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Supplementary Materials for

Hemagglutinin destabilization in H3N2 vaccine reference viruses skews antigenicity and prevents airborne transmission in ferrets

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This PDF file includes:

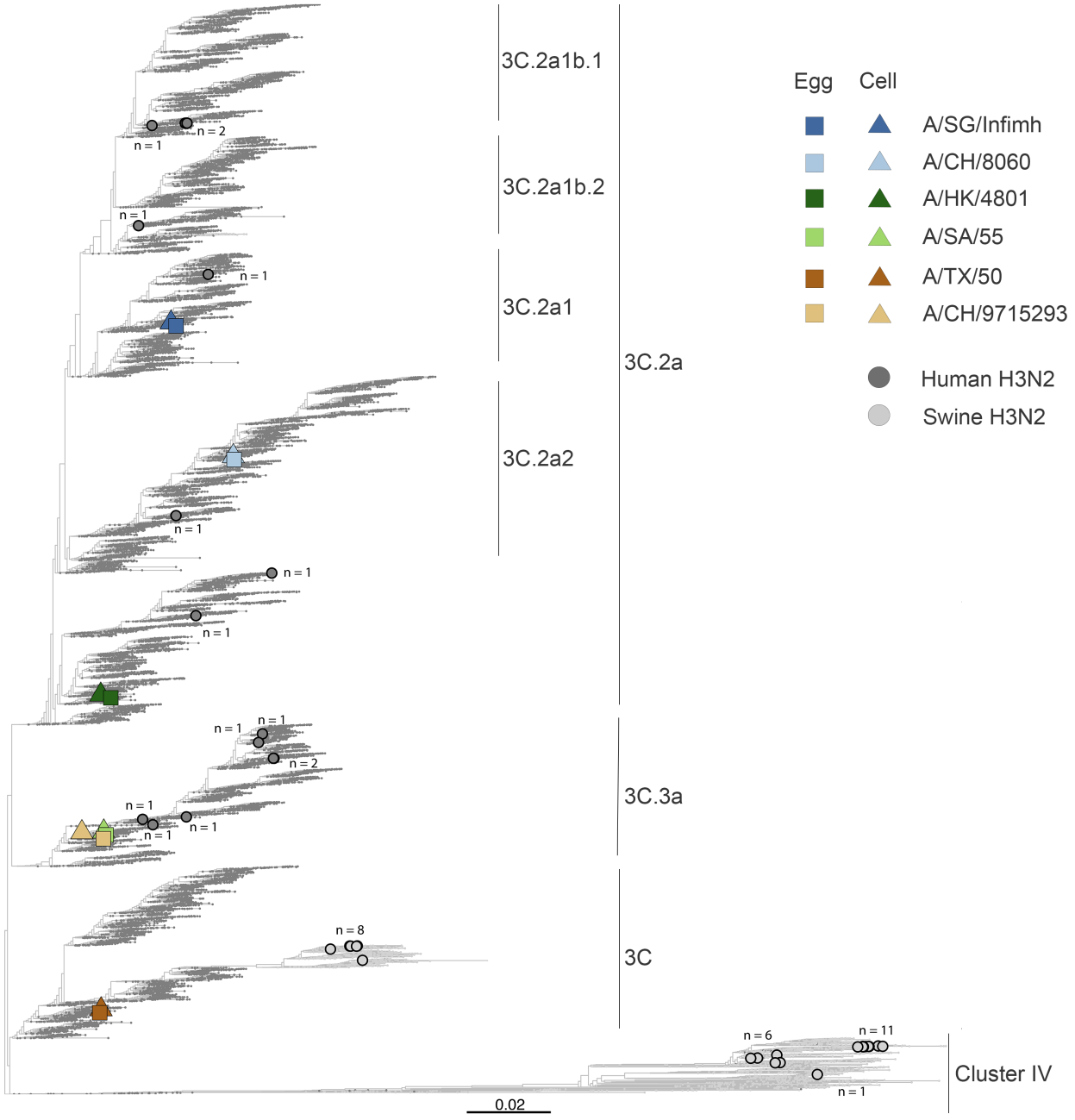


Fig. S1. Phylogenic analyses of HA segments of human and swine H3N2 IAVs. HA gene sequences of viruses used in this study were obtained by whole-genome sequencing. Full-length HA segments of representative human and swine isolates (2012-2019) were retrieved from Global Initiative on Sharing All Influenza Data (GISAID) or in-house sequencing. Human isolates in this study are dark grey circles, and swine isolates in this study are light grey circles. Six recent human vaccines reference viruses (utilized during 2013-2019 influenza vaccine seasons) grown in eggs (squares) and cells (triangles) are identified by colored symbols shown in the legend.

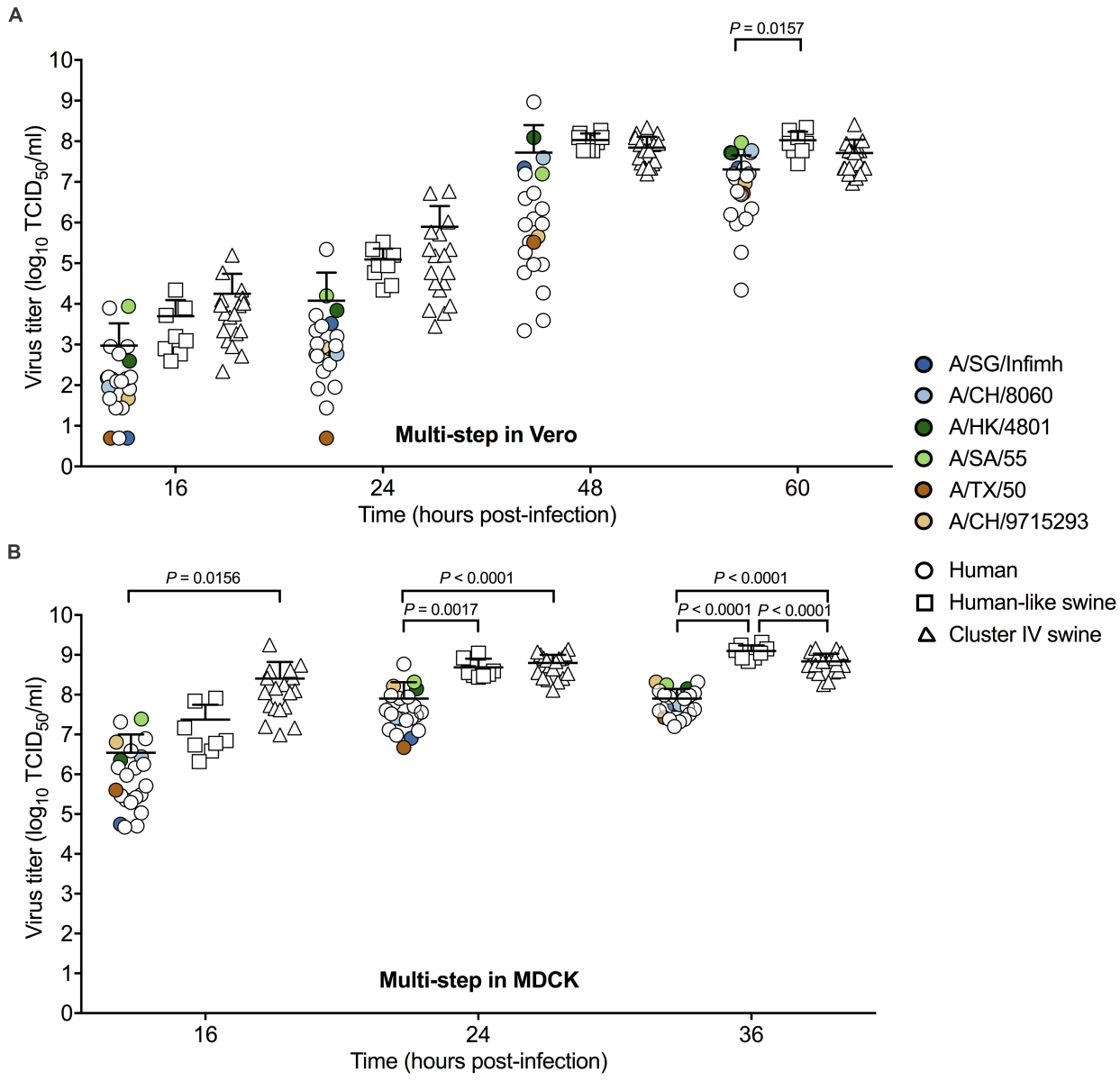


Fig. S2. Virus replication in Vero and MDCK cells. (**A**) Virus multi-step replication in Vero cells (MOI of 0.01 PFU/cell). (**B**) Virus multi-step replication in MDCK cells (MOI of 0.01 PFU/cell). Virus multi-step replication in Vero and MDCK cells were performed as in Fig. 2. *P* values were determined according to two-way ANOVA followed by Tukey's test.

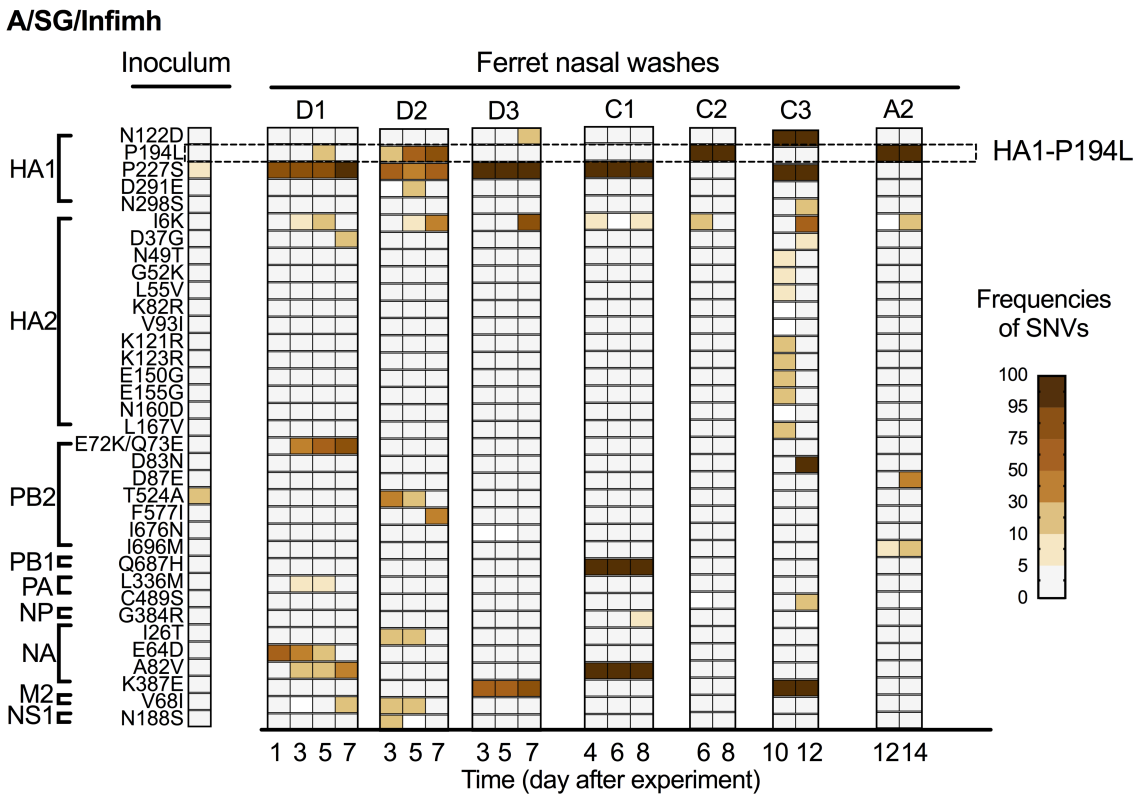
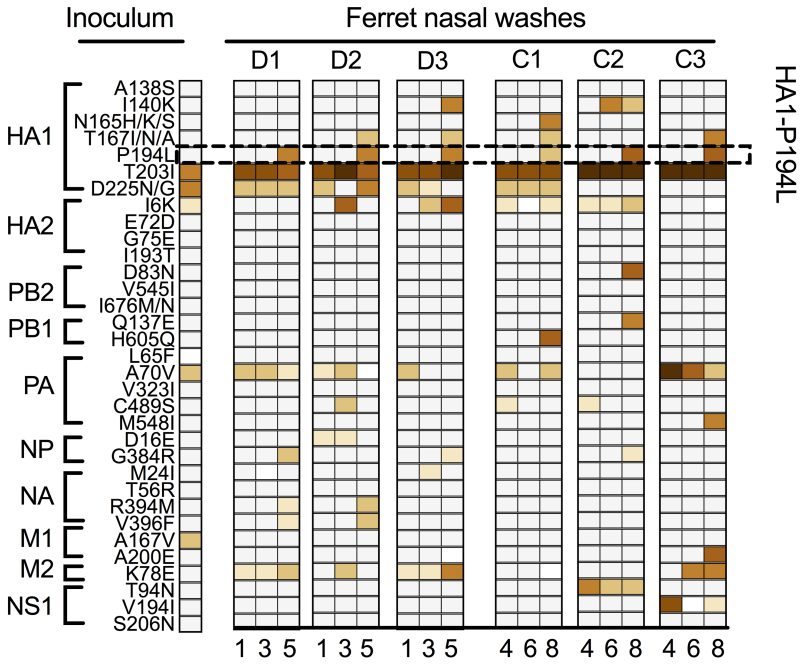


Fig. S3. Frequencies of genome variants of egg-based vaccine reference virus A/SG/Infimh in ferrets. The ferret experiment was performed as in Fig. 4. Whole genome sequences of the inoculum and the nasal washes from ferrets were obtained by the next generation sequencing. Each column represents the non-synonymous single nucleotide variants (SNVs) for one ferret nasal wash. SNVs with reads ≥ 10 are shown. For HA segment, SNV frequencies greater than 5% is shown. For other segments, SNV frequencies either greater than 50% or detected more than twice in nasal wash samples are shown. The frequencies of SNVs are represented by the colored scale.

A/HK/4801

Trial 1



Trial 2

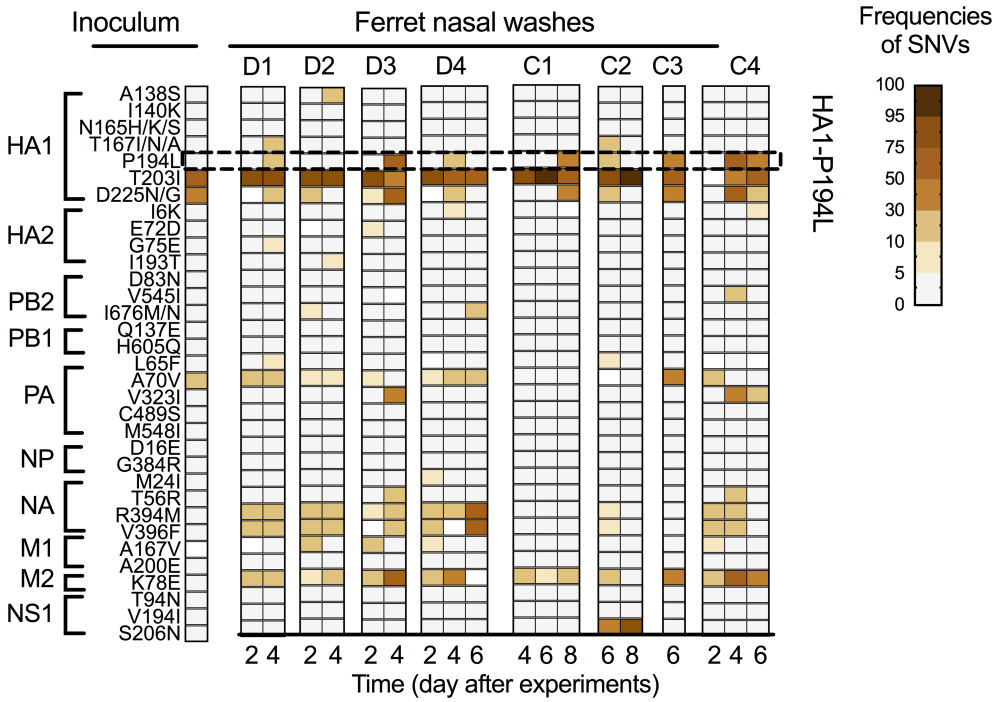
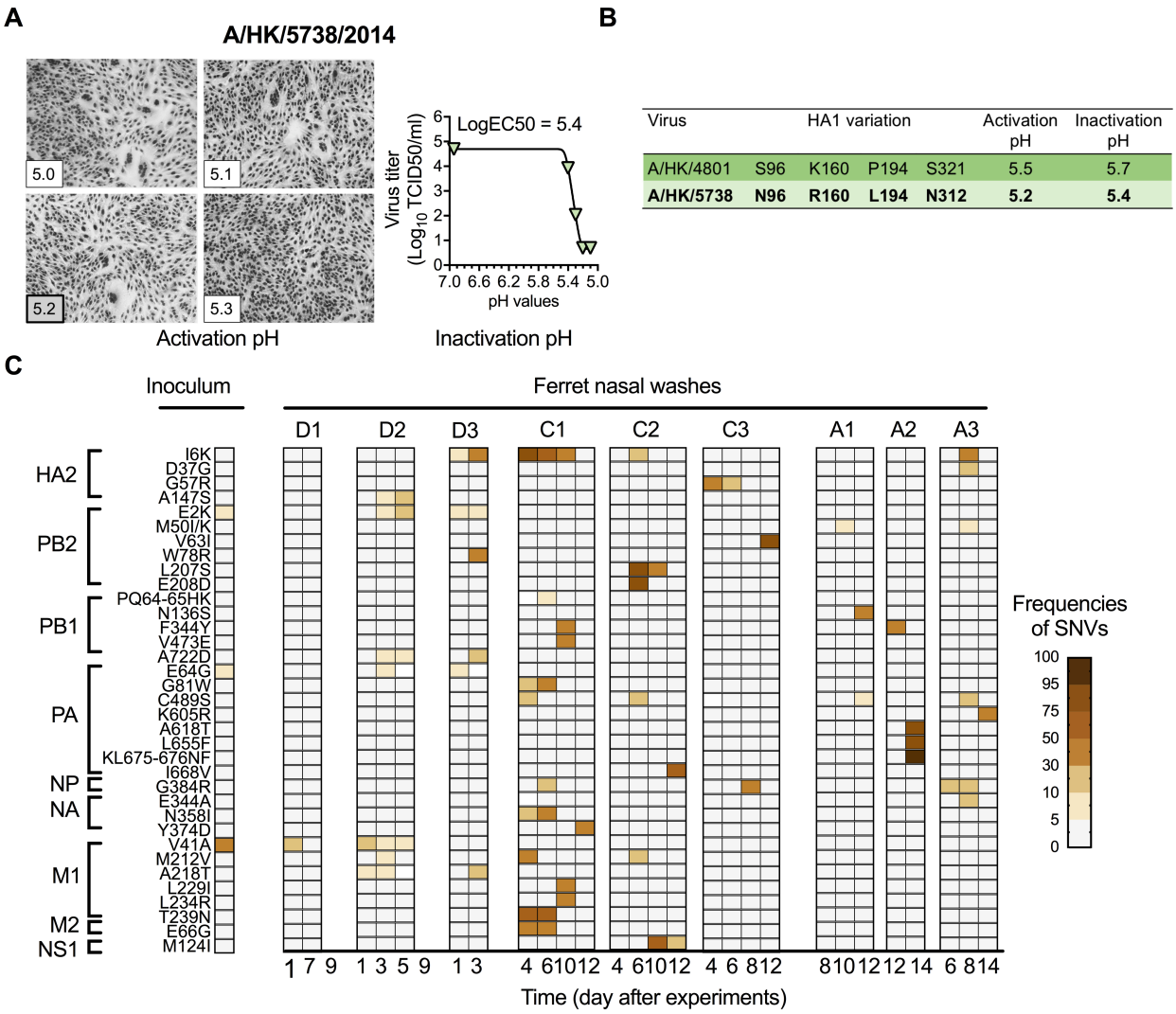


Fig. S4. Frequencies of genome variants of egg-based vaccine reference virus A/HK/4801 in ferrets. The ferret transmission experiment was performed as illustrated in Fig. 4. Next generation sequencing and SNV analysis were performed as in fig. S3.



Virus	HA1 variation				Activation pH	Inactivation pH
A/HK/4801	S96	K160	P194	S321	5.5	5.7
A/HK/5738	**N96**	**R160**	**L194**	**N312**	**5.2**	**5.4**

Fig. S5. Characterization of A/HK/5738 (an A/HK/4801-like virus) containing HA1-L194. (**A**) HA activation pH and virus inactivation pH values of the A/HK/5738 isolate. (**B**) Genotype and phenotype variations between A/HK/4801 and A/HK/5738. (**C**) Frequencies of genome variants of the A/HK/5738 in ferrets. The ferret transmission experiment was performed as illustrated in Fig. 4. Next generation sequencing and SNV analysis were performed as in fig. S3.

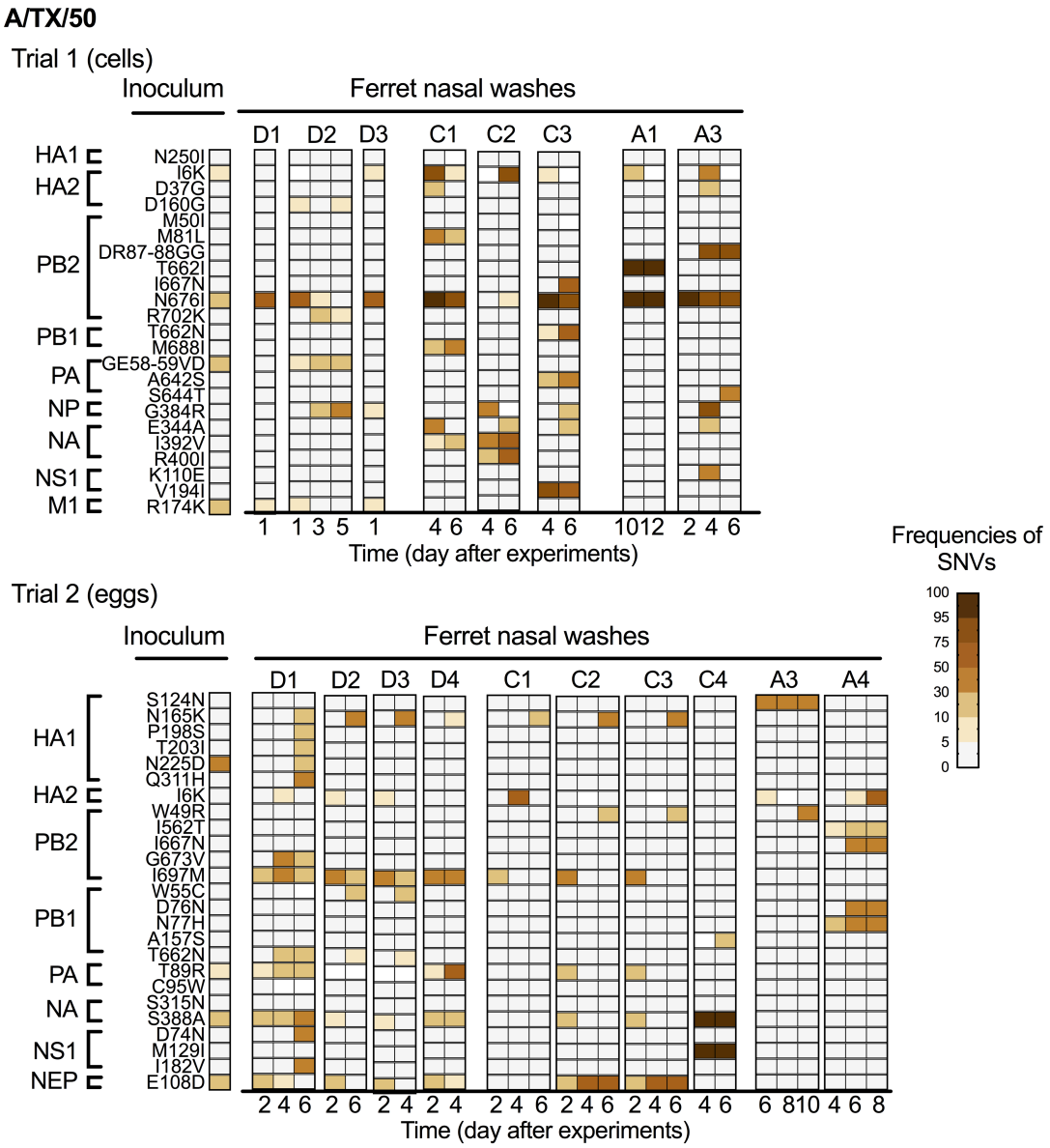


Fig. S6. Frequencies of genome variants for A/TX/50 with and without the egg adaptive mutation HA1-V186 in ferrets. The ferret transmission experiment was performed as illustrated in Fig. 4. Next generation sequencing and SNV analysis were performed as in fig. S3.

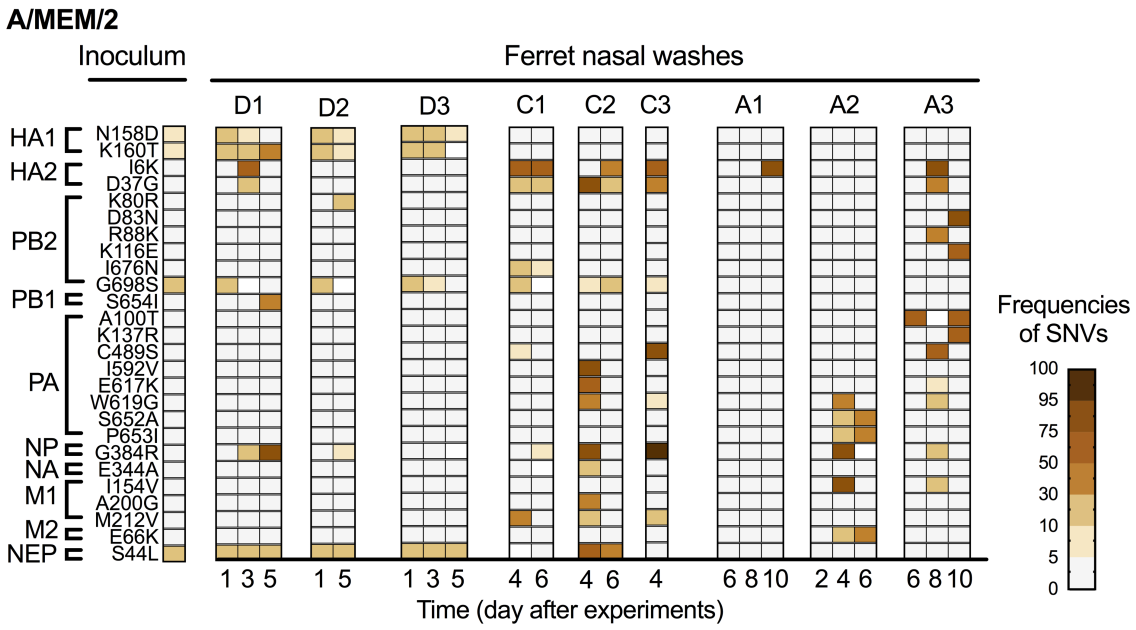


Fig. S7. Frequencies of genome variants for A/MEM/2 in ferrets. The ferret transmission experiment was performed as illustrated in Fig. 4. Next generation sequencing and SNV analysis were performed as in fig. S3.

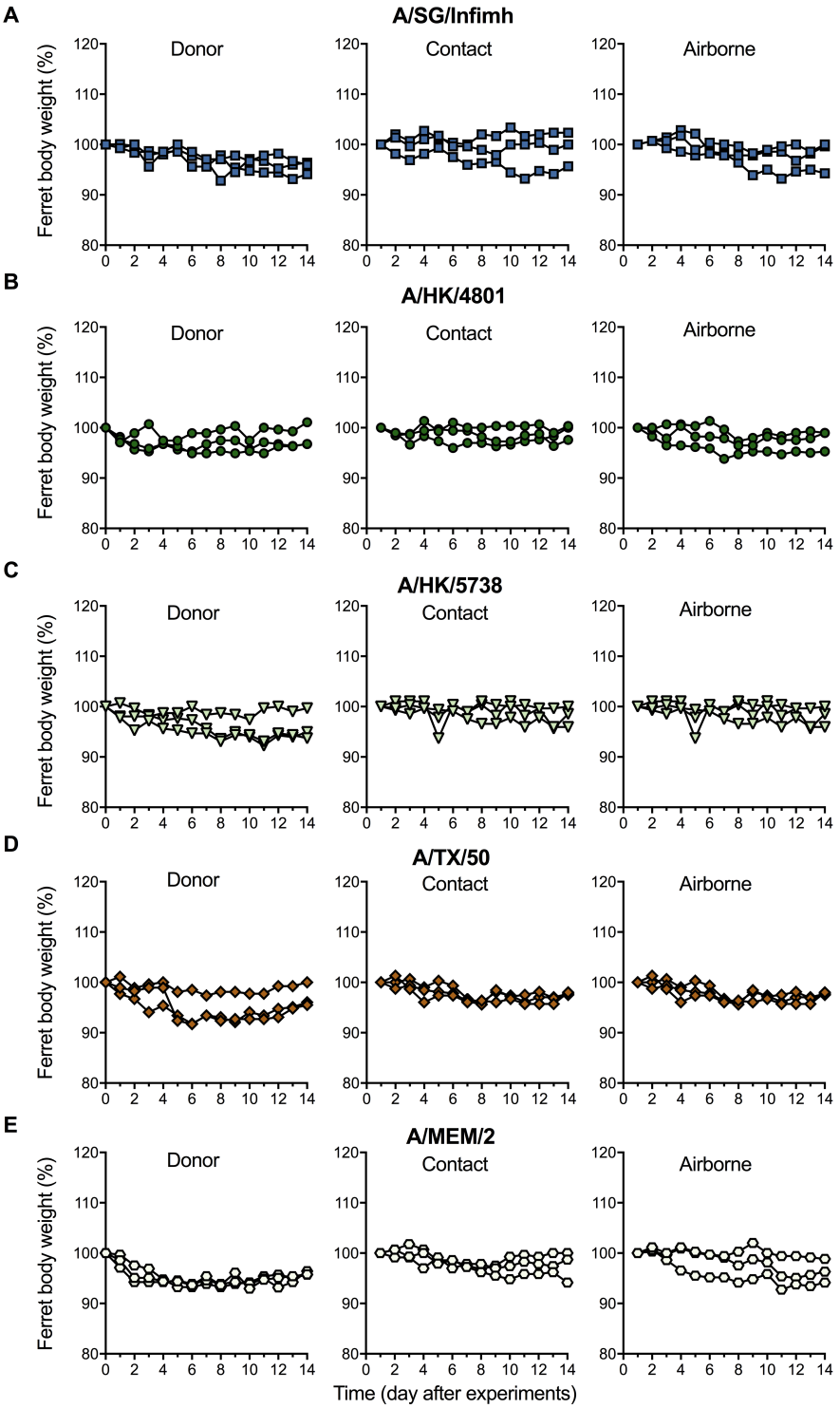


Fig. S8. Ferret body weights. Ferret body weights of A/SG/Infimh (**A**), A/HK/4801 trial 1 (**B**), A/HK/5738 (**C**). A/TX/50 trial 1 (**D**), and A/MEM/2 (**E**). Body weight was recorded daily.

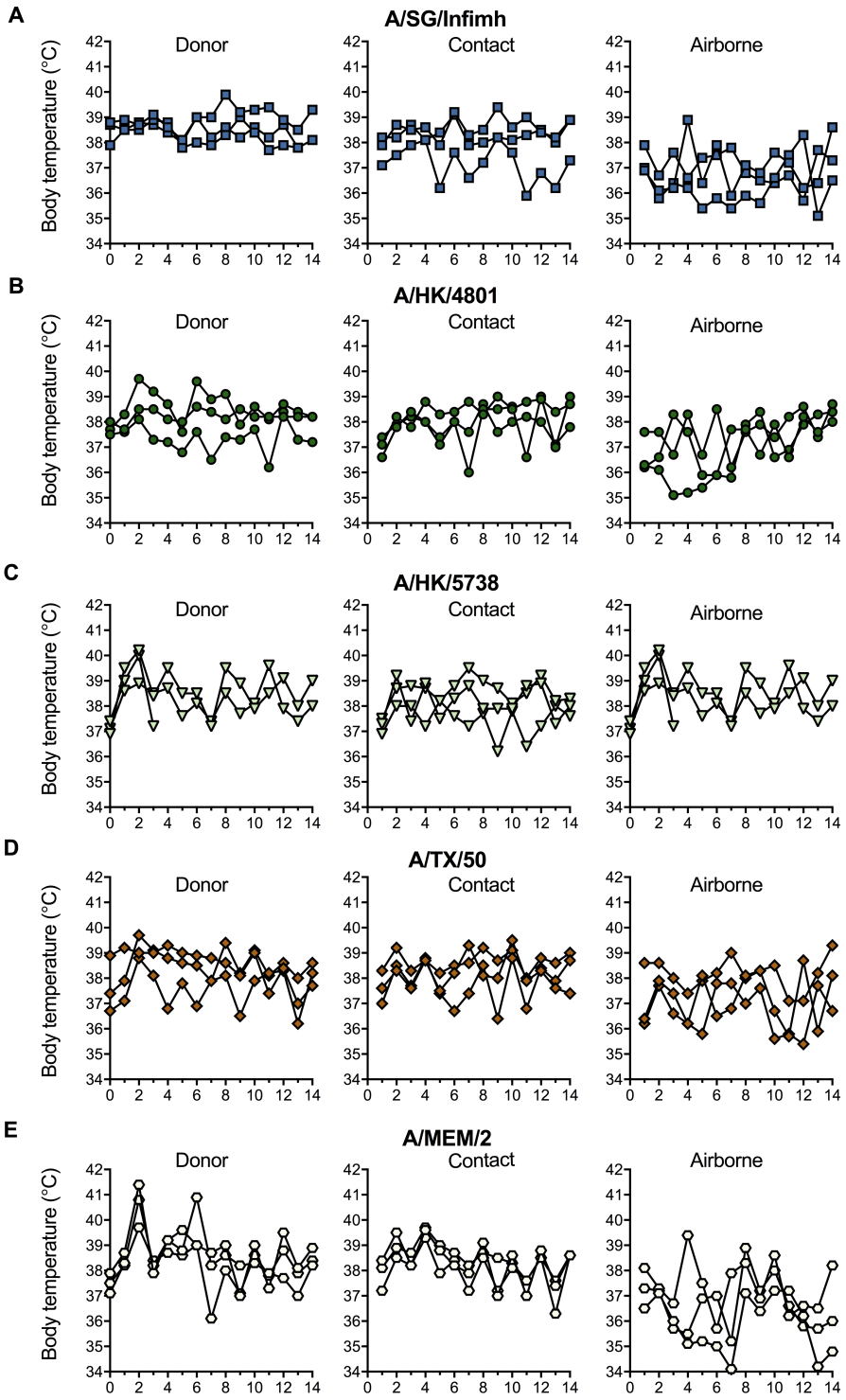


Fig. S9. Ferret body temperatures. Ferret body temperatures of A/SG/Infimh (**A**), A/HK/4801 trial 1 (**B**), A/HK/5738 (**C**). A/TX/50 trial 1 (**D**), and A/MEM/2 (**E**). Temperature was recorded daily.

Table S1. Genome variations for vaccine reference viruses cultured in cells and in eggs.

A/Singapore/INFIMH-16-0019/2016						
GISAID ID	Passage History	HA1 [b]			NP	NS1
EPI_ISL_16706695 [#a]	E5/E2/E1/C1	160K	194P	225G	311H	191T
EPI_ISL_1655936	E7	160K	194P	225G	X [c]	X
EPI_ISL_344420	E5/E1/E1	160K	194P	225G	X	X
EPI_ISL_335174	E5/E1	160K	194P	225G	X	X
EPI_ISL_284191	E5/E2	160K	194P	225G	X	191T
EPI_ISL_284136	E5/E2 CL-2	160K	194P	X	311H	191T
EPI_ISL_275709	E5/E1	160K	194P	X	311Q	191T
EPI_ISL_257001	E5/IR	160K	194P	X	311Q	191T
EPI_ISL_239803	E5	160K	194P	X	311Q	191T
EPI_ISL_284191	E5/E2	160K	194P	225G	X	191T
EPI_ISL_291274	E6	160K	194P	X	X	191T
EPI_ISL_344419	C1/SIAT3/SIAT1/SIAT2	160T	194L	225D	X	X
EPI_ISL_335173	C1/SIAT3/SIAT1	160T	194L	225D	X	X
EPI_ISL_330262	C1/SIAT3/SIAT4	160T	194L	225D	311Q	191T
EPI_ISL_311688	C1/SIAT3/SIAT3	160T	194L	225D	311Q	191T
EPI_ISL_296168	C1/SIAT3/SIAT1	160T	194L	225D	311Q	191T
EPI_ISL_285898	C1/SIAT3	160T	194L	225D	311Q	191T
EPI_ISL_285897	C1/SIAT3/SIAT2	160T	194L	225D	311Q	191I
EPI_ISL_225834	Clinical specimen	160T	194L	225D	X	X

A/Switzerland/8060/2017				
GISAID ID	Passage History	HA1		
EPI_ISL_16706701 [#]	E7/E1/C1	160K	194P	203I
EPI_ISL_344423	E5/E1/E1	160K	194P	203T
EPI_ISL_334556	E5/E1	160K	194P	203T
EPI_ISL_331821	E5/E2	160K	194P	203T
EPI_ISL_331220	E6	160K	194P	203T
EPI_ISL_330887	E5	160K	194P	203T
EPI_ISL_319720	E5/E1	160K	194P	203T
EPI_ISL_303951	SIAT1/SIAT1	160T	194L	203T
EPI_ISL_294252	SIAT1	160T	194L	203T
EPI_ISL_334555	SIAT2/SIAT2	160T	194L	203T
EPI_ISL_332305	SIAT2/SIAT1	160T	194L	203T
EPI_ISL_331924	SIAT2	160T	194L	203T

A/Hong Kong/4801/2014									
GISAID ID	Passage History	HA1					NP		NA
EPI_ISL_16706634 [#]	E5/E2/E2/E1/C1	96S	160K	194P	203T	260I	101G	454D	148T
EPI_ISL_16713985 [#]	E5/E2/E2/E1	96S	160K	194P	203T	260I	101G	454D	148T
EPI_ISL_2443866	E8	96S	160K	194P	203T	260I	101G	454D	148T
EPI_ISL_270160	E5/E3	96S	160K	194P	X	260I	101G	454D	148T
EPI_ISL_189814	E5/E2	96S	160K	194P	203T	260I	101G	454D	148T
EPI_ISL_176512	E5/E1	96S	160K	194P	203T	260I	101G	454D	148T
EPI_ISL_195755	E6 (Am1AI)/E1+1	96N	160K	194P	203I	260L	101G	454D	148T
EPI_ISL_215973 * [d]	E8	96S	160K	194P	203T	260I	X	X	148T
EPI_ISL_215974 *	E8	96S	160K	194P	X	260I	X	X	148T
EPI_ISL_165554	SIAT1	96N	160T	194L	203T	260I	101G	454D	148T
EPI_ISL_233740	C4/S4	96N	160T	194L	203T	260I	101D	454E	148K
EPI_ISL_202569	C4/S3	96N	160T	194L	203T	260I	101D	454E	148K
EPI_ISL_198222	C4/S2	96N	160T	194L	203T	260I	101D	454E	X

A/South Australia/55/2014								
GISAID ID	Passage History	PA		HA1				NP
EPI_ISL_16706071 [#]	E5/E2/E1/C1	V62	I669	N145	H156	V186	G225	N34
EPI_ISL_166891	E5/E1	V62	I669	N145	H156	X	G225	D34
EPI_ISL_166890	E5/E2	V62	I669	N145	H156	V186	G225	D34
EPI_ISL_166745	E5/E1	X	X	N145	H156	X	G225	X
EPI_ISL_166239	E5	X	X	N145	H156	G186	G225	X
EPI_ISL_165593	E5	X	X	N145	H156	G186	G225	X
EPI_ISL_207650 *	E6	X	X	S145	R156	E186	G225	X
EPI_ISL_288203	C1	V62	I669	S145	H156	G186	D225	D34
EPI_ISL_192859	C3	M62	I669	S145	H156	G186	D225	D34
EPI_ISL_189817	C1/SIAT3	V62	I669	S145	H156	G186	D225	D34
EPI_ISL_166892	C1/SIAT1	X	X	S145	H156	G186	D225	X
EPI_ISL_166846	C1/SIAT2	V62	V669	S145	H156	G186	D225	X
EPI_ISL_166746	C1/SIAT1	X	X	S145	H156	G186	D225	X
EPI_ISL_166238	C1	X	X	S145	H156	G186	D225	X
EPI_ISL_165592	C1	X	X	S145	H156	G186	D225	X

A/Texas/50/2012									
GISAID ID	Passage History	PB2	HA1				NP	NA	M1
EPI_ISL_16701302 [#]	C1/C2/C2	N676	K2	G186	S219	I226	G384	D304	R174
EPI_ISL_16713984 [#]	E6	I676	K2	V186	F219	I226	R384	D304	K174

EPI_ISL_2454729	C1/C2/C6/SIAT4	X	K2	G186	S219	I226	X	N304	X
EPI_ISL_122006	C1/C1	X	K2	G186	S219	I226	X	D304	K174
EPI_ISL_129858	C1/C2	I676	K2	G186	S219	I226	G384	D304	K174
EPI_ISL_170149	C1/C1/SIAT1	I676	K2	G186	S219	I226	G384	D304	K174
EPI_ISL_166860	C1/C2/SIAT1	X	K2	G186	S219	I226	X	D304	X
EPI_ISL_164401	C1/C1/SIAT2	I676	K2	G186	S219	I226	G384	D304	K174
EPI_ISL_2443864	E5/E2	X	Q2	V186	F219	N226	X	D304	X
EPI_ISL_138980	E6	X	K2	V186	F219	X	X	D304	X
EPI_ISL_132908	E5/E1	X	K2	V186	F219	I226	X	D304	X
EPI_ISL_129744	E5	I676	K2	V186	F219	I226	X	D304	K174
EPI_ISL_127832	E4	X	K2	V186	F219	I226	X	D304	K174
EPI_ISL_215972 *	E9	X	K2	V186	F219	N226	X	D304	X

A/Switzerland/9715293/2013					
GISAID ID	Passage History	PA	HA1		
EPI_ISL_16706632 [#]	E4/E3/E1/C1	PA-594S	R140	V186	S219
EPI_ISL_166858	E4/E1	X	R140	V186	S219
EPI_ISL_164719	E4/E1	X	R140	V186	S219
EPI_ISL_165829	E4/E2	PA-594S	R140	V186	X
EPI_ISL_169603	E4/E9/E1	X	R140	V186	F219
EPI_ISL_207641 *	E6	X	R140	V186	Y219
EPI_ISL_2443865	E7	X	R140	V186	Y219
EPI_ISL_202341	Clone123 E4	X	R140	V186	S219
EPI_ISL_166310	SIAT1/SIAT2/SIAT2	PA-594S	I140	G186	S219
EPI_ISL_189815	SIAT1/SIAT2/SIAT3	PA-594N	I140	G186	S219
EPI_ISL_230377	SIAT1/SIAT2/SIAT4	X	I140	G186	S219
EPI_ISL_2454697	SIAT1/SIAT8	X	R140	G186	S219
EPI_ISL_166859	SIAT1/SIAT2/SIAT1	X	I140	G186	S219
EPI_ISL_162149	SIAT2	X	I140	G186	S219

[a] #, wild type vaccine reference virus was obtained from the St. Jude Children's Research Hospital Center of Excellence for Influenza Research and Response (CEIRR), or kindly provided by the World Health Organizations (WHO)' Global Influenza Surveillance and Response System.
[b] H3 numbering.
[c] X, unknown.
[d] *, reassortant vaccine seed stocks.

Table S2. Human and swine H3N2 influenza viruses (2012-2019) used *in vitro* experiments.

Virus	Classification	Passage history	HA activation pH	Virus inactivation pH	GISAID ID
A/SWITZERLAND/9715293/2013	Human	E4/E3/E1/C1	5.30	5.51	EPI_ISL_16706632
A/HONG KONG/4801/2014	Human	E5/E2/E2/E1/C1	5.47	5.67	EPI_ISL_16706634
A/SOUTH AUSTRALIA/55/2014	Human	E5/E2/E1/C1	5.53	5.52	EPI_ISL_16706071
A/SINGAPORE/INFIMH-16-0019/2016	Human	E5/E2/E1/C1	5.67	5.78	EPI_ISL_16706695
A/PERU/4617/2017	Human	CX/C1/C1	5.40	5.48	EPI_ISL_16706702
A/SWITZERLAND/8060/2017	Human	E7/(Am2Al5)/E1/C1	5.70	5.65	EPI_ISL_16706701
A/MEMPHIS/256/2019	Human	C1/C1	5.40	5.51	EPI_ISL_16706703
A/MEMPHIS/16/2019	Human	C1/C1	5.13	5.49	EPI_ISL_16706704
A/MEMPHIS/26/2019	Human	C1/C1	5.30	5.41	EPI_ISL_16709533
A/MEMPHIS/23/2019	Human	C2/C1	5.40	5.46	EPI_ISL_16709534
A/MEMPHIS/33/2019	Human	C1/C1	5.40	5.39	EPI_ISL_16709535
A/TX/50/2012	Human	C1/C2/C2	5.27	5.53	EPI_ISL_16701302
A/HONG KONG/5738/2014	Human	C1/C2/SIAT2/C2	5.23	5.40	EPI_ISL_16706696
A/GREECE/35/2017	Human	E8/E1/C1	5.23	5.47	EPI_ISL_16706697
A/HONG KONG/2277/2017	Human	E6/E1/C1	5.23	5.44	EPI_ISL_16706698
A/MEMPHIS/23/2018	Human	C1/C1	5.67	5.51	EPI_ISL_16706700
A/MEMPHIS/2/2017	Human	C2/C1	5.27	5.31	EPI_ISL_16706635
A/MEMPHIS/11/2017	Human	C1/C1	5.47	5.47	EPI_ISL_16706636
A/MEMPHIS/19/2017	Human	C1/C1	5.27	5.45	EPI_ISL_16706638
A/MEMPHIS/25/2017	Human	C2/C1	5.70	5.50	EPI_ISL_16700249
A/HONG KONG/2286/2017	Human	E6/E1/C1	5.23	5.48	EPI_ISL_16706699
A/MEMPHIS/31/2017	Human	C1/C1	5.40	5.48	EPI_ISL_16706639
A/SWINE/OH/16TOSU4783/2016	Human-like Swine	C1/C1/C1	5.30	5.43	EPI_ISL_16706692
A/SWINE/OH/17TOSU1389/2017	Human-like Swine	X [a]/C1	5.27	5.33	EPI_ISL_16706693
A/SWINE/IL/TM1705DTW1/2017	Human-like Swine	C1/C1	5.37	5.41	EPI_ISL_16706640
A/SWINE/IL/TM1705DTW24/2017	Human-like Swine	C1/C1	5.40	5.44	EPI_ISL_16706641
A/SWINE/IL/TM1706DTW24/2017	Human-like Swine	C1/C1	5.33	5.47	EPI_ISL_16706642
A/SWINE/IL/TM1707DTW28/2017	Human-like Swine	C1/C1	5.27	5.48	EPI_ISL_16706643
A/SWINE/IL/TM1708DTW24/2017	Human-like Swine	C1/C1	5.30	5.49	EPI_ISL_16706666
A/SWINE/IL/TM1709DTW11/2017	Human-like Swine	C1/C1	5.30	5.38	EPI_ISL_16706694
A/SWINE/OH/12TOSU447/2012	Cluster IV Swine	X/C1	5.80	5.81	EPI_ISL_16706161
A/SWINE/OH/12TOSU527/2012	Cluster IV Swine	X/C1	5.57	5.52	EPI_ISL_16706690
A/SWINE/NE/4C-0813-G20/2013	Cluster IV Swine	C1/C1	5.87	5.74	EPI_ISL_16700814
A/SWINE/NE/4B-1113-P1/2013	Cluster IV Swine	C1/C1	5.70	5.69	EPI_ISL_16701460
A/SWINE/NE/4B-1113-P15/2013	Cluster IV Swine	C1/C1	5.87	5.79	EPI_ISL_16701536

A/SWINE/NE/4B-1113-P20/2013	Cluster IV Swine	C1/C1	5.90	5.88	EPI_ISL_16702403
A/SWINE/NE/4F-1113-P5/2013	Cluster IV Swine	C1/C1	5.80	5.85	EPI_ISL_16702404
A/SWINE/OH/13TOSU2194/2013	Cluster IV Swine	C1/C1/C1	5.37	5.45	EPI_ISL_16706630
A/SWINE/OH/13TOSU1238/2013	Cluster IV Swine	X/C1	5.87	5.68	EPI_ISL_16706165
A/SWINE/IN/14TOSU0364/2014	Cluster IV Swine	X/C1	5.70	5.75	EPI_ISL_16706691
A/SWINE/NE/4D-0114-P1/2014	Cluster IV Swine	C1/C1	5.87	5.71	EPI_ISL_16702842
A/SWINE/NE/4D-0114-P4/2014	Cluster IV Swine	C1/C1	5.87	5.85	EPI_ISL_16702922
A/SWINE/NE/4D-0114-P8/2014	Cluster IV Swine	C1/C1	5.90	5.93	EPI_ISL_16703274
A/SWINE/NE/4D-0114-P29/2014	Cluster IV Swine	C1/C1	5.87	5.88	EPI_ISL_16705252
A/SWINE/NE/4B-0114-P25/2014	Cluster IV Swine	C1/C1	5.80	5.77	EPI_ISL_16705321
A/SWINE/NE/4G-0314-P10/2014	Cluster IV Swine	C1/C1	5.80	5.83	EPI_ISL_16705322
A/SWINE/IL/1A-0314-G5/2014	Cluster IV Swine	C1/C1	5.57	5.57	EPI_ISL_16705685
A/SWINE/IL/1B-0614-P23/2014	Cluster IV Swine	C1/C1	5.50	5.53	EPI_ISL_16705686

[a] X, unknown.

Table S3. Binding of influenza viruses to regular and modified red blood cells (RBCs) from guinea pigs and turkeys.

Virus	HA titer (HAU/50 µl)			
	Guinea pig RBC (0.6%)	Turkey RBC (0.5%)	α2,6-Turkey RBC (0.5%)	α2,3-Turkey RBC (0.5%)
A/SG/Infimh (HA1-P194)	64	16	32	16
A/SG/Infimh (HA1-L194)	64	16	32	0
A/TX/50 (HA1-G186)	128	32	64	0
A/TX/50 (HA1-V186)	128	32	64	0
Pandemic H1N1 [a]	64	64	64	0
Recombinant H5N1 [b]	64	64	<2	64

[a] Pandemic H1N1 refers to A/TN/1-560/2009 (H1N1).
[b] Recombinant H5N1 refers to recombinant A/PR/08/1934 virus bearing the HA gene from A/mallard/Alberta/383/2009 (H5N1).

Table S4. Comparison of A/HK/4801 and A/HK/5738 isolates.

Virus		A/HK/4801	A/HK/5738
Passage history		E5/E2/E2/E1/C1	C1/C2/SIAT2/C2
HA activation pH		5.5	5.2
Virus inactivation pH		5.7	5.4
Genome variations	HA1 [a]	S96	N96
		K160	R160
		P194	L194
		S312	N312
	PB2	D408	G408
	PB1	T152	S152
		Y166	F166
	NP	101G	101D
		D454	E454
		D480	N480
	NA	T95	K95
		I231	V231

[a] H3 numbering.

Table S5. Data for virus growth based on HA activation pH and virus inactivation pH in ferrets.

Virus [a]		A/SG /Infimh	A/HK/4801		A/TX/50/2012		A/HK /5738	A/MEM /2
HA stability (pH)	Activation	5.67	5.47	5.48	5.27	5.30	5.23	5.27
	Inactivation	5.78	5.67	5.69	5.53	5.50	5.40	5.31
Virus replication in	Initial titer	2.20	3.88	3.13	5.67	4.75	4.76	6.02
Donor ($log_{10}TCID_{50}$/ml)	AUC [b]	5.96	5.47	4.91	5.38	5.05	6.24	5.75

[a] The values for HA activation pH, virus inactivation pH, and virus replication in Donor were the mean values for an individual isolate.
[b] AUC, area under shedding curves. The values were calculated by using GraphPad Prism version 7 software.