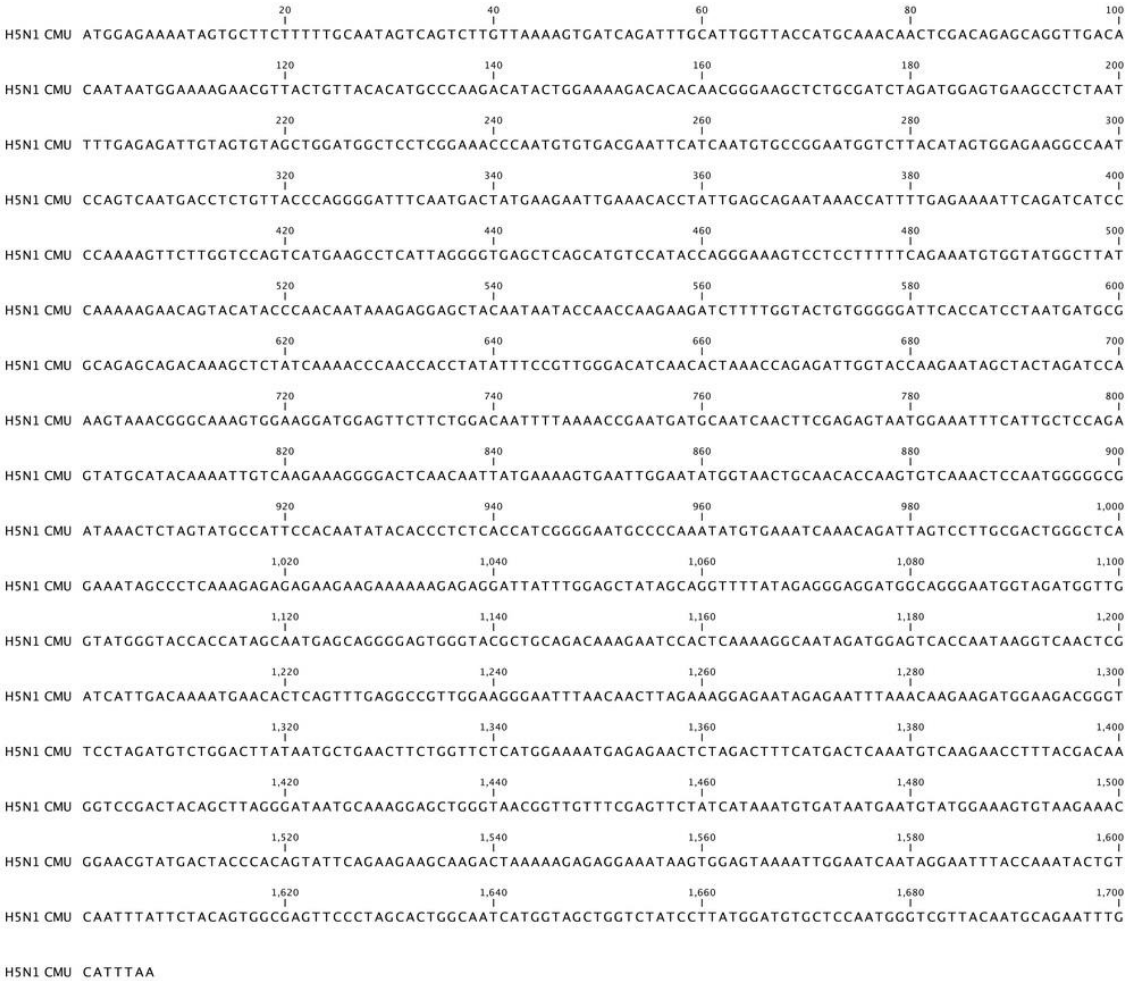
APPENDICES

APPENDIX A

1) The gene of haemagglutinin domain of avian influenza A virus strain CMU H5 (A/Chicken/Chiang Mai/1/2004 (H5N1))



2) The amino acid of haemagglutinin domain of avian influenza A virus strain CMU H5 (A/Chicken/Chiang Mai/1/2004 (H5N1))

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                                      20                  40
H5N1 CMU (+1)  MEKIVLLFAIVSLVKSDQICIGYHANNSTEQVDTIMEKNVTVTHAQDILE
                          60                  80                  100
H5N1 CMU (+1)  KTHNGKLCDLDGVKPLILRDCSVAGWLLGNPMCDEFINVPEWSYIVEKAN
                                     120                 140
H5N1 CMU (+1)  PVNDLCYPGDFNDYEELKHLLSRINHFEKIQIIPKSSWSSHEASLGVSSA
                          160                 180                 200
H5N1 CMU (+1)  CPYQGKSSFFRNVVWLIKKNSTYPTIKRSYNNTNQEDLLVLWGIHHPNDA
                                     220                 240
H5N1 CMU (+1)  AEQTKLYQNPTTYISVGTSTLNQRLVPRIATRSKVNGQSGRMEFFWTILK
                          260                 280                 300
H5N1 CMU (+1)  PNDAINFESNGNFIAPEYAYKIVKKGDSTIMKSELEYGNCNTKCQTPMGA
                                     320                 340
H5N1 CMU (+1)  INSSMPFHNIHPLTIGECPKYVKSNRLVLATGLRNSPQRERRRKKRGLFG
                          360                 380                 400
H5N1 CMU (+1)  AIAGFIEGGWQGMVDGWYGYHHSNEQGSGYAADKESTQKAIDGVTNKVNS
                                     420                 440
H5N1 CMU (+1)  IIDKMNTQFEAVGREFNNLERRIENLNKKMEDGFLDVWTYNAELLVLMEN
                          460                 480                 500
H5N1 CMU (+1)  ERTLDFHDSNVKNLYDKVRLQLRDNAKELGNGCFEFYHKCDNECMESVRN
                                     520                 540
H5N1 CMU (+1)  GTYDYPQYSEEARLKREEISGVKLESIGIYQILSIYSTVASSLALAIMVA
                          560
H5N1 CMU (+1)  GLSLWMCSNGSLQCRICI*
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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.

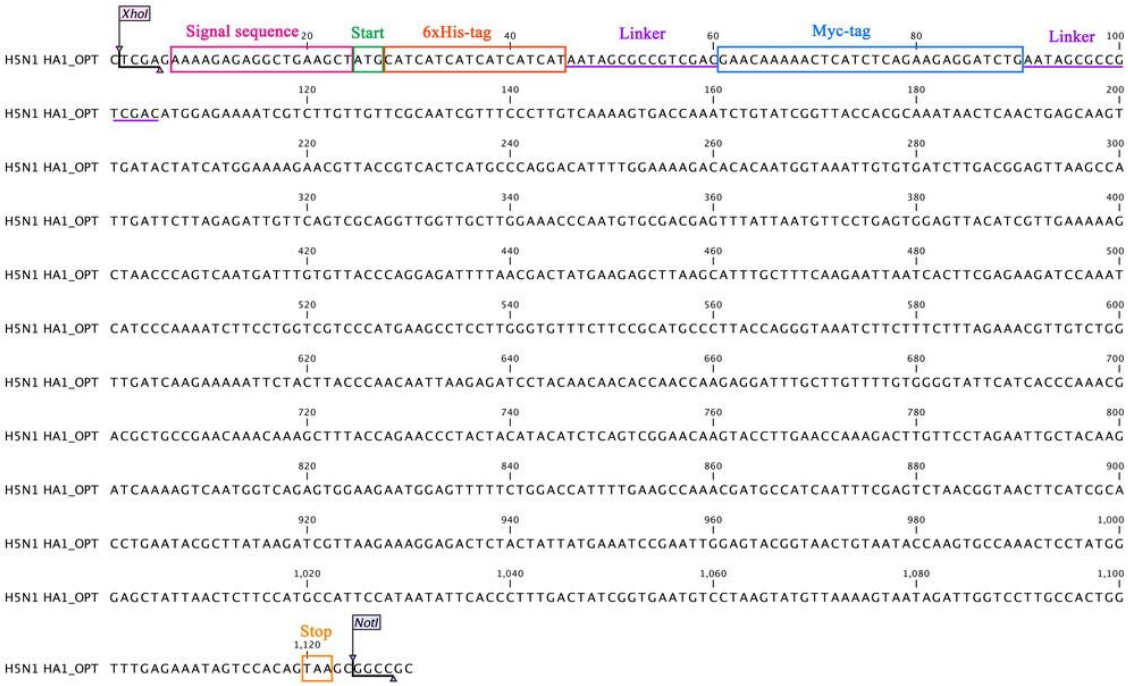
- The position of H5N1 HA2 is 355-537.

- The position of trans membrane is 559-568.

- The position of cytosal tail is 559-568.

APPENDIX B

Optimized sequence of H5N1 HA1_OPT gene (GenScript, USA.)



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 ± 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 Bufferred Glycerol-complex Medium (BMGY)

1% yeast extract

2% peptone

100 mM potassium phosphate, pH 6.0

1.34% YNB

4×10^{-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 $CaCl_2.H_2O$

- Dissolve 3.025 g of Tris base in deionized water (DI-H_2O).

- Adjust pH to 7.0 with 6 N HCl.

- Add 0.055 g of $CaCl_2$.

- Adjust volume to 250 ml with deionized water (DI-H_2O).

0.5 M EDTA, pH 8.0

- Dissolve 186.12 g of $Na_2EDTA.2H_2O$ 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 $NaOAC.3H_2O$ 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 $CaCl_2$

- Dissolve 1.48 g of $CaCl_2 \cdot 2H_2O$ 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.
- Adjuste 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

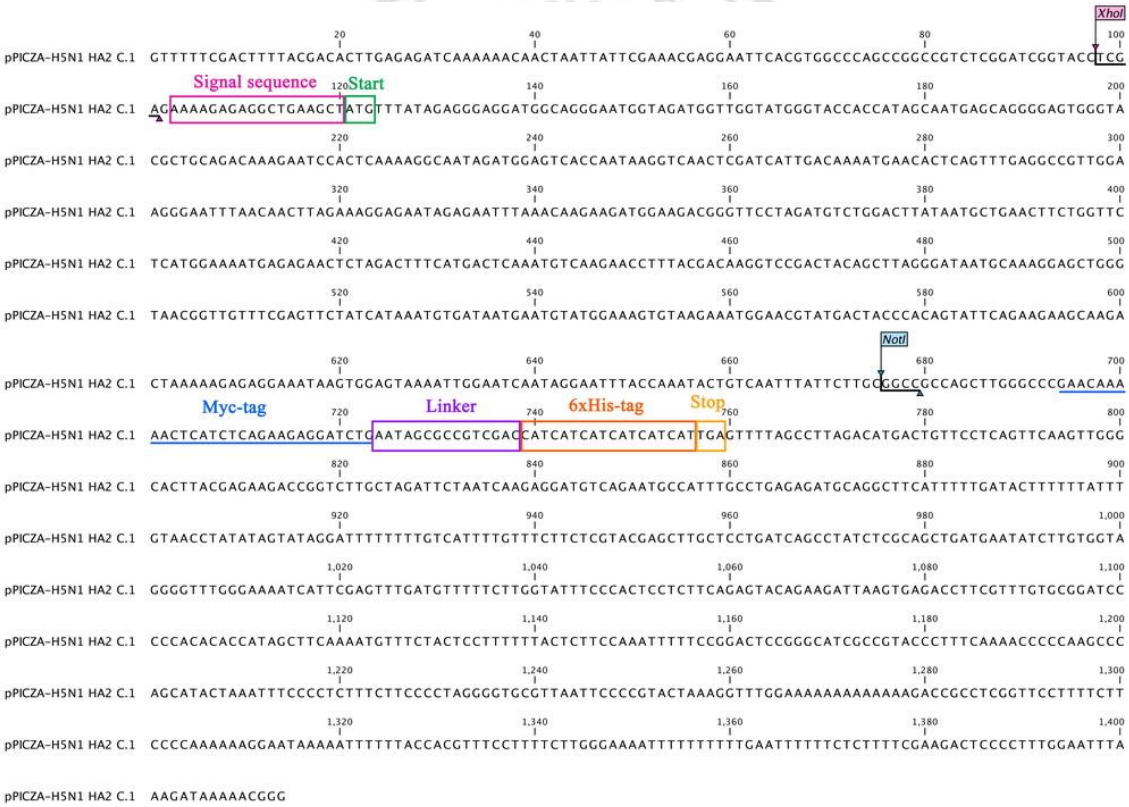
- Dissolve Glycine; 1.801 g, Tris-base; 0.378 g and 0.0625 g of SDS in distilled water.

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

APPENDIX E

The sequencing data of H5N1 HA2 gene

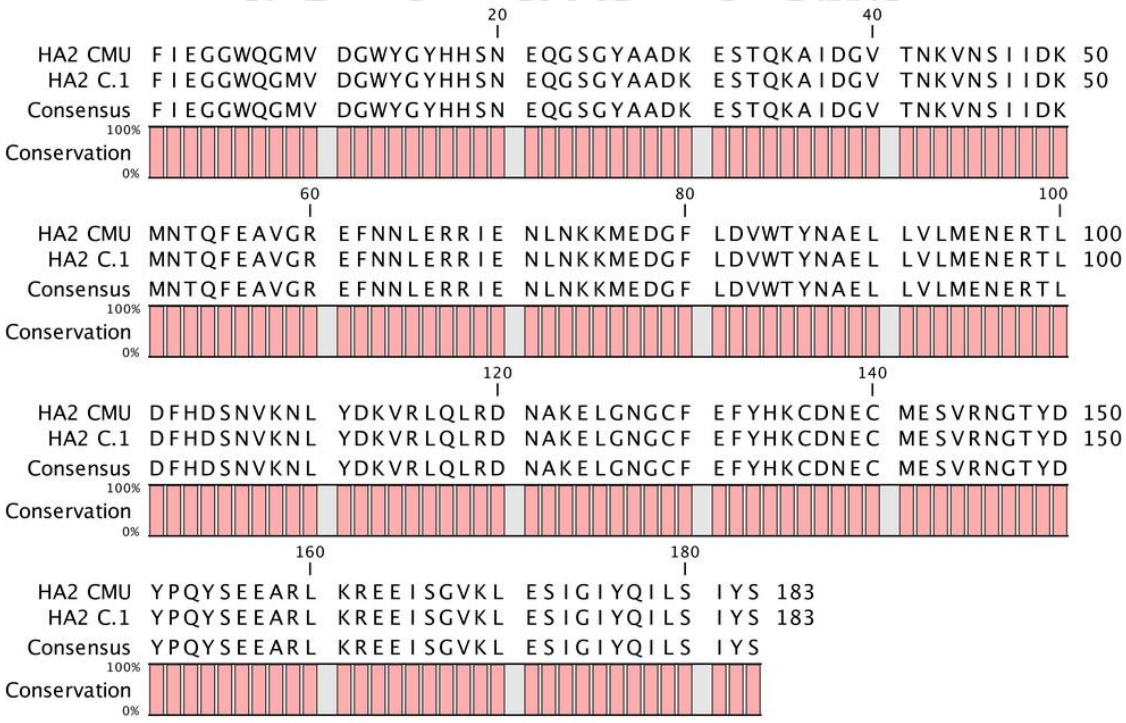
(1st_BASE_1106907_HA2_AOX1_forward)



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.



APPENDIX G

Calculation of transformation efficiency

Transformation efficiency was calculated by diving 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;

$$\text{DNA spread in µg} = \frac{\text{Total amount of DNA used in µl} \times \text{volume spread on plate (µl)}}{\text{Total sample volume in test tube (µl)}}$$

$$= (2.50 \times 100)/1100$$

$$= 0.227$$

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.

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

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Publication

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