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(54) **INFLUENZA VIRUS REPLICATION FOR VACCINE DEVELOPMENT**

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(58) **Field of Classification Search**

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See application file for complete search history.

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(57) **ABSTRACT**

The invention provides a composition useful to prepare high titer influenza viruses, e.g., in the absence of helper virus, which includes internal genes from an influenza virus vaccine strain or isolate, e.g., one that is safe in humans, for instance, one that does not result in significant disease, that confer enhanced growth in cells in culture, such as MDCK cells, or in eggs.

16 Claims, 53 Drawing Sheets

Specification includes a Sequence Listing.

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PR8(CAMBRIDGE)

PB2

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(SEQ ID NO:11)

Fig. 1A

PR8(CAMBRIDGE)

PB1

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(SEQ ID NO:10)

Fig. 1B

PR8(CAMBRIDGE)

PA

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(SEQ ID NO:12)

Fig. 1C

PR8(CAMBRIDGE)

NP

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GGAGACAATGCAGAGGAGTACGACAATTAAAGAAAAATACCCTTGTTTCTACT

(SEQ ID NO:13)

Fig. 1D

PR8(CAMBRIDGE)

M

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(SEQ ID NO:14)

NS

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(SEQ ID NO:15)

Fig. 1E

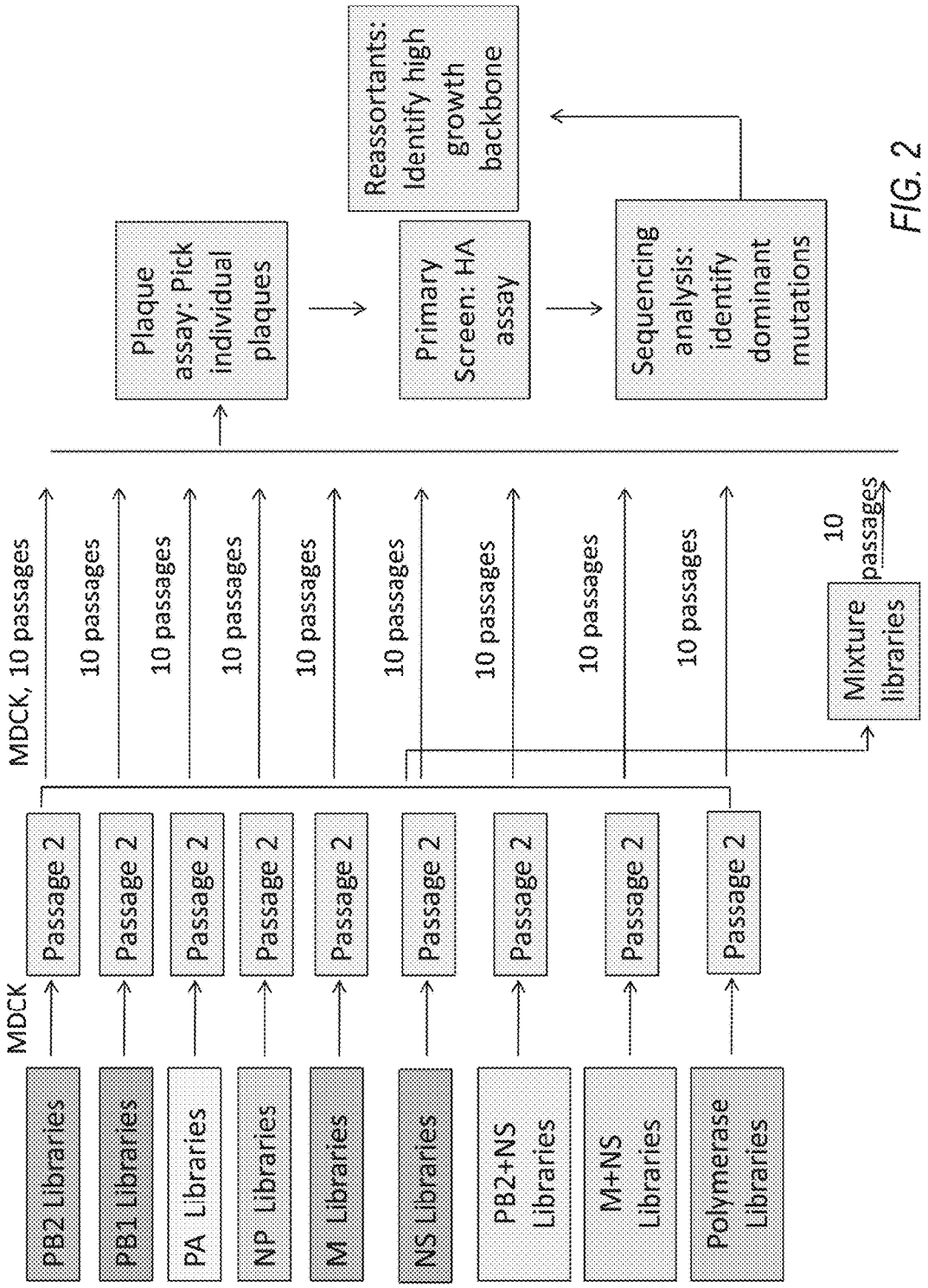

PB2 Libraries
PB1 Libraries
PA Libraries
NP Libraries
M Libraries
NS Libraries
PB2+NS Libraries
M+NS Libraries
Polymerase Libraries
MDCK
Passage 2
MDCK, 10 passages
10 passages
Mixture libraries
10 passages
Plaque assay: Pick individual plaques
Primary Screen: HA assay
Sequencing analysis: identify dominant mutations
Reassortants: Identify high growth backbone

FIG. 2

Figure 3 Summary of HA assay of 1434 individual clones

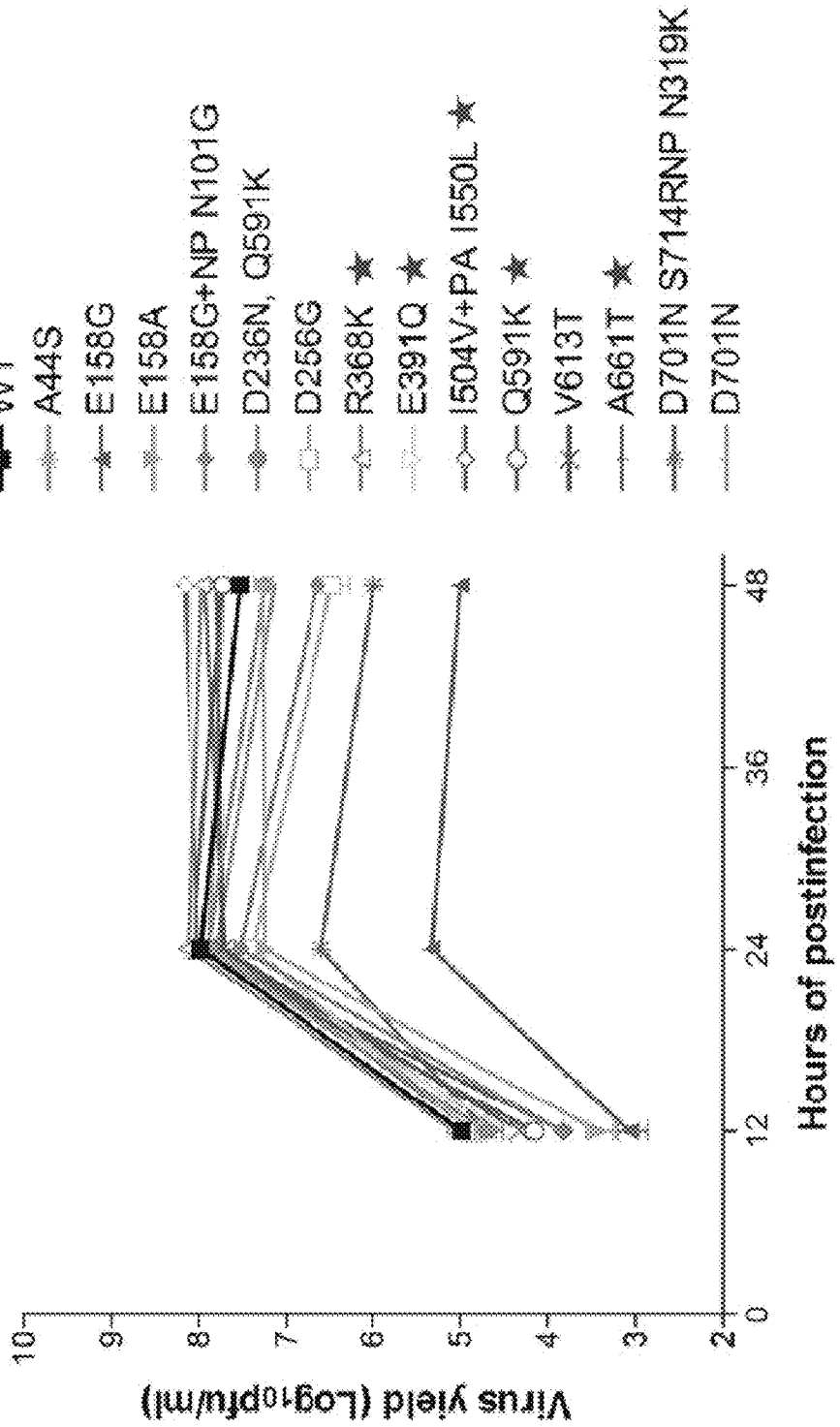
Groups	Numbers of clone	Fold change	%
WT HA titer = 2^{7}	-	-	-
HA titer =$2^{>9\text{-}9.5}$	8	>4	0.6%
HA titer =$2^{>8.5\text{-}9}$	23	>2.8 - 4	1.6%
HA titer =$2^{7\text{-}8.5}$	748	1 - 2.8	52.2%
HA titer < 2^{7}	655	<1	45.6%
Total	1434	-	100%

Figure 4 Recombinant viruses generated from dominant mutations

Viruses	Gene backbone								Virus stock titer	
	HA	NA	PB2	PB1	PA	NP	M	NS	2^n	Pfu/ml
WT	Indo/NC/09 delHA	Indo/NC/09 NA	PR8-wt	PR8-wt	PR8-wt	PR8-wt	PR8-wt	PR8-wt	7	3.0E+07
1			M202L F323L	M507V V644A		I116L		K55E	9~9.5	2.0E+08
2			M202L F323L	Q247H	R401K			T49A	9	1.0E+08
3			I504V	M507V V644A	I550L	R74K N417D		K55E	8~8.5	5.7E+07
4			I505V	E112G	I550L	R74K		S161T	9	1.6E+08
5			M202L F323L	E112G				S161T	8.5	1.3E+08
6			M66R	M40I G180W		R74K		S161T	8~8.5	2.3E+07

Figure 5A Growth curve – PB2 mutants

(MOI=0.001)



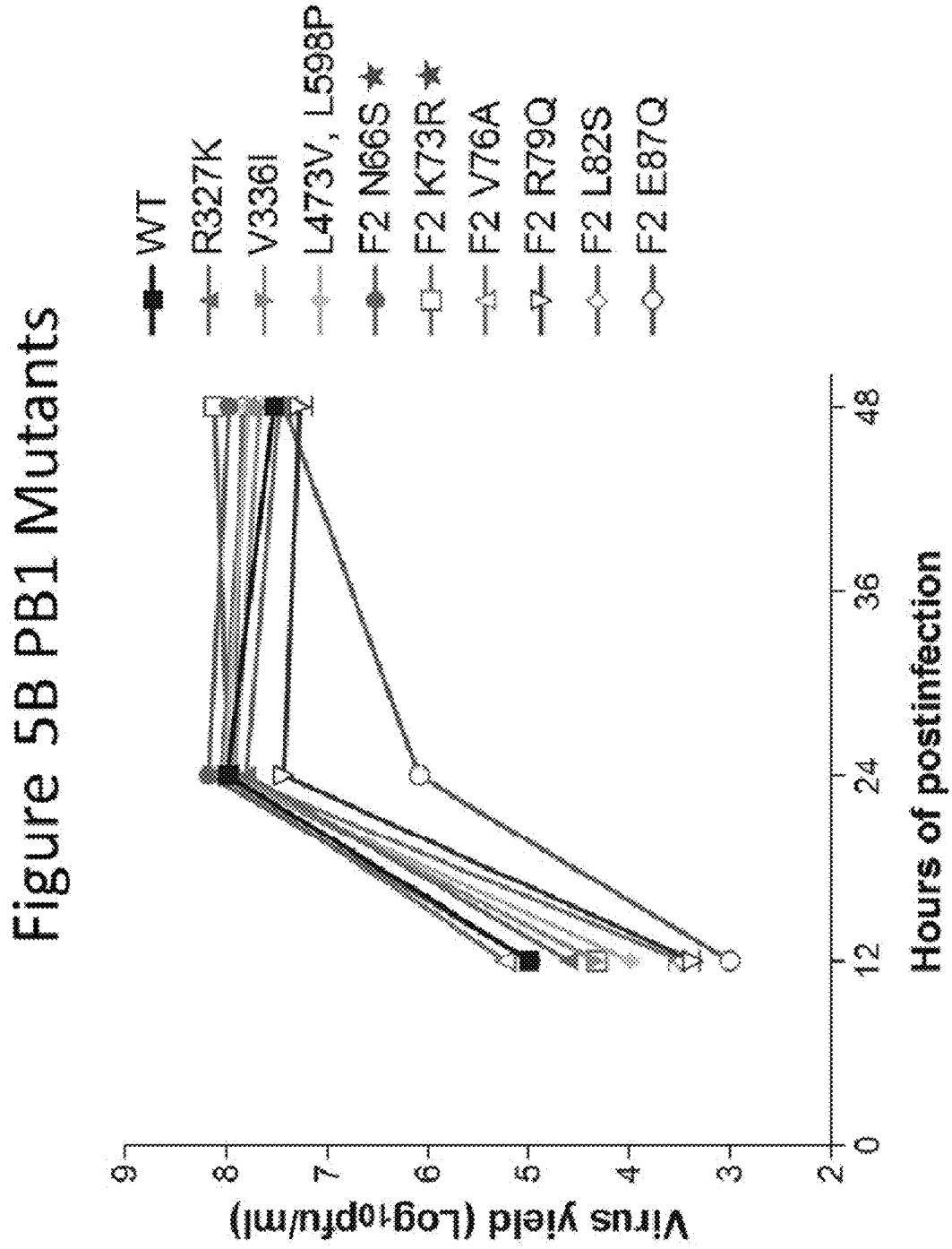
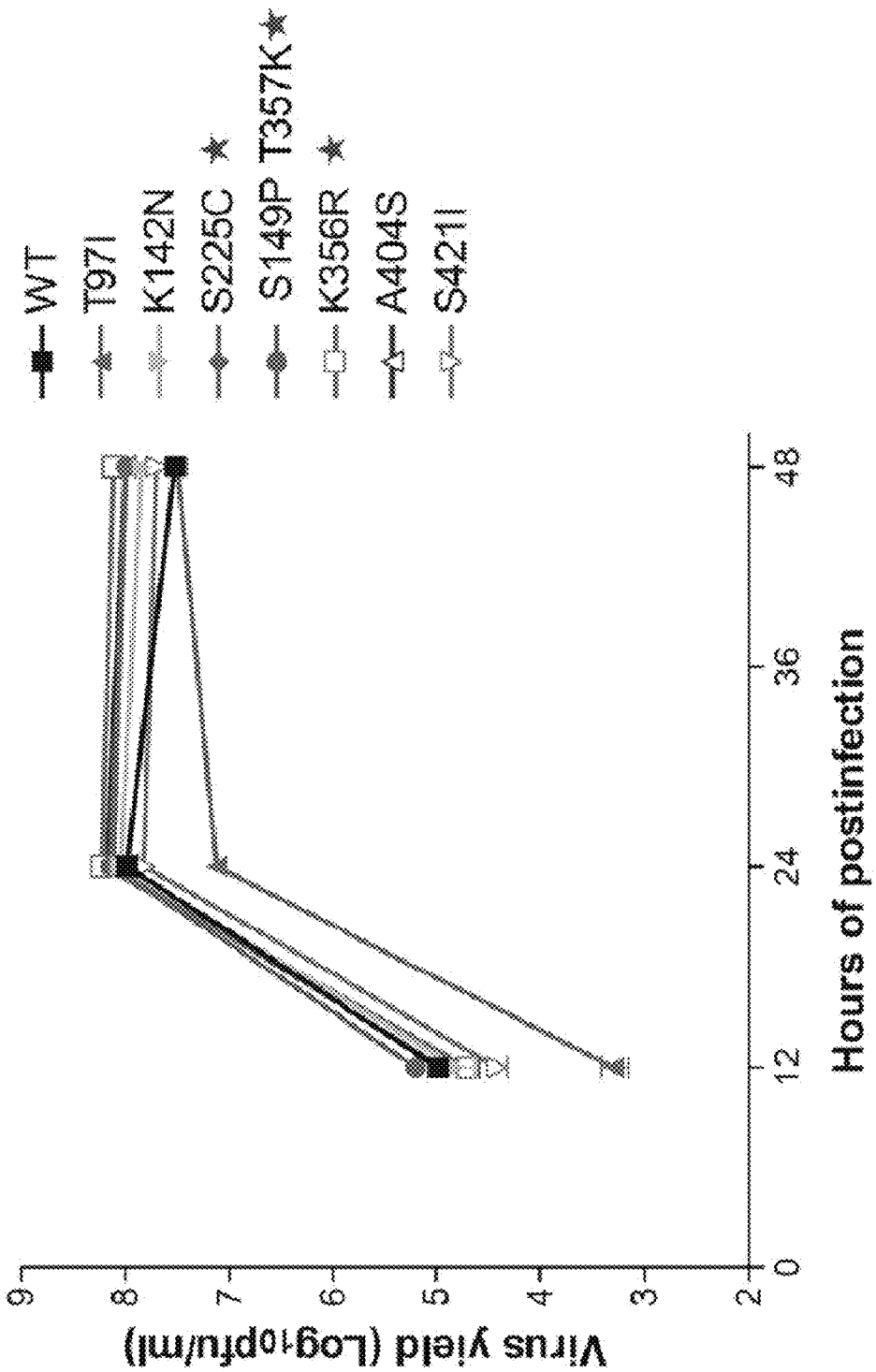


Figure 5B PB1 Mutants
WT
R327K
V336I
L473V, L598P
F2 N66S ★
F2 K73R ★
F2 V76A
F2 R79Q
F2 L82S
F2 E87Q
Virus yield (Log10pfu/ml)
Hours of postinfection
0
12
24
36
48
2
3
4
5
6
7
8
9

Figure 5C PA Mutants



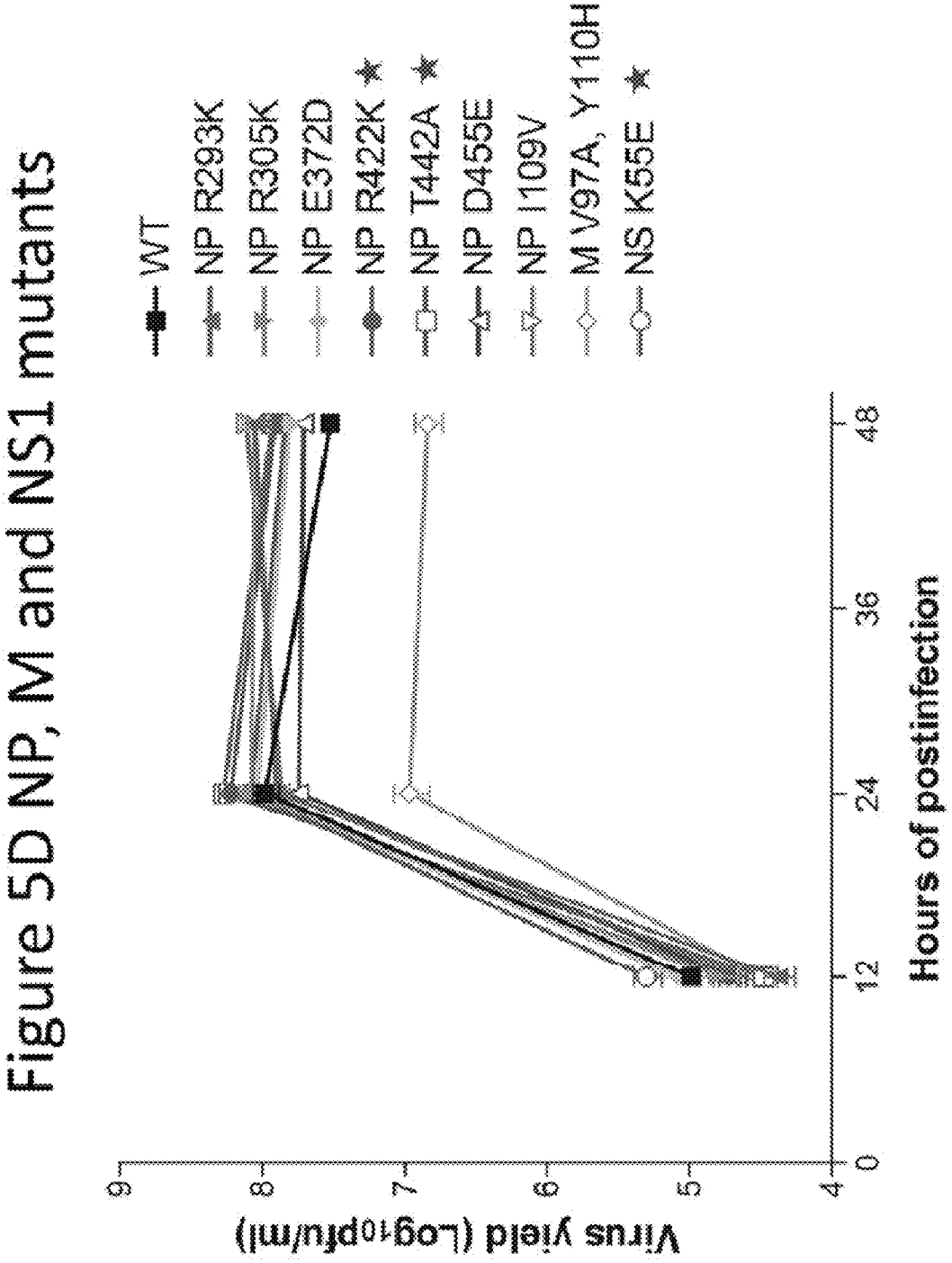


Figure 5D NP, M and NS1 mutants
WT
NP R293K
NP R305K
NP E372D
NP R422K ★
NP T442A ★
NP D455E
NP I109V
M V97A, Y110H
NS K55E ★
Virus yield (Log10pfu/ml)
9
8
7
6
5
4
0
12
24
36
48
Hours of postinfection

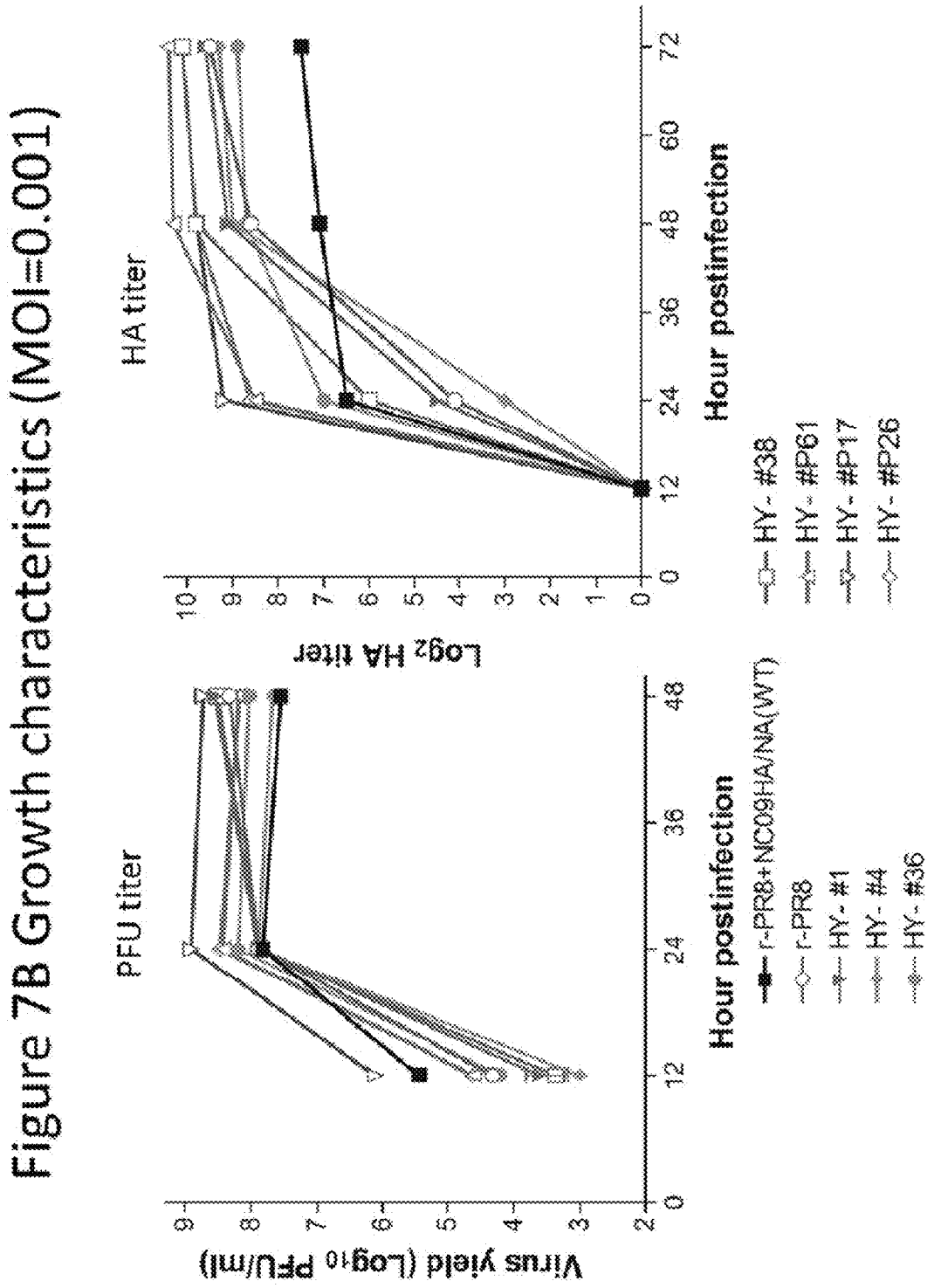
Figure 6 Confirmed high replicative mutations

Gene	Screened from viruses libraries	Described in literature
PB2	M202L F323L, I504V, M66R	A44S, E158G, E158A, D236N, D256G, R368K, E391Q, I504V, Q591K, V613T, A661T, D701N, D701N S714R
PB1	M507V V644A, V644A, R54I, Q247H, E112G, M40I G180W, I667T M714T	R327K, V336I, L473V L598P
PB1 F2	-	N66S, K73R, V76A, R79Q, L82S, E87Q
PA	F105C, R401K	T97I, K142N, S225C, S149PP T357K, K356R, A404S, S421I
NP	R293M, I116L, N224I, R74K, R74K N417D,	R293K, R305K, E372D, R422K, T442A, D455E, I109V, N101G, N319K
M	P90S	V97A, Y100H, V97A Y100H
NS	A30P, T49A, R140Q, S161T, A223E	K55E

Figure 7A Recombinant viruses generated by RGS

Virus #	Gene backbone								Virus stock titer	
	HA	NA	PB2	PB1	PA	NP	M	NS	2^n	Pfu/ml
wt	Inda/NC/09 delHA	Indo/NC/09 NA	wt	wt	wt	wt	wt	wt	7	3.0E+07
1			M202L F323L	M507V V644A		I116L		K55E	9~9.5	2.0E+08
4			M202L F323L	M507V V644A	K356R	T442A	V97A Y100H	K55E	10~10.5	1.6E+08
36			I504V	E112G	I550L	I112L	Y100H	R140Q	9.5	1.3E+08
38			M202L F323L	M507V V644A		I116L	Y100H	K55E	10~10.5	2.3E+08
HY-#17			I504V	E112G	S225C	R74K N417D	V97A Y100H	K55E	9.5~10	5.8E+08
HY-#61			M202L F323L	Q247H	K142N	R74K	V97A Y100H	K55E	10~10.5	2.0E+08
HY-#26			M202L F323L	M40L G180W	S225C	R422K	V97A Y100H	K55E	10	3.0E+08

Figure 7B Growth characteristics (MOI=0.001)



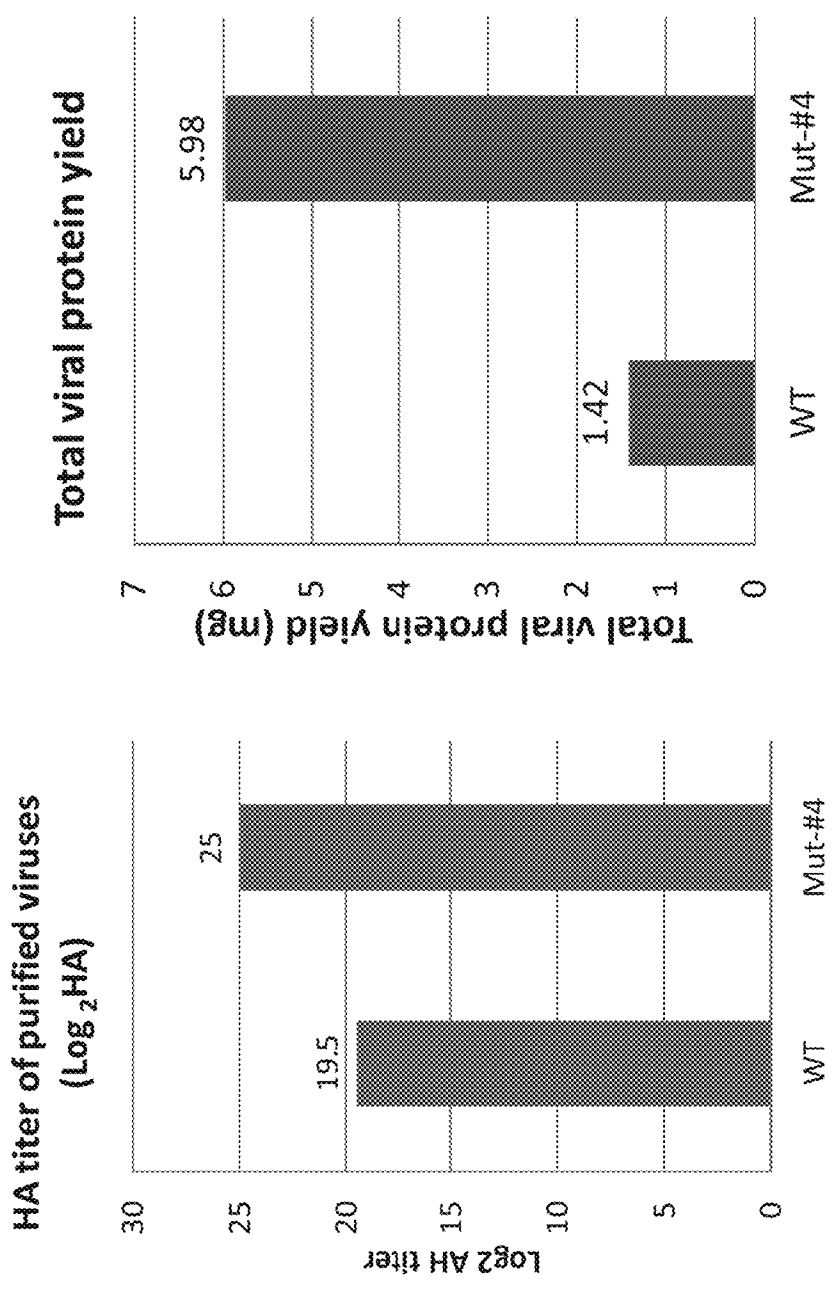
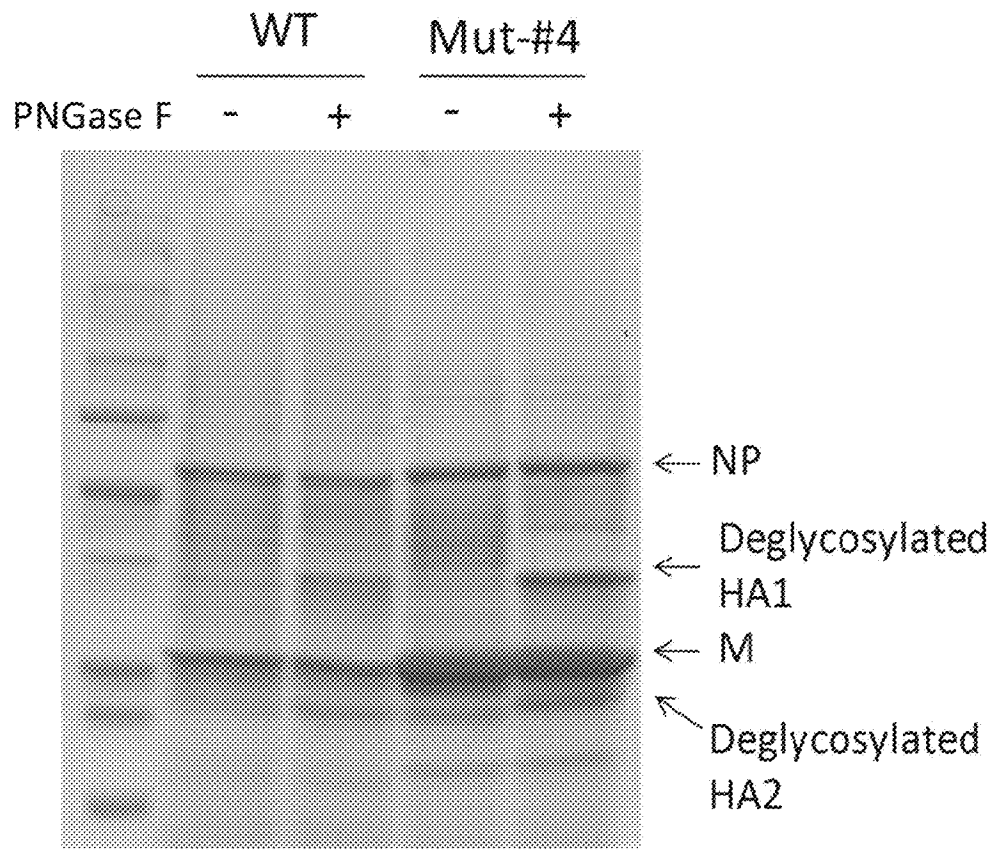


FIG. 8A Total viral protein yield: 4.2 fold FIG. 8B

Figure 8C SDS-PAGE analysis

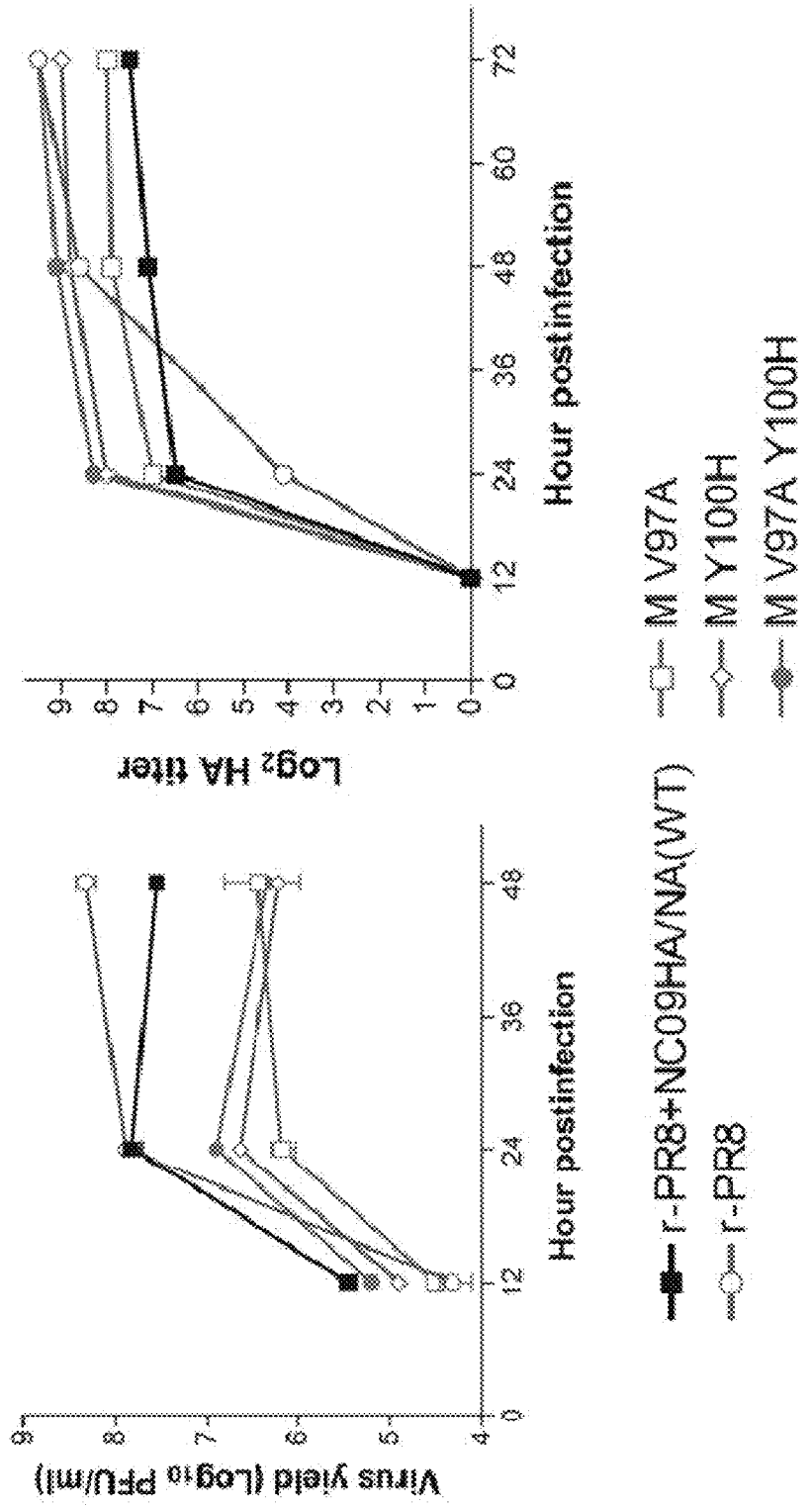


Mutant virus generates much more M1 and HA1 proteins than wild type.

Figure 9A Wild type VS. mutant

#	Gene backbone								Virus stock titer	
	HA	NA	PB2	PB1	PA	NP	M	NS	HA titer (2^n)	Pfu/ml
WT	Indo/NC/09 delHA	Indo/NC/09 NA	PR8-wt	PR8-wt	PR8-wt	PR8-wt	PR8-wt	PR8-wt	7	3.0E+07
4			M202L F323L	M507V V644A	K356R	T442A	V97A Y100H	K55E	10~10.5	1.6E+08

Figure 9B Growth kinetics (MOI=0.001)



CANIS FAMILIARIS [gbmam]: 1194 CDS's (559501 CODONS)

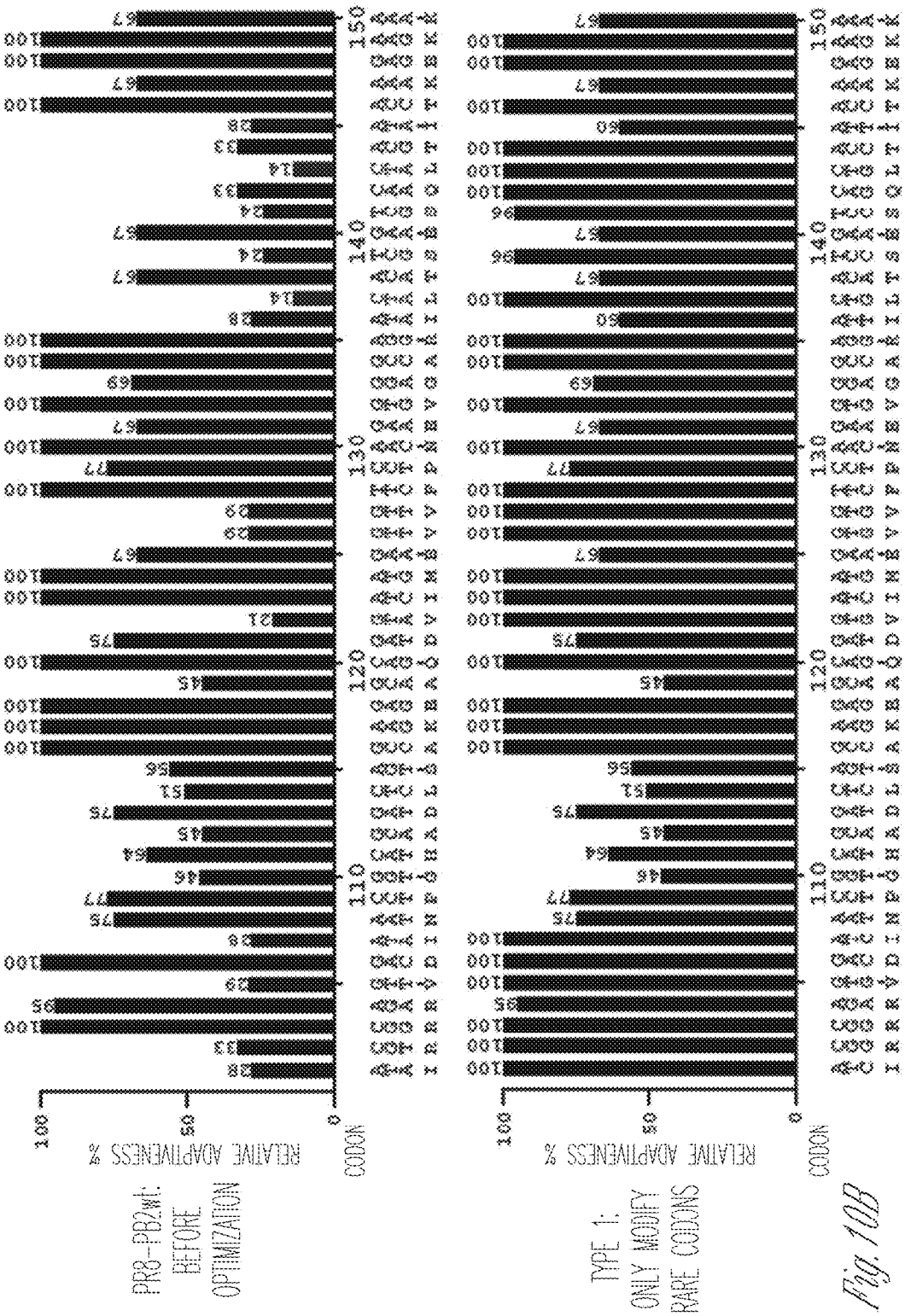
FIELDS: [TRIPLET] [AMINO ACID] [FRACTION] [FREQUENCY: PER THOUSAND] ([NUMBER])

UUU	F	0.41	17.1	(9540)	UCU	S	0.18	13.8	(7723)	UAU	Y	0.40	11.5	(6456)	UGU	C	0.42	10.1	(5665)
UUC	F	0.59	24.4	(13671)	UCC	S	0.24	18.4	(10299)	UAC	Y	0.60	17.5	(9786)	UGC	C	0.58	13.8	(7723)
UUA	L	0.06	5.8	(3270)	UCA	S	0.13	9.8	(5487)	UAA	*	0.27	0.6	(325)	UGA	*	0.53	1.1	(642)
UUG	L	0.12	11.8	(6627)	UCG	S	0.06	4.6	(2584)	UAG	*	0.21	0.5	(254)	UGG	W	1.00	13.8	(7704)
CUU	L	0.12	11.7	(6523)	CCU	P	0.27	15.6	(8713)	CAU	H	0.39	9.0	(5039)	CGU	R	0.07	3.9	(2163)
CUC	L	0.22	21.8	(12224)	CCC	P	0.35	20.4	(11422)	CAC	H	0.61	14.1	(7888)	CGC	R	0.20	10.6	(5943)
CUA	L	0.06	6.5	(3644)	CCA	P	0.25	14.6	(8157)	CAA	Q	0.25	11.0	(6149)	CGA	R	0.11	5.6	(3155)
CUG	L	0.43	42.8	(23966)	CCG	P	0.12	7.0	(3892)	CAG	Q	0.75	32.6	(18244)	CGG	R	0.21	11.0	(6132)
AUU	I	0.32	15.5	(8662)	ACU	T	0.22	12.3	(6886)	AAU	N	0.43	16.5	(9253)	AGU	S	0.14	10.8	(6029)
AUC	I	0.53	25.7	(14391)	ACC	T	0.39	21.4	(11979)	AAC	N	0.57	21.6	(12104)	AGC	S	0.25	18.9	(10595)
AUA	I	0.15	7.2	(4017)	ACA	T	0.26	14.2	(7972)	AAA	K	0.40	22.2	(12410)	AGA	R	0.20	10.5	(5847)
AUG	M	1.00	22.7	(12717)	ACG	T	0.13	7.2	(4005)	AAG	K	0.60	33.9	(18967)	AGG	R	0.21	11.1	(6228)
GUU	V	0.14	9.3	(5189)	GCU	A	0.25	17.2	(9609)	GAU	D	0.43	19.7	(11012)	GGU	G	0.16	11.3	(6298)
GUC	V	0.27	17.2	(9607)	GCC	A	0.44	30.3	(16927)	GAC	D	0.57	26.2	(14655)	GGC	G	0.35	24.2	(13513)
GUA	V	0.10	6.5	(3660)	GCA	A	0.20	13.7	(7651)	GAA	E	0.40	26.4	(14776)	GGA	G	0.24	16.9	(9465)
GUG	V	0.48	31.0	(17366)	GCG	A	0.11	7.9	(4431)	GAG	E	0.60	40.3	(22552)	GGG	G	0.25	17.4	(9718)

CODING GC 53.16% 1ST LETTER GC 55.35% 2ND LETTER GC 41.92% 3RD LETTER GC 62.22%

GENETIC CODE 1: STANDARD

Fig. 10A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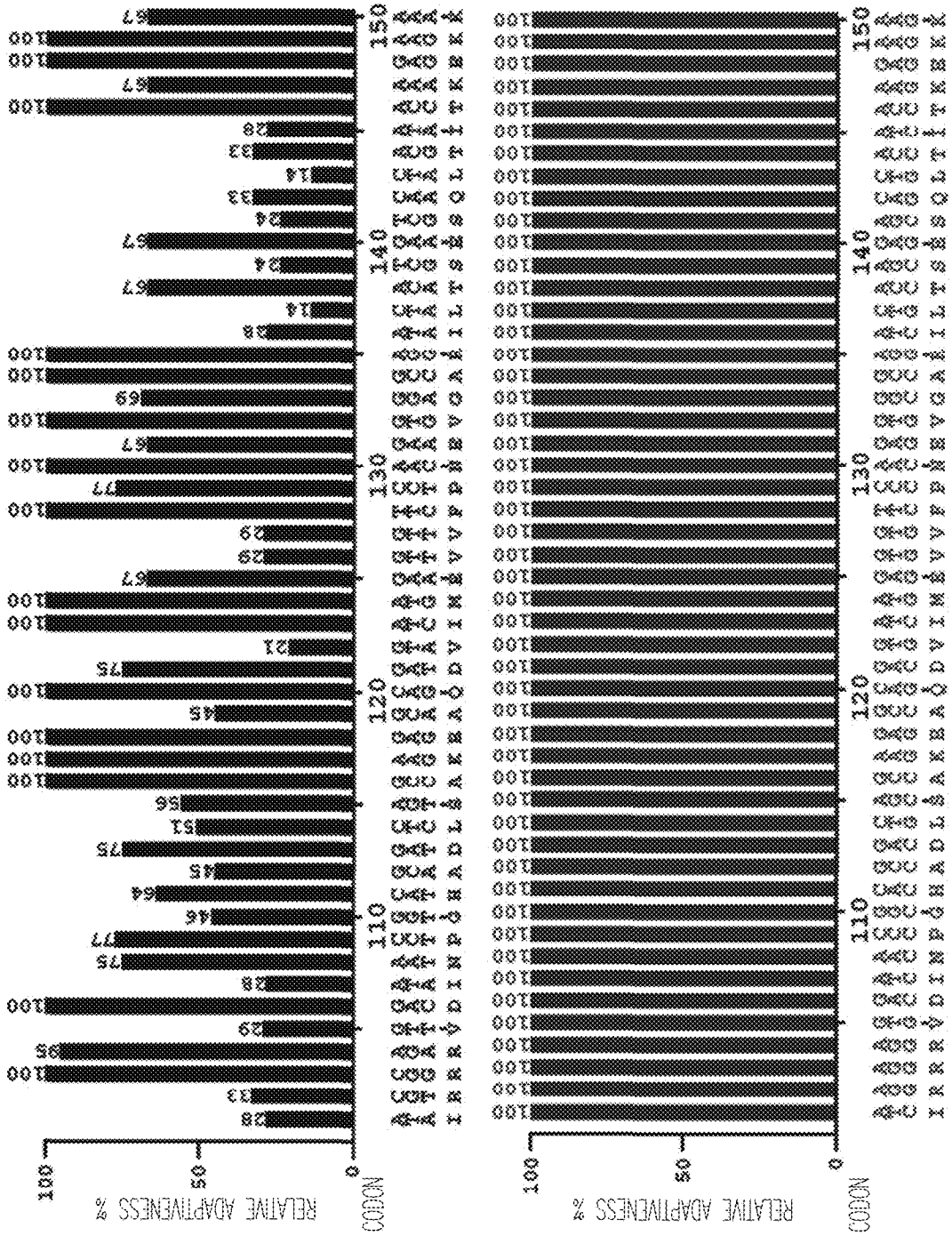
CODON

Fig. 10B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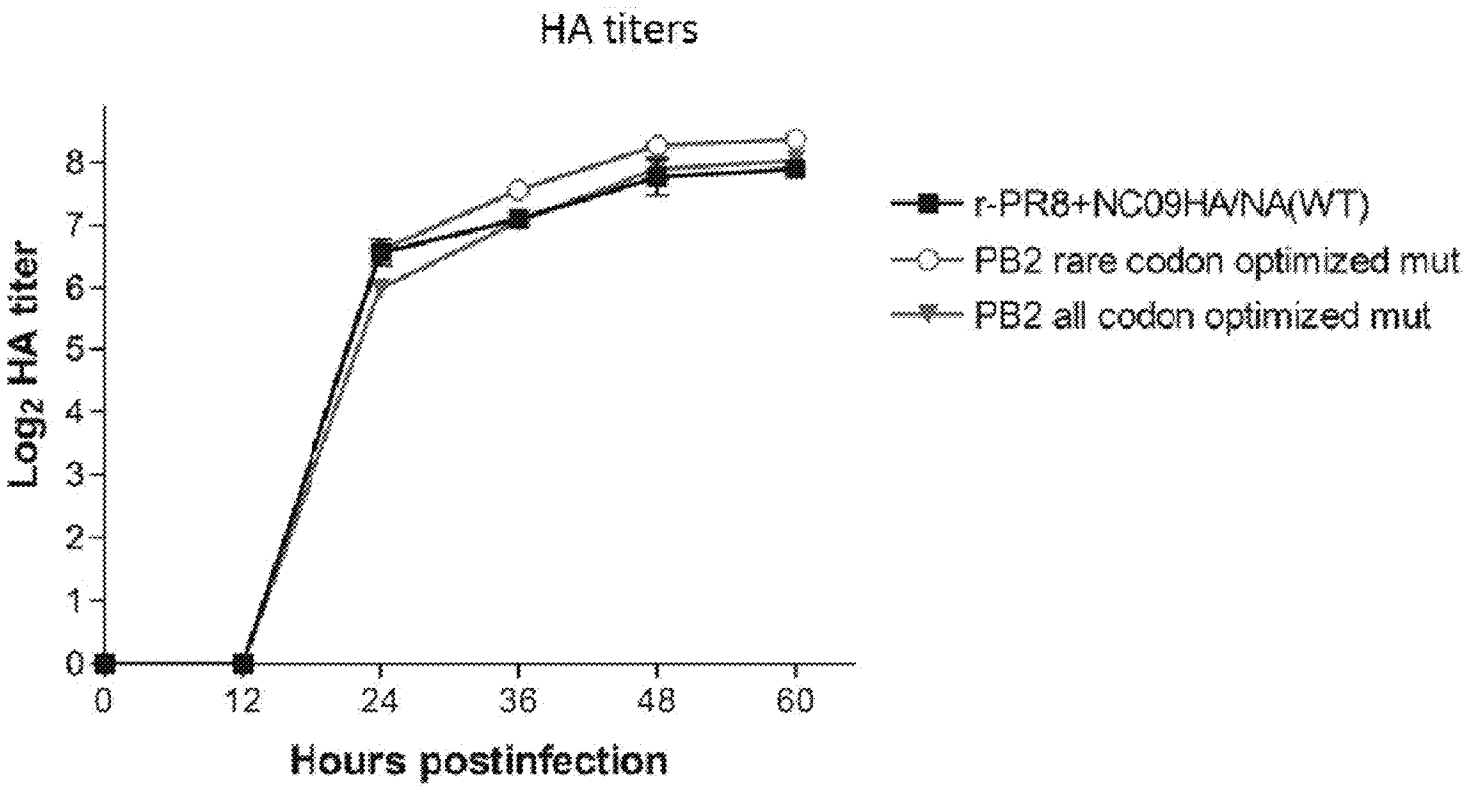
RELATIVE ADAPTIVENESS %
CODON

Fig. 10C

Figure 10D Growth kinetics in MDCK cells



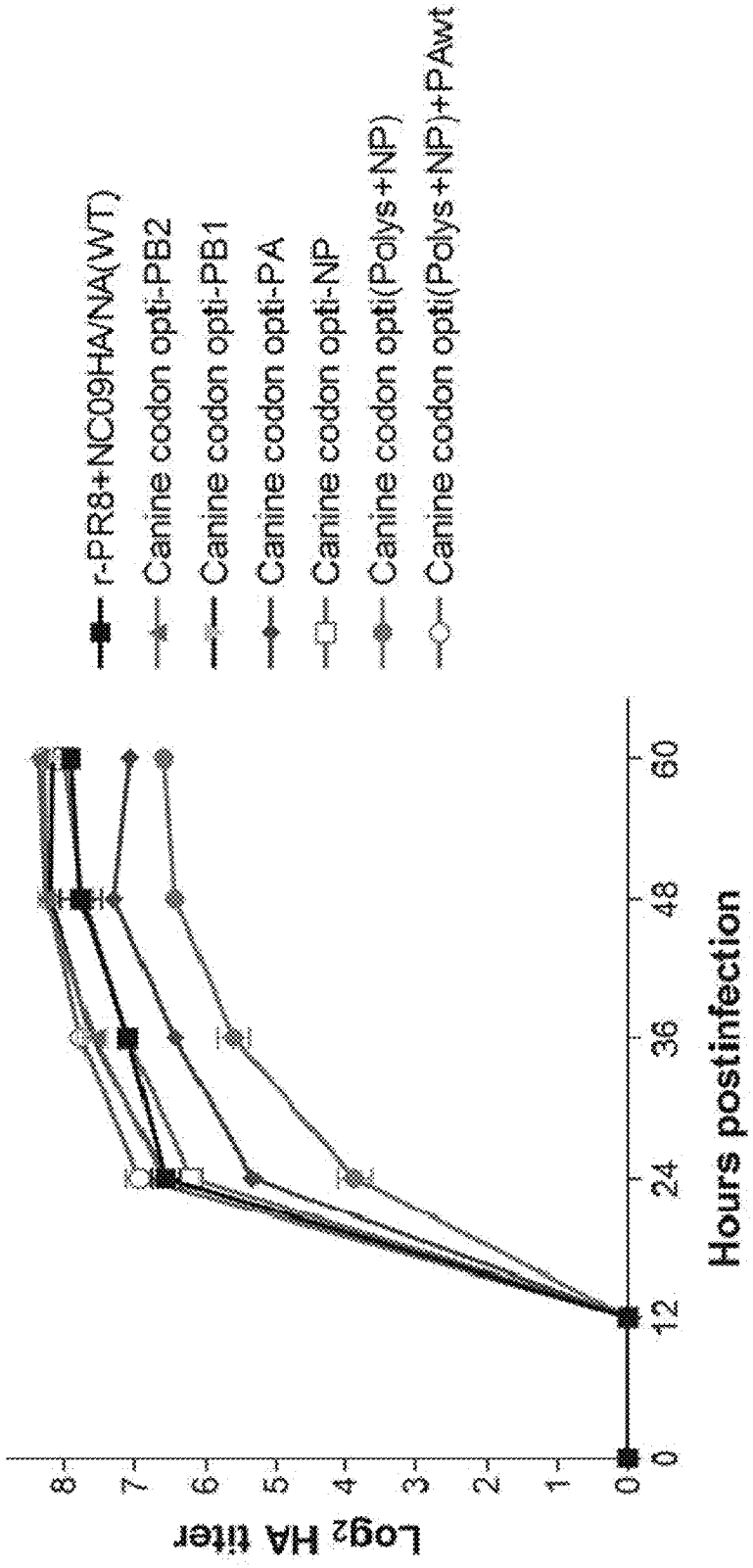

r-PR8+NC09HAVNA(WT)
Canine codon opti-PB2
Canine codon opti-PB1
Canine codon opti-PA
Canine codon opti-NP
Canine codon opti(Polys+NP)
Canine codon opti(Polys+NP)+PAwt
Log2 HA titer
0
1
2
3
4
5
6
7
8
0
12
24
36
48
60
Hours postinfection

Figure 10E

PR8-UW PB2:

AGCGAAAGCAGGTCAATTATATTCAATATGGAAAGAATAAAAGAACTACGAAATCTAATGTCGCAGTCTCGCACCCGCGA
GATACTCACAAAAACCACCGTGGACCATATGGCCATAATCAAGAAGTACACATCAGGAAGACAGGAGAAGAACCCAGCAC
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ATCGGAATCGCAACTAACGATAACCAAAGAGAAGAAAGAAGAACTCCAGGATTGCAAAATTTCTCCTTTGATGGTTGCAT
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CTTGATTATTGCTGCTAGGAACATAGTGAGAAGAGCTGCAGTATCAGCAGATCCACTAGCATCTTTATTGGAGATGTGCC
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CAAGAGAGAGGAAGAGGTGCTTACGGGCAATCTTCAAACATTGAAGATAAGAGTGCATGAGGGATATGAAGAGTTCACAA
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AAAAACGACCTTGTTTCTACT (SEQ ID NO:3)

FIG. 10F

Canine codon optimized PR8-PB2:

AGCGAAAGCAGGTCAATTATATTCAATATGGAAAGAATAAAAGAACTACGAAATCTAATGTCGCAGTCTCGCACCCGCGA
GATACTCACAAAAACCACCGTGGACCATATGGCCATAATCAAGAAGTACACATCAGGAAGACAGGAGAAGAACCCAGCAC
TGAGGATGAAATGGATGATGGCAATGAAATATCCAATTACAGCAGACAAGAGGATCACCGAAATGATTCCTGAGAGAAAT
GAGCAGGGACAGACTCTGTGGAGTAAAATGAATGATGCCGGATCAGACCGAGTGATGGTGTCACCTCTGGCTGTGACATG
GTGGAATAGGAATGGACCAATCACAAATACAGTGCATTATCCAAAAATCTACAAAACTTATTTTGAAAGAGTCGAAAGGC
TGAAGCATGGAACCTTTGGCCCTGTCCATTTTAGAAACCAGGTCAAAATCCGGCGGAGAGTGGACATCAATCCTGGTCAT
GCAGATCTCAGTGCCAAGGAGGCACAGGATGTGATCATGGAAGTGGTGTTCCCTAACGAAGTGGGAGCCAGGATTCTGAC
ATCCGAATCCCAGCTGACCATTACCAAAGAGAAGAAAGAAGAACTCCAGGATTGCAAAATTTCTCCTCTGATGGTGGCAT
ACATGCTGGAGAGAGAACTGGTCCGCAAAACAAGATTCCTCCCAGTGGCTGGTGGAACAAGCAGTGTGTACATTGAAGTG
CTGCATCTGACTCAGGGAACATGCTGGGAACAGATGTATACTCCAGGAGGGGAAGTGAGGAATGATGATGTGGATCAGAG
CCTGATTATTGCTGCTAGGAACATTGTGAGAAGAGCTGCAGTGTCAGCAGATCCACTGGCATCTCTGCTGGAGATGTGCC
ACAGCACACAGATTGGTGGAATTAGGATGGTGGACATCCTGAGGCAGAACCCAACAGAAGAGCAGGCCGTGGATATTTGC
AAGGCTGCAATGGGACTGAGAATTAGCTCATCCTTCAGTTTTGGTGGATTCACATTTAAGAGAACAAGCGGATCATCAGT
CAAGAGAGAGGAAGAGGTGCTGACCGGCAATCTGCAGACACTGAAGATCAGAGTGCATGAGGGATATGAAGAGTTCACAA
TGGTGGGGAGAAGAGCAACAGCCATCCTCAGAAAAGCAACCAGGAGACTGATTCAGCTGATCGTGAGTGGGAGAGACGAA
CAGTCCATTGCCGAAGCAATTATTGTGGCCATGGTGTTTTCACAGGAGGATTGTATGATTAAAGCAGTCAGAGGTGATCT
GAATTTCGTCAATAGGGCCAATCAGCGACTGAATCCTATGCATCAGCTGCTGAGACATTTTCAGAAGGATGCCAAAGTGC
TGTTTCAGAATTGGGGAGTGGAACCTATCGACAATGTGATGGGAATGATTGGGATCCTGCCCGACATGACTCCAAGCATC
GAGATGTCAATGAGAGGAGTGAGAATCAGCAAAATGGGTGTGGATGAGTACTCCAGCACCGAGAGGGTCGTGGTGAGCAT
TGACAGATTTCTGAGAATCCGGGACCAGCGAGGAAATGTGCTCCTGTCTCCCGAGGAGGTCAGTGAAACACAGGGAACAG
AGAAACTGACAATTACTTACTCATCCTCAATGATGTGGGAGATTAATGGTCCTGAATCAGTGCTGGTCAATACCTATCAG
TGGATCATCAGAAACTGGGAAACTGTGAAAATTCAGTGGTCCCAGAACCCTACAATGCTGTACAATAAAATGGAATTTGA
ACCATTTCAGTCTCTGGTGCCTAAGGCCATTAGAGGCCAGTACAGTGGGTTTGTGAGAACTCTGTTCCAGCAGATGAGGG
ATGTGCTGGGGACATTTGATACCGCACAGATTATTAAACTGCTGCCCTTCGCAGCCGCTCCACCAAAGCAGAGTAGAATG
CAGTTCTCCTCATTTACTGTGAATGTGAGGGGATCAGGAATGAGAATCCTGGTGAGGGGCAATTCTCCTGTGTTCAACTA
TAACAAGGCCACCAAGAGACTCACAGTGCTCGGAAAGGATGCTGGCACTCTGACTGAAGACCCAGATGAAGGCACAGCTG
GAGTGGAGTCCGCTGTGCTGAGGGGATTCCTCATTCTGGGCAAAGAAGACAAGAGATATGGGCCAGCACTGAGCATCAAT
GAACTGAGCAACCTGGCCAAAGGAGAGAAGGCTAATGTGCTAATTGGGCAAGGAGACGTGGTGTTGGTAATGAAACGGAA
ACGGGACTCTAGCATACTTACTGACAGCCAGACAGCGACCAAAAGAATTCGGATGGCCATCAATTAGTGTCGAATAGTTT
AAAAACGACCTTGTTTCTACT (SEQ ID NO:15)

FIG. 10G

PR8-UW PB1:

AGCGAAAGCAGGCAAACCATTTGAATGGATGTCAATCCGACCTTACTTTTCTTAAAAGTGCCAGCACAAAATGCTATAAG
CACAACTTTCCCTTATACTGGAGACCCTCCTTACAGCCATGGGACAGGAACAGGATACACCATGGATACTGTCAACAGGA
CACATCAGTACTCAGAAAAGGGAAGATGGACAACAAACACCGAAACTGGAGCACCGCAACTCAACCCGATTGATGGGCCA
CTGCCAGAAGACAATGAACCAAGTGGTTATGCCCAAACAGATTGTGTATTGGAGGCGATGGCTTTCCTTGAGGAATCCCA
TCCTGGTATTTTTGAAAACTCGTGTATTGAAACGATGGAGGTTGTTCAGCAAACACGAGTAGACAAGCTGACACAAGGCC
GACAGACCTATGACTGGACTCTAAATAGAAACCAACCTGCTGCAACAGCATTGGCCAACACAATAGAAGTGTTCAGATCA
AATGGCCTCACGGCCAATGAGTCTGGAAGGCTCATAGACTTCCTTAAGGATGTAATGGAGTCAATGAACAAAGAAGAAAT
GGGGATCACAACTCATTTTCAGAGAAAGAGACGGGTGAGAGACAATATGACTAAGAAAATGATAACACAGAGAACAATGG
GTAAAAAGAAGCAGAGATTGAACAAAAGGAGTTATCTAATTAGAGCATTGACCCTGAACACAATGACCAAAGATGCTGAG
AGAGGGAAGCTAAAACGGAGAGCAATTGCAACCCCAGGGATGCAAATAAGGGGGTTTGTATACTTTGTTGAGACACTGGC
AAGGAGTATATGTGAGAAACTTGAACAATCAGGGTTGCCAGTTGGAGGCAATGAGAAGAAAGCAAAGTTGGCAAATGTTG
TAAGGAAGATGATGACCAATTCTCAGGACACCGAACTTTCTTTCACCATCACTGGAGATAACACCAAATGGAACGAAAAT
CAGAATCCTCGGATGTTTTTTGGCCATGATCACATATATGACCAGAAATCAGCCCGAATGGTTCAGAAATGTTCTAAGTAT
TGCTCCAATAATGTTCTCAAACAAAATGGCGAGACTGGGAAAAGGGTATATGTTTGAGAGCAAGAGTATGAAACTTAGAA
CTCAAATACCTGCAGAAATGCTAGCAAGCATCGATTTGAAATATTTCAATGATTCAACAAGAAAGAAGATTGAAAAAATC
CGACCGCTCTTAATAGAGGGGACTGCATCATTGAGCCCTGGAATGATGATGGGCATGTTCAATATGTTAAGCACTGTATT
AGGCGTCTCCATCCTGAATCTTGGACAAAAGAGATACACCAAGACTACTTACTGGTGGGATGGTCTTCAATCCTCTGACG
ATTTTGCTCTGATTGTGAATGCACCCAATCATGAAGGGATTCAAGCCGGAGTCGACAGGTTTTATCGAACCTGTAAGCTA
CTTGGAATCAATATGAGCAAGAAAAAGTCTTACATAAACAGAACAGGTACATTTGAATTCACAAGTTTTTTCTATCGTTA
TGGGTTTGTTGCCAATTTCAGCATGGAGCTTCCCAGTTTTGGGGTGTCTGGGATCAACGAGTCAGCGGACATGAGTATTG
GAGTTACTGTCATCAAAAACAATATGATAAACAATGATCTTGGTCCAGCAACAGCTCAAATGGCCCTTCAGTTGTTCATC
AAAGATTACAGGTACACGTACCGATGCCATATAGGTGACACACAAATACAAACCCGAAGATCATTTGAAATAAAGAAACT
GTGGGAGCAAACCCGTTCCAAAGCTGGACTGCTGGTCTCCGACGGAGGCCCAAATTTATACAACATTAGAAATCTCCACA
TTCCTGAAGTCTGCCTAAAATGGGAATTGATGGATGAGGATTACCAGGGGCGTTTATGCAACCCACTGAACCCATTTGTC
AGCCATAAAGAAATTGAATCAATGAACAATGCAGTGATGATGCCAGCACATGGTCCAGCCAAAAACATGGAGTATGATGC
TGTTGCAACAACACACTCCTGGATCCCCAAAAGAAATCGATCCATCTTGAATACAAGTCAAAGAGGAGTACTTGAGGATG
AACAAATGTACCAAAGGTGCTGCAATTTATTTGAAAAATTCTTCCCCAGCAGTTCATACAGAAGACCAGTCGGGATATCC
AGTATGGTGGAGGCTATGGTTTCCAGAGCCCGAATTGATGCACGGATTGATTTCGAATCTGGAAGGATAAAGAAAGAAGA
GTTCACTGAGATCATGAAGATCTGTTCCACCATTGAAGAGCTCAGACGGCAAAAATAGTGAATTTAGCTTGTCCTTCATG
AAAAAATGCCTTGTTTCTACT (SEQ ID NO:2)

FIG. 10H

Canine codon optimized PR8 PB1:

AGCGAAAGCAGGCAAACCATTTGAATGGATGTCAATCCGACCTTACTTTTCTTAAAAGTGCCAGCACAAAATGCTATAAG
CACAACTTTCCCTTATACTGGAGACCCTCCTTACAGCCATGGGACAGGAACAGGATACACCATGGATACTGTCAACAGGA
CACATCAGTACTCAGAAAAGGGAAGATGGACAACAAACACCGAAACTGGAGCACCGCAACTCAACCCGATTGATGGGCCA
CTGCCAGAAGACAATGAACCAAGTGGTTATGCCCAAACAGATTGTGTATTGGAGGCGATGGCTTTCCTTGAGGAATCCCA
TCCTGGTATTTTTGAAAACTCGTGTATTGAAACGATGGAGGTTGTTCAGCAAACACGAGTGGACAAGCTGACACAGGGCC
GACAGACCTATGACTGGACTCTGAATAGAAACCAGCCTGCTGCAACAGCACTGGCCAACACAATCGAAGTGTTCAGATCA
AATGGCCTCACCGCCAATGAGTCTGGAAGGCTCATCGACTTCCTGAAGGATGTGATGGAGTCAATGAACAAAGAAGAAAT
GGGGATCACAACTCATTTTCAGAGAAAGAGACGGGTGAGAGACAATATGACTAAGAAAATGATTACACAGAGAACAATGG
GTAAAAAGAAGCAGAGACTGAACAAAAGGAGTTATCTGATTAGAGCACTGACCCTGAACACAATGACCAAAGATGCTGAG
AGAGGGAAGCTGAAACGGAGAGCAATTGCAACCCCAGGGATGCAGATTAGGGGGTTTGTGTACTTTGTGGAGACACTGGC
AAGGAGTATTTGTGAGAAACTGGAACAGTCAGGGCTGCCAGTGGGAGGCAATGAGAAGAAAGCAAAGCTGGCAAATGTGG
TGAGGAAGATGATGACCAATTCTCAGGACACCGAACTGTCTTTCACCATCACTGGAGATAACACCAAATGGAACGAAAAT
CAGAATCCTCGGATGTTTCTGGCCATGATCACATATATGACCAGAAATCAGCCCGAATGGTTCAGAAATGTGCTGAGTAT
TGCTCCAATTATGTTCTCAAACAAAATGGCCAGACTGGGAAAAGGGTATATGTTTGAGAGCAAGAGTATGAAACTGAGAA
CTCAGATTCCTGCAGAAATGCTGGCAAGCATCGATCTGAAATATTTCAATGATTCAACAAGAAAGAAGATTGAAAAAATC
CGACCCCTCCTGATTGAGGGGACTGCATCACTGAGCCCTGGAATGATGATGGGCATGTTCAATATGCTGAGCACTGTGCT
GGGCGTCTCCATCCTGAATCTGGGACAGAAGAGATACACCAAGACTACTTACTGGTGGGATGGTCTGCAGTCCTCTGACG
ATTTTGCTCTGATTGTGAATGCACCCAATCATGAAGGGATTCAGGCCGGAGTCGACAGGTTTTATCGAACCTGTAAGCTG
CTGGGAATCAATATGAGCAAGAAAAAGTCTTACATCAACAGAACAGGTACATTTGAATTCACAAGTTTTTTCTATCGCTA
TGGGTTTGTGGCCAATTTCAGCATGGAGCTGCCCAGTTTTGGGGTGTCTGGGATCAACGAGTCAGCCGACATGAGTATTG
GAGTGACTGTCATCAAAAACAATATGATCAACAATGATCTGGGTCCAGCAACAGCTCAGATGGCCCTGCAGCTGTTCATC
AAAGATTACAGGTACACCTACCGATGCCATATCGGTGACACACAGATTCAGACCCGAAGATCATTTGAAATCAAGAAACT
GTGGGAGCAGACCCGCTCCAAAGCTGGACTGCTGGTCTCCGACGGAGGCCCAAATCTGTACAACATTAGAAATCTCCACA
TTCCTGAAGTCTGCCTGAAATGGGAACTGATGGATGAGGATTACCAGGGGCGCCTGTGCAACCCACTGAACCCATTTGTC
AGCCATAAAGAAATTGAATCAATGAACAATGCAGTGATGATGCCAGCACATGGTCCAGCCAAAAACATGGAGTATGATGC
TGTGGCAACAACACACTCCTGGATCCCCAAAAGAAATCGATCCATCCTGAATACAAGTCAGAGAGGAGTGCTGGAGGATG
AACAGATGTACCAGAGGTGCTGCAATCTGTTTGAAAAATTCTTCCCCAGCAGTTCATACAGAAGACCAGTCGGGATCTCC
AGTATGGTGGAGGCTATGGTGTCCAGAGCCCGAATTGATGCACGGATTGATTTCGAATCTGGAAGGATCAAGAAAGAAGA
GTTCACTGAGATCATGAAGATCTGTTCCACCATTGAAGAGCTCAGACGGCAAAAATAGTGAATTTAGCTTGTCCTTCATG
AAAAAATGCCTTGTTTCTACT (SEQ ID NO:17)

FIG. 10I

PR8-UW PA:

AGCGAAAGCAGGTACTGATCCAAAATGGAAGATTTTGTGCGACAATGCTTCAATCCGATGATTGTCGAGCTTGCGGAAAA
AACAATGAAAGAGTATGGGGAGGACCTGAAAATCGAAACAAACAAATTTGCAGCAATATGCACTCACTTGGAAGTATGCT
TCATGTATTCAGATTTTCACTTCATCAATGAGCAAGGCGAGTCAATAATCGTAGAACTTGGTGATCCAAATGCACTTTTG
AAGCACAGATTTGAAATAATCGAGGGAAGAGATCGCACAATGGCCTGGACAGTAGTAAACAGTATTTGCAACACTACAGG
GGCTGAGAAACCAAAGTTTCTACCAGATTTGTATGATTACAAGGAGAATAGATTCATCGAAATTGGAGTAACAAGGAGAG
AAGTTCACATATACTATCTGGAAAAGGCCAATAAAATTAAATCTGAGAAAACACACATCCACATTTTCTCGTTCACTGGG
GAAGAAATGGCCACAAAGGCAGACTACACTCTCGATGAAGAAAGCAGGGCTAGGATCAAAACCAGACTATTCACCATAAG
ACAAGAAATGGCCAGCAGAGGCCTCTGGGATTCCTTTCGTCAGTCCGAGAGAGGAGAAGAGACAATTGAAGAAAGGTTTG
AAATCACAGGAACAATGCGCAAGCTTGCCGACCAAAGTCTCCCGCCGAACTTCTCCAGCCTTGAAAATTTTAGAGCCTAT
GTGGATGGATTCGAACCGAACGGCTACATTGAGGGCAAGCTGTCTCAAATGTCCAAAGAAGTAAATGCTAGAATTGAACC
TTTTTTGAAAACAACACCACGACCACTTAGACTTCCGAATGGGCCTCCCTGTTCTCAGCGGTCCAAATTCCTGCTGATGG
ATGCCTTAAAATTAAGCATTGAGGACCCAAGTCATGAAGGAGAGGGAATACCGCTATATGATGCAATCAAATGCATGAGA
ACATTCTTTGGATGGAAGGAACCCAATGTTGTTAAACCACACGAAAAGGGAATAAATCCAAATTATCTTCTGTCATGGAA
GCAAGTACTGGCAGAACTGCAGGACATTGAGAATGAGGAGAAAATTCCAAAGACTAAAAATATGAAGAAAACAAGTCAGC
TAAAGTGGGCACTTGGTGAGAACATGGCACCAGAAAAGGTAGACTTTGACGACTGTAAAGATGTAGGTGATTTGAAGCAA
TATGATAGTGATGAACCAGAATTGAGGTCGCTTGCAAGTTGGATTCAGAATGAGTTTAACAAGGCATGCGAACTGACAGA
TTCAAGCTGGATAGAGCTCGATGAGATTGGAGAAGATGTGGCTCCAATTGAACACATTGCAAGCATGAGAAGGAATTATT
TCACATCAGAGGTGTCTCACTGCAGAGCCACAGAATACATAATGAAGGGAGTGTACATCAATACTGCCTTGCTTAATGCA
TCTTGTGCAGCAATGGATGATTTCCAATTAATTCCAATGATAAGCAAGTGTAGAACTAAGGAGGGAAGGCGAAAGACCAA
CTTGTATGGTTTCATCATAAAAGGAAGATCCCACTTAAGGAATGACACCGACGTGGTAAACTTTGTGAGCATGGAGTTTT
CTCTCACTGACCCAAGACTTGAACCACATAAATGGGAGAAGTACTGTGTTCTTGAGATAGGAGATATGCTTATAAGAAGT
GCCATAGGCCAGGTTTCAAGGCCCATGTTCTTGTATGTGAGAACAAATGGAACCTCAAAAATTAAAATGAAATGGGGAAT
GGAGATGAGGCGTTGCCTCCTCCAGTCACTTCAACAAATTGAGAGTATGATTGAAGCTGAGTCCTCTGTCAAAGAGAAAG
ACATGACCAAAGAGTTCTTTGAGAACAAATCAGAAACATGGCCCATTGGAGAGTCCCCCAAAGGAGTGGAGGAAAGTTCC
ATTGGGAAGGTCTGCAGGACTTTATTAGCAAAGTCGGTATTCAACAGCTTGTATGCATCTCCACAACTAGAAGGATTTTC
AGCTGAATCAAGAAAACTGCTTCTTATCGTTCAGGCTCTTAGGGACAACCTGGAACCTGGGACCTTTGATCTTGGGGGGC
TATATGAAGCAATTGAGGAGTGCCTGATTAATGATCCCTGGGTTTTGCTTAATGCTTCTTGGTTCAACTCCTTCCTTACA
CATGCATTGAGTTAGTTGTGGCAGTGCTACTATTTGCTATCCATACTGTCCAAAAAAGTACCTTGTTTCTACT (SEQ ID NO:1)

FIG. 10J

Canine codon optimized PR8 PA:

AGCGAAAGCAGGTACTGATCCAAAATGGAAGATTTTGTGCGACAATGCTTCAATCCGATGATTGTCGAGCTTGCGGAAAA
AACAATGAAAGAGTATGGGGAGGACCTGAAAATCGAAACAAACAAATTTGCAGCAATATGCACTCACTTGGAAGTATGCT
TCATGTATTCAGATTTTCACTTCATCAATGAGCAAGGCGAGTCAATAATCGTAGAACTTGGTGATCCAAATGCACTTTTG
AAGCACAGATTTGAAATAATCGAGGGAAGAGATCGCACAATGGCCTGGACAGTAGTAAACAGTATTTGCAACACTACAGG
GGCTGAGAAACCAAAGTTTCTACCAGATTTGTATGATTACAAGGAGAATAGATTCATCGAAATTGGAGTAACAAGGAGAG
AAGTTCACATATACTATCTGGAAAAGGCCAATAAAATTAAATCTGAGAAAACACACATCCACATTTTCTCGTTCACTGGG
GAAGAAATGGCCACAAAGGCAGACTACACTCTCGATGAAGAAAGCAGGGCTAGGATCAAAACCAGACTATTCACCATAAG
ACAAGAAATGGCCAGCAGAGGCCTCTGGGATTCCTTTCGTCAGTCCGAGAGAGGAGAAGAGACAATTGAAGAAAGGTTTG
AAATCACAGGAACAATGCGCAAGCTTGCCGACCAAAGTCTCCCGCCGAACTTCTCCAGCCTTGAAAATTTTAGAGCCTAT
GTGGATGGATTCGAACCGAACGGCTACATTGAGGGCAAGCTGTCTCAAATGTCCAAAGAAGTAAATGCTAGAATTGAACC
TTTTCTGAAAACAACACCACGACCACTGAGACTGCCCAATGGGCCTCCCTGTTCTCAGCGGTCCAAATTCCTGCTGATGG
ATGCCCTGAAACTGAGCATTGAGGACCCAAGTCATGAAGGAGAGGGAATTCCCCTGTATGATGCAATCAAATGCATGAGA
ACATTCTTTGGATGGAAGGAACCCAATGTGGTGAAACCACACGAAAAGGGAATCAATCCAAATTATCTGCTGTCATGGAA
GCAGGTGCTGGCAGAACTGCAGGACATTGAGAATGAGGAGAAAATTCCAAAGACTAAAAATATGAAGAAAACAAGTCAGC
TGAAGTGGGCACTGGGTGAGAACATGGCACCAGAAAAGGTGGACTTTGACGACTGTAAAGATGTGGGTGATCTGAAGCAG
TATGATAGTGATGAACCAGAACTGAGGTCCCTGGCAAGTTGGATTCAGAATGAGTTTAACAAGGCATGCGAACTGACAGA
TTCAAGCTGGATTGAGCTCGATGAGATTGGAGAAGATGTGGCTCCAATTGAACACATTGCAAGCATGAGAAGGAATTATT
TCACATCAGAGGTGTCTCACTGCAGAGCCACAGAATACATCATGAAGGGAGTGTACATCAATACTGCCCTGCTGAATGCA
TCTTGTGCAGCAATGGATGATTTCCAGCTGATTCCAATGATCAGCAAGTGTAGAACTAAGGAGGGAAGGCGAAAGACCAA
CCTGTATGGTTTCATCATCAAAGGAAGATCCCACCTGAGGAATGACACCGACGTGGTGAACTTTGTGAGCATGGAGTTTT
CTCTCACTGACCCAAGACTGGAACCACATAAATGGGAGAAGTACTGTGTGCTGGAGATTGGAGATATGCTGATCAGAAGT
GCCATTGGCCAGGTGTCAAGGCCCATGTTCCTGTATGTGAGAACAAATGGAACCTCAAAAATTAAAATGAAATGGGGAAT
GGAGATGAGGCGCTGCCTCCTCCAGTCACTGCAGCAGATTGAGAGTATGATTGAAGCTGAGTCCTCTGTCAAAGAGAAAG
ACATGACCAAAGAGTTCTTTGAGAACAAATCAGAAACATGGCCCATTGGAGAGTCCCCCAAAGGAGTGGAGGAAAGTTCC
ATTGGGAAGGTCTGCAGGACTCTGCTGGCAAAGTCCGTGTTCAACAGCCTGTATGCATCTCCACAGCTGGAAGGATTTTC
AGCTGAATCAAGAAAACTGCTGCTGATCGTGCAGGCTCTGAGGGACAACCTGGAACCTGGGACCTTTGATCTGGGGGGGC
TGTATGAAGCAATTGAGGAGTGCCTGATTAATGATCCCTGGGTGCTGCTGAATGCTTCTTGGTTCAACTCCTTCCTTACA
CATGCATTGAGTTAGTTGTGGCAGTGCTACTATTTGCTATCCATACTGTCCAAAAAAGTACCTTGTTTCTACT (SEQ ID NO:18)

FIG. 10K

PR8-UW NP:

AGCAAAAGCAGGGTAGATAATCACTCACTGAGTGACATCAAAATCATGGCGTCTCAAGGCACCAAACGATCTTACGAACA
GATGGAGACTGATGGAGAACGCCAGAATGCCACTGAAATCAGAGCATCCGTCGGAAAAATGATTGGTGGAATTGGACGAT
TCTACATCCAAATGTGCACCGAACTCAAACTCAGTGATTATGAGGGACGGTTGATCCAAAACAGCTTAACAATAGAGAGA
ATGGTGCTCTCTGCTTTTGACGAAAGGAGAAATAAATACCTTGAAGAACATCCCAGTGCGGGGAAAGATCCTAAGAAAAC
TGGAGGACCTATATACAGGAGAGTAAACGGAAAGTGGATGAGAGAACTCATCCTTTATGACAAAGAAGAAATAAGGCGAA
TCTGGCGCCAAGCTAATAATGGTGACGATGCAACGGCTGGTCTGACTCACATGATGATCTGGCATTCCAATTTGAATGAT
GCAACTTATCAGAGGACAAGAGCTCTTGTTCGCACCGGAATGGATCCCAGGATGTGCTCTCTGATGCAAGGTTCAACTCT
CCCTAGGAGGTCTGGAGCCGCAGGTGCTGCAGTCAAAGGAGTTGGAACAATGGTGATGGAATTGGTCAGAATGATCAAAC
GTGGGATCAATGATCGGAACTTCTGGAGGGGTGAGAATGGACGAAAAACAAGAATTGCTTATGAAAGAATGTGCAACATT
CTCAAAGGGAAATTTCAAACTGCTGCACAAAAAGCAATGATGGATCAAGTGAGAGAGAGCCGGAACCCAGGGAATGCTGA
GTTCGAAGATCTCACTTTTCTAGCACGGTCTGCACTCATATTGAGAGGGTCGGTTGCTCACAAGTCCTGCCTGCCTGCCT
GTGTGTATGGACCTGCCGTAGCCAGTGGGTACGACTTTGAAAGGGAGGGATACTCTCTAGTCGGAATAGACCCTTTCAGA
CTGCTTCAAAACAGCCAAGTGTACAGCCTAATCAGACCAAATGAGAATCCAGCACACAAGAGTCAACTGGTGTGGATGGC
ATGCCATTCTGCCGCATTTGAAGATCTAAGAGTATTAAGCTTCATCAAAGGGACGAAGGTGCTCCCAAGAGGGAAGCTTT
CCACTAGAGGAGTTCAAATTGCTTCCAATGAAAATATGGAGACTATGGAATCAAGTACACTTGAACTGAGAAGCAGGTAC
TGGGCCATAAGGACCAGAAGTGGAGGAAACACCAATCAACAGAGGGCATCTGCGGGCCAAATCAGCATACAACCTACGTT
CTCAGTACAGAGAAATCTCCCTTTTGACAGAACAACCATTATGGCAGCATTCAATGGGAATACAGAGGGGAGAACATCTG
ACATGAGGACCGAAATCATAAGGATGATGGAAAGTGCAAGACCAGAAGATGTGTCTTTCCAGGGGCGGGGAGTCTTCGAG
CTCTCGGACGAAAAGGCAGCGAGCCCGATCGTGCCTTCCTTTGACATGAGTAATGAAGGATCTTATTTCTTCGGAGACAA
TGCAGAGGAGTACGACAATTAAAGAAAAATACCCTTGTTTCTACT (SEQ ID NO:4)

FIG. 10L

Canine codon optimized NP:

AGCAAAAGCAGGGTAGATAATCACTCACTGAGTGACATCAAAATCATGGCGTCTCAAGGCACCAAACGATCTTACGAACA
GATGGAGACTGATGGAGAACGCCAGAATGCCACTGAAATCAGAGCATCCGTCGGAAAAATGATTGGTGGAATTGGACGAT
TCTACATCCAGATGTGCACCGAACTCAAACTCAGTGATTATGAGGGACGGCTGATCCAGAACAGCCTGACAATCGAGAGA
ATGGTGCTCTCTGCTTTTGACGAAAGGAGAAATAAATACCTGGAAGAACATCCCAGTGCCGGGAAAGATCCTAAGAAAAC
TGGAGGACCTATCTACAGGAGAGTGAACGGAAAGTGGATGAGAGAACTCATCCTGTATGACAAAGAAGAAATCAGGCGAA
TCTGGCGCCAGGCTAATAATGGTGACGATGCAACCGCTGGTCTGACTCACATGATGATCTGGCATTCCAATCTGAATGAT
GCAACTTATCAGAGGACAAGAGCTCTGGTGCGCACCGGAATGGATCCCAGGATGTGCTCTCTGATGCAGGGTTCAACTCT
CCCTAGGAGGTCTGGAGCCGCAGGTGCTGCAGTCAAAGGAGTGGGAACAATGGTGATGGAACTGGTCAGAATGATCAAAA
GAGGGATCAATGATCGGAACTTCTGGAGGGGTGAGAATGGACGAAAAACAAGAATTGCTTATGAAAGAATGTGCAACATT
CTCAAAGGGAAATTTCAGACTGCTGCACAGAAAGCAATGATGGATCAGGTGAGAGAGAGCCGGAACCCAGGGAATGCTGA
GTTCGAAGATCTCACTTTTCTGGCACGGTCTGCACTCATCCTGAGAGGGTCCGTGGCTCACAAGTCCTGCCTGCCTGCCT
GTGTGTATGGACCTGCCGTGGCCAGTGGGTACGACTTTGAAAGGGAGGGATACTCTCTGGTCGGAATTGACCCTTTCAGA
CTGCTGCAGAACAGCCAGGTGTACAGCCTGATCAGACCAAA~GAGAATCCAGCACACAAGAGTCAGCTGGTGTGGATGGC
ATGCCATTCTGCCGCATTTGAAGATCTGAGAGTGCTGAGCTTCATCAAAGGGACCAAGGTGCTCCCAAGAGGGAAGCTGT
CCACTAGAGGAGTGCAGATTGCTTCCAATGAAAATATGGAGACTATGGAATCAAGTACACTGGAACTGAGAAGCAGGTAC
TGGGCCATCAGGACCAGAAGTGGAGGAAACACCAATCAGCAGAGGGCATCTGCCGGCCAGATCAGCATTCAGCCTACCTT
CTCAGTGCAGAGAAATCTCCCTTTTGACAGAACAACCATTATGGCAGCATTCAATGGGAATACAGAGGGGAGAACATCTG
ACATGAGGACCGAAATCATCAGGATGATGGAAAGTGCAAGACCAGAAGATGTGTCTTTCCAGGGGCGGGGAGTCTTCGAG
CTCTCGGACGAAAAGGCAGCGAGCCCGATCGTGCCTTCCTTTGACATGAGTAATGAAGGATCTTATTTCTTCGGAGACAA
TGCAGAGGAGTACGACAATTAAAGAAAAATACCCTTGTTTCTACT (SEQ ID NO:19)

FIG. 10M

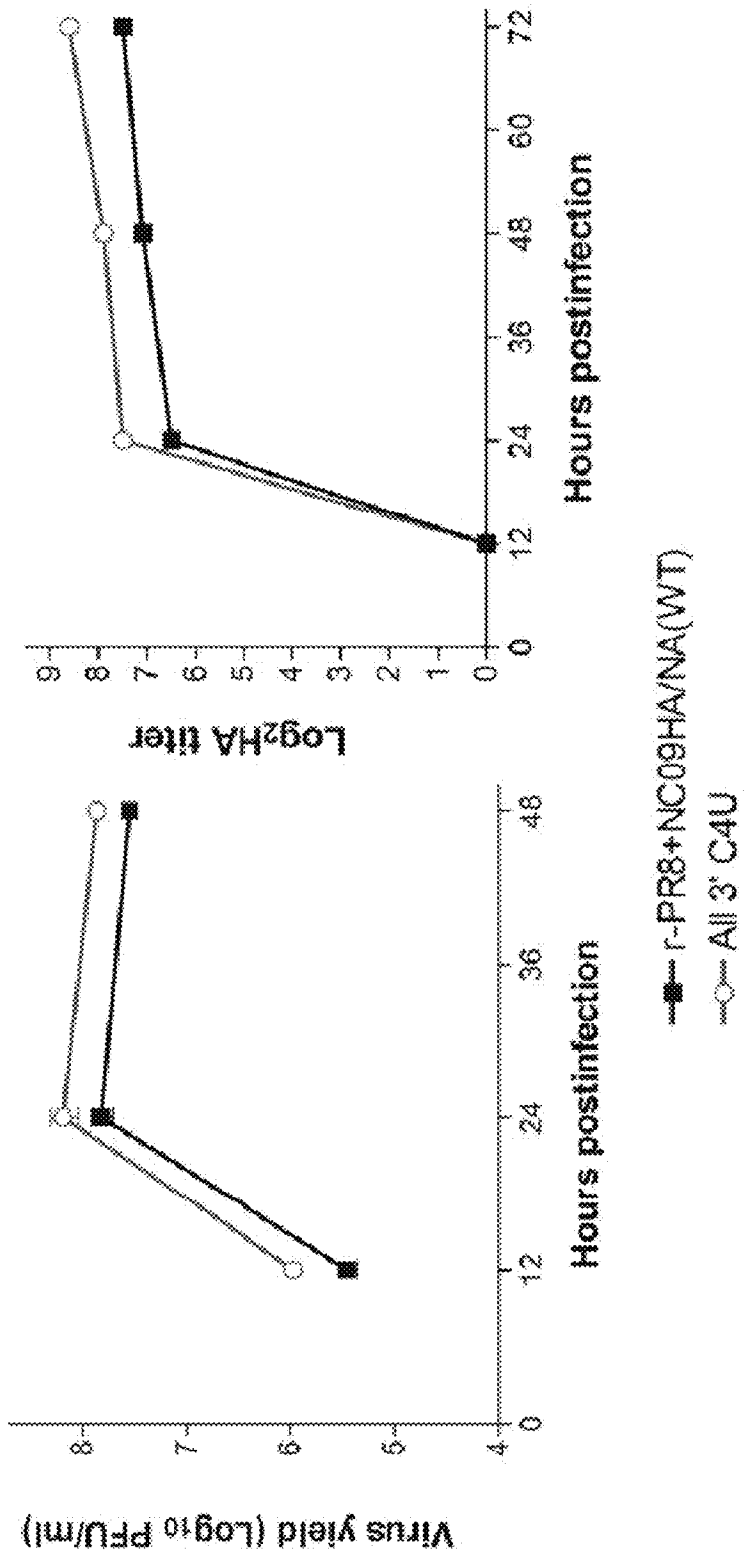
Figure 11 A Nucleotide mutation in position 4 of each gene of PR8 and Indo/NC/09.

Genes	Position 4 of vRNA	
PR8 PB2	C	
PR8 PB1	C	
PR8 PA	C	
PR8 NP		U
PR8 M		U
PR8 NS		U
Inda/NC/09 HA		U
Inda/NC/09 NA		U

Figure 11B All 3'C4U mutant

Genes	Position 4 of vRNA	
PR8 PB2	U	
PR8 PB1	U	
PR8 PA	U	
PR8 NP	U	
PR8 M	U	
PR8 NS	U	
India/NC/09 HA	U	
India/NC/09 NA	U	

Figure 11C Growth kinetics of 3' C4U mutant



HA

atgaacactcaaatcctggtattcgctctgattgcgatcattccaacaaatgcagacaaaatctgcctcggacatcatgccgtgtcaaacggaaccaaagtaaa
cacattaactgaaagaggagtggaagtcgtcaatgcaactgaaacagtggaacgaacaaacatccccaggatctgctcaaaagggaaaaggacagttgacc
tcggtcaatgtggactcctggggacaatcactggaccacctcaatgtgaccaattcctagaattttcagccgatttaattattgagaggcgagaaggaagtgatg
tctgttatcctgggaaattcgtgaatgaagaagctctgaggcaaattctcagagaatcaggcggaattgacaaggaagcaatgggattcacatacagtggaat
aagaactaatggagcaaccagtgcatgtaggagatcaggatcttcattctatgcagaaatgaaatggctcctgtcaaacacagataatgctgcattcccgcaga
tgactaagtcatataaaaatacaagaaaaagcccagctctaatagtatgggggatccatcattccgtatcaactgcagagcaaaccaagctatatgggagtgg
aaacaaactggtgacagttgggagttctaattatcaacaatcttttgtaccgagtccaggagcgagaccacaagttaatggtctatctggaagaattgacttttcat
tggctaatgctaaatcccaatgatacagtcacttcagtttcaatggggcttcatagctccagaccgtgcaagcttcctgagaggaaaatctatgggaatccag
agtggagtacaggttgatgccaattgtgaaggggactgctatcatagtggagggacaataataagtaacttgccatttcagaacatagatagcagggcagttg
gaaaatgtccgagatatgttaagcaaaggagtctgctgctagcaacagggatgaagaatgttcctgagattccaaagggaagaggcctatttggtgctatagc
gggtttcattgaaaatggatgggaaggcctaattgatggttggtatggtttcagacaccagaatgcacagggagagggaactgctgcagattacaaaagcact
caatcggcaattgatcaaataacaggaaaattaaaccggcttatagaaaaaaccaaccaacaatttgagttgatagacaatgaattcaatgaggtagagaag
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RKSPALIVWG IHHSVSTAEQ TKLYGSGNKL VTVGSSNYQQ SFVPSPGARP QVNGLSGRID
FHWLMLNPND TVTFSFNGAF IAPDRASFLR GKSMGIQSGV QVDANCEGDC YHSGGTIISN
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YGFRHQNAQG EGTAADYKST QSAIDQITGK LNRLIEKTNQ QFELIDNEFN EVEKQIGNVI
NWTRDSITEV WSYNAELLVA MENQHTIDLA DSEMDKLYER VKRQLRENAE EDGTGCFEIF
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FIG. 12A

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R EEAMQNRIQIDPVKLSSGYKDVILWFSFGASCFILLAIAMGLVFICVKNGNMRCTICI (SEQ ID NO:25)

NA

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GWSSTSCHDGKSRMSICISGPNNNASAVVWYNRRPVAEINTWARNILRTQESECVCHNGVCPVVFTDGSATGPADTRIYYFK

FIG. 12B

EGKILKWESLTGTAKHIEECSCYGERTGITCTCKDNWQGSNRPVIQIDPVAMTHTSQYICSPVLTDNPRPNDPNIGKCNDPYPG
NNNNGVKGFSYLDGANTWLGRTISTASRSGYEMLKVPNALTDDRSKPIQGQTIVLNADWSGYSGSFMDYWAEGDCYRACF
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FIG. 12C

Figure 13A Construct chimeric HA &NA to increase virus replication

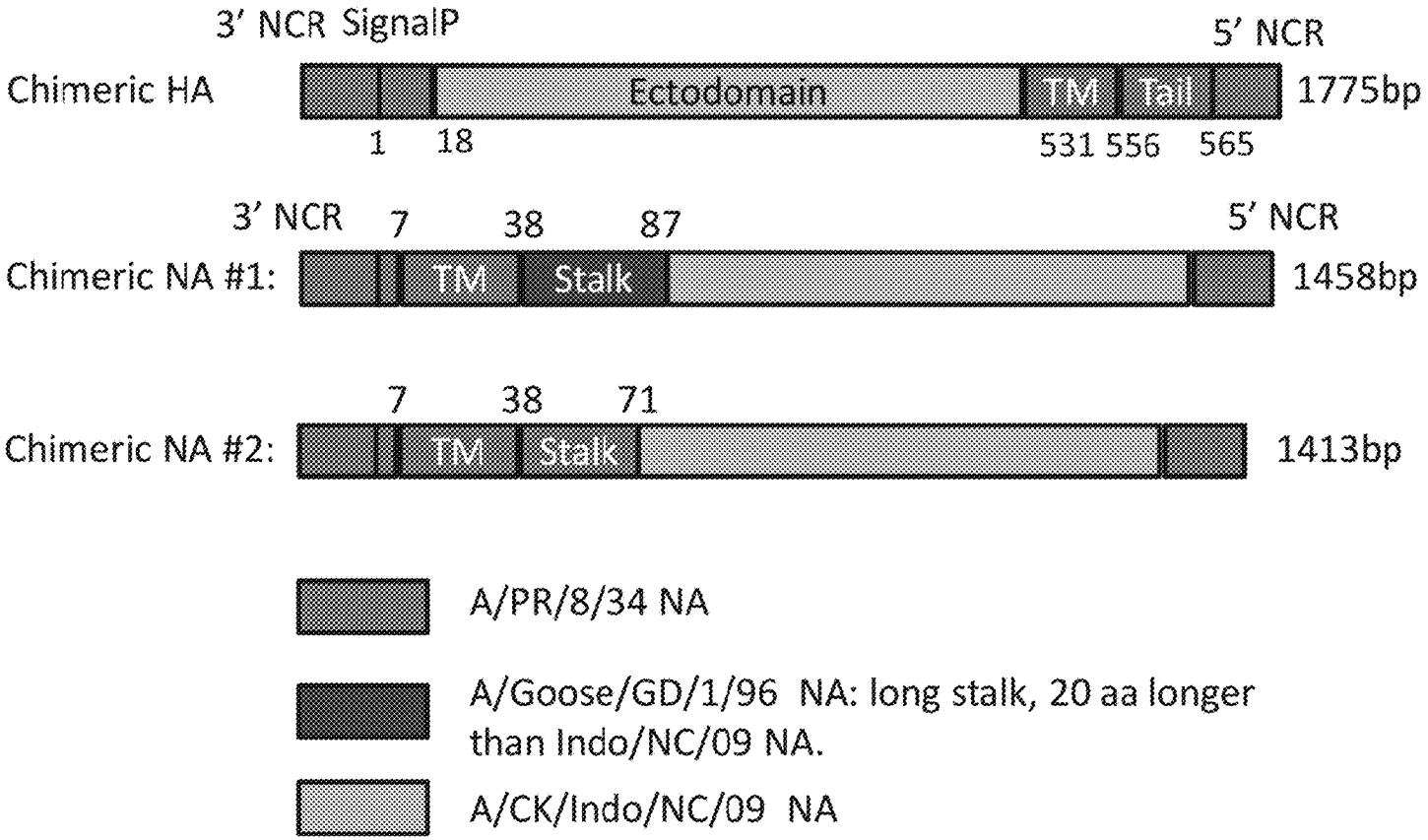


Figure 13B Growth kinetics in MDCK cells

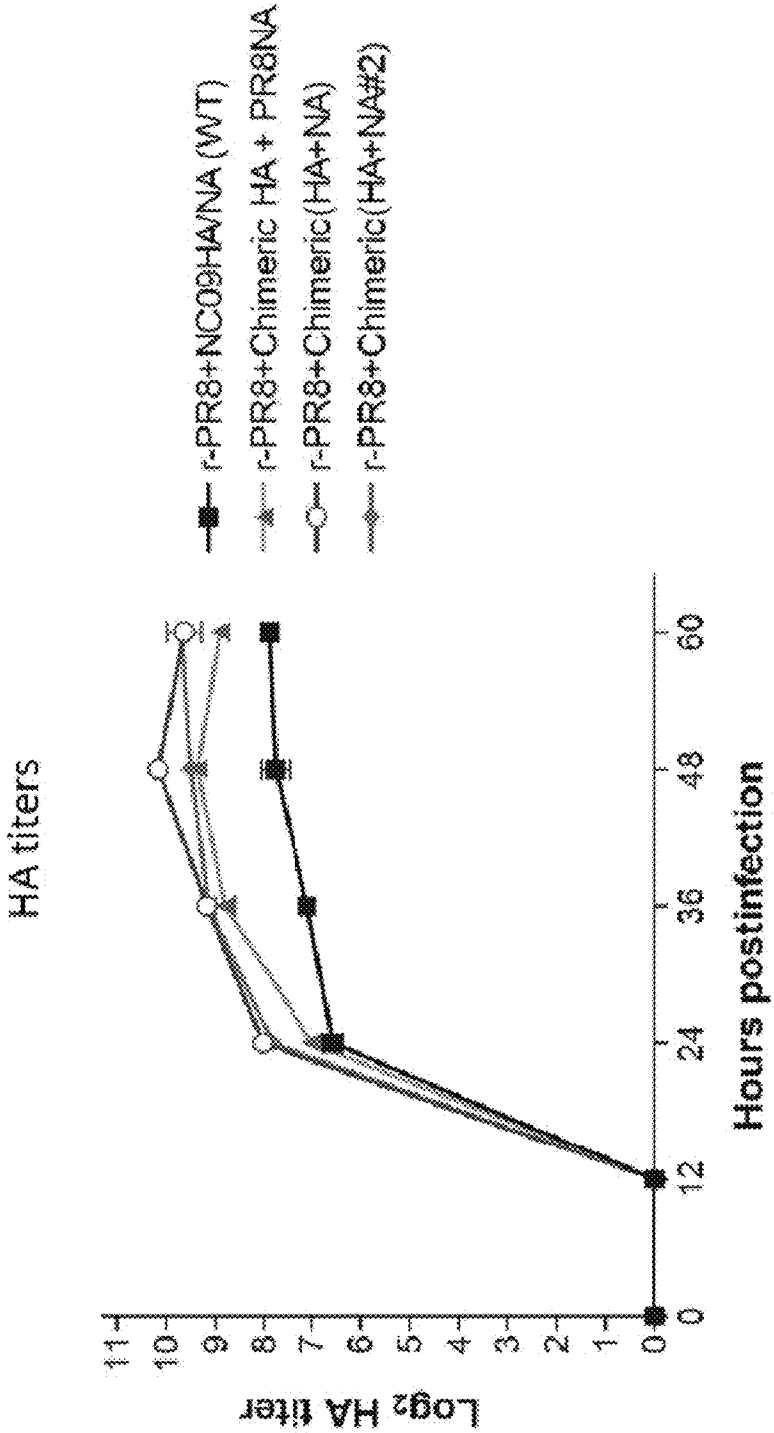
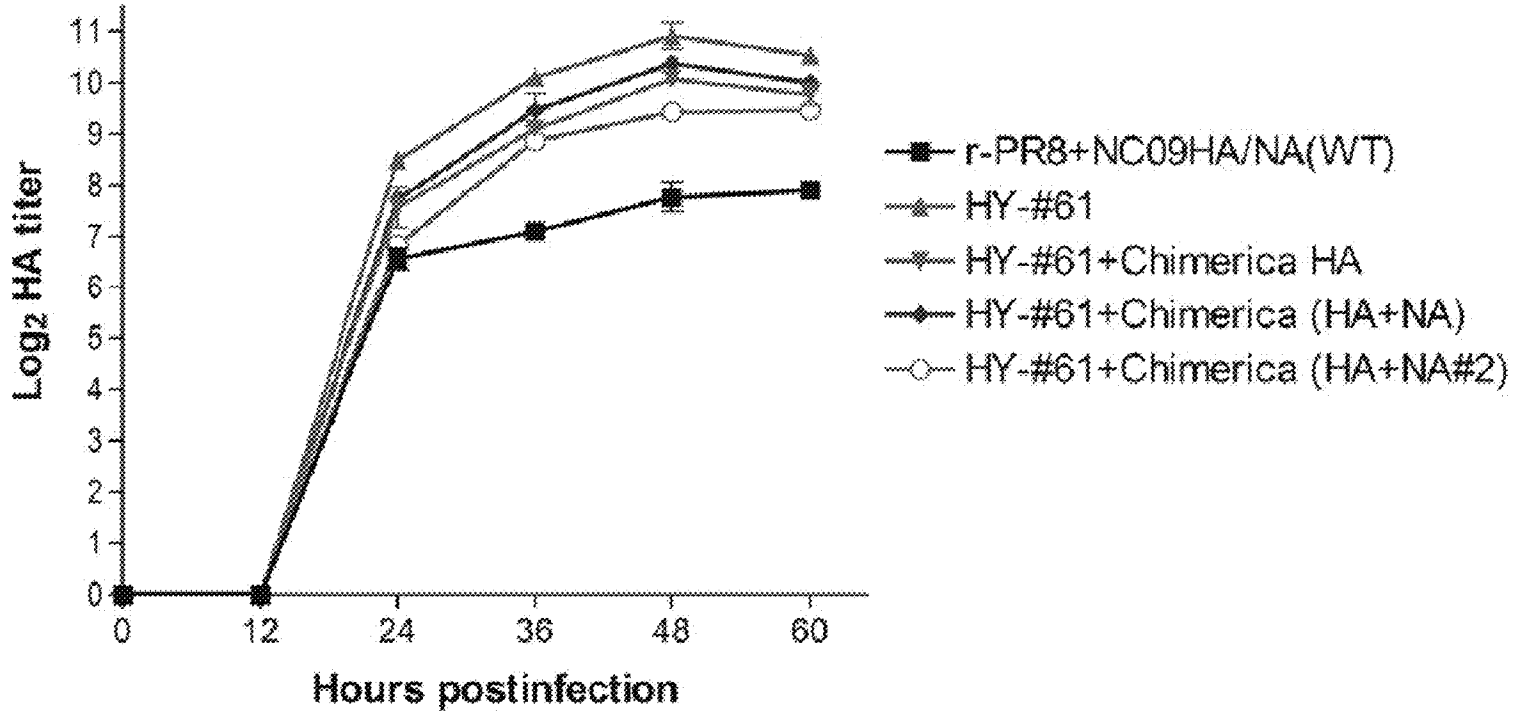


Figure 14A Growth kinetics in MDCK cells



HY-#61 includes PB2: M202L F323L, PB1 Q247H, PA K142N, NP R74K, M V97A Y100H and NS K55E mutations.

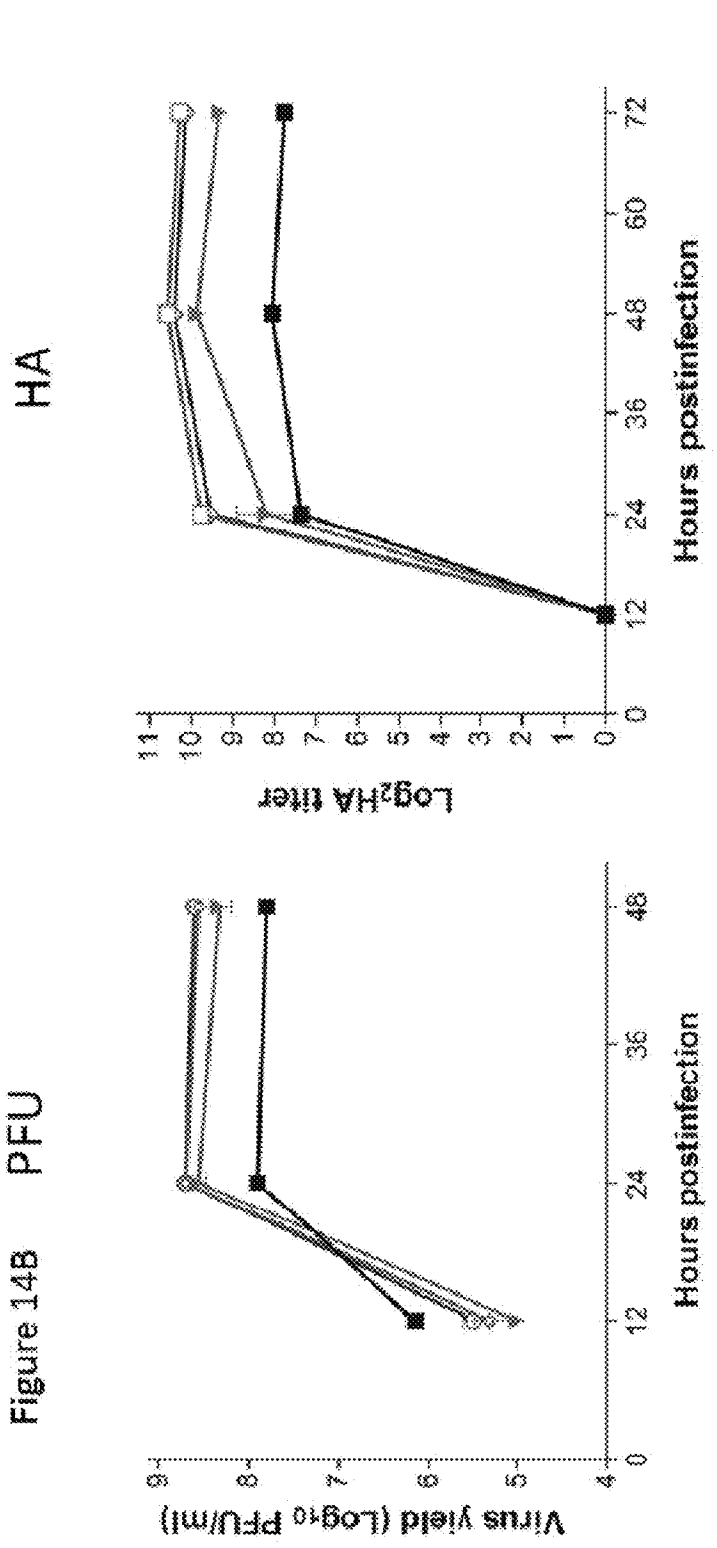


Figure 14B

HY mutations include PB2: M202L F323L, PB1 Q247H, PA K142N, NP R74K, M V97A Y100H and NS K55E mutations.

Figure 15 Schematic diagram of screening high growth mutations in eggs.

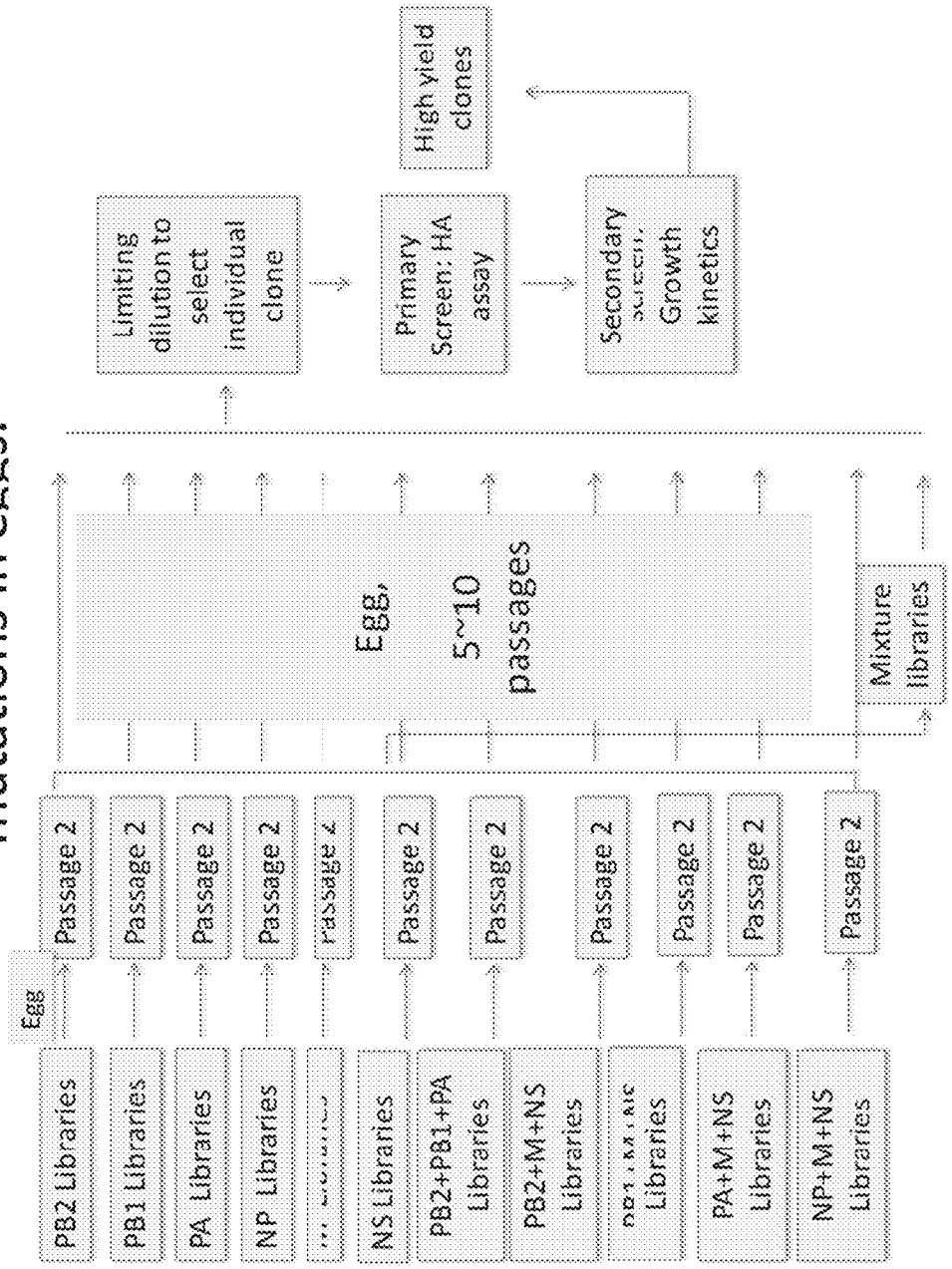


Figure 16 Summary of HA assay of individual clones purified from Vero cells

Groups	Numbers of clone	Fold change	%
WT HA titer = $2^{6.5}$	5	-	2.3%
HA titer =$2^{>9-9.5}$	16	>5.6	7.4%
HA titer =$2^{>8-9}$	91	>2.8 -5.6	42.2%
HA titer =$2^{6.5-8}$	80	1 - 2.8	37.0%
HA titer < $2^{6.5}$	24	<1	11.1%
Total	216	-	100%

Figure 17Recombinant viruses generated with different PR8 backbone mutants.

#	Del-HA & NA genes	PB2	PB1	PA	NP	M	NS
WT	CK/Indo/NC/09	WT	WT	WT	WT	WT	WT
HY-1		I504V	M40L G180W	R401K	I116L	WT	A30P R118K
HY-2		E391Q		I301 E31K K142N	R74K S377N	WT	S161T
HY-3		I504V		K142N	I116L	V97A Y100H	V136M S161T
HY-4		M202L F323L		K356R	I116L	V97A Y100H	K55E
HY-5				K356R	R422L	WT	K55E

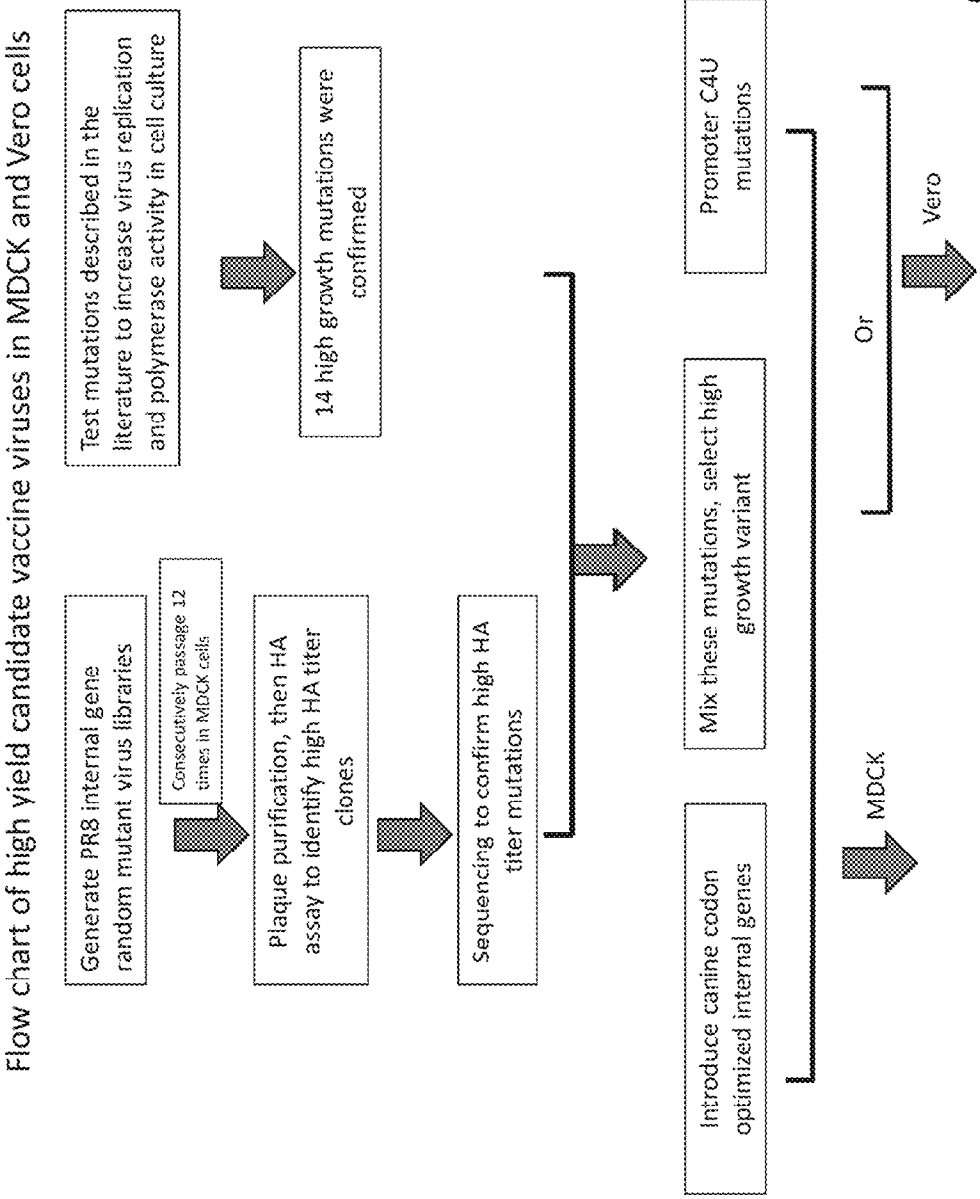

Flow chart of high yield candidate vaccine viruses in MDCK and Vero cells
Generate PR8 internal gene random mutant virus libraries
Consecutively passage 12 times in MDCK cells
Plaque purification, then HA assay to identify high HA titer clones
Sequencing to confirm high HA titer mutations
Test mutations described in the literature to increase virus replication and polymerase activity in cell culture
14 high growth mutations were confirmed
Introduce canine codon optimized internal genes
Mix these mutations, select high growth variant
Promoter C4U mutations
MDCK
Or
Vero

FIG. 18A

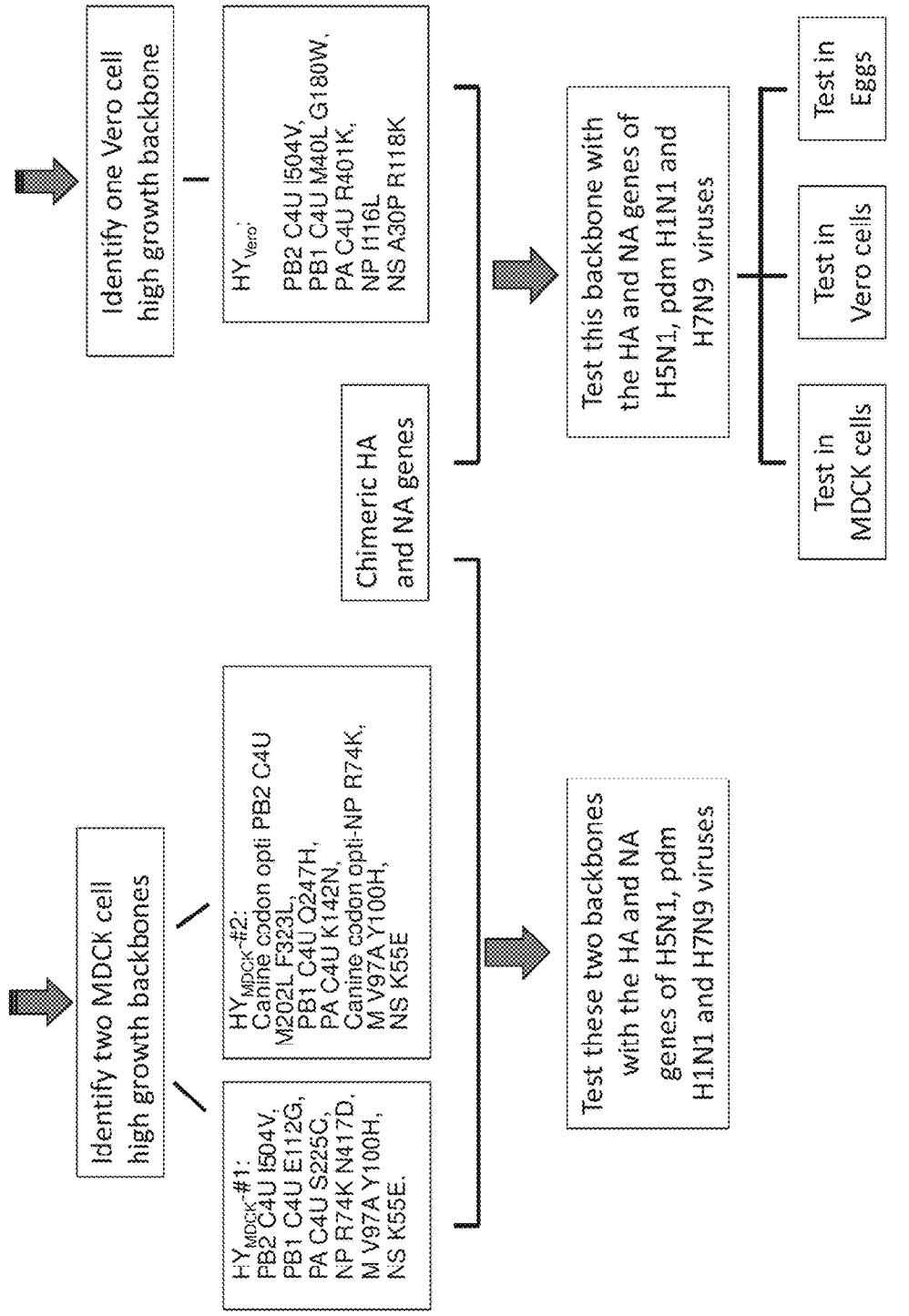

Identify two MDCK cell high growth backbones
HY MDCK #1: PB2 C4U I504V, PB1 C4U E112G, PA C4U S225C, NP R74K N417D, M V97A Y100H, NS K55E.
HY MDCK #2: Canine codon opti PB2 C4U M202L F323L, PB1 C4U Q247H, PA C4U K142N, Canine codon opti-NP R74K, M V97A Y100H, NS K55E
Test these two backbones with the HA and NA genes of H5N1, pdm H1N1 and H7N9 viruses
Identify one Vero cell high growth backbone
HY Vero: PB2 C4U I504V, PB1 C4U M40L G180W, PA C4U R401K, NP I116L NS A30P R118K
Chimeric HA and NA genes
Test this backbone with the HA and NA genes of H5N1, pdm H1N1 and H7N9 viruses
Test in MDCK cells
Test in Vero cells
Test in Eggs

FIG. 18B

Virus number	Surface genes		UW-PR8 internal gene						Growth substrate	Viral liter or HA liter	Fold change
	HA (H3 numbering)	NA	PB2	PB1	PA	NP	M1	NS1			
1	M476I		M202L F323L			R293M			MDCK	HA titer	2.8
2	F252I	L55S	M202L F323L			I116L			MDCK	HA titer	2.8
3			M202L F323L					A223E	MDCK	HA titer	2.8
4	L182V		M202L F323L						MDCK	HA titer	2.8~4
5				E112G (PB1-F2 R81G)[5]					MDCK	HA titer	2.8
6	E136D/ Q179L/ A194V			R54I					MDCK	HA titer	2.8~4
7	K162E			I667T/M714T					MDCK	HA titer	4
8	L182V					I116L		R140Q	MDCK	HA titer	2.8~4
9	L182V								MDCK	HA titer	2.8~4
10	L182V		M202L F323L						MDCK	HA titer	4
11	L182V		M66R						MDCK	HA titer	2.8~4
12	L182V		M202L F323L						MDCK	HA titer	4
13			M202L F323L	M507V/V644A					MDCK	HA titer	4
14	L182V		I504V			R74K/ N417D		A30P	MDCK	HA titer	2.8
15	K162E			E112G (PB1-F2-R81G)				S161T	MDCK	HA titer	2.8
16				I667T					MDCK	HA titer	2.8
17	K449E			M40L/G180W					MDCK	HA titer	2.8
18	K162E			E112G (PB1-F2-R81G)/ L624V				S161T	MDCK	HA titer	2.8~4

FIG. 19A

Virus number	Surface genes		UW-PRB internal gene						Growth substrate	Viral titer or HA titer	Fold change
	HA (H3 numbering)	NA	PB2	PB1	PA	NP	M1	NS1			
19				E112G (PB1-F2:R81G)					MDCK	HA titer	2.8
20				M40L G180W				S161T	MDCK	HA titer	2.8~4
21			M202L F323L M243I	R54I					MDCK	HA titer	4~5.7
22	V184I		M202L F323L		F105C		P90S		MDCK	HA titer	2.8~4
23			M202L F323L	Q247H					MDCK	HA titer	2.8~4
24			M202L F323L			N224I			MDCK	HA titer	2.8~4
25	M476I		I504V			R74K/ N417D			MDCK	HA titer	2.8~4
26	M476I		M202L F323L	V644A	R401K			T49A	MDCK	HA titer	4~5.7
27	F252I		I504V	V644A		M371V			MDCK	HA titer	2.8
28	M476I	A265V	I504V	T59I G62A A63P V644A N694K L695T		R74K/ N417D			MDCK	HA titer	2.8
29	M476I		I504V						MDCK	HA titer	2.8~4
30	F252I		I504V	E75V D76G E78P P79V S80G V644A E697P F699L F700L P701H S702R Y705T		R74K			MDCK	HA titer	2.8~4
31	L182V		I504V			R74K			MDCK	HA titer	2.8~4
32	F252I		M202L F323L	V644A					MDCK	HA titer	4

FIG. 19B

Virus number	Surface genes		UW-PR8 internal gene						Growth substrate	Viral titer or HA titer	Fold change
	HA (H3 numbering)	NA	PB2	PB1	PA	NP	M1	NS1			
33			I57V T58G A59V K61Q E677D D678E P679M	E112G (PB1-F2-R81G) S713C					MDCK	HA titer	2.8
34				M40I G180W				S161T	MDCK	HA titer	2.8
35			R368K						MDCK	Viral titer	2.8
36			E391K						MDCK	Viral titer	3.8
37			I504V		I550L				MDCK	Viral titer	4.3
38				PB1 F2 N66S					MDCK	Viral titer	2.78
39				PB1 F2 K73R					MDCK	Viral titer	3.9
40					K142N				MDCK	Viral titer	2.1
41					K356R				MDCK	Viral titer	3.9
42						R293K			MDCK	Viral titer	3.36
43						R442K			MDCK	Viral titer	2.4
44						T442A			MDCK	Viral titer	2.6
46							V97A		MDCK	HA titer	8
47							Y100H		MDCK	HA titer	9
48							V97A Y100H		MDCK	HA titer	9.5
49								K55E	MDCK	Viral titer	2.1
50			M202L F323L	M507V V644A		I116L		K55E	MDCK	Viral titer	9.9
										HA titer	4.3
51			M202L F323L	M507V V644A	K356R	T442A	V97A Y100H	K55E	MDCK	Viral titer	1.3
										HA titer	3.7
52			I504V	E112G (PB1-F2-R81G)	I550L	I112L	Y100H	R140O	MDCK	Viral titer	3
										HA titer	3

FIG. 19C

Virus number	Surface genes		UW-PR8 internal gene						Growth substrate	Viral titer or HA titer	Fold change
	HA (H3 numbering)	NA	PB2	PB1	PA	NP	M1	NS1			
53			M202L F323L	M507V V644A		I116L	Y100H	K55E	MDCK	Viral titer	9
										HA titer	6.5
54			M202L F323L	Q247H	K142N	R74K	V97A Y100H	K55E	MDCK	Viral titer	4.3
										HA titer	9.2
55			I504V	E112G (PB1-F2-R81G)	S225C	R74K N417D	V97A Y100H	K55E	MDCK	Viral titer	14.9
										HA titer	6.5
56			M202L F323L	M40L G180W	S225C	R422K	V97A Y100H	K55E	MDCK	Viral titer	4.2
										HA titer	6.5
57			*C4U*	*C4U*	*C4U*				MDCK	Viral titer	3.3
										HA titer	2.1
58	X-181 HA &NA (Derived from A/California/ 07/2009 pdmH1N1)			G180E S261G S361R Q621R N654S					MDCK	Viral titer	3.5
										HA titer	2.1
59	A/Chicken/Indonesia/NC/2009, A/Vietnam/1203/2004, A/Hubei/1/2010, A/Egypt/N03072/2010, A/Indonesia/5/2005, A/Anhui/1/2013, X-181, X-223A		*C4U* I504V	*C4U* M40L G180W	*C4U* R401K	I116L		A30P R118K	Vero	Viral titer	5.1~269
										HA titer	2.2~134
									MDCK	Viral titer	1.8~29
										HA titer	1.3~5.5
									Eggs	Viral titer	4.6~172
										HA titer	1.3~17.7
60	A/Chicken/Indonesia/NC/2009		C4U I504V	C4U M40L G180W	C4U R401K	I116L	*G1012C, A1013U, U1014A*	A30P R118K	Vero	Viral titer	2.8
										HA titer	1.2
									Egg	Viral titer	1.5
										HA titer	1.6

Note: 1. The HA and NA genes of #1-58 come from A/Chicken/Indonesia/NC/2009 (H5N1) or mutant HA and NA of A/Chicken/Indonesia/NC/2009.
2:All the H5N1 HAs used in this study were mutated to remove multibasic cleavage sites to create lowly pathogenicity mutants.

FIG. 19D

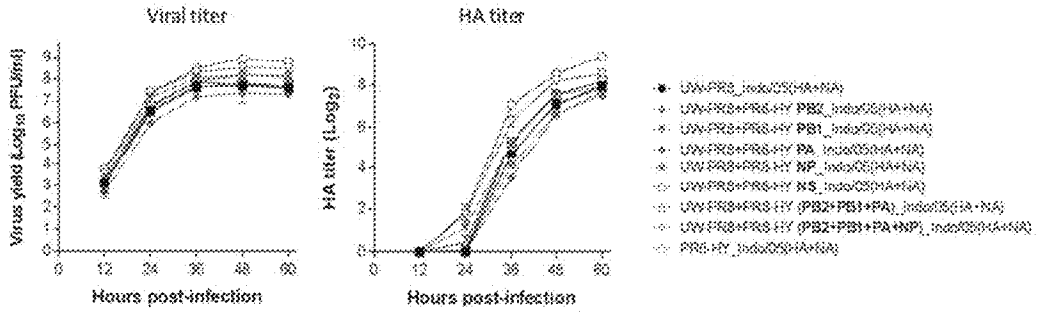

Viral titer
Virus yield (LOG10 PFU/ml)
Hours post-infection
HA titer
HA titer (LOG2)
Hours post-infection

Figure 20

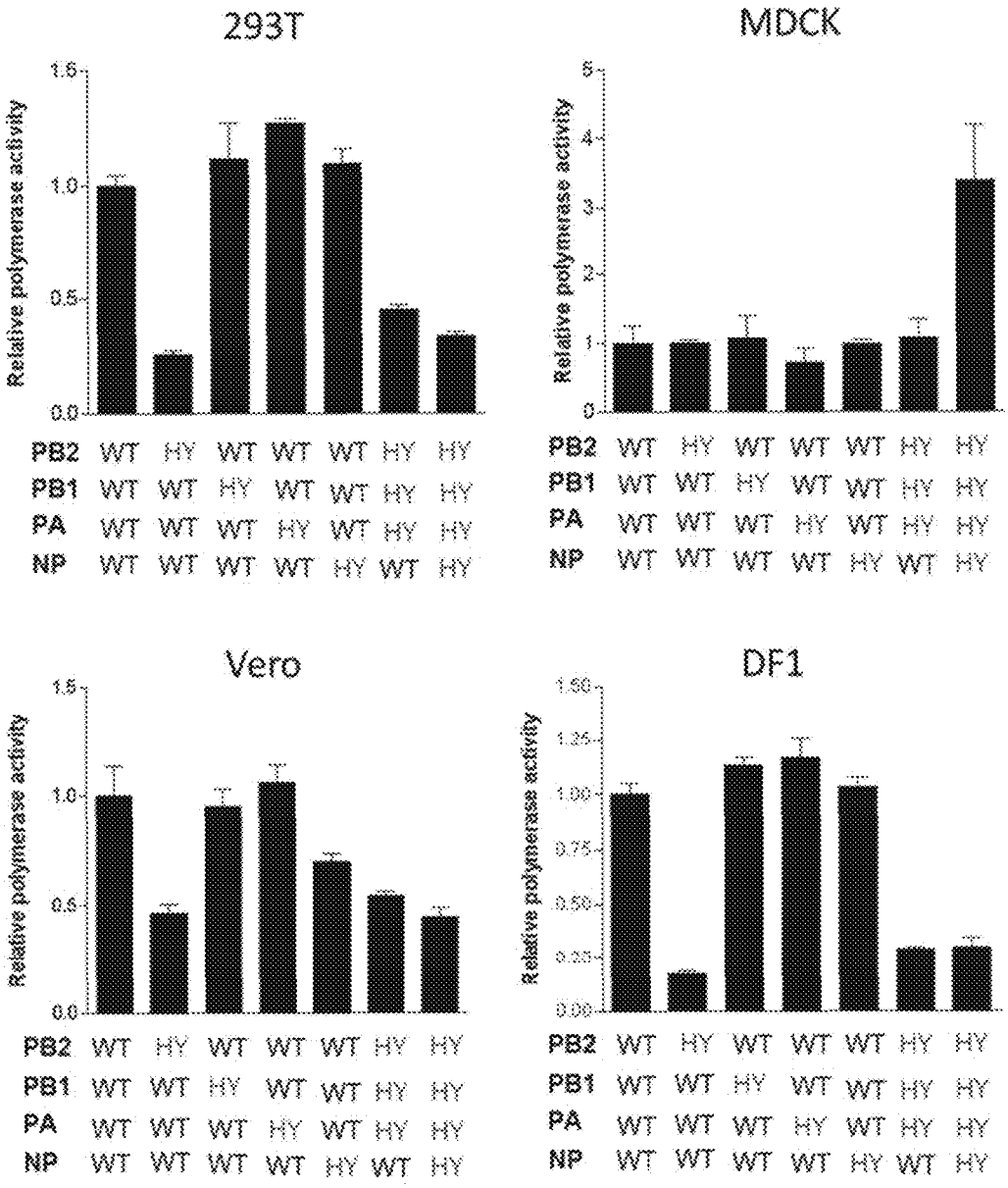

293T
MDCK
Vero
DF1
Relative polymerase activity
PB2 WT HY WT WT WT HY HY
PB1 WT WT HY WT WT HY HY
PA WT WT WT HY WT HY HY
NP WT WT WT WT HY WT HY

Figure 21

INFLUENZA VIRUS REPLICATION FOR VACCINE DEVELOPMENT

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation of and claims the benefit of priority to U.S. patent application Ser. No. 15/203,581, filed Jul. 6, 2016, which claims the benefit of the filing date of U.S. patent application Ser. No. 62/189,001, filed Jul. 6, 2015, the benefit of priority of each of which is claimed hereby, and which is incorporated by reference herein in its entirety.

STATEMENT OF GOVERNMENT RIGHTS

This invention was made with government support under HHSN272201400008C awarded by the National Institutes of Health. The government has certain rights in the invention.

BACKGROUND

Influenza is a major respiratory disease in some mammals including horses and is responsible for substantial morbidity and economic losses each year. In addition, influenza virus infections can cause severe systemic disease in some avian species, leading to death. The segmented nature of the influenza virus genome allows for reassortment of segments during virus replication in cells infected with two or more influenza viruses. The reassortment of segments, combined with genetic mutation and drift, can give rise to a myriad of divergent strains of influenza virus over time. The new strains exhibit antigenic variation in their hemagglutinin (HA) and/or neuraminidase (NA) proteins, and in particular the gene coding for the HA protein has a high rate of variability. The predominant current practice for the prevention of flu is vaccination. As the influenza HA protein is the major target antigen for the protective immune responses of a host to the virus and is highly variable, the isolation of influenza virus and the identification and characterization of the HA antigen in viruses associated with recent outbreaks is important for vaccine production. Based on prevalence and prediction, a vaccine is designed to stimulate a protective immune response against the predominant and expected influenza virus strains (Park et al., 2004).

There are three general types of influenza viruses, Type A, Type B and Type C, which are defined by the absence of serological crossreactivity between their internal proteins. Influenza Type A viruses are further classified into subtypes based on antigenic and genetic differences of their glycoproteins, the HA and NA proteins. Most of all the known HA and NA subtypes (H1 to H16 and N1 to N9) have been isolated from aquatic birds, which are though to act as a natural reservoir for influenza, although H17N10 and H18N11 were isolated from bats. The H1N1 pandemic virus caused a pandemic in 2009. The first vaccine candidates tested in 2009 did not grow to high titers, demonstrating the need to develop vaccine virus backbones that confer efficient replication to vaccine virus candidates.

SUMMARY OF THE INVENTION

Several strategies were employed (including random mutagenesis and the comprehensive testing of growth-enhancing mutations) to develop influenza A/Puerto Rico/8/34 (H1N1; the strain commonly used for the generation of inactivated influenza vaccines) viruses that replicate to high titers in cultured cells and/or embryonated chicken eggs. A number of growth-enhancing mutations were identified that increase the yield of influenza vaccine viruses. Individual growth-enhancing residues in an influenza virus polypeptide may be combined with one or more other growth-enhancing residues in the same influenza virus polypeptide, or with one or more other growth-enhancing residues in other influenza virus polypeptide(s), as well with growth-enhancing nucleotides in viral non-coding regions, e.g., promoter sequences. For example, one or more growth-enhancing residues in a polymerase protein, for instance, 1, 2, 3, 4, 5, 6, 7 or more, growth-enhancing residues in PB2, 1, 2, 3, 4, 5, 6, 7 or more, e.g., up to 12, 13, 14 or 15, growth-enhancing residues in PB1, 1, 2, 3, or 4 or more growth-enhancing residues in PA, or 1, 2, 3, or 4 growth-enhancing residues in NP, 1, 2, 3, or 4 growth-enhancing residues in M, e.g., 1, 2, or 3 growth-enhancing residues in M1, 1, 2, or 3 growth-enhancing residues in NS1, or any combination of growth-enhancing residues or nucleotides in viral non-coding, e.g., promoter sequences, may be combined when preparing influenza virus, e.g., for a vaccine, to enhance viral titers. In one embodiment, growth-enhancing nucleotides in viral promoter sequences may be introduced to a viral segment, or when present in a viral segment may be selected for inclusion in an influenza virus. In one embodiment, growth-enhancing residues in HA and/or in NA may be introduced into, or when present in a HA or NA selected for inclusion in, a HA viral segment or a NA viral segment in an influenza virus. In one embodiment, the one or more growth-enhancing residues may enhance viral growth by at least 1.2, 2, 2.8, 4, 3, 5, 6, 8, 10, 100, or 200 fold or more.

Mutations that increase the replicative ability of viruses in cell culture and/or embryonated chicken eggs are useful to amplify influenza viruses and to establish robust influenza vaccine platforms. Currently, most influenza vaccines are generated in embryonated chicken eggs. Influenza vaccines generated in MDCK cells are now approved for human use in the U.S. and in Europe, and influenza vaccines derived from Vero cells are approved for human use in Europe. Virus libraries possessing random mutations in the 'internal' viral genes (i.e., all viral genes except those encoding the viral surface glycoproteins HA and NA) of a vaccine virus isolate, e.g., UW-PR8, were generated and passaged in MDCK cells. The identified mutations result in higher virus titers in MDCK cells (and may also increase virus titers in Vero cells and/or embryonated chicken eggs), allowing more efficient influenza virus growth and more cost-effective vaccine production. Moreover, previously described mutations increased the replicative ability of UW-PR8 vaccine backbone virus. In addition to mutations in the coding regions of the six internal viral segments, mutations in non-coding regions were observed to increase viral titers, including promoter mutations, for instance, C-to-U mutations at position 4 from the 3' end of the PB2, PB1, and/or PA vRNA segments. The resulting sequences may be also codon-usage optimized, e.g., optimized for expression in mammalian cells such as canine cells or primate cells, or avian cells, e.g., chicken embryos. The mutations can be used in various combinations, with results influenced by the cell line (or egg) in use and the desired level of improvement in the replication of the virus.

The invention provides isolated recombinant, e.g., reassortant, influenza viruses with selected amino acid residues at one or more specified positions in one or more viral segments for PA, PB1, PB2, NP, M (encoding M1 and M2 proteins), and/or NS (encoding NS1 and NS2 proteins), e.g.,

in selected amino acid residues at specified positions of PB1. PB2 and NS1; PA, PB1, PB2, NP and NS1; PB1, PB2, NP, M, and NS1; PA, PB1, PB2, NP and NS1; or PA, PB1, PB2, NP, M, and NS1, and including HA and NA genes/proteins of interest, e.g., from annual and pandemic strains, which viruses are produced more efficiently and cost-effectively via cell culture (in MDCK or Vero cells) or in embryonated chicken eggs. As used herein, a "viral segment" in a virus means an influenza vRNA sequence and a "viral segment" in a transcription cassette for production of a viral segment means a sequence that when introduced into a cell or appropriate cell-free system and transcribed, yields influenza vRNA or cRNA. In one embodiment, the recombinant reassortant influenza virus has an amino acid residue at position 142 in PA that results in enhanced growth in cells including MDCK cells. Vero cells or eggs relative to a corresponding virus with, for instance, a lysine at position 142 in PA, i.e., the residue at position 142 in PA in the PA viral segment in the recombinant influenza virus is not lysine but is a residue that is correlated with enhanced replication in MDCK cells, Vero cells or eggs, as well as optionally selected amino acid residues at one or more specified positions in PB1, PB2, NP, M1 and/or NS1. In one embodiment, the recombinant reassortant influenza virus has an amino acid residue at position 142 in PA that results in enhanced interaction with one or more host proteins in MDCK cells, Vero cells or eggs relative to a corresponding virus with, for instance, a lysine at position 142 in PA. In one embodiment, the recombinant reassortant influenza virus has an asparagine or glutamine at position 142 in PA as well as optionally selected amino acid residues at one or more specified positions in PB1, PB2, NP, M1 and/or NS1. In one embodiment, the recombinant reassortant influenza virus has an amino acid residue at position 247 in PB1 that results in enhanced growth in cells including MDCK cells, Vero cells or eggs relative to a corresponding virus with, for instance, a glutamine at position 247 in PB1, i.e., the residue at position 247 in PB1 in the PB1 viral segment in the recombinant influenza virus is not glutamine but is a residue that is correlated with enhanced replication in MDCK cells, Vero cells or eggs, as well as optionally selected amino acid residues at one or more specified positions PA, PB2, NP, M1 and/or NS1 which have are described herein. In one embodiment, the recombinant reassortant influenza virus has an amino acid residue at position 247 in PB1 that results in enhanced interaction with one or more host proteins in MDCK cells, Vero cells or eggs relative to a corresponding virus with, for instance, a glutamine at position 247 in PB1. In one embodiment, the recombinant reassortant influenza virus has a histidine, arginine or lysine at position 247 in PB1 as well as optionally selected amino acid residues at one or more specified positions PA, PB2, NP, M1 and/or NS1 which are described herein. In one embodiment, the recombinant reassortant influenza virus has an amino acid residue at position 202 and/or position 323 in PB2 that results in enhanced growth in cells including MDCK cells, Vero cells or eggs relative to a corresponding virus with, for instance, a methionine at position 202 or a phenylalanine at position 323 in PB2, i.e., the residue at position 202 and/or 323 in PB2 in the PB2 viral segment in the recombinant influenza virus is not methionine or phenylalanine but is a residue that is correlated with enhanced replication in MDCK cells, Vero cells or eggs, as well as optionally selected amino acid residues at one or more specified positions PA, PB1, NP, M1 and/or NS which are described herein. In one embodiment, the recombinant reassortant influenza virus has an amino acid residue at position 323 in PB2 that results in an altered cap binding interaction relative to a corresponding virus with, for instance, a phenylalanine at position 323 in PB2. In one embodiment, the recombinant reassortant influenza virus has a leucine, alanine, threonine, valine, isoleucine, or glycine, at position 202 and/or position 323 in PB2 as well as optionally selected amino acid residues at one or more specified positions PA, PB1, NP, M1 and/or NS which are described herein. In one embodiment, the recombinant reassortant influenza virus has an amino acid residue at position 74 in NP that results in enhanced growth in cells including MDCK cells, Vero cells or eggs relative to a corresponding virus with, for instance, an arginine at position 74 in NP, i.e., the residue at position 74 in NP in the NP viral segment in the recombinant influenza virus is not arginine but is a residue that is correlated with enhanced replication in MDCK cells, Vero cells or eggs, as well as optionally selected amino acid residues at one or more specified positions PA, PB1, PB2, M1 and/or NS which are described herein. In one embodiment, the recombinant reassortant influenza virus has an amino acid residue at position 74 in NP that may alter folding, stability and/or interaction with other viral or host proteins relative to a corresponding virus with, for instance, an arginine at position 74 in NP. In one embodiment, the recombinant reassortant influenza virus has a lysine or histidine at position 74 in NP as well as optionally selected amino acid residues at one or more specified positions PA, PB1, PB2, M1 and/or NS which are described herein. In one embodiment, the recombinant reassortant influenza virus has an amino acid residue at position 97 and/or position 100 in M1 that results in enhanced growth in cells including MDCK cells. Vero cells or eggs relative to a corresponding virus with, for instance, a valine at position 97 or a tyrosine at position 100 in M1, i.e., the residue at position 97 and/or 100 in M1 in the M viral segment in the recombinant influenza virus is not valine or tyrosine, respectively, but is a residue that is correlated with enhanced replication in MDCK cells. Vero cells or eggs, as well as selected amino acid residues at one or more specified positions PA, PB1, PB2, NP and/or NS1 which are described herein. In one embodiment, the recombinant reassortant influenza virus has an amino acid residue at position 97 in M1 that may alter dimerization relative to a corresponding virus with, for instance, a valine at position 97 in M1. In one embodiment, the recombinant reassortant influenza virus has an amino acid residue at position 100 in M1 that may alter virus assembly relative to a corresponding virus with, for instance, a tyrosine at position 100 in M1. In one embodiment, the recombinant reassortant influenza virus has a leucine, threonine, isoleucine, alanine, or glycine, at position 97 and/or a lysine, arginine, or histidine at position 100 in M1 as well as selected amino acid residues at one or more specified positions PA, PB1, PB2, NP and/or NS1 which are described herein. In one embodiment, the recombinant reassortant influenza virus has an amino acid residue at position 55 in NS1 that results in enhanced growth in cells including MDCK cells, Vero cells or eggs relative to a corresponding virus with, for instance, a lysine at position 55 in NS1 as well as selected amino acid residues at one or more specified positions PA, PB1, PB2, NP and/or M1 which are described herein. In one embodiment, the recombinant reassortant influenza virus has an asparagine, aspartic acid, glutamic acid or glutamine at position 55 in NS1 as well as selected amino acid residues at one or more specified positions PA, PB1, PB2, NP and/or M1 which are described herein. In one embodiment, the invention provides an isolated recombinant reassortant influenza virus having six "internal" gene (viral) segments from a vaccine influenza virus with two or more of

the selected amino acid residues at specified positions described herein, and a NA viral segment selected from a first influenza virus isolate, and a HA viral segment from the same isolate or a different isolate.

In one embodiment, the influenza virus of the invention is a recombinant influenza virus having two or more of selected amino acid residues at specified positions in one or more viral segments for PA, PB1, PB2, NP, M1, and/or NS1, which can be employed with HA and NA genes of interest. In one embodiment, the recombinant reassortant influenza virus has an amino acid residue at position 142 in PA that results in enhanced growth in MDCK cells, Vero cells or eggs relative to a corresponding virus with, for instance, a lysine at position 142 in PA; an amino acid residue at position 247 in PB1 that results in enhanced growth in MDCK cells, Vero cells or eggs relative to a corresponding virus with, for instance, a glutamine at position 247 in PB1; an amino acid residue at position 202 and/or position 323 in PB2 that results in enhanced growth in MDCK cells, Vero cells or eggs relative to a corresponding virus with, for instance, a methionine at position 202 or a phenylalanine at position 323 in PB2; an amino acid residue at position 74 in NP that results in enhanced growth in MDCK cells, Vero cells or eggs relative to a corresponding virus with, for instance, a arginine at position 74 in NP; an amino acid residue at position 97 and/or position 100 in M1 that results in enhanced growth in MDCK cells, Vero cells or eggs relative to a corresponding virus with, for instance, a valine at position 97 or a tyrosine at position 100 in M1; or an amino acid residue at position 55 in NS1 that results in enhanced growth in MDCK cells, Vero cells or eggs relative to a corresponding virus with, for instance, a lysine at position 55 in NS1, or combinations thereof.

In one embodiment, the influenza virus of the invention is a recombinant influenza virus having two or more of selected amino acid residues at specified positions in one or more viral segments for PA, PB1, PB2, NP, M1, and/or NS1, which can be employed with HA and NA genes of interest. In one embodiment, the recombinant reassortant influenza virus has two or more of a lysine at position 142 in PA; a glutamine at position 247 in PB1; a leucine at position 202 and/or at position 323 in PB2; a lysine at position 74 in NP; an alanine at position 97 and an histidine at position 100 in M1; or a glutamic acid at position 55 in NS1.

The invention provides isolated recombinant, e.g., reassortant, influenza viruses with selected amino acid residues at one or more specified positions in one or more viral segments for PA, PB1, PB2, NP, M1, and/or NS1, e.g., in selected amino acid residues at specified positions PB1, PB2 and NS; PB1, PB2, NP and NS; PA, PB1, PB2, NP and NS; PB1, PB2, NP, M and NS; or PA, PB1, PB2, NP, M, and NS, that include one or more of the characteristic residues described herein. In one embodiment, the recombinant reassortant influenza virus has an amino acid residue at position 105 and/or 401 in PA that results in enhanced growth in cells, e.g., MDCK cells, relative to a corresponding virus with, for instance, a phenylalanine or arginine at position 105 or 401, respectively, in PA. In one embodiment, the recombinant reassortant influenza virus has an amino acid residue at position 40, 54, 59, 62, e.g., G62A, 63, 66 (F2), 73 (F2), 75, 76, 78, 79, 80, 112, 180, 261, 327, 361, 507, 621, 624, 644, 654, 667, 694, 695, 697, 699, 700, 701, 702, 705, 713, and/or 714 in PB1 that results in enhanced growth in cells, e.g., MDCK cells, relative to a corresponding virus with, for instance, a methionine, arginine, threonine, glycine, alanine, asparagine, lysine, glutamic acid, aspartic acid, glutamic acid, proline, serine, glutamic acid, glycine, serine, arginine, serine, methionine, glutamine, leucine, valine, asparagine, isoleucine, asparagine, leucine, glutamic acid, phenyalanine, phenylalanine, proline, serine, tyrosine, serine or methionine, at position 40, 54, 59, 62, 63, 66 (F2), 73 (F2), 75, 76, 78, 79, 80, 81(F2), 112, 180, 504, 507, 624, 644, 667, 694, 695, 697, 699, 700, 701, 702, 705, 713, or 714, respectively, in PB1. In one embodiment, the recombinant reassortant influenza virus has an amino acid residue at position 57, 58, 59, 61, 66, 202, 323, 368, 391, 504, 591, 677, 678, or 679 in PB2 that results in enhanced growth in cells, e.g., MDCK cells, relative to a corresponding virus with, for instance, an isoleucine, threonine, alanine, lysine, methionine, methionine, phenylalanine, arginine, glutamic acid, isoleucine, glutamine, glutamic acid, aspartic acid or phenylalanine, at position 57, 58, 59, 61, 66, 202, 323, 368, 391, 504, 591, 677, 678 or 679, respectively, in PB2. In one embodiment, the recombinant reassortant influenza virus has an amino acid residue at position 116, 224, 293, 371, 417, 422 or 442 in NP that results in enhanced growth in cells, e.g., MDCK cells, relative to a corresponding virus with, for instance, a leucine, asparagine, arginine, methionine, aspartic acid, arginine or threonine, at position 116, 224, 293, 371, 417, 422, or 442, respectively, in NP. In one embodiment, the recombinant reassortant influenza virus has an amino acid residue at position 90 in M1 that results in enhanced growth in cells relative to a corresponding virus with, for instance, a serine at position 90 in M1. In one embodiment, the recombinant reassortant influenza virus has an amino acid residue at position 30, 49, 118, 140, 161, or 223 in NS1 that results in enhanced growth in MDCK cells relative to a corresponding virus with, for instance, a proline, alanine, arginine, glutamine, threonine, or glutamic acid, respectively, at position 30, 49, 140, 161 or 223, respectively, in NS1. In one embodiment, the recombinant influenza virus does not have a valine at residue 504 in PB2 and a leucine at residue 550 in PA. In one embodiment, the recombinant reassortant influenza virus has an amino acid residue at position 136, 162, 179, 182, 184, 252, 449, and/or 476 (or any combination thereof) in HA (the numbers refer to the amino acid positions in H3 HA after removal of the N-terminal signal peptide) that results in enhanced growth in cells relative to a corresponding virus with, for instance, glutamic acid, lysine, glutamine, leucine, valine, phenylalanine, lysine or methionine at position 136, 162, 179, 182, 184, 252, 449, or 476 in HA. In one embodiment, the recombinant influenza virus has an amino acid residue at position 55 or 265, or both, in NA (the numbers refer to the amino acid positions in N1 NA) that results in enhanced growth in cells relative to a corresponding virus with, for instance, leucine or alanine at position 55 or 265, respectively, in NA.

In one embodiment, the influenza virus of the invention is a recombinant influenza virus having a particular amino acid residue at specified positions in one, two, three or more of PA, PB1, PB2, NP, M1 and/or NS1 and having an amino acid sequence with at least 80%, e.g., 90%, 92%, 95%, 97%, 98%, or 99%, including any integer between 80 and 99, contiguous amino acid sequence identity to a corresponding polypeptide encoded by one of SEQ ID Nos. 1-6 or 10-15, such as a polypeptide with a residue other than K142, S225, K356 or I550 in PA; other than E112. Q247, M507 or V644 in PB1; other than M202, F323 or I504 in PB2; other than R74, I112, I116, T442, or N417 in NP; other than V97 and/or Y100 in M1; and/or other than R140 or K55 in NS. The residue other than the specified residue may be conservative substitution. Conservative amino acid substitutions refer to the interchangeability of residues having similar side chains. For example, a group of amino acids having aliphatic side

chains is glycine, alanine, valine, leucine, and isoleucine; a group of amino acids having aliphatic-hydroxyl side chains is serine and threonine; a group of amino acids having amide-containing side chains is asparagine and glutamine; a group of amino acids having aromatic side chains is phenylalanine, tyrosine and tryptophan; a group of amino acids having basic side chains is lysine, arginine and histidine; and a group of amino acids having sulfur-containing side chain is cysteine and methionine. In one embodiment, conservative amino acid substitution groups are: threonine-valine-leucine-isoleucine-alanine; phenylalanine-tyrosine; lysine-arginine; alanine-valine; glutamic-aspartic; and asparagine-glutamine.

In one embodiment, the influenza virus of the invention is a recombinant influenza virus having a particular amino acid residue at specified positions in one, two, three or more of PB1, NS1, HA or NA which polypeptides have an amino acid sequence with at least 80%, e.g., 90%, 92%, 95%, 97%, 98%, or 99%, including any integer between 80 and 99, contiguous amino acid sequence identity to a corresponding polypeptide encoded by one of SEQ ID Nos. 2, 6 or 7-8, respectively, such as a polypeptide with a residue other than G62, S261, S361, Q621 or N654 in PB1; other than R118 in NS1; other than L55 or A265 in NA, or other than E136, K162, 0179, L182, V184, F252, K449 or M476 in HA. In one embodiment, the influenza virus of the invention is a recombinant influenza virus having a particular amino acid residue at specified positions in one or more of PA, PB1, PB2, NP, M1 and/or NS1 and an amino acid sequence with at least 80%, e.g., 90%, 92%, 95%, 97%, 98%, or 99%, including any integer between 80 and 99, contiguous amino acid sequence identity to a corresponding polypeptide encoded by one of SEQ ID Nos. 1-6 or 10-15, such as a polypeptide with a residue that is a conservative substitution relative to M202 in PB2, R74 in NP, and/or V97 in M1.

In one embodiment, the influenza virus of the invention is a recombinant influenza virus having a particular amino acid residue at specified positions in PA, PB1, PB2, NP, M1 and/or NS1 and an amino acid sequence with at least 80%, e.g., 90%, 92%, 95%, 97%, 98%, or 99%, including any integer between 80 and 99, contiguous amino acid sequence identity to a corresponding polypeptide encoded by one of SEQ ID Nos. 1-6 or 10-15, e.g., a polypeptide with a residue that is a non-conservative substitution relative to K142 in PA, Q247 in PB1, M202, F323 or I504 in PB2, R74 I112, I116, J442 or N417 in NP, V97 and/or Y100 in M1, and/or K55 or R140 in NS1.

In one embodiment, the influenza virus of the invention is a recombinant influenza virus having a particular amino acid residue at specified positions in PA, PB1, PB2, NP, M1 and/or NS1 and an amino acid sequence with at least 80%, e.g., 90%, 92%, 95%, 97%, 98%, or 99%, including any integer between 80 and 99, contiguous amino acid sequence identity to a corresponding polypeptide encoded by one of SEQ ID Nos. 1-6 or 10-15, e.g., a PB2 viral segment with a residue other than isoleucine and that is a conservative substitution for isoleucine at residue 504; a PB1 viral segment with a non-conservative substitution for E112; a PA viral segment with a substitution for S225; a NP viral segment with a conservative substitution for R74 and N417; a M viral segment with a conservative substitution for V97 and a non-conservative substitution for Y100; and a NS viral segment with a non-conservative substitution for K55.

In one embodiment, the influenza virus of the invention is a recombinant influenza virus having a particular amino acid residue at specified positions in PA, PB1, PB2, NP, M1 and/or NS1 and an amino acid sequence with at least 80%, e.g., 90%, 92%, 95%, 97%, 98%, or 99%, including any integer between 80 and 99, contiguous amino acid sequence identity to a corresponding polypeptide encoded by one of SEQ ID Nos. 1-6 or 10-15, e.g., a PB2 viral segment with a non-conservative substitution for M202 and F323; a PB1 viral segment with a non-conservative substitution for Q247; a PA viral segment with a non-conservative substitution for K142; a NP viral segment with a conservative substitution for R74; a M viral segment with a conservative substitution for V97 and a non-conservative substitution for Y100; and a NS viral segment with a conservative substitution for K55E.

In one embodiment, the influenza virus of the invention is a recombinant influenza virus having a particular amino acid residue at specified positions in PA, PB1, PB2, NP, M1 and/or NS1 and an amino acid sequence with at least 80%, e.g., 90%, 92%, 95%, 97%, 98%, or 99%, including any integer between 80 and 99, contiguous amino acid sequence identity to a corresponding polypeptide encoded by one of SEQ ID Nos. 1-6 or 10-15, e.g., a PB2 segment with a conservative substitution for I504; a PB1 segment with a conservative substitution for M40L and a non-conservative substitution for G180; a PA segment with a conservative substitution for R401; a NP segment with a conservative substitution for I116; a NS viral segment with a conservative substitution for A30 or R118.

In one embodiment, the influenza virus of the invention is a recombinant influenza virus having a particular amino acid residue at specified positions in one or more of PA, PB1, PB2, NP, M1 and/or NS1 and an amino acid sequence with at least 80%, e.g., 90%, 92%, 95%, 97% or 99%, including any integer between 80 and 99, contiguous amino acid sequence identity to a corresponding polypeptide encoded by one of SEQ ID Nos. 1-6 or 10-15, such as a polypeptide with a residue that is a non-conservative substitution relative to K142 in PA, Q247 in PB1, F323 in PB2, Y100 in M1, and/or K55 in NS1. In one embodiment, the amino acid residue that is replaced has an aliphatic side chain, amide-containing side chain, basic side chain, or sulfur containing side chain and the replacement of an aromatic side chain or acidic side chain (a nonconservative substitution). In one embodiment, the recombinant influenza virus has a residue that is a neutral or positively charged residue that is replaced with a polar or negatively charged residue.

Also included are any combination of the selected amino acid residues at specified positions described herein.

Viral segments for of PA, PB1, PB2, NP, M and/or NS that have the residues at the specified positions may be combined with a viral segment for HA. e.g., H1, H2, H3, H4, H5, H6, H7, H8, H9, H10, H11, H12, H13, H14, H15, H16, or H17 and a viral segment for NA, e.g., N1, N2, N3, N4, N5, N6, N7, N8, N9, or N10, and any combination of HA and NA, to provide the reassortant vaccine viruses of the invention. In one embodiment, the HA is H1, H5 or H7. In one embodiment the NA is N1 or N9. In one embodiment, the HA viral segment in the reassortant virus is heterologous to the viral segments for PA, PB1, PB2, NP, M and NS. In one embodiment, the NA viral segment in the reassortant virus is heterologous to the viral segments for PA, PB1, PB2, NP, M and NS. In one embodiment, the HA viral segment in the reassortant virus has viral segments for PA, PB1, PB2, NP, M and NS from one influenza virus isolate or strain ("parent"), or a variant thereof, e.g., one with viral segments encoding influenza virus proteins with at least 95%, 96%, 97%, 98%, 99%, or 99.5% amino acid sequence identity, or having 1, 2, 5, 10, or 20 substitutions relative, to sequences in a parent influenza virus isolate or strain. In one embodi-

ment, the parent strain has viral segments with sequences corresponding to SEQ ID Nos. 1-6 or 10-15. In one embodiment, the HA viral segment in the reassortant virus is a chimeric HA viral segment, e.g., a chimera of heterologous HA ectodomain sequences linked to HA signal peptide sequences and/or HA transmembrane domain sequences from the HA viral segment of the parent isolate or strain, or variant thereof. In one embodiment, the NA viral segment in the isolated recombinant virus is a chimeric NA viral segment e.g., a chimera of heterologous NA ectodomain sequences linked to NA transmembrane domain sequences from the NA viral segment of the parent isolate or strain, or variant thereof, and/or stalk sequences from the parent isolate or strain, or variant thereof. In one embodiment, the NA viral segment in the isolated recombinant virus is a chimeric NA viral segment e.g., a chimera of heterologous NA ectodomain sequences linked to NA transmembrane domain sequences from the NA viral segment of the parent isolate or strain, or variant thereof, and/or stalk sequences from a second isolate or strain, or variant thereof. In one embodiment, the isolated recombinant virus has a heterologous HA viral segment, a heterologous NA viral segment, a chimeric HA viral segment, a chimeric NA viral segment, or any combination thereof. The nucleic acid sequences employed to prepare vRNA may be ones that introduce the residues at the specified positions via recombinant methodology or may be selected as having the residues at the specified positions.

A/Puerto Rico/8/34 (HIN1), "PR8," virus serves as the genetic backbone for generation of inactivated influenza vaccines. Occasionally, vaccine strains based on PR8 backbone replicate to relatively low titers in eggs and cell culture resulting in delayed vaccine production and vaccine shortage. To determine if high yield vaccine strain backbones for propagation in MDCK cells, chicken eggs and Vero cells can be prepared to supply the demand of seasonal flu and highly pathogenic pandemic viruses, various mutagenesis strategies were employed. For example, PR8 backbone random mutant libraries were screened for high replicative mutants, e.g., by introducing random mutations to internal PR8 genes by error prone PCR, introducing mutations that confer high replication and high polymerase activity, and optimizing PR8 internal gene via codon bias. In another approach, the HA gene was optimized to increase virus replication and HA content, e.g., by optimizing the HA promoter to generate a strong promoter, optimizing the HA noncoding region, and/or optimizing the HA signal peptide.

As described herein, an influenza virus isolate useful as a vaccine virus (e.g., A/Puerto Rico/8/34, "PR8," including a specific isolate such as UW-PR8) to carry heterologous viral segments for NA and/or HA, was serially passaged in MDCK cells, e.g., about 10-12-times although fewer passages may be employed, to obtain virus with enhanced replication in those cells. In one embodiment, viruses obtained after serial passage which have enhanced replication, have titers that are at least 1 or 2 logs higher than viruses that were not serially passaged. In one embodiment, viruses obtained after serial passage had substitutions in two or more internal viral segments relative to the parent virus.

Thus, for vaccine viruses that are to be grown or passaged in cells in culture, e.g., MDCK or Vero cells or eggs, selection of sequences with, or replacement of, the disclosed residues at the specified positions in one or more of PA, PB1, PB2, NP, M1 and/or NS1, that confer enhanced growth of the virus in cultured cells when employed with HA and NA sequences of interest, can result in significantly higher viral titers. Thus, the invention provides a method to select for influenza viruses with enhanced replication in cell culture. The method includes providing cells suitable for influenza vaccine production; serially culturing one or more influenza virus isolates in the cells; and isolating serially cultured virus with enhanced growth relative to the one or more isolates prior to serial culture. In one embodiment, the cells are canine or primate, e.g., human or monkey, cells.

In one embodiment, the influenza virus of the invention is a recombinant influenza virus having two or more of selected amino acid residues at specified positions in one or more of PA, PB1, PB2, NP, M1, and/or NS1, which can be employed with HA and NA genes of interest. In one embodiment, the recombinant reassortant influenza virus has an asparagine or glutamine at position 142 in PA, a cysteine at position 225, an arginine or histidine at position 356 in PA, or a leucine, valine, threonine, or glycine at position 550 in PA; a histidine, arginine or lysine at position 247 in PB1, a valine, leucine, isoleucine, threonine, alanine or glycine at position 507 in PB1 and/or an alanine, glycine, leucine or isoleucine at position 644 in PB1; a leucine, alanine, valine, isoleucine, glycine, or threonine at position 202 and/or position 323 in PB2, or a valine, leucine, glycine, threonine, or alanine at position 504 in PB2; a lysine or a histidine at position 74 in NP or a leucine, valine, glycine or alanine at position 112, 116 or 442 in NP; a leucine, isoleucine, alanine, glycine, or threonine, at position 97 and/or a lysine, arginine or histidine position 100 in M1; or an asparagine, aspartic acid, glutamic acid or glutamine at position 55 or glutamine or asparagine at position 140 in NS1.

The invention provides a plurality of influenza virus vectors of the invention, e.g., those useful to prepare reassortant viruses including 6:1:1 reassortants, 6:2 reassortants and 7:1 reassortants. A 6:1:1 reassortant within the scope of the present invention is an influenza virus with 6 internal viral segments from a vaccine virus, a NA viral segment from a different (second) viral isolate, and a HA viral segment from a third isolate; a 6:2 reassortant within the scope of the present invention is an influenza virus with 6 internal viral segments from a vaccine virus, and a NA viral segment and a HA viral segment from a different (second) viral isolate; and a 7:1 reassortant within the scope of the present invention is an influenza virus with 6 internal viral segments and a NA viral segment from a vaccine virus, and a HA viral segment from a different viral source than the vaccine virus, or an influenza virus with 6 internal viral segments and a HA viral segment from the vaccine virus, and a NA viral segment is from a different viral source than the vaccine virus.

In one embodiment of the invention, the plurality includes vectors for vRNA production selected from a vector comprising a promoter operably linked to an influenza virus PA DNA linked to a transcription termination sequence, a vector comprising a promoter operably linked to an influenza virus PB1 DNA linked to a transcription termination sequence, a vector comprising a promoter operably linked to an influenza virus PB2 DNA linked to a transcription termination sequence, a vector comprising a promoter operably linked to an influenza virus HA DNA linked to a transcription termination sequence, a vector comprising a promoter operably linked to an influenza virus NP DNA linked to a transcription termination sequence, a vector comprising a promoter operably linked to an influenza virus NA DNA linked to a transcription termination sequence, a vector comprising a promoter operably linked to an influenza virus M DNA linked to a transcription termination sequence, and a vector comprising a operably linked to an influenza virus NS DNA linked to a transcription termination sequence. In one

embodiment, the DNAs for vRNA production of PB1, PB2, PA, NP, M, and NS, have sequences from an influenza virus that replicates to high titers in cultured mammalian cells such as MDCK cells, Vero cells or PER.C6® cells and also optionally embryonated eggs, and/or from a vaccine virus, e.g., one that does not cause significant disease in humans. The DNA for vRNA production of NA may be from any NA, e.g., any of N1-N10, and the DNA for vRNA production of HA may be from any HA, e.g., H1-H17. In one embodiment, the DNAs for vRNA production may be for an influenza B or C virus. The DNAs for vRNA production of NA and HA may be from different strains or isolates (6:1:1 reassortants) or from the same strain or isolate (6:2 reassortants), or the NA may be from the same strain or isolate as that for the internal genes (7:1 reassortant). The plurality also includes vectors for mRNA production selected from a vector encoding influenza virus PA, a vector encoding influenza virus PB1, a vector encoding influenza virus PB2, and a vector encoding influenza virus NP, and optionally one or more vectors encoding NP, NS, M, e.g., M1 and M2, HA or NA. The vectors encoding viral proteins may further include a transcription termination sequence.

Viruses that may provide the internal genes for reassortants within the scope of the invention include viruses that have high titers in MDCK cells, e.g., titers of at least about 10^5 PFU/mL, e.g., at least 10 PFU/mL, 10^7 PFU/mL or 10^8 PFU/mL; high titers in embryonated eggs, e.g., titers of at least about 10^7 EID_{50}/mL, e.g., at least 10^8 EID_{50}/mL, 10^9 EID_{50}/mL or 10^{10} EID_{50}/mL; high titers in cells such as MDCK cells, e.g., titers of at least about 10^7 PFU/mL, e.g., at least 10^8 PFU/mL, or high titers in two of more of those host cells.

In one embodiment, the titers of the reassortant viruses of the invention in cells such as MDCK cells or Vero cells may be over 1 log, 2 logs, 3 logs, or greater, than titers of the corresponding virus without particular residues at the specified positions.

Other reassortants with internal genes from other PR8 isolates or vaccine viruses may be employed in recombinant reassortant viruses of the invention. In particular, 5:1:2 reassortants having UW-PR8 PB1, PB2, PA, NP, and M ("5") and PR8(Cam) NS ("1"); 6:1:1 reassortants having UW-PR8 NA, PB1, PB2, PA, NP, and M ("6") and PR8 (Cam) NS ("1"); and 7:1 reassortants having UW-PR8 PB1, PB2, PA, NP, M. NA, and NS ("7") may be employed.

In one embodiment, the DNAs for the internal genes for PB1, PB2, PA, NP, M, and NS encode proteins with substantially the same activity as a corresponding polypeptide encoded by one of SEQ ID NOs:1-6 or 10-15. As used herein, "substantially the same activity" includes an activity that is about 0.1%, 1%, 10%, 30%, 50%, 90%, e.g., up to 100% or more, or detectable protein level that is about 80%, 90% or more, the activity or protein level, respectively, of the corresponding full-length polypeptide. In one embodiment, the nucleic acid a sequence encoding a polypeptide which is substantially the same as, e.g., having at least 80%, e.g., 90%, 92%, 95%, 97%, 98%, or 99%, including any integer between 80 and 99, contiguous amino acid sequence identity to, a polypeptide encoded by one of SEQ ID NOs:1-6 or 10-15. In one embodiment, the isolated and/or purified nucleic acid molecule comprises a nucleotide sequence which is substantially the same as, e.g., having at least 50%, e.g., 60%, 70%, 80% or 90%, including any integer between 50 and 100, or more contiguous nucleic acid sequence identity to one of SEQ ID NOs:1-6 or 10-15 and, in one embodiment, also encodes a polypeptide having at least 80%, e.g., 90%, 92%, 95%, 97%, 98%, or 99%, including any integer between 80 and 99, contiguous amino acid sequence identity to a polypeptide encoded by one of SEQ ID NOs:1-6 or 10-15. In one embodiment, the influenza virus polypeptide has one or more, for instance, 2, 5, 10, 15, 20 or more, conservative amino acids substitutions, e.g., conservative substitutions of up to 10% or 20% of 2, 5, 10, 15, 20 or more, of a combination of conservative and non-conservative amino acids substitutions, e.g., conservative substitutions of up to 10% or 20% of the residues, or relative to a polypeptide encoded by one of SEQ IS NOs:1-6 or 10-15, and has a characteristic residue in two or more of PA, PB1, PB2, NP, M1, and/or NS1 the residues, relative to a polypeptide encoded by one of SEQ ID NOs:1-6 or 10-15, and has a characteristic residue in two or more of the viral segments for PA, PB1, PB2, NP, M1, and/or NS1, e.g., there is an asparagine or glutamine at position 142 in PA; a histidine, arginine or lysine at position 247 in PB1; a leucine, alanine, valine, isoleucine, glycine, or serine at position 202 and/or position 323 in PB2; a lysine or a histidine at position 74 in NP; a leucine, isoleucine, alanine, glycine, or serine at position 202 and/or a lysine, arginine, or histidine position 100 in M1; or an asparagine, aspartic acid, glutamic acid or glutamine at position 44 in NS1. In one embodiment, the influenza virus polypeptide has one or more, for instance, 2, 3, 4, 5, 6, 7 or 8 conservative and/or nonconservative amino acid substitutions, relative to a polypeptide encoded by one of SEQ ID NOs:1-6 or 10-15, e.g., those in virus isolates 1, 4, 36, 38, P17, P25 or P61 in Table 4.

The invention thus includes the use of isolated and purified vectors or plasmids, which express or encode influenza virus proteins, or express or encode influenza vRNA, both native and recombinant vRNA. The vectors comprise influenza cDNA, e.g., influenza A (e.g., any influenza A gene including any of the 16 HA or 9 NA subtypes), B or C DNA (see Fields Virology (Fields et al. (eds.), Lippincott, Williams and Wickens (2006), which is specifically incorporated by reference herein). Any suitable promoter or transcription termination sequence may be employed to express a protein or peptide, e.g., a viral protein or peptide, a protein or peptide of a nonviral pathogen, or a therapeutic protein or peptide.

A composition or plurality of vectors of the invention may also comprise a heterologous gene or open reading frame of interest, e.g., a foreign gene encoding an immunogenic peptide or protein useful as a vaccine or in gene replacement, for instance, may encode an epitope useful in a cancer therapy or vaccine, or a peptide or polypeptide useful in gene therapy. When preparing virus, the vector or plasmid comprising the gene or cDNA of interest may substitute for a vector or plasmid for an influenza viral gene or may be in addition to vectors or plasmids for all influenza viral genes. Thus, another embodiment of the invention comprises a composition or plurality of vectors as described above in which one of the vectors is replaced with, or further comprises, 5' influenza virus sequences optionally including 5' influenza virus coding sequences or a portion thereof, linked to a desired nucleic acid sequence, e.g., a desired cDNA, linked to 3' influenza virus sequences optionally including 3' influenza virus coding sequences or a portion thereof. In one embodiment, the desired nucleic acid sequence such as a cDNA is in an antisense (antigenomic) orientation. The introduction of such a vector in conjunction with the other vectors described above to a host cell permissive for influenza virus replication results in recombinant virus comprising vRNA corresponding to the heterologous sequences of the vector.

The promoter in a vector for vRNA production may be a RNA polymerase I promoter, a RNA polymerase II promoter, a RNA polymerase III promoter, a T7 promoter, or a T3 promoter, and optionally the vector comprises a transcription termination sequence such as a RNA polymerase I transcription termination sequence, a RNA polymerase II transcription termination sequence, a RNA polymerase III transcription termination sequence, or a ribozyme. Ribozymes within the scope of the invention include, but are not limited to, tetrahymena ribozymes, RNase P, hammerhead ribozymes, hairpin ribozymes, hepatitis ribozyme, as well as synthetic ribozymes. In one embodiment, the RNA polymerase I promoter is a human RNA polymerase I promoter.

The promoter or transcription termination sequence in a vRNA or virus protein expression vector may be the same or different relative to the promoter or any other vector. In one embodiment, the vector or plasmid which expresses influenza vRNA comprises a promoter suitable for expression in at least one particular host cell, e.g., avian or mammalian host cells such as canine, feline, equine, bovine, ovine, or primate cells including human cells, or for expression in more than one host.

In one embodiment, at least one vector for vRNA comprises a RNA polymerase II promoter linked to a ribozyme sequence linked to viral coding sequences linked to another ribozyme sequences, optionally linked to a RNA polymerase II transcription termination sequence. In one embodiment, at least 2, e.g., 3, 4, 5, 6, 7 or 8, vectors for vRNA production comprise a RNA polymerase II promoter, a first ribozyme sequence, which is 5' to a sequence corresponding to viral sequences including viral coding sequences, which is 5' to a second ribozyme sequence, which is 5' to a transcription termination sequence. Each RNA polymerase II promoter in each vRNA vector may be the same or different as the RNA polymerase II promoter in any other vRNA vector. Similarly, each ribozyme sequence in each vRNA vector may be the same or different as the ribozyme sequences in any other vRNA vector. In one embodiment, the ribozyme sequences in a single vector are not the same.

In one embodiment, the invention provides a plurality of influenza virus vectors for a reassortant, comprising a vector for vRNA production comprising a promoter operably linked to an influenza virus PA DNA linked to a transcription termination sequence, a vector for vRNA production comprising a promoter operably linked to an influenza virus PB1 DNA linked to a transcription termination sequence, a vector for vRNA production comprising a promoter operably linked to an influenza virus PB2 DNA linked to a transcription termination sequence, a vector for vRNA production comprising a promoter operably linked to an influenza virus HA DNA linked to a transcription termination sequence, a vector for vRNA production comprising a promoter operably linked to an influenza virus NP DNA linked to a transcription termination sequence, a vector for vRNA production comprising a promoter operably linked to an influenza virus NA DNA linked to a transcription termination sequence, a vector for vRNA production comprising a promoter operably linked to an influenza virus M DNA linked to a transcription termination sequence, and a vector for vRNA production comprising a promoter operably linked to an influenza virus NS cDNA linked to a transcription termination sequence, wherein the DNAs for PB1, PB2, PA, NP, NS, and M are from one or more influenza vaccine seed viruses and contain two or more of the characteristic residues at the specified position(s); and a vector for mRNA production comprising a promoter operably linked to a DNA segment encoding influenza virus PA, a vector for mRNA production comprising a promoter operably linked to a DNA segment encoding influenza virus PB1, a vector for mRNA production comprising a promoter operably linked to a DNA segment encoding influenza virus PB2, and a vector for mRNA production comprising a promoter operably linked to a DNA segment encoding influenza virus NP, and optionally a vector for mRNA production comprising a promoter operably linked to a DNA segment encoding influenza virus HA, a vector for mRNA production comprising a promoter operably linked to a DNA segment encoding influenza virus NA, a vector for mRNA production comprising a promoter operably linked to a DNA segment encoding influenza virus M1, a vector for mRNA production comprising a promoter operably linked to a DNA segment encoding influenza virus M2, or a vector for mRNA production comprising a promoter operably linked to a DNA segment encoding influenza virus NS2. In one embodiment, at least one vector comprises sequences corresponding to those encoding PB1, PB2, PA, NP, M, or NS, or a portion thereof, having substantially the same activity as a corresponding polypeptide encoded by one of SEQ ID NOs:1-6 or 10-15, e.g., a sequence encoding a polypeptide with at least 80%, e.g., 85%, 90%, 92%, 95%, 98%, 99% or 100%, including any integer between 80 and 100, amino acid identity to a polypeptide encoded by one of SEQ ID NOs:1-6 or 10-15. Optionally, two vectors may be employed in place of the vector comprising a promoter operably linked to an influenza virus M cDNA linked to a transcription termination sequence, e.g., a vector comprising a promoter operably linked to an influenza virus M1 cDNA linked to a transcription termination sequence and a vector comprising a promoter operably linked to an influenza virus M2 cDNA linked to a transcription termination sequence.

A plurality of the vectors of the invention may be physically linked or each vector may be present on an individual plasmid or other, e.g., linear, nucleic acid delivery vehicle. In one embodiment, each vRNA production vector is on a separate plasmid. In one embodiment, each mRNA production vector is on a separate plasmid.

The invention also provides a method to prepare influenza virus. The method comprises contacting a cell with a plurality of the vectors of the invention, e.g., sequentially or simultaneously, in an amount effective to yield infectious influenza virus. The invention also includes isolating virus from a cell contacted with the plurality of vectors. Thus, the invention further provides isolated virus, as well as a host cell contacted with the plurality of vectors or virus of the invention. In another embodiment, the invention includes contacting the cell with one or more vectors, either vRNA or protein production vectors, prior to other vectors, either vRNA or protein production vectors. In one embodiment, the promoter for vRNA vectors employed in the method is a RNA polymerase I promoter, a RNA polymerase II promoter, a RNA polymerase III promoter, a T3 promoter or a T7 promoter. In one embodiment, the RNA polymerase I promoter is a human RNA polymerase I promoter. In one embodiment, each vRNA vector employed in the method is on a separate plasmid. In one embodiment, the vRNA vectors employed in the method are on one plasmid or on two or three different plasmids. In one embodiment, each mRNA vector employed in the method is on a separate plasmid. In one embodiment, the mRNA vectors for PA, PB1, PB2 and NP employed in the method are on one plasmid or on two or three different plasmids.

In one embodiment, the invention provides a method to select for influenza viruses with enhanced replication in cell culture. The method includes providing cells suitable for

influenza vaccine production; serially culturing one or more influenza virus isolates in the cells; and isolating serially cultured virus with enhanced growth relative to the one or more isolates prior to serial culture. In one embodiment, the cells are rodent or primate cells.

The methods of producing virus described herein, which do not require helper virus infection, are useful in viral mutagenesis studies, and in the production of vaccines (e.g., for AIDS, influenza, hepatitis B, hepatitis C, rhinovirus, filoviruses, malaria, herpes, and foot and mouth disease) and gene therapy vectors (e.g., for cancer, AIDS, adenosine deaminase, muscular dystrophy, ornithine transcarbamylase deficiency and central nervous system tumors). Thus, a virus for use in medical therapy (e.g., for a vaccine or gene therapy) is provided.

The invention also provides isolated viral polypeptides, and methods of preparing and using recombinant virus of the invention. The methods include administering to a host organism, e.g., a mammal, an effective amount of the influenza virus of the invention, e.g., an inactivated virus preparation, optionally in combination with an adjuvant and/or a carrier, e.g., in an amount effective to prevent or ameliorate infection of an animal such as a mammal by that virus or an antigenically closely related virus. In one embodiment, the virus is administered intramuscularly while in another embodiment, the virus is administered intranasally. In some dosing protocols, all doses may be administered intramuscularly or intranasally, while in others a combination of intramuscular and intranasal administration is employed. The vaccine may further contain other isolates of influenza virus including recombinant influenza virus, other pathogen(s), additional biological agents or microbial components, e.g., to form a multivalent vaccine. In one embodiment, intranasal vaccination, for instance containing with inactivated influenza virus, and a mucosal adjuvant may induce virus-specific IgA and neutralizing antibody in the nasopharynx as well as serum IgG.

The influenza virus of the invention may employed with other anti-virals, e.g., amantadine, rimantadine, and/or neuraminidase inhibitors, e.g., may be administered separately in conjunction with those anti-virals, for instance, administered before, during and/or after.

BRIEF DESCRIPTION OF THE FIGURES

FIGS. **1**A-**1**E. Nucleotide sequence for PR8(Cambridge) genes (SEQ ID NOs:10-15).

FIG. **2**: Overview of library passages and the identification of high-yield candidates.

FIG. **3**. Number of clones with random mutations having specified HA titers.

FIG. **4**. Titers of clones having selected mutations.

FIGS. **5**A, **5**B, **5**C and **5**D. Growth curves of UW-PR8 viruses possessing previously identified mutations in PB2 (A), PB1 (B), PA (C), and NP, M or NS1 (D).

FIG. **6**. Summary of mutations that confer high replicative property in MDCK cells.

FIGS. **7**A and **7**B. A) Virus stocks were tested for HA titers (in 2^n) and virus titers (in PFU/mL). B) Growth curves in MDCK cells.

FIGS. **8**A, **8**B and **8**C. A) HA titer of wild type (UW-PR8) and clone #4. B) Viral protein for wild type (UW-PR8) and #4. C) SDS-PAGE analysis of viral proteins of wild type and #4.

FIGS. **9**A and **9**B. A) Comparison of titers of wild type virus (UW-PR8) and high replicative virus with mutations in M1. B) Growth kinetics of wild type virus (UW-PR8) and high replicative virus with mutations in M1.

FIGS. **10**A, **10**B, **10**C, **10**D, **10**E, **10**F, **10**G, **10**H, **10**I, **10**J, **10**K, **10**L and **10**M. A) Codon usage table for canines. B) Relative adaptiveness of wild type (UW-PR8) and "rare" codon optimized PB2 viruses. C) Relative adaptiveness of wild type (UW-PR8) and "all" codon optimized PB2 viruses. D) Growth kinetics of PB2 codon optimized viruses. E) Growth kinetics of viruses with codon optimized PB2, PB1, PA, or NP viral segment or combinations of segments. F-M) Sequence of PB2, PB1, PA and NP viral segments of UW-PR8 and sequence of canine codon-usage optimized PB2, PB1, PA and NP viral segments of UW-PR8 (SEQ ID NOs:3, 16, 2, 17, 1, 18, 4, 19).

FIGS. **11**A, **11**B and **11**C. A) Nucleotide position 4 of each gene of PR8 and Indo/NC/09. B) All 3'C4U mutant. C) Growth kinetics of a recombinant UW-PR8 virus encoding 'C' at position 4 of the PB2, PB1, and PA genes (black), and a mutant encoding 'U' at position 4 of all eight segments (red).

FIG. **12**A-C. Nucleotide and amino acid sequences for H7 and N9 which are exemplary sequences for use with the internal viral segment sequences disclosed herein useful to provide high titer influenza viruses for vaccines (SEQ ID NOs: 20-27).

FIGS. **13**A and **13**B. A) Schematic of chimeric HA and NA genes to increase virus titer. B) Growth kinetics of chimeric viruses.

FIGS. **14**A and **14**B. A) Growth kinetics of viruses with combinations of mutations. B) PFU and HA titers of viruses with combinations of mutations.

FIG. **15**. Screening in eggs.

FIG. **16**. HA titers of 216 clones isolated from Vero cells.

FIG. **17**. Recombinant viruses generated with different PR8 backbone mutations.

FIGS. **18**A and **18**B. Overview of generation of viruses with enhanced growth in MDCK cells and Vero cells.

FIGS. **19**A, **19**B, **19**C and **19**D. Exemplary high yield substitutions (relative to PR8 (UW)).

FIG. **20**. Growth kinetics and HA titers of reassortant viruses possessing one or several vRNAs of PR8-HY virus.

FIG. **21**. Viral polymerase activity in mini-replicon assays in 293T, MDCK, Vero, and DF1 cells. The PB2, PB1, PA, and NP proteins were derived from UW-PR8 wild-type (WT) virus or from the high-yield PR8-HY (HY) variant.

DETAILED DESCRIPTION

Definitions

As used herein, the term "isolated" refers to in vitro preparation and/or isolation of a nucleic acid molecule, e.g., vector or plasmid, peptide or polypeptide (protein), or virus of the invention, so that it is not associated with in vivo substances, or is substantially purified from in vitro substances. An isolated virus preparation is generally obtained by in vitro culture and propagation, and/or via passage in eggs, and is substantially free from other infectious agents.

As used herein, "substantially purified" means the object species is the predominant species, e.g., on a molar basis it is more abundant than any other individual species in a composition, and preferably is at least about 80% of the species present, and optionally 90% or greater, e.g., 95%, 98%, 99% or more, of the species present in the composition.

As used herein, "substantially free" means below the level of detection for a particular infectious agent using standard detection methods for that agent.

A "recombinant" virus is one which has been manipulated in vitro, e.g., using recombinant DNA techniques, to introduce changes to the viral genome. Reassortant viruses can be prepared by recombinant or nonrecombinant techniques.

As used herein, the term "recombinant nucleic acid" or "recombinant DNA sequence or segment" refers to a nucleic acid, e.g., to DNA, that has been derived or isolated from a source, that may be subsequently chemically altered in vitro, so that its sequence is not naturally occurring, or corresponds to naturally occurring sequences that are not positioned as they would be positioned in the native genome. An example of DNA "derived" from a source, would be a DNA sequence that is identified as a useful fragment, and which is then chemically synthesized in essentially pure form. An example of such DNA "isolated" from a source would be a useful DNA sequence that is excised or removed from said source by chemical means, e.g., by the use of restriction endonucleases, so that it can be further manipulated, e.g., amplified, for use in the invention, by the methodology of genetic engineering.

As used herein, a "heterologous" influenza virus gene or viral segment is from an influenza virus source that is different than a majority of the other influenza viral genes or viral segments in a recombinant, e.g., reassortant, influenza virus.

The terms "isolated polypeptide", "isolated peptide" or "isolated protein" include a polypeptide, peptide or protein encoded by cDNA or recombinant RNA including one of synthetic origin, or some combination thereof.

The term "recombinant protein" or "recombinant polypeptide" as used herein refers to a protein molecule expressed from a recombinant DNA molecule. In contrast, the term "native protein" is used herein to indicate a protein isolated from a naturally occurring (i.e., a nonrecombinant) source. Molecular biological techniques may be used to produce a recombinant form of a protein with identical properties as compared to the native form of the protein.

Methods of alignment of sequences for comparison are well known in the art. Thus, the determination of percent identity between any two sequences can be accomplished using a mathematical algorithm.

Computer implementations of these mathematical algorithms can be utilized for comparison of sequences to determine sequence identity. Alignments using these programs can be performed using the default parameters. Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). The algorithm may involve first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence, which either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold. These initial neighborhood word hits act as seeds for initiating searches to find longer HSPs containing them. The word hits are then extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Cumulative scores are calculated using, for nucleotide sequences, the parameters M (reward score for a pair of matching residues; always >0) and N (penalty score for mismatching residues; always <0). For amino acid sequences, a scoring matrix is used to calculate the cumulative score. Extension of the word hits in each direction are halted when the cumulative alignment score falls off by the quantity X from its maximum achieved value, the cumulative score goes to zero or below due to the accumulation of one or more negative-scoring residue alignments, or the end of either sequence is reached.

In addition to calculating percent sequence identity, the BLAST algorithm may also perform a statistical analysis of the similarity between two sequences. One measure of similarity provided by the BLAST algorithm may be the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. For example, a test nucleic acid sequence is considered similar to a reference sequence if the smallest sum probability in a comparison of the test nucleic acid sequence to the reference nucleic acid sequence is less than about 0.1, more preferably less than about 0.01, and most preferably less than about 0.001.

The BLASTN program (for nucleotide sequences) may use as defaults a wordlength (W) of 11, an expectation (E) of 10, a cutoff of 100, M=5, N=−4, and a comparison of both strands. For amino acid sequences, the BLASTP program may use as defaults a wordlength (W) of 3, an expectation (E) of 10, and the BLOSUM62 scoring matrix. See http://www.ncbi.nlm.nih.gov. Alignment may also be performed manually by inspection.

For sequence comparison, typically one sequence acts as a reference sequence to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are input into a computer, subsequence coordinates are designated if necessary, and sequence algorithm program parameters are designated. The sequence comparison algorithm then calculates the percent sequence identity for the test sequence(s) relative to the reference sequence, based on the designated program parameters.

Influenza Virus Structure and Propagation

Influenza A viruses possess a genome of eight single-stranded negative-sense viral RNAs (vRNAs) that encode at least ten proteins. The influenza virus life cycle begins with binding of the hemagglutinin (HA) to sialic acid-containing receptors on the surface of the host cell, followed by receptor-mediated endocytosis. The low pH in late endosomes triggers a conformational shift in the HA, thereby exposing the N-terminus of the HA2 subunit (the so-called fusion peptide). The fusion peptide initiates the fusion of the viral and endosomal membrane, and the matrix protein (M1) and RNP complexes are released into the cytoplasm. RNPs consist of the nucleoprotein (NP), which encapsidates vRNA, and the viral polymerase complex, which is formed by the PA, PB1, and PB2 proteins. RNPs are transported into the nucleus, where transcription and replication take place. The RNA polymerase complex catalyzes three different reactions: synthesis of an mRNA with a 5' cap and 3' polyA structure, of a full-length complementary RNA (cRNA), and of genomic vRNA using the cRNA as a template. Newly synthesized vRNAs, NP, and polymerase proteins are then assembled into RNPs, exported from the nucleus, and transported to the plasma membrane, where budding of progeny virus particles occurs. The neuraminidase (NA) protein plays a crucial role late in infection by removing sialic acid from sialyloligosaccharides, thus releasing newly assembled virions from the cell surface and preventing the self aggregation of virus particles. Although virus assembly involves protein-protein and protein-vRNA interactions, the nature of these interactions is largely unknown.

Although influenza B and C viruses are structurally and functionally similar to influenza A virus, there are some differences. For example, influenza B virus does not have a

M2 protein with ion channel activity but has BM2 and has a viral segment with both NA and NB sequences. Influenza C virus has only seven viral segments.

Cell Lines that can be Used in the Present Invention

Any cell, e.g., any avian or mammalian cell, such as a human, e.g., 293T or PER.C6® cells, or canine, e.g., MDCK, bovine, equine, feline, swine, ovine, rodent, for instance mink, e.g., MvLu1 cells, or hamster, e.g., CHO cells, or non-human primate, e.g., Vero cells, including mutant cells, which supports efficient replication of influenza virus can be employed to isolate and/or propagate influenza viruses. Isolated viruses can be used to prepare a reassortant virus. In one embodiment, host cells for vaccine production are continuous mammalian or avian cell lines or cell strains. A complete characterization of the cells to be used, may be conducted so that appropriate tests for purity of the final product can be included. Data that can be used for the characterization of a cell includes (a) information on its origin, derivation, and passage history; (b) information on its growth and morphological characteristics; (c) results of tests of adventitious agents; (d) distinguishing features, such as biochemical, immunological, and cytogenetic patterns which allow the cells to be clearly recognized among other cell lines; and (e) results of tests for tumorigenicity. In one embodiment, the passage level, or population doubling, of the host cell used is as low as possible.

In one embodiment, the cells are WHO certified, or certifiable, continuous cell lines. The requirements for certifying such cell lines include characterization with respect to at least one of genealogy, growth characteristics, immunological markers, virus susceptibility tumorigenicity and storage conditions, as well as by testing in animals, eggs, and cell culture. Such characterization is used to confirm that the cells are free from detectable adventitious agents. In some countries, karyology may also be required. In addition, tumorigenicity may be tested in cells that are at the same passage level as those used for vaccine production. The virus may be purified by a process that has been shown to give consistent results, before vaccine production (see, e.g., World Health Organization, 1982).

Virus produced by the host cell may be highly purified prior to vaccine or gene therapy formulation. Generally, the purification procedures result in extensive removal of cellular DNA and other cellular components, and adventitious agents. Procedures that extensively degrade or denature DNA may also be used.

Influenza Vaccines

A vaccine of the invention includes an isolated recombinant influenza virus of the invention, and optionally one or more other isolated viruses including other isolated influenza viruses, one or more immunogenic proteins or glycoproteins of one or more isolated influenza viruses or one or more other pathogens, e.g., an immunogenic protein from one or more bacteria, non-influenza viruses, yeast or fungi, or isolated nucleic acid encoding one or more viral proteins (e.g., DNA vaccines) including one or more immunogenic proteins of the isolated influenza virus of the invention. In one embodiment, the influenza viruses of the invention may be vaccine vectors for influenza virus or other pathogens.

A complete virion vaccine may be concentrated by ultrafiltration and then purified by zonal centrifugation or by chromatography. Viruses other than the virus of the invention, such as those included in a multivalent vaccine, may be inactivated before or after purification using formalin or beta-propiolactone, for instance.

A subunit vaccine comprises purified glycoproteins. Such a vaccine may be prepared as follows: using viral suspensions fragmented by treatment with detergent, the surface antigens are purified, by ultracentrifugation for example. The subunit vaccines thus contain mainly HA protein, and also NA. The detergent used may be cationic detergent for example, such as hexadecyl trimethyl ammonium bromide (Bachmeyer, 1975), an anionic detergent such as ammonium deoxycholate (Laver & Webster, 1976); or a nonionic detergent such as that commercialized under the name TRITON X100. The hemagglutinin may also be isolated after treatment of the virions with a protease such as bromelin, and then purified. The subunit vaccine may be combined with an attenuated virus of the invention in a multivalent vaccine.

A split vaccine comprises virions which have been subjected to treatment with agents that dissolve lipids. A split vaccine can be prepared as follows: an aqueous suspension of the purified virus obtained as above, inactivated or not, is treated, under stirring, by lipid solvents such as ethyl ether or chloroform, associated with detergents. The dissolution of the viral envelope lipids results in fragmentation of the viral particles. The aqueous phase is recuperated containing the split vaccine, constituted mainly of hemagglutinin and neuraminidase with their original lipid environment removed, and the core or its degradation products. Then the residual infectious particles are inactivated if this has not already been done. The split vaccine may be combined with an attenuated virus of the invention in a multivalent vaccine.

Inactivated Vaccines.

Inactivated influenza virus vaccines are provided by inactivating replicated virus using known methods, such as, but not limited to, formalin or β-propiolactone treatment Inactivated vaccine types that can be used in the invention can include whole-virus (WV) vaccines or subvirion (SV) (split) vaccines. The WV vaccine contains intact, inactivated virus, while the SV vaccine contains purified virus disrupted with detergents that solubilize the lipid-containing viral envelope, followed by chemical inactivation of residual virus.

In addition, vaccines that can be used include those containing the isolated HA and NA surface proteins, which are referred to as surface antigen or subunit vaccines.

Live Attenuated Virus Vaccines.

Live, attenuated influenza virus vaccines, such as those including a recombinant virus of the invention can be used for preventing or treating influenza virus infection. Attenuation may be achieved in a single step by transfer of attenuated genes from an attenuated donor virus to a replicated isolate or reassorted virus according to known methods. Since resistance to influenza A virus is mediated primarily by the development of an immune response to the HA and/or NA glycoproteins, the genes coding for these surface antigens come from the reassorted viruses or clinical isolates. The attenuated genes are derived from an attenuated parent. In this approach, genes that confer attenuation generally do not code for the HA and NA glycoproteins.

Viruses (donor influenza viruses) are available that are capable of reproducibly attenuating influenza viruses, e.g., a cold adapted (ca) donor virus can be used for attenuated vaccine production. Live, attenuated reassortant virus vaccines can be generated by mating the ca donor virus with a virulent replicated virus. Reassortant progeny are then selected at 25° C. (restrictive for replication of virulent virus), in the presence of an appropriate antiserum, which inhibits replication of the viruses bearing the surface antigens of the attenuated ca donor virus. Useful reassortants are: (a) infectious, (b) attenuated for seronegative non-adult mammals and immunologically primed adult mammals, (c) immunogenic and (d) genetically stable. The immunogenicity of the ca reassortants parallels their level of replication.

Thus, the acquisition of the six transferable genes of the ca donor virus by new wild-type viruses has reproducibly attenuated these viruses for use in vaccinating susceptible mammals both adults and non-adult.

Other attenuating mutations can be introduced into influenza virus genes by site-directed mutagenesis to rescue infectious viruses bearing these mutant genes. Attenuating mutations can be introduced into non-coding regions of the genome, as well as into coding regions. Such attenuating mutations can also be introduced into genes other than the HA or NA, e.g., the PB2 polymerase gene. Thus, new donor viruses can also be generated bearing attenuating mutations introduced by site-directed mutagenesis, and such new donor viruses can be used in the production of live attenuated reassortants vaccine candidates in a manner analogous to that described above for the ca donor virus. Similarly, other known and suitable attenuated donor strains can be reassorted with influenza virus to obtain attenuated vaccines suitable for use in the vaccination of mammals.

In one embodiment, such attenuated viruses maintain the genes from the virus that encode antigenic determinants substantially similar to those of the original clinical isolates. This is because the purpose of the attenuated vaccine is to provide substantially the same antigenicity as the original clinical isolate of the virus, while at the same time lacking pathogenicity to the degree that the vaccine causes minimal chance of inducing a serious disease condition in the vaccinated mammal.

The viruses in a multivalent vaccine can thus be attenuated or inactivated, formulated and administered, according to known methods, as a vaccine to induce an immune response in an animal, e.g., a mammal. Methods are well-known in the art for determining whether such attenuated or inactivated vaccines have maintained similar antigenicity to that of the clinical isolate or high growth strain derived therefrom. Such known methods include the use of antisera or antibodies to eliminate viruses expressing antigenic determinants of the donor virus; chemical selection (e.g., amantadine or rimantidine); HA and NA activity and inhibition; and nucleic acid screening (such as probe hybridization or PCR) to confirm that donor genes encoding the antigenic determinants (e.g., HA or NA genes) are not present in the attenuated viruses.

Pharmaceutical Compositions

Pharmaceutical compositions of the present invention, suitable for inoculation, e.g., nasal, parenteral or oral administration, comprise one or more influenza virus isolates, e.g., one or more attenuated or inactivated influenza viruses, a subunit thereof, isolated protein(s) thereof, and/or isolated nucleic acid encoding one or more proteins thereof, optionally further comprising sterile aqueous or non-aqueous solutions, suspensions, and emulsions. The compositions can further comprise auxiliary agents or excipients, as known in the art. The composition of the invention is generally presented in the form of individual doses (unit doses).

Conventional vaccines generally contain about 0.1 to 200 μg, e.g., 30 to 100 μg, of HA from each of the strains entering into their composition. The vaccine forming the main constituent of the vaccine composition of the invention may comprise a single influenza virus, or a combination of influenza viruses, for example, at least two or three influenza viruses, including one or more reassortant(s).

Preparations for parenteral administration include sterile aqueous or non-aqueous solutions, suspensions, and/or emulsions, which may contain auxiliary agents or excipients known in the art. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Carriers or occlusive dressings can be used to increase skin permeability and enhance antigen absorption. Liquid dosage forms for oral administration may generally comprise a liposome solution containing the liquid dosage form. Suitable forms for suspending liposomes include emulsions, suspensions, solutions, syrups, and elixirs containing inert diluents commonly used in the art, such as purified water. Besides the inert diluents, such compositions can also include adjuvants, wetting agents, emulsifying and suspending agents, or sweetening, flavoring, or perfuming agents.

When a composition of the present invention is used for administration to an individual, it can further comprise salts, buffers, adjuvants, or other substances which are desirable for improving the efficacy of the composition. For vaccines, adjuvants, substances which can augment a specific immune response, can be used. Normally, the adjuvant and the composition are mixed prior to presentation to the immune system, or presented separately, but into the same site of the organism being immunized.

Heterogeneity in a vaccine may be provided by mixing replicated influenza viruses for at least two influenza virus strains, such as 2-20 strains or any range or value therein. Vaccines can be provided for variations in a single strain of an influenza virus, using techniques known in the art.

A pharmaceutical composition according to the present invention may further or additionally comprise at least one chemotherapeutic compound, for example, for gene therapy, immunosuppressants, anti-inflammatory agents or immune enhancers, and for vaccines, chemotherapeutics including, but not limited to, gamma globulin, amantadine, guanidine, hydroxybenzimidazole, interferon-α, interferon-β, interferon-γ, tumor necrosis factor-alpha, thiosemicarbarzones, methisazone, rifampin, ribavirin, a pyrimidine analog, a purine analog, foscamet, phosphonoacetic acid, acyclovir, dideoxynucleosides, a protease inhibitor, or ganciclovir.

The composition can also contain variable but small quantities of endotoxin-free formaldehyde, and preservatives, which have been found safe and not contributing to undesirable effects in the organism to which the composition is administered.

Pharmaceutical Purposes

The administration of the composition (or the antisera that it elicits) may be for either a "prophylactic" or "therapeutic" purpose. When provided prophylactically, the compositions of the invention which are vaccines are provided before any symptom or clinical sign of a pathogen infection becomes manifest. The prophylactic administration of the composition serves to prevent or attenuate any subsequent infection. When provided prophylactically, the gene therapy compositions of the invention, are provided before any symptom or clinical sign of a disease becomes manifest. The prophylactic administration of the composition serves to prevent or attenuate one or more symptoms or clinical signs associated with the disease.

When provided therapeutically, a viral vaccine is provided upon the detection of a symptom or clinical sign of actual infection. The therapeutic administration of the compound(s) serves to attenuate any actual infection. When provided therapeutically, a gene therapy composition is provided upon the detection of a symptom or clinical sign of the disease. The therapeutic administration of the compound(s) serves to attenuate a symptom or clinical sign of that disease.

Thus, a vaccine composition of the present invention may be provided either before the onset of infection (so as to prevent or attenuate an anticipated infection) or after the

initiation of an actual infection. Similarly, for gene therapy, the composition may be provided before any symptom or clinical sign of a disorder or disease is manifested or after one or more symptoms are detected.

A composition is said to be "pharmacologically acceptable" if its administration can be tolerated by a recipient mammal. Such an agent is said to be administered in a "therapeutically effective amount" if the amount administered is physiologically significant A composition of the present invention is physiologically significant if its presence results in a detectable change in the physiology of a recipient patient, e.g., enhances at least one primary or secondary humoral or cellular immune response against at least one strain of an infectious influenza virus.

The "protection" provided need not be absolute, i.e., the influenza infection need not be totally prevented or eradicated, if there is a statistically significant improvement compared with a control population or set of mammals. Protection may be limited to mitigating the severity or rapidity of onset of symptoms or clinical signs of the influenza virus infection.

Pharmaceutical Administration

A composition of the present invention may confer resistance to one or more pathogens, e.g., one or more influenza virus strains, by either passive immunization or active immunization. In active immunization, an attenuated live vaccine composition is administered prophylactically to a host (e.g., a mammal), and the host's immune response to the administration protects against infection and/or disease. For passive immunization, the elicited antisera can be recovered and administered to a recipient suspected of having an infection caused by at least one influenza virus strain. A gene therapy composition of the present invention may yield prophylactic or therapeutic levels of the desired gene product by active immunization.

In one embodiment, the vaccine is provided to a mammalian female (at or prior to pregnancy or parturition), under conditions of time and amount sufficient to cause the production of an immune response which serves to protect both the female and the fetus or newborn (via passive incorporation of the antibodies across the placenta or in the mother's milk).

The present invention thus includes methods for preventing or attenuating a disorder or disease, e.g., an infection by at least one strain of pathogen. As used herein, a vaccine is said to prevent or attenuate a disease if its administration results either in the total or partial attenuation (i.e., suppression) of a clinical sign or condition of the disease, or in the total or partial immunity of the individual to the disease. As used herein, a gene therapy composition is said to prevent or attenuate a disease if its administration results either in the total or partial attenuation (i.e., suppression) of a clinical sign or condition of the disease, or in the total or partial immunity of the individual to the disease.

A composition having at least one influenza virus of the present invention, including one which is attenuated and one or more other isolated viruses, one or more isolated viral proteins thereof, one or more isolated nucleic acid molecules encoding one or more viral proteins thereof, or a combination thereof, may be administered by any means that achieve the intended purposes.

For example, administration of such a composition may be by various parenteral routes such as subcutaneous, intravenous, intradermal, intramuscular, intraperitoneal, intranasal, oral or transdermal routes. Parenteral administration can be accomplished by bolus injection or by gradual perfusion over time.

A typical regimen for preventing, suppressing, or treating an influenza virus related pathology, comprises administration of an effective amount of a vaccine composition as described herein, administered as a single treatment, or repeated as enhancing or booster dosages, over a period up to and including between one week and about 24 months, or any range or value therein.

According to the present invention, an "effective amount" of a composition is one that is sufficient to achieve a desired effect. It is understood that the effective dosage may be dependent upon the species, age, sex, health, and weight of the recipient, kind of concurrent treatment, if any, frequency of treatment, and the nature of the effect wanted. The ranges of effective doses provided below are not intended to limit the invention and represent dose ranges.

The dosage of a live, attenuated or killed virus vaccine for an animal such as a mammalian adult organism may be from about 10^2-10^{15}, e.g., 10^3-10^{12}, plaque forming units (PFU)/kg, or any range or value therein. The dose of inactivated vaccine may range from about 0.1 to 1000, e.g., 30 to 100 μg, of HA protein. However, the dosage should be a safe and effective amount as determined by conventional methods, using existing vaccines as a starting point.

The dosage of immunoreactive HA in each dose of replicated virus vaccine may be standardized to contain a suitable amount, e.g., 30 to 100 μg or any range or value therein, or the amount recommended by government agencies or recognized professional organizations. The quantity of NA can also be standardized, however, this glycoprotein may be labile during purification and storage.

The dosage of immunoreactive HA in each dose of replicated virus vaccine can be standardized to contain a suitable amount, e.g., 1-50 pig or any range or value therein, or the amount recommended by the U.S. Public Health Service (PHS), which is usually 15 μg per component for older children (greater than or equal to 3 years of age), and 7.5 μg per component for children less than 3 years of age. The quantity of NA can also be standardized, however, this glycoprotein can be labile during the processor purification and storage (Kendal et al., 1980; Kerr et al., 1975). Each 0.5-ml dose of vaccine may contains approximately 1-50 billion virus particles, and preferably 10 billion particles.

Exemplary Embodiments

In one embodiment, the invention provides an isolated recombinant influenza virus having PA, PB1, PB2, NP, NS, and M viral segments from a first influenza vaccine virus isolate, a heterologous, recombinant or chimeric influenza virus NA viral segment, and a heterologous, recombinant or chimeric HA viral segment, wherein one, two or more of the PA, PB1, PB2, NP, NS, and M viral segments have selected amino acid residues at positions 30, 31, 105, 142, 149, 225, 356, 357, 401, and/or 550 in PA; positions 40, 54, 59, 62, e.g., 62A, 63, 75, 76, 78, 79, 80, 112, 180, 247, 261, e.g., 161G, 327, 361, e.g., 361R, 507, 621, e.g., 621R, 624, 644, 654, e.g., 654S, 667, 694, 695, 697, 699, 700, 701, 702, 705, 713, and/or 714 in PB1; positions 57, 58, 59, 61, 66, 202, 323, 368, 391, 504, 591, 677, 678, and/or 679, in PB2; positions 74, 112, 116, 224, 293, 371, 377, 417, 422 or 442 in NP; positions 90, 97 and/or 100 in M1; or positions 30, 49, 55, 118, e.g., 118K, 140, 161, and/or 223 in NS1, and optionally an HA with a residue other than glutamic acid, lysine, glutamine, leucine, valine, phenylalanine, lysine or methionine at position 136, 162, 179, 182, 184, 252, 449, or 476, respectively, e.g., a HA segment with one or more of 136D, 162E, 179L, 182V, 184I, 252I, 449E or 476I, or

optionally a NA with a residue other than leucine or alanine at residue 55 or 265, respectively, e.g., 55S or 265V. In one embodiment, the isolated virus has 142N, 225C, 356R, or 550L in PA; has one or more of 112G, 247H, 507V, or 644A in PB1; has one or more of 202L, 323L or 504V in PB2; has one or more of 74K, 112L, 116L, 417D, or 442A in NP; 97A and/or 100H in M1; and/or 55E and/or 140Q in NS1, or combinations thereof, e.g., has at least one of 202L and/or 323L in PB2, 247H in PB1 or 74K in NP and optionally at least one of 142N in PA1, 55K in NS1 or 97A and/or 100H in M1 or has at least one of 202L and/or 323L in PB2, 247H in PB1 or 74K in NP and at least one of 142N in PA1, 55K in NS1 or 97A and/or 100H in M1. In one embodiment, the virus has at least one of 202L and/or 323L in PB2, 247H in PB1 or 74K in NP and optionally at least one of 142N in PA1, 55K in NS1 or 97A and/or 100H in M1. In one embodiment, the virus has at least one of 202L and/or 323L in PB2, 247H in PB1 or 74K in NP and at least one of 142N in PA1, 55K in NS1 or 97A and/or 100H in M1. In one embodiment, the isolated virus has 202L and/or 323L in PB2, and optionally has 247H in PB1 and optionally 74K in NP. In one embodiment, the isolated virus has 247H in PB1 and optionally 74K in NP. In one embodiment, the isolated virus has 40I, 40L, 112G, 180W, 247H, 507V, or 644A in PB1 and optionally has 202L and/or 323L in PB2, and optionally has 74K, 112L, 116L, 377N, 417D, or 422L in NP, and optionally has 30P, 118K, 161T or 140Q in NS1, and optionally has 142N, 225C, 356R, 401K, or 550L in PA. In one embodiment, the isolated virus has 40I, 40L, 112G, 180W, 247H, 507V, or 644A in PB1. In one embodiment, the isolated virus has 202L and/or 323L in PB2. In one embodiment, the isolated virus has 74K, 112L, 116L, 377N, 417D, or 422L in NP. In one embodiment, the isolated virus has 30P, 118K, 161T or 1400 in NS1. In one embodiment, the isolated virus has 142N, 225C, 356R, 401K, or 550L in PA. In one embodiment, the selected amino acid residues at specified positions in the PA is/are at position(s) 97, 105, 142, 149, 225, 356, 357, 401, 404, and/or 421. In one embodiment, the selected amino acid residues at specified positions in the PB1 is/are at position(s) 12, 40, 54, 59, 62, 63, 66, 75, 76, 78, 79, 80, 180, 247, 507, 624, 644, 694, 695, 697, 699, 700, 701, 705, 713, 714, and/or 762. In one embodiment, the selected amino acid residues at specified positions in the PB2 is/are at position(s) 57, 58, 59, 61, 66, 202, 243, 323, 504, 677, 678, and/or 679. In one embodiment, the selected amino acid residues at specified positions in the NP is/are at position(s) 74, 112, 116, 224, 293, 417, and/or 442. In one embodiment, the selected amino acid residues at specified positions in the M1 is/are at position(s) 90, 97, and/or 100. In one embodiment, the selected amino acid residues at specified positions in the NS1 is/are at position(s) 49, 30, 55, 161, and/or 223. In one embodiment, the selected amino acid residues at specified positions in the PA is/are at position(s) 97, 105, 142, 149, 225, 356, 357, 401, 404, and/or 421; and optionally the selected amino acid residues at specified positions in the PB1 is/are at position(s) 12, 40, 54, 59, 62, 63, 66, 75, 76, 78, 79, 80, 180, 247, 507, 624, 644, 694, 695, 697, 699, 700, 701, 705, 713, 714, and/or 762, in any combination with the selected residues for PA; and optionally the selected amino acid residues at specified positions in the PB2 is/are at position(s) 57, 58, 59, 61, 66, 202, 243, 323, 504, 677, 678, and/or 679 in any combination with the selected residues for PA and/or PB1; and optionally the selected amino acid residues at specified positions in the NP is/are at position(s) 74, 112, 116, 224, 293, 417, and/or 442 any combination with the selected residues for PA, PB1 and/or PB2; and optionally the selected amino acid residues at specified positions in the M1 is/are at position(s) 90, 97, and/or 100 any combination with the selected residues for PA, PB1, PB2, and/or NP; and optionally the selected amino acid residues at specified positions in the NS1 is/are at position(s) 49, 30, 55, 161, and/or 223, or in any combination with the selected residues for PA. PB1, PB2, NP, and/or M1.

For any of the exemplary viruses disclosed above, in one embodiment, the PA, PB1, PB2, NP, NS, and M viral segments comprise sequences for at least one of the following: a PB1 having the amino acid sequence encoded by SEQ ID NO:2 or PB1 with at least 95% amino acid sequence identity to the PB1 encoded by SEQ ID NO:2; a PB2 having the amino acid sequence encoded by SEQ ID NO:3 or PB2 with at least 95% amino acid sequence identity to the PB2 encoded by SEQ ID NO:3; a PA having the amino acid sequence encoded by SEQ ID NO:1 or PA with at least 95% amino acid sequence identity to the PA encoded by SEQ ID NO:1; a NP having the amino acid sequence encoded by SEQ ID NO:4 or NP with at least 95% amino acid sequence identity to the NP encoded by SEQ ID NO:4; a M having the amino acid sequence encoded by SEQ ID NO:5 or M with at least 95% amino acid sequence identity to the M encoded by SEQ ID NO:5; or a NS having the amino acid sequence encoded by SEQ ID NO:6 or NS with at least 95% amino acid sequence identity to the NS encoded by SEQ ID NO:6, or the PA PB1, PB2, NP, NS, and M viral segments comprise sequences for at least one of the following: a PB1 having the amino acid sequence encoded by SEQ ID NO:10 or PB1 with at least 95% amino acid sequence identity to the PB1 encoded by SEQ ID NO:10; a PB2 having the amino acid sequence encoded by SEQ ID NO: 11 or PB2 with at least 95% amino acid sequence identity to the PB2 encoded by SEQ ID NO: 11; a PA having the amino acid sequence encoded by SEQ ID NO:12 or PA with at least 95% amino acid sequence identity to the PA encoded by SEQ ID NO:12; a NP having the amino acid sequence encoded by SEQ ID NO:13 or NP with at least 95% amino acid sequence identity to the NP encoded by SEQ ID NO:13; a M having the amino acid sequence encoded by SEQ ID NO:14 or M with at least 95% amino acid sequence identity to the M encoded by SEQ ID NO:14; or a NS having the amino acid sequence encoded by SEQ ID NO:15 or NS with at least 95% amino acid sequence identity to the NS encoded by SEQ ID NO:15.

For any of the exemplary viruses disclosed above, in one embodiment, at least one of the PA, PB1, PB2, NP, NS, and M viral segments has a C to U promoter mutation.

Any of the isolated viruses disclosed herein may be employed in a vaccine.

In one embodiment, the invention provides a plurality of influenza virus vectors for preparing a reassortant. In one embodiment, the plurality includes a vector for vRNA production comprising a promoter operably linked to an influenza virus PA DNA linked to a transcription termination sequence, a vector for vRNA production comprising a promoter operably linked to an influenza virus PB1 DNA linked to a transcription termination sequence, a vector for vRNA production comprising a promoter operably linked to an influenza virus PB2 DNA linked to a transcription termination sequence, a vector for vRNA production comprising a promoter operably linked to an influenza virus HA DNA linked to a transcription termination sequence, a vector for vRNA production comprising a promoter operably linked to an influenza virus NP DNA linked to a transcription termination sequence, a vector for vRNA production comprising a promoter operably linked to an influenza virus NA DNA linked to a transcription termination sequence, a

vector for vRNA production comprising a promoter operably linked to an influenza virus M DNA linked to a transcription termination sequence, and a vector for vRNA production comprising a promoter operably linked to an influenza virus NS cDNA linked to a transcription termination sequence, wherein the PB1, PB2, PA, NP, NS, and M DNAs in the vectors for vRNA production are from one or more influenza vaccine virus isolates, wherein the NA DNA in the vector for vRNA production of NA has sequences for a heterologous NA, and wherein the HA DNA in the vector for vRNA production of HA has sequences for a heterologous HA, 30, 31, 105, 142, 149, 225, 356, 357, 401, and/or 550 in PA; 40, 54, 59, 62, 63, 75, 76, 78, 79, 80, 112, 180, 247, 327, 507, 624, 644, 667, 694, 695, 697, 699, 700, 701, 702, 705, 713, or 714 and/or 247 in PB1; 57, 58, 59, 61, 66, 202, 323, 368, 391, 504, 591, 677, 678, or 679, 202 and/or 323 in PB2; 74, 112, 116, 224, 293, 371, 377, 417, 422 and/or 442 in NP; 90, 97 and/or 100 in M1; or 30, 49, 55, 118,140, 161 and/or 223 in NS; and a vector for mRNA production comprising a promoter operably linked to a DNA segment encoding influenza virus PA, a vector for mRNA production comprising a promoter operably linked to a DNA segment encoding influenza virus PB1, a vector for mRNA production comprising a promoter operably linked to a DNA segment encoding influenza virus PB2, and a vector for mRNA production comprising a promoter operably linked to a DNA segment encoding influenza virus NP, and optionally a vector for mRNA production comprising a promoter operably linked to a DNA segment encoding influenza virus HA, a vector for mRNA production comprising a promoter operably linked to a DNA segment encoding influenza virus NA, a vector for mRNA production comprising a promoter operably linked to a DNA segment encoding influenza virus M1, a vector for mRNA production comprising a promoter operably linked to a DNA segment encoding influenza virus M2, or a vector for mRNA production comprising a promoter operably linked to a DNA segment encoding influenza virus NS2. In one embodiment, the PB1, PB2, PA, NP, NS, and M DNAs in the vectors for vRNA production have a sequence corresponding to one that encodes a polypeptide having at least 95% amino acid sequence identity to a corresponding polypeptide encoded by SEQ ID NOs:1-6 or 10-15. In one embodiment, the promoter for vRNA vectors is a RNA polymerase I promoter, a RNA polymerase II promoter, a RNA polymerase III promoter, a T3 promoter or a T7 promoter. In one embodiment, the NA is N9. In one embodiment, the HA is H7. In one embodiment, the PA, PB1, PB2, NP, NS, and/or M viral segments has/have a promoter C to a mutation.

In one embodiment, the invention provides a method to prepare influenza virus. The method includes contacting a cell with: a vector for vRNA production comprising a promoter operably linked to an influenza virus PA DNA linked to a transcription termination sequence, a vector for vRNA production comprising a promoter operably linked to an influenza virus PB1 DNA linked to a transcription termination sequence, a vector for vRNA production comprising a promoter operably linked to an influenza virus PB2 DNA linked to a transcription termination sequence, a vector for vRNA production comprising a promoter operably linked to an influenza virus HA DNA linked to a transcription termination sequence, a vector for vRNA production comprising a promoter operably linked to an influenza virus NP DNA linked to a transcription termination sequence, a vector for vRNA production comprising a promoter operably linked to an influenza virus NA DNA linked to a transcription termination sequence, a vector for vRNA production comprising a promoter operably linked to an influenza virus M DNA linked to a transcription termination sequence, and a vector for vRNA production comprising a promoter operably linked to an influenza virus NS DNA linked to a transcription termination sequence, wherein the PB1, PB2, PA, NP, NS, and M DNAs in the vectors for vRNA production are from one or more influenza vaccine virus isolates, wherein the NA DNA in the vector for vRNA production of NA has sequences for a heterologous NA, and wherein the HA DNA in the vector for vRNA production of HA has sequences for a heterologous HA, 30, 31, 105, 142, 149, 225, 356, 357, 401, and/or 550 in PA; 40, 54, 59, 62, 63, 75, 76, 78, 79, 80, 112, 180, 247, 327, 507, 624, 644, 667, 694, 695, 697, 699, 700, 701, 702, 705, 713, and/or 714 and/or 247 in PB1; 57, 58, 59, 61, 66, 202, 323, 368, 391, 504, 591, 677, 678, and/or 679, 202 and/or 323 in PB2; 74, 112, 116, 224, 293, 371, 377, 417, 422 and/or 442 in NP; 90, 97 and/or 100 in M1; or 30, 49, 55, 118, 140, 161 or 223 in NS; and a vector for mRNA production comprising a promoter operably linked to a DNA segment encoding influenza virus PA, a vector for mRNA production comprising a promoter operably linked to a DNA segment encoding influenza virus PB1, a vector for mRNA production comprising a promoter operably linked to a DNA segment encoding influenza virus PB2, and a vector for mRNA production comprising a promoter operably linked to a DNA segment encoding influenza virus NP, and optionally a vector for mRNA production comprising a promoter operably linked to a DNA segment encoding influenza virus HA, a vector for mRNA production comprising a promoter operably linked to a DNA segment encoding influenza virus NA, a vector for mRNA production comprising a promoter operably linked to a DNA segment encoding influenza virus M1, a vector for mRNA production comprising a promoter operably linked to a DNA segment encoding influenza virus M2, or a vector for mRNA production comprising a promoter operably linked to a DNA segment encoding influenza virus NS2; in an amount effective to yield infectious influenza virus. In one embodiment, the cell is an avian cell or a mammalian cell, e.g., a Vero cell, a human cell or a MDCK cell. In one embodiment, the PB1, PB2, PA, NP, NS, and M DNAs in the vectors for vRNA productions have a sequence that corresponds to one that encodes a polypeptide having at least 95% amino acid sequence identity to a corresponding polypeptide encoded by SEQ ID NOs:1-6 or 10-15. In one embodiment, the method includes isolating the virus. In one embodiment, at least one of PA, PB1, or PB2 viral segments has a C to U promoter mutation.

Further provided is a vector for vRNA or mRNA expression of influenza virus PA having at least 95% amino acid sequence identity to a polypeptide encoded by SEQ ID NO:1 and having a threonine at position 30, a lysine at position 31, cysteine at position 105 or a lysine at position 401; a vector for vRNA or mRNA expression of influenza virus PB1 having at least 95% amino acid sequence identity to a polypeptide encoded by SEQ ID NO:2 and having a leucine at position 40, an alanine or isoleucine at position 54, glycine at position 112, histidine at position 247, valine at position 507, alanine at position 644, or cysteine at position 713; a vector for vRNA or mRNA expression of PB2 having at least 95% amino acid sequence identity to a polypeptide encoded by SEQ ID NO:3 and a leucine at position 202 and/or 323; a vector for vRNA or mRNA expression of influenza virus NP having at least 95% amino acid sequence identity to a polypeptide encoded by SEQ ID NO:4 and having a lysine at position 74, leucine at position 116, isoleucine at position 224, lysine at position 293, asparagine

at position 377, or aspartic acid at position 417; a vector for vRNA or mRNA expression of influenza virus NS1 having at least 95% amino acid sequence identity to a NS1 polypeptide encoded by SEQ ID NO:6 and having a proline at position 30, alanine at position 49, lysine at position 118, glutamine at position 140, threonine at position 161, or glutamic acid at position 223; and a vector for vRNA or mRNA expression of influenza virus M1 having at least 95% amino acid sequence identity to a M1 polypeptide encoded by SEQ ID NO:5 and having a serine at position 90.

The invention will be described by the following nonlimiting examples.

Example 1

Methods

Cells and Viruses

293T human embryonic kidney cells are maintained in Dulbecco's modified Eagle's minimal essential medium (DMEM) with 10% fetal calf serum and antibiotics. Madin-Darby canine kidney (MDCK) cells are grown in MEM with 5% newborn calf serum and antibiotics. African green monkey Vero WCB cells, which had been established after biosafety tests for use in human vaccine production (Sugawara et al., 2002), are maintained in serum-free VP-SFM medium (GIBCO-BRL) with antibiotics. Cells are maintained at 37° C. in 5% CO_2. A WHO-recommended vaccine seed virus is NIBRG-14.

Construction of Plasmids and Reverse Genetics

To generate reassortants of influenza A viruses, a plasmid-based reverse genetics (Neumann et al., 1999) is used. The full-length cDNAs were cloned into a plasmid under control of the human polymerase I promoter and the mouse RNA polymerase I terminator (Poll plasmids).

A previously produced series of Poll constructs, derived from A/WSN/33 (H5N1; WSN) or PR8 strains is used, for reverse genetics (Horimoto et al., 2006; Neumann et al., 1999). The World Health Organization (WHO) recommends A/Puerto Rico/8/34 (H1N1; PR8) as a donor virus, because of its safety in humans (Wood & Robertson, 2004; Webby & Webster, 2003).

Plasmids expressing WSN or PR8 NP, PA, PB1, or PB2 under control of the chicken actin, e.g., beta-actin, promoter are used for all reverse genetics experiments (Horimoto et al., 2006; Neumann et al., 1999). Briefly, Poll plasmids and protein expression plasmids are mixed with a transfection reagent, Trans-IT 293T (Panvera), incubated at room temperature for 15 minutes, and then added to 293T cells. Transfected cells are incubated in Opti-MEM I (GIBCO-BRL) for 48 hours. For reverse genetics in Vero WCB cells, an electroporator (Amaxa) is used to transfect the plasmid mixtures according to the manufacturer's instructions. Sixteen hours after transfection, freshly prepared Vero WCB cells were added onto the transfected cells and TPCK-trypsin (1 μg/mL) is added to the culture 6 hours later. Transfected cells are incubated in serum-free VP-SFM for a total of 4 days. Supernatants containing infectious viruses are harvested, and may be biologically cloned by limiting dilution.

A recombinant virus having the HA and NA genes from A/Hong Kong/213/2003 (H5N1) and the remainder of the type A influenza virus genes from PR8(UW) was prepared. The titer of the recombinant virus was $10^{10.67}$ EID_{50}/mL, and the HA titer was 1:1600

TABLE 1

Virus possessing PR8 genes together with the following HA and NA genes	HA titer (HAU/mL) in each dilition						
	10-2	10-3	10-4	10-5	10-6	10-7	10-8
WSN-HA NA	160	40	40	320	40	640	<1
HK-HAavir NA	400	800	400	400	400	800	<1

The sequences of PR8 (UW) genes are as follows:

PA

(SEQ ID NO: 1)

AGCGAAAGCA GGTACTGATC CAAAATGGAA GATTTTGTGC GACAATGCTT CAATCCGATG

ATTGTCGAGC TTGCGGAAAA AACAATGAAA GAGTATGGGG AGGACCTGAA AATCGAAACA

AACAAATTTG CAGCAATATG CACTCACTTG GAAGTATGCT TCATGTATTC AGATTTTCAC

TTCATCAATG AGCAAGGCGA GTCAATAATC GTAGAACTTG GTGATCCAAA TGCACTTTTG

AAGCACAGAT TTGAAATAAT CGAGGGAAGA GATCGCACAA TGGCCTGGAC AGTAGTAAAC

AGTATTTGCA ACACTACAGG GGCTGAGAAA CCAAAGTTTC TACCAGATTT GTATGATTAC

AAGGAGAATA GATTCATCGA AATTGGAGTA ACAAGGAGAG AAGTTCACAT ATACTATCTG

GAAAAGGCCA ATAAAATTAA ATCTGAGAAA ACACACATCC ACATTTTCTC GTTCACTGGG

GAAGAAATGG CCACAAAGGC AGACTACACT CTCGATGAAG AAAGCAGGGC TAGGATCAAA

ACCAGACTAT TCACCATAAG ACAAGAAATG GCCAGCAGAG GCCTCTGGGA TTCCTTTCGT

CAGTCCGAGA GAGGAGAAGA GACAATTGAA GAAAGGTTTG AAATCACAGG AACAATGCGC

AAGCTTGCCG ACCAAAGTCT CCCGCCGAAC TTCTCCAGCC TTGAAAATTT TAGAGCCTAT

GTGGATGGAT TCGAACCGAA CGGCTACATT GAGGGCAAGC TGTCTCAAAT GTCCAAAGAA

GTAAATGCTA GAATTGAACC TTTTTTGAAA ACAACACCAC GACCACTTAG ACTTCCGAAT

GGGCCTCCCT GTTCTCAGCG GTCCAAATTC CTGCTGATGG ATGCCTTAAA ATTAAGCATT

-continued

GAGGACCCAA GTCATGAAGG AGAGGGAATA CCGCTATATG ATGCAATCAA ATGCATGAGA

ACATTCTTTG GATGGAAGGA ACCCAATGTT GTTAAACCAC ACGAAAAGGG AATAAATCCA

AATTATCTTC TGTCATGGAA GCAAGTACTG GCAGAACTGC AGGACATTGA GAATGAGGAG

AAAATTCCAA AGACTAAAAA TATGAAGAAA ACAAGTCAGC TAAAGTGGGC ACTTGGTGAG

AACATGGCAC CAGAAAAGGT AGACTTTGAC GACTGTAAAG ATGTAGGTGA TTTGAAGCAA

TATGATAGTG ATGAACCAGA ATTGAGGTCG CTTGCAAGTT GGATTCAGAA TGAGTTTAAC

AAGGCATGCG AACTGACAGA TTCAAGCTGG ATAGAGCTCG ATGAGATTGG AGAAGATGTG

GCTCCAATTG AACACATTGC AAGCATGAGA AGGAATTATT TCACATCAGA GGTGTCTCAC

TGCAGAGCCA CAGAATACAT AATGAAGGGA GTGTACATCA ATACTGCCTT GCTTAATGCA

TCTTGTGCAG CAATGGATGA TTTCCAATTA ATTCCAATGA TAAGCAAGTG TAGAACTAAG

GAGGGAAGGC GAAAGACCAA CTTGTATGGT TTCATCATAA AAGGAAGATC CCACTTAAGG

AATGACACCG ACGTGGTAAA CTTTGTGAGC ATGGAGTTTT CTCTCACTGA CCCAAGACTT

GAACCACATA AATGGGAGAA GTACTGTGTT CTTGAGATAG GAGATATGCT TATAAGAAGT

GCCATAGGCC AGGTTTCAAG GCCCATGTTC TTGTATGTGA GAACAAATGG AACCTCAAAA

ATTAAAATGA AATGGGGAAT GGAGATGAGG CGTTGCCTCC TCCAGTCACT TCAACAAATT

GAGAGTATGA TTGAAGCTGA GTCCTCTGTC AAAGAGAAAG ACATGACCAA AGAGTTCTTT

GAGAACAAAT CAGAAACATG GCCCATTGGA GAGTCCCCCA AAGGAGTGGA GGAAAGTTCC

ATTGGGAAGG TCTGCAGGAC TTTATTAGCA AAGTCGGTAT TCAACAGCTT GTATGCATCT

CCACAACTAG AAGGATTTTC AGCTGAATCA AGAAAACTGC TTCTTATCGT TCAGGCTCTT

AGGGACAACC TGGAACCTGG GACCTTTGAT CTTGGGGGGC TATATGAAGC AATTGAGGAG

TGCCTGATTA ATGATCCCTG GGTTTTGCTT AATGCTTCTT GGTTCAACTC CTTCCTTACA

CATGCATTGA GTTAGTTGTG GCAGTGCTAC TATTTGCTAT CCATACTGTC CAAAAAAGTA

CCTTGTTTCT ACT

PB1

(SEQ ID NO: 2)

AGCGAAAGCA GGCAAACCAT TTGAATGGAT GTCAATCCGA CCTTACTTTT CTTAAAAGTG

CCAGCACAAA ATGCTATAAG CACAACTTTC CCTTATACTG GAGACCCTCC TTACAGCCAT

GGGACAGGAA CAGGATACAC CATGGATACT GTCAACAGGA CACATCAGTA CTCAGAAAAG

GGAAGATGGA CAACAAACAC CGAAACTGGA GCACCGCAAC TCAACCCGAT TGATGGGCCA

CTGCCAGAAG ACAATGAACC AAGTGGTTAT GCCCAAACAG ATTGTGTATT GGAGGCGATG

GCTTTCCTTG AGGAATCCCA TCCTGGTATT TTTGAAAACT CGTGTATTGA AACGATGGAG

GTTGTTCAGC AAACACGAGT AGACAAGCTG ACACAAGGCC GACAGACCTA TGACTGGACT

CTAAATAGAA ACCAACCTGC TGCAACAGCA TTGGCCAACA CAATAGAAGT GTTCAGATCA

AATGGCCTCA CGGCCAATGA GTCTGGAAGG CTCATAGACT TCCTTAAGGA TGTAATGGAG

TCAATGAACA AAGAAGAAAT GGGGATCACA ACTCATTTTC AGAGAAAGAG ACGGGTGAGA

GACAATATGA CTAAGAAAAT GATAACACAG AGAACAATGG GTAAAAAGAA GCAGAGATTG

AACAAAAGGA GTTATCTAAT TAGAGCATTG ACCCTGAACA CAATGACCAA AGATGCTGAG

AGAGGGAAGC TAAAACGGAG AGCAATTGCA ACCCCAGGGA TGCAAATAAG GGGGTTTGTA

TACTTTGTTG AGACACTGGC AAGGAGTATA TGTGAGAAAC TTGAACAATC AGGGTTGCCA

GTTGGAGGCA ATGAGAAGAA AGCAAAGTTG GCAAATGTTG TAAGGAAGAT GATGACCAAT

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CTAGCAAGCA TCGATTTGAA ATATTTCAAT GATTCAACAA GAAAGAAGAT TGAAAAAATC

CGACCGCTCT TAATAGAGGG GACTGCATCA TTGAGCCCTG GAATGATGAT GGGCATGTTC

AATATGTTAA GCACTGTATT AGGCGTCTCC ATCCTGAATC TTGGACAAAA GAGATACACC

AAGACTACTT ACTGGTGGGA TGGTCTTCAA TCCTCTGACG ATTTTGCTCT GATTGTGAAT

GCACCCAATC ATGAAGGGAT TCAAGCCGGA GTCGACAGGT TTTATCGAAC CTGTAAGCTA

CTTGGAATCA ATATGAGCAA GAAAAAGTCT TACATAAACA GAACAGGTAC ATTTGAATTC

ACAAGTTTTT TCTATCGTTA TGGGTTTGTT GCCAATTTCA GCATGGAGCT TCCCAGTTTT

GGGGTGTCTG GGATCAACGA GTCAGCGGAC ATGAGTATTG GAGTTACTGT CATCAAAAAC

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TCATTTGAAA TAAAGAAACT GTGGGAGCAA ACCCGTTCCA AAGCTGGACT GCTGGTCTCC

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TCCATCTTGA TACAAGTCA AAGAGGAGTA CTTGAGGATG AACAAATGTA CCAAAGGTGC

TGCAATTTAT TTGAAAAATT CTTCCCCAGC AGTTCATACA GAAGACCAGT CGGGATATCC

AGTATGGTGG AGGCTATGGT TTCCAGAGCC CGAATTGATG CACGGATTGA TTTCGAATCT

GGAAGGATAA AGAAAGAAGA GTTCACTGAG ATCATGAAGA TCTGTTCCAC CATTGAAGAG

CTCAGACGGC AAAAATAGTG AATTTAGCTT GTCCTTCATG AAAAAATGCC TTGTTTCTAC

T

PB2

(SEQ ID NO: 3)

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AAGAAGTACA CATCAGGAAG ACAGGAGAAG AACCCAGCAC TTAGGATGAA ATGGATGATG

GCAATGAAAT ATCCAATTAC AGCAGACAAG AGGATAACGG AAATGATTCC TGAGAGAAAT

GAGCAAGGAC AAACTTTATG GAGTAAAATG AATGATGCCG GATCAGACCG AGTGATGGTA

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CCTGTCCATT TTAGAAACCA AGTCAAAATA CGTCGGAGAG TTGACATAAA TCCTGGTCAT

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T

NP

(SEQ ID NO: 4)

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ATGGTGCTCT CTGCTTTTGA CGAAAGGAGA AATAAATACC TTGAAGAACA TCCCAGTGCG

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CTGATGCAAG GTTCAACTCT CCCTAGGAGG TCTGGAGCCG CAGGTGCTGC AGTCAAAGGA

GTTGGAACAA TGGTGATGGA ATTGGTCAGA ATGATCAAAC GTGGGATCAA TGATCGGAAC

TTCTGGAGGG GTGAGAATGG ACGAAAAACA AGAATTGCTT ATGAAAGAAT GTGCAACATT

CTCAAAGGGA AATTTCAAAC TGCTGCACAA AAAGCAATGA TGGATCAAGT GAGAGAGAGC

CGGAACCCAG GGAATGCTGA GTTCGAAGAT CTCACTTTTC TAGCACGGTC TGCACTCATA

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AGTCAACTGG TGTGGATGGC ATGCCATTCT GCCGCATTTG AAGATCTAAG AGTATTAAGC

TTCATCAAAG GGACGAAGGT GCTCCCAAGA GGGAAGCTTT CCACTAGAGG AGTTCAAATT

GCTTCCAATG AAAATATGGA GACTATGGAA TCAAGTACAC TTGAACTGAG AAGCAGGTAC

TGGGCCATAA GGACCAGAAG TGGAGGAAAC ACCAATCAAC AGAGGGCATC TGCGGGCCAA

ATCAGCATAC AACCTACGTT CTCAGTACAG AGAAATCTCC CTTTTGACAG AACAACCATT

ATGGCAGCAT TCAATGGGAA TACAGAGGGG AGAACATCTG ACATGAGGAC CGAAATCATA

AGGATGATGG AAAGTGCAAG ACCAGAAGAT GTGTCTTTCC AGGGGCGGGG AGTCTTCGAG

CTCTCGGACG AAAAGGCAGC GAGCCCGATC GTGCCTTCCT TTGACATGAG TAATGAAGGA

TCTTATTTCT TCGGAGACAA TGCAGAGGAG TACGACAATT AAAGAAAAAT ACCCTTGTTT

CTACT

M

(SEQ ID NO: 5)

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TGCAGGGAAG AACACCGATC TTGAGGTTCT CATGGAATGG CTAAAGACAA GACCAATCCT

GTCACCTCTG ACTAAGGGGA TTTTAGGATT TGTGTTCACG CTCACCGTGC CCAGTGAGCG

AGGACTGCAG CGTAGACGCT TTGTCCAAAA TGCCCTTAAT GGGAACGGGG ATCCAAATAA

CATGGACAAA GCAGTTAAAC TGTATAGGAA GCTCAAGAGG GAGATAACAT TCCATGGGGC

CAAAGAAATC TCACTCAGTT ATTCTGCTGG TGCACTTGCC AGTTGTATGG GCCTCATATA

CAACAGGATG GGGGCTGTGA CCACTGAAGT GGCATTTGGC CTGGTATGTG CAACCTGTGA

ACAGATTGCT GACTCCCAGC ATCGGTCTCA TAGGCAAATG GTGACAACAA CCAATCCACT

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GGTGCAAGCG ATGAGAACCA TTGGGACTCA TCCTAGCTCC AGTGCTGGTC TGAAAAATGA

TCTTCTTGAA AATTTGCAGG CCTATCAGAA ACGAATGGGG GTGCAGATGC AACGGTTCAA

GTGATCCTCT CACTATTGCC GCAAATATCA TTGGGATCTT GCACTTGACA TTGTGGATTC

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CTTCTACGGA AGGAGTGCCA AAGTCTATGA GGGAAGAATA TCGAAAGGAA CAGCAGAGTG

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TTCTACT

NS

(SEQ ID NO: 6)

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ATTGCTTTCT TTGGCATGTC CGCAAACGAG TTGCAGACCA AGAACTAGGC GATGCCCCAT

TCCTTGATCG GCTTCGCCGA GATCAGAAAT CCCTAAGAGG AAGGGGCAGT ACTCTCGGTC

TGGACATCAA GACAGCCACA CGTGCTGGAA AGCAGATAGT GGAGCGGATT CTGAAAGAAG

AATCCGATGA GGCACTTAAA ATGACCATGG CCTCTGTACC TGCGTCGCGT TACCTAACTG

ACATGACTCT TGAGGAAATG TCAAGGGACT GGTCCATGCT CATACCCAAG CAGAAAGTGG

CAGGCCCTCT TTGTATCAGA ATGGACCAGG CGATCATGGA TAAGAACATC ATACTGAAAG

CGAACTTCAG TGTGATTTTT GACCGGCTGG AGACTCTAAT ATTGCTAAGG GCTTTCACCG

AAGAGGGAGC AATTGTTGGC GAAATTTCAC CATTGCCTTC TCTTCCAGGA CATACTGCTG

-continued

AGGATGTCAA AAATGCAGTT GGAGTCCTCA TCGGAGGACT TGAATGGAAT GATAACACAG

TTCGAGTCTC TGAAACTCTA CAGAGATTCG CTTGGAGAAG CAGTAATGAG AATGGGAGAC

CTCCACTCAC TCCAAAACAG AAACGAGAAA TGGCGGGAAC AATTAGGTCA GAAGTTTGAA

GAAATAAGAT GGTTGATTGA AGAAGTGAGA CACAAACTGA AGATAACAGA GAATAGTTTT

GAGCAAATAA CATTTATGCA AGCCTTACAT CTATTGCTTG AAGTGGAGCA AGAGATAAGA

ACTTTCTCGT TTCAGCTTAT TTAGTACTAA AAAACACCCT TGTTTCTACT

HA

(SEQ ID NO: 7)

AGCAAAAGCAGGGGAAAATAAAAACAACCAAAATGAAGGCAAACCTACTGGTCCTGTTATGTGCACT

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ACAGTACTCGAGAAGAATGTGACAGTGACACACTCTGTTAACCTGCTCGAAGACAGCCACAACGGAA

AACTATGTAGATTAAAAGGAATAGCCCCACTACAATTGGGGAAATGTAACATCGCCGGATGGCTCTT

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TCTGAGAATGGAATATGTTATCCAGGAGATTTCATCGACTATGAGGAGCTGAGGGAGCAATTGAGCT

CAGTGTCATCATTCGAAAGATTCGAAATATTTCCCAAAGAAAGCTCATGGCCCAACCACAACACAAA

CGGAGTAACGGCAGCATGCTCCCATGAGGGGAAAAGCAGTTTTTACAGAAATTTGCTATGGCTGACG

GAGAAGGAGGGCTCATACCCAAAGCTGAAAAATTCTTATGTGAACAAAAAAGGGAAAGAAGTCCTTG

TACTGTGGGGTATTCATCACCCGCCTAACAGTAAGGAACAACAGAATCTCTATCAGAATGAAAATGC

TTATGTCTCTGTAGTGACTTCAAATTATAACAGGAGATTTACCCCGGAAATAGCAGAAAGACCCAAA

GTAAGAGATCAAGCTGGGAGGATGAACTATTACTGGACCTTGCTAAAACCCGGAGACACAATAATAT

TTGAGGCAAATGGAAATCTAATAGCACCAATGTATGCTTTCGCACTGAGTAGAGGCTTTGGGTCCGG

CATCATCACCTCAAACGCATCAATGCATGAGTGTAACACGAAGTGTCAAACACCCCTGGGAGCTATA

AACAGCAGTCTCCCTTACCAGAATATACACCCAGTCACAATAGGAGAGTGCCCAAAATACGTCAGGA

GTGCCAAATTGAGGATGGTTACAGGACTAAGGAACATTCCGTCCATTCAATCCAGAGGTCTATTTGG

AGCCATTGCCGGTTTTATTGAAGGGGGATGGACTGGAATGATAGATGGATGGTATGGTTATCATCAT

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CAAACAAGGTGAACACTGTTATCGAGAAAATGAACATTCAATTCACAGCTGTGGGTAAAGAATTCAA

CAAATTAGAAAAAAGGATGGAAAATTTAAATAAAAAAGTTGATGATGGATTTCTGGACATTTGGACA

TATAATGCAGAATTGTTAGTTCTACTGGAAAATGAAAGGACTCTGGATTTCCATGACTCAAATGTGA

AGAATCTGTATGAGAAAGTAAAAAGCCAATTAAAGAATAATGCCAAAGAAATCGGAAATGGATGTTT

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AGAGATATGAGGAAAAACACCCTTGTTTCTACT

NA

(SEQ ID NO: 8)

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CAAACTGGAAGTCAAAACCATACTGGAATATGCAACCAAAACATCATTACCTATAAAAATAGCACCT

GGGTAAAGGACACAACTTCAGTGATATTAACCGGCAATTCATCTCTTTGTCCCATCCGTGGGTGGGC

TATATACAGCAAAGACAATAGCATAAGAATTGGTTCCAAAGGAGACGTTTTTGTCATAAGAGAGCCC

-continued

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AGCATTCAAGTGGGACTGTTAAGGACAGAAGCCCTTATAGGGCCTTAATGAGCTGCCCTGTCGGTGA

AGCTCCGTCCCCGTACAATTCAAGATTTGAATCGGTTGCTTGGTCAGCAAGTGCATGTCATGATGGC

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GAACCGGCCATGGGTGTCTTTCGATCAAAACCTGGATTATCAAATAGGATACATCTGCAGTGGGGTT

TTCGGTGACAACCCGCGTCCCGAAGATGGAACAGGCAGCTGTGGTCCAGTGTATGTTGATGGAGCAA

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TTCCAGACATGGGTTTGAGATGATTTGGGATCCTAATGGATGGACAGAGACTGATAGTAAGTTCTCT

GTGAGGCAAGATGTTGTGGCAATGACTGATTGGTCAGGGTATAGCGGAAGTTTCGTTCAACATCCTG

AGCTGACAGGGCTAGACTGTATGAGGCCGTGCTTCTGGGTTGAATTAATCAGGGGACGACCTAAAGA

AAAAACAATCTGGACTAGTGCGAGCAGCATTTCTTTTTGTGGCGTGAATAGTGATACTGTAGATTGG

TCTTGGCCAGACGGTGCTGAGTTGCCATTCAGGATTGACAAGTAGTCTGTTCAAAAAACTCCTTGTT

TCTACT

High-titer A/PR/8/34 (H1N1, PR8(UW)) virus grows 10 times better than other A/PR/8/34 PR8 strains in eggs (10^{10} EID_{50}/mL; HA titer:1:8,000). Thus, replacement of the HA and NA genes of PR8(UW) with those of a currently circulating strain of influenza virus results in a vaccine strain that can be safely produced, and validates the use of PR8 (UW) as a master vaccine strain.

Genes that contribute to different growth properties between PR8(UW) and PR8 (Cambridge), which provides the non-HA and -NA genes of the NIBRG-14 vaccine strain (FIG. **1**A, **1**B, **1**C, **1**D, **1**E), were determined. Higher titers in eggs were obtained when the majority of internal genes were from PR8(UW). Highest titers were with the M viral segment of PR8(UW) and the NS gene of PR8 (Cambridge). The NS gene in PR8(UW) has a K (lysine) at residue 55 while the NS gene in PR8(Cam) has a E (glutamic acid). The polymerase subunit (PA, PB1, and PB2) and NP genes of PR8(UW) enhanced the growth of an H5N1 vaccine seed virus in chicken embryonated eggs, and the NS gene of PR8(Cambridge) enhanced the growth of an H5N1 vaccine seed virus in chicken embryonated eggs. A tyrosine (Y) at position 360 in PB2 of PR8(UW) likely contributes to the high growth rate of that virus in MDCK cells.

Example 2

To develop an high-yield A/PR/8/34 (H1N1; PR8) virus backbone for growth of vaccine virus in 50 specific host cells, random mutagenesis of the internal genes of PR8(HG) (PR8UW) was conducted. Random mutations were introduced into the UW-PR8 (Example 1) internal genes by error-prone PCR, after which plasmid libraries were prepared that possessed the random mutations in an individual UW-PR8 internal gene. Then virus libraries (PR8/H5N1) were generated that possessed random mutations in an individual UW-PR8 internal gene, along with the other wild type internal genes and the NA and 'detoxified' HA genes of A/chicken/Indonesia/NC/09 (H5N1) virus (Table 1), to generate "6+2" recombinant viruses. Consecutive passages of the virus in MDCK cells were employed to select for variants with high-growth properties.

TABLE 1

Virus libraries generated

Number	Internal genes: Gene library	Internal genes: Other internal genes	HA + NA	Titer of virus library (pfu/ml)
Control	PR8 wild type		NC/09/H5N1	3×10^6
1	PB2	5 UW-PR8 genes	NC/09/H5N1	2.1×10^2
2	PB1	5 UW-PR8 genes	NC/09/H5N1	1.6×10^5
3	PA	5 UW-PR8 genes	NC/09/H5N1	7×10^3
4	NP	5 UW-PR8 genes	NC/09/H5N1	1.5×10^3
5	M	5 UW-PR8 genes	NC/09/H5N1	1×10^6
6	NS	5 UW-PR8 genes	NC/09/H5N1	1.8×10^6
7	PB2 + PB1 + PA	3 UW-PR8 genes	NC/09/H5N1	75
8	PB2 + PB1 + PA + NP	2 UW-PR8 genes	NC/09/H5N1	33
9	PB2 + NS	4 UW-PR8 genes	NC/09/H5N1	2×10^2
10	M + NS	4 UW-PR8 genes	NC/09/H5N1	5.7×10^5

Virus libraries were passaged 12 times in MDCK cells or, after 2 passages, the libraries were mixed and 10 more passages were carried out (FIG. **2**).

After 10 to about 12 consecutive passages in MDCK cells, plaque assays were performed and over 1,400 individual plaques were picked. FIG. **3** shows the numbers of clones with various HA titers. Growth-enhancing mutations included: PB2: M202L, F323L, I504V, PB1: E112G, V644A, NP: R74K, N417D, I116L, and NS: S161T. FIG. **4** provides the titers of recombinant viruses generated from selected mutations.

36 viruses with the highest HA titers from the random mutagenesis libraries were sequenced (Table 2)

TABLE 2

Sequences of viruses with the highest HA titers

Clone #	Library	HA titer (2^n)	PB2	PB1	PA	HA (H3 numbering)	NP	NA	M	NS
WT		7								
329	Mix	9	M202L F323L			L182V				
154	Mix	8.5~9	M202L F323L			L182V				
347	Mix	9	M202L F323L			L182V				
94	Mix	8.5	M202L F323L			F252I	I116L	L55S		
1045	Mix	9	M202L F323L	V644A		F252I				
965	Mix	8.5~9	M202L F323L		F105C	V184I			P90S	
50	Mix	8.5	M202L F323L			M148I (HA2)	R293M			
1005	Mix	9~9.5	M202L F323L	V644A	R401K	M148I (HA2)				T49A
134	Mix	8.5	M202L F323L							A223E
387	Mix	9	M202L F323L	M507V V644A						
852	Mix	9~9.5	M202L F323L M243I	R54I						
981	Mix	8.5~9	M202L F323L	Q247H						
993	Mix	8.5~9	M202L F323L				N224I			
1043	Mix	8.5~9	I504V			L182V	R74K			
398	Mix	8.5	I504V			L182V	R74K, N417D			A30P
1007	Mix	8.5	I504V	V644A		F252I	M371V			
1042	Mix	8.5~9	I504V	E75V D76G E78P P79V S80G V644A E697P F699L F700L P701H S702R Y705T		F252I	R74K			
999	Mix	8.5~9	I504V			M148I (HA2)	R74K, N417D			
1014	Mix	8.5	I504V	T59I G62A A63P V644A N694K L695T		M1481 (HA2)	R74K, N417D	A265V		
1016	Mix	8.5~9	I504V			M148I (HA2)				
540	PB1	8.5		E112G (PB1-F2-R81G)		K162E				S161T
548	PB1	8.5~9		E112G (PB1-F2-R81G) L624V		K162E				S161T
191	PB1	88.5		E112G (PB1-F2-R81G)						
571	PB1	9~9.5		E112G (PB1-F2-R81G)						
572	PB1	8.5		E112G (PB1-F2-R81G)						

TABLE 2-continued

Sequences of viruses with the highest HA titers

Clone #	Library	HA titer (2^n)	PB2	PB1	PA	HA (H3 numbering)	NP	NA	M	NS
573	PB1	8.5		E112G (PB1-F2-R81G)						
1404	PB1	8.5	I57V T58G A59V K61Q E677D D678E P679M	E112G (PB1-F2-R81G) S713C						
1408	PB1	8.5		M40I G180W						S161T
582	PB1	8.5~9		M40L, G180W						S161T
545	PB1	8.5		M40L, G180W		K121E (HA2)				
543	PB1	8.5		I667T						
219	PB1	9		I667T, M714T		K162E				
344	Mix	8.5~9	M66R			L182V				
312	Mix	8.5~9				L182V	I116L			R140Q
320	Mix	8.5				L182V				
209	PB1	8.5~9		R54I		E136D, Q179L, A194V				

In a second approach, potentially growth-enhancing mutations described in the literature were introduced into the background of UW-PR8 virus (see Table 3 for virus stock titers) and tested for replicative ability. FIGS. **5**A, **5**B, **5**C and **5**D show growth curves for various viruses.

TABLE 3

UW-PR8 viruses possessing mutation(s) identified in the literature

Gene	Mutation(s)	Virus stock titer (Pfu/ml)
WT	—	2×10^7
PB2	A44S	4.5×10^7
	E158G	3.2×10^4
	E158G + NP N101G	7.5×10^4
	E158A	8.3×0^6
	D253N + Q591K	8.3×10^6
	D256G	2.8×10^7
	R368K	3.1×10^7
	E391Q	1.4×10^8
	I504V + PA I550L	1.1×10^8
	Q591K	4.4×10^7
	V613T	1.8×10^7
	A661T	2.2×10^7
	D701N + S714R + NP N319K	1×10^6
	D701N	2.1×10^7
PB1	R327K	1.3×10^7
	V336I	2.3×10^7
	L473V + L598P	3.9×10^6
PB1F2	F2 N66S	1.6×10^8
	F2 K73R	1.1×10^8
	F2 V76A	4.4×10^7

TABLE 3-continued

UW-PR8 viruses possessing mutation(s) identified in the literature

Gene	Mutation(s)	Virus stock titer (Pfu/ml)
	F2 R79Q	6.2×10^6
	F2 L82S	2.7×10^7
	F2 E87Q	1.5×10^6
PA	T97I	1.6×10^7
	K142N	3.3×10^7
	S225C	6.7×10^7
	S149P + T357K	3.4×10^8
	K356R	8.5×10^7
	A404S	5.2×10^7
	S421I	2.7×10^7
NP	R293K	4.7×10^7
	R305K	7.2×10^7
	E372D	2.2×10^7
	R422K	1.3×10^8
	T442A	5×10^7
	D455E	2.2×10^7
	I109V	3.9×10^7
M	V97A + Y100H	1.4×10^7
NS1	K55E	1.6×10^7

In a third approach, candidates from approaches 1 and 2 were combined and HA titers and PFU/mL determined (Table 4).

TABLE 4

High-growth candidates identified in approaches 1 and 2 were tested in various combinations.

	Gene origin								Virus stock titer	
#	HA	NA	PB2	PB1	PA	NP	M	NS	HA (2^n)	Pfu/ml
WT	Indo/NC/09	Indio/NC/09	UW-PR8	UW-PR8	UW-PR8	UW-PR8	UW-PR8	UW-PR8	7	3.00E+07
1	(detoxified)		M202L F323L	M507V V644A		I116L		K55E	9~9.5	2.00E+08
2			M202L F323L	R54I		N224I		K55E	5	1.00E+05
3			M202L F323L	Q247H	R401K			T49A	9	1.00E+08
4			M202L F323L	M507V V644A	K356R	T442A	V97A Y100H	K55E	10~10.5	1.60E+08
5			I504V	M507V V644A	I550L	R74K N417D		K55E	8~8.5	5.70E+07
6			I504V	M507V V644A	I550L	R74K N417D	V97A Y100H	K55E	9~9.5	4.40E+07
7			I505V	E112G	I550L	R74K		S161T	9	1.60E+08
8			M202L F323L	I667T M714T		I116L		R140Q	<1	<1E3
9			M202L F323L	E112G (PB1-F2-R81G)				S161T	8.5	1.30E+08
10			M66R	M40I G180W		R74K		S161T	8~8.5	2.30E+07
12			R368K	PB1 F2 N66S	K356R	R422K		K55E	5.5	9.00E+02
13			E391Q	R327K	S149P T357K	R293K			3	1.60E+06
14			Q591K	PB1 F2 K73R	S225C	R422K		K55E	7.5	2.00E+07
23							V97A		8.5~9	1.50E+07
24							Y100H		9~9.5	2.90E+07
25	NCR 15-19nt mut[1]	Indo/NC/09	M202L F323L	M507V V644A	K356R	R422K	V97A Y100H	K55E	9.5~10	7.50E+07
26	Indo/NC/09	Indo/NC/09						A30P	6.5~7	1.00E+07
27	(detoxified)							T49A	6.5~7	2.00E+07
28								R140Q	8	4.00E+07
29								S161T	7~7.5	1.40E+07
30								A223E	7.5	1.00E+07
31				I667T M714T					3.5	4.00E+05
32	NCR 15-19nt mut	UW-PR8	M202L F323L	V644A	K356R	T442A	Y100H	K55E	7~7.5	4.30E+06
33	Indo/NC/09 (detoxified)	Indio/NC/09	M202L F323L	E112G (PB1-F2-R81G)	K356R	R74K	Y100H	K55E	9~9.5	7.00E+07
34	NCR 15-19nt mut	UW-PR8	I504V	M507V V644A			V97A Y100H	K55E	7	2.00E+05
35	Indo/NC/09	Indo/NC/09	M202L F323L	M507V V644A	R401K	T442A	Y100H	R1400	9	3.20E+07
36	(detoxified)		I504V	E112G (PB1-F2-R81G)	I550L	I112L	Y100H	R140Q	9.5	1.30E+08
37			M202L F323L	E112G (PB1-F2-R81G)	S149P T357K	T442A	Y100H	K55E	0	0.00E+00
38			M202L F323L	M507V V644A		I116L	Y100H	K55E	10.1	2.30E+08
39			M202L F323L	M507V V644A	K356R	T442A	Y100H	K55E	9.8	1.00E+08
40			I504V	M507V V644A	I550L	T442A	Y100H	K55E	9.2	6.00E+07
41			I504V	I112E	I550L	R74K	Y100H	K55E	9.2	7.50E+07
P17			I504V	E112G (PB1-F2-R81G)	S225C	R74K N4170	V97A Y100H	K55E	9.5~10	5.80E+08

TABLE 4-continued

High-growth candidates identified in approaches 1 and 2 were tested in various combinations.

	Gene origin								Virus stock titer	
#	HA	NA	PB2	PB1	PA	NP	M	NS	HA (2^n)	Pfu/ml
P26			M202L F323L	M40L G180W	S225C	R422K	V97A Y100H	K55E	10	3.00E+08
P61		Indo/NC/09 NA P263T[2]	M202L F323L	Q247H	K142N	R74K	V97A Y100H	K55E	10~10.5	2.00E+08

[1]Mutation in the HA gene noncoding region;

[2]A P263T mutation was detected in the NA protein of this virus clone

As shown in Table 4, several recombinant viruses were identified that replicated better than wild type, such as #1, #4, #36, #38, P17, P16, and P61. To identify the growth characteristics of these viruses, growth kinetics in MDCK cells were determined (FIG. **7**). For one candidate, virus was purified on sucrose gradients and HA content and viral total protein evaluated. FIG. **8**A shows HA titer of wild type (UW-PR8) and #4, FIG. **8**B shows viral protein for wild type (UW-PR8) and #4, and FIGS. **8**B and **8**C is a SDS-PAGE analysis of viral proteins of wild type (UW-PR8) and #4. Further analysis demonstrated that viruses possessing the V97A/Y100H mutations in M1 yielded higher HA titers than the parental virus, although the virus titer was lower (see FIGS. **9**A and **9**B). The V97A/Y100H mutations in M1 may result in particles with a larger surface into which more HA protein can be incorporated. Since inactivated influenza viruses are dosed based on their HA content, variants with high HA content are attractive vaccine candidates.

To identify mutations in the influenza promoter region that provide for enhanced replication, viruses possessing a 'U' at position 4 at the 3' end of all eight vRNA segments were prepared in the UW-PR8 PA, PB1 and PB2 internal genes (the UW-PR8 PB2, PB1, and PA segments possess a 'C' at position 4). The growth curves of the resulting viruses are shown in FIG. **11**C.

Viruses possessing combinations of promoter mutations and amino acid changes were prepared and titers determined (Table 5).

TABLE 5

Virus titers of high-growth candidates.

	Gene backbone								Virus stock titer	
Viruses	HA	NA	PB2	PB1	PA	NP	M	NS	HA (2^n)	pfu/ml
Control	WT	WT	WT	WT	WT	WT	WT	WT	7	3.0E+07
1	WT	WT	3'C4U	3'C4U	3'C4U	R74K	V97A	K55E	10.5	2.2E+09
2	3' G3A U5C C8U & 5' U3C A8G		M202L F323L	Q247H	K142N		Y100H		8.5~9	5.6E+07
3	NCR 15-19nt mut								9~9.5	1.4E+09
4	3' G3A U5C C8U & 5' U3C A8G & NCR 15-19nt mut								7	7.0E+07

Note:

3'G3A, U5C, C8U and 5' U3C and A8G are nucleotide changes in the promoter of the HA viral RNA; NCR 15-19nt mut refers to mutations in the HA 3' non-coding region of positions 15-19 ("AAGUU" was replaced with "UUUAA").

Codon usage optimization was also conducted. Alteration of codons may increase protein expression but could also alter RNA structure and stability. For example, codon usage optimization of the PB2 viral segment was performed to reflect the codon usage in canine cells (since MDCK cells are of canine origin) (FIG. **10**A), while leaving the packaging signals (located at the 5' and 3' ends of the vRNA) unaltered. In one approach, codon optimization was performed for all codons in the 'internal' region of the PB2 gene (FIG. **10**C) and in another approach, codon optimization was performed for so-called 'rare' codons (FIG. **10**B) (used at significantly lower frequency compared to the codon used most frequently for a given amino acid) (see SEQ ID NO:25 in FIG. **10**F). Analyses were carried out using the "Graphical Codon Usage Analyzer" (www.gcua.de). The titers of those viruses are shown in Table 6 (see also FIGS. **10**B and **10**C).

TABLE 6

Titers of viruses encoding codon-optimized PB2 genes.

	Gene backbone								Virus stock titer	
Virus	HA	NA	PB2	PB1	PA	NP	M	NS	HA (2^n)	pfu/ml
Wild type	WT	WT	WT	WT	WT	WT	WT	W T	7~7.5	3.5E+07
PB2 codon optimization-1	WT	WT	Rare codon optimized PB2	WT	WT	WT	WT	WT	9	2.1E+08
PB2 codon optimization-2	WT	WT	All Codon optimized PB2	WT	WT	WT	WT	WT	3	9.0E+05

Optimization of rare codons in PB2 resulted in increased titers compared to wild type virus (UW-PR8) (see FIG. **10**D). Other viral segments were codon optimized and titers of viruses with those segments or combinations of optimized segments were determined (FIG. **10**E).

In another approach to increase virus titer in MDCK cells, chimeric HA and NA genes were prepared (FIG. **13**A) and titers of viruses having those genes were determined (FIG. **13**B).

Viruses with combinations of the above-mentioned mutations (high growth backbone mutations, promoter mutations, chimeric HA and NA genes and canine codon optimization) were prepared and growth kinetics, PFU and HA titers of those viruses were determined (see FIGS. **14**A and **14**B). An exemplary set of backbone mutations are canine codon opti-PB2+C4U+M202L, F323L; PB1: C4U+Q247H; PA: C4U+K142N; NP: Canine codon opti-NP+R74K; M: V97A, Y100H; and NS: K55E.

Any of the mutations described herein, or any combination thereof, may be combined with, for instance, seasonal H1N1 and H3N2, H3N2 Variant, PdmH1N1, H5N1, H7N9 or H9N2, or other clades or candidate vaccine strains. For example, HA and NA genes from A/California/04/2009(pdm H1N1) were combined with the six internal genes of UW-PR/8 to generate "6+2" recombinant viruses. Eleven virus libraries were generated and passaged 10 times in eggs. Three rounds of limiting dilution were performed to screen for high growth mutants (FIG. **15**). In one embodiment, a variant with high growth properties in MDCK cells has a PB2 viral segment with a promoter mutation (C4U) and a mutation that results in I504V (relative to the parental virus); a PB1 viral segment with a promoter mutation (C4U) and a mutation that results in E112G; a PA viral segment with a promoter mutation (C4U) and a mutation that results in S225C; a NP viral segment with mutations that result in R74K and N417D; a M viral segment with mutations that result in V97A and Y100H; and a NS viral segment with a mutation that results in K55E, where optionally the sequence of one or more viral segments, e.g., the NP viral segment, is modified to include canine codon optimized codons. In one embodiment, a variant with high growth properties in MDCK cells has a canine codon optimized PB2 viral segment with a promoter mutation (C4U) and mutations that result in M202L and F323L; a PB1 viral segment with a promoter mutation (C4U) and a mutation that results in Q247H; a PA viral segment with a promoter mutation (C4U) and a mutation that results in K142N; a canine codon optimized NP viral segment with a mutation that results in R74K; a M viral segment with mutations that result in V97A Y100H; and a NS viral segment with a mutation that results in K55E.

Similar experiments were conducted in Vero cells, e.g., after about 3 to 5 passages in Vero cells, using clones with high replicative properties in MDCK cells (see FIG. **16**). FIG. **17** shows 5 viruses likely to have high replicative properties in Vero cells. In one embodiment, a PR8(UW) variant with high-growth properties in Vero cells has the following mutations that may be used in various combinations to increase the replicative ability of PR8(UW) virus: PB2 segment: C4U (promoter mutation), I504V (amino acid change); PB1 segment: C4U (promoter mutation); M40L (amino acid change), G180W (amino acid change); PA segment: C4U (promoter mutation), R401K (amino acid change); NP segment: I116L (amino acid change); NS segment: A30P (amino acid change in NS1), or R118K (amino acid change in NS1).

In one embodiment, a PR8(UW) variant with high-growth properties has the following residues that may be used in various combinations with each other and/or other residues, e.g., those that enhance virus replication, to increase the replicative ability of reassortants having PR8(UW) based viral segment(s): a HA segment with one or more of 136D, 162E, 179L, 182V, 184I, 252I, 449E, and/or 476I: a NA segment with 55S and/or 265V; a NS segment with NS1 having 118K; F2 with 81G; a PB1 segment with 62A, 261G, 361R, 621R, and/or 654S, and/or viral segment promoters with the growth-enhancing nucleotides described herein, e.g., having one or more of the nucleotide changes G1012C, A1013U, or U1014A in the M viral segment.

Example 3

To assess the contribution of individual viral RNA (vRNA) segments to high-yield properties, a series of reassortant viruses was generated that possessed one or several vRNA segments of a high-yield PR8 (PR8-HY) variant in the background of the parental virus [UW-PR8_Indo/05 (HA+NA)]. Vero cells were infected in triplicate with the indicated viruses at a MOI of 0.005 and incubated at 37° C. in the presence of trypsin. At the indicated time points, virus titers and HA titers were determined by performing plaque or HA assays, respectively. The results are shown in FIG. **20**. These data indicated that several vRNA segments contribute to the properties of PR8-HY virus. In particular, the PB2+PB1+PA+NP vRNAs of PR8-HY virus conferred an appreciable increase in virus and HA titers, evidencing the enhanced replicative ability of this virus.

To further assess which component of the viral replication complex that provides for high-yield properties, wild-type or high-yield PB2, PB1, PA, and NP proteins were tested in various combinations in minireplicon assays in human 293T, canine MDCK, African green monkey Vero, and avian DF1 cells. The results are shown in FIG. **21**. Interestingly, the PB2, PB1, PA, and NP proteins of PR8-HY virus attenuated the viral replicative ability in 293T, Vero, and DF1 cells; this effect was primarily conferred by the PB2 protein. In contrast, the combination of PB2+PB1+PA+NP proteins derived from PR8-HY virus conferred a substantial increase in replicated ability in canine MDCK cells, which were used for the selection of PR8-HY virus. The findings suggested host-dependent mechanisms underlying the high yield of PR8-HY virus. For example, the combination of PB1+PA+NP proteins, or a subset thereof, derived from PR8-HY may confer enhanced viral replicative ability in 293T, Vero, and DF1 cells.

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All publications, patents and patent applications are incorporated herein by reference. While in the foregoing specification this invention has been described in relation to certain preferred embodiments thereof, and many details have been set forth for purposes of illustration, it will be apparent to those skilled in the art that the invention is susceptible to additional embodiments and that certain of the details described herein may be varied considerably without departing from the basic principles of the invention.

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acatgactct tgaggaaatg tcaagggact ggtccatgct catacccaag cagaaagtgg 360

caggccctct ttgtatcaga atggaccagg cgatcatgga taagaacatc atactgaaag 420

cgaacttcag tgtgattttt gaccggctgg agactctaat attgctaagg gctttcaccg 480

aagagggagc aattgttggc gaaatttcac cattgccttc tcttccagga catactgctg 540

aggatgtcaa aaatgcagtt ggagtcctca tcggaggact tgaatggaat gataacacag 600

ttcgagtctc tgaaactcta cagagattcg cttggagaag cagtaatgag aatgggagac 660

ctccactcac tccaaaacag aaacgagaaa tggcgggaac aattaggtca gaagtttgaa 720

gaaataagat ggttgattga agaagtgaga cacaaactga agataacaga gaatagtttt 780

gagcaaataa catttatgca agccttacat ctattgcttg aagtggagca agagataaga 840

actttctcgt ttcagcttat ttagtactaa aaaacaccct tgtttctact 890

<210> SEQ ID NO 7
<211> LENGTH: 1775
<212> TYPE: DNA
<213> ORGANISM: Influenza virus

<400> SEQUENCE: 7

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gtgcacttgc agctgcagat gcagacacaa tatgtatagg ctaccatgcg aacaattcaa 120

ccgacactgt tgacacagta ctcgagaaga atgtgacagt gacacactct gttaacctgc 180

tcgaagacag ccacaacgga aaactatgta gattaaaagg aatagcccca ctacaattgg 240

ggaaatgtaa catcgccgga tggctcttgg gaaacccaga atgcgaccca ctgcttccag 300

tgagatcatg gtcctacatt gtagaaacac caaactctga gaatggaata tgttatccag 360

gagatttcat cgactatgag gagctgaggg agcaattgag ctcagtgtca tcattcgaaa 420

gattcgaaat atttcccaaa gaaagctcat ggcccaacca caacacaaac ggagtaacgg 480

cagcatgctc ccatgagggg aaaagcagtt tttacagaaa tttgctatgg ctgacggaga 540

aggagggctc atacccaaag ctgaaaaatt cttatgtgaa caaaaaaggg aaagaagtcc 600

ttgtactgtg gggtattcat cacccgccta acagtaagga acaacagaat ctctatcaga 660

atgaaaatgc ttatgtctct gtagtgactt caaattataa caggagattt accccggaaa 720

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tagcagaaag acccaaagta agagatcaag ctgggaggat gaactattac tggaccttgc 780

taaaacccgg agacacaata atatttgagg caaatggaaa tctaatagca ccaatgtatg 840

ctttcgcact gagtagaggc tttgggtccg gcatcatcac ctcaaacgca tcaatgcatg 900

agtgtaacac gaagtgtcaa acacccctgg gagctataaa cagcagtctc ccttaccaga 960

atatacaccc agtcacaata ggagagtgcc caaaatacgt caggagtgcc aaattgagga 1020

tggttacagg actaaggaac attccgtcca ttcaatccag aggtctattt ggagccattg 1080

ccggttttat tgaaggggga tggactggaa tgatagatgg atggtatggt tatcatcatc 1140

agaatgaaca gggatcaggc tatgcagcgg atcaaaaaag cacacaaaat gccattaacg 1200

ggattacaaa caaggtgaac actgttatcg agaaaatgaa cattcaattc acagctgtgg 1260

gtaaagaatt caacaaatta gaaaaaagga tggaaaattt aaataaaaaa gttgatgatg 1320

gatttctgga catttggaca tataatgcag aattgttagt tctactggaa aatgaaagga 1380

ctctggattt ccatgactca aatgtgaaga atctgtatga gaaagtaaaa agccaattaa 1440

agaataatgc caaagaaatc ggaaatggat gttttgagtt ctaccacaag tgtgacaatg 1500

aatgcatgga aagtgtaaga aatgggactt atgattatcc caaatattca gaagagtcaa 1560

agttgaacag ggaaaaggta gatggagtga aattggaatc aatggggatc tatcagattc 1620

tggcgatcta ctcaactgtc gccagttcac tggtgctttt ggtctccctg ggggcaatca 1680

gtttctggat gtgttctaat ggatctttgc agtgcagaat atgcatctga gattagaatt 1740

tcagagatat gaggaaaaac acccttgttt ctact 1775

<210> SEQ ID NO 8
<211> LENGTH: 1413
<212> TYPE: DNA
<213> ORGANISM: Influenza virus

<400> SEQUENCE: 8

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gtctggtagt cggactaatt agcctaatat tgcaaatagg gaatataatc tcaatatgga 120

ttagccattc aattcaaact ggaagtcaaa accatactgg aatatgcaac caaaacatca 180

ttacctataa aaatagcacc tgggtaaagg acacaacttc agtgatatta accggcaatt 240

catctctttg tcccatccgt gggtgggcta tatacagcaa agacaatagc ataagaattg 300

gttccaaagg agacgttttt gtcataagag agccctttat ttcatgttct cacttggaat 360

gcaggacctt ttttctgacc caaggtgcct tactgaatga caagcattca agtgggactg 420

ttaaggacag aagcccttat agggccttaa tgagctgccc tgtcggtgaa gctccgtccc 480

cgtacaattc aagatttgaa tcggttgctt ggtcagcaag tgcatgtcat gatggcatgg 540

gctggctaac aatcggaatt tcaggtccag ataatggagc agtggctgta ttaaaataca 600

acggcataat aactgaaacc ataaaaagtt ggaggaagaa aatattgagg acacaagagt 660

ctgaatgtgc ctgtgtaaat ggttcatgtt ttactataat gactgatggc ccgagtgatg 720

ggctggcctc gtacaaaatt ttcaagatcg aaaaggggaa ggttactaaa tcaatagagt 780

tgaatgcacc taattctcac tatgaggaat gttcctgtta ccctgatacc ggcaaagtga 840

tgtgtgtgtg cagagacaat tggcatggtt cgaaccggcc atgggtgtct ttcgatcaaa 900

acctggatta tcaaatagga tacatctgca gtggggtttt cggtgacaac ccgcgtcccg 960

aagatggaac aggcagctgt ggtccagtgt atgttgatgg agcaaacgga gtaaagggat 1020

tttcatatag gtatggtaat ggtgtttgga taggaaggac caaaagtcac agttccagac 1080

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atgggtttga gatgatttgg gatcctaatg gatggacaga gactgatagt aagttctctg 1140

tgaggcaaga tgttgtggca atgactgatt ggtcagggta tagcggaagt ttcgttcaac 1200

atcctgagct gacagggcta gactgtatga ggccgtgctt ctgggttgaa ttaatcaggg 1260

gacgacctaa agaaaaaaca atctggacta gtgcgagcag catttctttt tgtggcgtga 1320

atagtgatac tgtagattgg tcttggccag acggtgctga gttgccattc agcattgaca 1380

agtagtctgt tcaaaaaact ccttgtttct act 1413

<210> SEQ ID NO 9

<400> SEQUENCE: 9

000

<210> SEQ ID NO 10
<211> LENGTH: 2342
<212> TYPE: DNA
<213> ORGANISM: Influenza virus

<400> SEQUENCE: 10

agcgaaagca ggcaaaccat ttgaatggat gtcaatccga ccttactttt cttaaaagtg 60

ccagcacaaa atgctataag cacaactttc ccttataccg gagaccctcc ttacagccat 120

gggacaggaa caggatacac catggatact gtcaacagga cacatcagta ctcagaaaag 180

ggaagatgga caacaaacac cgaaactgga gcaccgcaac tcaacccgat tgatgggcca 240

ctgccagaag acaatgaacc aagtggttat gcccaaacag attgtgtatt ggaagcaatg 300

gctttccttg aggaatccca tcctggtatt tttgaaaact cgtgtattga aacgatggag 360

gttgttcagc aaacactgag tagacaagct gacacaaggc cgacagacct atgactggac 420

tttaaataga aaccagcctg ctgcaacagc attggccaac acaatagaag tgttcagatc 480

aaatggcctc acggccaatg agtcaggaag gctcatagac ttccttaagg atgtaatgga 540

gtcaatgaaa aaagaagaaa tggggatcac aactcatttt cagagaaaga gacgggtgag 600

agacaatatg actaagaaaa tgataacaca gagaacaata ggtaaaagga aacagagatt 660

gaacaaaagg ggttatctaa ttagagcatt gaccctgaac acaatgacca aagatgctga 720

gagagggaag ctaaaacgga gagcaattgc aaccccaggg atgcaaataa gggggtttgt 780

atactttgtt gagacactgg caaggagtat atgtgagaaa cttgaacaat cagggttgcc 840

agttggaggc aatgagaaga aagcaaagtt ggcaaatgtt gtaaggaaga tgatgaccaa 900

ttctcaggac accgaacttt ctttcaccat cactggagat aacaccaaat ggaacgaaaa 960

tcagaatcct cggatgtttt tggccatgat cacatatatg accagaaatc agcccgaatg 1020

gttcagaaat gttctaagta ttgctccaat aatgttctca aacaaaatgg cgagactggg 1080

aaaagggtat atgtttgaga gcaagagtat gaaacttaga actcaaatac ctgcagaaat 1140

gctagcaagc attgatttga aatatttcaa tgattcaaca agaaagaaga ttgaaaaaat 1200

ccgaccgctc ttaatagagg ggactgcatc attgagccct ggaatgatga tgggcatgtt 1260

caatatgtta agcactgtat taggcgtctc catcctgaat cttggacaaa agagatacac 1320

caagactact tactggtggg atggtcttca atcctctgac gattttgctc tgattgtgaa 1380

tgcacccaat catgaaggga ttcaagccgg agtcgacagg ttttatcgaa cctgtaagct 1440

acttggaatc aatatgagca agaaaaagtc ttacataaac agaacaggta catttgaatt 1500

cacaagtttt ttctatcgtt atgggtttgt tgccaatttc agcatggagc ttcccagttt 1560

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tggggtgtct gggatcaacg agtcagcgga catgagtatt ggagttactg tcatcaaaaa 1620

caatatgata aacaatgatc ttggtccagc aacagctcaa atggcccttc agttgttcat 1680

caaagattac aggtacacgt accgatgcca tagaggtgac acacaaatac aaacccgaag 1740

atcatttgaa ataaagaaac tgtgggagca aacccgttcc aaagctggac tgctggtctc 1800

cgacggaggc ccaaatttat acaacattag aaatctccac attcctgaag tctgcctaaa 1860

atgggaattg atggatgagg attaccaggg gcgtttatgc aacccactga acccatttgt 1920

cagccataaa gaaattgaat caatgaacaa tgcagtgatg atgccagcac atggtccagc 1980

caaaaacatg gagtatgatg ctgttgcaac aacacactcc tggatcccca aaagaaatcg 2040

atccatcttg aatacaagtc aaagaggagt acttgaagat gaacaaatgt accaaaggtg 2100

ctggaattta tttgaaaaat tcttccccag cagttcatac agaagaccag tcgggatatc 2160

cagtatggtg gaggctatgg tttccagagc ccgaattgat gcacggattg atttcgaatc 2220

tggaaggata aagaaagaag agttcactga gatcatgaag atctgttcca ccattgaaga 2280

gctcagacgg caaaaatagt gaatttagct tgtccttcat gaaaaaatgc cttgtttcta 2340

ct 2342

<210> SEQ ID NO 11
<211> LENGTH: 2341
<212> TYPE: DNA
<213> ORGANISM: Influenza virus

<400> SEQUENCE: 11

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tcgcagtctc gcacccgcga gatactcaca aaaaccaccg tggaccatat ggccataatc 120

aagaagtaca catcaggaag acaggagaag aacccagcac ttaggatgaa atggatgatg 180

gcaatgaaat atccaattac agcagacaag aggataacgg aaatgattcc tgagagaaat 240

gagcaaggac aaactttatg gagtaaaatg aatgatgccg gatcagaccg agtgatggta 300

tcacctctgg ctgtgacatg gtggaatagg aatggaccaa tgacaaatac agttcattat 360

ccaaaaatct acaaaactta ttttgaaaga gtcgaaaggc taaagcatgg aacctttggc 420

cctgtccatt ttagaaacca agtcaaaata cgtcggagag ttgacataaa tcctggtcat 480

gcagatctca gtgccaagga ggcacaggat gtaatcatgg aagttgtttt ccctaacgaa 540

gtgggagcca ggatactaac atcggaatcg caactaacga taaccaaaga gaagaaagaa 600

gaactccagg attgcaaaat ttctccttta atggttgcat acatgttgga gagagaactg 660

gtccgcaaaa cgagattcct cccagtggct ggtggaacaa gcagtgtgta cattgaagtg 720

ttgcatttga ctcaaggaac atgctgggaa cagatgtata ctccaggagg ggaagtgaag 780

aatgatgatg ttgatcaaag cttgattatt gctgctagga acatagtgag aagagctgca 840

gtatcagcag acccactagc atctttattg gagatgtgcc acagcacaca gattggtgga 900

attaggatgg tagacatcct taagcagaac ccaacagaag agcaagccgt ggatatatgc 960

aaggctgcaa tgggactgag aattagctca tccttcagtt ttggtggatt cacatttaag 1020

agaacaagcg gatcatcagt caagagagag gaagaggtgc ttacgggcaa tcttcaaaca 1080

ttgaagataa gagtgcatga gggatctgaa gagttcacaa tggttgggag aagagcaaca 1140

gccatactca gaaaagcaac caggagattg attcagctga tagtgagtgg gagagacgaa 1200

cagtcgattg ccgaagcaat aattgtggcc atggtatttt cacaagagga ttgtatgata 1260

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aaagcagtta gaggtgatct gaatttcgtc aatagggcga atcagcgact gaatcctatg 1320

catcaacttt taagacattt tcagaaggat gcgaaagtgc tttttcaaaa ttggggagtt 1380

gaacctatcg acaatgtgat gggaatgatt gggatattgc ccgacatgac tccaagcatc 1440

gagatgtcaa tgagaggagt gagaatcagc aaaatgggtg tagatgagta ctccagcacg 1500

gagagggtag tggtgagcat tgaccggttc ttgagagtca gggaccaacg aggaaatgta 1560

ctactgtctc ccgaggaggt cagtgaaaca cagggaacag agaaactgac aataacttac 1620

tcatcgtcaa tgatgtggga gattaatggt cctgaatcag tgttggtcaa tacctatcaa 1680

tggatcatca gaaactggga aactgttaaa attcagtggt cccagaaccc tacaatgcta 1740

tacaataaaa tggaatttga accatttcag tctttagtac ctaaggccat tagaggccaa 1800

tacagtgggt ttgtaagaac tctgttccaa caaatgaggg atgtgcttgg gacatttgat 1860

accgcacaga taataaaact tcttcccttc gcagccgctc caccaaagca aagtagaatg 1920

cagttctcct catttactgt gaatgtgagg ggatcaggaa tgagaatact tgtaaggggc 1980

aattctcctg tattcaacta caacaaggcc acgaagagac tcacagttct cggaaaggat 2040

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aggggattcc tcattctggg caaagaagac aggagatatg ggccagcatt aagcatcaat 2160

gaactgagca accttgcgaa aggagagaag gctaatgtgc taattgggca aggagacgtg 2220

gtgttggtaa tgaaacgaaa acgggactct agcatactta ctgacagcca gacagcgacc 2280

aaaagaattc ggatggccat caattagtgt cgaatagttt aaaaacgacc ttgtttctac 2340

t 2341

<210> SEQ ID NO 12
<211> LENGTH: 2234
<212> TYPE: DNA
<213> ORGANISM: Influenza virus

<400> SEQUENCE: 12

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attgtcgagc ttgcggaaaa aacaatgaaa gagtatgggg aggacctgaa aatcgaaaca 120

aacaaatttg cagcaatatg cactcacttg gaagtatgct tcatgtattc agatttccac 180

ttcatcaatg agcaaggcga gtcaataatc gtagaacttg gtgatcctaa tgcacttttg 240

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agtatttgca acactacagg ggctgagaaa ccaaagtttc taccagattt gtatgattac 360

aaggaaaata gattcatcga aattggagta acaaggagag aagttcacat atactatctg 420

gaaaaggcca ataaaattaa atctgagaaa acacacatcc acattttctc gttcactggg 480

gaagaaatgg ccacaagggc cgactacact ctcgatgaag aaagcagggc taggatcaaa 540

accaggctat tcaccataag acaagaaatg gccagcagag gcctctggga ttcctttcgt 600

cagtccgaga gaggagaaga gacaattgaa gaaaggtttg aaatcacagg aacaatgcgc 660

aagcttgccg accaaagtct cccgccgaac ttctccagcc ttgaaaattt tagagcctat 720

gtggatggat tcgaaccgaa cggctacatt gagggcaagc tgtctcaaat gtccaaagaa 780

gtaaatgcta gaattgaacc ttttttgaaa acaacaccac gaccacttag acttccgaat 840

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gaggacccaa gtcatgaagg agagggaata ccgctatatg atgcaatcaa atgcatgaga 960

acattctttg gatggaagga acccaatgtt gttaaaccac acgaaaaggg aataaatcca 1020

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aaaattccaa agactaaaaa tatgaaaaaa acaagtcagc taaagtgggc acttggtgag 1140

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tatgatagtg atgaaccaga attgaggtcg cttgcaagtt ggattcagaa tgagttcaac 1260

aaggcatgcg aactgacaga ttcaagctgg atagagcttg atgagattgg agaagatgtg 1320

gctccaattg aacacattgc aagcatgaga aggaattatt tcacatcaga ggtgtctcac 1380

tgcagagcca cagaatacat aatgaagggg gtgtacatca atactgcctt acttaatgca 1440

tcttgtgcag caatggatga tttccaatta attccaatga taagcaagtg tagaactaag 1500

gagggaaggc gaaagaccaa cttgtatggt ttcatcataa aaggaagatc ccacttaagg 1560

aatgacaccg acgtggtaaa ctttgtgagc atggagtttt ctctcactga cccaagactt 1620

gaaccacaca aatgggagaa gtactgtgtt cttgagatag gagatatgct tctaagaagt 1680

gccataggcc aggtttcaag gcccatgttc ttgtatgtga ggacaaatgg aacctcaaaa 1740

attaaaatga aatggggaat ggagatgagg cgttgtctcc tccagtcact tcaacaaatt 1800

gagagtatga ttgaagctga gtcctctgtc aaagagaaag acatgaccaa agagttcttt 1860

gagaacaaat cagaaacatg gcccattgga gagtctccca aaggagtgga ggaaagttcc 1920

attggggaag gtctgcagga ctttattagc aaagtcggta tttaacagct tgtatgcatc 1980

tccacaacta gaaggatttt cagctgaatc aagaaaactg cttcttatcg ttcaggctct 2040

tagggacaat ctggaacctg ggacctttga tcttgggggg ctatatgaag caattgagga 2100

gtgcctaatt aatgatccct gggttttgct taatgcttct tggttcaact ccttccttac 2160

acatgcattg agttagttgt ggcagtgcta ctatttgcta tccatactgt ccaaaaaagt 2220

accttgtttc tact 2234

<210> SEQ ID NO 13
<211> LENGTH: 1565
<212> TYPE: DNA
<213> ORGANISM: Influenza virus

<400> SEQUENCE: 13

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accaaacggt cttacgaaca gatggagact gatggagaac gccagaatgc cactgaaatc 120

agagcatccg tcggaaaaat gattggtgga attggacgat tctacatcca aatgtgcaca 180

gaacttaaac tcagtgatta tgagggacgg ttgatccaaa acagcttaac aatagagaga 240

atggtgctct ctgcttttga cgaaaggaga aataaatacc tggaagaaca tcccagtgcg 300

gggaaagatc ctaagaaaac tggaggacct atatacagaa gagtaaacgg aaagtggatg 360

agagaactca tcctttatga caaagaagaa ataaggcgaa tctggcgcca agctaataat 420

ggtgacgatg caacggctgg tctgactcac atgatgatct ggcattccaa tttgaatgat 480

gcaacttatc agaggacaag ggctcttgtt cgcaccggaa tggatcccag gatgtgctct 540

ctgatgcaag gttcaactct ccctaggagg tctggagccg caggtgctgc agtcaaagga 600

gttggaacaa tggtgatgga attggtcagg atgatcaaac gtgggatcaa tgatcggaac 660

ttctggaggg gtgagaatgg acgaaaaaca agaattgctt atgaaagaat gtgcaacatt 720

ctcaaaggga aatttcaaac tgctgcacaa aaagcaatga tggatcaagt gagagagagc 780

cggaacccag ggaatgctga gttcgaagat ctcacttttc tagcacggtc tgcactcata 840

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gccagtgggt acgactttga aagagaggga tactctctag tcggaataga ccctttcaga 960

ctgcttcaaa acagccaagt gtacagccta atcagaccaa atgagaatcc agcacacaag 1020

agtcaactgg tgtggatggc atgccattct gccgcatttg aagatctaag agtattgagc 1080

ttcatcaaag ggacgaaggt ggtcccaaga gggaagcttt ccactagagg agttcaaatt 1140

gcttccaatg aaaatatgga gactatggaa tcaagtacac ttgaactgag aagcaggtac 1200

tgggccataa ggaccagaag tggaggaaac accaatcaac agagggcatc tgcgggccaa 1260

atcagcatac aacctacgtt ctcagtacag agaaatctcc cttttgacag aacaaccgtt 1320

atggcagcat tcactgggaa tacagagggg agaacatctg acatgaggac cgaaatcata 1380

aggatgatgg aaagtgcaag accagaagat gtgtctttcc aggggcgggg agtcttcgag 1440

ctctcggacg aaaaggcagc gagcccgatc gtgccttcct ttgacatgag taatgaagga 1500

tcttatttct tcggagacaa tgcagaggag tacgacaatt aaagaaaaat acccttgttt 1560

ctact 1565

<210> SEQ ID NO 14
<211> LENGTH: 1027
<212> TYPE: DNA
<213> ORGANISM: Influenza virus

<400> SEQUENCE: 14

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ctctatcatc ccgtcaggcc ccctcaaagc cgagatcgca cagagacttg aagatgtctt 120

tgcagggaag aacaccgatc ttgaggttct catggaatgg ctaaagacaa gaccaatcct 180

gtcacctctg actaagggga ttttaggatt tgtgttcacg ctcaccgtgc ccagtgagcg 240

aggactgcag cgtagacgct ttgtccaaaa tgcccttaat gggaacgggg atccaaataa 300

catggacaaa gcagttaaac tgtataggaa gctcaagagg gagataacat tccatggggc 360

caaagaaatc tcactcagtt attctgctgg tgcacttgcc agttgtatgg gcctcatata 420

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ggtgcaagcg atgagaacca ttgggactca tcctagctcc agtgctggtc tgaaaaatga 720

tcttcttgaa aatttgcagg cctatcagaa acgaatgggg gtgcagatgc aacggttcaa 780

gtgatcctct cgctattgcc gcaaatatca ttgggatctt gcacttgata ttgtggattc 840

ttgatcgtct ttttttcaaa tgcatttacc gtcgctttaa atacggactg aaaggagggc 900

cttctacgga aggagtgcca aagtctatga gggaagaata tcgaaaggaa cagcagagtg 960

ctgtggatgc tgacgatggt cattttgtca gcatagagct ggagtaaaaa actaccttgt 1020

ttctact 1027

<210> SEQ ID NO 15
<211> LENGTH: 890
<212> TYPE: DNA
<213> ORGANISM: Influenza virus

<400> SEQUENCE: 15

agcaaaagca gggtgacaaa gacataatgg atccaaacac tgtgtcaagc tttcaggtag 60

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attgctttct ttggcatgtc cgcaaacgag ttgcagacca agaactaggt gatgccccat 120

tccttgatcg gcttcgccga gatcagaaat ccctaagagg aaggggcagc actcttggtc 180

tggacatcga gacagccaca cgtgctggaa agcagatagt ggagcggatt ctgaaagaag 240

aatccgatga ggcacttaaa atgaccatgg cctctgtacc tgcgtcgcgt tacctaaccg 300

acatgactct tgaggaaatg tcaagggaat ggtccatgct catacccaag cagaaagtgg 360

caggccctct ttgtatcaga atggaccagg cgatcatgga taaaaacatc atactgaaag 420

cgaacttcag tgtgattttt gaccggctgg agactctaat attgctaagg gctttcaccg 480

aagagggagc aattgttggc gaaatttcac cattgccttc tcttccagga catactgctg 540

aggatgtcaa aaatgcagtt ggagtcctca tcggaggact tgaatggaat gataacacag 600

ttcgagtctc tgaaactcta cagagattcg cttggagaag cagtaatgag aatgggagac 660

ctccactcac tccaaaacag aaacgagaaa tggcgggaac aattaggtca gaagtttgaa 720

gaaataagat ggttgattga agaagtgaga cacaaactga aggtaacaga gaatagtttt 780

gagcaaataa catttatgca agccttacat ctattgcttg aagtggagca agagataaga 840

actttctcat ttcagcttat ttaataataa aaaacaccct tgtttctact 890

<210> SEQ ID NO 16
<211> LENGTH: 2341
<212> TYPE: DNA
<213> ORGANISM: Influenza virus

<400> SEQUENCE: 16

agcgaaagca ggtcaattat attcaatatg gaaagaataa aagaactacg aaatctaatg 60

tcgcagtctc gcacccgcga gatactcaca aaaaccaccg tggaccatat ggccataatc 120

aagaagtaca catcaggaag acaggagaag aacccagcac tgaggatgaa atggatgatg 180

gcaatgaaat atccaattac agcagacaag aggatcaccg aaatgattcc tgagagaaat 240

gagcagggac agactctgtg gagtaaaatg aatgatgccg gatcagaccg agtgatggtg 300

tcacctctgg ctgtgacatg gtggaatagg aatggaccaa tcacaaatac agtgcattat 360

ccaaaaatct acaaaactta ttttgaaaga gtcgaaaggc tgaagcatgg aacctttggc 420

cctgtccatt ttagaaacca ggtcaaaatc cggcggagag tggacatcaa tcctggtcat 480

gcagatctca gtgccaagga ggcacaggat gtgatcatgg aagtggtgtt ccctaacgaa 540

gtgggagcca ggattctgac atccgaatcc cagctgacca ttaccaaaga gaagaaagaa 600

gaactccagg attgcaaaat ttctcctctg atggtggcat acatgctgga gagagaactg 660

gtccgcaaaa caagattcct cccagtggct ggtggaacaa gcagtgtgta cattgaagtg 720

ctgcatctga ctcagggaac atgctgggaa cagatgtata ctccaggagg ggaagtgagg 780

aatgatgatg tggatcagag cctgattatt gctgctagga acattgtgag aagagctgca 840

gtgtcagcag atccactggc atctctgctg gagatgtgcc acagcacaca gattggtgga 900

attaggatgg tggacatcct gaggcagaac ccaacagaag agcaggccgt ggatatttgc 960

aaggctgcaa tgggactgag aattagctca tccttcagtt ttggtggatt cacatttaag 1020

agaacaagcg gatcatcagt caagagagag gaagaggtgc tgaccggcaa tctgcagaca 1080

ctgaagatca gagtgcatga gggatatgaa gagttcacaa tggtggggag aagagcaaca 1140

gccatcctca gaaaagcaac caggagactg attcagctga tcgtgagtgg gagagacgaa 1200

cagtccattg ccgaagcaat tattgtggcc atggtgtttt cacaggagga ttgtatgatt 1260

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aaagcagtca gaggtgatct gaatttcgtc aatagggcca atcagcgact gaatcctatg 1320

catcagctgc tgagacattt tcagaaggat gccaaagtgc tgtttcagaa ttggggagtg 1380

gaacctatcg acaatgtgat gggaatgatt gggatcctgc ccgacatgac tccaagcatc 1440

gagatgtcaa tgagaggagt gagaatcagc aaaatgggtg tggatgagta ctccagcacc 1500

gagagggtcg tggtgagcat tgacagattt ctgagaatcc gggaccagcg aggaaatgtg 1560

ctcctgtctc ccgaggaggt cagtgaaaca cagggaacag agaaactgac aattacttac 1620

tcatcctcaa tgatgtggga gattaatggt cctgaatcag tgctggtcaa tacctatcag 1680

tggatcatca gaaactggga aactgtgaaa attcagtggt cccagaaccc tacaatgctg 1740

tacaataaaa tggaatttga accatttcag tctctggtgc ctaaggccat tagaggccag 1800

tacagtgggt ttgtgagaac tctgttccag cagatgaggg atgtgctggg gacatttgat 1860

accgcacaga ttattaaact gctgcccttc gcagccgctc caccaaagca gagtagaatg 1920

cagttctcct catttactgt gaatgtgagg ggatcaggaa tgagaatcct ggtgaggggc 1980

aattctcctg tgttcaacta taacaaggcc accaagagac tcacagtgct cggaaaggat 2040

gctggcactc tgactgaaga cccagatgaa ggcacagctg gagtggagtc cgctgtgctg 2100

aggggattcc tcattctggg caaagaagac aagagatatg ggccagcact gagcatcaat 2160

gaactgagca acctggccaa aggagagaag gctaatgtgc taattgggca aggagacgtg 2220

gtgttggtaa tgaaacggaa acgggactct agcatactta ctgacagcca gacagcgacc 2280

aaaagaattc ggatggccat caattagtgt cgaatagttt aaaaacgacc ttgtttctac 2340

t 2341

<210> SEQ ID NO 17
<211> LENGTH: 2341
<212> TYPE: DNA
<213> ORGANISM: Influenza virus

<400> SEQUENCE: 17

agcgaaagca ggcaaaccat ttgaatggat gtcaatccga ccttactttt cttaaaagtg 60

ccagcacaaa atgctataag cacaactttc ccttatactg gagaccctcc ttacagccat 120

gggacaggaa caggatacac catggatact gtcaacagga cacatcagta ctcagaaaag 180

ggaagatgga caacaaacac cgaaactgga gcaccgcaac tcaacccgat tgatgggcca 240

ctgccagaag acaatgaacc aagtggttat gcccaaacag attgtgtatt ggaggcgatg 300

gctttccttg aggaatccca tcctggtatt tttgaaaact cgtgtattga aacgatggag 360

gttgttcagc aaacacgagt ggacaagctg acacagggcc gacagaccta tgactggact 420

ctgaatagaa accagcctgc tgcaacagca ctggccaaca caatcgaagt gttcagatca 480

aatggcctca ccgccaatga gtctggaagg ctcatcgact tcctgaagga tgtgatggag 540

tcaatgaaca aagaagaaat ggggatcaca actcattttc agagaaagag acgggtgaga 600

gacaatatga ctaagaaaat gattacacag agaacaatgg gtaaaaagaa gcagagactg 660

aacaaaagga gttatctgat tagagcactg accctgaaca caatgaccaa agatgctgag 720

agagggaagc tgaaacggag agcaattgca accccaggga tgcagattag ggggtttgtg 780

tactttgtgg agacactggc aaggagtatt tgtgagaaac tggaacagtc agggctgcca 840

gtgggaggca atgagaagaa agcaaagctg gcaaatgtgg tgaggaagat gatgaccaat 900

tctcaggaca ccgaactgtc tttcaccatc actggagata acaccaaatg gaacgaaaat 960

cagaatcctc ggatgtttct ggccatgatc acatatatga ccagaaatca gcccgaatgg 1020

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ttcagaaatg tgctgagtat tgctccaatt atgttctcaa acaaaatggc cagactggga   1080

aaagggtata tgtttgagag caagagtatg aaactgagaa ctcagattcc tgcagaaatg   1140

ctggcaagca tcgatctgaa atatttcaat gattcaacaa gaaagaagat tgaaaaaatc   1200

cgacccctcc tgattgaggg gactgcatca ctgagccctg gaatgatgat gggcatgttc   1260

aatatgctga gcactgtgct gggcgtctcc atcctgaatc tgggacagaa gagatacacc   1320

aagactactt actggtggga tggtctgcag tcctctgacg attttgctct gattgtgaat   1380

gcacccaatc atgaagggat tcaggccgga gtcgacaggt tttatcgaac ctgtaagctg   1440

ctgggaatca atatgagcaa gaaaaagtct tacatcaaca gaacaggtac atttgaattc   1500

acaagttttt tctatcgcta tgggtttgtg gccaatttca gcatggagct gcccagtttt   1560

ggggtgtctg ggatcaacga gtcagccgac atgagtattg gagtgactgt catcaaaaac   1620

aatatgatca acaatgatct gggtccagca acagctcaga tggccctgca gctgttcatc   1680

aaagattaca ggtacaccta ccgatgccat atcggtgaca cacagattca gacccgaaga   1740

tcatttgaaa tcaagaaact gtgggagcag acccgctcca aagctggact gctggtctcc   1800

gacggaggcc caaatctgta caacattaga aatctccaca ttcctgaagt ctgcctgaaa   1860

tgggaactga tggatgagga ttaccagggg cgcctgtgca acccactgaa cccatttgtc   1920

agccataaag aaattgaatc aatgaacaat gcagtgatga tgccagcaca tggtccagcc   1980

aaaaacatgg agtatgatgc tgtggcaaca acacactcct ggatccccaa aagaaatcga   2040

tccatcctga atacaagtca gagaggagtg ctggaggatg aacagatgta ccagaggtgc   2100

tgcaatctgt ttgaaaaatt cttccccagc agttcataca gaagaccagt cgggatctcc   2160

agtatggtgg aggctatggt gtccagagcc cgaattgatg cacggattga tttcgaatct   2220

ggaaggatca agaaagaaga gttcactgag atcatgaaga tctgttccac cattgaagag   2280

ctcagacggc aaaaatagtg aatttagctt gtccttcatg aaaaaatgcc ttgtttctac   2340

t                                                                   2341
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<210> SEQ ID NO 18
<211> LENGTH: 2233
<212> TYPE: DNA
<213> ORGANISM: Influenza virus

<400> SEQUENCE: 18

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agcgaaagca ggtactgatc caaaatggaa gattttgtgc gacaatgctt caatccgatg     60

attgtcgagc ttgcggaaaa aacaatgaaa gagtatgggg aggacctgaa aatcgaaaca    120

aacaaatttg cagcaatatg cactcacttg gaagtatgct tcatgtattc agattttcac    180

ttcatcaatg agcaaggcga gtcaataatc gtagaacttg gtgatccaaa tgcacttttg    240

aagcacagat ttgaaataat cgagggaaga gatcgcacaa tggcctggac agtagtaaac    300

agtatttgca acactacagg ggctgagaaa ccaaagtttc taccagattt gtatgattac    360

aaggagaata gattcatcga aattggagta acaaggagag aagttcacat atactatctg    420

gaaaaggcca ataaaattaa atctgagaaa acacacatcc acattttctc gttcactggg    480

gaagaaatgg ccacaaaggc agactacact ctcgatgaag aaagcagggc taggatcaaa    540

accagactat tcaccataag acaagaaatg gccagcagag gcctctggga ttcctttcgt    600

cagtccgaga gaggagaaga gacaattgaa gaaaggtttg aaatcacagg aacaatgcgc    660

aagcttgccg accaaagtct cccgccgaac ttctccagcc ttgaaaattt tagagcctat    720
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gtggatggat tcgaaccgaa cggctacatt gagggcaagc tgtctcaaat gtccaaagaa 780

gtaaatgcta gaattgaacc ttttctgaaa acaacaccac gaccactgag actgcccaat 840

gggcctccct gttctcagcg gtccaaattc ctgctgatgg atgccctgaa actgagcatt 900

gaggacccaa gtcatgaagg agagggaatt cccctgtatg atgcaatcaa atgcatgaga 960

acattctttg gatggaagga acccaatgtg gtgaaaccac acgaaaaggg aatcaatcca 1020

aattatctgc tgtcatggaa gcaggtgctg gcagaactgc aggacattga gaatgaggag 1080

aaaattccaa agactaaaaa tatgaagaaa acaagtcagc tgaagtgggc actgggtgag 1140

aacatggcac cagaaaaggt ggactttgac gactgtaaag atgtgggtga tctgaagcag 1200

tatgatagtg atgaaccaga actgaggtcc ctggcaagtt ggattcagaa tgagtttaac 1260

aaggcatgcg aactgacaga ttcaagctgg attgagctcg atgagattgg agaagatgtg 1320

gctccaattg aacacattgc aagcatgaga aggaattatt tcacatcaga ggtgtctcac 1380

tgcagagcca cagaatacat catgaaggga gtgtacatca atactgccct gctgaatgca 1440

tcttgtgcag caatggatga tttccagctg attccaatga tcagcaagtg tagaactaag 1500

gagggaaggc gaaagaccaa cctgtatggt ttcatcatca aaggaagatc ccacctgagg 1560

aatgacaccg acgtggtgaa ctttgtgagc atggagtttt ctctcactga cccaagactg 1620

gaaccacata aatgggagaa gtactgtgtg ctggagattg gagatatgct gatcagaagt 1680

gccattggcc aggtgtcaag gcccatgttc ctgtatgtga gaacaaatgg aacctcaaaa 1740

attaaaatga aatggggaat ggagatgagg cgctgcctcc tccagtcact gcagcagatt 1800

gagagtatga ttgaagctga gtcctctgtc aaagagaaag acatgaccaa agagttcttt 1860

gagaacaaat cagaaacatg gcccattgga gagtccccca aaggagtgga ggaaagttcc 1920

attgggaagg tctgcaggac tctgctggca aagtccgtgt tcaacagcct gtatgcatct 1980

ccacagctgg aaggattttc agctgaatca agaaaactgc tgctgatcgt gcaggctctg 2040

agggacaacc tggaacctgg gacctttgat ctgggggggc tgtatgaagc aattgaggag 2100

tgcctgatta atgatccctg ggtgctgctg aatgcttctt ggttcaactc cttccttaca 2160

catgcattga gttagttgtg gcagtgctac tatttgctat ccatactgtc caaaaaagta 2220

ccttgtttct act 2233

<210> SEQ ID NO 19
<211> LENGTH: 1565
<212> TYPE: DNA
<213> ORGANISM: Influenza virus

<400> SEQUENCE: 19

agcaaaagca gggtagataa tcactcactg agtgacatca aaatcatggc gtctcaaggc 60

accaaacgat cttacgaaca gatggagact gatggagaac gccagaatgc cactgaaatc 120

agagcatccg tcggaaaaat gattggtgga attggacgat tctacatcca gatgtgcacc 180

gaactcaaac tcagtgatta tgagggacgg ctgatccaga acagcctgac aatcgagaga 240

atggtgctct ctgcttttga cgaaaggaga aataaatacc tggaagaaca tcccagtgcc 300

gggaaagatc ctaagaaaac tggaggacct atctacagga gagtgaacgg aaagtggatg 360

agagaactca tcctgtatga caaagaagaa atcaggcgaa tctggcgcca ggctaataat 420

ggtgacgatg caaccgctgg tctgactcac atgatgatct ggcattccaa tctgaatgat 480

gcaacttatc agaggacaag agctctggtg cgcaccggaa tggatcccag gatgtgctct 540

ctgatgcagg gttcaactct ccctaggagg tctggagccg caggtgctgc agtcaaagga 600

-continued

gtgggaacaa tggtgatgga actggtcaga atgatcaaaa gagggatcaa tgatcggaac 660

ttctggaggg gtgagaatgg acgaaaaaca agaattgctt atgaaagaat gtgcaacatt 720

ctcaaaggga aatttcagac tgctgcacag aaagcaatga tggatcaggt gagagagagc 780

cggaacccag ggaatgctga gttcgaagat ctcacttttc tggcacggtc tgcactcatc 840

ctgagagggt ccgtggctca caagtcctgc ctgcctgcct gtgtgtatgg acctgccgtg 900

gccagtgggt acgactttga aagggaggga tactctctgg tcggaattga ccctttcaga 960

ctgctgcaga acagccaggt gtacagcctg atcagaccaa atgagaatcc agcacacaag 1020

agtcagctgg tgtggatggc atgccattct gccgcatttg aagatctgag agtgctgagc 1080

ttcatcaaag ggaccaaggt gctcccaaga gggaagctgt ccactagagg agtgcagatt 1140

gcttccaatg aaaatatgga gactatggaa tcaagtacac tggaactgag aagcaggtac 1200

tgggccatca ggaccagaag tggaggaaac accaatcagc agagggcatc tgccggccag 1260

atcagcattc agcctacctt ctcagtgcag agaaatctcc cttttgacag aacaaccatt 1320

atggcagcat tcaatgggaa tacagagggg agaacatctg acatgaggac cgaaatcatc 1380

aggatgatgg aaagtgcaag accagaagat gtgtctttcc aggggcgggg agtcttcgag 1440

ctctcggacg aaaaggcagc gagcccgatc gtgccttcct ttgacatgag taatgaagga 1500

tcttatttct tcggagacaa tgcagaggag tacgacaatt aaagaaaaat acccttgttt 1560

ctact 1565

<210> SEQ ID NO 20
<211> LENGTH: 1684
<212> TYPE: DNA
<213> ORGANISM: Influenza virus

<400> SEQUENCE: 20

atgaacactc aaatcctggt attcgctctg attgcgatca ttccaacaaa tgcagacaaa 60

atctgcctcg gacatcatgc cgtgtcaaac ggaaccaaag taaacacatt aactgaaaga 120

ggagtggaag tcgtcaatgc aactgaaaca gtggaacgaa caaacatccc caggatctgc 180

tcaaaaggga aaaggacagt tgacctcggt caatgtggac tcctggggac aatcactgga 240

ccacctcaat gtgaccaatt cctagaattt tcagccgatt taattattga gaggcgagaa 300

ggaagtgatg tctgttatcc tgggaaattc gtgaatgaag aagctctgag gcaaattctc 360

agagaatcag gcggaattga caaggaagca atgggattca catacagtgg aataagaact 420

aatggagcaa ccagtgcatg taggagatca ggatcttcat tctatgcaga aatgaaatgg 480

ctcctgtcaa acacagataa tgctgcattc ccgccagatg actaagtcat ataaaaatac 540

aagaaaaagc ccagctctaa tagtatgggg gatccatcat tccgtatcaa ctgcagagca 600

aaccaagcta tatgggagtg gaaacaaact ggtgacagtt gggagttcta attatcaaca 660

atcttttgta ccgagtccag gagcgagacc acaagttaat ggtctatctg gaagaattga 720

ctttcattgg ctaatgctaa atcccaatga tacagtcact ttcagtttca atggggcttt 780

catagctcca gaccgtgcaa gcttcctgag aggaaaatct atgggaatcc agagtggagt 840

acaggttgat gccaattgtg aaggggactg ctatcatagt ggagggacaa taataagtaa 900

cttgccattt cagaacatag atagcagggc agttggaaaa tgtccgagat atgttaagca 960

aaggagtctg ctgctagcaa cagggatgaa gaatgttcct gagattccaa agggaagagg 1020

cctatttggt gctatagcgg gtttcattga aaatggatgg gaaggcctaa ttgatggttg 1080

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tcaatcggca attgatcaaa taacaggaaa attaaaccgg cttatagaaa aaaccaacca 1200

acaatttgag ttgatagaca atgaattcaa tgaggtagag aagcaaatcg gtaatgtgat 1260

aaattggacc agagattcta taacagaagt gtggtcatac aatgctgaac tcttggtagc 1320

aatggagaac cagcatacaa ttgatctggc tgattcagaa atggacaaac tgtacgaacg 1380

agtgaaaaga cagctgagag agaatgctga agaagatggc actggttgct ttgaaatatt 1440

tcacaagtgt gatgatgact gtatggccag tattagaaat aacacctatg atcacagcaa 1500

atacagggaa gaggcaatgc aaaatagaat acagattgac ccagtcaaac taagcagcgg 1560

ctacaaagat gtgatacttt ggtttagctt cggggcatca tgtttcatac ttctagccat 1620

tgtaatgggc cttgtcttca tatgtgtaaa gaatggaaac atgcggtgca ctatttgtat 1680

ataa 1684

<210> SEQ ID NO 21
<211> LENGTH: 560
<212> TYPE: PRT
<213> ORGANISM: Influenza virus

<400> SEQUENCE: 21

Met Asn Thr Gln Ile Leu Val Phe Ala Leu Ser Lys Gly Lys Arg Thr
 1   5   10   15

Val Asp Leu Gly Ile Ala Ile Ile Pro Thr Asn Ala Asp Lys Gln Cys
            20   25   30

Gly Leu Leu Gly Thr Ile Thr Gly Ile Cys Leu Gly His His Ala Val
        35   40   45

Ser Asn Pro Pro Gln Cys Asp Gln Phe Leu Glu Phe Gly Thr Lys Val
    50   55   60

Asn Thr Leu Thr Glu Arg Ser Ala Asp Leu Ile Ile Glu Arg Arg Glu
65   70   75   80

Gly Val Glu Val Val Asn Ala Thr Glu Thr Gly Ser Asp Val Cys Tyr
                85   90   95

Pro Gly Lys Phe Val Glu Arg Thr Asn Ile Pro Arg Ile Cys Val Asn
            100   105   110

Glu Glu Ala Leu Arg Gln Ile Leu Arg Glu Ser Gly Gly Ile Asp Lys
        115   120   125

Glu Ala Met Gly Phe Thr Tyr Ser Gly Ile Arg Thr Asn Gly Ala Thr
    130   135   140

Ser Ala Cys Arg Arg Ser Gly Ser Ser Phe Tyr Ala Glu Met Lys Trp
145   150   155   160

Leu Leu Ser Asn Thr Asp Asn Ala Ala Phe Pro Gln Met Thr Lys Ser
                165   170   175

Tyr Lys Asn Thr Arg Lys Ser Pro Ala Leu Ile Val Trp Gly Ile His
            180   185   190

His Ser Val Ser Thr Ala Glu Gln Thr Lys Leu Tyr Gly Ser Gly Asn
        195   200   205

Lys Leu Val Thr Val Gly Ser Ser Asn Tyr Gln Gln Ser Phe Val Pro
    210   215   220

Ser Pro Gly Ala Arg Pro Gln Val Asn Gly Leu Ser Gly Arg Ile Asp
225   230   235   240

Phe His Trp Leu Met Leu Asn Pro Asn Asp Thr Val Thr Phe Ser Phe
                245   250   255

Asn Gly Ala Phe Ile Ala Pro Asp Arg Ala Ser Phe Leu Arg Gly Lys

```
            260                 265                 270

Ser Met Gly Ile Gln Ser Gly Val Gln Val Asp Ala Asn Cys Glu Gly
        275                 280                 285

Asp Cys Tyr His Ser Gly Gly Thr Ile Ile Ser Asn Leu Pro Phe Gln
    290                 295                 300

Asn Ile Asp Ser Arg Ala Val Gly Lys Cys Pro Arg Tyr Val Lys Gln
305                 310                 315                 320

Arg Ser Leu Leu Leu Ala Thr Gly Met Lys Asn Val Pro Glu Ile Pro
                325                 330                 335

Lys Gly Arg Gly Leu Phe Gly Ala Ile Ala Gly Phe Ile Glu Asn Gly
            340                 345                 350

Trp Glu Gly Leu Ile Asp Gly Trp Tyr Gly Phe Arg His Gln Asn Ala
        355                 360                 365

Gln Gly Glu Gly Thr Ala Ala Asp Tyr Lys Ser Thr Gln Ser Ala Ile
    370                 375                 380

Asp Gln Ile Thr Gly Lys Leu Asn Arg Leu Ile Glu Lys Thr Asn Gln
385                 390                 395                 400

Gln Phe Glu Leu Ile Asp Asn Glu Phe Asn Glu Val Glu Lys Gln Ile
                405                 410                 415

Gly Asn Val Ile Asn Trp Thr Arg Asp Ser Ile Thr Glu Val Trp Ser
            420                 425                 430

Tyr Asn Ala Glu Leu Leu Val Ala Met Glu Asn Gln His Thr Ile Asp
        435                 440                 445

Leu Ala Asp Ser Glu Met Asp Lys Leu Tyr Glu Arg Val Lys Arg Gln
    450                 455                 460

Leu Arg Glu Asn Ala Glu Glu Asp Gly Thr Gly Cys Phe Glu Ile Phe
465                 470                 475                 480

His Lys Cys Asp Asp Asp Cys Met Ala Ser Ile Arg Asn Asn Thr Tyr
                485                 490                 495

Asp His Ser Lys Tyr Arg Glu Glu Ala Met Gln Asn Arg Ile Gln Ile
            500                 505                 510

Asp Pro Val Lys Leu Ser Ser Gly Tyr Lys Asp Val Ile Leu Trp Phe
        515                 520                 525

Ser Phe Gly Ala Ser Cys Phe Ile Leu Leu Ala Ile Val Met Gly Leu
    530                 535                 540

Val Phe Ile Cys Val Lys Asn Gly Asn Met Arg Cys Thr Ile Cys Ile
545                 550                 555                 560


<210> SEQ ID NO 22
<211> LENGTH: 1398
<212> TYPE: DNA
<213> ORGANISM: Influenza virus

<400> SEQUENCE: 22

atgaatccaa atcagaagat tctatgcact tcagccactg ctatcataat aggcgcaatc     60

gcagtactca ttggaatagc aaacctagga ttgaacatag gactgcatct aaaaccgggc    120

tgcaattgct cacactcaca acctgaaaca accaacacaa gccaaacaat aataaacaac    180

tattataatg aaacaaacat caccaacatc caaatggaag agagaacaag caggaatttc    240

aataacttaa ctaaagggct ctgtactata aattcatggc acatatatgg gaaagacaat    300

gcagtaagaa ttggagagag ctcggatgtt ttagtcacaa gagaacccta tgtttcatgc    360

gacccagatg aatgcaggtt ctatgctctc agccaaggaa caacaatcag agggaaacac    420

tcaaacggaa caatacacga taggtcccag tatcgcgccc tgataagctg gccactatca    480
```

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tcaccgccca cagtgtacaa cagcagggtg gaatgcattg ggtggtcaag tactagttgc    540

catgatggca aatccaggat gtcaatatgt atatcaggac caaacaacaa tgcatctgca    600

gtagtatggt acaacagaag gcctgttgca gaaattaaca catgggcccg aaacatacta    660

agaacacagg aatctgaatg tgtatgccac aacggcgtat gcccagtagt gttcaccgat    720

gggtctgcca ctggacctgc agacacaaga atatactatt ttaaagaggg gaaaatattg    780

aaatgggagt ctctgactgg aactgctaag catattgaag aatgctcatg ttacggggaa    840

cgaacaggaa ttacctgcac atgcagggac aattggcagg gctcaaatag accagtgatt    900

cagatagacc cagtagcaat gacacacact agtcaatata tatgcagtcc tgttcttaca    960

gacaatcccc gaccgaatga cccaaatata ggtaagtgta atgaccctta tccaggtaat   1020

aataacaatg gagtcaaggg attctcatac ctggatgggg ctaacacttg gctagggagg   1080

acaataagca cagcctcgag gtctggatac gagatgttaa aagtgccaaa tgcattgaca   1140

gatgatagat caaagcccat tcaaggtcag acaattgtat taaacgctga ctggagtggt   1200

tacagtggat ctttcatgga ctattgggct gaaggggact gctatcgagc gtgtttttat   1260

gtggagttga tacgtggaag acccaaggaa gataaagtgt ggtggaccag caatagtata   1320

gtatcgatgt gttccagtac agaattcctg ggacaatgga actggcctga tggggctaaa   1380

atagagtact tcctctaa                                                 1398
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<210> SEQ ID NO 23
<211> LENGTH: 464
<212> TYPE: PRT
<213> ORGANISM: Influenza virus

<400> SEQUENCE: 23

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Met Asn Pro Asn Gln Lys Ile Leu Cys Thr Ser Ala Thr Ala Ile Ile
 1               5                  10                  15

Ile Gly Ala Ile Ala Val Leu Ile Gly Ile Ala Asn Leu Gly Leu Asn
            20                  25                  30

Ile Gly Leu His Leu Lys Pro Gly Cys Asn Cys Ser His Ser Gln Pro
        35                  40                  45

Glu Thr Thr Asn Thr Ser Gln Thr Ile Ile Asn Asn Tyr Tyr Asn Glu
    50                  55                  60

Thr Asn Ile Thr Asn Ile Gln Met Glu Glu Arg Thr Ser Arg Asn Phe
65                  70                  75                  80

Asn Asn Leu Thr Lys Gly Leu Cys Thr Ile Asn Ser Trp His Ile Tyr
                85                  90                  95

Gly Lys Asp Asn Ala Val Arg Ile Gly Glu Ser Ser Asp Val Leu Val
            100                 105                 110

Thr Arg Glu Pro Tyr Val Ser Cys Asp Pro Asp Glu Cys Arg Phe Tyr
        115                 120                 125

Ala Leu Ser Gln Gly Thr Ile Ile Arg Gly Lys His Ser Asn Gly Thr
    130                 135                 140

Ile His Asp Arg Ser Gln Tyr Arg Ala Leu Ile Ser Trp Pro Leu Ser
145                 150                 155                 160

Ser Pro Pro Thr Val Tyr Asn Ser Arg Val Glu Cys Ile Gly Trp Ser
                165                 170                 175

Ser Thr Ser Cys His Asp Gly Lys Ser Arg Met Ser Ile Cys Ile Ser
            180                 185                 190

Gly Pro Asn Asn Asn Ala Ser Ala Val Val Trp Tyr Asn Arg Arg Pro
        195                 200                 205
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Val Ala Glu Ile Asn Thr Trp Ala Arg Asn Ile Leu Arg Thr Gln Glu
    210                 215                 220

Ser Glu Cys Val Cys His Asn Gly Val Cys Pro Val Val Phe Thr Asp
225                 230                 235                 240

Gly Ser Ala Thr Gly Pro Ala Asp Thr Arg Ile Tyr Phe Lys Glu Gly
                245                 250                 255

Lys Ile Leu Lys Trp Glu Ser Leu Thr Gly Thr Ala Lys His Ile Glu
            260                 265                 270

Glu Cys Ser Cys Tyr Gly Glu Arg Thr Gly Ile Thr Cys Thr Cys Arg
        275                 280                 285

Asp Asn Trp Gln Gly Ser Asn Arg Pro Val Ile Gln Ile Asp Pro Val
    290                 295                 300

Ala Met Thr His Thr Ser Gln Tyr Ile Cys Ser Pro Val Leu Thr Asp
305                 310                 315                 320

Asn Pro Arg Pro Asn Asp Pro Asn Ile Gly Lys Cys Asn Asp Pro Tyr
                325                 330                 335

Pro Gly Asn Asn Asn Asn Gly Val Lys Gly Phe Ser Tyr Leu Asp Gly
            340                 345                 350

Ala Asn Thr Trp Leu Gly Arg Thr Ile Ser Thr Ala Ser Arg Ser Gly
        355                 360                 365

Tyr Glu Met Leu Lys Val Pro Asn Ala Leu Thr Asp Asp Arg Ser Lys
    370                 375                 380

Pro Ile Gln Gly Gln Thr Ile Val Leu Asn Ala Asp Trp Ser Gly Tyr
385                 390                 395                 400

Ser Gly Ser Phe Met Asp Tyr Trp Ala Glu Gly Asp Cys Tyr Arg Ala
                405                 410                 415

Cys Phe Tyr Val Glu Leu Ile Arg Gly Arg Pro Lys Glu Asp Lys Val
            420                 425                 430

Trp Trp Thr Ser Asn Ser Ile Val Ser Met Cys Ser Ser Thr Glu Phe
        435                 440                 445

Leu Gly Gln Trp Asn Trp Pro Asp Gly Ala Lys Ile Glu Tyr Phe Leu
    450                 455                 460


<210> SEQ ID NO 24
<211> LENGTH: 1683
<212> TYPE: DNA
<213> ORGANISM: Influenza virus

<400> SEQUENCE: 24

atgaacactc aaatcctggt attcgctctg attgcgatca ttccaacaaa tgcagacaaa     60

atctgcctcg gacatcatgc tgtgtcaaac ggaaccaaag taaacacatt aactgaaaga    120

ggagtggaag tcgtcaatgc aactgaaaca gtggaacgaa caaacatccc caggatctgc    180

tcaaaaggga aaaggacagt tgacctcggt caatgtggac tcctggggac aatcactgga    240

ccacctcaat gtgaccaatt cctagaattt tcagccgatt taattattga gaggcgagaa    300

ggaagtgatg tctgttatcc tgggaaattc gtgaatgaag aagctctgag gcaaattctc    360

agagaatcag gcggaattga caaggaagca atgggattca catacagtgg aataagaact    420

aatggagcaa ccagttcatg taggagatca ggatcttcat tctatgcaga aatgaaatgg    480

ctcctgtcaa acacagataa tgctgcattc ccgcagatga ctaagtcata taaaaataca    540

agaaaaaacc cagctctaat agtatggggg atccatcatt ccggatcaac tgcagagcaa    600

accaagctat atgggagtgg aaacaaactg gtgacagttg ggagttctaa ttatcaacaa    660
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tcttttgtac cgagtccggg agcgagaaca caagttaatg gtcaatctgg aagaattgac    720

tttcattggc taatgctaaa tcccaatgat acagtcactt tcagtttcaa tggggctttc    780

atagctccag accgtgcaag cttcctgaga ggaaaatcta tgggaatcca gagtggagta    840

caggttgatg ccgattgtga aggggactgc tattatagtg gagggacaat aataagtaac    900

ttgccatttc agaacataga tagcagggca gttggaaaat gtccgagata tgttaagcaa    960

aggagtctgc tgctagcaac agggatgaag aatgttcctg agattccaaa gggaagaggc   1020

ctatttggtg ctatagcggg tttcattgaa aatggatggg aaggcctaat tgatggttgg   1080

tatggtttca gacaccagaa tgcacaggga gagggaactg ctgcagatta caaaagcact   1140

caatcggcaa ttgatcaaat aacaggaaaa ttaaaccggc ttatagaaaa aaccaaccaa   1200

caatttgagt tgatagacaa tgaattcact gaggtagaga agcaaatcgg taatgtgata   1260

aattggacca gagattctat aacagaagtg tggtcataca atgctgaact cttggtagca   1320

atggagaacc agcatacaat tgatctggct gattcagaaa tggacaaact gtacgaacga   1380

gtgaaaagac agctgagaga gaatgctgaa gaagatggca ctggttgctt tgaaatattt   1440

cacaagtgtg atgatgactg tatggccagc attagaaata acacctatga tcacagcaaa   1500

tacagggaag aggcaatgca aaatagaata cagattgacc cagtcaaact aagcagcggc   1560

tacaaagatg tgatactttg gtttagcttc ggggcatcat gtttcatact tctagccatt   1620

gcaatgggcc ttgtcttcat atgtgtaaag aatggaaaca tgcggtgcac tatttgtata   1680

taa                                                                 1683


<210> SEQ ID NO 25
<211> LENGTH: 560
<212> TYPE: PRT
<213> ORGANISM: Influenza virus

<400> SEQUENCE: 25

Met Asn Thr Gln Ile Leu Val Phe Ala Leu Ile Ala Ile Ile Pro Thr
 1               5                  10                  15

Asn Ala Asp Lys Ile Cys Leu Gly His His Ala Val Ser Asn Gly Thr
            20                  25                  30

Lys Val Asn Thr Leu Thr Glu Arg Gly Val Glu Val Val Asn Ala Thr
        35                  40                  45

Glu Thr Val Glu Arg Thr Asn Ile Pro Arg Ile Cys Ser Lys Gly Lys
    50                  55                  60

Arg Thr Val Asp Leu Gly Gln Cys Gly Leu Leu Gly Thr Ile Thr Gly
65                  70                  75                  80

Pro Pro Gln Cys Asp Gln Phe Leu Glu Phe Ser Ala Asp Leu Ile Ile
                85                  90                  95

Glu Arg Arg Glu Gly Ser Asp Val Cys Tyr Pro Gly Lys Phe Val Asn
            100                 105                 110

Glu Glu Ala Leu Arg Gln Ile Leu Arg Glu Ser Gly Gly Ile Asp Lys
        115                 120                 125

Glu Ala Met Gly Phe Thr Tyr Ser Gly Ile Arg Thr Asn Gly Ala Thr
    130                 135                 140

Ser Ser Cys Arg Arg Ser Gly Ser Ser Phe Tyr Ala Glu Met Lys Trp
145                 150                 155                 160

Leu Leu Ser Asn Thr Asp Asn Ala Ala Phe Pro Gln Met Thr Lys Ser
                165                 170                 175

Tyr Lys Asn Thr Arg Lys Asn Pro Ala Leu Ile Val Trp Gly Ile His
            180                 185                 190
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His Ser Gly Ser Thr Ala Glu Gln Thr Lys Leu Tyr Gly Ser Gly Asn
        195                 200                 205

Lys Leu Val Thr Val Gly Ser Ser Asn Tyr Gln Gln Ser Phe Val Pro
    210                 215                 220

Ser Pro Gly Ala Arg Thr Gln Val Asn Gly Gln Ser Gly Arg Ile Asp
225                 230                 235                 240

Phe His Trp Leu Met Leu Asn Pro Asn Asp Thr Val Thr Phe Ser Phe
                245                 250                 255

Asn Gly Ala Phe Ile Ala Pro Asp Arg Ala Ser Phe Leu Arg Gly Lys
            260                 265                 270

Ser Met Gly Ile Gln Ser Gly Val Gln Val Asp Ala Asp Cys Glu Gly
        275                 280                 285

Asp Cys Tyr Tyr Ser Gly Gly Thr Ile Ile Ser Asn Leu Pro Phe Gln
    290                 295                 300

Asn Ile Asp Ser Arg Ala Val Gly Lys Cys Pro Arg Tyr Val Lys Gln
305                 310                 315                 320

Arg Ser Leu Leu Leu Ala Thr Gly Met Lys Asn Val Pro Glu Ile Pro
                325                 330                 335

Lys Gly Arg Gly Leu Phe Gly Ala Ile Ala Gly Phe Ile Glu Asn Gly
            340                 345                 350

Trp Glu Gly Leu Ile Asp Gly Trp Tyr Gly Phe Arg His Gln Asn Ala
        355                 360                 365

Gln Gly Glu Gly Thr Ala Ala Asp Val Lys Ser Thr Gln Ser Ala Ile
    370                 375                 380

Asp Gln Ile Thr Gly Lys Leu Asn Arg Leu Ile Glu Lys Thr Asn Gln
385                 390                 395                 400

Gln Phe Glu Leu Ile Asp Asn Glu Phe Thr Glu Val Glu Lys Gln Ile
                405                 410                 415

Gly Asn Val Ile Asn Trp Thr Arg Asp Ser Ile Thr Glu Val Trp Ser
            420                 425                 430

Tyr Asn Ala Glu Leu Leu Val Ala Met Glu Asn Gln His Thr Ile Asp
        435                 440                 445

Leu Ala Asp Ser Glu Met Asp Lys Leu Tyr Glu Arg Val Lys Arg Gln
    450                 455                 460

Leu Arg Glu Asn Ala Glu Glu Asp Gly Thr Gly Cys Phe Glu Ile Phe
465                 470                 475                 480

His Lys Cys Asp Asp Asp Cys Met Ala Ser Ile Arg Asn Asn Thr Tyr
                485                 490                 495

Asp His Ser Lys Tyr Arg Glu Glu Ala Met Gln Asn Arg Ile Gln Ile
            500                 505                 510

Asp Pro Val Lys Leu Ser Ser Gly Tyr Lys Asp Val Ile Leu Trp Phe
        515                 520                 525

Ser Phe Gly Ala Ser Cys Phe Ile Leu Leu Ala Ile Ala Met Gly Leu
    530                 535                 540

Val Phe Ile Cys Val Lys Asn Gly Asn Met Arg Cys Thr Ile Cys Ile
545                 550                 555                 560


<210> SEQ ID NO 26
<211> LENGTH: 1398
<212> TYPE: DNA
<213> ORGANISM: Influenza virus

<400> SEQUENCE: 26

atgaatccaa atcagaagat tctatgcact tcagccactg ctatcataat aggcgcaatc     60
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gcagtactca ttggaatagc aaacctagga ttgaacatag gactgcatct aaaaccgagc 120

tgcaattgct cacactcaca acctgaaaca accaacacaa gccaaacaat aataaacaac 180

tattataatg aaacaaacat caccaacatc caaatggaag agagaacaag caggaatttc 240

aataacttaa ctaaagggct ctgtactata aattcatggc acatatatgg gaaagacaat 300

gcggtaagaa ttggagagag ctcggatgtt ttagtcacaa gagaacccta tgtttcatgc 360

gacccagatg aatgcaggtt ctatgctctc agccaaggaa caacaatcag aggaaaacac 420

tcaaacggaa caatacacga taggtcccag tatcgcgccc tgataagctg gccactatca 480

tcaccgccca cagtgtacaa cagcagggtg gaatgcattg ggtggtcaag tactagttgc 540

catgatggca aatccaggat gtcaatatgt atatcaggac caaacaacaa tgcatctgca 600

gtagtatggt acaacagaag gcctgttgca gaaattaaca catgggcccg aaacatacta 660

agaacacagg aatctgaatg tgtatgccac aacggcgtat gcccagtagt gttcaccgat 720

gggtctgcca ctggacctgc agacacaaga atatactatt ttaaagaggg gaaaatattg 780

aaatgggagt ctctgactgg aactgctaag catattgaag aatgctcatg ttacggggaa 840

cgaacaggaa ttacctgcac atgcaaggac aattggcagg gctcaaatag accagtgatt 900

cagatagatc cagtagcaat gacacacact agtcagtata tatgcagtcc tgttcttaca 960

gacaatcccc gaccgaatga cccaaatata ggtaagtgta atgacccttа tccaggtaat 1020

aataacaatg gagtcaaggg attctcatac ctggatgggg ctaacacttg gctagggagg 1080

acaataagca cagcctcgag gtctggatac gagatgttaa aagtgccaaa tgcattgaca 1140

gatgatagat caaagcccat tcaaggtcag acaattgtat taaacgctga ctggagtggt 1200

tacagtggat ctttcatgga ctattgggct gaggggact gctatcgagc gtgtttttat 1260

gtggaattga tacgtggaag acccaaggag gataaagtgt ggtggaccag caatagtata 1320

gtatcgatgt gttccagtac agaattcctg ggacaatgga actggcctga tggggctaaa 1380

atagagtact tcctctaa 1398

<210> SEQ ID NO 27
<211> LENGTH: 462
<212> TYPE: PRT
<213> ORGANISM: Influenza virus

<400> SEQUENCE: 27

Met Asn Pro Asn Gln Lys Ile Leu Cys Thr Ser Ala Thr Ala Ile Ile
 1   5   10   15

Ile Gly Ala Ile Ala Val Leu Ile Gly Ile Ala Asn Leu Gly Leu Asn
            20   25   30

Ile Gly Leu His Leu Lys Pro Ser Cys Asn Cys Ser His Ser Gln Pro
        35   40   45

Glu Thr Ile Asn Thr Ser Gln Thr Ile Ile Asn Asn Tyr Tyr Asn Glu
    50   55   60

Thr Asn Ile Thr Asn Ile Gln Met Glu Glu Arg Thr Ser Arg Asn Phe
65   70   75   80

Asn Asn Leu Thr Lys Gly Leu Cys Thr Ile Asn Ser Trp His Ile Tyr
                85   90   95

Gly Lys Asp Asn Ala Val Arg Ile Gly Glu Ser Ser Asp Val Leu Val
            100   105   110

Thr Arg Glu Pro Tyr Val Ser Cys Asp Pro Asp Glu Cys Arg Phe Tyr
        115   120   125

-continued

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Ala Leu Ser Gln Gly Thr Ile Ile Arg Gly Lys His Ser Asn Gly Thr
    130                 135                 140

Ile His Asp Arg Ser Gln Tyr Arg Ala Leu Ile Ser Trp Pro Leu Ser
145                 150                 155                 160

Ser Pro Pro Thr Val Tyr Asn Ser Arg Val Glu Cys Ile Gly Trp Ser
                165                 170                 175

Ser Thr Ser Cys His Asp Gly Lys Ser Arg Met Ser Ile Cys Ile Ser
            180                 185                 190

Gly Pro Asn Asn Asn Ala Ser Ala Trp Trp Tyr Asn Arg Arg Pro Val
        195                 200                 205

Ala Glu Ile Asn Thr Trp Ala Arg Asn Ile Leu Arg Thr Gln Glu Ser
    210                 215                 220

Glu Cys Val Cys His Asn Gly Val Cys Pro Trp Phe Thr Asp Gly Ser
225                 230                 235                 240

Ala Thr Gly Pro Ala Asp Thr Arg Ile Tyr Tyr Phe Lys Glu Gly Lys
                245                 250                 255

Leu Lys Trp Glu Ser Leu Thr Gly Thr Ala Lys His Ile Glu Glu Cys
            260                 265                 270

Ser Cys Tyr Gly Glu Arg Thr Gly Ile Thr Cys Thr Cys Lys Asp Asn
        275                 280                 285

Trp Gln Gly Ser Asn Arg Pro Val Ile Gln Ile Asp Pro Val Ala Met
    290                 295                 300

Thr His Thr Ser Gln Tyr Ile Cys Ser Pro Val Leu Thr Asp Asn Pro
305                 310                 315                 320

Arg Pro Asn Asp Pro Asn Ile Gly Lys Cys Asn Asp Pro Tyr Pro Gly
                325                 330                 335

Asn Asn Asn Asn Gly Val Lys Gly Phe Ser Tyr Leu Asp Gly Ala Asn
            340                 345                 350

Thr Trp Leu Gly Arg Thr Ile Ser Thr Ala Ser Arg Ser Gly Tyr Glu
        355                 360                 365

Met Leu Lys Val Pro Asn Ala Leu Thr Asp Asp Arg Ser Lys Pro Ile
    370                 375                 380

Gln Gly Gln Thr Ile Val Leu Asn Ala Asp Trp Ser Gly Tyr Ser Gly
385                 390                 395                 400

Ser Phe Met Asp Tyr Trp Ala Glu Gly Asp Cys Tyr Arg Ala Cys Phe
                405                 410                 415

Tyr Val Glu Leu Ile Arg Gly Arg Pro Lys Glu Asp Lys Val Trp Trp
            420                 425                 430

Thr Ser Asn Ser Ile Val Ser Met Cys Ser Ser Thr Glu Phe Leu Gly
        435                 440                 445

Gln Trp Asn Trp Pro Asp Gly Ala Lys Ile Glu Tyr Phe Leu
    450                 455                 460
```

What is claimed is:

1. An isolated recombinant, reassortant influenza virus having PA, PB1, PB2, NP, NS, and M viral segments from a first influenza vaccine virus isolate, a heterologous, recombinant or chimeric influenza virus NA viral segment, and a heterologous, recombinant or chimeric HA viral segment, wherein the PB1 viral segment encodes a polypeptide having an alanine at residue 62, a glycine at residue 261, an arginine at residue 361, an arginine at residue 621, a serine at residue 654 or a glycine at residue 81 in F2, or a combination thereof, which PB1 viral segment encodes a PB1 having at least 85% amino acid sequence identity to a polypeptide encoded by SEQ ID NO:2; or wherein the PB2 viral segment encodes a polypeptide having a lysine at residue 391 and having at least 85% amino acid sequence identity to a polypeptide encoded by SEQ ID NO:3; or wherein the HA viral segment encodes a HA having an aspartic acid at position 136, a glutamic acid at position 162 or position 449, a leucine at position 179, a valine at position 182, an isoleucine at any of positions 184, 252 or 476, or any combination thereof; or wherein the NA viral segment encodes a NA having a serine at position 55 or a valine at position 265, or a combination thereof; and/or wherein the NS viral segment encodes a NS1 polypeptide having a lysine

at position 118, and having at least 85% amino acid sequence identity to a NS1 polypeptide encoded by SEQ ID NO:6.

2. The isolated recombinant, reassortant influenza virus of claim **1** wherein the PB2 viral segment encodes a PB2 polypeptide having a lysine at residue 391 and having at least 90% amino acid sequence identity to a polypeptide encoded by SEQ ID NO:3.

3. The isolated recombinant, reassortant influenza virus of claim **1** wherein the PB1 viral segment encodes a PB1 having at least 90% amino acid sequence identity to a polypeptide encoded by SEQ ID NO:2.

4. The isolated recombinant, reassortant influenza virus of claim **1** wherein the NS viral segment encodes a NS1 polypeptide having at least 90% amino acid sequence identity to a NS1 polypeptide encoded by SEQ ID NO:6.

5. The isolated recombinant, reassortant influenza virus of claim **1** wherein the HA is H1, H3 or H5.

6. The isolated recombinant, reassortant influenza virus of claim **1** wherein the NA is N1, N2, N3, N7, or N9.

7. The isolated recombinant, reassortant influenza virus of claim **1** wherein at least one of the PA, PB1, PB2, NP, NS, and M viral segments comprise: a PB1 having the amino acid sequence encoded by SEQ ID NO:2 or PB1 with at least 95% amino acid sequence identity to the PB1 encoded by SEQ ID NO:2; a PB2 having the amino acid sequence encoded by SEQ ID NO:3 or PB2 with at least 95% amino acid sequence identity to the PB2 encoded by SEQ ID NO:3; a PA having the amino acid sequence encoded by SEQ ID NO:1 or PA with at least 95% amino acid sequence identity to the PA encoded by SEQ ID NO: 1; a NP having the amino acid sequence encoded by SEQ ID NO:4 or NP with at least 95% amino acid sequence identity to the NP encoded by SEQ ID NO:4; a M having the amino acid sequence encoded by SEQ ID NO:5 or M with at least 95% amino acid sequence identity to the M encoded by SEQ ID NO:5; or a NS having the amino acid sequence encoded by SEQ ID NO:6 or NS with at least 95% amino acid sequence identity to the NS encoded by SEQ ID NO:6 or wherein at least one of the PA, PB1, PB2, NP, NS, and M viral segments comprise: a PB1 having the amino acid sequence encoded by SEQ ID NO: 10 or PB1 with at least 95% amino acid sequence identity to the PB1 encoded by SEQ ID NO: 10; a PB2 having the amino acid sequence encoded by SEQ ID NO: 11 or PB2 with at least 95% amino acid sequence identity to the PB2 encoded by SEQ ID NO: 11; a PA having the amino acid sequence encoded by SEQ ID NO: 12 or PA with at least 95% amino acid sequence identity to the PA encoded by SEQ ID NO: 12; a NP having the amino acid sequence encoded by SEQ ID NO: 13 or NP with at least 95% amino acid sequence identity to the NP encoded by SEQ ID NO: 13; a M having the amino acid sequence encoded by SEQ ID NO:14 or M with at least 95% amino acid sequence identity to the M encoded by SEQ ID NO: 14; or a NS having the amino acid sequence encoded by SEQ ID NO: 15 or NS with at least 95% amino acid sequence identity to the NS encoded by SEQ ID NO: 15.

8. The isolated recombinant, reassortant influenza virus of claim **1** further comprising wherein at least one of the PA, PB1, PB2, NP, NS, and M viral segments has a C to U promoter mutation.

9. The isolated recombinant, reassortant influenza virus of claim **1** further comprising 142N, 225C, 356R, or 550L in PA, one or more of 112G (PB1-F2-R81G), 247H, 507V, or 644A in PB1, one or more of 202L, 323L or 504V in PB2, one or more of 74K, 112L, 116L, 417D, or 442A in NP, 97A and/or 100H in M1, and/or 55E and/or 140Q in NS1, or combinations thereof; further comprising at least one of 202L and/or 323L in PB2, 247H in PB1 or 74K in NP and optionally at least one of 142N in PA1, 55K in NS1 or 97A and/or 100H in M1; further comprising 202L and/or 323L in PB2, 247H in PB1, has 74K in NP, 202L and/or 323L in PB2 and 247H in PB1, or 202L and/or 323L in PB2, 247H in PB1, and 74K in NP; or further comprising 40I, 40L, 112G (PB1-F2-R81G), 180W, 247H, 507V, or 644A in PB1, 202L and/or 323L in PB2, 74K, 112L, 116L, 377N, 417D, or 422L in NP, 30P, 118K, 161T or 140Q in NS1, 142N, 225C, 356R, 401K, or 550L in PA.

10. The isolated recombinant, reassortant influenza virus of claim **1** further comprising 40I, 40L, 112G (PB1-F2-R81G), 180W, 247H, 507V, or 644A in PB1 and 202L and/or 323L in PB2; 40I, 40L, 112G (PB1-F2-R81G), 180W, 247H, 507V, or 644A in PB1, 202L and/or 323L in PB2 and 74K, 112L, 116L, 377N, 417D, or 422L in NP; 40I, 40L, 112G (PB1-F2-R81G), 180W, 247H, 507V, or 644A in PB1, has 202L and/or 323L in PB2, 74K, 112L, 116L, 377N, 417D, or 422L in NP, and has 30P, 118K, 161T or 140Q in NS1; 40I, 40L, 112G (PB1-F2-R81G), 180W, 247H, 507V, or 644A in PB1, 202L and/or 323L in PB2, 74K, 112L, 116L, 377N, 417D, or 422L in NP, 30P, 118K, 161T or 140Q in NS1, and 142N, 225C, 356R, 401K, or 550L in PA; or 40I, 40L, 112G, 180W, 247H, 507V, or 644A in PB1, 202L and/or 323L in PB2, 74K, 112L, I16L, 377N, 417D, or 422L in NP, 30P, 118K, 161T or 140Q in NS1, and 142N, 225C, 356R, 401K, or 550L in PA.

11. A method to prepare influenza virus, comprising: contacting a cell with:

a vector for vRNA production comprising a promoter operably linked to an influenza virus PA DNA linked to a transcription termination sequence, a vector for vRNA production comprising a promoter operably linked to an influenza virus PB1 DNA linked to a transcription termination sequence, a vector for vRNA production comprising a promoter operably linked to an influenza virus PB2 DNA linked to a transcription termination sequence, a vector for vRNA production comprising a promoter operably linked to an influenza virus HA DNA linked to a transcription termination sequence, a vector for vRNA production comprising a promoter operably linked to an influenza virus NP DNA linked to a transcription termination sequence, a vector for vRNA production comprising a promoter operably linked to an influenza virus NA DNA linked to a transcription termination sequence, a vector for vRNA production comprising a promoter operably linked to an influenza virus M DNA linked to a transcription termination sequence, and a vector for vRNA production comprising a promoter operably linked to an influenza virus NS DNA linked to a transcription termination sequence, wherein the PB1, PB2, PA, NP, NS, and M DNAs in the vectors for vRNA production are from one or more influenza vaccine virus isolates, wherein the NA DNA in the vector for vRNA production of NA has sequences for a heterologous, recombinant or chimeric NA, and wherein the HA DNA in the vector for vRNA production of HA has sequences for a heterologous, recombinant or chimeric HA, wherein the PB1 DNA encodes a PB1 polypeptide having an alanine at residue 62, a glycine at residue 261, an arginine at residue 361, an arginine at residue 621, a serine at residue 654 or a glycine at residue 81 in F2, or a combination thereof, having at least 85% amino acid sequence identity to a polypeptide encoded by SEQ ID NO:2; or wherein the PB2 DNA encodes a

polypeptide having a lysine at residue 391 and having at least 85% amino acid sequence identity to a polypeptide encoded by SEQ ID NO:3; or wherein the HA DNA encodes a HA having an aspartic acid at position 136, a glutamic acid at position 162 or 449, a leucine at position 179, a valine at position 182, an isoleucine at position 184, 252 or 476, or any combination thereof; or wherein the NA DNA encodes a NA having a serine at position 55 or a valine at position 265, or a combination thereof; and/or wherein the NS DNA encodes a NS1 polypeptide having a lysine at position 118 and having at least 85% amino acid sequence identity to a NS1 polypeptide encoded by SEQ ID NO:6; or a combination thereof; and

a vector for mRNA production comprising a promoter operably linked to a DNA segment encoding influenza virus PA, a vector for mRNA production comprising a promoter operably linked to a DNA segment encoding influenza virus PB1, a vector for mRNA production comprising a promoter operably linked to a DNA segment encoding influenza virus PB2, and a vector for mRNA production comprising a promoter operably linked to a DNA segment encoding influenza virus NP, and optionally a vector for mRNA production comprising a promoter operably linked to a DNA segment encoding influenza virus HA, a vector for mRNA production comprising a promoter operably linked to a DNA segment encoding influenza virus NA, a vector for mRNA production comprising a promoter operably linked to a DNA segment encoding influenza virus M1, a vector for mRNA production comprising a promoter operably linked to a DNA segment encoding influenza virus M2, or a vector for mRNA production comprising a promoter operably linked to a DNA segment encoding influenza virus NS2;

in an amount effective to yield infectious influenza virus.

12. The method of claim **11** wherein the cell is an avian cell or a mammalian cell.

13. The method of claim **11** wherein the cell is a Vero cell, a human cell or a MDCK cell.

14. The method of claim **11** wherein the PB1, PB2, PA, NP, NS, and M DNAs in the vectors for vRNA productions have a sequence that corresponds to one that encodes a polypeptide having at least 95% amino acid sequence identity to a corresponding polypeptide encoded by SEQ ID NOs: 1-6 or 10-15.

15. The method of claim **11** further comprising isolating the virus.

16. The method of claim **11** wherein at least one of PA, PB1, or PB2 viral segments has a C to U promoter mutation.

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