Electronic Supplementary Material (ESI) for Chemical Communications

HPAI 9G DNAChip: Discrimination of Highly Pathogenic Influenza Virus Genes

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Supporting Information

1. Materials and methods

All chemicals were purchased from Sigma-Aldrich Chemicals, Korea. All the oligonucleotides were purchased from Bioneer, Korea. The RT-PCR pre-mix and RNA extractions kits were obtained from the Introgen, Korea. Glass slides (2.5x7.5 cm) were purchased from Paul Marienfeld GmbH & Co. KG, Germany. All washing solvents for the substrates are of HPLC grade from SK Chemicals, Korea. Ultrapure water (18 M Ω/cm) was obtained from a Milli-Q purification system (Millipore). The DNA chips with modified with the under mentioned probes (**Table 1**) were obtained from Biometrix technology Inc., Korea. Oligonucleotides were spotted using Qarray2 microarrayer (Genetix Technologies, Inc.) to produce DNA Chips used for the experiments. The HPAI genomic DNA's used in this study for PCR amplification were obtained from Department of Veterinary Medicine, Kangwon National University (Korea). However, the HPAI genomic DNA's used in this study for PCR amplification were obtained by RT-PCR of the clinical samples and standardized by the sequencing analysis (Table S2). Virus strains used: feces/Korea/CSM2/02 (H5N3); A/duck/Hokkaido/Vac-3/2007(H5N1); A/chicken/Korea/IS2/2006(H5N1); A/duck/Hokkaido/HY57/2005(H9N4); A/duck/Shantou/163/2004 (H9N2); Influenza A virus (A/duck/Hong Kong/147/1977(H9N6). Influenza RNA was extracted as described previously[1] and stored at -70℃. All DNA chips used in this study were obtained by following the previously reported method.[2]

2. Composition of different solutions used for hybridization and washing on 9G DNAChips

1. Immobilization solution (pH = 7.4): 15% glycerol, 50mM butyl amine, 600mM NH4Cl
2. Blocking buffer solution (pH = 7.4): 0.5% milk casein in 4xSSC
3. Hybridization buffer (pH = 7.4): 25% Formamide, 0.1% Triton X-100, 6xSSC
4. Washing buffer solution A (pH = 7.4): 0.1% SDS in 4xSSC
5. Washing buffer solution B (pH = 7.4): 4xSSC

3. Probes and Target oligonucleotides:

Table 1. Sequences and the nomenclature of probe and target oligonucleotides

Probes	Probe Description	Probe starting position	HPAI subtype	Sequence
Original HPAI Probes (without mutations)				
Probe1	H5N3	-3	H5N3	5'-GGGGGGGGG CTTTATCTT GAG GAC TAT TCG GGG CC-3'
Probe2	H5N1	-3	H5N1	5'-GGGGGGGGG CTTTATCTT GAG GAC TAT TTG GGG CC-3'
Probe3	H5N1(K)	-3	H5N1 (K)	5'-GGGGGGGGG CTTTATCTT GAG GAC TAT TTG GAG CT-3'
Probe4	H7N1	-3	H7N1	5'-GGGGGGGGG CTTTATCTT GAG GCC TAT TTG GTG CC-3'
Probe5	H7N3	-3	H7N3	5'-GGGGGGGGG CTTTATCTT GAG GAC TCT TCG GGG CA-3'
Probe6	H7N7	-4	H7N7	5'-GGGGGGGGG CTTTATCTT AGG CTT ATT TGG TGC CA-3'
Probe7	H9N4	-3	H9N4	5'-GGGGGGGGG CTTTATCTT GAG GGT TGT TTG GTG CT-3'
Probe8	H9N2	-3	H9N2	5'-GGGGGGGGG CTTTATCTT GAG GGC TCT TCG GTG CC-3'
Probe9	H9N6	-3	H9N6	5'-GGGGGGGGG CTTTATCTT GAG GGC TAT TTG GTG CC-3'
HPAI Probes with one artificial mutation				
Probes	**Probe Description**	**Mutation position**	**HPAI subtype**	**Sequence**
Probe10	H5N3	13T	H5N3	5'-GGGGGGGGG CTTTATCTT GAG GTC TAT TCG GGG CC-3'
Probe11	H5N1	10T	H5N1	5'-GGGGGGGGG CTTTATCTT GAG GAC TTT TTG GGG CC-3'
Probe12	H5N1(K)	10T	H5N1 (K)	5'-GGGGGGGGG CTTTATCTT GAG GAC TTT TTG GAG CT-3'
Probe13	H7N1	5T	H7N1	5'-GGGGGGGGG CTTTATCTT GAG GCC TAT TTG TTG CC-3'
Probe14	H7N3	5T	H7N3	5'-GGGGGGGGG CTTTATCTT GAG GAC TCT TCG TGG CA-3'
Probe15	H7N7	4T	H7N7	5'-GGGGGGGGG CTTTATCTT GAG GAC TCT TCG TTG CA-3'
Probe16	H9N4	5T	H9N4	5'-GGGGGGGGG CTTTATCTT GAG GGT TGT TTG TTG CT-3'
Probe17	H9N2	5T	H9N2	5'-GGGGGGGGG CTTTATCTT GAG GGC TCT TCG TTG CC-3'
Probe18	H9N6	5T	H9N6	5'-GGGGGGGGG CTTTATCTT GAG GGC TAT TTG TTG CC-3'
	HC		HC	5'-GGGGGGGGG TTTTATCCT AG TGG CTC TAT GGT AAC-3'
	NC		NC	5'-GGGGGGGGG AAA GCT GCT GCT CGT CGT CGT CGT
	Target1		HC-Cy5-T1	3'- GGA TCA CCG AGA TAC CAT TG GAG ACT GCG -Cy5-5'
	H5(FP)	Primer	H5-918F	5'- CCA RTR GGK GCK ATA AAY TC -3'
	H7(FP)	Primer	MMU31 C	5'-GGGGCYTTYATAGCTCCWGAYCGKGC-3'
	H9(FP)	Primer	H9-943F	5'- CAC AAT GTM AGY AAR TAT GC -3'
	URP	Primer	RP-Cy5	5'-Cy5-C WAT GAA DCC HGC TAT RGC- 3'

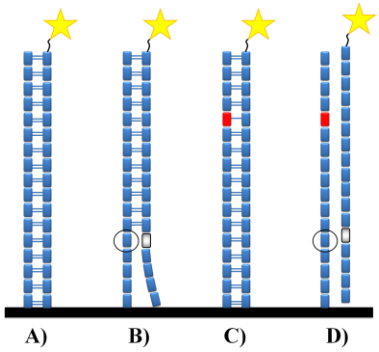
HC – probe for the Hybridization control, HC-Cy5-T1 – Target oligonucleotide for HC probe, GGGGGGGGG – 9G for immobilization of the probes on the AMCA slides, CTT TAT – vertical spacer group, FP: Forward primer, URP: Universal reverse primer

Table 2. Sequences and the nomenclature of probe and Accession strain.

HA	Probe	Subtype	Accession	Host	Reference Strains	Country	Year
H5	H5N3	H5N3	AB241626	Avian	Influenza A virus (A/duck/Hokkaido/299/04(H5N3)	Japan	2004
		H5N3	AB241626		feces/Korea/CSM2/02 (H5N3)	S.Korea	2002
	H5N1	H5N1	GU186680	Avian	Influ. A (A/environment/Hong Kong/674.15/2002-H5N1	Hong Kong	2002
		H5N1	GU052019		Influenza A virus (A/goose/Hong Kong/437-6/1999-H5N1	Hong Kong	1999
		H5N1	AY075027		Influenza A virus (A/Chicken/Hong Kong/317/2001-H5N1	Hong Kong	2001
		H5N1	AB212054		Influenza A virus (A/Hong Kong/213/2003(H5N1))	Hong Kong	2003
		H5N1	FJ265565		Influenza A virus (A/quail/Thailand/Phichit-1/2004)	Thailand	2004
		H5N1	EF541415		Influenza A virus (A/chicken/Laos/44/2004(H5N1))	Laos	2002
		H5N1	EF473081		Influenza A virus (A/chicken/Indonesia/11/2003(H5N1))	Indonesia	2005
		H5N1	EF541404		Influenza A virus (A/Viet Nam/1204/2004(H5N1))	Viet Nam	
		H5N1	AY585368		Influenza A virus (A/duck/Shanghai/35/2002(H5N1))	China	2008
		H5N1	CY021397		Influenza A virus (A/mallard/Italy/3401/2005(H5N1))	Italy	2006-2007
		H5N1	GU052473		Influenza A virus (A/avian/Italy/1485/1997(H5N1))	Italy	
		H5N1	AM408214		Influenza A virus (A/teal/Germany/WV632/2005(H5N1))	Germany	
		H5N1	AJ971297		Influenza A virus (A/duck/France/05066b/2005(H5N1))	France	
		H5N1	CY046182		Influenza A virus (A/duck/France/080036/2008(H5N1))	France	
		H5N1	AB355929		Influenza A virus (A/duck/Hokkaido/Vac-3/2007(H5N1)	Japan	
		H5N1	AB530992		Influenza A virus (A/duck/Hokkaido/Vac-3/2007(H5N1)	Japan	
		H5N1	DQ767725		Influenza A virus (A/chicken/Shandong/K01/2004(H5N1	China	
	H5N1(K)	H5N1	EU233675	Avian	Influenza A virus (A/chicken/Korea/IS/2006(H5N1))	South Korea South Korea	2006-2007
		H5N1	EU233683		Influenza A virus (A/chicken/Korea/IS2/2006(H5N1))	South Korea South Korea	2008 2008
		H5N1	EU233739		Influenza A virus (A/environment/Korea/W150/2006	South Korea South Korea	2008 2008
		H5N1	EU233723		Influenza A virus (A/quail/Korea/KJ4/2006(H5N1))	South Korea South Korea	2008
		H5N1	EU233699		Influenza A virus (A/chicken/Korea/IS3/2006(H5N1))	South Korea South Korea	
		H5N1	GQ412053		Influenza A virus (A/chicken/Korea/Gimje/08)		
		H5N1	GQ412056		Influenza A virus		

		H5N1	GQ412055		(A/chicken/Korea/YAQ173/2008) Influenza A virus (A/chicken/Korea/USQ284/2008)		
		H5N1	GQ412052		Influenza A virus (A/duck/Korea/JEQ149/2008(H5N1))		
		H5N1	GQ412051		Influenza A virus (A/chicken/Korea/ISQ250/2008(H5N1)		
H7	H7N1	H7N1	AJ493472	Avian	Influenza A virus (A/turkey/Italy/3775/99	Italy	1999
				Avian	(H7N1)	Italy	1999
		H7N1	CY025189	Avian	Influenza A virus (A/turkey/Italy/1265/1999(H7N1)	Italy	2002
		H7N1	GU052976		Influenza A virus (A/turkey/Italy/3283/1999(H7N1)		
	H7N3	H7N3	FLAAHAX	Avian	Influenza A virus (A/turkey/Oregon/71 (H7N3)	USA, Chile	2002
	H7N7	H7N7	FJ750854	Avian	Influenza A virus (A/magpie/Korea/YJD174/2007(H7N7)	South Korea	2007
						South Korea	2007
			FJ750864		Influenza A virus (A/mallard/Korea/GH170/2007(H7N7)	South Korea	2007
						South Korea	2007
			FJ750862		Influenza A virus (A/mallard/Korea/GG2/2007(H7N7)	South Korea	2007
			FJ750863		Influenza A virus (A/mallard/Korea/GG3/2007(H7N7))		
			FJ767719		Influenza A virus (A/mallard/Geumgang/1/2007(H7N7))		
H9	H9N4	H9N4	AB455035	Avian	Influenza A virus (A/duck/Hokkaido/ HY57/2005(H9N4)	Japan	2005
	H9N2	H9N2	AF508560	Avian	Influenza A virus (A/Pheasant/Ireland/PV18/97(H9N2))	Ireland	1997
		H9N2	AJ781819		Influenza A virus (A/turkey/Germany/R113/95(H9N2))	Germany	
		H9N2	AF508557		Influenza A virus (A/Duck/Germany/113/95(H9N2))	Germany	
		H9N2	AJ781818		Influenza A virus (A/turkey/Germany/R90/95(H9N2))	Germany	
		H9N2	CY041274		Influenza A virus (A/Bewick's swan/Netherlands/5/2007	Netherland	
		H9N2	CY024016		Influenza A virus (A/duck/Shantou/163/2004(H9N2))	China	
		H9N2	AF156377		Influenza A virus (A/Duck/Hong Kong/Y439/97(H9N2))	Hong Kong	
		H9N2	AB538969		Influenza A virus (A/duck/Mongolia/564/2003(H9N2))	Mongolia	
	H9N6	H9N6	AY206671	Avian	Influenza A virus (A/duck/Hong Kong/147/1977(H9N6))	Hong Kong	1977

4. Generalized probe selection method:



Scheme S0: Hybridizations of the immobilized probes with the Cy5 labeled target DNAs A) original probe-target specific hybridization, B) original probe-target non-target hybridization, C) original probe (with one artificial mutation)-target specific hybridization, D) original probe (with one artificial mutation)-target no non-target hybridization

5. Methods and experimental procedures:

5a. Typical method for the preparation of the pre-HPAI 9G DNAChip and the HPAI 9G DNAChip:

The pre-HPAI 9G DNAChip and the HPAI 9G DNAChips were prepared by spotting the immobilization solution containing oligonucleotide probes Probe1-Probe9 and Probe10-Probe18, resepctively, with the microarrayer, and the spots were arranged to make 6 by 3 pixels on the 9G slides. The microarrayed 9G slides were then kept in the incubator (25^0C, 50% humidity) for 4h to immobilize the oligonucleotides. The slides were then suspended in the blocking buffer solution at 25^0C for 30 min, in order to remove the excess oligonucleotides and to deactivate the non-spotted area. Then the slides were rinsed with washing buffer solutions A and B for 5min each, and then dried with commercial centrifuge to obtain the pre-HPAI 9G DNAChip and the HPAI 9G DNAChip. Before hybridization, the pre-HPAI 9G DNAChip and the HPAI 9G DNAChip were covered with Secure-Seal™ hybridization chambers**.**

5b. Typical hybridization and washing method:

Hybridizations were done by using the 100fmol of the Cy5 labeled PCR products of the 9 HPAI genotypes at 25^0C for 30min in the commercial incubator. Then pre-HPAI 9G DNAChip (or the HPAI 9G DNAChip) was rinsed with washing buffer solutions A and B successively for 2 min each, in order to remove the excess target DNA, and dried with commercial centrifuge (1000 rpm). The fluorescence signal of the microarray was measured on ScanArrayLite, and the images were analyzed by Quant Array software (Packard Bioscience).

5c. Amplification of the HPAI Gene:

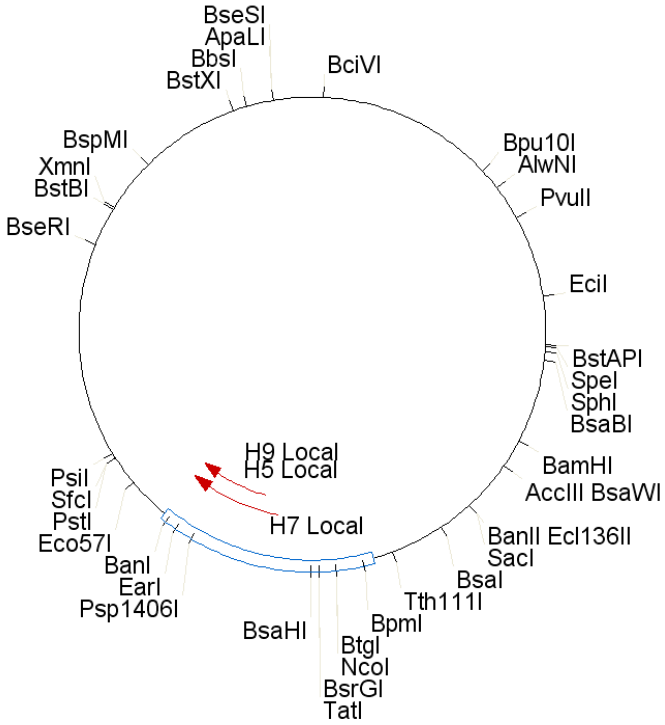


Figure S1: Genomic map with different regions on the HPAI genotype, H5, H7, H9 gene position lies between the 700-1055.

5d. Amplification of HPAI RNA by RT-PCR:

The H5, H7, H9 genotype samples were amplified by RT-PCR to generate amplicons of approximately 166bp, 280bp and 135bp. HPAI RNA was amplified with the multiplex primer set containing U1055R (Universal reverse primer) and H5, H7, and H9 (forward primer) (**Table S1**). 10µl of the HPAI RNA sample, 10µl (20pmol/µl) of U1055R, and 10µl (10pmol/µl) of 3 FP were added to the RT-PCR premix. All tubes were incubated for 2 min at 50℃ before PCR was started. Amplification was performed with the following steps: reverse transcription at 50℃ for 30min, transcriptase denaturation at 95℃ for 5 min and then DNA polymerization for 45 cycles of 94℃ for 15 s, 57℃ for 30 s, and 72℃. Then 5µl of PCR product was subjected to agarose gel electrophoresis, using a 2% agarose standard run in 1X Tris borate EDTA (Figure S2). 5µl of Cy5 labeled PCR product was used for the further experiments.

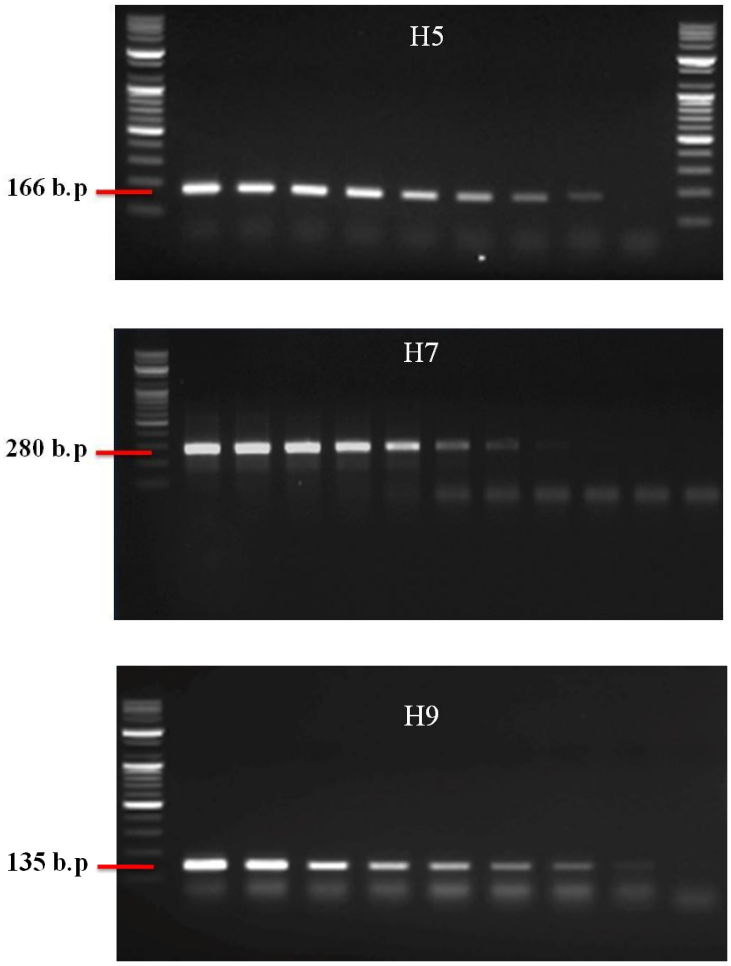
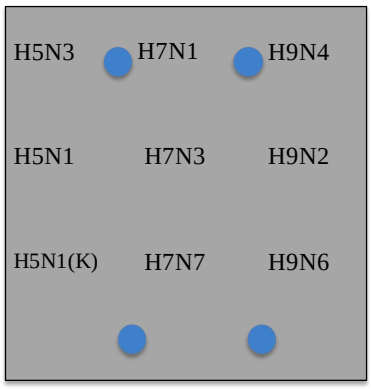


Figure S2. Electrophoresis gel images of HPAI RT-PCR products

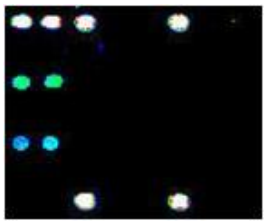
6. Results for hybridization of the probes (without ARTIFICIAL mutations)

6a. Hybridization with Cy5 labeled PCR product of H5N3



PMT gian 58

Original Probe		
Probes	**Sequences**	**T_M**
H5N3	**5'-GGGGGGGGG CTTTATCTT GAG GAC TAT TCG GGG CC-3'**	**51**
H5N1	**5'-GGGGGGGGG CTTTATCTT GAG GAC TAT TTG GGG CC-3'**	**49**
H5N1 (K)	**5'-GGGGGGGGG CTTTATCTT GAG GAC TAT TTG GAG CT-3'**	**45**
H7N1	5'-GGGGGGGGG CTTTATCTT GAG GCC TAT TTG GTG CC-3'	49
H7N3	5'-GGGGGGGGG CTTTATCTT GAG GAC TCT TCG GGG CA-3'	47
H7N7	5'-GGGGGGGGG CTTTATCTT AGG CTT ATT TGG TGC CA-3'	49
H9N4	5'-GGGGGGGGG CTTTATCTT GAG GGT TGT TTG GTG CT-3'	47
H9N2	5'-GGGGGGGGG CTTTATCTT GAG GGC TCT TCG GTG CC-3'	49
H9N6	5'-GGGGGGGGG CTTTATCTT GAG GGC TAT TTG GTG CC -3'	51



PCR H5N3

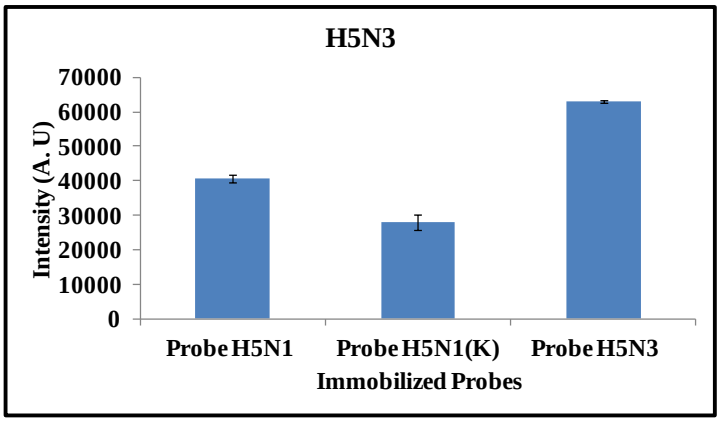
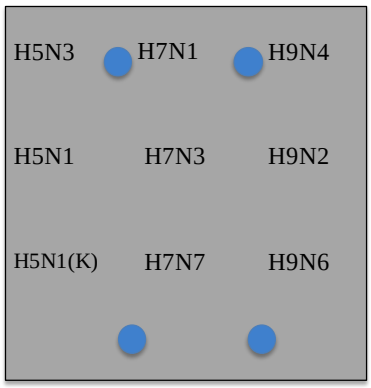


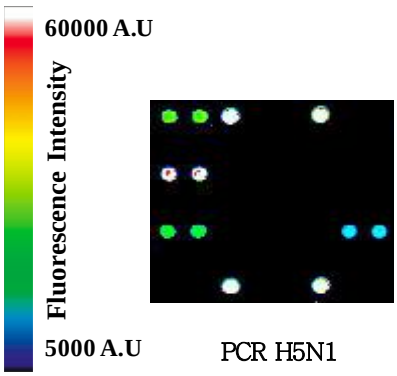
Figure S3: Fluorescence image after hybridization of Pre-HPAI 9GDNAChip with Cy5 labeled PCR product of H5N3. PMT gain = 58.

6b. Hybridization with Cy5 labeled PCR product of H5N1



PMT gian 58

Original Probe		
Probes	**Sequences**	**T_M**
H5N1	**5'-GGGGGGGGG CTTTATCTT GAG GAC TAT TTG GGG CC-3'**	**49**
H5N3	**5'-GGGGGGGGG CTTTATCTT GAG GAC TAT TCG GGG CC-3'**	**51**
H5N1 (K)	**5'-GGGGGGGGG CTTTATCTT GAG GAC TAT TTG GAG CT-3'**	**45**
H9N6	**5'-GGGGGGGGG CTTTATCTT GAG GGC TAT TTG GTG CC -3'**	**51**
H7N1	5'-GGGGGGGGG CTTTATCTT GAG GCC TAT TTG GTG CC-3'	49
H7N3	5'-GGGGGGGGG CTTTATCTT GAG GAC TCT TCG GGG CA-3'	47
H7N7	5'-GGGGGGGGG CTTTATCTT AGG CTT ATT TGG TGC CA-3'	49
H9N4	5'-GGGGGGGGG CTTTATCTT GAG GGT TGT TTG GTG CT-3'	47
H9N2	5'-GGGGGGGGG CTTTATCTT GAG GGC TCT TCG GTG CC-3'	49



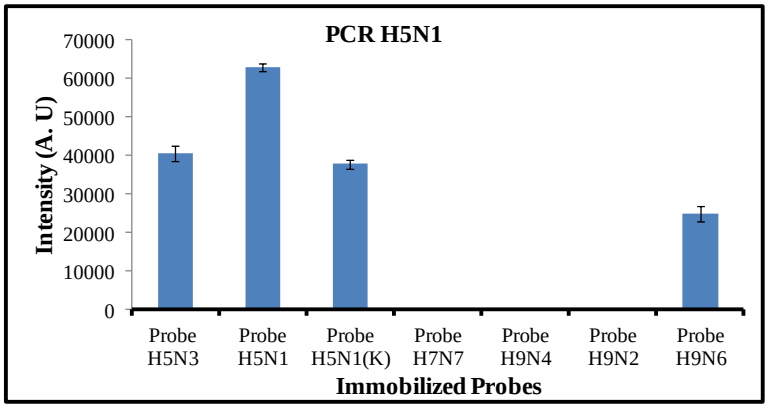
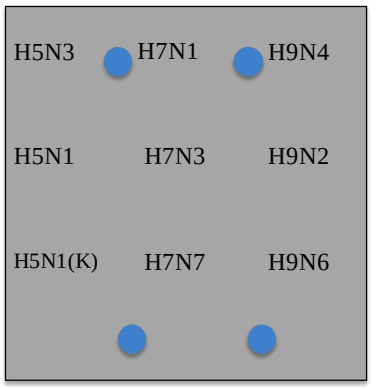


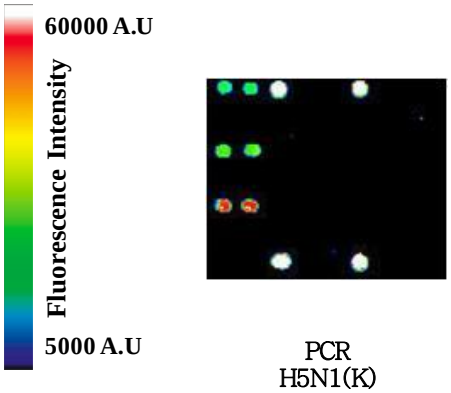
Figure S4: Fluorescence image after hybridization of Pre-HPAI 9GDNAChip with Cy5 labeled PCR product of H5N1. PMT gain = 58.

6c. Hybridization with Cy5 labeled PCR product of H5N1 (K)



PMT gian 58

Original Probe		
Probes	**Sequences**	**T_M**
H5N1 (K)	**5'-GGGGGGGGG CTTTATCTT GAG GAC TAT TTG GAG CT-3'**	**45**
H5N3	**5'-GGGGGGGGG CTTTATCTT GAG GAC TAT TCG GGG CC-3'**	**51**
H5N1	**5'-GGGGGGGGG CTTTATCTT GAG GAC TAT TTG GGG CC-3'**	**49**
H7N1	5'-GGGGGGGGG CTTTATCTT GAG GCC TAT TTG GTG CC-3'	49
H7N3	5'-GGGGGGGGG CTTTATCTT GAG GAC TCT TCG GGG CA-3'	47
H7N7	5'-GGGGGGGGG CTTTATCTT AG GCT TAT TTG GTG CCA-3'	49
H9N4	5'-GGGGGGGGG CTTTATCTT GAG GGT TGT TTG GTG CT-3'	47
H9N2	5'-GGGGGGGGG CTTTATCTT GAG GGC TCT TCG GTG CC-3'	49
H9N6	5'-GGGGGGGGG CTTTATCTT GAG GGC TAT TTG GTG CC -3'	51



PCR
H5N1(K)

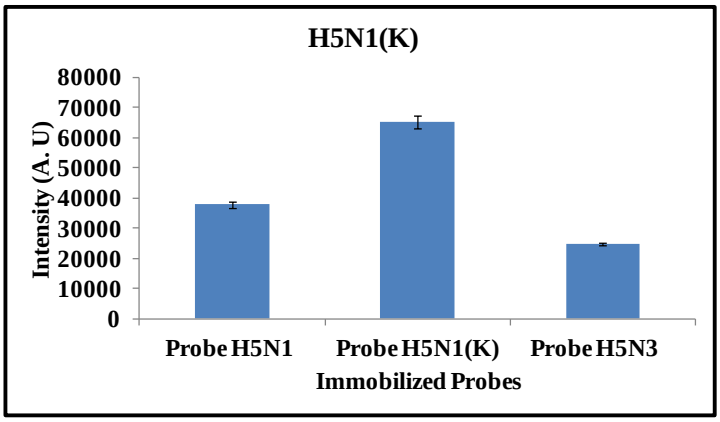
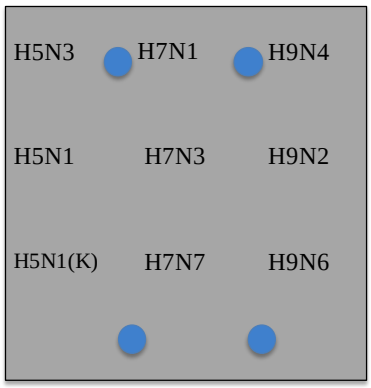


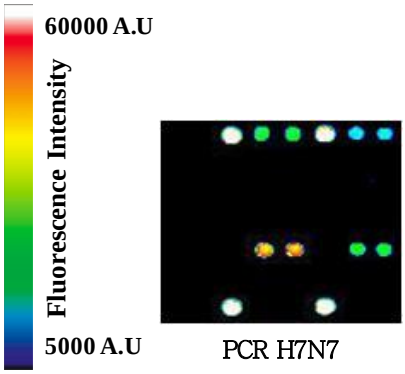
Figure S5: Fluorescence image after hybridization of Pre-HPAI 9GDNAChip with Cy5 labeled PCR product of H5N1 (K). PMT gain = 58.

6d. Hybridization with Cy5 labeled PCR product of H7N7



PMT gian 58

Original Probe		
Probes	**Sequences**	**T_M**
H7N7	**5'-GGGGGGGGG CTTTATCTTAG GCT TAT TTG GTG CC A-3'**	**49**
H7N1	**5'-GGGGGGGGG CTTTATCTT GAG GCC TAT TTG GTG CC-3'**	**49**
H9N4	**5'-GGGGGGGGG CTTTATCTT GAG GGT TGT TTG GTG CT-3'**	**47**
H9N6	**5'-GGGGGGGGG CTTTATCTT GAG GGC TAT TTG GTG CC -3'**	**51**
H5N3	5'-GGGGGGGGG CTTTATCTT GAG GAC TAT TCG GGG CC-3'	51
H5N1	5'-GGGGGGGGG CTTTATCTT GAG GAC TAT TTG GGG CC-3'	49
H5N1 (K)	5'-GGGGGGGGG CTTTATCTT GAG GAC TAT TTG GAG CT-3'	45
H7N3	5'-GGGGGGGGG CTTTATCTT GAG GAC TCT TCG GGG CA-3'	47
H9N2	5'-GGGGGGGGG CTTTATCTT GAG GGC TCT TCG GTG CC-3'	49



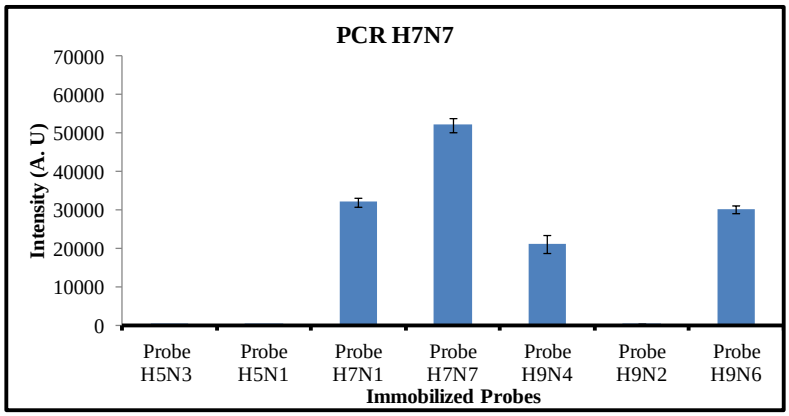
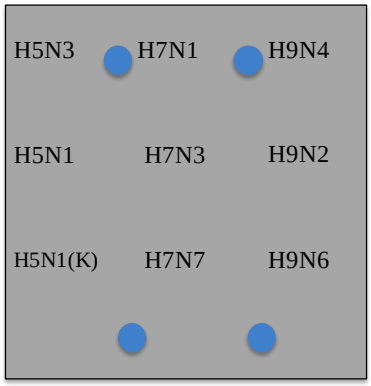


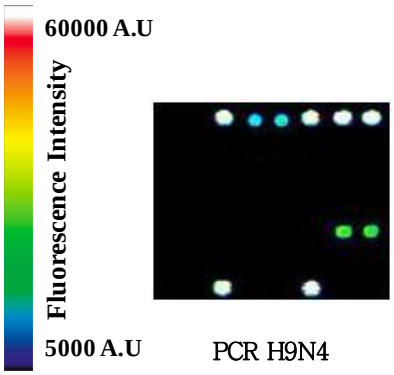
Figure S6: Fluorescence image after hybridization of Pre-HPAI 9GDNAChip with Cy5 labeled PCR product of H7N7. PMT gain = 58.

6e. Hybridization with Cy5 labeled PCR product of H9N4



PMT gian 58

Original Probe		
Probes	**Sequences**	**T_M**
H9N4	**5'-GGGGGGGGG CTTTATCTT GAG GGT TGT TTG GTG CT-3'**	**47**
H9N6	**5'-GGGGGGGGG CTTTATCTT GAG GGC TAT TTG GTG CC -3'**	**51**
H7N1	**5'-GGGGGGGGG CTTTATCTT GAG GCC TAT TTG GTG CC-3'**	**49**
H9N2	5'-GGGGGGGGG CTTTATCTT GAG GGC TCT TCG GTG CC-3'	49
H5N3	5'-GGGGGGGGG CTTTATCTT GAG GAC TAT TCG GGG CC-3'	51
H5N1	5'-GGGGGGGGG CTTTATCTT GAG GAC TAT TTG GGG CC-3'	49
H5N1 (K)	5'-GGGGGGGGG CTTTATCTT GAG GAC TAT TTG GAG CT-3'	45
H7N3	5'-GGGGGGGGG CTTTATCTT GAG GAC TCT TCG GGG CA-3'	47
H7N7	5'-GGGGGGGGG CTTTATCTT AG GCT TAT TTG GTG CC A-3'	49



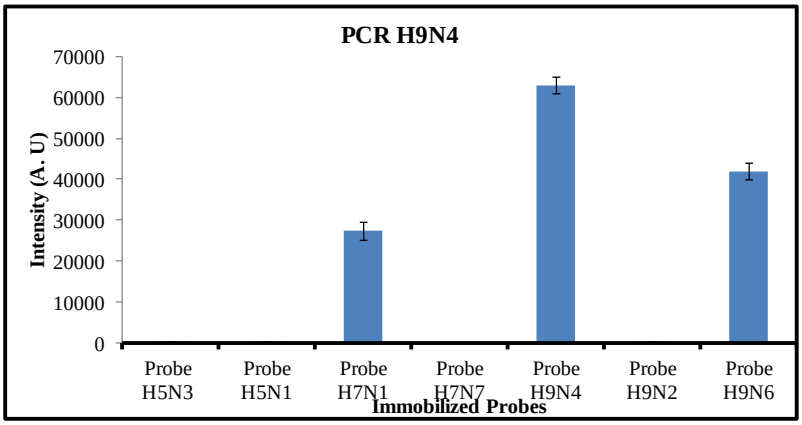
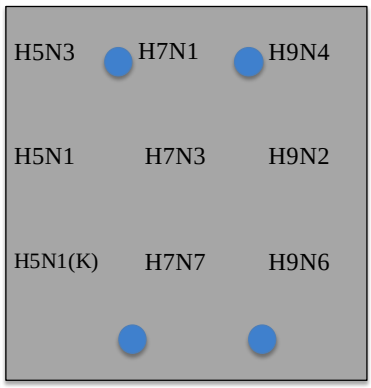


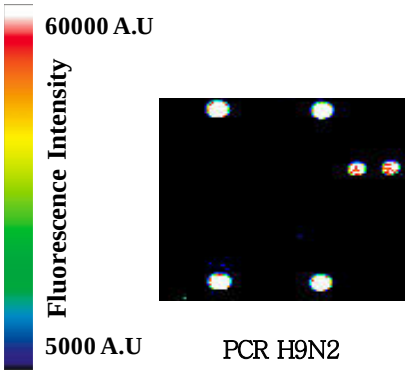
Figure S7: Fluorescence image after hybridization of Pre-HPAI 9GDNAChip with Cy5 labeled PCR product of H9N4. PMT gain = 58.

6f. Hybridization with Cy5 labeled PCR product of H9N2



PMT gian 58

Original Probe		
Probes	**Sequences**	**T_M**
H9N2	**5'-GGGGGGGGG CTTTATCTT GAG GGC TCT TCG GTG CC-3'**	**49**
H5N3	5'-GGGGGGGGG CTTTATCTT GAG GAC TAT TCG GGG CC-3'	51
H5N1	5'-GGGGGGGGG CTTTATCTT GAG GAC TAT TTG GGG CC-3'	49
H5N1 (K)	5'-GGGGGGGGG CTTTATCTT GAG GAC TAT TTG GAG CT-3'	45
H7N1	5'-GGGGGGGGG CTTTATCTT GAG GCC TAT TTG GTG CC-3'	49
H7N3	5'-GGGGGGGGG CTTTATCTT GAG GAC TCT TCG GGG CA-3'	47
H7N7	5'-GGGGGGGGG CTTTATCTT AGG CTT ATT TGG TGC CA-3'	49
H9N4	5'-GGGGGGGGG CTTTATCTT GAG GGT TGT TTG GTG CT-3'	47
H9N6	5'-GGGGGGGGG CTTTATCTT GAG GGC TAT TTG GTG CC -3'	51



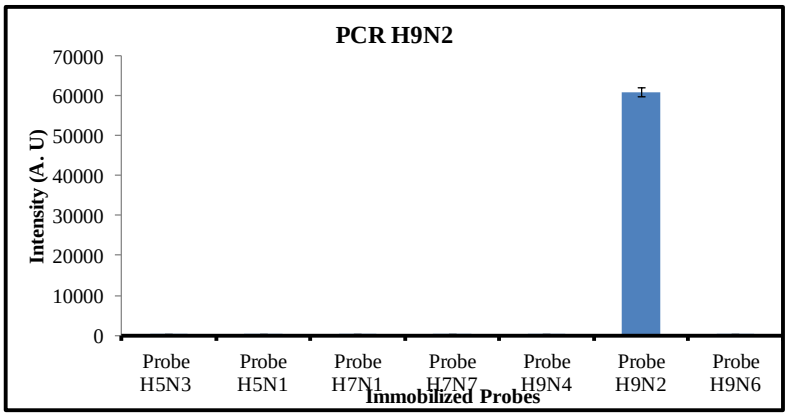
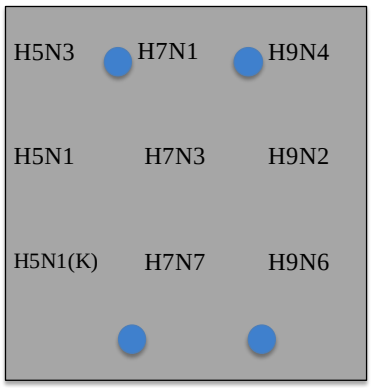


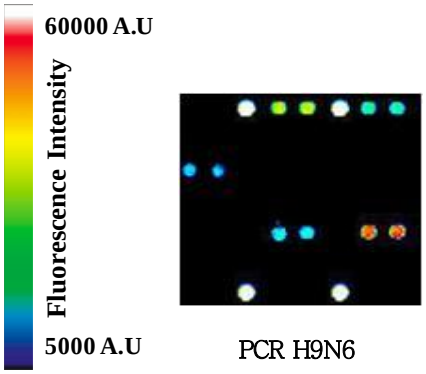
Figure S8: Fluorescence image after hybridization of Pre-HPAI 9GDNAChip with Cy5 labeled PCR product of H9N2. PMT gain = 58.

6g. Hybridization with Cy5 labeled PCR product of H9N6



PMT gian 58

Original Probe		
Probes	**Sequences**	**T_M**
H9N6	**5'-GGGGGGGGG CTTTATCTT GAG GGC TAT TTG GTG CC -3'**	**51**
H7N1	**5'-GGGGGGGGG CTTTATCTT GAG GCC TAT TTG GTG CC-3'**	**49**
H9N4	**5'-GGGGGGGGG CTTTATCTT GAG GGT TGT TTG GTG CT-3'**	**47**
H7N7	**5'-GGGGGGGGG CTTTATCTT AG GCT TAT TTG GTG CC A-3'**	**49**
H5N1	**5'-GGGGGGGGG CTTTATCTT GAG GAC TAT TTG GGG CC-3'**	**49**
H5N3	5'-GGGGGGGGG CTTTATCTT GAG GAC TAT TCG GGG CC-3'	51
H5N1 (K)	5'-GGGGGGGGG CTTTATCTT GAG GAC TAT TTG GAG CT-3'	45
H7N3	5'-GGGGGGGGG CTTTATCTT GAG GAC TCT TCG GGG CA-3'	47
H9N2	5'-GGGGGGGGG CTTTATCTT GAG GGC TCT TCG GTG CC-3'	49



PCR H9N6

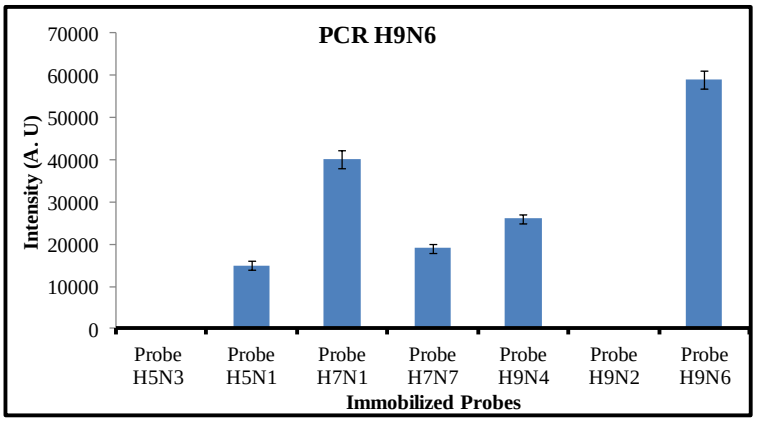
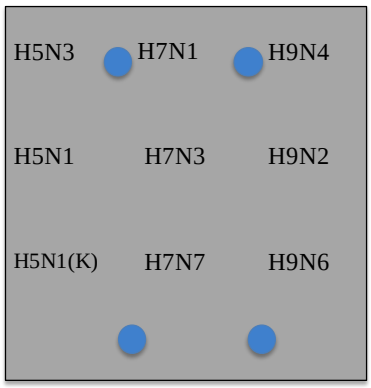


Figure S9: Fluorescence image after hybridization of Pre-HPAI 9GDNAChip with Cy5 labeled PCR product of H9N6. PMT gain = 58.

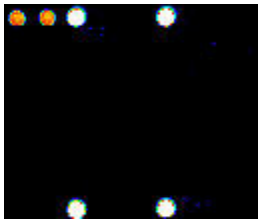
7. Results for hybridization of the probes (with one artifical mutations)

7a. Hybridization with Cy5 labeled PCR product of H5N3



PMT gian 58

Artificial Probe		
Probes	**Sequences**	**T_M**
H5N3	**5'-GGGGGGGGG CTTTATCTT GAG GTC TAT TCG GGG CC-3'**	**51**
H5N1	**5'-GGGGGGGGG CTTTATCTT GAG GAC TTT TTG GGG CC-3'**	**49**
H5N1 (K)	**5'-GGGGGGGGG CTTTATCTT GAG GAC TTT TTG GAG CT-3'**	**45**
H7N1	5'-GGGGGGGGG CTTTATCTT GAG GCC TAT TTG TTG CC-3'	**47**
H7N3	5'-GGGGGGGGG CTTTATCTT GAG GAC TCT TCG TGG CA-3'	**45**
H7N7	5'-GGGGGGGGG CTTTATCTT AGG CTT ATT TGG TTC CA-3'	**47**
H9N4	5'-GGGGGGGGG CTTTATCTT GAG GGT TGT TTG TTG CT-3'	**45**
H9N2	5'-GGGGGGGGG CTTTATCTT GAG GGC TCT TCG TTG CC-3'	**51**
H9N6	5'-GGGGGGGGG CTTTATCTT GAG GGC TAT TTG TTG CC -3'	**47**



PCR H5N3

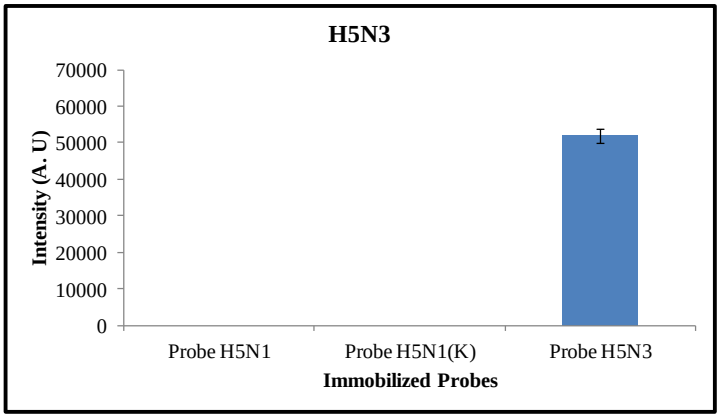
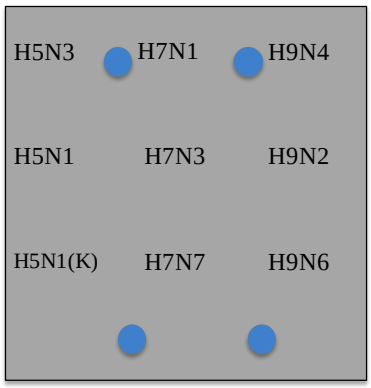


Figure S10: Fluorescence image after hybridization of final HPAI 9GDNAChip with Cy5 labeled PCR product of H5N3. PMT gain = 58.

7b. Hybridization with Cy5 labeled PCR product of H5N1



PMT gian 58

Artificial Probe		
Probes	**Sequences**	**T_M**
H5N1	**5'-GGGGGGGGG CTTTATCTT GAG GACTTT TTG GGG CC-3'**	**49**
H5N3	**5'-GGGGGGGGG CTTTATCTT GAG GTC TAT TCG GGG CC-3'**	**51**
H5N1 (K)	**5'-GGGGGGGGG CTTTATCTT GAG GACTTT TTG GAG CT-3'**	**45**
H9N6	**5'-GGGGGGGGG CTTTATCTT GAG GGC TAT TTG TTG CC -3'**	**49**
H7N1	**5'-GGGGGGGGG CTTTATCTT GAG GCC TAT TTG TTG CC-3'**	47
H7N3	**5'-GGGGGGGGG CTTTATCTT GAG GAC TCT TCG TGG CA-3'**	45
H7N7	**5'-GGGGGGGGG CTTTATCTTAGG CTTATT TGG TTC CA-3'**	47
H9N4	**5'-GGGGGGGGG CTTTATCTT GAG GGT TGT TTG TTG CT-3'**	45
H9N2	**5'-GGGGGGGGG CTTTATCTT GAG GGC TCT TCG TTG CC-3'**	47

PCR H5N1

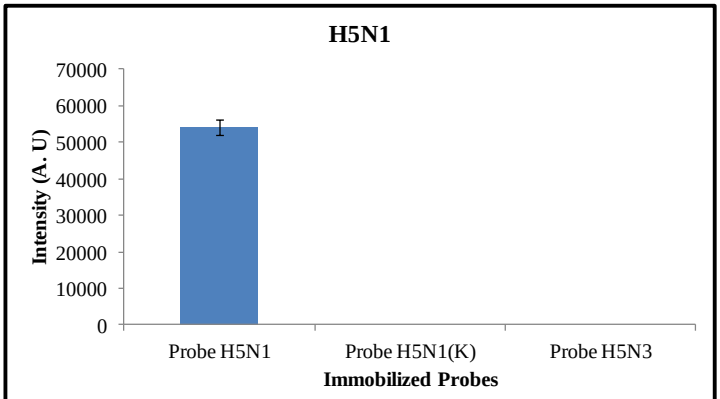
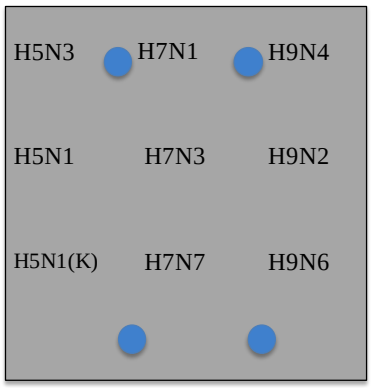


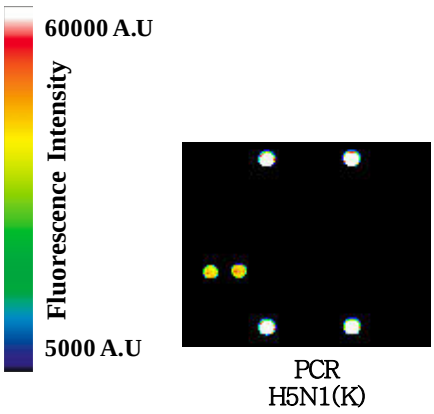
Figure S11: Fluorescence image after hybridization of final HPAI 9GDNAChip with Cy5 labeled PCR product of H5N1. PMT gain = 58.

7c. Hybridization with Cy5 labeled PCR product of H5N1(K)



PMT gian 58

Artificial Probe		
Probes	**Sequences**	**T_M**
H5N1 (K)	5'-GGGGGGGGG CTTTATCTT GAG GAC TTT TTG GAG CT-3'	45
H5N3	5'-GGGGGGGGG CTTTATCTT GAG GTC TAT TCG GGG CC-3'	51
H5N1	5'-GGGGGGGGG CTTTATCTT GAG GAC TTT TTG GGG CC-3'	49
H7N1	5'-GGGGGGGGG CTTTATCTT GAG GCC TAT TTG TTG CC-3'	47
H7N3	5'-GGGGGGGGG CTTTATCTT GAG GAC TCT TCG TGG CA-3'	45
H7N7	5'-GGGGGGGGG CTTTATCTT AGG CTT ATT TGG TTC CA-3'	47
H9N4	5'-GGGGGGGGG CTTTATCTT GAG GGT TGT TTG TTG CT-3'	45
H9N2	5'-GGGGGGGGG CTTTATCTT GAG GGC TCT TCG TTG CC-3'	47
H9N6	5'-GGGGGGGGG CTTTATCTT GAG GGC TAT TTG TTG CC -3'	49



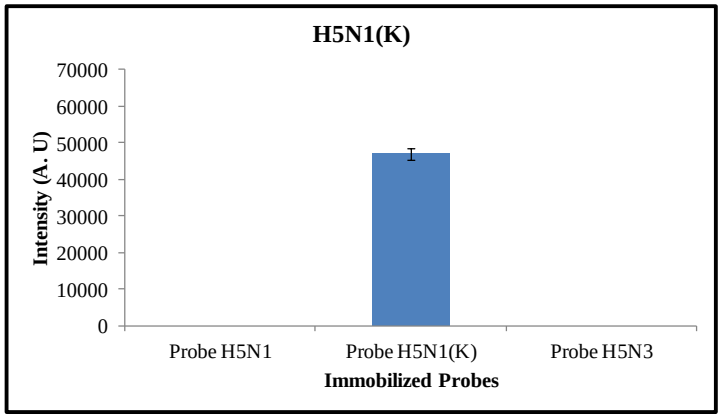
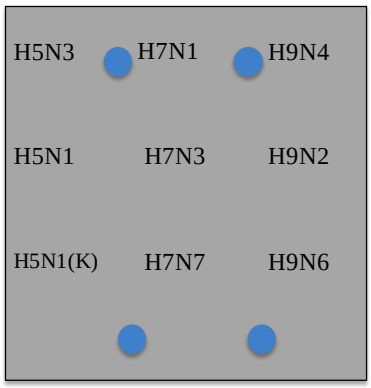


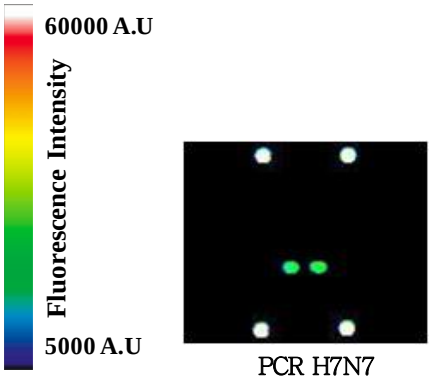
Figure S12: Fluorescence image after hybridization of final HPAI 9GDNAChip with Cy5 labeled PCR product of H5N1(K). PMT gain = 58.

7d. Hybridization with Cy5 labeled PCR product of H7N7



PMT gian 58

Artificial Probe		
Probes	**Sequences**	**T_M**
H7N7	**5'-GGGGGGGGG CTTTATCTTAG GCT TAT TTG GTT CC A-3'**	**47**
H7N1	**5'-GGGGGGGGG CTTTATCTT GAG GCC TAT TTG TTG CC-3'**	**47**
H9N4	**5'-GGGGGGGGG CTTTATCTT GAG GGT TGT TTG TTG CT-3'**	**45**
H9N6	**5'-GGGGGGGGG CTTTATCTT GAG GGC TAT TTG TTG CC -3'**	**49**
H5N3	5'-GGGGGGGGG CTTTATCTT GAG GTC TAT TCG GGG CC-3'	51
H5N1	5'-GGGGGGGGG CTTTATCTT GAG GAC TTT TTG GGG CC-3'	49
H5N1 (K)	5'-GGGGGGGGG CTTTATCTT GAG GAC TTT TTG GAG CT-3'	45
H7N3	5'-GGGGGGGGG CTTTATCTT GAG GAC TCT TCG TGG CA-3'	45
H9N2	5'-GGGGGGGGG CTTTATCTT GAG GGC TCT TCG TTG CC-3'	47



PCR H7N7

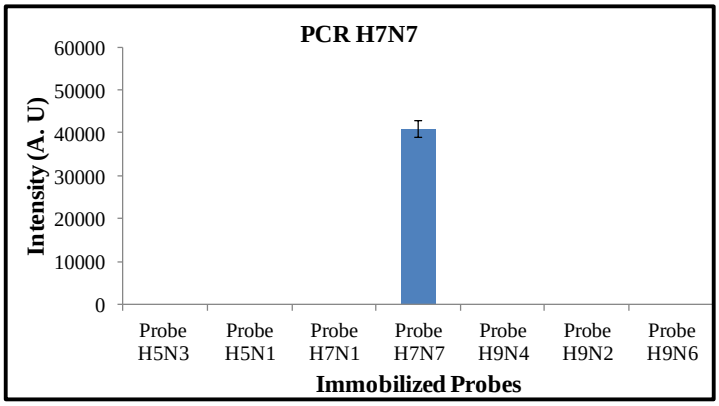
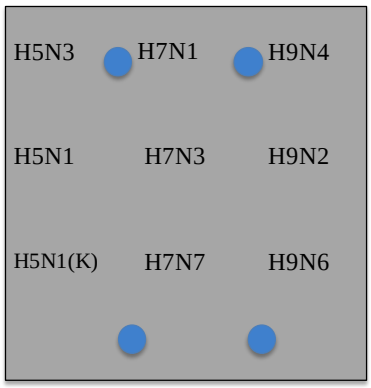


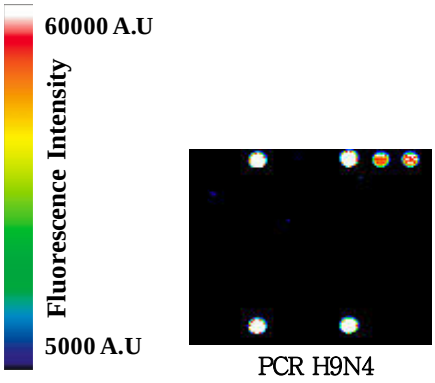
Figure S13: Fluorescence image after hybridization of final HPAI 9GDNAChip with Cy5 labeled PCR product of H7N7. PMT gain = 58.

7e. Hybridization with Cy5 labeled PCR product of H9N4



PMT gian 58

Artificial Probe		
Probes	**Sequences**	**T_M**
H9N4	**5'-GGGGGGGGG CTTTATCTT GAG GGT TGT TTG TTG CT-3'**	**45**
H9N6	**5'-GGGGGGGGG CTTTATCTT GAG GGC TAT TTG TTG CC -3'**	**49**
H7N1	**5'-GGGGGGGGG CTTTATCTT GAG GCC TAT TTG TTG CC-3'**	**47**
H9N2	5'-GGGGGGGGG CTTTATCTT GAG GGC TCT TCG TTG CC-3'	47
H5N3	5'-GGGGGGGGG CTTTATCTT GAG GTC TAT TCG GGG CC-3'	51
H5N1	5'-GGGGGGGGG CTTTATCTT GAG GAC TTT TTG GGG CC-3'	49
H5N1 (K)	5'-GGGGGGGGG CTTTATCTT GAG GAC TTT TTG GAG CT-3'	45
H7N3	5'-GGGGGGGGG CTTTATCTT GAG GAC TCT TCG TGG CA-3'	45
H7N7	5'-GGGGGGGGG CTTTATCTT AG GCT TAT TTG TTG CC A-3'	47



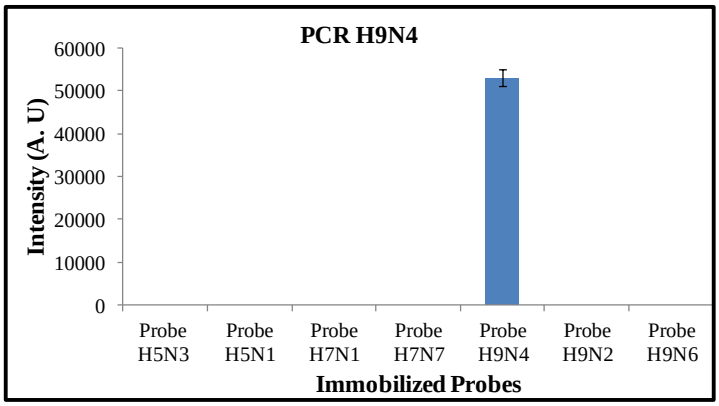
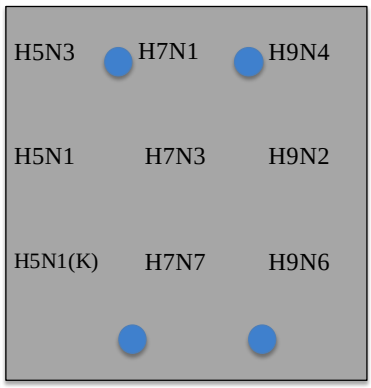


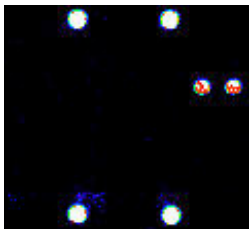
Figure S14: Fluorescence image after hybridization of final HPAI 9GDNAChip with Cy5 labeled PCR product of H9N4. PMT gain = 58.

7f. Hybridization with Cy5 labeled PCR product of H9N2



PMT gian 58

Artificial Probe		
Probes	**Sequences**	**T_M**
H9N2	5'-GGGGGGGGG CTTTATCTT GAG GGC TCT TCG TTG CC-3'	47
H5N3	5'-GGGGGGGGG CTTTATCTT GAG GTC TAT TCG GGG CC-3'	51
H5N1	5'-GGGGGGGGG CTTTATCTT GAG GAC TTT TTG GGG CC-3'	49
H5N1 (K)	5'-GGGGGGGGG CTTTATCTT GAG GAC TTT TTG GAG CT-3'	45
H7N1	5'-GGGGGGGGG CTTTATCTT GAG GCC TAT TTG TTG CC-3'	47
H7N3	5'-GGGGGGGGG CTTTATCTT GAG GAC TCT TCG TGG CA-3'	45
H7N7	5'-GGGGGGGGG CTTTATCTT AGG CTT ATT TGG TTC CA-3'	47
H9N4	5'-GGGGGGGGG CTTTATCTT GAG GGT TGT TTG TTG CT-3'	45
H9N6	5'-GGGGGGGGG CTTTATCTT GAG GGC TAT TTG TTG CC -3'	49



PCR H9N2

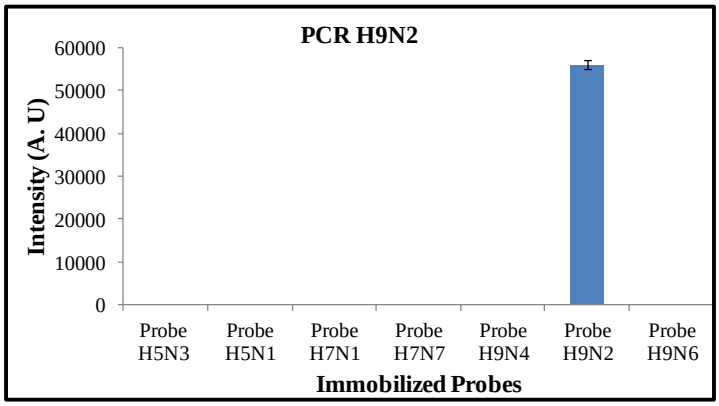
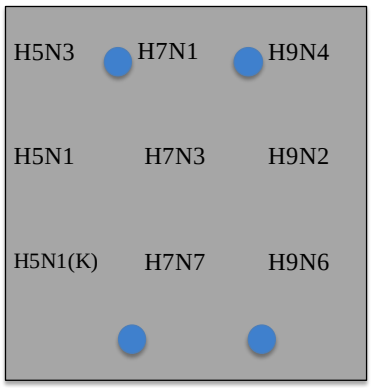


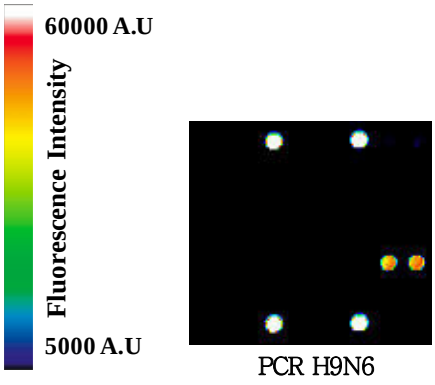
Figure S15: Fluorescence image after hybridization of final HPAI 9GDNAChip with Cy5 labeled PCR product of H9N2. PMT gain = 58.

7g. Hybridization with Cy5 labeled PCR product of H9N6



PMT gian 58

Artificial Probe		
Probes	**Sequences**	**T_M**
H9N6	**5'-GGGGGGGGG CTTTATCTT GAG GGC TAT TTG TTG CC -3'**	**49**
H7N1	**5'-GGGGGGGGG CTTTATCTT GAG GCC TAT TTG TTG CC-3'**	**47**
H9N4	**5'-GGGGGGGGG CTTTATCTT GAG GGT TGT TTG TTG CT-3'**	**45**
H7N7	**5'-GGGGGGGGG CTTTATCTT AG GCT TAT TTG GTT CC A-3'**	**47**
H5N1	**5'-GGGGGGGGG CTTTATCTT GAG GAC TTT TTG GGG CC-3'**	**49**
H5N3	5'-GGGGGGGGG CTTTATCTT GAG GTC TAT TCG GGG CC-3'	51
H5N1 (K)	5'-GGGGGGGGG CTTTATCTT GAG GAC TTT TTG GAG CT-3'	45
H7N3	5'-GGGGGGGGG CTTTATCTT GAG GAC TCT TCG TGG CA-3'	45
H9N2	5'-GGGGGGGGG CTTTATCTT GAG GGC TCT TCG TTG CC-3'	47



PCR H9N6

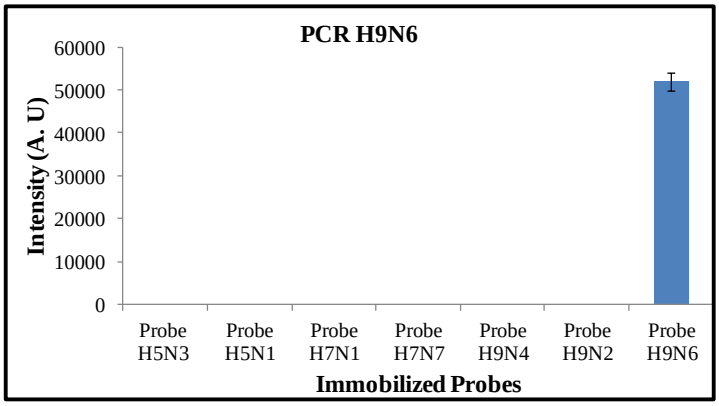
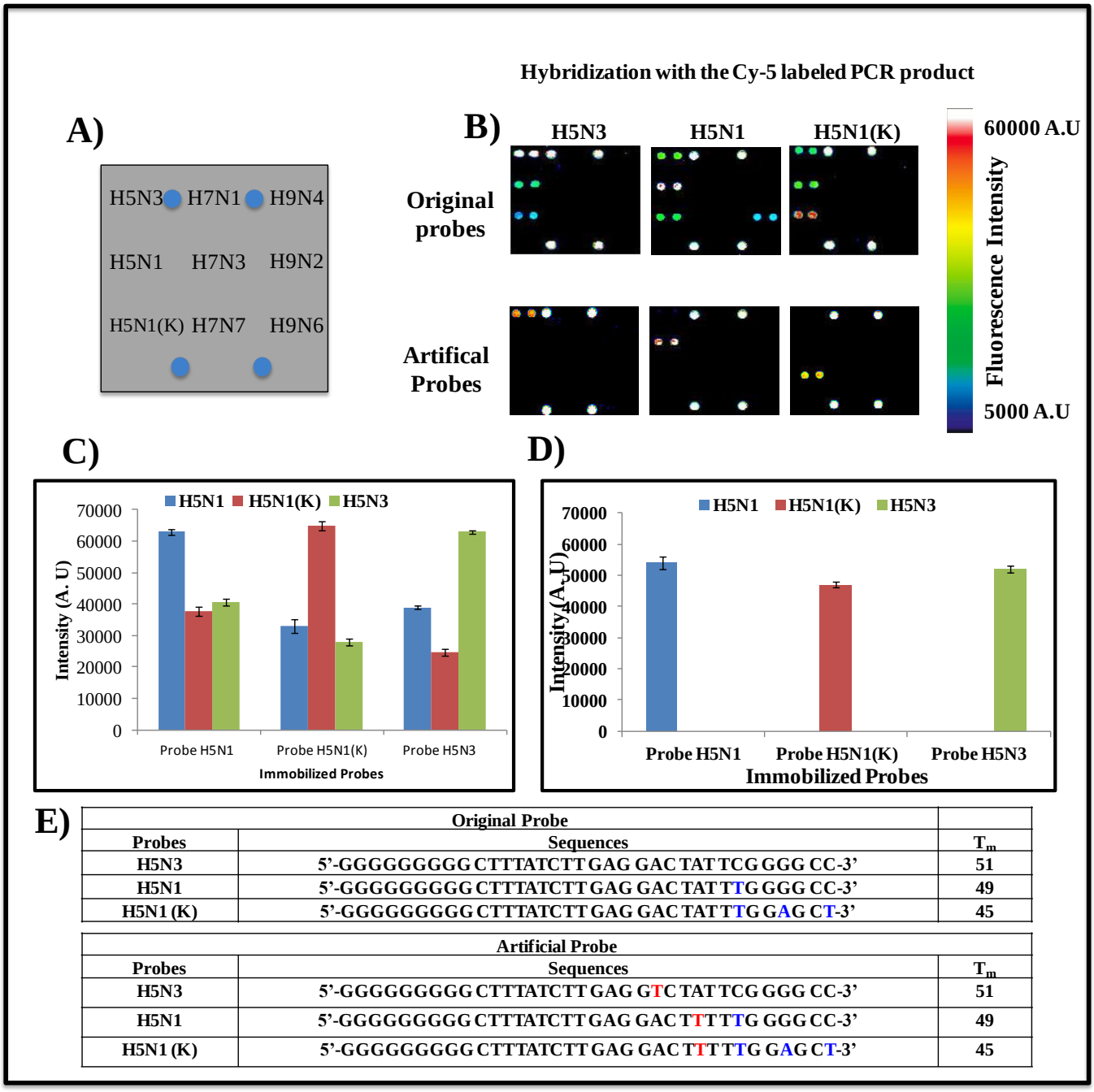


Figure S16: Fluorescence image after hybridization of final HPAI 9GDNAChip with Cy5 labeled PCR product of H9N6. PMT gain = 58.

8. Comparison of the results after hybridization of the original probes (without mutation) and final probes (with one artificial mutation)

8a. Hybridization with Cy5 labeled PCR products of H5N3, H5N1, and H5N1 (K)



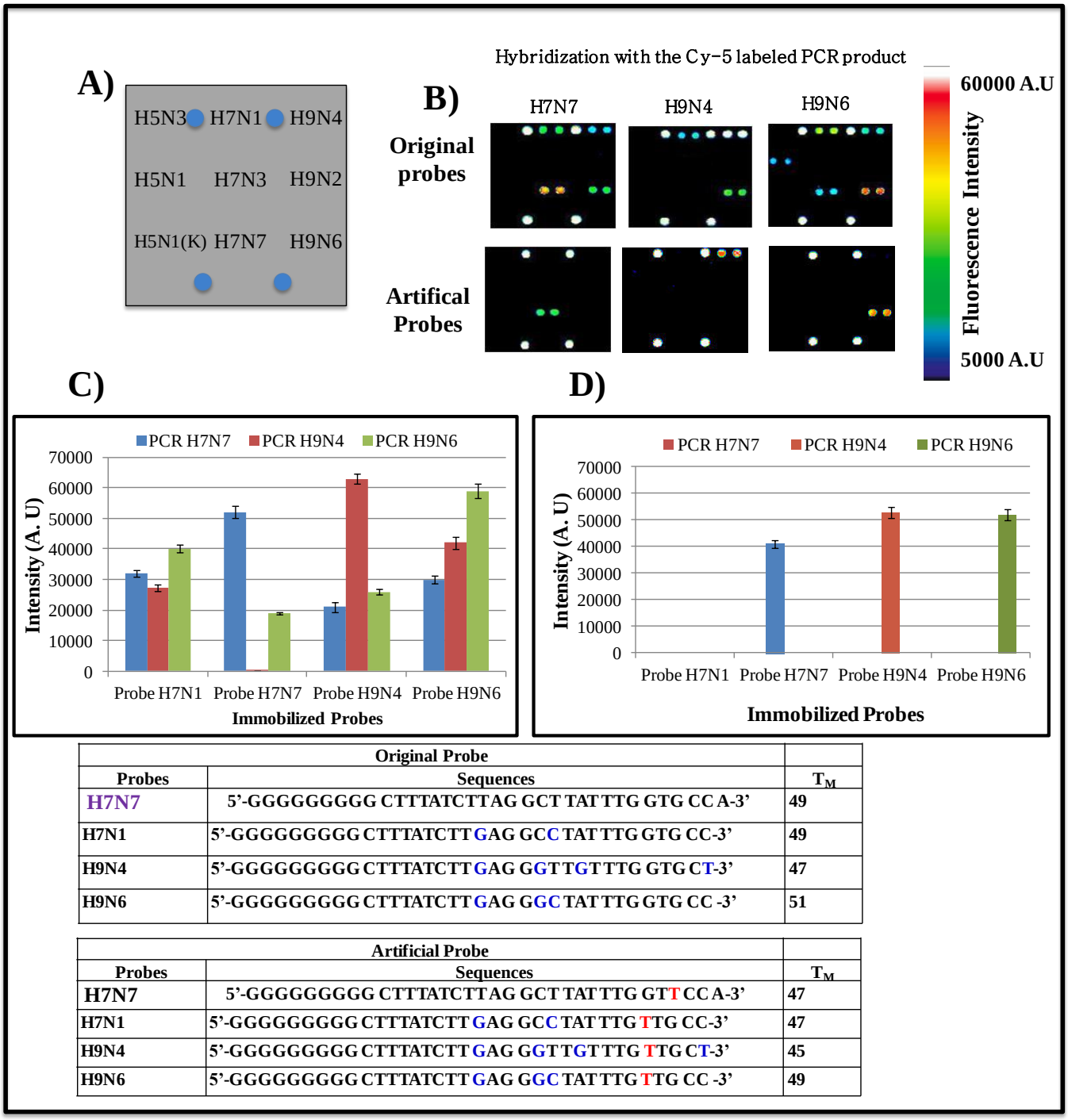
E)

Original Probe		
Probes	**Sequences**	**T_m**
H5N3	5'-GGGGGGGGG CTTTATCTT GAG GAC TAT TCG GGG CC-3'	51
H5N1	5'-GGGGGGGGG CTTTATCTT GAG GAC TAT TTG GGG CC-3'	49
H5N1 (K)	5'-GGGGGGGGG CTTTATCTT GAG GAC TAT TTG GAG CT-3'	45

Artificial Probe		
Probes	**Sequences**	**T_m**
H5N3	5'-GGGGGGGGG CTTTATCTT GAG GTC TAT TCG GGG CC-3'	51
H5N1	5'-GGGGGGGGG CTTTATCTT GAG GAC TTT TTG GGG CC-3'	49
H5N1 (K)	5'-GGGGGGGGG CTTTATCTT GAG GAC TTT TTG GAG CT-3'	45

Figure S17: Fluorescence image after hybridization of HPAI 9G DNAChip with Cy5 labeled PCR product of H5N3, H5N1, and H5N1 (K). PMT gain = 58.

8b. Hybridization with Cy5 labeled PCR products of H7N7, H9N4, and H9N6.

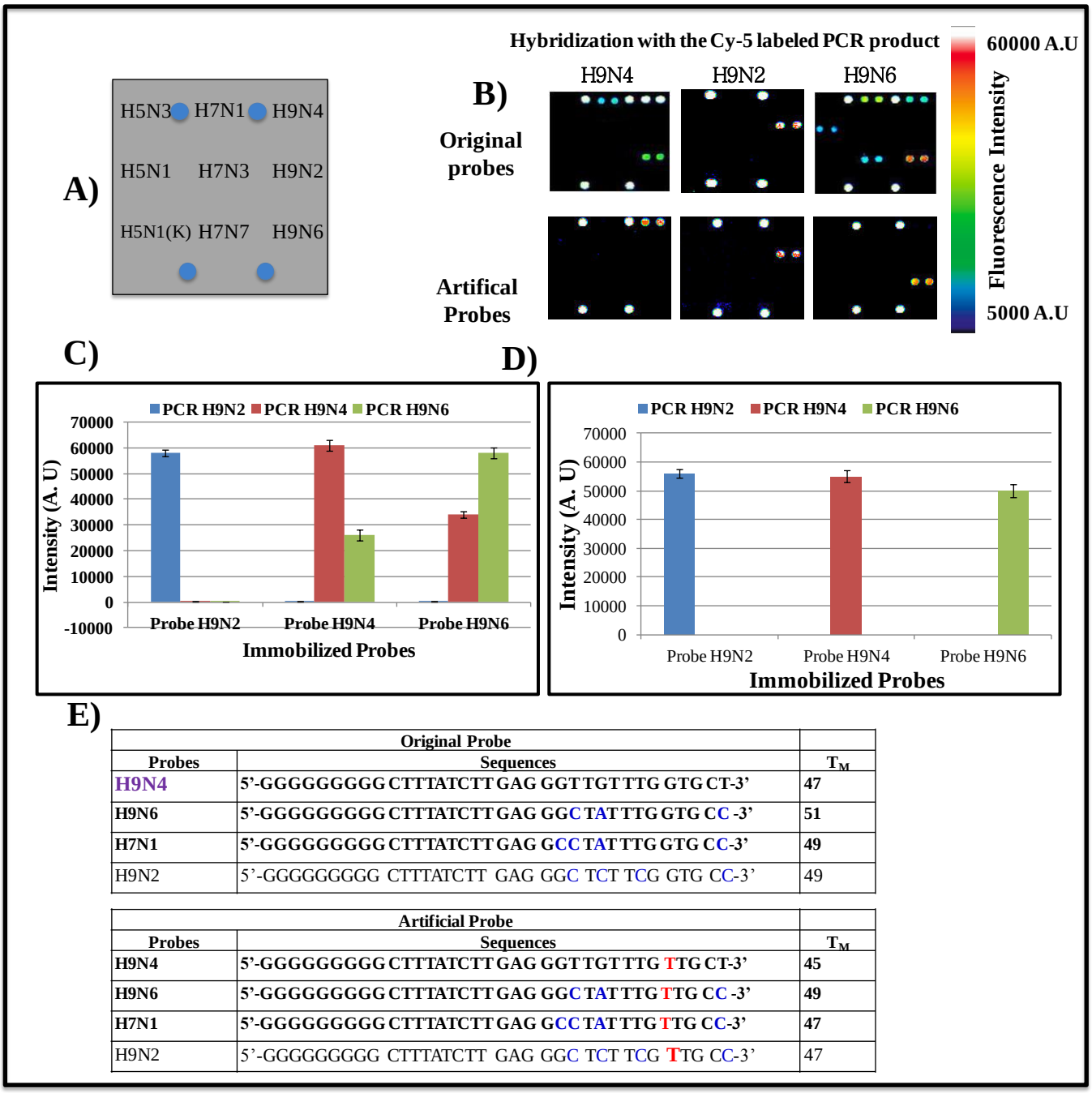


Original Probe		
Probes	**Sequences**	**T_M**
H7N7	**5'-GGGGGGGGG CTTTATCTTAG GCT TAT TTG GTG CC A-3'**	**49**
H7N1	**5'-GGGGGGGGG CTTTATCTT GAG GCC TAT TTG GTG CC-3'**	**49**
H9N4	**5'-GGGGGGGGG CTTTATCTT GAG GGT TGT TTG GTG CT-3'**	**47**
H9N6	**5'-GGGGGGGGG CTTTATCTT GAG GGC TAT TTG GTG CC -3'**	**51**

Artificial Probe		
Probes	**Sequences**	**T_M**
H7N7	**5'-GGGGGGGGG CTTTATCTTAG GCT TAT TTG GTT CC A-3'**	**47**
H7N1	**5'-GGGGGGGGG CTTTATCTT GAG GCC TAT TTG TTG CC-3'**	**47**
H9N4	**5'-GGGGGGGGG CTTTATCTT GAG GGT TGT TTG TTG CT-3'**	**45**
H9N6	**5'-GGGGGGGGG CTTTATCTT GAG GGC TAT TTG TTG CC -3'**	**49**

Figure S18: Fluorescence image after hybridization of HPAI 9G DNAChip with Cy5 labeled PCR product of H7N7, H9N4, and H9N6. PMT gain = 58.

8c. Hybridization with Cy5 labeled PCR products of H9N2, H9N4, and H9N6



E)

Original Probe		
Probes	**Sequences**	**T_M**
H9N4	**5'-GGGGGGGGG CTTTATCTT GAG GGT TGT TTG GTG CT-3'**	**47**
H9N6	**5'-GGGGGGGGG CTTTATCTT GAG GGC TAT TTG GTG CC -3'**	**51**
H7N1	**5'-GGGGGGGGG CTTTATCTT GAG GCC TAT TTG GTG CC-3'**	**49**
H9N2	5'-GGGGGGGGG CTTTATCTT GAG GGC TCT TCG GTG CC-3'	49

Artificial Probe		
Probes	**Sequences**	**T_M**
H9N4	**5'-GGGGGGGGG CTTTATCTT GAG GGT TGT TTG TTG CT-3'**	**45**
H9N6	**5'-GGGGGGGGG CTTTATCTT GAG GGC TAT TTG TTG CC -3'**	**49**
H7N1	**5'-GGGGGGGGG CTTTATCTT GAG GCC TAT TTG TTG CC-3'**	**47**
H9N2	5'-GGGGGGGGG CTTTATCTT GAG GGC TCT TCG TTG CC-3'	47

Figure S19: Fluorescence image after hybridization of HPAI 9G DNAChip with Cy5 labeled PCR product of H9N2, H9N4, and H9N6. PMT gain = 58.

9. Comparison of the probes with and without artificial mutations:

The probes immobilized on the Pre-HPAI 9G DNAChip (original probes without mutations) and the HPAI 9G DNAChip (probes with one artificial mutation) were hybridized with the Cy5 labeled PCR products of the H5N3, H5N1, H5N1(K), H7N7, H9N4, H9N2, and H9N6, repsectively. After washing and drying the respective chips were scanned and the results are presented in the Figure S20.

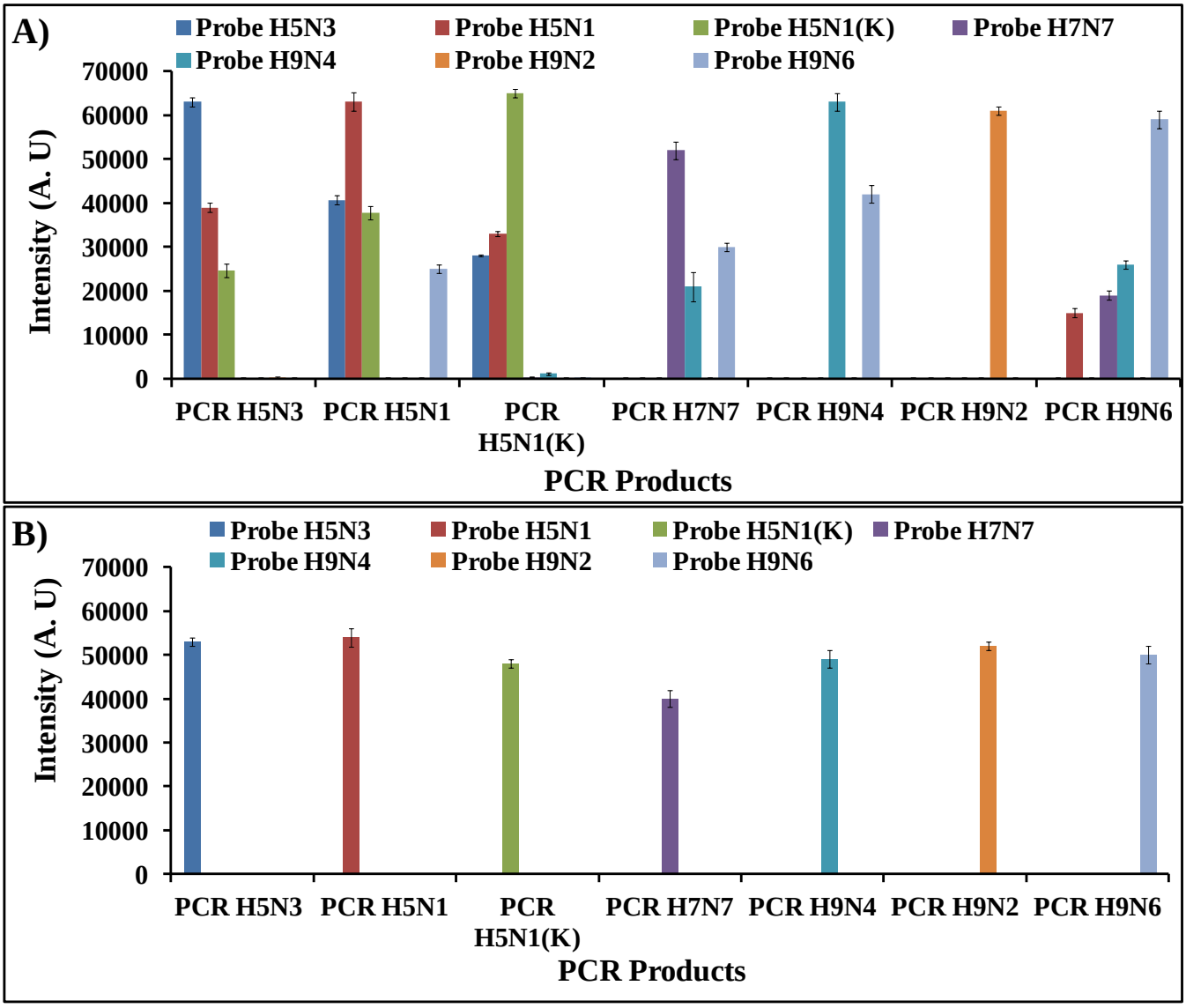


Figure S20: Fluorescence intensities after the hybridization with the Cy5 labeled PCR products of H5N3, H5N1, H5N1(K), H7N7, H9N4, H9N2, and H9N6, A) Graph of fluorescence intensities after the hybridization of the probes (original probes) on the pre-HPAI 9G DNAChip, B) Graph of fluorescence intensities after the hybridization of the probes (with one artificial mutation) on the HPAI 9G DNAChip. PMT gain = 58.

10. Sensitivity of the HPAI 9G DNAChip

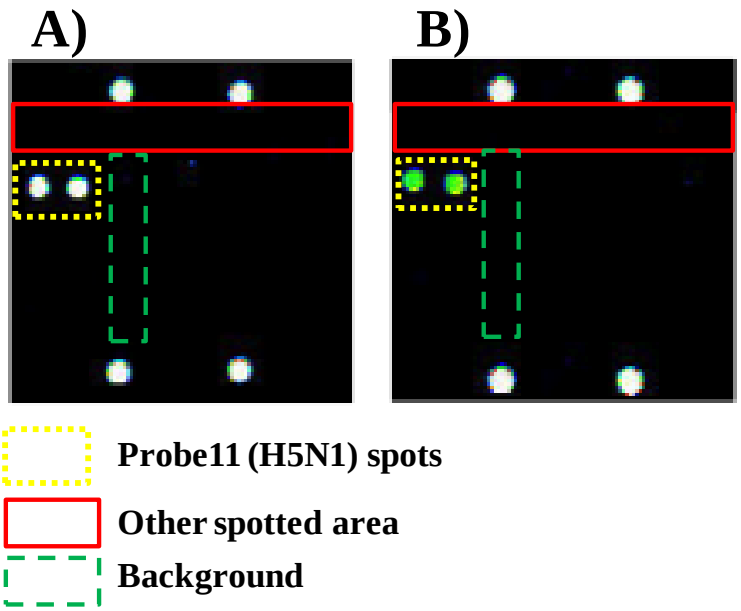


Figure S21: Sensitivity of the HPAI 9G DNAChip, A) fluorescence image after the hybridization of the probes on the HPAI 9G DNAChip with the Cy5 labeled PCR product of H5N1 obtained by using 10^8 copies of PCR template, B) fluorescence image after the hybridization of the probes on the HPAI 9G DNAChip with the Cy5 labeled PCR product of H5N1 obtained by using 10^5 copies of PCR template. MPT gain = 58.

11. Detection limit of HPAI 9G DNAchip

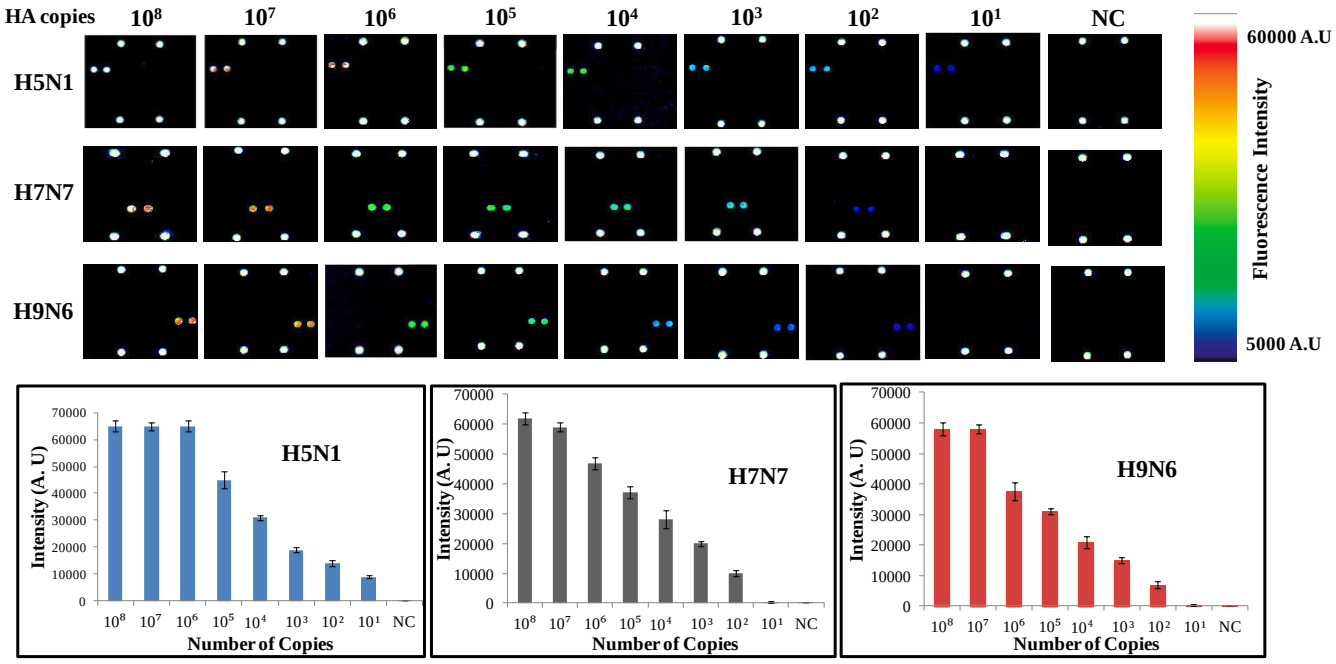


Figure S22: Fluorescence images and the corresponding graphs after the hybridization of the probes (with one artificial mutation) on the HPAI 9G DNAChip with the Cy5 labeled PCR product of H5N1, H7N7, and H9N6 obtained by using different concentrations of the PCR templates. PMT gain = 58.

1. Zhang, W. D., and D. H. Evans. 1991. Detection and identification of human influenza viruses by the polymerase chain reaction. J. Virol. Methods. **33**: 165–189.

2. (a) K. Song, S. B. Nimse, J. Kim, J. Kim, V. Nguyen, V. Ta, T. Kim, *Chem. Commun.*, 2011, **47**, 7101; (b) K. Song, S. B. Nimse, J. Kim, J. Kim, V. Nguyen, V. Ta, T. Kim, *Chem. Commun.*, 2011, **47**, 7616; (c) S. B. Nimse, K. Song, J. Kim, V. Ta, V. Nguyen, T. Kim, *Chem. Commun.*, 2011, **47**, 12444.