CHAPTER V
RESULTS

1. Complementary (cDNA) synthesis

Complementary DNA was produced from the total RNA extracted from the influenza A virus, subtype H5N1 (A/duck/144/Thailand/2005(H5N1)) by RT-PCR using a ThermoScript^{TM} Revers Transcriptase (Invitrogen). The quality of the cDNA was checked by PCR using specific primers for detecting segment of gene encoding hemagglutinin subtype 5 (HA5 or H5). The PCR amplicon of the H5 gene segment was seen as a band of about 545 bp (**Figure 14**).

2. PCR amplification of DNA sequences encoding N-terminal halves of PA, PB1 and PB2 polymerase proteins

The cDNA was used as a template for PCR amplification of the 5'-halves of the PA, PB1 and PB2-coding DNA sequences using specific primers for individual genes. The PA-5', PB1-5' and PB2-5' amplicons were seen as a band of about 720, 1,134, 1,212 bp, respectively, as shown in **Figure 15**.

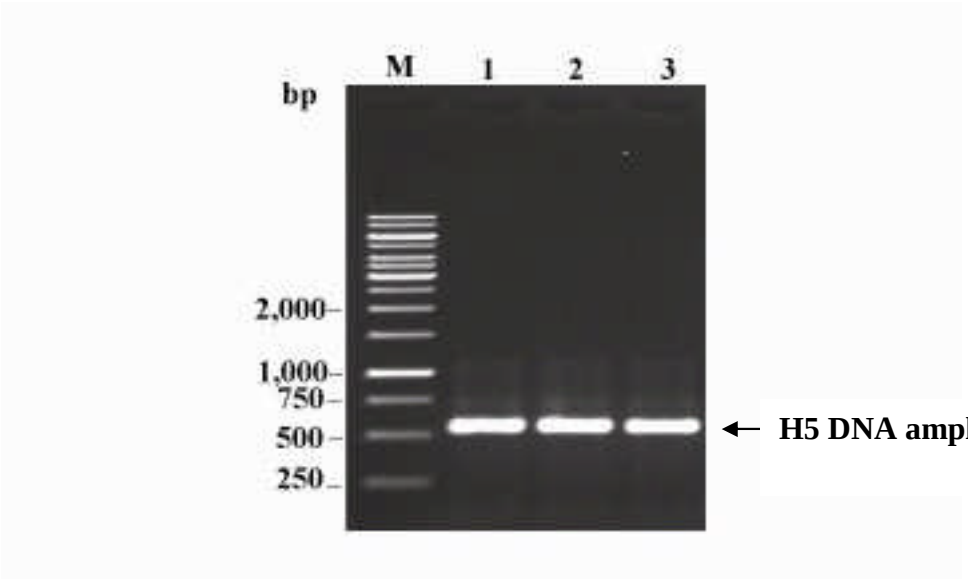


Figure 14 PCR amplified product of H5 gene segment of influenza A virus, H5N1 subtype

Lane M, 1 kb DNA marker

Lanes 1-3, H5 gene PCR product of H5 gene segment (~545 bp)

Numbers at the left of the figure are DNA sizes in base pairs (bp).

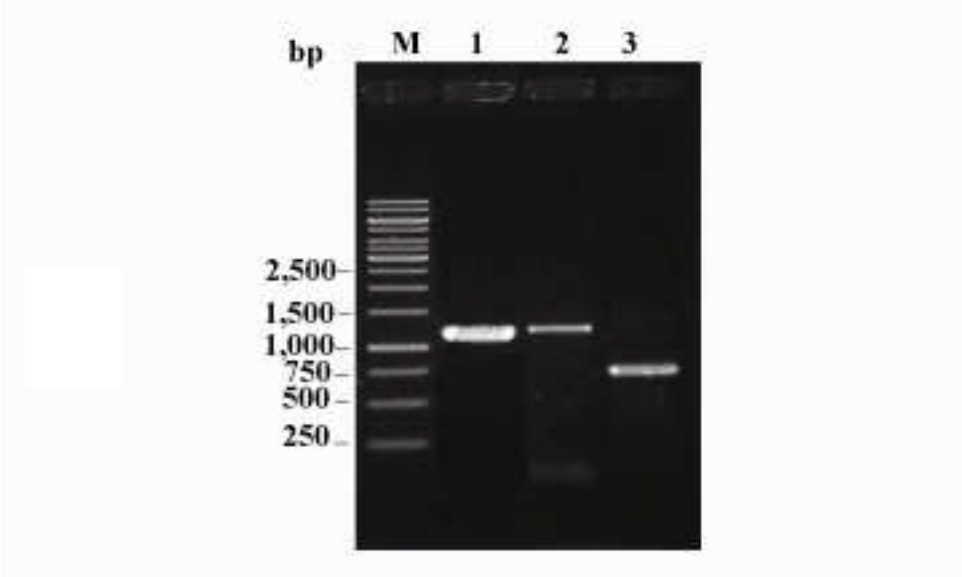


Figure 15 PCR amplicons of the *PB1-5'*, *PB2-5'* and *PA-5'* sequences

Lane M, 1 kb DNA marker

Lane 1, PCR amplicon of *PB1-5*' at of 1,134 bp

Lane 2, PCR amplicon of *PB2-5'* at of 1,212 bp

Lane 3, PCR amplicon of *PA-5'* at of 720 bp

Numbers at the left of the figure are DNA sizes in bp.

3. Cloning of DNA sequences encoding N-terminal halves of the PA, PB1 and PB2-polymerases, *i.e.*, *PA-5'*, *PB1-5'* and *PB2-5'* sequences, into cloning vectors

The *PA-5'*, *PB1-5'* and *PB2-5'* amplicons encoding the N-ternimal halves of the PA, PB1 and PB2 polymerase proteins were individually purified from agarose gels. The eluted *PA-5'*, *PB1-5'* and *PB2-5'* fragments with adenine (A) nucleotides at the 3' end were ligated with pTZ57R/T vector *via* the overhang T of the vector. The ligation product of each gene was introduced into competent JM109 *E. coli* cells by using chemical transformation protocol. The white colonies of the transformed JM109 *E. coli* colonies (**Figure 16**) were randomly picked from the overnight selective agar plates for plasmid extraction. The recombinant plasmids were then doubly digested with restriction endonucleases. The digested products were subjected to 1% agarose gel electrophoresis to verify the inserted DNA sizes. The sizes of the inserted fragments of PA-5', PB1-5' and PB2-5' genes cut from the respective recombinant plasmids were 720, 1,134, 1,212 bp, respectively, as shown in **Figure 17**.

4. DNA sequencing

The recombinant plasmids carrying inserted DNA fragments were extracted from the selected JM109 *E. coli* clones. The DNA sequencing was performed by Macrogen (Korea). The results of sequence comparison showed that there were 100% identity of nucleotide sequences between the prepared *PB1-5'*in this study and that of the PB1 gene sequence of database (**Figure 18**). Also, 100% identity of nucleotide sequences were found between the cloned *PB2-5'* and PB2 gene sequences of the database (**Figure 19**). However, there were only 99 % identity of nucleotide sequences between the cloned *PA-5'* and the PA gene sequences of the database (**Figure 20**).

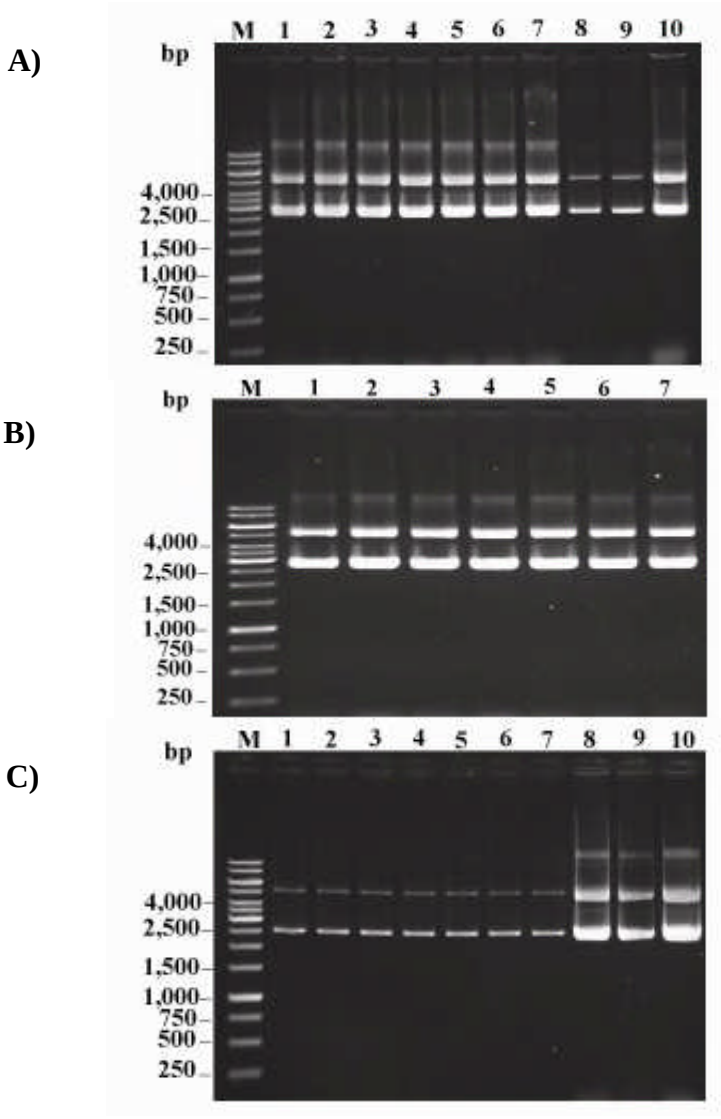


Figure 16 Patterns of recombinant plasmids extracted from JM109 *E. coli* clones after 1% agarose gel electrophoresis and ethidium bromide staining

A, Lanes 1-10 are extracted recombinant *PB1-5'* plasmids of *E. coli* clones 1-10, respectively.

B, Lanes 1-7 are extracted recombinant *PB2-5'* plasmids of *E coli* clones 1-7, respectively.

C, Lanes 1-10 are extracted recombinant *PA-5'* plasmids of *E. coli* clones 1-10, respectively.

Lane M, 1 kb DNA marker

Numbers at the left of A, B, and C are DNA sizes in bp.

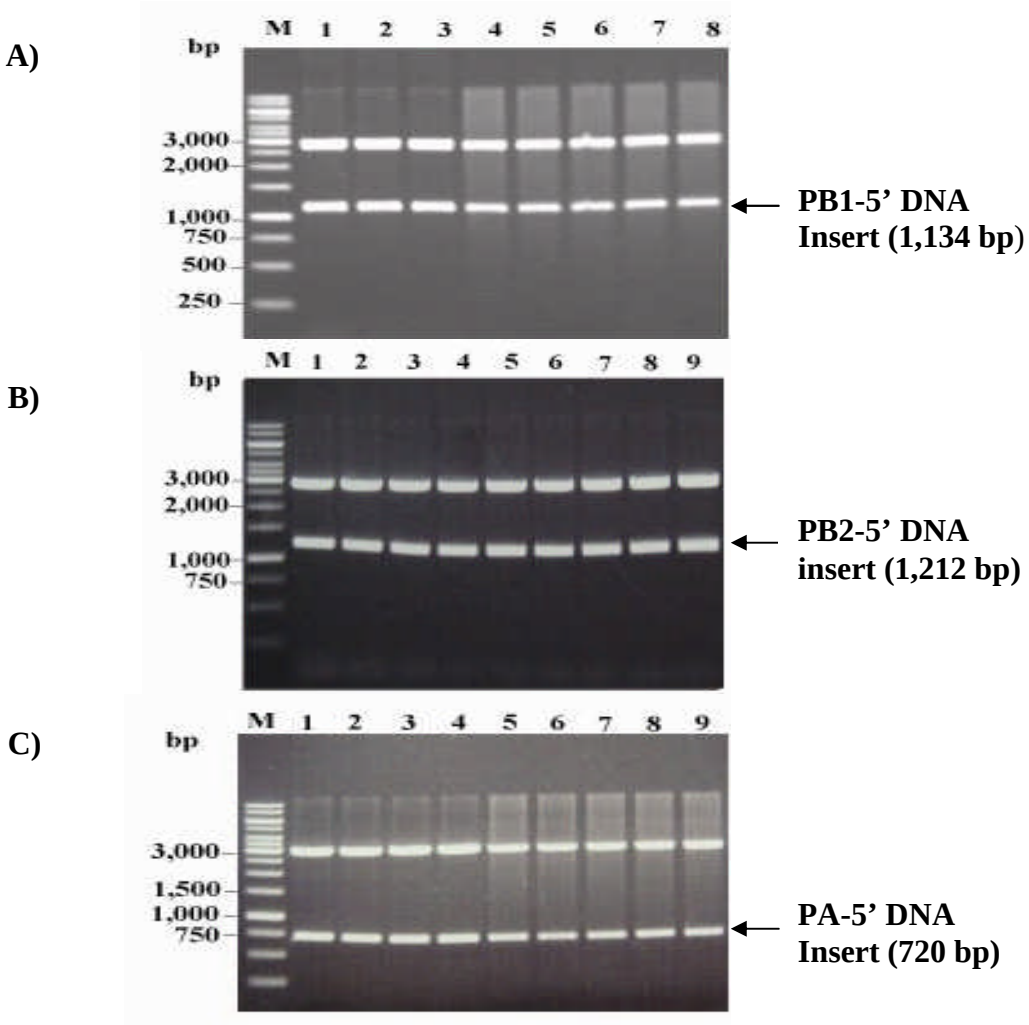


Figure 17 Patterns of DNA of recombinant plasmids extracted from JM109 *E. coli* and cut by restriction enzymes after 1% agarose gel electrophoresis and ethidium bromide staining

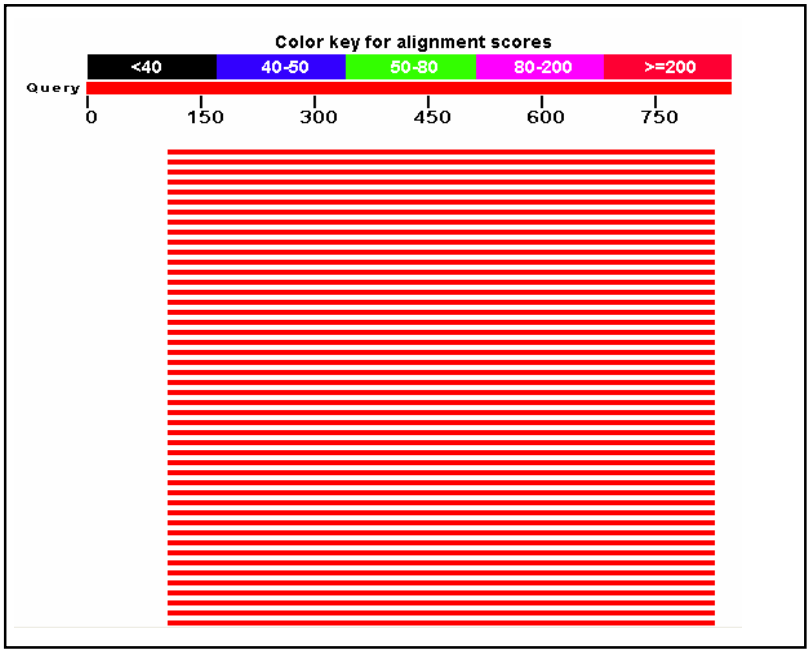
A, Lanes 1-8 are cut recombinant *PB1-5'* plasmids of *E. coli* clones 1-8, respectively.

B, Lanes 1-9 cut recombinant *PB2-5'* plasmids of *E. coli* clones 1-9, respectively.

C, Lanes 1-9 are cut recombinant *PA-5'* plasmid of *E. coli* clones 1-9, respectively.

Lane M, 1 kb DNA marker

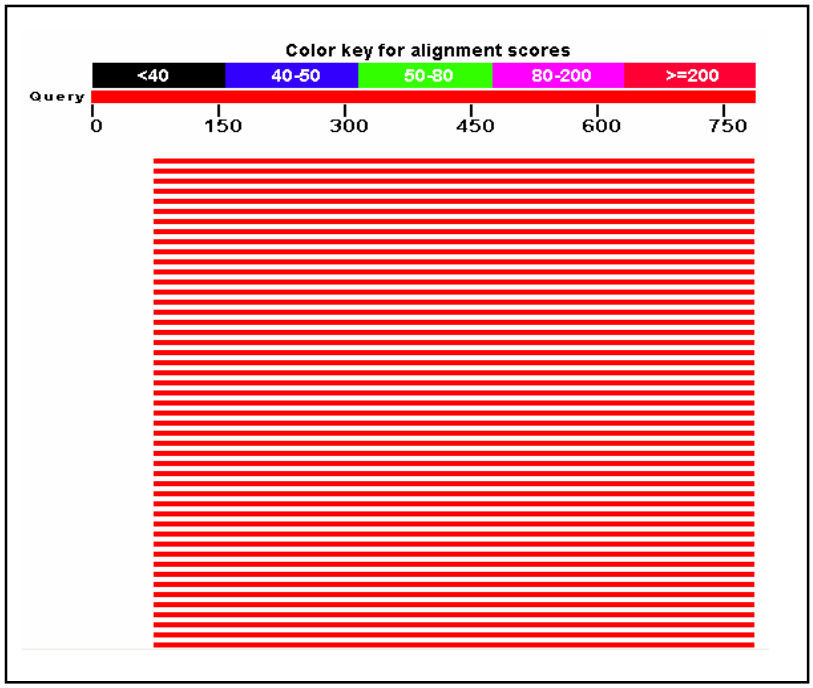
Numbers at the left of A, B and C are DNA sizes in bp.



Sequences producing significant alignments:

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
EF112246.1	Influenza A virus (A/open-billed stork/Nakhonsawan/BBA0627May/05	1051	1051	88%	0.0	100%
EF112245.1	Influenza A virus (A/open-billed stork/Nakhonsawan/BBD3616M/05(H	1051	1051	88%	0.0	100%
EF112238.1	Influenza A virus (A/open-billed stork/Nakhonsawan/BBA2711M/05(H!	1051	1051	88%	0.0	100%
EF112230.1	Influenza A virus (A/open-billed stork/Nakhonsawan/BBD2316F/05(H5	1051	1051	88%	0.0	100%
EF112227.1	Influenza A virus (A/open-billed stork/Nakhonsawan/BBD1621J/05(H5	1051	1051	88%	0.0	100%
EF112226.1	Influenza A virus (A/open-billed stork/Nakhonsawan/BBD1421J/05(H5	1051	1051	88%	0.0	100%
EF112225.1	Influenza A virus (A/open-billed stork/Nakhonsawan/BBD1221J/05(H5	1051	1051	88%	0.0	100%
DQ989993.1	Influenza A virus (A/open-billed stork/Nakhonsawan/BBD2616F/2005(	1051	1051	88%	0.0	100%
EF178509.1	Influenza A virus (A/tree sparrow/Thailand/VSMU-16-RBR/2005(H5N1	1051	1051	88%	0.0	100%
DQ530171.1	Influenza A Virus (A/dog/Thailand-Suphanburi/KU-08/04(H5N1)) polyr	1051	1051	88%	0.0	100%
AY770994.1	Influenza A virus (A/chicken/Ayutthaya/Thailand/CU-23/04(H5N1)) pc	1051	1051	88%	0.0	100%
DQ321330.1	Influenza A virus (A/quail/Malaysia/6309/2004(H5N1)) polymerase ba	1051	1051	88%	0.0	100%
DQ321329.1	Influenza A virus (A/chicken/Malaysia/5858/2004(H5N1)) polymerase	1051	1051	88%	0.0	100%
AY972554.1	Influenza A virus (A/tiger/Thailand/CU-T7/2004(H5N1)) polymerase b	1051	1051	88%	0.0	100%
AY972550.1	Influenza A virus (A/tiger/Thailand/CU-T3/2004(H5N1)) polymerase b	1051	1051	88%	0.0	100%
EF112242.1	Influenza A virus (A/open-billed stork/Nakhonsawan/BBD4211M/05(H	1046	1046	88%	0.0	99%
EF112236.1	Influenza A virus (A/open-billed stork/Nakhonsawan/BBA2111M/05(H!	1046	1046	88%	0.0	99%

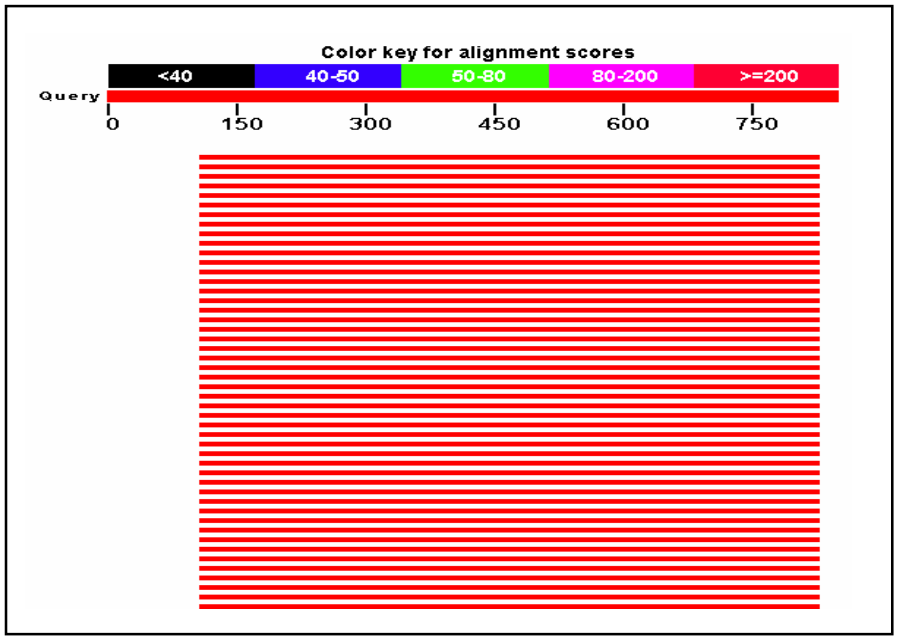
Figure 18 BLAST search across multiple nucleotide sequences of the database showing nucleotide sequence of the cloned PB1-5' gene sequence matched with 100% identity to the PB1 gene sequences of influenza A viruses of the database (accession numbers: EF112238.1, EF112245.1 and EF112246.1)



Sequences producing significant alignments:

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
EF112220.1	Influenza A virus (A/open-billed stork/Nakhonsawan/BBD3516M/05(H	1312	1312	90%	0.0	100%
EF112219.1	Influenza A virus (A/open-billed stork/Nakhonsawan/BBD4411M/05(H	1312	1312	90%	0.0	100%
EF112217.1	Influenza A virus (A/open-billed stork/Nakhonsawan/BBD4011M/05(H	1312	1312	90%	0.0	100%
EF112213.1	Influenza A virus (A/open-billed stork/Nakhonsawan/BBA2611M/05(H	1312	1312	90%	0.0	100%
EF112211.1	Influenza A virus (A/open-billed stork/Nakhonsawan/BBD3509M/05(H	1312	1312	90%	0.0	100%
EF112210.1	Influenza A virus (A/open-billed stork/Nakhonsawan/BBD3309M/05(H	1312	1312	90%	0.0	100%
EF112209.1	Influenza A virus (A/open-billed stork/Nakhonsawan/BBD3209M/05(H	1312	1312	90%	0.0	100%
EF112208.1	Influenza A virus (A/open-billed stork/Nakhonsawan/BBD3009M/05(H	1312	1312	90%	0.0	100%
EF112207.1	Influenza A virus (A/open-billed stork/Nakhonsawan/BBD2416F/05(H5	1312	1312	90%	0.0	100%
EF112206.1	Influenza A virus (A/open-billed stork/Nakhonsawan/BBD2316F/05(H5	1312	1312	90%	0.0	100%
EF112203.1	Influenza A virus (A/open-billed stork/Nakhonsawan/BBD1621J/05(H5	1312	1312	90%	0.0	100%
EF112201.1	Influenza A virus (A/open-billed stork/Nakhonsawan/BBD1221J/05(H5	1312	1312	90%	0.0	100%
DQ990000.1	Influenza A virus (A/open-billed stork/Nakhonsawan/BBA3011M/2005	1312	1312	90%	0.0	100%
EF112218.1	Influenza A virus (A/open-billed stork/Nakhonsawan/BBD4211M/05(H	1306	1306	90%	0.0	99%
EF112204.1	Influenza A virus (A/open-billed stork/Nakhonsawan/BBD1721J/05(H5	1306	1306	90%	0.0	99%

Figure 19 BLAST search across multiple nucleotide sequences of the database showing nucleotide sequence of the cloned PB2-5' gene sequence matched with 100% identity with the PB2 gene sequences of influenza A viruses of the database (accession numbers: EF112217.1, EF112219.1 and EF112220.1)



Sequences producing significant alignments:

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
EF112270.1	Influenza A virus (A/open-billed stork/Nakhonsawan/BBA0627May/05	1327	1327	84%	0.0	99%
EF112269.1	Influenza A virus (A/open-billed stork/Nakhonsawan/BBD3616M/05(H	1327	1327	84%	0.0	99%
EF112268.1	Influenza A virus (A/open-billed stork/Nakhonsawan/BBD3516M/05(H	1327	1327	84%	0.0	99%
EF112266.1	Influenza A virus (A/open-billed stork/Nakhonsawan/BBD4211M/05(H	1327	1327	84%	0.0	99%
EF112260.1	Influenza A virus (A/open-billed stork/Nakhonsawan/BBA2111M/05(H	1327	1327	84%	0.0	99%
EF112253.1	Influenza A virus (A/open-billed stork/Nakhonsawan/BBD1821J/05(HE	1327	1327	84%	0.0	99%
EF112252.1	Influenza A virus (A/open-billed stork/Nakhonsawan/BBD1721J/05(HE	1327	1327	84%	0.0	99%
EF112251.1	Influenza A virus (A/open-billed stork/Nakhonsawan/BBD1621J/05(HE	1327	1327	84%	0.0	99%
EF112249.1	Influenza A virus (A/open-billed stork/Nakhonsawan/BBD1221J/05(HE	1327	1327	84%	0.0	99%
DQ989994.1	Influenza A virus (A/open-billed stork/Nakhonsawan/BBD2616F/2005(	1327	1327	84%	0.0	99%
DQ989986.1	Influenza A virus (A/open-billed stork/Nakhonsawan/BBD1521J/2005(	1327	1327	84%	0.0	99%
EF112262.1	Influenza A virus (A/open-billed stork/Nakhonsawan/BBA2711M/05(H	1321	1321	84%	0.0	99%
EF112250.1	Influenza A virus (A/open-billed stork/Nakhonsawan/BBD1421J/05(HE	1321	1321	84%	0.0	99%
EF112267.1	Influenza A virus (A/open-billed stork/Nakhonsawan/BBD4411M/05(H	1310	1310	84%	0.0	99%
EF112265.1	Influenza A virus (A/open-billed stork/Nakhonsawan/BBD4011M/05(H	1310	1310	84%	0.0	99%
EF112264.1	Influenza A virus (A/open-billed stork/Nakhonsawan/BBA2911M/05(H	1310	1310	84%	0.0	99%

Figure 20 BLAST search across multiple nucleotide sequences of the database showing nucleotide sequence of the cloned PA-5' gene sequence matched with 99% identity with the PA gene sequences of influenza A viruses of database (accession numbers: EF112268.1, EF112269.1 and EF112270.1)

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

The extracted recombinant plasmids with PA-5', PB1-5' and PB2-5' DNA inserts and expression vector (pET-20b(+), pQE31 or pQE30) were subjected to double digestion with appropriate restriction endonucleases. The digested products were subjected to 1% agarose gel electrophoresis. The PA-5', PB1-5' and PB2-5' gene segments were purified from agarose gels by using GENECLEAN II kit. PB1-5' gene segments were ligated into pET-20b(+) protein expression vector pre-cut with the same enzymes. PA-5' and PB2-5' gene fragments were ligated into pQE31 and pQE30 protein expression vectors, respectively that were pre-cut with the same endonucleases. The ligation product of each gene was introduced into competent JM109 *E. coli* cells by using chemical transformation protocol. Transformed colonies were randomly picked from an overnight LB-A plate for PCR amplification. **Figure 21** shows the PCR products of PA-5', PB1-5' and PB2-5' gene segments from the selected transformed *E. coli* colonies. The sizes of *PA-5', PB1-5'* and *PB2-5'* amplicons were 720, 1,134, 1,212 bp, respectively. The transformed *E. coli* colonies carrying the target genes were subjected to plasmid extraction. The extracted recombinant *PB1-5'*- plasmids were introduced into BL21(DE3)pLysS *E. coli* cells. While the extracted recombinant *PA-5'*- and *PB2-5'* plasmids were introduced into M15 *E. coli* cells. Transformed *E. coli* colonies were randomly picked for PCR amplification. **Figure 22** shows the PCR products of PA-5', PB1-5' and PB2-5' gene segments from the *E. coli* transformants.

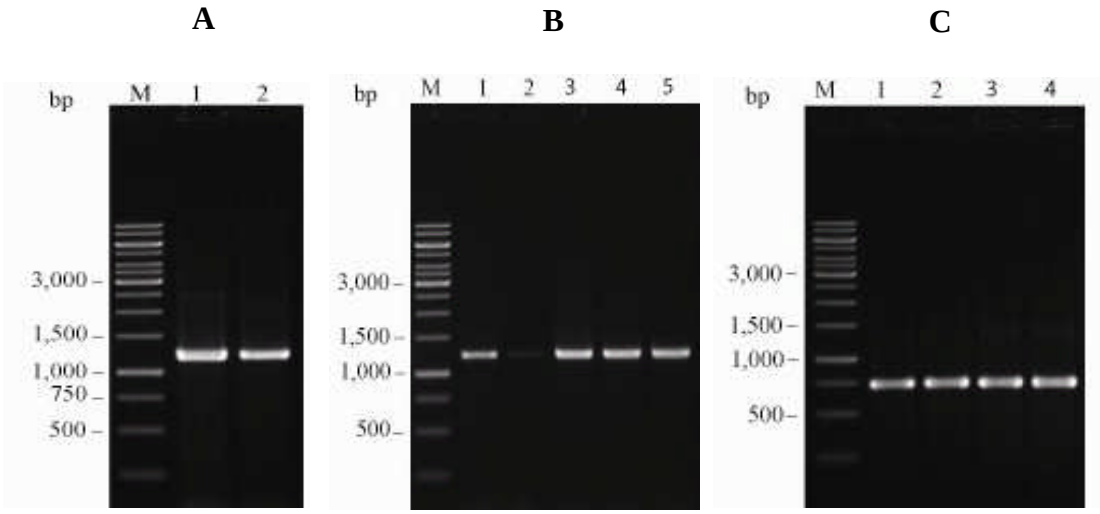


Figure 21 PCR amplicons of *PB1-5'*, *PB2-5'* and *PA-5'* segments from transformed JM 109 *E. coli* colonies after 1% agarose gel electrophoresis and ethidium bromide staining

A, Lanes 1-2 are PCR amplicons of *PB1-5'* segment (1,134 bp) of *E. coli* clones 1 and 2.

B, Lanes 1-5 are PCR amplicons of *PB2-5'* segment (1,212 bp) of *E. coli* clones 1-5.

C, Lanes 1-4 are PCR amplicons of *PA-5'* segment (720 bp) of *E. coli* clones 1-4.

Lane M, 1 kb DNA marker

Numbers at the left of A, B, and C are DNA sizes in bp.

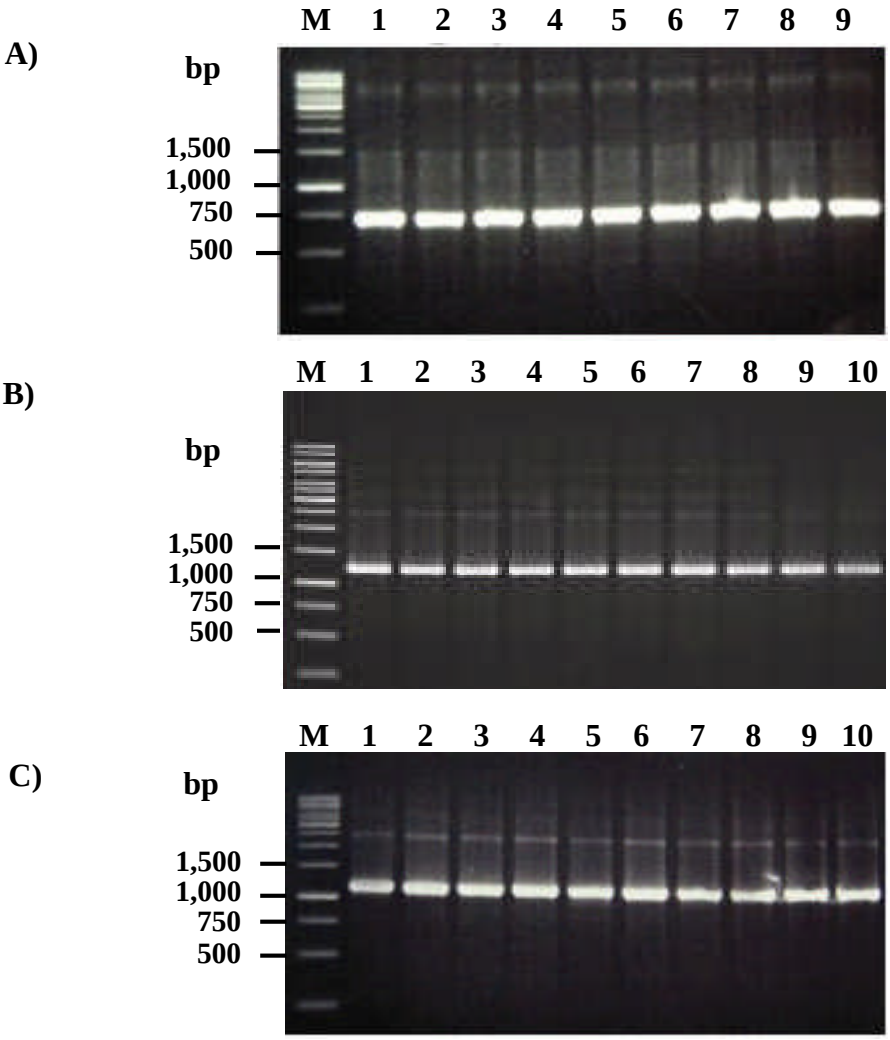


Figure 22 PCR amplicons of *PB1-5'*, *PB2-5'* and *PA-5'* sequences from transformed expression hosts after 1% agarose gel electrophoresis and ethidium bromide staining.

A, Lanes 1-10 are PCR amplicons of *PB1-5'* segments (~1,100 bp) of BL21 (DE3)pLysS *E. coli* clones 1-10, respectively.

B, Lanes 1-10 are PCR amplicons of *PB2-5'* segments (~1,200 bp) of M15 *E. coli* clones 1-10, respectively.

C, Lanes 1-9 are PCR amplicons of *PA-5'* segments (~720 bp) of M15 *E. coli* clones 1-9, respectively

Lane M, 1 kb DNA marker

Numbers at the left of **A**, **B**, and **C** are DNA sizes in bp.

6. N-terminal PA, PB1 and PB2 protein expression and purification

The recombinant *PB1-5'*-plasmid-transformed *E. coli* BL21(DE3)pLysS and the *PB2-5'* / *PA-5'*-plasmid-transformed M15 *E. coli* clones carrying the target genes were individually grown and induced to express the respective recombinant proteins by using IPTG. The expressed recombinant N-terminal PB1, PB2 and PA proteins were determined by SDS-PAGE and WB analysis using anti-His antibody as shown in **Figures 23, 24, 25 and 26**. The recombinant N-terminal PB1 and PB2 proteins had relative molecular masses (*Mr*) of approximately 43 and 41, respectively. They were detected in the insoluble cytoplasmic fractions (**Figures 23, 24 and 25**). The recombinant N-terminal PA protein had *Mr* of approximately 26. The protein was found in both soluble and insoluble cytoplasmic fractions but the latter showed more intense band (**Figure 26**).

The recombinant N-terminal PA protein was purified by using nickel column under natured and denatured conformation using batch elution with various concentrations of imidazole ranging form 50 mM to 500 mM. Three ml fractions were collected. The fractions were analyzed by SDS-PAGE and WB analysis using anti-His antibody as shown in **Figures 27 and 28**. The fractions containing the recombinant N-terminal PA protein were pooled and concentrated. The protein was used in the bio-panning process (**Figure 29**).

The recombinant N-terminal PB1 and PB2 proteins were also purified by using the nickel column but under a denatured conformation. The SDS-PAGE and WB analysis of the purified recombinant N-terminal-PB1 and -PB2 proteins are shown in **Figures 30 and 31,** respectively.

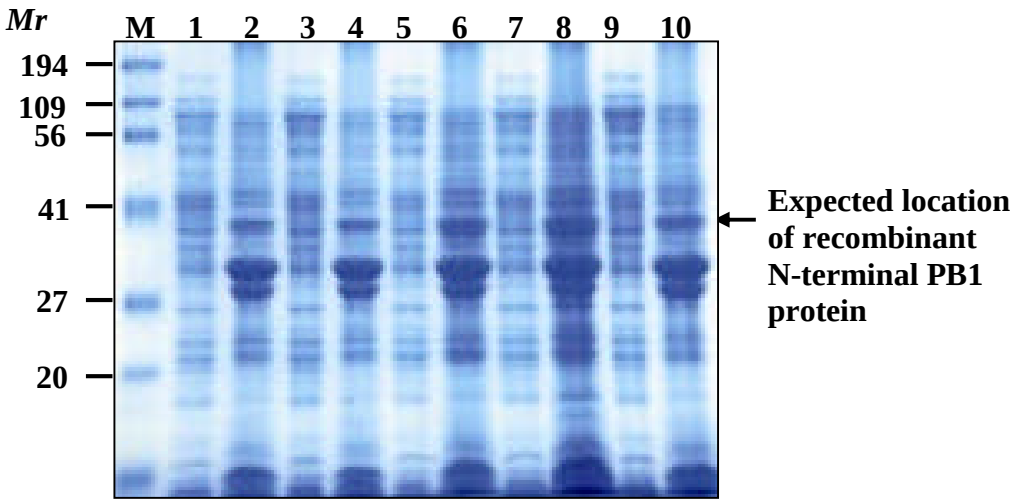


Figure 23 SDS-PAGE patterns run under reducing condition of recombinant N-terminal PB1 protein

Lanes 1 and 2; 3 and 4; 5 and 6; 7 and 8; and 9 and 10 are samples from soluble and insoluble fractions, respectively, of transformed BL21(DE3)pLysS *E. coli* clones no. 1-5, respectively.

Lane M, Pre-strained protein marker

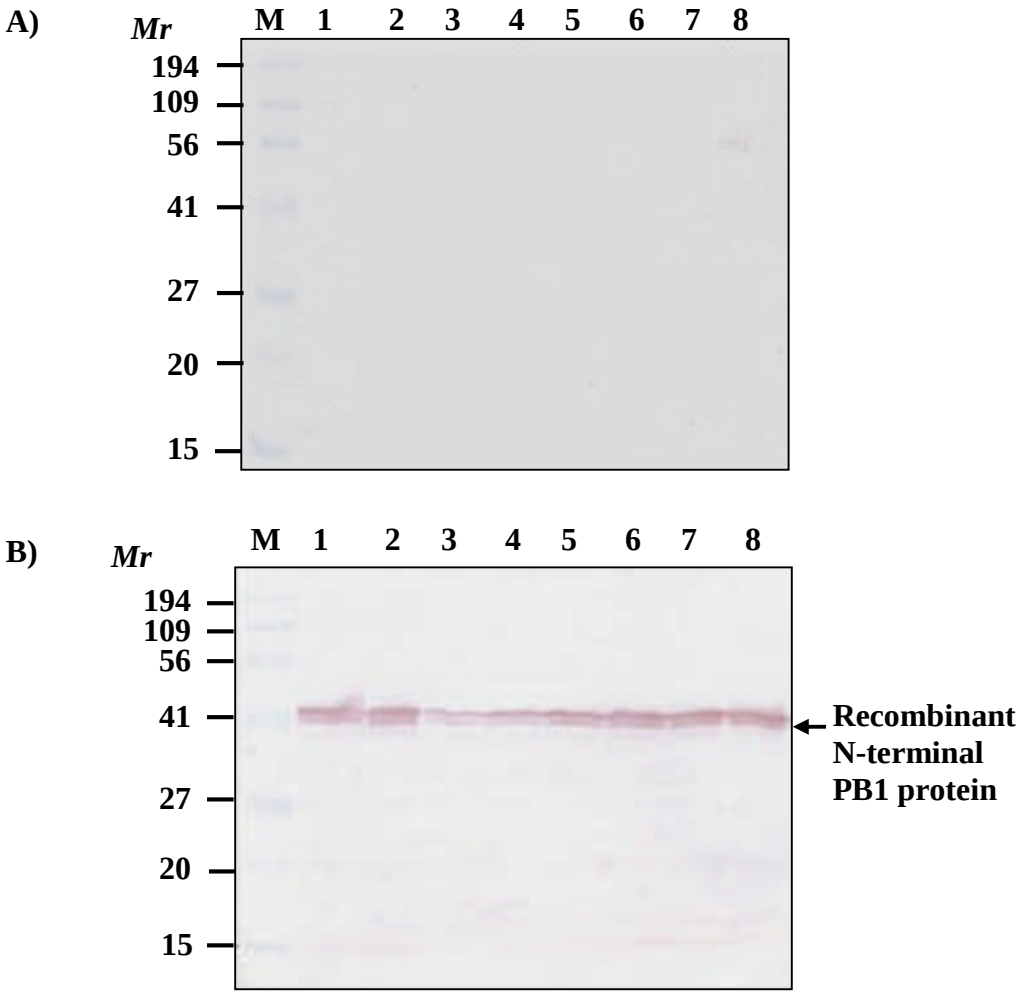


Figure 24 Results of Western blot analysis for screening of positive BL21(DE3) pLysS *E. coli* clones expressing recombinant N-terminal PB1 protein

A, WB patterns of SDS-PAGE separated-soluble fractions probed with anti-His antibody

B, WB patterns of SDS-PAGE separated-insoluble cytoplasmic fractions probed with anti-His antibody

Lane M, Pre-strained protein marker

Lanes 1-8, samples from transformed *E. coli* clones no. 1-8, respectively

Location of the recombinant N-terminal PB1 protein is indicated by arrow in **B**.

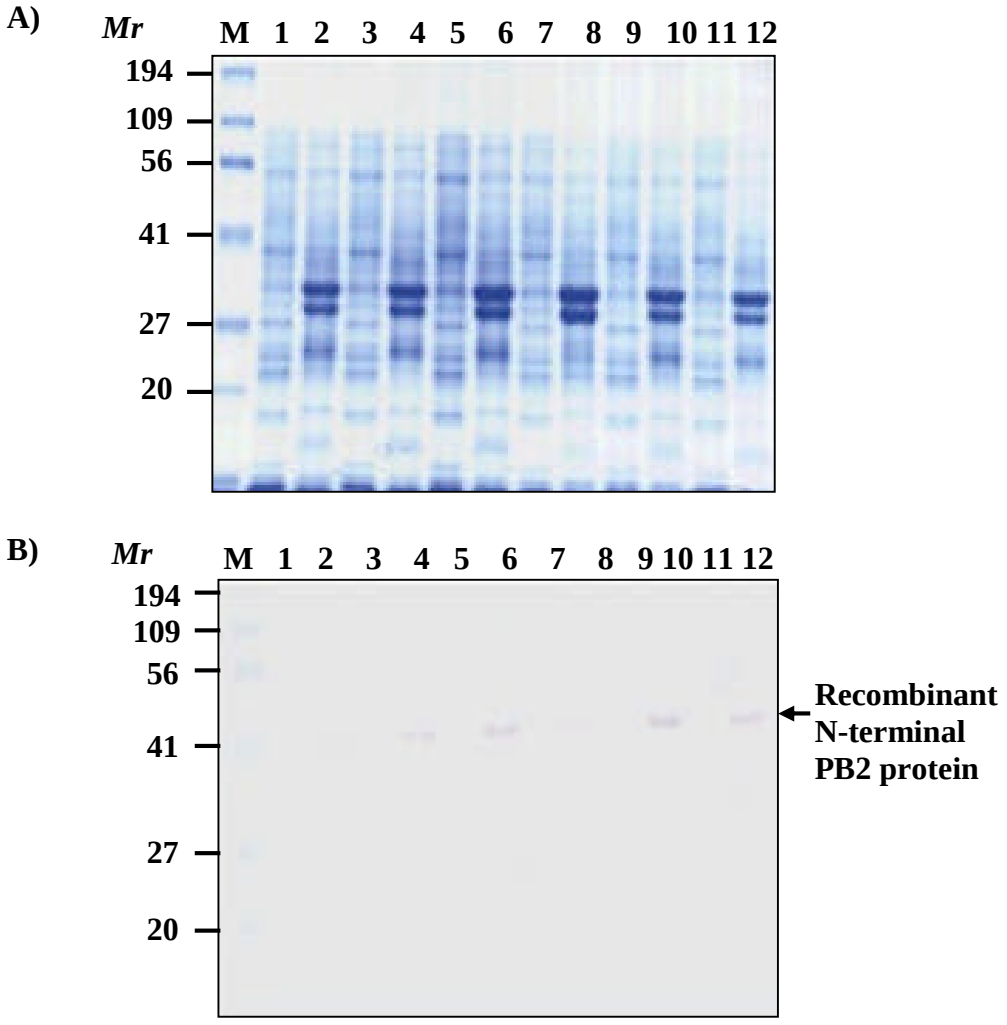


Figure 25 SDS-PAGE and Western blot results for screening of positive M15 *E. coli* clones expressing recombinant N-terminal PB2 protein

A, SDS-PAGE separated-soluble and insoluble cytoplasmic fractions of *E. coli* homogenates stained by CBB dye

B, WB of SDS-PAGE separated-soluble and insoluble cytoplasmic fractions in **A** probed with anti-His antibody

Lane M, Pre-strained protein marker

Lanes 1 and 2; 3 and 4; 5 and 6; 7 and 8; 9 and 10; 11 and 12 are samples from clones no. 1-6, respectively. Lanes with odd numbers are soluble cytoplasmic fractions and lanes with even numbers are insoluble cytoplasmic fractions.

Location of the recombinant PB2 protein is indicated by arrow in **B**.

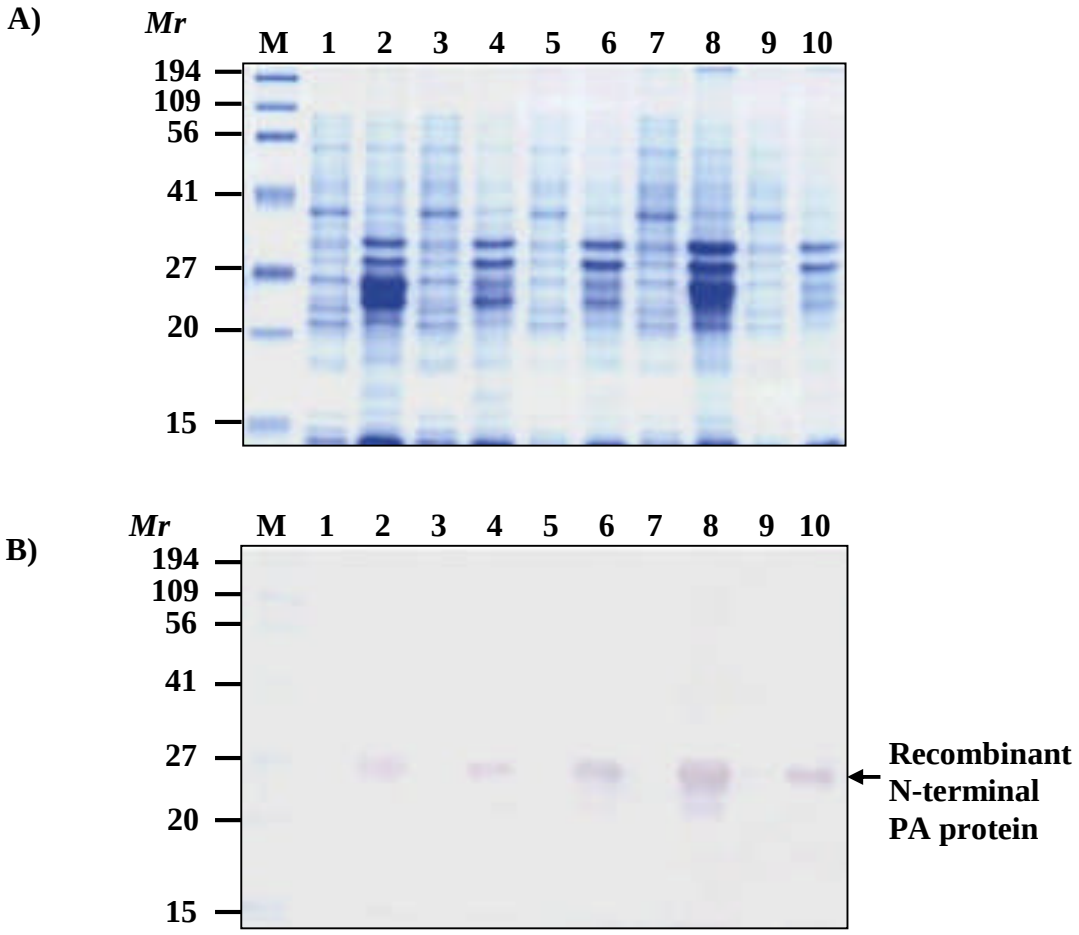


Figure 26 SDS-PAGE and Western blot results for screening of positive *E. coli* clones expressing recombinant N-terminal PA protein

A, SDS-PAGE separated-soluble and insoluble cytoplasmic fractions of transformed *E. coli* clones no. 1-5, respectively, stained by Coomassie Brilliant Blue G-250 (CBB)

B, WB of SDS-PAGE separated-soluble and insoluble cytoplasmic fractions of transformed *E. coli* clones no. 1-5, respectively, probed with anti-His antibody

Lane M, Pre-strained protein marker

Lanes 1 and 2; 3 and 4; 5 and 6; 7 and 8; and 9 and 10 are samples from transformed *E. coli* clones no. 1-5, respectively. Lanes with odd numbers are soluble cytoplasmic fractions of clones no. 1-5, respectively. Lanes with even numbers are insoluble cytoplasmic fractions of clones no. 1-5, respectively.

Location of the recombinant N-terminal PA protein is indicated by arrow.

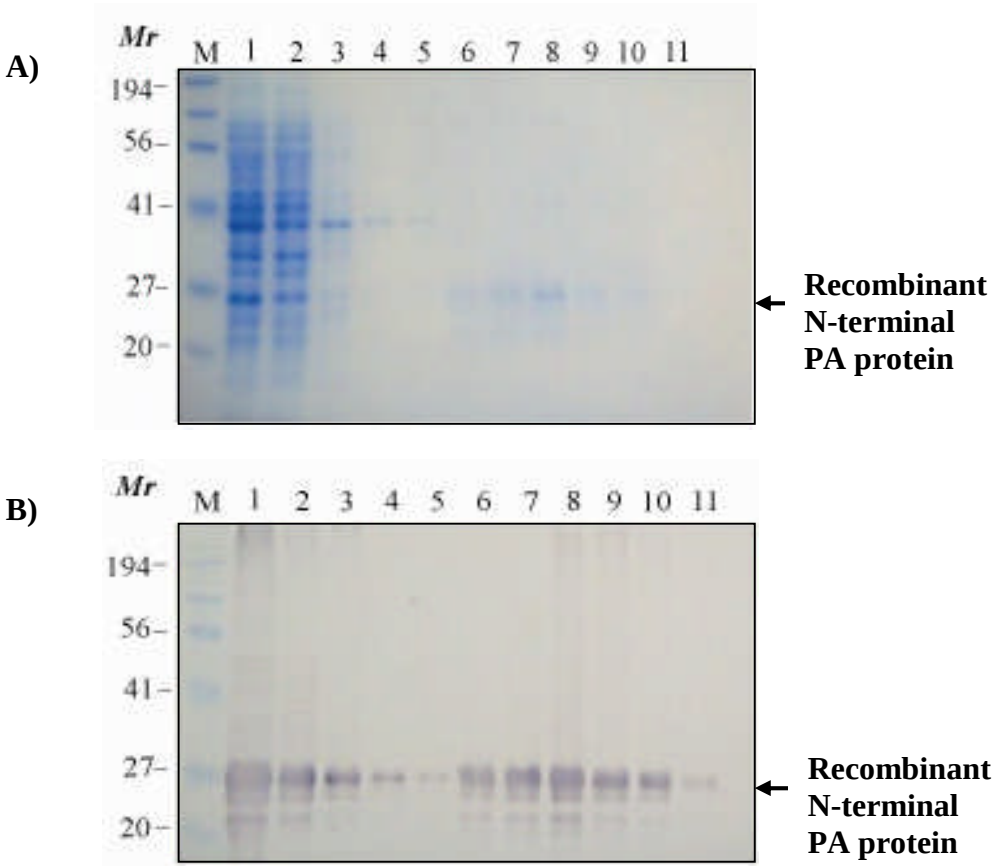


Figure 27 Results of the purification of the recombinant N-terminal PA protein by using a nickel column under denatured conformation using batch elution with various concentrations of imidazole ranging form 50 mM to 500 mM

A, SDS-PAGE patterns of proteins in the eluted fractions stained by CBB dye

B, WB patterns of the recombinant N-terminal PA protein in the eluted fractions of probed with anti-His antibody

Lane M, Pre-strained protein marker; Lane 1, bacterial lysate; Lanes 2 and 3, wash fractions; Lanes 4-11, fractions eluted with 50 mM, 100 mM, 150 mM, 200 mM and 250 mM, 300 mM and 500 mM imidazole, respectively

Locations of the recombinant N-terminal PA protein are indicated by arrows in **A** and **B**.

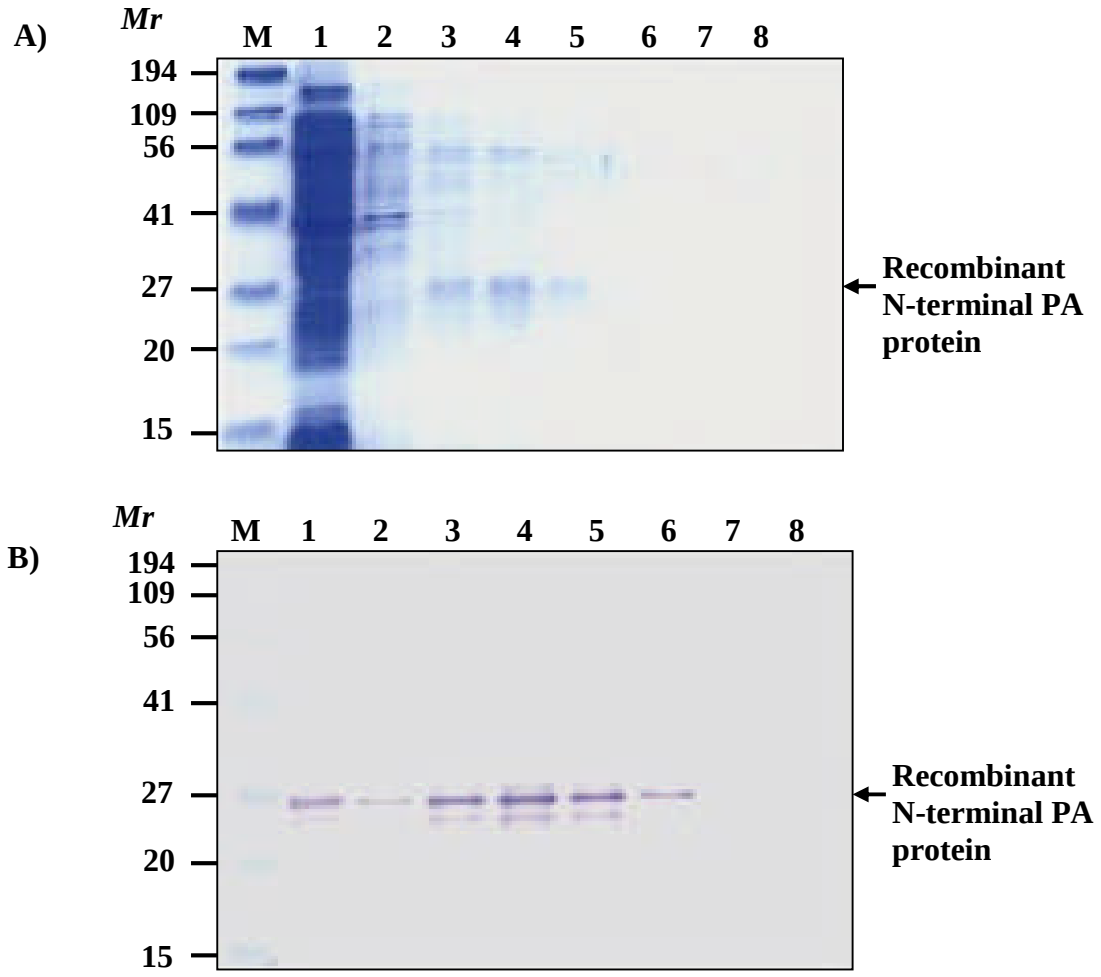


Figure 28 Results of purification of the recombinant N-terminal PA protein by nickel column under natural conformation using batch elution with various concentrations of imidazole ranging form 50 mM to 500 mM

A, SDS-PAGE separated-proteins in the eluted fractions of stained by CBB dye

B, WB of recombinant N-terminal PA protein in the eluted fractions probed with anti-His antibody

Lane M, Pre-strained protein marker; Lane 1, unbound fraction (flow through fluid); Lane 2, wash fraction; Lanes 3-8, fractions eluted with 50 mM, 100 mM, 150 mM, 200 mM, 250 mM, and 500 mM imidazole, respectively

Locations of the recombinant N-terminal PA protein are indicated by arrows in **A** and **B**.

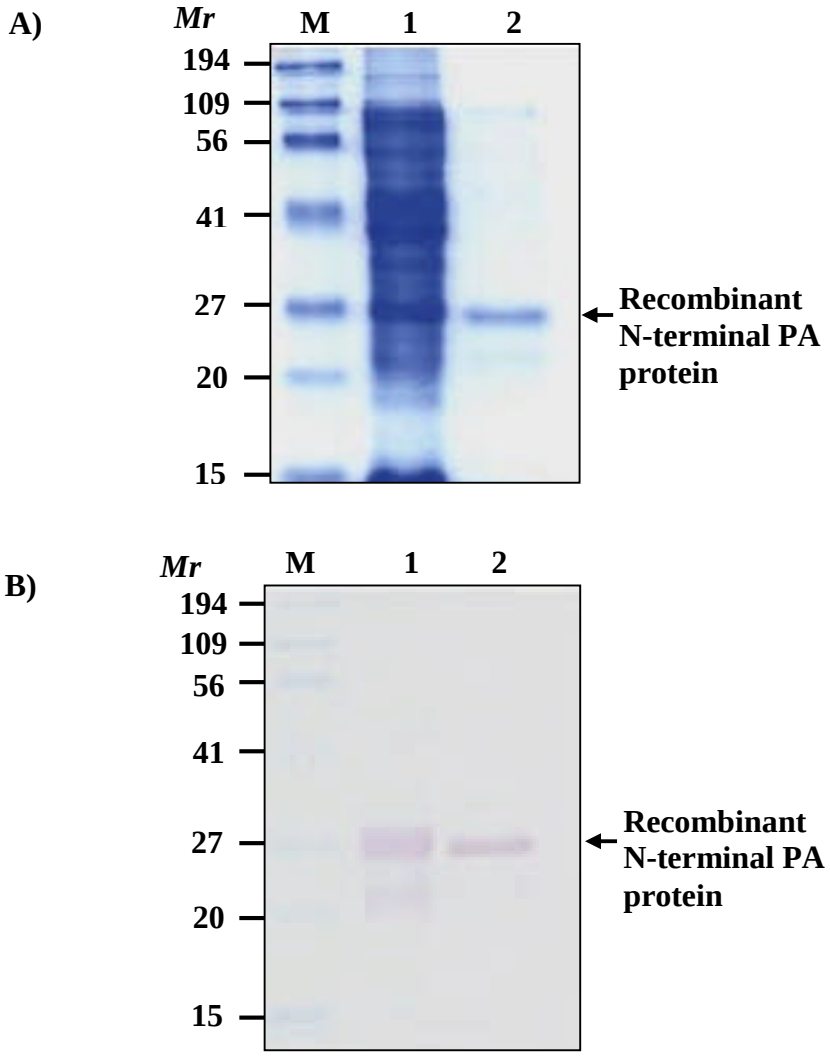


Figure 29 Results of purification of the recombinant N-terminal PA protein by using a nickel column under denatured conformation

A, SDS-PAGE separated-patterns of the recombinant N-terminal PA protein preparations

B, WB of SDS-PAGE of purified recombinant N-terminal PA protein probed with anti-His antibody

Lane M, Pre-strained protein marker; Lane 1, transformed *E. coli* lysate in lysis buffer-2; Lane 2, purified recombinant N-terminal PA protein

Locations of recombinant N-terminal PA protein are indicated by arrows in (**A**) and (**B**).

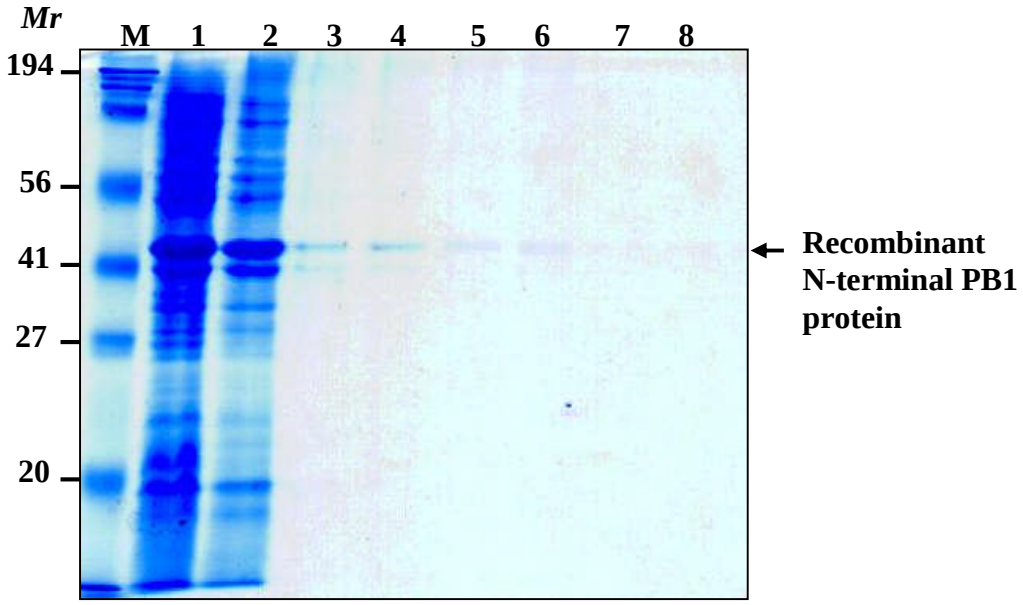


Figure 30 Results of SDS-PAGE patterns from purification of the recombinant N-terminal PB1 protein by using nickel column under denatured conformation

Lane M, Pre-strained protein marker

Lane 1, transformed *E. coli* lysate in lysis buffer-2

Lane 2, unbound fraction (flow through fluid)

Lanes 3-8, fractions eluted with 50 mM, 100 mM, 150 mM, 200 mM, 300 mM, and 500 mM imidazole, respectively

Locations of recombinant N-terminal PA protein are indicated by arrows.

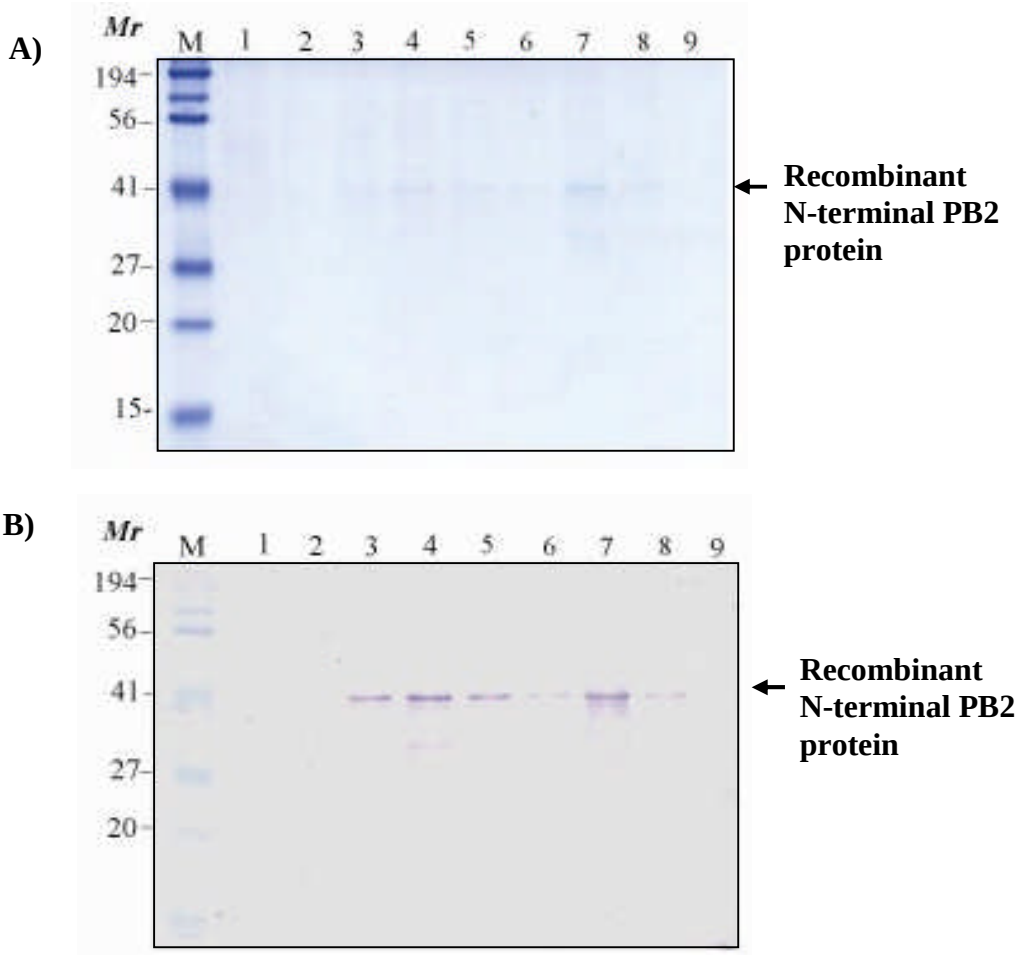


Figure 31 Results of purification of recombinant N-terminal PB2 protein purification by using a nickel column under denatured conformation using elution buffer (buffer D, pH 5.9 and buffer E, pH 4.5)

A, SDS-PAGE patterns of protein in the eluted fractions stained by CBB dye

B, WB of SDS-PAGE separated-recombinant N-terminal PB2 protein probed with anti-His antibody

Lane M, Pre-strained protein marker; Lane 1, wash fraction; Lanes 2-5, fractions eluted with buffer D, pH 5.9; Lanes 6-9, fractions eluted with buffer E, pH 4.5

Locations of recombinant N-terminal PB2 protein are indicated by arrows in **A** and **B**.

7. Selection of phage clones displaying HuScFv to the polymerase proteins from the phage display human antibody library

7.1 Phage display human single pot library

This phage display library was constructed in our laboratory from peripheral blood B lymphocytes of 60 healthy Thai blood donors. The library had approximately 3.0 x 10^8 antibody (VH-linker-VL; HuScFv) diversity**.**

7.2 Bio-panning

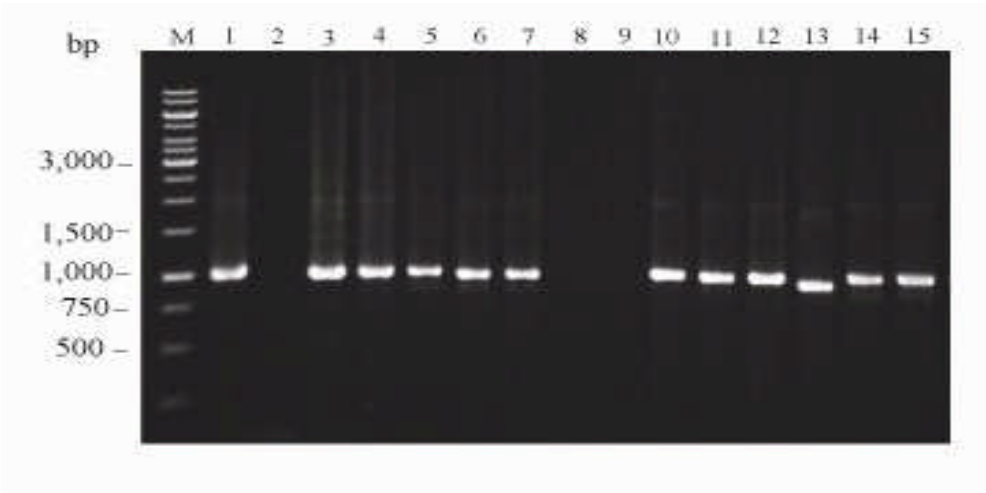
One microgram of purified recombinant protein was used to coat well of an ELISA plate. The coated protein was used as antigen to select the human antibody in the form of ScFv (HuScFv) molecule from phage display library**.** The human antibody phage library was added to the well. After removal of the unbound phages, the bound phages were eluted and they were allowed to infect HB2151 *E. coli*. The transformed bacterial cells were grown on two 2x YT-AG plates at 37ºC overnight. For bio-panning with recombinant N-terminal PA protein, a total of 315 transformed *E. coli* colonies appeared on the two agar plates. There were 252 transformed *E. coli* colonies from the phage bio-panning with recombinant N-terminal PB1. There were 268 transformed *E. coli* colonies from the phage bio-panning with recombinant N-terminal PB2 protein.

8. Screening of transformed HB2151 *E. coli* clones carrying the *huscFv*-phagemids

Thirty colonies of HB2151 *E. coli* transformants were randomly picked from the selective agar plate for checking the presence of the *huscFv*-phagemids by PCR. The PCR amplicons of *huscFv* gene from the randomly selected transformed *E. coli* colonies derived from bio-panning with recombinant N-terminal PA protein are shown in **Figure 32**. Twenty-five of the 30 clones (83.3%) (clones no. 1, 3, 4, 5, 6, 7, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 27, 28, 29, and 30) revealed DNA band at ~1,000 bp which is the expected size of the *huscFv*. The colonies carrying *huscFv* were used for HuScFv protein expression (please see **Section 9** below).

There were 19 of the 30 clones (63.3%) (clones no. 1, 2, 3, 4, 5, 6, 9, 11, 12, 13, 16, 18, 19, 20, 21, 25, 26, 27, and 28) carrying the *huscFv* obtained from bio-panning using recombinant N-terminal PB1 protien as antigen (**Figure 33**).

A)



B)

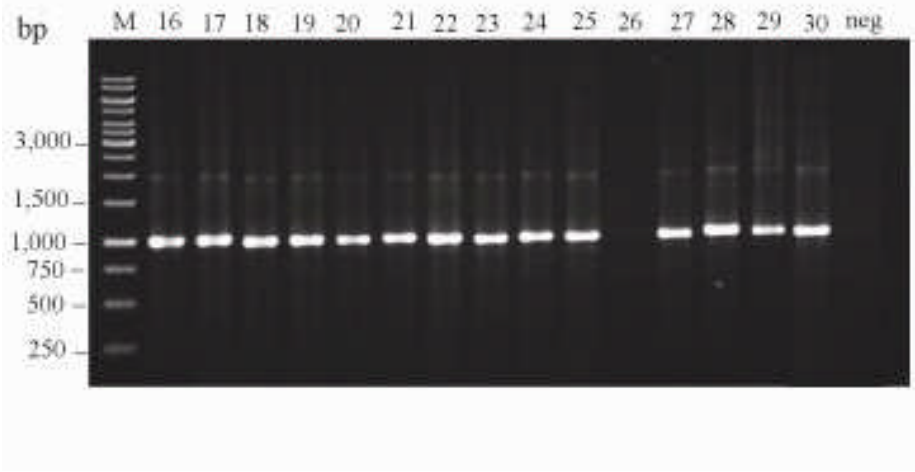


Figure 32 PCR amplicons of the *huscFv* sequences from transformed HB2151 *E. coli* derived from phage bio-panning using recombinant N-terminal PA protein as antigen

A, Lanes 1-15 are PCR products of *huscFv* sequences from clones no. 1-15, respectively.

B, Lanes 16-30 are PCR products of *huscFv* sequences from clones no. 16-30, respectively.

Lane M, 1 kb DNA marker

neg, negative control which had no DNA template in PCR mixture

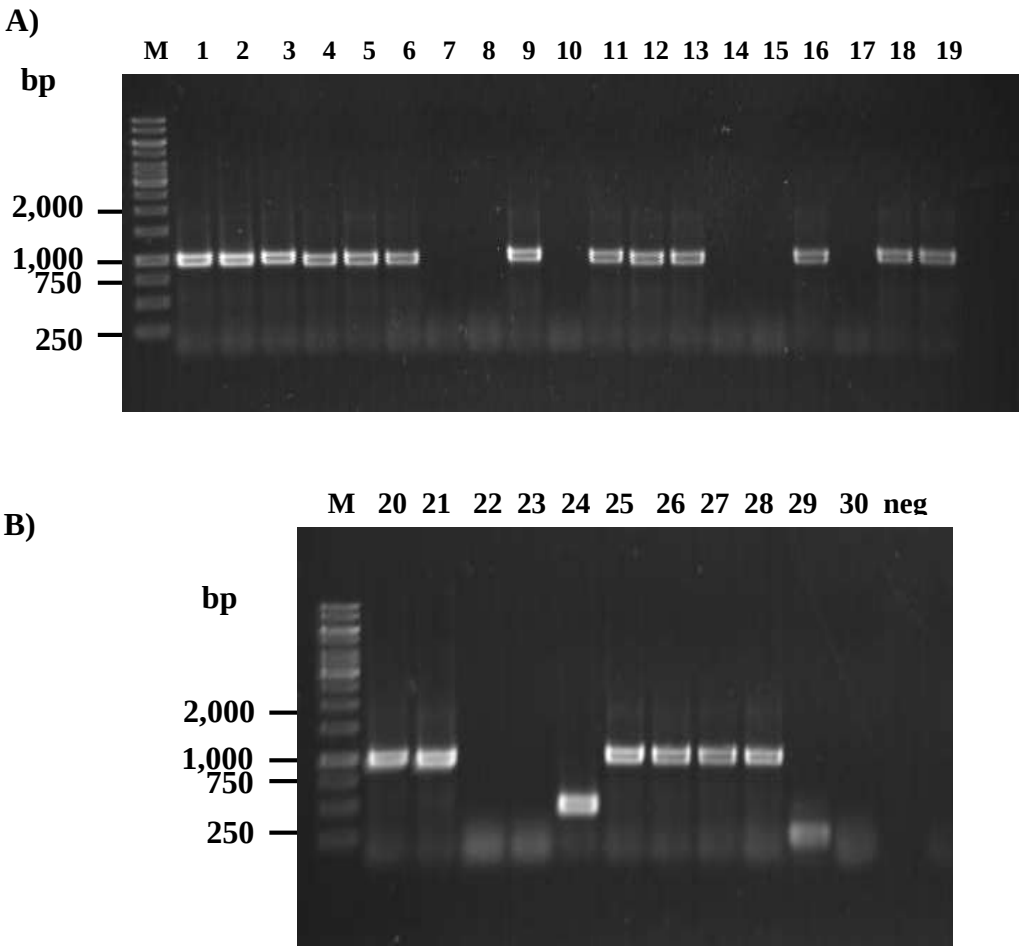


Figure 33 PCR amplicons of *huscFv* sequences from transformed HB2151 *E. coli* derived from phage bio-panning using recombinant N-terminal PB1 protein as antigen

A, Lanes 1-19 are PCR amplicons of *huscFv* sequences from clones no. 1-19, respectively.

B, Lanes 20-30 are PCR product of *huscFv* sequences from clones no. 20-30, respectively.

Lane M, 1 kb DNA marker

neg, negative control which had no DNA template in PCR mixture

From the bio-panning using recombinant N-terminal PB2 protein as antigen, 25 clones of the 30 randomly selected transformants carrying *huscFv* (83.3%) were obtained (clones no. 1, 3, 4, 5, 6, 9, 10, 11, 12 13, 14, 15, 17, 18, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, and 30) (**Figure 34**).

9. Screening of HuScFv-expressing transformed HB2151 *E. coli* clones

The *E. coli* colonies carrying *huscFv*-phagemids from **Section 8** above were induced to express the HuScFv by using IPTG. After induction with IPTG for 5 hours, the whole cell lysates of *E. coli* clones were prepared and subjected to SDS-PAGE and Western blot analysis by using mouse anti-E-Tag, goat anti-mouse immunoglobulin-alkaline phosphatase conjugate and phosphatase substrate as the detection reagents. There were 20 of the 25 screened colonies (80%) expressing HuScFv protein to the recombinant N-terminal PA protein (clones no. 3, 5, 6, 7, 10, 11, 12, 14, 15, 18, 19, 20, 21, 22, 23, 24, 27, 28, 29, and 30) (**Figure 35**). There were 12 of the 19 screened colonies (63.2%) expressing HuScFv protein to the recombinant N-terminal PB1 protein (clones no. 1, 2, 5, 6, 13, 18, 19, 20, 21, 25, 27, and 28). **Figure 36** shows HuScFv to recombinant N-terminal PB1 protein prepared from 8 representative clones of *E. coli* transformants.

Seventeen of the screened 25 colonies to recombinant N-terminal PB2 protein (68%) expressed HuScFv (clones no. 1, 3, 4, 5, 6, 9, 10, 11, 14, 15, 17, 22, 23, 24, 27, 29, and 30). **Figure 37** shows HuScFv to the recombinant N-terminal PB2 protein prepared from 8 representative clones of *E. coli* transformants.

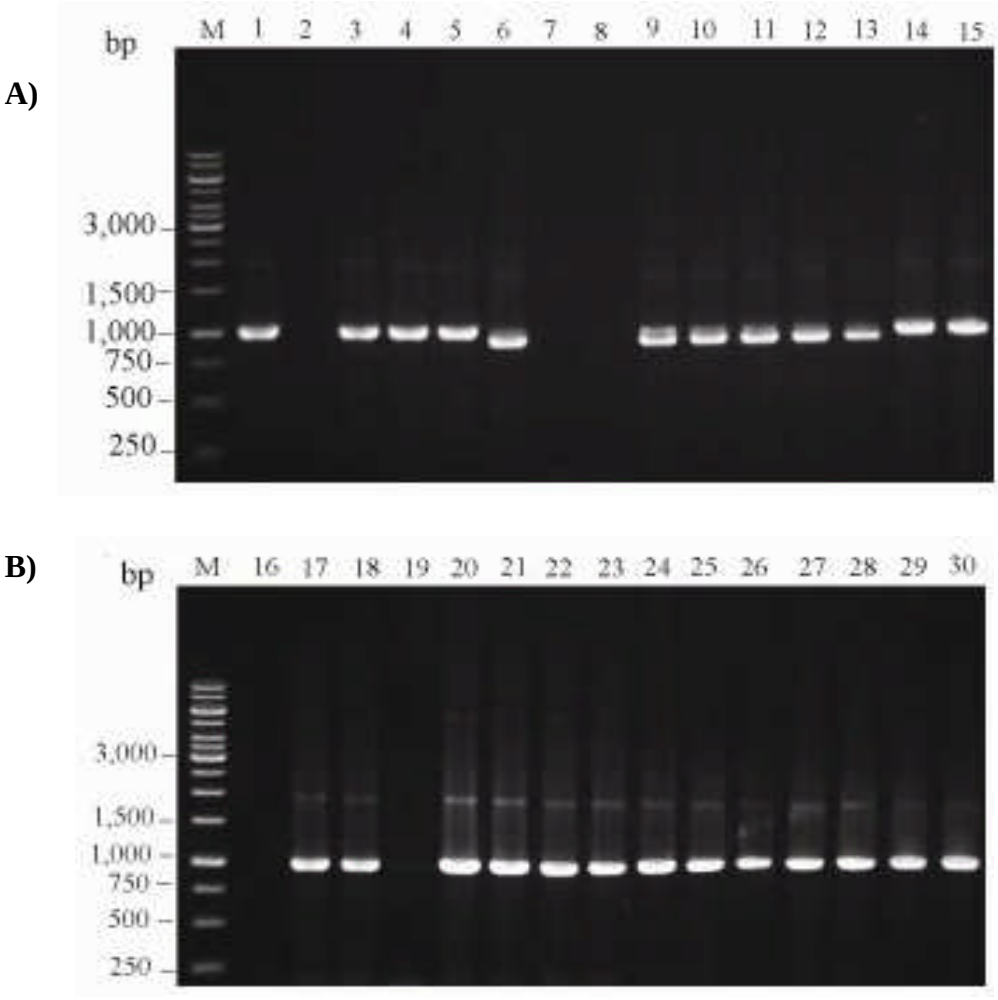


Figure 34 PCR amplicons of *huscFv* sequences from transformed HB2151 *E. coli* derived from phage bio-pannig using recombinant N-terminal PB2 protein as antigen

A, Lanes 1-15 are PCR amplicons of *huscFv* sequences from clones no. 1-15, respectively.

B, Lanes 16-30 are PCR product of *huscFv* sequences from clones no. 16-30, respectively.

Lane M, 1 kb DNA marker

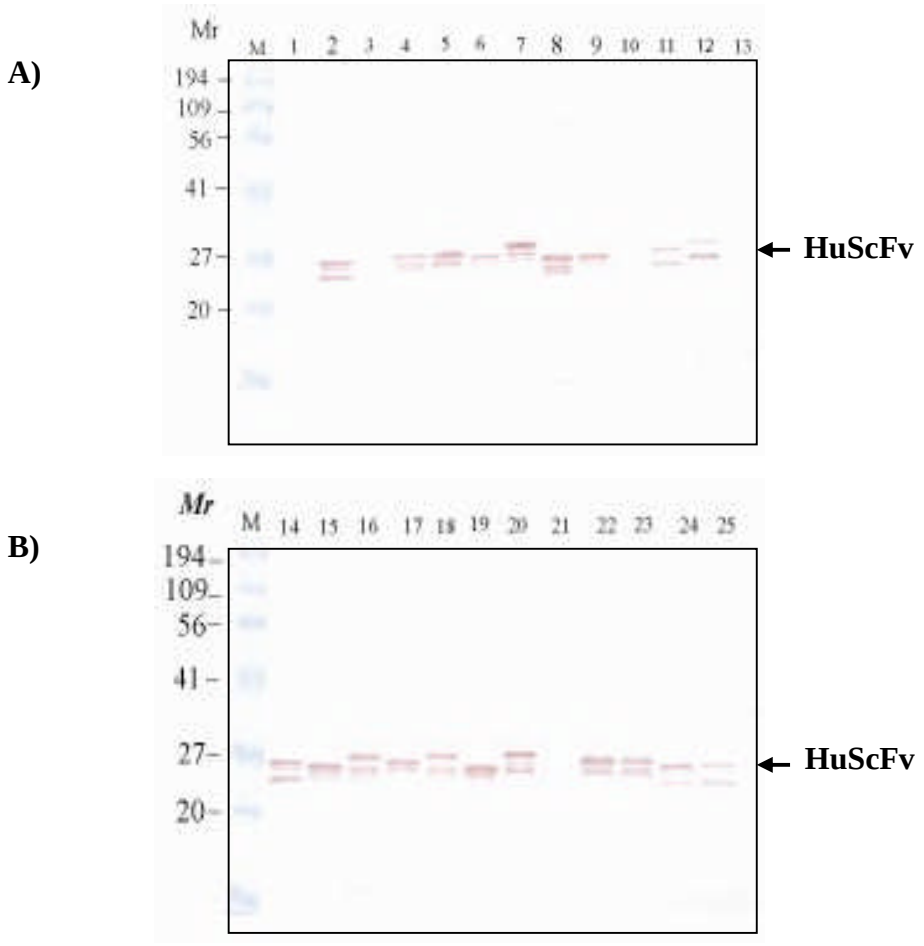


Figure 35 Western blot analysis for screening of HuScFv-expressing HB2151 *E. coli* clones from bio-panning using recombinant N-terminal PA protein (**A** and **B**)

Whole cell lysates of HB2151 *E. coli* clones carrying recombinant *huscFv*-phagemids were separated in a 12% polyacrylamide gel under reducing condition and the SDS-PAGE-separated-components were transblotted onto NC membrane.

A, Lanes 1-13 are lysates of *E. coli* clones no. 1, 3, 4, 5, 6, 7, 10, 11, 12, 13, 14, 15, 16, and 17, respectively.

B, Lanes 14-25 are lysates of *E. coli* clones no. 18, 19, 20, 21, 22, 23, 24, 25, 27, 28, 29, and 30, respectively.

Lane M, Pre-strained protein marker

The arrows indicate the expected sizes of the HuScFv which were *Mr* ~25-30.

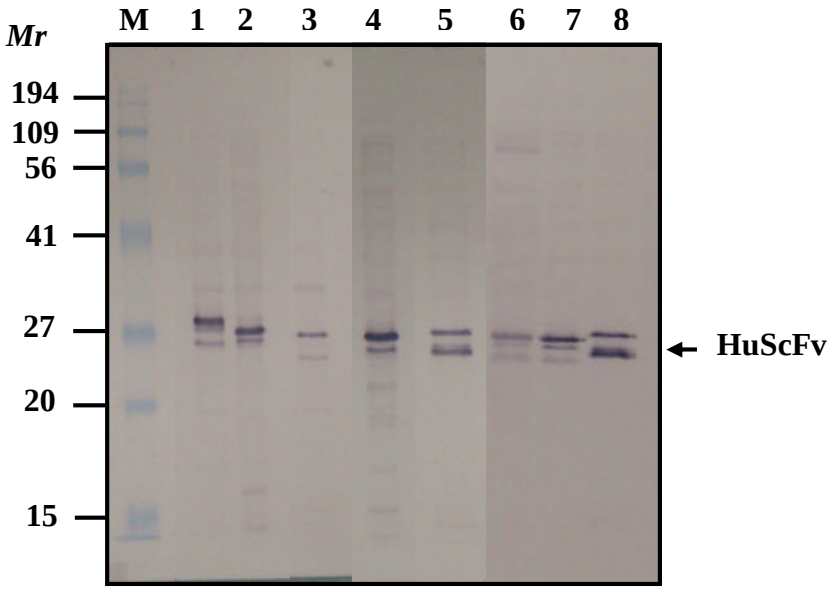


Figure 36 Western blot analysis of HuScFv-expressing HB2151 *E. coli* clones derived from phage bio-panning using recombinant N-terminal PB1 protein as antigen

Whole cell lysates of HB2151 *E. coli* clones carrying *huscFv*-phagemids were individually separated in a 12% polyacrylamide gel under reducing condition and the SDS-PAGE-separated-components were transblotted onto NC membrane.

Lanes 1-8 are lysates of *E. coli* clones no. 1, 2, 6, 9, 13, 18, 19, and 28, respectively.

Lane M, Pre-strained protein marker

The arrow indicates the expected sizes of HuScFv which were *Mr* ~ 25-30.

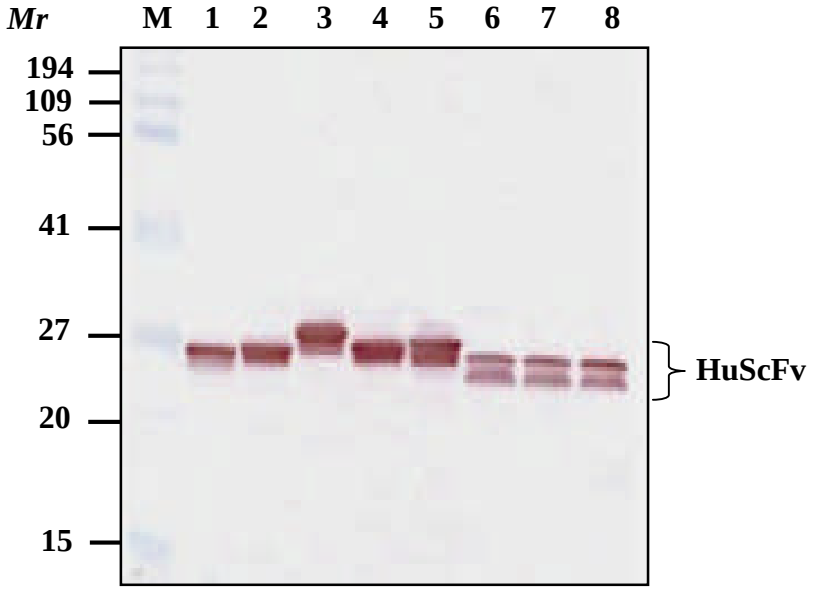


Figure 37 Western blot analysis of HuScFv-expressing HB2151 *E. coli* clones derived from phage bio-panning using recombinant N-terminal PB2 protein as antigen

Whole cell lysates of HB2151 *E. coli* clones carrying *huscFv*-phagemids were individually separated in a 12% polyacrylamide gel under reducing condition and the SDS-PAGE-separated-components were transblotted onto NC membrane.

Lanes 1-8 are lysates of *E. coli* clones no. 1, 3, 4, 5, 6, 9, 10, and 11, respectively.

Lane M, Pre-strained protein marker

The arrow indicates the expected sizes of HuScFv which were *Mr* ~ 25-30.

10. Determination of binding of HuScFv to recombinant proteins by using HuScFv-ELISA, Western blot analysis and dot ELISA

From phage bio-panning using recombinant N-terminal PA protein as antigen, whole cell lysates of *E. coli* transformants producing HuScFv (**Section 9**) were tested by HuScFv-ELISA to detect their binding specificity to recombinant protein. Whole cell lysate of original HB2151 *E. coli*, whole cell lysate of *E. coli* producing HuScFv specific to hemolysin of *Leptospira* and PBS were also included in the test as the respective controls. One microgram of BSA was used as a baseline control. It was found that all of the whole cell lysates from 20 *E. coli* clones (100%) (clones no. 3, 5, 6, 7, 10, 11, 12, 14, 15, 18, 19, 20, 21, 22, 23, 24, 27, 28, 29, and 30) that could produce HuScFv could bind to the homologous recombinant N-terminal PA protein with significant ELISA OD value when compared with that of the BSA (**Figure 38**). Whole cell lysate of original HB2151 *E. coli*, whole cell lysate of *E. coli* producing HuScFv specific to hemolysin of *Leptospira* and PBS showed low OD value. Western blotting of recombinant N-terminal PA protein reacted with whole cell lysates of *E. coli* transformants producing HuScFv showed that HuScFv from all *E. coli* could bind specifically to recombinant N-terminal PA protein at the expected size which were *Mr* 26 (**Figure 39**).

From phage bio-panning using recombinant N-terminal PB1 protein as antigen, seven of the whole cell lysates from 12 *E. coli* clones (58.3%) (clones no. 1, 9, 13, 18, 19, 25, and 28) producing HuScFv could bind to the homologous recombinant N-terminal PB1 protein with significant ELISA OD value when compared with that of the BSA (**Figure 40**). The positive bands from Western blotting of SDS-PAGE separated-recombinant N-terminal PB1 protein reacted with whole cell lysates of *E. coli* containing the ScFv to recombinant N-terminal PB1 protein (clones no. 1, 9, 13, 18, 19, 25, and 28) were not found. For dot-ELISA, 3 µl of the recombinant PB1-5' protein were applied onto NC membrane and then whole cell lysates of *E. coli* clones containing HuScFv were individually applied onto the dotted antigen. Six from seven *E. coli* (85.7%) showed positive signal (clones no. 9, 13, 18, 19, 25, and 28) (**Figure 41**).

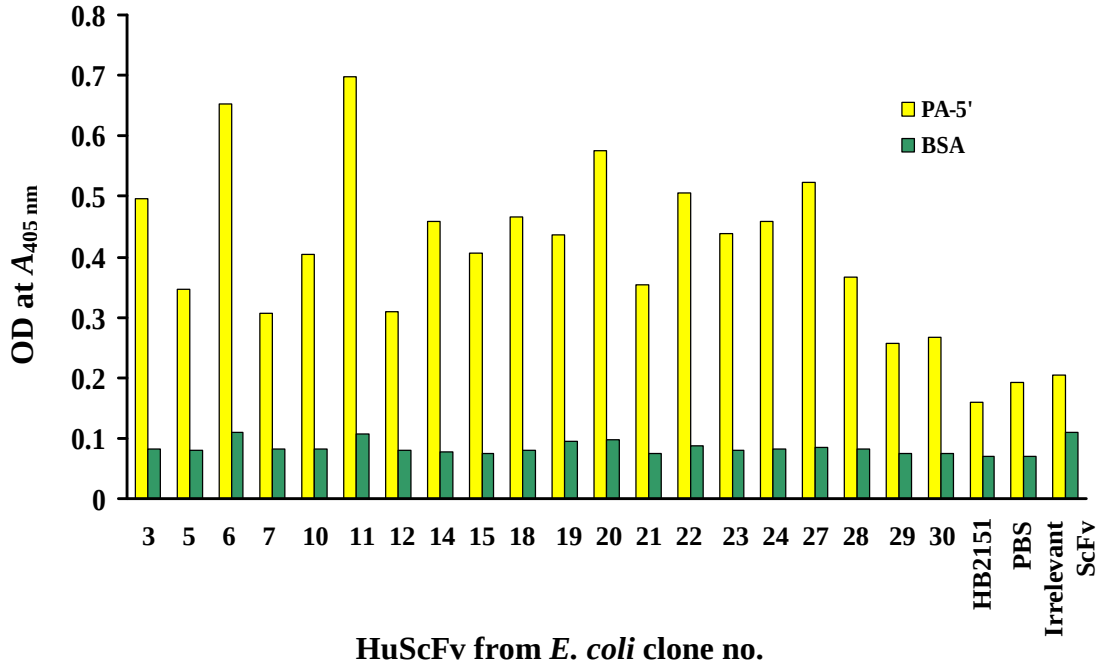


Figure 38 Results of HuScFv ELISA for detecting of the binding of HuScFv derived from different *E. coli* transformants to recombinant N-terminal PA protein

One microgram of purified recombinant N-terminal PA protein was used to coated each well of the ELISA plate. The wells were incubated with *E. coli* whole cell lysate containing HuScFv. Whole cell lysate of original HB2151 *E. coli* and the whole cell lysate of *E. coli* containing HuScFv specific to Tly A hemolysin of *Leptospira* and the PBS served as controls. The bound HuScFv were then detected by anti-E-Tag. The BSA was used as negative control to determine non-specific binding of HuScFv.

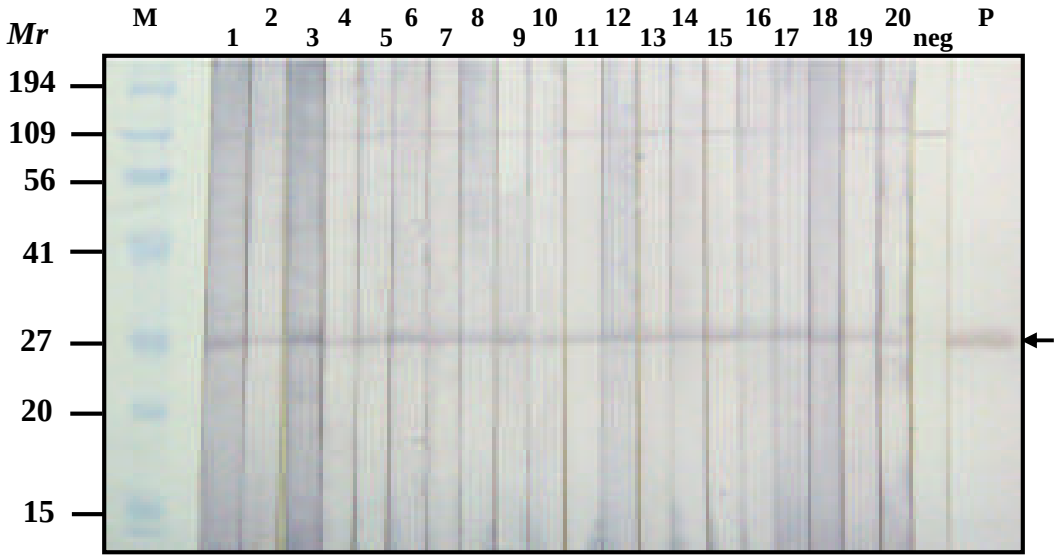


Figure 39 Results of Western blotting for detection of the binding of the HuScFv to recombinant N-terminal PA protein

Recombinant N-terminal PA protein (100 μg) was separated in a 12% polyacrylamide gel under reducing condition and SDS-PAGE-separated-components were transblotted onto an NC membrane. The membrane was probed with whole cell lysates of *E. coli* containing HuScFv, whole cell lysate of original HB2151 *E. coli* (negative control, neg) and anti-His antibody (positive control, P) were included. Anti-E-Tag was used as the detection reagent.

Lanes 1-20 are whole cell lysates of *E. coli* clones no. 3, 5, 6, 7, 10, 11, 12, 14, 15, 18, 19, 20, 21, 22, 23, 24, 27, 28, 29, and 30, respectively.

Lane M, Pre-strained protein marker

The arrow indicates the reactive band of HuScFv and recombinant PA-5’ protein.

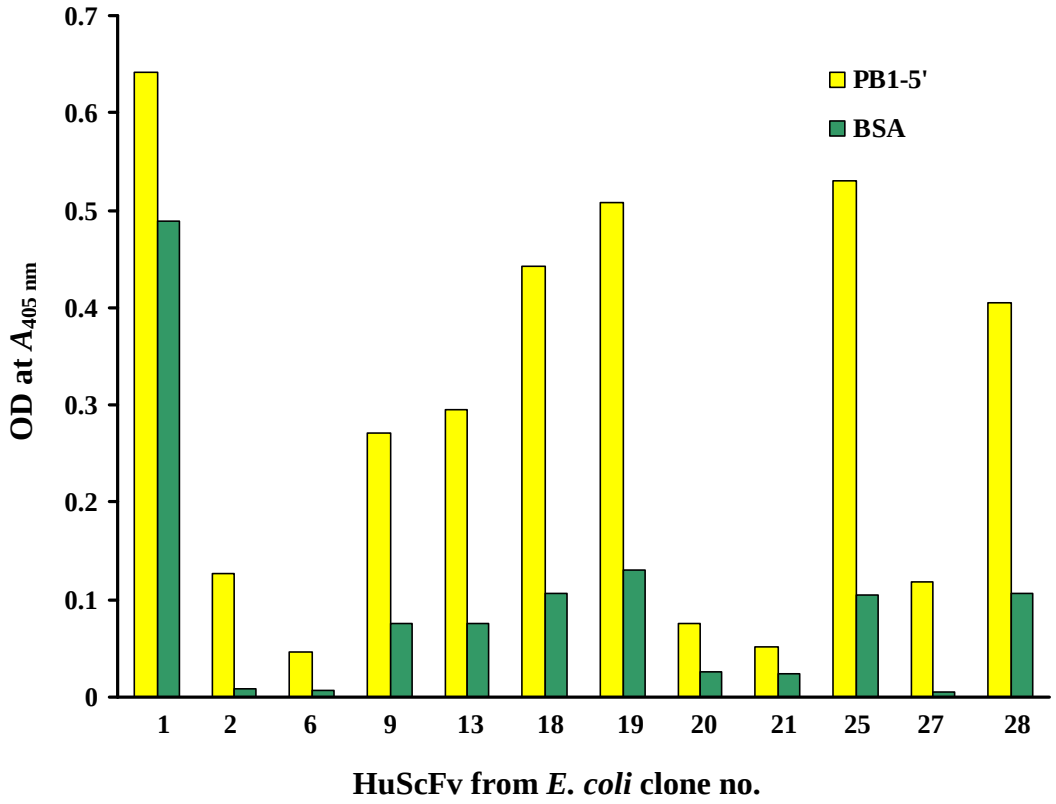


Figure 40 Results of HuScFv-ELISA for detection of the binding of the HuScFv derived from different *E. coli* transformants to recombinant N-terminal PB1 protein

One microgram of purified recombinant N-terminal PB1 protein was used to coated each well of the ELISA plate. The wells were incubated with *E. coli* whole cell lysate containing HuScFv. The bound HuScFv were then detected by anti-E-Tag. The BSA was used as negative control to determine non-specific binding of HuScFv.

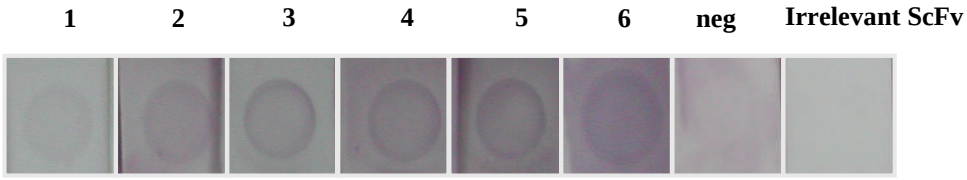


Figure 41 Dot-ELISA results for determining the binding of HuScFv to recombinant N-terminal PB1 protein

Three microliters aliquots of purified recombinant N-terminal PB1 protein were dotted onto the NC membrane. The individual NC squares were incubated with individual whole cell lysates of *E. coli* containing HuScFv. The binding was detected by using anti-E-Tag.

No. 1-6 are whole cell lysates of *E. coli* clones no. 9, 13, 18, 19, 25, and 28, respectively.

neg, whole cell lysate of original HB2151 *E. coli* which used as negative control

Irrelevant ScFv is the whole cell lysate of *E. coli* containing HuScFv specific to Tly A hemolysin of *Leptospira*.

From phage bio-panning using recombinant N-terminal PB2 protein as antigen, five of the whole cell lysates from 17 *E. coli* clones (29.4%) (clones no. 1, 4, 9, 10, and 12) showed significant OD value when compared with BSA (**Figure 42**). The positive band form Western blotting of SDS-PAGE separated- recombinant N-terminal PB2 protein reacteds with whole cell lysates of *E. coli* containing the HuScFv to the protein (clones no. 1, 3, 4, 5, 6, 9, 10, 11, 14, 15, 17, 22, 23, 24, 27, 29, and 30) were not detected. For dot-ELISA, Thirteen from 17 *E. coli* (76.5%) showed positive signal (clones no. 1, 3, 4, 5, 6, 9, 10, 14, 15, 17, 22, 27, and 29) (**Figure 43**).

11. Determination of *huscFv* diversity of the HuScFv by RFLP

The PCR products of *huscFv* sequences from phage bio-panning using recombinant respective proteins were cut with restriction endonuclease enzyme, *Mva*I. The digested product of each clone was loaded onto 12 % acrylamide gel in 0.5% TBE buffer for electrophoresis. The gel was then stained by ethidium bromide and visualized under a UV transilluminator.

From bio-panning with the recombinant N-terminal PA protein, twenty PCR products of *scFv* sequences from clones no. 3, 5, 6, 7, 10, 11, 12, 14, 15, 18, 19, 20, 21, 22, 23, 24, 27, 28, 29, and 30) were determined for *huscFv* diversity by RFLP. There were 16 RFLP patterns from the 20 digested *huscFv* sequences (80%) of the 20 *E. coli* clones as shown in **Figure 44**.

From bio-panning using recombinant N-temianl PB1 protein as antigen, six PCR products of *huscFv* sequences from clones no. 9, 13, 18, 19, 25, and 28 were used for diversity analysis. Five different RFLP patterns (83.3%) were observed as shown in **Figure 45**.

Thirteen PCR products of *huscFv* sequences (clones no. 1, 3, 4, 5, 6, 9, 10, 14, 15, 17, 22, 27, and 29) from phage bio-panning using recombinant N-terminal PB2 protein were tested for *huscFv* diversity by RFLP. Thirteen different RFLP patterns from the13 cut *huscFv* sequences (100%) were found as shown in **Figure 46**.

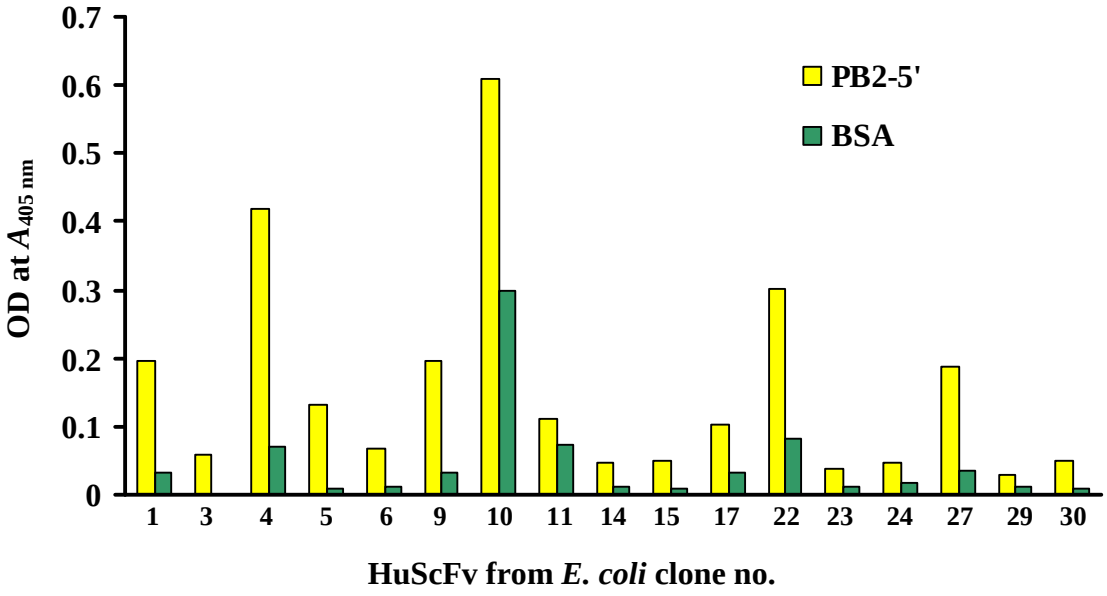


Figure 42 Results of HuScFv-ELISA for detection of the binding of HuScFv derived from different *E. coli* transformants to recombinant N-terminal PB2 protein

One microgram of purified recombinant N-terminal PB2 protein was used to coated each well of the ELISA plate. The wells were incubated with individual transformed HB2151 *E. coli* whole cell lysate containing HuScFv. The bound HuScFv were then detected by anti-E-Tag. The BSA was used as negative control to determine non-specific binding of HuScFv.

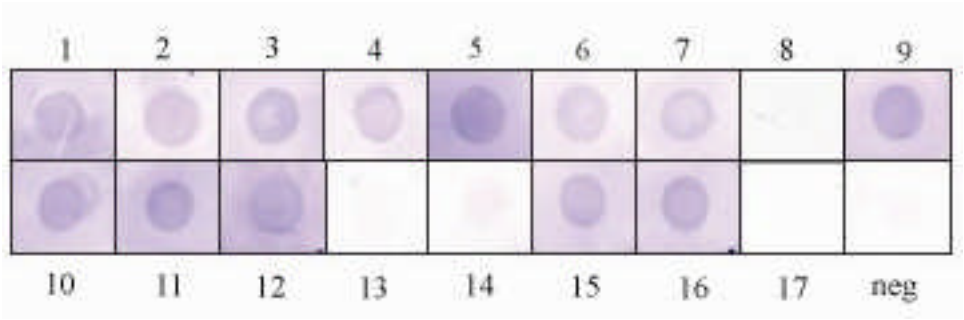


Figure 43 Dot-ELISA results for determining the binding of HuScFv to recombinant N-terminal PB2 protein

Row 1, no.1-9 are whole cell lysates of *E. coli* clones no. 1, 3, 4, 5, 6, 9, 10, 11, and 14, respectively.

Row 2, no. 10-17 are whole cell lysates of *E. coli* clones no. 15, 17, 22, 23, 24, 27, 29, and 30, respectively.

neg, whole cell lysate of original HB2151 *E. coli* which was used as negative control.

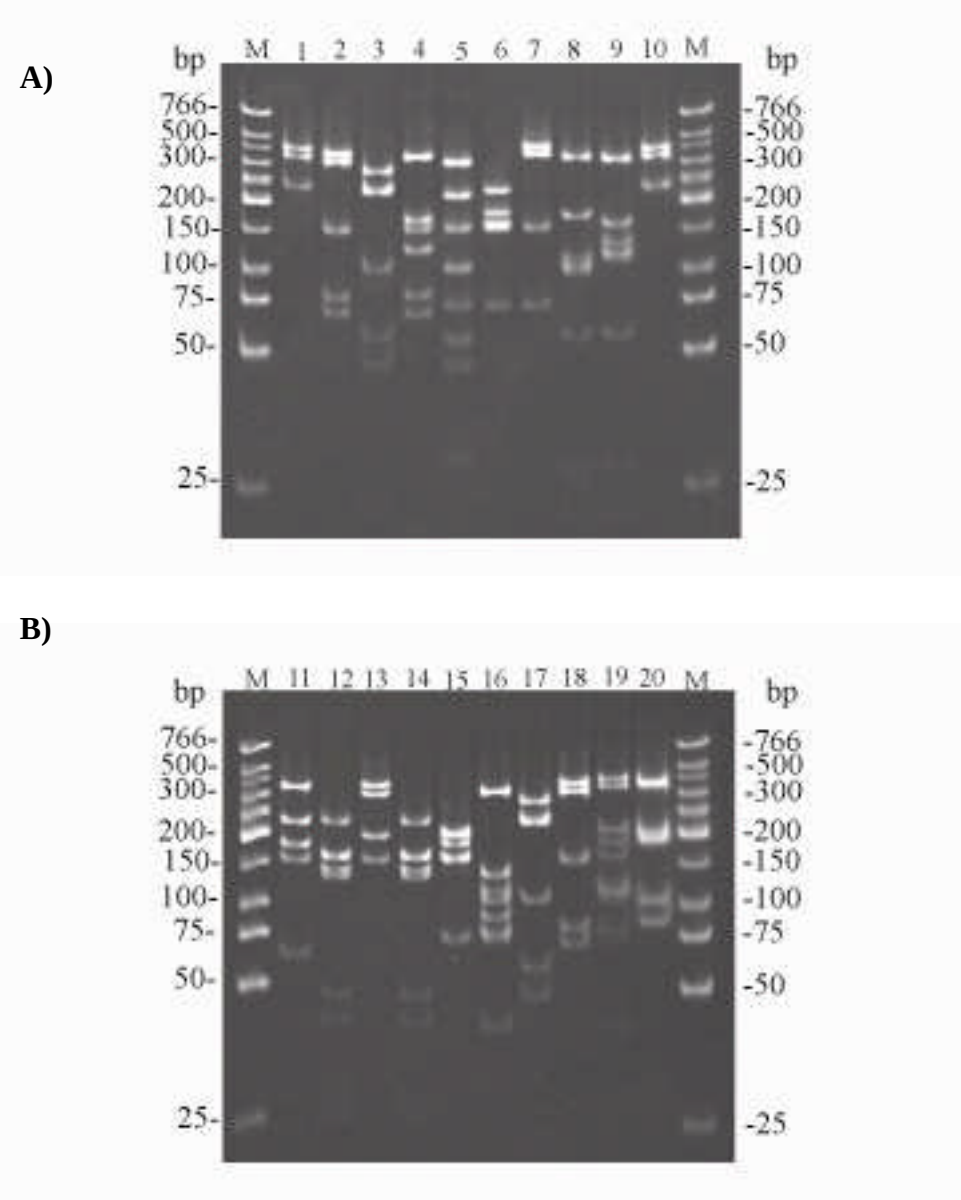


Figure 44 RFLP banding patterns of *huscFv* sequences from bio-pannig using recombinant N-terminal PA protein as antigen

Lane M, low molecular weight DNA ladder

Lanes 1-10, digested *huscFv* sequences of clones no. 3, 5, 6, 7, 10, 11, 12, 14, 15, and 18**,** respectively; Lanes 11-20, digested *huscFv* sequences of clones no. 19, 20, 21, 22, 23, 24, 27, 28, 29, and 30**,** respectively

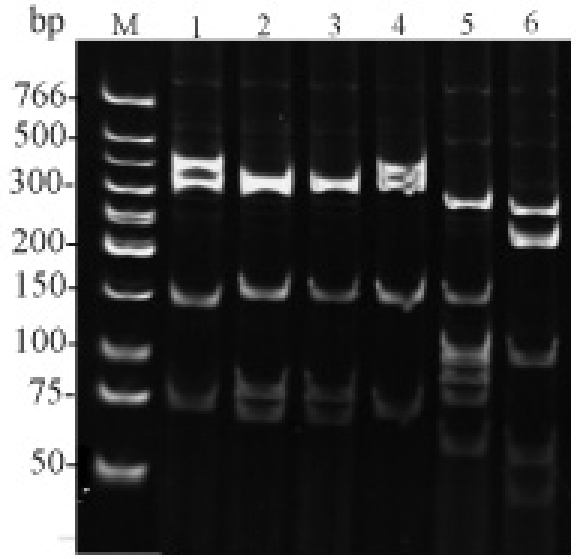


Figure 45 RFLP banding patterns of *huscFv* sequences from bio-panning using recombinant N-terminal PB1protein as antigen

Lane M, low molecular weight DNA ladder

Lanes 1-6, digested *huscFv* sequences of clones no. 9, 13, 19, 20, 25, and 28, respectively

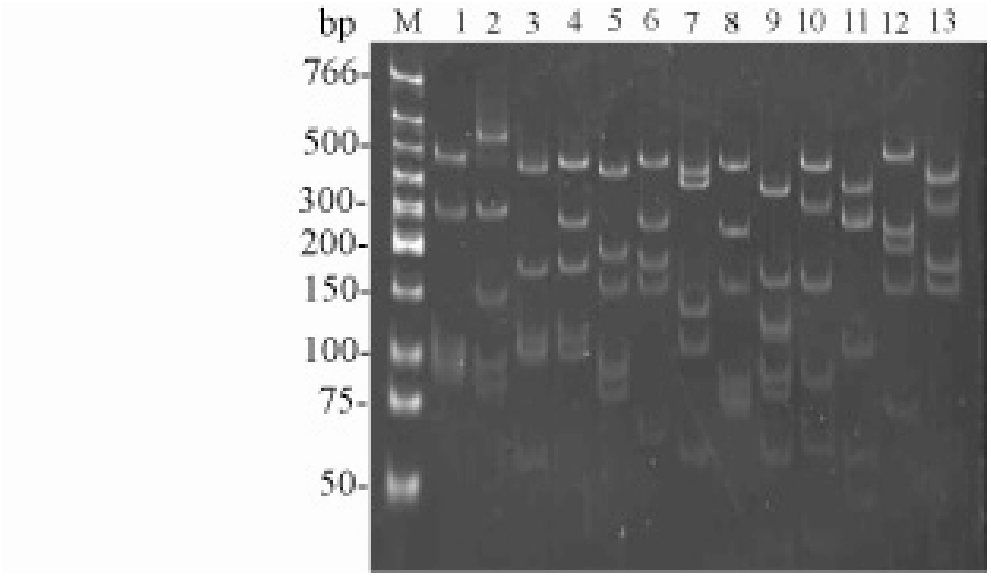


Figure 46 RFLP banding patterns of *huscFv* sequences from bio-pannig using recombinant N-terminal PB2 protein as antigen

Lane M, low molecular weight DNA ladder

Lanes 1-13, digested *huscFv* sequences of clones no. 1, 3, 4, 5, 6, 9, 10, 14, 15, 17, 22, 27, and 29, respectively

12. Functional assay of the recombinant N-terminal PA protein

12.1 Nuclear import assay

The ability of the recombinant N-terminal PA which contains nuclear localization signals, region I and partial region II in nuclear entry was determined by using a nuclear import assay. The nuclear import assays were carried out in digitonin-treated Vero cells cultured on glass cover slips as described in **Section 23.1.2**, **Chapter IV**. The cells on the glass cover slips were stained with anti-His antibody and incubated with by Alexa Fluor® 488 labeled chicken anti-mouse IgG (H+L). The stained cells were visualized under a confocal microscope. As shown in **Figure 47 D**, recombinant N-terminal PA efficiently accumulated in the nucleus of digitonin-permeabilized cells whereas the signal was not found in a negative control which the recombinant N-terminal PA was not added into the nuclear import reaction mixture (**Figure 47 A**). The recombinant NP protein which was used as the positive control was found in both the nucleus and throughout the cytoplasm (**Figur 47 C**). As shown in **Figure 47 B**, antibody to nucleolin binds efficiently to the nuclear materials in the nuclei of the permeabilized Vero cells.

12.2 RNA binding assay

The recombinant N-terminal PA protein was tested for its specific binding to 5'vRNA promoter by using the UV cross-linking assay as described in **Section 23.1.3**, **Chapter IV**. The recombinant NP protein was used as positive control because it has been known that the NP of the influenza virus which is the most abundant protein of the viral core could bind efficiently to the viral RNA (Kingsbury *et al*., 1887). **Figure 48** shows that the recombinant NP proteins could be cross-linked to the biotinylated 5'-vRNA as seen as a band at the expected location of the recombinant NP (*Mr* ~ 56) implying that the recombinant NP protein which was used as the control influenza virus protein could bind to the biotinylated 5'-vRNA. Nevertheless, no band was seen at the expected position of recombinant N-terminal PA protein (*Mr* ~ 26) implying that the recombinant N-terminal PA could not bind to the biotinylated 5'-vRNA. No band was seen in the negative control experiment to which the biotinylated 5' vRNA was not added into the reaction. It was concluded that in order to bind to the vRNA, the PA must be complexed within other subunit(s) and/or NP.

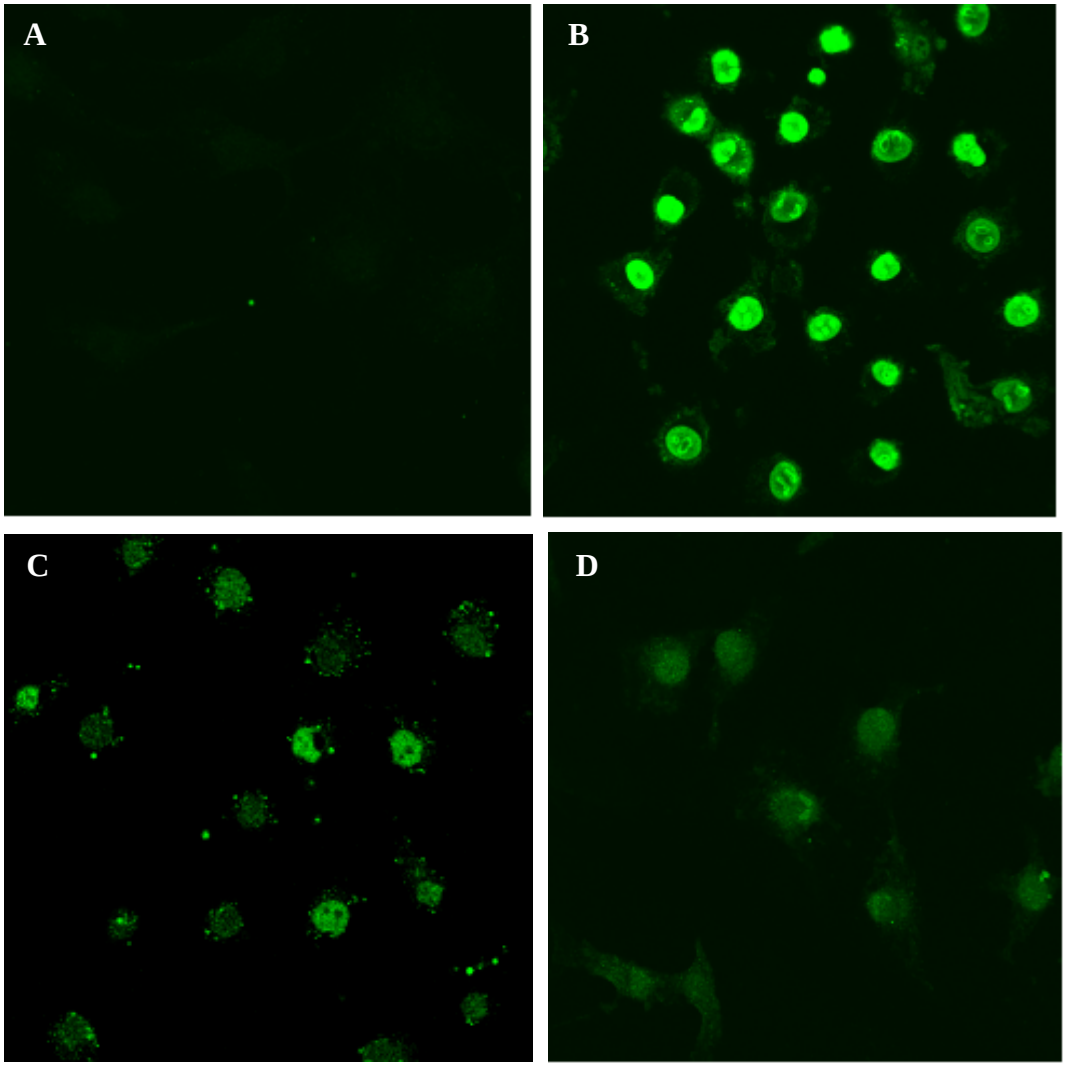


Figure 47 Nuclear import of recombinant N-terminal PA protein

Digitonin-treated Vero cells were directly stained with anti-His antibody (**A**). The permeabilized Vero cells were stained with anti-nucleolin antibody (**B**). Localization of influenza virus recombinant NP protein in the digitonin permeabilized Vero cells, in both cytoplasm and nucleus (**C**). Recombinant N-terminal PA protein was seen in the digitonin permeabilized Vero cells, both in nucleus and in cytoplasm implying the intact function of nuclear localization signal (**D**).

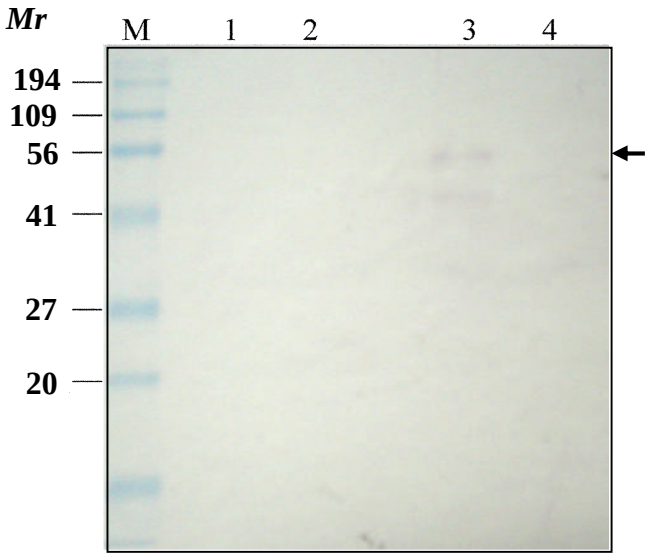


Figure 48 Results of the RNA binding assay of recombinant N-terminal PA protein

Recombinant protein was UV-cross linked with biotinylated 5'-vRNA and the UV-cross linked product was analyzed by Western blotting using streptavidin-AP conjugate as a detection reagent.

Lane M, Pre-strained protein marker

Lane 1, Mixture containing recombinant N-terminal PA protein and biotinylated 5'-vRNA

Lane 2, Recombinant N-terminal PA protein alone

Lane 3, Mixture containing recombinant NP protein and biotinylated 5'-vRNA

Lane 4, Recombinant NP protein alone

The arrow indicates location of positive band of recombinant NP that cross-linked with biotinylated 5'-vRNA after the UV-irradiation. No band is seen at the expected location of the N-terminal PA implying that the PA could not bind directly to the vRNA.

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

Recombinant N-terminal PA was incubated with digitonin permeabilized Vero cells followed by addition of specific HuScFv to PA or irrelevant HuScFv. As shown in **Figures 49 B and E**, the recombinant N-terminal PA alone or incubated with the irrelevant HuScFv could efficiently accumulated in the nucleus of digitonin-permeabilized Vero cells. Almost complete inhibition of the nuclear localization of the PA were found when the HuScFv of clone no. 18 was added to the PA exposed Vero cells (**Figure 49 D**). The HuScFv of clones no. 10, 11 and 15 could mediate significant reduction of the nuclear localization of the PA. **Figure 49 C** shows inhibitory activity of the HuScFv from clone no. 15.

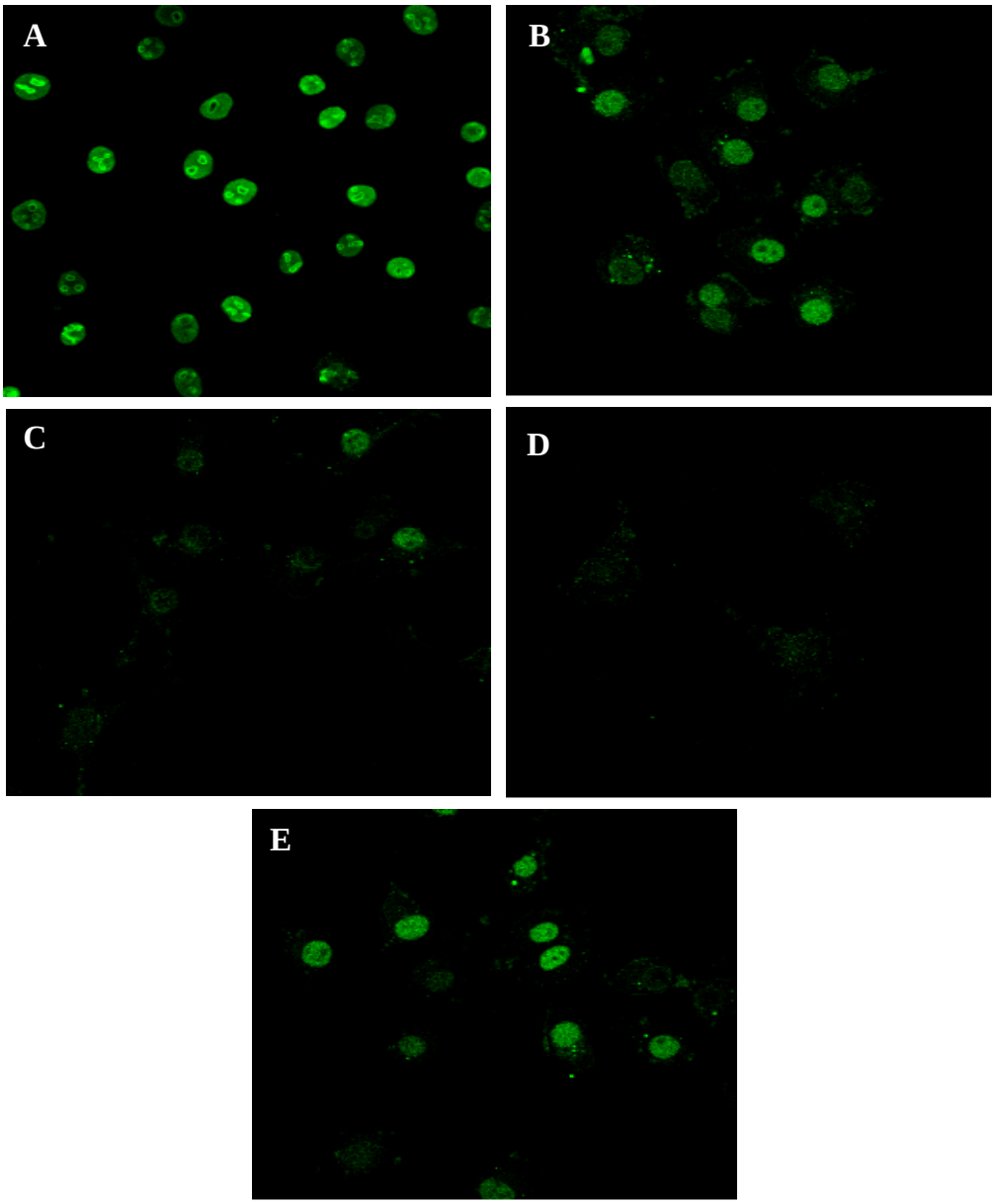


Figure 49 Inhibition of recombinant N-terminal PA nuclear import

The permeabilized Vero cells were stained with anti-nucleolin antibody (**A**). Recombinant N-terminal PA protein was seen in the digitonin permeabilized Vero cells, both in nucleus and in cytoplasm implying the intact function of nuclear localization signal (**B**). Significant reduction of PA nuclear import was seen when HuScFv of clone no. 15 was added (**C**). Almost complete abrogation of the PA nuclear import was obtained when the HuScFv of clone no.18 was added (**D**). The recombinant N-terminal PA was accumulated in the nucleus of digitonin permeablilized cells when irrelevant HuScFv was added (**E**).