

**USE OF SEMICONDUCTOR-BASED OLIGONUCLEOTIDE
MICROARRAYS FOR IDENTIFICATION AND MONITORING
OF H5N1 AVIAN INFLUENZA A VIRUS MUTATION**

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SUPAPORN KAEWPONGSRI

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OF THE REQUIREMENTS FOR
THE DEGREE OF MASTER OF SCIENCE
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Thesis

Entitled

**USE OF SEMICONDUCTOR-BASED OLIGONUCLEOTIDE
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OF H5N1 AVIAN INFLUENZA A VIRUS MUTATION**

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USE OF SEMICONDUCTOR-BASED OLIGONUCLEOTIDE MICROARRAYS FOR IDENTIFICATION AND MONITORING OF H5N1 AVIAN INFLUENZA A VIRUS MUTATION

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ABSTRACT

Global surveillance of influenza A/H5N1 is critical for improvement in disease management, and is especially important for early detection, rapid intervention, and a possible reduction of the impact of an influenza pandemic. There is a great demand for new technological methods for viral discovery. We described a semiconductor-base oligonucleotide microarray for influenza A virus identification and monitoring. Central to this approach was microarray designed to identify influenza A virus hemagglutinin subtypes 1 through 16 and neuraminidase subtypes 1 through 9. To further characterize this influenza A virus, we proposed Bioedit program for accurately identifying viral sequences by discovering sequence signature, which was enough distinctive information for the sequence identification.

The 8 H5N1-RNA samples were collected and extracted from birds and mammal samples between 2003 to 2006, which were used to analyze H5N1 genotypic testing with semiconductor-base oligonucleotide microarray. One hundred percent (8/8) of the samples tested were subtyped correctly as H5N1. After absolutely correct subtyping from microarray signal intensity result, the method used microarray output combined with Bioinformatic tool for identification and monitoring of genetic variations of H5N1. The outstanding feature of this assay is its capability to distinguish different strains of H5N1. Ninety percent HA (4/5) and ninety percent NA (4/5) genes were sequenced correctly in accordance with previous examinations performed by classical diagnostic methods.

This assay was a convenient and practical tool for the study of avian influenza viruses, providing important HA and NA data more rapidly than conventional methods. However, the protocol monitoring of the new strain H5N1 is recommended for further study.

KEY WORDS: H5N1 VIRUS / SEMICONDUCTOR-BASED OLIGONUCLEOTIDE / MICROARRAY / MUTATION

101 pp.

การตรวจติดตาม และบ่งชี้การกลายพันธุ์ของเชื้อไวรัสไข้หวัดนก สายพันธุ์ เอช5เอ็น1 โดยเทคนิคของเซมิคอนดักเตอร์ เบสโอลิโกนิวคลีโอไทด์ ไมโครอะเรย์

(USE OF SEMICONDUCTOR-BASED OLIGONUCLEOTIDE MICROARRAYS FOR IDENTIFICATION AND MONITORING OF H5N1 AVIAN INFLUENZA A VIRUS MUTATION)

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บทคัดย่อ

โรคไข้หวัดนกสายพันธุ์ เอช 5 เอ็น 1 เป็นโรคติดต่อที่ พบการระบาดทุกภูมิภาคทั่วโลก การเฝ้าระวังและการจัดการควบคุมการแพร่ระบาดที่ได้ผล ต้องอาศัยการตรวจวัดที่รวดเร็วและทันการ จึงจะสามารถควบคุมการระบาดที่รุนแรงของไวรัสไข้หวัดนก ดังนั้นเพื่อจัดการและควบคุมการแพร่ระบาดที่รุนแรงของไวรัสไข้หวัดนกอย่างได้ผล จะต้องอาศัยเทคโนโลยีในการตรวจจับที่รวดเร็ว การนำเทคโนโลยีเซมิคอนดักเตอร์ เบสโอลิโกนิวคลีโอไทด์ ไมโครอะเรย์ เป็นเทคโนโลยีร่วมของการผลิตชิปวงจรรวมและสารกึ่งตัวนำ มาใช้ระบุสายพันธุ์และครอบคลุมการติดตามการกลายพันธุ์ของไวรัสไข้หวัดนก โดยอาศัยเทคโนโลยีนี้สามารถระบุชนิดของไวรัสอินฟลูเอนซาตามคุณสมบัติแอนติเจนฮีมากลูตินินได้ 16 ชนิด และ นิวรามินิเดสได้ 9 ชนิด

โดยการศึกษาได้นำตัวอย่าง อาร์เอ็นเอเชื้อไวรัสไข้หวัดนกสายพันธุ์ เอช 5 เอ็น 1 ที่สกัดจากสิ่งส่งตรวจจากนกและสัตว์เลี้ยงลูกด้วยนมที่ระบาดในปี 2003-2006 มาทำการตรวจวิเคราะห์ด้วยเทคโนโลยีดังกล่าว ซึ่งสามารถระบุซับไทป์ของตัวอย่างทั้ง 8 ได้อย่างถูกต้อง แต่เพื่อให้ครอบคลุมการตรวจติดตามการเกิดการกลายพันธุ์ของเชื้อไวรัสไข้หวัดนกระดับโมเลกุล จึงมีการใช้เทคโนโลยีชีวสารสนเทศ ร่วมกับผลการตรวจจับสัญญาณของเซมิคอนดักเตอร์ เบสโอลิโกนิวคลีโอไทด์ เพื่อที่จะหาลำดับเบสที่จะบ่งถึงความจำเพาะต่อสายพันธุ์เชื้อไวรัสไข้หวัดนกสายพันธุ์ เอช 5 เอ็น 1 โดยวิธีดังกล่าวสามารถถอดรหัสพันธุกรรมของฮีมากลูตินินและนิวรามินิเดสได้ถูกต้องและสอดคล้องกับผล จากวิธีการหาลำดับเบสด้วยวิธีดั้งเดิม จากตัวอย่าง 4 ใน 5 ตัวอย่าง

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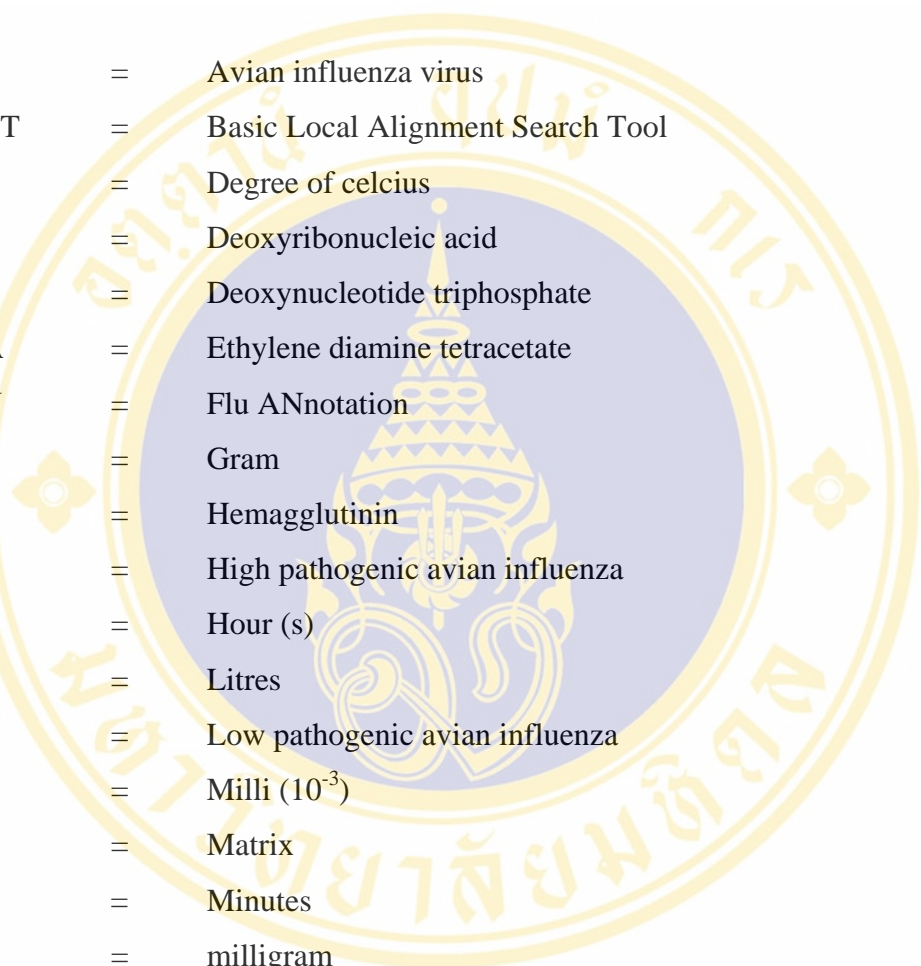
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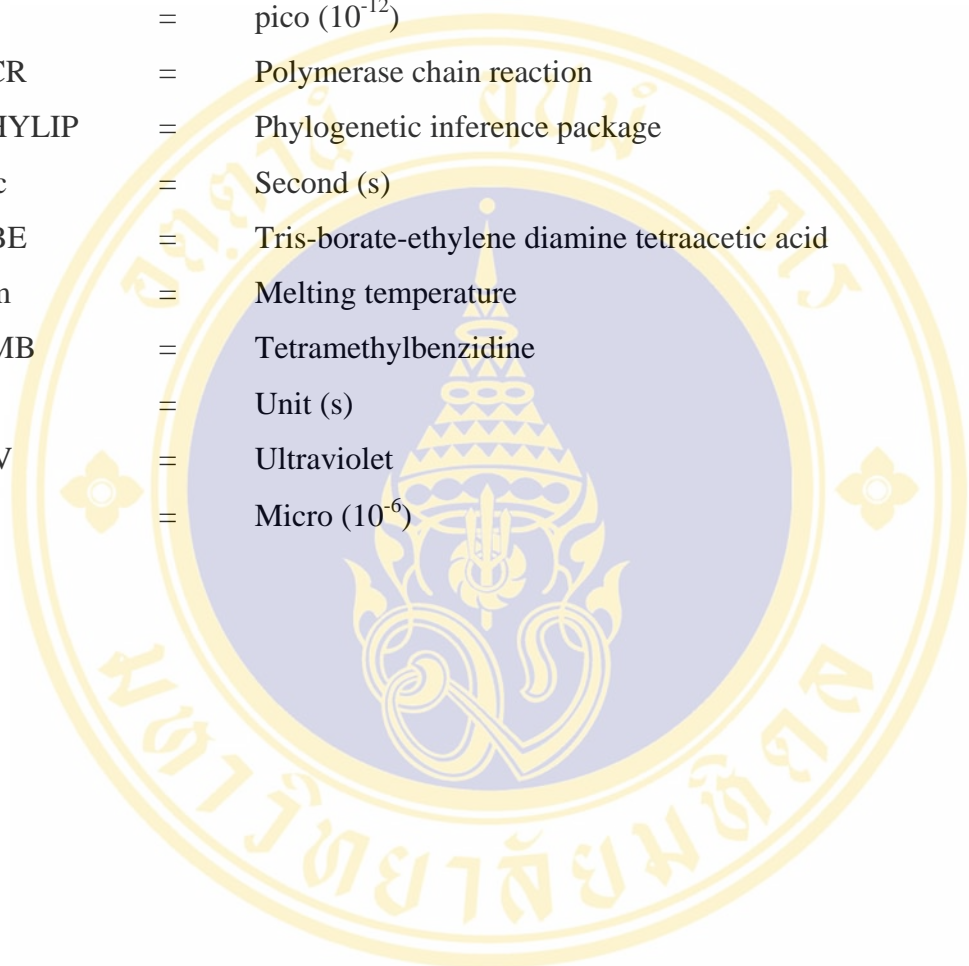
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LIST OF ABBREVIATIONS



| | | |
|-------------------|---|-----------------------------------|
| AIV | = | Avian influenza virus |
| BLAST | = | Basic Local Alignment Search Tool |
| °C | = | Degree of celcius |
| DNA | = | Deoxyribonucleic acid |
| dNTP | = | Deoxynucleotide triphosphate |
| EDTA | = | Ethylene diamine tetracetate |
| FLAN | = | Flu ANnotation |
| g | = | Gram |
| HA | = | Hemagglutinin |
| HPAI | = | High pathogenic avian influenza |
| hr | = | Hour (s) |
| L | = | Litres |
| LPAI | = | Low pathogenic avian influenza |
| m | = | Milli (10 ⁻³) |
| M | = | Matrix |
| min | = | Minutes |
| mg | = | milligram |
| MgCl ₂ | = | Magnesium chloride |
| NA | = | Neuraminidase |
| NaCl | = | Sodium chloride |
| NP | = | Neucleoprotein |

LIST OF ABBREVIATIONS (Continued)

| | | |
|----------------|---|---|
| RNP | = | Ribonucleoprotein |
| p | = | pico (10^{-12}) |
| PCR | = | Polymerase chain reaction |
| PHYLIP | = | Phylogenetic inference package |
| sec | = | Second (s) |
| TBE | = | Tris-borate-ethylene diamine tetraacetic acid |
| T _m | = | Melting temperature |
| TMB | = | Tetramethylbenzidine |
| U | = | Unit (s) |
| UV | = | Ultraviolet |
| μ | = | Micro (10^{-6}) |

CHAPTER I

INTRODUCTION

Bird flu is a complex and dangerous disease that recognizes no borders. It is being watched anxiously by the entire world. The most important fact about avian influenza is that it is caused by the same virus that causes influenza among humans. Influenza A viruses infecting poultry can be divided into two distinct groups on the basis of their ability to cause disease highly pathogenic avian influenza (HPAI) and low pathogenic avian influenza (LPAI). HPAI are highly virulent and may result in mortality as high as 100%. These viruses have been restricted to subtypes H5 and H7, although not all viruses of these subtypes cause HPAI. LPAI viruses are generally lower virulence but these viruses can serve as progenitors to HPAI viruses (1, 2).

Influenza viruses are segmented, negative-sense, RNA viruses that exhibit considerable antigenic diversity. They are divided into types, either A, B or C by variations in nucleoprotein (NP) and matrix protein (M) antigens. Type A viruses are further subtyped by the antigenic differences in two proteins, hemagglutinin (HA) and neuraminidase (NA), which are present on the virus particle surface. Sixteen HA subtypes (designated H1 to H16) and nine NA subtypes (designated N1 to N9) have been identified so this virus has many strains (3). Influenza B and C viruses are not divided into subtype. Although all HA and NA subtypes are found in aquatic birds, the number of subtypes that have crossed the species barrier and established stable lineages in mammals is limited. Only three HA and two NA subtypes (i.e. H1–3 and N1–2) have circulated in human since 1918. In horses, only two influenza A subtypes (H7N7 and H3N8) are found, while, despite susceptibility to all avian subtypes in experimental settings, the only subtypes recovered from pigs in nature are H1, H3, N1, and N2. The molecular, biological or ecological factors determining the apparent subtype-specific ability of viruses to cross species barriers and spread among a range of hosts remain largely unresolved (2). Different strains of virus are composed of different combinations of Hemagglutinin and Neuraminidase. Some strains lead to

severe symptoms. The strain that has spread through Thailand and other countries in the past two years causes severe symptoms. Its scientific name is H5N1 (4).

From a diagnostic standpoint, influenza is often described as a “moving target” due to the high mutation rate of HA and NA (5). But it is known that antigenic analysis of influenza A/H5N1 viruses from 2002-2003 have also shown significant antigenic drift compared to viruses from 1997 to 2001. In addition, the evolution of the HA gene of A/H5N1 since 2004 has resulted in several different lineages, and according to phylogenetic analyses (6). As this particular virus continues to mutate, it is important to have a diagnostic tool capable of detecting as many of the A/H5N1 variants as possible for reliable global strain surveillance efforts. Preparedness in diagnostic laboratories means recognition of the particular agent in good time, but obtaining information on the characteristics of the detected pathogen is also of increasing importance. The latter might help to trace the source of the infection, predict its spread and the potential epidemiological consequences by identifying virulence markers, receptor binding motifs, drug resistance traits, reassorting variants, etc., and elaborate the strategies to control it. The data regarding the above considerations is most accurately generated by in-depth nucleotide sequence analysis of the pathogen of concern (2, 7-9).

A number of diagnostic methods are available for the detection and subtype of influenza viruses. The current “gold standard” for complete influenza A subtyping (determination of both HA and NA) involves virus replication in egg or tissue culture followed by a hemagglutination inhibition test. This method is tedious and requires several days, with the analysis time often extended to several weeks for antigenically novel viruses (3, 10-12). Although these methods are excellent for determining subtypes, they do not give detailed genetic information when antigenic shifts occur. RT-PCR techniques depend on specific primers, which may fail when corresponding viral sequences mutate. By overcoming this limitation, microarrays have become valuable tools for viral discovery, detection, and genotyping (5, 12-17).

Recently, the molecular diagnostic methods have also gained ground and traditional as well as real-time PCR methods targeting the conserved region of the matrix protein gene are widely used for the detection of influenza A viruses. In positive cases the subtype of the detected virus is determined with other PCRs

targeting the haemagglutinin and neuraminidase genes. In case of H5 or H7 subtypes it is followed by the pathotyping, i.e. the characterization of the nucleotide composition of the region flanking the HA cleavage site. If the detected virus turns out to be HPAI (that is subsequently confirmed by biological assays as well), strictly regulated measures must be applied as detailed in the respective National Contingency Plans. Although the above steps carried out in National Reference laboratories accomplish the compulsory laboratory investigations, thorough analysis of every isolate has become a routine in the more sophisticated laboratories in order to be better prepared for a possible outbreak. In the case of the highly pathogenic H5N1 avian influenza, full-length sequencing protocols are being used in the diagnostic laboratories. These protocols can quickly provide comprehensive data on a detected H5N1 avian influenza virus to animal and public health authorities facilitating their actions (18, 19).

A Semiconductor-based oligonucleotide microarray platform can simultaneously detect and characterize many H1-H16 and N1-N9 subtypes of influenza A viruses. Oligonucleotide microarrays do this by simultaneously interrogating hundreds to thousands of immobilized oligonucleotide probes, where each probe provides a single query for a known sequence that is unique for each H gene and N gene. The micro-array chip can be read fluorescently or using a simple electrochemical detection (direct reading of the chip through an electrochemical sensing process). RNA samples are prepared, labeled and hybridized with arrays. Arrays are scanned and images are produced and analysed to obtain an intensity value for each probe. These intensities represent how much hybridization occurred for each oligonucleotide probe (12, 20).

This semiconductor-based microarray chip detected and accurately typed flu strains with hemagglutinin subtypes 1-16 and neuraminidase subtypes 1-9 but do not provide strain sequence information. It's very difficult or ambiguous to tested genetic drift or shift in a flu virus within a chip. To simplify the assay procedure for running the influenza assays, we demonstrated the bioinformatic's ability to distinguish different strain of each isolates.

CHAPTER II

OBJECTIVES

1. To determine the performance of Semiconductor-Based Oligonucleotide Microarrays for detection and subtyping the Avian Influenza virus (H5N1) circulating in Thailand.
2. To describe the development of an algorithm to find potential oligonucleotide probe that would enable the nucleotide sequencing of different influenza A/H5N1 viruses on a microarray.
3. To monitor the antigenic differences of H5N1 during 2003-2006.

CHAPTER III

LITERATURE REVIEW

3.1 General Description of Avian Influenza

Influenza viruses hold generic status in the *Orthomyxoviridae* family and are classified into types A, B or C based on antigenic differences of their nucleo- and matrix proteins. Avian influenza viruses (AIV) belong to type A. Influenza A viruses are lipid-enveloped viruses containing a genome composed of eight strands of negative-sense RNA that encode ten viral proteins. These gene segments are encapsidated in a virally encoded nucleoprotein (NP), and the ribonucleoprotein (21) structures are associated with the three subunits of the viral polymerase (PB1, PB2, and PA). Virus particle formation occurs at the surface membrane of infected cells, where budding occurs from regions of the membrane at which the viral glycoproteins, hemagglutinin (HA) and neuraminidase (NA) have accumulated. The viral matrix protein (M1) is the most abundant component of the virion and is thought to play a pivotal role in the process of assembly and budding. Details on the mechanism of assembly are still forthcoming, but it is often assumed that M1 interacts with the RNPs and the cytoplasmic domains of HA, NA, and possibly the third integral membrane protein M2. The two other viral proteins, NS1 and NS2, were initially designated as nonstructural proteins, but there is now evidence for the presence of NS2 in virions (22, 23). Figure 1 is a schematic representation of Influenza A virion.

The main antigenic determinants of influenza A and B viruses are the haemagglutinin (H or HA) and the neuraminidase (N or NA) transmembrane glycoproteins, capable of eliciting subtype-specified and immune responses which are fully protective within, but only partially protective across, different subtypes. On the basis of the antigenicity of these glycoproteins, influenza A viruses currently cluster into sixteen H (H1 - H16) and nine N (N1 - N9) subtypes. These clusters are

substantiated when phylogenetically analysing the nucleotide and deduced amino acid sequences of the HA and NA genes, respectively

3.1.1 Description of the Gene Segments

Using the A/Puerto Rico/8/34 virus (PR8) as an example, the influenza A viral gene segments range in size from 890 to 2341 nucleotides and contain from 20 to 45 noncoding nucleotides at the 3' end and 23 to 61 nucleotides at the 5' end, depending on the segment. With the exception of position 4 at the 3' end of viral gene segments, which displays U/C heterogeneity, the 12 nucleotides at the 3' end and the 13 nucleotides at the 5' end are completely conserved for all segments in all strains of influenza A virus. These terminal RNA regions are partially complementary and viral promoter activity has been mapped to these domains, but with the exception of the polyadenylation signal, the functional significance of most of the noncoding sequences beyond the conserved domain remains unresolved. There is common speculation that these sequences are involved in binding of NP and/or polymerase complexes, or that they may contribute to packaging signals.

The three largest gene segments encode the subunits of the viral polymerase, PB2, PB1, and PA, which are so named because of their basic (PB2, PB1) or acidic (PA) properties on isoelectric focusing gels. These are responsible for transcribing messenger RNAs (mRNAs), for synthesizing positive-sense antigenomic template RNAs (cRNAs), and for transcribing the cRNAs into the gene segments (vRNAs) that are incorporated into progeny viruses. Segment 4 encodes the hemagglutinin glycoprotein, which is responsible for binding virus to sialic acid-containing cell-surface receptors and for membrane fusion during virus entry into host cells. It is also the principal target for neutralizing antibodies. The nucleoprotein is the product of the fifth gene segment. This is the protein that encapsidates cRNAs and vRNAs, which is necessary for them to be recognized as templates for the viral polymerase. Segment 6 encodes the neuraminidase, which cleaves sialic acid from virus and host cell glycoconjugates at the end of the virus life cycle to allow mature virions to be released. Segment 7 generates two gene products, the matrix protein, M1, and the M2

protein. M1 mRNA is a collinear transcript, and its product has a structural role in the virion and it is thought to play a fundamental role in virus assembly. The M2 is a small transmembrane protein derived from spliced mRNA. It has proton channel activity that aids in virus disassembly during the initial stages of infection. The eighth gene segment also encodes two proteins due to alternative splicing. These proteins were originally referred to as NS1 and NS2 because they were thought to be nonstructural, but NS2 has since been shown to be a component of virions. The NS1 has numerous functions. It is a regulator of both mRNA splicing and translation, and it also plays a critical role in the modulation of interferon responses to viral infection. The NS2 functions to mediate the export of newly synthesized RNPs from the nucleus and as such, it is also referred to as the nuclear export protein (NEP) (23).

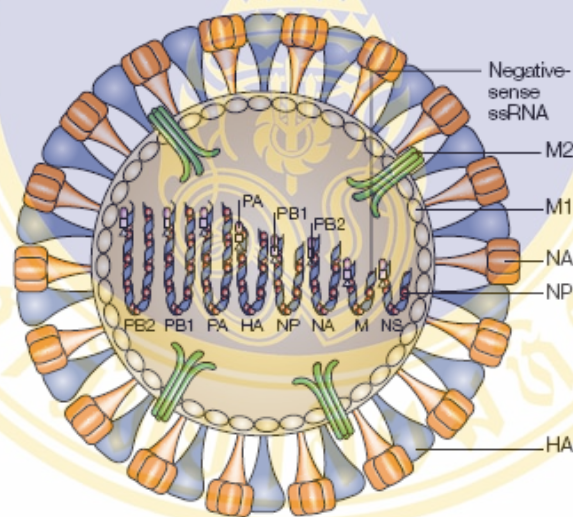


Figure 1 Schematic diagram of an influenza A virus virion. Two surface glycoproteins, haemagglutinin (HA) and neuraminidase (NA), and the M2 ion-channel protein are embedded in the viral envelope, which is derived from the host plasma membrane. The ribonucleoprotein complex comprises a viral RNA segment associated with the nucleoprotein (NP) and three polymerase proteins (PA, PB1 and PB2). The matrix (M1) protein is associated with both ribonucleoprotein and the viral envelope. A small amount of non-structural protein 2 is also present, but its location within the virion is unknown (24).

3.1.2 Antigenic Influenza A virus

The main antigenic determinants of influenza A and B viruses are the haemagglutinin and the neuraminidase transmembrane glycoproteins. Hemagglutinin (H) is a glycoprotein containing either 2 or 3 glycosylation sites, with a molecular weight of approximately 76,000. It spans the lipid membrane so that the major part, which contains at least 5 antigenic domains, is presented at the outer surface. HA serves as a receptor by binding to sialic acid (N-acetylneuraminic acid) and induces penetration of the interior of the virus particle by membrane fusion. Haemagglutinin is the main influenza virus antigen; the antigenic sites being A, B (carrying the receptor binding site), C, D, and E. The antigenic sites are presented at the head of the molecule, while the feet are embedded in the lipid layer. The body of the HA molecule contains the stalk region and the fusiogenic domain which is needed for membrane fusion when the virus infects a new cell. At low pH, the fusion peptide is turned to an interior position. The HA forms trimers and several trimers form a fusion pore.

Neuraminidase is a glycoprotein, which is also found as projections on the surface of the virus. It forms a tetrameric structure with an average molecular weight of 220,000. The NA molecule presents its main part at the outer surface of the cell, spans the lipid layer, and has a small cytoplasmic tail. NA acts as an enzyme, cleaving sialic acid from the HA molecule, from other NA molecules and from glycoproteins and glycolipids at the cell surface. It also serves as an important antigenic site, and in addition, seems to be necessary for the penetration of the virus through the mucin layer of the respiratory epithelium (23, 25, 26).

3.1.3 Influenza virus replication cycle

The influenza virus binds to the cell surface by fixing the outer top of the HA to the sialic acid of a cell's glycoproteins and glycolipids. The sialic acid linkage to the penultimate galactose, either alpha 2,3 (in birds) or alpha 2,6 (in humans), determines host specificity (27, 28). Since sialic acid-presenting carbohydrates are present on several cells of the organism, the binding capacity of the

HA explains why multiple cell types in an organism may be infected. After attachment, the virus is taken up by the cell via a clathrin-coated receptor mediated endocytosis process. When internalised, the clathrin molecules are liberated and the vesicle harbouring the whole virus fuses with endosomes. The contents of the vesicle are usually digested through a stepwise lowering of the pH within the phagosome. When a certain level is reached, the lowering of the pH is stopped by the action of the M2 protein which induces the partial liberation of the fusion peptide of the HA (23). This allows the fusion of the HA with the membrane of the vesicle and liberation of the ribonucleoproteins (RNPs) into the cytoplasm, as described above. The ion influx from the endosome to the virus particle leads to disconnection of the different viral proteins; M1-protein aggregation is disrupted and RNPs no longer adhere to the M1-protein complex. Uncoating is completed within 20-30 min of virus attachment. The RNPs are transported to the nucleus, where the polymerase complex binds to viral RNA, cleaves viral RNA by its endonuclease activity, and simultaneously leads to elongation. The production of viral RNA is limited by the NP in favour of mRNA. Both are transported to the cytoplasm, where viral proteins are generated at the ribosome. Part of the viral mRNA is spliced by cellular enzymes so that finally viral proteins, such as M1 and NS2, can be synthesized without any further cleavage. Some of the newly synthesized viral proteins are transported to the nucleus where they bind to viral RNA to form RNPs. Other newly synthesized viral proteins are processed in the endoplasmic reticulum and the Golgi apparatus where glycosylation occurs. These modified proteins are transported to the cell membrane where they stick in the lipid bilayer. When they reach a high enough concentration at the plasma membrane, RNPs and M1 proteins aggregate and condense to produce the viral particle. Finally, the particle is extruded from the membrane and will be liberated by the neuraminidase activity. The time from entry to production of new virus is on average 6 h (26, 29, 30).

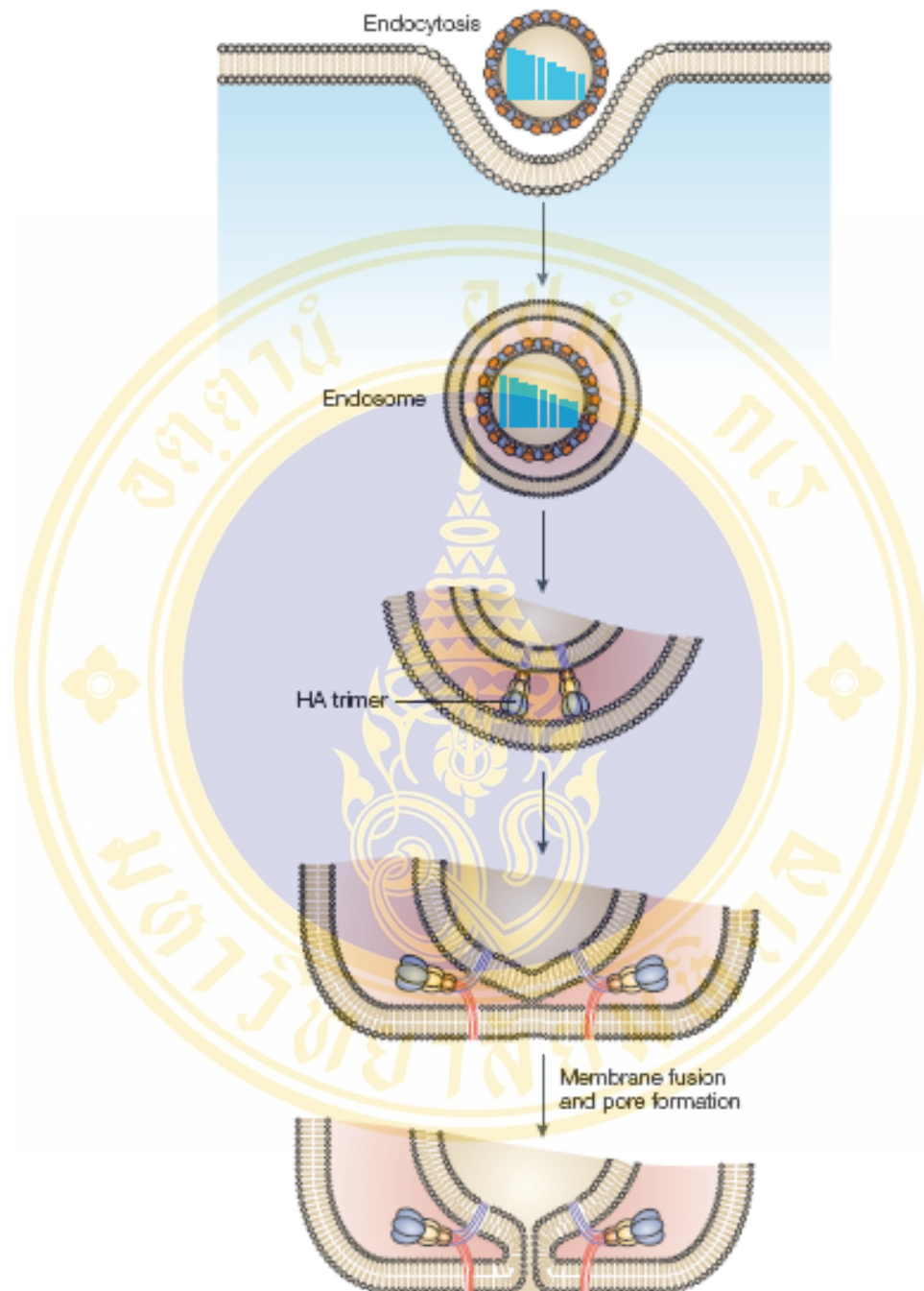


Figure 2 Schematic representation of mechanism of action of influenza A haemagglutinin (HA). HA mediates virus binding to sialic-acid-containing host-cell receptors. Following binding, the virus is internalized by endocytosis. Acidification of the endosome environment induces conformational changes in the HA trimer, mediating fusion between the viral envelope and the endosomal membrane, which allows delivery of the viral ribonucleoprotein into host cells (3).

3.1.4 Genetic variation

RNA viruses are extremely mutable and use very efficient strategies for generating viral diversity during evolution (Figure 3). RNA viruses have few or no proofreading mechanisms and many mutations are introduced during replication. As such, RNA viruses exist as a quasispecies comprising viruses of slightly different genetic composition. Thus, when selective conditions arise, such as the conditions present after jumping to a new host, variants with an advantage are quickly amplified. It can therefore take only limited replication under suboptimal conditions before an adapted virus emerges. Unfortunately, the evolution of viruses is not only confined to point mutations inserted during replication. Viruses can also undergo leaps in evolution through the processes of recombination and reassortment. These processes achieve population heterogeneity in viruses through the acquisition of large sections of genomic material from other viruses. Reassortment occurs in viruses with segmented genomes when sections of different viruses are exchanged on dual infection. Both the Asian (1957) and Hong Kong (1968) human influenza pandemics arose through reassortment of human and avian strains (31).

Global health authorities are concerned that the same may happen with the H5N1 viruses that are currently circulating in birds in southeastern Asia, with the result being a virus that is transmissible in humans. Unfortunately, we know little about the conditions for successful reassortment and it is difficult to assess the possibility of this happening. The available evidence from influenza A viruses is that there is a selective rather than random mechanism for packaging RNA segments into infectious virions³⁶ and not all combinations of viral genes are compatible. Recent findings from vaccine development have shown that the surface proteins of the H5N1 influenza viruses are at least compatible with older human strains, suggesting that reassortants of H5N1 and human influenza virus may well be viable. Arenaviruses are also capable of reassortment, although reassortment again does not seem to be completely random and some combinations of genes do not seem to be compatible (38). Nevertheless, there has been some speculation that reassortment may have contributed to the emergence of new arenaviruses with disease associations (32). Recombination leads to the same end as reassortment—that is, the acquisition of

foreign genetic material—but through a different route. Rather than through the acquisition of gene segments, recombination occurs through ‘splicing’ of the foreign component into the virus genome. Recombination is often observed in positive-sense RNA viruses such as picornaviruses and coronaviruses (33, 34) although it also occurs to a limited extent in negative-sense viruses such as influenza A (35). As with reassortment, recombination has the capacity to change the phenotype of a virus markedly and has the potential to aid in the transmission of viruses between species (36).

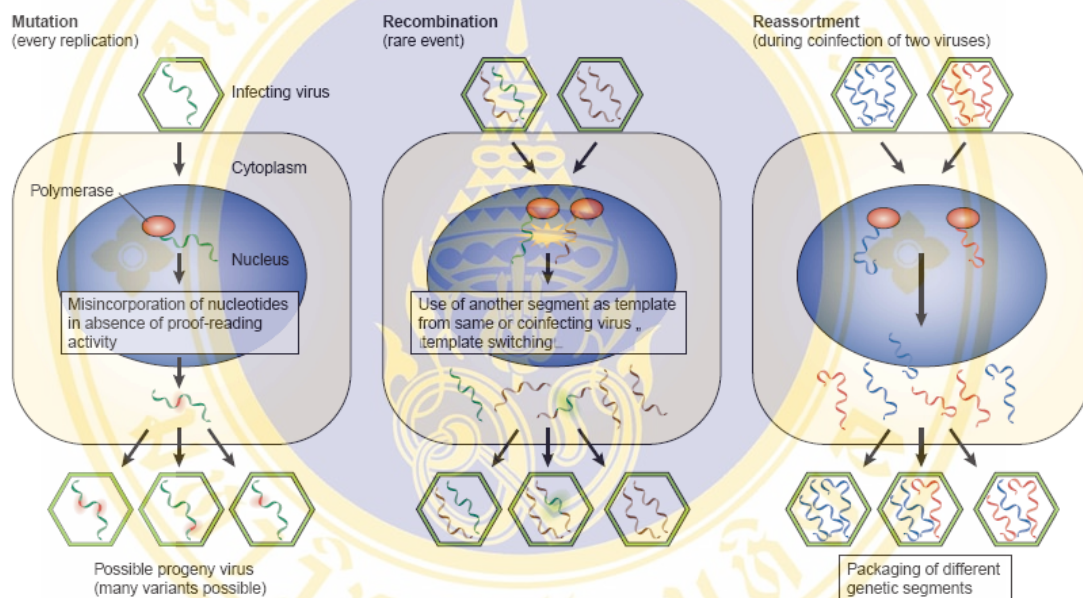


Figure 3 Molecular mechanisms for generating viral diversity (37)

3.2 History and origin of H5N1 viruses

Avian influenza viruses have now been identified as the source of novel hemagglutinin and neuraminidases (38) associated with pandemics. Two mechanisms can be responsible for the adaptation of avian viruses with novel HA and NA into viruses with the capacity to spread in human populations, genetic reassortment, or direct transmission. The segmented nature of the influenza virus genome allows for the exchange of genetic material between two subtypes of the virus coinfecting a host.

Such genetic reassortment can result in a virus with a genetic makeup different from either of the coinfecting subtypes. Sequence analysis has identified the viruses of both 1957 and 1968 pandemics as products of reassortment (39).

Because human influenza virus was first identified in 1933, it is harder to pinpoint the origin of the 1918 pandemic. However, the genetic material of the 1918 virus has been amplified and sequenced from formalin-fixed pathological specimens of the lung tissue (40). Although phylogenetic analysis of the HA from the 1918 virus places the virus firmly in the mammalian clade of the influenza viruses, the sequences of the NA, NP (nucleo protein), and polymerase genes are close to the avian consensus sequences and strongly suggest that the 1918 virus was a direct introduction into human populations of an avian virus, possibly with a period of adaptation before it emerged in pandemic form (41).

Sequence analysis of H5N1 and other avian viruses has suggested that the current H5N1 virus originated by reassortment between multiple cocirculating avian influenza strains prevalent in Hong Kong in 1997. In addition, certain unique structural features like a 19 amino acid sequence deletion in the stalk of the NA and an additional glycosylation site on the head of the HA were found in the 1997 isolate of the H5N1 virus. Although these features are characteristic of highly pathogenic viruses infecting chicken, they are not common to the avian influenza viruses circulating among wild birds (42).

3.3 Avian Influenza pathogenicity

Avian influenza viruses can be sorted on the basis of virulence: highly pathogenic avian influenza viruses cause systemic lethal infection, killing birds as soon as 24 hours post-infection, and usually within one week, these viruses have been restricted to subtypes H5 and H7, although not all viruses of these subtype cause HPAI, (43) whereas low-pathogenic avian influenza viruses rarely generate outbreaks of severe disease in the field, and their associated morbidity and mortality rates are lower than those of HPAI viruses (44). Phylogenetic studies of avian influenza viruses have revealed two geographically separate sublineages (Eurasian and American),

which probably reflects the separation of viruses owing to the distinct migratory patterns of the host birds in these two regions. Both HPAI and LPAI viruses are found within these two sublineages, indicating that viral pathogenicity is not determined by geographical distribution. Without exception, all of the HPAI viruses belong to the H5 or H7 subtype, for reasons that are still unclear. There do not seem to be any associations of specific NA subtypes with HPAI viruses (26). Sometimes other infections or environmental conditions may cause exacerbation of influenza infections leading to much more serious disease.

Virulence of influenza viruses is a polygenic trait and has been attributed to several viral genes; however, the HA protein is a major virulence factor in poultry (45). It has been demonstrated that the HA0 precursor of the main functional HA glycoprotein requires cleavage by host proteases before virus particles are infectious. HA0 proteins of avian influenza viruses of low virulence for poultry are limited to cleavage by host proteases such as trypsin and trypsin-like enzymes and thus restricted to replication at sites in the host where such enzymes are found, i.e. the respiratory and intestinal tracts. In contrast virulent viruses appear to be cleavable by a ubiquitous protease(s), which remains to be fully identified but appears to be one or more proprotein-processing subtilisin-related endoproteases of which furin is the leading candidate and this enables these viruses to replicate throughout the animal, damaging vital organs and tissues which brings about disease and death in the infected bird (43, 46, 47).

Comparisons of the amino acid sequences at the HA0 cleavage site of avian influenza viruses of high and low pathogenicity revealed that while viruses of low virulence have only two basic amino acids, at positions $\alpha 1$ and $\alpha 4$ from the cleavage site for H5 and at positions $\alpha 1$ and $\alpha 3$ for H7 subtype, all HPAI viruses possessed multiple basic amino acids (arginine and lysine) adjacent to the cleavage site either as a result of apparent insertion or apparent substitution. The additional basic amino acids resulting in a motif recognised and cleavable by the putative ubiquitous protease(s) (48-50). Mammals, including humans, also have furinlike proteases capable of cleaving at multiple basic amino acid motifs. However, the question as to whether or not viruses with multiple basic amino acids at the H0 cleavage site could cause systemic infections and highly pathogenic disease in humans remained unanswered up to 1997,

because the viruses known to have infected humans (H1, including the 1918 pandemic virus, H2 and H3 subtypes) all have motifs at the cleavage site indicating they would be cleaved only by trypsin like enzymes (43).

3.4 Human infection with influenza A (H5N1) viruses and Clinical Feature

In aquatic birds, the natural hosts of influenza viruses, infection is usually asymptomatic and localized to the intestinal tract. H5N1 viruses have been actively reassorting and crossing interspecies-host barriers, moving from aquatic poultry to land-based poultry and, more recently, to wild terrestrial birds and humans (43, 51, 52) Avian and human viruses preferentially bind sialic acid molecules with specific oligosaccharide side chains with α -2,3 and α -2,6 linkages, respectively. Receptor specificity was thought to be an important determinant of viral host range, as demonstrated by the fact that reports of experimental human infections with avian influenza viruses were rare (53).

Also, recent studies with differentiated human airway epithelial cells have demonstrated that cells bearing α -2,3 and α -2,6 linked sialic acids are present in human respiratory epithelium (54). Pigs are susceptible to both avian and human influenza viruses, due to the presence of sialic acid molecules with both α 2,3 and α 2,6 linkages to galactose on their tracheal epithelium, and could potentially serve as mixing vessels for the reassortment of avian and human viruses (55).

H5 viruses can also be associated with high cleavability and lethality in poultry and have caused many severe outbreaks of disease in poultry. The first known transmissions of H5N1 viruses from birds to humans took place in April–May 1997,(56) shortly after the recognition of an outbreak in sick geese on a farm in Guangdong Province of China (57). Human cases have typically followed outbreaks in poultry. Concerns about H5N1 infection becoming a global threat were raised when H5N1 infection was detected in migratory waterfowl in Qinghai Lake in western China in April 2005 (58). The virus has since spread from its original geographic focus in Southeast Asia along the flyways of migratory birds to more than 30 countries

worldwide, including Kazakhstan, Russia, parts of Europe, the Middle East, and Africa. As of August 2006, there have been a total of 238 human cases with 139 deaths (58%) with additions to the list of countries reporting confirmed human cases like Turkey, Iraq, Azerbaijan, Egypt, and Djibouti (59). In many of these countries, close human contact with poultry is common due to practices such as maintenance of backyard flocks of birds, live animal markets, and a thriving poultry industry. This combination of these factors has resulted in continued opportunities for human transmission, increasing the likelihood of further human adaptation and transmission between humans Figure 4.

To date, person-to-person transmission has been rare. Fifteen family clusters of infection involving 2 family members have been documented between January 2004 and July 2005. The largest cluster identified thus far has been in the village of Kubu Sembelang in northern Sumatra in Indonesia in May 2006 where seven confirmed cases occurred in extended family members of a 37-year-old woman who died of an acute respiratory illness and was buried prior to the establishment of a diagnosis. Sequence comparisons of viruses from human cases and those of concurrent avian cases have established that at least some of the cases in the cluster represented person-to-person transmission. In addition, there is one well-documented transmission of virus from an ill child in Thailand to her mother and a suggestive report of transmission to a health care worker (39, 52).

The available data regarding the signs and symptoms of H5N1 infection are mostly from hospitalized patients (39). The frequency of subclinical and mild forms of infection is currently unknown. Most patients present with the nonspecific complaints of a fever 38°C along with cough and shortness of breath. In many of the patients, there is a progression of symptoms leading to respiratory failure requiring ventilation and other supportive measures. Atypical symptoms like nausea, vomiting, encephalopathy, and bleeding gums and nose have been reported. Watery diarrhea may be present prior to the onset of respiratory symptoms. The majority of patients have an abnormal chest x-ray with diffuse and multifocal or patchy infiltration, but pleural effusions are rare. Laboratory abnormalities include significant lymphopenia and leucopenia, mild to moderate thrombocytopenia, and elevated transaminases. These abnormalities are poor prognostic signs (60-62). Most people have negative

sputum and blood bacteriological cultures. Pathological changes show diffuse alveolar damage in the lungs, reactive hemophagocytosis in the marrow, and lymphoid depletion with atypical lymphocytosis in the spleen and lymphoid tissues. Centrilobular hepatic necrosis and acute tubular necrosis have been reported (26, 63, 64).

3.5 Evolution of H5N1 viruses

A key concept in influenza virus evolution is that there is a marked difference in evolutionary dynamics between those viruses that infect aquatic birds and those from other host species. The evolution of H5N1 viruses in recent years has been associated with increasing virulence and an expanding host range, which beside terrestrial poultry and wild birds. Also includes mammals. While all H5N1 viruses isolated from ducks in China between 1999 and 2002 were highly pathogenic in chickens, an increasing level of pathogenicity was observed in mice with the progression of time: virus isolated in 1999 and 2000 were less pathogenic than those isolated in 2001 and 2002 (65). It has been suggested that the increasing ability to replicate in mammals has resulted from transmission between ducks and pigs as Figure 4. The expanding host range is also illustrated by successful experimental infection of domestic cats, and natural infections of tigers and leopards with recent H5N1 strains (21, 66, 67).

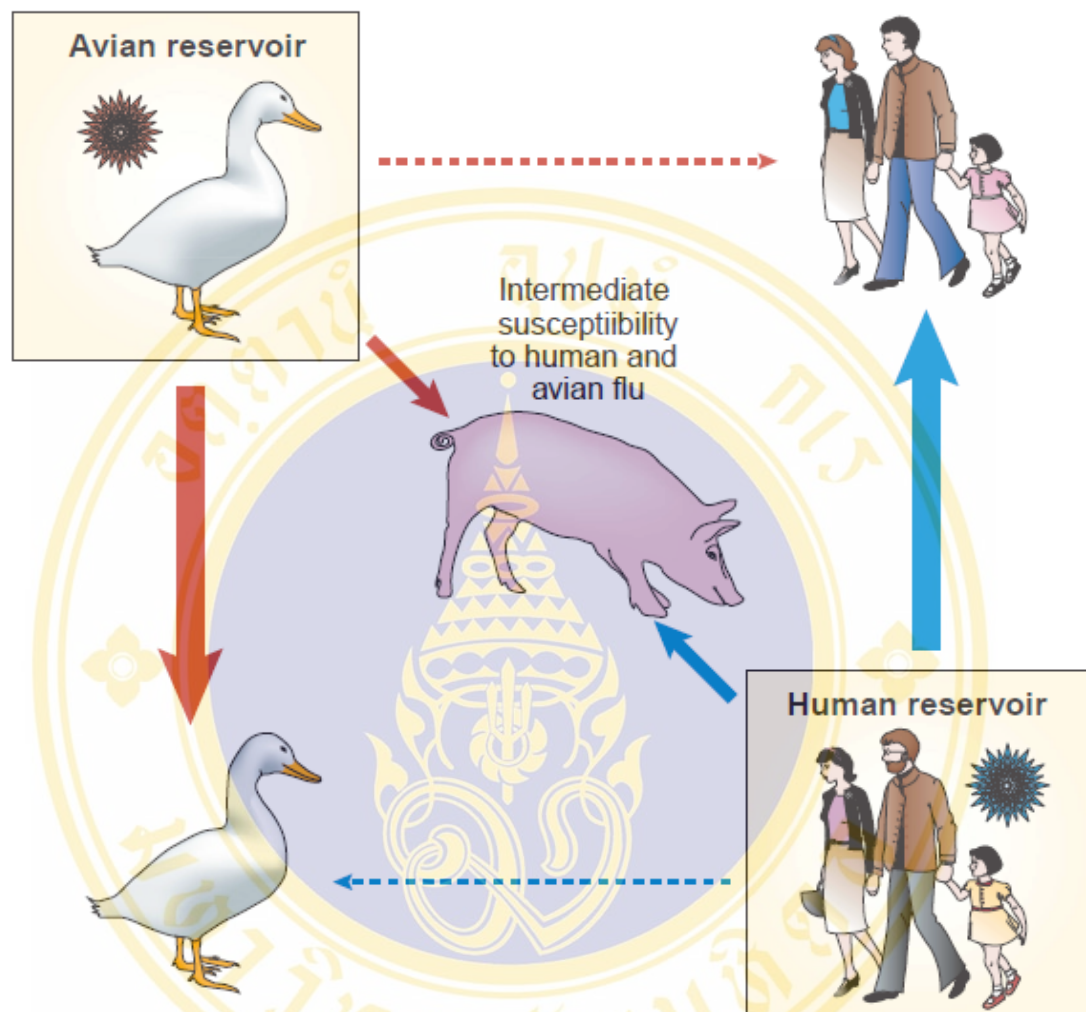


Figure 4 Possible cross-species transmission of H5N1 virus (37)

3.6 Avian influenza viruses and pandemic influenza

Evidence of bird flu spreading to humans caused enormous concern among scientists and among national and international health organizations. The reason for the concern was the fear that the virus might one day evolve into a form that spreads easily from human to human. If that were to happen, it would be difficult to avoid a pandemic causing hundreds of millions of illnesses, and millions of deaths. Pandemics of this magnitude have occurred six or seven times in the past two centuries. Avian species constitute the origin of the human H5N1 avian influenza

virus. The virus has not yet manifested effective human-to-human transmission, but the situation may change if the virus continues to mutate and assort during an epidemic (43, 68).

The viruses that cause bird flu and human flu store their genetic code in RNA. These viruses frequently mutate; in other words, changes occur in their genetic code. The mutations often alter the characteristics of the viruses. The immune systems of individuals infected by earlier strains of the virus do not necessarily recognize the new strains. Thus the same individuals can become infected for a second time. Mutations in the influenza virus, including avian and human influenza, can occur either gradually or quickly. Either type of mutation can give rise to new strains of the virus. These new strains may increase or decrease the severity of the symptoms. The effects are impossible to predict (22, 69, 70).

3.7 Characteristic of H5N1 isolated in Thailand review prevalence and incidence in Thailand

The 2005 H5N1 viruses circulating in Thailand were genetically comparable with 2004 H5N1 isolates in Thailand and Vietnam, but distinct from the H5N1 virus strains circulating in Asia such as Hong Kong, China, Indonesia (1996–2005) and Europe (2005) as well as in Africa (2006). The 2005 H5N1 viruses harbor multiple insertions of basic amino acids at the HA cleavage site, which by definition is characteristic for highly pathogenic avian influenza (HPAI) (71-73). Interestingly, the HA cleavage site of 2005 H5N1 viruses in this study contained one basic amino acid “SPQREKRRKKR” differing from that of 2004 H5N1 viruses in Thailand, Indonesia, Vietnam and Eastern China “SPQRERRRKKR” (arginine (R) to lysine (K)). It is noted that HA cleavage site of the 2005 H5N1 viruses from Vietnam (mallard/347/05) and Indonesia (duck/Parepare/BBVM/05) remain unchanged “SPQRERRRKK” (57, 72). On the other hand, the HA cleavage site variability have been previously observed in some wild bird species during earlier outbreaks in Thailand in 2004 (open-bill/CU the significance of genetic characterization presenting as examples of 4 H5N1 viruses isolated from human, chickens and quail which isolated from Thailand in 2005. Sequence analysis of eight gene segments revealed that the 2005 H5N1 viruses

isolated in October 2005 were related to those recovered from chicken, tiger(s) and human(s) in January and July 2004 but not related to the viruses from the recent outbreaks in Western China, Eurasia and Africa. Interestingly, amino acid substitution especially at the HA cleavage site has been observed. However, the amino acid substitution still basic protein which can cause highly pathogenic to host. It shows that H5N1 viruses have continued to evolve since early 2004–2005 of Thailand with minor change, alternatively unchanged in pathogenicity (68). The Thailand viruses contained more avian-specific residues than the 1997 Hong Kong H5N1 viruses, suggesting that the virus may have adapted to allow a more efficient spread in avian species (72). It is still not certain how bird flu first came to Thailand. However, current evidence points towards migratory water birds such as ducks and geese. Experts believe that the virus H5N1 must have been evolving among bird populations for many years, to the point where it can infect birds without causing disease. Migratory birds are therefore a natural reservoir of disease, which they pass to other species along their migration routes (22, 42).

3.8 Laboratory diagnosis of influenza A/H5N1

Influenza A/H5N1 is HPAIV and cause disastrous epidemic disease in poultry (74, 75). Identification of a virus subtype and molecular identification of the subtype of the viral hemagglutinin and neuraminidase genes can help create a global surveillance program to monitor outbreaks. The goal of surveillance is to gather information on the influenza virus subtypes that are circulating in human and animal populations so that recommendations can be made on the content of vaccines for the next season. Surveillance systems of WHO Animal Influenza Network for detection of influenza viruses procedures used include detection of virus by direct virus direct virus isolation, by detection of specific genes or gene products by amplification procedures of polymerase chain reaction (PCR), rapid diagnostic kits or by detection of serological response (76). There are two methodologies for Influenza A/H5N1 monitoring: Subtype determination (“subtyping”) and nucleotide sequencing.

Subtype determination is essential for tracking emerging viruses and for designing appropriate influenza vaccines. The current “gold standard” for complete influenza A subtyping (determination of both HA and NA) involves virus replication in egg or tissue culture followed by a hemagglutinin inhibition assay. This method is tedious and requires several days, with the analysis time often extended to several weeks for antigenically novel viruses (76).

Influenza diagnostic method based on reverse transcription polymerase chain reaction (RT-PCR) and real-time RT-PCR (RRT-PCR) are currently available for typing or as presumptive tests for a specific subtype (77-81). RRT-PCR-based typing of influenza uses a single primer pair that targets a short, well conserved portion of the M gene segment (7, 76, 82-84). RRT-PCR-based presumptive tests for a specific subtype use a single primer pair that targets a portion of the HA gene that is well-conserved only for a particular subtype. However, a major limitation of these singleplex PCR-based presumptive tests in terms of influenza surveillance is that they do not provide complete subtype information and are limited to a specific subtype. Clearly, the speed of this method for identifying specific A/H5 viruses could be extremely useful during an influenza pandemic. In addition, the inclusion of internal control in RT-PCR assays is highly desirable to monitor for false-negative results due to inefficient nucleic acid extraction, cDNA synthesis, or amplification (2, 11).

Nucleotide sequencing assays use further analysis of PCR-positive samples by automated sequencing. The PCR products were extracted and examined by agarose gel electrophoresis and further enzymatic reactions. The DNA sequencing reaction was performed, using a commercially available kit and automate (85, 86). The end of this assay is use specific software that facilitates the process of sequence alignment. However, a positive amplification can be verified only by subsequent assays to elaborate sequence information.

According to global surveillance, thus rapid and specific identification of influenza A/H5N1 subtype and accurate sequence information are crucial for proper treatment. But traditional methods are problematic in scenarios where new strains of virus emerge or a mixture of viruses exist. Because of their sensitivity, specificity, and accuracy, DNA microarrays have become an acceptable technology for screening

samples for the presence or absence of a large variety of viruses simultaneously and identifying the genotype of and unknown specimen (10, 20, 83, 87-89).

3.9 Microarray Technology

A microarray is a series of nucleic acid targets immobilized on a solid substrate, such as a silicon wafer or glass slide, on which DNA or oligonucleotides from either host or pathogen are attached. These nucleic acids are complementary to thousands of genes of both known and unknown function. Hybridisation of fluorescently labeled probes made from nucleic acids in the test sample to these targets allows analysis of the relative concentrations of mRNA or DNA in the sample. There are two main types of microarray: spotted DNA and oligonucleotide arrays (14, 20, 89-91).

3.9.1 CombiMatrix Oligonucleotide microarray

CombiMatrix microarrays are based on a specially modified semiconductor adapted for biological applications, which contains arrays of platinum microelectrodes. The semiconductor logic circuitry directs digitally controlled simultaneous synthesis of different oligonucleotides at thousands of electrodes in response to a computer software program (20).

The ElectraSense™ 12 K microarray consists of 12,544 individually addressable electrodes linked by the semiconductor circuitry. The contact pogo pads on the array side provide the electrical contact with an external device to control synthesis or ElectraSense™ detection. Each working electrode is made of high purity platinum with a circular geometry (44 µm diameter), and coated with a bio-membrane, a porous reaction layer. The working electrodes are surrounded with platinum grid that is used as a counter electrode. Voltage between the working and counter electrode is set at 0 V. A principle of action of ElectraSense™ platform of DNA/RNA hybridization assays is depicted in Figure 5. An oligonucleotide probe on an electrode specifically captures a biotin labeled target molecule from the hybridization solution. This bound target is subsequently labeled with HRP using biotin-avidin

chemistry. HRP then catalyses the oxidation of tetramethylbenzidine (TMB). Due to the close proximity of HRP to the electrode surface, the oxidized TMB is easily reduced by application of a reducing potential. The generated current flux enables to quantify the HRP label and, therefore, to assay the labeled bio-components bound to the microarray (12, 17, 20).

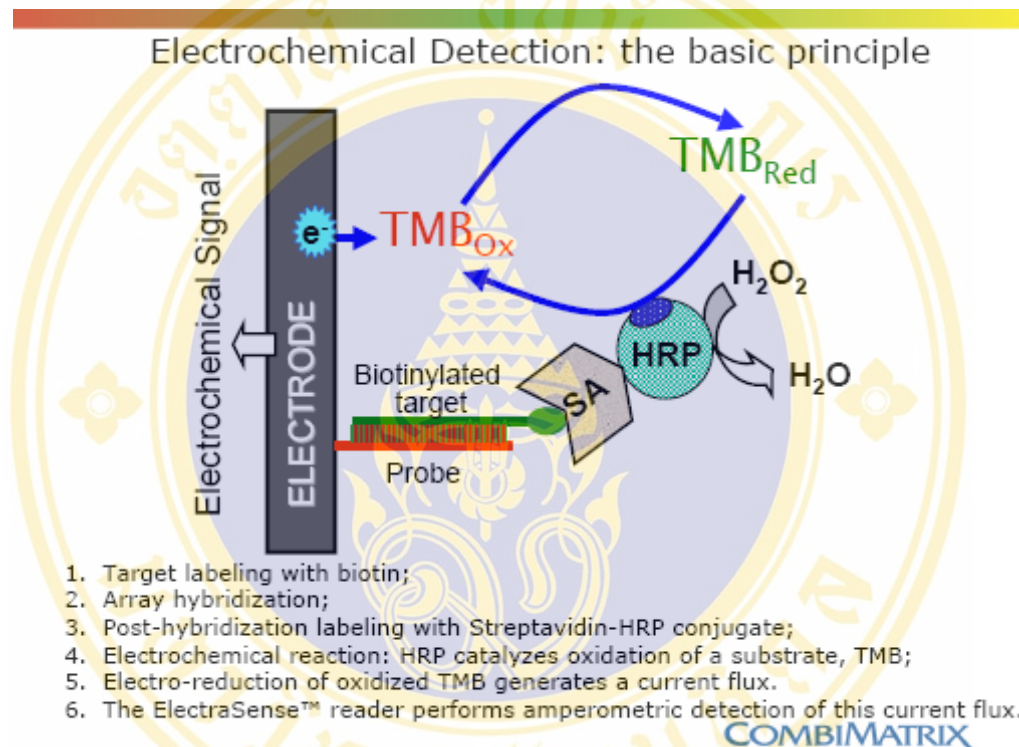


Figure 5 A general scheme of electrochemical detection for CombiMatrix ElectraSense™ microarray (20)

3.10 Bioinformatic Tool

3.10.1 Basic Local Alignment Search Tool (BLAST)

BLAST takes a query sequence and aligns it with each sequence in the database, looking for segments of high degrees of similarities. It picks out from the database those sequences that contain a segment so similar to part or the entire query that such similarity is deemed statistically significant (i.e., unlikely to occur by

chance). A BLAST search is often the most convenient method for detecting homology of a biological sequence to existing characterized sequences. BLAST looks for homology by searching for locally aligned regions of identity and/or similarity between a query sequence and sequences in a database (92).

3.10.2 Genotype nomenclature

The genotype of an influenza A viral strain is the sequential aggregate of the eight assigned gene segment lineages. A nomenclature for influenza A viral genotypes will allow researchers to unequivocally describe influenza A viral genotypes to analyze, compare and communicate the molecular epidemiology of the virus. Define a nomenclature for influenza A viral genotypes and describe a web tool developed for genotyping influenza A viruses from genome sequences. This tool facilitates identification of reassortment events between divergent lineages.

Two nomenclature conventions are used routinely in influenza research: (i) the eight segments in the influenza A genome are numbered from 1 to 8 for PB2, PB1, PA, HA, NP, NA, M and NS, respectively; (ii) There are currently 16 alleles of the HA gene termed subtypes. Likewise, there are nine alleles for NA, and two alleles for non-structural (NS) proteins. Since influenza A viruses have an unusual genomic structure, we approached the genotyping problem by first analyzing each gene segment separately. According to the above, conventions and considering that the evolutionary rate varies from segment to segment here defined a genotype as a sequential combination of the lineages for each of the eight segments in a genome. A letter was assigned to each lineage of PB2, PB1, PA, NP and M, and a number followed by a letter was assigned to each lineage of HA, NA and NS with the number representing the subtype or allele. For example, [A, D, B, 3A, A, 2A, B, 1A] is the genotype of a human seasonal subtype H3N2 virus with PB2 lineage A, PB1 lineage D, PA lineage B, HA subtype 3, lineage A and so on, following the convention for numbering of influenza genome segments. With this nomenclature, identifying genotypes and reassortment becomes an easy task accomplished by comparing the predicted genotype against all genomes that have been classified previously (92, 93).

3.10.3 FLAN (short for FLu ANnotation), the NCBI

Web server for genome annotation of influenza virus (<http://www.ncbi.nlm.nih.gov/genomes/FLU/Database/annotation.cgi>) is a tool for user-provided influenza A virus or influenza B virus sequences. It can validate and predict protein sequences encoded by an input flu sequence. The input sequence is BLASTed against a database containing influenza sequences to determine the virus type (A or B), segment (1 through 8) and subtype for the hemagglutinin and neuraminidase segments of influenza A virus. For each segment/subtype of the viruses, a set of sample protein sequences is maintained. The input sequence is then aligned against the corresponding protein set with a 'Protein to nucleotide alignment tool' (ProSplign). The translated product from the best alignment to the sample protein sequence is used as the predicted protein encoded by the input sequence. The output can be a feature table that can be used for sequence submission to GenBank (by Sequin or tbl2asn), a GenBank flat file, or the predicted protein sequences in FASTA format (94).

3.10.4 The Flu Genome database

It contains three tables: Segment, Genome and Genotype. The Segment table contains information-related to sequences, including assigned lineage, strain name, segment, serotype, host, country, year, GenBank accession number, nucleotide sequence and sequence length. The Genome table contains the information for complete genomes, including assigned genotype and accession numbers of each gene segment. When more than one sequence was available for a gene segment, the longer of the two sequences was kept for the genome accession. Unique genotypes are stored in the Genotype table along with the total number of genomes that have that genotype. The Genotype table was created by querying the Genome table for distinct genotypes. Host categories were created to separate the genomes of each genotype, which include Human (Hu), Avian (Av), Swine (61), Equine (Eq), Canine (Ca) and Others (ONHM). The FluGenome database is updated automatically every night. New sequences are downloaded from the NCBI Influenza Virus Resource

(<ftp://ftp.ncbi.nih.gov/genomes/INFLUENZA/>) and added into the FluGenome database. The lineage information predicted for new sequences is used to update Segment, Genome and Genotype tables if necessary. For sequences already in the database, the script checks to see what information needs to be updated, and the sequences entries are flagged for further validation (93, 95).



CHAPTER IV

MATERIALS AND METHODS

4.1 Study design

The 8 H5N1-RNA samples were collected and extracted from birds and mammals samples, which were used to analyze H5N1 genotypic testing with semiconductor-base oligonucleotide microarray. The results were used to evaluate tendency for using the Bioedit.

Avian influenza A subtype H5N1 was analyzed by using the CombiMatrix Influenza A Sub-typing Primer Kit. This test kit is based on reverse transcription and PCR Technology used to target preparation for hybridization with the Influenza A research Microarray. There are 3 major processes as followed: for each sample, the two amplified biotin-labeled targets are combined and hybridized to the same Influenza A Research Microarray 12K and finally fluorescent detection of the oligonucleotide probe-bound amplified DNA by electrochemical detection.

4.2 Influenza A/H5N1 samples

H5N1 samples were originally received from the Faculty of Veterinary and Faculty of Science, Mahidol University, Nakorn Prathom, Thailand, Department of Pathology, Faculty of Veterinary Medicine, Kasetsart University, Nakorn Prathom, Thailand, and Department of Livestock Development, National Institute of Animal Health, Bangkok, Thailand.

4.3 Statistic Analysis

The quantitative detection of influenza viruses used different method of oligoneucleotide signal intensity. The tests to identify H5N1 intensity were measured by the mean and the median. The best cut off intensity for each test was assessed by statistical optimisation of sensitivity; specificity and the kappa value which available procedures from STATA[®].

4.4 The CombiMatrix Microarray

CombiMatrix microarrays are based on silicon chip technology-the integrated circuits contain arrays of microelectrodes that are individually addressable using embedded logic circuitry on the chip. Placed in a specially designed fluidic chamber, the chip directs the synthesis of DNA in response to a digital command.

During synthesis each microelectrode is addressed to selectively generate chemical reagents by means of an electrochemical reaction. The electrochemical reaction is used to generate acid during the detritylation step of standard phosphoramidite synthesis. DNA is synthesized above a platinum electrode, within a boundary layer that coats the chip. Under the direction of a PC the CustomArray chip rapidly synthesizes 12544 different DNA molecules in parallel, each above a distinct electrode. Since a different product can be synthesized at each site, the technology eliminates the need to synthesize 12544 different oligos by conventional methods. This means arrays can be made inexpensively to any sorts of organism (e.g. flu) and bioinformatics approaches can be tested and optimized easily without having to purchase expensive oligo sets.

A generic PCR based amplification (designed to be pan-influenza strain specific) followed by specific hybridization on a microarray designed to precisely type influenza. Probes on the chip are designed to differentiate viral strain type based on the HA (types 1-16) and NA (types 1-9) serotype classifications of influenza strains. Chip designed against 1614 animal and 1937 human hemagglutinin sequences, and

against isolates 522 human and 831 animal neuraminidase sequences. Chip is laid out in a pattern to aid visual identification of HA and NA type. Chip can be read fluorescently or using a simple electrochemical detection (direct reading of the chip through an electrochemical sensing process)

4.5 Influenza A Nucleotide Sequence database

There are three groups of database for bioinformatic analysis

1. Data of Hemagglutinin and Neuraminidase genes influenza A viruses nucleotide sequences were retrieved from the NCBI web server for genome annotation of influenza virus (<http://www.ncbi.nlm.nih.gov/genomes/FLU/Database/annotation.cgi>). This web server is a tool for user-provided influenza A virus or influenza B virus sequences. Total cases of influenza A virus are HA gene 6,463 sequences and NA gene 5,403 sequences. The database contains all of subtypes of the hemagglutinin and neuraminidase nucleotide sequences.
2. 168 Nucleotide probe sequences of H5 and 168 Nucleotide probe sequences of N1 from CombiMetrix array.
3. Sample sequences.

4.6 Nucleotide sequence analysis with Bioedit version 7.0.0

Program Bioedit version 7.0.0 was used for nucleotide sequence analysis including sequence comparison and local BLAST with the ability to compile local databases from Fasta files. BioEdit is a biological sequence editor that runs in Windows 95/98/NT/2000/XP and is intended to provide basic functions for protein and nucleic sequence editing, alignment, manipulation and analysis. They offer many quick and easy functions for sequence editing, annotation and manipulation, as well as a few links to external sequence analysis programs. The Bioedit is offered free of charge.

Table 1 Samples for this study

| Sample no. | Virus name |
|------------|---|
| 11 | Influenza A (A/Tiger/Thailand (Supanburi)/vsmu-1/2004 (H5N1) |
| 17 | Influenza A (A/Tiger/Thailand (Chonburi)/vsmu-23/2004 (H5N1) |
| 25 | Influenza A (A/ Brown-headed Gull /Thailand (Samutprakarn)/ vsmu-28/2005 (H5N1) |
| 27 | Influenza A (A/Open-bill strok/Thailand (Nakornsawan)/vsmu-30/2005(H5N1) |
| 31 | Influenza A (A/duck/Thailand(Bangkok)/2003 (H5N1) |
| 37 | Influenza A (A/duck/Thailand(Bangkok)/2003 (H5N1) |
| 47 | Influenza A/duck/Thailand (Bangkok)/2006(H5N1) |
| 48 | Influenza A/Chicken/Thailand (Bangkok)/2006(H5N1) |
| H1N1 | Isolate from culture |
| H4N8 | Isolate from culture |
| H5N1 | Isolate from culture |

4.7 Method

4.7.1 The Low Resolution (genotyping)

4.7.1.1 Single step preparation

For target preparation, total RNA isolated from virus-containing material is reverse-transcribed into cDNA, which is then PCR-amplified to produce biotinylated single-strand DNA. Since the influenza-specific reverse primers are grouped into two different pools (to increase sensitivity of the multiplex reactions), each sample is amplified in two tubes, one for HA subtypes 1 to 7, and another one for HA subtypes 8 to 16, and NA subtypes 1 to 9. Amplification is performed as a single-step procedure including reverse transcription and PCR. The reverse transcription conditions were 10-min at 40°C, 20-min at 50°C and 3-min at 94°C. 1st PCR thermal cycling condition were 30 cycles of a 30-s 94°C denaturation step, a 45-s 50°C annealing step and a 30-s 72°C extension. Then 2nd PCR consisted of 40 cycles of a 30-s 94°C denaturation step, a 60-s 65°C annealing and extension, and finally, a 5-min extension at 72°C and 4°C after cycling was performed. The presence of PCR

products was confirmed on 4% Nusieve Seakem 3:1 agarose gels (FMC, Roeland) in 0.5XTBE (0.089 M Tris-borate, 0.089 M Boric acid and 0.002 MEDTA, pH 8), and stained with 0.5 µg/ml ethidium bromide. Gels were run at 100 V for 45 min. PCR yields were estimated by comparison with a low DNA mass ladder (Promega, USA).

Table 2 Two reaction mixtures for single step RT-PCR

| Reagent | H1-7 (50 µl) | H8-16+N1-9 (50 µl) |
|--|--------------|--------------------|
| Nuclease-free water | 13.5 µl | 13.5 µl |
| Template (total RNA) | 2.5 µl | 2.5 µl |
| 2X Reaction Mix | 25 µl | 25 µl |
| 0.4 mM Biotin-14-dCTP | 5.0 µl | 5.0 µl |
| Influenza Forward primer (Tube 1) | 1 µl | 1 µl |
| Influenza Reverse Primer Pool (Tubes 2 and 3) | H1-7: 2 µl | H8-16+N1-9: 2 µl |
| RT/ Platinum® Taq Enzyme Mix | 1 µl | 1 µl |
| Total Volume | 50 µl | 50 µl |

4.7.1.2 Pre-hybridization

The single-stranded target was heated to 95°C for 5 min and place on ice. The chip microarray was incubated in the hybridization chamber with nuclease-free water at 65°C for 10 min. Remove the microarray from the incubator and bring to room temperature. Fill the hybridization chamber with the Pre-hybridization Solution (50X Denhardt's solution, 1%SDS, 6X SSPE, 0.05% Tween-20, 20mM EDTA and nuclease free water) and loaded onto the rotisserie in the hybridization oven and incubate at 45°C for 15 min with gentle rotation.

4.7.1.3 Hybridization

For each sample, mix together 10 µl aliquots of the purified target samples (H1-7 and H8-16+N1-9). Denature the HA-NA target mixture by incubating at 95°C for 5 min and place on ice. Each of samples were diluted in hybridization solution (2X Hyb Solution Stock, 1% SDS and Positive Hybridization

Control from the CombiMatrix Influenza A Sub-typing Primer Kit) to a final volume of 50 μ l and load the hybridization chamber onto the rotisserie in the hybridization oven and follow by hybridization of biotinylated target in pre-block solution for 1 hour at 45°C with gentle rotation.

4.7.1.4. Hybridization Washing

The array were then washed once for 5 min with 6X SSPET (6X SSPE, 0.05% Tween-20) at 45°C and then 30 s each with 3X SSPET, 0.5X SSPET, PBST and PBS at room temperature.

4.7.1.5 Post-hybridization Blocking, Labeling, and Washing

The hybridized array was then blocked with 5X PBS-Casein Blocking Buffer for 15 min at room temperature and labeled for 30 min with the Fluorolink™ Cy5®-labeled streptavidin diluted 1:1000 dilution (v/v, 1 μ l per 1 ml) in the 5X PBS-Casein Blocking Buffer and incubate the microarray at room temperature for 30 minutes. Protect the microarray from light by covering with aluminum foil. Remove the Dye Labeling Solution from the hybridization chamber. The arrays were washed twice with 2X PBST and twice with 2X PBS.

4.7.1.6. Imaging of Microarray 12K

The microarrays were scanned for probe and target fluorescence intensity with a scanner. After complete the scan then, save the image as a .tiff image file. The data can be extracted from the image using the CombiMatrix Microarray Imager Software. After imaging is completed, we can proceed to stripping of the microarray for subsequent rehybridization using the CombiMatrix CustomArray™ Stripping Kit (see Appendix A). Do not allow the semiconductor microarray surface to dry; keep the microarray wet, either in a tube or slide-holder containing 1X PBS, or in the Imaging Solution with the LifterSlip™ attached. Avoid prolonged storage of hybridized microarrays prior to stripping; instead, first strip the

microarray, then store wet in Imaging Solution or 1X PBS at 4°C for a maximum of 2 weeks.

4.7.1.7 Data analysis

Image intensities were quantified with Microarray Imager (Combimatrix Corp.) and graphed with Microsoft Excel software. Subtype identification was accomplished by averaging HA and NA subtype DNA sequence information was generated from array intensity data with an Excel and then associate the most intense signal with the nucleotide represented by that probe. Sequence strings were then used to search the GenBank nonredundant database with the BLASTN program.

4.7.2 Microarray output combined with bioinformatics tools

Our common identification framework of influenza A/H5N1 sequences is depicted in Figure 6.

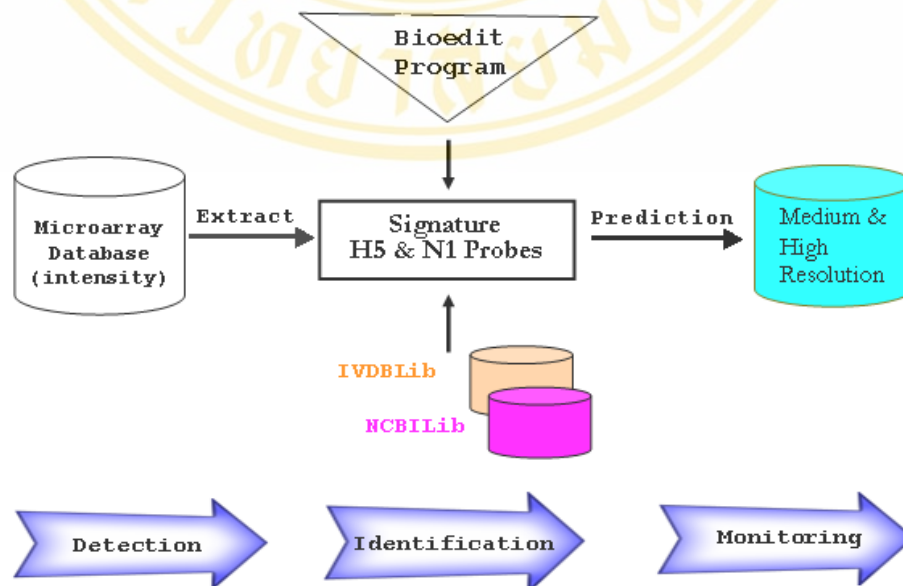


Figure 6 Identification framework of influenza A/H5N1 sequences

4.7.2.1 Statistic analysis to indicate the cut off value

Sensitivity, specificity positive and negative predictive values, and Kappa are the measures for assessing the results of diagnostic according to signal intensity. Sensitivity represents the proportion of truly diseased avian animals in a screened population who are identified as being diseased by the test. It is a measure of the probability of correctly diagnosing a condition. Specificity is the proportion of truly non-diseased avian animals that are so identified by the screening test. It is a measure of the probability of correctly identifying a non-diseased animal. Predictive value of tests in screening and diagnostic tests is the probability, that a person with a positive test is a true positive (i.e., has the disease), is referred to as the predictive value of a positive test (PPV: Positive predictive value); whereas, the predictive value of a negative (PNV) test is the probability that the animal with a negative test does not have the disease. Predictive value is related to the sensitivity and specificity of the test.

Kappa is a measure of the degree of non-random agreement between observers or measurements of the same categorical variable. Kappa is the proportion of agreement after chance agreement has been removed. If $\kappa=1$, there is perfect agreement. If $\kappa=0$ the agreement is the same as would be expected by chance. The stronger the agreement, the higher the value of kappa, Negative values occur when agreement is weaker than expected by chance, but this rarely happens. Depending on the application, kappa less than 0.6 may indicate that the measurement system needs to be improved. Kappa values that greater than 0.8 are generally considered very well. The observed prevalence is the total proportion of animal oligonucleotides found positive by both methods, and the true prevalence is the proportion of animal whose oligonucleotides are avian flu.

Table 3 Basic contingency table and formulae

| | | Flu oligoneucleotide | | | |
|----------------|--|----------------------|-------|---------------------------|-----------|
| Cut off signal | | | | Sensitivity | $a/(a+c)$ |
| intensity test | | T = + | F = - | Specificity | $d/(b+d)$ |
| T = + | | a | b | Observed prevalence | $(a+b)/N$ |
| F = - | | c | d | True prevalence | $(a+c)/N$ |
| N=a+b+c+d | | | | | |
| | | | | Positive predictive value | $a/(a+b)$ |
| | | | | Negative predictive value | $d/(c+d)$ |

4.7.2.2 Sequencing model

The experiment microarray data used in the present analysis were obtained using a variety of purified nucleic acid templates as oligonucleotide probes. To circumvent problems of analyzing unknown sequences (de novo sequence analysis), PCR products with a known reference sequence (resequencing analysis) were examined. But resequencing model provides base call resolution by comparing the intensities of H5 and N1 oligonucleotide probes was established. The files of scanned intensities were transferred to the ElectraSense™ for subsequent analysis. To analyze the results, we needed to determine whether a particular intensity value was indicative of hybridization. For this purpose, Base calls were made based on the intensity data of each probe. The oligonucleotide probes were represented in FASTA format.

Resequencing model is to transform the H5 and N1 oligonucleotide probes FASTA format into Bioedit program as the data representation of Influenza A/H5N1 sequences. In this study, we divided the oligonucleotide probes of each sample into ten groups by rank order of intensity (1000-10,000) and use them to generate signature “consensus” sequences for each group of intensity. This process is divided into three parts.

4.7.2.3. Overlapping probes

After genotype was determined by hybridization signal intensity produced by colourimetric assay at each strain specific probe. To test the sequencing approach in high density oligonucleotide arrays with the appropriate Bioedit program is use to evaluate oligonucleotide probe for all possible sequence H5N1 variation in Thailand during 2003-2006. Although it is possible to use oligonucleotide microarrays in mutation screen, overlapping probe must be improved as Figure 7. Initially, all sequences (the 168 oligo H5 probes and the 168 oligo N1 probe) of the oligonucleotide probes are considered as candidate sample sequence. Sets of oligo probe sequences that were collected in FASTA format. To analyze data sets from FASTA file, each oligo is first examined for the presence of complementary with H5 and N1 consensus. After that, set of H5 and N1 complementary oligo probes were rely by order of base position corresponding with H5 and N1 consensus.

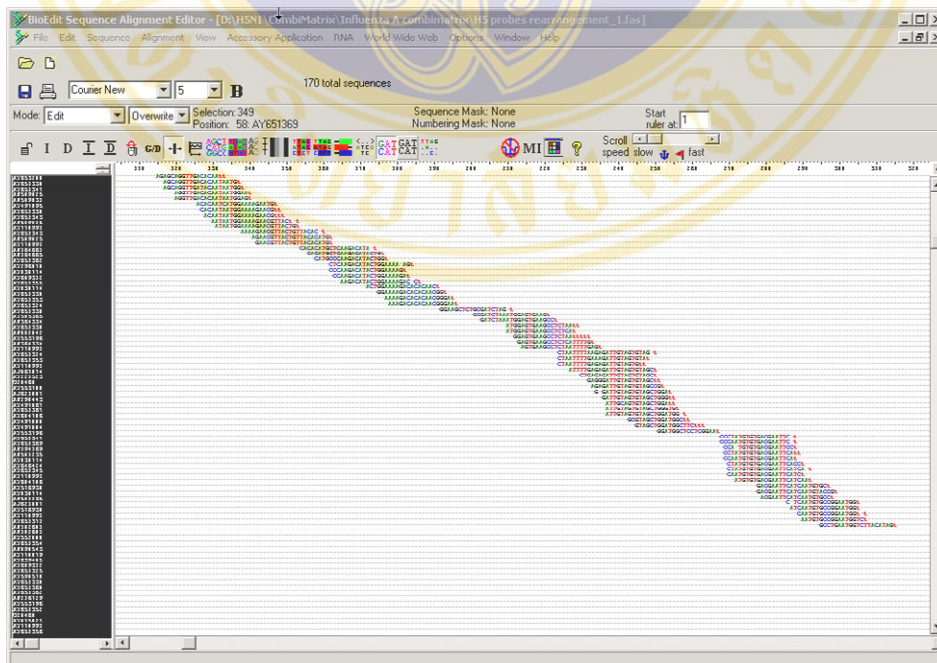


Figure 7 Overview of overlapping probe

4.7.2.4 Rank and group of probes intensity

For each sample, we divided positive signal intensity into ten groups, 1000-10,000 as Figure 8. To create consensus sequence, the oligonucleotide of each group of influenza sequences is taken multiple alignments using the Bioedit program as the pre-process of systems for finding a “consensus” sequence. The consensus sequence is nearly a signature, but it is created by taking the majority bases in the multiple alignments of a sequence collection.

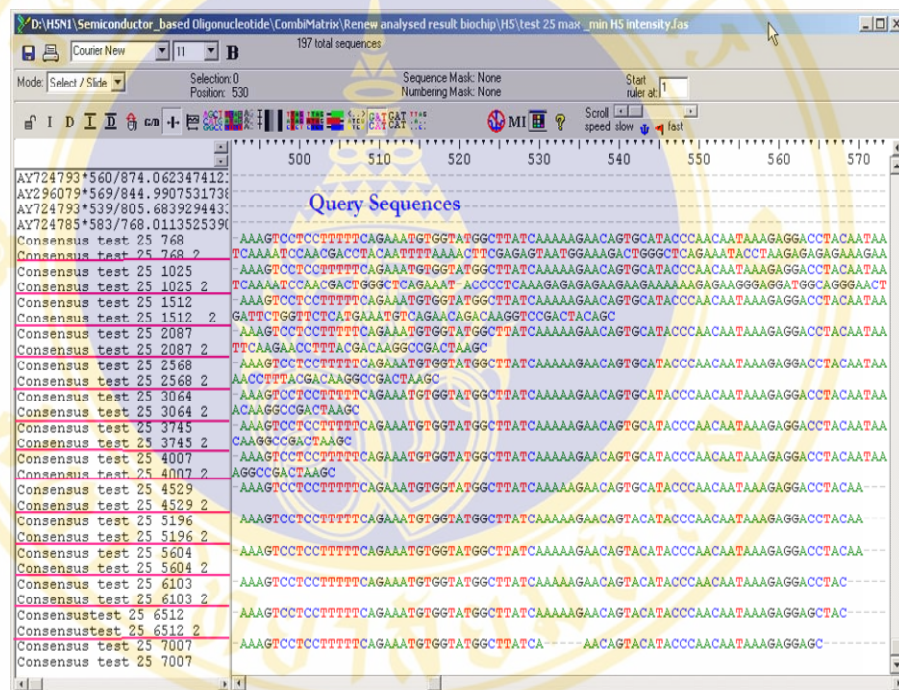


Figure 8 Overview of rank and group of probes intensity

4.7.3 Database preparation

Our Sequence databases are selected from the Genbank genome database of National Center for Biotechnology Information (NCBI). In this study, we use influenza A virus is distinctly separated into Hemagglutinin (HA) and Neuraminidase (NA) segments. We picked 6,463 HA gene sequences, 5,403 NA gene sequences. For our sample, we picked 5 HA and 5 NA genes, then inserted into the

sequence database which is used for performance evaluation of signature “consensus” sequence. Figure 9 show scheme of database preparation.

4.7.4 Identification task

It is process of creating Identifiers using the signature “consensus” sequences and Bioedit program. The identification task is divided into two consecutive parts: Medium resolution and High resolution, to properly correct sequence of influenza A/H5N1 in each sample in term of % identity and E-value of BLAST tool, data for which we know the “truth’ is required. In this study we examine three data sets for which assessments can be performed where specific results are expected. Data set A provides probes for which we can assume the measurements are entirely due to non-specific binding. This permits us to study the variety intensity measures. Data set B provides the results of a spike-in experiment where gene fragments have been added at known sequence. These data can be used to assess correctly group of influenza A/H5N1 in Thailand during 2003-2006 (medium resolution). Data set C provides the results from a study in which samples were hybridize at different host. This permits us to assess similarity between consensus template and set of subsequences in each sample. To reduce the impact of repeated nucleotide of sequence in each sequence of Influenza A virus database, this resulted in database of Influenza A virus for HA and NA sequences. The robustness of each sequence was made manually editing.

4.7.4.1 Medium resolution.

Finding an oligonucleotide representation of DNA sequences is performed in a resequencing model. The oligonucleotide representation, called base call, is a substring of the DNA sequences. Members of the oligonucleotide representation were transfer to Bioedit program and then pass to multiple alignments generates the signature “consensus” sequences. Each sample generates the 8-10 consensus sequences. Sequence alignment were created using the Bioedit programs and the adjusted manually using the edit alignment according to nucleotide sequences.

Finally, All subsequence candidates passing the previous tests are examined for correctly group. The database query subtask performed a batch similarity search of a database using subsequence as the queries. The BLAST program used was the NCBI blastn version 2.12 via Bioedit with a defined set of parameters (figure 9). The masking of low complex regions was performed for the seeding phase to speed up the query; however, low complexity repeats were included in the actual scoring. The entire nucleotide database from NCBI acquired on February 7, 2006 was used as the reference database. The default gap penalty and nucleotide match score were used. The nucleotide mismatch penalty, -q, parameter was set to -1 rather than the default. The results of any BLAST query with an expect value <0.0001 were returned in tabular format from the blast all program. The information about each return (bit score, expect value, mismatches, length of match) was placed in the Return hash using the subsequence identity as the hash key for further analysis.

4.7.4.2 High resolution

The goal is to learn how RNA population differs in sequence in each sample in different host which was designed to identify unique sequence. The signature consensus is to measure the significance of each sample, called “% Identity” using variously different percent and select a set of the consensus sequence highest percent as our “Signatures”. We then pass the signatures through the pairwise process which uses the signatures for formulating a sequence identity score. If there is a query sequence, the highest identity scoring is to detect whether it is the target (influenza A/H5N1, for our experiments) or not.

When there is a query sequence, the scoring is used to measure a similarity value between the query sequence and target sequences, If the score is higher than a threshold, the query is identified as a member of the target sequences. Assume that we pass a query sequence through and identification system; subsequences that only have returns with highest identity score with pairwise alignment in Bioedit program.

NCBI Local BLAST

BLAST is government software obtained from the NCBI. For reference see:
 Altschul, Stephen F., Thomas L. Madden, Alejandro A. Schaffer, Jinghui Zhang, Zheng Zhang, Webb Miller, and David J. Lipman (1997). "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs", Nucleic Acids Res. 25:3389-3402.

Program: Nucleotide Database:
 Protein Database:

Query:

Output file name: (default = file opened but not saved)

Open output
 Filter sequences for low-complexity regions
 Do Gapped BLAST (not available for tblastx)
 Show GI's in defines
 Tabular output

Expectation Value (E):
 Matrix:

Max number of hits to report: Effective database size: (0 = real size)
 Max number of alignments to show:
 Threshold for extending hit:

Additional parameters:

Warning! The complete combined command line (including file paths and auto-set parameters) cannot exceed 128 characters Under DOS. I have not yet found a way around this. If the program doesn't run, try saving the query file to C:\Temp first.

Usage

```
blastall arguments:
-p Program Name [String] (set internally with BioEdit)
-d Database [String] (set internally with BioEdit)
-i Query File [File In] (set internally with BioEdit)
```

Figure 9 Screen short for Local BLAST using in Medium resolution

CHAPTER V

RESULTS

5.1 Genotyping

To assess the capacity of the semiconductor-base oligonucleotide for detection and typing of influenza A/H5N1 virus, we tested 8 influenza A/H5N1 references strains of animal origin and two non-H5N1 subtypes (H4N8 and H1N1). The avian strains represented chicken, duck, Brown-headed Gull and Open-bill stork and mammal strains represented tiger. The amplification of known influenza A virus isolates was accomplished with a pool of reverse primers at a concentration of 100 μ M each. Two pools were used: one pool for the HA gene and one pool for the NA gene. All known targets were successfully amplified, as revealed by agarose gel electrophoresis (Figure 10). The reaction show one or more bands at approximately 500-600 bp. And when they were hybridized to the arrays, they were identified as subtype H5N1. The hybridization patterns of the eight known samples indicated that the HA and NA sequences from samples no. 11, 17, 25, 27, 31, 37,47 and 48 were very similar. We also tested H1N1 and H4N8 viruses isolated from culture for negative control. Avian and mammal influenza virus strains were accurately detected. All three HA (H1, H4 and H5) and two NA (N1 and N8) influenza A subtypes tested were correctly identified. Very distinct hybridization patterns were also seen for hemagglutinins H1, H4 and neuraminidase N1, N8, indicating that the arrays could potentially be used to identify subgroupings of influenza virus subtypes (Figure 12, 13). Average signal was vary from 2,000 to 10,000 for H5 and 1,000 to 10,000 for N1 (Table 4).

Visual HA and NA subtype determinations were made in two ways: (i) by marking the array images with the location of subtype-specific probes and (ii) by graphing of the subtype probe intensities. In addition, bar graphs of mean subtypes

intensity values could be used to predict the correct HA and NA subtypes. All 3 HA subtypes generated from known isolates could be correctly identified with the array, as could as 3 NA subtypes. The results of this genotyping assay are presented in Figure 11-21.

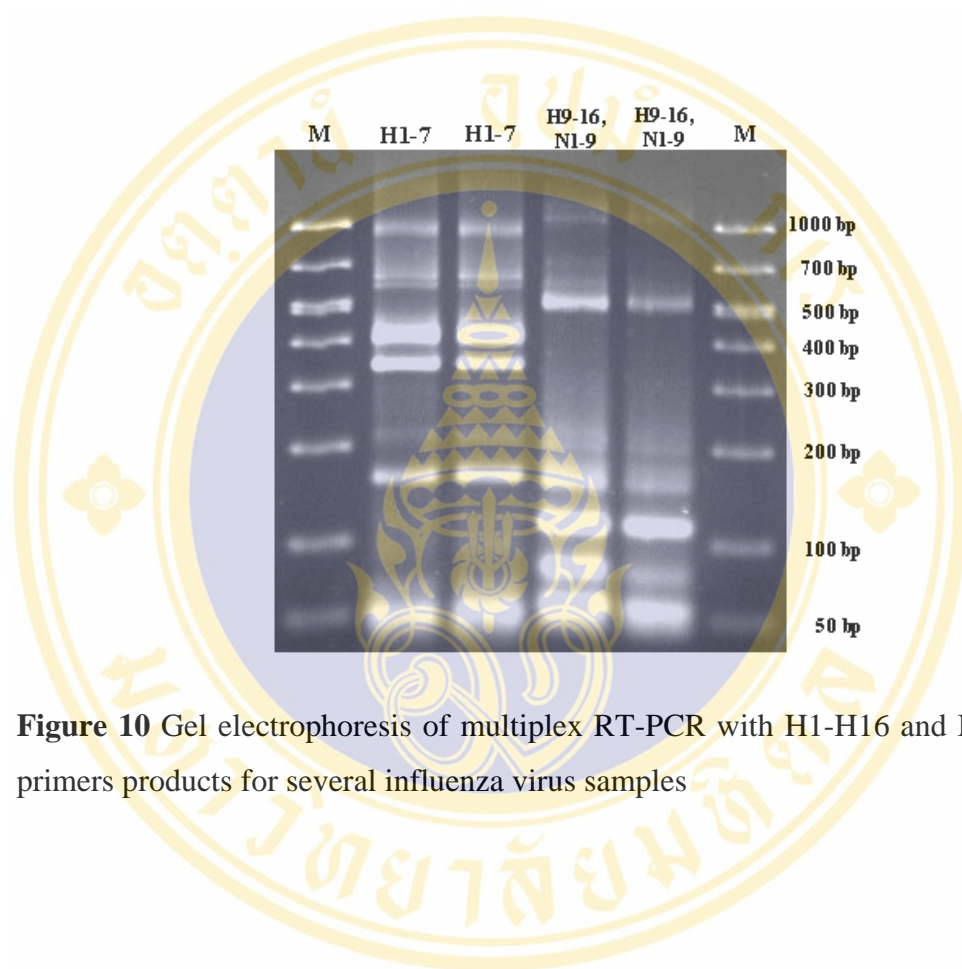


Figure 10 Gel electrophoresis of multiplex RT-PCR with H1-H16 and N1-N9 of primers products for several influenza virus samples

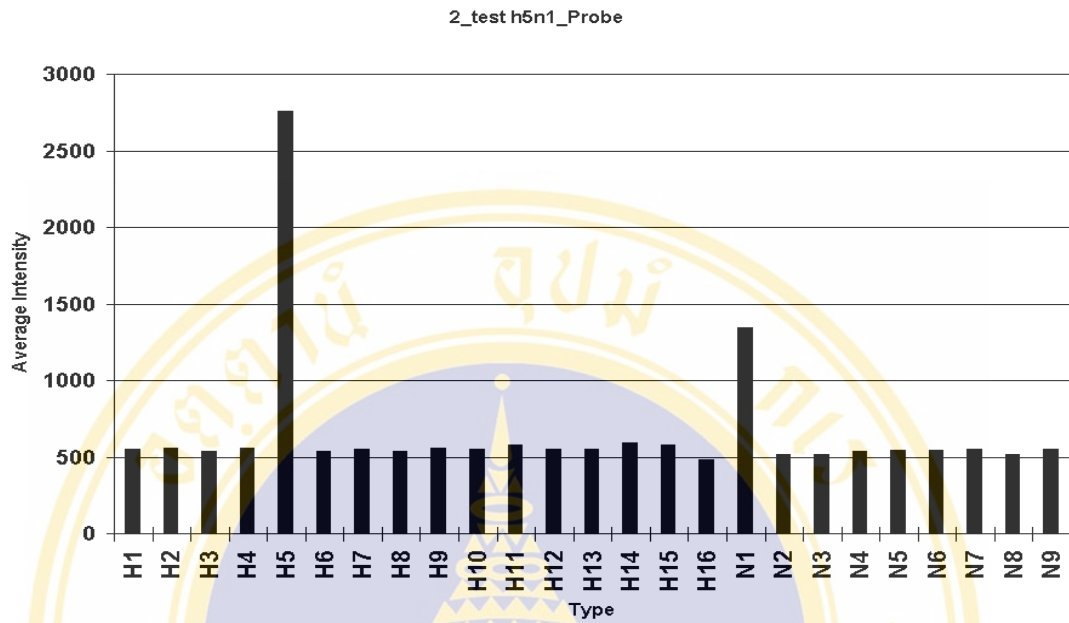


Figure 11 The average signal intensity of subtype probe group in positive control H5N1

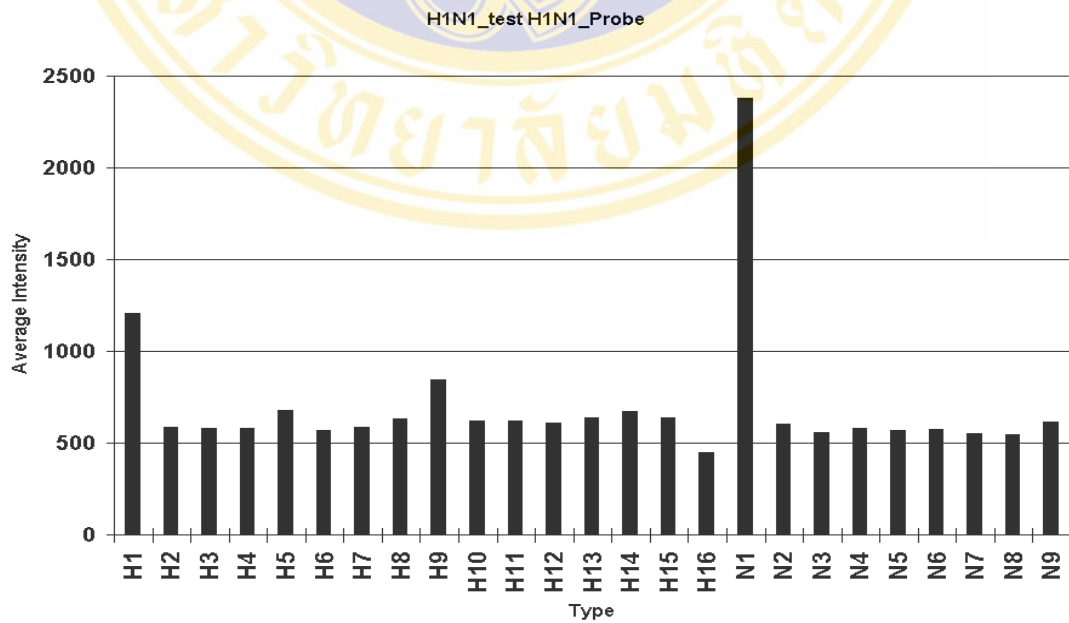


Figure 12 The average signal intensity of subtype probe group in negative control H1N1

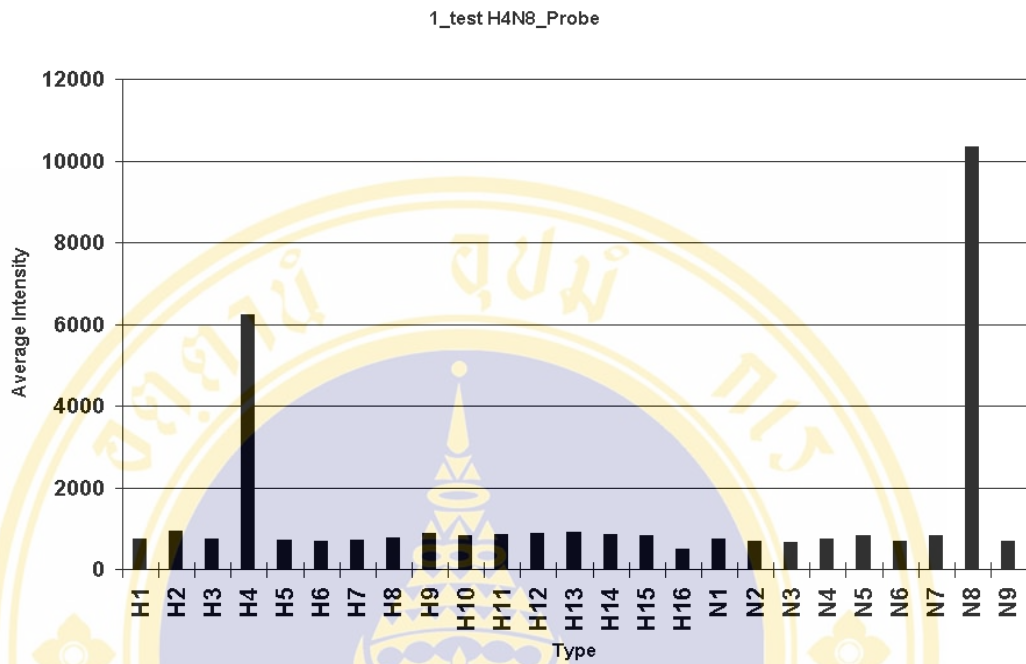


Figure 13 The average signal intensity of subtype probe group in negative control H4N8

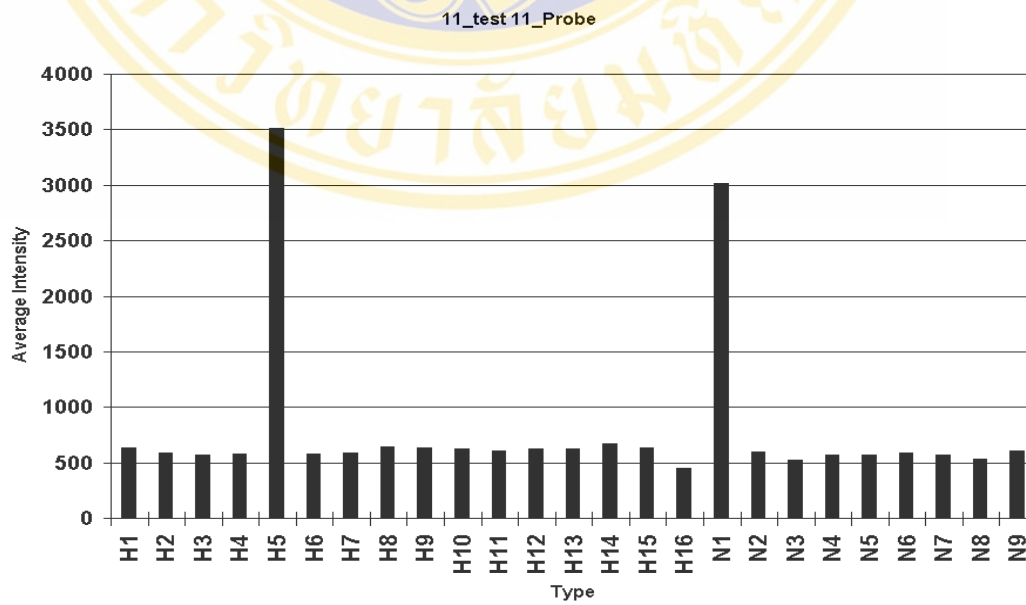


Figure 14 The average signal intensity of subtype probe group in sample no. 11

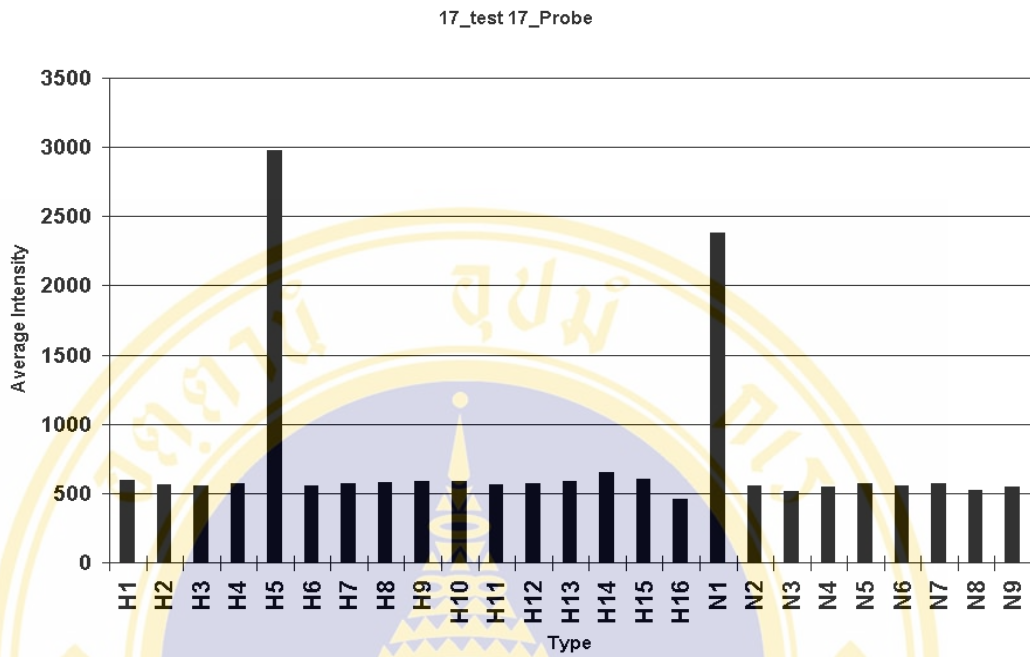


Figure 15 The average signal intensity of subtype probe group in sample no. 17

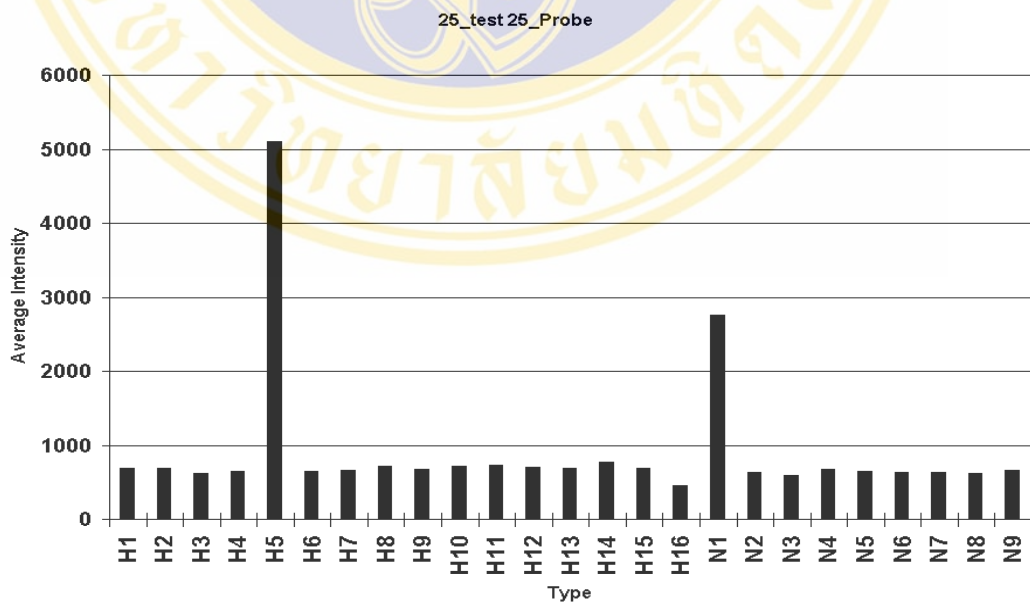


Figure 16 The average signal intensity of subtype probe group in sample no. 25

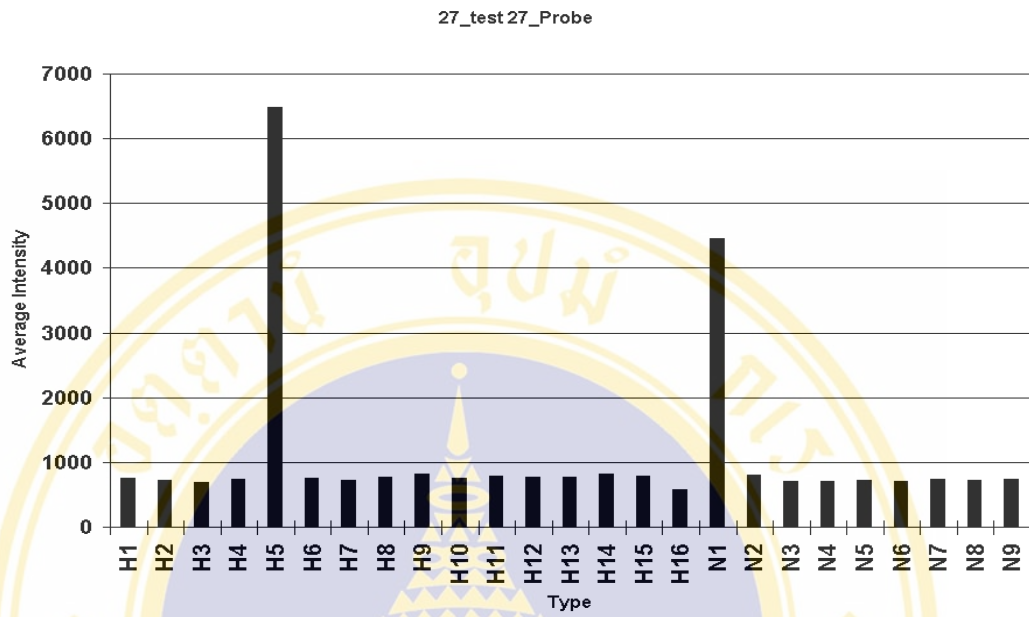


Figure 17 The average signal intensity of subtype probe group in sample no. 27

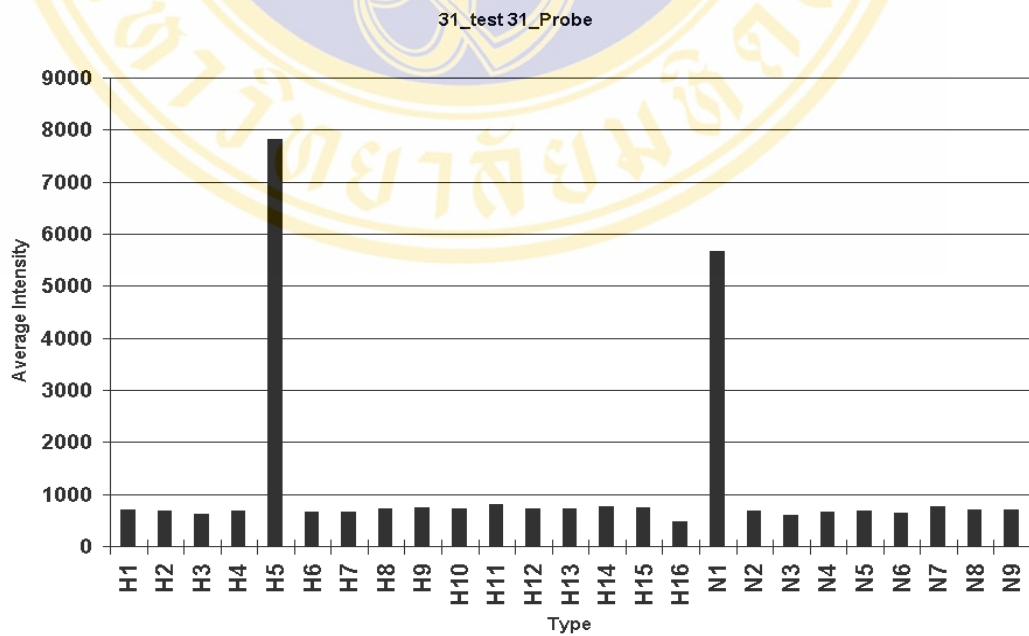


Figure 18 The average signal intensity of subtype probe group in sample no. 31

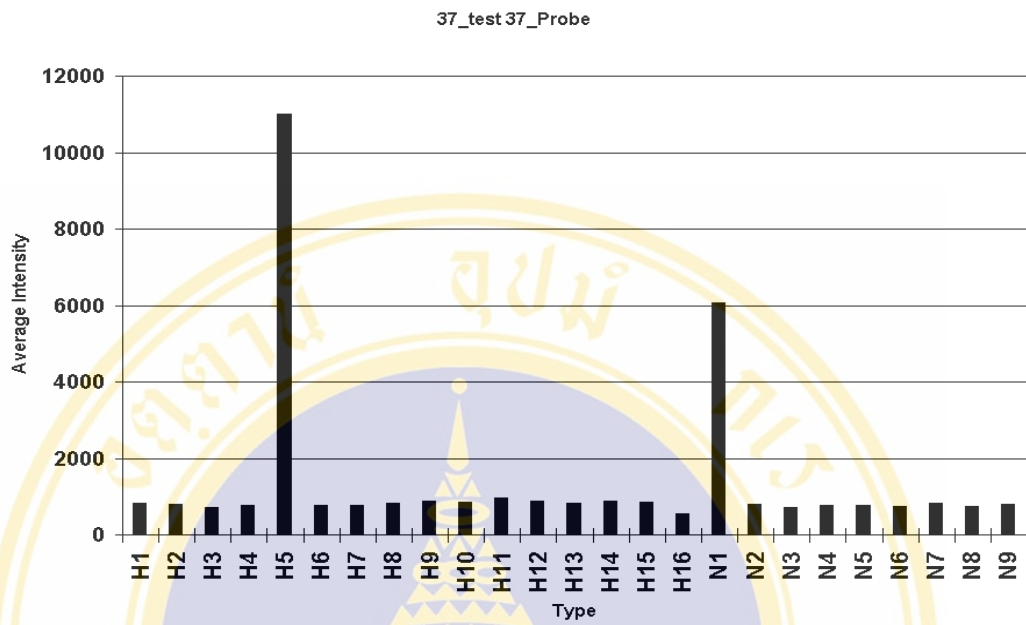


Figure 19 The average signal intensity of subtype probe group in sample no.37

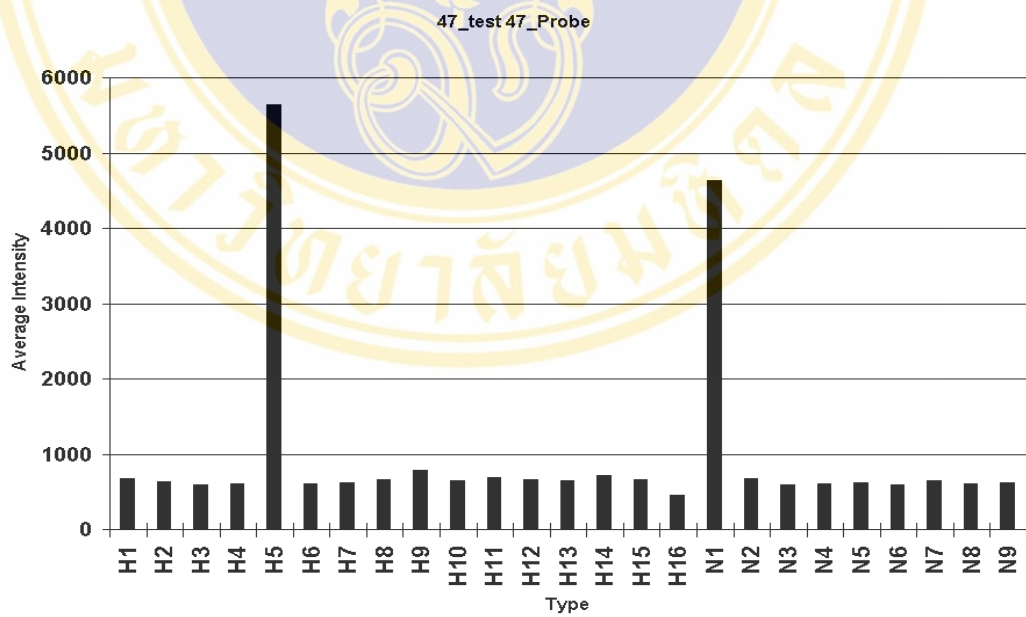


Figure 20 The average signal intensity of subtype probe group in sample no.47

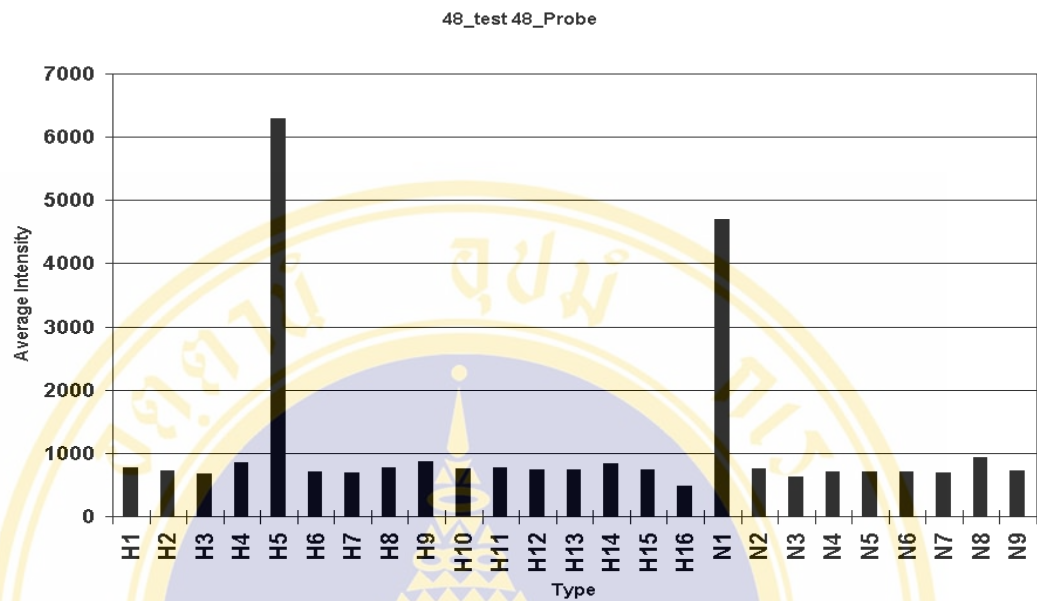


Figure 21 The average signal intensity of subtype probe group in sample no. 48

Table 4 Summary of the genotyping result using Combimatrix chip

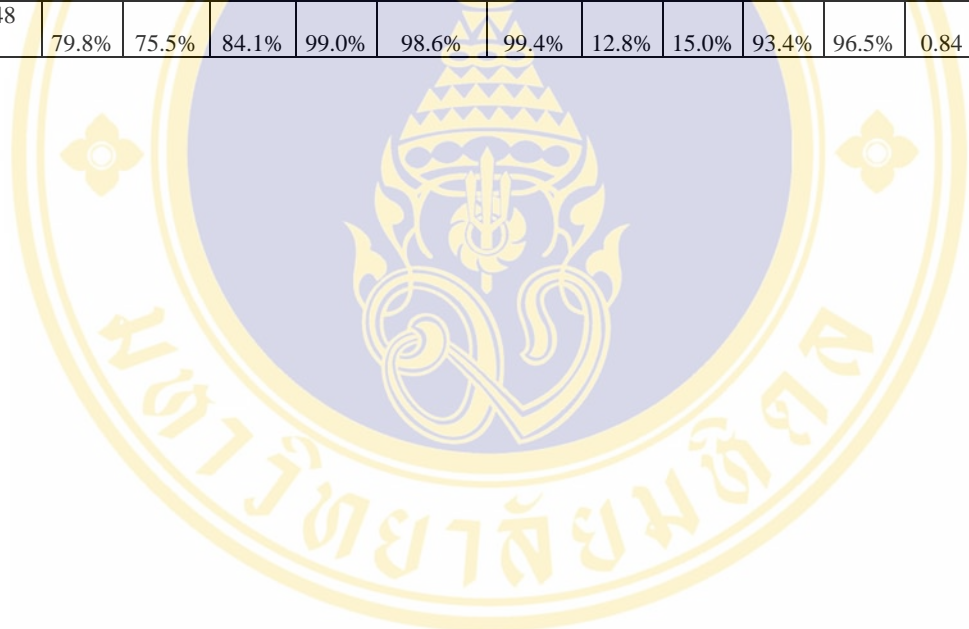
| Sample no. | Call | Assay Baseline | Type | Average Signal | Average/ Baseline | Median Signal | Median / Baseline | StDev |
|----------------|------|----------------|------|----------------|-------------------|---------------|-------------------|---------|
| H5N1 test | H5N1 | 377 | H5 | 2760 | 7.31 | 2245 | 5.95 | 2134.41 |
| | | | N1 | 1342 | 3.56 | 634 | 1.68 | 1595.46 |
| Non- H5N1 test | H4N8 | 424 | H4 | 6244 | 14.73 | 4028 | 9.5 | 5556.93 |
| | | | N8 | 10339 | 24.39 | 11039 | 26.05 | 4356.03 |
| Non- H5N1 test | H1N1 | 373 | H1 | 1206 | 3.23 | 701 | 1.88 | 1409.15 |
| | | | N1 | 2378 | 6.38 | 651 | 1.74 | 3475.35 |
| No.11 | H5N1 | 386 | H5 | 4255 | 11.02 | 4591 | 11.89 | 2252.7 |
| | | | N1 | 3146 | 8.15 | 2249 | 5.82 | 2468.45 |
| No.17 | H5N1 | 436 | H5 | 3277 | 7.52 | 2855 | 6.55 | 2229.48 |
| | | | N1 | 2618 | 6.01 | 1491 | 3.42 | 2299.18 |
| No.25 | H5N1 | 413 | H5 | 5846 | 14.14 | 6133 | 14.83 | 3051.13 |
| | | | N1 | 3119 | 7.54 | 2171 | 5.25 | 2648.6 |
| No.27 | H5N1 | 451 | H5 | 6488 | 14.38 | 6841 | 15.16 | 3260.92 |
| | | | N1 | 4460 | 9.89 | 3481 | 7.72 | 3784.1 |
| No.31 | H5N1 | 373 | H5 | 7826 | 21 | 7726 | 20.74 | 4235.37 |
| | | | N1 | 5672 | 15.22 | 5673 | 15.23 | 4523.03 |
| No.37 | H5N1 | 422 | H5 | 11001 | 26.09 | 11553 | 27.4 | 4886.61 |
| | | | N1 | 6080 | 14.42 | 5325 | 12.63 | 4859.51 |
| No.47 | H5N1 | 377 | H5 | 5643 | 14.96 | 5511 | 14.61 | 3140.53 |
| | | | N1 | 4636 | 12.29 | 3672 | 9.73 | 3524.38 |
| No.48 | H5N1 | 433 | H5 | 6286 | 14.52 | 6353 | 14.67 | 3692.81 |
| | | | N1 | 4701 | 10.86 | 3335 | 7.7 | 3848.1 |

5.2 Sensitivity and Specificity

The best method to assess H5N1 was the 47_test_47. This test was associated with a high sensitivity (87.2%, 95%CI 83.6%-90.8%), and specificity (98.2%, 95%CI 97.6%-98.8%) and a kappa of 0.86 (95%CI 0.83-0.89) showing a very good agreement of the test for a cut off mean signal intensity of 1100. For the sample no. 11, 17, 25, 27, 31, 37, 48 the best Kappa were 0.83 (cut off mean signal intensity of 900), 0.76 (cut off mean signal intensity of 1100), 0.82 (cut off mean signal intensity of 1100), 0.84 (cut off mean signal intensity of 1400), 0.82 (cut off mean signal intensity of 1400), 0.83 (cut off mean signal intensity of 1700), 0.84 (cut off mean signal intensity of 1400), respectively.

Table 5 Optimised cut off and results for different tests to identify H5N1

| Optimized cut off | Sensitivity | | | Specificity | | | Obs. Prev. | True prev. | PPV | NPV | Kappa | | |
|----------------------------|-------------|-------------|-------------|-------------|-------------|-------------|------------|------------|-------|-------|-------|-------------|-------------|
| | % | Lower 95%CI | Upper 95%CI | % | Lower 95%CI | Upper 95%CI | | | | | value | Lower 95%CI | Upper 95%CI |
| Sample no. 11 mean 900 | 81.0% | 76.8% | 85.2% | 98.4% | 97.8% | 98.9% | 13.5% | 15.0% | 89.8% | 96.7% | 0.83 | 0.79 | 0.86 |
| Sample no. 17 mean 900 | 59.9% | 65.0% | 74.8% | 98.7% | 98.2% | 99.2% | 11.6% | 15.0% | 90.7% | 94.9% | 0.76 | 0.72 | 0.80 |
| Sample no. 25 mean 1100 | 78.0% | 73.5% | 82.4% | 98.7% | 98.2% | 99.2% | 12.8% | 15.0% | 91.6% | 96.2% | 0.82 | 0.78 | 0.85 |
| Sample no. 27 mean 1400 | 80.4% | 76.1% | 84.6% | 99.1% | 98.6% | 99.5% | 12.9% | 15.0% | 93.8% | 96.6% | 0.84 | 0.81 | 0.88 |
| Sample no. 31 mean 1400 | 78.9% | 74.5% | 83.2% | 98.5% | 98.0% | 99.1% | 13.1% | 15.0% | 90.4% | 96.4% | 0.82 | 0.78 | 0.85 |
| Sample no. 37 mean 1700 | 81.5% | 77.4% | 85.7% | 98.3% | 97.7% | 98.9% | 13.7% | 15.0% | 89.5% | 96.8% | 0.83 | 0.80 | 0.86 |
| Sample no. 47 mean 1100 | 87.2% | 83.6% | 90.8% | 98.2% | 97.6% | 98.8% | 14.6% | 15.0% | 89.6% | 97.8% | 0.86 | 0.83 | 0.89 |
| Sample no.48 mean 1400 | 79.8% | 75.5% | 84.1% | 99.0% | 98.6% | 99.4% | 12.8% | 15.0% | 93.4% | 96.5% | 0.84 | 0.80 | 0.87 |



5.3 Microarray output combined with bioinformatics tools

We directly recovered hybridized viral sequence in each sample from the surface of microarray by using Bioedit program. To analysis of signature subsequences for each sample, we divided the protocol into two levels: Medium resolution and High resolution. Medium resolution experiment uses a signature sequence that can be submitted as a query to BLAST. We used the subsequence to search the own influenza A virus database using BLASTn. This approach identified target sequences of a length expected to contribute to hybridization. The BLASTn result table was processed to identify, for each hit, the oligonucleotide, and the following virus characteristic: the virus type, the source host, the RNA segment and, if applicable, it's HA and NA subtypes. This model was designed to be comfortable tool to predict novo sequences. Signature oligonucleotides were defined as the oligonucleotides that recognized only one of the possible characteristics for each sample. After alignment with Local BLAST, the data from Signature sequences were selected by high intensity signal. Specially, (535-702) the 168 oligo H5 probes and (1457-1624) the 168 oligo N1 probe were chosen to enable identification of influenza A virus H5N1 that all come from Thailand and recently isolated (2003-2006). But for first Hit for BLASTn analysis were present in table. Two output of sample no. 17 and 47 were hit in BLASTn result as Table 6.

Table 6 Analysis of H5N1 isolates during 2003- 2006 From Thailand**Hemagglutinin**

| Sample | Accession no. | Strain Identification by medium resolution |
|---------------|----------------------|---|
| Sample 11 | DQ497672 | Avian/4(HA)/H5N1/Viet Nam/2003 |
| Sample 17 | salaya17 | Influenza A virus (A/tiger/Thailand/VSM). |
| Sample 25 | DQ201829 | Avian/4(HA)/H5N1/China/2000 |
| Sample 47 | Arunee001 | 49-AI-009-E-HA-TS |
| Sample 48 | DQ497689 | Avian/4(HA)/H5N1/Viet Nam/2005 |

Neuraminidase

| Sample | Accession no. | Strain Identification by medium resolution |
|---------------|----------------------|---|
| Sample 11 | AF098551 | Avian/6(NA)/H11N1/Hong Kong/1998 |
| Sample 17 | EF124300 | Avian/6(NA)/H5N1/China/2005 |
| Sample 25 | DQ334770 | Avian/6(NA)/H5N1/Thailand/2005 |
| Sample 47 | EF124300 | Avian/6(NA)/H5N1/China/2005 |
| Sample 48 | DQ182482 | Avian/6(NA)/H5N1/Belgium/2004 |

Table 7 Summary of signature H5 consensus Nucleotide sequence of each sample test.

| Signature consensus of H5 sample | Length (bp) | Nucleotide sequence |
|----------------------------------|-------------|--|
| Sample no. 11 | 138 | ACAATAATGGAAAAGAACGTTACTGAGTGAAGCCTCTAATTTTGAG AGATTGTAGTGTAGCCTATTGAGCAGAATAAACCATTTTGAGAGAA ATGTGGTATGGCTTATCAAACAGTACATACCCAACAATAAAGAGGA |
| Sample no. 17 | 479 | AGAGCAGGTTGACACAATAATGGAAAAGAACGTTACTGTTACACAT GCCCAAGACATACTGGAAAAGACACACAACGGGAAGCTCTGCGATC TAGATGGAGTGAAGCCTCTAATTTTGAGAGATTGTAGTGTAGCTGGA TGGCTTCTCGAAACCAATGTGTGACGAATTCATCAATGTGCCGGAA TGGTCTTACATAGAGTCAATGACCTCTGTTACCCAGGAGAACTGAAA CACCTATTGAGCAGAACAACCATTTTGAGAAAATTCAGATCATCCC CAAAAGTTCCTGGTCCAGTCATGAAGCCTCAAAGCTCAGCATGTCCA TACAAAAGTCCTCTTTTTCAGAAATGTGGTATGGCTTATCAAAAAG AACATACCCAACAACAATAAAGAGGACCTACAATAATACCAGGGA GGAAGAAGTCTGGTCTCATGGAAATCAAGAACCTTTACGACAAG GCCGACTAAGCC |
| Sample no. 25 | 308 | AGCAGGTTGACACAATAATGGAAAAGAACGTTACTGTTACACATGC CCAAGACATACTGGAAAAGACACACAACGGGAAATGGAGTGAAGC CTCTAATTTTGAGAGATTGTAGTGTAGCTGGATGGCCCAATGTGTGA CGAATTCATCAATGTGCCGGAATGGTCAATTGAAACACCTATTGAGC AGAATAAACCATTTTGAGAAAATTCAGATCATCCCCAAAAGTCTTG GTCCAGTCAAAGTCTCTCTTTTTCAGAAATGTGGTATGGCTTATCAA ACAGTACATACCCAACAATAAAGAGGAGC |
| Sample no. 47 | 305 | AGAGCAGGTTGACACAATAATGGAAAAGAACGTTACTGCCCAAGAC ATACTGGAAAAGACACACAACGGATGGAGTGAAGCCTCTAATTTTG AGAGATTGTAGTGTAGCTGGATGGCCCAATGTGTGACGAATTCATCA ATGTGCCGGAATGGAATTGAAACACCTATTGAGCAGAATAAACCAT TTTGAGAAAATTCAGATCATCCCCAAAAGTCTTGCTCCTTTTTCAG AAATGTGGTATGGCTTATCAAACAGTACATACCCAACAATAAAGAG GAGCTACAATGTCAAGAACCTTTACGA |
| Sample no. 48 | 336 | AGAGCAGGTTGACACAATAATGGAAAAGAACGTTACTGTTACACAT GCCCAAGACATACTGGAAAAGACACACAACGGATGGAGTGAAGCCT CTAATTTTGAGAGATTGTAGTGTAGCTGGATGGCCCAATGTGTGACG AATTCATCAATGTGCCGGAATGGTCAATTGAAACACCTATTGAGCAG AATAAACCATTTTGAGAAAATTCAGATCATCCCCAAAAGTCTTGGT CCAGTCAAAGTCTCTCTTTTTCAGAAATGTGGTATGGCTTATCAAAA AAGAACAGTACATACCCAACAATAAAGAGGACCTACAATGTCAAGA ACCTTTACGA |

Table 8 Summary of signature N1 consensus Nucleotide sequence of each sample test.

| Signature consensus of N1 sample | Length (bp) | Nucleotide sequence |
|----------------------------------|-------------|---|
| Sample no. 11 | 503 | ATAACCATTGGATCAATCTGTATGGTAATTGGAATAGTTAGCTTAATGTTACAAA TTGGGAACATTATCTCAATATGGGTTAGTCATTCAATCATTGTAACATTAGCGGG CAATTCATCTCTTTGTCCTATTAGTGGATGGGCTATATACAGTAAGGACAACRGT ATAACAAGGGGGATGTGTTGTTATAAGAGAGCCATTATCTCATGCTCCCACTT GGAATGCAGAACCTTCCGTCAAAGACAGAAGCCCTTATAGCTCCCATATAACTC AAGGTTTGAGTCTGTTGCTTGGTCAGCAGTTGGTTGACAATTGGAATTTCTGGCTG GCTGTATTGAAATACAATGGCATAATAACAGACACAGTCTGAATGTGCATGTGTA AATGGCAGATCTTCAAATGGAAGGAAAGGGAAAGTGGTTAAATCCTGTTATCCTGA TGCCGGTGGGTATCTTCAATCAAAAATTTGGAGTATCAAATAGGGTGGAGTTTC GGAGACA |
| Sample no. 17 | 311 | ATAACCATCGGATCAATCTGTATGGTAATTGGAATGGTTAGCTTAATGTTACAAA TTGGGAACATCTCAATATGGGTTAGTCATTCAATCAATTAGCGGGCAATTCATCT CTTTGTCCCTATATACAGTAAGGACAACAGTATAACAAGGGGGATGTGTTGTTA TAAGAGAGCCATTATCTCATGCTCCCACTTGAATGCAGAACCCCATATAACT CAAGGTTTGAGTCTGTTGCTTGGTCAGCAGTTGGTTGACAATTGGAATTTCTGGCT GGCTGTATTGAAATACAATGGCATAATAACAGAC |
| Sample no. 25 | 254 | ATCTCTTTGCTTAGGGAGGCTTACAGAAGGACAAGTATAACAAGGGGATTGTTA AAGAGAGCATTATCTCAGCACTTGAATGAGTTGACCTCAGACAGAAGCCCCTCC CCATATAACTCAAGGTTTGAGTCTGTTGCTTGGTCAGCAGTTGGTTGACAATTGG AATTTCTGGCTGGCTGTATTGAAATACAATGGCATAATAACAGACACTATCAAGA GTTGGAGGAATAACATACAGTCTGAATGTGCATG |
| Sample no. 47 | 312 | ATAACCATCGGATCAATCTGTATGGTAATTGGAATAGTTAGCTTAATGTTACAAA TTGGGAACATCTCAATATGGGTTAGTCATTCAATCAATTAGCGGGCAATTCATCT CTTTGTCCATTAGTATATACAGTAAGGACAACAGTATAAAAGGGGATGTGTTG TTATAAGAGAGCCATTATCTCATGCTCCCACTTGAATGCAGAACCCCTCCCAT ATAACTCAAGGTTTGAGTCTGTTGCTTGGTCAGCAGTTGGTTGACAATTGGAATTT CTGGCGTATTGAAATACAATGGCATAATAACAGAC |
| Sample no. 48 | 667 | TAACCATTGGATCAATCTGTATGGTAATTGGAATAGTTAGCTTAATGTTACAAATT GGGAACATAATCTCAATATGGGTTAGTCATTCAATTCAGTAACATTAGCGGGCAA TTCATCTCTTTGTCCTATTAGTGGATGGGCTATATACAGTAAGGACAACAGTATA ACAAGGGGGATGTGTTTGTATAAGAGAGCCATTATCTCATGCTCCCACTTGGGA ATGCAGAACCTTCGACCGTCAAAGACAGAAGCCCTTATAGCTCCCATATAACTC AAGGTTTGAGTCTGTTGCTTGGTCAGCAGTTGGTTGACAATTGGAATTTCTGGCTG GCTGTATTGAAATACAATGGCATAATAACAGACACTATCAAGAGTTGGAGGAAT AACATACAGTCTGAATGTGCATGTGTAATGGCTCTTGCTTTACTGTAATGACTG ACGGACCAAGTAATGGAGATCTTCAAAAATGGAAGGAAAGTGGTTAAATCA GTGCAATTGGATGCTCCTAATTATCACTATGAGGAATGCTCCTGTTATCCTGATGC CGGGATAATTGGCATGGCTCGAATCGGCCATGGGTATCTTCAATCAAAAATTTGG AGTATCAAAATAGGGTGGAGTTTCGGAGACAATCCACGCCCAATGATGGAACC AAAAGC |

5.4 The high resolution

We further validated the high resolution by testing 5 HA sequences and 5 NA sequences of our Influenza A/H5N1 samples. After proof-of-concept of medium resolution we used the oligonucleotide sequences from probe set to search the identity for each sample. The results of Identity percent in 5 sequences of H5 gene and 5 sequences of N1 gene were shown in Figure 22-26.

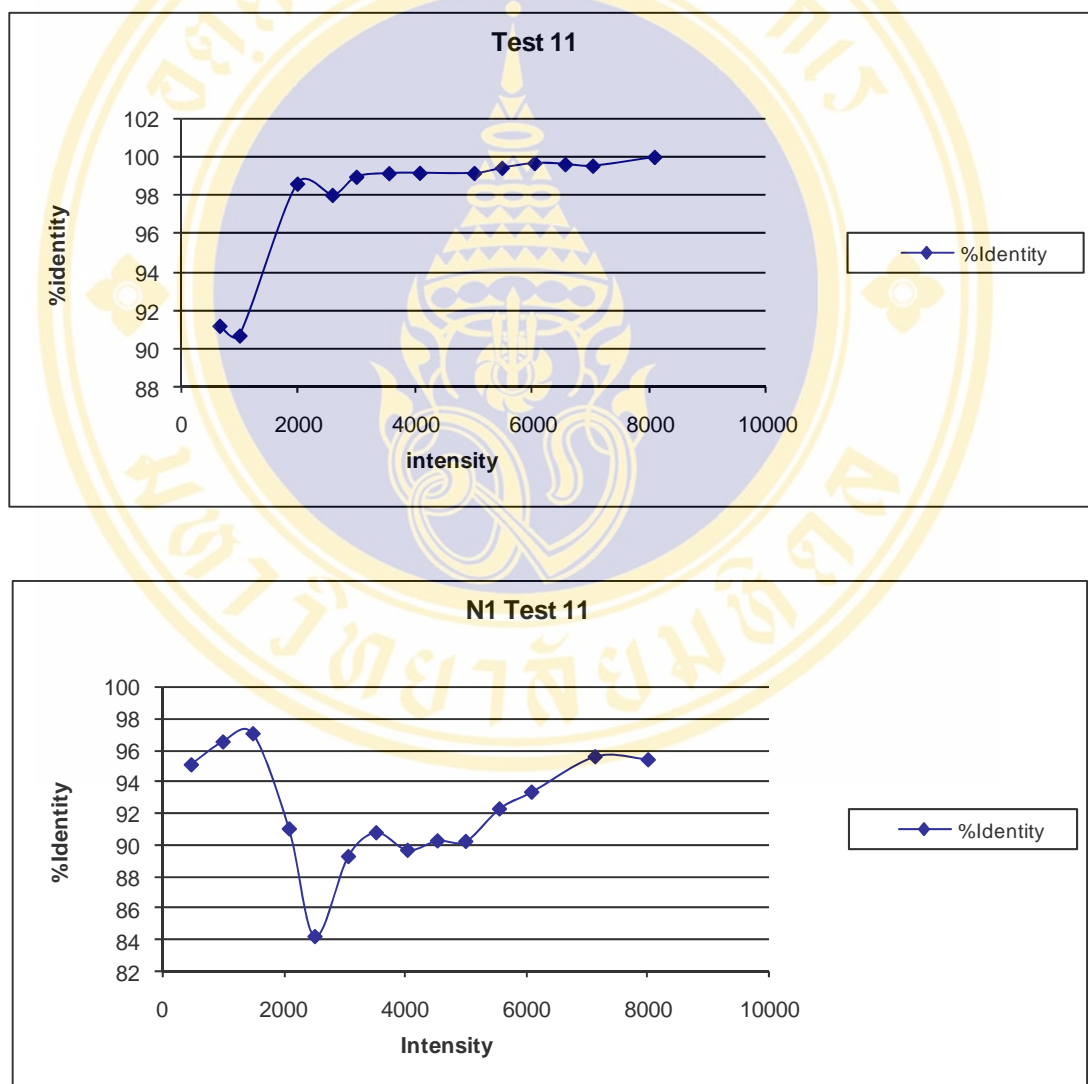


Figure 22 Identity percent sample no.11 for H5 and N1 sequences

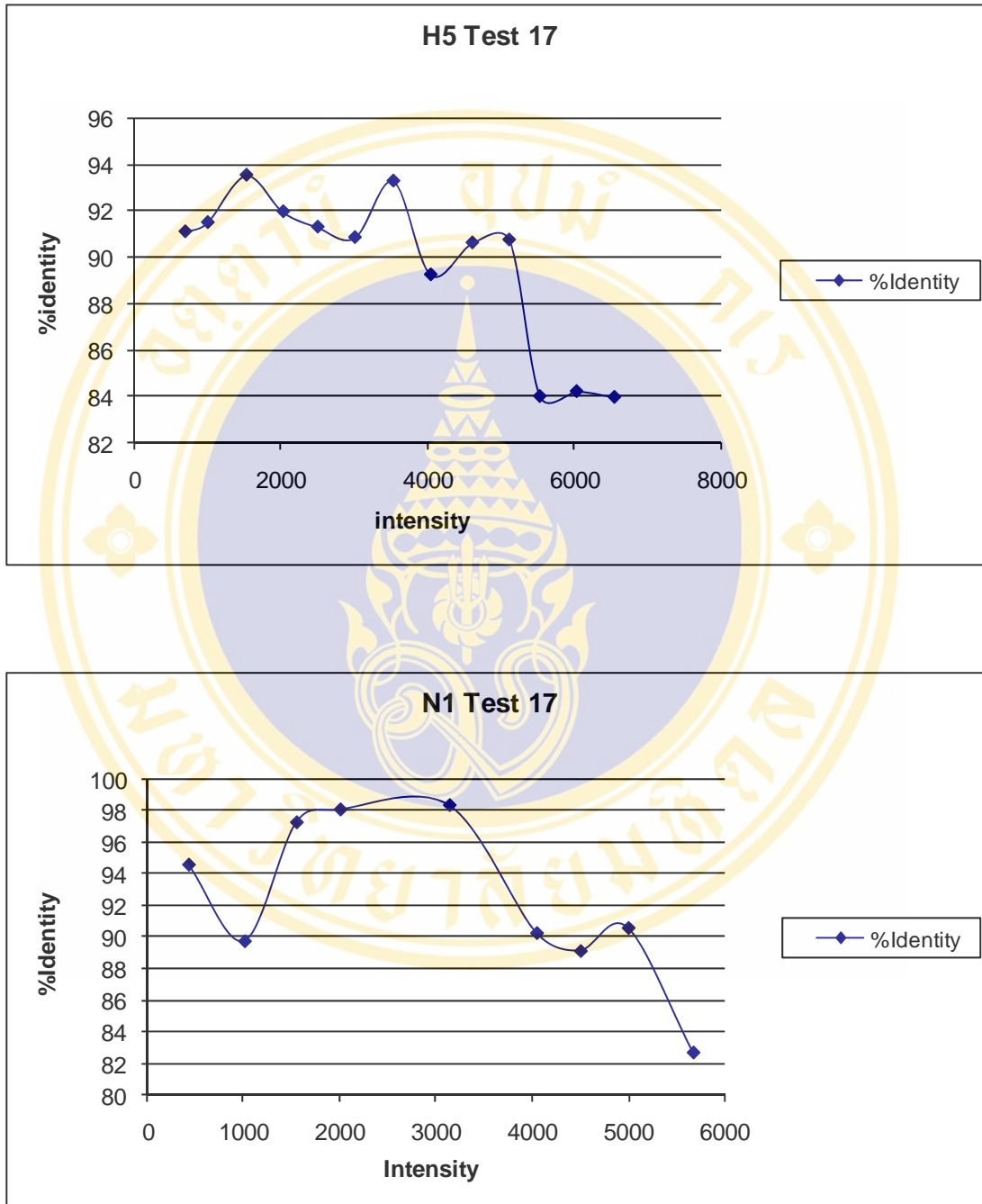


Figure 23 Identity percent sample no.17 for H5 and N1 sequences

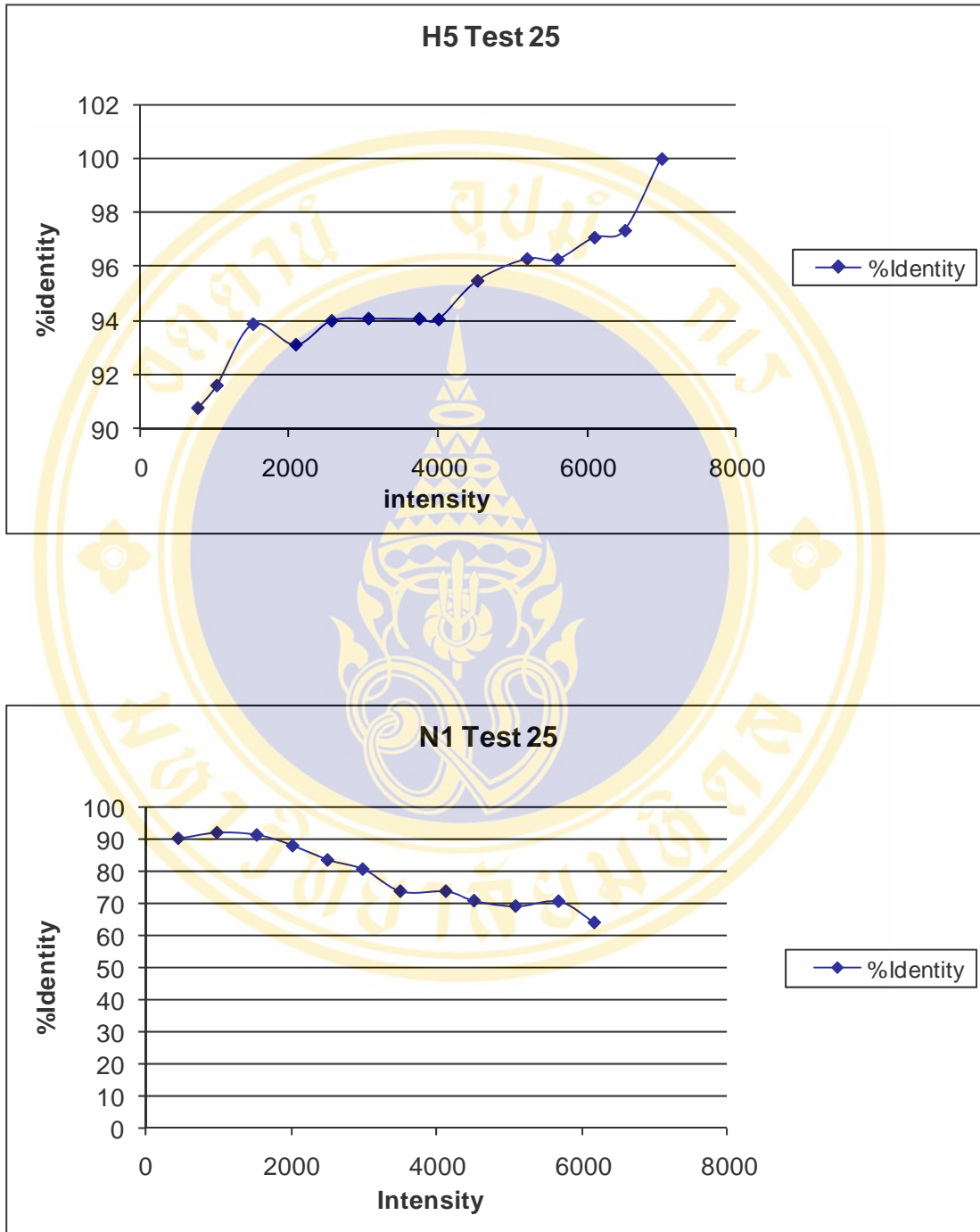


Figure 24 Identity percent sample no.125 for H5 and N1 sequences

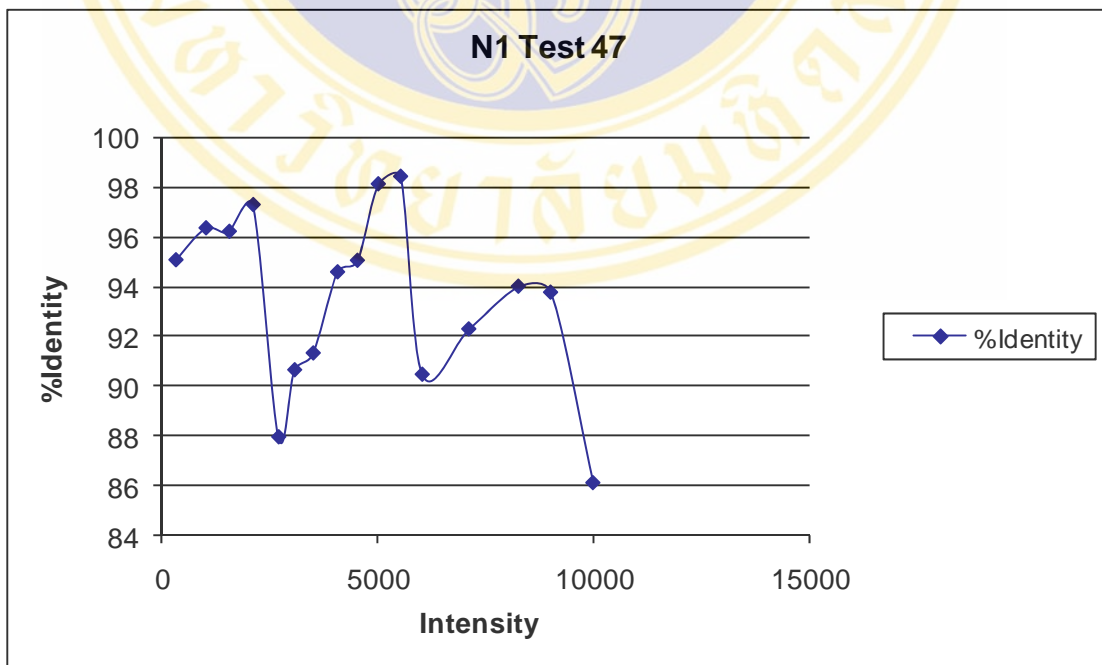
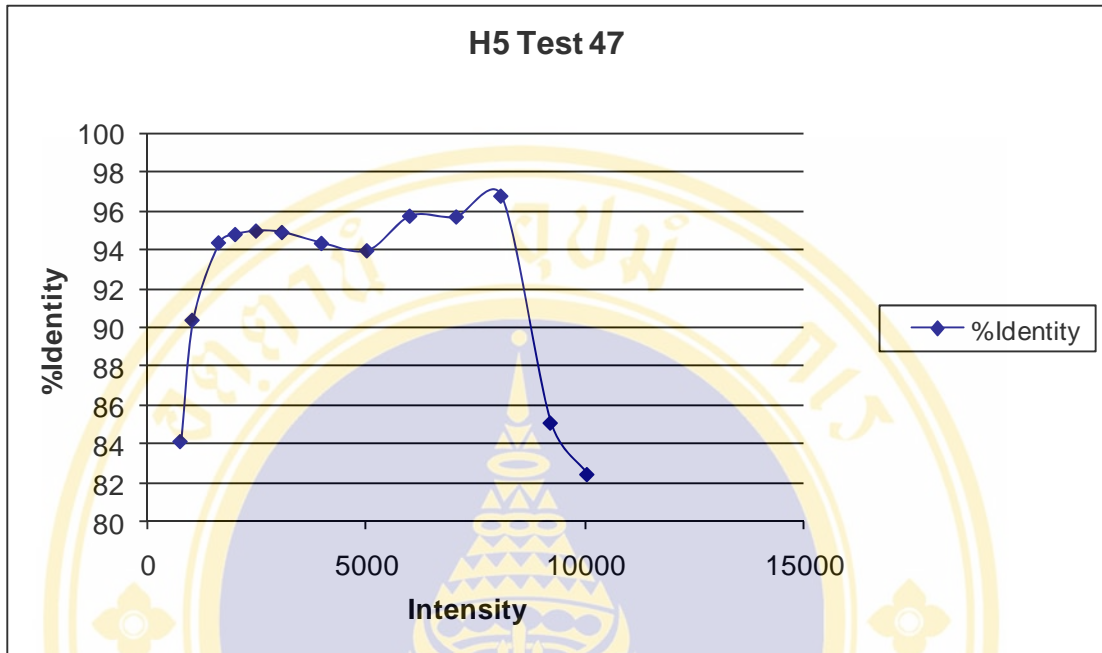


Figure 25 Identity percent sample no.47 for H5 and N1 sequences

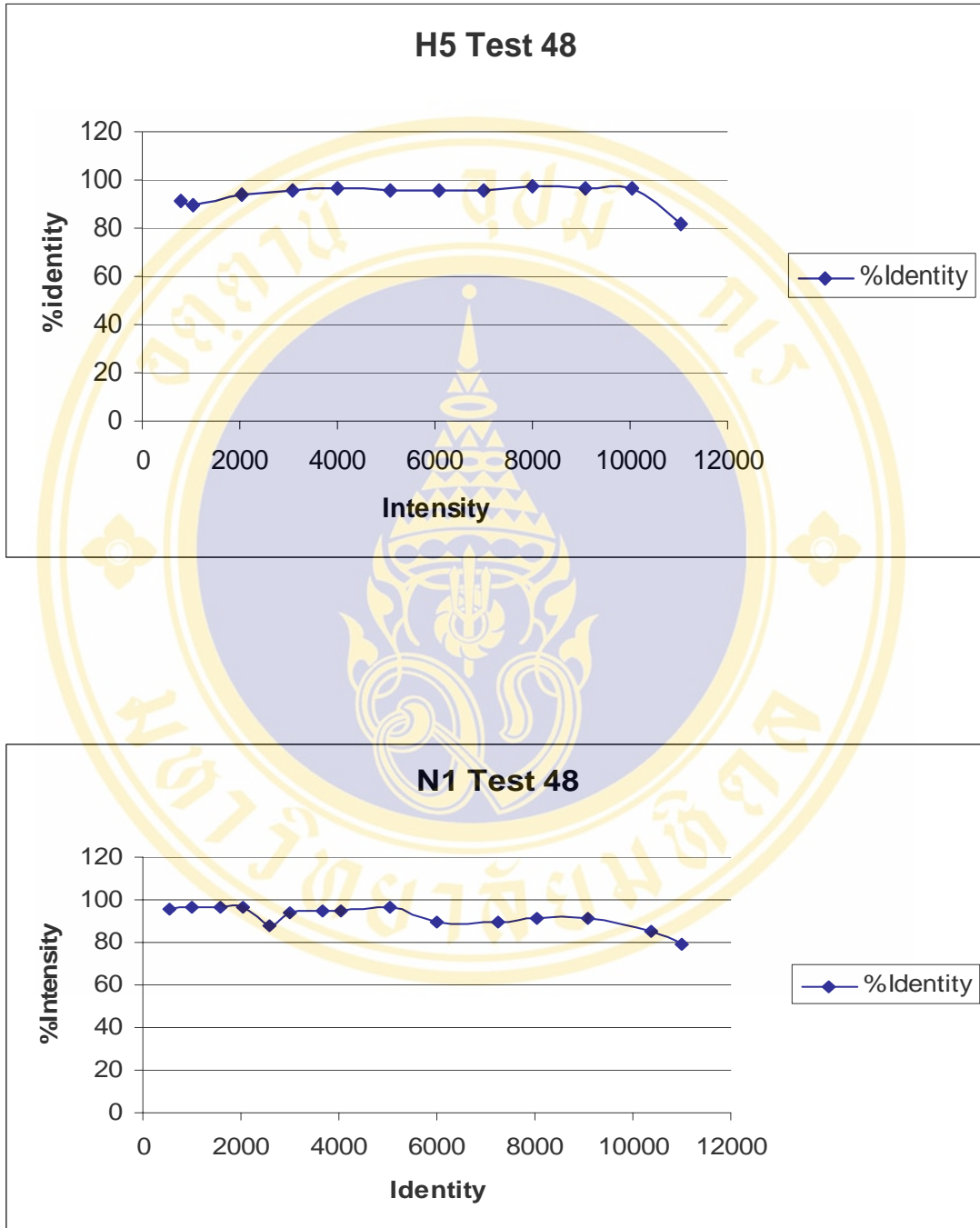


Figure 26 Identity percent sample no.48 for H5 and N1 sequences

5.5 Combine medium and high resolution result for monitoring avian influenza A H5N1

After proof-of-concept experiments, we combine medium and high resolution results to discriminate H5N1 strain. It is important not only to distinguish subtypes but also to identify the differences associated with significant shifts of the subtype from year to year by medium resolution. Not all of isolate corresponded to identification made based on the sequence obtained using the conventional DNA sequencing. For every isolate identified as influenza A virus H5 and N1 whose sequence was obtained using conventional DNA sequencing methods, the two methods identified strains that corresponded with each other except N1 in sample no.11 and no.48 in part of unEdit Sequence. After we create new database for H5 and N1 sequences, we can found all representative sequences were match against sequences in database (Table 9).

Sequence-based medium and high resolution analysis revealed the identity of the virus to be most nearly identical to each other that had been circulating during 2003-2006 in Thailand.

Table 9 Comparison of Local Blast result between unedited and edited H5 signature consensus sequence combine medium and high resolution

| Sample No. | % Identity | Local Blast Result | | | | | |
|------------|------------|--------------------|-----|----------|---------------|-----|----------|
| | | UnEdit Sequence | | | Edit Sequence | | |
| | | order | Bit | E-value | order | Bit | E-value |
| H5 No.11 | 100 | 2nd | 74 | 7.00E-14 | 2nd | 74 | 3.00E-14 |
| H5 No.17 | 93.52818 | 1st | 373 | E-103 | 1st | 373 | E-103 |
| H5 No.25 | 100 | 2nd | 157 | 2.00-38 | 2nd | 157 | 7.00E-39 |
| H5 No.47 | 97.04918 | 1st | 159 | 4.00-39 | 1st | 135 | 3.00E-32 |
| H5 No.48 | 97.02381 | 2nd | 155 | 7.00E-38 | 2nd | 155 | 3.00E-38 |
| N1 No.11 | 97.01789 | Not found | - | - | 8th | 177 | 8.00E-45 |
| N1 No.17 | 98.39744 | 1st | 145 | 1.00E-35 | 11th | 125 | 2.00E-29 |
| N1 No.25 | 92.2449 | 1st | 137 | 5.00E-33 | 1st | 125 | 2.00E-29 |
| N1 No.47 | 98.39744 | Not found | - | - | 18th | 117 | 5.00E-27 |
| N1 No.48 | 96.875 | 1st | - | - | 4th | 188 | 3.00E-48 |

CHAPTER VI

DISCUSSION

Mutations in the influenza virus, including avian and human influenza, can occur either gradually or quickly. Either type of mutation can give rise to new strains of the virus. The mutations often alter the characteristics of the viruses. These new strains may increase or decrease the severity of illness. The immune systems of individuals infected by earlier strains of the virus do not necessarily recognize the new strains (22, 96). From a diagnostics standpoint, influenza is often described as a “moving target” due to the high mutation rate of HA and NA. The HA and NA genes evolve rapidly at 6.7×10^{-3} and 3.2×10^{-3} nucleotide substitutions per nucleotide per year, respectively, and are genetically diverse between subtypes (5, 67, 97). The effects are impossible to predict. For influenza virus strains, it is important not only to distinguish subtypes but also to identify the differences associated with significant shifts of the subtype from year to year. Subtype determination (“subtyping”) is essential for tracking emerging viruses and for designing appropriate influenza vaccines (53, 98). The current “gold standard” for complete influenza A subtyping (determination of both HA and NA) involves virus replication in egg or tissue culture followed by a hemagglutinin inhibition assay. This method is tedious and requires several days, with the analysis time often extended to several weeks for antigenically novel viruses. RT-PCR techniques depend on specific primers, which may fail when corresponding viral sequences mutate (74, 81, 82, 99, 100). We are using a semiconductor-based oligonucleotide array technology that can be used with fluorescent labels and traditional optical scanning devices or used as a biodetector using electrochemical techniques for analysis. This platform is extremely flexible, which allows array designs to be rapidly and easily modified and synthesized, thus permitting oligonucleotides of interest to be tested empirically. In addition, the ability to use electrochemical detection with semiconductor microchips eliminates the need

for expensive optical scanning equipment. In contrast to the fluorescent detection, the ElectraSense™ format has fewer limitations for further miniaturization of geometrical sizes of individual elements on the microarray. Clearly, the cost per assay and the associated costs of equipment needed to run and analyze the assay are critical for diagnostic applications such as these (12, 17, 20).

However, the influenza virus subtyping array is also compatible with traditional labeling, hybridization, and washing protocols that can be completed within 1.0 to 1.5 h. However, use of this method allows the array to be “stripped” and reused multiple times since there is no covalent coupling of the label to the array. In addition, this array can also benefit from the sectoring approach mentioned above to further bring costs to minimum. This platform is a viable alternative to RT-PCR because of the combination of assay speed, array sectoring, which would allow multiple assays on one array, the potential to strip and reuse the adaptability to inexpensive electrochemical scanning devices. The target sample preparation system used for standard RT-PCR-based method, except that it uses a very redundant consensus priming system that maximizes the chance that novel strains of influenza virus will be amplified and thus minimizes false-negative results. The chip contains multiple probes that correspond to key distinguishing elements of each HA or NA subtype. It is laid out in a visual pattern so that it can be read visually for quick identification as well as analyzed with more advanced algorithms. The system can also identify rare versus more commonly seen genetic variants based on the organization of subtype-specific probes (i.e., the probes are arranged in order from more universal to more specific for each subtype).

In many cases, the detection and identification of influenza A subtype is not sufficient for making decisions on vaccine development, patient treatment, and general surveillance, and specific sequence is needed. For example, mutations in the sequence coding the haemagglutinin receptor binding site of subtype H5N1 have been shown to be important for replication of avian viruses in humans (31, 51, 101). In this study, viral HA and NA subtype sequencing was accomplished with a bioinformatic tool. After scanning the array, intensity data were exported to an Excel worksheet. Sequence information was extracted with a routine design to associate the correct base

with the highest signal from sets of probes that were tiled by one nucleotide to cover the sequence of interest. Although microarrays are excellent for determining subtypes, they do not give detailed genetic information or information when antigenic drift occurs (102-105). The identification of HA and NA subtypes followed by sequencing with bioinformatic tools will significantly decrease the time and cost for the genetic monitoring of potential lethal virus strains (91, 102, 104, 106-109). This study demonstrates that the detection, identification and monitoring of viral genomes in samples by using a bioinformatic tool in combination with electrochemical detection is a viable rapid approach that can complement traditional method. Two level resolutions were used to make detection and identification. Finding the signature oligonucleotide used the exclusivity of BLASTn hit to determine specificity (medium resolution). It is unambiguous but ignores the information in oligonucleotides that react with multiple characteristics, for many HA and NA subtypes there were no uniquely diagnostic probes. To compensate for the lack of signature oligonucleotides, an attempt was made to use the overrepresentation of some categories among BLASTn hits of the oligonucleotides and the underrepresentation of others to aid in the characterization.

The protocol we have developed successfully in medium resolution which provided pathogen identification to maximum level of detail possible (species or strain) depending on the quality of each signature sequence and local database. This identification capability requires minimal input on the identity of the pathogens, making non-expert use feasible. The crucial feature incorporated that allowed complete automation was the use of taxonomic databases, which classify organisms into ordered groups and provide relationships between organism entries, allowing removal of redundancies, comparison of different related signature consensus and simplification of data presentation. This allows databases such as NCBI or IVDB, which are redundant and subject to minimal curation but which constantly receive update and new sequence information, to be used with great success. For more variable or rapidly mutating H5N1, low resolution still provided accurate detailed identification, but medium and high resolution were unable to report fine scale discrimination. The comparison of the conventionally sequenced influenza A/H5N1 gene sequences illustrated that the algorithm is capable of automatically adjusting for

updates in databases. The protocol demonstrated the local database's capability to properly distinguish hybridization caused by the specified influenza A/H5N1 from those caused by genetically close (near neighbor) strains and to not make incorrect identifications, eliminating one potential cause of false positive. Improving on the level of detail reported in medium and high resolution will require more information about an individual pathogen and may have to be developed for each specific pathogen or class of pathogens. This information is also required for the protocol to identify which differences between a sample and database entries represent significant mutations.

Microarray designed to analysis of influenza virus strains have previously been study by Li et al. (16), Kessler et al (110), and Sengupta et al. (15). Wang et al. (14), incorporated influenza virus-specific probes in a microarray designed for analysis of a wide range of viruses but did not test specifically for influenza virus. On the other hand, our study, we have non-influenza virus species probes in a microarray to test specifically for influenza virus. For medium and high resolution, we need more number of samples to test and validate our algorithm. Just as we pointed to limitations of previous task, it is important to point out limitations of this study. One limitation is that, sequences were initially selected primarily for their biological interest, but then some of these candidates were excluded. This introduces a possible bias for local database into our medium resolution results. Sequences with every average intensity signal set were not excluded. These factors resulted in the selection of primarily low to high signal intensity. In medium resolution has suggested that difficulties with methods that subtract sequences arise for low intensity signal due to extreme variability. The remaining criteria used to select signature oligonucleotide were the agreement of kappa analysis. We initially indicate cut off of intensity signal of H5 and N1 oligonucleotide probe. Kappa analysis result produced the best agreement between H5N1 oligonucleotide and non-H5N1 oligonucleotides. And other criteria were existing knowledge of an appropriate bioinformatics tools and update database influenza A virus library. We are unaware of any biases produced by these latter selection criteria.

However, high resolution task shows the “signature sequences” really not depend on high signal intensity. For example, Figure 22-26 (except H5 Test 11 and Test 25) show that this is exactly what happens. For set of oligonucleotides at low level of intensity, high resolutions that use identity percent have been found to extremely variable. They give high identity percent but local blast results were poor. We also found poor reliability for high resolution using set of high signal intensity oligonucleotide set. Because the signature sequence in each sample by using high resolution are very long which produced high mismatch tolerance (15, 89).

These studies are useful and important, but are not the end of the story. We need more number of influenza A strain to use for validation. A criterion often not considered in the spike-in studies is the accuracy of measurements across genes. In addition to the extremely high degree of accuracy of the method microarray is to detect RNA rather than the intermediate cDNA, absence of amplification and a more sensitivity (3). Our result suggest a more complicated scenario that each of these low-medium-high resolution perform well if combined with particular other appropriate statistic analysis (111, 112), proper bioinformatics tools (93-95, 113-117) and information of oligonucleotide probes in microarray (90, 118, 119). Future work will involve improving the use of the current taxonomic database or potentially developing a new relational database that is specific to our needs and then incorporating more specific information of target pathogens. The hierarchal design of the data analysis makes it easy to incorporate analysis that build upon the analysis already performed (120).

CHAPTER VII

CONCLUSION

In conclusion, we report exploratory analyses of signal intensity oligonucleotide array data from the semiconductor base oligonucleotide microarray with the objective of detection, identification and monitoring of the currently used measures of hemagglutinin and neuraminidase genes of H5N1 avian influenza circulating in Thailand during 2003-2006. Our analyses make use of three levels of typing (i) low resolution (detection or genotyping), experimental study consisting of single step reverse transcription, hybridization and imaging microarray, (ii) medium resolution and (iii) high resolution.

Finally, we evaluated the algorithms in term of their ability to detect known DNA sequences using the signal intensities data and bioinformatic (Bioedit program) analysis of the five hemagglutinin segments and five neuraminidase segments of five RNA H5N1 avian influenza viruses. The experiment results showed that detection, using almost all scoring algorithms achieved good results, except the use of high resolution which was produces less accurate than the others.

We have two areas of concern to date. We found that for medium and high resolution typing, it was time consuming to create the HA and NA database. Also, the specific gene sequence of each H5N1 strain is subjective, so the protocol monitoring of the new strain H5N1 is recommended for further study.

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APPENDIX

1. Instruments and laboratory supplies for single-step target preparation

- Top-bench microcentrifuge : JOUAN, France
- Automated pipette, P2/10/20/100/200/1000 μ l : Gilson, France
- Thermocycler 9700 : Perkin Elmer Cetus, USA
- Microcentrifuge tube (0.5 ml and 1.5 ml in sizes) : Treff, Switzerland
- Pipette tips 2-10 μ l : Gilson (white)
20-200 μ l : Coster (yellow)
1000 μ l : Treff (blue)
- Horizontal gel electrophoresis : Mupid, Japan
- UV- transilluminator with camera computer processing : Stratagene, Eagle eye II Still Video System, USA
- Power supply : Eric line Co.Ltd.

2. Instruments and laboratory supplies for hybridization and imaging of influenza A research Microarray 12K

- Influenza A Research Microarray 12K
- Hybridization Cap (50 μ l Volume)
- Clips for Hybridization Cap
- LifterSlip™ coverslip
- Imaging Solution Standard, high-resolution fluorescent microarray scanner (CombiMatrix recommends the Axon Instruments Genepix® 4000B and 4200A, and the Perkin Elmer ScanArray® 4000, 5000, Lite and Express microarray scanners).
- Adhesive tape: Scotch® Brand Magic® Transparent Tape is suitable for hybridization temperatures of 50°C or less; for extended incubations at higher temperatures use a PCR sealing tape such as Nunc

Brand PN 232702 (clear polyolefin liner) or PN 276014 (aluminum liner)

- Rotisserie oven for array hybridization
- CombiMatrix CustomArray™ holders for rotisserie oven
- 95°C Heating Block
- Pipettors and nuclease-free tips
- Sterile plastic ware
- Gloves (powder-free)

3. Reagents and materials for single-step target preparation

- Total RNA sample (10 to 100 ng)
- CombiMatrix Influenza A Sub-typing Primer Kit for Single-Step Target Preparation, Product Number 610028
- Nuclease-free water
- 0.4 mM Biotin-14-dCTP, Invitrogen, Cat. #19518-018
- SuperScript™ One-Step RT-PCR System containing the RT/Platinum® Taq Mix (a mixture of SuperScript™ II Reverse Transcriptase and Platinum® Taq DNA Polymerase) and the 2X Reaction Mix (a buffer system optimized for reverse transcription and PCR amplification with Mg²⁺, and deoxyribonucleotide triphosphates), Invitrogen Cat. #10928-034

4. Reagents for agarose gel electrophoresis

- 1X TBE buffer : 0.089 M Tris-borate, 0.89 M Boric acid, 0.02 M EDTA
- Gel loading dye solution
- Gel marker : contain linear double stranded DNA band of 1000, 700, 525, 500, 400, 300, 200, 100 and 50 base pairs (bp) : Bioactive, USA

5. Reagent and materials for hybridization and imaging of influenza A research Microarray 12K

- Nuclease-free water
- 50x Denhardt's solution
- 0.5 M EDTA (pH 8.0)
- 10% Tween-20
- 20x SSPE Buffer
- 1% SDS
- 10x Phosphate Buffered Saline (PBS: 1.37M Sodium Chloride, 0.027M Potassium Chloride, 0.08M Sodium Phosphate dibasic, 0.02M Sodium Phosphate monobasic, pH 7.4; Ambion, Cat.# 9625)

5X PBS-Casein Blocking Buffer, BioFX Laboratories, Cat.# PBSC-0100-01 Biotin detection reagent: Fluorolink™ Cy5®-labeled streptavidin, GE Healthcare/Amersham Biosciences Cat. # PA45001

6. H5 oligonucleotide sequence on microarray for medium and high resolution

| Probe No. | Name | Oligonucleotide sequence |
|-----------|-------------|---------------------------|
| 535 | H5 AY296080 | GTACCCAACAATAAAGAGGAt |
| 536 | H5 AY728894 | CCCAACAATAAAGAGGAGCt |
| 537 | H5 AY296079 | CCAACAATAAAGAGGACCTACt |
| 538 | H5 AY684706 | ATTGTAGTGTAGCTGGATGGt |
| 539 | H5 AY724793 | GAGTTCTTCTGGACAATTTTAAt |
| 540 | H5 AY830774 | CCAATGTGTGACGAATTCAt |
| 541 | H5 AJ621807 | GGATTGTAGTGTAGCTGGAAt |
| 542 | H5 AY651369 | CCCAATGTGTGACGAATTCt |
| 543 | H5 U37179 | CCATTTTGAAAAAATTCAGATAttt |
| 544 | H5 AY296077 | GCACAAACCATTTTGAGAAAAt |
| 545 | H5 AJ867074 | ATTTTGAGAGATTGTAGTGTAGCt |
| 546 | H5 AY590571 | AGAGAGAAGAAGAAAAAAGAGAt |
| 547 | H5 AJ305306 | CACAAACCATTTAGAGAAAATTCt |
| 548 | H5 AY651373 | CCTTTTTCAGGAATGTGGtt |
| 549 | H5 AY651371 | GAAATGTGGTATGGCTTATCAAt |
| 550 | H5 AF164662 | ACAATAAAGAGGACCTACAAt |
| 551 | H5 AF509025 | AGGTTGACACAATAATGGAAt |
| 552 | H5 AY770991 | CTAATTTTGAGAGATTGTAGTgt |
| 553 | H5 AY497095 | ACACAATCATGGAAAAGAATGt |
| 554 | H5 AY651362 | CTATTGAGCAGAATAAACCAttt |
| 555 | H5 AF164659 | ATCCAACAATAAAGAGGACCTc |
| 556 | H5 AY553787 | AAACCATTTTGAGAAAACCTCAAt |
| 557 | H5 AF509031 | AGGTTGACACAATAATGGAGt |
| 558 | H5 AY724795 | CCAACCAAGAAGATCCTTTTgt |
| 559 | H5 AY651326 | CTCCTTTTTCAGAAATGTGGt |
| 560 | H5 AY724793 | ACTTCGAGAGTAATGGAAAAttt |
| 561 | H5 AY497070 | CAAACAATAAAGAGGACCTACAAt |
| 562 | H5 AY497072 | GAACAATAAAGAGGACCTACAAt |
| 563 | H5 U37167 | AAACAATAAAGAGGACCTACAAt |
| 564 | H5 AY497084 | GTAGCTGGATGGCTTCtt |
| 565 | H5 M18001 | CAACAATAAAGAGGACTTACAAt |
| 566 | H5 AY770991 | GCAGAATAAACCATTTTGAGAt |
| 567 | H5 AY684706 | GAGCAGAATAAACCATTTTGAAt |
| 568 | H5 AY830774 | AGAAAATTCAGATCATCCCCt |
| 569 | H5 AY296079 | AGCACAAACCATTTTGAGAt |
| 570 | H5 AY609312 | AAGAACTGAAACACCTATTGAAt |
| 571 | H5 AY651330 | CCTATTGAGCAGAATAAACCAAt |
| 572 | H5 AY770991 | CACCTATTGAGCAGAATAAACc |
| 573 | H5 M10243 | ACAAACCTTTTGAGAAAATTAGt |
| 574 | H5 AF164665 | CACATGCTCAAGACATACTGt |
| 575 | H5 AY497067 | ATTGCAGTGTAGCTGGAt |

7. H5 oligonucleotide sequence on microarray for medium and high resolution

| Probe No. | Name | Oligonucleotide sequence |
|-----------|-------------|---------------------------|
| 576 | H5 AY684706 | GAAAATTCAGATCATCCCAAt |
| 577 | H5 AY684706 | ATGTGTGACGAATTCATCAAt |
| 578 | H5 AY651325 | AGAACTGAAACACCTATTGAGt |
| 579 | H5 AY590570 | GAACTGAAACACCTATTGAGCt |
| 580 | H5 AY075027 | GAGCAGAACAAACCATTTTGt |
| 581 | H5 AY553796 | AAACACCTATTGAGCAGAAAt |
| 582 | H5 AY651330 | CAGAAATGTGGTATGGCttt |
| 583 | H5 AY724785 | AACTTCGAGAGTAATGGAAAtt |
| 584 | H5 AY651345 | CTATGTGTGACGAATTCATCAAt |
| 585 | H5 AY651362 | CCCTCCTTTTTTCAGAAATGtt |
| 586 | H5 AF082042 | CAGTACAAACCATTTTGAGAAAt |
| 587 | H5 U37176 | GCTCAGCATGTCCATACAAt |
| 588 | H5 AY728894 | ACAGTACATACCCAACAATAAt |
| 589 | H5 AY653200 | AGAGCAGGTTGACACAAtt |
| 590 | H5 AF046097 | AATTCAGATCATCCCCAAAAt |
| 591 | H5 AY728894 | ATTCAGATCATCCCCAAAAGt |
| 592 | H5 AY639405 | CAAGAACTGAAACACCTATTGt |
| 593 | H5 AY684706 | ATCATCCCCAAAAGTTCTTGt |
| 594 | H5 AF164665 | AACAATAAAGAAGACCTACAATAAt |
| 595 | H5 AF164661 | CACACATGCTCAAGACATAAt |
| 596 | H5 AY296070 | CTCAAGACATACTGGAAAAAGt |
| 597 | H5 AF290443 | GATTGTAGTGTAGCTGGGtt |
| 598 | H5 AY553807 | CAGATCATCCCCAAAAGTTt |
| 599 | H5 AY651362 | CTGAAACACCTATTGAGCAAt |
| 600 | H5 AF509025 | AATAATGGAAAAGAACGTTACtt |
| 601 | H5 AF216729 | AAACACCTATTGAGCAGAAAt |
| 602 | H5 AY770991 | ATAATGGAAAAGAACGTTACTGt |
| 603 | H5 AY770991 | CAATGTGTGACGAATTCATCt |
| 604 | H5 AY221523 | CTGAGAGATTGTAGTGTAGCt |
| 605 | H5 AY684706 | GTCCAATCATGAAGCCTCAAt |
| 606 | H5 AY651368 | ACTGAAACACCTATTGAGCt |
| 607 | H5 AY651330 | AGCAGGTTGACACAATAATGt |
| 608 | H5 AY651360 | ATTCAGATCATCCCCAAAAt |
| 609 | H5 AY651349 | CCAATCATGAAGCCTCATCt |
| 610 | H5 AY576930 | ATCAATGTGCCGGAATGGt |
| 611 | H5 AY651356 | ACTTATTGAGCAGAATAAACCAAt |
| 612 | H5 AY651330 | CACAATAATGGAAAAGAACGtt |
| 613 | H5 AY651352 | AAACATCTATTGAGCAGAATAAAAt |
| 614 | H5 AY651367 | ATTGTAGTGTAGCTGGGTGt |
| 615 | H5 AY651343 | ACAATAATGGAAAAGAACGttt |
| 616 | H5 AY651362 | ATCATGAAGCCTCATCAGGt |

8. H5 oligonucleotide sequence on microarray for medium and high resolution

| Probe No. | Name | Oligonucleotide sequence |
|-----------|-------------|----------------------------|
| 617 | H5 AY590572 | CAGAAATACCCCTCAAAGAGt |
| 618 | H5 AY576930 | GACGAATTCATCAATGTGCt |
| 619 | H5 AF501235 | ACGAATTCATCAATGTGCCt |
| 620 | H5 AY651330 | AATTGAAACACCTATTGAGCAt |
| 621 | H5 AF501235 | CCTATGTGTGACGAATTCAtt |
| 622 | H5 AY770079 | AATGACCTCTGTTACCCAGt |
| 623 | H5 AY590568 | GATCAGATTTGCATTGGTTACt |
| 624 | H5 AF509032 | CAAAAAGAACAGTGCATACct |
| 625 | H5 AY651362 | CATGCCCAAGACATACTGGt |
| 626 | H5 AY830774 | CCCAAGACATACTGGAAAAGt |
| 627 | H5 AY770991 | AGTGAAGCCTCTAATTTTGAGt |
| 628 | H5 U20460 | GAGAAAATTCAAATCATCCCCt |
| 629 | H5 AY651367 | AGAAAATTCAAATCATCCCCAt |
| 630 | H5 AY609312 | CCAAGACATACTGGAAAAGAt |
| 631 | H5 AY651364 | CTTATCAAAAAGAACAATGCAtt |
| 632 | H5 AY651367 | GGTCCAATCATGAAGCCtt |
| 633 | H5 AY553796 | GGAGTGAAGCCTCTAAAtttt |
| 634 | H5 AY651354 | AGCCAATGACCTCTGTTACt |
| 635 | H5 AF046088 | CCAGATCATCCCCAAAAGtt |
| 636 | H5 AY770079 | AAAGTGATCAGATTTGCATTGt |
| 637 | H5 AY651347 | AAAAGTGATCAGATTTGCAtt |
| 638 | H5 AY651366 | GCTTATCAAAAAGAACAATGCt |
| 639 | H5 AY651324 | CTAATTTTAAGAGATTGTAGTGTAGt |
| 640 | H5 AY590576 | AACTTCTGGTTCTCATGGAt |
| 641 | H5 AY497080 | GCGTAGCTGGATGGCtt |
| 642 | H5 AF509025 | AGAACGTTACTGTTACACATGt |
| 643 | H5 AY585370 | AAAATTCAGATCATACCCAAAAt |
| 644 | H5 AY590576 | AATGTCAAGAACCTTTACGAt |
| 645 | H5 AY651347 | CCCTATGTGTGACGAATTCt |
| 646 | H5 AY651343 | AAAAGAACGTTACTGTTACACt |
| 647 | H5 AY651324 | AAAGACACACAACGGGAAt |
| 648 | H5 AY552000 | AGTCAATGACCTCTGTTACCt |
| 649 | H5 AY651330 | ATGGAGTGAAGCCTCTAAtt |
| 650 | H5 AY830774 | ACTGAAAAGACACACAACt |
| 651 | H5 AY651325 | AAAGTCCTCTTTTTTAGAAATGt |
| 652 | H5 AY553788 | AGAGATTGTAGTGTAGCCGt |
| 653 | H5 AF216737 | GTGATCAGATTTGCATTGGtt |
| 654 | H5 J02160 | CCAGATTTGCATTGGTTACCt |
| 655 | H5 AF102681 | AGCCAATGACCTCTGTTAtt |
| 656 | H5 AY651353 | AAAAGACACACAACGGGAAt |
| 657 | H5 AY646424 | CTATGTGTGACGAATTCACct |

9. H5 oligonucleotide sequence on microarray for medium and high resolution

| Probe No. | Name | Oligonucleotide sequence |
|-----------|--------------|--------------------------|
| 658 | H5 S68489 | GAAAATTCAAATCATCCCCAGt |
| 659 | H5 AY553808 | ATGTGGTATGGCTTATCCA |
| 660 | H5 AF364334 | GAGTGAAGCCTCTCATTTTg |
| 661 | H5 AY651355 | AAGACATACTGGAAAAGACt |
| 662 | H5 AY590573 | ACCCAACAATCAAGAGGAGt |
| 663 | H5 AY651371 | AATGTGCCGGAATGGTc |
| 664 | H5 AY590571 | AGGGAGGATGGCAGGGt |
| 665 | H5 AY651350 | CAGATCATCCCCAAAAATTCt |
| 666 | H5 AJ621807 | CTCAATGTGCCGGAATGGt |
| 667 | H5 AY770991 | CAATGTGCCGGAATGGt |
| 668 | H5 AF364334 | GATCTAAATGGAGTGAAGCCt |
| 669 | H5 AF046097 | AAGCTCAGCATGTCCATa |
| 670 | H5 U20460 | GAGGGATTGTAGTGTAGCt |
| 671 | H5 U20460 | AAACACCTATTGAGCAGTACt |
| 672 | H5 U79451 | AGTAATGAATACCCAACAATAAa |
| 673 | H5 AY770991 | GAACGTTACTGTTACACATGt |
| 674 | H5 ISDN49024 | ACCAGATTTGCATTGGTTACt |
| 675 | H5 AY590572 | GACTGGGCTCAGAAATACc |
| 676 | H5 AY651323 | CCTCCTTTTTTAGAAATGTGGt |
| 677 | H5 AY590573 | AACAGTACATACCCAACAATCt |
| 678 | H5 AY651330 | GGAAAAGACACACAACGGt |
| 679 | H5 AY553796 | AAAAGTTCTTGGTCCAGTc |
| 680 | H5 AY585365 | GCGATCTAAATGGAGTGAAGt |
| 681 | H5 AF102681 | GCCTGAATGGTCTTACATAGt |
| 682 | H5 AF082042 | ATGGAGTGAAGCCTCTCA |
| 683 | H5 AY585365 | ATCAGTCTTGTTAAAAGTGATc |
| 684 | H5 AY651330 | GGAAGCTCTGCGATCTAGt |
| 685 | H5 AF098543 | AATGACCTCTGTTATCCAGGt |
| 686 | H5 AY553796 | GGATGGCTCCTCGGAa |
| 687 | H5 AY075027 | ACACCTATTGAGCAGAACA |
| 688 | H5 AF046097 | CAGAATAAGCCATTTTGAGAA |
| 689 | H5 AY609312 | CATGAAGCCTCATCAGGGt |
| 690 | H5 AY651325 | ATGAAGCCTCATCAGGGGt |
| 691 | H5 AF194169 | CCATGTGTGACGAATTCCt |
| 692 | H5 AY651352 | GAAAATTCAGATCTTCCCCa |
| 693 | H5 AY651367 | CTAAAAGTGATCAGATTTGCA |
| 694 | H5 AY651352 | AGGAATGTGGTATGGCtt |
| 695 | H5 AY590575 | AAAGTTCTTGGTCCAGTCA |
| 696 | H5 AF509037 | ATTGAGACAATAAACCAttt |
| 697 | H5 AY576930 | AAAGCTCTATCAAAATCCAAC |
| 698 | H5 AF509025 | AAATGTGGTATGGCTCATC |
| 699 | H5 AY830774 | GACGAATTCATCAATGTACCG |
| 700 | H5 AY651353 | CTAATTTGAAAGATTGTAGTGA |
| 701 | H5 AY651347 | AGCAGGTTGATACAATAATGG |
| 702 | H5 AY590576 | ACAAGGTCCGACTACAGC |

10. N1 oligonucleotide sequence on microarray for medium and high resolution

| Probe No. | Name | Oligonucleotide sequence |
|-----------|-------------|-----------------------------|
| 1457 | N1 AY553818 | GTATTGAAATACAATGGCATAATAt |
| 1458 | N1 AY553838 | ATACAATGGCATAATAACAGACt |
| 1459 | N1 AY575892 | ATTGAAATACAACGGCATAATAt |
| 1460 | N1 AY553826 | GCATAATAACAGACACTATCAAAt |
| 1461 | N1 AY553812 | AATCAAAATTTGGAGTATCAAATAt |
| 1462 | N1 AY553826 | CCTAATTATCACTATGAGGAATGt |
| 1463 | N1 AY553818 | AATGGCATAATAACAGACACtt |
| 1464 | N1 AY553813 | CAAAATTTGGAGTATCAAATAGGt |
| 1465 | N1 AY553826 | TGGCTGTATTGAAATACAATGt |
| 1466 | N1 AY553838 | CTGTATTGAAATACAATGGCAAt |
| 1467 | N1 AY574188 | CCACTTGAATGCAGAAcT |
| 1468 | N1 AY180836 | TCACTTGAATGCAGAACcT |
| 1469 | N1 AF398869 | CTCACTTGAATGCAGAAcT |
| 1470 | N1 AY553828 | TTGCTTTACTGTAATGACTGAt |
| 1471 | N1 AY553826 | GACACTATCAAGAGTTGGAGt |
| 1472 | N1 AY553816 | ACAGACACTATCAAGAGTTGt |
| 1473 | N1 AY553812 | TGCTTTACTGTAATGACTGACt |
| 1474 | N1 AY553828 | AGTCTGAATGTGCATGTGtt |
| 1475 | N1 AY576928 | TTGACAATTGGAATTTCTGGCt |
| 1476 | N1 AF494254 | CTTGAATGCAGAACcTttt |
| 1477 | N1 AF509092 | ATTGGATCAATCTGTATGGTAAAt |
| 1478 | N1 K01031 | AACCATTGGATCAATCTGTcT |
| 1479 | N1 AY553812 | GCTCCTAATTATCACTATGAGGt |
| 1480 | N1 AY576928 | TCAAGAGTTGGAGGAACAAt |
| 1481 | N1 AY623431 | TGACAATTGGAATTTCTGGCt |
| 1482 | N1 AF028708 | TTGGAATGCAGAACcTttt |
| 1483 | N1 AF509092 | CAATATGGGTTAGTCATTCAAtt |
| 1484 | N1 AY553813 | TATCTTTCAATCAAAATTTGGAGt |
| 1485 | N1 AF057292 | TTTGAATGCAGAACcTttt |
| 1486 | N1 AY651480 | GGATCAATCTGTATGGTAATTGt |
| 1487 | N1 AY553812 | GGGTATCTTTCAATCAAAATTTGt |
| 1488 | N1 Z30275 | CATTCACCATTGACAAGTAAtt |
| 1489 | N1 AY590565 | CGGGCAATTCATCTCTTTGt |
| 1490 | N1 AF305216 | TGGCTGTATTAATAACAACGt |
| 1491 | N1 AY590567 | GGGATGTGTTTGTATAAGAGt |
| 1492 | N1 AY653195 | GGGGATGTGTTTGTATAAGAAt |
| 1493 | N1 AJ416629 | TTGGAATGCAGAACCTTCtt |
| 1494 | N1 AY553826 | ATGTGCATGTGTAATGGCt |
| 1495 | N1 AY553828 | TGGGTATCTTTCAATCAAAAttt |
| 1496 | N1 AY553828 | CCTGTTATCCTGATGCCGt |
| 1497 | N1 AY619960 | AAATTGGAAATATAATCTCAATATGGt |

11. N1 oligonucleotide sequence on microarray for medium and high resolution

| Probe No. | Name | Oligonucleotide sequence |
|-----------|----------------|-----------------------------|
| 1498 | N1 AJ416628 | AACATAATCTCAATATGGGTTAGt |
| 1499 | N1 ISD3BF2A798 | CGGCAATTCATCTCTTTGtt |
| 1500 | N1 AF398872 | CAATTCATCTCTTTGCCct |
| 1501 | N1 AY575883 | CAATCTGTATGGTAATTGGAAt |
| 1502 | N1 AY590565 | TTTGAGTCTGTTGCTTGGtt |
| 1503 | N1 AY574188 | GGTTTGAGTCTGTTGCTTgT |
| 1504 | N1 AF250481 | ATTTGGAATGCAGAACCttt |
| 1505 | N1 AY575881 | GGAACATAATCTCAATATGGGtt |
| 1506 | N1 AY651434 | ATCAATCTGTATGGTAATTGGAAt |
| 1507 | N1 AY553828 | TAAATGGCTCTTGCTTTACtt |
| 1508 | N1 AJ518091 | GGCAATTCATCTCTTTGTTcT |
| 1509 | N1 AY609314 | ATAACCATCGGATCAATCTGt |
| 1510 | N1 M73976 | GGCAATTCATCTCTTTGTCCt |
| 1511 | N1 AF509092 | GAACATAATCTCAATATGGGttt |
| 1512 | N1 AJ410876 | AAATTGGAAACATAATCTCAATATGt |
| 1513 | N1 AJ410561 | CATTTGGAATGCAGAACCtt |
| 1514 | N1 AF509114 | ATTGGATCAATCTGTATGATAAttt |
| 1515 | N1 AY553813 | GTGGAGTTTTTCGGAGACAt |
| 1516 | N1 AY660557 | TTATAAGAGAGCCATTCATCTcT |
| 1517 | N1 AY585409 | GTGGATGGGCTATATACAGtt |
| 1518 | N1 AJ410560 | CAAGGTTTGAGTCTGTTGcT |
| 1519 | N1 AY651476 | CCCATATAACTCAAGGTTTGAAt |
| 1520 | N1 AJ416627 | ATAATCTCAATATGGGTTAGCCt |
| 1521 | N1 AJ410875 | TAGTGGATGGGCTATATACAGt |
| 1522 | N1 AY575883 | CTCAATATGGGTTAGTCATTCAt |
| 1523 | N1 AY585403 | ATCTCAATATGGGTTAGTCAttt |
| 1524 | N1 AY646176 | CCCACTTGGAAATGCAGAt |
| 1525 | N1 AJ412689 | TGGAATGCAGAACCCTTcTt |
| 1526 | N1 AY651473 | CATAATCTCAATATGGGTTAGTCt |
| 1527 | N1 AY590567 | ATTAGCGGGCAATTCATCtt |
| 1528 | N1 AJ410560 | GATCAATCTGTATGGTAGTTGGt |
| 1529 | N1 AJ410878 | TCTCCATATAACTCAAGGTTTgT |
| 1530 | N1 AY553826 | AGAGTTGGAGGAATAACATACt |
| 1531 | N1 AY576928 | TTGAGTCTGTTGCTTGGtt |
| 1532 | N1 AJ416625 | GGAAACATAATCTCAATATGGGt |
| 1533 | N1 AY585403 | ATTGGAAACATAATCTCAATATGGt |
| 1534 | N1 AY553812 | CTGTTATCCTGATGCCGGt |
| 1535 | N1 AJ410558 | AATAATTAGCTTAATGTTACAAATTGt |
| 1536 | N1 AF250481 | GGATGGGCTATATACAGTAAAt |
| 1537 | N1 AY553828 | TATCACTATGAGGAATGCTCCt |
| 1538 | N1 D31947 | GTTCTCACTTGGAAATGCAGt |

12. N1 oligonucleotide sequence on microarray for medium and high resolution

| Probe No. | Name | Oligonucleotide sequence |
|-----------|-------------|----------------------------|
| 1539 | N1 AY633118 | CTCTCACTTGGAAATGCAGGt |
| 1540 | N1 AY553814 | TTAAATCAGTCGAATTGAATGCt |
| 1541 | N1 AF389120 | GCAATTCATCTCTTTGTCCct |
| 1542 | N1 AY651473 | TATGGGTTAGTCATTCAATTCat |
| 1543 | N1 AY575881 | GTTAGCTTGATGTTACAAATTGt |
| 1544 | N1 AJ410875 | ATTCATCTCATGCTCCCACt |
| 1545 | N1 AY651462 | ATAGTTAGCTTAATGTTACAAATTGt |
| 1546 | N1 AY526746 | TGGATGCTCCTAATTATCACct |
| 1547 | N1 AY701754 | GATAATTGGCATGGCTCGt |
| 1548 | N1 AY059485 | TAATCTCAATATGGGTTAGCCt |
| 1549 | N1 AY576928 | ATGCTCCCCTTGGAAATGt |
| 1550 | N1 AY651473 | TAGCGGGCAATTCATCTCt |
| 1551 | N1 AY590567 | GAGTCTGTTGCTTGGTCat |
| 1552 | N1 AY790269 | TCTCACTTGGAAATGCAGGt |
| 1553 | N1 AY651449 | GATCAATCTGTATGGTAACTGGt |
| 1554 | N1 AY633286 | GTGGATGGGCTATATACAGCt |
| 1555 | N1 AY553816 | AGATCTTCAAAAATGGAAAAGGt |
| 1556 | N1 K01003 | GATCAATCTGTATGGTAGTCGt |
| 1557 | N1 AJ412692 | GTGTTTGTCTATAAGAGAGCCt |
| 1558 | N1 K01033 | AATATTATCTCAATATGGGTCAGCt |
| 1559 | N1 AY623431 | TTCATCTCATGCTCCCACt |
| 1560 | N1 AY575884 | CGTCAAAGACAGAAGCCct |
| 1561 | N1 AY526746 | CTGACGGACCAAGTAATGGt |
| 1562 | N1 AY779049 | GTTAGCTTAATGTTACAAATTGGt |
| 1563 | N1 AY651479 | GAGTCTGTTGCTTGGTCGt |
| 1564 | N1 AJ410564 | GCAGGCAATTCATCTCttt |
| 1565 | N1 AY590567 | TACAGTAAGGACAACAGTATAAt |
| 1566 | N1 AF250359 | CTCACTTGGAAATGCAGGAt |
| 1567 | N1 AY553816 | TCAAGAGTTGGAGGAATAACt |
| 1568 | N1 AY653195 | CATCTCATGCTCCCACttt |
| 1569 | N1 K02252 | TATTACAAATTGGAAACATAATCTCt |
| 1570 | N1 AY651434 | TGCTCTCACTTGGAAATGCt |
| 1571 | N1 AY553814 | GAATTGAATGCTCCTAATTATCAt |
| 1572 | N1 AY535028 | GATCGGGAGAACCAAAAAGCt |
| 1573 | N1 AY553812 | TTAAATCAGTCGAATTGGATGt |
| 1574 | N1 AY651482 | GCGGGCAATTCATCTCtt |
| 1575 | N1 AY221546 | ATCAATCTGTATGGTAGTTGGt |
| 1576 | N1 AY651434 | GGGGGATGTGTTTGTTAAtt |
| 1577 | N1 AY585410 | ATAGTTAGCTTGATGTTACAAAtt |
| 1578 | N1 AY553826 | TGTAAATGGCTCTTGCttt |
| 1579 | N1 AF509097 | TAGCTTAATGTTACAAATTGGGt |

13. N1 oligonucleotide sequence on microarray for medium and high resolution

| Probe No. | Name | Oligonucleotide sequence |
|-----------|-------------|----------------------------|
| 1580 | N1 AJ518095 | AATTCATCTCTTTGTTCTATCAGt |
| 1581 | N1 AY553826 | GAAAAAGGGAAAGTGGTTAAAt |
| 1582 | N1 AF057292 | ATTCATCTCATGCTCCCAtt |
| 1583 | N1 AJ410557 | AAGGGGATGTGTTTGTATGt |
| 1584 | N1 AY623431 | GACCGTCAAAGACAGAAGCt |
| 1585 | N1 AY553813 | TTGGCATGGCTCAAATCGt |
| 1586 | N1 AF208598 | TACAAATTGGAAACATAATCTCAt |
| 1587 | N1 AY526746 | AAAAGGGAAAGTGGTTAAATCt |
| 1588 | N1 AY553818 | TGGCTCTTGCTTTACTGtt |
| 1589 | N1 AY646425 | AATAGTTAGCTTGATGTTACAAAt |
| 1590 | N1 AY575888 | ATCTGTATGGTAATTGGAATAGtt |
| 1591 | N1 AY574188 | CAAGGGGGATGTGTTTGtt |
| 1592 | N1 AY261521 | TCATGCTCTCACTGGAAAt |
| 1593 | N1 AY590564 | TCTTGTTATCCTGATGCCGt |
| 1594 | N1 AY575884 | TTGATGTTACAAATTGGGAACt |
| 1595 | N1 AF208598 | CACTTGGAATGCAGGACct |
| 1596 | N1 AF250362 | GATGTTACAAATTGGAAATATAATCt |
| 1597 | N1 AY619960 | TAAAGACAGAAGCCCTTATAGt |
| 1598 | N1 AY590564 | CATATTACTACTATGAGGAATGttt |
| 1599 | N1 AY649383 | TTAATGTTACAAATTGGGAACtt |
| 1600 | N1 AY553813 | AATCCACGCCCAATGt |
| 1601 | N1 AY576928 | GTTCACTCATGCTCCCAt |
| 1602 | N1 AJ410565 | GGTCAATCTGTATGGTAATTGt |
| 1603 | N1 AY122326 | ATCAATCTGTATGGTAGTCGGt |
| 1604 | N1 AJ410564 | TCATCTCTTTGCCCTATTAGtt |
| 1605 | N1 AJ410558 | CTATTAGTGGATGGGCTATATACt |
| 1606 | N1 AY651482 | GTAACATTAGCGGGCAAtt |
| 1607 | N1 AY770992 | GGTTAGCTTAATGTTACAAATTGt |
| 1608 | N1 AF398422 | ATAATCTCAATATGGGTTACTCAt |
| 1609 | N1 AY576928 | CTCCCATATAACTCAAGGtt |
| 1610 | N1 AY649383 | ATCAATCTGTATGGTAACTGGt |
| 1611 | N1 AY553813 | GGCCATGGGTATCTTTCAt |
| 1612 | N1 AF036357 | GTTACAAATTGGAAACATAATATCAt |
| 1613 | N1 AF046081 | TTATAAGAGAACCATTCTCATt |
| 1614 | N1 AY553812 | TGCATGTGTAATGGCTCt |
| 1615 | N1 AJ518095 | AGTGGATGGGCTATATACACt |
| 1616 | N1 AF144304 | ATAATTAGCTTGATGTTACAAAtt |
| 1617 | N1 AY180836 | CAGTGGATGGGCTATATACAt |
| 1618 | N1 AY585409 | TATATACAGTAAGGACAACGGt |
| 1619 | N1 AY518363 | GGGGATGTGTTTGTATTAGAt |
| 1620 | N1 AY651467 | ACCGTCAAAGACAGAAGCt |
| 1621 | N1 AY651479 | GATCAATCTGTATGATAATTGGAt |
| 1622 | N1 AF046081 | ACATTAGCGGGCAATTCCt |
| 1623 | N1 AY651437 | TTCATCTCATGCTCCCAttt |
| 1624 | N1 AY590567 | CAGTTGGTTGACAATTGGAAt |

14. Distribution and signal intensity of the oligoneucleotides used to detect H5N1 sample no.11

| Group | Mean | N | SD | Minimum | Maximum |
|-----------|-------------|------------|----------------|------------|-------------|
| H1 | 633 | 168 | 126.2 | 350 | 1187 |
| H10 | 634 | 50 | 95.23 | 361 | 820 |
| H11 | 634 | 50 | 121.41 | 346 | 924 |
| H12 | 623 | 50 | 137.12 | 337 | 1044 |
| H13 | 625 | 50 | 109.98 | 424 | 870 |
| H14 | 673 | 50 | 107.73 | 422 | 899 |
| H15 | 645 | 50 | 110.9 | 412 | 892 |
| H16 | 478 | 18 | 138.81 | 232 | 724 |
| H2 | 602 | 128 | 111.8 | 352 | 1133 |
| H3 | 581 | 168 | 124.76 | 216 | 1371 |
| H4 | 598 | 50 | 108.32 | 358 | 1045 |
| H5 | 3211 | 168 | 1675.88 | 576 | 7569 |
| H6 | 599 | 50 | 117.64 | 280 | 858 |
| H7 | 600 | 168 | 106.06 | 249 | 1006 |
| H8 | 649 | 50 | 108.52 | 382 | 911 |
| H9 | 648 | 168 | 215.04 | 193 | 1912 |
| N1 | 2677 | 168 | 2055.97 | 181 | 8348 |
| N2 | 600 | 168 | 289.92 | 271 | 2268 |
| N3 | 541 | 50 | 102.02 | 358 | 734 |
| N4 | 585 | 50 | 144.83 | 235 | 916 |
| N5 | 597 | 50 | 118.56 | 303 | 833 |
| N6 | 585 | 50 | 103.28 | 336 | 865 |
| N7 | 590 | 168 | 157.98 | 288 | 1729 |
| N8 | 559 | 50 | 153.92 | 317 | 1169 |
| N9 | 614 | 50 | 126.63 | 295 | 962 |
| Total | 958 | 2240 | 1119.15 | 181 | 8348 |

15. Distribution and signal intensity of the oligoneucleotides used to detect H5N1 sample no.17

| Group | Mean | N | SD | Minimum | Maximum |
|-----------|-------------|------------|----------------|------------|-------------|
| H1 | 632 | 168 | 133.75 | 255 | 1133 |
| H10 | 632 | 50 | 100.43 | 403 | 842 |
| H11 | 608 | 50 | 117.44 | 304 | 906 |
| H12 | 604 | 50 | 124.62 | 256 | 877 |
| H13 | 618 | 50 | 118 | 347 | 898 |
| H14 | 662 | 50 | 108.82 | 480 | 855 |
| H15 | 666 | 50 | 134.25 | 323 | 978 |
| H16 | 466 | 18 | 106.16 | 327 | 719 |
| H2 | 598 | 128 | 109.77 | 319 | 823 |
| H3 | 581 | 168 | 128.21 | 230 | 885 |
| H4 | 627 | 50 | 117.07 | 385 | 878 |
| H5 | 2403 | 168 | 1444.01 | 425 | 7190 |
| H6 | 588 | 50 | 123.32 | 336 | 860 |
| H7 | 603 | 168 | 114.79 | 250 | 968 |
| H8 | 642 | 50 | 121.79 | 376 | 915 |
| H9 | 618 | 168 | 110.29 | 332 | 896 |
| N1 | 1789 | 168 | 1392.01 | 203 | 6208 |
| N2 | 573 | 168 | 166.37 | 267 | 1451 |
| N3 | 556 | 50 | 122.94 | 298 | 847 |
| N4 | 590 | 50 | 108.87 | 301 | 824 |
| N5 | 594 | 50 | 142.96 | 254 | 833 |
| N6 | 586 | 50 | 122 | 321 | 844 |
| N7 | 579 | 168 | 142.19 | 269 | 1225 |
| N8 | 563 | 50 | 166.01 | 234 | 1215 |
| N9 | 615 | 50 | 148.97 | 247 | 1033 |
| Total | 826 | 2240 | 783.55 | 203 | 7190 |

16. Distribution and signal intensity of the oligoneucleotides used to detect H5N1 sample no.25

| Group | Mean | N | SD | Minimum | Maximum |
|-----------|-------------|------------|----------------|------------|--------------|
| H1 | 733 | 168 | 183.02 | 367 | 1290 |
| H10 | 767 | 50 | 157 | 443 | 1142 |
| H11 | 784 | 50 | 215.67 | 424 | 1289 |
| H12 | 743 | 50 | 225.64 | 321 | 1305 |
| H13 | 729 | 50 | 158.55 | 446 | 1090 |
| H14 | 828 | 50 | 176.41 | 497 | 1313 |
| H15 | 743 | 50 | 167.32 | 281 | 1201 |
| H16 | 530 | 18 | 217.02 | 187 | 1083 |
| H2 | 735 | 128 | 198.54 | 248 | 1577 |
| H3 | 677 | 168 | 176.13 | 207 | 1122 |
| H4 | 688 | 50 | 127.96 | 404 | 1025 |
| H5 | 5202 | 168 | 2634.97 | 608 | 12071 |
| H6 | 695 | 50 | 157.19 | 342 | 1026 |
| H7 | 717 | 168 | 176.5 | 301 | 1345 |
| H8 | 754 | 50 | 139.94 | 410 | 1194 |
| H9 | 737 | 168 | 214.91 | 268 | 1721 |
| N1 | 2819 | 168 | 2220.19 | 291 | 8305 |
| N2 | 718 | 168 | 335 | 364 | 2322 |
| N3 | 639 | 50 | 175.41 | 310 | 1088 |
| N4 | 731 | 50 | 213.25 | 367 | 1215 |
| N5 | 710 | 50 | 208.79 | 327 | 1267 |
| N6 | 656 | 50 | 158.19 | 310 | 1007 |
| N7 | 710 | 168 | 227.66 | 214 | 2133 |
| N8 | 685 | 50 | 250.44 | 284 | 1506 |
| N9 | 731 | 50 | 182.25 | 293 | 1333 |
| Total | 1213 | 2240 | 1586.91 | 187 | 12071 |

17. Distribution and signal intensity of the oligoneucleotides used to detect H5N1 sample no.27

| Group | Mean | N | SD | Minimum | Maximum |
|-----------|-------------|------------|----------------|------------|--------------|
| H1 | 769 | 168 | 211.44 | 229 | 2064 |
| H10 | 747 | 50 | 152.73 | 419 | 1155 |
| H11 | 798 | 50 | 210.24 | 422 | 1336 |
| H12 | 775 | 50 | 228.75 | 393 | 1422 |
| H13 | 775 | 50 | 159.46 | 378 | 1211 |
| H14 | 833 | 50 | 158.69 | 600 | 1291 |
| H15 | 790 | 50 | 184.23 | 464 | 1322 |
| H16 | 567 | 18 | 218.81 | 204 | 1005 |
| H2 | 739 | 128 | 150.94 | 312 | 1240 |
| H3 | 701 | 168 | 167.52 | 309 | 1406 |
| H4 | 734 | 50 | 133.35 | 452 | 1011 |
| H5 | 6463 | 168 | 3234.05 | 585 | 13182 |
| H6 | 750 | 50 | 158.71 | 350 | 1059 |
| H7 | 733 | 168 | 145.17 | 317 | 1151 |
| H8 | 779 | 50 | 164.42 | 456 | 1248 |
| H9 | 812 | 168 | 282.57 | 296 | 2450 |
| N1 | 4455 | 168 | 3754.27 | 414 | 13274 |
| N2 | 812 | 168 | 711.72 | 236 | 4921 |
| N3 | 695 | 50 | 160.24 | 420 | 1140 |
| N4 | 727 | 50 | 180.68 | 246 | 1143 |
| N5 | 717 | 50 | 176.18 | 313 | 1113 |
| N6 | 710 | 50 | 169.6 | 304 | 1173 |
| N7 | 741 | 168 | 440.21 | 253 | 5869 |
| N8 | 745 | 50 | 458.69 | 362 | 3705 |
| N9 | 746 | 50 | 187.17 | 355 | 1259 |
| Total | 1461 | 2240 | 2210.65 | 204 | 13274 |

18. Distribution and signal intensity of the oligoneucleotides used to detect H5N1 sample no.31

| Group | Mean | N | SD | Minimum | Maximum |
|-----------|-------------|------------|----------------|------------|--------------|
| H1 | 716 | 168 | 266.48 | 324 | 2787 |
| H10 | 714 | 50 | 172.58 | 313 | 1128 |
| H11 | 821 | 50 | 441.54 | 338 | 2633 |
| H12 | 730 | 50 | 225.74 | 318 | 1378 |
| H13 | 702 | 50 | 191.76 | 285 | 1300 |
| H14 | 759 | 50 | 208.19 | 344 | 1394 |
| H15 | 743 | 50 | 185.71 | 319 | 1250 |
| H16 | 486 | 18 | 211.2 | 160 | 897 |
| H2 | 699 | 128 | 218.18 | 351 | 1960 |
| H3 | 628 | 168 | 188.29 | 131 | 1146 |
| H4 | 678 | 50 | 138.81 | 476 | 1123 |
| H5 | 7869 | 168 | 4248.99 | 451 | 16066 |
| H6 | 665 | 50 | 185.66 | 404 | 1138 |
| H7 | 653 | 168 | 170.1 | 313 | 1061 |
| H8 | 725 | 50 | 191.73 | 372 | 1281 |
| H9 | 743 | 168 | 262.21 | 351 | 2022 |
| N1 | 5739 | 168 | 4565.9 | 226 | 15485 |
| N2 | 701 | 168 | 489.08 | 144 | 2944 |
| N3 | 613 | 50 | 181.07 | 367 | 1112 |
| N4 | 665 | 50 | 217.32 | 296 | 1095 |
| N5 | 688 | 50 | 207.99 | 352 | 1267 |
| N6 | 643 | 50 | 187.67 | 311 | 1135 |
| N7 | 767 | 168 | 639.57 | 187 | 4908 |
| N8 | 719 | 50 | 718.79 | 182 | 5539 |
| N9 | 697 | 50 | 215.29 | 284 | 1339 |
| Total | 1616 | 2240 | 2814.06 | 131 | 16066 |

19. Distribution and signal intensity of the oligoneucleotides used to detect H5N1 sample no.37

| Group | Mean | N | SD | Minimum | Maximum |
|--------------|--------------|------------|---------------|----------------|----------------|
| H1 | 837 | 168 | 358.17 | 303 | 4020 |
| H10 | 852 | 50 | 302.56 | 429 | 2473 |
| H11 | 969 | 50 | 589.95 | 354 | 3648 |
| H12 | 878 | 50 | 354.54 | 303 | 2072 |
| H13 | 818 | 50 | 219.88 | 381 | 1545 |
| H14 | 888 | 50 | 232.25 | 349 | 1607 |
| H15 | 866 | 50 | 241.95 | 400 | 1544 |
| H16 | 540 | 18 | 242.74 | 140 | 985 |
| H2 | 819 | 128 | 380.36 | 79 | 3526 |
| H3 | 719 | 168 | 232.94 | 215 | 1405 |
| H4 | 785 | 50 | 193.74 | 514 | 1501 |
| H5 | 11104 | 168 | 4941.8 | 375 | 20898 |
| H6 | 774 | 50 | 241.91 | 348 | 1496 |
| H7 | 777 | 168 | 214.15 | 345 | 1555 |
| H8 | 829 | 50 | 253.46 | 417 | 1763 |
| H9 | 872 | 168 | 380.3 | 233 | 2984 |
| N1 | 6138 | 168 | 4898.2 | 291 | 15887 |
| N2 | 813 | 168 | 572.46 | 278 | 3571 |
| N3 | 732 | 50 | 256.72 | 329 | 1401 |
| N4 | 782 | 50 | 271.01 | 154 | 1445 |
| N5 | 774 | 50 | 259.19 | 229 | 1479 |
| N6 | 734 | 50 | 202.64 | 347 | 1233 |
| N7 | 826 | 168 | 521.97 | 205 | 5238 |
| N8 | 776 | 50 | 560.82 | 241 | 4309 |
| N9 | 806 | 50 | 301.19 | 289 | 2016 |
| Total | 1981 | 2240 | 3526.55 | 79 | 20898 |

20. Distribution and signal intensity of the oligoneucleotides used to detect H5N1 sample no.47

| Group | Mean | N | SD | Minimum | Maximum |
|-----------|-------------|------------|----------------|------------|--------------|
| H1 | 670 | 168 | 220.83 | 288 | 2450 |
| H10 | 654 | 50 | 130.03 | 366 | 930 |
| H11 | 698 | 50 | 198 | 426 | 1259 |
| H12 | 651 | 50 | 205.03 | 221 | 1271 |
| H13 | 657 | 50 | 146.06 | 430 | 986 |
| H14 | 713 | 50 | 161.18 | 446 | 1177 |
| H15 | 670 | 50 | 175.65 | 308 | 1204 |
| H16 | 480 | 18 | 159.25 | 239 | 763 |
| H2 | 632 | 128 | 148.33 | 155 | 1092 |
| H3 | 597 | 168 | 146.86 | 238 | 999 |
| H4 | 602 | 50 | 119.89 | 343 | 988 |
| H5 | 5652 | 168 | 3116.73 | 529 | 13028 |
| H6 | 621 | 50 | 154.65 | 305 | 978 |
| H7 | 615 | 168 | 129.31 | 273 | 1035 |
| H8 | 668 | 50 | 150.95 | 429 | 1104 |
| H9 | 787 | 168 | 917.97 | 296 | 7202 |
| N1 | 4668 | 168 | 3546.97 | 249 | 14235 |
| N2 | 684 | 168 | 629.73 | 286 | 4433 |
| N3 | 593 | 50 | 169.05 | 288 | 1111 |
| N4 | 612 | 50 | 177.26 | 276 | 945 |
| N5 | 619 | 50 | 152.69 | 319 | 956 |
| N6 | 592 | 50 | 145.93 | 317 | 989 |
| N7 | 649 | 168 | 349.37 | 163 | 2996 |
| N8 | 614 | 50 | 438.89 | 275 | 3470 |
| N9 | 631 | 50 | 177.08 | 228 | 1196 |
| Total | 1328 | 2240 | 2101.36 | 155 | 14235 |

21. Distribution and signal intensity of the oligoneucleotides used to detect H5N1 sample no.48

| Group | Mean | N | SD | Minimum | Maximum |
|-----------|-------------|------------|----------------|------------|--------------|
| H1 | 766 | 168 | 239.38 | 341 | 2828 |
| H10 | 752 | 50 | 130.14 | 408 | 1100 |
| H11 | 785 | 50 | 206.19 | 429 | 1309 |
| H12 | 748 | 50 | 212.62 | 412 | 1385 |
| H13 | 757 | 50 | 171.1 | 419 | 1254 |
| H14 | 827 | 50 | 159.78 | 479 | 1155 |
| H15 | 752 | 50 | 172.33 | 417 | 1233 |
| H16 | 500 | 18 | 213.1 | 156 | 833 |
| H2 | 730 | 128 | 160.67 | 250 | 1222 |
| H3 | 684 | 168 | 189.8 | 274 | 1869 |
| H4 | 853 | 50 | 192.11 | 537 | 1339 |
| H5 | 6297 | 168 | 3689.5 | 627 | 15047 |
| H6 | 707 | 50 | 149.59 | 417 | 1028 |
| H7 | 691 | 168 | 152.06 | 267 | 1065 |
| H8 | 769 | 50 | 154.43 | 329 | 1098 |
| H9 | 876 | 168 | 917.06 | 158 | 7031 |
| N1 | 4726 | 168 | 3858.87 | 369 | 13729 |
| N2 | 764 | 168 | 792.83 | 249 | 5747 |
| N3 | 655 | 50 | 171.44 | 424 | 1123 |
| N4 | 712 | 50 | 208.72 | 264 | 1117 |
| N5 | 711 | 50 | 156.95 | 363 | 1066 |
| N6 | 697 | 50 | 166.2 | 290 | 1025 |
| N7 | 698 | 168 | 259.81 | 219 | 2789 |
| N8 | 943 | 50 | 397.82 | 438 | 2682 |
| N9 | 733 | 50 | 193.14 | 213 | 1354 |
| Total | 1463 | 2240 | 2292.29 | 156 | 15047 |

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