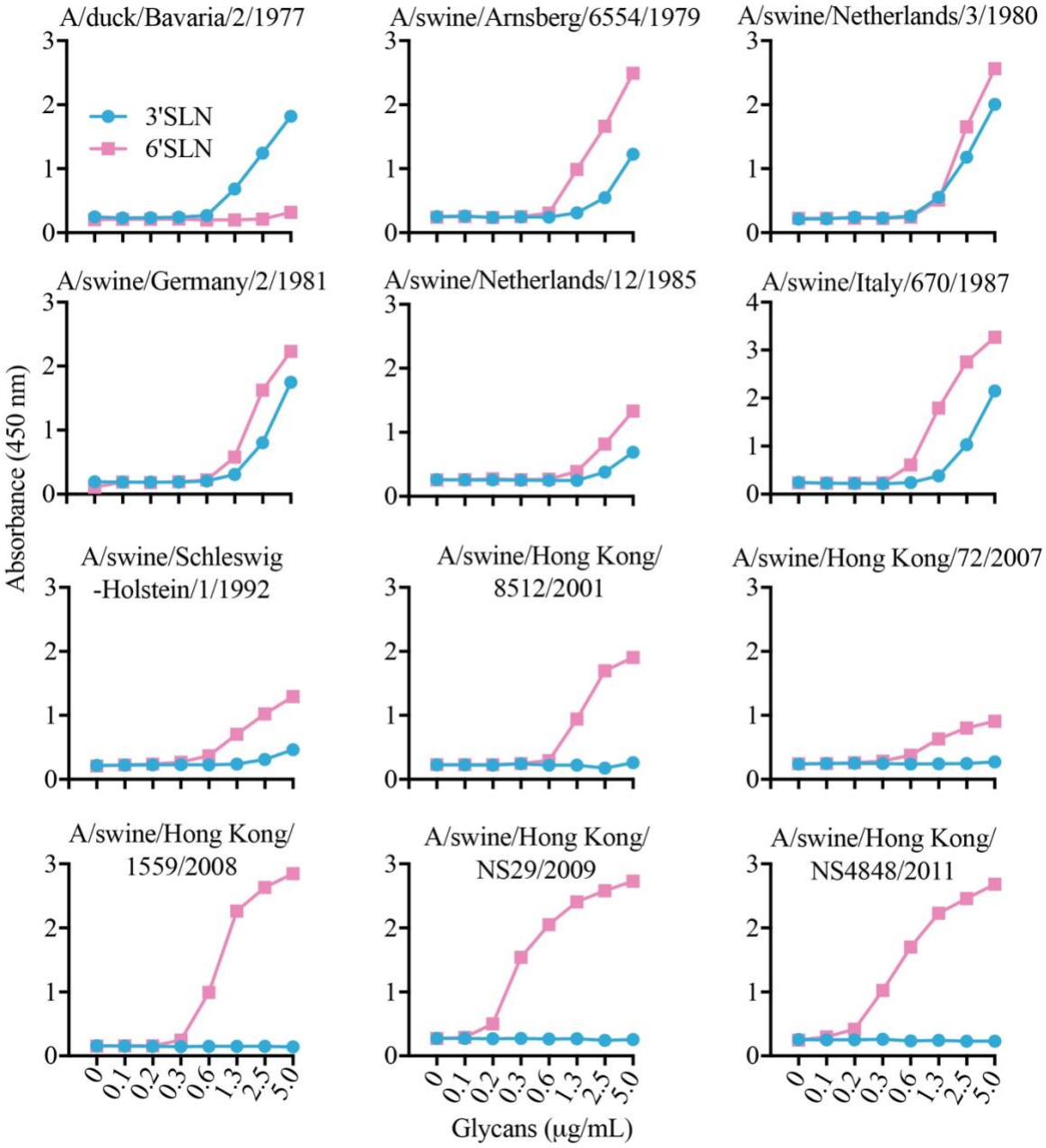
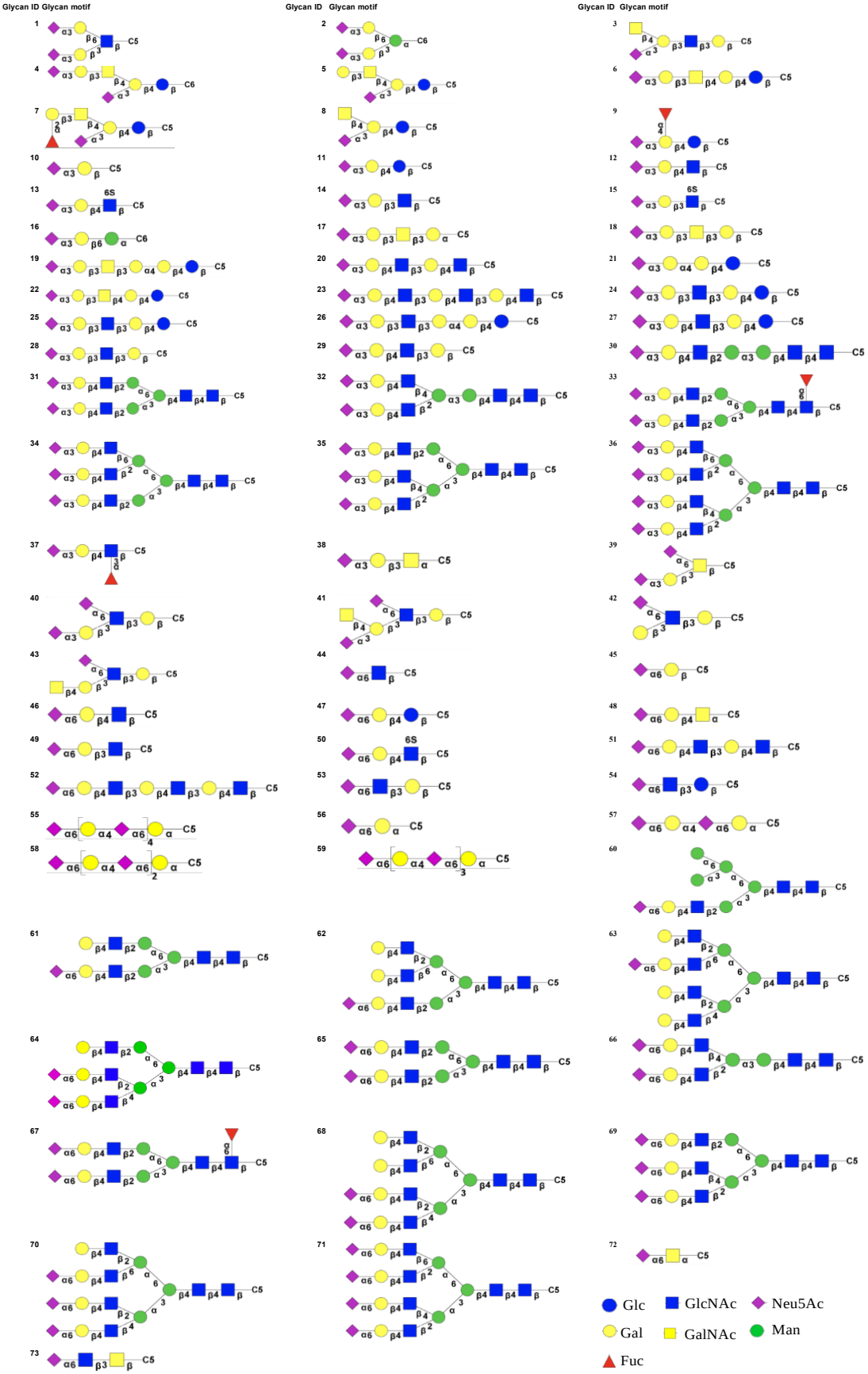
SUPPLEMENTARY INFORMATION

Supplementary Fig. 1. Receptor binding profile of EA swine influenza A viruses. An ELISA-based assay was employed to investigate receptor binding preference of H1N1 influenza viruses. 3'SLN (shown in cyan circles) and 6'SLN (shown in pink squares) were coated in 96 well plates at concentrations of 5 to 0.1 µg/mL. Infectious viruses (diluted to 64 HAU/50µL) were added to each well and co-incubated with 3'SLN and 6'SLN. The plates were washed with PBS and the detection of bound virus was done using a rabbit polyclonal antibody against HA from A/Duck/NZL/160/1976(H1N3) (Sino Biological Inc., Beijing, China) followed by polyclonal Goat Anti-Rabbit Immunoglobulins/HRP (Dako, Denmark). The absorbance at 450 nm was plotted against glycan concentration. Results are shown with the mean and SD absorbance from 3 replicates in one of two independently performed experiment.



Supplementary Fig. 2 Maximum likelihood phylogenies of PB2 (N = 459), PB1 (N = 432), PA (N = 402), HA (N = 415), NP (N = 422), NA (N = 448), M1 (N = 500), M2 (N = 500), NS1 (N = 405) and NS2 (N = 405) genes. Sequences of avian and EA swine influenza viruses were retrieved from NCBI. The phylogenetic trees were generated by RAxML (Version 8) and were rooted to the sequence of the oldest strain. Avian influenza viruses are shown in black; swine influenza viruses are shown in blue. The deduced nodal sequences for generating four recombinant viruses are shown in red. Representative wild-type EA swine influenza viruses used in the study are shown in pink. Enriched amino acid substitutions (>80% frequency) among EA swine influenza viruses were mapped using the treesub programme and are labelled at the nodes of the trees. H1N1 numbering was used for HA and NA trees.

Supplementary Figure 3. Structure of glycans used for glycan array assay.



Supplementary Table 1 Passage history and sequence information of H1N1 subtype avian influenza virus and Eurasian avian-like influenza viruses tested in this study.

Virus Name	Passage history[a]	HAU/50μL	Store virus titer (pfu/mL)	pH fusion [b]	GISAID accession numbers
A/duck/Bavaria/2/1977	Ex/C2	256	1.66E+06	5.4	EPI_ISL_539860
A/swine/Arnsberg/6554/1979	Ex/C2	256	3.60E+07	5.5	EPI_ISL_539869
A/swine/Netherlands/3/1980	Ex/C2	128	1.00E+06	ND	EPI_ISL_539861
A/swine/Germany/2/1981	Ex/C2	512	1.27E+07	5.6	EPI_ISL_539862
A/swine/Netherlands/12/1985	Ex/C2	256	4.77E+05	ND	EPI_ISL_539863
A/swine/Italy/670/1987	Ex/E1	1,024	ND	ND	EPI_ISL_68983
A/swine/Schleswig-Holstein/1/1992	Xx/C2	32	4.80E+05	5.8	EPI_ISL_539864
A/swine/Hong Kong/8512/2001	C3/C2	32	1.93E+05	ND	EPI_ISL_539865
A/swine/Hong Kong/72/2007	E2/C2	256	8.57E+07	5.2	EPI_ISL_539866
A/swine/Hong Kong/1559/2008	C2/C2	64	3.53E+04	ND	EPI_ISL_539867
A/swine/Hong Kong/NS29/2009	C2/C2	256	7.93E+07	5.7	EPI_ISL_29774
A/swine/Hong Kong/NS4848/2011	C2/C2	256	9.20E+07	ND	EPI_ISL_539868

[a] Passage histories in eggs (E) and MDCK cells (C) are shown. Ex denotes unknown passage history in eggs, Xx indicates not passaged in eggs.

[b] Syncytium formation assays were performed on Vero cells infected with each virus at a MOI of 10 plaque forming units (pfu)/ cell. The pH of activation was recorded as the highest pH value at which syncytia were observed.

ND, not determined.

Supplementary Table 2 Recombinant EA swine H1N1 viruses resurrected using ancestral sequence reconstruction.

Virus Name	Passage history[a]	Store virus titer (pfu/mL)	pH fusion [b]	NA activity(Mean ± SD)[c]		GISAID accession numbers
				Vmax (μM/min)	Km(μM)	
RG-EA1	C2	1.3E+07	5.9	0.9±0.07	16.48±3.93	EPI_ISL_539852
RG-EA2	C2	2.2E+07	5.8	0.09±0.01	7.38±1.21	EPI_ISL_539853
RG-EA3	C2	1.4E+07	5.9	0.14±0.02	16.19±2.04	EPI_ISL_539854
RG-EA4	C2	5.9E+07	5.8	0.16±0.002	17.24±4.7	EPI_ISL_539855

[a] Recombinant viruses were passaged at a MOI of 0.001-0.005 in MDCK cells after rescued from 293T cells.

[b] Syncytium formation assays were performed on Vero cells infected with each virus at a MOI of 10. The pH of activation was recorded as the highest pH value at which syncytia were observed.

[c] NA kinetics analysis were performed in flat-bottom 96-well opaque black plates where the 10^5pfu of each virus was incubated with 2′-(4-Methylumbelliferyl)-α-D-*N*-acetylneuraminic acid (MUNANA) substrate at 6.59 – 2,000 μM . Fluorescence was monitored every 45 seconds for 60 min at 37 °C. Enzyme kinetics data were fitted to the Michaelis–Menten equation by using nonlinear regression to determine the Michaelis constant and maximum velocity (V_{max}) of substrate conversion.

Supplementary Table 3 Substitutions presented in the nasal swabs collected from contact pigs exposed to RG-EA2$^{NP\text{-}R351K}$ or RG-EA2$^{PB1\text{-}Q621K, NP\text{-}R351K}$ ∗.

Virus	Cubicle	Segment	Nucleotide position	Reference	Mutation	Amino acid change	Frequency (%) in different contact pigs	
RG-EA2 $^{NP\text{-}R351K}$	Group 1	PA	1211	A	T	D396V	**52.2**	<
		HA1	508	G	A	R142H	**99.3**	**99.8**
		NA	880	A	G	E287G	57.3	<
	Group 2	PB2	1006	G	A	G327S	<	12.5
			1142	C	A	A372D	7.6	10.7
			1771	T	G	S582A	18.8	<
		HA1	507	C	T	R142C	7.2	11.4
RG-EA2 $^{PB1\text{-}Q621R, NP\text{-}R351K}$	Group 1	PB1	622	G	A	V200I	<	**64.2**
		NP	70	T	C	S9P	**99.9**	17.8
	Group 2	PB2	209	A	G	K61R	**50.3**	<
			1142	C	A	A372D	8.7	15.6
		PA	1665	C	A	D547E	<	11.1
		NP	58	G	A	G5S	9.2	<

* The nasal swabs with peak viral titers detected from contact pigs were RT-PCR amplified and analysed by iseq100 and CLC Main Workbench. The read depth for the full genome was > 1,000 and variants detected at a frequency of 5% or more are listed in the table. H1N1 was used for HA and NA numbering.

<, No variant was detected at frequency greater than 3% variant calling threshold.

Supplementary Table 4. Amino acid changes in recombinant viruses serially propagated three times in chicken embryonated eggs*.

Strains	Segment	Egg replicate 1		Egg replicate 2		Egg replicate 3	
		Amino acid change	Frequency (%)	Amino acid change	Frequency (%)	Amino acid change	Frequency (%)
RG-Be02-lung^{SG} x EA2^{IG}	PB2	V686M	3.2	<		<	
	PB1	<		<		<	
	PA	<		<		<	
	HA1	Q51K	15.9	Q51K	14.0	Q51K	14.1
		<		<		C42F	12.7
		<		R155G	61.0	R155G	19.1
	HA2	<		N28H	7.7	N28H	5.4
		<		<		H111P	5.2
	NP	<		M440I	5.8	M189I	6.4
	NA	<		L24I	10.8	<	
	M1	<		<		<	
	M2	<		<		<	
	NS1	<		<		<	
	NS2	<		<		<	
RG-EA1	PB2	<		E72V	3.7	<	
	PB1	<		<		<	
	PA	Q180H	49.2	T32A	3.0	T32A	3.4
	HA1	L105M	6.1	L105M	19.0	K153E	52.0
		K153E	62.7	F111L	3.6	T267M	51.1
		<		L312M	7.4	<	
	HA2	N28H	5.0	N28H	6.8	N28H	5.2
		G67S	3.2	D37N	4.3	V52M	4.2
		N79K	60.3	A44S	3.1	<	
		<		G67S	7.1	<	
	NP	<		<		<	
	NA	N28S	4.5	N28S	5.0	N28S	4.9
	M1	<		<		<	
	M2	<		<		<	
	NS1	<		<		<	
	NS2	<		<		E36G	42.4
RG-EA2	PB2	<		<		<	
	PB1	<		<		D70A	7.4
	PA	G631C	3.7	<		H452R	3.0
	HA1	Q51K	3.4	Q51K	6.1	Q51K	3.8
		<		R142H	4.8	<	
	HA2	N28H	5.6	N28H	5.9	N28H	6.5
	NP	<		<		<	
	NA	N28S	5.3	N28S	4.8	N28S	5.4
	M1	<		<		<	
	M2	<		<		<	

	NS1		<		<		<
	NS2		<		<		<
RG-EA3	PB2		<		<		<
	PB1	S732T	3.2		<		<
	PA	C489S	16.0	C489S	16.3	C489S	16.1
	HA1	R142L	3.7		<		<
	NP	I377S	6.0		<		<
	NA		<		<		<
	M1		<		<		<
	M2		<		<		<
	NS1		<		<		<
	NS2		<		<		<
RG-EA4	PB2	R355S	3.9		<		<
	PB1	Q202K	22.2	Q202K	8.3	D464N	3.9
		D619G	3.7		<		<
	PA	C489S	34.2	C489S	46.9	C489S	36.0
	HA1	N185D	26.1	R142L	80.2	R142L	10.4
	HA2		<		<		<
	NP	D375G	67.8	D375G	64.4	R31T	3.2
	NA	H126Q	5.8		<		<
	M1	F251S	5.5	F251S	9.5	F251S	6.6
		K252R	11.5	K252R	19.9	K252R	14.2
	M2	S23G	11.5	S23G	19.9	S23G	14.2
	NS1	S195Y	4.6		<	R37C	13.1
	NS2		<		<		<

* Each virus was serially passaged three times in 9-10-day old embryonated chicken eggs at 10^4 pfu in 0.1 mL in three replicates. The passage 3 sequences were determined by NGS and compared to that determined from the transfection supernatant. Mutational frequency detected above 3% were shown, <, below 3% cut-off of mutational frequency.

Supplementary Table 5. Amino acid changes in recombinant viruses serially propagated three times in MDCK cells*.

Strains	Segment	MDCK replicate 1		MDCK replicate 2		MDCK replicate 3	
		Amino acid change	Frequency (%)	Amino acid change	Frequency (%)	Amino acid change	Frequency (%)
RG-Be02-lung^{SG} x EA2^{IG}	PB2	N148S	71.3	<		<	
		M575V	3.6	<		<	
	PB1	I674S	10.9	<		I18L	3.3
	PA	<		S49P	7.9	<	
		<		A660G	16.0	<	
	HA1	N156D	96.0	N156D	96.6	N156D	96.8
	HA2	N28H	4.0	N28H	3.8	N28H	4.6
	NP	<		<		<	
	NA	T48R	5.0	<		<	
		F74S	7.0	<		<	
	M1	<		<		<	
	M2	<		<		<	
	NS1	<		<		<	
	NS2	<		<		<	
RG-EA1	PB2	L10P	3.2			K663R	16.8
	PB1	I667T	4.9	V709I	4.3	C87Y	6.4
		<		<		R190S	5.5
		<		<		R192K	5.6
		<		<		D193N	5.9
		<		<		V200I	12.3
		<		<		N532K	3.1
		<		<		N533K	3.4
	PA	N96H	5.6	<		S93N	3.3
	HA1	<		<		I79K	5.4
		<		<		T299A	3.0
		L312M	6.4	L312M	33.5	L312M	10.8
		L318P	26.4	<		<	
		V321D	31.4	<		<	
	HA2	<		I6V	3.3	<	
		N28H	5.3	N28H	4.7	N28H	5.1
		A96V	12.5	I56V	6.4	A44T	12.7
		<		G67D	22.4	G67D	12.5
		N114K	5.5	<		D85N	15.2
		E97K	16.8	E97K	17.0	E97K	18.7
		R116G	4.0	T107A	14.9	R116G	14.9
	NP	<		<		<	
	NA	<		<		<	
	M1	<		<		<	
	M2	<		<		<	

	NS1	F14L	15.1		<		F14L	6.5
	NS2		<		<		<	
RG-EA2	PB2		<	S688Y	10.9		<	
			<	N701I	3.5		<	
			<	R703K	3.4		<	
	PB1	Q582K	11.2	Q582K	4.3	I181T	3.8	
			<		<	Q582K	3.2	
			<		<	I674T	3.8	
			<		<	P100H	4.0	
	PA		<	T639I	12.9	V5M	3.1	
			<		<	S65T	36.1	
	HA1	R142H	7.1	S143I	3.1	R142S	4.5	
			<		<	R142H	3.3	
			<		<	S143I	6.3	
	HA2	N28H	28.5	N28H	21.4	N28H	25.4	
		V100F	3.2		<		<	
	NP		<		<		<	
	NA		<		<		<	
	M1		<		<		<	
	M2		<		<		<	
	NS1		<		<		<	
	NS2		<		<		<	
RG-EA3	PB2		<		<		<	
	PB1		<		<		<	
	PA	L42Q	4.6	C489S	7.6	H74L	14.2	
		E56K	11.4	E684K	8.3	C489S	6.2	
		R75K	30.4		<		<	
		C489S	9.1		<		<	
	HA1		<		<	W60R	7.0	
	HA2		<		<		<	
	NP		<		<		<	
	NA		<		<	V75A	4.9	
			<		<	A76S	4.6	
	M1		<		<		<	
	M2		<		<		<	
	NS1		<		<		<	
	NS2		<		<		<	
RG-EA4	PB2	A624D	4.4		<	L298I	13.6	
			<		<	L665I	16.0	
	PB1	I18K	3.9	V88G	9.0		<	
		M655R	3.8		<		<	
		R707K	3.4		<		<	
	PA	C489S	24.1	C489S	22.8	C489S	25.3	
		M607K	3.5		<		<	
	HA1	K160N	98.4	N125D	12.4	R142H	4.5	

		<		K160N	85.8	K154E	33.4
		<			<	K160N	61.3
	HA2	<			<		<	
	NP	<			<		<	
	NA	<			<		<	
	M1	F251S	3.7	F251S	4.1	F251S	3.6
		K252R	8.5	K252R	9.5	K252R	8.2
	M2	S23G	8.5	S23G	9.5	S23G	8.2
	NS1	<			<		<	
	NS2	<			<		<	

* Each virus was serially passaged three times in MDCK cells at a MOI of 0.001-0.005 in three replicates. The passage 3 sequences were determined by NGS and compared to that determined from the transfection supernatant. Mutational frequency detected above 3% were shown, <, below 3% cut-off of mutational frequency.

Supplementary Table 6. Amino acid changes in recombinant viruses serially propagated three times in NPTr cells*.

Strains	Segment	NPTr replicate 1		NPTr replicate 2		NPTr replicate 3	
		Amino acid change	Frequency (%)	Amino acid change	Frequency (%)	Amino acid change	Frequency (%)
RG-Be02-lung^{SG} x EA2^{IG}	PB2	<		<		<	
	PB1	<		<		<	
	PA	C489S	5.6	F53V	68.3	W577R	15.1
		<		C489S	4.3	C489S	5.8
	HA1	N125D	90.6	N125D	98.6	N125D	96.7
		R155G	7.5	<		<	
	HA2	<		N28H	2.4	<	
	NP	<		<		<	
	NA	<		<		<	
	M1	<		<		<	
	M2	<		<		<	
	NS1	<		V230A	68.3	<	
	NS2	<		F73L	68.3	<	
RG-EA1	PB2	V686G	12.8			<	
	PB1	<		G71E	57.5	<	
		<		L282V	17.9	<	
		<		S654I	16.9	<	
	PA	G684R	14.9	<		<	
	HA1	S105G	15.3	S105G	14.8	S105G	13.8
		<		S263T	3.8	S263T	4.5
		<		<		S263F	3.4
	HA2	N28H	11.2	N28H	15.4	N28H	12.4
		I48L	39.9	I48L	47.1	I48L	38.5
		I48T	6.9	I48T	5.4	I48T	7.2
		K51I	5.1	<		K51I	4.2
		D85N	4.2	D85N	6.1	D85N	3.8
		D90N	6.4	D90N	3.9	D90N	4.5
		A96T	3.5	A96T	3.1	<	
		D109Y	9.8	D109Y	7.8	D109Y	7.9
		<		<		S113Y	6.4
		N114Y	7.7	N114Y	7.0	N114Y	5.4
	NP	<		Y10H	3.5	Y10H	4.1
	NA	<		<		G336D	4.9
	M1	<		<		<	
	M2	<		<		<	
	NS1	<		<		<	
	NS2	<		<		<	
RG-EA2	PB2	<		R62T	11.3	<	

	PB1		<		<			<
	PA		<		<		E31V	4.2
		C489S	6.2	C489S	6.2		C489S	8.0
		A651T	3.8		<			<
	HA1	A216D	5.2		<			<
	HA2	N28H	26.4	N28H	28.7		N28H	30.5
			<	Q174E	15.1			<
	NP		<		<			<
	NA		<		<			<
	M1		<		<			<
	M2		<		<			<
	NS1		<		<			<
	NS2		<		<			<
RG-EA3	PB2		<	I67T	24.4		M756R	17.5
	PB1	S732T	4.7		<			<
		R754G	3.5		<		S732T	4.3
	PA	C489S	14.1	C489S	16.1		L187I	3.7
			<		<		C489S	16.4
	HA1	N125D	15.4		<		G155E	6.8
		R142L	5.2	R142L	24.5		R142L	22.9
	HA2		<		<			<
	NP		<		<			<
	NA		<		<		S110F	3.2
			<		<		N200S	7.5
			<		<		N146K	7.6
	M1		<		<			<
	M2		<		<			<
	NS1		<		<			<
	NS2		<		<			<
RG-EA4	PB2		<	R389K	11.4		E472G	4.4
	PB1		<		<			<
	PA	C489S	28.9	V636A	10.7		C489S	27.8
			<	C489S	30.6			<
	HA1		<		<			<
	HA2	Y94H	25.9		<			<
	NP		<	R422G	8.4			<
	NA		<	S31T	13.3		I29T	8.1
			<	I34T	12.6		R331K	5.5
			<	I30N	13.0			<
	M1	F251S	4.0	F251S	3.8		F251S	4.5
		K252R	8.8	K252R	8.7		K252R	9.7
	M2	S23G	8.8	S23G	8.7		S23G	9.7
	NS1		<		<			<

NS2	<	<	<

* Each virus was serially passaged three times in NPTr cells at a MOI of 0.001-0.005 in three replicates. The passage 3 sequences were determined by NGS and compared to that determined from the transfection supernatant. Mutational frequency detected above 3% were shown, <, below 3% cut-off of mutational frequency.