CHAPTER IV
MATERIALS AND METHODS

1. Avian influenza A (H5N1) virus, Thailand isolate and total RNA isolation

A highly pathogenic avian influenza (HPAI) H5N1 virus [influenza A/duck/144/ Thailand/2005(H5N1)] isolated from lungs of infected duck from Nakhon-pathom province, Thailand, in 2005 was used for total RNA isolation. TRIzol reagent (Invitrogen, USA) was used to isolate the viral RNA.

Two hundred microliters of inactivated virus sample was mixed with 1 ml of TRIzol® Reagent (Invitrogen, CA, USA), then 200 µl of chloroform was added to the mixture and mixed by shaking the tube vigorously for 15 seconds. The mixture was kept at 25ºC for 3 minutes and centrifuged at 12,000 × *g*, 4ºC, for 15 minutes. The top layer containing RNA was transferred to a new tube and the RNA was precipitated by adding 0.5 ml of cold isopropranol, mixed well, and incubated at 25ºC for 10 minutes. The tube was centrifuged at 10,000 × *g*, 4ºC for 10 minutes. The RNA pellet was washed once with 1 ml of 75% DEPC-treated ethanol. After centrifugation at 7,500 × *g*, 4ºC for 15 minutes, the total RNA pellet was air-dried. The preparation was added with 10 µl of DEPC-treated water to completely dissolve. The RNA was kept at -70^{o}C until use.

2. Complementary DNA (cDNA) synthesis

Complementary DNA (cDNA) was produced from the extracted total RNA by RT-PCR using ThermoScript™ Revers Transcriptase (Invitrogen, USA). The first strand cDNA synthesis was performed in an RNase-free microcentrifuge tube by preparing the following reaction mixture in a tube kept on ice: 9 µl of total RNA, 2 µl of uni12 primer (AGCAAAAGCAGG) (25 µM) and 2 µl of dNTPs (10 mM). The preparation was mixed gently, centrifuged for 3-5 seconds, and incubated at 65^{o}C for 5 minutes. The tube was placed on ice for 1 minute and the following components were sequentially added: 4 µl of 5x cDNA synthesis buffer, 1 µl of 0.1 M dithiothreitol (DTT), 1 µl of RNaseOUT™ (40 units) and 1 µl of ThermoScript™ Reverse Transcriptase (15 units). The mixture was incubated at 65^{o}C for 1 hour and followed

by 85^{o}C for 5 minutes. The synthesized first strand-cDNA was used as a template for amplification of the polymerase gene segments of the influenza A virus (H5N1). The quality of the cDNA was checked by PCR amplification of H5 gene sequence using the cDNA as a template. The specific primers for amplifying the hemagglutinin 5 gene sequence were: forward, 5'-ACACATGCYCARGACATACT-3' and reverse, 5'-CTYTGRTTYAGTGTTGATGT -3'. The PCR reaction mixture (25 µl) consisted of: 2 µl of cDNA, 0.5 µl of each primer (20 µM), 2.5 µl of 10x buffer with KCl, 3 µl of 25 mM $MgCl_2$, 2 µl of 10 mM dNTPs, 0.2 µl of 5 units/µl *Taq* DNA polymerase (Fermentas), and ultra-pure distilled water (UDW). PCR condition consisted of 1 cycle denaturing at 95ºC for 3 minutes, 35 cycles of annealing at 50ºC for 30 seconds, extending at 72ºC for 45 seconds, and denaturing at 95ºC for 30 seconds. The final chain elongation was performed at 72^{o}C for 10 minutes. After amplification, the PCR product was analyzed by 1% agarose gel electrophoresis and ethidium bromide staining.

3. Primer design for amplification of 5'-halves of PA, PB1 and PB2-coding DNA sequences

The primers for amplification of 5'-halves of PA, PB1 and PB2-coding DNA sequences (designated: *PA-5', PB1-5'* and *PB2-5'*, respectively) were designed using avian influenza A (H5N1) virus sequences that were available in Genbank database, *i.e.,* accession no. AY551934.1, AY626147.1, AY626148.1, AY646179.1, AY627897.1, AY651713.1, AY651715.1, and AY651716.1. The primer sequences are shown in **Table 4**.

Table 4 Primer names, their sequences, and sizes of DNA amplicons.

Primer name	**Sequence**	**PCR product (base pairs; bp)**
PA-5’;		720
Forward	5’-AGGATCCAATGGAAGACTTTGTG-3’	
Reverse	5’-AAAGCTTGCCGTTCGGTTCG-3’	
PB1-5’;		1,134
Forward	5’-AGGATCCAATGGATGTCAATC-3’	
Reverse	5’-ACTCGAGAAGATCAATGTTTGCAAGC-3’	
PB2-5’;		1,212
Forward	5’-TGTCGACATGGAGAGAATAAAAG-3’	
Reverse	5’-ACAAGCTTGAACACCATTGCTAC-3’	

4. PCR amplification of *PA-5', PB1-5'* and *PB2-5'* sequences

PA-5', PB1-5' and *PB2-5'* sequences were amplified using a Thermo Cycler (Eppendorf, Germany). The first strand cDNA in (**2**) and the primers in (**3**) were used. The PCR reaction mixture (25 µl) consisted of: 2 µl of cDNA, 0.5 µl of each primer (20 µM), 2.5 µl of 10x pfu buffer with $MgSO_4$, 2 µl of 10 mM dNTPs, 0.25 µl of 2.5 units/µl pfu DNA polymerase (Fermentas), and UDW. PCR condition consisted of denaturing at 94ºC for 3 minutes, 35 cycles of annealing at 45°C for 1 minute, extending at 72ºC for 1 minute, and denaturing at 94ºC for 1 minute. The chain elongation was performed at 72°C for 10 minutes. The PCR product was verified as in section (**2**) above.

5. Agarose gel electrophoresis

PCR product or extracted plasmid was electrophoresed in 1% agarose gel (USB Corp, USA) (**Section 7, Appendix A**) in 1x TAE buffer (**Section 3, Appendix A**). The melted agarose was casted, allowed to solidify for at least 20 minutes, and submerged in 1x TAE buffer contained in a minicell electrophoresis set (Mupid, Tokyo, Japan). DNA samples were mixed in a ratio of 10:1 with triple dye loading buffer (**Section 1, Appendix A**). The gel was run at a constant voltage, *i.e.*, 100 Volts for ~30 minutes or until the loading dye reached ~1 cm from the lower rim of the gel. The gel was then removed from the tray, it was stained with 0.5 µg/ml ethidium bromide solution (**Section 6, Appendix A**) for 10 minutes, then destained with distilled water for 5-10 minutes. The DNA band was visualized under a UV transilluminator (Biodoc-It™ Imaging system, UVP Transilluminator, Cambridge, UK). The size of DNA segments were estimated by comparing with the bands of known sizes of the 1 kb DNA ladder (Fermentas) run concurrently in the same gel.

6. Cloning of *PA-5', PB1-5'* and *PB2-5'* sequences

6.1 DNA purification

The *PA-5', PB1-5'* and *PB2-5'* amplicons were purified from the respective agarose gels by using GENECLEAN II kit (Bio 101, USA). The gel pieces containing the respective gene segments were excised from the gel slabs and the gel pieces were individually mixed with 3 volumes of 6 M NaI in a 2 ml-microcentrifuge tube

(for 0.1 g of the gel slice, 100 µl of the NaI solution was used according to the instruction manual) and the agar was melted at 55°C for 5-10 minutes. Five microliters of glass milk suspension were added to each tube and the mixture was incubated at 25°C for at least 10 minutes with occasional mixing to allow binding of the DNA to the silica matrix. The tube was centrifuged at 12,000 × *g* for approximately 10 seconds and the supernatant was discarded. The glass milk pellet was washed thoroughly with 500 µl of a wash buffer (Bio 101) by centrifugation. After washing, the pellet was allowed air-dried. The dried pellet was dissolved in 20 µl of sterile UDW and centrifuged at 12,000 × *g* for 30 seconds; the supernatant containing the eluted DNA was carefully collected and placed into a new tube.

6.2 Ligation of the *PA-5', PB1-5'* and *PB2-5'* sequences to cloning vectors

Cloning of the individually purified *PA-5', PB1-5'* and *PB2-5'* segments into the cloning vector was performed by means of the A-tailing of each gene segment. The reaction mixture (10 µl) consisted of 1 µl of 10x buffer with KCl, 1 µl of 25 mM $MgCl_2$, 1 µl of 2 mM dATP, 1 µl of 5 units/µl *Taq* DNA polymerase (Fermentas), and 6 µl of the purified DNA segment. The mixture was incubated at 70°C for 30 minutes. The *PA-5', PB1-5'* or *PB2-5'* segments were individually ligated with pTZ57R/T vector (Fermentas) *via* the overhang T of the vector and the A of the DNA amplicon. The ligation mixture (30 µl) contained: 6 µl of 5x ligation buffer, 3 µl of pTZ57R/T vector, 6 µl of the *PA-5', PB1-5'* or *PB2-5'* segments, 1 µl of T4 DNA ligase (5 units/µl) and UDW. The mixture was incubated at 22°C overnight. The recombinant-pTZ57R/T cloning vectors with *PA-5', PB1-5'* or *PB2-5'* insert were prepared.

6.3 Preparation of competent cells

JM109 *E. coli* was used as a host of the recombinant-pTZ57R/T cloning vectors. One colony of the bacteria grown overnight on an agar plate was inoculated into 5 ml of LB broth (**Section 1, Appendix B**) and incubated in a shaking incubator at 37°C overnight. One hundred microliters of the overnight culture were inoculated into a tube containing 10 ml of fresh LB broth and the tube was incubated at 37°C with shaking until the culture reached log phase (OD_{600nm} = 0.5). The bacterial culture was centrifuged at 4,000 × *g*, 4°C for 10 minutes, and the bacteria in the pellet were gently resuspended in 5 ml of ice-cold 100 mM $MgCl_2$ (**Section 1, Appendix C**). The

bacterial suspension was centrifuged at 4,000 × *g*, 4°C for 10 minutes; the cells in the pellet were resuspended in 5 ml of ice-cold 100 mM $CaCl_2$ (**Section 2, Appendix C**) and kept in an ice-bath for 1 hour. The suspension was re-centrifuged and 225 µl of 80% glycerol were added to the bacterial pellet to re-suspend the cells. The preparation was aliquoted into 0.6 ml microcentifuge tubes, snap frozen in dry-ice-ethanol, and kept at -70°C until use.

6.4 Transformation of JM 109 *E. coli* with the recombinant cloning vectors

The ligation products from (**6.2**) which were the recombinant cloning vectors were used individually to transform the competent *E. coli* cells of (**6.3**) by means of the chemical transformation protocol (Hanahan, 1983) which was performed as the following: 5 µl of the ligation product (recombinant vectors) were added to 100 µl of competent *E. coli* cells. After gently mixing, the tube was kept in an ice-bath for 20 minutes to allow attachment of the vectors to the competent bacterial cells. Subsequently, the mixture was heated at 42°C in a water bath for 2 minutes, and immediately placed in an ice-bath for 2 minutes. Then, 900 µl of 2x YT broth (**Section 9, Appendix B**) were added to the mixture and the preparation was incubated at 37°C in a shaking incubator for 1 hour. Two hundred microliters of the culture were spread onto LB agar plate containing 100 µg ampicillin/ml, 100 mM IPTG and 5% X-Gal (**Section 5, Appendix B**). The plate was incubated at 37°C overnight.

6.5 Screening of the *E. coli* clones carrying the recombinant plasmids

White colonies of the transformed JM109 *E. coli* on the overnight culture plate were randomly picked. They were individually inoculated into 5 ml of LB-ampicillin (100 µg/ml), *i.e.*, LB-A broth (**Section 3, Appendix B**) and incubated at 37°C with shaking at 250 rpm overnight. The cells were harvested by centrifugation at 4,000 × *g*, 25°C for 5 minutes.

The plasmids were extracted from the *E. coli* cell pellet: cell pellet in each tube was added with 200 µl of solution I (50 mM glucose, 25 mM Tris-HCl, pH 8.0, 10 mM EDTA) (**Section 1, Appendix D**) containing ribonuclease A (RNase A, 20 µg/ml). The tube was inverted few times; 400 µl of solution II (0.1 N NaOH, 1% SDS) (**Section 2, Appendix D**) were then added, and the preparation was placed in an ice-bath for 5 minutes. The mixture was added with 300 µl of solution III (3 M

potassium acetate, pH 5.2) (**Section 3, Appendix D**), kept in an ice-bath for 5 minutes, and centrifuged at 12,000 × *g* for 5 minutes. The supernatant was collected and the plasmids were precipitated from the supernatant by adding with 0.6 volumes of isopropranol. The mixture was centrifuged at 12,000 × *g*, 25°C for 5 minutes. The plasmid pellet was air-dried at 25°C before dissolving in UDW. The integrity of the plasmids was checked by 1% agarose gel electrophoresis and ethidium bromide staining. These extracted recombinant plasmids were subjected to the below restriction endonuclease digestions.

6.6 Restriction endonuclease digestions of the recombinant plasmids

The recombinant plasmids were doubly digested with restriction endonuclease enzymes (Fermentas). The reactions of restriction digestion were set up in 0.6 microcentrifuge tube with a total volume of 20 µl.

The mixture for restriction digestion of the recombinant *PA-5'*-plasmids consisted of 2 µl of 10x *Bam*HI buffer, 5 µl of the recombinant *PA-5'*-plasmids, 0.5 µl of *Bam*HI (10 units/µl), 0.5 µl of *Hin*dIII (10 units/µl), and 12 µl of UDW.

The mixture for restriction digestion of the recombinant *PB1-5'*-plasmids consisted of 2 µl of 10x *Bam*HI buffer, 5 µl of the recombinant *PB1-5'*-plasmids, 0.5 µl of *Bam*HI (10 units/µl), 0.5 µl of *Xho*I (10 units/µl), and 12 µl of UDW.

The mixture for restriction digestion of the recombinant *PB2-5'*-plasmids consisted of 4 µl of 10x Tango™ buffer, 5 µl of the recombinant *PB2-5'*-plasmid, 0.5 µl of *Sal*I (10 units/µl), 0.5 µl of *Hin*dIII (10 units/µl) and 10 µl of UDW.

The reaction mixtures were incubated at 37°C for 6 hours. The individually digested products were subjected to 1 % agarose gel electrophoresis and ethidium bromide staining in order to ensure that the plasmids contained DNA insert of correct sizes. The recombinant plasmids with the DNA insert were then sequenced.

7. Plasmid extraction for DNA sequencing and verification

The recombinant plasmids were extracted from the JM109 *E. coli* cells by using plasmid extraction kit (Qiagen, Germany) following the manufacturer's instruction. Single *E. coli* colony containing recombinant vectors (transformed *E. coli*) was inoculated into 5 ml of LB-A and incubated at 37°C with shaking at 250 rpm overnight. Two milliliters of the culture were transferred to a tube and centrifuged at

10,000 × *g* for 5 minutes. The cells in the pellet were resuspended in 250 μl of buffer P1 and Vortex mixed. Two hundred and fifty microliters of buffer P2 were added and the tube was gently inverted 4-6 times. Three hundred and fifty microliters of the buffer N3 were added and the tube was gently inverted 4-6 times. The tube was centrifuged at 12,000 × *g* for 10 minutes. The supernatant was transferred into a QIAprep column. The column was centrifuged at 13,000 × *g* for 1 minute. Seven hundred and fifty μl of buffer PE were added into the column and centrifuged at 13,000 × *g* for 1 minute. After discard the flow though, the column was centrifuged at 13,000 × *g* for 2 minute. The column was placed in a clean 1.5 ml microcentrifuge tube. Fifty microliters of buffer EB or UDW were added to the center of column. The column was allowed to stand for 2 minutes and then centrifuged at 13,000 × *g* for 1 minute. Integrity of the plasmid was verified by 1% agarose gel electrophoresis and ethidium bromide staining.

8. DNA sequencing

DNA sequencing for verification of the inserted *PA-5', PB1-5'* and *PB2-5'* DNA sequences was performed by Macrogen (Korea). The nucleotide sequences were compared to the relevant sequences of the influenza A viruses in the GenBank of National Center for Biotechnology Information using the BLAST network services.

9. Subcloning of *PA-5', PB1-5'* and *PB2-5'* sequences into protein expression vectors

The recombinant plasmids carrying *PB1-5'* sequences were subcloned into a pET-20b(+) protein expression vector (Novagen, Germany), whereas the recombinant plasmids carrying *PA-5'* and *PB2-5'* sequences were subcloned into pQE31 and pQE30 protein expression vectors (Qiagen, Germany), respectively.

9.1 Restriction endonuclease digestions of recombinant plasmids and the protein expression vectors

9.1.1 Recombinant *PA-5'*-plasmids and the pQE31 protein expression vectors

The mixture consisted of 2 µl of 10x *Bam*HI buffer, 5 µl of recombinant *PA-5'*-plasmids (pQE31 protein expression vectors), 0.5 µl of *Bam*HI (10 units/µl), 0.5 µl of *Hin*dIII (10 units/µl), and 12 µl of UDW. The mixture was incubated at 37^{o}C for 6 hours. The digested product was subjected to 1% agarose gel electrophoresis and DNA staining by 0.5 µg/ml ethidium bromide.

9.1.2 Recombinant *PB1-5'*-plasmids and the pET-20b(+) protein expression vectors

The mixture consisted of 2 µl of 10x *Bam*HI buffer, 5 µl of recombinant *PB1-5'*-plasmids (pET-20b(+) protein expression vectors), 0.5 µl of *Bam*HI (10 units/µl), 0.5 µl of *Xho*I (10 units/µl) and 12 µl of UDW. The mixture was incubated at 37^{o}C for 6 hours. The digested product was verified as above (**Section 9.1.1**).

9.1.3 Recombinant *PB2-5'*-plasmids and the pQE30 protein expression vectors

The mixture consisted of 4 µl of 10x Tango^{TM} buffer, 5 µl of recombinant *PB2-5*'-plasmids (pQE30 protein expression vectors), 0.5 µl of *Sal*I (10 units/µl), 0.5 µl of *Hin*dIII (10 units/µl) and 10 µl of UDW. The mixture was incubated at 37^{o}C for 6 hours. The digested product was verified as above.

9.2 DNA purification

The *PA-5', PB1-5'* and *PB2-5'* segments and the linear pET-20b(+) (Novagen, Germany), pQE30 and pQE31 vectors (Qiagen, Germany) were individually purified from the agarose gels by using GENECLEAN II kit (Bio 101) as described in **Section 6.1.**

9.3 Ligation of *PA-5'*, *PB1-5'* and *PB2-5'* sequences to the protein expression vectors

PB1-5' segments were ligated into pET-20b(+) protein expression vector pre-cut with the same enzymes. The *PA-5'* and *PB2-5'* segments were ligated into pQE31 and pQE30 protein expression vectors, respectively, which were pre-cut with the appropriate endonucleases. Each ligation mixture consisted of: 2 µl of 10x buffer, 1 µl of cut- protein expression vectors (~50 ng), 4 µl of cut-gene segments (~150 ng), 1 µl of T4 ligase (400,000 units/ml) (Gibcobrl, USA), and UDW to a final volume of 20 µl. The mixture was incubated at 16^{o}C overnight.

9.4 Preparation of competent cells

9.4.1 Competent JM109 *E. coli*

The competent JM109 *E. coli* cells were used as storage host for recombinant vectors, *i.e.,* pET-20b(+), pQE30 or pQE31, and they were prepared as described in **Section 6.3.**

9.4.2 Competent *E. coli* BL21(DE3)pLysS cells

The BL21(DE3)pLysS *E. coli* cells were used as a protein expression host for recombinant *PB1-5'*-pET-20b(+) vectors. A colony of the *E. coli* BL21(DE3) pLysS cell was inoculated into 5 ml of LB-broth (**Section 1, Appendix B**). The culture was incubated at 37^{o}C with shaking at 250 rpm overnight. One hundred microliters of the overnight *E. coli* cultures were inoculated into 10 ml of fresh LB broth. The bacteria were grown for approximately 3 hours or until the growth reached an $OD_{600\ nm}$ = 0.5. The culture was then centrifuged at 4,000 × *g*, 4^{o}C for 10 minutes and bacterial cells in the pellet were gently resuspended in 5 ml of ice-cold 100 mM $MgCl_2$ (**Section 1, Appendix C**). The suspension was centrifuged at 4,000 × *g*, 4^{o}C for 10 minutes. The cell pellet was resuspended in 5 ml of ice-cold 100 mM $CaCl_2$ (**Section 2, Appendix C**) and kept in an ice-bath for 1 hour. The suspension was then centrifuged at 4,000 × *g*, 4^{o}C for 10 minutes. Two hundred and twenty five microliters of 80% glycerol were added to the suspension and gently mixed. One hundred microliters of the mixture was aliquoted into 0.6 ml microcentifuge tubes, snap-frozen in dry-ice- ethanol and kept at -70^{o}C until use.

9.4.3 Competent M15 *E. coli* cells

The M15 *E. coli* cells were used as a protein expression host for recombinant *PB2-5'*-pQE30 and *PA-5'*-pQE31 vectors. A colony of the M15 *E. coli* was inoculated into 5 ml of LB-broth containing 25 µg/ml of kanamycin (LB-K broth) (**Section 6, Appendix B**). The cultures were incubated at 37°C with shaking at 250 rpm overnight. One hundred microliters of the overnight *E. coli* culture were inoculated into 10 ml of LB-K broth. The bacteria were grown for approximately 3 hours or until the growth reached an $A_{600\ nm} = 0.5$. The culture was then centrifuged at 4,000 × *g*, 4°C for 10 minutes and bacterial cells in the pellet were gently resuspended in 5 ml of ice-cold 100 mM $MgCl_2$ (**Section 1, Appendix C**). The mixture was centrifuged at 4,000 × *g*, 4°C for 10 minutes. The bacterial cells in the pellet were resuspended in 5 ml of ice-cold 100 mM $CaCl_2$ (**Section 2, Appendix C**) and kept in an ice-bath for 1 hour. The suspension was then centrifuged at 4,000 × *g*, 4°C for 10 minutes. Two hundred and twenty five microliters of 80% glycerol were added to the suspension and gently mixed. One hundred microliters of the mixture was aliquoted into 0.6 ml microcentifuge tubes, snap frozen in dry-ice- ethanol and kept at -70°C until use.

9.5 Transformation of JM109 *E. coli* cells which were used as DNA storage host

9.5.1 DNA transformation into the JM109 *E. coli* cells

The ligation products from section 9.3 were individually introduced into the storage JM 109 *E. coli* cells by chemical transformation protocol (Hanahan, 1983) as described in **Section 6.4.** The bacterial cultures were spread onto LB agar plate containing 100 µg ampicillin/ml (LB-A agar) (**Section 4, Appendix B**) and incubated at 37 ºC overnight. Thereafter, individual transformed *E. coli* colonies were randomly picked and checked for the presence of the recombinant plasmids with *PA-5', PB1-5'* and *PB2-5'* inserts by using PCR amplification. Individual transformed *E. coli* colonies were randomly picked and inoculated into 100 µl of UDW. One microliter of each solution was used as DNA template for the PCR amplification. The primers as shown in **Table 4** were used. The PCR reaction mixture (25 µl) consisted of: 1 µl of DNA template, 0.5 µl of each primer (20 µM), 2.5 µl of 10x buffer with KCl, 3 µl of

25 mM $MgCl_2$, 2 µl of 10 mM dNTPs, 0.2 µl of 5 units/µl *Taq* DNA polymerase (Fermentas), and UDW. PCR condition consisted of 1 cycle denaturing at 94ºC for 5 minutes, 35 cycles of annealing at 45ºC for 1 minute, extending at 72ºC for 1 minute, denaturing at 94ºC for 1 minute. The chain elongation was done at 72°C for 10 minutes. After amplification, the PCR product was analyzed by 1% agarose gel electrophoresis and ethidium bromide staining. Recombinant plasmids with the correct DNA inserts carried by the transformed *E. coli* colonies were extracted as in **Section 6.5.**

9.5.2 Transformation of the *E. coli* expression host

9.5.2.1 Recombinant *PB1-5'*-pET-20b(+) plasmids

The extracted recombinant *PB1-5'*-pET-20b(+) plasmids were introduced into BL21(DE3)pLysS *E. coli* cells. Two microliters of extracted recombinant plasmids were used to transform the competent *E. coli* cells of **Section 9.4.2** by using a chemical transformation protocol (Hanahan, 1983) as in **Section 6.4**. The BL21(DE3)pLysS *E. coli* cultures were spread onto LB-A agar plates. The agar plates were incubated at 37 ºC overnight. Individual transformed *E. coli* colonies were randomly picked and inoculated into 100 µl of UDW. One microliter of the solution was used as DNA template for PCR amplification. The primers as shown in **Table 4** were used. The PCR reaction mixture (25 µl) consisted of: 1 µl of DNA template, 0.5 µl of each primer (20 µM), 2.5 µl of 10x buffer with KCl, 3 µl of 25 mM $MgCl_2$, 2 µl of 10 mM dNTPs, 0.2 µl of 5 units/µl *Taq* DNA polymerase (Fermentas), and UDW. PCR condition consisted of 1 cycle denaturing at 94ºC for 5 minutes, 35 cycles of annealing at 45ºC for 1 minute, extending at 72ºC for 1 minute, and denaturing at 94ºC for 1 minute. Additional chain elongation was allowed at 72°C for 10 minute. After amplification, the PCR product was verified as above.

9.5.2.2 Recombinant *PB2-5'*-pQE30 and *PA-5'*-pQE31 plasmids

The extracted recombinant pQE31 plasmids with insert *PA-5'* insert and the extracted recombinant pQE30 plasmids with *PB2-5'* insert were individually introduced into the competent M15 *E. coli* cells. Two microliters of each extracted recombinant plasmids were used to transform the competent *E. coli* cells of **Section 9.4.3** by using a chemical transformation protocol (Hanahan, 1983) as in section (6.4).

The M15 *E. coli* cultures were spread onto LB agar plate containing 100 µg ampicillin/ml and 25 µg kanamycin/ml (LB-AK agar) (**Section 8, Appendix B**). The agar plates were incubated at 37 ºC overnight. Individual transformed *E. coli* colonies were randomly picked and inoculated into 100 µl of UDW. One microliter of the solution was used as DNA template for PCR amplification of the DNA inserts. The primers as shown in **Table 4** were used. The PCR reaction mixture and the condition, and the PCR product verification were similar to those of the *PB1-5'* in **Section 9.5.2.1.**

10. Recombinant N-terminal PA, N-terminal PB1, and N-terminal PB2 protein expression

10.1 Screening of appropriate recombinant *E. coli* clones for the recombinant protein expression

The recombinant *E. coli* BL21(DE3)pLysS colonies carrying *PB1-5'* recombination vectors were individually and appropriately inoculated into 2 ml of LB-A broth (**Section 3, Appendix B**); whereas the recombinant M15 *E. coli* colonies carrying *PA-5'* or *PB2-5'* recombination vectors were individually and appropriately inoculated into 2 ml of LB broth containing 100 µg ampicillin/ml and 25 µg kanamycin/ml (LB-AK broth) (**Section 7, Appendix B**). The bacteria were grown at 37^{o}C with shaking at 250 rpm overnight. One hundred microliliters of the overnight BL2(DE3)pLysS and M15 *E. coli* cultures were individually added into fresh 10 ml of LB-A and LB-AK broth, respectively. The preparation was incubated at 37^{o}C with shaking at 250 rpm until A_{600nm} reached 0.6. Then, IPTG (Amersham, USA) was added to each culture to the final concentration of 1 mM and the cultures were further incubated for 3 hours. The bacterial cells were harvested by centrifugation at 4,000 × *g*, 4^{o}C for 10 minutes. The cell pellet was added with a lysis buffer (50 mM Tris-HCl, pH 8.0) and sonicated (Sartorius, Germany) at 40% amplitude and 0.5 cycles, in an ice-bath. The bacterial homogenate was centrifuged at 12,000 × *g*, 4^{o}C for 15 minutes. The supernatant (soluble cytoplasmic fraction) and the sediment (insoluble cytoplasmic fraction) were separately collected. Each of the sediment was washed 3 times with the lysis buffer. One hundred microliters of supernatant and the washed sediment were added with 100 µl of sample buffer (**Section 1, Appendix F**),

boiled for 10 minutes, centrifuged, and the debris was discarded. The *E. coli* supernatant (soluble cytoplasmic fraction) and the extract of the sediment (insoluble cytoplasmic fraction) were subjected to SDS-PAGE to localize the target protein within the *E. coli* cells. The gels containing electrophoresed products were stained with Coomassie™ Brilliant Blue G-250 (**Section 11, Appendix F**). Western blotting was performed to confirm the presence of the N-terminal PA, N-terminal PB1, and N-terminal PB2 proteins using anti-His antibody (Serotec, USA) as a detection reagent.

10.2 Large scale expression of the recombinant proteins

The transformed BL21(DE3)pLysS *E. coli* colony carrying recombinant *PB1-5'*-vectors was inoculated into 5 ml of LB-A broth, whereas the transformed M15 *E. coli* colony carrying either recombinant *PA-5'*- or *PB2-5'*-vectors was inoculated into 5 ml of LB-AK broth. The culture was incubated at 37°C with shaking at 250 rpm overnight. Five milliliters of each overnight *E. coli* culture were added into fresh 500 ml of appropriate medium and incubated at 37°C with shaking until $A600_{nm}$ reached 0.6. IPTG was added to the final concentration of 1 mM and the cultures were incubated further at 37°C for 3 hours. The bacterial cells were harvested by centrifugation at 4,000 × *g*, 4°C for 20 minutes. The cell pellets were separately kept at -20°C until use in **Section 11** below.

11. Purification of recombinant polymerase proteins

Recombinant N-terminal PA, N-terminal PB1 and N-terminal PB2 proteins were purified by using nickel column (Invitrogen, USA).

11.1 Preparation of cell lysate for purification of the recombinant protein expressed under the natural conformation

Transformed bacterial cells carrying recombinant *PA-5'* vectors in one gram of the pellet from **Section 10.2** were resuspended in 5 ml of lysis buffer-1 (**Section 1, Appendix E**). The cells were lysed by sonication at 40% amplitude and 0.5 cycles in an ice-bath. The cell debris was removed from the homogenate by centrifugation at 12,000 × *g*, 4°C for 20 minutes. The supernatant (cell lysate) was transferred to a new tube and used for purification in **Section 11.4.**

11.2 Preparation of cell lysates for purification of the recombinant protein expressed under the denatured conformation

Transformed bacterial cells carrying recombinant *PB1-5'*, *PB2-5* or *PA-5'* vectors in one gram of the pellet from section 10.2 were resuspended in 5 ml of lysis buffer-2 (**Section 2, Appendix E**). Each suspension was rotated at 25^{o}C for 1 hour. The cell debris was removed from the homogenate by centrifugation at 12,000 × *g*, 4^{o}C for 20 minutes. The supernatant (cell lysate) was transferred to a new tube and used for purification as in **Section 11.5** below.

11.3 Preparation of the Ni-NTA Agarose

The Ni-NTA Agarose resin (Invitrogen, USA) was resuspended by gently inverting and tapping the bottle repeatedly. Two milliliters of the resin were added into a 50 ml-Falcon tube. Eight milliliters of UDW were added into the tube. The preparation was centrifuged at 2,000 × *g* for 2 minutes. The supernatant was discarded. The wet resin was washed one more time with UDW.

11.4 Purification of the recombinant protein expressed under the natural conformation

The preparation from **Section 11.1** was added into the tube containing the prepared Ni-NTA Agarose resin of section 11.3. The tube was rotated at 4^{o}C for 1 hour to keep the resin suspended in the bacterial lysate solution. The suspension was loaded into a 10 ml polypropylene column and the resin was allowed to set by gravity. The column was washed twice with 4 ml of washing buffer-1 (**Section 3, Appendix E**). The flow-through fluid was collected. The bound proteins were batch-eluted with various concentrations of imidazole: 0-500 mM in the washing buffer-1 (**Section 5, Appendix E**). Three milliliter fractions were collected and subsequently the flow-through fluid and the eluted fractions were analyzed by SDS-PAGE and Western blot analysis.

11.5 Purification of the recombinant proteins expressed under denatured conformation

The suspensions from **Section 11.2** were individually added into the prepared Ni-NTA agarose resin of **Section 11.3.** The mixture was rotated at 25^{o}C for 1 hour. The suspension was loaded into a 10 ml polypropylene column. The resin was allowed

to set by gravity. The column was washed twice with 4 ml of washing buffer-2 (**Section 4, Appendix E**). The flow-through fluid was collected for subsequent SDS-PAGE analysis. The bound proteins in the column were batch-eluted with various concentrations of imidazole: 0-500 mM in wash buffer-2 (**Section 4, Appendix E**) or eluted with elution buffer, pH 5.9 (**Section 7, Appendix E**) and 4.5 (**Section 8, Appendix E**). Three milliliter fractions were collected and subsequently analyzed by SDS-PAGE and Western blot analysis.

12. Protein determination

The Bio-Rad protein assay, based on the method of Bradford, which is a simple and accurate procedure for determining a concentration of solubilized protein was used. The method is a dye binding assay in which a differential color change of a dye occurs in response to various concentrations of proteins (Bradford, 1976).

Five dilutions of bovine serum albumin (BSA), which is a protein standard, were prepared. Ten microliters of each BSA dilution and sample solutions were individually placed into separate microtiter plate wells. Two hundred microliters of diluted dye reagent [one part of Dye Reagent Concentrate (Bio-Rad, USA) was added with four parts of deionized distilled water] were added to each well, mixed thoroughly using a microplate mixer, and the plate was kept at 25^{o}C for 5 minutes. The absorbance of the content in each well was determined at 595 nm (A_{595nm}) using microplate reader (Multiskan EX, Labsystems, Helsinki, Finland). The protein content of the sample was determined from a standard BSA curve constructed by plotting the OD of the diluted BSA solutions at A_{595nm} against the respective concentrations in the same assay. The buffer which was used to dissolve the sample served as a blank.

13. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was used to analyze the complexity of protein profiles. The separated components were visualized by staining or further subjected to Western blot analysis (WB). The technique described by Laemmli (1970) was followed with some modifications. The 8.0 × 7.3 cm vertical slab gel was prepared in a casting apparatus (Mini PROTEAN® 3 Cell, Bio-Rad, USA) while electrophoresis was done in an electrophoretic chamber and with an electric power supply (model 3,000/300, Bio-

Rad). A 4% acrylamide stacking gel and 12% acrylamide separating gel were used in this process (**Appendix F**).

13.1 Preparation of sample for loading into the slab gel

Sample to be separated by the SDS-PAGE was denatured by diluting with four volumes of the sample buffer (**Section 1, Appendix F**) and heating at 100^{o}C for 4 minutes before carefully loaded into the slots in the stacking polyacrylamide gel. Pre-stained SDS-PAGE broad range standard (Bio-Rad) was included in one slot of each gel slab. Care was taken not to contaminate the adjacent wells with the samples.

13.2 Electrophoresis

Electrophoresis was carried out in an electrophoretic chamber at 10 mAmp/gel during the first 15 minutes and at 20 mAmp/gel until the tracking dye reached the lower edge of the gel.

After electrophoresis, the gel was removed from the glass. It was then either stained and destained for direct visualization of the separated components or proceeded to the electro-transblotting onto the nitrocellulose membrane for Western blot analysis.

13.3 Staining and destaining of protein bands

Protein bands were revealed by soaking the gel in Coomassie^{TM} Brilliant Blue G-250 (**Section 11, Appendix F**) as follows.

The slab gel from SDS-PAGE containing separated proteins was placed in a fixing solution (**Section 11.1, Appendix F**) at 25^{o}C for 1 hour on a rotating plateform. After that, the fixed gel was stained in Coomassie^{TM} Brilliant Blue G-250 overnight and the gel was destained in UDW until the background color was adequately reduced. When destaining was completed, the gel was either immediatedly photographed, scanned and save to a computer, or dried on a paper (Cellophane membrane backing, Bio-Rad).

14. Western blot analysis (WB)

The component(s) resolved in the polyacrylamide gel after electrophoresis was/were electrobloted onto nitrocellulose membranes (NC). After blotting, the empty sites on the nitrocellulose membrane were blocked by soaking in a blocking buffer [3% BSA in 0.01 M phosphate buffered saline, pH 7.4 (PBS)] at 25^{o}C with gently

agitating for 1 hour. The nitrocellulose blot was then washed 3 times for at least 15 minutes with washing buffer (0.05% PBS-T) to remove the excess BSA. After washing, the nitrocellulose membrane was placed in a solution of 1:4,000 diluted anti-His antibody (Serotec, USA) and gently agitated at 25^{o}C for 1 hour. The membrane was then washed 3 times with washing buffer and incubated with 1:4,000 diluted goat anti-mouse immunoglubolin-HRP conjugate (secondary antibody) (Southern Biotech, AL, USA) with gentle agitating at 25^{o}C for 1 hour. The membrane was then washed 3 times with washing buffer (0.05% PBS-T) and equilibrated in 1/15 M phosphate buffer, pH 7.6. The 2, 6-dichlorophenolindophenol (**Section 9.2, Appendix G**) was used as a chromogenic substrate. When goat anti-mouse immunoglobulin-AP conjugate (Southern Biotech, AL, USA) was used as secondary antibody, the nitrocellulose membrane was equilibrated in 0.15 M Tris pH, 9.6 and the color was developed by using BCIP/NBT phosphatase substrate (KPL, USA) (**Section 9.1, Appendix G**). The enzymatic reaction was stopped by washing the membrane with DW and the membrane was allowed to air-dry.

15. The human antibody phage display library

The human antibody phage display library used in this study had been constructed in our laboratory by using immunoglobulin genes of B lymphocytes from 60 Thai blood donors. The M13 bacteriophages were used for displaying individual ScFv. This large phage library has ~3x10^{8} antibody diversity.

16. Selection of phage clones displaying specific ScFv to the target antigens by "phage bio-panning"

The purified recombinant proteins (N-terminal PA, N-terminal PB1, and N-terminal PB2) were individually used in the form of immobilized antigens in wells of ELISA plate to select the phage clones displaying human ScFv (HuScFv) specific to the proteins from the human antibody phage library. In the bio-panning process, 1 μg of individual purified recombinant protein in coating buffer (carbonate-bicarbonate buffer, pH 9.6) was immobilized in each ELISA well (EIA/RIA 8 Well Strip Flat Bottom Corning, NY, USA). The plate was incubated at 37ºC overnight. The wells were washed three times with PBS-T. Two hundred microliters of a blocking solution (5% skim milk in PBS, pH 7.4) were added into each well and

incubated at 25°C for 1 hour. The blocking solution was discarded and the wells were washed three times with PBS-T. The phage library (1.1 x 10^9 plaques forming units; pfu) was diluted three times the wash buffer. Two hundred microliters of diluted phages were added into each antigen coated well and the plate was kept at 25°C for 1 hour. During the incubation, the contents in each well were mixed by pipitting every 15 minutes. After discarding the fluid, each well was washed three times with PBS-T. Fifty microliters of 0.1 M HCl-glycine pH 2.2 (**Section 5, Appendix H**) were added into the well and incubated at 25ºC for 5 minutes. The solution was transferred into 1.5 ml tube containing 3 µl of 2 M Tris-base. The mixture was added to a tube containing 200 µl of HB2151 *E. coli* which had been grown to log-phase. The mixture (250 µl) was incubated at 37ºC for 20 minutes. One hundred and twenty five microliters were aliquoted and spread onto two 2x YT agar plates containing 100 µg/ml of ampicillin and 2% glucose (2x YT-AG) (**Section 12, Appendix B**) and the plates were incubated at 37ºC overnight.

17. Screening of HB2151 *E. coli* clones carrying recombinant phagemids with *huscFv* insert

The bacterial colonies on 2x YT-AG plate were randomly picked for checking the presence of *huscFv* by PCR. The colonies were inoculated into 100 µl of UDW. PCR was carried out using a specific pair of primers for *huscFv c*onsisting of 26 bp forward, 5'-CCATGATTACGCCAAGCTTTGGAGCC-3' and 25 bp reverse, 5'-GCTAGATTTCAAAACAGCAGAAAGG-3'sequences. One microliter of the DNA solution was used as a DNA template to amplify the *huscFv* sequences in a 25 µl reaction mixture containing 2.5 µl of 10x PCR buffer with KCl, 1.5 µl of $MgCl_2$, 2 µl of 10 mM dNTPs, 0.5 µl of each 25 µM primer, 0.2 µl of *Taq* DNA polymerase (5 units/µl) (Fermentas) and UDW. PCR amplification was carried out by using a Thermo Cycler (Eppendorf, Germany). PCR condition consisted of 1 cycle denaturing at 94ºC for 5 minutes, 30 cycles of annealing at 50ºC for 1 minute, extending at 72ºC for 1.5 minutes, and denaturing at 94ºC for 1 minute. Chain elongation was done at 72°C for 10 minutes. The *huscFv* amplicons were verified by agagrose gel electrophoresis and ethidium bromide staining.

18. Expression of soluble human monoclonal single chain antibody fragments (HuScFv)

The transformed HB2151 *E. coli* colonies giving *huscFv* amplicons of the expected size were picked from 2x YT-AG culture plate, individually inoculated into 5 ml of 2x YT-AG broth (**Section 10, Appendix B**) and incubated at 37ºC with shaking at 250 rpm overnight. One milliliter of the overnight culture was inoculated into 10 ml of fresh 2x YT-AG broth and incubated at 37ºC with shaking at 250 rpm for 1 hour. The culture was centrifuged at 4000 × *g* for 10 minutes. The cells in the pellet were resuspended in 2x YT broth containing 100 μg/ml of ampicillin and 1 mM of IPTG (2x YT-AI) (**Section 13, Appendix B**). The culture was incubated at 37ºC with shaking at 250 rpm for 5 hours. The *E. coli* cells were collected by centrifugation at 4,000 × *g*, 4ºC for 10 minutes. The bacterial cells in the pellet were resuspended with 1 ml of PBS, pH 7.4 and then sonicated by using ultrasonic-homogenizer LABSONIC® P (Satorius AG, Germany) at 40% amplitude and 0.5 cycles in an ice-bath. The whole cell homogenate was centrifuged at 12,000 × *g*, 4ºC for 10 minutes. The supernatant (cell lysate) was collected and 10 μl of the preparation was subjected to SDS-PAGE and stained with the Coomassie Brilant BlueG-250 dye (**Section 11, Appendix F**). Western blotting was performed as in **Section 14** to confirm the presence of HuScFv by using anti-E-Tag (Amersham, USA) as a detection reagent.

19. Detection of binding of the HuScFv to the respective recombinant proteins by indirect ELISA (HuScFv-ELISA)

Soluble HuScFv preparations were tested for their binding activity to the respective recombinant proteins of the influenza A (H5N1) by the HuScFv-ELISA.

For the HuScFv-ELISA, one microgram of purified recombinant protein in coating buffer (carbonate-bicarbonate buffer, pH 9.6) was used to coat each well of an ELISA plate. The plate was incubated at 37ºC overnight. The wells were washed three times with PBS-T. Two hundred microliters of a blocking buffer (5% skim milk in PBS, pH 7.4) were added into each well and incubated for 1 hour. The blocking buffer was discarded and the wells were washed three times with the washing buffer. One hundred microliters of HuScFv preparation contained in 50 μl of the whole cell lysate of recombinant *E. coli* were added and the plate was incubated at 25ºC for

1 hour. Whole cell lysate of normal HB2151 *E. coli* (negative HuScFv control), whole cell lysate of *E. coli* containing HuScFv specific to hemolysin of *Leptospira* spp. (irrelevant HuScFv control) and PBS (blank) were included in the same ELISA plate and served as respective controls. After incubation, the wells were then washed with the PBS-T. The bound HuScFv in each well were detected by adding 1:5,000 diluted mouse anti-E-Tag monoclonal antibody (Amersham, USA). After the incubation at 25ºC for 1 hour, all wells were washed three times with washing buffer and incubated individually with 100 µl of 1:4,000 diluted goat anti-mouse immunoglobulin-HRP conjugate (Southern Biotech, AL, USA). The wells were then washed and 50 µl of ABTS [2, 2 azino-bis(3-ethylbenzthiazoline-6-suulphonic acid)] solution (ZYMED CA, USA) was added into each well and the plate was kept at 25ºC for 30 minutes with light protection. The OD of the content in each well was measured at A_{405nm} against blank (PBS) by using ELISA reader (Multiskan® EX, Labsystem).

20. Detection of binding of HuScFv to the respective recombinant proteins by immunoblotting

The NC membrane containing SDS-PAGE separated-recombinant protein was incubated with the blocking buffer for 30 minutes. The membrane was washed three times with PBS-T and incubated with 1 ml of HuScFv solution (500 µl of transformed *E. coli* whole cell lysate + 500 µl of PBS, pH 7.4) at 25ºC for 1 hour with gentle agitating and then kept at 4ºC overnight. The membrane was washed three times with the washing buffer and allowed to react with 1:5,000 diluted mouse anti-E-Tag monoclonal antibody (Amersham, USA) with gentle agitating at 25 ºC for 1 hour. The membrane was washed three times with PBS-T and was incubated with 1:4,000 diluted goat anti-mouse immunoglubulin-alkaline phosphatase conjugate (Southern Biotech, AL, USA) with gentle agitating at 25ºC for 1 hour. After washing, the NC was equilibrated in 0.15 M Tris-HCl, pH 9.6 and incubated with the chromogenic substrate, *i.e.,* BCIP/NBT phosphatase (KPL, USA). The reaction was stopped by washing the membrane with DW and the membrane was allowed to air-dry.

21. Detection of binding of HuScFv to the respective recombinant proteins by dot-ELISA

Three microliters of purified recombinant protein were applied onto NC. The membrane was air-dried. The NC dotted with the recombinant protein was blocked as above, incubated with HuScFv preparation from whole cell lysate of transformed HB2151 *E. coli*. The binding of the HuScFv to the homologous recombinant polymerase protein was detected by using mouse anti-E-Tag monoclonal antibody, goat anti-mouse immunoglobulin-alkaline phosphatase and BCIP/NBT phosphatase substrate as for the immunoblotting.

22. RFLP patterns of the *huscFv* sequences

The PCR products of *huscFv* sequences from positive clones that their expressed HuScFv could bind to the respective recombinant proteins as tested in **Section 19, 20** and **21** were subjected to RFLP analysis for determining the *huscFv* DNA banding diversity.

The PCR products were digested with restriction endonuclease enzyme, M*va*I. The digestion mixture consisted of 2 µl of *huscFv* PCR product, 2.5 µl of 10x R buffer, 0.5 µl of *Mva*I (10 units/µl), 20 µl of UDW. The mixture was incubated at 37^{o}C for 6 hours. The digested *huscFv* preparation was subjected to electrophoresis in a 12% acrylamide gel in 0.5% TBE buffer. The gel with separated DNA fragments was then stained with ethidium bromide and DNA banding patterns were visualized under a UV transilluminator (Biodoc-It™ Imaging system, UVP Transilluminator, Cambridge, UK). The sizes of the DNA bands were estimated by comparison to the bands of the low molecular weight DNA ladder (New England Biolabs) run concurrently in the same gel.

23. Functional analysis of the recombinant N-terminal PA

23.1 Nuclear import assay using digitonin-permeabilized Vero cells

23.1.1 Vero cell culture

African green monkey kidney (Vero) cells were grown to late log phase in Dulbecco's modified Eagle medium (DMEM) (Gibco, USA) supplemented with 10% fetal bovine serum (FBS) (HyClone, Utah), 1 unit penicillin-streptomycin (Gibco), and 2 mM L-glutamine (Gibco) and incubated at 37°C in a 5% CO_2 incubator. One ml of the Vero cell suspension (5x 10^4 cells/ml) was added to each well of the 24-well tissue culture plate (Costar, USA) which glass cover slips had been placed into individual wells. After 24 hours of incubation at 37ºC in the 5% CO_2 incubator, Vero cells of ~ 80% confluent growth on individual glass cover slips were used for the nuclear import assay.

23.1.2 Nuclear import assay

The nuclear import assay was performed as previously described (Adam *et al.*, 1990). The glass cover slips with attached Vero cells were gently washed three times with an import buffer (20 mM HEPES, pH 7.4, 110 mM postassium acetate, 1 mM EGTA, 5 mM sodium acetate, 2 mM magnesium acetate, and 2 mM dithiothreitol). Individual slips were lifted and the cells were allowed to face to a drop (50 µl) of 50 µg/ml digitonin, and incubated at 25ºC for 5 minutes. Each glass cover slip was then washed with the import buffer and then exposed to a drop (30 µl) of an import mixture containing 200 ng of recombinant N-terminal PA, 0.4 mM ATP, 0.45 mM GTP, 4.5 mM phosphocreatine, 18 units/ml phosphocreatine kinase (all from Sigma), 20% rabbit reticulocyte lysate (Promega), protease inhibitors (10 µg/ml Leupeptin, 10 µg/ml Aprotinin, 1 mM PMSF (all from sigma), 1x complete protease inhibitor cocktail (Roche) and 1.6 mg/ml BSA, and incubated at 37°C for 45 minutes. Each cover glass was washed with the import buffer. The cells on the slip were fixed with 4% paraformaldehyde in the import buffer at 25ºC for 1 hour before washing with PBS.

23.1.3 Cell staining and confocal microscopy

The fixed cells from **Section 23.1.2** were permeabilized by placing the glass slip into 1 ml of 2% Tritron-X100 (Sigma) for 10 minutes. The Vero cells on the glass cover slip were then incubated with 2% fetal bovine serum in PBS for 1 hour at 25ºC, washed with PBS, and faced to a drop (50 µl) of 1:100 diluted anti-His antibody (Southern Biotech, AL, USA). After the incubation at 25ºC for 1 hour, each glass cover slip was washed, placed with the cell side down to a drop (50 µl) of Alexa Fluor® 488 chicken anti-mouse IgG (H+L) (Invitrogen, USA), and incubated at 25ºC for 1 hour. Each glass cover slips was washed with PBS before mounting with 50% glycerol and sealing with nail polish on a microscopic glass slide. The cells were observed by using laser scanning confocal microscope (LSM 510 META, Carl Zeiss, Germany). Recombinant NP protein was used as a positive import substrate control. Moreover, to ensure that the cell staining process was working properly, a nuclear stain control was performed. Vero cells were permeabilized with 1 ml of 2% Tritron-X100 (Sigma) for 10 minutes and washed with PBS, then they were incubated with rabbit polyclonal anti-nucleolin antibody (Santa Cruz Biotechnology, USA) followed by Alexa Fluor® 488 goat anti-rabbit IgG (H+L) (Invitrogen).

23.2 RNA binding assay

The RNA polymerase complex (NP + PB1, PB2, and PA) of influenza A virus binds to viral RNA promoter for initiating the viral RNA transcription. The PB1 subunit and NP in the complex have been shown previously to bind to the viral RNA (Kingsbury *et al*., 1887). In order to test whether the PA alone could directly bind to the viral promoter or not, the RNA binding assay of the recombinant N-terminal PA was performed.

The binding reactions mixture was prepared in a total volume of 20 µl containing 1 µg of recombinant N-terminal PA (or recombinant NP which served as positive control), 10 µM biotinylated 5'-vRNA (5'-AGUAGAACAAGGCC-3'), 25 mM HEPES (pH 7.5), 125 mM KCl, 2 mM $MgCl_2$, 1 mM DTT, 0.5 mM EGTA, 0.05 mM EDTA, 1 mM PMSF, 8 units of RNasin (Fermentus), and 15% glycerol,. Reaction mixtures were incubated at 37°C for 1 hour. The reaction tube was carefully placed on the surface of a UV transilluminator and the UV-irradiation was performed at 302 nm. Six microliters of a reducing sample buffer were added to the cross-linked

product and the preparation was boiled for 5 minutes. The sample was subjected to 12% SDS-PAGE and then Western blot analysis was performed as in **Section 14** of **Chapter IV.** The NC membrane was blocked with the blocking solution for 30 minutes. The membrane was washed three times with PBS-T and incubated with 1:500 diluted streptavidin-AP conjugate for 1 hour, washed with PBS-T, equilibrated in 0.15 M Tris-HCl, pH 9.6 and incubated with the chromogenic substrate, *i.e.,* BCIP/NBT phosphatase (KPL, USA). The enzymatic reaction was stopped by washing the membrane with DW and the NC was allowed to air-dry.

24. Inhibition of nuclear import of the recombinant N-terminal PA by the HuScFv

The nuclear import assay for the recombinant PA was set as for the **Section 23.2.2** but the permeablized Vero cells were allowed to incubate with the PA in the import mixture for only 15 minutes. Thereafter the glass cover slips were individually incubated with HuScFv specific to the recombinant PA prepared from different HB2151 transformed *E. coli* clones (clones 10, 11, 15 and 18) using HuScFv specific to Tly A of *Leptospira* spp. as irrelevant HuScFv control. After 30 minutes of incubation at 37°C, the cells on the cover glass were thoroughly washed with the import buffer, fixed with 4% paraformaldehyde, washed and stained as above for the confocal microscopy as in **Section 23.1.3.**