CHAPTER 4

RESULTS

4.1 Construction of recombinant vectors

4.1.1 Preparation of expression vectors

Fifty microliters of expression vector pPICZA and pPICZαB were successfully isolated from *Escherichia coli* harboring the vector using Favor Prep ™ Plasmid DNA Extraction Mini Kit (FAVORGEN ® BIOTECH CORP., Taiwan) (section 3.5.1). DNA sample was eluted with 50 µl of ddH_2O. Plasmid was electrophoresed on 1% agarose, and shown in Figure 4.1(a) and 4.1(b), respectively. The size of pPICZA and pPICZαB vector was estimated as 2.5 kb and 3.0 kb, respectively. The vector contains at least two topologically-different forms of DNA, corresponding to supercoiled forms and nicked circles. It was the cause that circular form of vector migrates in agarose differently from linear vector of the same mass (McMaster and Carmichael, 1977). The concentration of pPICZA and pPICZαB vector was estimated to be 180 ng/µl.

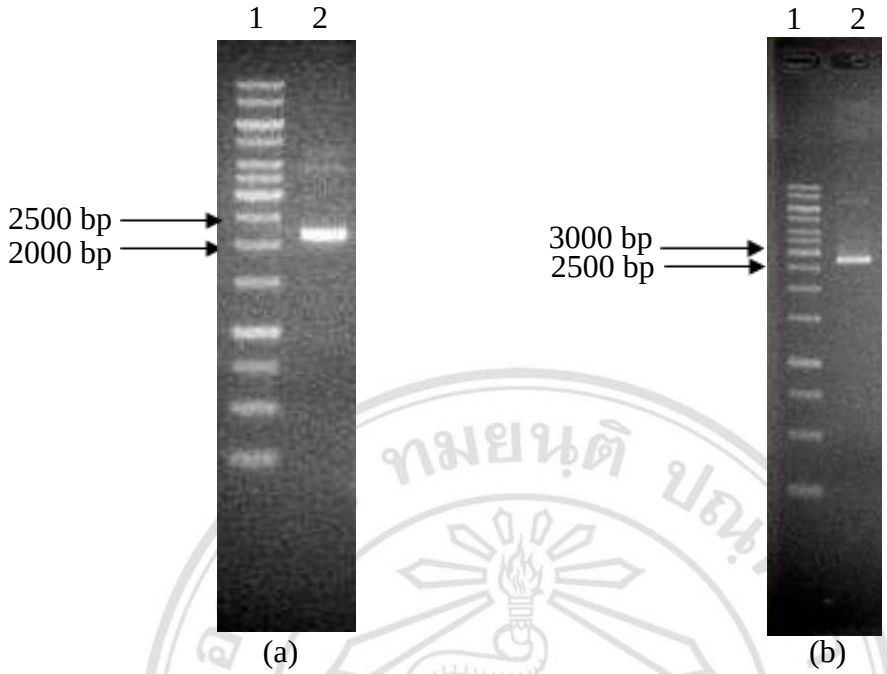


Figure 4.1 Agarose gel electrophoresis of expression vectors. Lane 1 is 1 kb DNA ladder, lane 2 is the vector isolated form *Escherichia coli*. (a) Agarose gel electrophoresis of expression vectors pPICZA vector. (b) Agarose gel electrophoresis of expression vectors pPICZαB vector

4.1.2 Construction of recombinant plasmid containing H5N1 HA genes

4.1.2.1 Construction of pPICZA-H5N1 HA1_OPT and transformation into *Escherichia coli* XL1-blue

The original Haemagglutinin domain 1 (H5N1 HA1) gene was CMU H5 (A/Chicken/Chiang Mai/1/2004). The sequence was codon optimized, synthesized and cloned into pPICZA vector by GenScript, USA. The 6xHIS-tag and other tags were inserted in frame in H5N1 HA1_OPT gene as illustrated in Figure 4.2. Next, the pPICZA plasmid containing H5N1 HA1_OPT gene was transformed to *Escherichia coli* XL1-blue. More than 200 colonies were appeared on selective medium (Figure 4.3).

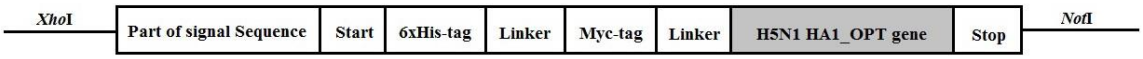


Figure 4.2 H5N1 HA1_OPT gene and fusion tags

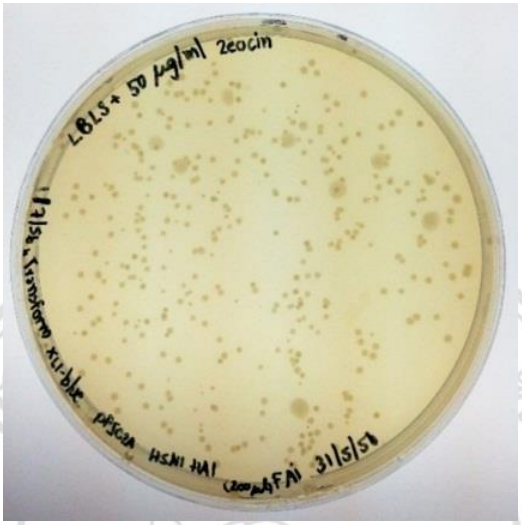


Figure 4.3 The colonies of *Escherichia coli* containing recombinant plasmid (pPICZA-H5N1 HA1_OPT) on LB low salt agar containing of 50 µg/ml zeocin.

Eleven colonies of *Escherichia coli* grown on selective medium were randomly selected and tested for the present of recombinant plasmid (pPICZA-H5N1 HA1_OPT) by colony PCR using specific primers as described in section 3.5.8. The PCR products were electrophoresed on 1% agarose gel. It was found that 9 colonies of the selected colonies were positive as shown in Figure 4.4.

Next, clone 1 were arbitrarily selected for further confirmation (restriction analysis). Plasmids isolated from clone 1 was digested with *Xho*I and *Not*I (section 3.5.9). After digestion, two bands of DNA were visualized on agarose gel, top band was 3.3 kb and bottom band was approximately 1,130 bp which were similar to the size of pPICZA vector and H5N1 HA1_OPT gene, respectively (Figure 4.5). These results indicated that recombinant plasmid (pPICZA-H5N1 HA1_OPT) was successfully transformed into *E. coli* XL1-blue.

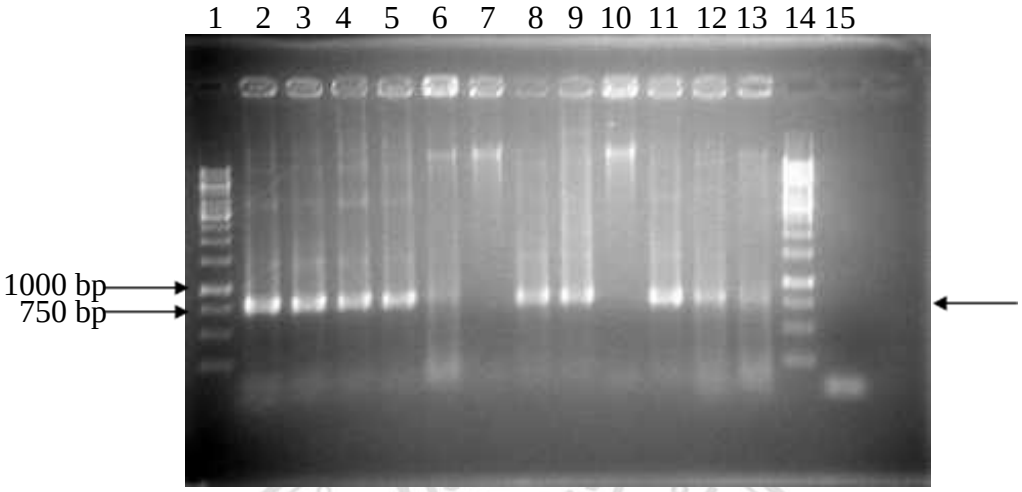


Figure 4.4 Analysis of the recombinant plasmid (pPICZA H5N1 HA1_OPT) by colony PCR technique using specific primer. Lane 1 and 14 are 1 kb DNA ladder. Lane 2-12 are recombinant plasmid isolated from colonies of recombinant *Escherichia coli* XL1-blue (clone 1-12, respectively).Lane 13 is positive control (H5N1 HA1_OPT gene). Lane 15 is negative control. Arrow indicates the PCR product which is approximate 750 bp.

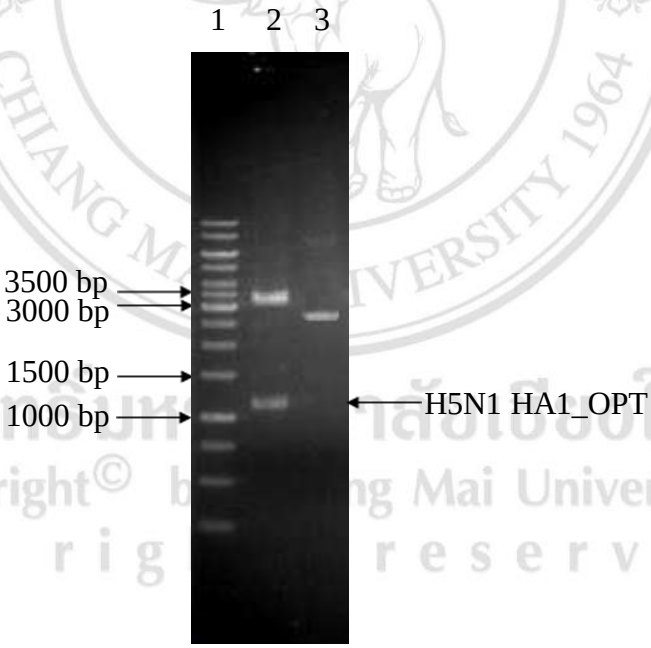


Figure 4.5 Agarose gel electrophoresis of recombinant plasmid (pPICZA-H5N1 HA1_OPT) with *Xho*I and *Not*I restriction analysis. Lane 1 is 1 kb DNA ladder, lane 2 is recombinant plasmid (pPICZA-H5N1 HA1_OPT) digested with *Xho*I and *Not*I, lane 3 is un-digested recombinant plasmid (pPICZA-H5N1 HA1_OPT). Arrow indicates the band of H5N1 HA1_OPT gene, which is approximate 1,100 bp.

In summary, the pPICZA plasmid containing H5N1 HA1_OPT gene which optimized and synthesized by GenScript, USA was transformed to *E. coli* stain XL1-blue. The clones were selected on LB low salt agar containing 50 µg/ml zeocin. PCR and restriction analysis results indicated that the H5N1 HA1_OPT gene was successfully cloned into *E. coli* stain XL1-blue. After comfirmation, the recombinant vector containing H5N1 HA1_OPT gene was renamed pPICZA-H5N1 HA1_OPT.

4.1.2.2 Construction of pPICZαB-H5N1 HA1_OPT and transformation into *Escherichia coli* XL1-blue

To prepare H5N1 HA1_OPT gene for cloning into pPICZαB vector, the recombinant plasmid (pPICZA-H5N1 HA1_OPT) was extracted from *Escherichia coli* XL1-blue containing such vector (section 3.5.1). The plasmid was digested with *Xho*I and *Not*I. The band containing H5N1 HA1_OPT gene was excised from agarose gel and purified (section 3.5.3). The product was analyzed using 0.8% (w/v) agarose gel electrophoresis and only a single band of approximately 1,100 bp H5N1 HA1_OPT gene was observed. The concentration of purified H5N1 HA1_OPT was estimated to be 45 ng/µl (Figure 4.6).

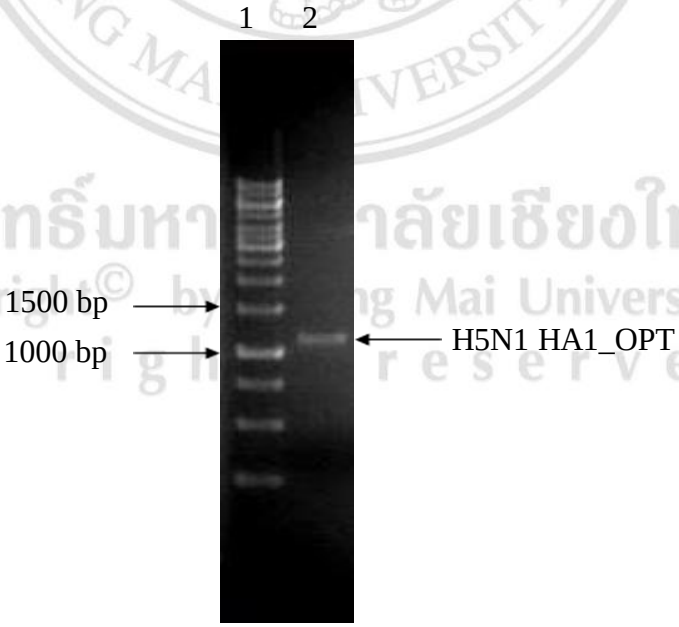


Figure 4.6 Agarose gel electrophoresis of H5N1 HA1_OPT gene purification. Lane 1 is 1 kb DNA ladder. Lane 2 is purified H5N1 HA1_OPT. Arrow indicates the band of H5N1 HA1_OPT gene, which as approximate 1,100 bp.

In the meantime, the expression vector (pPICZαB) was also separately double digested with *Xho*I and *Not*I (section 3.5.4).The band containing pPICZαB plasmid was purified (section 3.5.3) (data not shown).

After purification, the digested H5N1 HA1_OPT gene and pPICZαB expression vector were ligated by using T4 ligase (section 3.5.5). Then, the ligation products were transformed into *Escherichia coli*. Thirty colonies of transformants were observed on LB low salt agar containing zeocin (50 µg/ml) as shown in Figure 4.7, suggesting successful transformation. The *E. coli* transformed with vector control did not grow on LB low salt agar containing of 50 µg/ml zeocin (data not shown).

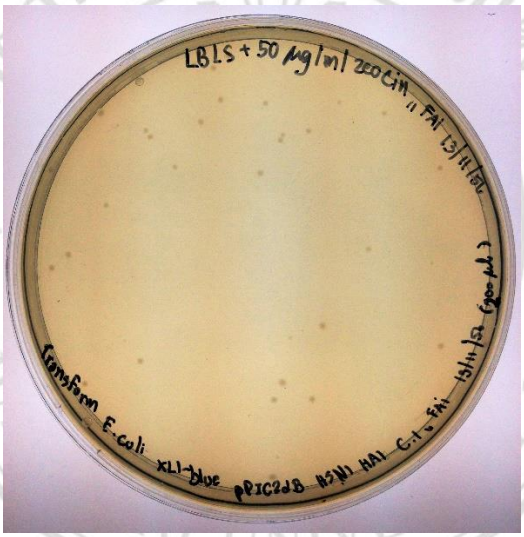


Figure 4.7 The colonies of *Escherichia coli* XL1-blue containing recombinant plasmid (pPICZαB-H5N1 HA1_OPT) on LB low salt agar containing 50 µg/ml zeocin.

Twelve colonies of *Escherichia coli* grown on selective medium were then selected and tested for the present of recombinant plasmid by PCR using hanging primers as described in section 3.5.8. The PCR products were electrophoresed on 1% agarose gel. It was found that 10 selected colonies were positive as shown in Figure 4.8. Clone 1 was arbitrarily selected for further confirmation (restriction analysis). Plasmids isolated from clone 1 (lane 2) was digested with *Xho*I and *Not*I electrophoresed on 1% agarose gel (Figure 4.9). It was found that the sample generated a band with the size similar to H5N1 HA1_OPT gene and pPICZαB vector when digested with *Xho*I and *Not*I. These results indicated that the H5N1 HA1_OPT gene was successfully cloned into pPICZαB vector.

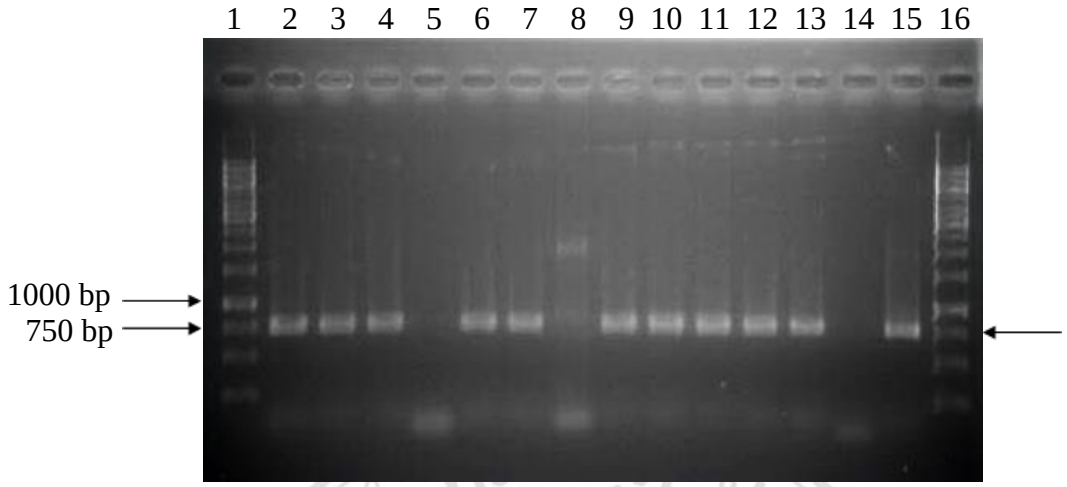


Figure 4.8 Analysis of the recombinant plasmid (pPCZαB-H5N1 HA1_OPT) by colony PCR technique. Lane 1 and 16 are 1 kb DNA ladder. Lane 2-13 are recombinant plasmid isolated from colonies of recombinant *Escherichia coli* XL1-blue (clone 1-12, respectively). Lane 14 is a negative control (ddH_2O) and lane 15 is a positive control (H5N1 HA1_OPT gene). Arrow indicates the band PCR product, which as approximate 750 bp.

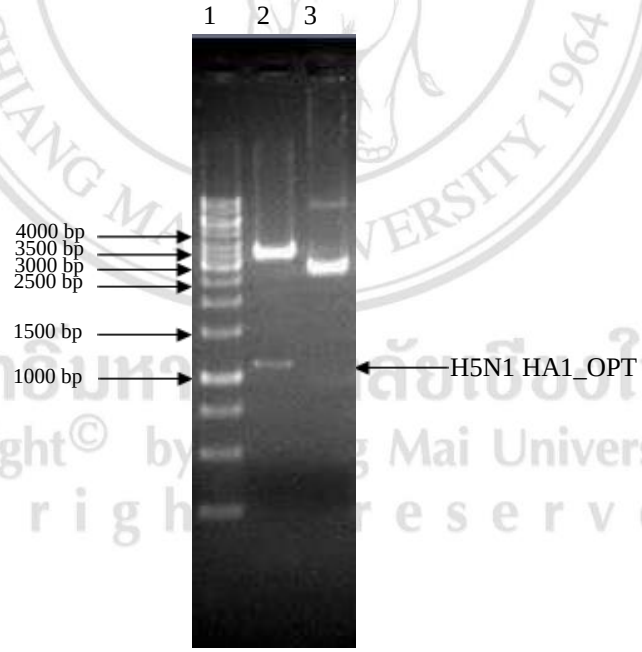


Figure 4.9 Agarose gel electrophoresis of recombinant plasmid (pPICZαB-H5N1 HA2) with *Xho*I and *Not*I restriction analysis. Lane 1 is 1 kb DNA ladder, lane 2 is recombinant plasmid (pPICZαB-H5N1 HA2) digested with *Xho*I and *Not*I, lane 3 is un-digested recombinant plasmid (pPICZαB-H5N1 HA2). Arrow indicates the band of H5N1 HA1_OPT gene, which is approximate 1,100 bp.

In summary, the H5N1 HA1_OPT genes was purified, digested with *Xho*I and *Not*I, ligated into pPICZαB vector, and used to transform to *E. coli* stain XL1-blue. The clones were selected on LB low salt agar containing 50 µg/ml zeocin. PCR and restriction analysis result indicated that the H5N1 HA1_OPT gene was successfully cloned into pPICZαB vector. After comfirmation, the pPICZαB vector containing HA1_OPT gene was renamed pPICZαB-H5N1 HA1_OPT.

4.1.2.3 Construction of pPICZA-H5N1 HA2 and transformation into *Escherichia coli* XL1-blue

The H5N1 HA2 gene was amplified by High Fidelity PCR (section 3.5.2) using hanging primers was designed to add restriction enzymes sites *Xho*I and part of signal sequence to the 5' end. The reverse primer was designed to add the restriction enzymes sites *Not*I in frame to the 3' end. So, the PCR products which amplified should be contained sequence of H5N1 HA2 gene and restriction enzymes sites (Figure 4.10). After inframe ligate onto pPICZA vector, the H5N1 HA2 would have myc epitope and 6xHIS tag attached to the C terminal of HA2 product.

Figure 4.10 H5N1 HA2 gene and restriction enzymes sites

The PCR product was electrophoresed on 1% agarose (as described in section 3.8). PCR product then was purified using GF-1 Ambiclean Kit (PCR & Gel) (Vivantis, Poland) (section 3.5.3). The size and concentration of purified PCR product was estimated by software Quality One 4.6 (BioRad, USA), which were approximate 600 bp and 120 ng/µl, respectively (Figure 4.11).

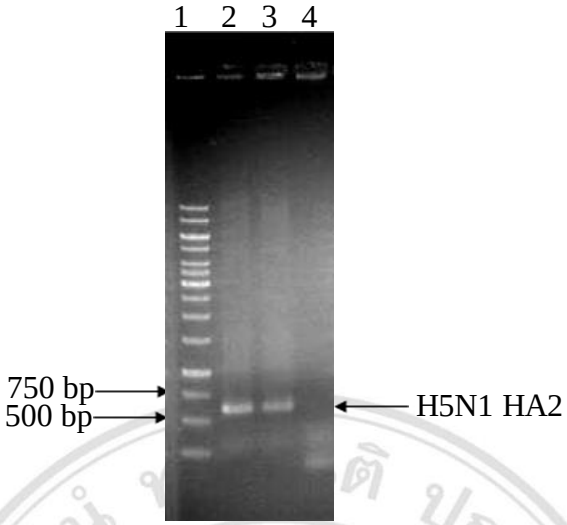


Figure 4.11 Agarose gel electrophoresis of PCR products (H5N1 HA2 gene) amplified by using High Fidelity PCR. Lane 1 is 1 kb DNA ladder, lane 2 and 3 are H5N1 HA2 genes, and lane 4 is negative control (ddH_2O). Arrow indicates the band of H5N1 HA2 gene, which is approximate 600 bp.

The H5N1 HA2 gene and the expression vector (pPICZA) were separately double digested with *Xho*I and *Not*I (section 3.5.4). After digestion, DNA samples were electrophoresed on 1% agarose gel. It was found that cut and uncut vectors were different in relative mobility. The uncut vector appeared as multiple bands due to different configuration with major band. The cut vector, on the other hand was a single band resulting from successfully digestion. Typically, the uncut vector contains at least two topologically-different forms of DNA, corresponding to supercoiled forms and nicked circles. It was the cause of circular forms of vector migrates in agarose differently from linear vector of the same mass (McMaster and Carmichael, 1977). Since the expression vector (pPICZA) and H5N1 HA2 gene were digested in the exact conditions, it can be assumed the H5N1 HA2 gene were also cut.

After purification, the digested H5N1 HA1_OPT or H5N1 HA2 gene and expression vector were ligated by using T4 ligase (section 3.5.5). Then, the ligation products were transformed into *Escherichia coli*. More than 200 colonies of tranformants were observed on LB low salt agar containing zeocin (50 µg/ml), suggesting successful transformation (Figure 4.12). The *E. coli* transformed with vector control did not grow on LB low salt agar containing of 50 µg/ml zeocin (data not shown).

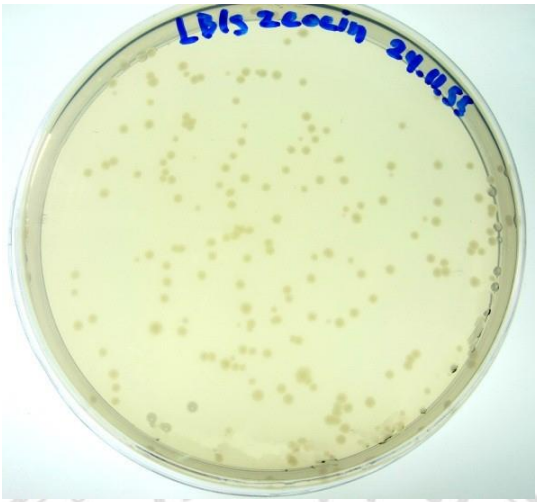


Figure 4.12 The colonies of *Escherichia coli* XL1-blue containing pPICZA-H5N1 HA2 on LB low salt agar containing 50 µg/ml zeocin.

The colonies of *Escherichia coli* grown on selective medium were selected and tested for the present of recombinant plasmid by PCR using hanging primers as described in section 3.5.8. The PCR products were electrophoresed on 1% agarose gel. It was found that 10 clones of selected colonies were positive as shown in Figure 4.13.

Clone 1 and 2 were arbitrarily selected for further confirmation (restriction analysis). Plasmids isolated from clone 1 and 2 (Figure 4.13; lane 2 and 4, respectively) were digested with *Xho*I and *Not*I electrophoresed on 1% agarose gel. It was found that all the samples generated a band with the size similar to H5N1 HA2 gene and pPICZA vector when digested with *Xho*I and *Not*I. These results indicated that the H5N1 HA2 gene was successfully cloned into pPICZA vector.

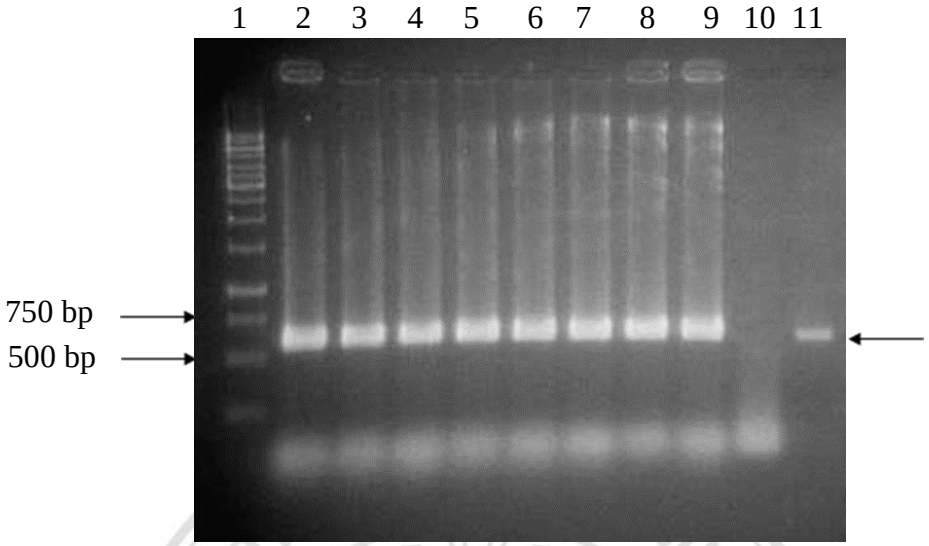


Figure 4.13 Analysis of the recombinant plasmid (pPICZA-H5N1 HA2) by colony PCR technique. Lane 1 is 1 kb DNA ladder, lane 2-9 are recombinant plasmid isolated from colonies of recombinant *E. coli* XL1-blue (clone 1-8, respectively). Lane 10 is a negative control (ddH_2O) and lane 11 was a positive control (H5N1 HA2 gene). Arrow indicates the band of H5N1 HA2 gene, which is approximately 600 bp.

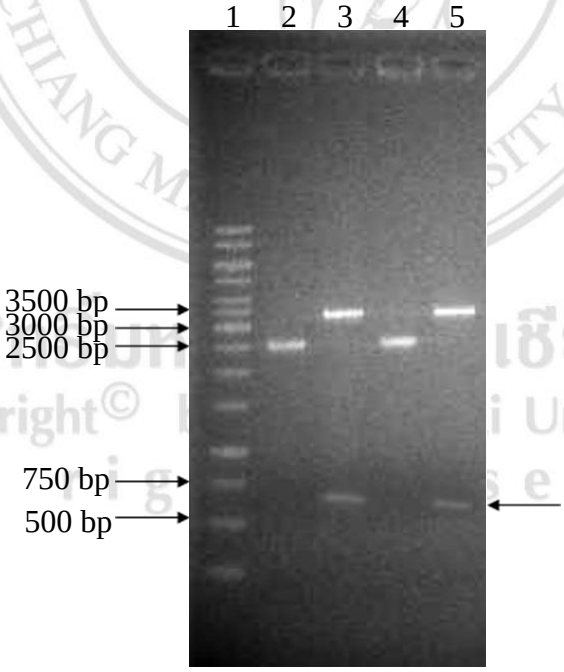


Figure 4.14 Agarose gel electrophoresis of recombinant plasmid (pPICZA-H5N1 HA2) with *Xho*I and *Not*I restriction analysis. Lane 1 is 1 kb DNA ladder, lane 2 and 4 are un-digested plasmid, lane 3 and 5 are recombinant plasmid digested. Arrow indicates the band of H5N1 HA2 gene, which is approximate 600 bp.

Next, the expression vector containing H5N1 HA2 gene (clone 1) was sent to 1st BASE Pte. LtA., in order to determine the nucleotide sequence using primer shown in Table 3.2. The raw sequencing data are shown in appendix E. The sequencing result showed that there was one base shift from C to T at position 438 (Figure 4.15). However, the amino acid sequence was not affected (appendix F).

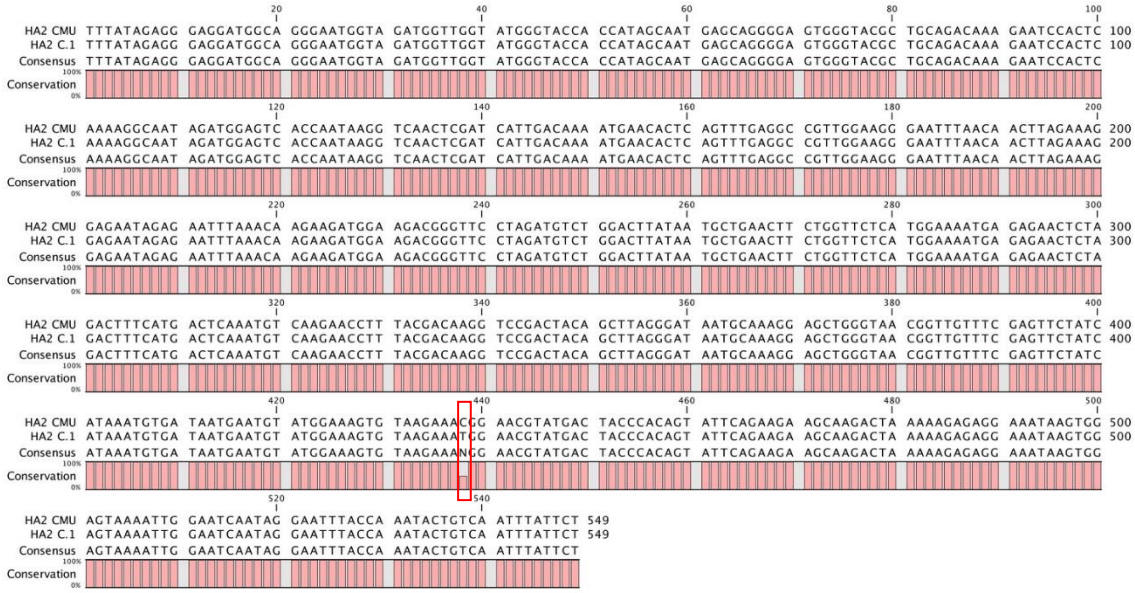


Figure 4.15 Nucleotide sequence of the H5N1 HA2 gene from recombinant plasmid (pPICZA-H5N1 HA2) compared with the haemagglutinin domain gene of avian influenza A (H5N1) virus strain CMU H5 (A/Chicken/Chiang Mai/1/2004).

In summary, the H5N1 HA2 genes were PCR-amplified, digested with *Xho*I/*Not*I, ligated into pPICZA vector, and transformed to *E. coli* XL1-blue. The clones were selected on LB low salt agar containing 50 µg/ml zeocin. PCR, restriction analysis, and sequencing result indicated that the H5N1 HA2 gene was successfully cloned into pPICZA vector. After comfirmation, the pPICZA vector containing H5N1 HA2 gene was renamed pPICZA-H5N1 HA2.

4.1.2.4 Construction of pPICZαB -H5N1 HA2 and transformation into *Escherichia coli* XL1-blue

To prepare H5N1 HA2 gene for cloning into pPICZαB vectors, the recombinant plasmid (pPICZA-H5N1 HA2) was extracted from *Escherichia coli* XL1-blue containing such vector (section 3.5.1). The plasmid was digested with *Xho*I and *Not*I. The band containing H5N1 HA2 gene was excised from agarose gel and purified (section 3.5.3). The product was analyzed using 1% (w/v) agarose gel electrophoresis and only a single band of approximately 600 bp H5N1 HA2 gene was observed. The concentration of purified H5N1 HA2 was estimated to be 50 ng/µl (Figure 4.16).

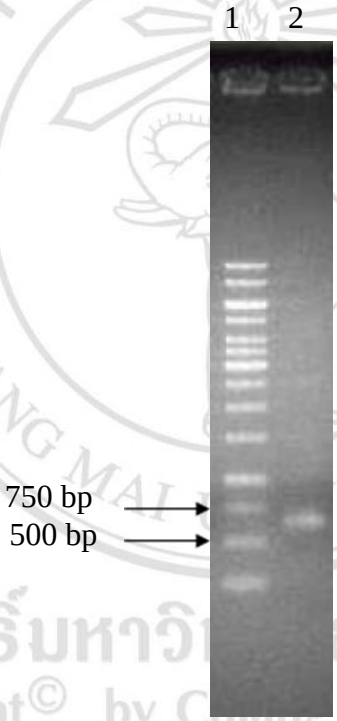


Figure 4.16 Agarose gel electrophoresis of H5N1 HA2 gene purification. Lane 1 is 1 kb DNA ladder. Lane 2 is purified H5N1 HA2. Arrow indicates the band of H5N1 HA2 gene, which is approximate 600 bp.

In the meantime, the expression vector (pPICZαB) was double digested with *Xho*I and *Not*I (section 3.5.4). The band containing pPICZαB plasmid was purified (section 3.5.3) (data not shown).

After purification, the digested H5N1 HA2 gene and pPICZαB expression vector were ligated by using T4 ligase (section 3.5.5). Then, ligation product as transformed into *E. coli*. More than 300 colonies of transformants were observed on LB low salt agar containing zeocin (50 µg/ml) as shown in Figure 4.17, suggesting successful transformation. The *E. coli* transformed with vector control did not grow on LB low salt agar containing of 50 µg/ml zeocin (data not shown).

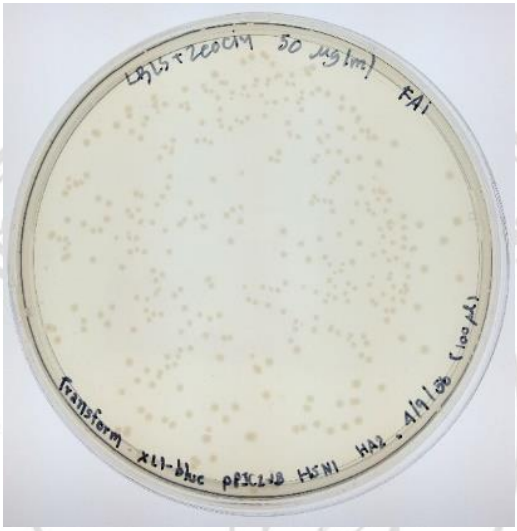


Figure 4.17 The colonies of *Escherichia coli* XL1-blue containing recombinant plasmid (pPICZαB-H5N1 HA2) on low salt LB agar containing 50 µg/ml zeocin.

Twelve colonies of *E. coli* grown on selective medium were selected and tested for the present of recombinant plasmid by PCR using hanging primers as described in section 3.5.8. The PCR products were electrophoresed on 1% agarose gel. It was found that 11 selected colonies were positive as shown in Figure 4.18. Clone 1 was arbitrarily selected for restriction analysis. Plasmid isolated from clone 1 (lane 2) was digested with *Xho*I and *Not*I and was electrophoresed on 1% agarose gel (Figure 4.19). It was found that the samples generated a band with the size similar to H5N1 HA2 gene and pPICZαB vector when digested with *Xho*I and *Not*I. These results indicated that the H5N1 HA2 gene was successfully cloned into pPICZαB vector.

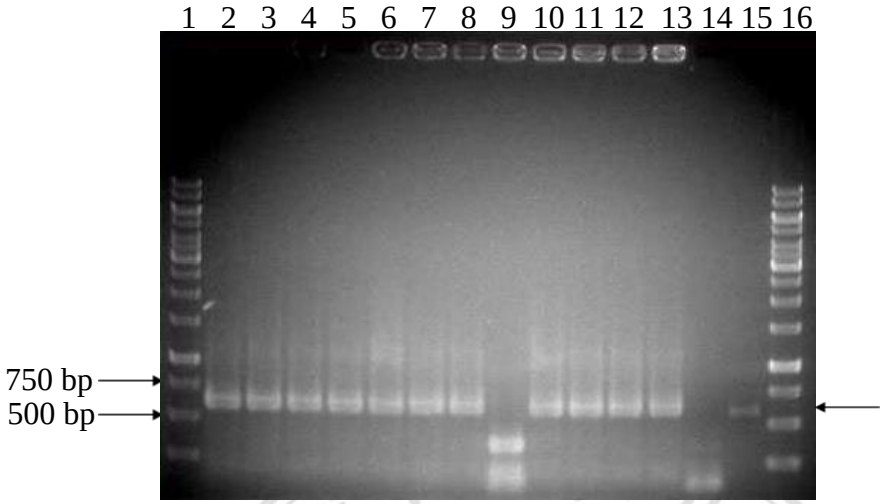


Figure 4.18 Analysis of the recombinant plasmid (pPCZαB-H5N1 HA2) by colony PCR technique. Lane 1 and 16 were 1 kb DNA ladder, lane 2-13 were recombinant plasmid isolated from colonies of recombinant *Escherichia coli* XL1-blue (clone 1-12, respectively). Lane 14 was a negative control (ddH_2O) and lane 15 was a positive control (H5N1 HA2 gene). Arrow indicated that the band of H5N1 HA2 gene, which were approximately 600 bp.

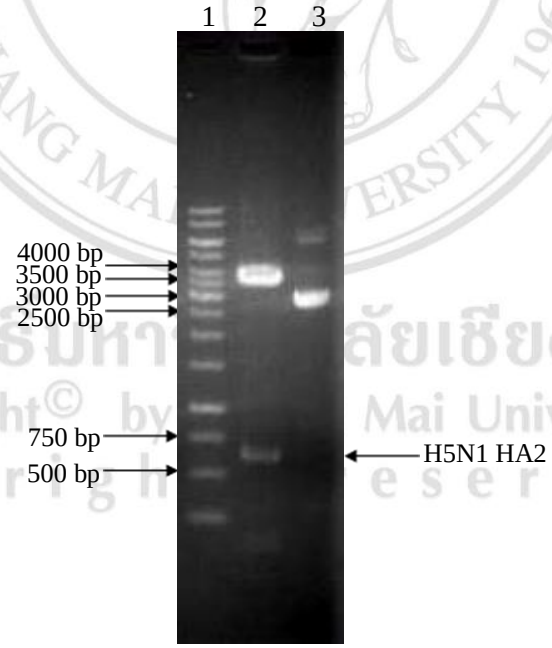


Figure 4.19 Restriction analysis of recombinant plasmid (pPICZαB-H5N1 HA2) with *Xho*I and *Not*I. Lane 1 is 1 kb DNA ladder, lane 2 is recombinant plasmid digested with *Xho*I and *Not*I, lane 3 is un-digested plasmid. Arrow indicates the band of H5N1 HA2 gene, which is approximate 600 bp.

In summary, the H5N1 HA2 genes was excised, purified, ligated into pPICZαB vector, and transformed to *E. coli* stain XL1-blue. The clones were selected on LB low salt agar containing 50 µg/ml zeocin. PCR and restriction analysis result indicated that the H5N1 HA2 gene was successfully cloned into pPICZαB vector. After comfirmation, the pPICZαB vector containing H5N1 HA2 gene was renamed pPICZαB-H5N1 HA2.

4.2 Linearization of recombinant vector with *Sac*I

To prepare the vector for transformation, *Escherichia coli* harboring the vector was grown and the vector was isolated using Favor Prep ™ Plasmid DNA Extraction Mini Kit (FAVORGEN ® BIOTECH CORP., Taiwan) (section 3.5.1). The total amount of vector obtained from two runs of each recombinant vector was approximately 10 µg in 60 µl of ddH_2O. After that, the recombinant vector was digested with *Sac*I (section 3.6.2). The digestion was performed in two reactions with each containing 15 µl of vector. After that they were electrophoresed on 1% agarose gel.

Figure 4.20(a), 4.20(b), 4.20(c) and 4.20(d) shows that cut and uncut vector were different in relative mobility. The cut vector (lane 3 and 4) appears as a single band resulting from successfully digestion. On the other hand, uncut vector appeared as multiple bands since it contains at least two topologically-different forms of DNA, corresponding to supercoiled forms and nicked circles. The circular forms of vector migrate in agarose distinctly differently from linear vector of the same mass. The plasmid was then isopropanol precipitated (section 3.6.3) and reconstituted in 20 µl of TE buffer.

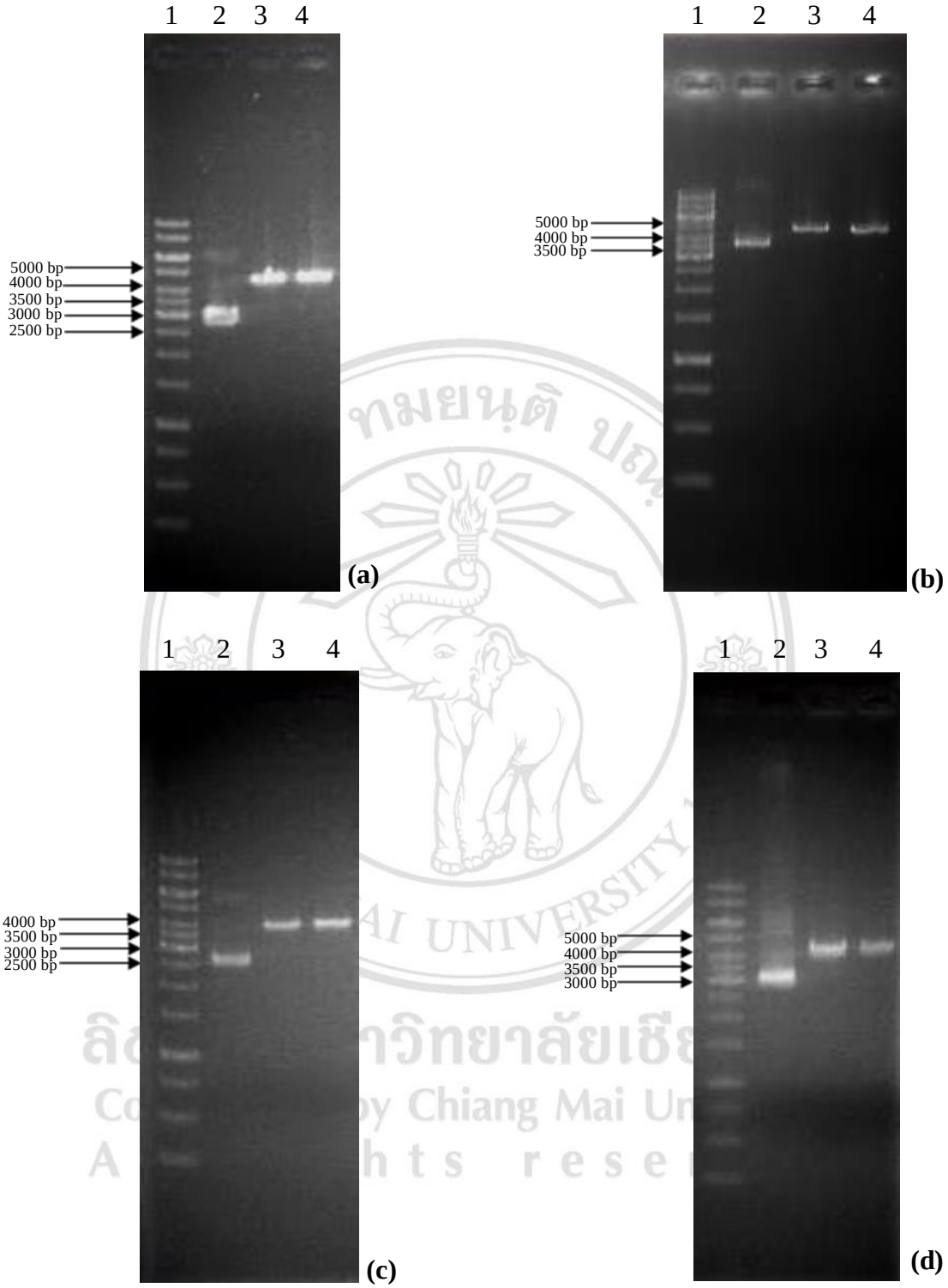
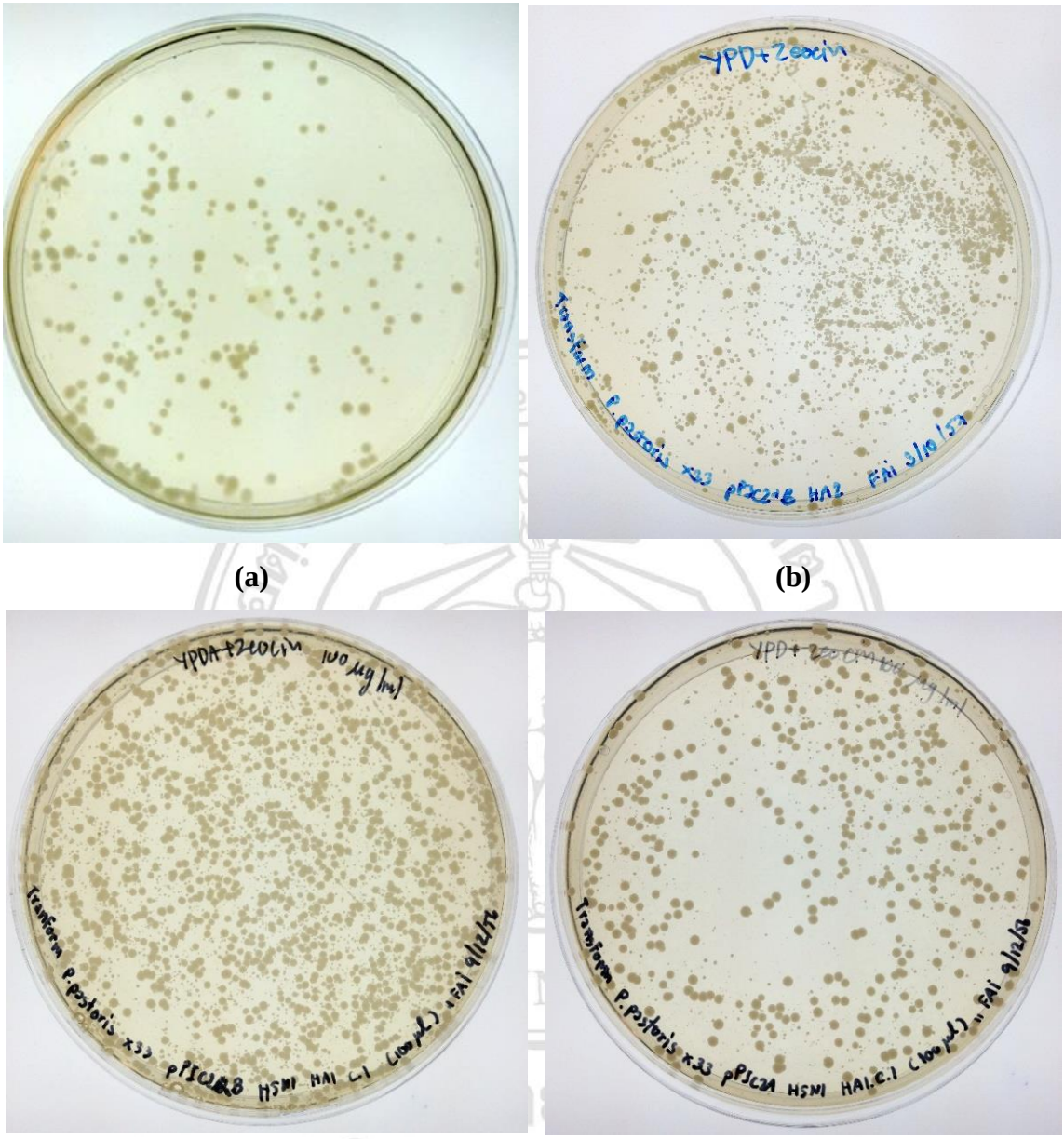


Figure 4.20 Agarose gel electrophoresis of the recombinant vectors digested with *Sac*I. Lane 1 is 1 kb DNA ladder. Lane 2 is un-digested vector; lane 3 and 4 are digested vector. Agarose gel electrophoresis of the pPICZA-H5N1 HA1_OPT vector (a) digested, pPICZαB-H5N1 HA1_OPT vector (b) digested, pPICZA-H5N1 HA2 vector (c) digested and pPICZαB-H5N1 HA2 vector (d) digested, respectively.

4.3 Transformation of recombinant vectors into *Pichia pastoris*

The linearized recombinant vector (pPICZA-H5N1 HA1_OPT or pPICαB-H5N1 HA1_OPT or pPICZA-H5N1 HA2 or pPICZαB-H5N1 HA2) were transformed into *Pichia pastoris* using electroporation technique (section 3.6.5). After three days incubation, the transformants were selected on YPD agar containing 100 µg/ml zeocin, and shown in Figure 4.20(a), 4.20(b), 4.20(c) and 4.20(d), respectively. The *P. pastoris* strain GS115 or *P. pastoris* strain X33 which used as control did not grow on selective medium (data not shown). This shows that the *P. pastoris* was successfully transformed with recombinant vector (pPICZA-H5N1 HA1_OPT or pPICαB-H5N1 HA1_OPT or pPICZA-H5N1 HA2 or pPICZαB-H5N1 HA2).

The total number of transformant of *P. pastoris* strain GS115 containing recombinant vector pPICZA-H5N1 HA2 was 251 cells and the total number of transformant of *P. pastoris* strain X33 containing recombinant vector pPICZA-H5N1 HA1_OPT and pPICZαB-H5N1 HA1_OPT was 458 cells and 1,179 cells, respectively. Then, the transformation efficiency was calculated as described in Appendix G. The transformation efficiency of transformant of *P. pastoris* strain GS115 containing recombinant vector (pPICZA-H5N1 HA2) was 1.11×10^3 cells/µg DNA and the transformation efficiency of transformant of *P. pastoris* strain X33 containing recombinant vector (pPICZA-H5N1 HA1_OPT, pPICZαB-H5N1 HA1_OPT) was 2.02×10^3 cells/µg DNA and 5.19×10^3 cells/µg DNA, respectively. While the total number of the colonies of *Pichia pastoris* strain X33 containing recombinant vector (pPICZαB-H5N1 HA2) could not counted because they were a lot of colonies of *P. pastoris* grown on selective medium. So, the transformation efficiency could not be calculated too.



(a) **(b)**

(c) **(d)**

Figure 4.21 The colonies of *Pichia pastoris* containing recombinant plasmid on selective medium using 100 µl of transformant suspension on YPD agar containing 100 µg/ml zeocin. The colonies of *P. pastoris* strain GS115 containing pPICZA-H5N1 HA2 on selective medium (a). The colonies of *P. pastoris* strain X33 containing pPICZαB-H5N1 HA2 on selective medium (b). The colonies of *P. pastoris* strain X33 containing pPICZA-H5N1 HA1_OPT on selective medium (c). The colonies of *P. pastoris* strain X33 containing pPICZαB-H5N1 HA1_OPT on selective medium (d).

4.4 PCR analysis of *Pichia* Integrants

Twelve colonies of recombinant *Pichia pastoris* grown on selective medium were randomly selected for confirmation of integration. Colony was resuspended in 30 µl ddH_2O and was heated at 80°C for 10 minutes. Supernatant was then used as template in PCR using specific primers (for H5N1-HA1_OPT) and hanging primers (for H5N1 HA2) (section 3.7). The PCR products were electrophores on 1% agarose gel**.**

4.4.1 Analysis of recombinant *Pichia* containing H5N1 HA1_OPT gene

Figure 4.22 and 4.23 show the result of PCR analysis of genomic DNA from twelve selected colonies using specific primers (Table 3.1) to the H5N1 HA1_OPT gene.

The PCR analysis of *Pichia* integrants was performed twice (section 3.7). It was found that the selected samples from *Pichia* colonies containing pPICZA-H5N1 HA1_OPT vector (clone 1-3) were positive (Figure 4.22). *Pichia* colonies containing pPICZαB-H5N1 HA1_OPT vector (clone 1-6) were positive (Figure 4.23). This indicates that the H5N1 HA1_OPT gene had been integrated into the *Pichia* genome.

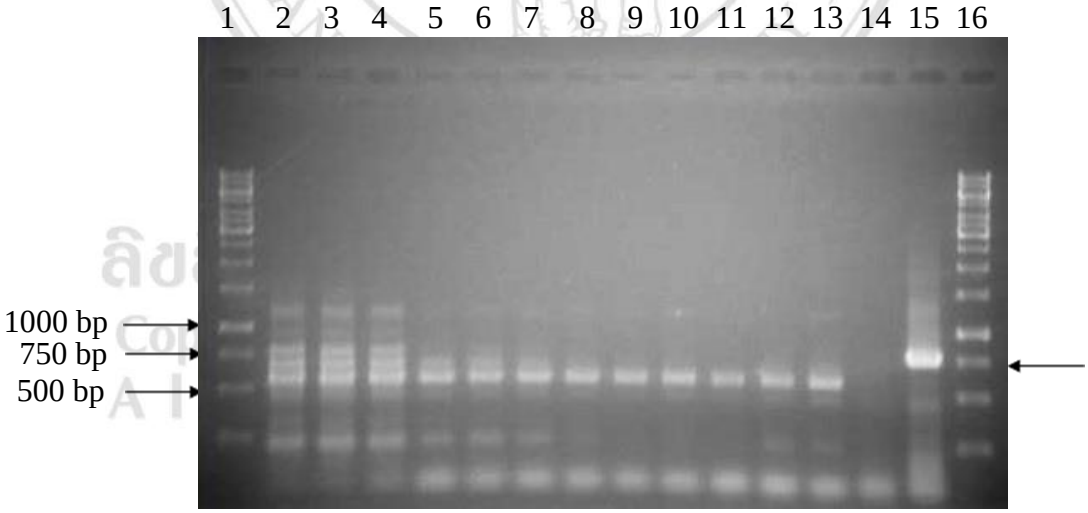


Figure 4.22 PCR products of twelve selected recombinant *Pichia* colonies containing pPICZA-H5N1 HA1_OPT vector. Lane 1 and 16 are 1 kb DNA ladder, lane 2-13 are PCR products of H5N1 HA1_OPT genes of recombinant *P. pastoris*, lane 14 was a negative control (ddH_2O) and lane 15 is a positive control (H5N1 HA1_OPT gene), respectively. Arrow indicates the band of the PCR product which is approximate 750 bp.

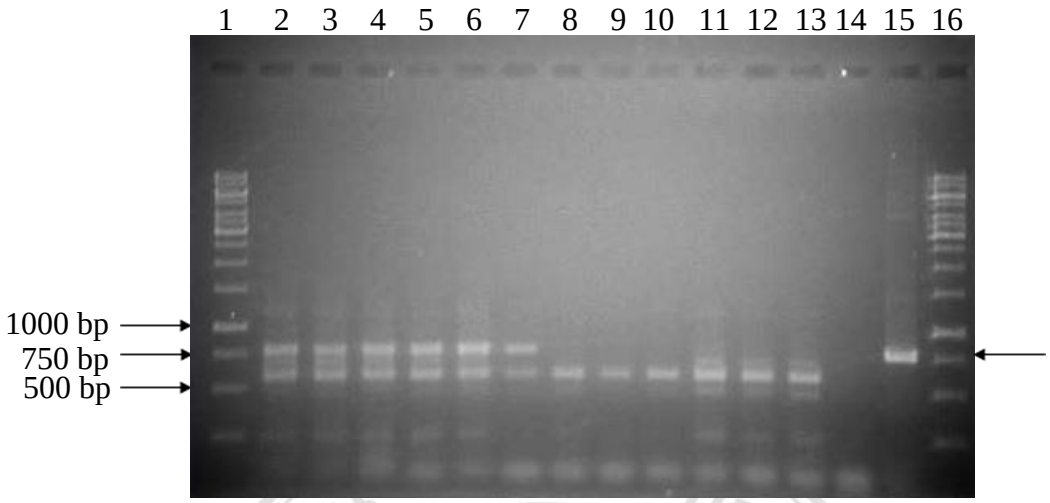


Figure 4.23 PCR products of twelve selected recombinant *Pichia* colonies containing pPICZαB-H5N1 HA1_OPT vector. Lane 1 and 16 were 1 kb DNA ladder, lane 2-13 were PCR products of H5N1 HA1_OPT genes of recombinant *P. pastoris*, lane 14 was a negative control (ddH_2O) and lane 15 was a positive control (H5N1 HA1_OPT gene), respectively. Arrow indicates the band of the PCR product which is approximate 750 bp.

4.4.2 Analysis of recombinant *Pichia* containing H5N1 HA2 gene

Figures 4.24 and 4.25 show the result of PCR analysis of genomic DNA from twelve selected colonies using hanging primers (Table 3.2) to the H5N1 HA2 gene.

To confirm the result of the PCR analysis of *Pichia* integrants, the analysis were repeated twice (section 3.7). It was found that all of the selected samples form *Pichia* colonies containing pPICZA-H5N1 HA2 vector (clone 1-12) were positive with PCR (Figure 4.24). But, the selected samples from *Pichia* colonies containing pPICZαB-H5N1 HA2 were positive with PCR only three clones (clone 1 (lane 2), 3 (lane 4) and 8 (lane 9), respectively) (Figure 4.25). This indicates that the H5N1 HA2 gene had been integrated into the *Pichia* genome.

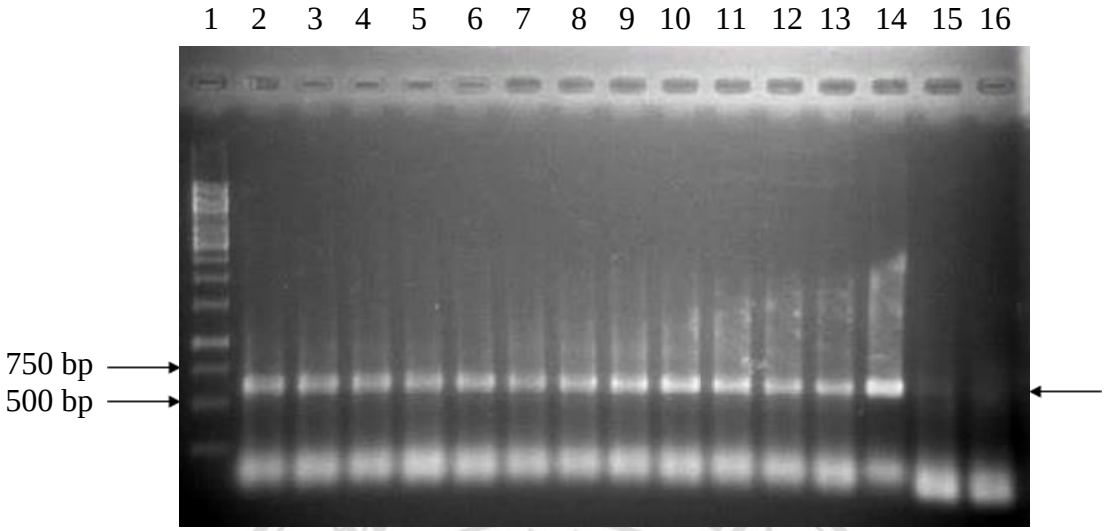


Figure 4.24 PCR products of twelve selected recombinant *Pichia* colonies containing pPICZA-H5N1 HA2 vector. Lane 1 is 1 kb DNA ladder, lane 2-13 are PCR products of H5N1 HA2 genes of recombinant *P. pastoris*, lane 14 is H5N1 HA2 gene (positive control), lane 15 and 16 are distilled water and *Pichia* with explin expression vector (pPICZA) (negative control), respectively. Arrow indicates the band of the PCR product which is approximate 600 bp.

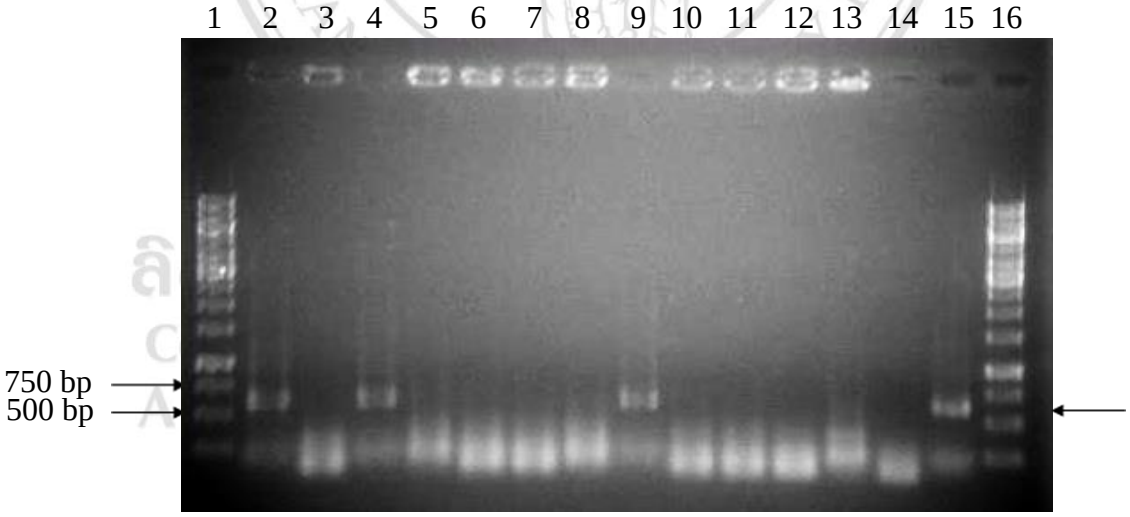


Figure 4.25 PCR products of twelve selected recombinant *Pichia* colonies containing pPICZαB-H5N1 HA2 vector. Lane 1 and 16 are 1 kb DNA ladder, lane 2-13 are PCR products of H5N1 HA2 genes of recombinant *P. pastoris*, lane 14 is a negative control (ddH_2O) and lane 15 is a positive control (H5N1 HA2), respectively. Arrow indicates the band of the PCR product which is approximately 600 bp.

4.5 Expression of H5N1 HA gene by recombinant *Pichia pastoris*

In this experiment, the colonies of the PCR positive clone (*Pichia* containing H5N1 HA gene) were cultured and induced. The HA1 or HA2 were analyzed by SDS-PAGE and western blot using HisDetector™ and anti-Myc antibody.

4.5.1 Expression of H5N1 HA1_OPT gene by recombinant *Pichia pastoris*

4.5.1.1 Expression of H5N1 HA1_OPT gene by recombinant *Pichia pastoris* containing pPICZA-H5N1 HA1_OPT vector

In this experiment, the colonies (clone 1-3) of the PCR positive clone (*Pichia* containing H5N1 HA1_OPT gene) were selected for cultivated in BMGY and induced with methanol to final concentration of 1% (v/v) every 12 hours for 3 days. The cells were collected and the endogenous protein was extracted by ultrasonicator. Then, the sample supernatant was used for analysis of recombinant H5N1 HA1_OPT protein by SDS-PAGE and western blot using HisDetector™ Western Blot Kits (KPL, Inc., USA) (section 3.12). It was found that the sample supernatant generated many bands of the protein (data not shown). So, the sample supernatant should be purify using HisPur ™ Ni-NTA Spin Column (Thermo Fisher Scientific Inc., USA) under native condition before use to detect the present of recombinant protein.

Next, the H5N1 HA1_OPT protein supernatant was purified from using HisPur ™ Ni-NTA Spin Column (Thermo Fisher Scientific Inc., USA) under native condition. The present of recombinant protein was analyzed by SDS-PAGE and western blot similar to the previous experiment. It was found that *Pichia pastoris* strain X33 containing pPICZA-H5N1 HA1_OPT was not expressed a single band of the protein (data not shown).

4.5.1.2 Expression of H5N1 HA1_OPT gene by recombinant *Pichia pastoris* containing pPICZαB-H5N1 HA1_OPT vector

In this experiment, the colonies (clone 1-6) of the PCR positive clone (the recombinant *Pichia* containing H5N1 HA1_OPT gene) were selected for cultivated in

BMGY and induced with methanol to final concentration of 1% (v/v) every 12 hours for 3 days. The culture supernatant was collected and used for analysis of recombinant H5N1 HA1_OPT protein by SDS-PAGE and western blot using HisDetector™ Western Blot Kits (KPL, Inc., USA) (section 3.12). It was found that the sample supernatant did not generated a band of the protein (data not shown). So, the sample supernatant used for purify using HisPur ™ Ni-NTA Spin Column (Thermo Fisher Scientific Inc., USA) under native condition before use to detect the present of recombinant protein again.

Next, the supernatant was used for purify the H5N1 HA1_OPT protein using HisPur ™ Ni-NTA Spin Column (Thermo Fisher Scientific Inc., USA) under native condition. The present of recombinant protein was analyzed by western blot and SDS-PAGE similar to the previous experiment. For colorimetric Assay of AP Western blotting detection, HisDetector™ Western Blot Kits (KPL, Inc., USA) was used for detected the protein on membrane. It was found that the recombinant *Pichia pastoris* strain X33 containing pPICZαB-H5N1 HA1_OPT did not express the protein as no the protein band was found on the membrane (data not shown).

Next, 1.5% casein amino acid was added into BMGY for prevent protease enzyme which might affected to the secreted protein during cultivation process. After cultivated and induced with methanol every 12 hours for 3 days, the culture supernatant were collected and used for purification similar to the previous experiment. Then, the purified protein was analysed with HisDetector™ Western Blot Kits (KPL, Inc., USA). It was found that *P. pastoris* strain X33 containing pPICZαB-H5N1 HA1_OPT did not express the protein (data not shown).

From the result of the previous experiment, it was possible that the recombinant *P. pastoris* could not secrete protein outside the cells. So, the endogenous protein from *Pichia* cell was extracted by ultrasonicator. Next, the supernatant was used for purify the H5N1 HA1_OPT protein and the purified protein was analyzed with western blot analysis by HisDetector™ Western Blot Kits (KPL, Inc., USA). It was found that *Pichia pastoris* strain X33 containing pPICZαB-H5N1 HA1_OPT did not express a single band of the protein (data not shown).

4.5.2 Expression of H5N1 HA2 gene by recombinant *Pichia pastoris*

4.5.2.1 Expression of H5N1 HA2 gene by recombinant *Pichia pastoris* containing pPICZA-H5N1 HA2 vector

In this experiment, the colonies of the PCR positive clone (*Pichia* containing H5N1 HA2 gene) were selected to cultivate in BMGY and induced with methanol to final concentration of 1% (v/v) every 12 hours for 3 days. The cells were collected and the endogenous protein was extracted by ultrasonicator. Next, the supernatant was used for purify the H5N1 HA2 protein using HisPur ™ Ni-NTA Spin Column (Thermo Fisher Scientific Inc., USA) under native condition. The present of recombinant protein was analyzed by SDS-PAGE and western blot (section 3.12).

For colorimetric Assay of Western blotting detection, HisDetector™ Western Blot Kits (KPL, Inc., USA) and Colorimetric Assay of Anti-myc antibody and patient serum (positive and negative serum) were used for detection of the protein on the membrane.

The SDS-PAGE of H5N1 HA2 protein was carried out and result was shown in Figure 4.26. The single band of protein was observed with approximate molecular weight 35-40 kDa.

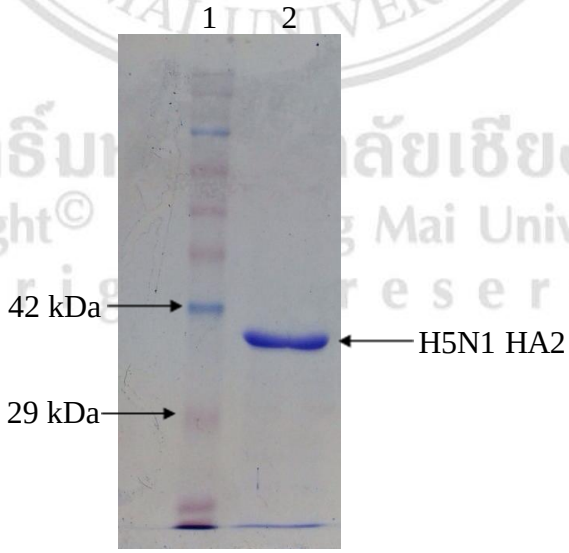


Figure 4.26 SDS-PAGE analysis of H5N1 HA2 protein with coomassie blue staining. Lane 1 is protein molecular weight marker, lane 2 is purified H5N1 HA2 protein. Arrow indicates the band of the recombinant HA2 protein which is approximately 35-40 kDa.

The western blotting analysis using HisDetector™ Western Blot Kits (KPL, Inc., USA) was carried out and the result is shown in Figure 4.27. The purified H5N1 HA2 protein was observed with molecular weight approximate 35-40 kDa.

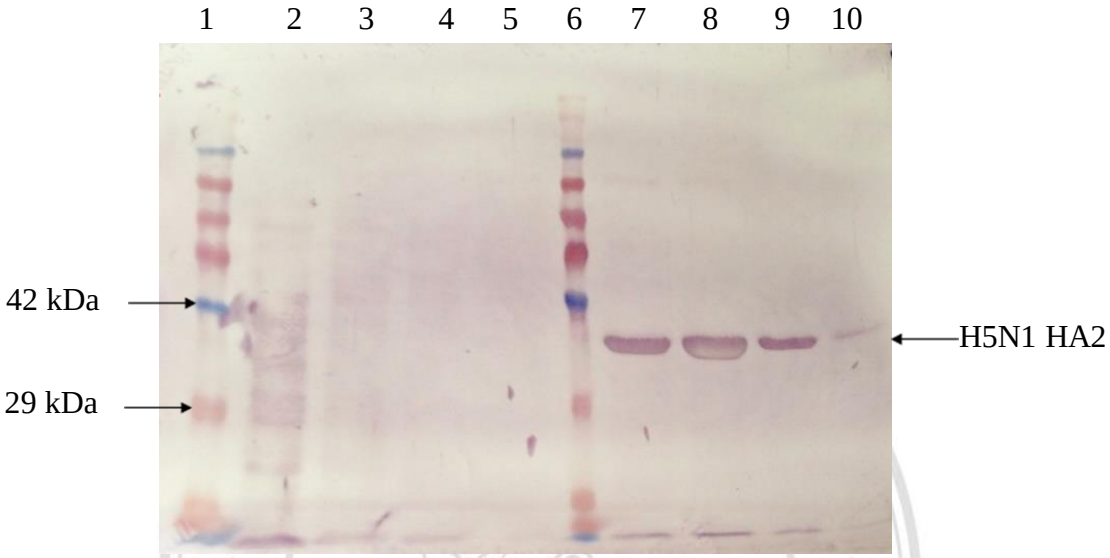


Figure 4.27 Western blot analysis of H5N1 HA2 protein detected by HisDetector™ Western Blot Kits (KPL, Inc., USA). Lane 1 is molecular weight marker, lane 2 is supernatant from cell lysate, lane 3 is unabsorbed of cell lysates, lane 4-5 are first and second wash, respectively, lane 6 is molecular weight marker, lane 7-10 are eluted fraction 1, 2, 3 and 4 respectively. Arrow indicates the band of the recombinant HA2 protein which is approximately 35-40 kDa.

The western blotting analysis using Anti-myc antibody was carried out and the result is shown in Figure 4.28. The purified H5N1 HA2 protein was observed with molecular weight approximate 35-40 kDa.

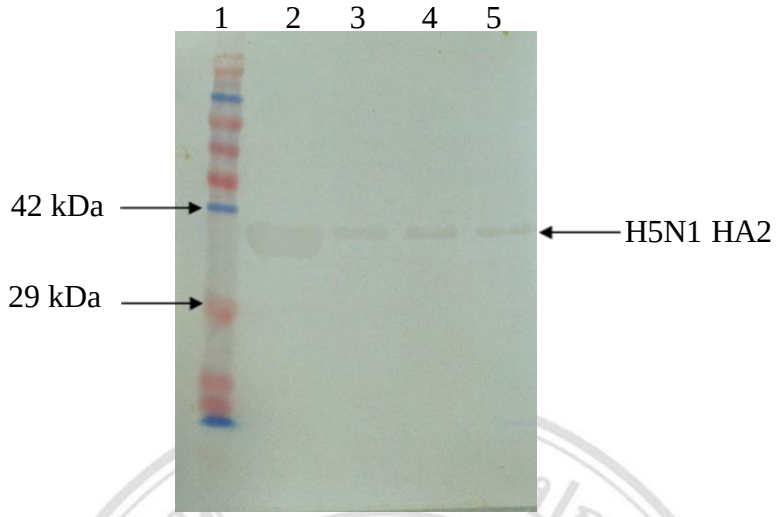


Figure 4.28 Western blot analysis of H5N1 HA2 protein detected by Anti-myc antibody. Lane 1 was protein molecular weight marker, lane 2-5 were eluted fraction 1,2,3 and 4 respectively. Arrow indicates the band of the recombinant HA2 protein which is approximately 35-40 kD.

The human serum samples which was used to detect the recombinant H5N1 HA protein were corrected at the same time of avian influenza transmission occurred in 2004. Serum sample from patients recovered from avian influenza infection who had their HI titer more than 1:40 and renamed as PO3 (positive serum) were tested against recombinant H5N1 HA protein. The non-exposed individual serum and renamed as CS (negative serum) were used as negative control. All of the human serum samples were aliquot and stored at -70^{o}C.

The western blotting analysis using PO3 (positive serum) was carried out and the result is shown in Figure 4.29. The H5N1 HA2 protein which was purified using HisPur^{TM} Ni-NTA Spin Column (Thermo Fisher Scientific Inc., USA) under native condition were presented with immunostained bands with molecular weight approximately 35-40 kDa and the western blotting analysis using CS (negative serum) was carried out and the result is shown in Figure 4.30. No immunostained band was observed.

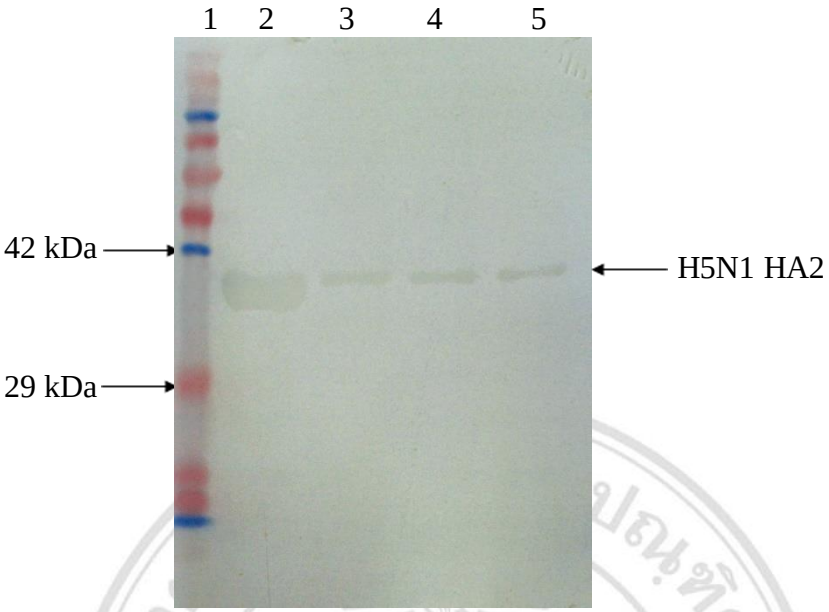


Figure 4.29 Western blot analysis of H5N1 HA2 protein detected by PO3 (positive serum). Lane 1 is protein molecular weight marker, lane 2-5 are eluted fraction 1,2,3 and 4 respectively. Arrow indicates the band of the recombinant HA2 protein which is approximately 35-40 kD.

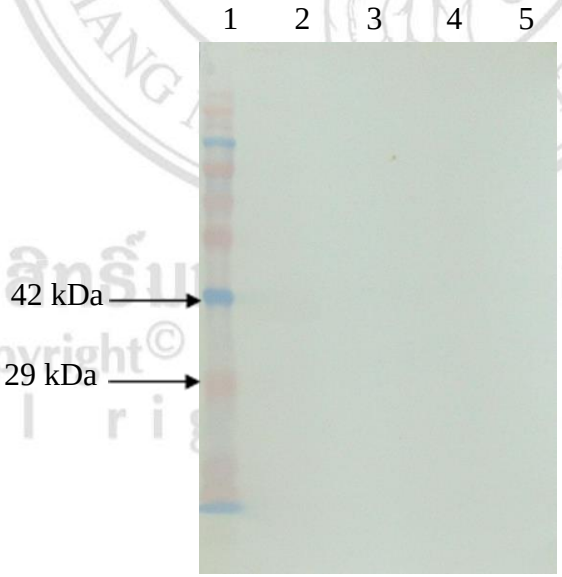


Figure 4.30 Western blot analysis of H5N1 HA2 protein detected by CS (serum negative). Lane 1 is protein molecular weight marker, lane 2-5 are eluted fraction 1,2,3 and 4 respectively.

4.5.2.2 Expression of H5N1 HA2 gene by recombinant *Pichia pastoris* containing pPICZαB-H5N1 HA2 vector

In this experiment, the colonies of the PCR positive clone (the recombinant *Pichia* containing H5N1 HA2 gene) were selected for cultivated in BMGY and induced with methanol to final concentration of 1% (v/v) every 12 hours for 3 days. The culture supernatant as collected and used to purify the H5N1 HA2 protein using HisPur ™ Ni-NTA Spin Column (Thermo Fisher Scientific Inc., USA) under native condition. The present of recombinant protein was analyzed by SDS-PAGE and western blot (section 3.12).

For colorimetric Assay of Western blotting detection, HisDetector™ Western Blot Kits (KPL, Inc., USA) was used to detect the protein on membrane. It was found that the recombinant *Pichia pastoris* strain X33 containing pPICZαB-H5N1 HA2 did not express the H5N1 HA2 protein as no the protein band was seen on the membrane (data not shown).

Next, 1.5% casein amino acid was added into BMGY for prevent protease enzyme which might affect the secreted protein during cultivation process. After cultivated and induced with methanol every 12 hours for 3 days, the culture supernatant were collected and used for purification similar to the previous experiment. Then, the purified protein was analyzed with HisDetector™ Western Blot Kits (KPL, Inc., USA). It was found that *P. pastoris* strain X33 containing pPICZαB-H5N1 HA2 did not expressed the protein (data not shown).

From the result of the previous experiment, it was possible that the recombinant *P. pastoris* could not secreted protein to outside cells. So, the the endrogenous protein from recombinant *Pichia* was extracted by ultrasonicator. Next, the supernatant was used for purify the H5N1 HA2 protein and the purified protein was detected with western blot analysis by HisDetector™ Western Blot Kits (KPL, Inc., USA). It was found that *Pichia pastoris* strain X33 containing pPICZαB-H5N1 HA2 did not express a single band of the protein (data not shown).

4.6 Estimation of protein concentration

Protein concentration of the five fractions of H5N1 HA2 protein purified by HisPur™ Ni-NTA Spin Column under native condition (section 4.8.2) was estimated using absorbance at 280 nm. (Table 4.1).

Table 4.1 Estimated protein concentration of H5N1 HA2 protein purified by HisPur™ Ni-NTA Spin Column under native condition.

	Calculated the amount of protein HA2, mg/ml				
	Elute 1	**Elute 2**	**Elute 3**	**Elute 4**	**Elute 5**
Total	0.5038	0.5038	0.3602	0.3327	0.2350

The concentration of five eluted fractions of H5N1 HA2 protein which was purified by HisPur™ Ni-NTA Spin Column under native condition was 1.9355 mg/5ml or 0.3871 mg/ml.