



**APPENDICES**

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# APPENDIX A

## 1) The gene of haemagglutinin domain of avian influenza A virus strain CMU H5

(A/Chicken/Chiang Mai/1/2004 (H5N1))

```

      20      40      60      80      100
      |      |      |      |      |
H5N1 CMU ATGGAGAAAATAGTGCCTCTTTTGGCAATAGTCAGTCTTGTTAAAAGTGATCAGATTTGCATTGGTTACCATGCAAACAACCTCGACAGAGCAGGTTGACA
      120      140      160      180      200
      |      |      |      |      |
H5N1 CMU CAATAATGGAAAAGAAGCTTACTGTTACACATGCCCAAGACATACTGGAAAAGACACACAACGGGAAGCTCTGGCATCTAGATGGAGTGAAGCCTCTAAT
      220      240      260      280      300
      |      |      |      |      |
H5N1 CMU TTTGAGAGATTGTAGTGTAGCTGGATGGCTCCTCGGAAACCAATGTGTGACGAATTCATCAATGTGCCGGAATGGTCTTACATAGTGGAGAAGGCCAAT
      320      340      360      380      400
      |      |      |      |      |
H5N1 CMU CCAGTCAATGACCTCTGTTACCCAGGGGATTTCAATGACTATGAAGAATTGAAACACCTATTGAGCAGAATAAACCATTTTGAGAAAAATTCAGATCATCC
      420      440      460      480      500
      |      |      |      |      |
H5N1 CMU CCAAAAGTTCTTGGTCCAGTCATGAAGCCTCATTAGGGGTGAGCTCAGCATGTCCATACCAGGGAAGTCTCTCTTTTTCAGAAATGTGGTATGGCTTAT
      520      540      560      580      600
      |      |      |      |      |
H5N1 CMU CAAAAAGAACAGTACATACCCAACAATAAAGAGGAGCTACAATAATACCAACCAAGAAGATCTTTTGGTACTGTGGGGGATTCCACCATCCTAATGATGCG
      620      640      660      680      700
      |      |      |      |      |
H5N1 CMU GCAGAGCAGACAAAGCTCTATCAAAACCCAACCACTATATTTCCGTTGGGACATCAACACTAAACCAGAGATTGGTACCAAGAATAGCTACTAGATCCA
      720      740      760      780      800
      |      |      |      |      |
H5N1 CMU AAGTAAACGGGCAAAGTGGGAAGGATGGAGTTCTTCTGGACAATTTTAAAACCGAATGATGCAATCAACTTCGAGAGTAATGGAATTTTCATTGCTCCAGA
      820      840      860      880      900
      |      |      |      |      |
H5N1 CMU GTATGCATACAAAATTTGTCAAGAAAGGGGACTCAACAATTTATGAAAAGTGAATGGAAATATGGTAACTGCAACACCAAGTGTCAAACCTCCAATGGGGGG
      920      940      960      980      1,000
      |      |      |      |      |
H5N1 CMU ATAAACTCTAGTATGCCATTCACAATATACACCCTCTCACCATCGGGGAATGCCCAAAATATGTGAAATCAAACAGATTAGTCCTTGGGACTGGGCTCA
      1,020      1,040      1,060      1,080      1,100
      |      |      |      |      |
H5N1 CMU GAAATAGCCCTCAAAGAGAGAGAAGAAGAAAAAGAGAGGATTATTTGGAGCTATAGCAGGTTTTATAGAGGGAGGATGGCAGGGAATGGTAGATGGTTG
      1,120      1,140      1,160      1,180      1,200
      |      |      |      |      |
H5N1 CMU GTATGGGTACCACCATAGCAATGAGCAGGGGAGTGGGTACGCTGCAGACAAGAATCCACTCAAAGGCAATAGATGGAGTCACCAATAAGGTCAACTCG
      1,220      1,240      1,260      1,280      1,300
      |      |      |      |      |
H5N1 CMU ATCATTGACAAAATGAACACTCAGTTTGAGGCCGTTGGAAGGGAATTTAACAACTTAGAAAGGAGAATAGAGAATTTAAACAAGAAGATGGAAGACGGGT
      1,320      1,340      1,360      1,380      1,400
      |      |      |      |      |
H5N1 CMU TCCTAGATGTCTGGACTTATAATGCTGAACTTCTGGTTCTCATGGAAAATGAGAGAACTCTAGACTTTTCATGACTCAAATGTCAAGAACCTTTACGACAA
      1,420      1,440      1,460      1,480      1,500
      |      |      |      |      |
H5N1 CMU GGTCCGACTACAGCTTAGGGATAATGCAAAGGAGCTGGGTAAACGGTTGTTTCGAGTTCTATCATAAATGTGATAATGAATGTATGGAAAAGTGAAGAAAC
      1,520      1,540      1,560      1,580      1,600
      |      |      |      |      |
H5N1 CMU GGAACGTATGACTACCCACAGTATTCAGAAGAAGCAAGACTAAAAAGAGAGGAAATAAGTGGAGTAAAATTGGAATCAATAGGAATTTACCAAACTACTGT
      1,620      1,640      1,660      1,680      1,700
      |      |      |      |      |
H5N1 CMU CAATTTATTCTACAGTGGCGAGTTCCCTAGCACTGGCAATCATGGTAGCTGGTCTATCCTTATGGATGTGCTCCAATGGGTCGTACAATGCAGAATTTG
      1,700
H5N1 CMU CATTAA

```

## 2) The amino acid of haemagglutinin domain of avian influenza A virus strain

### CMU H5 (A/Chicken/Chiang Mai/1/2004 (H5N1))

```

                20                               40
H5N1 CMU (+1) MEKIVLLFAIVSLVKSDQICIGYHANNSTEQVDTIMEKNVTVTHAQDILE
                60                               80                               100
H5N1 CMU (+1) KTHNGKLCDLGDKVPLILRDCSVAGWLLGNPMCDEFINVPESYIVEKAN
                120                               140
H5N1 CMU (+1) PVNDLCYPGDFNDYEELKHLISRINHFEKIQIIPKSSWS SHEASLGVSSA
                160                               180                               200
H5N1 CMU (+1) CPYQGKSSFFRNVVWLIKKNSTYPTIKRSYNNTNQEDLLVLWGIHHPNDA
                220                               240
H5N1 CMU (+1) AEQTKLYQNPTTYISVGTSTLNQRLVPR IATRSKVNGQSGRMEFFWTILK
                260                               280                               300
H5N1 CMU (+1) PNDAINFESNGNFI APEYAYKIVKKG DSTIMKSELEYGNCNTKCQTPMGA
                320                               340
H5N1 CMU (+1) INSMPFHN IHPLTIG ECPKYVKS NRLLV LATGLRNSPQRERRRKRGLFG
                360                               380                               400
H5N1 CMU (+1) A IAGFIEGGWQGMVDGWYGYHHSNEQSGYAADKESTQKAIDGVTNKVNS
                420                               440
H5N1 CMU (+1) I IDKMNTQFEAVGREFNNLERRIENLNKKMEDGFLDVWTYNAELLVLMEN
                460                               480                               500
H5N1 CMU (+1) ERTLDFHDSNVKNLYDKVRLQLRDNAKELGNGCFEFYHKCDNECME SVRN
                520                               540
H5N1 CMU (+1) GTYDYPQYSEEARLKREEISGVKLESIGIYQILSIYSTVASSLALAIMVA
                560
H5N1 CMU (+1) GLSLWMC SNGSLQCRICI*
```

**Note:** - The position of H5N1 HA1 is 1-338.

- The position of cleavage site is 339-346.

- The position of fusion site is 374-354.

- The position of H5N1 HA2 is 355-537.

- The position of trans membrane is 559-568.

- The position of cytosol tail is 559-568.

## APPENDIX B

### Optimized sequence of H5N1 HA1\_OPT gene (GenScript, USA.)

XhoI  
 H5N1 HA1\_OPT CTTCGACAAAAGAGAGGCTGAAGCTATGCATCATCATCATCATCAATAGCGCGTCGACGAACAAAACTCATCTCAGAAAGGATCTGAATAGCGCGC  
Signal sequence Start 6xHis-tag Linker Myc-tag Linker  
 H5N1 HA1\_OPT TCGACATGGAGAAAATCGTCTTGTGTTGCGCAATCGTTTCCCTTGTCAAAAGTGACCAAATCTGTATCGGTTACCACGCAAATAACTCAACTGAGCAAGT  
 H5N1 HA1\_OPT TGATACTATCATGAAAAGAACGTTACCGTCACTCATGCCCAGGACATTTGGAAAAGACACACAATGGTAAATTGTTGATCTTGACGGAGTTAAGCCA  
 H5N1 HA1\_OPT TTGATTCCTAGAGATTGTTCCAGTCGCAGGTTGGTTGCTTGGAAACCAATGTGCGACGAGTTTATTAAATGTTCTTGAGTGGAGTTACATCGTTGAAAAG  
 H5N1 HA1\_OPT CTAACCCAGTCAATGATTGTGTTACCCAGGAGATTTAACGACTATGAAGAGCTTAAGCATTTGCTTTCAAGAATTAATCACTTCGAGAAGATCCAAT  
 H5N1 HA1\_OPT CATCCCAAAATCTTCTGGTCGTCATGAAGCCTCCTTGGGTGTTTCTCCGCATGCCCTTACCAGGTAATCTTCTTTTAGAAACGTTGCTCG  
 H5N1 HA1\_OPT TTGATCAAGAAAAATTTACTTACCAACAATTAAGAGATCCTACAACAACCAACCAAGAGGATTGCTTGTGTTGGGGTATTTCACCCAAACG  
 H5N1 HA1\_OPT ACGCTGCCGAACAACAAGCTTTACCAGAACCCTACTACATACATCTCAGTCGGAACAAGTACCTTGAACCAAGACTTGTTCCTAGAATTGCTACAAG  
 H5N1 HA1\_OPT ATCAAAAGTCAATGGTCAGAGTGAAGAATGGAGTTTTCTGGACCATTTGAAGCCAACGATGCCATCAATTCGAGTCTAACGGTAACCTCATCGCA  
 H5N1 HA1\_OPT CCTGAATACGCTTATAAGATCGTTAAGAAAGGAGACTCTACTATTGAAATCCGAATTTGGAGTACGGTAACGTAATACCAAGTGCCAAACTCCTATGG  
 H5N1 HA1\_OPT GAGCTATTAACCTTCCATGCCATTCCATAATATTACCCTTTGACTATCGGTGAATGTCCTAAGTATGTTAAAAGTAATAGATTGGTCTTGCCACTGG  
 H5N1 HA1\_OPT TTTGAGAAATAGTCCACAGTAACTGGCCGC  
Stop NotI  
 H5N1 HA1\_OPT TTTGAGAAATAGTCCACAGTAACTGGCCGC

  
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## APPENDIX C

### Media recipes

#### 1. Stock solution, reagents and media

##### 1.1 Stock solutions

##### **10X YNB (13.4% yeast nitrogen base with ammonium sulfate without amino acids)**

Dissolve 134 g of yeast nitrogen base (YNB) with ammonium sulfate and without amino acids in 1000 ml of water heat the solution to dissolved YNB completely and filter sterilize. Store at 4°C.

##### **500X B (0.02% Biotin)**

Dissolve 20 mg of biotin in 100 ml of water and filter sterilize. Store at 4°C. The shelf life of this solution is approximately one year.

##### **10X GY (10% Glycerol)**

Mix 100 ml of glycerol with 900 ml of water. Sterilize either by filtering or autoclaving. Store at room temperature. The shelf life of this solution is greater than one year.

##### **1 M potassium phosphate buffer, pH 6.0**

Combine 132 ml of 1 M  $K_2HPO_4$  with 868 ml of 1 M  $KH_2PO_4$  and confirm that the  $pH=6.0 \pm 0.1$  (if the pH needs to be adjusted, use phosphoric acid or KOH). Autoclave and store at room temperature. The shelf life of this solution is greater than one year.

## 2. *Pichia media* recipe.

### 2.1 Buffered Glycerol-complex Medium (BMGY)

1% yeast extract

2% peptone

100 mM potassium phosphate, pH 6.0

1.34% YNB

$4 \times 10^{-5}$  % biotin

1% glycerol

1. Dissolve 10 g of yeast extract, 20 g of peptone in 700 ml of water.

2. Autoclave 20 minutes on liquid cycle.

3. Cool to room temperature, then add the following and mix well:

- 100 ml 1M potassium phosphate buffer, pH 6.0

- 100 ml 10X YNB

- 2 ml 500X B

- 100 ml 10X GY

4. Store media at 4°C. The shelf life of this solution is approximately two months.

### 2.2 Yeast Extract Peptone Dextrose Medium (YPD 1 liter)

1% yeast extract

2% peptone

2% dextrose (glucose)

1. Dissolve 10 g of yeast extract and 20 g of peptone in 900 ml of water.

Note: Add 20 g of agar if making YPD slants or plates.

2. Autoclave 20 min. on liquid cycle.

Note: Cool solution to 55°C and add zeocin (If require)

Store the liquid medium at room temperature. Store YPD slants or plates at 4°C. The shelf life of this solution is several months.

### 3. *Escherichia coli* Media Recipes.

#### 3.1 Low salt-LB medium with zeocin

10 g tryptone

5 g NaCl

5 g yeast extract

1. Combine the dry reagents above and add distilled water to 950 ml. Adjust pH to 7.5 with 1 N NaOH. Bring the volume up to 1 liter. For plates, add 15 g/l agar before autoclaving.

2. Autoclave on liquid cycle at 15 psi and 121°C for 20 minutes.

3. Allow the medium to cool at least 55°C before adding the zeocin to 50 µg/ml final concentration.

4. Store plates at 4°C in the dark. Plates containing zeocin are stable for up to 2 weeks.

## APPENDIX D

### Reagents and buffers preparation

#### 0.1 M Tris-HCl buffer (pH 7.0) supplemented with 2 mM CaCl<sub>2</sub>·H<sub>2</sub>O

- Dissolve 3.025 g of Tris base in deionized water (DI-H<sub>2</sub>O).
- Adjust pH to 7.0 with 6 N HCl.
- Add 0.055 g of CaCl<sub>2</sub>.
- Adjust volume to 250 ml with deionized water (DI-H<sub>2</sub>O).

#### 0.5 M EDTA, pH 8.0

- Dissolve 186.12 g of Na<sub>2</sub>EDTA·2H<sub>2</sub>O in 800 ml of distilled water with magnetic stirring plate.
- Adjust pH to 8.0 with 1 M NaOH
- Adjust volume to 1 liter with distilled water.
- Sterilize by autoclaving and store at room temperature.

#### 50X TAE buffer, pH 8.0

- Dissolve 242 g of Tris base in 500 ml of distilled water.
- Add glacial acetic acid and 0.5 M EDTA.
- Adjust volume to 1 liter with distilled water.
- Store at room temperature.



### **1. 3M sodium acetate**

- Dissolve 408.1 g of NaOAC.3H<sub>2</sub>O in 800 ml of distilled water.
- Adjust pH to 5.2 with glacial acetic acid.
- Adjust volume to 1 liter with distilled water.
- Store at room temperature.

### **2. 0.1 M CaCl<sub>2</sub>**

- Dissolve 1.48 g of CaCl<sub>2</sub>•2H<sub>2</sub>O in 100 ml of distilled water.
- Sterilize by autoclaving and store at 4°C.

### **3. 1 M sorbitol**

- Dissolve 18.2 g of sorbitol in 100 ml of distilled water.
- Sterilize by autoclaving and store at 4°C.

### **4. 30% Acrylamide mix**

- Dissolve 29 g of Acrylamide and 1 g of Bis-acrylamide in 100 ml of distilled water.
- Store in the dark at 4°C.

### **5. 10% APS**

- Dissolve 100 mg of ammonium persulfate (APS) in 1 ml of distilled water.
- Store in the dark at 4°C

### **6. 1.5 M Tris-HCl pH 8.8**

- Dissolve 18.15 g of Tris base in distilled water.
- Adjust to pH 8.8 with 6 M HCl.
- Adjust volume to 100 ml of distilled water.
- Store at 4 °C

### **7. 0.5 M Tris-HCl pH 6.8**

- Dissolve 6.06 g of Tris base in distilled water.
- Adjust to pH 6.8 with 6 M HCl.
- Adjust volume to 100 ml with distilled water.
- Store at 4°C

### **8. Running buffer 1X**

- Dissolve 7.21 g of Glycine and 1.51 g of Tris base in distilled water.
- Add 0.5 g of SDS (sodium dodecyl sulfate)
- Adjust volume to 500 ml with distilled water.
- Filtrate by passing through a 0.2 µm filter

### **9. Coomassie Blue protein stain**

- Dissolve 0.5 g of Coomassie Brilliant Blue R-250 in distilled water.
- Add 500 ml of methanol and 100 ml of acetic acid into solution
- Adjust volume to 1 l with distilled water.

## 10. Blotting buffer

- Dissolve Glycine; 1.801 g, Tris-base; 0.378 g and 0.0625 g of SDS in distilled water.

- Add 25 ml of methanol and adjust to 100 ml with distilled water.




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## APPENDIX E

### The sequencing data of H5N1 HA2 gene

(1st\_BASE\_1106907\_HA2\_AOX1\_forward)



```

pPICZA-H5N1 HA2 C.1      20      40      60      80      100
GTTTTTCGACTTTTACGACACTTGAGAGATCAAAAAACAACATAATTATTCGAAACGAGGAATTCACGTGGCCAGCCGGCCGCTCTCGGATCGGTACCTCC
pPICZA-H5N1 HA2 C.1      120     140     160     180     200
AGAAAAGAGAGGCTGAAGCTATGTTTATAGAGGAGGATGGCAGGGAATGGTAGATGGTTGGTATGGTACCACCATAGCAATGAGCAGGGGAGTGGGTA
pPICZA-H5N1 HA2 C.1      220     240     260     280     300
CGCTGCAGACAAAGAATCCACTCAAAGGCAATAGATGGAGTCAACAATAAGGTCAACTCGATCATTGACAAAATGAACACTCAGTTTGAGGCCGTGGGA
pPICZA-H5N1 HA2 C.1      320     340     360     380     400
AGGGAATTTAACAACCTTAGAAAGGAGAATAGAGAATTTAAACAAGAAGATGGAAGACGGGTTCTAGATGTCTGGACTTATAATGCTGAACCTCTGGTTC
pPICZA-H5N1 HA2 C.1      420     440     460     480     500
TCATGGAAAATGAGAGAACTCTAGACTTTTATGACTCAAATGTCAAGAACCTTTACGACAAGGTCGGACTACAGCTTAGGGATAATGCAAAGGAGCTGGG
pPICZA-H5N1 HA2 C.1      520     540     560     580     600
TAACGGTTGTTTCGAGTTCTATCATAAATGTGATAATGATGTAAGAAAGTGAAGAAATGGAACGTATGACTACCCACAGTATTGAGAAGAAGCAAGA
pPICZA-H5N1 HA2 C.1      620     640     660     680     700
CTAAAAAGAGAGAAATAAGTGGAGTAAAATTGGAATCAATAGGAATTTACCAAACTACTGTCAATTTATCTTGGCGCCAGCTTGGGCCCGAACAAA
pPICZA-H5N1 HA2 C.1      720     740     760     780     800
AACTCATCTCAGAAGAGGATCTCAATAGCCCGTCGACCATCATCATCATCATTTGAGTTTTAGCCCTAGACATGACTGTTCTCAGTTCAAGTTGGG
pPICZA-H5N1 HA2 C.1      820     840     860     880     900
CACTTACGAGAAGACCGGCTTTGCTAGATTCTAATCAAGAGGATGTCAGAATGCCATTTGCCTGAGAGATGCAGGCTTCATTTTTGATACTTTTTTATTT
pPICZA-H5N1 HA2 C.1      920     940     960     980     1,000
GTAACCTATATAGTATAGGATTTTTTTTGTGCTTTTGTCTTCTCGTACGAGCTTGCTCCTGATCAGCCTATCTCGCAGCTGATGAATATCTTGTGGTA
pPICZA-H5N1 HA2 C.1      1,020    1,040    1,060    1,080    1,100
GGGGTTTGGGAAATCATTGAGTTTGTATTTTTCTTGGTATTTCCCACTCCTCTTCAGAGTACAGAAGATTAAGTGAAGACCTCGTTTGTGCGGATCC
pPICZA-H5N1 HA2 C.1      1,120    1,140    1,160    1,180    1,200
CCCACACCATAGCTTCAAATGTTTCTACTCCTTTTTACTCTTCAAATTTTTCCGGACTCCGGCATCGCCGTACCCCTTTCAAACCCCAAGCCC
pPICZA-H5N1 HA2 C.1      1,220    1,240    1,260    1,280    1,300
AGCATACTAAATTTCCCTCTTTCTCCCTAGGGTGGTAAATCCCGTACTAAAGTTTGGAAAAAAGAACCCGCTCGGTTCTTTTTCTT
pPICZA-H5N1 HA2 C.1      1,320    1,340    1,360    1,380    1,400
CCCCAAAAAGGAATAAAAATTTTTACCAGTTTCCTTTCTTGGGAAAATTTTTTTTTTGAATTTTTTCTTTTTCGAAGACTCCCTTTGGAATTTA
pPICZA-H5N1 HA2 C.1      AAGATAAAAACGGG

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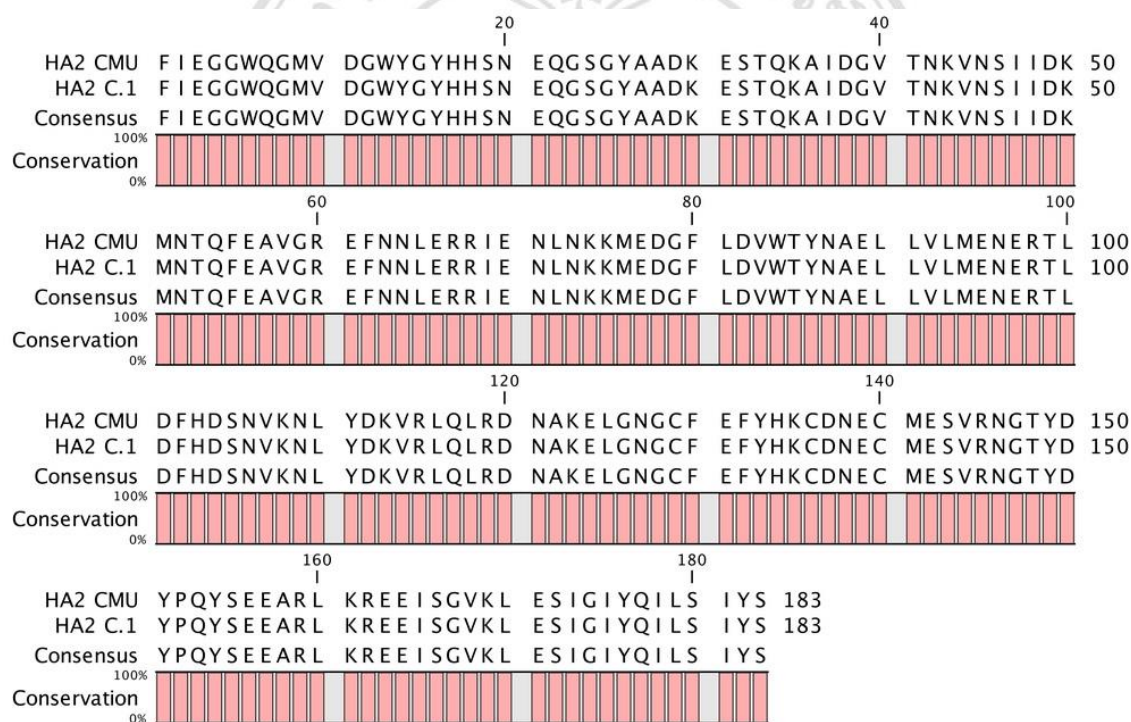
Signal sequence Start XhoI  
Myc-tag Linker 6xHis-tag Stop NotI

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## APPENDIX F

### Comparison of amino acids

Comparison of amino acid between the haemagglutinin domain of avian influenza A virus strain CMU H5 (A/Chicken/Chiang Mai/1/2004 (H5N1)) and the recombinant vector (pPICZA-H5N1 HA1 clone1) from *Escherichia coli* strain XL1-blue.



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## APPENDIX G

### Calculation of transformation efficiency

Transformation efficiency was calculated by dividing the number of successful transformants by the amount of DNA used during a transformation procedure (Tu *et al.*, 2004). In this study, 2.50 µg of H5N1 HA2 gene (concentration of 1 µg/µl) was used. Then, 100 µl of cells suspension containing DNA from a test tube containing a total volume of 1100 µl of solution were spread on YPDS plate with zeocin, so the total amount of H5N1 HA2 gene that spread on plate was calculated using the following formula;

$$\begin{aligned} \text{DNA spread in } \mu\text{g} &= \frac{\text{Total amount of DNA used in } \mu\text{l} \times \text{volume spread on plate } (\mu\text{l})}{\text{Total sample volume in test tube } (\mu\text{l})} \\ &= \frac{(2.50 \times 100)}{1100} \\ &= 0.227 \end{aligned}$$

Each colony on the YPDS plate can be assumed to be derived from a single cell. The total number of yeast cell was determined by counting the colonies on the plates. So, these represented the total number of yeast cell that express the H5N1 HA2 gene, divided by the amount of H5N1 HA2 gene used in this study. The transformation efficiency was calculated using the following formula;

$$\text{Transformation efficiency} = \frac{\text{Total number of cells growing on the plate}}{\text{Amount of DNA spread on the plate}}$$

The 251 colonies were grown on YPDS agar containing 100 µg/ml of zeocin.

$$\text{Transformation efficiency} = \frac{251}{0.227} = 1.11 \times 10^3 \text{ cells}/\mu\text{g}$$

## CURRICULUM VITAE

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<b>Publication</b>	Pornprasert, S., Thanuthamcharoen, W., Pawichai, S., Jannoi, P., Jannoi, S. (2007). Antibacterial activity of <i>Eupatorium odoratum</i> leaf extract. <i>Journal of Medical Technology Association of Thailand</i> , 35, 2010-2017.



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