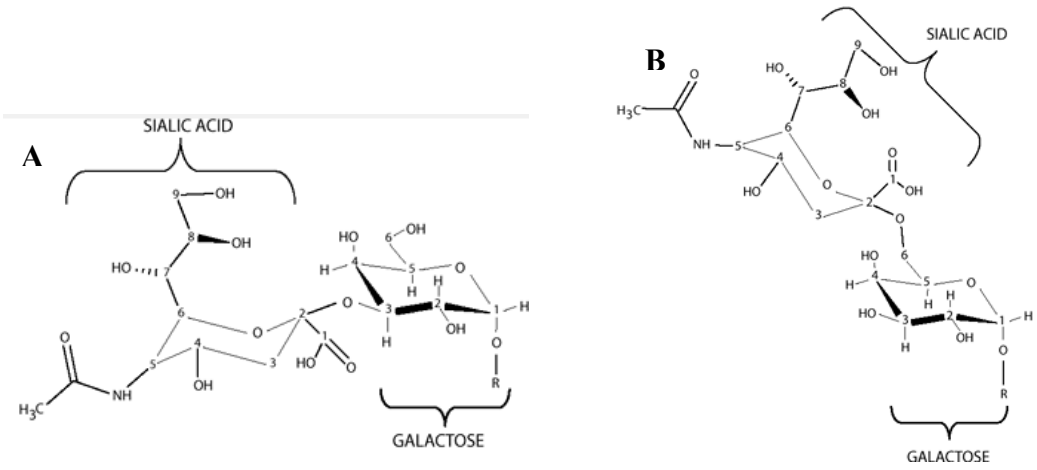

Figure 3.10 Sialic acid linkages preferred by avian or human influenza viruses. (A) SA α 2,3 Gal (avian). (B) SA α 2,6 Gal (human) [22]

Therefore, the receptor specificity influences the anatomical site of replication and affects the transmissibility of virus. Base on the fact that an essential characteristic of a pandemic virus is the ability to recognize SA α 2,6 Gal receptor for pandemic potential. Thus, the lack of ability to bind with human receptor on the human upper respiratory tract contributes to inefficient transmission of H5N1 influenza virus between humans [6, 36].

The NA cleavage specificity should be compatible with the specificity of HA. The knowledge of substrate specificity is still not unclear, but in the previous study of N8 NA was found that it associated with the amino acid at position 275, close to the active site and with glycosylation at Asn144 [37, 38]. A layer of mucin, which covers the respiratory epithelium, is a barrier to prevent the viral penetration before virus attaches to the cell surface [16, 39]. A mucin is rich in sialic acid, which is a SA α 2,6 Gal in human. Therefore, human influenza virus has efficiency to cleave and promote the virus spreading in the respiratory tract of human. In contrast, the NA of avian viruses preferentially recognize the SA α 2,3 Gal linkage rather than the SA α 2,6 Gal mammalian linkage [16], suggesting the inefficient infection of H5N1 influenza virus in human.

Another host range barrier is the viral polymerase function at different temperature. Human influenza virus can replicate well at the lower temperature like in human upper respiratory tract, which is the first site for viral infection, but at the elevated temperature of about 40ºC the viral growth is restricted. Avian influenza H5N1 virus replicates well at higher temperature, like in human lower respiratory tract, but at lower temperature the virus exhibits a cold-sensitive phenotype [8]. It has been well characterized that the different activity of viral polymerase between avian and human viruses was determined by PB2 residue 627 [1, 8]. Avian influenza viruses typically have Glu627 in the PB2, while most human viruses contain Lys at this position [16]. Several H5N1 isolates which were collected from human cases in Vietnam and Thailand possessed Lys in PB2 627, which has been shown to enhance ability to replicate at lower temperature and upper respiratory tract of mice [40]. This observation can suggest that the presence of a Lys at position 627 of PB2 is a strong determinant of adaptation to mammals by improving ability of replication at upper

respiratory tract, which allows the virus spreading more efficiently by sneezing and coughing and contributes to enhance human to human transmission [6].

Table 3.3 WHO cumulative number of H5N1 confirmed cases (21 April 2010) [7].

Country	2003		2004		2005		2006		2007		2008		2009		2010		Total	
	cases	deaths	cases	deaths	cases	deaths	cases	deaths	cases	deaths	cases	deaths	cases	deaths	cases	deaths	cases	deaths
Azerbaijan	0	0	0	0	0	0	8	5	0	0	0	0	0	0	0	0	8	5
Bangladesh	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0
Cambodia	0	0	0	0	4	4	2	2	1	1	1	0	1	0	0	0	9	7
China	1	1	0	0	8	5	13	8	5	3	4	4	7	4	0	0	38	25
Djibouti	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0
Egypt	0	0	0	0	0	0	18	10	25	9	8	4	39	4	19	7	109	34
Indonesia	0	0	0	0	20	13	55	45	42	37	24	20	21	19	1	1	163	135
Iraq	0	0	0	0	0	0	3	2	0	0	0	0	0	0	0	0	3	2
Lao People's Democratic Republic	0	0	0	0	0	0	0	0	2	2	0	0	0	0	0	0	2	2
Myanmar	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0
Nigeria	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	1	1
Pakistan	0	0	0	0	0	0	0	0	3	1	0	0	0	0	0	0	3	1
Thailand	0	0	17	12	5	2	3	3	0	0	0	0	0	0	0	0	25	17
Turkey	0	0	0	0	0	0	12	4	0	0	0	0	0	0	0	0	12	4
Viet Nam	3	3	29	20	61	19	0	0	8	5	6	5	5	5	7	2	119	59
Total	4	4	46	32	98	43	115	79	88	59	44	33	73	32	27	10	495	292

3.4 H5N1 transmission

There are several manners that H5N1 virus uses for transmission to human as described below.

3.4.1 Avian to human transmission

Most of human infection cases are result of zoonotic infection. Sick poultry are a major source of human infection, and large amount of infectious virions were excreted in the feces and other secretions [41]. The direct contact with poultry is main route of infection, including slaughtering or preparing sick poultry for consumption, consumption of uncooked poultry products such as raw blood, or close contact with infected poultry [15, 16, 42, 43].

Moreover, H5N1 can survive in the environment, so this is a possible theory for another mode of virus transmission to human, including oral ingestion of contaminated water during swimming, direct intranasal or conjunctival inoculation during exposure to water. Another potential mode is self-inoculation, for example, hands expose to infected formites and take the virus to entry the body. Using of untreated poultry feces as a fertilizer or fish feed has been suspected to be a source of human infection cases who were no history in direct contact with sick poultry [15, 16].

3.4.2 Human to human transmission

Confirmed cases in human to human transmission are rare. The first confirmed case was found in the rural northern Sumatra, Indonesia and reported by the World Health Organization (WHO) on June 23, 2006. In this case, seven members of a family were infected with H5N1 and six died. Later, another confirmed case, which reported by WHO on December 27, 2007, was found in Pakistan. The human to human transmission of both cases occurred via prolong close contact without using the precautions [16, 18]. In addition, New England Journal of Medicine had reported the probably human to human transmission in last September on 2005 of Thailand. In this case, the 11 year olds Thai girl most likely picked up the virus from the infected chickens, and probably transmit to her mother during the taking care in hospital. She became ill herself four days later and died 12 days after her daughter.

3.5 Clinical features of H5N1 infection

The age of cases ranges from 3 months to 75 years, with a median age of 18 years [16]. Most patients have initial symptoms of high fever, which has typically a temperature more than 38ºC**,** and influenza-like illness with lower respiratory tract symptoms, while upper respiratory tract symptoms are present only sometimes. In some patients, symptoms of diarrhea, vomiting, abdominal pain, pleuritic pain, and bleeding from the nose and gums have been reported. Central nervous system (CNS) manifestation in human cases is rarely to occur, but in one report, the patient shows the symptoms of CNS involvement with diarrhea, convulsion, and progressive coma. For the symptoms in the course of illness, lower respiratory tract manifestation including: respiratory distress, tachypnea, and inspiratory crackles are common, is usually found in most patients. Almost all of patients have primary viral pneumonia and abnormalities in chest radiographs, which are often bilateral and include diffuse, patchy, or interstitial infiltrates and segmental or lobular consolidation with air brochogram. The one important character of H5N1 infection in human is rapid progression of lower respiratory tract disease and associated with manifestation of the acute respiratory distress syndrome (ARDS). The tendency of H5N1 viruses to cause lower respiratory tract disease because in this area contains receptor for avian virus (SA α 2,3 Gal), which virus can recognize and bind for entry and replication in the host cell, and also the function of viral polymerase of avian viruses is optimal at higher temperature [15, 16].

The HA molecule of H5N1 containing multiple basic amino acid are cleaved by ubiquitous intracellular proteases, allowing the virus to replicate beyond its usual sites of infection and contributes to systematic infection. The multi-organ failure with the signs of renal dysfunction, cardiac dysfunction, Reye's syndrome, pneumothorax, pulmonary hemorrhage, and ventilator-associated pneumonia, common found in most patients [15, 16].

3.6 Dysregulation of cytokines and chemokines correlated with the high fever

As in many other infectious diseases, the results of host immune response contribute substantially to clinical signs and symptoms of influenza and to the control the infection. Avian H5N1 virus differs from human influenza virus in term of virus dissemination beyond the respiratory tract, high viral load, and induction of hypercytokinemia [11]. In many previous studies, cases of H5N1 infection have high viral load in pharyngeal specimen and elevated serum level of pro-inflammatory cytokines and chemokines, therefore, the researchers suggest that high viral replication competency may induce hypercytokinemia and hyperchemokine. Higher serum levels of interlukin-6 (IL-6), interlukin-8 (IL-8), tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), chemokines interferon-inducible protein 10 (IP-10), macrophage inflammatory protein-1α (MIP-1α), etc. can be found in patients with H5N1 disease, which are secreted from infected immune cells (Figure 3.11) [15, 16]. The IL-8 is believed to play a role in the development of ARDS [44]. Several important cytokine serve as endogenous pyrogens and involve in the pathogenesis of fever including IL-6, IL-8, TNF-α, IFN- γ, MIP-1α etc [45].

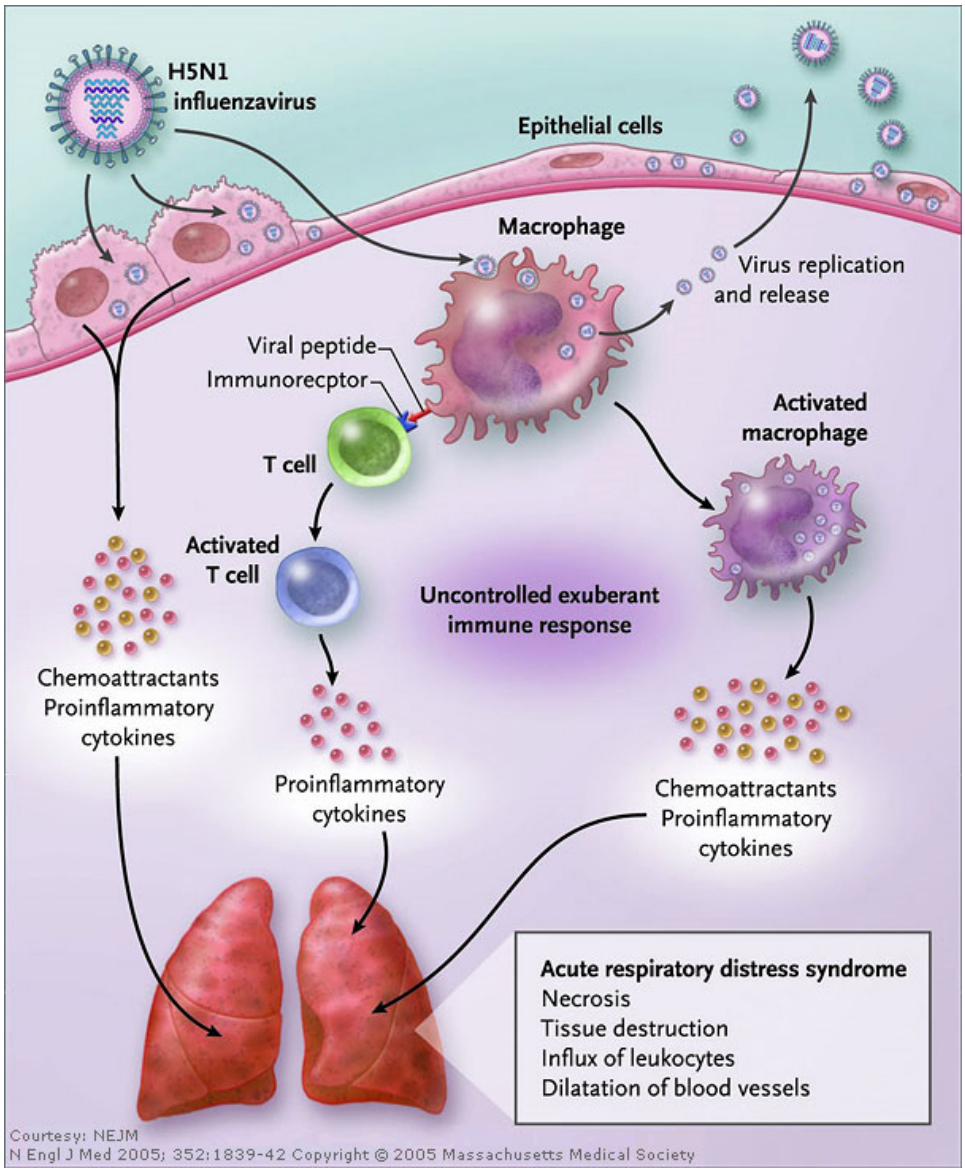

Figure 3.11 Illustration of the mechanism of H5N1 virus induces hypercytokinemia and hypercytokinemia. H5N1 infects immune cells and induces the releasing of inflammatory cytokines and chemoattractants which will stimulate leukocytes into the lung resulting in inflammation and tissue necrosis.
(Available from URL: http://planetflu.com/images/cytokine-storm.jpg)

Fever is a clinical indicator of a host immune response, which uses for elimination of the invading pathogens [46, 47]. The cytokines are produced, when the immune cells interact with an exogenous pyrogens, like an influenza viruses. The

virion components of influenza virus can activate host immune response to produce cytokines, such as dsRNA [48, 49], and virosome, which is the vRNA depleted particle but the other remain components including viral lipid, hemagglutinin (HA), and neuraminidase (NA) [13, 45].

The result of this interaction stimulates the production of prostaglandin E_2 (PGE_2), which is the inflammatory mediator and synthesized from essential fatty acids. PGE_2 is transported across the cell wall, enter the circulation, and reach to the hypothamus in central nervous system. The hypothalamus is in close contact with the general circulation in cerebral by a cluster of neurons termed the circumventricular organ system. This structure dilates the blood brain barrier and allows direct contact with both exogenous and endogenous pyrogens in the circulation [46, 47]. At this site, PGE_2 plays an important role in establishing a specific thermoregulatory change [50]. The high concentration of PGE_2 receptor in pre-optic area of the hypothalamus responsible for the genesis of fever (Figure 3.12) [51]. The consequence of direct interaction of PGE_2 to PGE_2 receptor is an increasing in the body temperature “set point”, which believes that it is a normal physiology. The new body temperature “set point” will remain as long as there is a production of endogenous pyrogens [46, 47].

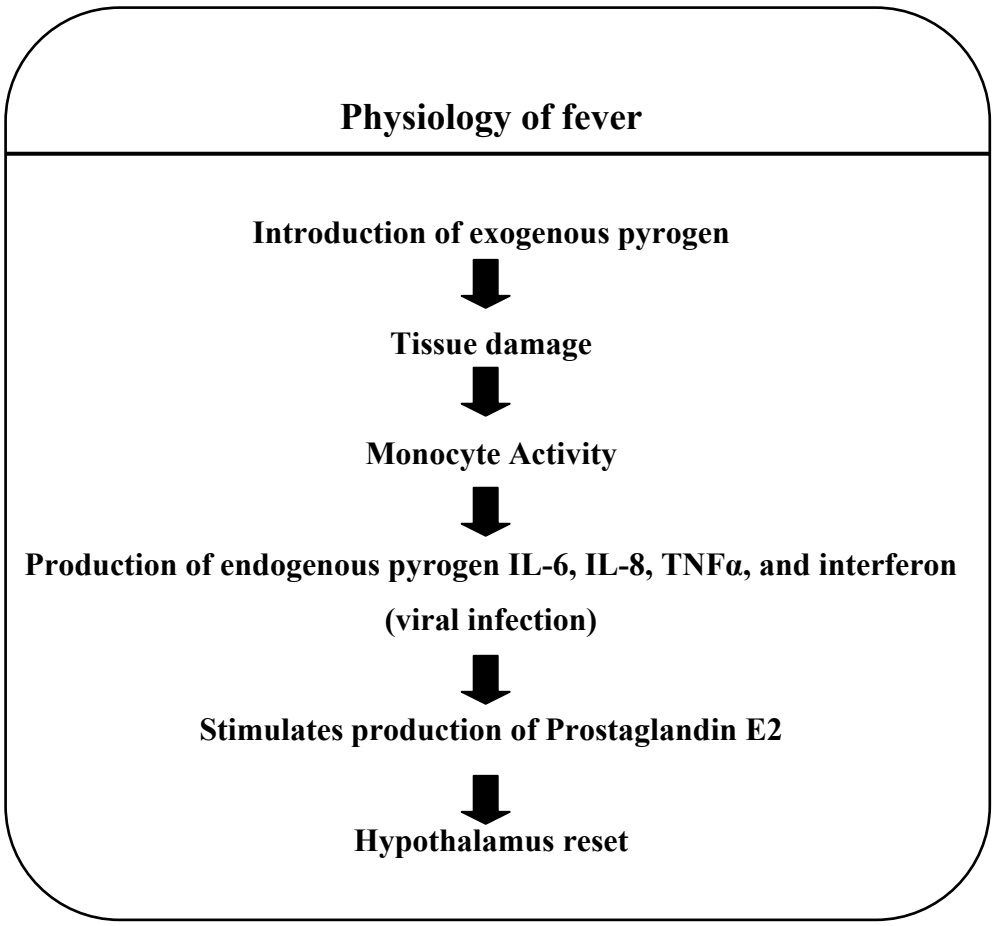

Figure 3.12 Physiology of fever.

Like other viruses, most human influenza viruses are sensitive to increasing the body temperature and slow down their rate of replication [46, 47]. However, the highly pathogenic avian influenza H5N1 virus can tolerate and replicate well in the body's natural response, which may involve with the genetic of viral polymerase complex that is responsible for thermal tropism.

3.7 Temperature sensitive lesions

Influenza, like other RNA viruses, displays a high mutation rate due to the error-prone nature of the viral polymerase, so mutant viruses are easy to isolate. The advantage of mutant virus is often used in study of functional individual gene of influenza virus. To study, the researchers may select only some particular phenotype of mutant virus, and the generation of desired mutant virus has been carried out by relying on naturally occurring variants, or force mutation by using chemically or physically etc [28].

In the late 1960s and early 1970s, a large collection of a particular phenotype of influenza virus, temperature sensitive (*ts*), derived from different strains, and was generated in several laboratories worldwide. These *ts* mutants were selected for growth at a permissive temperature (usually between 31ºC and 36ºC, depending on the study), but were significantly inhibited for replication at a higher nonpermissive temperature(usually between 38ºC and 42ºC) [28]. Studying the phenotype of temperature sensitive (*ts*) mutants with a defect in a particular gene at the non- or semi-permissive temperature might provide them with information about the function of the corresponding gene product [52]. Here I offer some examples of these studies.

For example, mutant *ts* 263 of the H7N1 A/fowl plaque/Rostock/34 (FPV) carried a single amino acid mutation in the PA gene in position 671: alanine changed to valine (PA A671V). This mutation located in a completely conserved region of the PA protein [53], was responsible for the *ts* phenotype. In this study, they found that this lesion in ts263 virus altered regulation of viral protein synthesis by over production of the M1 protein, so they concluded the result that function of PA protein involved in the regulation of viral protein synthesis at the level of expression of mRNA [53].

The lesions in another gene segments, *ts* mutants of segment 5, encoded for the NP protein. Medcalf et al. using the *ts* mutants viruses from the H1N1 A/WSN/33 (WSN) *ts*56: serine changed to asparagines at position 314, and H7N1 A/fowl plaque/Rostock/34 (FPV) *ts*G81: alanine changed to threonine at position 332, which have been shown the specific defect in replicative transcription at the nonpermissive temperature. To identify the functional defect of both lesions by introducing the mutations into the NP gene of influenza strain A/PR/8/34, they

reported the functional defect in replicative transcription that it involved in the disrupt interaction between NP and RNA, but not affect to NP-NP or NP-polymerase protein-protein contact. They suggested that the RNA-binding activity of NP was essensial for replicative transcription [54]. In year 2008, another study of *ts* lesion in NP from H7N1 A/chicken/Rostock/34 (FPV) US3 *ts* contained a single mutation in position 239: methionine to leucine (M239L). The introducing of this lesion into influenza strain A/PR/8/34 virus contributed to a reduction of viral titer of around 100-fold and showed the morphological abnormalities to form excessive pleomorphic shapes or apparent protrusion of material from the virion interior beyond the glycoprotein fringe. The conclusion of this study was NP US3 *ts* mutation (M239L) affect virion assembly and/or stability, which most likely involving in the altering interaction with M1 as a partner, thus it could indicate the new role of NP in viral morphogenesis [55].

The properties of *ts*5l was the influenza A/WSN/33 (WSN), which had a single *ts* lesion in RNA segment 7, encoding for M protein, in amino acid position 79 from phenylalanine to serine (M F79S). This mutation was responsible for both aberrant nuclear accumulation of Ml and low virus production at non permissive temperature. The researcher suggested that it could be due to M1 protein failure to exit into the cytoplasm, increased entry of Ml into the nucleus, or a combination of both. Although, they showed that M1 had a nuclear retention, it did not effect in nuclear export of vRNPs into the cytoplasm. In this study, thus, they could exhibit the role of M1 protein in late phase of viral replication, most likely at the stage of particle assembly and budding [56]. Another study of *ts* lesion in M protein, in previous study of Liu et al., they altered both two regions on M1 gene, which is RNA-binding domains, comprised of zinc finger motif (amino acid position 148 to 162) and substitution of serine for arginine at either position 101 or 105 of the RKLKR (basic amino acid domain), resulting in slightly reduction of viral growth and partially reduce the nuclear export of RNP and viral replication, respectively. Therefore, in this study, they wanted to understand the role of two RNA-binding domains of M1 protein by introducing multiple mutations to M gene of influenza virus A/WSN/33 (WSN). The result showed that a double mutations of R101S and R105S of RKLKR exhibited *ts* phenotype. The *ts* phenotype conferred a reduced incorporation of M1 into viral

particles, and susceptibility of M1/RNP complex to salt dissociation, so it suggested the role of this domain in control viral replication [57].

The master donor virus-A (MDV-A) strain, cold-adapted (*ca*) A/Ann Arbor/6/60 (A/AA/6/60), was used in the development of FluMist vaccine, which is a live attenuated vaccine. FluMist vaccine strains contain HA and NA gene segments derived from the currently circulating wild-type strains along with six gene segments, PB2, PB1, PA, NP, M, and NS from MDV. The MDV-A of FluMist was created by serial passage of the wt A/AA/6/60 strain in primary chicken kidney tissue culture at lower temperature [58], resulting in it replicates efficiently at 25ºC (*ca*), but its growth is restricted at 38 and 39ºC (*ts*), and not replicates in the lung of infected ferrets. The *ts* phenotype in MDV-A was determined by combination of ts lesions in three genes, PB2, PB1, and NP: PB2 N265S, PB1 K391E, PB1 E581G, PB1 A661T, and NP D34G [59].

In addition, there are many other reports of *ts* lesion in the other genes such as neuraminidase (NA) or nonstructural-1 (NS1) genes, which can explain the understanding in the viral life cycle of influenza virus.

3.8 Possible role of NP in RNA synthesis

NP has long been known to be the major protein component of influenza vRNPs. This complex involves in vRNA synthesis. The function of NP during vRNA synthesis is its potential role in the switch from mRNA transcription to genome replication. Several hypotheses have been proposed for this role as shown in Figure 3.13. The encapsidation hypothesis proposes that NP dose not have regulatory function as such but is only an essential co-factor. For this hypothesis, other factors alter polymerase activity to change the modes of transcription initiation and termination. The NP is required to co-transcriptionally coat the nascent cRNA segments [73]. Alternatively, the template modification hypothesis holds that the interaction of soluble NP with the template RNA alters its structure and mode of transcription. This is plausible, since the terminal sequences of the vRNA template are partially base-paired to form a panhandle structure and recognition of this structure by

the polymerase is intimately connected with the mechanism of mRNA transcription initiation and polyadenylation [73].

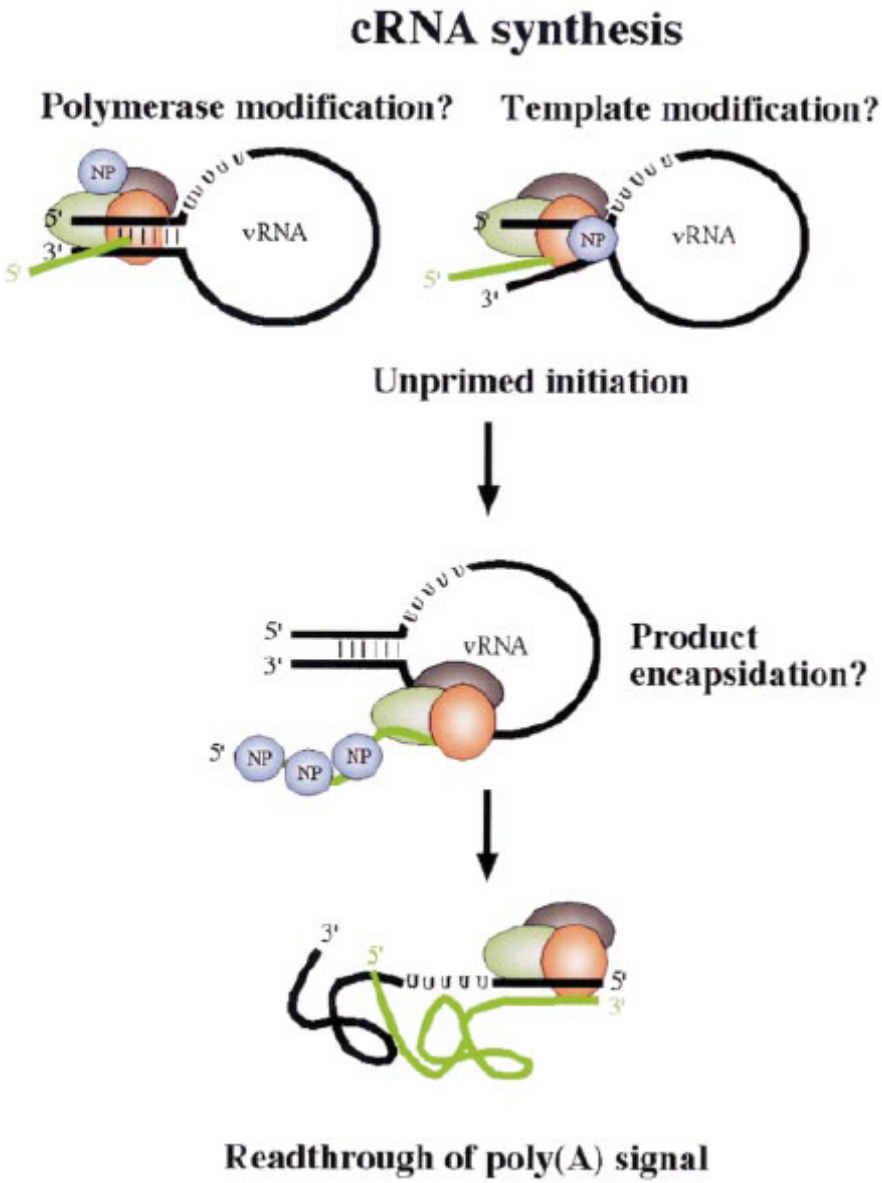

Figure 3.13 Possible role of NP in RNA synthesis. In the template modification hypothesis, NP disrupts the panhandle structure and so biases the polymerase towards unprimed transcription initiation. The product encapsidation hypothesis predicts that NP is requires to polyuridine stretch to produce a full-length copy of the template [73].

3.9 Molecular techniques

In this chapter, some molecular techniques that are important for the experiment were described as below.

3.9.1 The Overlap extension polymerase chain reaction (OE-PCR)

This technique is a variant of PCR and can be applied for inserting mutations at the specific points in a specific points in a sequence further than ~55 nucleotides from either end or produce full-length polynucleotides from smaller fragments [60]. The joining of two DNA sequences does not need restriction enzyme, briefly, two sequences that have region of short sequence complementarity between the 3'end of the first sequence and the 5'end of the other are joined and fused during PCR, as the overlapping sequences will hybridize and extend to produce a full-length sequence.

3.9.2 Gene cloning

The development of DNA-cloning technology in the 1970s provided a means of amplifying a given DNA fragment to extend large amounts of identical DNA fragments. A million copies of interested gene are generated by PCR reaction along with specific primers. These PCR products are separated and purified using gel electrophoresis, and inserted into an autonomously replicating DNA molecule, called a cloning vector. There are many types of vector as shown in Table 3.3, but the plasmid, which is a small, circular, extrachromosomal dsDNA of the bacteria, is often used as vector for genes transportation. The insertion of interested gene into plasmid vector is ligated together using DNA ligase to create phosphodiester bond between them. The recombinant plasmids are transferred into host cells, called "transformation", for gene amplification. To select the interested clones, generally, plasmids carry genes for antibiotic resistance, so transformed cells can be selected by their ability to grow in the presence of the antibiotic (Figure 3. 14) [61].

Vector type	Maximum length of cloned DNA (kb)
Plasmid	20
Bacteriophage λ	25
Cosmid	45
Bacteriophage P1	100
Bacterial artificial chromosome (BAC)	100–300
Yeast artificial chromosome (YAC)	>1000

Table 3.4 Vectors and maximum length of DNA that they can carry [61].

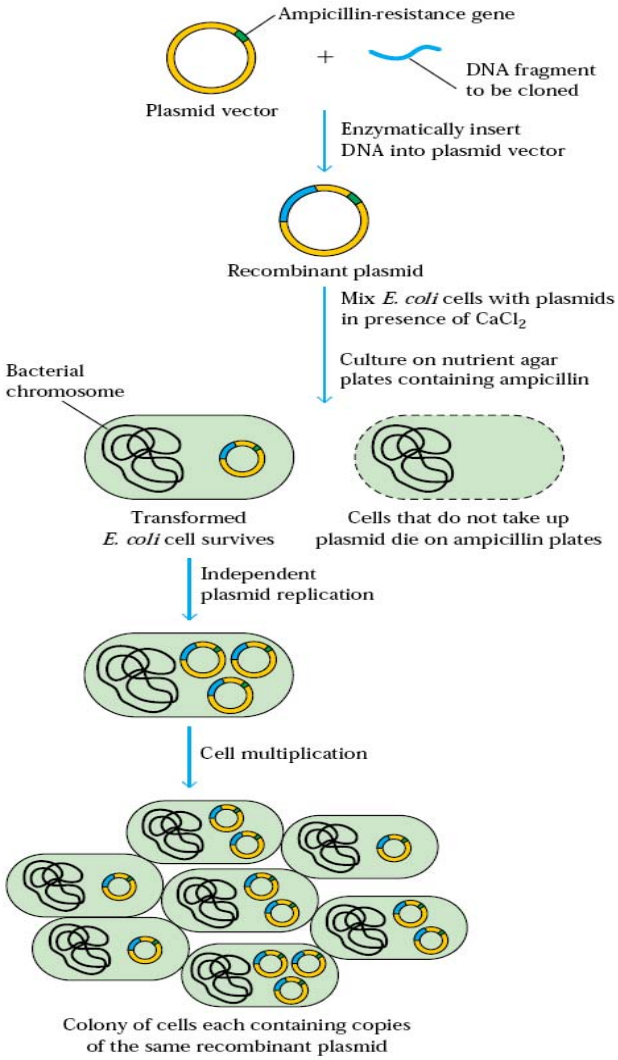

Figure 3.14 Schematic representation of gene cloning using a plasmid vector (Available from URL: http://www.bio. davidson. edu/Courses/ Molbio/ MolStudents/ spring2003/ Keogh/ cloning.gif)

3.9.3 Reverse genetics

The reverse genetics is a highly efficient system for the artificial generation of influenza viruses. The influenza virus has negative ssRNA eight segmented genomes that must be expressed and packaged to complete the virus replication cycle, therefore, the introduction of the genomic RNAs into cells does not result in the formation of infectious virus [22].

The system for generation influenza virus in in vitro was developed, and until in 1999, the breakthrough of reverse genetics system was established by two research groups, Fodor et al. and Neumann et al. [62, 63] , relying on the cellular enzyme RNA polymerase I for the synthesis of influenza viral RNAs. The abundance of RNA polymerase I in the nucleus utilizes for ribosomal RNA transcription. This cellular enzyme is an ideal for generation of influenza vRNA because the RNA transcript does not contain both of capped and polyadenylated [64]. In the system of Fodor et al., cDNA from each of the eight genome segments was cloned in negative orientation between the RNA polymerase I promoter and the RNA polymerase I terminator or hepatitis delta virus ribozyme, then eight vRNA-encoding plasmids were transfected into Vero cells along with four polII-driven plasmids expressing NP and the polymerase complex (PB1, PB2, PA) required for vRNA replication and transcription to generate infectious virus (Figure 3.15) [63]. The improvement to this system includes using cocultured of two cell lines of 293T cells (Human Embryonic Kidney) and MDCK cells (Mardin-Darby canine kidney) for transfection. The 293T cell is the species specificity of the human RNA polymerase I promoter and gives high transfection efficiency, and MDCK cell can support the high level of viral replication [22, 64]. Although this system requires 12 plasmids for recovering the infectious virions, it is high efficient to provide yielding of 10^8 plaque-forming units (PFU) per ml of cell culture supernatant [22].

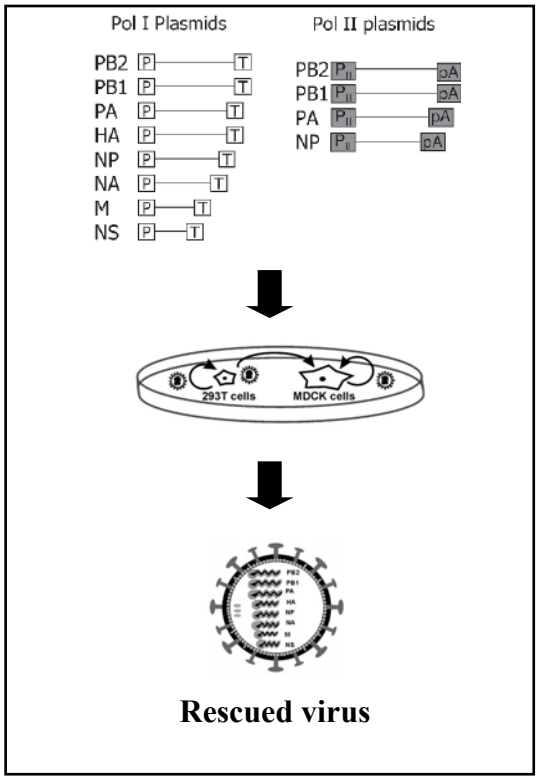

Figure 3.15 Schematic representation of the 12 plasmids-based reverse genetics system for influenza virus generation [64, 65].

Further improvements to this system were reported, in which both of vRNA and mRNA were synthesized together from the same template [66]. This system requires only eight plasmids, each contains cDNA of genomic segment cloned in negative orientation with a human RNA polymerase I promoter at the 5'end and the mouse RNA polymerase I terminator at the 3'end, and in the pol I transcription unit is flanked by the pol II promoter (pIICMV) of the human cytomegalovirus and the polyadenylation signal (aIIBGH) of the gene encoding bovine growth hormone [22, 65]. After transfection of the eight expression plasmids, negative sense vRNA is synthesized by cellular RNA polymerase I enzyme from human pol I promoter, whereas the viral mRNA is synthesized by cellular RNA polymerase I I enzyme from pol II promoter. The molecule of vRNA contains the noncoding regions (NCR) at the 5' and 3'ends, but viral mRNA contains the 5' cap structure and 3' poly(A) tails and is translated into viral protein [65]. Ultimately, all vRNA and mRNA molecules are assembled in the transfected cells and resulting in the generation of infectious particle

of influenza viruses (Figure 3.16). Recently, *de novo* synthesis of influenza virus was achieved by using only a single plasmid containing the cDNAs of all eight RNAs for transfection into cells. The transcription of mRNA-like molecules occurred from this plasmid, which contributed to the formation of the complementing viral polymerase proteins, and along with the full-length vRNAs allowing rescued of fully infectious particles [66].

Reverse genetics technique is the most powerful genetic tool in modern virology and can be applied or coupled with in vitro mutagenesis for widely beneficial, for example, to study of structure or function of difference influenza genes and their proteins for progress in understanding the influenza virus life cycle, the generation of live-attenuated vaccines and the use of influenza virus as vaccine and gene delivery vectors [64].

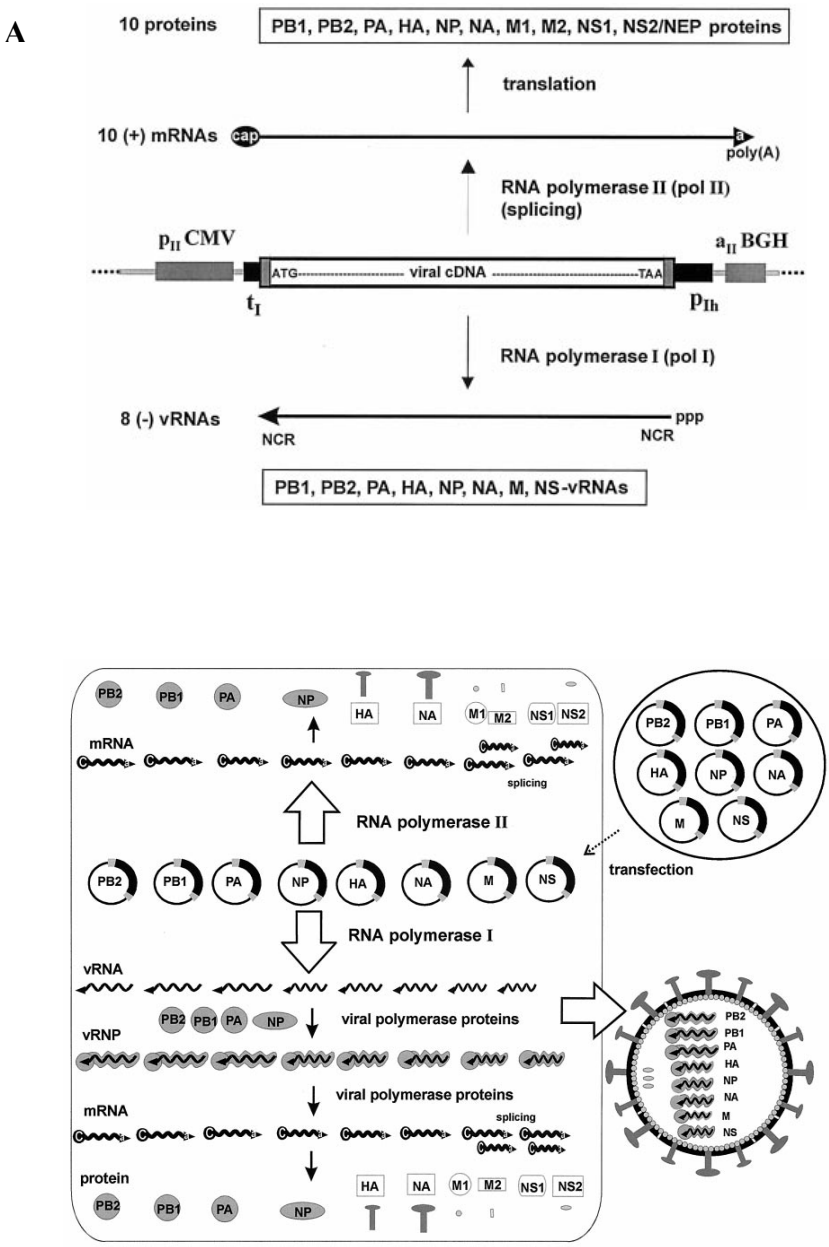

Figure 3.16 Schematic representation of the eight plasmids-based reverse genetics system. (A) The pol I-pol II transcription system for synthesis of vRNA and mRNA. (B) The generation of influenza A virus in transfected cell [65].

3.9.4 *Dpn*I mediated site-directed mutagenesis

The *Dpn*I mediated site-directed mutagenesis is based on PCR technique and usually focused on the plasmid templates. The oligonucleotide primers contain the desired mutation, which should has a mis-match during the first cycle in the binding the DNA template strand. After the first cycle, the newly synthesized strand containing the mutation would be at equal concentration to the original template, and after this cycle, it would exponentially grow to outnumber the original in the ratio of about 8 million to 1, resulting in a nearly homogeneous solution of mutated amplified products.

Before transformation of mutated plasmids into the competent cells, the DNA template is eliminated by enzymatic digestion with *Dpn*I**,** a restriction enzyme which cleaves only the methylated DNA, to prevent mixed population of two types plasmid. The DNA template is derived from an alkaline lysis plasmid extracted competent cells, so it is methylated and destroyed in this step. In contrast, the mutated plasmid, which is synthesized by in vitro assay, is unmethylated so it is preserved (Figure 3.17) [67].

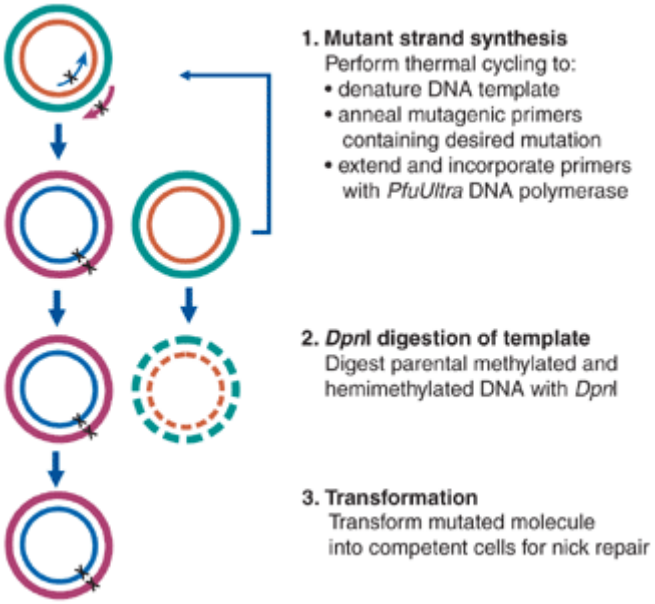

Figure 3.17 Schematic representation of *Dpn*I mediated site-directed mutagenesis. (Available from URL: www.stratagene.com)

3.9.5 Immunofluorescence

The immunofluorescence technique was known in year 1994 by Albert Coons, which in his study showed that antibodies could be labeled with molecules that have the property of fluorescence. The property of fluorescent molecules can absorb light of one wavelength (excitation) and emit light of another wavelength (emission). If antibody molecules are tagged with a fluorescent dye, or fluorochrome, immune complexes containing these fluorescence-labeled antibodies (FA) can be detected by colored light emission when excited by light of the appropriate wavelength and viewed with fluorescence microscope or confocal microscope. The molecule of fluorochrome can be conjugated to the Fc portion of an antibody molecule without affecting the specificity of the antibody. There are many kinds of fluorochrome such as fluorescein, rhodamine, phycoerythrin etc., and each kind will absorb light at one below wavelength and emit light at a longer wavelength as shown in Table 3.5.

Fluorescent-antibody staining of cell membrane molecules or tissue sections can be direct or indirect (Figure 3.18). In direct staining, the specific antibody (the primary antibody) is directly conjugated with dye-fluorescence and is used to against the antigen of interest, while the indirect staining, the primary antibody is unlabeled and is detected with an additional dye fluorescence-coupled antibody. In this pattern, the researchers can create several primary antibodies that recognize various antigens, and all of antibodies share a common constant region, resulting in may be recognized by a single dye-coupled secondary antibody. The most common used of secondary antibody is a dye-coupled antibody made in one species against antibodies of another species, such as fluorescein-labeled goat anti-mouse immunoglobulin.

Indirect immunofluorescence staining has two advantages over direct staining. First, the primary antibody does not need to be conjugated with a fluorochrome. Because the supply of primary antibody is often a limiting factor, indirect methods avoid the loss of antibody that usually occurs during the conjugation reaction. Second, indirect methods increase the sensitivity of staining because multiple molecules of the fluorochrome can bind to each primary antibody molecule, increasing the amount of light emitted at the location of each primary antibody molecule. However, in some cases, the direct staining has advantages over indirect staining, for

example, the direct labeling decreases the multiple-steps in the staining procedure, and more importantly, it avoids cross-reactivity and high background problems.

Table 3.5 Example of fluorochromes and their absorbed and emitted light.

Dye	Wavelength	
	Absorb light(nm)	**Emitted light (nm)**
Fluorescein	Blue light (490 nm)	Yellow-green (517 nm).
Rhodamine	Yellow-green range (515nm)	Deep red (546 nm).
Phycoerythrin	~30-fold greater than fluorescein	Red fluorescence

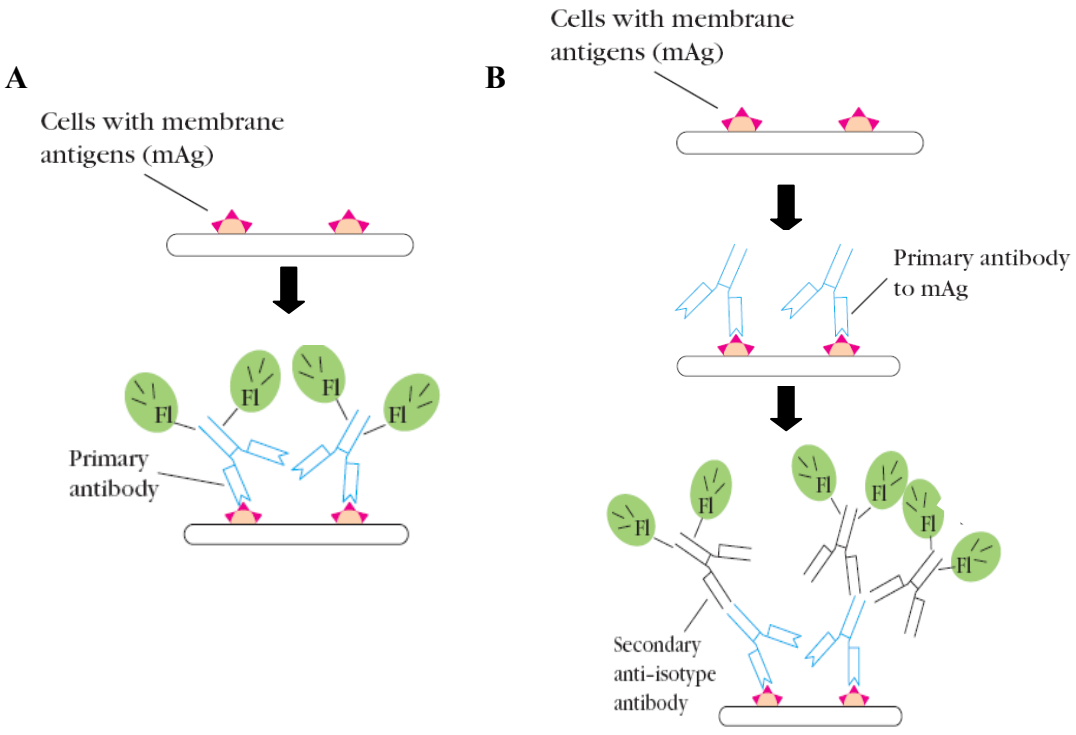

Figure 3.18 Direct (A) and indirect (B) immunofluorescence staining of membrane antigen (mAg).

CHAPTER IV
MATERIALS AND METHODS

Part I Materials

4.1 Virus

4.1.1 Virus Strains

H5N1 influenza virus strain A/Thailand/3 (SP-83)/2004 (SP83wt) and its variant (SP83ts20) were used in this thesis. H5N1 A/Thailand/3 (SP-83)/2004 virus was isolated from a 58 year-old women. It was kindly obtained from Dr. Pathom Sawanpanyalert (Director, National Institute of Health, Department of Medical Sciences) through Professor Pilaipan Puthavathana. The *ts* variant of H5N1 was kindly provided by Dr. Ornpreya Suptawiwat from a temperature shift experiment. This method was used for selection the temperature sensitive (*ts*) phenotype The starting stock for propagation the *ts* mutant virus was SP83wt passage 8 in MDCK cells. Briefly, SP83wt virus was serially cultured in MDCK cells at 30ºC for 5 passages, then shifted to 27ºC for 9 passages and finally 25ºC for 6 passages, after 20 passages the virus is called SP83ts20. The experiments of H5N1 virus were performed in biosafety level 3 (BSL-3) facilities.

4.1.2 Virus propagation

SP83ts20 was propagated in T75 flask, which was seeded with $5x10^6$ Mardin-Darby canine kidney (MDCK) cells. Cells were maintained in 10% heat-inactivated fetal calf serum MEM media and incubated at 37ºC for 24 hours. Before the inoculation, MDCK cells were washed two times with serum free media 1X MEM containing 1µg/ml of L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK) trypsin. Ten HA unit of SP83wt was absorbed for 1.5 hour at 30, 27, and 25ºC in 5%

CO_2 incubator, respectively. Serum free media (1X MEM containing 1ug/ml TPCK trypsin) was added to adjust the volume to 15 ml, and incubated at 30, 27, and 25ºC in 5% CO_2 incubator for 48-96 hours. After 48-96 hours, viral supernatant was titrated by hemagglutination (HA) assay, and harvested by centrifugation at 1500 g for 5 minutes and aliquot in volume of 300 µl in viral collection. The viral sample was kept at -80ºC.

4.1.3 Virus titration

Hemagglutination (HA) assay was used to titrate viral output. Hemagglutinin protein (HA) of influenza virus can agglutinate red blood cells (RBC) by forming lacttice which prevents them from setting out of suspension. Geese blood was collected and stored in Alsever solution (National Laboratory Animal Center, Mahidol University). Geese red blood cells (GRBC) were washed to prepare 10% GRBC. Five to six hundred microliters of geese blood was transferred into a 1.5 ml clean microcentrifuge tube. Normal saline (NSS) was added into tube to adjust volume to 1.5 ml. Geese blood was resuspended by gentle mixing, and centrifuged at 5,000 rpm for 1 minute at 4ºC. The supernatant was removed by pipetting. The packed GRBC was repeated this step for washing two times with NSS and 1X PBS pH 7.2, respectively. Then, 1X PBS pH 7.2 was added into packed GRBC to adjust volume to 1 ml for making 10% GRBC. Before using, 10% packed GRBC was diluted to 20 ml with 1X PBS pH 7.2 to obtain a 0.5% GRBC suspension. The HA assay was carried out according to a standard method. Briefly, 50 µ1 of serial two folds dilution of virus in phosphate buffered saline (PBS) was prepared in 96 well U-microtiter plate, then 50 µ1 of 0.5% of goose RBC suspension in PBS was added into each wells. The mixture was incubated at 4ºC for 30 minutes. The HA titer was calculated as the reciprocal value of the highest virus dilution that caused complete hemagglutination.

4.2 Cell culture

4.2.1 Mardin-Darby canine kidney (MDCK) cell lines were used for virus propagation and kinetic growth curve experiment. MDCK cells were grown with Minimum Essential Medium (MEM) supplemented with 10% heat-inactivated fetal calf serum (FBS) (GIBCO). Human Embryonic Kidney (HEK) 293T cells, which were used for transfection of reverse genetic plasmids, were grown in Dulbecco's Modified Eagle Medium (DMEM) (GIBCO, USA) supplemented with 10% heat-inactivated fetal calf serum (FBS) (GIBCO).

4.2.2 Primary Chicken Embryo Fibroblasts (CEF) for kinetic growth curve experiment were grown in Minimum Essential Medium (MEM) supplemented with 10% heat-inactivated fetal calf serum (FBS) (GIBCO).

Part II Methods

In this study, the genetic determinants of temperature sensitive (*ts*) phenotype in avian influenza virus were studied by using A/Thailand/3 (SP-83)/2004 and its variant, SP83ts20. DNA sequences of these two strains were compared to determine the candidate mutations for genetic determinants of temperature sensitive (*ts*) phenotype by evaluating growth phenotype.

4.3 Construction of the reverse genetic plasmid

Preparation of plasmids carrying genomic segments of both strains, SP83 wt and SP83ts20, comprised these steps:

4.3.1 Viral RNA extraction

Culture supernatants of SP83wt and SP83 ts20 viruses were extracted using a High Pure Viral Nucleic acid kit (Roche) according to the manufacture's instruction. Viral culture supernatant (200µ1) was mixed into 200 µ1 of binding buffer supplemented with poly(A) RNA and 50 µ1 of proteinase K by vortex immediately and incubated at 72ºC for 10 min. The mixture was tranferred to a high purefilter tube in a collection tube and centrifuged at 8,000x g for 1 minute. The high purefilter tube was placed into new collection tube. Five hundred microlites of inhibitor removal buffer was added to High Pure filter tube and the tube was centrifuged at 8,000x g for 1 minute. The high purefilter tube was placed into new collection tube. For removal of residue comtaminants, 450 µ1 of wash buffer was added into high purefilter tube and the tube was centrifuged at 8,000x g for 1 minute. The washing steps were repeated once. The High Pure filter tube was dried by centrifuged at 13,000x g for 1 minute. The high purefilter tube was transferred into a clean 1.5 ml microcentrifuge tube. Viral RNA was eluted from high purefilter tube by adding 50 µ1 of elution buffer. The column in high purefilter tube was equilibrated for 1 minute at room temperature and centrifuged at 8,000x g for 1 minute. The concentration of viral RNA was determined by a UV spectrophotometer and the RNA was stored at -80ºC until used.

4.3.2 cDNA synthesis

One microgram of viral RNA was used to synthesize cDNA by using SuperScript^{TM}III First-Strand Synthesis System for RT-PCR (Invitrogen) according to the manufacture's instruction (Figure 4.1). Reaction were amplified in a total volume of 20 µ1 containing 1 µg of total RNA, 1 µ1 of 10mM dNTP, 1 µ1 of Random hexamer, 10 µ1 of cDNA synthesis mixture and DEPC water. The cDNA was synthesized using the following cycle; denature 5 min at 65ºC, annealing 10 min at 25ºC, cDNA synthesis 50 min at 50ºC and terminate reaction min at 85ºC. For removal of residual viral RNA, 1 µ1 of RNase H (2U/µ1) was added and the reaction incubated at 37ºC for 20 minutes.

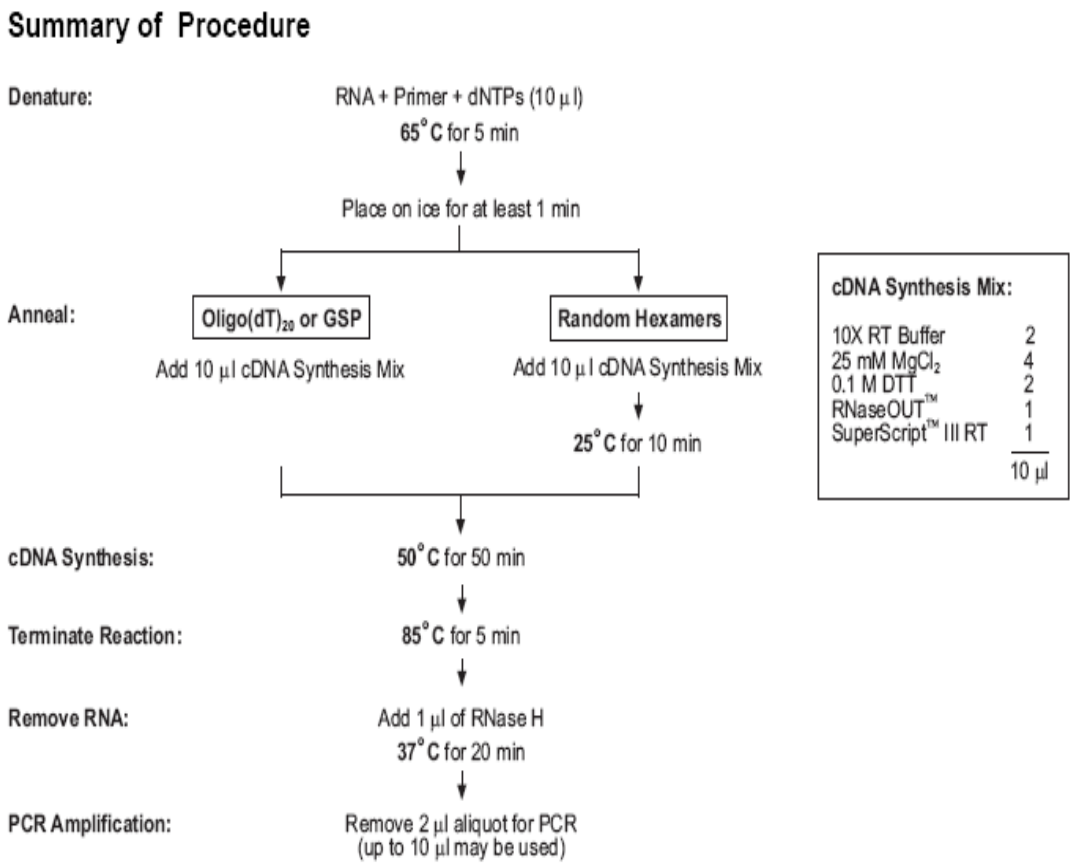

Figure 4.1 Summary of cDNA synthesis procedure**.**
(Available from URL: www.invitrogen.com)

4.3.3 Viral gene amplification

The cDNA were used for amplification of avian influenza genes as follow:

4.3.3.1 Platinum Taq DNA polymerase High Fidelity (Invitrogen) was used for amplification of PB2, PB1, PA, NP, M, and NS genes. Reactions were amplified full-length of NP, M, and NS genes and two fragments of each vial polymerase gene in total volume of 50 µ1 containing 2 µ1 of cDNA, 5 µ1 of 10x high fedility buffer, 2 µ1 of 25mM $MgSO_4$, 5 µ1 of 10mM dNTP, 2 µ1 of each specific forward and reward primers, 0.2 µ1 of HIFI Taq and RNase free distilled water. These viral genes were amplified using specific primers shown in Table 4.1. These primers contain the BsmBI or BsaI restriction site for cloning purposes. Reaction were carried out with an initial denaturation step of 94ºC for 2 min, then 3 cycles of 94ºC for 30 seconds, 45ºC for 30 seconds and 68ºC for 5 min and 32 cycles of 94ºC for 30 seconds, 55ºC for 30 seconds and 68ºC for 5 min and final extension at 72ºC for 10 min. The viral polymerase genes (PB2, PB1, PA) were amplified into two overlapping fragments and then were annealed into full-length genes using Overlap extension polymerase chain reaction. The PCR reactions contain 5 µ1 of each cDNA fragments, 5 µ1 of 10x buffer, 2 µ1 of 25mM $MgSO_4$, 5 µ1 of 10mM dNTP, 1 µ1 of each specific forward and reward primers, 0.2 µ1 of Taq polymerase and RNase free distilled water in final volume of 50 µ1. The total amplification cycles were 35 but divided into two parts; the first of 3 cycles composed of 94ºC for 30 seconds, 45ºC for 30 seconds and 68ºC for 3 min and remained cycles composed of 94ºC for 30 seconds, 50ºC for 30 seconds and 68ºC for 3 min and final extension at 72ºC for 10 min. The PCR products were separated on 1% agarose gel by electrophoresis, and amplicon of the appropriate size were subsequently excised from the gel and extracted using Qiagen gel extraction kit (QIAGEN) according to procedures recommended by the menufacture's instructions. A 3 volume of QG buffer was added into 1 volume of excised gel in 1.5 ml microcentrifuge tube and gel was melted at 50ºC until gel had completely dissolved. The solution was mixed then transferred to QIAquick spin column in a 2 ml collection tube and centrifuged at 13,000 rpm at 4ºC for 1 minute. The flow-through was discarded and the QIAquick spin column was placed back in the same collection tube. 750 µ1 of PE buffer was added into the spin column to wash the DNA then the column was centrifuged at 13,000 rpm at 4ºC for 1 minute. The

QIAquick spin column was transferred into a clean 1.5 ml microcentrifuge tube. DNA was eluted from the QIAquick spin column by adding 50 µ1 of EB buffer. The column was equilibrated for 1 minute at room temperature and centrifuged at 13,000 rpm at 4ºC for 1 minute. The PCR product was kept at -20ºC until used. All amplified viral cDNA fragments were sequenced using fluorescent-labeled dideoxyterminator.

4.3.3.2 One Step RNA PCR kit (TaKaRa, Japan) was used for amplification of HA and NA genes. The extracted viral RNA was converted to cDNA by using AMV RT (reverse transcriptase) before amplification process. Reaction were amplified in total volume of 50 µ1 containing 1µg of extracted viral RNA, 5 µ1 of 10x one step RNA PCR buffer, 10 µ1 of 25mM $MgSO_4$, 5 µ1 of 10mM dNTP, 1 µ1 of RNase inhibitor, 1 µ1 of AMV RT, 1 µ1 of AMV optimized Taq, 2 µ1 of each specific forward and reward primers, and RNase free distilled water. These viral genes were amplified using universal primers shown in Table 4.1 These primers contain segment-specific nucleotides and sequences for the type IIs restriction endonucleases *Bsm*BI or *Bsa*I for cloning purposes. These genes were amplified using following cycling parameters; reverse transcription at 50ºC for 30 min, inactivation of RNase at 94ºC for 2 min, 3 PCR cycles of 94ºC for 30 seconds, 45ºC for 30 seconds and 72ºC for 5 min and 32 cycles of 94ºC for 30 seconds, 55ºC for 30 seconds and 72ºC for 5 min and final extension at 72ºC for 10 min. The PCR products were separated on 1% agarose gel by electrophoresis and amplicon of the appropriate size were subsequently excised from the gel and extracted using Qiagen gel extraction kit (QIAGEN) according to procedures recommended by the menufacture’s instruction same as above and kept the product at -20ºC. All amplified viral cDNA fragments were sequenced using fluorescent-labeled dideoxyterminator.

Table 4.1 Primers used for influenza A viral gene amplification

Genes	Primers	Sequences of primer (5' to 3')
PB2 fragment 1	Ba-PB2-1 [68]	TATTGGTCTCAGGGAGCGAAAGCAGGTC
	PB2-1384R	GGTTCAATTCCCCAATTCTG
PB2 fragment 2	PB2-1208-1227F	CGCTGAGGCAATCAT
	Ba-PB2-2341R [68]	ATATGGTCTCGTATTAGTAGAAACAAGGTCTTT
PB1 fragment 1	Bm-PB1-1 [68]	TATTCGTCTCAGGGAGCGAAAGCAGGCA
	PB1-1369R	GGATTGGAGTCCGTCCCACC
PB1 fragment 2	PB1-1250-1268F	GAGCCCTGGAATGATGATGG
	Bm-PB1-2341R [68]	ATATCGTCTCGTATTAGTAGAAACAAGGCATTT
PA fragment 1	Bm-PA-1 [68]	TATTCGTCTCAGGGAGCGAAAGCAGGTAC
	PA-1359R	CTCATACTTGCAATGTGCTC
PA fragment 2	PA-1141-1160F	CGGTGAGAACATGGCACCAG
	Bm-PA-2233R [68]	ATATCGTCTCGTATTAGTAGAAACAAGGTACTT
HA	Bm-HA-1 [68]	TATTCGTCTCAGGGAGCAAAAGCAGGGG
	BM-NS-890R [68]	ATATCGTCTCGTATTAGTAGAAACAAGGGTGTTTT
NP	Bm-NP-1 [68]	TATTCGTCTCAGGGAGCAAAAGCAGGGTA
	Bm-NP-1565R [68]	ATATCGTCTCGTATTAGTAGAAACAAGGGTATTTTT
NA	Ba-NA-1 [68]	TATTGGTCTCAGGGAGCAAAAGCAGGAGT
	Ba-NA-1413R [68]	ATATGGTCTCGTATTAGTAGAAACAAGGAGTTTTTT
M	Bm-M-1 [68]	TATTCGTCTCAGGGAGCAAAAGCAGGTAG
	Bm-M-1027R [68]	ATATCGTCTCGTATTAGTAGAAACAAGGTAGTTTTT
NS	Bm-NS-1 [68]	TATTCGTCTCAGGGAGCAAAAGCAGGGTG
	Bm-NS-890R [68]	ATATCGTCTCGTATTAGTAGAAACAAGGGTGTTTT

The recognition sequences for the restriction endonucleases *Bsm*BI (Bm) or *Bsa*I (Ba) were shown in underlined.

4.3.4 Gene cloning

To generate the reverse genetic influenza virus entirely from cloned cDNA by using eight plasmid system, eight transcription plasmids were constructed as follow:

4.3.4.1 Cloning into the pGEM-T easy vector. For both strains of virus, eight of individual either Bs*m*BI or *Bsa*I viral cDNA fragments were cloned into the pGEM-T easy vector (Promega, USA) by insertion into multiple cloning sites between T7 RNA polymerase promoter and SP6 promoter (Figure 4.2). Ligation reaction mixture consisted of 7 µ1 of viral cDNA, 1 µ1 of pGEM-T easy vector, 1 µ1 of 10X ligation buffer (Promega, USA) and 1 µ1 of T4 DNA ligase (Promega, USA). The reaction mixture was incubated overnight at 16ºC. Recombinant plasmid was introduced into competent cells of *Escherichia coli* (*E. coli*) strain JM 109 using transformation process. 5 µ1 of ligation product was added into 100 µ1 of ice-thawed competent cells and incubated on ice for 30 minutes. Competent cells were heat shocked at 42ºC for 30 seconds then incubated on ice for 3 minutes. Eight hundred micoliters of SOC medium was added into cells and incubated with shaking for 1 hour at 37ºC. Cells were centrifuged at 13,000 rpm for 1 minute. Eight hundred micoliters of supernatant were discarded then the cell pellet was resuspended. Fifty microliters of cell suspension were spreaded on LB/ampicillin/IPTG/X-Gal agar plates, which were used for clone selection, and then incubated overnight at 37ºC. Recombinant plasmids were extracted from *E. coli* by alkaline lysis procedure. A single colony of *E. coli* was inoculated into 2 ml of LB media with 50 µg/ml ampicillin broth and incubated in shaking incubator overnight at 37ºC. One milliliter of bacterial culture was transferred to a 1.5 ml microcentrifuge tube then centrifuged at 13,000 rpm for 1 minute at 4ºC. After discarding the supernatant, cell pellet was resuspended in 100 µ1 of ice-cold solution I. Two hundred microliters of freshly prepared solution II were added into cell suspension and mixed by inverting. One hundred fifity microliters of solution III were added and mixed by inverting then the tube was centrifuged at 13,000 rpm for 10 minute at 4ºC. Aqueous phase of sample were transferred to a clean tube. Plasmid DNA from the aqueous phase was precipitated by adding 2 volume of absolute ethanol. The reaction was incubated for 2 minutes at room temperature then centrifuged at 13,000 rpm for 10 minute at 4ºC. After discarding the supernatant, DNA pellet was

washed with 70% ethanol then centrifuged at 13,000 rpm for 3 minute at 4ºC. After discarding the ethanol and drying, DNA pellet was dissolved in 20 µ1 of elution buffer and stored at -20ºC until used. The extracted plasmid was examined for presence of the insert using restriction enzyme digestion. Once clones with the correct inserts were identified, a miniprep using the Qiaprep Spin Miniprep Kit (QIAGEN) was performed to collect plasmid DNA according to the manufacture's instructions. The recombinant bacteria with each of eight viral gene insert was inoculated into 5 ml of LB broth with 50 µ1 of 50 mg/ml ampicillin and incubated with shaking overnight at 37ºC. Bacterial cultures were centrifuged at 13,000 rpm for 1 minute at 4ºC. After discarding the supernatant, cell pellet was resuspened in 250 µ1 of P1 buffer. Two hundred fifty microliters of P2 buffer was added into cell suspension and mixed by gently inverting tube 4-6 times until the color of mixture turned blue. Three hundred fifty microliters of N3 buffer was added into the mixture and mixed immediately by inverting tube until color of mixture was changed into white, and then the tube was centrifuged at 13,000 rpm for 10 minutes at 4ºC. Aqueous phase of sample was transferred to QIAprep spin column and centrifuged at 13,000 rpm for 1 minute at 4ºC. The flow-through was discarded and QIAprep spin column was placed back in the same collection tube. 500 µ1 of PB buffer was added into the spin column then centrifuged at 13,000 rpm for 1 minute at 4ºC. The flow-through was discarded and QIAprep spin column was placed back in the same collection tube. 750 µ1 of PE buffer was added into the spin column to washed DNA then centrifuged at 13,000 rpm for 1 minute at 4ºC. The spin column was transferred into a clean 1.5 ml microcentrifuge tube. DNA was eluted from the spin column by adding 50 µ1 of EB buffer. The column was equilibrated for 1 minute at room temperature and centrifuged at 13,000 rpm at 4ºC for 1 minute. The concentration of purified plasmids was determined by a UV spectrophotometer and the DNA sample was kept at -20ºC until used.

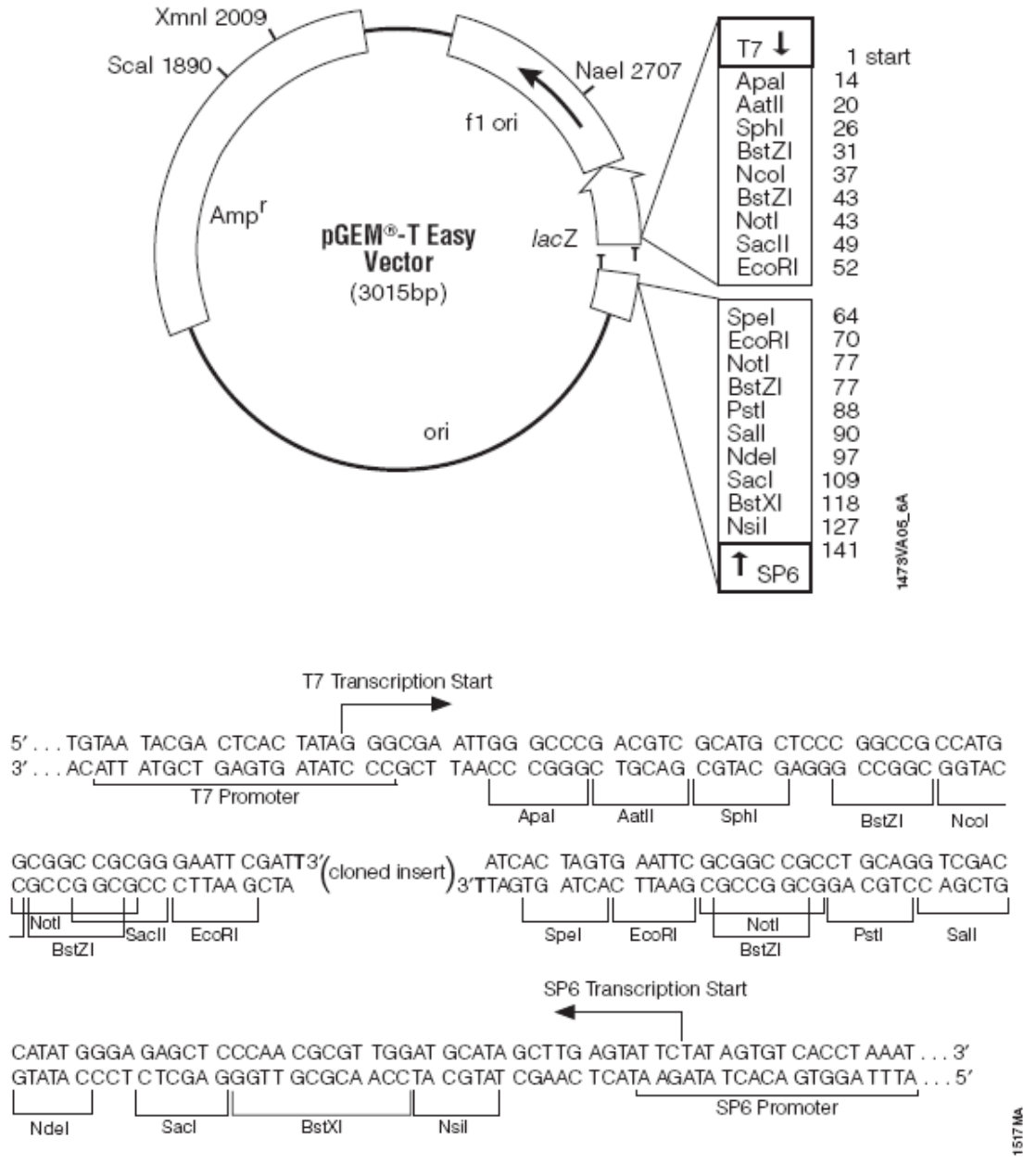

Figure 4.2 pGem-T Easy Vector circle map and multiple cloning sequence of the pGem-T Easy Vector
(Available from URL: www.promega.com)

4.3.4.2 Cloning into the pHW2000 vector. The eight viral cDNA fragments were digested with *Bsm*BI or *Bsa*I and inserted into pHW2000 (Hoffmann et al., 2000) (Figure 4.3). Viral cDNA fragments except PB2 and NA were digested by *Bsm*BI restriction enzyme. The reaction mixture consisted of 13-15 µ1 of

recombinant plasmid, 5 µ1 of 10X Buffer Tango™ (Fermentas), 1 µ1 of 10 U/µ1 Esp3I (*Bsm*BI) restriction enzyme (Fermentas) and the final volume of reaction mixture was adjusted to 50 µ1 with nuclease-free water. The reaction mixture was incubated for 6 hours at 37ºC. PB2 and NA cDNA fragments were digested by *Bsa*I restriction enzyme. The reaction mixture consisted of 10-20 µ1 of recombinant plasmid, 5 µ1 of 10X NEB buffer 3 (New England Biolabs, USA), 0.2 µ1 of 10 U/µ1 *Bsa*I restriction enzyme (New England Biolabs, USA) and the final volume of reaction mixture was adjusted to 50 µ1 with nuclease-free water. The reaction mixture was incubated for 5 minutes at 50ºC. Digested eight viral cDNA fragments were separated on 1% agarose gel by electrophoresis, and insert of the appropriate size were subsequently excised from the gel and extracted using Qiagen gel extraction kit (QIAGEN) according to procedures recommended by the menufacture's instruction and kept the product at -20ºC. After digestion process, eight viral cDNA fragments were ligated into pHW2000 vector. Ligation reaction mixture consisted of 7 µ1 of viral cDNA, 1 µ1 of pHW2000 vector, 1 µ1 of 10X ligation buffer (Promega, USA) and 1 µ1 of T4 DNA ligase (Promega, USA). The reaction mixture was incubated overnight at 16ºC. After ligation process, recombinant plasmids were transformed to *E. coli* strain JM 109 by using same protocol as above process but cell suspension were spreaded on LB/ampicillin not LB/ampicillin/IPTG/X-Gal agar plates. The plasmids were then extracted by alkali lysis as described above. The extracted plasmid was examined for presence of the insert using restriction enzyme digestion. Once clones with the correct inserts were identified then these plasmids were prepared in a large scale for using in reverse genetics method. Zero point one microliter of extracted plasmid was transformed into *E. coli* strain JM 109 and a single colony of recombinant bacteria was inoculated in 100 ml of LB with 50 µg/ml ampicillin broth and incubated in shaking incubator overnight at 37ºC. One hundred milliliters of bacterial cultures were transferred into clean 2 x 50 ml tubes. The bacterial cells were harvested by centrifuged at 3,000 g at 4ºC for 30 minutes and extracted by using the Qiaprep Spin Midiprep Kit (QIAGEN) for collect clone according to the manufacture's instructions. Two milliliters of P1 buffer was added into bacterial cells to resuspended then pooled bacterial suspension into one 50 ml tube. Four milliliters of P2 buffer was added into cell suspension and mixed thoroughly by inverting tube for 4-6 times until the color of

solution turned blue and the reaction was further incubated at room temperature for 5 minutes. Four milliliters of P3 buffer was added into the mixture and mixed thoroughly by inverting tube for 4-6 times until the color of solution turned white. The mixture was transferred into QIAfilter Midi Cartridge and incubated at room temperature for 10 minutes. During the incubation time, QIAGEN-tip 100 was equilibrated by adding 10 ml of QBT buffer and allowed the column to empty by gravity flow. The cap from QIAfilter Midi Cartridge was removed and Cartridge was transferred into QIAGEN-tip 100 and the plunger was inserted into the Cartridge. Cell lysate was filtered into QIAGEN-tip 100 and cleared lysate was allowed to enter the resin by gravity flow. 2 x 10 ml of QC buffer was added into QIAGEN-tip 100 to wash the QIAGEN-tip and QIAGEN-tip was transferred into a clean 50 ml tube to eluted DNA. DNA was eluted by adding 5 ml of QF buffer and precipitated by adding 3.5 ml of isopropanol (room temperature). This mixture was mixed thoroughly by inverting tube. 8.5 ml of eluted DNA was aliquoted into a clean 1.5 ml microcentrifuge tube then centrifuged at 13,000 rpm for 10 minutes at 4ºC. The supernatant was discarded and DNA pellet was washed by adding 500 µ1 of 70% alcohol (room temperature). After discarding the supernatant, DNA pellet was dried and resuspended by adding 100 µ1 of EB buffer. The concentration of purified plasmid was determined by a UV spectrophotometer and the DNA plasmids were kept at -20ºC until used. All eight midiprep plasmids were sequenced, and any nucleotide changes introduced during the cloning process were corrected to represent the authentic concensus sequence from RNA templates by using *Dpn*I-site directed mutagenesis protocol.

4.3.5 Sequence alignment

Sequencing of H5N1 strain A/Thailand/3 (SP-83)/2004 derived from GenBank, SP83wt at 8 passages in MDCK cells, and SP83ts20 were aligned using Bioedit Sequence Alignment Editor program. The GenBank accession numbers of each gene of A/Thailand/3 (SP-83)/2004 that were aligned are: EF456776.1 (A/Thailand/3 (SP-83)/2004, PB2 gene), EF467804.1 (A/Thailand/3 (SP-83)/2004, PB1 gene), EF541451.1 (A/Thailand/3 (SP-83)/2004, PA gene), EF541410.1 (A/Thailand/3 (SP-83)/2004, HA gene), EF467815.1 (A/Thailand/3 (SP-83)/2004, NP

gene), EF541472.1 (A/Thailand/3 (SP-83)/2004, NA gene), EF541445.1 (A/Thailand/3 (SP-83)/2004, M gene), EF467816.1 (A/Thailand/3 (SP-83)/2004, NS gene).

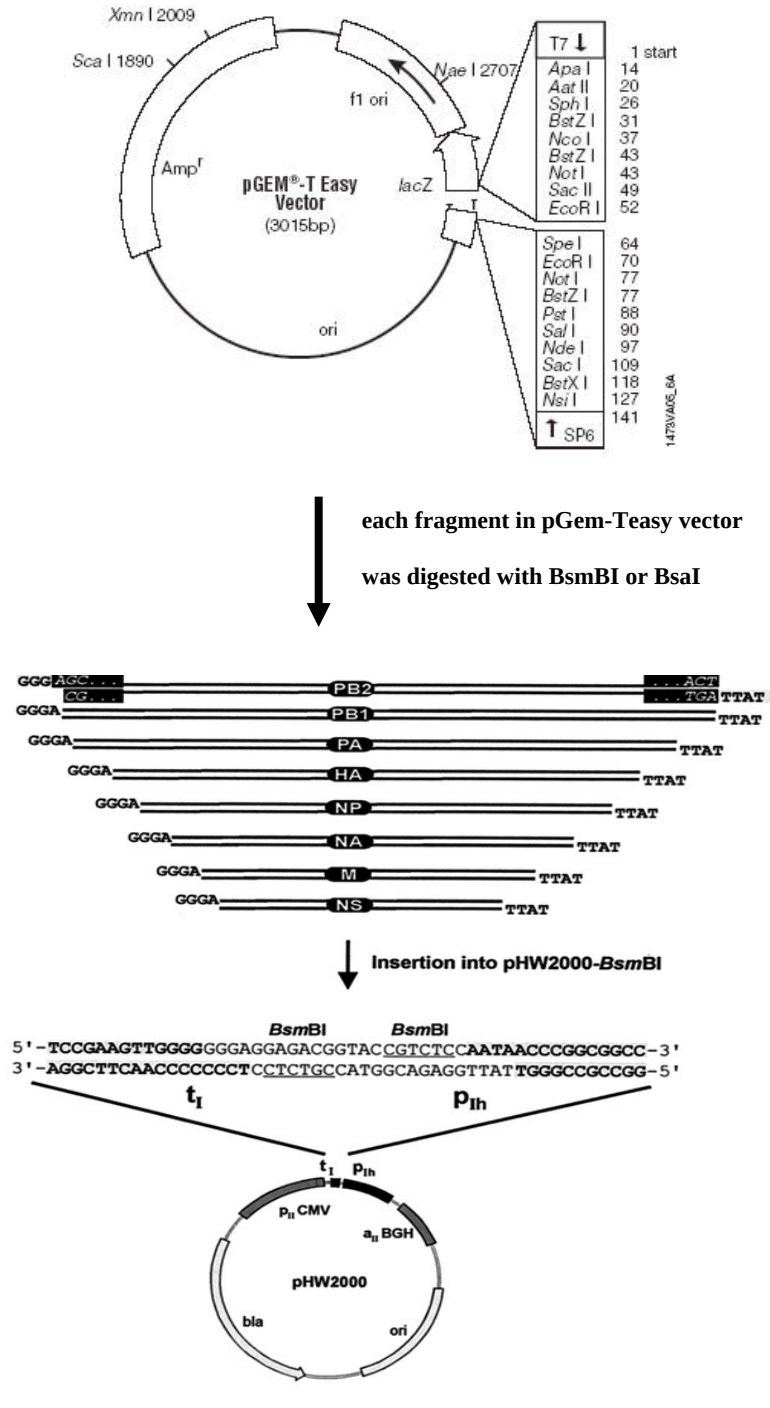

Figure 4.3 Schematic of gene cloning in pGEM-T easy vector and pHW2000 vector

4.3.6 *Dpn*I-site directed mutagenesis

After cloning process, the several cloning artifact were found in the viral polymerase complex and NP plasmids derived from SP83wt. Beside, some extra mutations were found in *ts* plasmids as shown in Chapter V, Table 5.2. Therefore, the extra mutations in *ts* plasmid were corrected to match the consensus direct sequence by using *Dpn*I-mediate site directed mutagenesis. Then, I decided to use newly synthesized SP83ts20 plasmids as a template for PCR mutagenesis to generate wild type viral polymerase complex and NP plasmids.

*Dpn*I-site directed mutagenesis is consisted of three main steps: plasmid DNA denaturation, PCR reaction and *Dpn*I digestion (Figure 4.4). The viral *ts* plasmids were used as templates and specific primers were designed for *Dpn*I-site directed mutagenesis as shown in Table 4.2. Begin with plasmid DNA denaturation process, 5 µg of recombinant plasmid was transferred into mixture of 40 µ1 steriled distilled water and 10 µ1 of 1 M NaOH/ 1mM EDTA then incubated at 37ºC for 15 minutes. Five microliters of 3M sodium acetate (pH 4.8) was added into the mixture and DNA was precipitated by adding 150 µ1 of ice-cold absolute ethanol then centrifuged at 13,000 rpm at 4ºC for 10 minutes. The supernatant was discarded and pellet was washed by adding 150 µ1 of 70% ethanol then centrifuged at 13,000 rpm at 4ºC for 2 minutes. Pellet was dried and resuspended by adding 20 µ1 of steriled distilled water. The denatured plasmid was kept at -20ºC until used. PCR reaction was used to mutate DNA sequence. The PCR reaction mixture consisted of 5 µ1 of 5 ng/µ1 DNA template, 5 µ1 of 10X buffer (Promega, USA), 5 µ1 of each forward and reward primers, 5 µ1 of 2.5 mM dNTPs, 0.8 µ1 of *pfu* DNA polymerase (Promega, USA) and the total volume was adjusted to 50 µ1 with nuclease-free water. Reaction were carried out with an initial denaturation step of 95ºC for 1 min, then 15 cycles of 95ºC for 30 seconds, 40ºC for 1 minute and 68ºC for 10 min and 1 cycle of 94ºC for 1 minute, 40ºC for 1 minute and final extension at 72ºC for 10 min. The PCR product was detected by 1% agarose gel electrophoresis with ethidium bromide staining. The final step, DNA template which contained methylation on sequence was destroyed by *Dpn*I restriction enzyme. Digestion reaction mixture consisted of 10 µ1 of PCR product, 2 µ1 of 10X NEB buffer 4 (New England Biolabs, USA), 0.2 µ1 of *Dpn*I (New England Biolabs, USA) and total volume was adjusted to 20 µ1 with nuclease-free water. The

reaction was incubated overnight at 37ºC for 16 hours. Finally, reaction was transformed into competent cells (*E. coli* strain JM 109), and mutated plasmid was extracted by using the same protocol as described above.

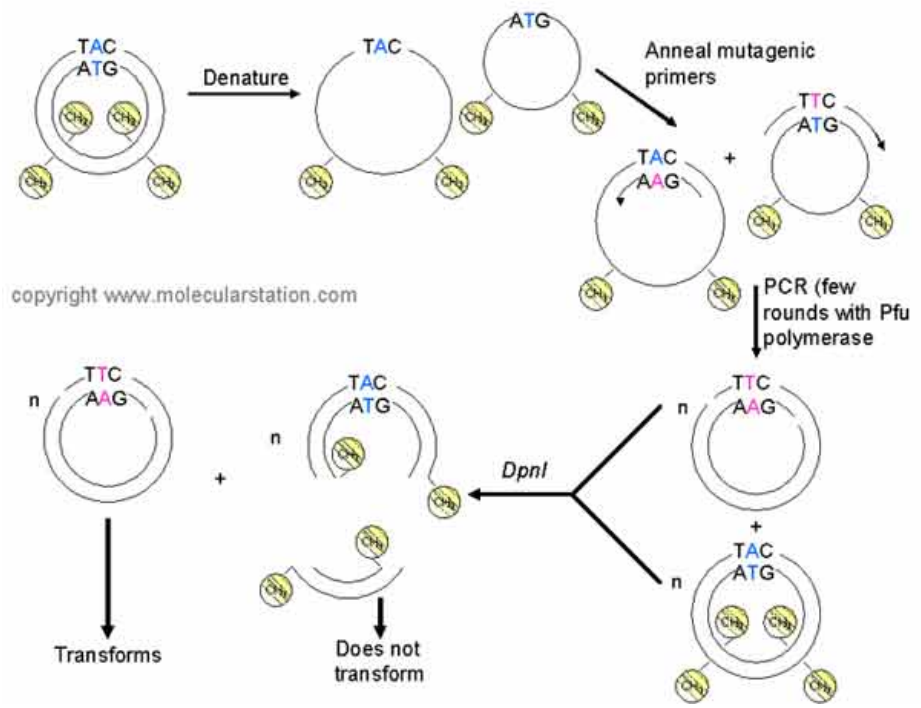

Figure 4.4 The principle of *Dpn*I mutagenesis
(Available from URL: www. molecularstation.com**)**

Table 4.2 Primers used for *Dpn*I-site directed mutagenesis

Primers	Sequences of primer (5' to 3')
SP83-PB2 H194Q F	GAAAGAAGATCTCCAATATTGTAAGATTGCTC
SP83-PB2 H194Q R	GAGCAATCTTACAATATTGGAGATCTTCTTTC
SP83-PB2 Y195D F	GAAAGAAGATCTCCATGATTGTAAGATTGCTC
SP83-PB2 Y195D R	GAGCAATCTTACAATCATGGAGATCTTCTTTC
SP83-PB2 R591Q F	AGGCTGCCAGAGGTCAATACAGTGGATTTGT
SP83-PB2 R591Q R	ACAAATCCACTGTATTGACCTCTGGCAGCCT
SP83-PB2 H194Q, Y195D F	GAAAGAAGATCTCCAAGATTGTAAGATTGCTC
SP83-PB2 H194Q, Y195D R	GAGCAATCTTACAATCTTGGAGATCTTCTTTC
SP83-PB1 V397I F	AATAAGGCCTCTATTAATAGATGGTACAGCCTC
SP83-PB1 V397I R	GAGGCTGTACCATCTATTAATAGAGGCCTTATT
SP83-PB1 N554D F	CAGTTATTCATCAAGGACTACAGATACACAT
SP83-PB1 N554D R	ATGTGTATCTGTAGTCCTTGATGAATAACTG
SP83-PA E448A F	GGAACTATTTTACAGCGGAAGTATCCCATTG
SP83-PA E448A R	CAATGGGATACTTCCGCTGTAAAATAGTTCC
SP83-PA P550L F	TAGGAGACATGCTCCTCCGGACTGCAGTAGG
SP83-PA P550L R	CCTACTGCAGTCCGGAGGAGCATGTCTCCTA
SP83-NP N314S F	CAGCCAGGTCTTTAGTCTCATTAGACCAA
SP83-NP N314S R	TTGGTCTAATGAGACTAAAGACCTGGCTG

The underlined nucleotide represented the position of designed mutation.

4.4 Generation of reassortant influenza viruses from plasmids

Reassortant influenza viruses were generated from plasmids using reverse genetic method (Figure 4.5). Transfection efficiency eukaryotic cell lines were used for expressed viral RNAs and mRNAs from plasmids. The eight expression plasmids with a pol I promoter and a pol II promoter contain one copy of each of the viral cDNAs of the eight segments. The cellular RNA polymerase I enzyme was used for the synthesis of influenza viral RNAs and cellular RNA polymerase II enzyme was used for the synthesis of influenza viral mRNAs resulted in the interaction of all synthesized molecules to generated infectious influenza A virus. Reverse genetic viruses were generated using these steps:

4.4.1 Preparation of cells

HEK- 293T cells were cocultured together with MDCK cells. Viruses produced in the 293T cells after transfection would infect into MDCK cells and replicated. The day before transfection, 293T cells and MDCK were cocultured at a density of approximately 1.3 x 10^5 cells of each cell line in six-well plates. The cells were maintained in Opti-MEM® I Reduced Serum Medium (GIBCO, USA) without serum.

4.4.2 Transfection

The compositions of the reassortant viruses are shown in Table 4.3. Each wild type plasmid and mutated plasmid from SP83 and SP83ts20 viruses (pHW2000-PB2wt, pHW2000-PB1wt, pHW2000-PAwt, pHW2000-NPwt; pHW2000-PB2ts20, pHW2000-PB2194ts, pHW2000-PB2195ts, pHW2000-PB2591ts, pHW2000-PB1ts20, pHW2000-PAts20, pHW2000-NPts20, respectively) were reassorted into plasmid backbone of a mouse adapted influenza virus A/Puerto Rico/8/34 (H1N1) (pHW2000-PB2, -PB1, -PA, -NP, -M, -NS, -HA, -NA), which kindly provided by Hoffmann et al. A set of eight plasmids were transfected into the cocultured 293T/MDCK cells using Lipofectamine™ 2000 (Invitrogen, USA). The transfection was initiated by diluting 1 µg of each plasmid and 2 µ1 of Lipofectamine™ 2000 reagent per 1 µg of each plasmid in 100 µ1 of Opti-MEM® I Reduced Serum Medium in separated 1.5 ml microcentrifuge tubes. The reaction was incubated for 5 minutes at room temperature,

then pooled into one 1.5 ml microcentrifuge tube and further incubated for 45 minutes to allow the plasmid-Lipofectamine 2000 complex to form. After finishing the incubation time, the mixture of 200 µ1 of plasmid-Lipofectamine 2000 complex was added to the wells containing cocultured cells and volume was adjusted to 1 ml by adding the Opti-MEM® I Reduced Serum Medium. The plate was then gently rocked back and forth. The cocultured 293T/MDCK cells were incubated at 37ºC in 5% CO2 incubator for 24 hours. After 24 hours, viruses were rescued from 293T cell by adding 1 ml of Opti-MEM® I Reduced Serum Medium containing 2X Trypsin-TPCK to final concentration 1µg/ml. The plate was then incubated at 37ºC for 24-48 hours. After 24-48 hours of incubation, the presence of rescued virus was determined by observing cytopathic effect (CPE) and testing the hemagglutination assay. The supernatant was collected and stored at -80ºC. Rescued virus was passaged in MDCK cells. Before inoculating reverse genetic virus, monolayer of 5.5 x 10^5 MDCK cells were washed two times with serum free media containing 1µg/ml of Trypsin-TPCK. 500 µ1 of supernatant was inoculated into cells and allowed to adsorbed for 1 hour at 37ºC in 5% CO_2 incubator then media was added to adjusted the volume to 2 ml and incubated at 37ºC in 5% CO_2 incubator for 48 hours. After 48 hours, supernatant was collected and titrated by HA.

4.4.2.1 Cytopathic effect (CPE) of influenza virus was detected by microscopic examination of the monolayer cell culture.

4.4.2.2 Hemagglutination (HA) assay was done by using the same process in viral titration.

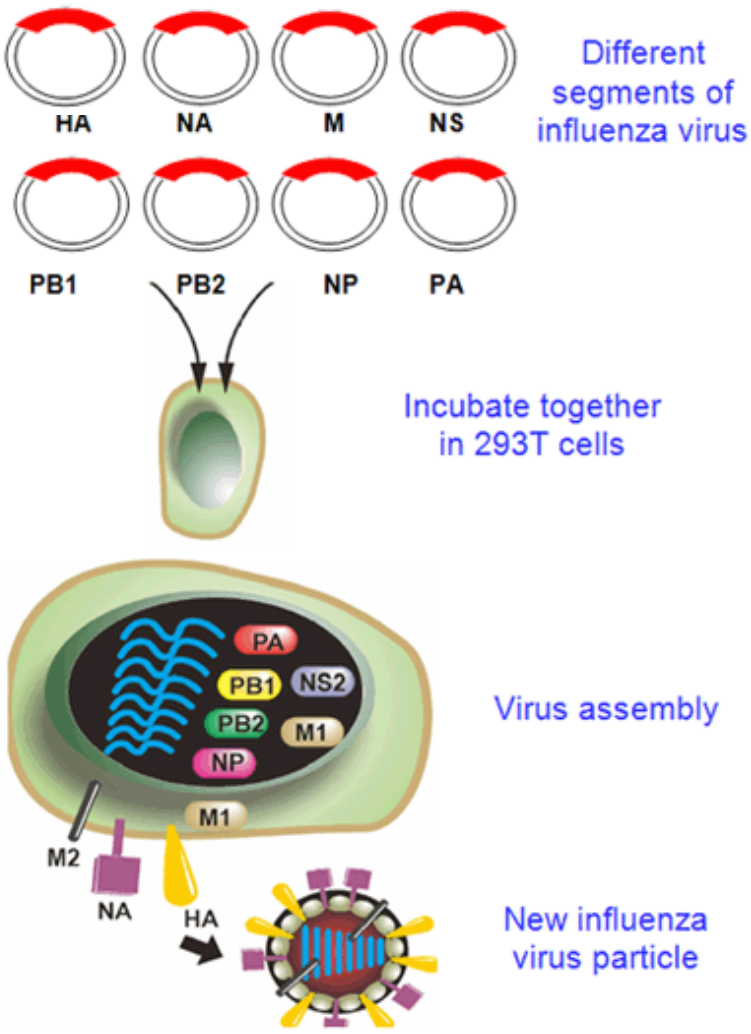

Figure 4.5 Schematic representation of reverse genetics for generation of influenza A virus.

(Available from URL: http://www.aht.org.uk/images/flu6.gif)

Table 4.3 The pattern of reassortant viruses in PR8 backbone

Gene substitution	Reassortant PR8 virus with	
	SP83 wt	**SP83 ts20**
PB1	R-PR8-PB1 wt	R-PR8-PB1 ts20
PB2	R-PR8-PB2 wt	R-PR8-PB2 ts20
		R-PR8-PB2 194ts
		R-PR8-PB2 195ts
		R-PR8-PB2 591ts
PA	R-PR8-PA wt	R-PR8-PA ts20
NP	R-PR8-NP wt	R-PR8-NP ts20

4.4.3 Collection of reverse genetic virus stock

Reverse genetic viruses were propagated in T25 or T75 flask, which was seeded with 2.5 x 10^6 or 5.5 x 10^6 MDCK cells, respectively, and maintained in 10% heat-inactivated fetal calf serum MEM media. Before the inoculation, MDCK cells were washed two times with serum free media containing 1µg/ml of Trypsin-TPCK. 200 µ1 of viral supernatant was inoculated onto cells and allowed to adsorbed for 1 hour at 37ºC in 5% CO_2 incubator then media was added to adjusted the volume to 5 or 15 ml for T25 and T75, respectively, and incubated at 37ºC in 5% CO_2 incubator for 48 hours. After 48 hours, viral supernatant was titrated by HA, collected, and aliquoted in volume of 150 and 300 µ1 in viral collection tube. The stock virus was quantified in a 50% tissue culture infectious dose (TCID50) assay before used in experiments.

4.4.3.1 Determination of 50% tissue culture infectious dose (TCID50). On the day before the titration, 200 µ1 of 3.3 x 10^6 MDCK cells in 20 ml of MEM medium supplemented with 10% heat-inactivated fetal calf serum was seeded to each well of 96 well-flat bottom plate and incubated overnight at 37ºC in 5% CO_2 incubator. Cells were washed two time with serum free MEM containing 2X Trypsin-treated TPCK. To inoculate virus in MDCK cells, 1:10 of diluted virus was serial

diluted in MEM containing 2X Trypsin-treated TPCK. The titration was performed in 96 well-U bottom plate by adding 146 µ1 of the diluted virus in lane 1 and added 100 µ1 of media to each well except lanes 1 and 12, then 46 µ1 of the diluted virus was transferred from lane 1 to 11 and discarded. 100 µ1 of media was added in lane 1 to 11 and 100 µ1 of each dilution was transferred to MDCK plate, then 100 µ1 of media was added in each well, the plate was then incubated overnight at 37ºC. After the incubation, cells were washed two times by 200 µ1 of 1X PBS pH 7.2 and fixed with 200 µ1 of 80% cold acetone at 4ºC for 1 hour. An ELISA was used to determine the result according to a standard protocol. MDCK cells were washed four times with 200 µ1 of washing buffer to each well. The endogenous peroxidase was blocked by adding 200 µ1 of 3% H_2O_2 to each well, the plate was then incubated for 30 minutes at room temperature. The 3% H_2O_2 was discarded and 50 µ1 of primary antibody specific to Influenza A nucleoprotein (mouse anti-Influenza A monoclonal Ab) (MILLIPORE, USA) was added to each well. The plate was then incubated for 1 hour at 37ºC in a humidified incubator. Cells were washed four times with 200 µ1 of washing buffer to each well. Fifty microliters of secondary antibody (HRP-coupled goat anti-mouse) (Southern Biotech) was added to each well, and the plate was then incubated for 1 hour at 37ºC in a humidified incubator. The reaction was developed by adding 50 µ1 of mixture of 1:1 TMB peroxidase substrate and peroxidase substrate solution B (KPL, USA) and incubating for 10 minutes at room temperature in the dark. The reaction was stopped by adding 50 µ1 of 1M H_2SO_4 to each well, and the optical density of reaction was measured spectrophotometrically at 450/630 on an ELISA reader. The data was analyzed using Reed and Muench equation.

4.5 Determination of temperature sensitive phenotype

The reassortant viruses containing mutated plasmids from SP83ts20 were determined for the temperature sensitive phenotype by evaluating growth property in comparison with the virus containing wide type plasmid.

4.5.1 Growth kinetic assay

The day before infection virus, 5.5 x 10^5 MDCK and CEF cells were seeded in six well plate and maintained with 10% heat-inactivated fetal calf serum MEM media and incubated overnight at 37ºC. Cells were washed two times with serum free media MEM containing 2X Trypsin-treated TPCK. 55 TCID50 of each virus in total volume of 200 µ1 was inoculated to cells and allowed to adsorb for 1 hour at 33 or 40ºC for MDCK cells and only at 40ºC for CEF cells in 5% CO_2 incubator, then media was added to adjusted the volume to 3 ml. The cells were incubated at 33 or 40ºC in 5% CO_2 incubator. At 6, 24, 30, and 48 hours, supernatants were collected and HA titers and TCID50 were determined by using the same protocol as above. The growth kinetic assay was performed in triplicate.

4.6 Determination of the effect in nuclear transport

Because of the significant titer reduction of SP83ts20 when compared to SP83wt at 40ºC, possible mechanism which may affect viral replication was studied. Indirect immunofluorescence assay was performed to determine subcellular localization and nuclear transport of viral protein.

4.6.1 Preparation of cell

The day before transfection, HEK-293T cells were seeded at approximately 1.3 x 10^5 cells in each well of 24 well plate and maintained in 10% heat-inactivated fetal calf serum DMEM media and incubated overnight at 37ºC in 5% CO_2 incubator.

4.6.2 Transfection

The NP plasmid from SP83ts20, which was shown to contain genetic determinant of temperature sensitive phenotype, and wild type NP plasmid were transfected into 293T cells using DMRIE-C reagent (Invitrogen, USA). The transfection was initiated with diluting 1 µg of plasmid and 2 µ1 of DMRIE-C reagent in 125 µ1 of Opti-MEM® I Reduced Serum Medium in separated 1.5 ml microcentrifuge tubes. The DNA and transfection reagent were incubated for 5

minutes at room temperature, then pooled into one 1.5 ml microcentrifuge tube and further incubated for 45 minutes to allow the plasmid- DMRIE-C complex to form. During the incubation time, 293T cells were gently washed two times with serum free DMEM media, then the mixture of 250 µ1 of plasmid- DMRIE-C complex was added to the wells. Plate was gently rocked back and forth and incubated at 33 or 40ºC in 5% CO_2 incubator for 4 hours. Two hundred fifty microliters of 20% heat-inactivated fetal calf serum DMEM media was then added to each well. Cells were incubated further at 33 or 40ºC in 5% CO_2 incubator for 48 hours.

4.6.3 Indirect immunofluorescence

After 48 hours of incubation time, cells were gently washed two times with 500 µ1 of 95% normal saline (NSS) and resuspended in 200 µ1 of NSS, and transferred into a clean 1.5 ml microcentrifuge tube. The cells were spun down and 100 µ1 of supernatant was discarded. The residual cell pellets were resuspended. Ten microliters of cell suspension was spotted on glass slide and dried. Cells were fixed with 80% cold acetone and incubated at 4ºC for 30 minutes. Cells were incubated with 20 µ1 of primary antibody specific to Influenza A nucleoprotein (mouse anti-Influenza A monoclonal Ab) (MILLIPORE, USA) at dilution 1:30 at 37ºC for 1 hour in stainless moist chamber. After incubation with primary antibody, cells were washed two times with 1X PBS pH 7.2 for 10 minutes and incubated for further 1 hour at 37ºC in stainless moist chamber with 20 µ1 of the dilution mixture of 1:100 Hoechst dye nuclear staining (Invitrogen, USA) and FITC-coupled IgG anti-mouse secondary antibody (MILLIPORE, USA). Cells were washed with 1X PBS pH 7.2 for 10 minutes and distilled water for 3 minutes and then the glass slide was covered by coverslip and mounted. Cells were observed under the laser scanning confocal microscope (LSM 510 Meta, Zeiss, Jena, Germany) at the Division of Medical Molecular Biology, Department of Research and Development, Faculty of Medicine Siriraj Hospital, Mahidol University. This experiment was performed in triplicate.

CHAPTER V
RESULTS

5.1 Identification of the mutations in *ts* mutant SP83ts20

To evaluate the mutations of the *ts* mutant of H5N1 A/Thailand/3 (SP-83)/2004 called SP83ts20, both of SP83wt and SP83ts20 viruses were extracted for change to cDNA by reverse transcription. Each of gene segments was amplified by PCR along with specific primers, and a set of viral genome products were purified and sequenced for further analysis. By direct sequencing, mutations were found in the viral polymerase complex and NP gene of SP83ts20 as shown in Figure 5.1. and summarized in Table 5.1. Then, the eight cDNAs corresponding to the viral gene segments were inserted into multiple cloning sites of pGEM-T easy vector between T7 RNA polymerase promoter and SP6 promoter, then corrected clones of each gene segment were extracted for collect the plasmids. The eight cDNA fragments in this vector were digested using *Bsm*BI or *Bsa*I restriction enzymes and inserted into pHW2000 vector (Hoffmann et al., 2000), then plasmid of each viral gene segments were extracted by a midiprep using the Qiaprep Spin Midiprep Kit (QIAGEN) and determine the correction of these plasmids by evaluating on the pattern of restriction cut size as shown in Figure 5.2. Then, these plasmids were sequenced. Several extra nucleotide mutations were found in the wild type viral polymerase complex and NP plasmids, while some extra mutations were found in *ts* plasmids as shown in Table 5.2. Therefore, the extra mutations in *ts* plasmid were corrected to match the consensus direct sequence by using *Dpn*I-mediate site directed mutagenesis. Then, the newly synthesized SP83ts20 plasmids were used as a template for PCR mutagenesis to generate wild type viral polymerase complex and NP plasmids. Therefore, both sequences, which derived from PCR or direct sequencing and cloning sequencing, were compared together using Bioedit Sequence Alignment Editor Program. Each gene from SP83wt and SP83ts20 was aligned, and the results were shown in Figure 5.1.

The comparison of two full genome sequences revealed the mutations in SP83ts20, which occurred in the viral polymerase complex (PB2, PB1, PA) and the nucleoprotein (NP) genes as shown in Figure 5.1 A, B, C, and D. These results suggested the optimal temperature for viral polymerase function play an important role in determining the phenotype.

A

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                                        10        20        30        40        50        60        70        80        90       100
                                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
gi|126361883|gb|EF456776.1| In  MERIKELRDLMSQSRTREILTKTTVDHMAIIKKYTSGRQEKNPALRMKWMMAMKYPITADKRIIEMIPERNEQGQTLWSKTNDAGSDRVMVSPLAVTWWN
direct seq. PB2-wt              ..................................................................................................
direct seq. PB2-ts              ..................................................................................................
pHW SP83wt-PB2                  ..................................................................................................
pHW SP83ts20-PB2                ..................................................................................................

                                       110       120       130       140       150       160       170       180       190       200
                                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
gi|126361883|gb|EF456776.1| In  RNGPATSAVHYPKVYKTYFEKVERLKHGTFGPVHFRNQVKIRRRVDINPGHADLSAKEAQDVIMEVVFPNEVGARILTSESQLTITKEKKEDLQDCKIAP
direct seq. PB2-wt              ..................................................................................................
direct seq. PB2-ts              ..................................................HY..............................................
pHW SP83wt-PB2                  ..................................................................................................
pHW SP83ts20-PB2                ..................................................HY..............................................

                                       210       220       230       240       250       260       270       280       290       300
                                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
gi|126361883|gb|EF456776.1| In  LMVAYMLERELVRKTRFLPVAGGTSSVYIEVLHLTQGTCWEQMYTPGGEVRNDDVDQSLIIAARNIVRRATVSADPLASLLEMCHSTQIGGIRMVDILRQ
direct seq. PB2-wt              ..................................................................................................
direct seq. PB2-ts              ..................................................................................................
pHW SP83wt-PB2                  ..................................................................................................
pHW SP83ts20-PB2                ..................................................................................................

                                       310       320       330       340       350       360       370       380       390       400
                                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
gi|126361883|gb|EF456776.1| In  NPTEEQAVDICKAAMGLRISSSFSFGGFTFKRTSGSSVKKEEEVLTGNLQTLKIRVHEGYEEFTMVGRRATAILRKATRRLIQLIVSGRDEQSIAEAIIV
direct seq. PB2-wt              ..................................................................................................
direct seq. PB2-ts              ..................................................................................................
pHW SP83wt-PB2                  ..................................................................................................
pHW SP83ts20-PB2                ..............................................................P...................................

                                       410       420       430       440       450       460       470       480       490       500
                                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
gi|126361883|gb|EF456776.1| In  AMVFSQEDCMIKAVRGDLNFVNRANQRLNPMHQLLRHFQKDARVLFQNWGIEPIDNVMGMIGILPDMTPSTEMSLRGVRVSKMGVDEYSSTERVVVSIDR
direct seq. PB2-wt              ..........................................K...........................................................
direct seq. PB2-ts              ..........................................K...........................................................
pHW SP83wt-PB2                  ..........................................K...........................................................
pHW SP83ts20-PB2                ..........................................K...........................................................
```

Figure 5.1 A The comparison of sequence alignment of PB2 gene segments of SP83wt and SP83ts20.

A (cont.)

```
                                        510       520       530       540       550       560       570       580       590       600
                                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
gi|126361883|gb|EF456776.1| In  FLRVRDQRGNVLLSPEEVSETQGTEKLTITYSSSMMWEINGPESVLVNTYQWIIRNWETVKIQWSQDPTMLYNKMEFEPFQSLVPKAARGQYSGFVRTLF
direct seq. PB2-wt              ..................................................................................................
direct seq. PB2-ts              ..................................................................................................
pHW SP83wt-PB2                  ..................................................................................................
pHW SP83ts20-PB2                ..................................................................................................

                                        610       620       630       640       650       660       670       680       690       700
                                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
gi|126361883|gb|EF456776.1| In  QQMRDVLGTFDTVQIIKLLPFAAAPPEQSRMQFSSLTVNVRGSGMRILVRGNSPVFNYNKATKRLTVLGKDAGALTEDPDEGTAGVESAVLRGFLILGKE
direct seq. PB2-wt              ..................................................................................................
direct seq. PB2-ts              ..................................................................................................
pHW SP83wt-PB2                  ..................................................................................................
pHW SP83ts20-PB2                ..................................................................................................


                                        710       720       730       740       750       760
                                ....|....|....|....|....|....|....|....|....|....|....|...
gi|126361883|gb|EF456776.1| In  DKRYGPALSINELSNLAKGEKANVLIGQGDVVLVMKRKRDSSILTDSQTATKRIRMAINCRII
direct seq. PB2-wt              ............................................................
direct seq. PB2-ts              ............................................................
pHW SP83wt-PB2                  ............................................................
pHW SP83ts20-PB2                ............................................................
```

Figure 5.1 A (cont.) The comparison of sequence alignment of PB2 gene segments of SP83wt and SP83ts20.

B

```
                                        10        20        30        40        50        60        70        80        90       100
                                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
gi|126598539|gb|EF467804.1| In  MDVNPTLLFLKVPVQNAISTTFPYTGDPPYSHGTGTGYTMDTVNRTHQYSEKGKWTTNTETGAPQLNPIDGPLPEDNEPSGYAQTDCVLEAMAFLEESHP
direct seq. PB1-wt              ..................................................................................................
direct seq. PB1-ts              ..................................................................................................
pHW Sp83wt-PB1                  ..................................................................................................
pHW Sp83ts20-PB1                ..................................................................................................

                                       110       120       130       140       150       160       170       180       190       200
                                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
gi|126598539|gb|EF467804.1| In  GIFENSCLETMEIVQQTRVDKLTQGRQTYDWTLNRNQPAATALANTIEIFRSNGLTANESGRLIDFLKDVMESMDKEEMEITTHFQRKRRVRDNMTKKMV
direct seq. PB1-wt              ..................................................................................................
direct seq. PB1-ts              ..................................................................................................
pHW Sp83wt-PB1                  ..................................................................................................
pHW Sp83ts20-PB1                ..................................................................................................

                                       210       220       230       240       250       260       270       280       290       300
                                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
gi|126598539|gb|EF467804.1| In  TQRTIGKKKQRLNKKSYLIRALTLNTMTKDAERGKLKRRAIATPGMQIRGFVYFVETLARSICEKLEQSGLPVGGNEKKAKLANVVRKMMTNSQDTELSF
direct seq. PB1-wt              ..................................................................................................
direct seq. PB1-ts              ..................................................................................................
pHW Sp83wt-PB1                  ..................................................................................................
pHW Sp83ts20-PB1                ..................................................................................................

                                       310       320       330       340       350       360       370       380       390       400
                                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
gi|126598539|gb|EF467804.1| In  TITGDNTKWNENQNPRMFLAMITYITRNQPEWFRNVLSIAPIMFSNKMARLGKGYMFESKSMKLRTQIPAEMLANIDLKYFNELTKKKIEKIRPLLIDGT
direct seq. PB1-wt              ..................................................................................................
direct seq. PB1-ts              ..................................................................................................
pHW Sp83wt-PB1                  ..................................................................................................
pHW Sp83ts20-PB1                ..................................................................................................

                                       410       420       430       440       450       460       470       480       490       500
                                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
gi|126598539|gb|EF467804.1| In  ASLSPGMMMGMFNMLSTVLGVSILNLGQKRYTKTTYWWDGLQSSDDFALIVNAPNHEGIQAGVDRFYRTCKLVGINMSKKKSYINRTGTFEFTSFFYRYG
direct seq. PB1-wt              ..................................................................................................
direct seq. PB1-ts              ..................................................................................................
pHW Sp83wt-PB1                  ..................................................................................................
pHW Sp83ts20-PB1                ..................................................................................................
```

Figure 5.1 B The comparison of sequence alignment of PB1 gene segments of SP83wt and SP83ts20.

B (cont.)

```
                                        510       520       530       540       550       560       570       580       590       600
                                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
gi|126598539|gb|EF467804.1| In  FVANFSMELPSFGVSGINESADMSIGVTVIKNNMINNDLGPATAQMALQLFIKDYRYTYRCHRGDTQIQTRRSFELKKLWEQTRSKAGLLISDGGPNLYN
direct seq. PB1-wt              ..................................................|...|.....................................V.........
direct seq. PB1-ts              ..................................................N...|.....................................V.........
pHW Sp83wt-PB1                  ..................................................|...|.....................................V.........
pHW Sp83ts20-PB1                ..................................................N...|.....................................V.........

                                        610       620       630       640       650       660       670       680       690       700
                                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
gi|126598539|gb|EF467804.1| In  IRNLHIPEVCLKWELMDEDYQGRLCNPLNPFVSHKEIESVNNAVVMPAHGPAKSMEYDAVATTHSWIPKRNRSILNTSQRGILEDEQMYQKCCNLFEKFF
direct seq. PB1-wt              ..................................................|...|.....................................V.........
direct seq. PB1-ts              ..................................................N...|.....................................V.........
pHW Sp83wt-PB1                  ..................................................|...|.....................................V.........
pHW Sp83ts20-PB1                ..................................................N...|.....................................V.........

                                        710       720       730       740       750
                                ....|....|....|....|....|....|....|....|....|....|..
gi|126598539|gb|EF467804.1| In  PSSSYRRPVGISSMVEAMVSRARIDARIDFESGRIKKEEFAEIMKICSTIEELRRQK
direct seq. PB1-wt              ..................................................
direct seq. PB1-ts              ..................................................
pHW Sp83wt-PB1                  ..................................................
pHW Sp83ts20-PB1                ..................................................
```

Figure 5.1 B (cont.) The comparison of sequence alignment of PB1 gene segments of SP83wt and SP83ts20.

C

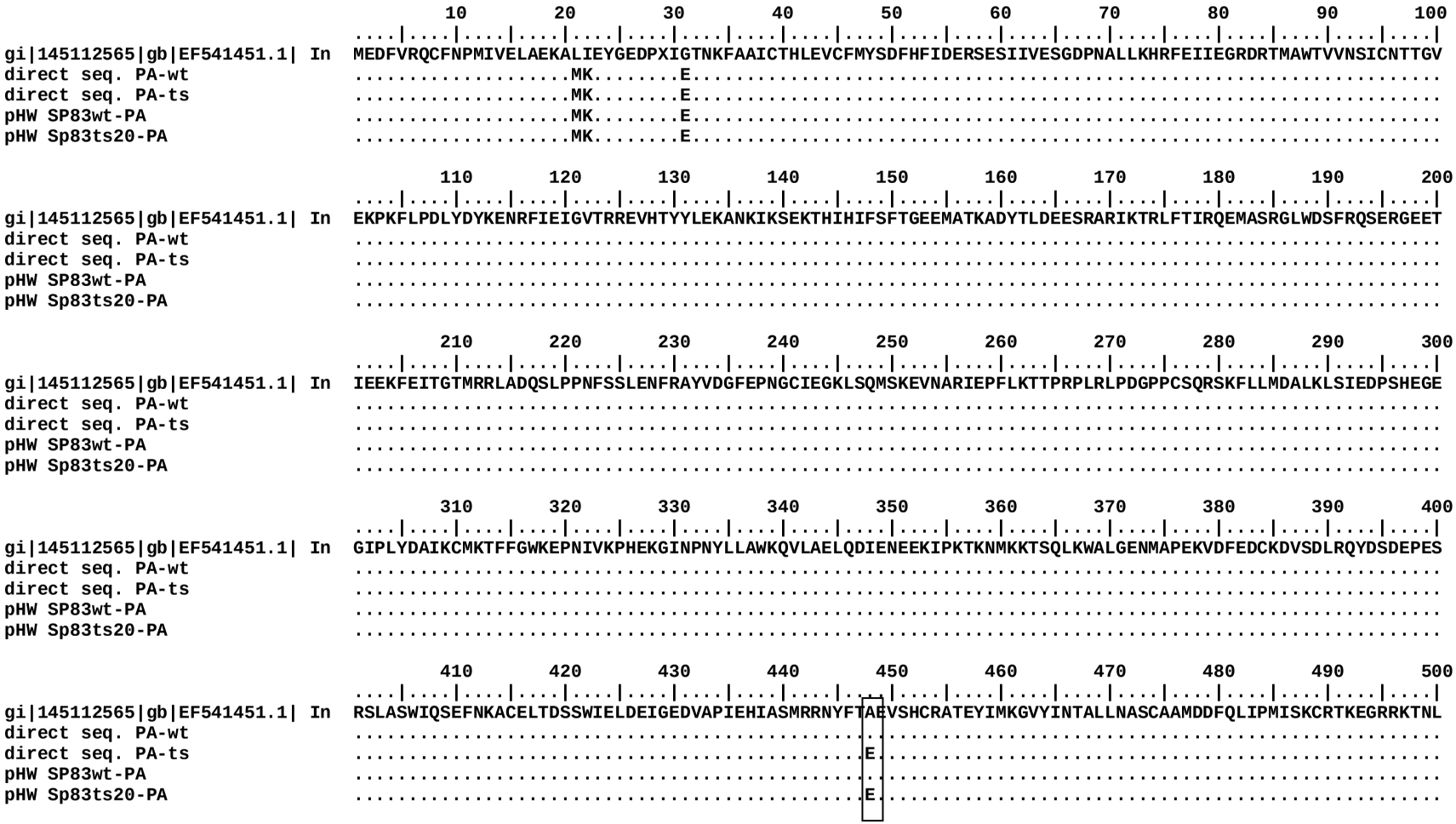


Figure 5.1 C The comparison of sequence alignment of PA gene segments of SP83wt and SP83ts20.

C (cont.)

```
                                        510       520       530       540       550       560       570       580       590       600
                                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
gi|145112565|gb|EF541451.1| In  YGFIIKGRSHLRNDTDVVNFVSMEFSLTDPRLEPHKWEKYCVLEIGDMLLRTAVGQVSRPMFLYVRTNGTSKIKMKWGMEMRRCLLQSLQQIESMIEAES
direct seq. PA-wt               ..........................................................................................................
direct seq. PA-ts               ..........................................................................................................
pHW SP83wt-PA                   ..........................................................................................................
pHW Sp83ts20-PA                 ..........................................................................................................

                                        610       620       630       640       650       660       670       680       690       700
                                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
gi|145112565|gb|EF541451.1| In  SVKEKDMTKEFFENKSETWPIGESPKGVEEGSIGKVCRTLLAKSVFNSLYASPQLEGFSAESRKLLLIAQALRDNLEPGTFDLGGLYEAIEECLINDPWV
direct seq. PA-wt               ..........................................................................................................
direct seq. PA-ts               ..........................................................................................................
pHW SP83wt-PA                   ..........................................................................................................
pHW Sp83ts20-PA                 ..........................................................................................................


                                ....|
gi|145112565|gb|EF541451.1| In  LLNAS
direct seq. PA-wt               .....
direct seq. PA-ts               .....
pHW SP83wt-PA                   .....
pHW Sp83ts20-PA                 .....
```

Figure 5.1 C (cont.) The comparison of sequence alignment of PA gene segments of SP83wt and SP83ts20.

D

```
                                        10        20        30        40        50        60        70        80        90       100
                                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
gi|126598907|gb|EF467815.1| In  MASQGTKRSYEQMETGGERQNATEIRASVGRMVSGIGRFYIQMCTELKLSDYEGRLIQNSITIERMVLSAFDERRNRYLEEHPSAGKDPKKTGGPIYRRR
direct seq. NP-wt               ....................................................................................................
direct seq. NP-ts               ....................................................................................................
pHW Sp83wt-NP                   ....................................................................................................
pHW Sp83ts20-NP                 ....................................................................................................

                                       110       120       130       140       150       160       170       180       190       200
                                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
gi|126598907|gb|EF467815.1| In  DGKWVRELILYDKEEIRRIWRQANNGEDATAGLTHLMIWHSNLNDATYQRTRALVRTGMDPRMCSLMQGSTLPRRSGAAGAAVKGVGTMVMELIRMIKRG
direct seq. NP-wt               ....................................................................................................
direct seq. NP-ts               ....................................................................................................
pHW Sp83wt-NP                   ....................................................................................................
pHW Sp83ts20-NP                 ....................................................................................................

                                       210       220       230       240       250       260       270       280       290       300
                                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
gi|126598907|gb|EF467815.1| In  INDRNFWRGENGRRTRIAYERMCNILKGKFQTAAQRAMMDQVRESRNPGNAEIEDLIFLARSALILRGSVAHKSCLPACVYGLAVASGYDFEREGYSLVG
direct seq. NP-wt               ....................................................................................................
direct seq. NP-ts               ....................................................................................................
pHW Sp83wt-NP                   ....................................................................................................
pHW Sp83ts20-NP                 ....................................................................................................

                                       310       320       330       340       350       360       370       380       390       400
                                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
gi|126598907|gb|EF467815.1| In  IDPFRLLQNSQVFSLIRPNENPAHKSQLVWMACHSAAFEDLRVSSFIRGTRVVPRGQLSTRGVQIASNENMEAMDSNTLELRSRYWAIRTRSGGNTNQQR
direct seq. NP-wt               ....................................................................................................
direct seq. NP-ts               ............N.....................................................................................
pHW Sp83wt-NP                   ....................................................................................................
pHW Sp83ts20-NP                 ............N.....................................................................................

                                       410       420       430       440       450       460       470       480       490
                                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|...
gi|126598907|gb|EF467815.1| In  ASAGQISVQPTFSVQRNLPFERATIMAAFTGNTEGRTSDMRTEIIRMMESARPDDVSFQGRGVFELSDEKATNPIVPSFDMNNEGSYFFGDNAEEYDN
direct seq. NP-wt               ....................................................................................................
direct seq. NP-ts               ....................................................................................................
pHW Sp83wt-NP                   ....................................................................................................
pHW Sp83ts20-NP                 ....................................................................................................
```

Figure 5.1 D The comparison of sequence alignment of NP gene segments of SP83wt and SP83ts20.

E

```
                                        10        20        30        40        50        60        70        80        90       100
                                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
gi|145284479|gb|EF541410.1| In  MEKIVLLFAIVSLVKSDQICIGYHANNSTEQVDTIMEKNVTVTHAQDILEKTHNGKLCDLDGVKPLILRDCSVAGWLLGNPMCDEFINVPEWSYIVEKAN
direct seq. HA-wt               ....................................................................................................
direct seq. HA-ts               ....................................................................................................
pHW Sp83wt-HA                   ....................................................................................................
pHW Sp83ts20-HA                 ...........................................................S........................................

                                       110       120       130       140       150       160       170       180       190       200
                                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
gi|145284479|gb|EF541410.1| In  PVNDLCYPGDFNDYEELKHLLSRINHFEKIQIIPKSSWSSHEASLGVSSACPYQGKSSFFRNVVWLIKKNSTYPTIKRSYNNTNQEDLLVLWGIHHPNDA
direct seq. HA-wt               ....................................................................................................
direct seq. HA-ts               ....................................................................................................
pHW Sp83wt-HA                   ....................................G...............................................................
pHW Sp83ts20-HA                 ....F...............................................................................................

                                       210       220       230       240       250       260       270       280       290       300
                                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
gi|145284479|gb|EF541410.1| In  AEQTKLYQNPTTYISVGTSTLNQRLVPRIATRSKVNGQSGRMEFFWTILKPNDAINFESNGNFIAPEYAYKIVKKGDSTIMKSELEYGNCNTKCQTPMGA
direct seq. HA-wt               ....................................................................................................
direct seq. HA-ts               ....................................................................................................
pHW Sp83wt-HA                   ....................................................................................................
pHW Sp83ts20-HA                 ....................................................................................................

                                       310       320       330       340       350       360       370       380       390       400
                                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
gi|145284479|gb|EF541410.1| In  INSSMPFHNIHPLTIGECPKYVKSNRLVLATGLRNSPQRERRRKKRGLFGAIAGFIEGGWQGMVDGWYGYHHSNEQGSGYAADKESTQKAIDGVTNKVNS
direct seq. HA-wt               ....................................................................................................
direct seq. HA-ts               ....................................................................................................
pHW Sp83wt-HA                   ..........................................................................................P.........
pHW Sp83ts20-HA                 ....................................................................................................

                                       410       420       430       440       450       460       470       480       490       500
                                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
gi|145284479|gb|EF541410.1| In  IIDKMNTQFEAVGREFNNLERRIENLNKKMEDGFLDVWTYNAELLVLMENERTLDFHDSNVKNLYDKVRLQLRDNAKELGNGCFEFYHKCDNECMESVRN
direct seq. HA-wt               ....................................................................................................
direct seq. HA-ts               ....................................................................................................
pHW Sp83wt-HA                   ....................................................................................................
pHW Sp83ts20-HA                 ....................................................................................................
```

Figure 5.1 E The comparison of sequence alignment of HA gene segments of SP83wt and SP83ts20.

E (cont.)

```
                                        510       520       530       540       550       560       570
                                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
gi|145284479|gb|EF541410.1| In  GTYDYPQYSEEARLKREEISGVKLESIGIYQILSIYSTVASSLALAIMVAGLSLWMCSNGSLQCRICIICEFRLL
direct seq. HA-wt               ............................................................................
direct seq. HA-ts               ............................................................................
pHW Sp83wt-HA                   ............................................................................
pHW Sp83ts20-HA                 ............................................................................
```

Figure 5.1 E (cont.) The comparison of sequence alignment of HA gene segments of SP83wt and SP83ts20.

F

```
                                  10        20        30        40        50        60        70        80        90       100
                                  ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
gi|145284418|gb|EF541472.1| In   MNPNKKIITIGSICMVTGMVSLMLQIGNLISIWVSHSIHTGNQQKAEPISNTNFLTEKAVASVKLAGNSSLCPINGWAVYSKDNSIRIGSKGDVFVIREP
direct seq. NA-wt                 ....................................................................................................
direct seq. NA-ts                 ....................................................................................................
pHW Sp83wt-NA                     ....................................................................................................
pHW Sp83ts20-NA no.               ....................................................................................................

                                 110       120       130       140       150       160       170       180       190       200
                                  ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
gi|145284418|gb|EF541472.1| In   FISCSHLECRTFFLTQGALLNDKHSNGTVKDRSPHRTLMSCPVGEAPSPYNSRFESVAWSASACHDGTSWLTIGISGPDSGAVAVLKYNGIITDTIKSWR
direct seq. NA-wt                 ....................................................................................................
direct seq. NA-ts                 ....................................................................................................
pHW Sp83wt-NA                     ....................................................................................................
pHW Sp83ts20-NA no.               ....................................................................................................

                                 210       220       230       240       250       260       270       280       290       300
                                  ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
gi|145284418|gb|EF541472.1| In   NNILRTQESECACVNGSCFTVMTDGPSNGQASHKIFKMEKGKVVKSVELDAPNYHYEECSCYPDAGEITCVCRDNWHGSNRPWVSFNQNLEYQIGYICSG
direct seq. NA-wt                 ....................................................................................................
direct seq. NA-ts                 ....................................................................................................
pHW Sp83wt-NA                     ....................................................................................................
pHW Sp83ts20-NA no.               ....................................................................................................

                                 310       320       330       340       350       360       370       380       390       400
                                  ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
gi|145284418|gb|EF541472.1| In   VFGDNPRPNDGTGSCGPVSSNGAYGVKGFSFKYGNGVWIGRTKSTNSRSGFEMIWDPNGWIETDSSFSVKQDIVAITDWSGYSGSFVQHPELTGLDCIRP
direct seq. NA-wt                 ....................................................................................................
direct seq. NA-ts                 ....................................................................................................
pHW Sp83wt-NA                     ....................................................................................................
pHW Sp83ts20-NA no.               ....................................................................................................

                                 410       420       430       440
                                  ....|....|....|....|....|....|....|....|....|...
gi|145284418|gb|EF541472.1| In   CFWVELIRGRPKESTIWTSGSSISFCGVNSDTVGWSWPDGAELPFTIDK
direct seq. NA-wt                 ...........................................
direct seq. NA-ts                 ...........................................
pHW Sp83wt-NA                     ...........................................
pHW Sp83ts20-NA no.               ...........................................
```

Figure 5.1 F The comparison of sequence alignment of NA gene segments of SP83wt and SP83ts20.

G

```
                                     10        20        30        40        50        60        70        80        90       100
                                    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
gi|145112587|gb|EF541445.1| In  MSLLTEVETYVLSIIPSGPLKAEIAQKLEDVFAGKNTDLEALMEWLKTRPILSPLTKGILGFVFTLTVPSERGLQRRRFVQNALNGNGDPNNMDRAVKLY
direct seq. M-wt                    ....................................................................................................
direct seq. M-ts                    ....................................................................................................
pHW Sp83wt-M                        ....................................................................................................
pHW Sp83ts20-M                      ....................................................................................................

                                           110       120       130       140       150       160       170       180       190       200
                                    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
gi|145112587|gb|EF541445.1| In  KKLKREITFHGAKEVALSYSTGALASCMGLIYNRMGTVTTEVAFGLVCATCEQIADSQHRSHRQMATITNPLIRHENRMVLASTTAKAMEQMAGSSEQAA
direct seq. M-wt                    ....................................................................................................
direct seq. M-ts                    ....................................................................................................
pHW Sp83wt-M                        ....................................................................................................
pHW Sp83ts20-M                      ....................................................................................................

                                           210       220       230       240       250       260       270       280       290       300
                                    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
gi|145112587|gb|EF541445.1| In  EAMEIANQARQMVQAMRTIGTHPNSSAGLRDNLLENLQAYQKRMGVQMQRFKSYCCCRKYHWDLALDIVDSSSFLQMHLSSPIRFEKRACYGRGTVYEGR
direct seq. M-wt                    ....................................................................................................
direct seq. M-ts                    ....................................................................................................
pHW Sp83wt-M                        ..................................................................................G.................
pHW Sp83ts20-M                      ....................................................................................................

                                           310       320
                                    ....|....|....|....|....|....
gi|145112587|gb|EF541445.1| In  VPAGTAECCGCRWSFCQHRIGVKNYLVST
direct seq. M-wt                    ............................
direct seq. M-ts                    ............................
pHW Sp83wt-M                        ............................
pHW Sp83ts20-M                      ............................
```

Figure 5.1 G The comparison of sequence alignment of M gene segments of SP83wt and SP83ts20.

H

```
                                        10        20        30        40        50        60        70        80        90       100
                                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
gi|126598924|gb|EF467816.1| In  MDSNTVSSFQVDCFLWHVRKRFADQELGDAPFLDRLRRDQKSLRGRGNTLGLDIETATRAGKQIVERILEEESDKALKMPASRYLTDMTLEEMSRDWFML
direct seq. NS-wt               ..................................................................................................
direct seq. NS-ts               ..................................................................................................
pHW Sp83wt-NS                   ..................................................................................................
pHW Sp83ts20-NS                 ..................................................................................................

                                       110       120       130       140       150       160       170       180       190       200
                                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
gi|126598924|gb|EF467816.1| In  MPKQKVAGSLCIKMDQAIMDKVIILKANFSVIFDRLETLILLRAFTEEGAIVGEISPLPSLPGHTGEDVKNAIGVLIGGLEWNDNTVRVTETIQRFAWRS
direct seq. NS-wt               ..................................................................................................
direct seq. NS-ts               ..................................................................................................
pHW Sp83wt-NS                   ..................................................................................................
pHW Sp83ts20-NS                 ..................................................................................................

                                       210       220
                                ....|....|....|....|....|
gi|126598924|gb|EF467816.1| In  SDEDGRLPLPPNQKRKMARTIESEV
direct seq. NS-wt               .........................
direct seq. NS-ts               .........................
pHW Sp83wt-NS                   .........................
pHW Sp83ts20-NS                 .........................
```

Figure 5.1 H The comparison of sequence alignment of NS gene segments of SP83wt and SP83ts20.

Table 5.1 Summary the sequence comparison of H5N1 strain A/Thailand/3 (SP-83) /2004 from database in the GenBank, SP83wt, and SP83ts20.

Gene	**Amino acid position**	**Strain**		
		SP83-GenBank	**SP83wt**	**SP83ts20**
PB2	194	Q (Gln)	Q (Gln)	H (His)
	195	D (Asp)	D (Asp)	Y (Tyr)
	591	Q (Gln)	Q (Gln)	R (Arg)
PB1	554	D (Asp)	D (Asp)	N (Asn)
PA	448	A (Ala)	A (Ala)	E (Glu)
NP	314	S (Ser)	S (Ser)	N (Asn)

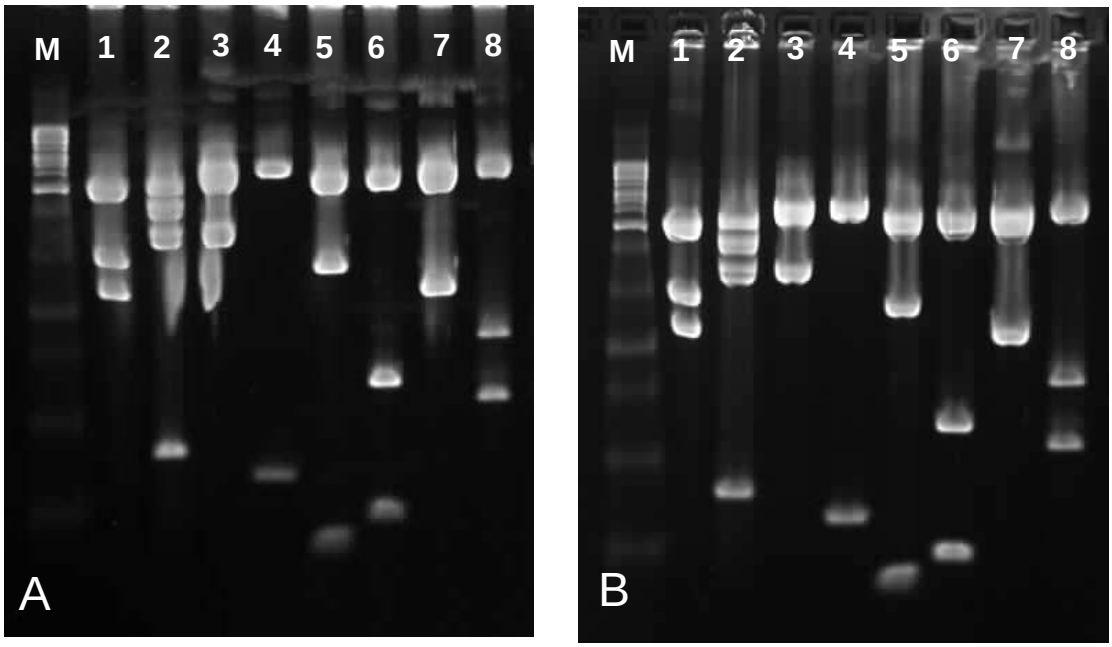


Figure 5.2 The digestion by restriction enzymes for examination of the presence of gene from (A) SP83wt and (B) SP83ts20 insert

Lane M : 1Kb DNA ladder (Fermentas, USA)
Lane 1 : PB2 plasmid digested by *Nco*I and *EcoR*I
Lane 2 : PB1 plasmid digested by *Nco*I
Lane 3 : PA plasmid digested by *Nco*I
Lane 4 : NS plasmid digested by *Nco*I and *Hind*III
Lane 5 : NP plasmid digested by *Nco*I and *PSt*I
Lane 6 : M plasmid digested by *Nco*I
Lane 7 : NA plasmid digested by *Nco*I
Lane 8 : HA plasmid digested by *Nco*I and *EcoR*I

Table 5.2 The extra point mutations in SP83ts20 plasmids during cloning process

Plasmid	Extra Mutations
pHW SP83ts20-PB1	N 554 D
pHW SP83ts20-PA	P 550 L

5.2 Determination of *ts* lesion in SP83ts20

To determine which mutations in SP83ts20 was responsible for temperature sensitive (*ts*) phenotype, the reverse genetics technique was used to generate the reassortant viruses.

5.2.1 Construct a single gene reassortant virus

A single gene reassortant bearing the mutated gene (PB2, PB1, PA, and NP) of SP83ts20 or wild type gene from SP83wt virus in the background of the mouse adapted influenza virus A/Puerto Rico/8/34 (H1N1) were constituted to compare the phenotype. Each set of eight viral plasmids was transfected into coculture of 293T/MDCK cells using Lipofectamine reagent for uptake the plasmids into the nucleus and generate the rescued virus. However, all of the single gene reassortant viruses could be constructed except the virus containing PB2 gene derived from SP83ts20. To solve this problem, the *Dpn*I-site directed mutagenesis was used to generate plasmids containing a single mutation in PB2 of SP83ts20, and these plasmids were taken to do the reverse genetic again, resulting in ten reassortant viruses as shown in Table 5.3.

Table 5.3 The reassortant viruses in PR8 backbone.

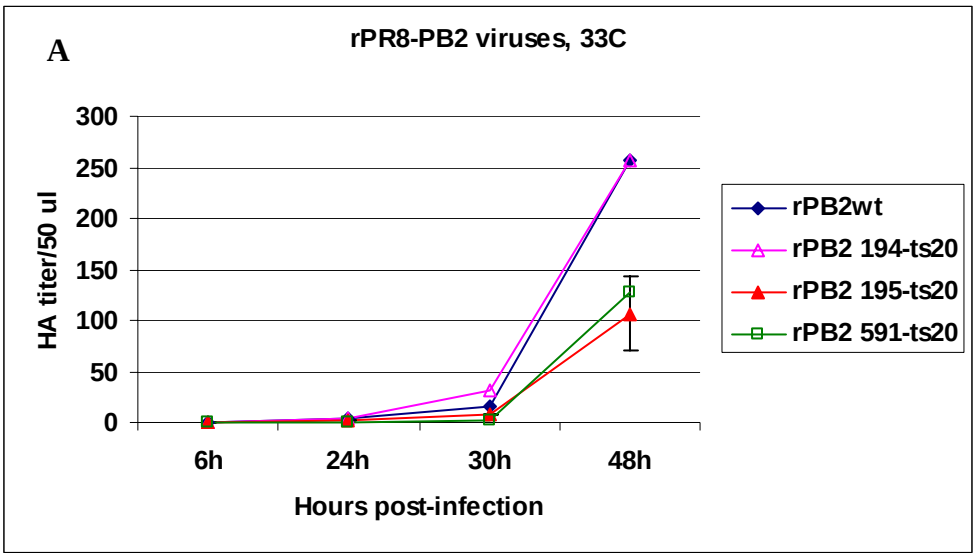
Gene substitution	**Reassortant PR8 virus with**	
	SP83 wt	**SP83 ts20**
PB1	R-PR8-PB1 wt	R-PR8-PB1 ts20
PB2	R-PR8-PB2 wt	R-PR8-PB2 194ts
		R-PR8-PB2 195ts
		R-PR8-PB2 591ts
PA	R-PR8-PA wt	R-PR8-PA ts20
NP	R-PR8-NP wt	R-PR8-NP ts20

5.2.2 Comparison the growth property of reassortant viruses

All of reassortant viruses were evaluated for the temperature sensitive (*ts*) phenotype, relying on the kinetic growth curve. MDCK cells were infected with virus at 55 TCID50 and incubated at 33 or 40ºC. Aliquots of the supernatants were collected at 6, 24, 30, 48 hours post-infection and titrated by HA as shown in Figure 5.3-5.6. At 33ºC, most of all reassortant viruses grew to higher titers than at 40ºC, except PB2-195, PB2-591, and PB1 gene reassortant viruses as shown in Figure 5.3A-5.6A. Beside, most of each virus showed the similar kinetic growth curve with its pair. Thus, this temperature was a permissive temperature for their replication. However, the kinetic growth curve of these viruses changed, when studied at 40ºC. Most of viruses containing gene derived from SP83ts20 showed lower titers when compared with their pair, except the reassortant virus of PB2 gene (rPR8-PB2 viruses) as shown in Figure 5.3B-5.6B. Three PB2 gene reassortant viruses (rPB2-194,-195,-591 ts20) showed similar kinetic growth curves (Figure 5.3B). At each time post infection, they grew to similar titers as compared with wild type PB2 virus (rPB2 wt, blue line). The kinetic growth curve of PB1-ts20 gene reassortant virus (rPB1-ts20) as shown in Figure 5.4B, it grew to similar titers as compared with wild type PB1 virus at 6-30 h p.i., but it showed around 3 fold lower viral titer at 40ºC than its pair (rPB1wt) at 48 h p.i. For the result of PA gene reassortant viruses, rPA-ts20 virus showed more defect in its replication at 40ºC. It could not replicate at 6-24 h p.i. Its viral titer was decline from rPA wt virus at 30 and 48 h p.i. around 10 and 2 fold, respectively. The NP-ts20 gene reassortant virus (rNP-ts20) showed the most interesting result of *ts* phenotype. As shown in Figure 5.6B, rNP-ts20 virus could not grow at 40ºC at 6-30 h p.i., when compared with its pair (rNP wt). At 48 h p.i., it showed appreciably around 40 fold lower viral titers than rNP wt.

To confirm the kinetic growth curve derived from NP gene reassortant viruses, the supernatant of viruses at each time post-infection at 33 and 40ºC was titrated by TCID50 assay. As shown in Figure 5.7A, the kinetic growth curve of these viruses was similar at 33ºC. In contrast, the kinetic growth curve of rNP-ts20 virus showed a clear defect in its replication at higher temperature (40ºC) as shown in Figure 5.7B. The pink line of rNP-ts20 virus grew to lower viral titer than rNP wt (blue line) by average more than 2-3 log at each time post infection.

Thus, the defect in viral growth of rNP-ts20 virus at higher temperature in MDCK cells suggested that NP gene of SP83ts20 determined the temperature sensitive (*ts*) phenotype. In addition, a single mutation in NP-SP83ts20, S314N, is indicated as the *ts* lesion.



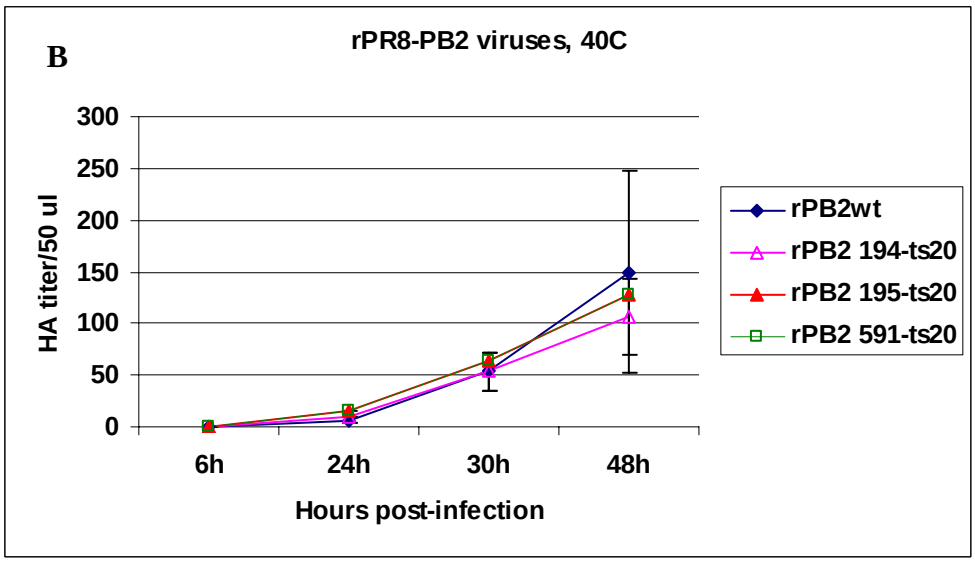


Figure 5.3 Growth kinetics of PB2 gene reassortant viruses in MDCK cells at 33ºC (A), and 40ºC (B). MDCK cells were infected with virus at 55 TCID50 and incubated at 33ºC or 40ºC. Aliquots of the supernatants were titrated by HA at each time point. The values were means (± SD) of the triplicate determinations.

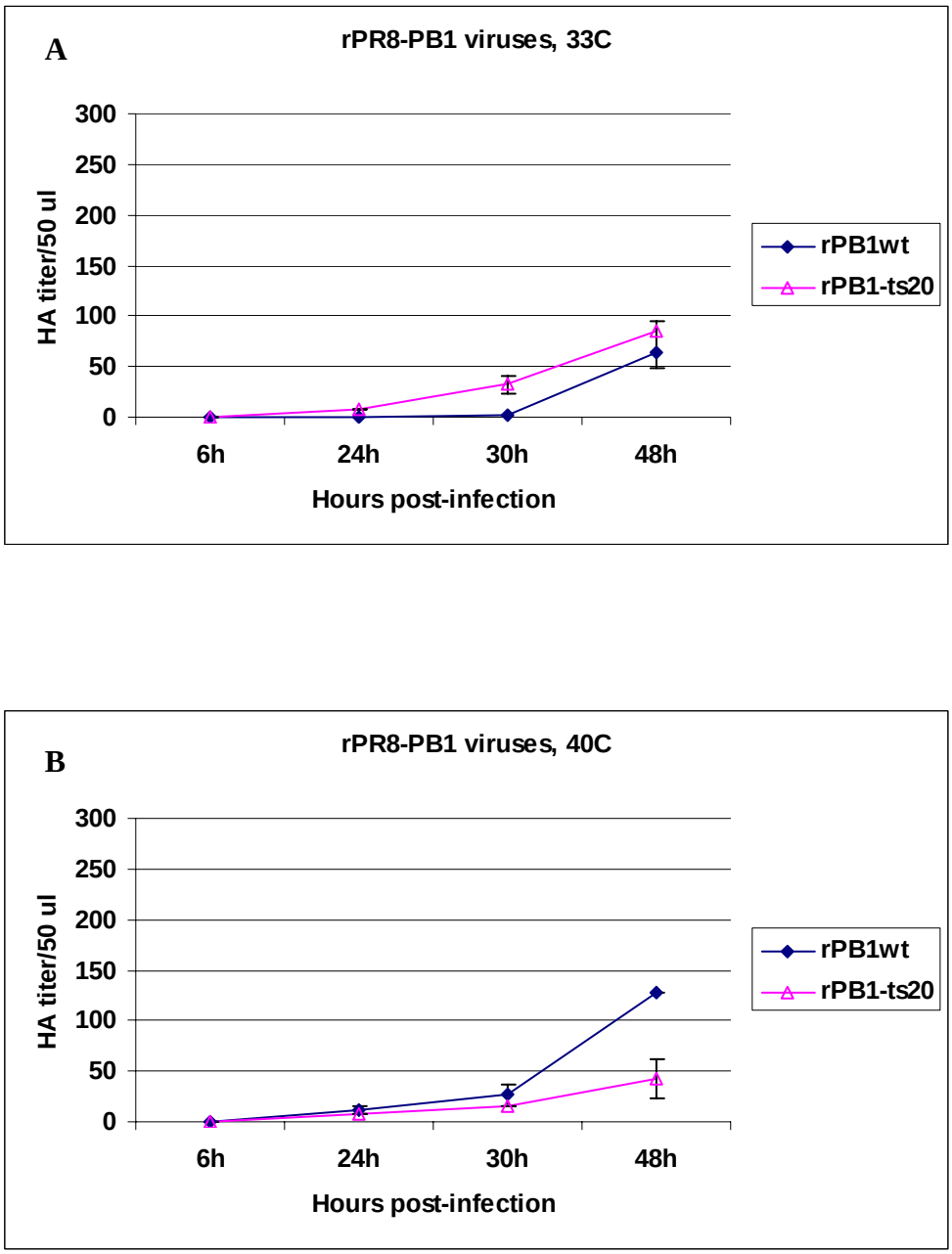
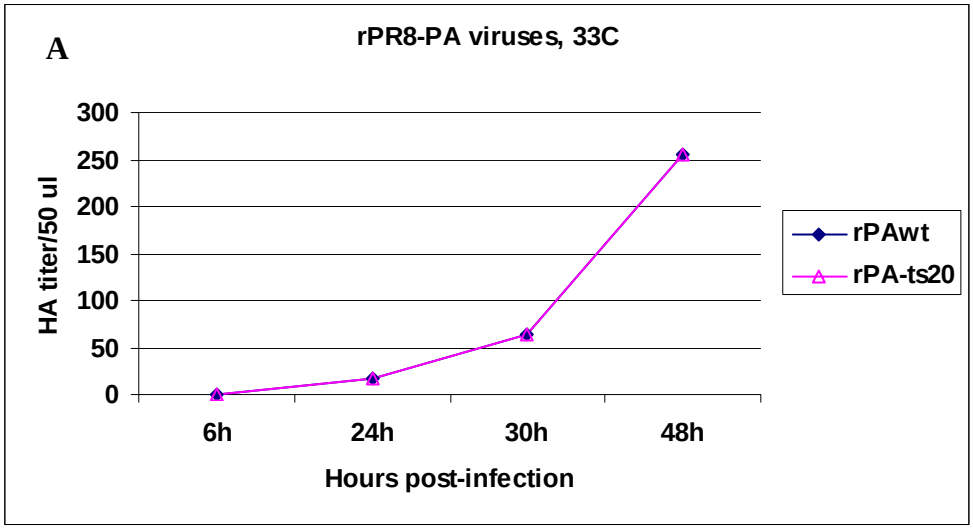


Figure 5.4 Growth kinetics of PB1 gene reassortant viruses in MDCK cells at 33ºC (A), and 40ºC (B). MDCK cells were infected with virus at 55 TCID50 and incubated at 33ºC or 40ºC. Aliquots of the supernatants were titrated by HA at each time point. The values were means (± SD) of the triplicate determinations.



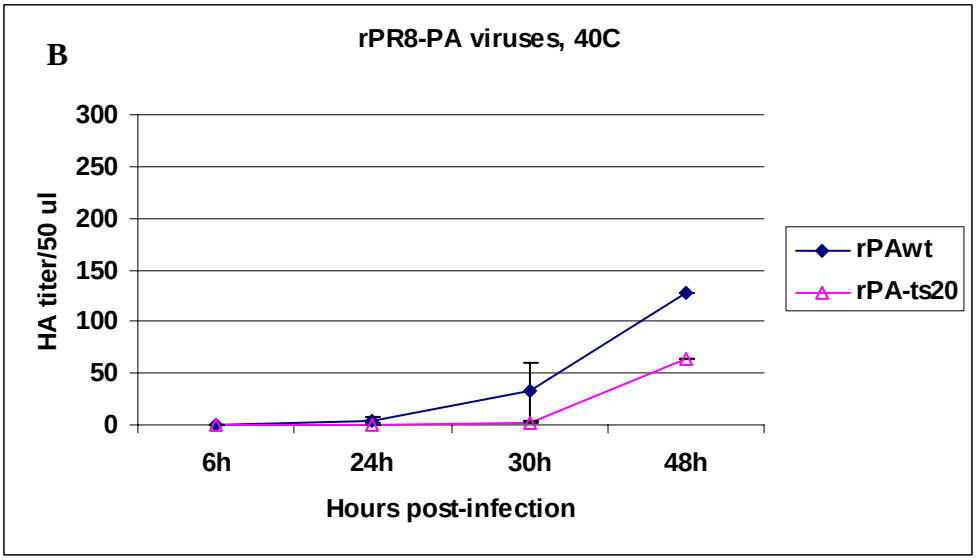


Figure 5.5 Growth kinetics of PA gene reassortant viruses in MDCK cells at 33ºC (A), and 40ºC (B). MDCK cells were infected with virus at 55 TCID50 and incubated at 33ºC or 40ºC. Aliquots of the supernatants were titrated by HA at each time point. The values were means (± SD) of the triplicate determinations.

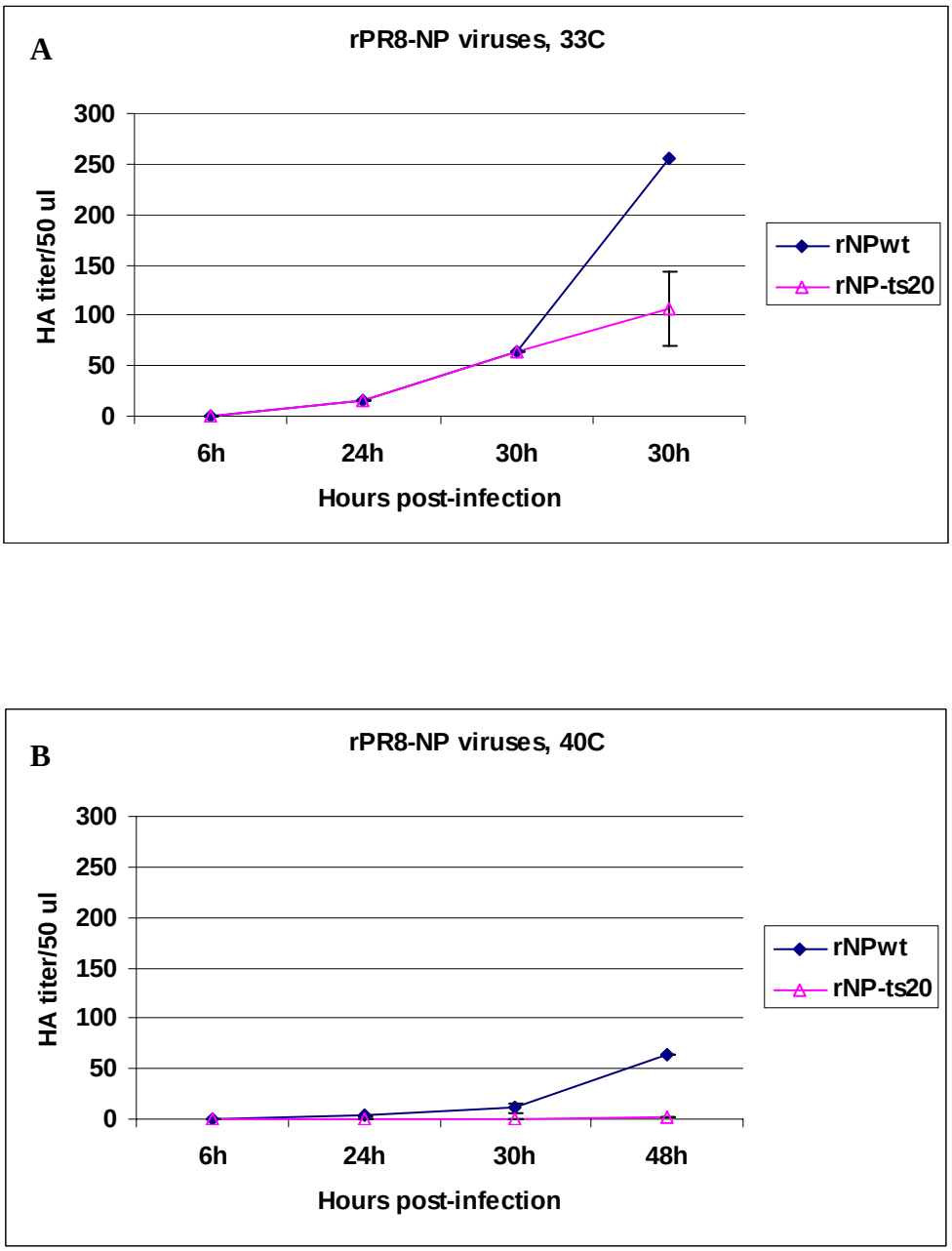
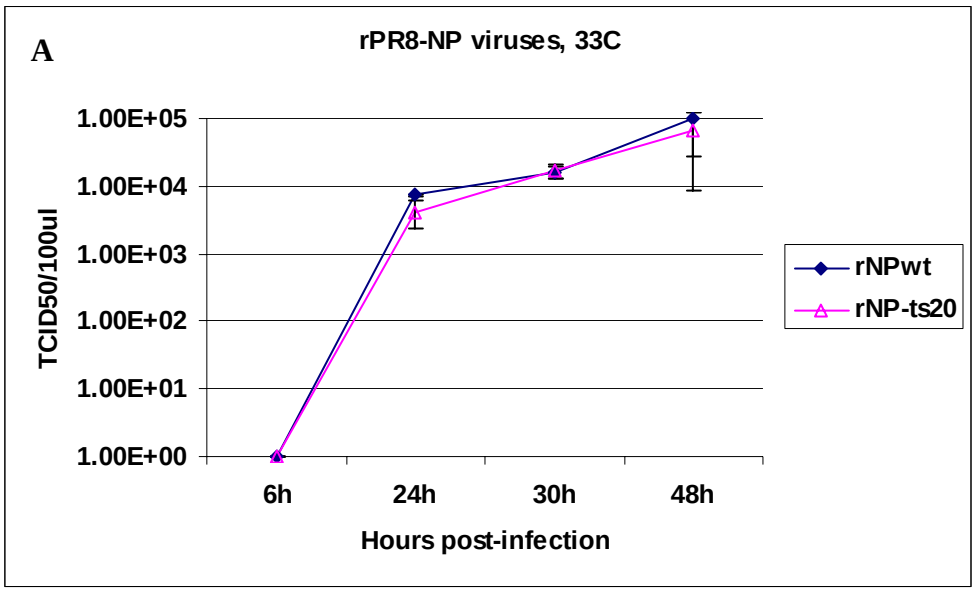


Figure 5.6 Growth kinetics of NP gene reassortant viruses in MDCK cells at 33ºC (A), and 40ºC (B). MDCK cells were infected with virus at 55 TCID50 and incubated at 33ºC or 40ºC. Aliquots of the supernatants were titrated by HA at each time point. The values were means (± SD) of the triplicate determinations.



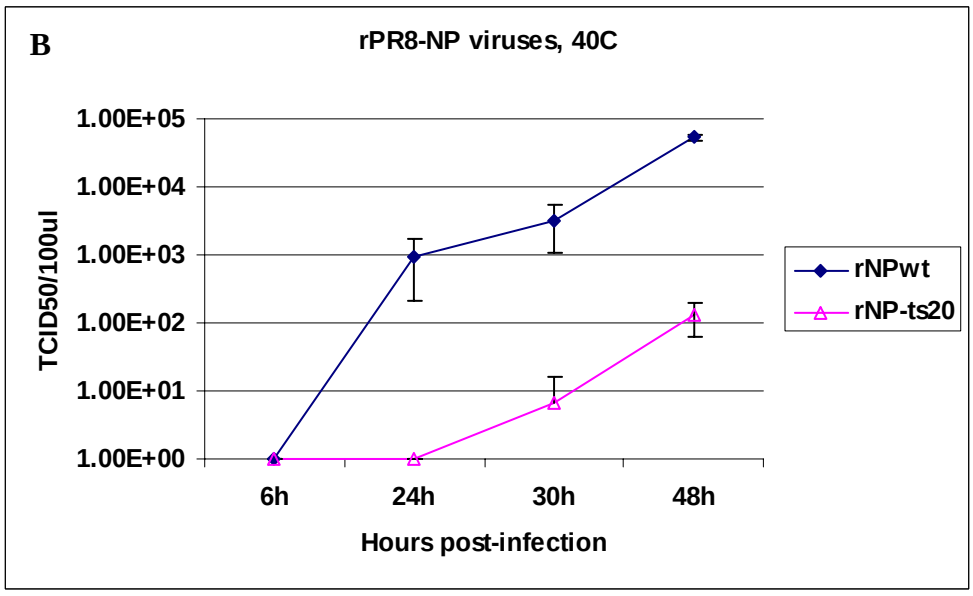
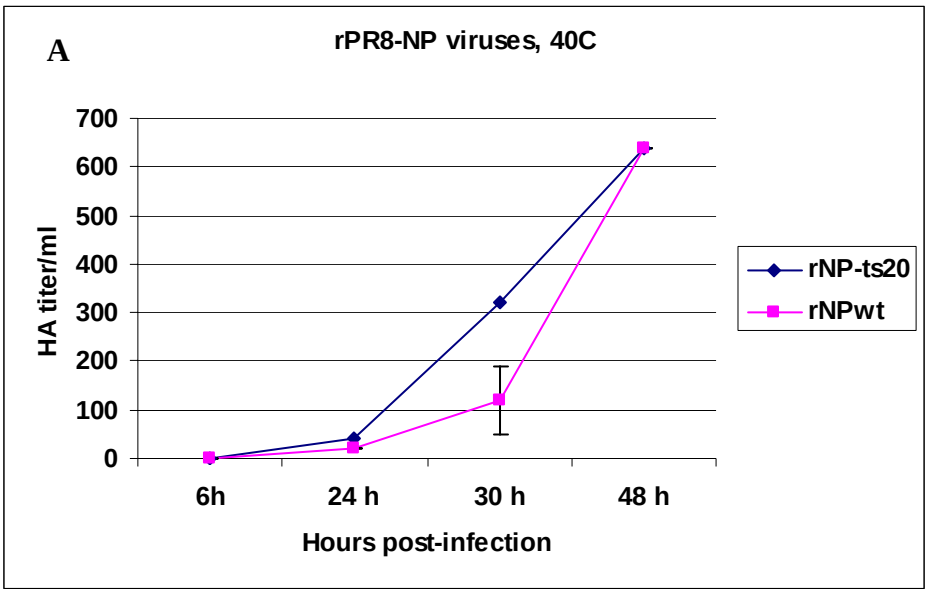


Figure 5.7 Growth kinetics of NP gene reassortant viruses in MDCK cells at 33ºC (A) and 40ºC (B). MDCK cells were infected with virus at 55 TCID50 and incubated at 33 or 40ºC. Aliquots of the supernatants were titrated by TCID50 assay. The values were mean ± SD of the triplicate determinations.

5.3 Determine the effect of the *ts* lesion in avian cells

To test whether *ts* lesion in NP gene, S314N, affect the temperature sensitive phenotype in avian cells, another type of cell lines, CEF cells, was used to examine the growth property of rPR8-NP viruses: rNP-wt and rNP-ts20.

Avian cell lines, CEF cells, were infected with virus at 55 TCID50 and incubated at 40ºC. Aliquots of the supernatants were collected at 6, 24, 30, and 48 hours post-infection and titrated by HA and TCID50. As shown in Figure 5.8A, viral growth in avian cells (CEF cells) of the two reassortant viruses of NP-wt and ts20 was similar. The viral supernatants were also titrated by TCID50 assay to confirm the results, which were similar in the HA test (Figure 5.8B). In addition, the growth ability of virus containing NP-ts20 gene in CEF cells was seemingly better than in MDCK at 40ºC, so these results indicated that the *ts* lesion of NP S314N did not take effect in avian cells.



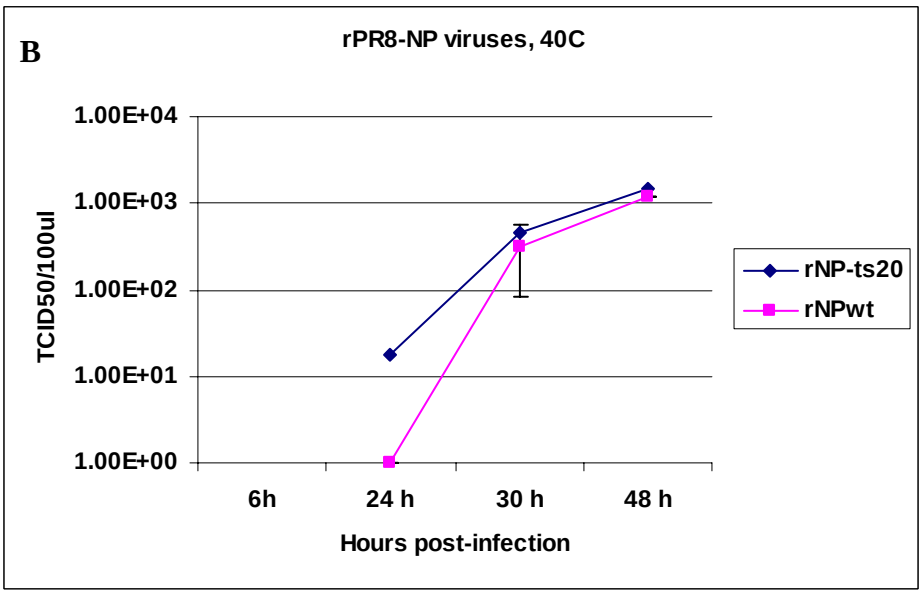


Figure 5.8 Growth kinetics of rPR8-NP viruses in CEF cells at 40ºC. Cells were infected with virus at 55 TCID50 and incubated at 40ºC. Aliquots of the supernatants were titrated by HA (A) and TCID50 (B) at each time point. The values were means (± SD) of the triplicate determinations.

5.4 Analyses of NP subcellular localization

The NP protein has been shown to play important roles at the many steps of the virus life cycle. As well as in the step of import the vRNPs into the nucleus of infected cells, NP is capable of binding with cellular protein, importin α, and the interaction of two proteins contribute to nuclear uptake of vRNPs. In the previous study of Gabriel et al. [69], the mutation of NP N319K of SC35M (H7N7) showed increasing ability in binding with importin α in mammalian cells, but not avian cells, resulting in higher viral replication.

To determine whether the result of lower viral titer of virus containing *ts* lesion (NP S314N) was associated with the defect in nuclear importation, the experiment was done by comparing the NP import into the nucleus of both wild type and mutant NP. 293T cells were transfected with the plasmid of NPwt or NPts20 and incubated at 33 or 40ºC for 48 hours. Then, cells were fixed with 80% acetone and determined the subcellular localization of NP by indirect immunofluorescence assay. As shown in Figure 5.10, NP localization in transfected cells was markedly different between NP plasmids derived from wild type and mutant gene. At 40ºC, the specific monoclonal antibody to NP protein could predominately detect the localization of NP wild type in the nucleus more than in cytoplasm (Figure 5.10 B), while the signal detection of NP from SP83ts20 was almost exclusively present only in the cytoplasm (Figure 5.10 A). Moreover, at 33ºC, both of NP wild type and SPts20 proteins could detect the NP localization in the nucleus of transfected cells as shown in Figure 5.9A and B. Therefore, these results suggested that the nuclear accumulation of NPwt protein correlated with the efficiency of viral replication, resulting in get a high viral titer, while lower viral titer of virus bearing single gene of NP-SP83ts20 involved with the mutation in NP (S314N), which may affect to the interaction of NP and importin α that contributed to the defect of vRNPs transport into the nucleus.

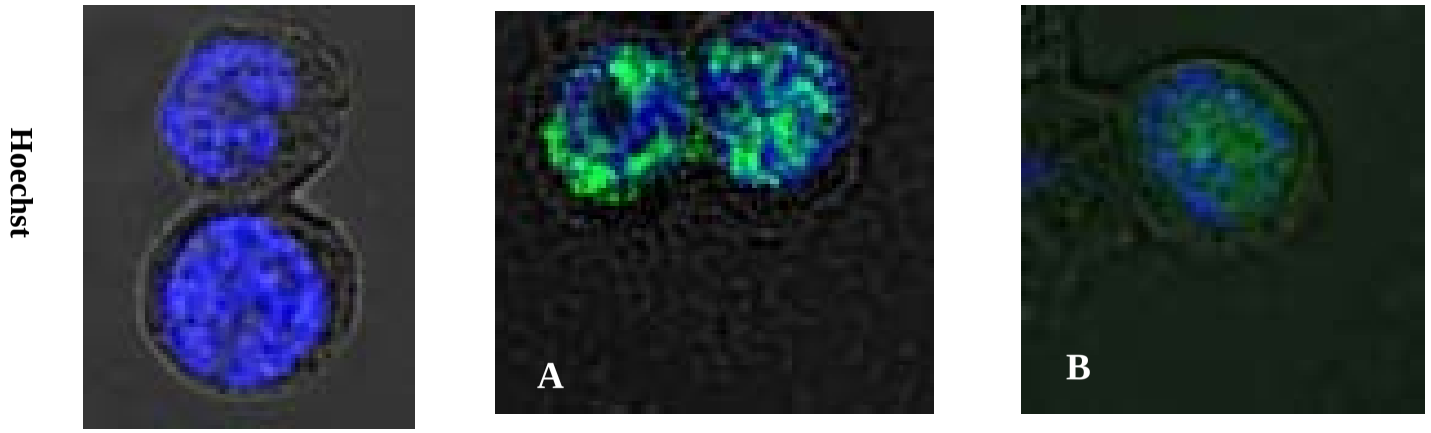


Figure 5.9 Localization of (A) NPts20 and (B) NPwt in 293T cells at 33ºC. 293T cells were transfected with 1 µg of plasmid and analyzed by indirect immunofluorescence assay. For detection of NP protein, FITC secondary antibody was used. Cells nuclei were stained with Hoechst dye.

Figure 5.10 Localization of (A) NPts20 and (B) NPwt in 293T cells at 40ºC. 293T cells were transfected with 1 μg of plasmid and analyzed by indirect immunofluorescence assay. For detection of NP protein, FITC secondary antibody was used. Cells nuclei were stained with Hoechst.

CHAPTER VI
DISCUSSION

This thesis studied the genetic determinant of temperature sensitivity of SP83ts20: the variant of H5N1 virus strain A/Thailand/3 (SP-83)/2004. The reverse genetic technique was used to generate a single gene reassortant carrying the mutated gene of SP83ts20 virus in the background of a mouse adapted influenza virus A/Puerto Rico/8/34 (H1N1). These reassortant viruses were used to test kinetic growth curve at 33 or 40ºC for determining *ts* phenotype. Here, it showed that a single mutation in NP gene: serine to asparagines at position 314 (S314N), was responsible for *ts* phenotype in mammalian cells. At 33ºC or permissive temperature, both rNP-ts20 and rNP-wt viruses were grew in quite similar titer, while at 40ºC or non-permissive temperature, rNP-ts20 virus showed a clear defect in kinetic growth curve.

Interestingly, the kinetic growth curve between rPR8-NPwt and rPR8-NPts20 at 40ºC in avian cells was not difference. Thus, it was possible that this mutation did not interfere the efficiency of viral polymerase in avian cells and may involve in the cell type specific. This observation supported the concept that the virus goes through a phase that allows gradual acquisition of the genetic mutations without losing fitness for the natural host [69].

As many previous studies, the researcher reported the *ts* lesion in NP gene [54, 70, 71, 72]. There is abundant knowledge showing that NP protein plays some important role in many steps of viral life cycle. Influenza A virus RNA segment 5 encodes NP (a polypeptide of 498 amino acids in length), which is a 56 kDa and rich in arginine, glycine and serine residues. The NP structure contains several functional domains (as refered to Figure 3.5, Chapter III) that are able to interact with a variety of macromolecules of both viral and cellular origins as summarized in Table 3.2, Chapter III. It seems reasonable to conclude that the functions of NP in the virus life cycle are mediated through these binding interactions [73]. The role of NP during the virus life

cycle comprises of viral RNA synthesis, RNA trafficking, and posttranscriptional steps [55, 73].

Most NP *ts* mutants that have been previously characterized show defects in vRNA synthesis at the nonpermissive temperature [70, 71]. It is believed that NP plays a role in the switch from mRNA transcription to genome replication. Medcalf et al. 1999, studied the functional defect in two NP *ts* lesions, which were derived from temperature sensitive viruses A/WSN/33 *ts*56 (NP S314N) and A/FPV/Rostock/34/Giessen *ts*G81 (NP A332T). They introduced these mutations into the NP of influenza virus strain A/PR8/8/34. The results of their analysis showed that the defect in replicative transcription involved disruption of RNP-binding activity of NP. This result suggested that an NP-RNA interaction was essential to support genome replication. There are two hypotheses that support this result in the switching mechanism: the encapsidation and template modification (refer to Figure 3.13, Chapter III 6). The encapsidation hypothesis proposes that NP does not have any regulatory function in determining between cap-primed and unprimed transcription initiation, but NP is required to co-transcriptionally coat the nascent c- and vRNA segments [54, 72]. Alternatively, the template modification hypothesis holds that the interaction of free NP (that is not already present in the RNP structure) with the promoter element of template RNA can alter mode of transcription initiation and termination by disrupting the partially base paired at terminal sequence of vRNA to form panhandle structure [74, 75]. The viral polymerase recognizes the panhandle structure of vRNA, stimulates the cap-snatching and endonuclease activities to generate mRNA transcription initiation and polyadenylation [54].

Although, in the study result of Medcalf et al. 1999, they showed that two *ts* lesions of NP S314N and A332T did not substantially alter the cellular NP accumulation or distribution of NP, the result derived from NP S314N in H5N1 variant (SP83ts20) of this study showed defect in the NP import into the nucleus. Analysis of subcellular localization of NP in tranfected cell by indirect immunofluorescence assay found that the predominant location of NP of SP83ts20 was in the cytoplasm, while NP of wild type virus was detected mostly in the nucleus. However, their study did not compare cellular distribution of NP wild type at either temperature. Thus, the suggestion of conflictive result may involve in these difference

factors: origin of NP (human and avian viruses), type of transfected cell (BHK and 293T cells), dose of NP plasmid (3 or 300 ng and 1 μg), time of incubation (4 and 48 hours), and temperature of incubation (31 or 37ºC and 33 or 40ºC). This suggestion could support by the review article of Portela et al. 2002.They showed that the distribution of NP depended on cell type and, possibly, virus strain [73]. So, it is plausible that *ts* lesion of NP S314N of SP83ts20 affected RNA trafficking and transport into the nucleus.

The vRNPs crossing of nuclear membrane by NP is a key event for influenza virus life cycle. NP is shown to be sufficient to mediate the nuclear import of vRNAs [27]. The NP can interact with karyopherin (importin) α because of its nuclear localization signal (NLS). The presence of three NLSs on the NP of influenza A viruses as shown in Figure 3.5, Chapter III. An unconventional NLS (M_1ASQGTKRSYEQM_{13}) is at the very N-terminus. The second NLS residue (K_{198}RGINDRNFWRGFNGRRTR$_{216}$) in the central part of NP is the bipartite signal appears to be weaker than the unconventional NLS [76]. The third NLS proposed to be located between amino acid 320 and 400 [77, 78]. Karyopherin alpha 1 is thought to interact with the vRNP, recruiting karyopherin beta into a trimeric complex that docks at the nuclear pore complex (NPC). Nuclear translocation requires Ran and P_{10} in the presence of energy provided by an ATP-generating system as shown in Figure 3.7, Chapter III.

The study of Gabriel et al. 2008 [69], reported the effect of adaptive mutation in NP gene of SC35M (H7N7 virus) at position 319: asparagines to lysine (NP N319K). This mutation was responsible for enhancing polymerase activity and viral replication in mammalian cell, but not avian cell. They demonstrated that adaptation of NP could enhance binding of this protein to importin α1 of mammalian origin, resulting in improvement of vRNPs transport into the nucleus. Although, this mutation was not included in three NLSs, suggesting that NP N319K may modulate one of the NLSs identify on NP by an allosteric mechanism. Thus, I proposed that the defect in viral replication of SP83ts20 at higher temperature may explain by same mechanism. The *ts* lesion of NP S314N of SP83ts20 may give the opposite result with NP N319K by interfering the binding with importin α1 in mammalian cells. Therefore, the possibility of interaction between them was difficult to occur, and the importation of vRNPs into the nucleus would decline. This mechanism reflected to the low

efficiency of viral replication and reducing of viral titer. However, in order to clarify the interaction of NP S314N with importin α1 needs further studies.

This thesis succeeded in identifying the genetic determinant of temperature sensitivity of *ts* mutant H5N1 virus strain A/Thailand/3 (SP-83)/2004. The current knowledge of *ts* lesion can be applied widely, for example, make us understand the influenza virus life cycle or to generate live-attenuated vaccines [64]. Nowadays, the vaccination is the most effective means to prevent influenza epidemic. Large scale clinical studies in young children have demonstrated that live attenuated influenza vaccine significantly reduced the number of influenza cases compared to inactivated vaccine [79]. The *ts* phenotype is the one important character of live attenuated influenza vaccine other than cold-adapted, *ca* and attenuated *att* phenotype. This property confers the safety of vaccine in humans by restricting its replication in the upper respiratory tract but not in the lower respiratory tract. Thus, the mutation of NP S314N indicated as the genetic marker of *ts* lesion in both of human and avian viruses, may be useful in development of live attenuated influenza vaccine for protecting us. In addition, the *ts* determinant may provide an insight into the mechanism of the viral thermal tropism.

CHAPTER VII
CONCLUSION

The *ts* mutant virus (SP83ts20) was derived from a wild type A/Thailand/3 (SP-83)/2004 (SP83wt) by serial adaptation at low temperature. The SP83ts20 virus did not grow at 40ºC, thus, this thesis had the aim to identify its genetic determinant of temperature sensitivity. Single gene reassortant viruses were generated using reverse genetic system. Each virus carried one mutated gene from SP83ts20 or wild type gene from SP83wt in a backbone of a mouse adapted influenza virus, A/Puerto Rico/8/34 (H1N1). These viruses were characterized for the *ts* phenotype using kinetic growth curve assay at 33 or 40ºC. NP gene derived from SP83ts20 conferred *ts* phenotype in mammalian, but not avian cells. Moreover, the NP gene contained only a single point mutation at the position 314: serine to asparagine (NP S314N). The plausible mechanism to explain the *ts* phenotype was vRNPs nuclear importation. The NP S314N protein showed a defect in nuclear uptake at 40^{o}C.

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APPENDICES

A. Reagent for polymerase chain reaction (PCR) and gel electrophoresis

1. 10 mM Deoxynucleotide triphosphate (dNTPs) mixture

Each dATP, dGTP, dCTP, and dTTP is supplied in a vial of concentration 100 mM

dATP	25	µ1
dGTP	25	µ1
dCTP	25	µ1
dTTP	25	µ1
DW	900	µ1

Store 10 mM dNTPs mixture at -20ºC.

2. 6x gel loading dye

Bromphenol blue	0.25%
Xylene cyanol FF	0.25%
Ficoll in water	15%

Store 6x gel loading dye at 4ºC.

3. TBE buffer

3.1 TBE stock solution (5X)

Tris-base	54	g
Boric acid	27.5	g
0.5 M EDTA, pH 8.0	20	ml

Adjust the volume to 1 liter with distilled water and store at room temperature.

3.2 TBE working solution (0.5X)

TBE stock solution	100	ml
DW	900	ml

Store at room temperature.

4. 1% agarose gel

Agarose gel	0.2	g
TBE working solution (0.5X)	20	ml

5. Ethidium bromide

Ethidium bromide(10mg/ml)	40	µ1
DW	400	ml

B. Reagents for molecular cloning

1. Competent cells preparation

1.1 Reagent preparation

CM1 (25 ml)

10 mM NaOAC pH 5.6	0.25 ml	of	1M NaOAC stock
50 mM $MnCl_2$	1.25 ml	of	1M $MnCl_2$ stock
5 mM NaCl	0.125 ml	of	NaCl stock
DW	23.375 ml		

CM2 (10 ml)

10 mM NaOAC pH 5.6	0.1 ml	of	1M NaOAC stock
70 mM $CaCl_2$	0.7 ml	of	1M $CaCl_{2\ stock}$
5 mM $MnCl_2$	0.05 ml	of	1M $MnCl_2$ stock
5% glycerol	0.5 ml	of	glycerol
DW	8.65 ml		

CM1 and CM2 reagent were filtered by using 0.45 µm millipore membrane before used.

1.2 Competent cell

Streak frozen bacteria stock onto LB plate and incubate overnight. Pick 1 single colony for inoculate in 2 ml LB broth and shake overnight at 37ºC. Transfer 1 ml into 100 ml LB broth and shake at 37ºC for 2-3 hours until reaching the OD of 0.45-0.5. After that transfer 100 ml LB broth to 2x50 ml tube, incubate on ice for 5

miniutes and centrifuge at 3,000 rpm for 10 minutes at 4ºC. Discard supernatant and add CM1 10ml/50 ml culture. This suspension incubates on ice for 45 minutes, meanwhile, cool tubes in -80ºC freezer. After that, centrifuge at 3,000 rpm for 5 minutes at 4ºC, discard supernatant and add 1 ml CM2/50 ml culture. Finally, aliquots 100 µ1 cells/ tube and store at -80ºC freezer.

2. Ampicillin 50 mg/ml

Ampicillin	500	mg

Add sterile distrilled, deionized water to 10 ml. Aliquot 1 ml and store at-20ºC

3. Lauria Bertani (LB) medium

3.1 LB agar plate with 50 µg/ml ampicillin

Bacto peptone	10	g
Yeast Extract	5	g
NaCl	10	g
Bacteria agar	15	g

Adjust the volume to 1 liter with distilled water. Sterilize by autoclaving at 121ºC under pressure of 15lb/square inch for 15 minutes. After medium has been autoclaved, allow it to cool to 60ºC or less then add 1 ml of 50 mg/ml ampicillin and pour the plate.

3.2 LB broth

Bacto peptone	10	g
Yeast Extract	5	g
NaCl	10	g

Adjust the volume to 1 liter with distilled water. Sterilize by autoclaving at 121ºC under pressure of 15lb/square inch for 15 minutes. Before using, add ampicillin to be 50 µg/ml ampicillin.

4. X-Gal plate

X-Gal (50mg/ml)	20	µ1
0.1 M IPTG	100	µ1

Add two reagents and spread on LB/Amp plate.

5. SOC medium

Bacto peptone	20	g
Yeast Extract	5	g
NaCl	0.5	g
250 mM KCl	10	ml

Adjust the volume to 1 liter with distilled water. Sterilize by autoclaving at 121ºC under pressure of 15lb/square inch for 15 minutes.

Before used add

2M $MgCl_2$	5	ml
1M Glucose	20	ml

Sterile through filteration with 0.2 µm millipore membrane and store at 4 ºC.

C. Reagent for alkaline lysis plasmid extraction

1. Solution I

50 mM Glucose
25 mM Tris-HCl (pH 8.0)
10 mM EDTA (pH 8.0)

Sterilize by autoclaving at 121ºC under pressure of 15lb/square inch for 15 minutes and store at 4ºC.

2. Solution II (freshly prepare)

10 N NaOH
10% SDS
DW

3. Solution III

5M potassium acetate	60	ml
Glacial acetic acid	11.5	ml
DW	28.5	ml

The resulting solution is 3M with respect to potassium and 5M with respect to acetate. Store at 4ºC.

D. Reagent for restriction enzyme digestion

1. Check for colonies with insert

Enzyme	0.2	µ1
10x buffer	2	µ1
100x BSA	Depending on enzyme	
Miniprep DNA	1-2	µ1
DW	Volume up to 15 µ1	

Incubate the reaction at 37ºC for 1-3 hour.

2. For digest PCR fragment

Enzyme	~ 1-2 U/ 1µg DNA
10x buffer	10% of total volume
100x BSA	Depending on enzyme
DNA	1-5 µg
DW	Volume up to 50 µ1

Incubate the reaction at 37ºC for 1 hour to overnight.

E. Reagent for cell culture

1. Minimal Essential Medium (MEM)

1.1 Stock solution 10X

MEM powder	93.5	g

Add sterile distilled deionized water to 1 liter and sterile through filtration with 0.45 µm Millipore membrane. Aliquot 100ml/bottle and store at -20ºC.

1.2 Working solution 1X

MEM 10X	50	ml
Penicillin 40,000 U/ml	2.5	ml
Gentamycin 4 mg/ml	2.5	ml
Fungizone 1mg/ml	0.5	ml
5% $NaHCO_3$	10	ml

Add sterile distilled deionized water to 500 ml and store at 4ºC.

1.3 Growth Medium (10% FBS in MEM)

MEM (working solution 1X)	90	ml
Fetal bovine serum	10	ml

Store at 4ºC.

1.4 Maintainance media for influenza virus infection

MEM (working solution 1X)	100	ml
Trypsin-TPCK 500 µg/ml	0.5	ml

Store at 4ºC.

2. 0.125% Trypsin-EDTA

0.25% Trypsin-EDTA (GIBCO, USA)	50	ml
PBS	50	ml

Store at 4ºC.

3. Phosphate buffer saline (PBS), pH 7.2

3.1 PBS stock solution (10X)

NaCl	80.0	g
KCl	2.0	g
KH_2PO_4 (anhydrous)	1.2	g
Na_2HPO_4 (anhydrous)	9.1	g

Add the sterile distilled deionized water to 1 liter and adjust the pH to 7.2 by 1N NaOH or 1N HCl. Sterile by autoclaving at 121ºC under pressure of 15lb/square inch for 15 minutes and store at room temperature.

3.2 PBS working solution (1X)

PBS stock solution (10X)	100	ml
Sterile DW	900	ml

Store at 4ºC.

4. Trypsin-TPCK 500 µg/ml

TPCK-trypsin	10	ml
(Sigma-Aldrich, USA)		
MEM 1X	20	ml

Steriled through filtration with 0.45 µm Millipore membrane. Aliquot 200 µ1/tube and store at -20ºC.

5. 5% $NaHCO_3$

$NaHCO_3$	25	g

Add the sterile distilled deionized water to 1 liter and sterile through filtration with 0.45 µm Millipore membrane. Aliquot 50 m1/tube and store at 4ºC.

6. Antibiotics

6.1 Penicillin 40,000 U/ml (10X)

Penicillin	1,000,000 U/ bottle

Add the sterile distilled deionized water to 25 ml. Aliquot 100 m1/tube and store at 4ºC.

6.2 Gentamycin 4 µg/ml (10X)

Gentamycin	80	mg

Add the sterile distilled deionized water to 20 ml. Aliquot 100 m1/tube and store at 4ºC.

6.3 Fungizone 1mg/ml (10X)

Fungizone	50	mg

Add the sterile distilled deionized water to 50 ml. Aliquot 100 m1/tube and store at 4ºC.

F. Reagent for ELISA in TCID50

1. Washing buffer (0.05% Tween)

1X PBS pH 7.2	1000	ml
Stock Tween 20	0.5	ml

Store at 4ºC.

2. Blocking reagent (3% H_2O_2)

1X PBS pH 7.2	100	ml
BSA	1	g
Tween 20	100	µ1

Store at 4ºC.

3. Primary Ab in blocking buffer (freshly prepare)

Blocking buffer	5	ml
Primary Ab	1	µ1

4. Secondary Ab in blocking buffer (freshly prepare)

Blocking buffer	5	ml
Secondary Ab	2.5	µ1

5. TMB substrate and peroxidase (freshly prepare)

TMB substrate	3	ml
Peroxidase	3	ml

6. Stop reaction reagent (1M H_2SO_4)

Stock H_2SO_4	5	ml
DW	85	ml

Store at room temperature.

G. Reagent for immunofluorescent assay

1. Fix reagent (80% acetone)

1X PBS pH 7.2	20	ml
100% acetone	80	ml

Store at 4ºC.

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