

Electrochemical Microarray as a Rapid Tool for Identification of Mutations in Influenza Virus Genes

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Influenza viruses, as etiological agent of acute respiratory-related diseases, cause seasonal epidemics and/or less frequently pandemics with substantial mortality across the world. They display high mutation rates and complex evolutionary patterns inducing often significant resistance to antiviral drugs. We considered an electrochemical detection method, so called CombiMatrix ElectraSense core technology, to create one influenza array containing probes of all genes encodable by different subtypes of influenza type A genome. We detected particularly: neuraminidase, hemagglutinin, nucleocapsid protein, matrix protein 1, matrix protein 2, polymerase PB1, polymerase PA and nuclear export protein. Moreover, we succeeded to detect *in vitro* prepared mutations of the mentioned genes and it was found that the suggested array might be a suitable method not only for detection of the expression of the genes but also for detection of their mutations.

Keywords: Influenza Virus; Microarray; Mutation Sequence; Antiviral Drug; Electrochemistry

1. INTRODUCTION

Influenza is an infectious disease caused by ssRNA viruses of the family *Orthomyxoviridae*, and is considered one of the life threatening infectious diseases. Every year, approximately 10-20% of the world's population is infected by influenza viruses, therefore resulting to a significant number of outpatients and inpatients accompanied by substantial economic burden both on health care systems and society [1,2]. The risk of complications following influenza, e.g. lower respiratory tract infection, admission to hospital, and death, vary depending on several factors, such as age and the type of

comorbidity with bacterial infection that may be present. There is a significant morbidity through the all age groups of the human population and higher mortality in high-risk group (children, adults over 60 years old, patients with chronic illnesses and pregnant women). During the influenza A (H1N1) pandemic in 2009, pregnant women were at risk for severe influenza illness [3,4]. Moreover, the mentioned pandemic disproportionately affected children and resulted in a substantially increased number of hospitalizations and deaths among them [5,6]. The threat of an influenza pandemic onset, which could be virulent and highly contagious, has motivated an escalating research effort of identifying transmissible genotypes of avian influenza viruses that cross over into the human population (avian-human transmission) and sustain human-human transmission. In May 2013, the world's first human-infected case of H6N1 bird flu was reported in Taiwan. A novel avian-origin influenza A(H6N1) was confirmed by the National Influenza Centre, Centers for Disease Control, Taiwan, and the patient has already recovered [7]. Infections with H7 subtypes, such as H7N2, H7N3, and H7N7, usually related to outbreaks of poultry have been reported to be transmitted to human in several countries [8-10], whereas China reported high severity and fatality of human infections caused by avian influenza A(H7N9) [9,11].

Each year influenza A viruses cause significant mortality worldwide. Antiviral inhibitors have become important alternate means as constraints of influenza spreading. The neuraminidase inhibitors and the M2 protein blockers belong to the contemporary antivirals. The neuraminidase inhibitors (NAIs) are the most commonly used class of influenza antiviral drugs for the treatment of infected patients [12]. The M2 protein blockers are effective only against influenza A viruses, and the resistance arises rapidly. Many of the H5N1 strains circulating in Southeast Asia, especially in Vietnam and Thailand, are also resistant to M2 inhibitors [13-15]. The most common NAIs drugs are zanamivir, oseltamivir, peramivir, and a long-acting NAIs, laninamivir [16]. However, mutations in the influenza viruses induce resistance toward antiviral drugs. Resistance was more likely to arise to oseltamivir, due to the structural changes needed for oseltamivir to bind with high affinity [17]. The resistance toward oseltamivir was reported for the first time in A(H3N2) virus strains during the 2011-2012 influenza season [18]. Detection of one mutation in the virus neuraminidase (NA) gene within the first 2 days of oseltamivir treatment, in the first reported human infection by avian A(H7N9) influenza virus, raised concern about the emergence of resistance occurrence during the treatment with neuraminidase inhibitors [19]. In spite of the resistance toward adamantane antiviral drugs evidenced in 2011 for more than 99% of influenza A viruses circulating in the Asia-Pacific region, over, the large majority of influenza A and B strains remained susceptible toward the neuraminidase inhibitors oseltamivir and zanamivir [20]. Recently, a study reported that an I223R/H275Y double mutant of neuraminidase (NA) creates a multidrug resistant form of the pandemic influenza A (H1N1) virus [20].

Based on the above-mentioned facts, new molecular techniques are required for rapid detection of the influenza mutation to monitor its spreading through the population. Application of oligonucleotide microarrays in different areas of molecular biology and clinical studies has been rapidly growing during the last decade [21-24]. The microarrays are currently combined with fluorescent detection. However, the underlying electronics employed for the oligonucleotide synthesis can also be utilized for electrochemical detection (CombiMatrix ElectraSense™) of target molecules bound to the microarray [25-29]. CombiMatrix ElectroSense™ is designed to provide data on the

presence of biological material deposited above the electrodes of a CustomArray microarray after a material sample has been hybridized on the microarray. The ElectraSense reader is able to detect electric current values for an array of 12,544 electrodes [30]. The electrochemical detection is based on the 3,3',5,5'-tetramethylbenzidine (TMB) oxidation reaction catalyzed by horseradish peroxidase (HRP), generated a current flux. (Fig. 1). The CombiMatrix influenza detection system is effective for influenza A and B subtype analysis [28,30]. In this study, the electrochemical method (CombiMatrix ElectraSense™ platform) was used to develop nucleic acid assays for highly accurate genotyping of a variety of genes in influenza A virus and the mutations detection within the sequences of the different genes belonging to influenza virus.

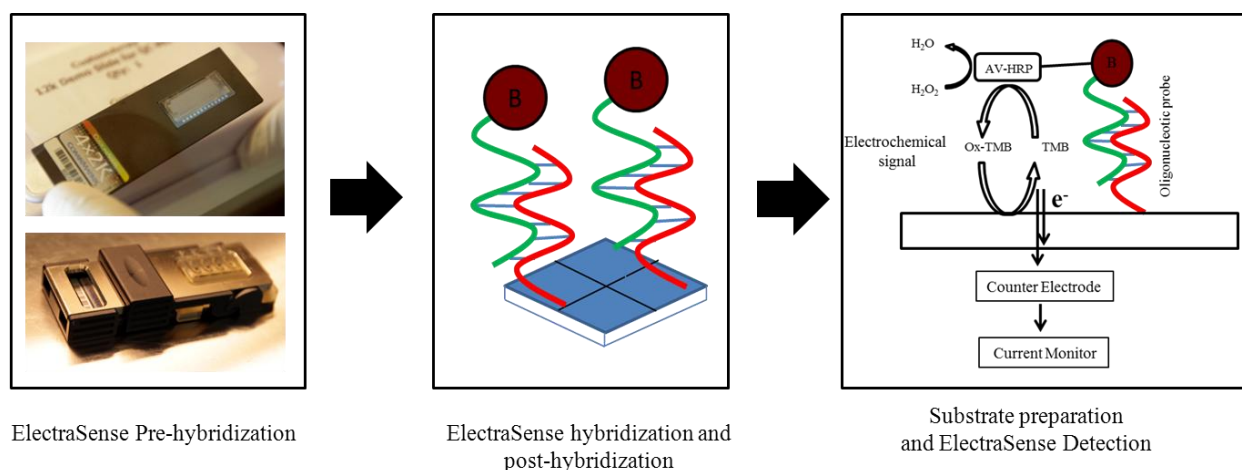


Figure 1. Scheme of different steps for ElectroSense CombiMatrix: Pre-hybridization (preparation of the chip to be hybridized), hybridization and post-hybridization (incubated chip in hybridization oven with rotation and hybridization wash), substrate preparation (labeling biotin (B) with avidin horseradish peroxidase (AV-HRP) and ElectroSense detection). The AV-HRP binds to the biotinylated targets that have been hybridized to influenza oligonucleotides probes (ODN). HRP catalyzes the oxidation of a substrate, TMB (3,3',5,5'-tetramethylbenzidine). The oxidized TMB is subsequently reduced at the surface of the electrode generating a current flux. The ElectraSense Reader performs amperometric detection of this current flux for each individual spot.

2. EXPERIMENTAL PART

2.1 Chemicals

Buffers or standard solutions for hybridization were prepared daily by dilution of the precursor stock solutions. The chemicals for standard operating procedure were prepared according the manufacturer's instructions and were purchased from CombiMatrix ElectraSense™ (USA). All working solutions as buffers were prepared using deionised water obtained by an reverse osmosis apparatus, Aqual 25 (Czech Republic) and subsequently autoclaved (120°C, 1 h). The obtained deionised water was further purified by using the apparatus MiliQ Direct QUV equipped with an UV

lamp. The final water resistance was 18 M Ω . The pH was measured using a pH meter WTW inoLab (Weilheim, Germany). During the hybridization and electrochemical detection, trials the following reagents were used: Sodium chloride, potassium chloride, sodium phosphate dibasic and sodium phosphate monobasic and tween-20 (Sigma–Aldrich, St. Louis, USA). Detection Kit used in the hybridization was CombiMatrix ElectraSense™ and contained Biotin Blocking Solution, Biotin Wash Solution, TMB Rinse, and TMB Substrate (CombiMatrix Corporation, USA). Oligonucleotide targets labeled on the 3' end with biotin was obtained from Metabions International AG (Martinsried, Germany). For the preparation of 3' Biotin Oligonucleotide probes, standard solutions in the nuclease-free water were used. Each oligonucleotide probe stock had a concentration of 100 μ M.

2.2 Designing and Preparation of Oligonucleotides Probes for Microarrays

Detail description of sequences are given in supplementary data. The sequences showed in Table S1 were used for probe design Genbank® number (the NIH genetic sequence database) of genes. A total of 80 oligonucleotide probes were synthesized and printed onto the CombiMatrix CustomArray™ 4 \times 2K microarray support to generate the prototype chips for the different influenza viruses and different sequences of genes of influenza viruses. Likewise, the optimized chips were designed, using a subset of 10 probes of either type for each of the viruses that had been selected based on the specificity and intensity of the positive signals. 4 \times 2K microarray chip has four identical array sectors that can be hybridized with different target samples using the sectorized hybridization cap provided. Biotinylated-oligonucleotide probes, which were hybridized on arrays, containing probe specific sequences unique to influenza subtypes and other sequences with one mutation to influenza subtypes, are shown in Table S2. Each 100 μ M oligonucleotide probe was prepared with nuclease-free water. For this study, a mixture of 80 oligonucleotide probes (3' Biotin Oligonucleotides Mix) was prepared, with a final concentration of each oligonucleotide of 100 nM. To study the changing signal due to the increase of sequence mutations, it was necessary to introduce 8 different specific probes of different influenza viruses with one, two, three and four mutations each within the microarray. The selected viruses were A(H1N1) Neuraminidase (1), HPAI A(H5N1) Matrix protein 1 (38), H2N2 Matrix protein 1 (33), Seasonal A(H1N1) matrix protein 2 (41), A(H3N2) hemagglutinin (15), Seasonal A(H1N1) polymerase PB1 (51), HPAI A(H5N1) nucleocapsid (28) and H2N2 polymerase PA (63). The mutations in the sequences are listed in Table S3.

2.3 ElectroSense Microarrays: Hybridization and Electrochemical Detection

The chips were activated at 65 °C for 10 min with nuclease-free water (30 μ L), followed by a further pre-hybridization at room temperature for 3 min (three time) using a pre-hybridization solution (1X PBS). Pre-hybridization and post hybridization rinsing and detection conditions are in detail in the ElectraSense manual, available at (http://www.combimatrix.com/support_docs.htm). Then, the 3' Biotin Oligonucleotide probes Mix microarrays were hybridized for 1 h at 40 °C in a hybridization solution and the slip was loaded onto the rotisserie of the hybridization oven. The 3' biotin

oligonucleotide probes mix was prepared in PBST hybridization solution (Phosphate Buffered Saline (PBS) with 0.05% Tween-20). Then, hybridization occurred at room temperature for 1 minute incubated with PBST solution. For post-hybridization the CombiMatrix ElectraSense™ Detection Kit was used. After removing the PBST Wash Solution from the hybridization chamber, the Blocking Buffer was added into the hybridization chamber and the array was incubated at room temperature for 15 minutes. Later on, the Blocking Buffer was removed, and the Biotin Labeling Solution was added to the hybridization chamber. The array was incubated at room temperature for 30 min, followed by removing of the Biotin Labeling Solution from the hybridization chamber, using the Biotin Wash Solution. The hybridization chamber, was rinsed, filled and incubated at room temperature for 5 minutes (the procedure was repeated twice for a total of three washes), meanwhile the Biotin Wash Solution was retained in the hybridization chamber during preparation of the ElectraSense™ Reader and until electrochemical detection was performed. For the preparation of the hybridized ElectraSense™ aimed for the electrochemical detection, a TMB (3, 3', 5, 5'-tetramethylbenzidine) substrate solution was used. The oxidized TMB is reduced at the surface of the electrode generating a current flux. The ElectraSense Reader performs amperometric detection of this current flux for each individual spot. The collected data were processed by CombiMatrix ElectroSense software Version 6.2.6 (2009).

3. RESULTS AND DISCUSSION

Microarrays have risen to the forefront of preferred analytical technologies for a variety of molecular applications including genetic studies and pathogens detection. Nowadays the application of oligonucleotide microarrays as a monitoring step in different areas of molecular biology, bio-defense, and infectious disease is rapidly growing during the last decade [21,30]. Mostly, the employed DNA microarrays consist of several oligonucleotide probes that have been immobilized on a solid glass support, and the technique used has great potential for the discrimination of closely related strains by employing oligonucleotides specific for each target organism [23].

3.1 Influenza Array Assay Validation

The use of this method and its respective apparatus is already reported in literature [28,29]. In the present study, the influenza array contained probes representing all genes belonging to the influenza virus: i.e. neuraminidase, hemagglutinin, nucleocapsid protein, matrix protein 1, matrix protein 2, polymerase PB1, polymerase PA and nuclear export protein (NEP). A list of 80 oligonucleotide probes are shown in Table S1. All biotinylated-oligonucleotide probes hybridized correctly with their respective complementary probes present in microarray support, revealing an obviously high and similar signal between each family of probes. Results of array validation studies are shown in Figs. 2a and 2b. The average signal for the following compounds: neuraminidase, hemagglutinin, nucleocapsid protein, matrix protein 1, matrix protein 2, polymerase PB1, polymerase

PA and NEP, oligonucleotides was respectively 8617.6, 10235.07, 9382.5, 9347.3, 11870.5, 9082.9, 10651.77, 11088.5, with RSD < 5% (Fig. 2a). These data emphasize the high specificity of the detection system, suggesting therefore CombiMatrix influenza microarray system as an effective method for influenza subtype analysis.

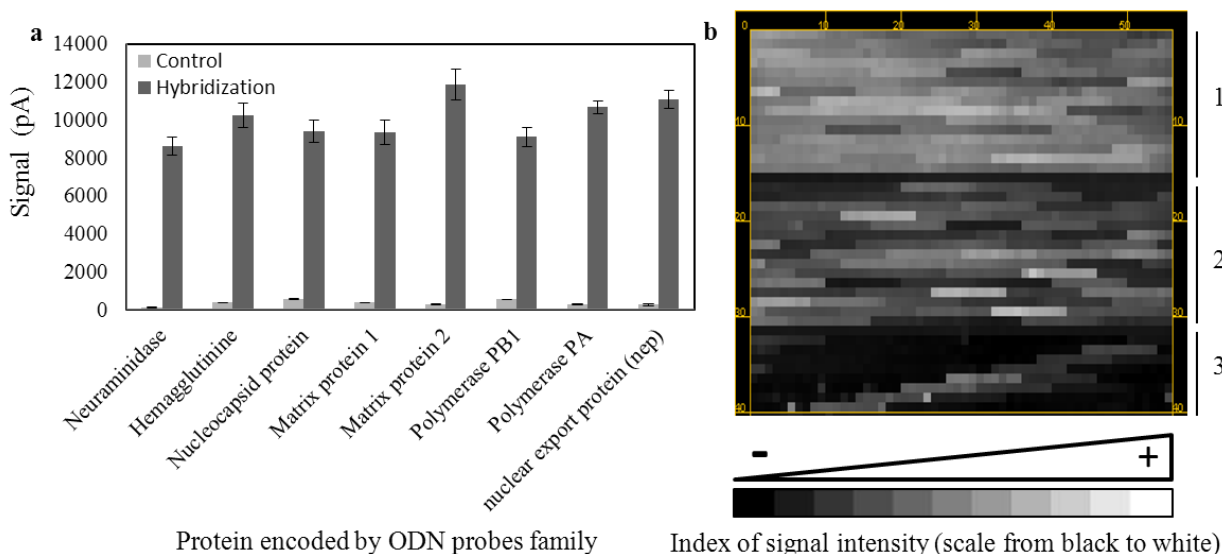


Figure 2. (a) The average signal for neuraminidase, hemagglutinin, nucleocapsid protein, matrix protein 1, matrix protein 2, polymerase PB1, polymerase PA and nuclear export protein (nep) probes oligonucleotides family and \pm error standard after hybridization. (b) Image of microarray of influenza: 1) the position and intensity signal of 80 normal oligonucleotide probes, 2) 80 oligonucleotides probes with one mutation and 3) 32 oligonucleotides probes with one, two, three and four mutations in the sequences. All probes have 10 repetitions within an array. Control is intensity of signal without hybridization oligonucleotides probes.

3.2 The effect of one Mutation within the Sequences of Oligonucleotide Probes in the Hybridization Signal

Although influenza virus infection is drug-treatable, high rates of mutation of viral genome can result in reduced effectiveness of antiviral drugs. Certain mutations in NA segment of influenza viral genome confer resistance to NAIs. Thus, the emergence and global spread of oseltamivir-resistant seasonal H1N1 viruses in 2007–2009 demonstrated the ability of some drug-resistant variants which are rapidly become predominant worldwide [31]. The NAI-resistant virus widely spread in the population, known as the A/Brisbane/59/2007 (H1N1) strain, contained permissive mutations that restored the detrimental effect caused by the H275Y change [32,33]. Influenza virus variants carrying NA substitutions, which emerge in response to drug treatment, are commonly present along with some wild-type viruses [34]. Moreover, influenza A H1N1 2009 virus were the first to cause pandemic in an era when neuraminidase inhibitor antiviral drugs were available in many countries [35]. The precise role of genetic changes in the efficient transmission and maintenance of resistant viruses acting in the absence of drug pressure, is still poorly understood [31].

Mutations are interlaced with different consequences in addition to the emergence of their resistance. Nowadays, in spite of the evident suppression of human-to-human transmission of avian influenza viruses, their evolution to new variants is still a continuous thread of a new pandemic. The avian influenza A(H5N1) along with the A(H7N9) viruses, and the polymerase basic 2 protein (PB2) E627K mutation appears to be of key importance for human adaptation, during the A(H7N7) virus outbreak in the Netherlands in 2003. The A(H7N7) virus isolated from a fatal human case, contained the PB2 E627K mutation as well as a hemagglutinin (HA) K416R mutation [36].

All the above facts strongly appeal for new influenza mutation detection methods. Phenotypic and genotypic assays compounds can be used for assessing of the resistance to anti-influenza. For the NAIs, enzymatic assays are preferred and have shown their reliably to assess drug susceptibilities [37]. Different types of NA assays can be performed using fluorescent [38], chemiluminescent [39,40], or colorimetric NA substrates [41]. The basic approach for genotypic testing is related to the amplification of the targeted gene by RT-PCR as M2 in the case of the adamantanes and NA for the NAIs, followed by conventional DNA Sanger sequencing (gold standard) or pyrosequencing.

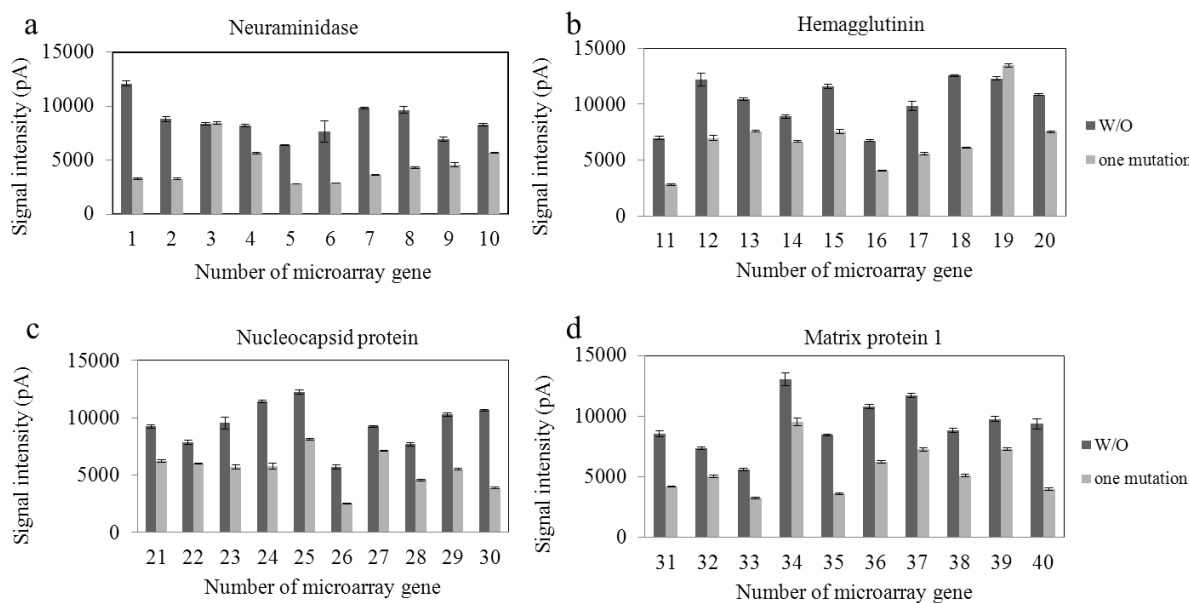


Figure 3. The recorded signal after the hybridization of oligonucleotide probes of influenza viruses without or with one mutation in the sequences of gene probes of (a) neuraminidase, (b) hemagglutinin, (c) nucleocapsid protein and (d) matrix protein 1.

Resistance mutations differ according to the type of NAI used and they have been found to be sub-type specific. In the present study, a change of a nucleotide (for one adenine) is performed in the sequence of all oligonucleotides probes. Therefore, the oligonucleotides probes with one mutation in the sequence (Tab. S2) were synthesized and printed onto the CombiMatrix CustomArray™ 4×2K microarray support to generate the prototype chips for the different influenza viruses. These mixtures of biotinylated-oligonucleotides probes were hybridized on the prototype microarrays influenza. The

collected results show that the samples with one mutation in the sequence are generally associated by signal declination (Figs. 3 and 4). Moreover, a signal decrease was observed for samples with mutations belonging to nucleocapsid protein 100% and matrix protein 1 family (Figs. 3c and 3d). Neuraminidase, hemagglutinin, polymerase protein PA and nuclear export protein family probes showed 90% (Figs. 3a and 3b, and Figs. 4c and 4d), while matrix protein 2 and polymerase PB1 was merely 80% (Figs. 3a and 3b). The evident signal decrease expressed by electrochemical CombiMatrix technology demonstrates the considerable reliability of this novel electrochemical method for the detection of mutation within the sequences of influenza virus.

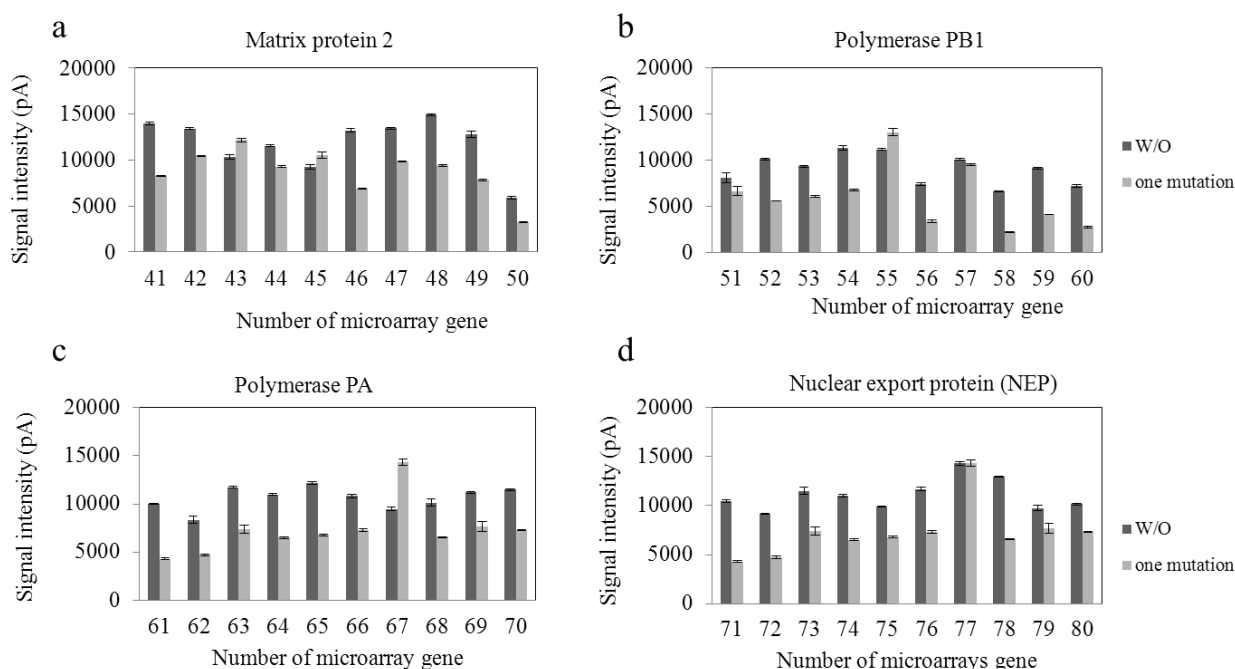


Figure 4. The recorded signal after the hybridization of oligonucleotide probes of influenza viruses without (W/O) or with one mutation in the sequences of genes probes of (a) matrix protein 2, (b) polymerase PB1, (c) Polymerase PA and (d) nuclear export protein (NEP).

3.3 The effect caused by the Increase of Mutations Occurrence within the Sequences of Oligonucleotide Probes in the Hybridization Signal

Eight different specific probes of different influenza virus with one, two, three and four mutations respectively in the sequence, within the microarray (A(H1N1) Neuraminidase (1), HPAI A(H5N1), Matrix protein 1 (38), H2N2 Matrix protein 1 (33), Seasonal A(H1N1) matrix protein 2 (41), A(H3N2) hemagglutinin (15), Seasonal A(H1N1) polymerase PB1 (51), HPAI A (H5N1) nucleocapsid (28) and H2N2 polymerase PA (63)) showed a signal decrease by increasing the number of mutations in the sequences (Fig. 5). Thus, all probes had the same performance when the number of mutations in the sequences increased. These results make CombiMatrix ElectroSense™ influenza a new useful method for the identification of influenza mutations in sequence. Furthermore, it provides

an approximation of the number of mutations, often present in influenza virus and complex evolutionary patterns, inducing antiviral drug resistance.

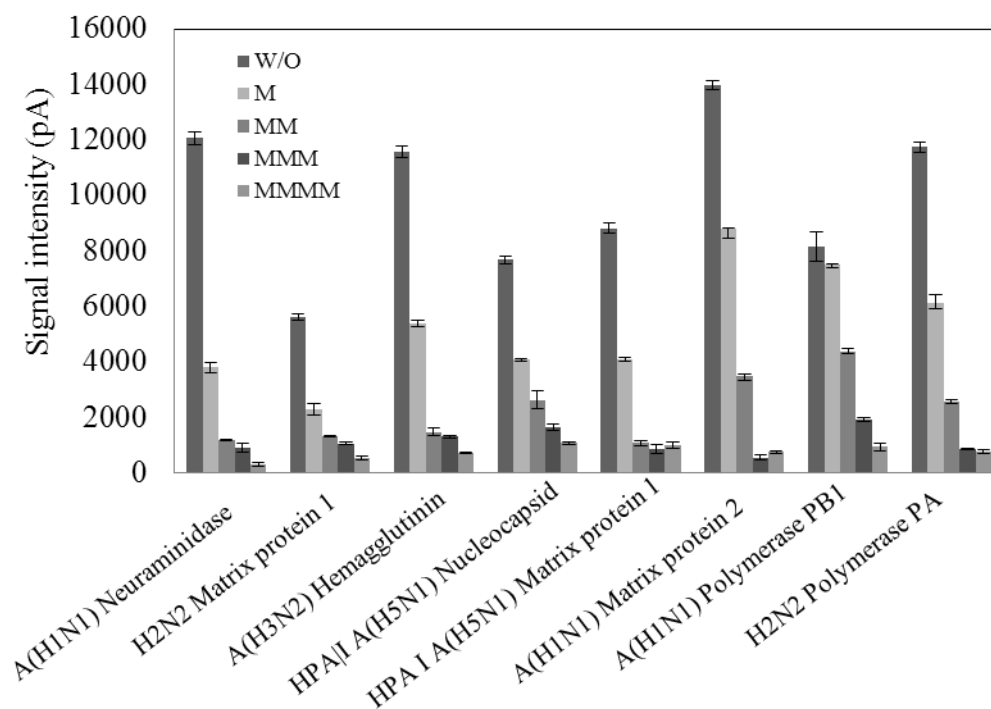


Figure 5. Signal intensity of different genes a function of the increasing of sequence mutations. W/O- without mutation, M- one, MM- two, MMM- three, and MMMM- four mutations. A(H1N1) neuraminidase, H2N2 Matrix protein 1, A(H3N2) Hemagglutinin, HPAI A(H5N1) Nucleocapsid, HPAI A(H5N1) Matrix protein 1, Seasonal A(H1N1) Matrix protein 2, Seasonal A(H1N1) polymerase PB1, and H2N2 polymerase PA.

4. CONCLUSIONS

This study shed light on a reliable and sensitive electrochemical method for the identification of different subtypes of influenza viruses and mutations in their genome. This novel electrochemical technique enhances assay the performance due to its easiness and fast detection of the mutations within a sequence of the influenza virus. This finding is expected to have a global impact on the monitoring of influenza resistance, as well as the monitoring of the mutations in the sequences of influenza virus and the acquisition of resistance toward antiviral drugs by microarrays.

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Conflict of interest

The authors declare no conflicts of interest.

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SUPPLEMENTARY DATA:

Table S1. Summary of sequences of the selected specific microarray probes and the respective number assigned to the microarray.

Viruses	Genes	GenBank accession number	Sequence	Number of position inside of Arrays
Seasonal A(H1N1)	neuraminidase	CY076797	5'-TCCTCATAATGAAAATTGGG-3'	1
Pandemic A(H1N1)	neuraminidase	GQ377078	5'-TCCTCATAGTGATAATTAGG-3'	2
H2N2	neuraminidase	CY125896.1	5'-TAGCAATTGCAGGGATCCTA-3'	3
H2N8	neuraminidase	HM589207.1	5'-TGGATCAAGAGGCCATGTTT-3'	4
A(H3N2)	neuraminidase	GQ293082	5'-ACACATAAGGTTCTCTTGT-3'	5
H3N8	neuraminidase	JQ433881.1	5'-GTTGAAATCAGTTTCAATGG-3'	6
Seasonal A(H5N1)	neuraminidase	CY053327.1	5'-ACCGTTAAAGACAGAAGC C-3'	7
HPAI A(H5N1)	neuraminidase	EU263982.1	5'-AGGGAAAGTAGTTAAATCAG-3'	8
H7N7	neuraminidase	AY340079.1	5'-CCAGAAACATTCTGAGGACT-3'	9
H9N2	neuraminidase	NC 004909.1	5'-TACATGATAGGAGTCCCCAT-3'	10
Seasonal A(H1N1)	hemagglutinin	JN017181.1	5'-AGAGAAGAAATAGATGGGGTA-3'	11
Pandemic A(H1N1)	hemagglutinin	JX625498.1	5'-ACCCAAAGCTCAGCAAATC-3'	12
H2N2	hemagglutinin	CY125918.1	5'-TGTGTGGACATAACAATGCTG-3'	13
H2N8	hemagglutinin	HM589205.1	5'-ATCAACACTAAATCAGAGGTC-3'	14

A(H3N2)	hemagglutinin	JX844665.1	5'-TTTTGTTGAACGCAGCAAAG-3'	15
H3N8	hemagglutinin	JQ433879.1	5'-GACCTTAAAAGCACTCAAGC-3'	16
Seasonal A(H5N1) HPAI	hemagglutinin	CY053325.1	5'-GGGAATACACCACCCTAAT-3'	17
A(H5N1)	hemagglutinin	EU263981.1	5'-CTCCAGAATATGCATACAAAA-3'	18
H7N7	hemagglutinin	AY338459.1	5'-GAAACGGTGGAACGAACAAA-3'	19
H9N2	hemagglutinin	NC_004908.1	5'-GTAGTGCAATGTCAGACTGA-3'	20
Seasonal A(H1N1)	nucleocapsid protein	CY087027.1	5'-ATAGACGGAAAATGGATGAG-3'	21
Pandemic A(H1N1)	nucleocapsid protein	JX625388.1	5'-GACGACTAATCCAGAATAGC-3'	22
H2N2	nucleocapsid protein	CY125897.1	5'-AGAGAGTAAATGGAAAGTGGA-3'	23
H2N8	nucleocapsid protein	HM589206.1	5'-AATGGAGAAGACGCAACC-3'	24
A(H3N2)	nucleocapsid protein	CY113840.2	5'-GATTATGAAGGGCGGTTGAT-3'	25
H3N8	nucleocapsid protein	JQ433880.1	5'-CTTCAGTCTTATCAGACCAAA-3'	26
Seasonal A(H5N1) HPAI	nucleocapsid protein	CY053328.1	5'-GCGAATAATGGAGAAGACG-3'	27
A(H5N1)	nucleocapsid protein	EU263985.1	5'-ACATATCAGAGAACGAGAGC-3'	28
H7N7	nucleocapsid protein	AY342425.1	5'-CTAATTCGGATGATAAAGCG-3'	29
H9N2	nucleocapsid protein	AF255743.1	5'-GGAAGGTTGATCCAGAAC-3'	30
Seasonal A(H1N1) Pandemic A(H1N1)	matrix protein 1	CY087025.1	5'-ACTATACAAGAAGCTCAAAAG-3'	31
H2N2	matrix protein 1	JX625390.1	5'-ATGAAAACAGAATGGTGCTG-3'	32
H2N8	matrix protein 1	CY125895.1	5'-TTAAGAGGGAGATAACATTC-3'	33
A(H3N2)	matrix protein 1	HM589208.1	5'-CTTTGTCCAAAATGCCCTTAA-3'	34
H3N8	matrix protein 1	CY113838.2	5'-AACATGTGAACAGATTGCTG-3'	35
Seasonal A(H5N1) HPAI	matrix protein 1	JQ433882.1	5'-TACAGGAAGCTTAAAAGGGA-3'	36
A(H5N1)	matrix protein 1	CY053326.1	5'-TCCTGTACCTCTGACTA A-3'	37
H7N7	matrix protein 1	EU263984.1	5'-ACTGCAGCGTAGACGTTT T-3'	38
H9N2	matrix protein 1	GU053111.1	5'-CCCCTAATCAGGCATGAA-3'	39
	matrix protein 1	AJ278646.1	5'-CAAGAAGCTGAAGAGGGAAA-3'	40
Seasonal A(H1N1) Pandemic A(H1N1)	matrix protein 2	CY087025.1	5'-TCGCTTTAAATACGGTTTGAA-3'	41
H2N2	matrix protein 2	JX625390.1	5'-TCATTGGGATCTTGACCT-3'	42
H2N8	matrix protein 2	CY125895.1	5'-AACACGGTCTGAAAAGAGGG-3'	43
A(H3N2)	matrix protein 2	HM589208.1	5'-ATCATTGGGATCTTGCACTT-3'	44
H3N8	matrix protein 2	CY113838.2	5'-TTCAAACACGGTCTGAAAAGA-3'	45
Seasonal A(H5N1) HPAI	matrix protein 2	JQ433882.1	5'-GCCTTAAATACGGTTTGAA-3'	46
A(H5N1)	matrix protein 2	CY053326.1	5'-TATCATTGGGATCTTGCACT-3'	47
H7N7	matrix protein 2	EU263984.1	5'-TCGCCTTAAATACGGTTTGAA-3'	48
H9N2	matrix protein 2	GU053111.1	5'-CGCCTTAAATACGGTTTGAA-3'	49
	matrix protein 2	AJ278646.1	5'-CTTCAAATGCATTTATCGTC-3'	50
Seasonal A(H1N1) Pandemic A(H1N1)	polymerase PB1	CY125673.1	5'-AAGATGATGACCAACTCCCA-3'	51
H2N2	polymerase PB1	HQ240702.2	5'-AGTGAATGAAAATCAAATC-3'	52
H2N8	polymerase PB1	CY125900.1	5'-TGGAATGAGAATCAAATCCT-3'	53
A(H3N2)	polymerase PB1	HM589203.1	5'-GGATAAAGAGGAAATGGAAAT-3'	54
	polymerase PB1	CY112971.2	5'-TGAATCTTGGGCAAAAAGAAA-3'	55

H3N8 Seasonal A(H5N1) HPAI	polymerase PB1	JQ433877.1	5'-GATGACTAATTCACAAGACA-3'	56
A(H5N1) HPAI	polymerase PB1	JF758819.1	5'-AGCATTGACACTGAACACTA-3'	57
A(H5N1) H7N7	polymerase PB1	EU263987.1	5'-AGAAAATGGTAACACAAAGAA-3'	58
H7N7	polymerase PB1	AY340083.1	5'-AATTACTGGAGACAACACCA-3'	59
H9N2	polymerase PB1	NC_004911.1	5'-AGTAAGAGCATGAAGCTACG-3'	60
Seasonal A(H1N1) Pandemic A(H1N1)	polymerase PA	CY125671.1	5'-AAACCAGACTATTCACCATAA-3'	61
H2N2	polymerase PA	JQ433878.1	5'-ATTCCTGGAGAGGAGATGG-3'	62
H2N8	polymerase PA	CY125923.1	5'-AGAACATTCTTTGGATGGAA-3'	63
A(H3N2)	polymerase PA	HM589204.1	5'-GAAAGCCAACAAGATAAAATC-3'	64
H3N8	polymerase PA	CY113858.2	5'-CAGAGCCACTGAGTACATAAT-3'	65
Seasonal A(H5N1) HPAI	polymerase PA	JQ433878.1	5'-AGGCGAAGAGACAATTGAAG-3'	66
A(H5N1) H7N7	polymerase PA	CY053330.1	5'-TGCATTGAGGGCAAGCTT T-3'	67
H7N7	polymerase PA	EU263986.1	5'-GGGCAAGCTTTCTCAAAT GT-3'	68
H9N2	polymerase PA	AY342418.1	5'-ACAACACCACGCCCTCT-3'	69
	polymerase PA	NC_004912.1	5'-CTGAAATTAAGCATTGAGGAC-3'	70
Seasonal A(H1N1) Pandemic A(H1N1)	nuclear export protein (nep)	CY125670.1	5'-GATTGAAGAAGTGAGACACAA-3'	71
H2N2	nuclear export protein (nep)	HQ240288.2	5'-TGGTTAATTGAAGAAATGCGG-3'	72
H2N8	nuclear export protein (nep)	CY125898.1	5'-ACGGAAAATGGCGAGAACAA-3'	73
A(H3N2)	nuclear export protein (nep)	HM589209.1	5'-TGATTGAGGAAGTACGACATA-3'	74
H3N8	nuclear export protein (nep)	CY103967.1	5'- AGAACAGTTAGGTCAAAAAGT-3'	75
Seasonal A(H5N1) HPAI	nuclear export protein (nep)	JQ433883.1	5'-TGGCTGATTGAAGAAGTGC-3'	76
A(H5N1)	nuclear export protein (nep)	CY053329.1	5'-ACCTCCACTCCCTCCAAAA-3'	77
H7N7	nuclear export protein (nep)	CY098618.1	5'-GCTGATTGAAGAAGTACGACA-3'	78
H9N2	nuclear export protein (nep)	GU053125.1	5'-AAGTGCACATAGGTTGAAG-3'	79
	nuclear export protein (nep)	FJ793288.1	5'-TGGCTGATTGAAGAAGTGC-3'	80

Table S2. Summary of sequences of the selected specific array probes with one mutation in the sequence and number assigned to the microarray.

Viruses	Genes	GenBank accession number	Oligonucleotides probes with one mutations
Seasonal A(H1N1) Pandemic A(H1N1)	neuraminidase	CY076797	5'-TCCTCATAATAAAAATTGGG-3'
H2N2	neuraminidase	GQ377078	5'-TCCTCAAGTGATAATTAGG-3'
H2N8	neuraminidase	CY125896.1	5'-TAGCAAATGCAGGGATCCTA-3'
A(H3N2)	neuraminidase	HM589207.1	5'-TGGATCAAAGG CCATGTTT-3'
H3N8 seasonal A(H5N1)	neuraminidase	GQ293082	5'-ACACATAAAGTTCTCTTGT-3'
HPAI A(H5N1)	neuraminidase	JQ433881.1	5'-GTTGAAAACAGT TTCAATGG-3'
	neuraminidase	CY053327.1	5'-ACCGTTAAAACAGAAGCC-3'
	neuraminidase	EU263982.1	5'-AGGGAAAATAGTTAAATCAG-3'

H7N7	neuraminidase	AY340079.1	5'-CCAGAAAAATTCTGAGGACT-3'
H9N2	neuraminidase	NC_004909.1	5'-TACATGAAA GGAGTCCCCAT-3'
Seasonal A(H1N1)	hemagglutinin	JN017181.1	5'-AGAGAAA AAATAGATGGGGTA-3'
Pandemic A(H1N1)	hemagglutinin	JX625498.1	5'-ACCCAAA ACTCAGCAAATC-3'
H2N2	hemagglutinin	CY125918.1	5'-TGTGTGGA AATACAATGCTG-3'
H2N8	hemagglutinin	HM589205.1	5'-ATCAACACA AAATCAGAGGTC-3'
A(H3N2)	hemagglutinin	JX844665.1	5'-TTTTGTTGAA AGCAGCAAAG-3'
H3N8	hemagglutinin	JQ433879.1	5'-GACCTTAAAA CACTCAAGC-3'
seasonal A(H5N1)	hemagglutinin	CY053325.1	5'-GGGAATA ACCACCCTAA T-3'
HPAI A(H5N1)	hemagglutinin	EU263981.1	5'-CTCCAGAAA ATGCATACAAAA-3'
H7N7	hemagglutinin	AY338459.1	5'-GAAACGGTGAACGAAA AAA-3'
H9N2	hemagglutinin	NC_004908.1	5'-GTAGTGCAA AGTCAGACTGA-3'
Seasonal A(H1N1)	nucleocapsid protein	CY087027.1	5'-ATAGACG AAAATGGATGAG-3'
Pandemic A(H1N1)	nucleocapsid protein	JX625388.1	5'-GACGACTAA CCAGAATAGC-3'
H2N2	nucleocapsid protein	CY125897.1	5'-AGAGAGA AAATG GAAAGTGA-3'
H2N8	nucleocapsid protein	HM589206.1	5'-AATGGAGAA ACGCAACC-3'
A(H3N2)	nucleocapsid protein	CY113840.2	5'-GATTATA AAGGGCGG TTGAT-3'
H3N8	nucleocapsid protein	JQ433880.1	5'-CTTCAGTCTTATCA AACCAA-3'
seasonal A(H5N1)	nucleocapsid protein	CY053328.1	5'-GCG AAAAATGGAGAAGACG-3'
HPAI A(H5N1)	nucleocapsid protein	EU263985.1	5'-ACATATCAGAA AACGAGAGC-3'
H7N7	nucleocapsid protein	AY342425.1	5'-CTAATTCGGATGAAA AAGCG-3'
H9N2	nucleocapsid protein	AF255743.1	5'-GGAAA GTTGATCCAGAAC-3'
Seasonal A(H1N1)	matrix protein 1	CY087025.1	5'-ACTATACAAA AAGCT CAAAAG-3'
Pandemic A(H1N1)	matrix protein 1	JX625390.1	5'-ATGAAAA AGAATGGTGCTG-3'
H2N2	matrix protein 1	CY125895.1	5'-TTAAA AGGGAGATAACATTC-3'
H2N8	matrix protein 1	HM589208.1	5'-CTT TGTC AAAAATGCCCTTAA-3'
A(H3N2)	matrix protein 1	CY113838.2	5'-AACATGTGAAA AGATTGCTG-3'
H3N8	matrix protein 1	JQ433882.1	5'-TACAGGAAGCT AAAAAGGGA-3'
seasonal A(H5N1)	matrix protein 1	CY053326.1	5'-TCCTGTACCTCT ACTA A-3'
HPAI A(H5N1)	matrix protein 1	EU263984.1	5'-ACTGCAGCGTAA ACGTTT T-3'
H7N7	matrix protein 1	GU053111.1	5'-CCCCTAAA CAGGCATGAA-3'
H9N2	matrix protein 1	AJ278646.1	5'-CAAGAAGCTGAAA AGGGA AA-3'
Seasonal A(H1N1)	matrix protein 2	CY087025.1	5'-TCGCTTTAAA ACGGTTTGAA-3'
Pandemic A(H1N1)	matrix protein 2	JX625390.1	5'-TCATTGGGA ACTTGACCT-3'
H2N2	matrix protein 2	CY125895.1	5'-AACACGGTCTGAAAA AAGGG-3'
H2N8	matrix protein 2	HM589208.1	5'-ATCATTGGGA ACTTGACTT-3'
A(H3N2)	matrix protein 2	CY113838.2	5'-TTCAAACACGGTCTGAAAA AA-3'

H3N8 seasonal A(H5N1)	matrix protein 2	JQ433882.1	5'-GCCTTAAAACGGTTTGAA-3'
HPAI A(H5N1)	matrix protein 2	CY053326.1	5'-TATCATTGGGAACCTGCACT-3'
H7N7	matrix protein 2	EU263984.1	5'-TCGCCTTAAAACGGTTTGAA-3'
H9N2	matrix protein 2	GU053111.1	5'-CGCCTTAAAACGGTTTGAA-3'
H9N2	matrix protein 2	AJ278646.1	5'-CTTCAAAAGCATTATCGTC-3'
Seasonal A(H1N1)	polymerase PB1	CY125673.1	5'-AAAATG ATGACCAACTCCCA-3'
Pandemic A(H1N1)	polymerase PB1	HQ240702.2	5'-AGTGGAAATGAAAAACAAAATC-3'
H2N2	polymerase PB1	CY125900.1	5'-TGGAATGAGAATAAAAATCCT-3'
H2N8	polymerase PB1	HM589203.1	5'-GGATAAAGAGGAAAAAGGAAAT-3'
A(H3N2)	polymerase PB1	CY112971.2	5'-TGAATCTTGGGCAAAAATAA-3'
H3N8 seasonal A(H5N1)	polymerase PB1	JQ433877.1	5'-GATGACTAATTCAAAGACA-3'
HPAI A(H5N1)	polymerase PB1	JF758819.1	5'-AGCATTGACACTGAAACTA-3'
H7N7	polymerase PB1	EU263987.1	5'-AGAAAAAGGTAACACAAAGAA-3'
H9N2	polymerase PB1	AY340083.1	5'-AATTACTGGAGAAACACCA-3'
H9N2	polymerase PB1	NC_004911.1	5'-AGTAAAGCATGAAGCTACG-3'
Seasonal A(H1N1)	polymerase PA	CY125671.1	5'-AAAACAGACTATTCACCATAA-3'
Pandemic A(H1N1)	polymerase PA	JQ433878.1	5'-ATTCCTGGAAAGGAGATGG-3'
H2N2	polymerase PA	CY125923.1	5'-AGAAATTCTTTGGATGGAA-3'
H2N8	polymerase PA	HM589204.1	5'-GAAAGCCAAAGATAAAATC-3'
A(H3N2)	polymerase PA	CY113858.2	5'-CAGAGCCACTGAGTACAAT-3'
H3N8 seasonal A(H5N1)	polymerase PA	JQ433878.1	5'-AGGCGAAAGACAATTGAAG-3'
HPAI A(H5N1)	polymerase PA	CY053330.1	5'-TGCATTGAGGGCAAACCTT T-3'
H7N7	polymerase PA	EU263986.1	5'-GGGCAAGCTTCTCAAAAGT-3'
H9N2	polymerase PA	AY342418.1	5'-ACAAACCACGCCCTCT-3'
H9N2	polymerase PA	NC_004912.1	5'-CTGAAAATAAGCATTGAGGAC-3'
Seasonal A(H1N1)	nuclear export protein (nep)	CY125670.1	5'-GATTGAAAGTGAGACACAA-3'
Pandemic A(H1N1)	nuclear export protein (nep)	HQ240288.2	5'-TGGTTAATTGAAAATGCGG-3'
H2N2	nuclear export protein (nep)	CY125898.1	5'-ACGGAAAAAGGCGAGAACAA-3'
H2N8	nuclear export protein (nep)	HM589209.1	5'-TGATTGAGGAAATACGACATA-3'
A(H3N2)	nuclear export protein (nep)	CY103967.1	5'-AGAACAGTTAGGTAAAAAGT-3'
H3N8 seasonal A(H5N1)	nuclear export protein (nep)	JQ433883.1	5'-TGGCTGATTGAAAAGTGC-3'
HPAI A(H5N1)	nuclear export protein (nep)	CY053329.1	5'-ACCTCCACTCCCTCAAAA-3'
H7N7	nuclear export protein (nep)	CY098618.1	5'-GCTGATTGAAAAGTACGACA-3'
H9N2	nuclear export protein (nep)	GU053125.1	5'-AAGTGCGACAAGGTTGAAG-3'
H9N2	nuclear export protein (nep)	FJ793288.1	5'-TGGCTGATTGAAAAGTGC-3'

Table S3. Summary of specific sequences belonging to specific probes of different influenza virus containing one, two, three and four mutations, respectively within the microarray. Selected viruses were A(H1N1) Neuraminidase (1), HPAI A(H5N1) Matrix protein 1 (38), H2N2 Matrix protein 1 (33), Seasonal A(H1N1) Matrix protein 2 (41), A(H3N2) Hemagglutinin (15), Seasonal A(H1N1) Polymerase PB1 (51), HPAI A(H5N1) Nucleocapsid (28) and H2N2 Polymerase PA (63). W/O – without mutation, M- one mutations, MM – two mutations, MMM – three mutations and MMMM – four mutations.

	A(H1N1) Neuraminidase	HPAI A(H5N1) Matrix protein 1
	Sequence of gene 1	Sequence of gene 38
W/O	5'-TCCTCATAATGAAAATTGGG-3'	5'-ACTGCAGCGTAGACGTTTT-3'
M	5'-TCCTCATAAT A AAAATTGGG-3'	5'-ACTGCAGCGTAA A ACGTTTT-3'
MM	5'-TCCTCATAAA AA AAAATTGGG-3'	5'-ACTGCAGCG AA AAACGTTTT-3'
MMM	5'-TCCTCATAAA AAAA AATTGGG-3'	5'-ACTGCAGCG AAAA AGTTTT-3'
MMMM	5'-TCCTCATAAA AAAAA AGGG-3'	5'-ACTGCAGCG AAAAA ATTTTT-3'
	H2N2 Matrix protein 1	Seasonal A(H1N1) Matrix protein 2
	Sequence of gene 33	Sequence of gene 41
W/O	5'-TTAAGAGGGAGATAACATTC-3'	5'-TCGCTTTAAATACGGTTTGAA-3'
M	5'-TTAA A AGGGAGATAACATTC-3'	5'-TCGCTTTAAA A ACGGTTTGAA-3'
MM	5'-TTAA AA AGGGAGAA A AACATTC-3'	5'-TCGCTTTAAA AA AGGGTTTGAA-3'
MMM	5'-TTAA AAA AGGGAGAA AA AATTC-3'	5'-TCGCTT AAAA AAAGGGTTTGAA-3'
MMMM	5'-TTAA AAAA AGGGAA AAA AATTC-3'	5'-TCGCTT AAAAA AAAGTTTGAA-3'
	A(H3N2) Hemagglutinin	Seasonal A(H1N1) polymerase PB1
	Sequence of gene 15	Sequence of gene 51
W/O	5'-TTTTGTTGAACGCAGCAAAG-3'	5'-AAGATGATGACCAACTCCCA-3'
M	5'-TTTTGTTGAA A GCAGCAAAG-3'	5'-AA A ATGATGACCAACTCCCA-3'
MM	5'-TTTTGTTGAA AA CAGCAAAG-3'	5'-AA AA AGATGACCAACTCCCA-3'
MMM	5'-TTTTGTTGAA AAA CAGCAAA-3'	5'-AA AAA AATGACCAACTCCCA-3'
MMMM	5'-TTTTGTTGAA AAAA AGCAAAA-3'	5'-AA AAAA AGACCAACTCCCA-3'
	HPAI A(H5N1) nucleocapsid	H2N2 polymerase PA
	Sequence of gen 28	Sequence of gene 63
W/O	5'-ACATATCAGAGAACGAGAGC-3'	5'-AGAACATTCTTTGGATGGAA-3'
M	5'-ACATATCAGA A AACGAGAGC-3'	5'-AGAA A ATTCTTTGGATGGAA-3'
MM	5'-A AA ATATCAGA AA AACGAGAGC-3'	5'-A AAAA ATTCTTTGGATGGAA-3'
MMM	5'-A AAA AATCAGA AA AACGAGAGC-3'	5'-A AAAAA ATCTTTGGATGGAA-3'
MMMM	5'-A AAAA AACAGA AA AACGAGAGC-3'	5'-A AAAAA ATCTTTGGATG AAA -3'