

## Supplementary Information

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Fig.S17. Duration of FICT in dry condition.

**Table.S1. List of virus in Wonkwang University**

Avian influenza viruses in Wonkwang University		
1	H1N1	A/H1N1/2009/CA
2	H3N2	Influenza A/H3N2 isolated from humans in 2014 was donated from the Korean National Research Resource Center (Registration number: KBPV-VR-85)
3	H5N3	A/spot-billed duck/Korea/KNU SYG06/2006(H5N3)
4	H7N1	A/common teal/Korea/KNU YSR12/2012(H7N1)
5	H7N7	A/mallard/Korea/KNU GPH12/2011(H7N7)
6	H9N2	A/chicken/Korea/KNUSWR09/2009(H9N2)

**Table.S2. List of virus in Chungbuk University**

Avian influenza viruses in Chungbuk University		
1	H1	A/California/04/2009 (H1N1)
2	H2	A/Em/Korea/ W357/2008 (H2N3)
3	H3	A/Perth/16/2009 (H3N2)
4	H4	A/aquatic bird/Korea/W137/2006 (H4N7)
5	H5	W149 (H5N1) A/EM/Korea/W149/2006(H5N1)
6	H5	W452 (H5N8) A/MD/Korea/W452/2014(H5N8)
7	H6	A/Em/Korea/W502/2015 (H6N2)
8	H7	W557 (H7N7) A/duck/Korea/557/2016 (H7N7)
9	H8	A/Em/Korea/W563/ 2016 (H8N6)
10	H9-L428	A/Chicken/Korea/LPM428/2016 (H9N2)
12	H9-L95	A/Chicken/Korea/LPM95/2006 (H9N2)
13	H9-W757	A/Em/Korea/W757/2019 (H9N2)

14	H9-SU-11	A/Em/Korea/Su-11/2019 (H9N2)
15	H9-HCO09	A/chicken/Korea/HC09/2009 (H9N2)
16	H9-L429	A/Chicken/Korea/LPM429/2016 (H9N2)
17	H10	A/aquatic bird/Korea/w296/2007 (H10N4)
18	H11	A/Em/Korea/W552/2016 (H11N9)
19	H12	A/Em/Korea/W373/2008 (H12N5)
20	Influenza B	B/Brisbane/60/2008 (Victoria lineage)
21	Influenza B	B/Phuket/3073/2013 (Yamagata lineage)



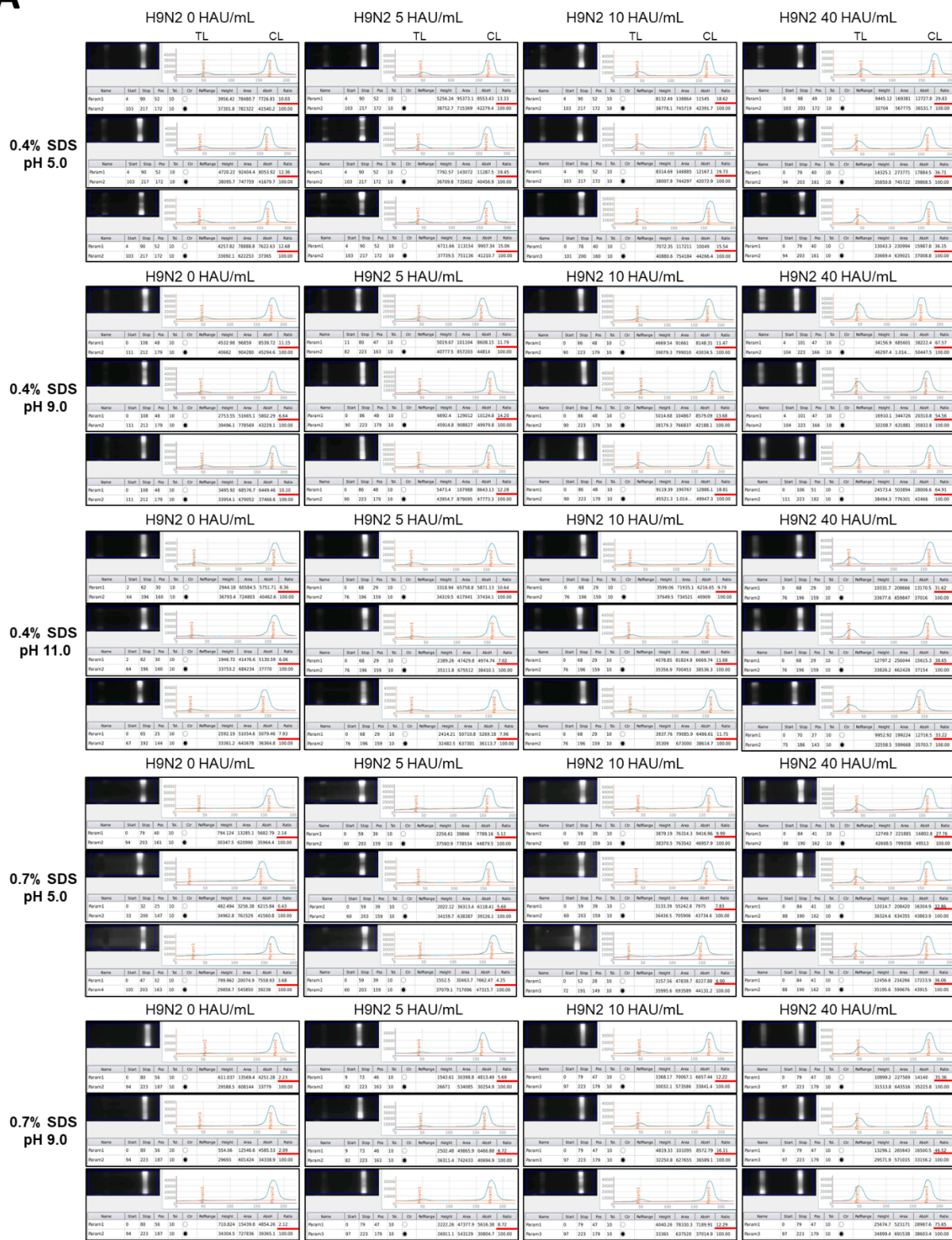
**Table.S3. Real time RT PCR (rRT PCR) with clinical specimens (A/Chicken/Korea/LPM429/2016 (H9N2) strain).**

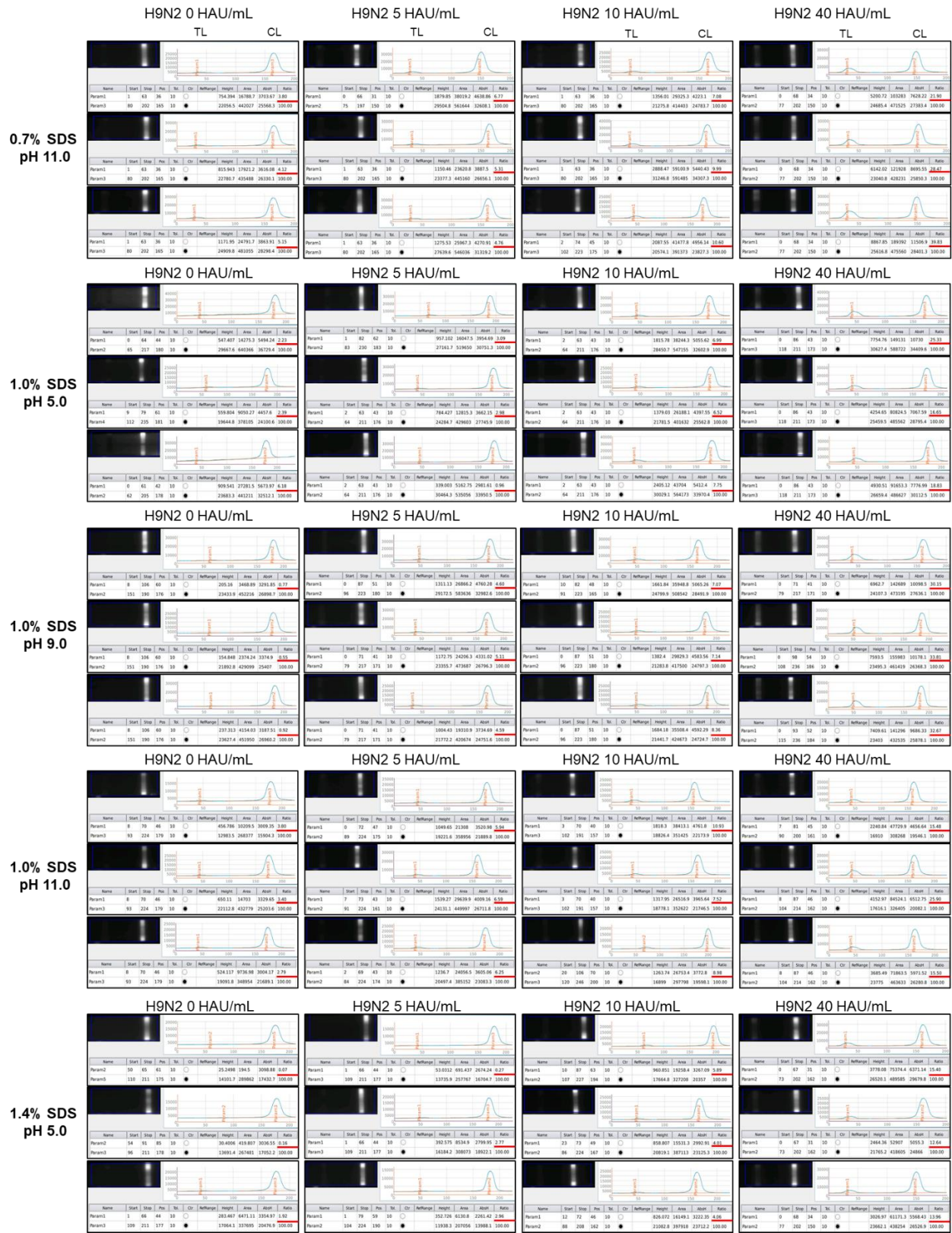
	Clinical specimens		2 dpi					4 dpi					6 dpi				
			Ct	FICT (Ratio of TL/CL)				Ct	FICT (Ratio of TL/CL)				Ct	FICT (Ratio of TL/CL)			
				D5 pair		G10 pair			D5 pair		G10 pair			D5 pair		G10 pair	
Cloacal	C	N	N/A	-	10.47	-	0.12	N/A	-	0.69	-	0.32	N/A	-	8.52	-	0.13
	C	N		-	54.30	-	4.66		-	30.20	-	6.47		-	32.74	-	2.19
	C	N		-	32.04	-	3.61		-	29.59	-	4.91		-	26.66	-	7.11
	C	1	N/A	-	5.11	-	0.06	27.91	-	13.79	-	0.06	N/A	-	7.20	-	0.00
	C	2	N/A	-	10.29	-	0.00	26.91	-	8.74	-	0.54	35.38	-	10.33	-	0.00
	C	3	N/A	-	9.37	-	0.64	N/A	-	12.13	-	0.45	N/A	-	23.26	-	0.05
	C	4	N/A	-	11.31	-	0.37	29.9	-	11.72	-	0.40	34.7	-	14.66	-	0.24
	C	5	N/A	-	11.85	-	0.03	N/A	-	4.51	-	0.91	N/A	-	18.77	-	0.12
	C	6	N/A	-	8.18	-	0.36	N/A	-	12.85	-	1.57	37.01	-	15.01	-	0.48
Stool	S	N	N/A	-	3.31	-	0.15	N/A	-	3.61	-	4.81	N/A	-	4.15	-	0.82
	S	N		-	2.50	-	4.17		-	5.42	-	2.55		-	4.46	-	5.30
	S	N		-	1.45	-	0.00		-	2.52	-	5.30		-	3.37	-	5.42
	S	1	37.17	+	6.82	-	0.00	29.48	-	3.12	-	1.99	33.72	+	11.80	-	2.16
	S	2	38.06	+	25.02	-	0.00	N/A	-	5.53	+	137.85	28	+	12.86	-	0.98
	S	3	N/A	+	11.67	+	6.18	N/A	-	0.53	+	6.51	26.8	+	18.98	-	0.42
	S	4	N/A	+	9.18	-	0.01	26.61	-	4.61	-	0.70	31.96	+	17.11	-	0.50
	S	5	N/A	+	11.72	-	0.22	37.32	+	10.84	-	0.60	30.33	+	10.48	-	0.02
	S	6	N/A	-	3.87	-	0.03	27.47	+	11.15	-	2.84	30.84	+	10.17	-	0.06
	S	7	38.11	-	2.99	-	2.71	35.94	+	14.13	-	5.55	33.55	+	9.25	-	0.00
	S	8	N/A	+	6.83	-	0.43	20.33	-	0	+	31.47	31.79	+	17.31	-	0.74
Control	H9N2		22.33					23.02					21.1				
	Neg Ctrl		N/A					N/A					N/A				

**Table.S4. Real time RT PCR (rRT PCR) with clinical specimens (A/chicken/Korea/KNUSWR09/2009(H9N2)).**

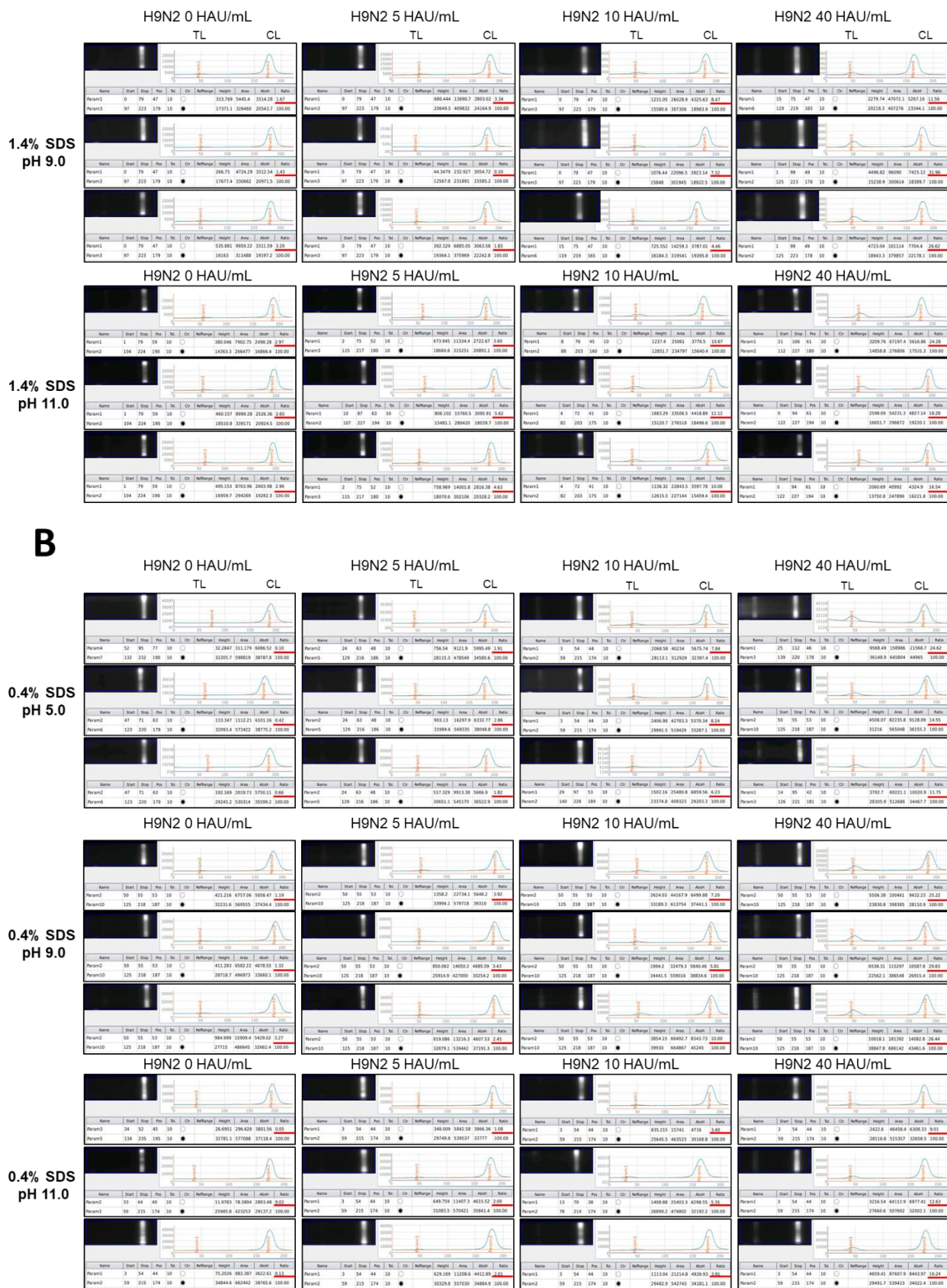
	Clinical specimens		2 dpi					4 dpi					6 dpi				
			Ct	FICT (Ratio of TL/CL)				Ct	FICT (Ratio of TL/CL)				Ct	FICT (Ratio of TL/CL)			
				D5 pair		G10 pair			D5 pair		G10 pair			D5 pair		G10 pair	
Cloacal	C	NC-1	N/A	-	2.8	-	0.02	N/A	-	3.26	-	0.18	N/A	-	3.56	-	0.16
	C	NC-2	N/A	-	2.45	-	0.18	N/A	-	1.91	-	1.12	N/A	-	2.38	-	0.24
	C	NC-3	N/A	-	2.49	-	0.35	N/A	-	2.59	-	0.57	N/A	-	4.38	-	0.21
	C	H9N2-1	N/A	-	3.03	-	0.28	38.22	+	15.75	-	2.47	30.49	+	33.84	-	3.95
	C	H9N2-2	N/A	-	2.03	-	0.6771	N/A	-	3.73	-	0.76	N/A	-	4.52	-	0.05
	C	H9N2-3	38.89	-	2.87	-	0.40	39.26	-	2.34	-	0.15	32.79	+	7.24	-	0.08
Stool	S	NC-1	N/A	-	2.97	-	0.17	N/A	-	1.09	-	0.75	N/A	-	3.44	-	0.28
	S	NC-2	N/A	-	2.73	-	0.06	N/A	-	0.09	-	0.12	N/A	-	3.55	-	0.26
	S	NC-3	N/A	-	2.20	-	1.32	N/A	-	1.49	-	0.58	N/A	-	3.42	-	1.97
	S	H9N2-1	35.02	-	3.48	-	0.60	31.44	+	1094.3	+	394.16	23.1	+	675.84	+	466.25
	S	H9N2-2	N/A	-	3.01	-	0.71	33.74	-	1.07	-	3.09	27.14	+	7.61	-	0.91
	S	H9N2-3	N/A	-	2.53	-	0.24	35.35	+	15.92	-	0.21	26	+	866.35	+	125.12
Control	H9N2		19.24														
	Neg Ctrl		N/A														

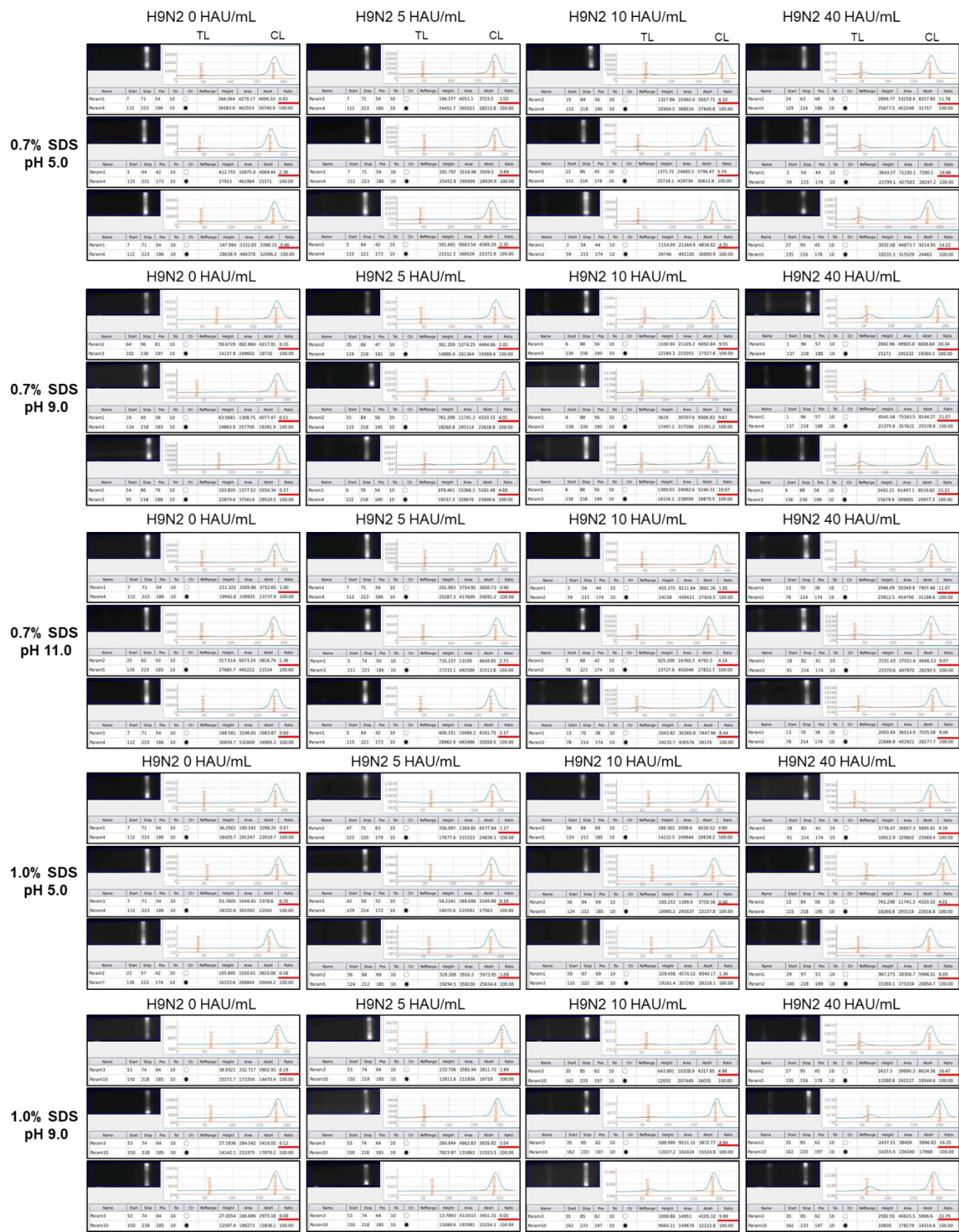
A



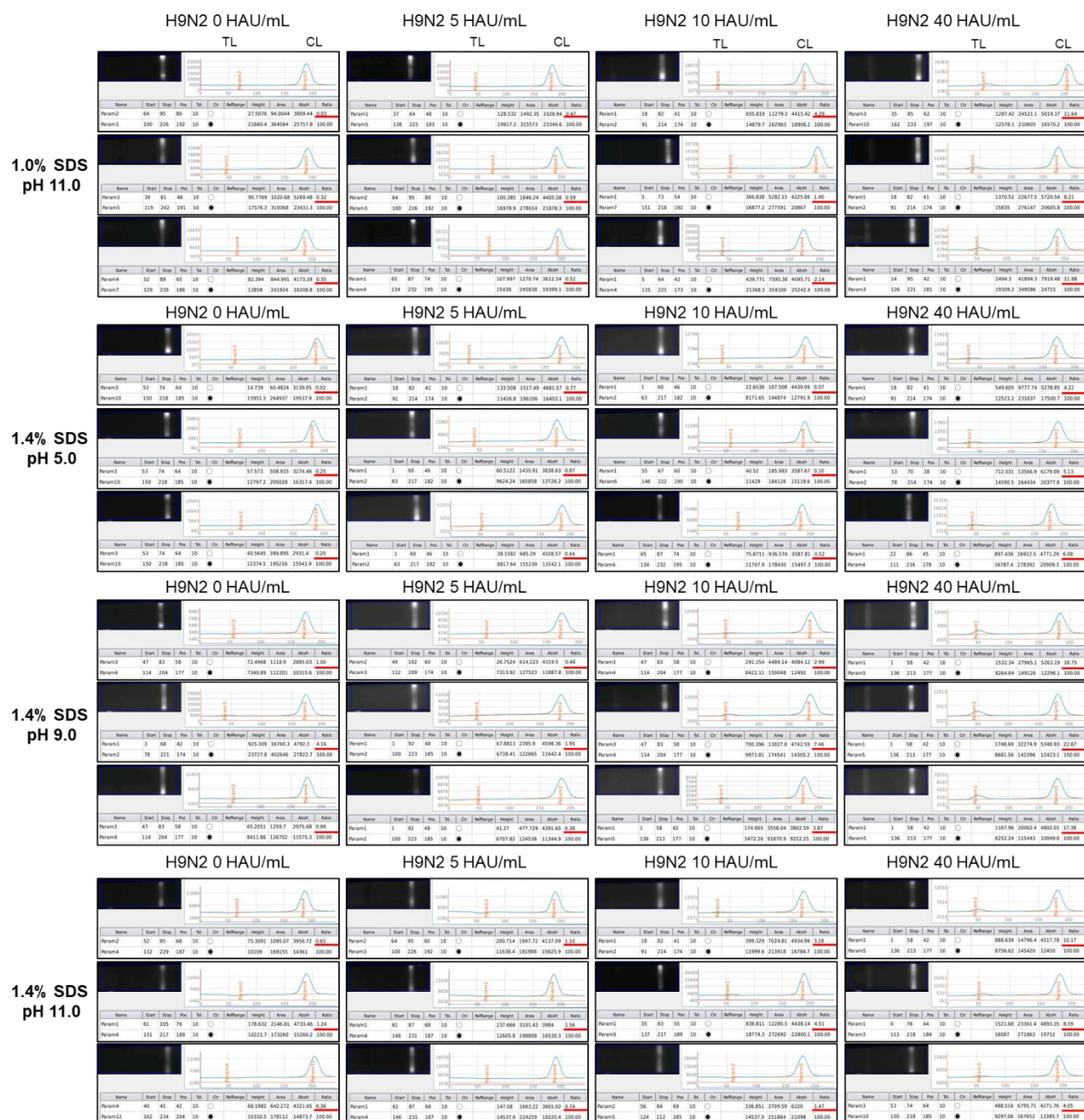




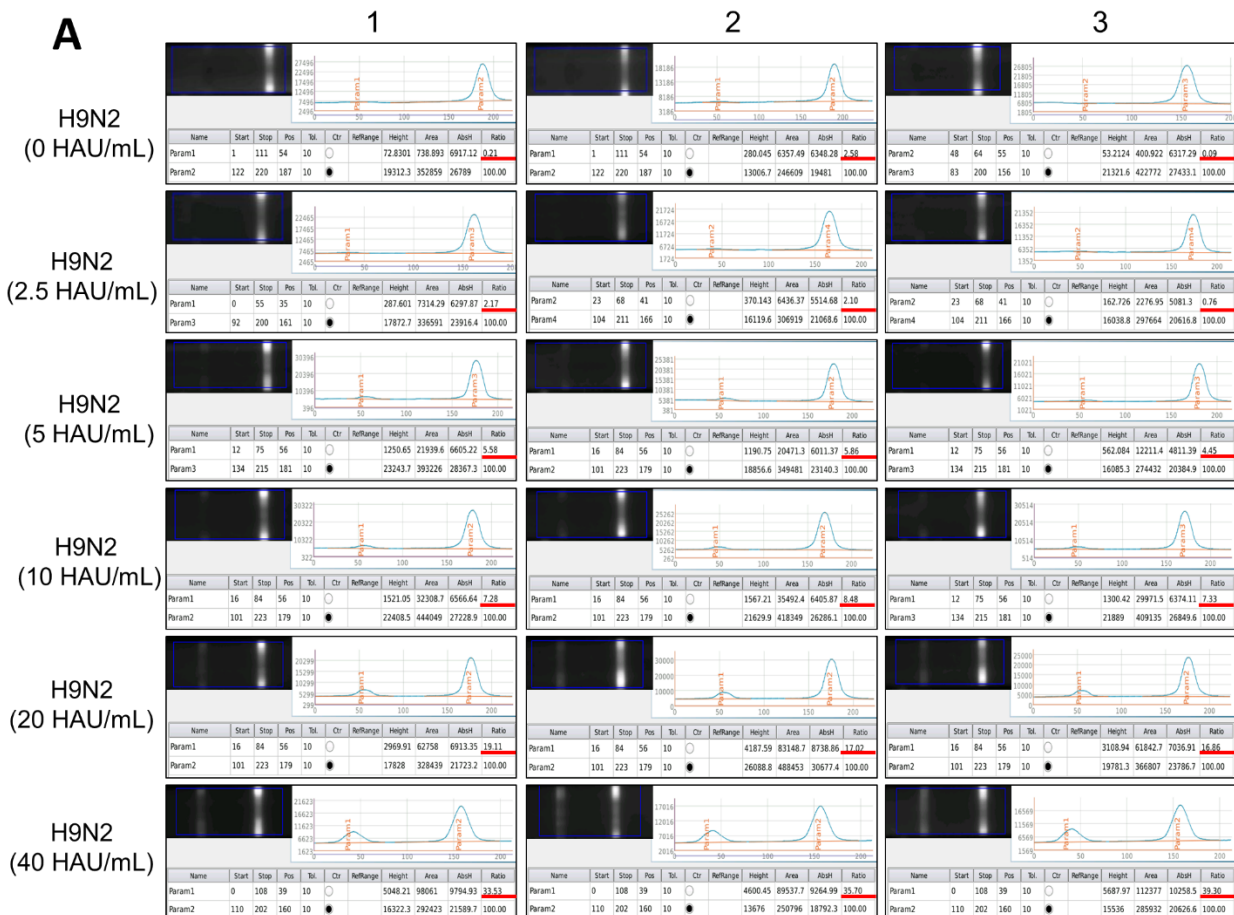




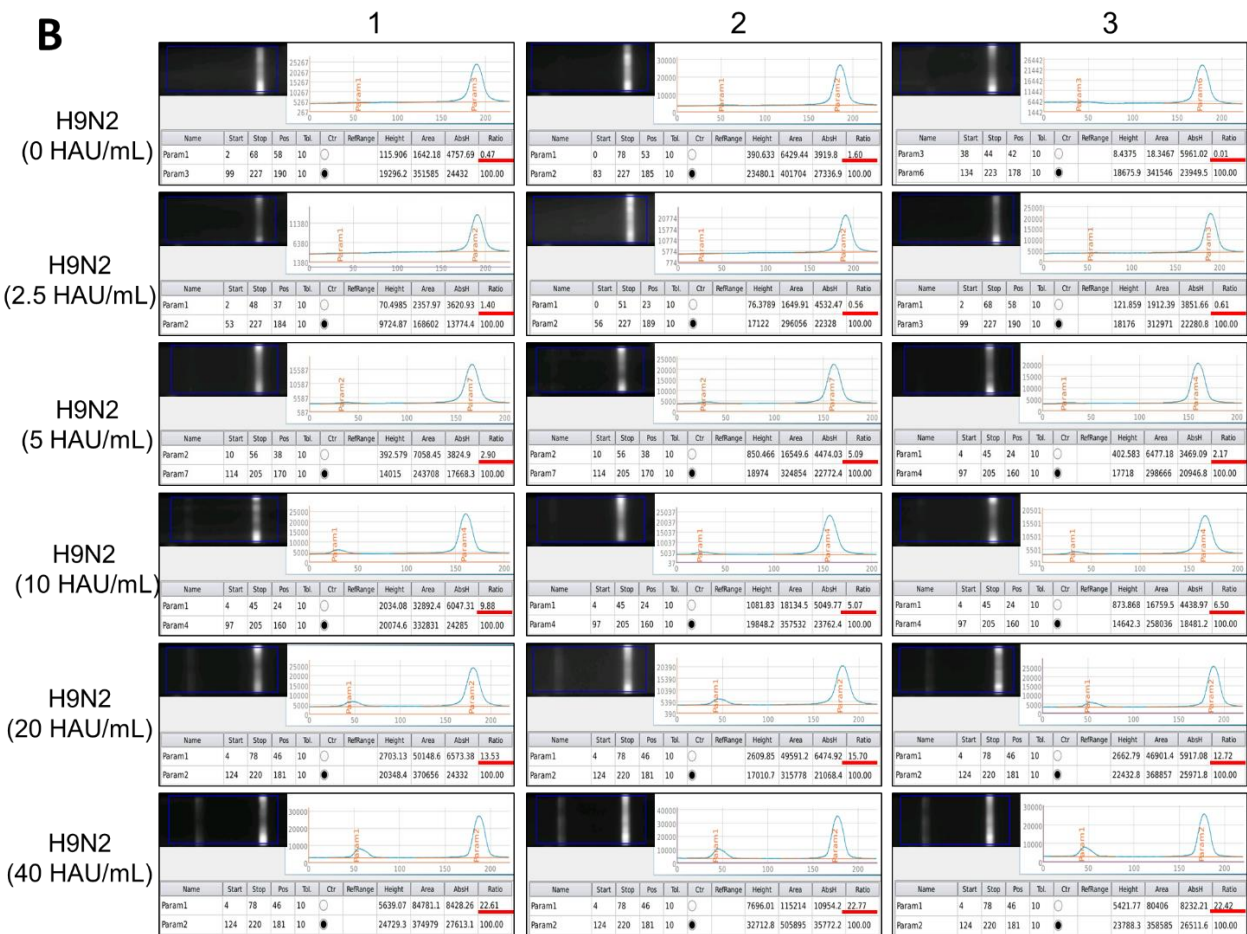




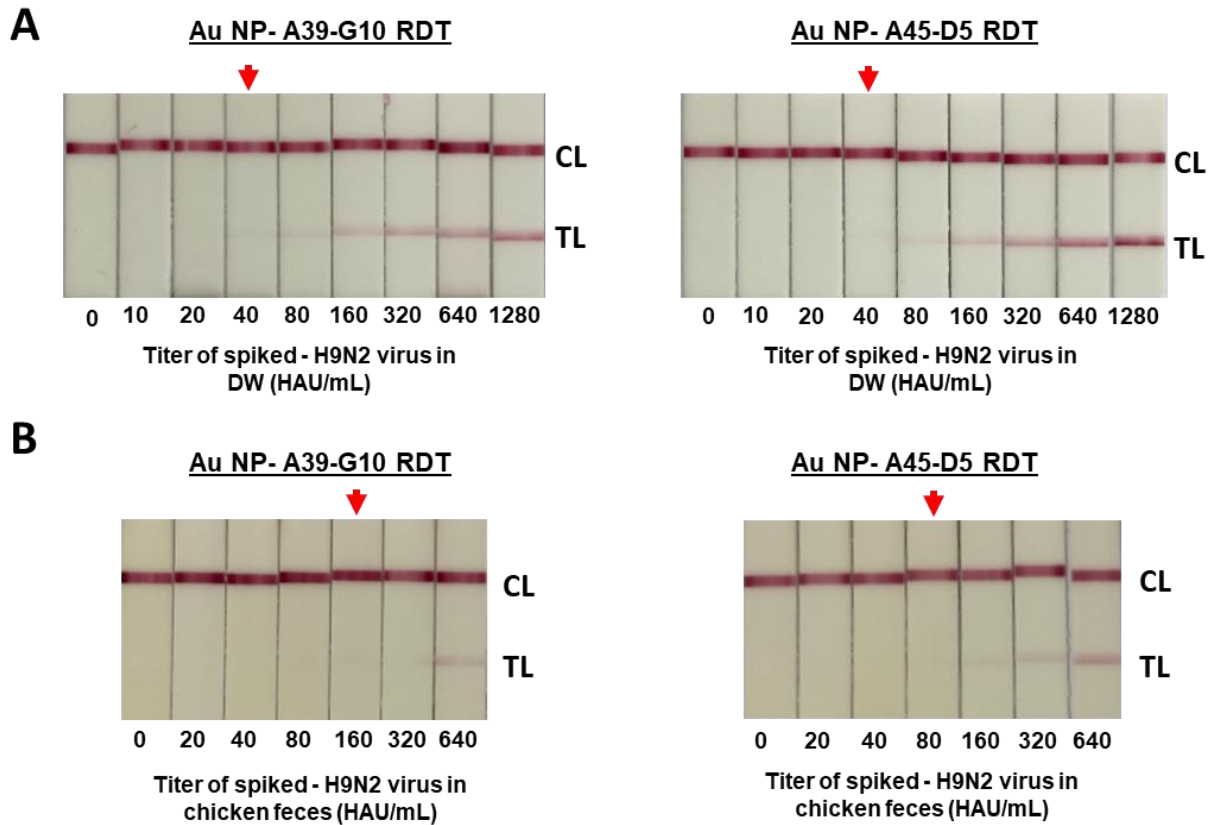
**Fig.S1. Raw data of lysis buffer optimization. (A) Europium A45-D5 FICT, (B) Europium A39-G10 FICT. TL/CL is underlined with red color. (H9N2: A/chicken/Korea/KNUSWR09/2009(H9N2))**







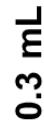
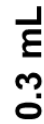
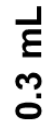
**Fig.S2. Raw data of limit of detection for spiked-H9N2 virus in distilled water (DW). (A) Europium A45-D5 FICT, (B) Europium A39-G10 FICT. TL/CL is underlined with red color. (H9N2: A/chicken/Korea/KNUSWR09/2009(H9N2))**



**Fig.S3. Limit of detection of conventional rapid diagnostic test (RDT).** The A45-D5 and A39-G10 McAbs conjugated with colloidal gold nanoparticle (Au NP) was captured on the TL (A27-9 McAb) if there was virus during lateral flow reaction. After 20 min, the strip was read with the naked eye for a binary decision. Detection limit of FICT assay targeting influenza A virus H9N2 hemagglutinin (HA) were tested with the two-fold serially diluted from 10 to 1280 HAU/mL in deionized sterile water (DW) (A) and the two-fold serially diluted from 10 to 1280 HAU/mL in in normal chicken feces (B). The arrow indicates the LOD.



**Fig.S4. Raw data of specificity for spiked-H9N2 virus in DW. (A) Europium A45-D5 FICT, (B) Europium A39-G10 FICT. TL/CL is underlined with red color.**

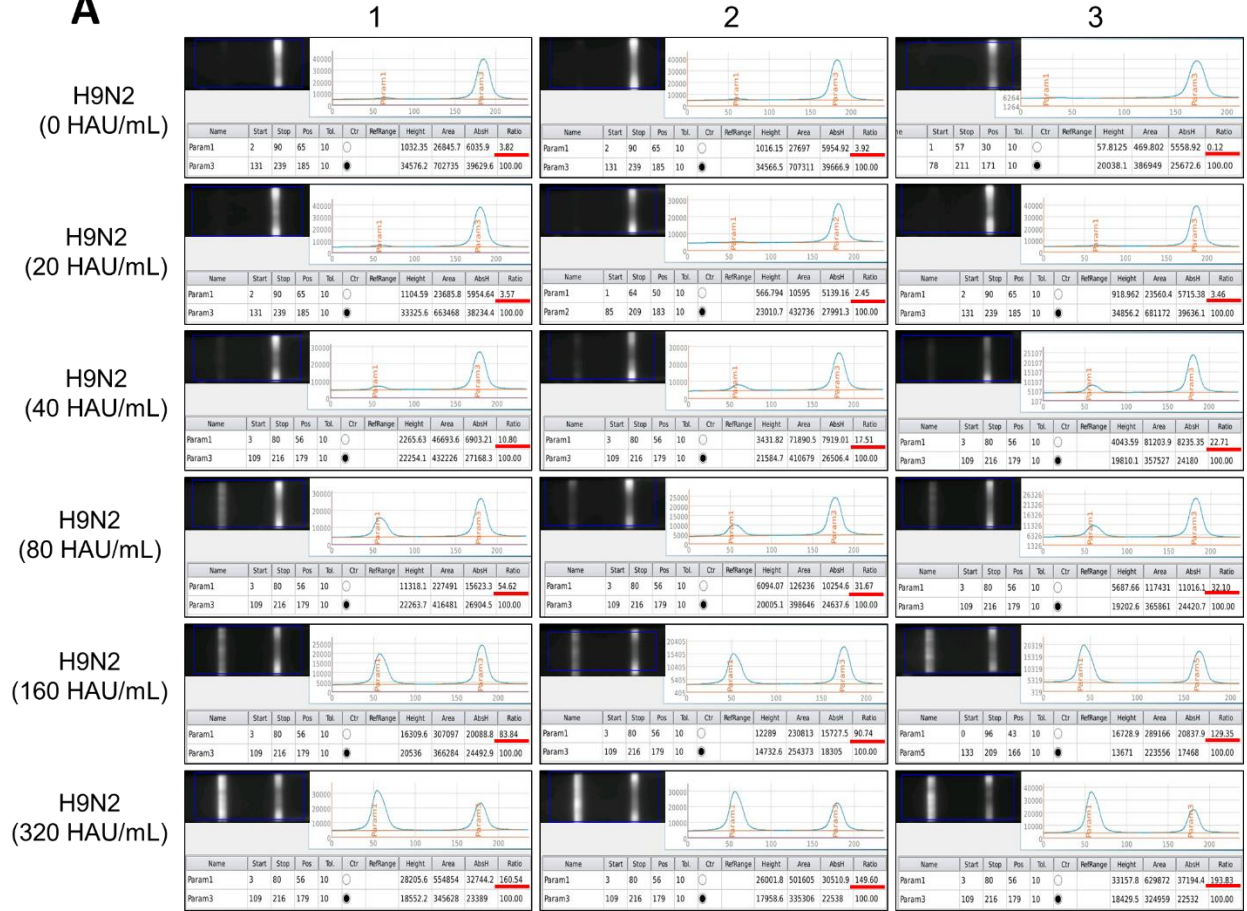


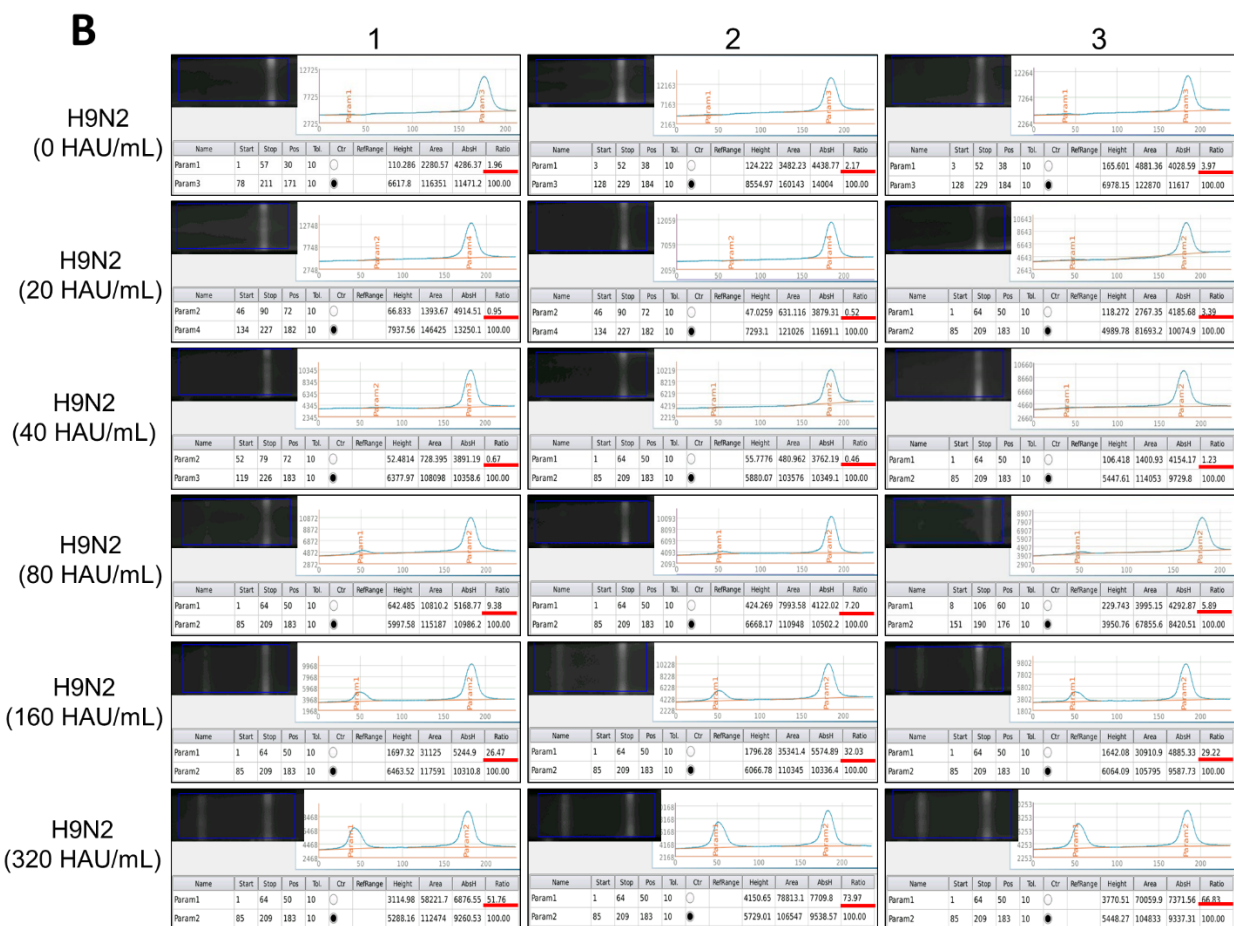




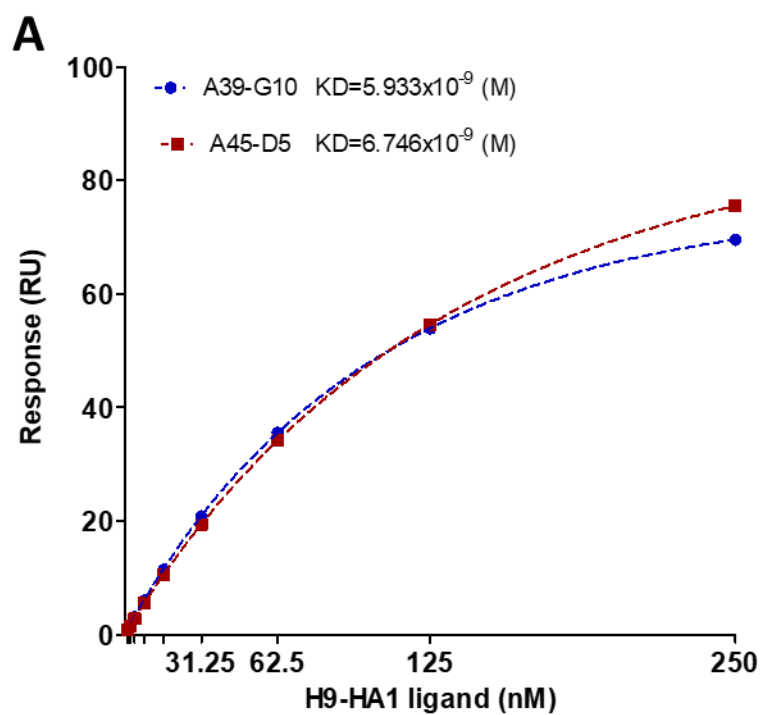
**Fig.S5: Optimization amount of lysis buffer for feces condition.** Lysis buffer (0.1M Tris pH9.0, 0.1M EDTA, 1.0%SDS and 0.5% Triton X-100) with various volume (0.3mL, 0.5mL and 1mL) were tested. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ . TL/CL is underlined with red color. (H9N2: A/chicken/Korea/KNUSWR09/2009(H9N2))

**A**





**Fig.S6. Raw data of limit of detection for spiked-virus in feces.** (A) Europium A45-D5 FICT, (B) Europium A39-G10 FICT. TL/CL is underlined with red color. (H9N2: A/chicken/Korea/KNUSWR09/2009(H9N2))



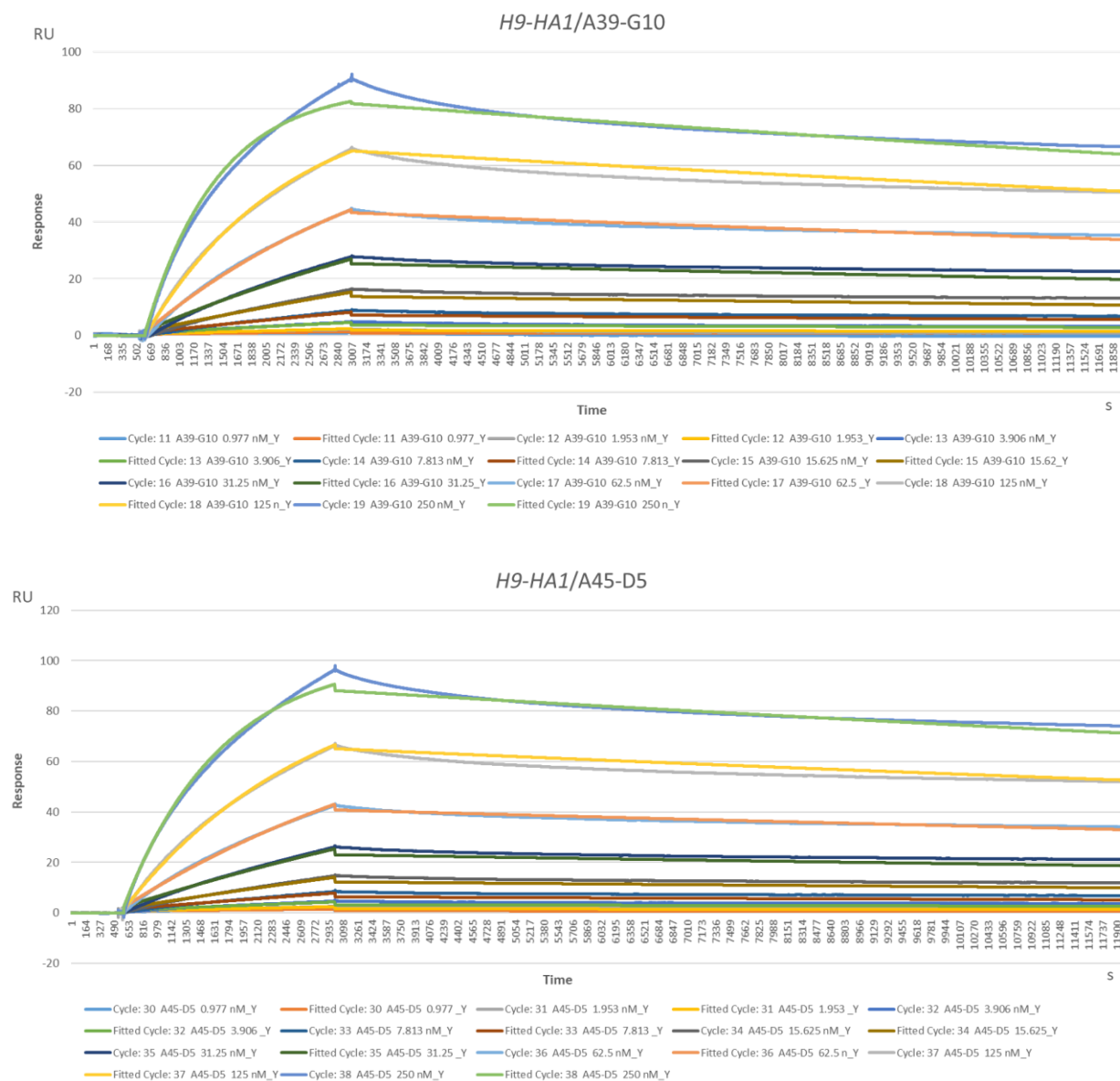
Ligand	Analyte	Conc.	ka (1/Ms)	kd (1/s)	KD (M)	Rmax (RU)	Chi <sup>2</sup> (RU <sup>2</sup> )
<i>H9-HA1</i>	A39-G10	0.977, 1.953, 3.906, 7.813, 15.625, 31.25, 62.5, 125, 250 nM	4.641E+04	2.753E-04	5.933E-09	88.84	2.16
	A45-D5	0.977, 1.953, 3.906, 7.813, 15.625, 31.25, 62.5, 125, 250 nM	3.513E+04	2.370E-04	6.746E-09	102.2	1.96

KD (Equilibrium dissociation rate constant)

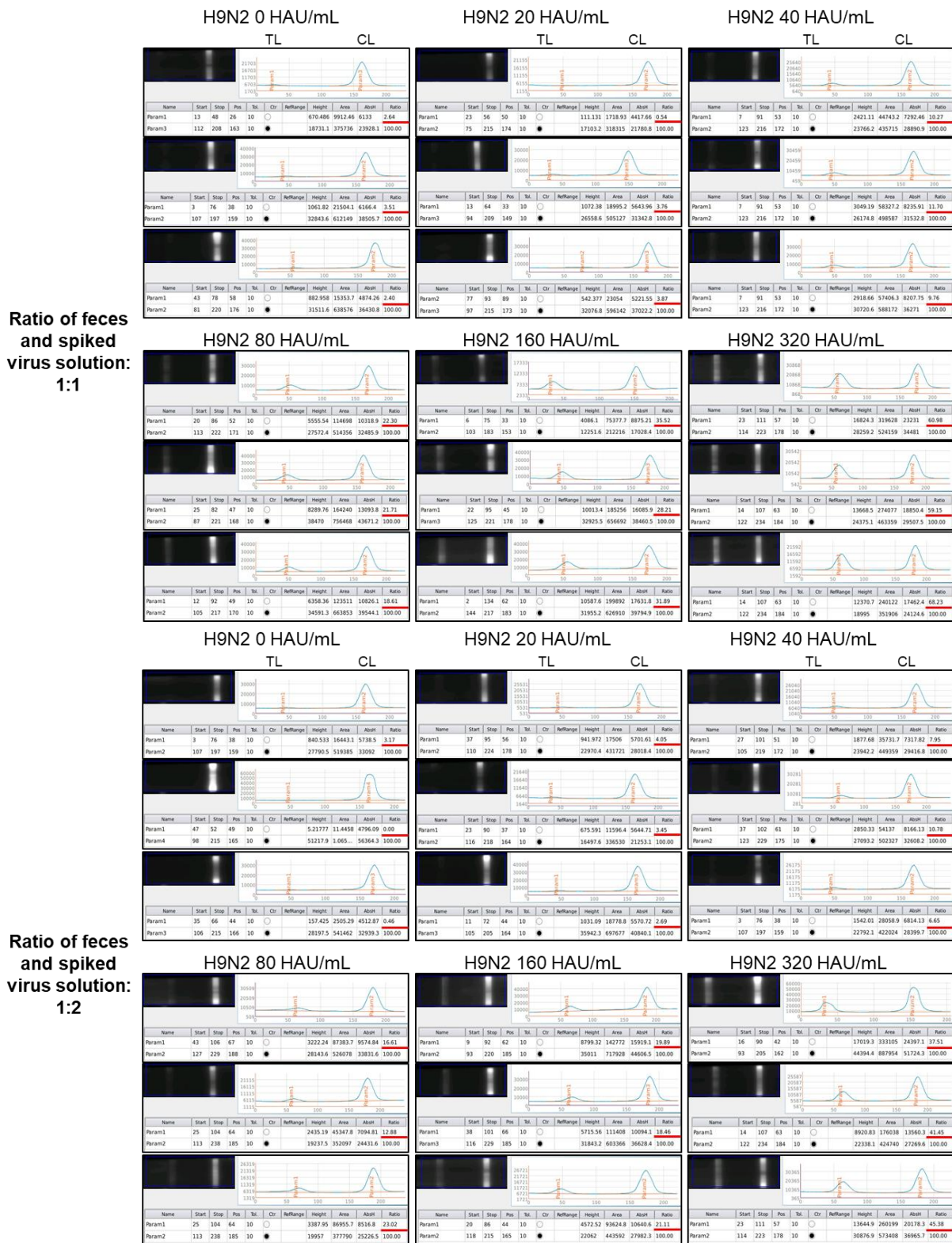
ka (association rate constant)

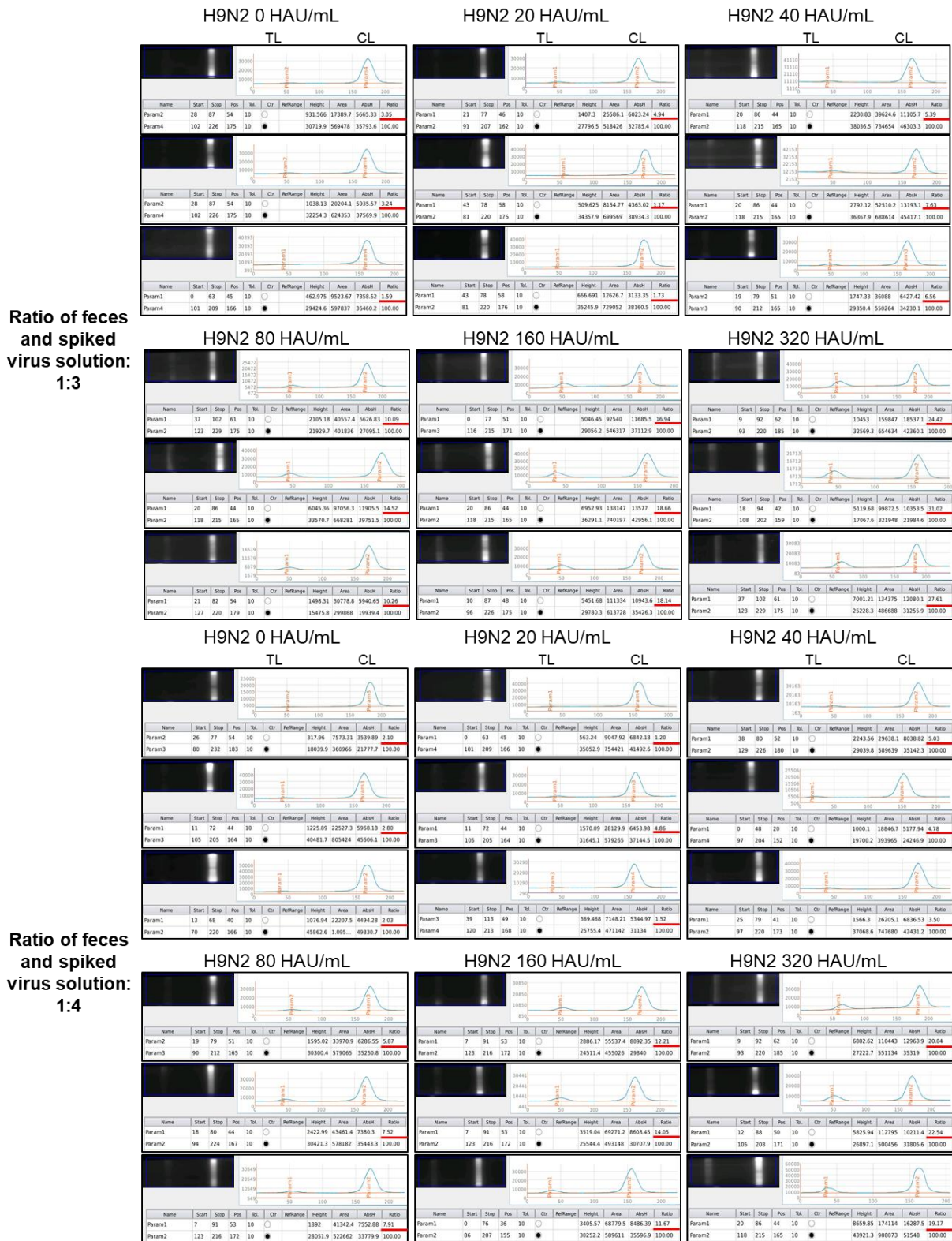
kd (dissociation rate constant)



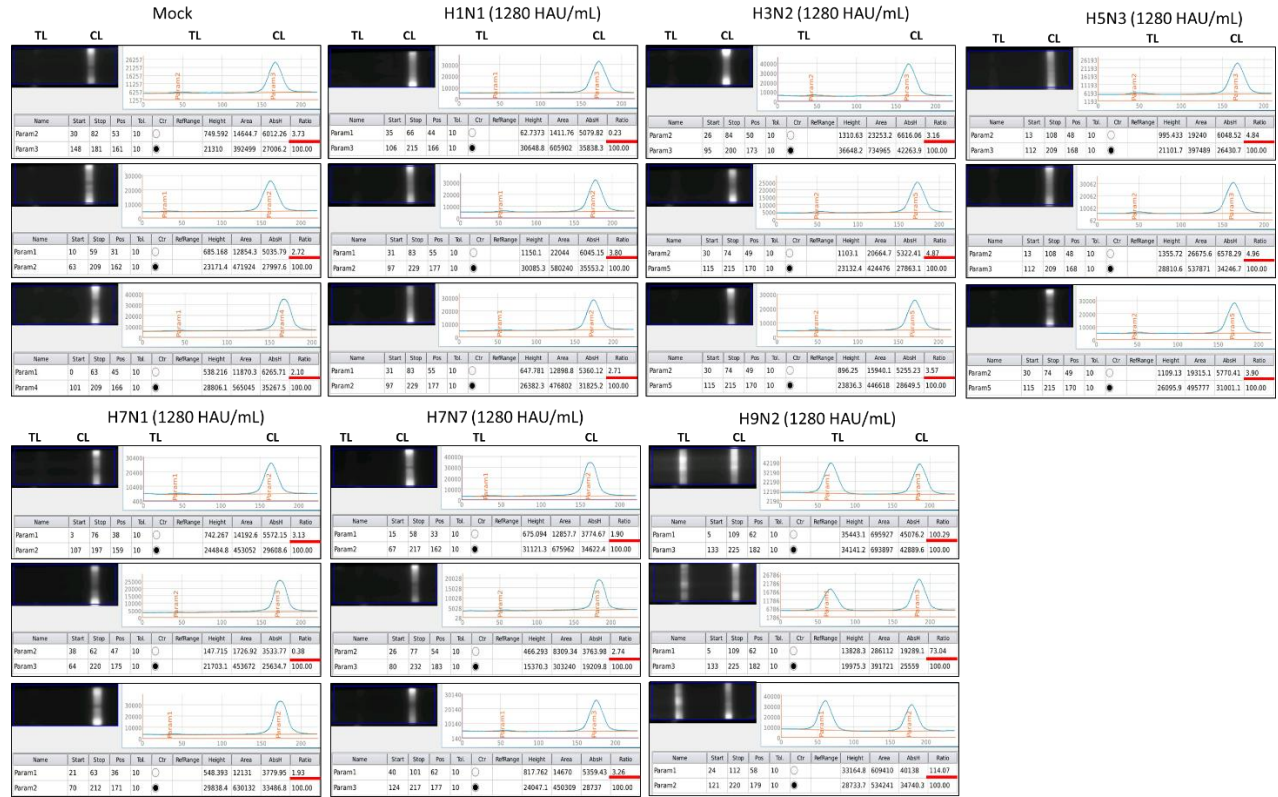
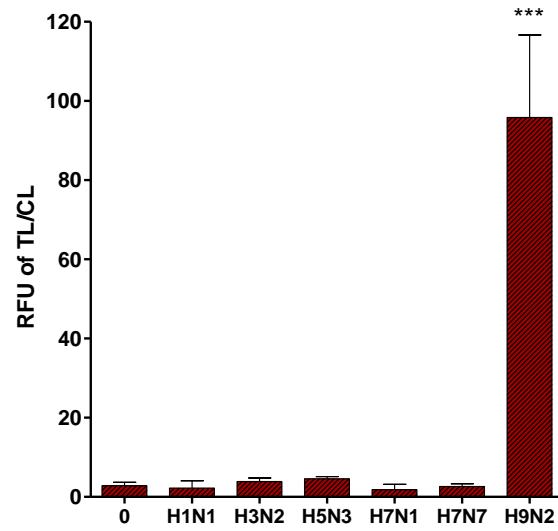
**B**

**Fig.S7. Measurement of KD values for two antibodies (A39-G10 and A45-D5) by surface plasmon resonance (SPR) with Biacore T200 (GE Healthcare (Sweden) of WOOJUNG BIO, Inc Company. (A) The H9N2 HA1 recombinant protein express in E.coli system was used for KD measurement. The Graph was re-draw with raw data by GraphPad Prism5.0. (B) Raw data of KD values measurement.**



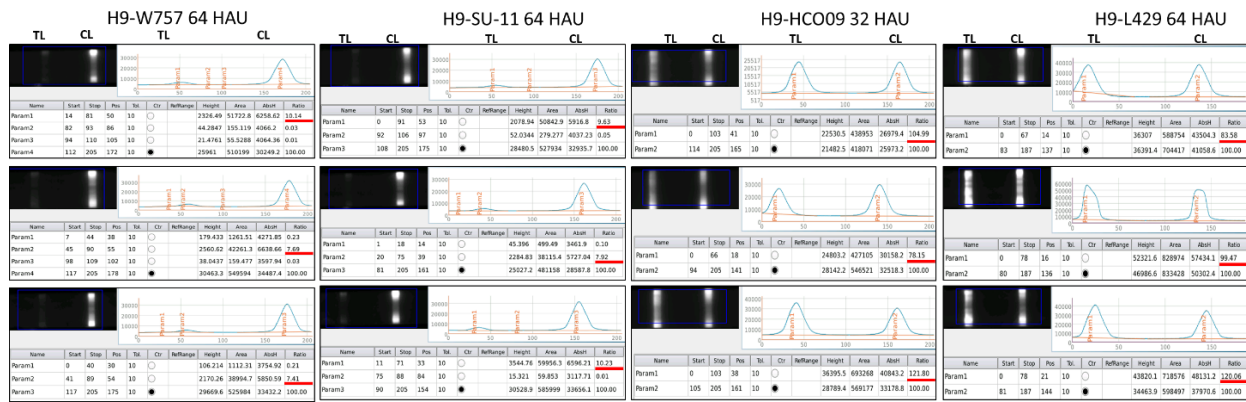




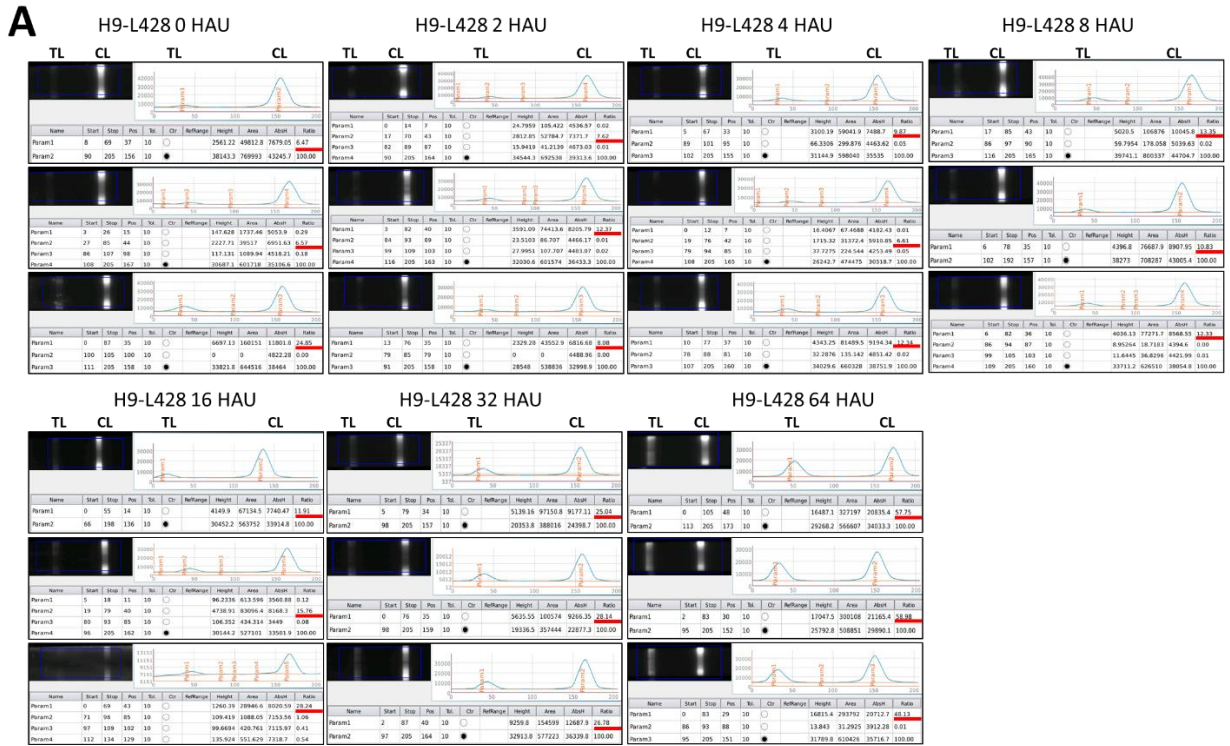
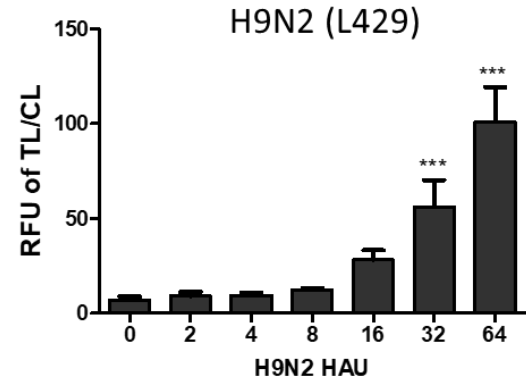
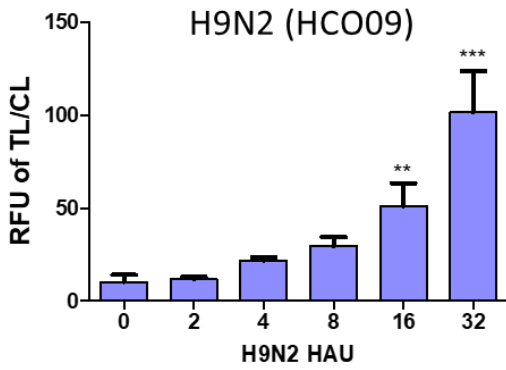
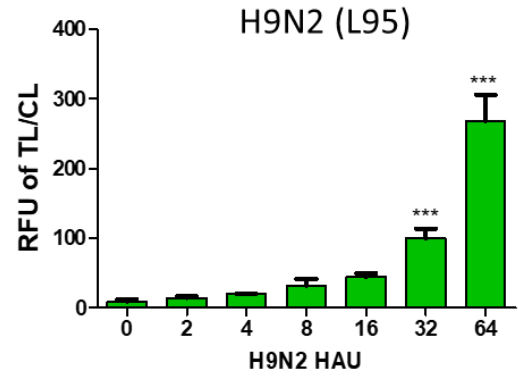
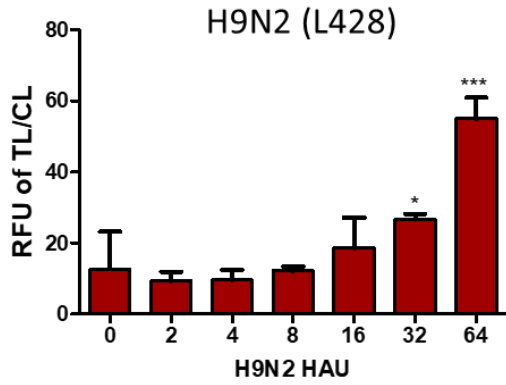


**Fig.S9. The specificity of FICT for maximum feces condition.** The specificity of FICT was evaluated by using influenza A virus in Wonkwang (H1N1, H3N2, H5N3, H7N1, H7N7 and H9N2) that spiked in chicken feces which were applied in high titer (1280 HAU/mL). \*\*\*,  $P < 0.001$ . TL/CL is underlined with red color.





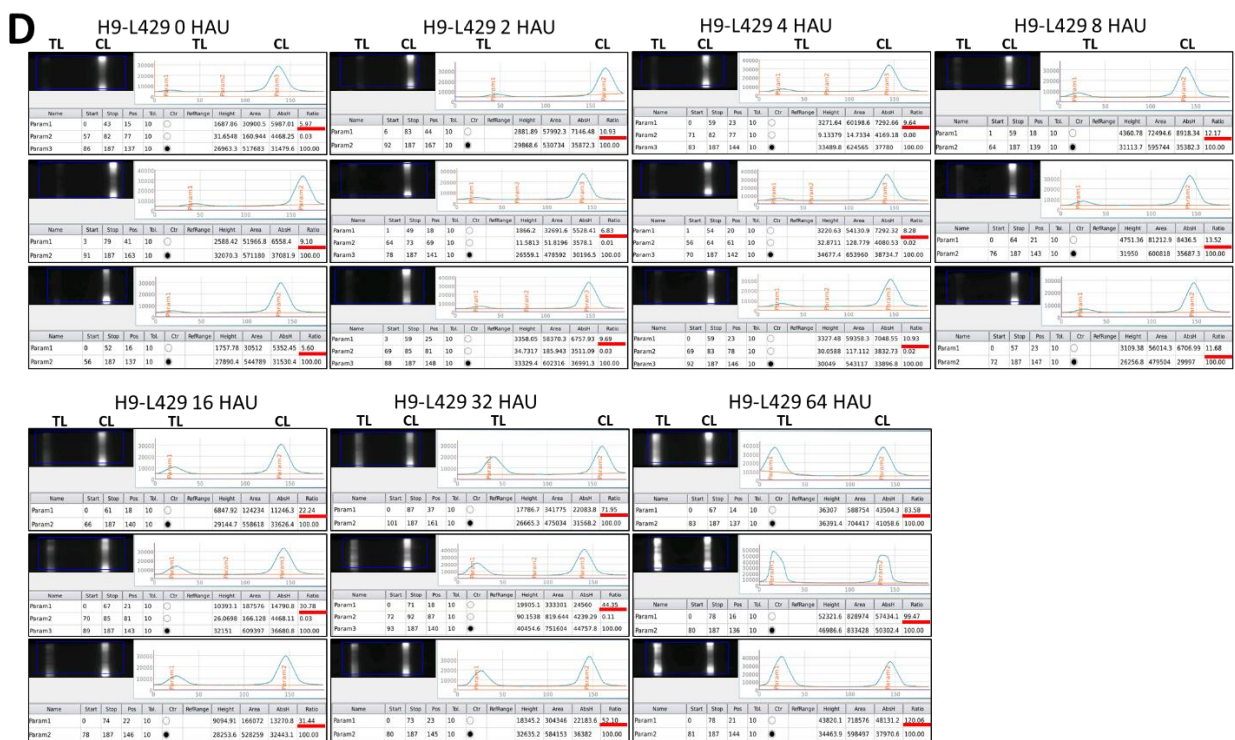
**Fig.S10. Raw data of specificity of FICT for influenza A/B virus in Chungbuk. TL/CL is underlined with red color.**



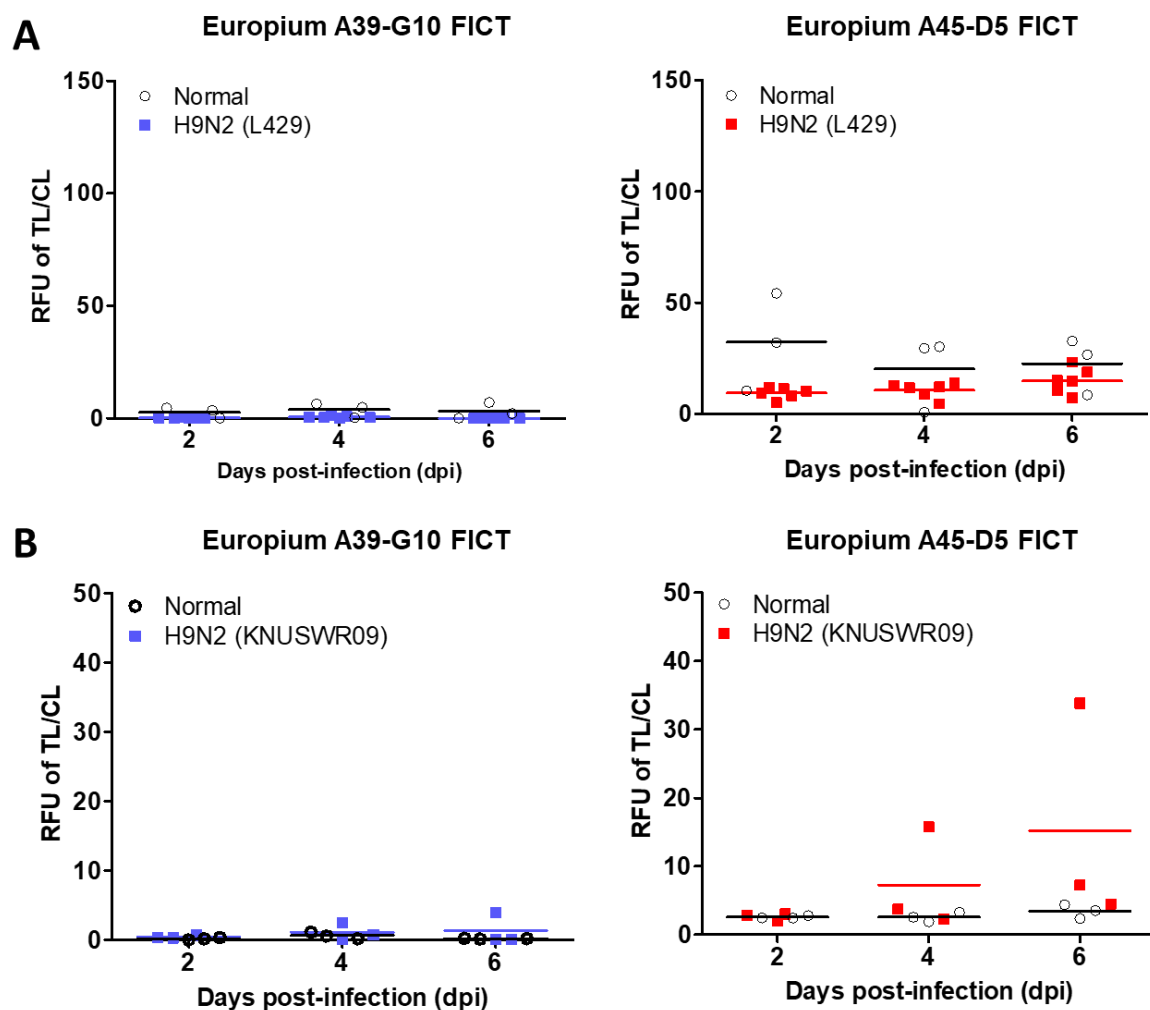








**Fig.S11. The limit of detection of four H9N2 virus strains in Chungbuk.** TL/CL is underlined with red color.



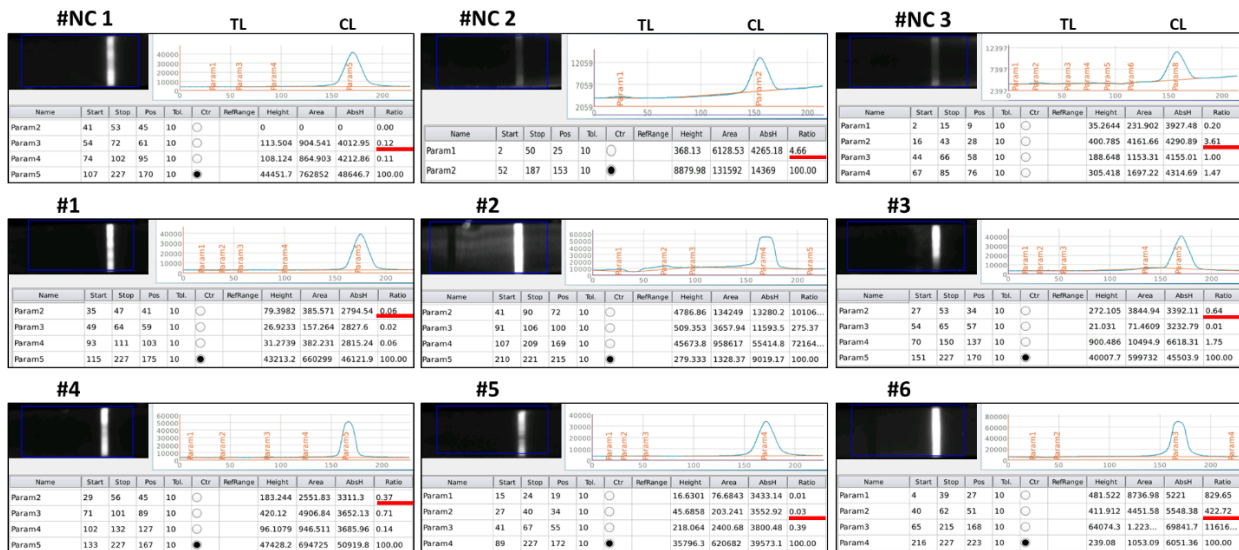
**Fig.S12. Analysis of Europium A45-D5 FICT and Europium A39-G10 FICT using cloacal samples from SPF chickens at different day post inoculation with two H9N2 strains. The cut-off value used for detection of H9N2 virus was applied to determine the present of the virus in fecal samples.** (A) A/Chicken/Korea/LPM429/2016 (H9N2) strain, (B) A/chicken/Korea/KNUSWR09/2009(H9N2) strain

A

## Europium A39-G10 FICT - Cloacal

2 dpi

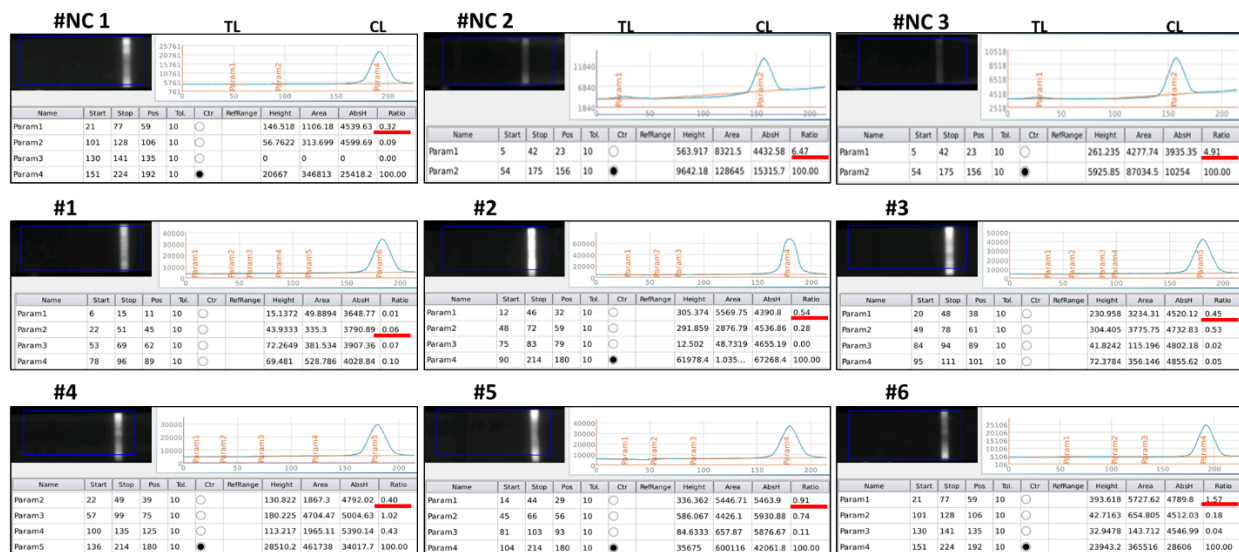
A/Chicken/Korea/LPM429/2016 (H9N2)



## Europium A39-G10 FICT - Cloacal

4 dpi

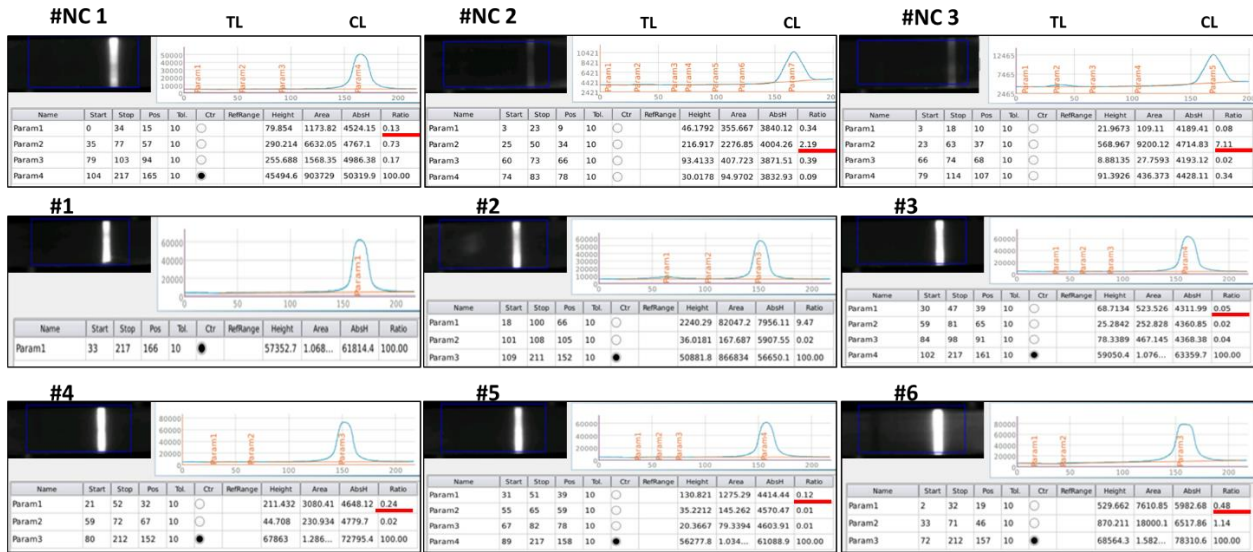
A/Chicken/Korea/LPM429/2016 (H9N2)



Europium A39-G10 FICT - Cloacal

6 dpi

A/Chicken/Korea/LPM429/2016 (H9N2)



Europium A45-D5 FICT - Cloacal

2 dpi

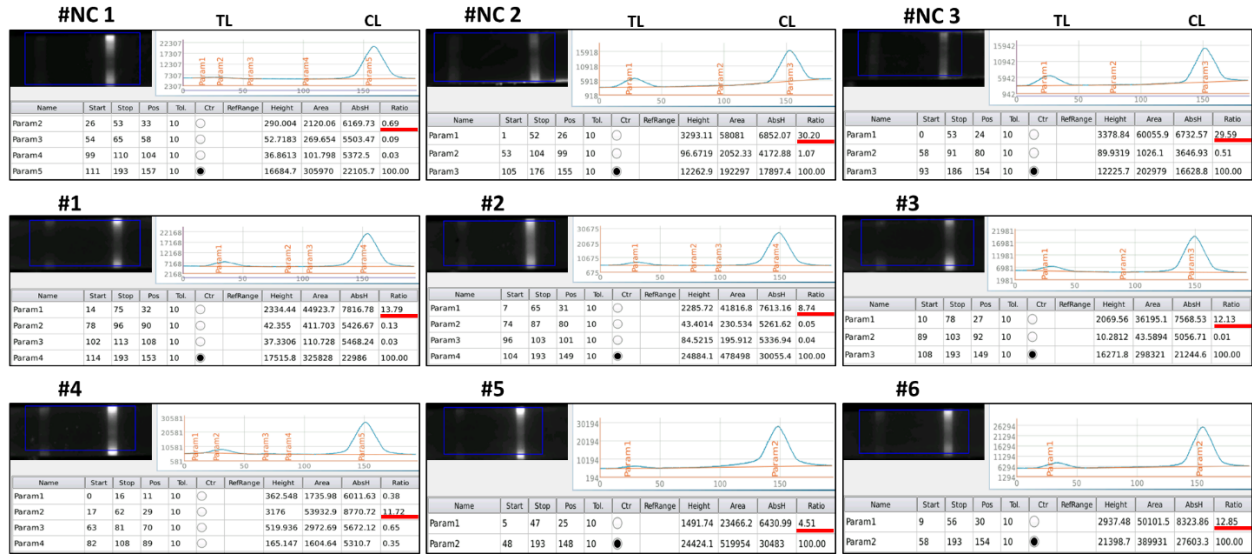
A/Chicken/Korea/LPM429/2016 (H9N2)



# Europium A45-D5 FICT - Cloacal

4 dpi

A/Chicken/Korea/LPM429/2016 (H9N2)



# Europium A45-D5 FICT - Cloacal

6 dpi

A/Chicken/Korea/LPM429/2016 (H9N2)



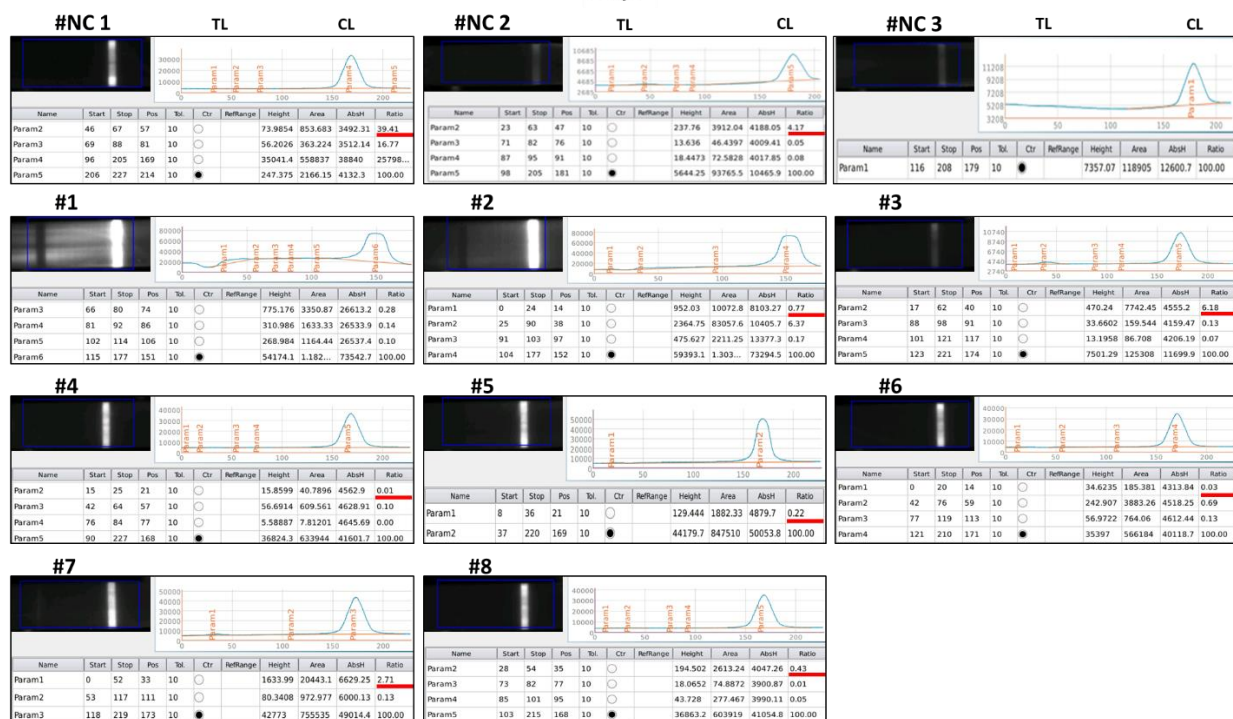


B

## Europium A39-G10 FICT - Feces

2 dpi

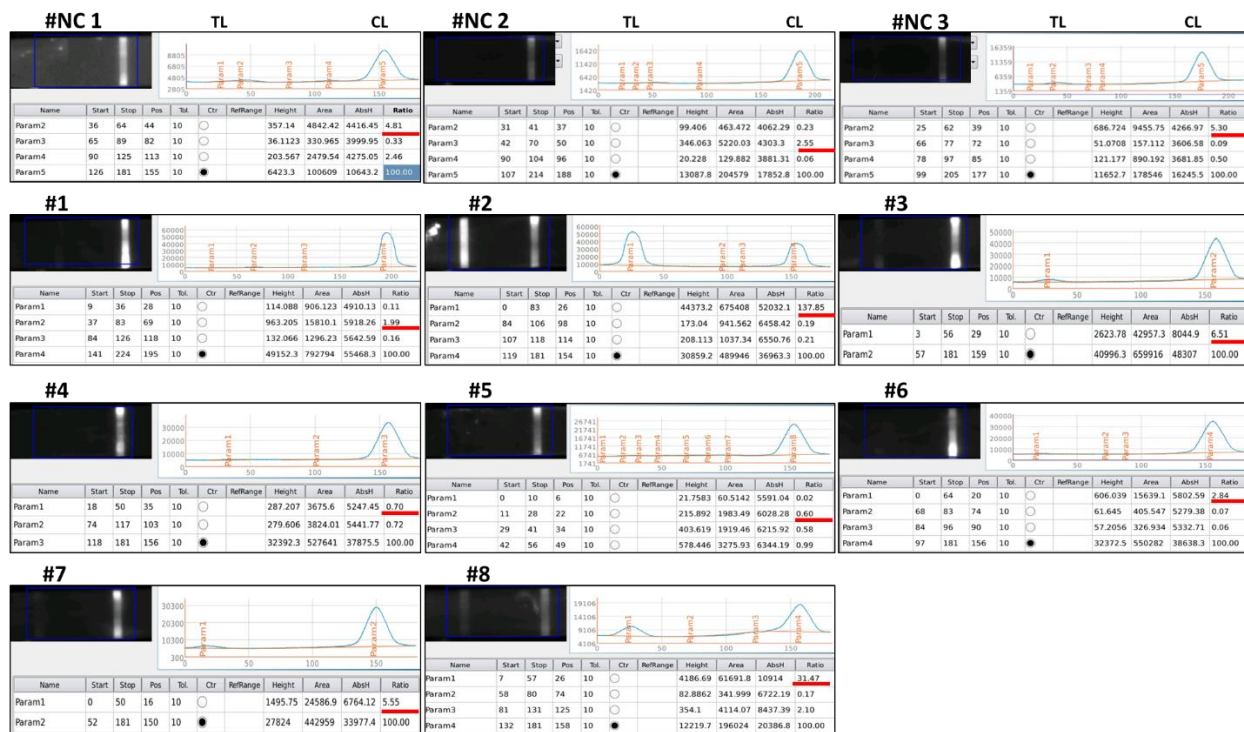
A/Chicken/Korea/LPM429/2016 (H9N2)



## Europium A39-G10 FICT - Feces

4 dpi

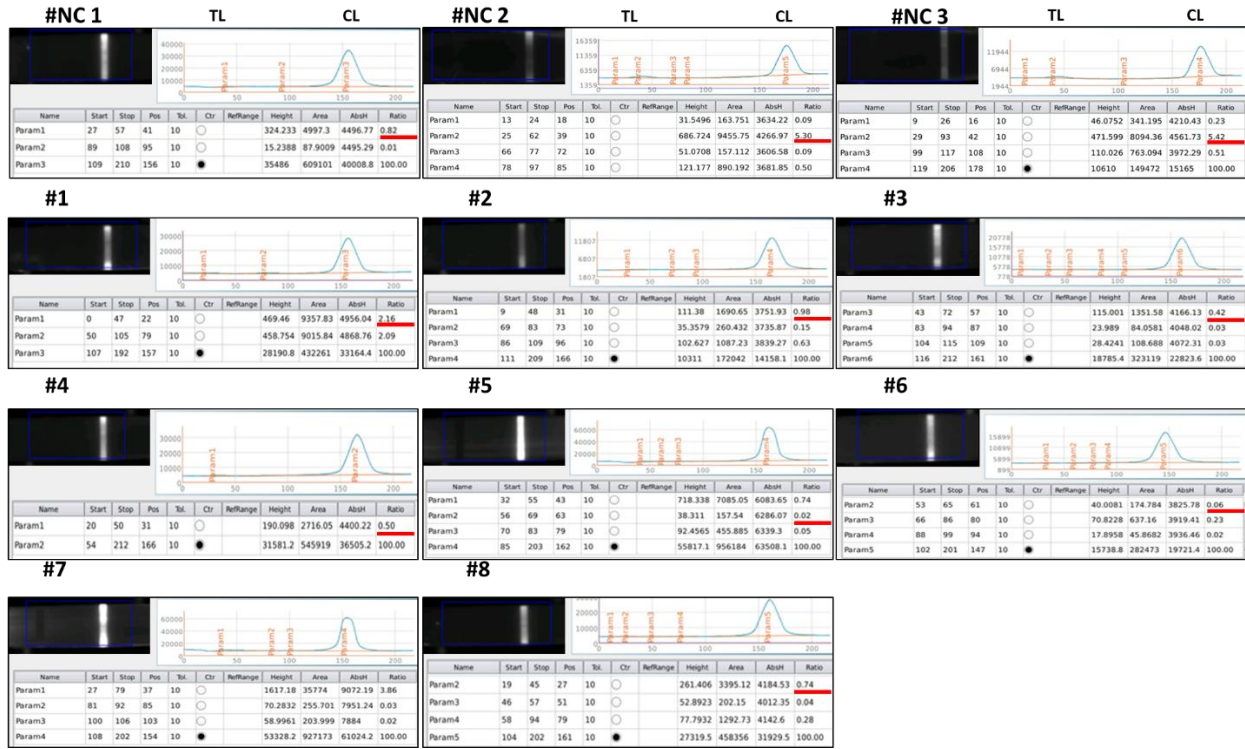
A/Chicken/Korea/LPM429/2016 (H9N2)



## Europium A39-G10 FICT - Feces

6 dpi

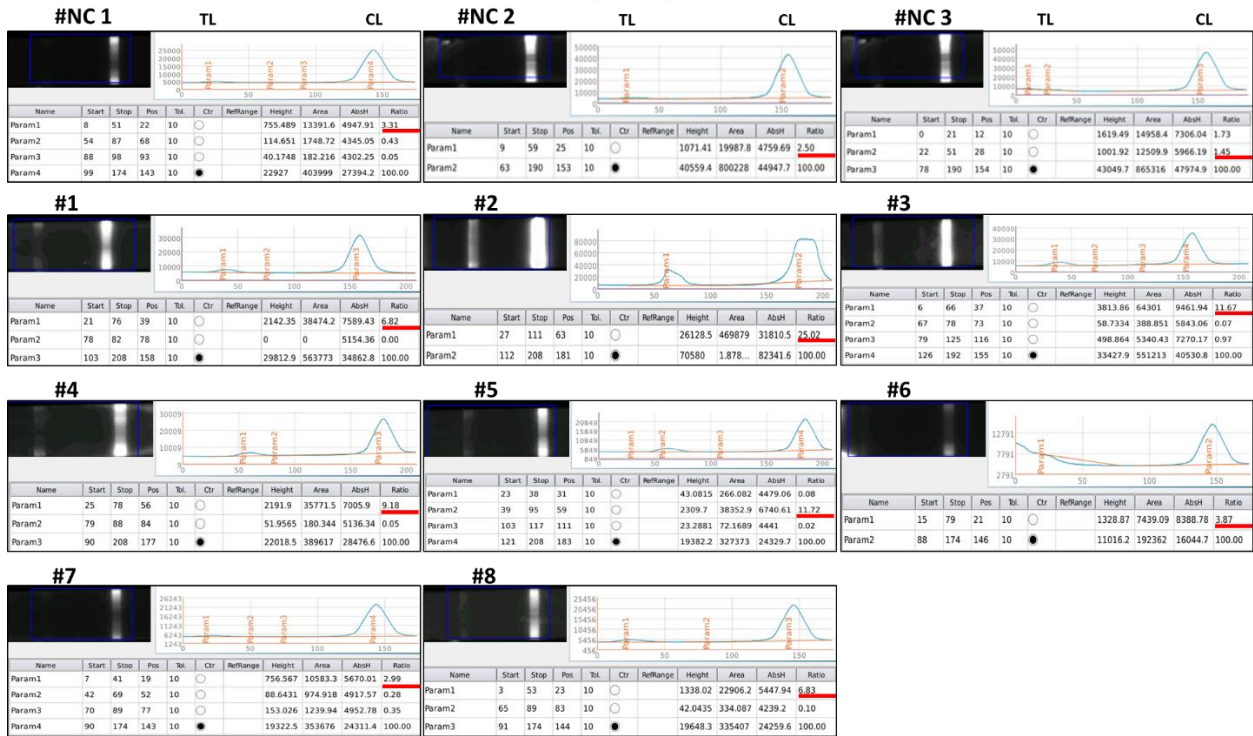
A/Chicken/Korea/LPM429/2016 (H9N2)

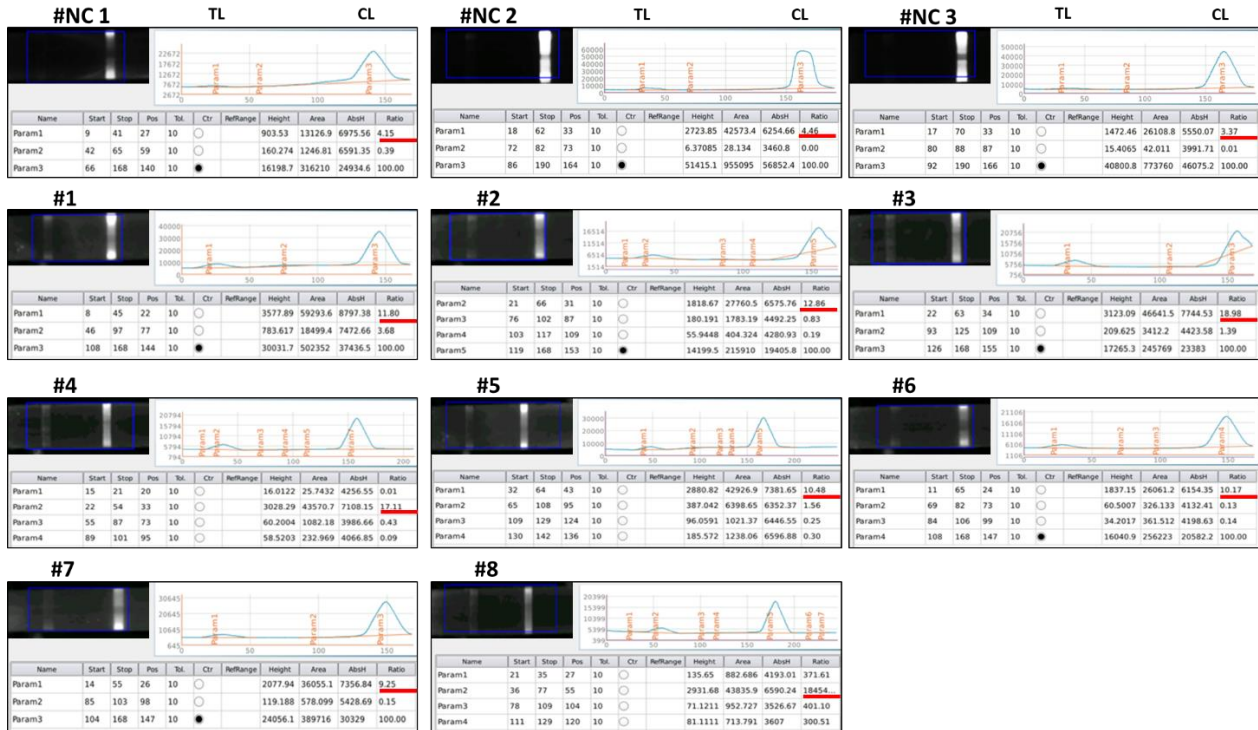
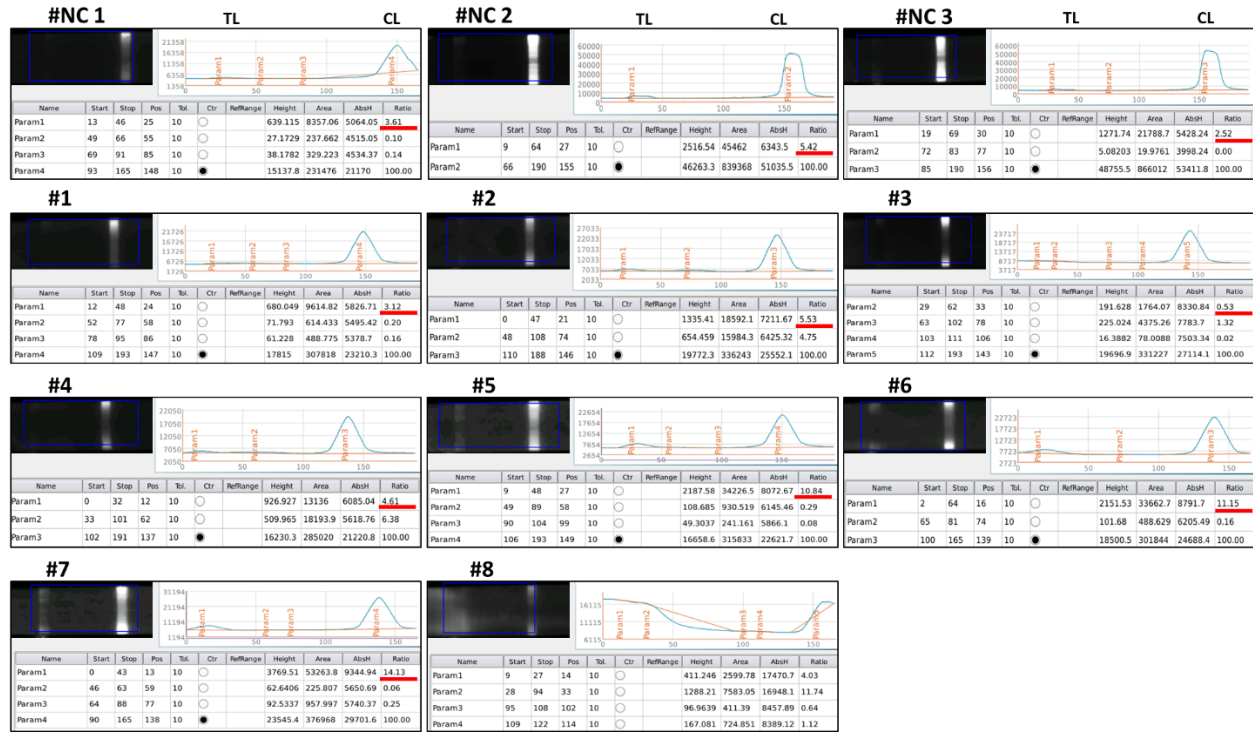


## Europium A45-D5 FICT - Feces

2 dpi

A/Chicken/Korea/LPM429/2016 (H9N2)







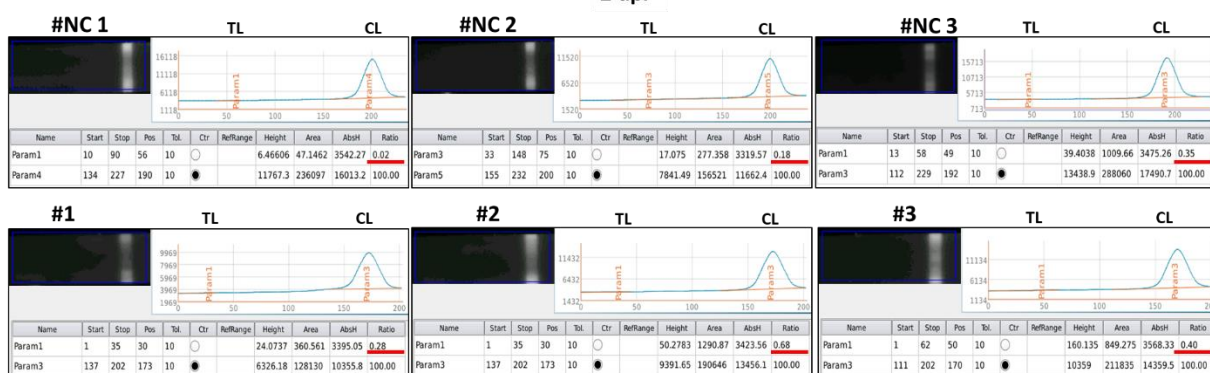
**A/Chicken/Korea/LPM429/2016 (H9N2) strain** (A) Cloacal swabs, (B) feces samples. TL/CL is underlined with red color.

A

## Europium A39-G10 FICT - Cloacal

2 dpi

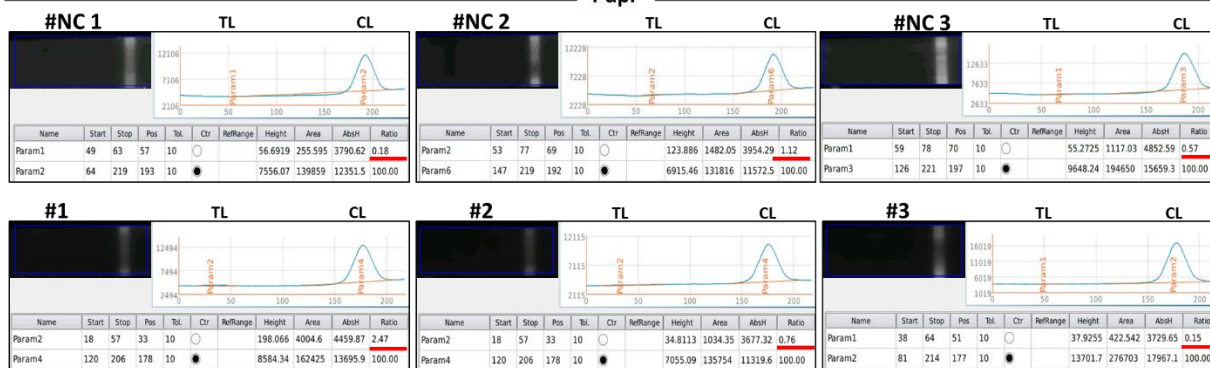
A/chicken/Korea/KNUSWR09/2009(H9N2)



## Europium A39-G10 FICT - Cloacal

4 dpi

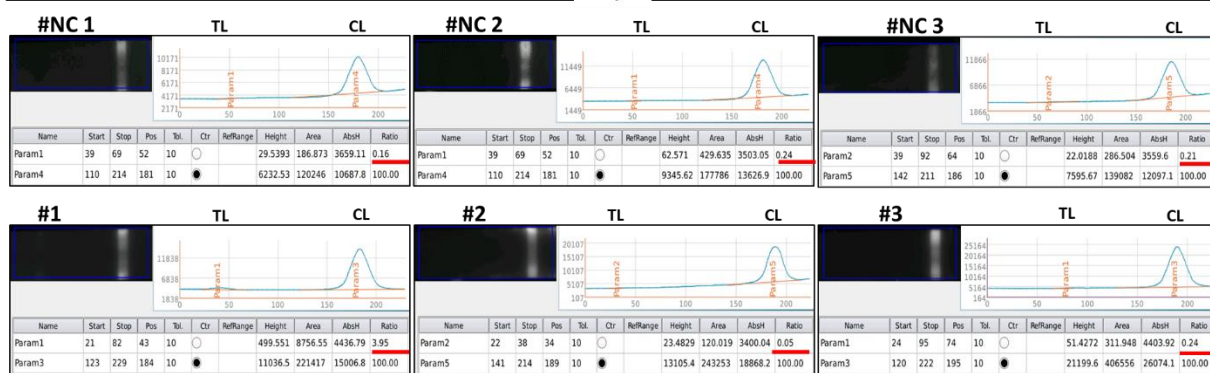
A/chicken/Korea/KNUSWR09/2009(H9N2)



## Europium A39-G10 FICT - Cloacal

6 dpi

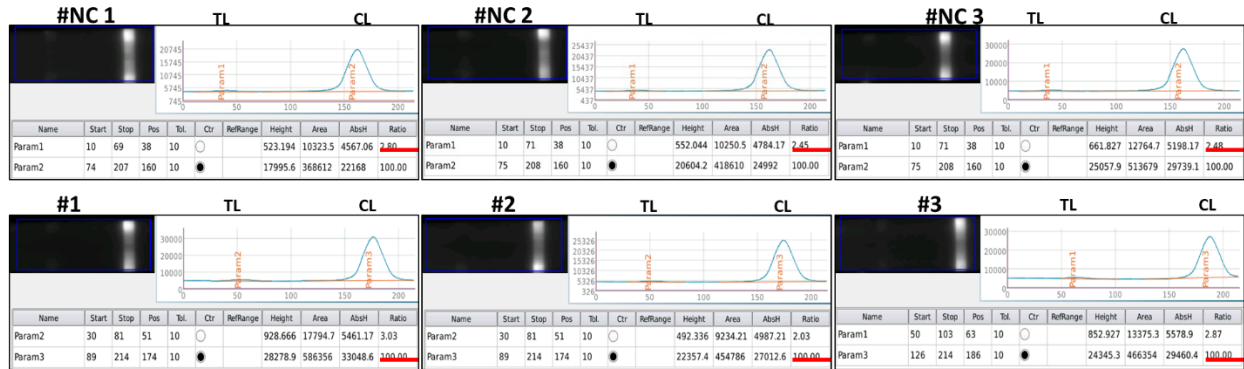
A/chicken/Korea/KNUSWR09/2009(H9N2)



Europium A45-D5 FICT - Cloacal

2 dpi

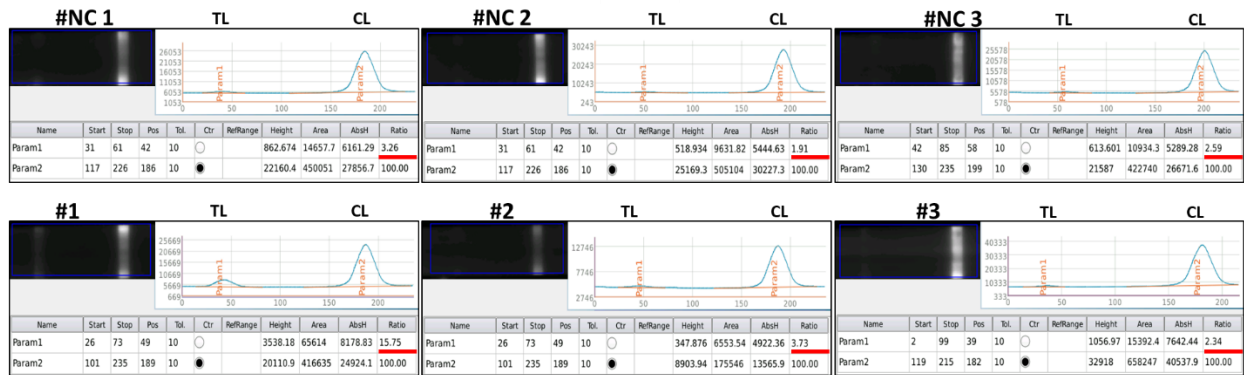
A/chicken/Korea/KNUSWR09/2009(H9N2)



Europium A45-D5 FICT - Cloacal

4 dpi

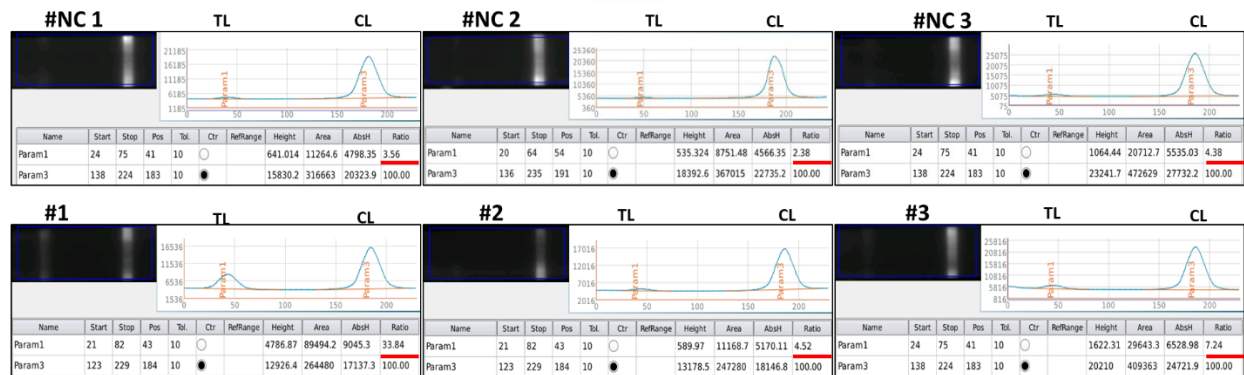
A/chicken/Korea/KNUSWR09/2009(H9N2)



Europium A45-D5 FICT - Cloacal

6 dpi

A/chicken/Korea/KNUSWR09/2009(H9N2)

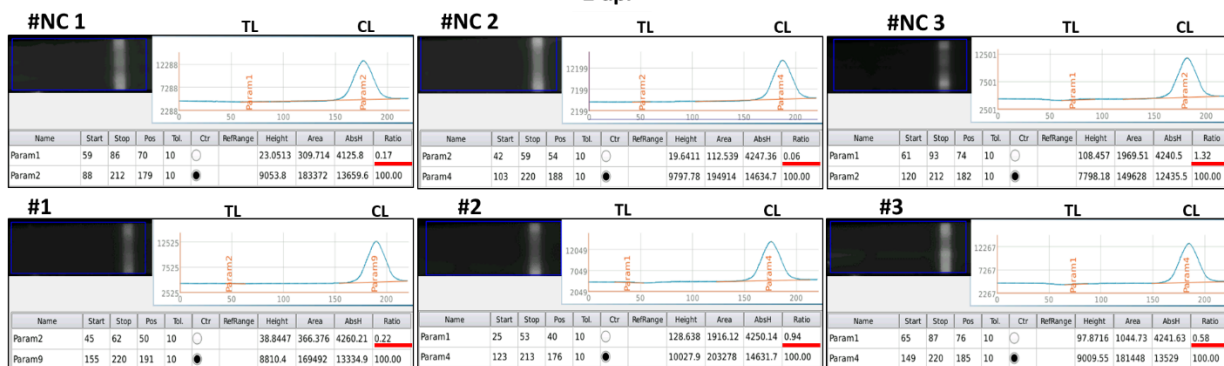


B

## Europium A39-G10 FICT - Feces

2 dpi

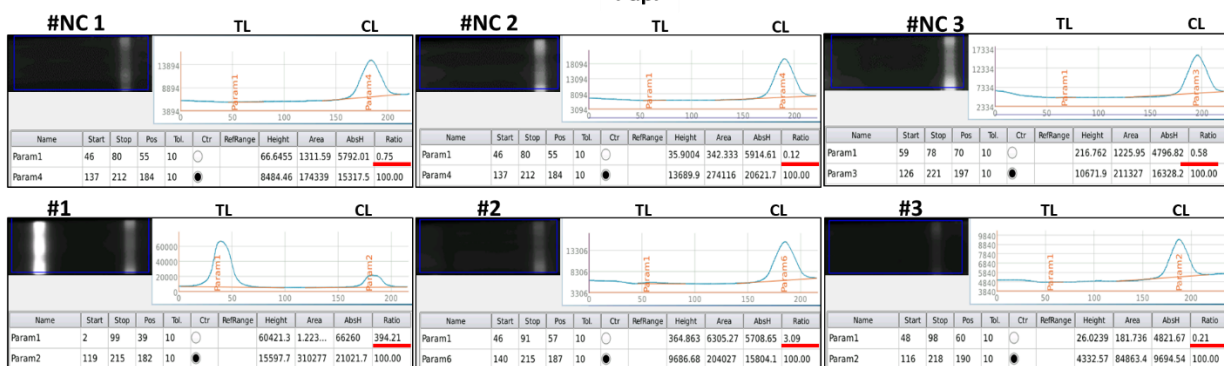
A/chicken/Korea/KNUSWR09/2009(H9N2)



## Europium A39-G10 FICT - Feces

4 dpi

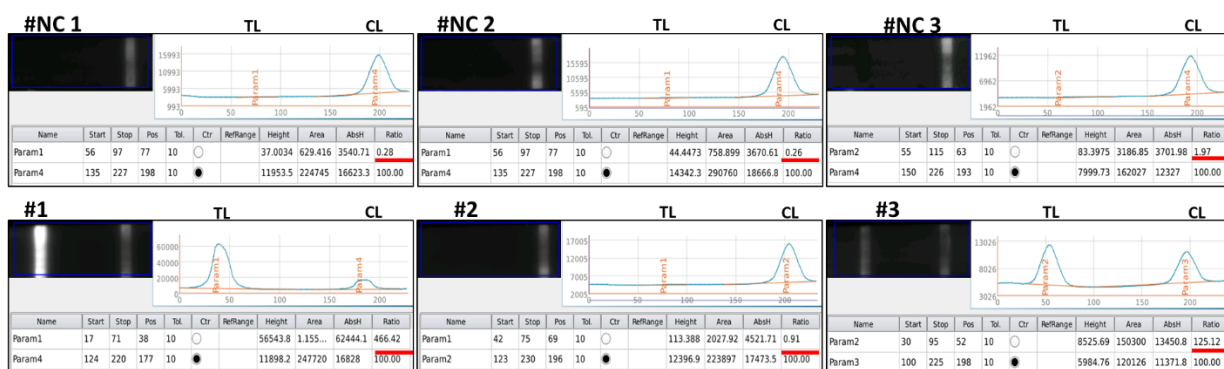
A/chicken/Korea/KNUSWR09/2009(H9N2)



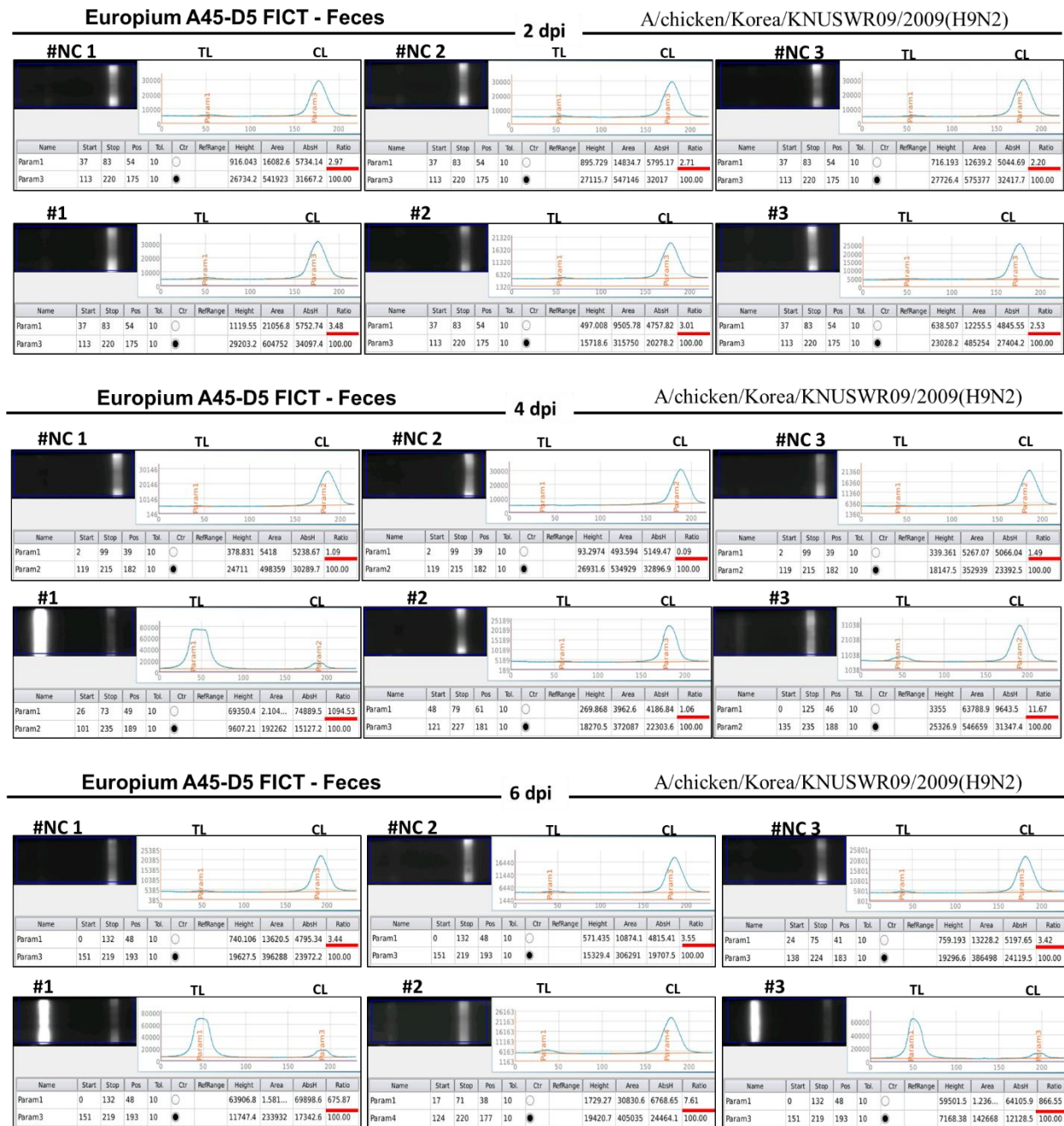
## Europium A39-G10 FICT - Feces

6 dpi

A/chicken/Korea/KNUSWR09/2009(H9N2)



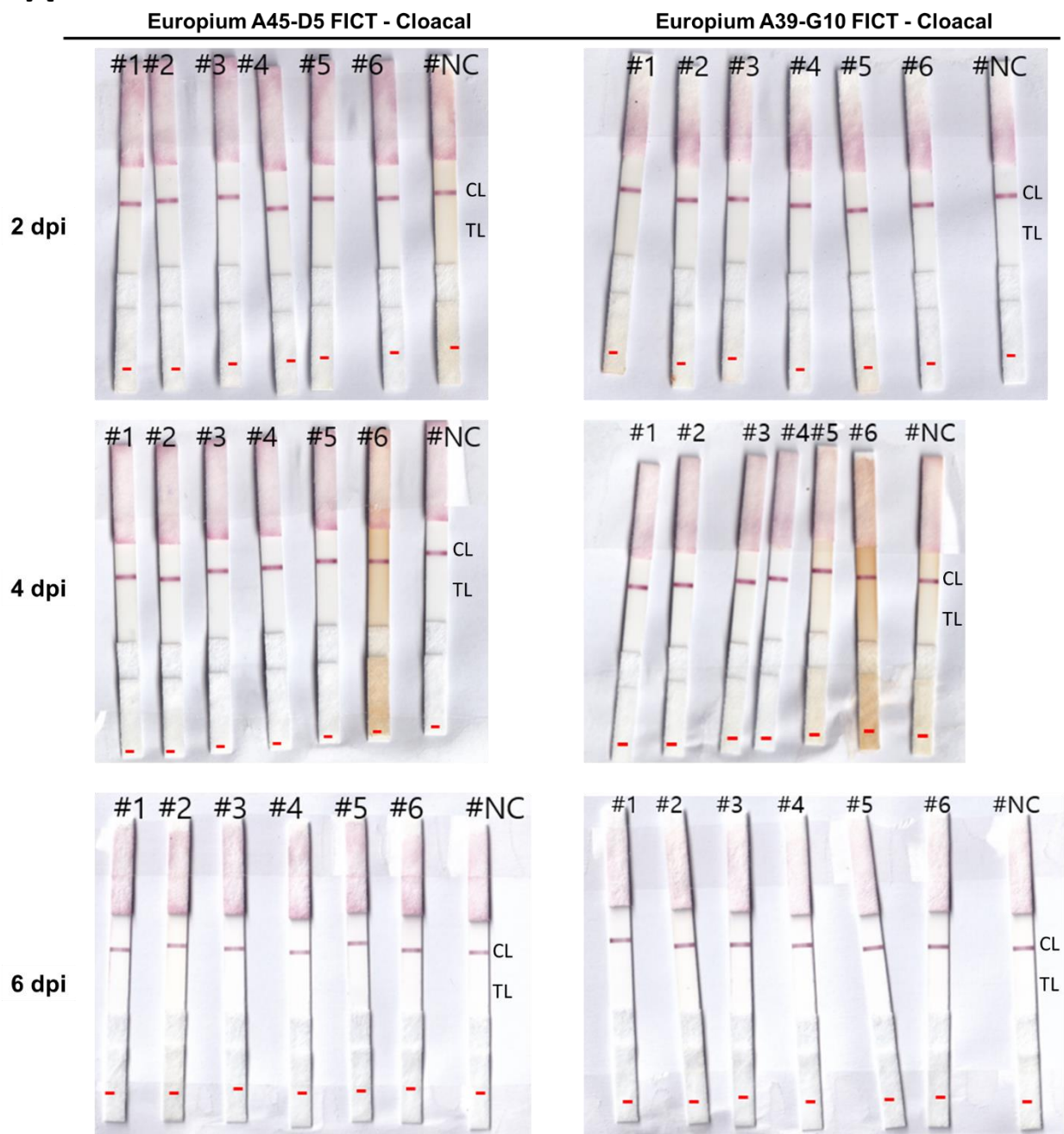




**Fig.S14. Raw data of Europium A45-D5-FICT and Europium A39-G10 FICT using clinical specimens from SPF chickens at different day post-infection with A/chicken/Korea/KNUSWR09/2009(H9N2) strain. (A) Cloacal swabs, (B) feces samples. TL/CL is underlined with red color.**

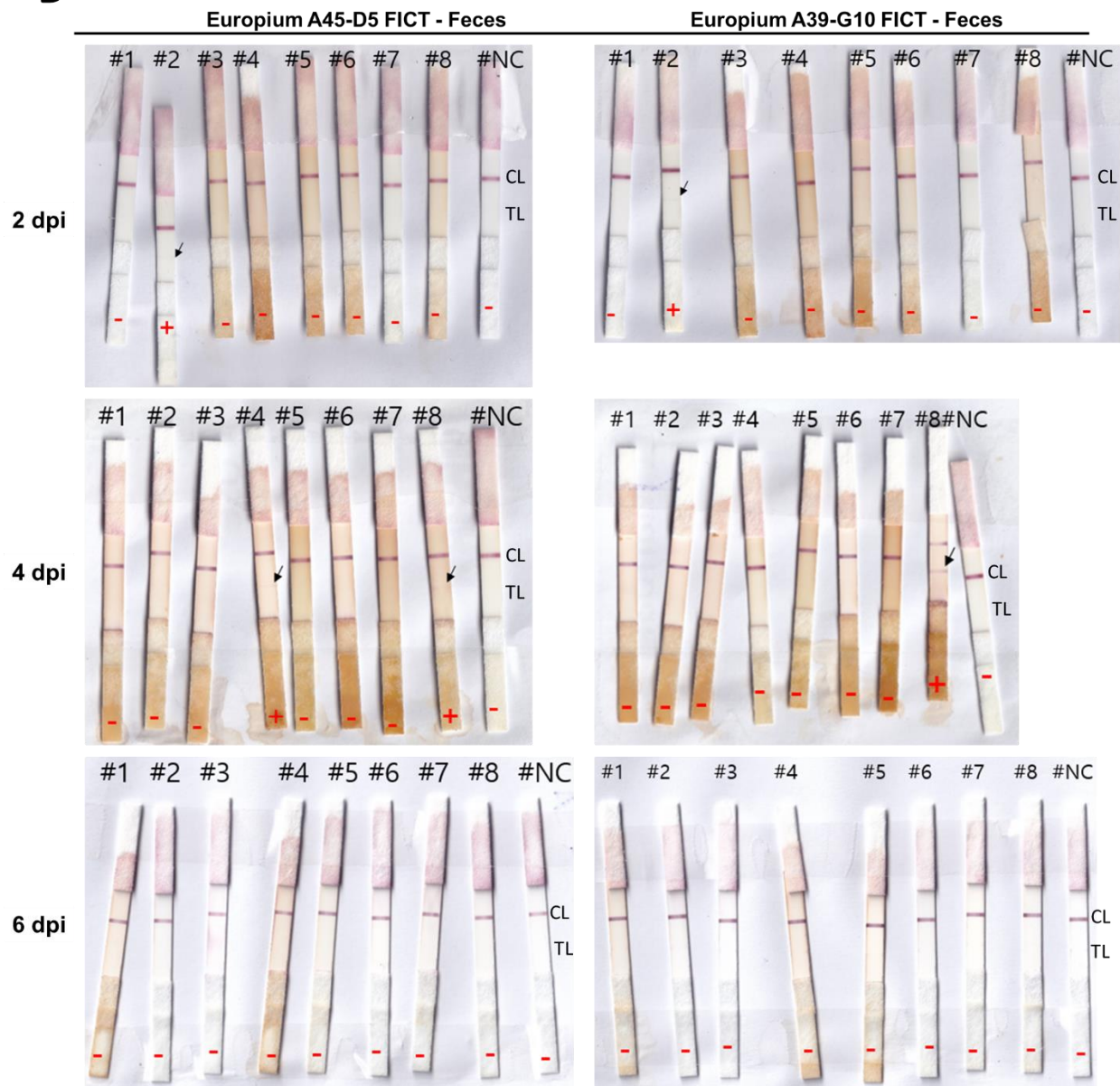
**A**

A/Chicken/Korea/LPM429/2016 (H9N2)



**B**

A/Chicken/Korea/LPM429/2016 (H9N2)

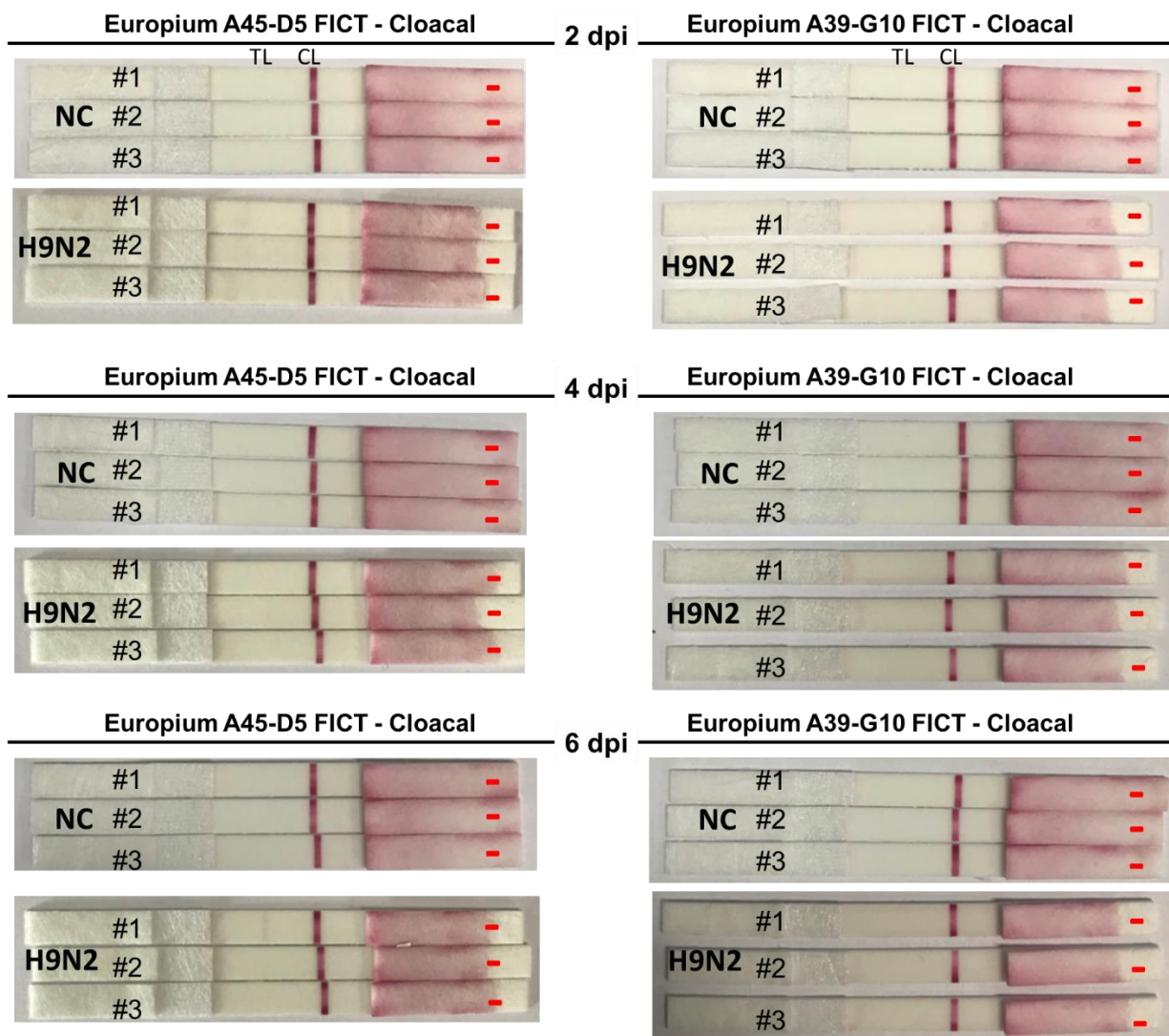


**Fig.S15. RDT with clinical specimens from SPF chickens at different day post-infection with A/Chicken/Korea/LPM429/2016(H9N2) strain. (A) Cloacal swabs, (B) feces samples. TL, test line of influenza A H9N2; CL, control line**

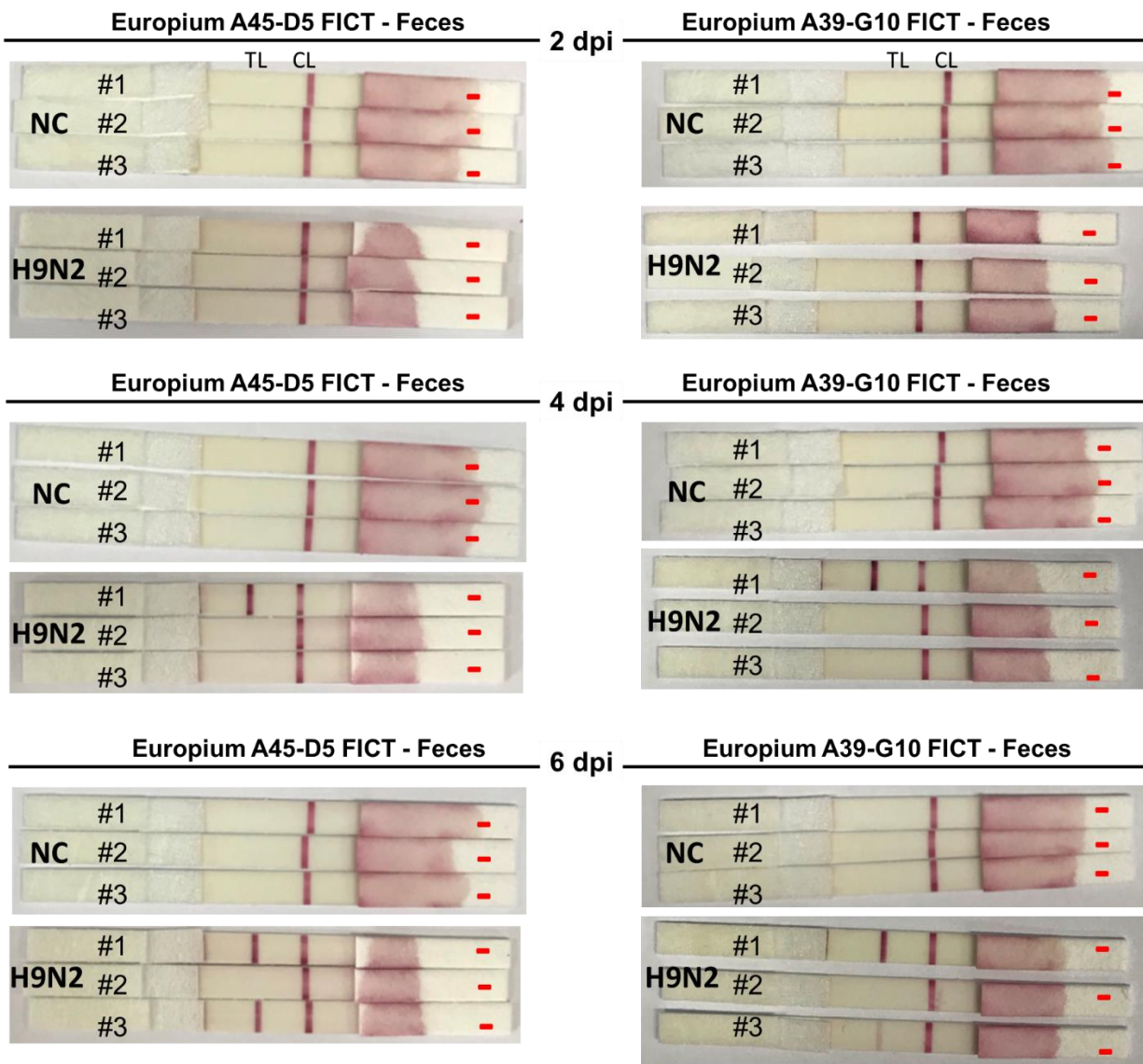


**A**

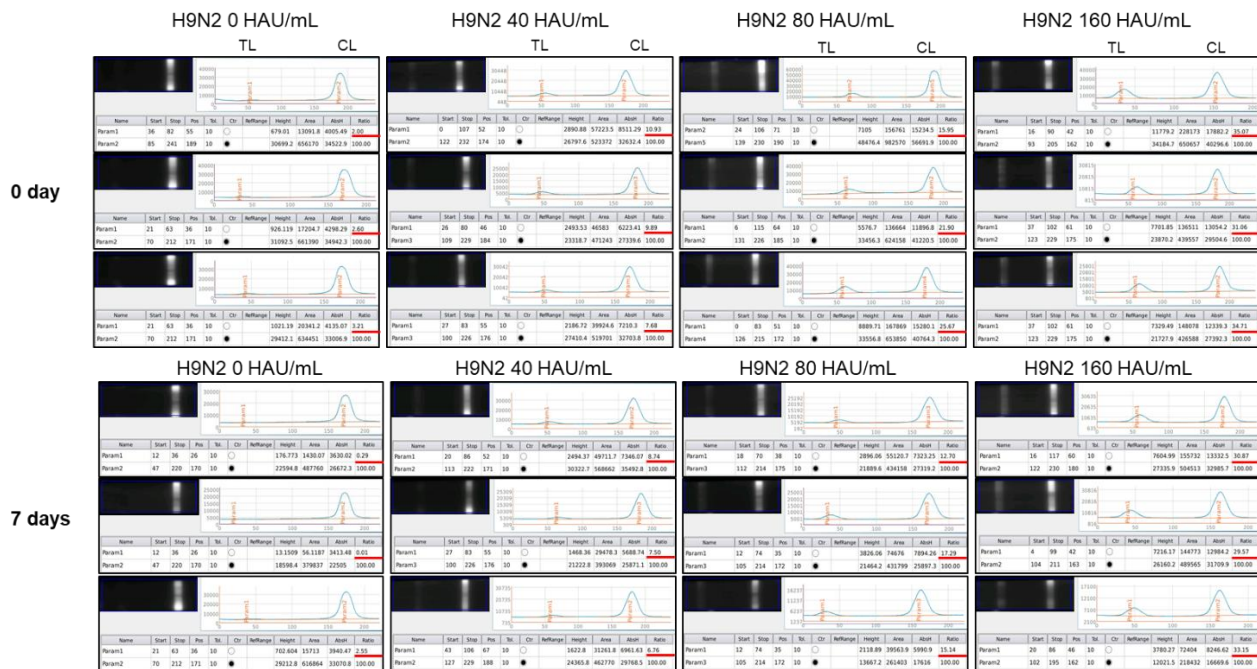
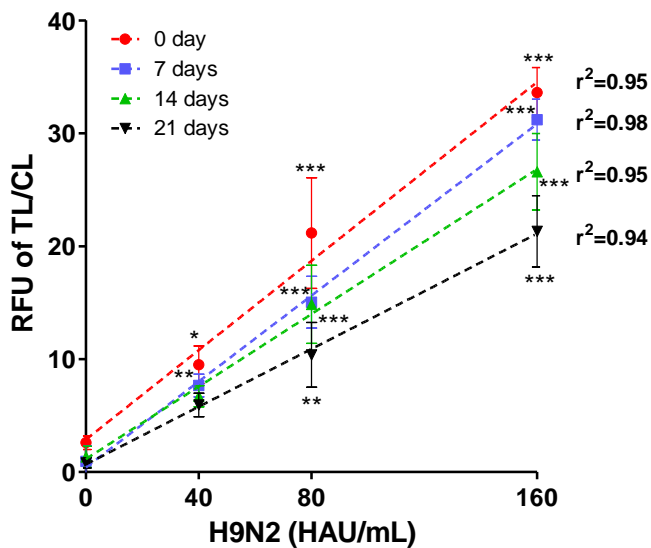
A/chicken/Korea/KNUSWR09/2009(H9N2)

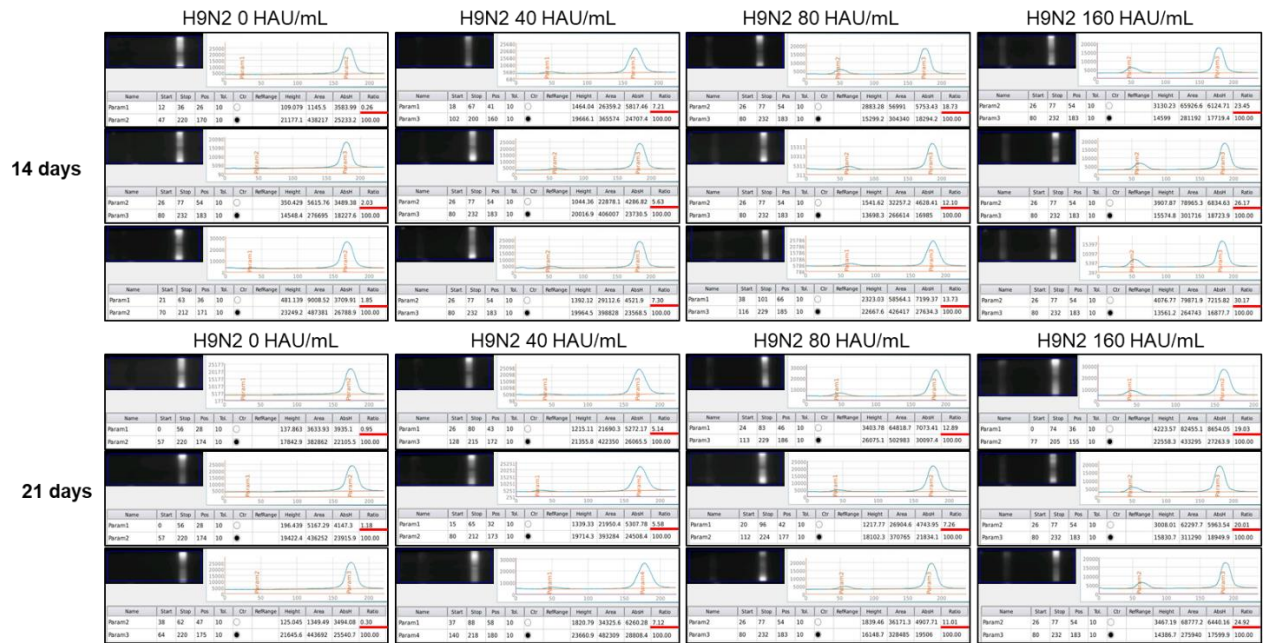




**B***A/chicken/Korea/KNUSWR09/2009(H9N2)*

**Fig.S16. RDT with clinical specimens from SPF chickens at different day post-infection with *A/chicken/Korea/KNUSWR09/2009(H9N2)* strain. (A) Cloacal swabs, (B) feces samples. TL, test line of influenza A H9N2; CL, control line**






**Fig.S17. Duration of FICT in dry condition.** Two-fold serially diluted spiked-H9N2 (A/chicken/Korea/KNUSWR09/2009(H9N2)) virus in chicken feces was tested using 0-, 7-, 14-, 21-day-old conjugates. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ .


Dear editor of MDPI,


Juwon Jeong, Younjeong Kim, Suyeon Yoon, and Gumju Bae in Wonkwang university endorse that they supported the documentation to perform this work and their names are written in Acknowledgement section of this article.:

Article title: Development of a Rapid Fluorescent Diagnostic System to Detect Subtype H9 Influenza A Virus in Chicken Feces.

Date: August 13, 2021

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