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(12) **United States Patent**
Kawaoka et al.(10) **Patent No.:** **US 9,890,363 B2**(45) **Date of Patent:** **Feb. 13, 2018**(54) **INFLUENZA VIRUS REPLICATION FOR
VACCINE DEVELOPMENT**(71) Applicant: **Wisconsin Alumni Research
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6, 2015.(51) **Int. Cl.****A61K 39/145** (2006.01)**C12N 7/00** (2006.01)**C12N 7/01** (2006.01)**C12N 7/02** (2006.01)**C07K 14/005** (2006.01)**C12N 15/86** (2006.01)(52) **U.S. Cl.**CPC **C12N 7/00** (2013.01); **A61K 39/145**
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2760/16151 (2013.01)(58) **Field of Classification Search**CPC C12N 7/00; C12N 2760/16134; A61K
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See application file for complete search history.(56) **References Cited****U.S. PATENT DOCUMENTS**4,071,618 A 1/1978 Konobe et al.
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Woessner, P.A.(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 vac-
cine 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.**19 Claims, 53 Drawing Sheets**

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PR8(CAMBRIDGE)

PB2

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ACTTTATGGAGTAAATGAATGATGCCGATCAGACCGAGTGATGGTATCACCTCTGGCTGTGACATGGTGGAATAGGAATGGA
CCAATGACAAATACAGTTCAATATCCAAAATCTACAAAATTTTGAAGAGTCGAAAGGCTAAGCATGGAACCTTTGGC
CCTGTCCATTTAGAAACCAAGTCAAAATACGTGGAGAGTIGACATAAATCTGGTCATGCGAGTCTCAGTGCCCAAGGAGGCA
CAGGATGTAATCATGGAAAGTTGTTTCCCTAACGAAGTGGAGCCAGGATACCTAACATCGGAATCGCAACTAACGATAACCAA
GAGAAGAAAGAACTCCAGGATGCAAAATTTCTCTTGAATGGTTCATACATGTTGGAGAGAACTGGTCCGCAAAACG
AGATTCTCCAGTGGCTGGTGGAAACAGCAGTGTGTACATIGAAGTGTGCATTTGACTCAAGGAACATGCTGGGAACAGATG
TATACTCCAGGAGGGAAGTGAAGATGATGTTGATCAAAAGCTTGATTATGCTGCTAGGAACATAGTGAGAAGAGCTGCA
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CAGAACCCCAACAGAAAGACAGCCGTGGATATATGCAAGGCTGCAATGGGACTGAGAAATTAGCTCATCTTCAGTTTGGTGGGA
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GTGAGCATTGACCGGTTCTTGAGAGTCAGGACCAACGAGGAAATGTACTACTGTCTCCGAGGAGGTGAGTGAAACACAGGGA
ACAGAGAACTGACAAATAACTTACTCATCGTCAATGATGTGGAGATTAAATGGTCTCTGAATCAGTGTGGTCAATACCTATCAA
TGGATCATCAGAACTGGGAACTGTTAAATTCAGTGGTCCAGAACTTACAAATGCTATACAAATAAAATGGAAATTTGAACCA
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GAGAAGGCTAATGTGCTAATTTGGGCAAGGAGACGTGGTGTGGTAAATGAACGAAACGAGGACTCTAGCATACTTACTGACAGC
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(SEQ ID NO:11)

Fig. 1A

PR8(CAMBRIDGE)

PB1

AGCGAAGCAGGCAAAACCATTTGAAATGGATGTCAATCGACCTTACTTTTCTTAAAGTGCCAGCACAAAATGCTATAAGCACAACTTTCCCTTATACCGGAGACCTCTTACAGCCATGGGACAGGAACAGGATACACCATGGATACTGTCAACAGGACACATCAGTACTCAGAAAAGGGAAGATGGACAAACAAACACCGAACTGGAGCACCGCAACTCAACCCGATTGATGGCCACTGCCAGAAACAAATGAACCAAGTGTTATGCCCAAACAGATTGTGATTGGAAAGCAATGGCTTTCTTTGAGGAATCCCATCTCTGGTATTTTGGAAACTCGTGTATTGAACCGATGGAGTTGTTTCAGCAACACAGTAGACAAGCTGACACAAGGCCGACAGACCTATGACTGGACTTTAAATAGAAACCCAGCTGCTGCAACAGCATTTGGCCAAACAATAGAAAGTGTTCAGATCAAAATGGCTCAGGGCCAAATGAGTCAAGAGGCTCATAGACTTCTTAAGGATGTAATGGAGTCAATGAAGAAAGAAATAGGATCAAGGATCAAAATGAAAGGGGTAGACGGGTGAGAGACAAATATGACTAAGAAAATGATAACAGAGAAACAAATAGGTAAAGGAAACAGAGATTGAACAAAGGGGTATCTAATTAGAGCAATTGACCTGAAACACAATGACCAAAAGATGCTGAGAGAGGAAGCTAAACCGGAGAGCAATTGCAACCCCAAGGATGCAAAATAGGGGTTTGATATCTTTGTGAGACACTGGCAAGGAGTATATGTGAGAAACTTGAACAATCAGGGTTGCGCATGTGGAGGCAATGAGAAAGCAAGTTGGCAAAATGTTGTAAGGAAGATGATGACCAATTTCTCAGGACACCGAACTTTCCTTCACCATCACTGGAGATAACACCAATGGAACGAAAATCAGAAATCTCGGATGTTTGGCCATGATCATATATGACCAAGAAATCAGCCCCGAATGGTTCAGAAATGTTCTAAGTATTTGCTCCAATATGTTCTCAAAACAATGGCGAGACTGGGAAAGGGTATATGTTGAGAGCAAGAGTATGAAACTTAGAACTCAAACTACCTGCAGAAAATGCTAGCAAGCATTTGAAATATTTCAATGATTCAACAAGAAAGAGATTGAAAATAATCCGACCGCTCTTAATAGAGGGGACTGCATCATTTGAGCCCTGGAATGATGATGGCATGTTCAATATGTTAAGCACTGTATTAGGCGTCTCCATCTGAAATCTTGGACAAAGAGATACACCAAGACTACTTACTGGTGGATGGTCTTCAATCCTCTGACGATTTGCTCTGATGTGAATGCACCAATCATGAAGGATTCAGGCGGAGTTCGACAGGTTTATCGAACCTGTAAAGCTACTTGGAAATCAATATGAGCAAGAAAAGTCTTACATAAACAGAAACAGGTACATTTGAATTCACAAAGTTTTCATATCGTTATGGGTTTGTGGCAATTCAGCATGGAGCTTCCAGTTTGGGGTGTCTGGGATCAACGAGTCAGCGGACATGAGTATTGGAGTTACTGTATCAAAACAATATGATAACAATATGATAAACAATGATCTTGGTCCAGCAACAGCTCAAAATGGCCCTTCAGTTGTTCATCAAGATTACAGGTACACGTACCGATGCCATAGAGGTGACACACAAATACAAACCCGAAAGATCATTTGAAATAAAGAAACTGTGGGAGCAACCCGTTCCAAAGCTGGACTGCTGGTCTCCGACGGAGGCCAAATTTATACAAATTAGAAATCTCCACATTCCTTGAAATGCTGCCCTAAATGGGAATTGATGGATGAGGATTACCGGGGCTTTATGCAACCCACTGAACCATTTTGTGAGCCATAAGAAATTGAAATCAATGAACAATGCAGTATGCCAGCACATGGTCCAGCCAAACATGGAGTATGATGCTGTGCAACCAACACATCTCTGGATCCCCAAAGAAATCGATCCATCTTGAATACAAGTCAAGAGGAGTACTTGAAGATGAACAAATGTACCAAGGTGCTGCAATTTATTTGAAAATTTCTTCCCGACGATTCATACAGAAAGACCGTGGGATATCCAGTATGTTGGAGGCTATGGTTCCAGGCCCCGAATTTGATGACCGGATTTGGAATCTGGAAGGATAAGAAAGAGAGTTCACTGAGATCATGAAGATCTGTTCACCATTTGAAGAGCTCAGACGGCAAAAATAGTGAATTTAGCTTGTCTTCATGAAGAAAATGSCCTTGTCTACT

(SEQ ID NO:10)

Fig. 1B

PR8(CAMBRIDGE)

PA

AGCGAAAGCAGGTAAGTCAAAATGGAAGATTTGTGGGACAATGCTTCAATCCGATGATTGTGAGCTTGGGAAAAACA
ATGAAAGAGTATGGGAGGACCTGAAATCGAAACAAACAAATTTGCAGCAATATGCACTCACTTGGAAATGCTTCAATGAT
TCAGATTTCCACTTCATCAATGAGCAAGGCGAGTCAATAATCGTAGAATTTGGTGAATCTTAAAGCACAGATTT
GAAATAATCGAGGGAAGAGATCGCACAAATGGCCTGGACAGTAGTAAACAGTATTTGCAACACTACAGGGCTGAGAAACCAAG
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GAAAGGCCAATAAAATTAATCTGAGAAACACACATCCATTTCTCGTTCACTGGGGAAGAAATGGCCAGCAGAGGCTCTGG
TACACTCTCGATGAAGAAAGCAGGCTAGGATCAAAACCGCTATTTCAACATAAGACAAGAAATGGCCAGCAGAGGCTCTGG
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CAAGTCTCCCGCCGAACTTTCTCCAGCTTTGAAATTTTAGAGCTTATGGAATGGAATCGAACCGAACGGTACATTGAGGGC
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GGGCTCCCTGTTCTCAGCGGTCCAAATTCCTGCTGATGGATGCCTTAAATTAAGCATTTGAGGACCCAAAGTCATGAAGGAG
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(SEQ ID NO:12)

Fig. 1C

PR8(CAMBRIDGE)

NP

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CCAGCACACAAGAGTCAACTGGTGTGGATGGCATGCCATTCTGCCGATTTGAAGATCTAAGAGTATTGAGCTTCAACAAGGG
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GAGGGAGAACATCTGACATGAGGACCGAAATCATAGGATGATGGAAAGTGCAGACCAAGAGATGTGCTTTCCAGGGGCGG
GGAGTCTTCGAGCTCTCGGACGAAAGGCAGCGGCCGATCGTGCTTCCTTTGACATGAGTAATGAAGGATCTTATTCTTC
GGAGACAAATGCAGAGGAGTACGACAAATTAAGAAATAATACCTTGTCTTCTACT

(SEQ ID NO:13)

Fig. 1D

PR8(CAMBRIDGE)

M

AGCAAAGCAGGTAGATATTGAAGATGAGTCTTCTAACCGAGGTGAAACGTACGTTCTCTCTATCATCCGTCAGGCCCCCT
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TAGACGCTTTGTCCAAATGCCCTTAATGGGAACGGGATCCAAATAACATGGACAAAGCAGTTAAACTGTATAGGAAGCTCAA
GAGGAGATAACATTCATGGGGCCAAAGAAATCTCACTCAGTTATCTGTGTGTCACCTTGCCAGTTGTATGGGCTCATATA
CAACAGGATGGGGCTGTGACCACTGAAGTGGCATTTGGCCTGGTATGTGCAACCTGTGAACAGATTGCTGACTCCAGCATCG
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TTGATCGTCTTTTTCAAATGCAATTTACCGTCTGCTTTAAATACGGACTGAAAGGAGGGCTTCTACGGAAGGAGTGCCAAAGT
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AAACTACCTTGTCTACT

(SEQ ID NO:14)

NS

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CACTTAAATGACCAATGGCCTCTGTACCTGTGCTGCGTTACTTAACCGACATGACTCTTGAGGAAATGTCAAGGGAATGGTCCA
TGCTCATACCCAAGCAGAAAGTGGCAGGGCCCTCTTTGTATCAGAAATGGACAGGCGATCATGGATAAAAACATCATCTGAAAG
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CAAAACAGAAACGAGAAATGGCGGGAACAATAGGTGAGAAATTAAGATGGTTGATTGAAGAAGTGAGACACAAA
CTGAAGGTAACAGAGAAATAGTTTGGAGCAAAATAACATTTATGCAAGCCTTACATCTATTGCTTGAAGTGGAGCAAGAGATAAGA
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(SEQ ID NO:15)

Fig. 1E

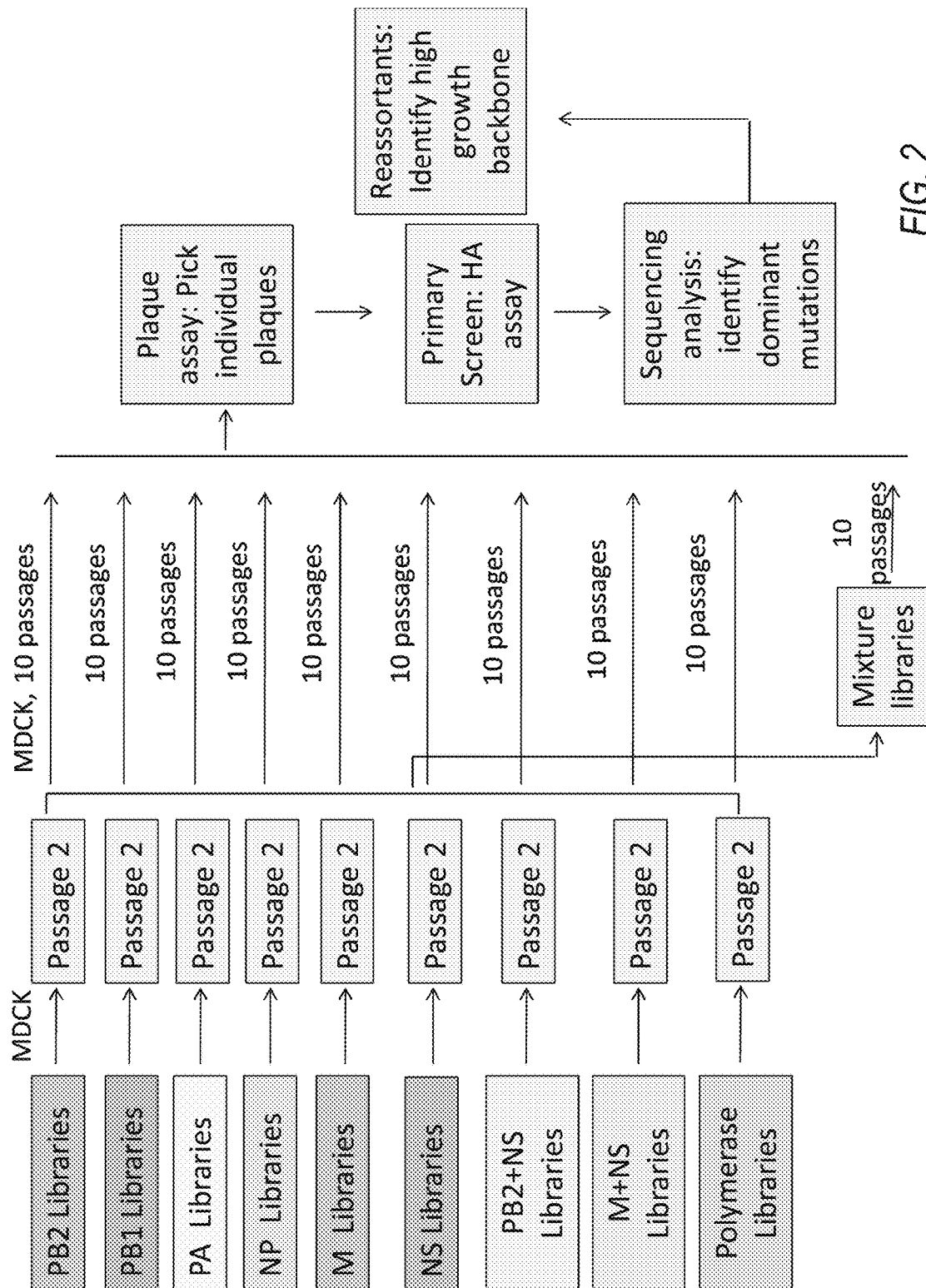


Figure 3 Summary of HA assay of 1434 individual clones

Groups	Numbers of clone	Fold change	%
WT HA titer = 2^7	-	-	-
HA titer = $2^{>9-9.5}$	8	>4	0.6%
HA titer = $2^{>8.5-9}$	23	>2.8 - 4	1.6%
HA titer = $2^{7-8.5}$	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			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	Indo/NC /09 delHA	Indo/NC /09 NA	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 5B PB1 Mutants

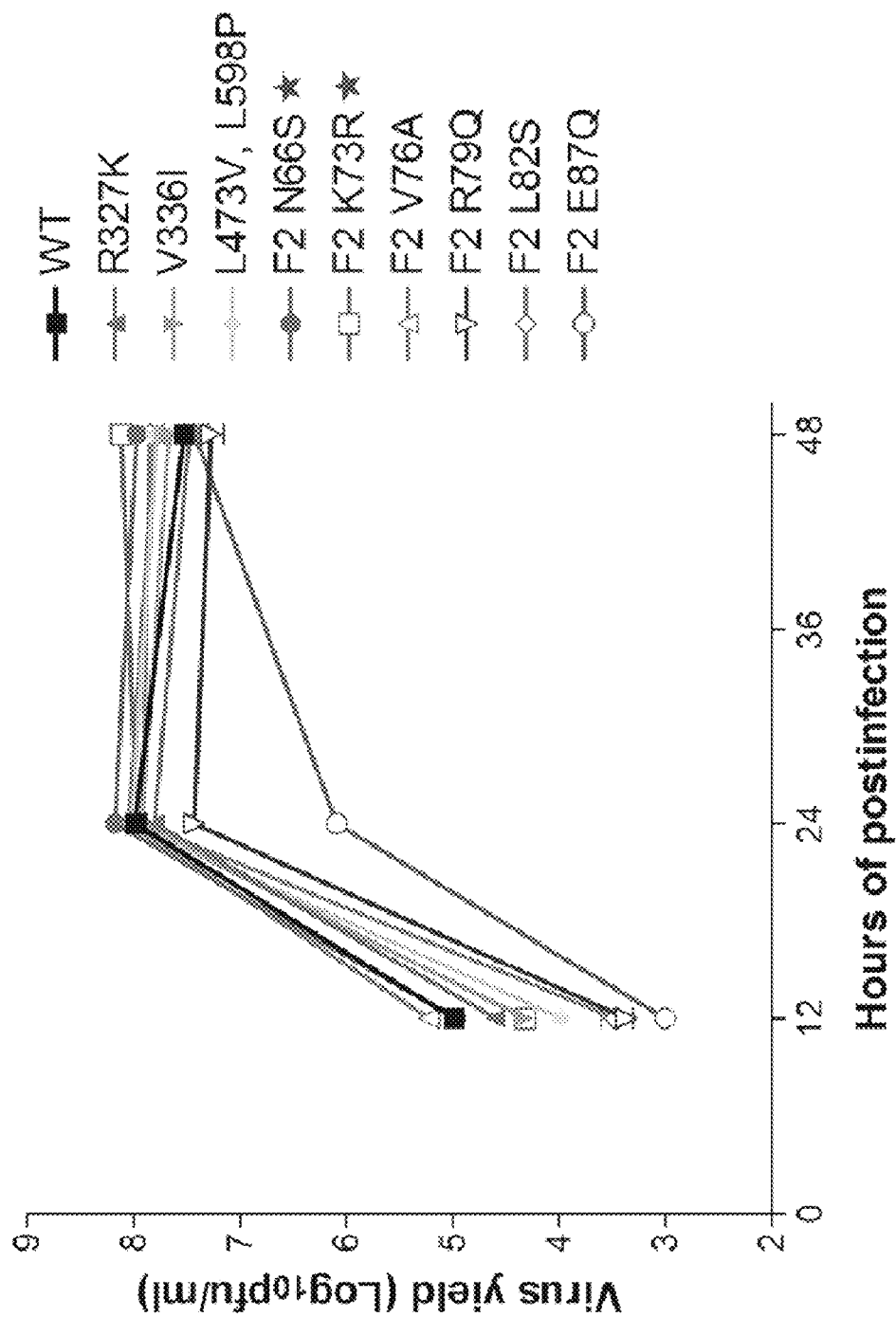


Figure 5C PA Mutants

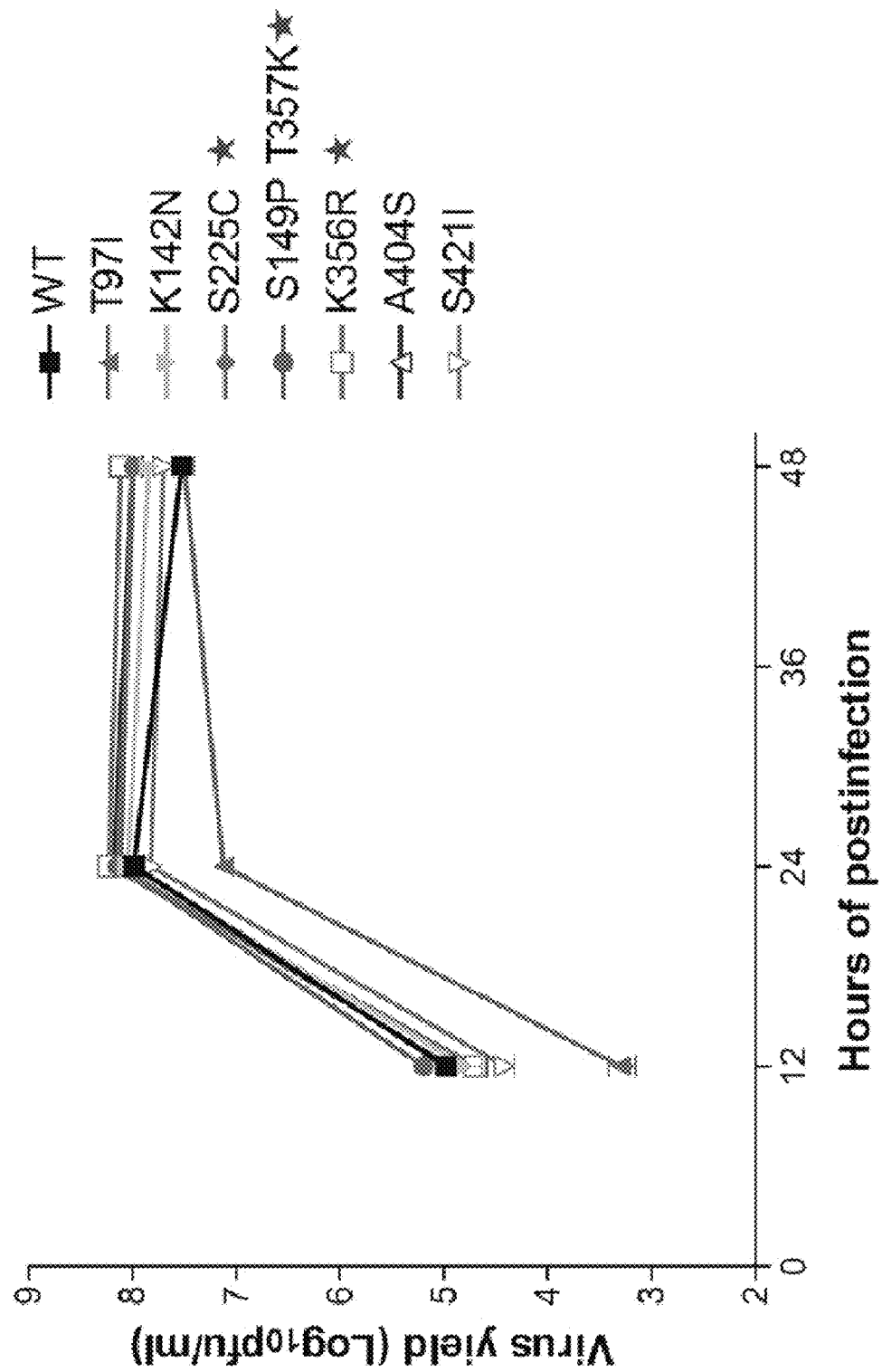


Figure 5D NP, M and NS1 mutants

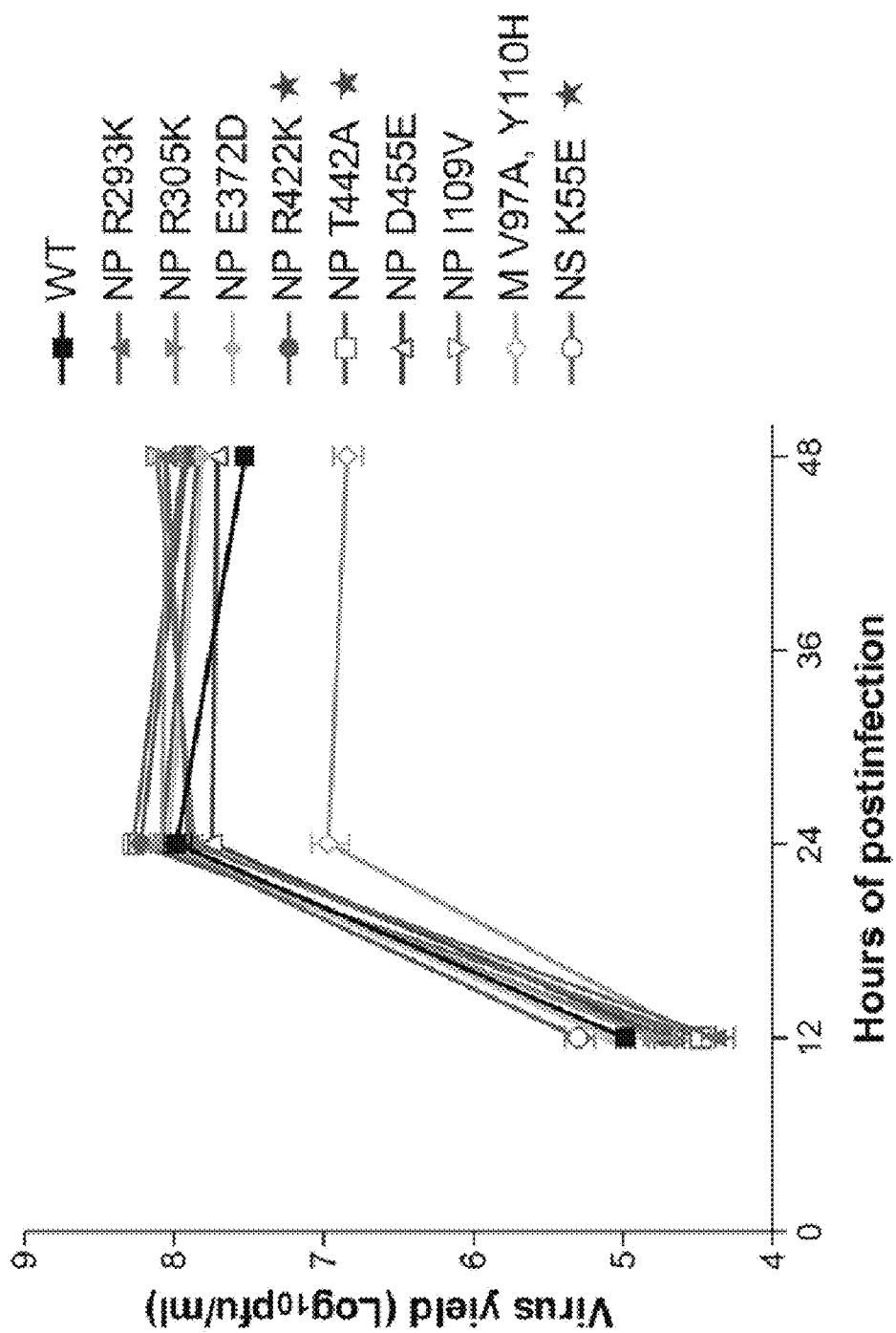


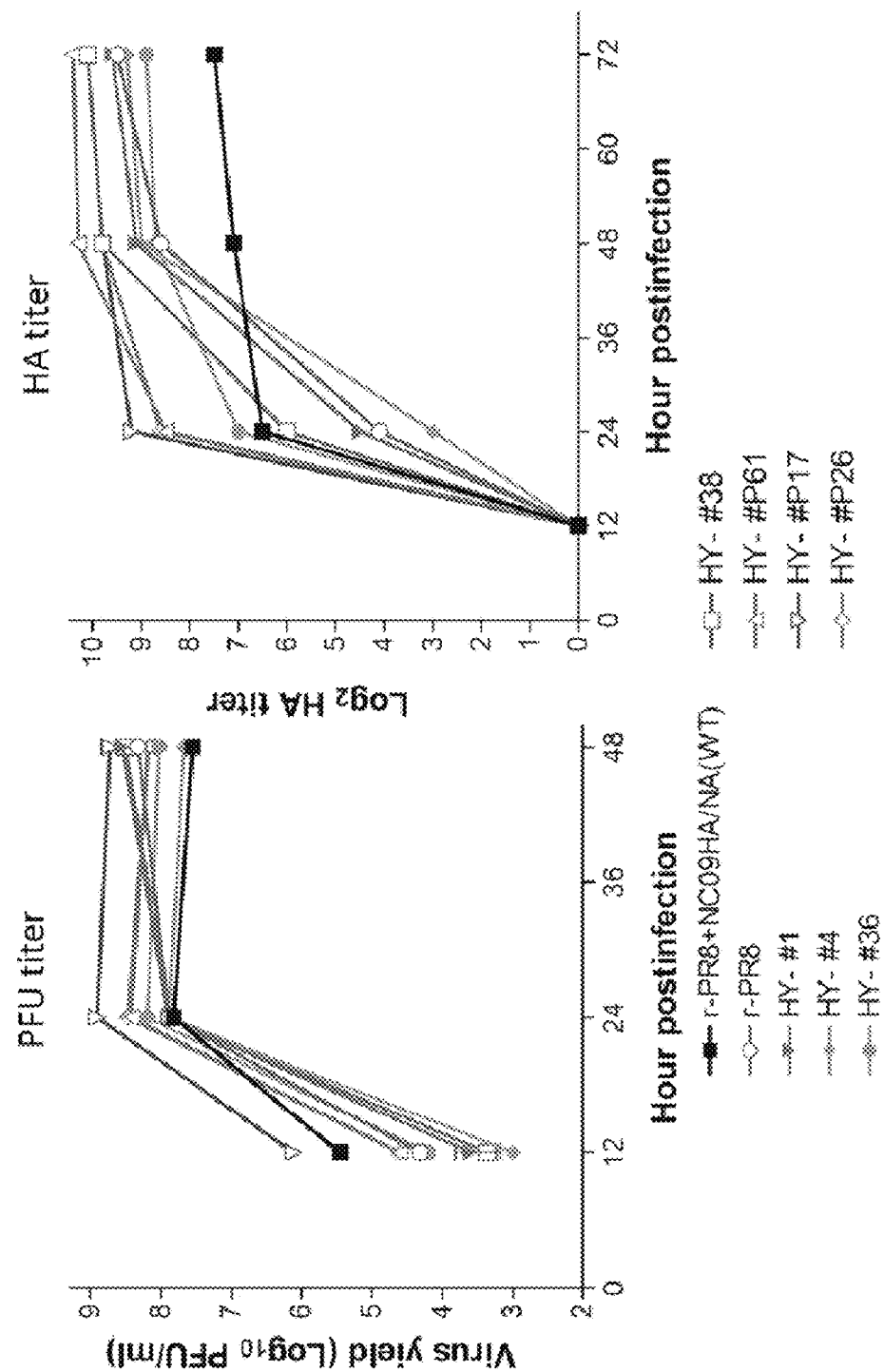
Figure 6 Confirmed high replicative mutations

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

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)



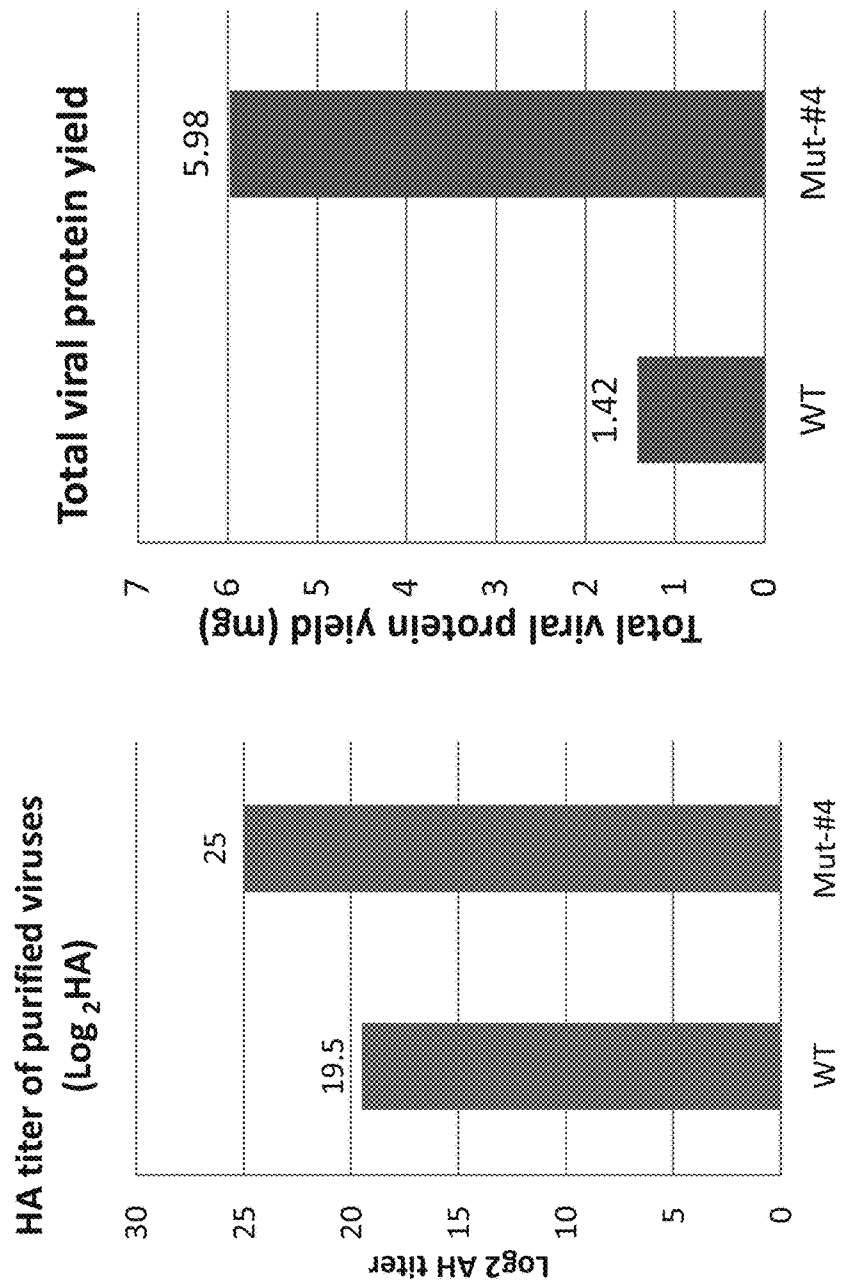
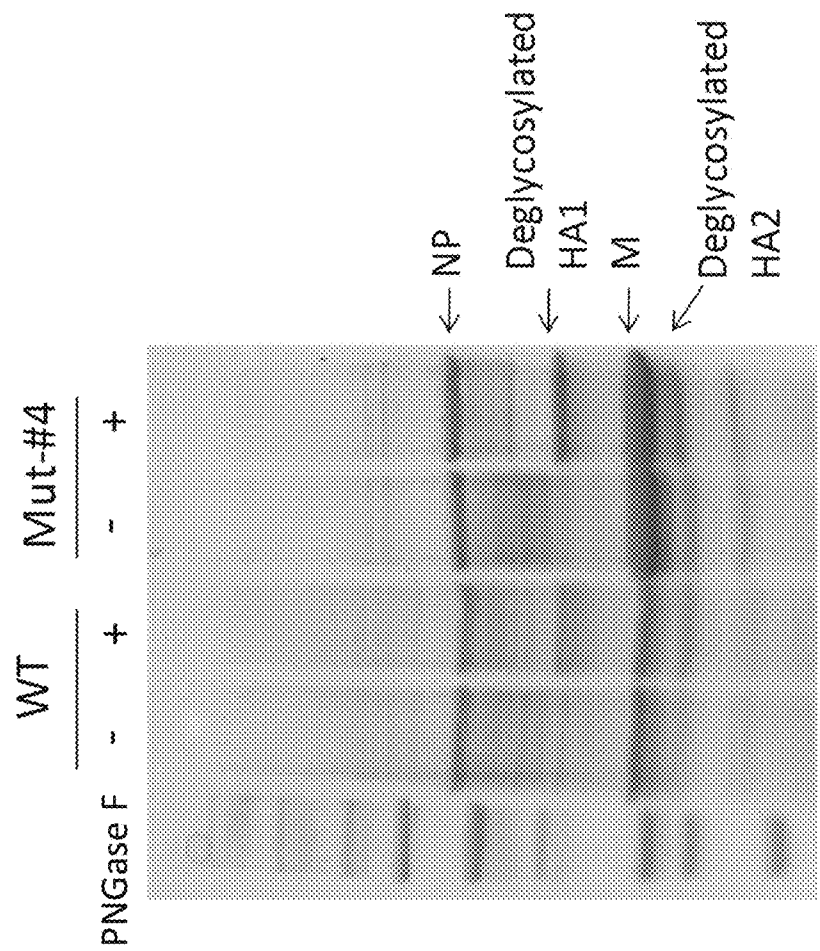


FIG. 8A Total viral protein yield: 4.2 fold FIG. 8B

Figure 8C SDS-PAGE analysis

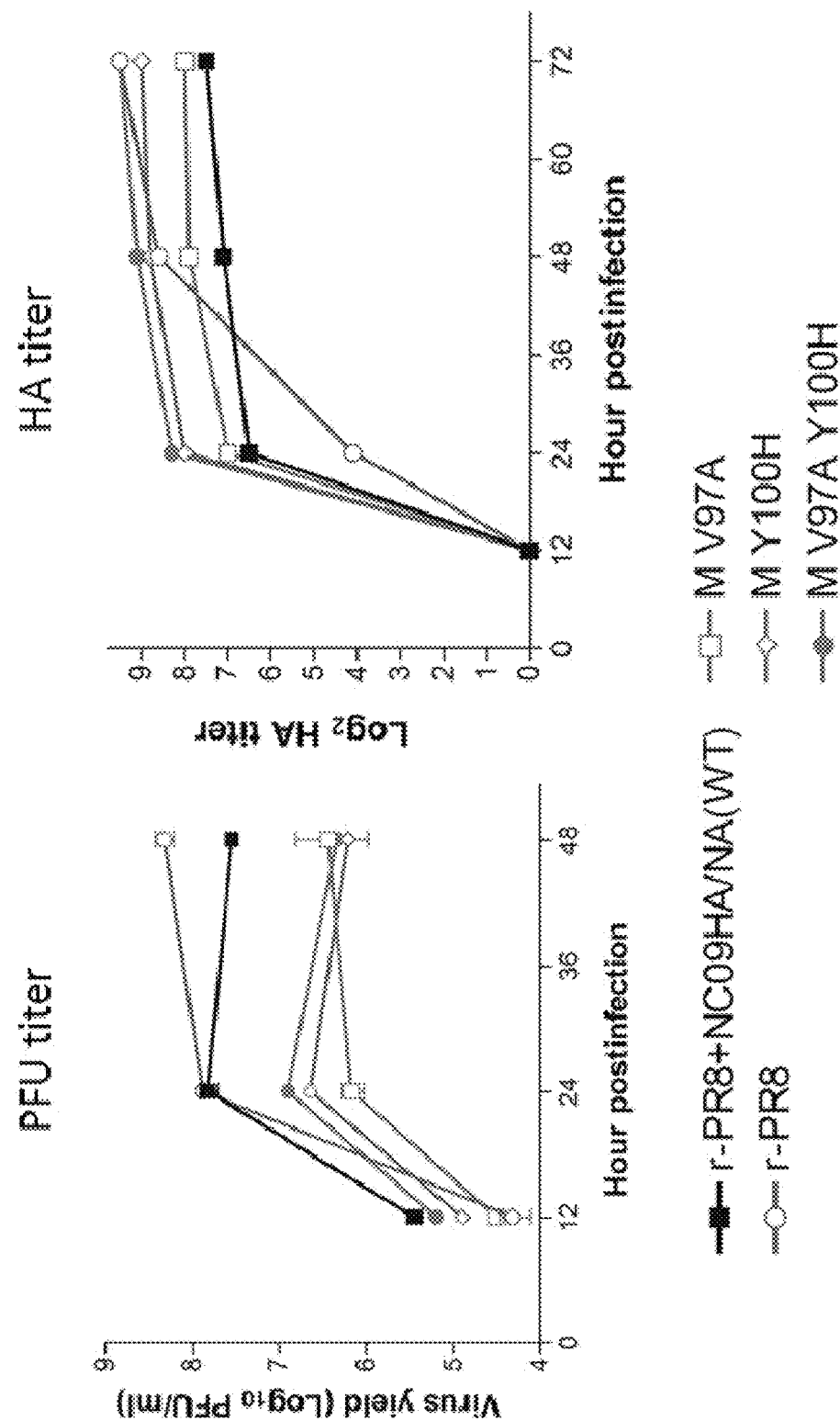


Mutant virus generates much more M1 and HA1 proteins than wild type.

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

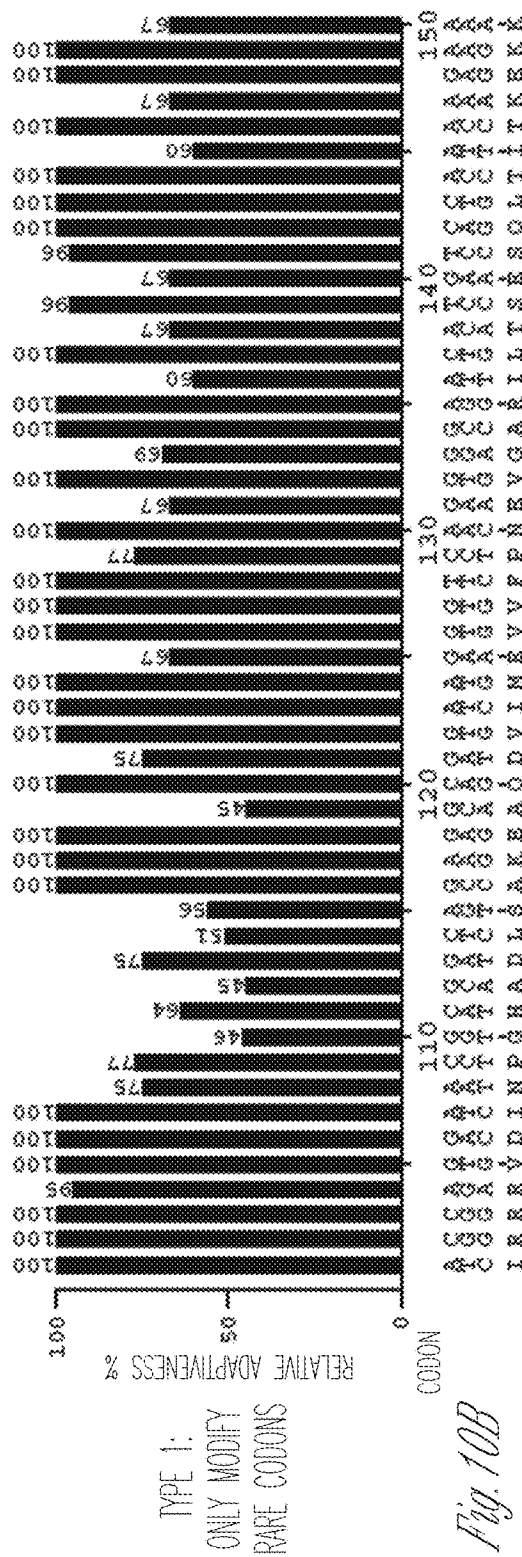
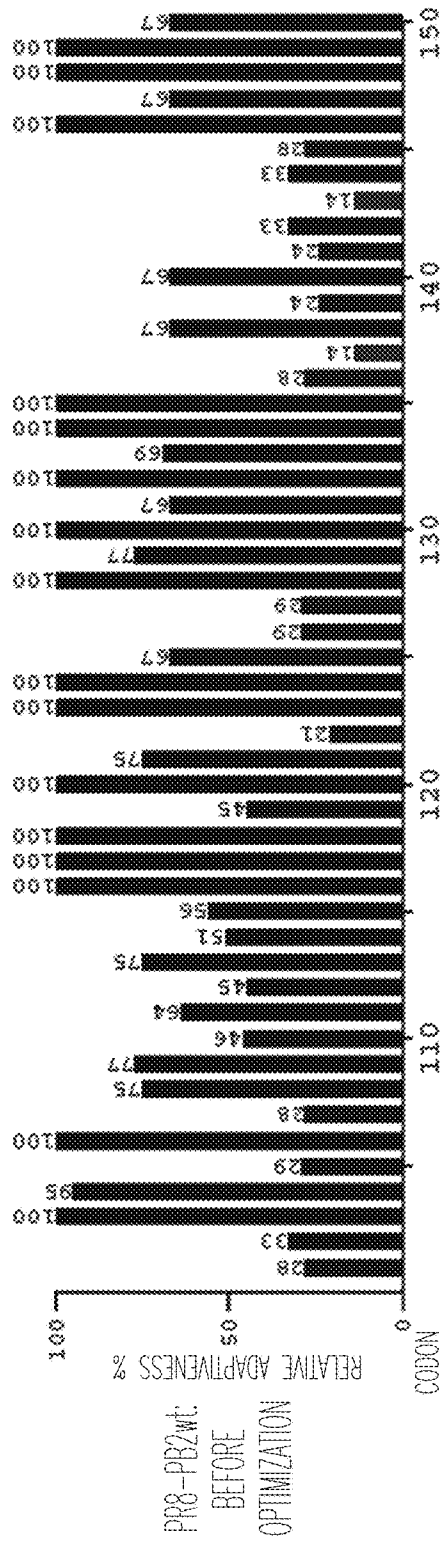


Fig. 10B

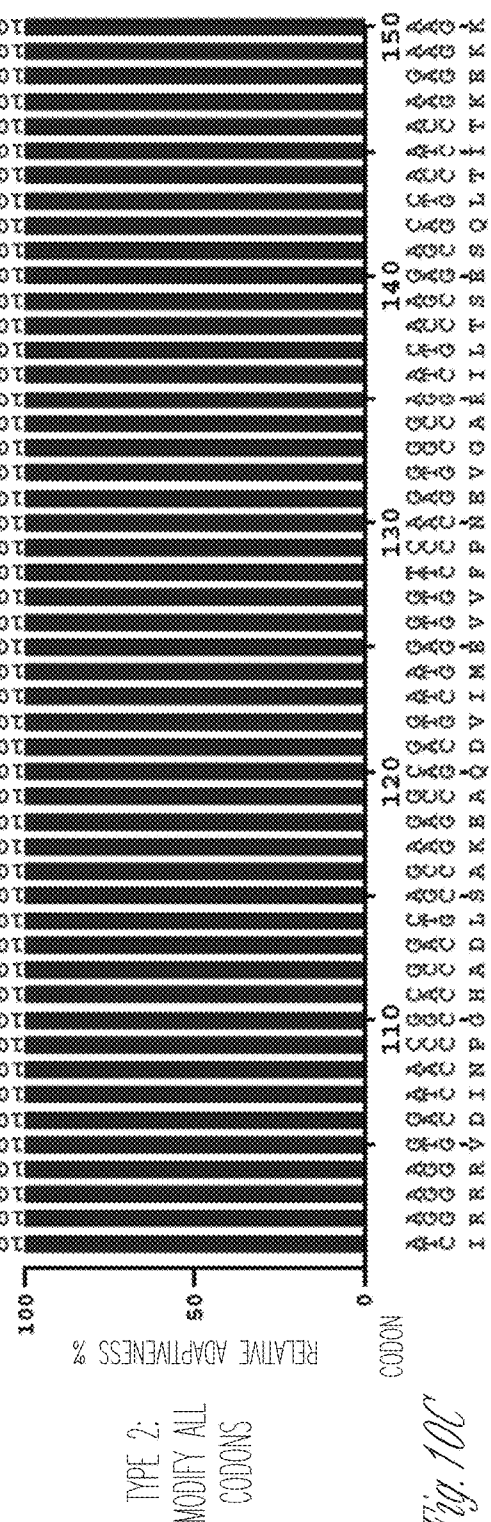
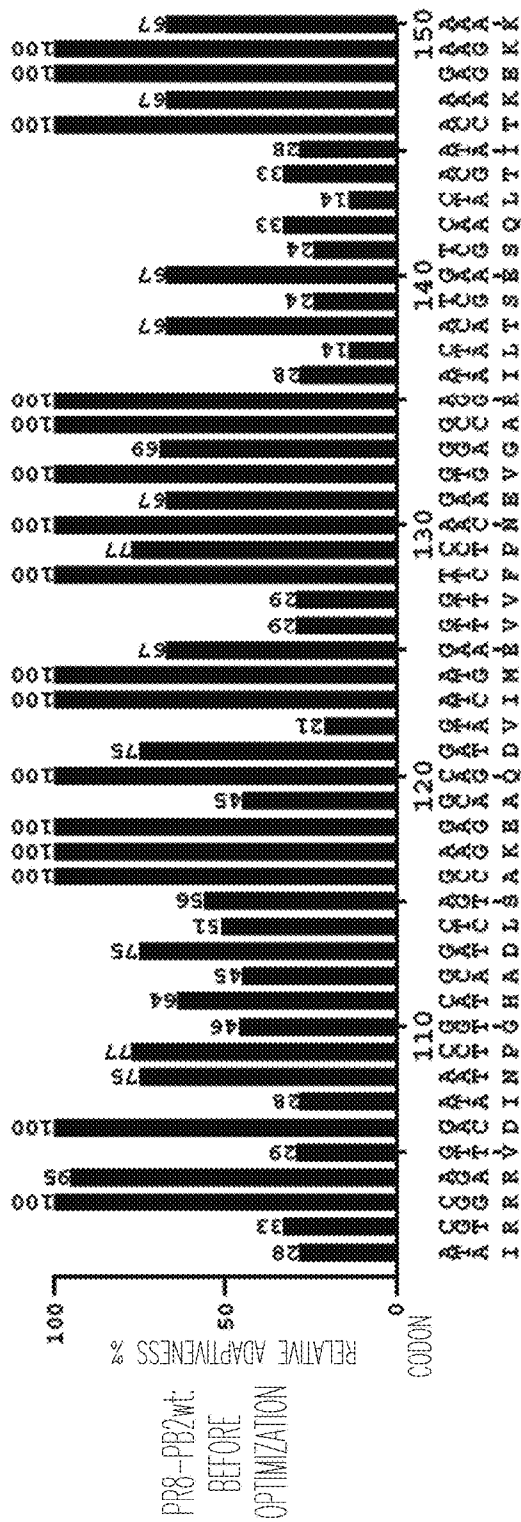


Figure 10D Growth kinetics in MDCK cells

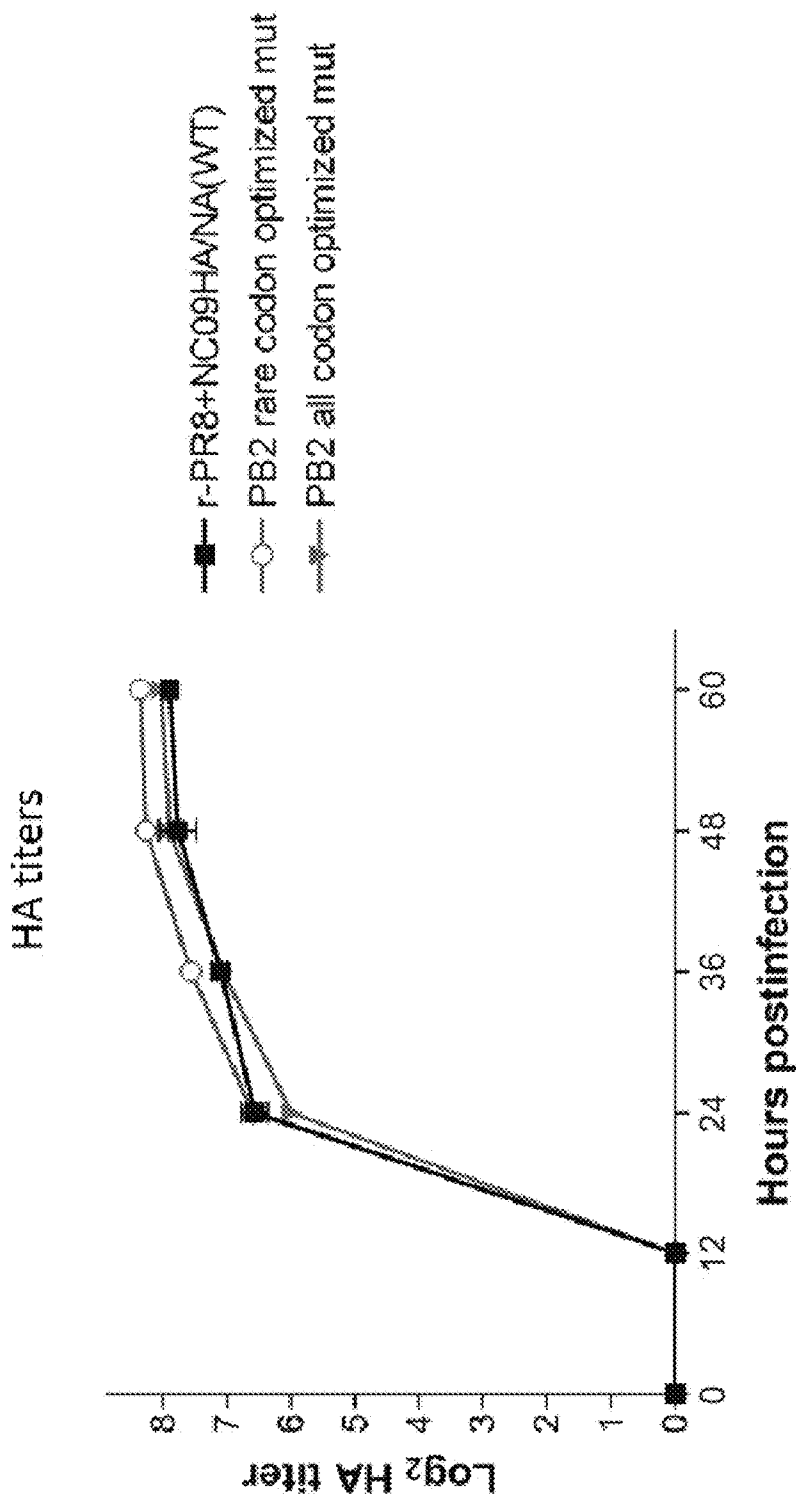
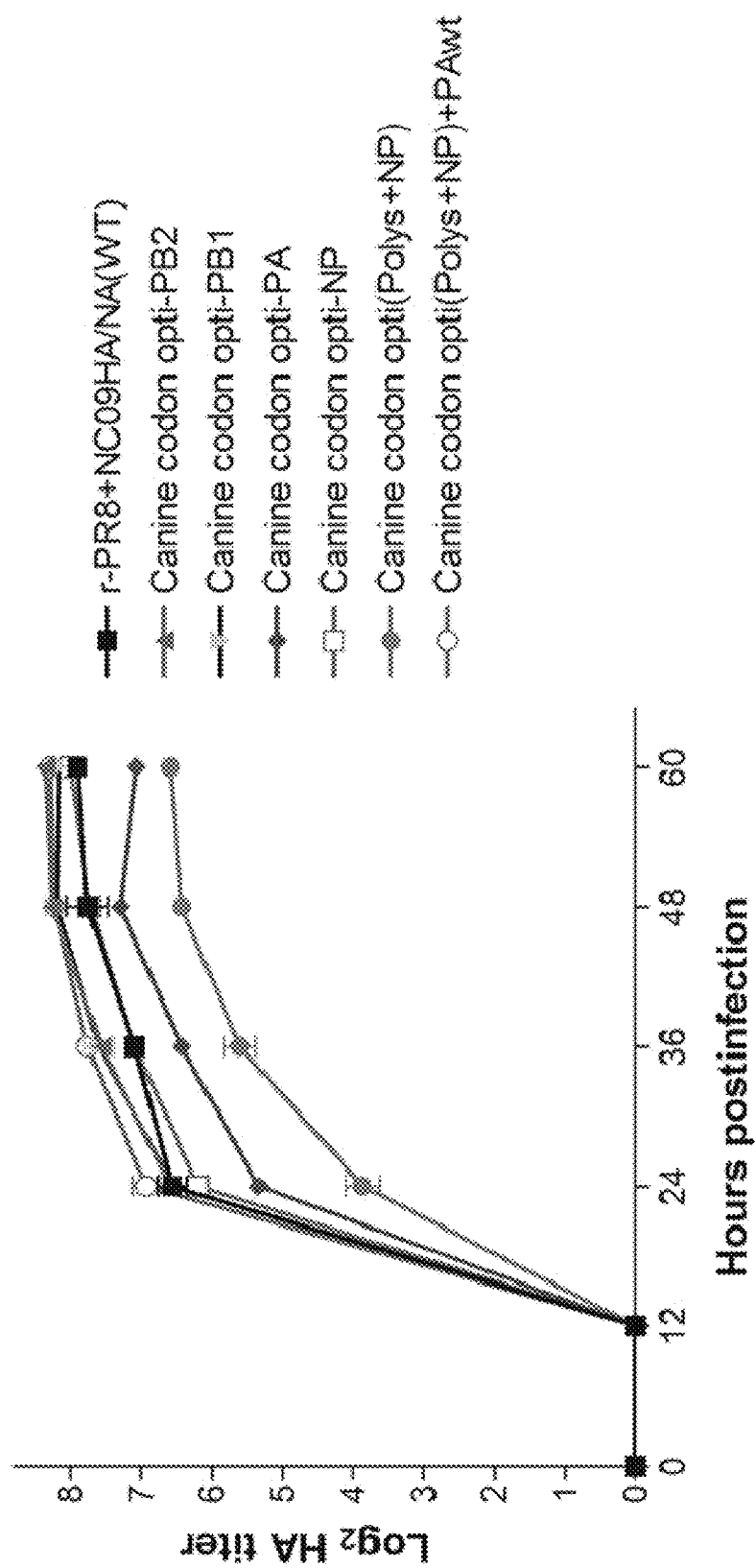


Figure 10E



PR8-UW PB2:

AGCGAAAGCAGGTCAATTATATTC AATATGGAAGAATAAAAGAACTACGAAATCTAATGTCGCAGTCTCGCACCCGCGA
GATACTCACAAAAACCACCGTGGACCATATGGCCATAATCAAGAAGTACACATCAGGAAGACAGGAGAAGAACCCAGCAC
TTAGGATGAAATGGATGATGGCAATGAAATATCCAATTACAGCAGACAAGAGGATAACGGAAATGATTCCTGAGAGAAAT
GAGCAAGGACAACTTTATGGAGTAAATGAATGATGCCGGATCAGACCGAGTGATGGTATCACCTCTGGCTGTGACATG
GTGGAATAGGAATGGACCAATAACAAATACAGTTCAATTATCCAAAATCTACAAAATCTATTTTGAAAGAGTCGAAAGGC
TAAAGCATGGAACCTTTGGCCCTGTCCATTTAGAAACCAAGTCAAAATACGTCGGAGAGTTGACATAAATCCTGGTCAT
GCAGATCTCAGTGCCAAGGAGGCACAGGATGTAATCATGGAAGTTGTTTTCCCTAACGAAGTGGGAGCCAGGATACTAAC
ATCGGAATCGCAACTAACGATAACCAAGAGAAGAAAGAAGAACTCCAGGATTGCAAAATTTCTCCTTGATGGTTGCAT
ACATGTTGGAGAGAGAAGTGGTCCGCAAAACGAGATTCTCCAGTGGCTGGTGGGAACAAGCAGTGTGTACATTGAAGTG
TTGCATTTGACTCAAGGAACATGCTGGGAACAGATGTATACTCCAGGAGGGGAAGTGAGGAATGATGATGTTGATCAAAG
CTTGATTATTGCTGCTAGGAACATAGTGAGAAGAGCTGCAGTATCAGCAGATCCACTAGCATCTTTATTGGAGATGTGCC
ACAGCACACAGATTGGTGGAAATTAGGATGGTAGACATCCTTAGGCAGAACCAACAGAAGAGCAAGCCGTGGATATATGC
AAGGCTGCAATGGGACTGAGAATTAGCTCATCTTCAGTTTTGGTGGATTACATTTAAGAGAACAAGCGGATCATCAGT
CAAGAGAGAGGAAGAGGTGCTTACGGGCAATCTTCAACATTGAAGATAAGAGTGCATGAGGGATATGAAGAGTTCACAA
TGTTTGGGAGAAGAGCAACAGCCATACTCAGAAAAGCAACCAGGAGATTGATTGAGTGTAGTGAGTGGGAGAGACGAA
CAGTCGATTGCCAAGCAATAATTGTGGCCATGGTATTTTACAAGAGGATTGTATGATAAAAGCAGTCAGAGGTGATCT
GAATTTGTC AATAGGGCGAATCAACGATTGAATCCTATGCATCAACTTTTAAGACATTTTCAGAAGGATGCGAAAGTGC
TTTTTCAAATTTGGGAGTTGAACCTATCGACAATGTGATGGGAATGATTGGGATATTGCCGACATGACTCCAAGCATC
GAGATGTCAATGAGAGGAGTGAGAATCAGCAAAATGGGTGTAGATGAGTACTCCAGCACGGAGAGGGTAGTGGTGAGCAT
TGACCGTTTTTTGAGAATCCGGGACCAACGAGGAAATGTACTACTGTCTCCGAGGAGGTGAGTGAACACAGGGAACAG
AGAACTGACAATAACTTACTCATCGTCAATGATGTGGGAGATTAATGGTCCTGAATCAGTGTGGTCAATACCTATCAA
TGGATCATCAGAACTGGGAACTGTTAAATTCAGTGGTCCAGAACCTACAATGCTATACAATAAAATGGAATTTGA
ACCATTTAGTCTTTAGTACCTAAGGCCATTAGAGGCCAATACAGTGGGTTTGTAAGAACTCTGTTCCAACAAATGAGGG
ATGTGCTTGGGACATTTGATACCGCACAGATAATAAACTTCTTCCCTTCGCAGCCGCTCCACCAAAGCAAAGTAGAATG
CAGTTCTCTCATTTACTGTGAATGTGAGGGGATCAGGAATGAGAATACTTGAAGGGGCAATTCTCCTGTATTCAACTA
TAACAAGGCCACGAAGAGACTCACAGTTCTCGGAAAGGATGCTGGCACTTTAACTGAAGACCCAGATGAAGGCACAGCTG
GAGTGGAGTCCGCTGTTCTGAGGGGATTCTCATTCTGGGCAAAGAAGACAAGAGATATGGGCCAGCACTAAGCATCAAT
GAACTGAGCAACCTTGCGAAAGGAGAGAAGGCTAATGTGCTAATTGGGCAAGGAGACGTGGTGTGGTAATGAAACGGAA
ACGGGACTCTAGCATACTTACTGACAGCCAGACGACCAAAAGAATTTCGGATGGCCATCAATTAGTGTGAATAGTTT
AAAAACGACCTTGTCTTACT (SEQ ID NO:3)

FIG. 10F

Canine codon optimized PR8-PB2:

AGCGAAAGCAGGTCAATTATATCAATATGGAAAGAATAAAGAAGTACGAAATCTAATGTCGCAGTCTCGACCCGCGA
GATACTCAGAAAAACACCGTGGACCATATGGCCATAATCAAGAAGTACACATCAGGAAGACAGGAGAAGAACCAGCAC
TGAGGATGAAATGGATGATGGCAATGAAATATCCAATTACAGCAGACAAGAGGATCACCGAAATGATTCCTGAGAGAAAT
GAGCAGGGACAGACTCTGTGGAGTAAATGAATGATGCCGGATCAGACCGAGTGATGGTGTACCTCTGGCTGTGACATG
GTGGAATAGGAATGGACCAATCAGAAATACAGTGCATTATCCAAAAATCTACAAACTTATTTTGAAAGAGTCGAAAGGC
TGAAGCATGGAACCTTTGGCCCTGTCCATTTAGAAACCAGGTCAAATCCGGCGGAGAGTGGACATCAATCCTGGTCAT
GCAGATCTCAGTGCCAAGGAGGCACAGGATGTGATCATGGAAGTGGTGTCCCTAACGAAGTGGGAGCCAGGATTCTGAC
ATCCGAATCCAGCTGACCATTACCAAAGAGAAGAAAGAAGAACTCCAGGATTGCAAAATTTCTCCTCTGATGGTGGCAT
ACATGCTGGAGAGAGAAGTGGTCCGCAAAACAAGATTCTCCAGTGGCTGGTGGAAACAAGCAGTGTGTACATTGAAGTG
CTGCATCTGACTCAGGGAACATGCTGGGAACAGATGTATACTCCAGGAGGGGAAGTGAGGAATGATGATGTGGATCAGAG
CCTGATTATTGCTGCTAGGAACATTGTGAGAAGAGCTGCAGTGTGAGCAGATCCACTGGCATCTCTGCTGGAGATGTGCC
ACAGCACACAGATTGGTGGAATTAGGATGGTGGACATCCTGAGGCAGAACCAACAGAAGAGCAGGCCGTGGATATTTGC
AAGGCTGCAATGGGACTGAGAATTAGCTCATCCTTCAGTTTTGGTGGATTACATTTAAGAGAACAAGCGGATCATCAGT
CAAGAGAGAGGAAGAGGTGCTGACCGGCAATCTGCAGACACTGAAGATCAGAGTGCATGAGGGATATGAAGAGTTCACAA
TGGTGGGGAGAAGAGCAACAGCCATCCTCAGAAAAGCAACCAGGAGACTGATTAGCTGATCGTGAGTGGGAGAGACGAA
CAGTCCATTGCCGAAGCAATTATTGTGCCATGGTGTTCACAGGAGGATTGTATGATTAAAGCAGTCAGAGGTGATCT
GAATTCGTCAATAGGGCCAATCAGCGACTGAATCCTATGCATCAGCTGCTGAGACATTTTCAGAAGGATGCCAAAGTGC
TGTTTCAGAATTGGGGAGTGGAACCTATCGACAATGTGATGGGAATGATTGGGATCCTGCCGACATGACTCCAAGCATC
GAGATGTCAATGAGAGGAGTGAGAATCAGCAAAATGGGTGTGGATGAGTACTCCAGCACCGAGAGGGTCTGGTGAGCAT
TGACAGATTTCTGAGAATCCGGGACCAGCGAGGAAATGTGCTCCTGTCTCCCGAGGAGGTGAGTGAACACAGGGAACAG
AGAACTGACAATTACTTACTCATCTCAATGATGTGGGAGATTAATGGTCTGAATCAGTGTGGTCAATACCTATCAG
TGGATCATCAGAACTGGGAACTGTGAAAATTCAGTGGTCCAGAACCTACAATGCTGTACAATAAAATGGAATTTGA
ACATTTTCAGTCTCTGGTGCCTAAGGCCATTAGAGGCCAGTACAGTGGGTTTGTGAGAACTCTGTTCCAGCAGATGAGGG
ATGTGCTGGGGACATTTGATACCGCACAGATTATTAAGTGTGCTGCTCCGAGCGGCTCCACCAAAGCAGAGTAGAATG
CAGTTCTCCTCATTTACTGTGAATGTGAGGGGATCAGGAATGAGAATCCTGGTGAGGGGCAATTCTCCTGTGTTCACTA
TAACAAGGCCACCAAGAGACTCACAGTGTCTGGAAAGGATGCTGGCACTCTGACTGAAGACCCAGATGAAGGCACAGCTG
GAGTGGAGTCCGCTGTGCTGAGGGGATTCTCATTCTGGGCAAGAAGACAAGAGATATGGGCCAGCACTGAGCATCAAT
GAACTGAGCAACCTGGCCAAAGGAGAGAAGGCTAATGTGCTAATTGGGCAAGGAGACGTGGTGTGTAATGAAACGGAA
ACGGGACTCTAGCATACTTACTGACAGCCAGACAGCGACCAAAAGAATTCGGATGGCCATCAATTAGTGTGCAATAGTTT
AAAAACGACCTTGTCTTACT (SEQ ID NO:16)

FIG. 10G

PR8-UW PB1:

AGCGAAAGCAGGCAAACCATTTGAATGGATGTCAATCCGACCTTACTTTTCTTAAAAGTGCCAGCACAAAATGCTATAAG
CACAACTTTCCCTTATACTGGAGACCTCCTTACAGCCATGGGACAGGAACAGGATACACCATGGGATACTGTCAACAGGA
CACATCAGTACTCAGAAAAGGGAAGATGGACAACAAACACCGAAACTGGAGCACCGCAACTCAACCCGATTGATGGGCCA
CTGCCAGAAGACAATGAACCAAGTGGTTATGCCCAAACAGATTGTGTATTGGAGGCGATGGCTTTCCTTGAGGAATCCCA
TCCTGGTATTTTTGAAAACCTCGTGATTGAAACGATGGAGGTTGTTAGCAACACGAGTAGACAAGCTGACACAAGGCC
GACAGACCTATGACTGGACTCTAAATAGAAACCAACCTGCTGCAACAGCATTGGCCAAACACAATAGAAGTGTTCAGATCA
AATGGCCTCACGGCCAATGAGTCTGGAAGGCTCATAGACTTCCCTAAGGATGTAATGGAGTCAATGAACAAAGAAGAAAT
GGGGATCACAACTATTTTCAGAGAAAGAGACGGGTGAGAGACAATATGACTAAGAAAATGATAACACAGAGAACAATGG
GTAAAAAGAAGCAGAGATTGAACAAAAGGAGTTATCTAATTAGAGCATTGACCCTGAACACAATGACCAAAGATGCTGAG
AGAGGGAAGCTAAACGGAGAGCAATTGCAACCCAGGGATGCAAATAAGGGGGTTTGATACTTTGTTGAGACACTGGC
AAGGAGTATATGTGAGAACTTGAAACATCAGGGTTGCCAGTTGGAGGCAATGAGAAGAAAGCAAAGTTGGCAAATGTTG
TAAGGAAGATGATGACCAATTCTCAGGACACCGAACTTTCTTCCACCATCACTGGAGATAACACCAAATGGAACGAAAAAT
CAGAATCCTCGGATGTTTTTGGCCATGATCACATATGACCAGAAATCAGCCCGAATGGTTCAGAAATGTTCTAAGTAT
TGCTCCAATAATGTTCTCAACAAAATGGCGAGACTGGGAAAAGGGTATATGTTTGAGAGCAAGAGTATGAAACTTAGAA
CTCAAATACCTGCAGAAATGTAGCAAGCATCGATTTGAAATATTTCAATGATTCAACAAGAAAGAAGATTGAAAAAATC
CGACCGCTCTTAATAGAGGGGACTGCATCATTGAGCCCTGGAATGATGATGGGCATGTTCAATATGTTAAGCACTGTATT
AGGCGTCTCCATCCTGAATCTTGGACAAAAGAGATACACCAAGACTACTTACTGGTGGGATGGTCTTCAATCCTCTGACG
ATTTTGCTCTGATTGTGAATGCACCAATCATGAAGGGATTCAAGCCGGAGTCGACAGGTTTTATCGAACCTGTAAGCTA
CTTGAATCAATATGAGCAAGAAAAAGTCTTACATAAACAGAACAGGTACATTTGAATTCACAAGTTTTTCTATCGTTA
TGGGTTTGTTGCCAATTTCAAGCATGGAGCTTCCAGTTTTGGGGTGTCTGGGATCAACGAGTCAGCGGACATGAGTATTG
GAGTTACTGTCATCAAAAACAATATGATAAACAATGATCTTGGTCCAGCAACAGCTCAAATGGCCCTTCAGTTGTTATC
AAAGATTACAGGTACACGTACCGATGCCATATAGGTGACACACAAAATACAAACCCGAAGATCATTTGAAATAAGAAACT
GTGGGAGCAAACCGTTCCAAAGCTGGACTGCTGGTCTCCGACGGAGGCCAAATTTATACAACATTAGAAATCTCCACA
TTCCTGAAGTCTGCCTAAAAATGGGAATTGATGGATGAGGATTACCAGGGGCGTTTATGCAACCCACTGAACCCATTGTCT
AGCCATAAAGAAATTGAATCAATGAACAATGCAGTGATGATGCCAGCACATGGTCCAGCCAAAACATGGAGTATGATGC
TGTTGCAACAACACACTCCTGGATCCCCAAAAGAAATCGATCCATCTTGAATACAAGTCAAAGAGGAGTACTTGAGGATG
AACAAATGTACCAAAGGTGCTGCAATTTATTTGAAAAATCTTCCCCAGCAGTTCATACAGAAGACCAGTCGGGATATCC
AGTATGGTGGAGGCTATGGTTCCAGAGCCCGAATTGATGCACGGATTGATTTGCAATCTGGAAGGATAAAGAAAGAAGA
GTTCACTGAGATCATGAAGATCTGTTCCACCATGAAGAGCTCAGACGGCAAAAATAGTGAATTTAGCTTGCTCTCATG
AAAAATGCCTTGTTTCTACT (SEQ ID NO:2)

FIG. 10H

Canine codon optimized PR8 PB1:

AGCGAAAGCAGGCAAACCATTTGAATGGATGTCAATCCGACCTTACTTTTCTAAAAGTGCCAGCACAAATGCTATAAG
CACAACTTTCCCTTATACTGGAGACCCTCCTTACAGCCATGGGACAGGAACAGGATACACCATGGATACTGTCAACAGGA
CACATCAGTACTCAGAAAAGGGAAGATGGACAACAAACACCGAAACTGGAGCACCGCAACTCAACCCGATTGATGGGCCA
CTGCCAGAAGACAATGAACCAAGTGGTTATGCCCAAACAGATTGTGTATTGGAGGCGATGGCTTTCCTTGAGGAATCCCA
TCCTGGTATTTTTGAAAACCTGTGTATTGAAACGATGGAGGTTGTTTCAGCAAACACGAGTGGACAAGCTGACACAGGGCC
GACAGACCTATGACTGGACTCTGAATAGAAACCAGCCTGCTGCAACAGCACTGGCCAACACAATCGAAGTGTTCAGATCA
AATGGCCTCACCGCCAATGAGTCTGGAAGGCTCATCGACTTCCTGAAGGATGTGATGGAGTCAATGAACAAAGAAGAAAT
GGGGATCACAACCTATTTTCAGAGAAAGAGACGGGTGAGAGACAATATGACTAAGAAAATGATTACACAGAGAACAAATGG
GTAAAAAGAAGCAGAGACTGAACAAAAGGAGTTATCTGATTAGAGCACTGACCCTGAACACAATGACCAAAGATGCTGAG
AGAGGGAAGCTGAAACGGAGAGCAATTGCAACCCCAAGGATGCGAGATTAGGGGGTTTGTACTTTGTGGAGACACTGGC
AAGGAGTATTTGTGAGAACTGGAACAGTCAGGGCTGCCAGTGGGAGGCAATGAGAAGAAAGCAAAGCTGGCAAATGTGG
TGAGGAAGATGATGACCAATTCTCAGGACACCGAACTGTCTTTCACCATCACTGGAGATAACACCAAATGGAACGAAAT
CAGAATCCTCGGATGTTTCTGGCCATGATCACATATATGACCAGAAATCAGCCGAATGGTTCAGAAATGTGCTGAGTAT
TGCTCCAATTATGTTCTCAACAAAATGGCCAGACTGGGAAAAGGGTATATGTTTGAGAGCAAGAGTATGAAACTGAGAA
CTCAGATTCTGCAGAAATGCTGGCAAGCATCGATCTGAAATATTTCAATGATTCAACAAGAAAGAAGATTGAAAAATC
CGACCCCTCCTGATTGAGGGGACTGCATCACTGAGCCCTGGAATGATGATGGGCATGTTCAATATGCTGAGCACTGTGCT
GGGCGTCTCCATCTGAATCTGGGACAGAAGAGATACACCAAGACTACTTACTGGTGGGATGGTCTGCAGTCTCTGACG
ATTTTGCTCTGATTGTGAATGCACCAATCATGAAGGGATTGAGGCCGAGTCGACAGGTTTTATCGAACCTGTAAGCTG
CTGGGAATCAATATGAGCAAGAAAAAGTCTTACATCAACAGAACAGGTACATTTGAATTCACAAGTTTTTTCTATCGCTA
TGGGTTTGTGGCCAATTCAGCATGGAGCTGCCAGTTTTGGGGTGTCTGGGATCAACGAGTCAGCCGACATGAGTATTG
GAGTGACTGTATCAAAAACAATATGATCAACAATGATCTGGGTCCAGCAACAGCTCAGATGGCCCTGCAGCTGTTTCATC
AAAGATTACAGGTACACCTACCGATGCCATATCGGTGACACACAGATTGAGACCCGAAGATCATTGAAATCAAGAACT
GTGGGAGCAGACCCGCTCAAAGCTGGACTGCTGGTCTCCGACGGAGGCCCAAATCTGTACAACATTAGAAATCTCCACA
TTCCTGAAGTCTGCCTGAAATGGGAACCTGATGGATGAGGATTACCAGGGGCGCCTGTGCAACCCACTGAACCCATTGTC
AGCCATAAAGAAATTGAATCAATGAACAATGCAGTGATGATGCCAGCACATGGTCCAGCCAAAAACATGGAGTATGATGC
TGTGGCAACAACACACTCCTGGATCCCCAAAAGAAATCGATCCATCCTGAATACAAGTCAGAGAGGAGTGCTGGAGGATG
AACAGATGTACCAGAGGTGCTGCAATCTGTTTGAATAATCTTCCCCAGCAGTTCATACAGAAGACCAGTCGGGATCTCC
AGTATGGTGGAGGCTATGGTGCCAGAGCCGAATTGATGCACGGATTGATTCGAATCTGGAAGGATCAAGAAAGAAGA
GTTCACTGAGATCATGAAGATCTGTTCCACCATGAAGAGCTCAGACGGCAAAAATAGTGAATTTAGCTTGTCTTCTATG
AAAAATGCCTTGTCTTCTACT (SEQ ID NO:17)

FIG. 10I

PR8-UW PA:

AGCGAAAGCAGGTA CTGATCCAAAATGGAAGATTTTGTGCGACAATGCTTCAATCCGATGATTGTCGAGCTTGCGGAAAA
AACAAATGAAAGAGTATGGGGAGGACCTGAAAATCGAAACAAACAAATTTGCAGCAATATGCACTCACTTGGAAGTATGCT
TCATGTATTGAGATTTTCACTTCATCAATGAGCAAGGCGAGTCAATAATCGTAGAACTTGGTGATCCAAATGCACCTTTG
AAGCACAGATTTGAAATAATCGAGGGAAGAGATCGCACAAATGGCCTGGACAGTAGTAAACAGTATTTGCAACACTACAGG
GGCTGAGAAACCAAAGTTTCTACCAGATTTGTATGATTACAAGGAGAATAGATTCATCGAAATTGGAGTAACAAGGAGAG
AAGTTCACATATACTATCTGGAAAAGGCCAATAAAATTAATCTGAGAAAACACATCCACATTTTCTCGTTCACTGGG
GAAGAAATGGCCACAAAGGCAGACTACACTCTCGATGAAGAAAGCAGGGCTAGGATCAAAACCAGACTATTCACCATAAG
ACAAGAAATGGCCAGCAGAGGCCCTCTGGGATTCTTTCTGTCAGTCCGAGAGAGGAGAAGAGACAATTGAAGAAAGGTTTG
AAATCACAGGAACAATGCGCAAGCTTGCCGACCAAGTCTCCCGCGAACTTCTCCAGCCTTGAAAAATTTAGAGCCTAT
GTGGATGGATTGGAACCGAACGGCTACATTGAGGGCAAGCTGTCTCAAATGTCAAAGAAGTAAATGCTAGAATTGAACC
TTTTTTGAAAACAACACCAGCAGCACTTAGACTTCCGAATGGGCCCTCCCTGTTCTCAGCGGTCCAAATTCCTGCTGATGG
ATGCCTTAAATTAAGCATTGAGGACCCAAGTCATGAAGGAGAGGGAATACCGCTATATGATGCAATCAAATGCATGAGA
ACATTTCTTTGGATGGAAGGAACCCAATGTTGTTAAACCACAGAAAAGGGAATAAATCCAAATTATCTTCTGTCATGGAA
GCAAGTACTGGCAGAACTGCAGGACATTGAGAATGAGGAGAAAATTCAAAGACTAAAAATATGAAGAAAACAAGTCAGC
TAAAGTGGGCACTTGGTGAGAACATGGCACCAGAAAAGGTAGACTTTGACGACTGTAAAGATGTAGGTGATTGAAGCAA
TATGATAGTGATGAACCAGAATTGAGGTGCTTGAAGTTGGATTGAGAATGAGTTTACAAGGCATGCGAACTGACAGA
TTCAAGCTGGATAGAGCTCGATGAGATTGGAGAAGATGTGGCTCCAATTGAACACATTGCAAGCATGAGAAGGAATTATT
TCACATCAGAGGTGTCTCACTGCAGAGCCACAGAATACATAATGAAGGGAGTGATACATCAATACTGCCTTGCTTAATGCA
TCTTGTGCAGCAATGGATGATTTCGAATTAATCCAATGATAAGCAAGTGTAAGTAAGGAGGGAAGGCGAAAGACCAA
CTTGATGTTTCATCATAAAAGGAAGATCCCACTTAAGGAATGACACCGACGTGGTAACTTTGTGAGCATGGAGTTTT
CTCTCACTGACCCAAGACTTGAACCACATAAATGGGAGAAGTACTGTGTTCTTGAGATAGGAGATATGCTTATAAGAAGT
GCCATAGGCCAGGTTTCAAGGCCCATGTTCTGTATGTGAGAACAAATGGAACCTCAAAAATTAATGAATGGGGAAT
GGAGATGAGGCGTTGCCTCTCCAGTCACTTCAACAAATGAGAGTATGATTGAAGCTGAGTCTCTGTCAAAGAGAAAAG
ACATGACCAAAGAGTTCTTTGAGAACAAATCAGAAACATGGCCATTGGAGAGTCCCCAAAGGAGTGGAGGAAAGTTCC
ATTGGAAGGTCTGCAGGACTTTATTAGCAAAGTCGGTATTCAACAGCTTGATGCATCTCCCAACTAGAAGGATTTTC
AGCTGAATCAAGAAAAGTCTTCTATCGTTAGGCTCTTAGGGACAACCTGGAACCTGGGACCTTTGATCTTGGGGGGC
TATATGAAGCAATTGAGGAGTGCCTGATTAATGATCCCTGGGTTTTGCTTAATGCTTCTTGGTTCAACTCCTTCTTACA
CATGCATTGAGTTAGTTGTGGCAGTGCTACTATTTGCTATCCATACTGTCCAAAAAGTACCTTGTCTTCTACT (SEQ ID NO:1)

FIG. 10J

Canine codon optimized PR8 PA:

AGCGAAAGCAGGTACTGATCCAAAATGGAAGATTTTGTGCGACAATGCTTCAATCCGATGATTGTGCGAGCTTGCAGAAAA
AACAAATGAAAGAGTATGGGGAGGACCTGAAAATCGAAACAAACAAATTTGCAGCAATATGCACTCACTTGGAAAGTATGCT
TCATGTATTCAGATTTTCACTTCATCAATGAGCAAGGCGAGTCAATAATCGTAGAACTTGGTGATCCAAATGCACCTTTG
AAGCACAGATTTGAAATAATCGAGGGAAGAGATCGCACAAATGGCCTGGACAGTAGTAAACAGTATTTGCAACACTACAGG
GGCTGAGAAACCAAAGTTTCTACCAGATTTGTATGATTACAAGGAGAATAGATTTCATCGAAATTGGAGTAACAAGGAGAG
AAGTTCACATATACTATCTGGAAAAGGCCAATAAAATTAATCTGAGAAAACACACATCCACATTTTCTCGTTCACTGGG
GAAGAAATGGCCACAAAGGCAGACTACACTCTCGATGAAGAAAGCAGGGCTAGGATCAAAACCAGACTATTCACCATAAG
ACAAGAAATGGCCAGCAGAGGCCTCTGGGATTCCTTTCTGTCAGTCCGAGAGAGGAGAAGAGACAATTGAAGAAAAGGTTTG
AAATCACAGGAACAATGCGCAAGCTTGCCGACCAAAGTCTCCCGCCGAACCTCTCCAGCCTGAAAAATTTAGAGCCTAT
GTGGATGGATTGCAACCGAACGGCTACATTGAGGGCAAGCTGTCTCAAATGTCCAAAGAAGTAAATGCTAGAATTGAACC
TTTTCTGAAAACAACACCAGACCACTGAGACTGCCAATGGGCCTCCCTGTTCTCAGCGGTCCAAATTCCTGCTGATGG
ATGCCCTGAAACTGAGCATTGAGGACCCAAGTCATGAAGGAGAGGGAATCCCCTGTATGATGCAATCAAATGCATGAGA
ACATTTCTTTGGATGGAAGGAACCCAATGTGGTGAAACCACACGAAAAGGGAATCAATCCAAATTTATCTGCTGTCATGGAA
GCAGGTGCTGGCAGAACTGCAGGACATTGAGAATGAGGAGAAAATCCAAAGACTAAAAATATGAAGAAAACAAGTCAGC
TGAAGTGGGCACTGGGTGAGAACATGGCACCAGAAAAGGTGGACTTTGACGACTGTAAAGATGTGGGTGATCTGAAGCAG
TATGATAGTGATGAACCAGAACTGAGGTCCCTGGCAAGTTGGATTGAGAATGAGTTTAACAAGGCATGCGAACTGACAGA
TTCAAGCTGGATTGAGCTCGATGAGATTGGAGAAGATGTGGCTCCAATTGAACACATTGCAAGCATGAGAAGGAATTATT
TCACATCAGAGGTGTCTCACTGCAGAGCCACAGAATACATCATGAAGGGAGTGTACATCAATACTGCCCTGCTGAATGCA
TCTGTGTCAGCAATGGATGATTTCCAGCTGATTCCAATGATCAGCAAGTGTAAGAACTAAGGAGGGAAGGCGAAAGACCAA
CCTGTATGGTTTCATCATCAAAGGAAGATCCACCTGAGGAATGACACCGACGTGGTGAACTTTGTGAGCATGGAGTTTT
CTCTCACTGACCCAAGACTGGAACCACATAAATGGGAGAAGTACTGTGTGCTGGAGATTGGAGATATGCTGATCAGAAGT
GCCATTGGCCAGGTGTCAAGGCCCATGTTCTGTATGTGAGAACAAATGGAACCTCAAAAATTAATGAAATGGGGAAT
GGAGATGAGGCGCTGCCTCCTCCAGTCACTGCAGCAGATTGAGAGTATGATTGAAGCTGAGTCCTCTGTCAAAGAGAAAG
ACATGACCAAAGAGTTCTTTGAGAACAAATCAGAAACATGGCCCATTTGGAGAGTCCCCAAAGGAGTGGAGGAAAGTTCC
ATTGGGAAGGTCTGCAGGACTCTGCTGGCAAAGTCCGTGTTCAACAGCCTGTATGCATCTCCACAGCTGGAAGGATTTTC
AGCTGAATCAAGAAAAGTCTGCTGATCGTGCAGGCTCTGAGGGACAACCTGGAACCTGGGACCTTTGATCTGGGGGGG
TGTATGAAGCAATTGAGGAGTGCCTGATTAATGATCCCTGGGTGCTGCTGAATGCTTCTTGGTTCAACTCCTTCCTTACA
CATGCATTGAGTTAGTTGTGGCAGTGCTACTATTTGCTATCCATACTGTCCAAAAAGTACCTTGTTTCTACT (SEQ ID NO:18)

FIG. 10K

PR8-UW NP:

AGCAAAAGCAGGGTAGATAATCACTCACTGAGTGACATCAAATCATGGCGTCTCAAGGCACCAAACGATCTTACGAACA
GATGGGAGACTGATGGAGAACGCCAGAATGCCACTGAAATCAGAGCATCCGTCGGAAAAATGATTGGTGAATTGGACGAT
TCTACATCCAAATGTGCACCGAACTCAAACCTCAGTGATTATGAGGGACGGTTGATCCAAACAGCTTAACAATAGAGAGA
ATGGTGCTCTGCTTTTGACGAAAGGAGAAATAAATACCTTGAAGAACATCCAGTGCGGGGAAAGATCCTAAGAAAAC
TGGAGGACCTATATACAGGAGAGTAAACGGAAAGTGGATGAGAGAACTCATCCTTTATGACAAAGAAGAAATAAGGCGAA
TCTGGCGCCAAGCTAATAATGGTGACGATGCAACGGCTGGTCTGACTCACATGATGATCTGGCATTCCAATTTGAATGAT
GCAACTTATCAGAGGACAAGAGCTCTTGTTGACCCGGAATGGATCCCAGGATGTGCTCTCTGATGCAAGGTTCAACTCT
CCCTAGGAGGTCTGGAGCCGAGGTGCTGCAGTCAAAGGAGTTGGAACAATGGTGTGGAATTGGTCAGAATGATCAAAC
GTGGGATCAATGATCGGAACTTCTGGAGGGGTGAGAATGGACGAAAAACAAGAATTGCTTATGAAAGAATGTGCAACATT
CTCAAAGGGAATTTCAAACCTGCTGCACAAAAAGCAATGATGGATCAAGTGAGAGAGAGCCGGAACCCAGGGAATGCTGA
GTTTCAAGATCTCACTTTTCTAGCACGGTCTGCACTCATATTGAGAGGGTCGGTTGCTCACAAGTCCTGCCTGCCTGCCT
GTGTGTATGGACCTGCCGTAGCCAGTGGGTACGACTTTGAAAGGGAGGGATACTCTCTAGTCGGAATAGACCTTTTCTAGA
CTGCTTCAAACAGCCAAGTGTACAGCCTAATCAGACCAAATGAGAATCCAGCACACAAGAGTCAACTGGTGTGGATGGC
ATGCCATTCTGCCGATTTGAAGATCTAAGAGTATTAAGCTTCATAAAGGGACGAAGGTGCTCCCAAGAGGGAAGCTTT
CCACTAGAGGAGTTCAAATTGCTTCCAATGAAAATATGGAGACTATGGAATCAAGTACACTTGAAGTGAAGCAGGTAC
TGGGCCATAAGGACCAGAAGTGGAGGAAACCAATCAACAGAGGGCATCTGCGGGCCAAATCAGCATACAACCTACGTT
CTCAGTACAGAGAAATCTCCCTTTTGACAGAACCACTTATGGCAGCATTCAATGGGAATACAGAGGGGAGAACATCTG
ACATGAGGACCGAAATCATAAGGATGATGGAAAGTGCAAGACCAGAAGATGTGTCTTTCCAGGGGCGGGGAGTCTTCGAG
CTCTCGGACGAAAAGGCAGCGAGCCCGATCGTGCCTTCTTTGACATGAGTAATGAAGGATCTTATTTCTTCGAGACAA
TGCAGAGGAGTACGACAATTAAGAAAAATACCCTTGTCTACT (SEQ ID NO:4)

FIG. 10L

Canine codon optimized NP:

AGCAAAAGCAGGGTAGATAATCACTCACTGAGTGACATCAAAATCATGGCGTCTCAAGGCACCAAACGATCTTACGAACA
GATGGAGACTGATGGAGAACGCCAGAATGCCACTGAAATCAGAGCATCCGTCGGAAAAATGATTGGTGGAATTGGACGAT
TCTACATCCAGATGTGCACCGAACTCAAACCTCAGTGATTATGAGGGACGGCTGATCCAGAACAGCCTGACAATCGAGAGA
ATGGTGCTCTCTGCTTTTGACGAAAGGAGAAATAAATACCTGGAAGAACATCCCAGTGCCGGGAAAGATCCTAAGAAAAC
TGGAGGACCTATCTACAGGAGAGTGAAACGGAAAGTGGATGAGAGAACTCATCCTGTATGACAAAGAAGAAATCAGGCGAA
TCTGGCGCCAGGCTAATAATGGTGACGATGCAACCGCTGGTCTGACTCACATGATGATCTGGCATTCCAATCTGAATGAT
GCAACTTATCAGAGGACAAGAGCTCTGGTGCACCCGGAATGGATCCCAGGATGTGCTCTCTGATGCAGGGTTCAACTCT
CCCTAGGAGGTCTGGAGCCGAGGTGCTGCAGTCAAAGGAGTGGGAACAATGGTGATGGAACTGGTCAGAATGATCAAAA
GAGGGATCAATGATCGGAACCTCTGGAGGGGTGAGAATGGACGAAAAACAAGAATTGCTTATGAAAGAATGTGCAACATT
CTCAAAGGGAAATTCAGACTGCTGCACAGAAAGCAATGATGGATCAGGTGAGAGAGAGCCGGAACCCAGGGAATGCTGA
GTTGGAAGATCTCACTTTTCTGGCACGGTCTGCACTCATCCTGAGAGGGTCCGTGGCTCACAAGTCCTGCCTGCCTGCCT
GTGTGTATGGACCTGCCGTGGCCAGTGGGTACGACTTTGAAAGGGAGGGGATACTCTCTGGTCGGAATTGACCCTTTCAGA
CTGCTGCAGAACAGCCAGGTGTACAGCCTGATCAGACCAATGAGAATCCAGCACACAAGAGTCAGCTGGTGTGGATGGC
ATGCCATTCTGCCGCAATTTGAAGATCTGAGAGTGCTGAGCTTCATCAAAGGGACCAAGGTGCTCCCAAGAGGGAAGCTGT
CCACTAGAGGAGTGCAGATTGCTTCCAATGAAAATATGGAGACTATGGAATCAAGTACACTGGAACTGAGAAGCAGGTAC
TGGGCCATCAGGACCAGAAGTGGAGGAAACCAATCAGCAGAGGGCATCTGCCGGCCAGATCAGCATTACAGCTACCTT
CTCAGTGACAGAGAAATCTCCCTTTTGACAGAAACACCATATGGCAGCATTCAATGGGAATACAGAGGGGAGAACATCTG
ACATGAGGACCGAAATCATCAGGATGATGGAAGTGCAAGACCAGAAGATGTGTCTTCCAGGGGCGGGGAGTCTTCGAG
CTCTCGGACGAAAAGGCAGCGAGCCCGATCGTGCTTCCTTTGACATGAGTAATGAAGGATCTTATTTCTCGGAGACAA
TGCAGAGGAGTACGACAATTAAAGAAAAATACCCTTGTTTCTACT (SEQ ID NO:19)

FIG. 10M

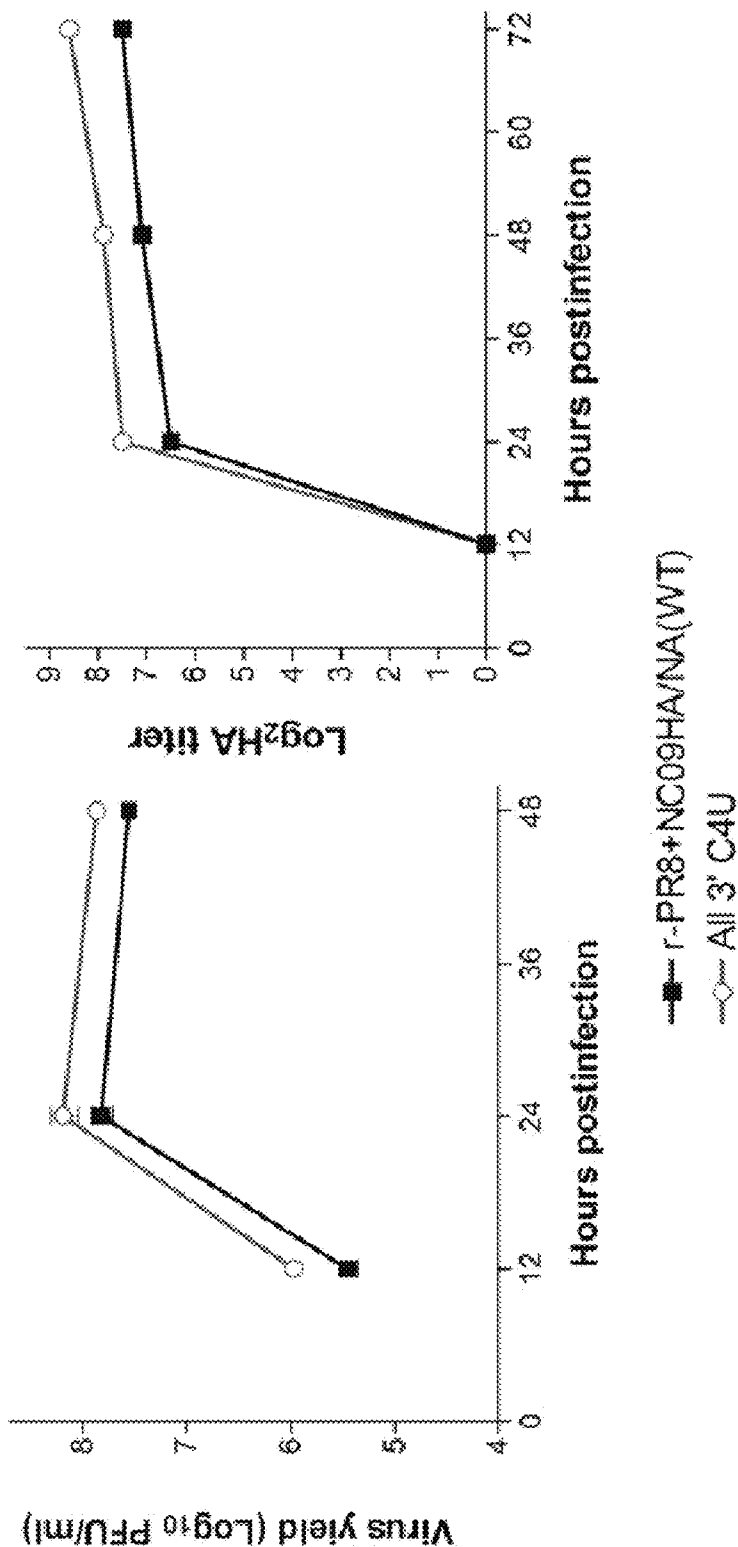
Figure 11 A Nucleotide mutation in position 4 of each gene of PR8 and Inda/NC/09.

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
Inda/NC/09 HA		U
Inda/NC/09 NA		U

Figure 11C Growth kinetics of 3' C4U mutant



HA

atgaacactcaaatcctgggtattcgtctgattgcatcattccaacaaatgcagacaaaatctgctcggacatcatgccgtgtcaaacggaaccaaagtaaa
cacattaactgaaagaggagtggaagtcgtcaatgcaactgaaacagtggaacgaacaaacatccccaggtatctgtcaaaaagggaagacagtgacc
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catcggtgcactattttatataa (SEQ ID NO:20)

MNTQILVFAL	IAIIPTNADK	ICLGHHAVSN	GTKVNTLIER	GVEVFNATET	VERTNIPRIC
SKGKKTVDLG	QCGLLGTITG	PPQCDQFLEF	SADLI IERRE	GSDVCYPGKF	VNEEALRQIL
RESGGIDKEA	MGFTYSGIRT	NGATSACRRS	GSSFYAEMKW	LLSNTDNAAF	PQMTKSYKNT
RKSPALIVWG	IHHSVSTAEQ	TKLYGSGNKL	VTVGSSNYQQ	SFVPSPGARP	QVNLGSGRID
FHWLMLNPND	TVIFSFGAF	IAPDRASFRL	GKSMGIQSGV	QVDANCEGDC	YHSGGT IISN
LPFQNIIDSRA	VGKCPRYVKQ	RSLLLATGMK	NVPEIPRGRG	LFGAIAGFIE	NGWEGLIDGW
YGRHQNAQG	EGTAADYKST	QSAIDQITGK	LNRLIEKTNQ	QFELIDNEFN	EVEKQIGNVI
NWTRDSITEV	WSYNAELIVA	MENQHTIDLA	DSEMDKLYER	VKRQLRENAE	EDGTGCFEIF
HKDDDDCMAS	IRNNTYDHSK	YREEAMQNRI	QIDPVKLSSG	YKDVILWFSF	GASCFILLAI

VMGLVFIQVK NGNMRCTICI (SEQ ID NO:21)

NA

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MNPNQKILCTSATAIIIGIAVLIGIANLGLNIGLHLKPGCNCSHSQPETTNTSQTIINYYNETNITNIQMEERTSRNFNNLTKGL
CTINSWHIYKDNVAVRIGESSDLVLTREPYVSCDPDECRFALSQGTIRG KHSNGTIHDSRQYRALISWPLSSPPTVYNSRVECI
GWSSTSCHDGKSRMSICISGPNNNASAVVWYNRRPVAEINTWARNILRTQSECVCHNGVCPVVFDTGSATGPADTRIYYFK

FIG. 12A

EGKILKWESLTGTAKHIEECSCYGERTGITCTCRDNWQGSNRPVIQIDPVAMTHTSQYICSPVLTNDNPRNDPNIGKNDPYPG
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Y VELIRGRPKEDKVVWTSNSIVSMCSSTFLGQWNWPDGAKIEYFL (SEQ ID NO:23)

HA

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c atgctgctgactattgtatataa (SEQ ID NO:24)

MNTQILVFALIAIIPNADKICLGHHAIVSNGTKVNTLTERGVEVNVNATETVERTNIPRICSKGKRTVDLGQCGLLGTITGPPQCDQ
FLEFSADLIHERRGSDVCYPGKFVNEALRQJLRESGGIDKEAMGFTYSG IRTNGATSSCRRSGSSFYAEMKWLLSNTDAAFP
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R EEAMQNRQIDPVKLSSGKYDVLWFSFGASCIFILLAIAMGLVFCVKNNGNMRTCTICI (SEQ ID NO:25)

NA

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MNPNQKILCTSATAIIGAIIVLIGIANLGLNIGLHLKPSNCNSHSQPETTNTSQTIIINYYNETNITNIQMEERTSRNFNNLTGKL
CTINSWH:YKGDNAVRIGESSDVLVTREPYVSCDPDECFYALSQGTITRG KHSNGTIHDSQYRALISWPLSSPPTVYNSRVECI
GWSSTSCHDGKSRMSICISGPNNNASAVVWYNRRPVAEINTWARNILRTQESECCHNGVCPVVFTDGSATGPADTRIYYFK

FIG. 12B

EGKILKWESLTGTAKHIEECSCYGERTGITCTCKDNWQGSNRPVIQIDPVAMTHTSQYICSPVLTDNPRPNDPNIGKCNDPYPG
NNNNGVKGFSYLDGANTWLGRITISTASRSGYEMLKVPNALTD DRSKPIQGQTIVLNADWSGYSGSFMDYWAEGDCYRACF
Y VELIRGRPKEDKVWWTNSIVSMCSSTFLGQWNWPDGAKIEYFL (SEQ. ID NO:27)

FIG. 12C

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

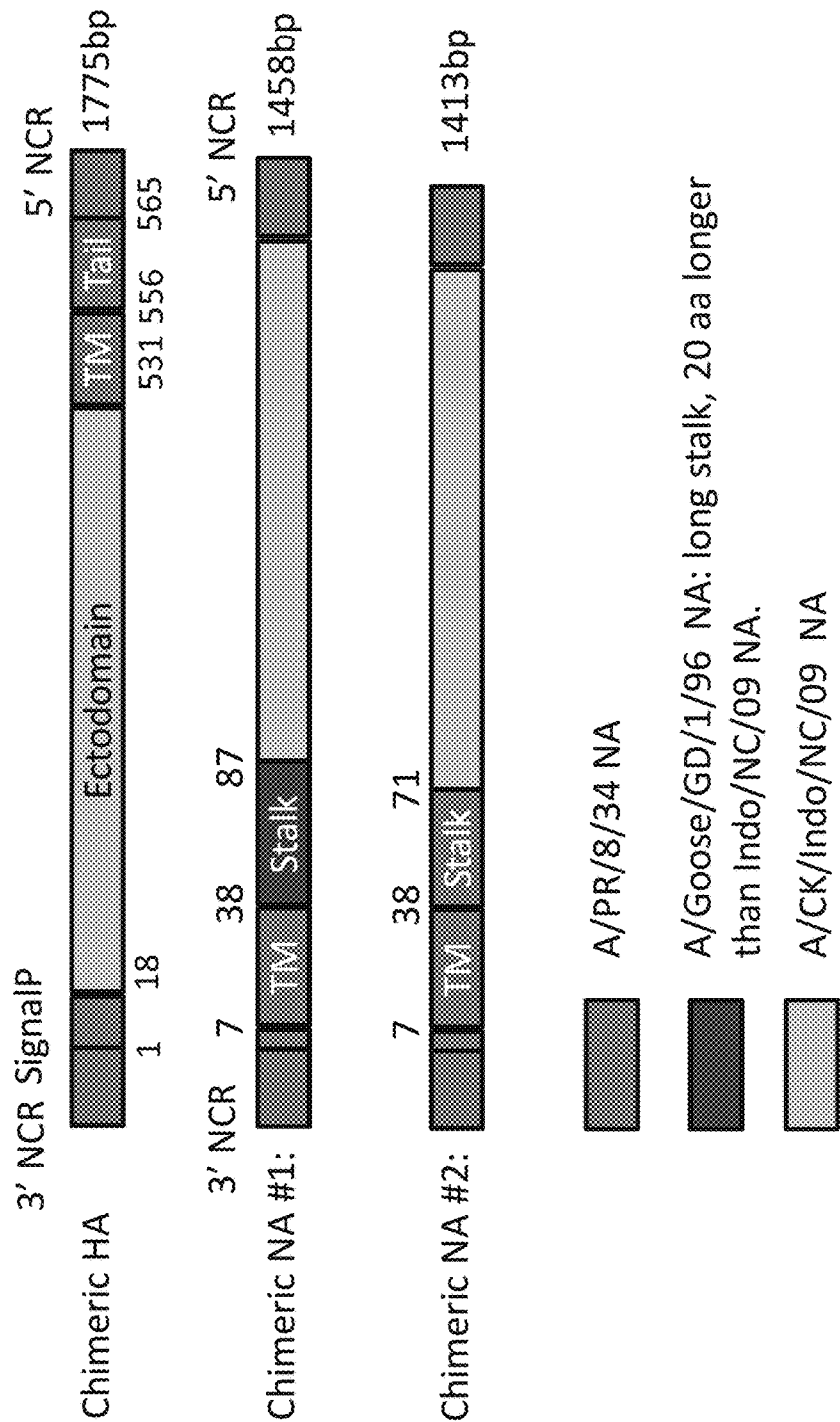


Figure 13B Growth kinetics in MDCK cells

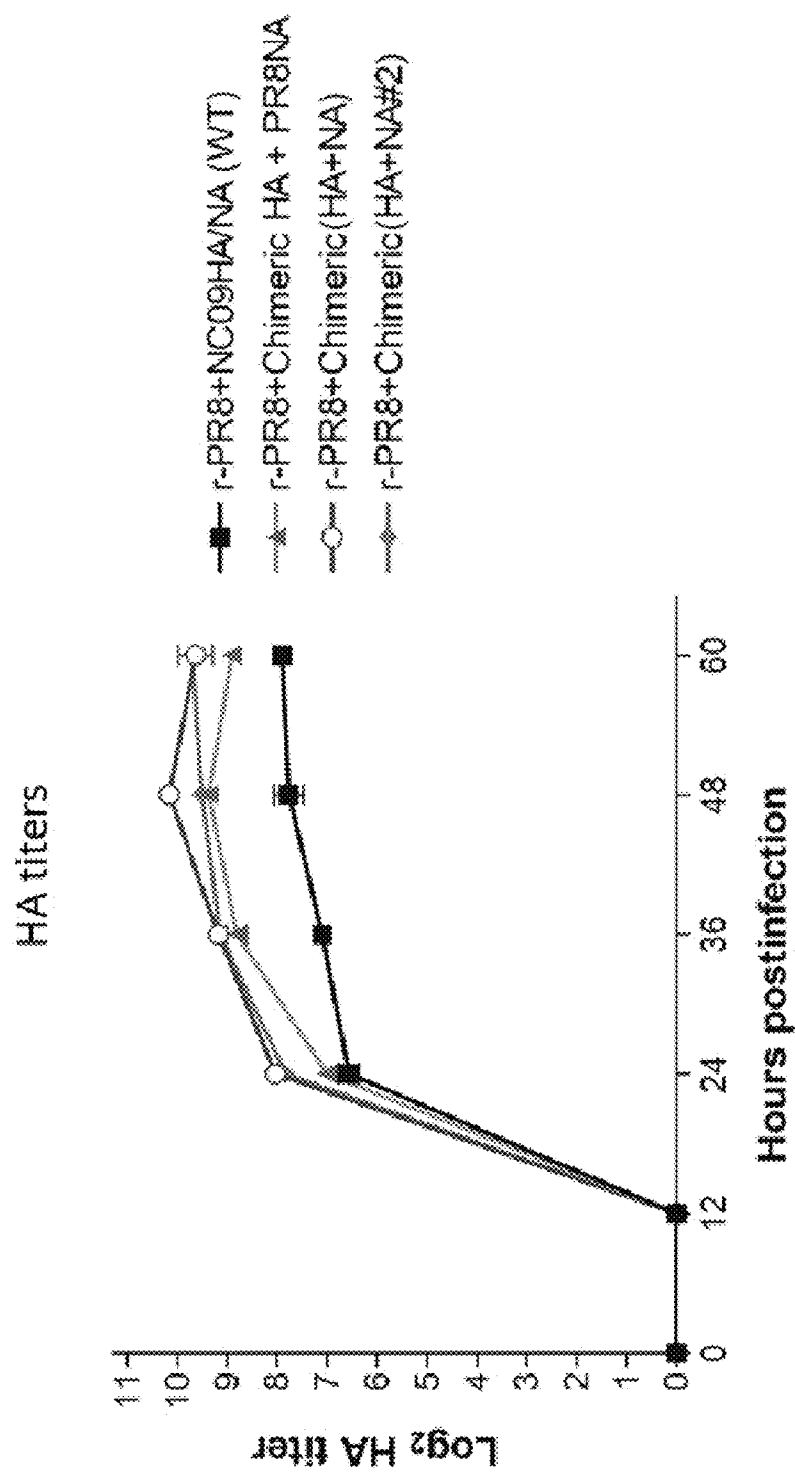
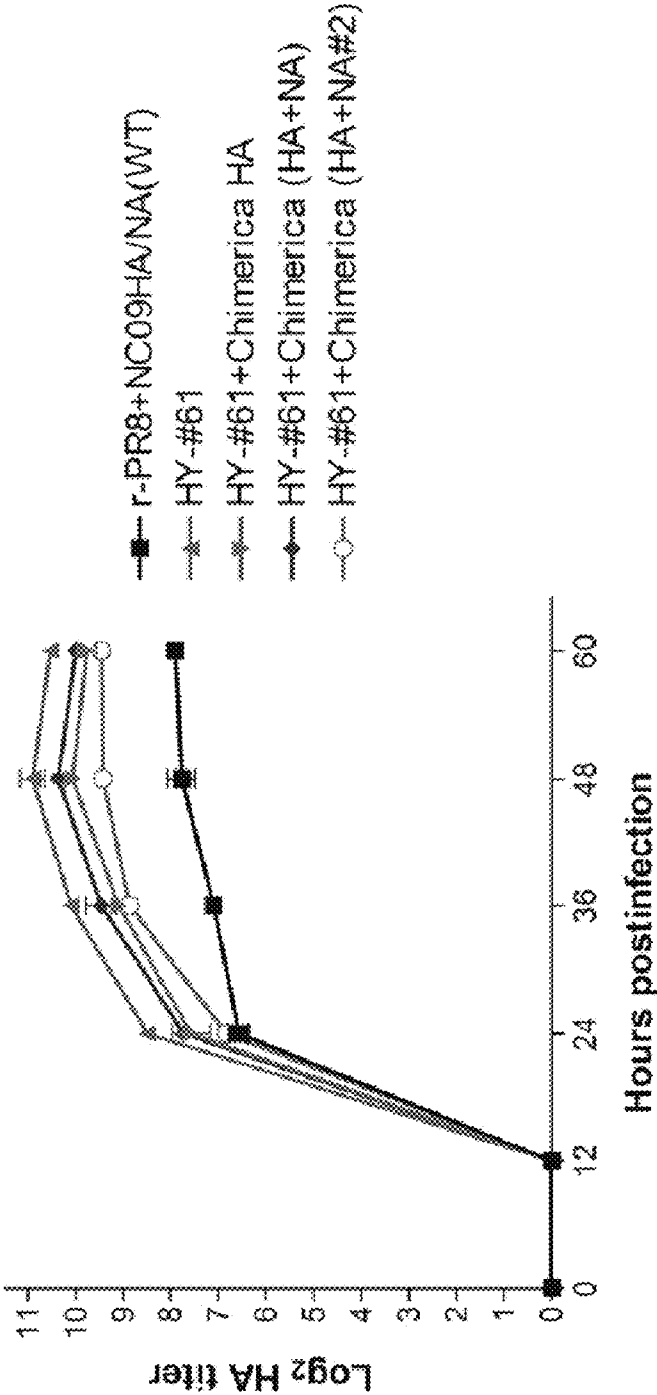
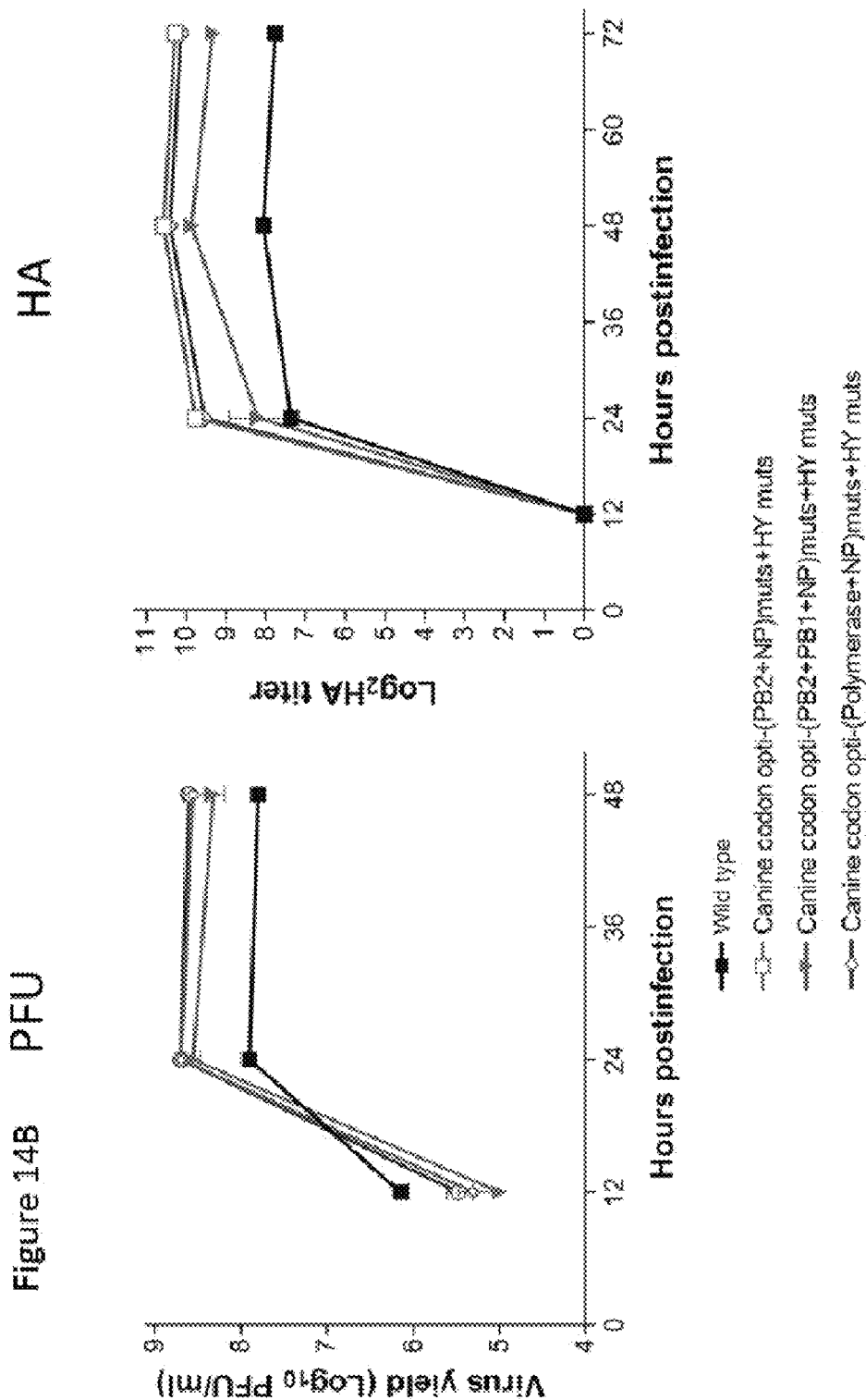


Figure 14A Growth kinetics in MDCK cells



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



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.

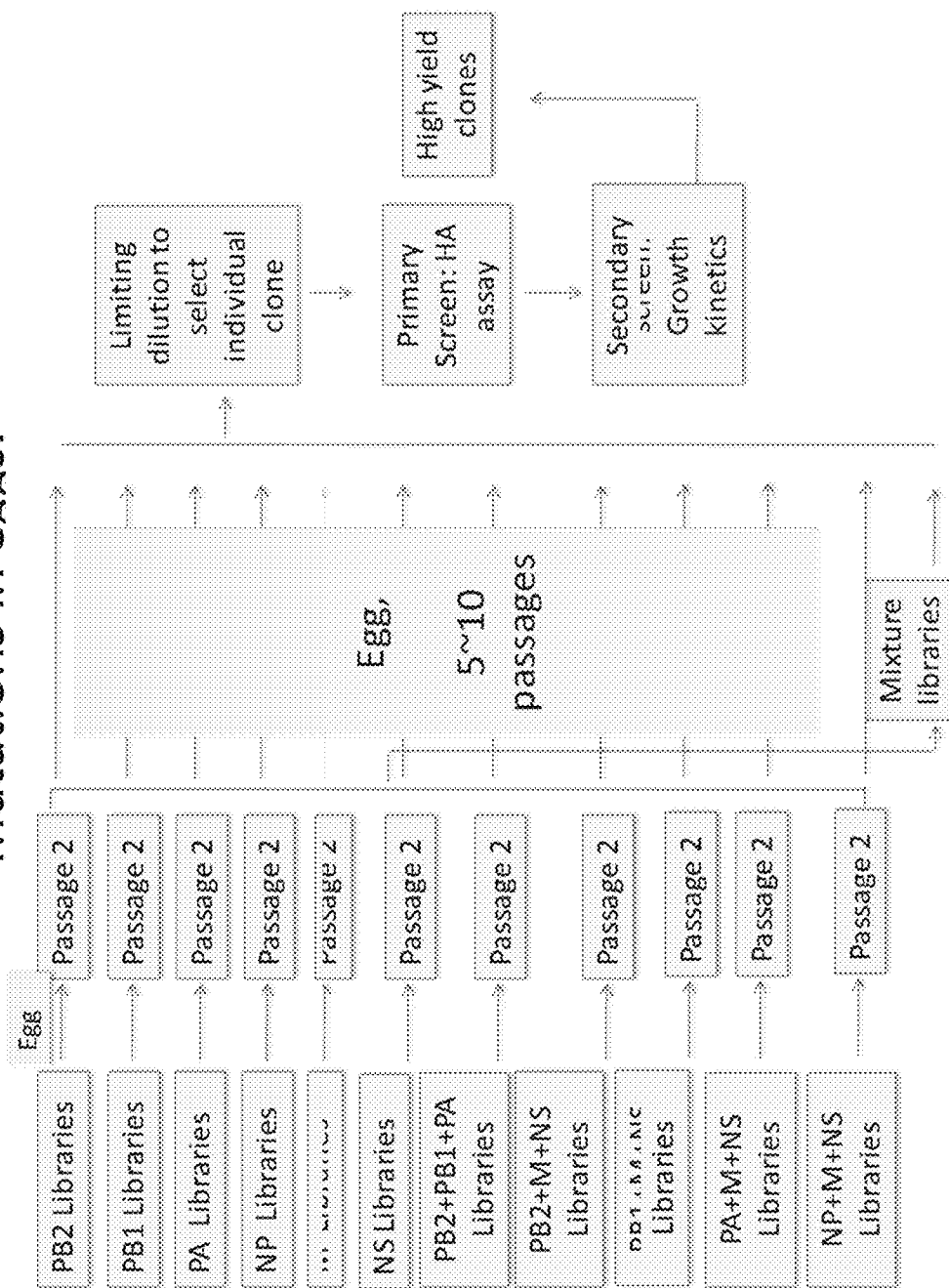


Figure 16 Summary of HA assay of individual clones purified from Vero cells

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 17 Recombinant 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		I30T 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

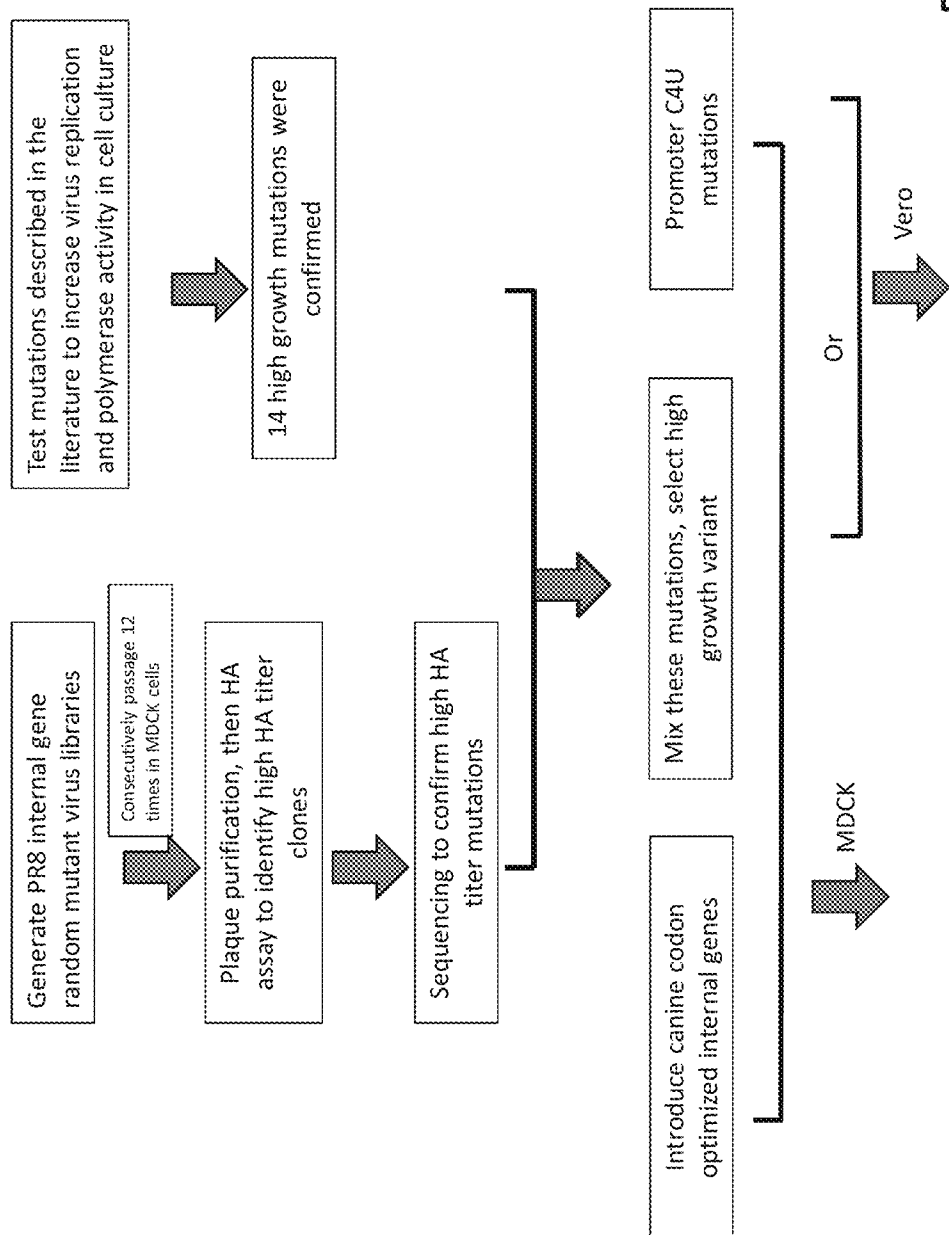


FIG. 18A

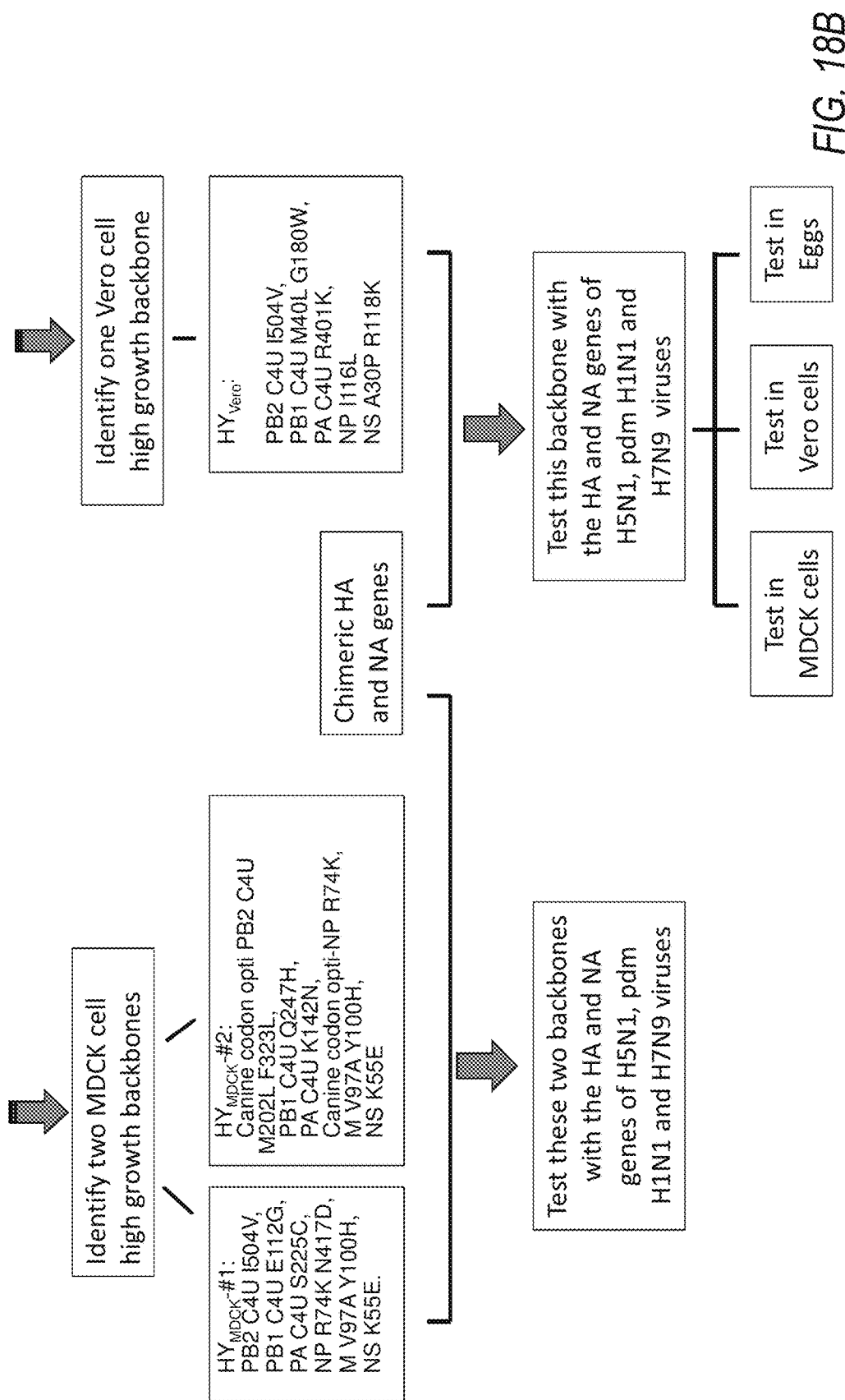


FIG. 18B

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			
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/V644 A					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-PR8 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	R140Q		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/Calfornia/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/NO3072/2010, 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
60	A/Chicken/Indonesia/NC/2009		C4U I504V	C4U M40L G180W	C4U R401K	I116L	G1012C, A1013U, U1014A	A30P R118K	Eggs	Viral titer	4.6~172	
										HA titer	1.3~17.7	
										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

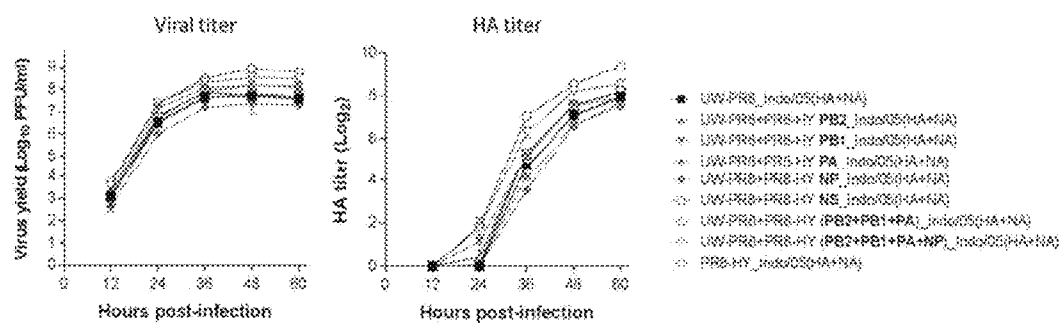


Figure 20

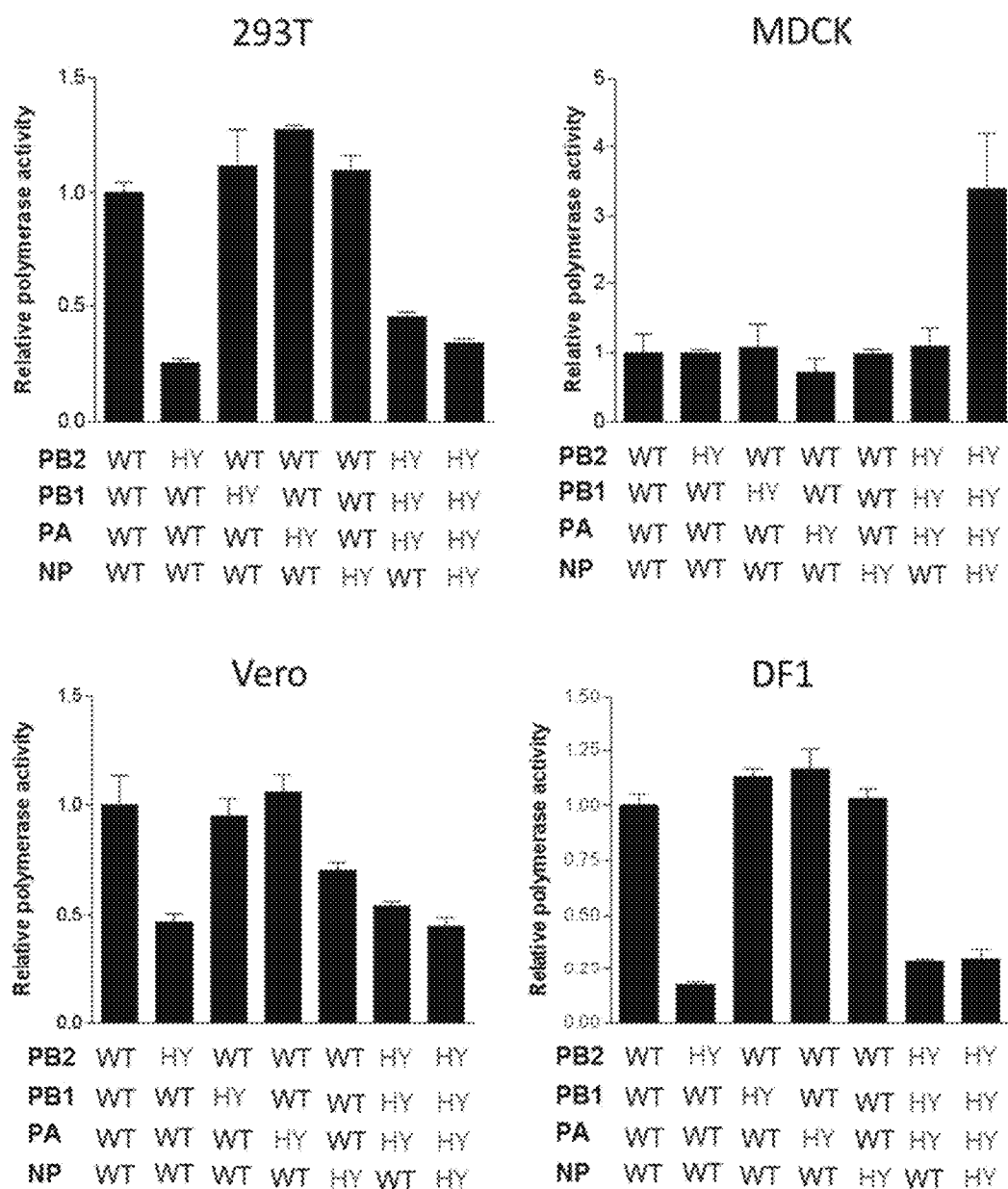


Figure 21

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INFLUENZA VIRUS REPLICATION FOR VACCINE DEVELOPMENT

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of the filing date of U.S. application Ser. No. 62/189,001, filed Jul. 6, 2015, the disclosure of which is incorporated by referenced herein.

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 thought 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

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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

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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

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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.

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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, an 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, phenylalanine, phenylalanine, proline, serine, tyrosine, serine or methionine, at position 40, 54, 59, 62, 63, 66 (F2),

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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, Q179, 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 embodiment, 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 (H1N1), "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 virus 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 promoter 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 10^6 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 EID₅₀/mL, e.g., at least 10^8 EID₅₀/mL, 10^9 EID₅₀/mL or 10^{10} EID₅₀/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 ID 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

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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, hammer-head 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 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

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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.

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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. 1A, 1B, 1C, 1D and 1E. 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. 5A, 5B, 5C and 5D. 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. 7A and 7B. A) Virus stocks were tested for HA titers (in 2") and virus titers (in PFU/mL). B) Growth curves in MDCK cells.

FIGS. 8A, 8B and 8C. 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. 9A and 9B. 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. 10A, 10B, 10C, 10D, 10E, 10F, 10G, 10H, 10I, 10J, 10K, 10L and 10M. A) Codon usage table for canines. B) Relative adaptiveness of wild type (UW-PR8) and "rare" codon optimized PB2 viruses. C) Relative adaptiveness of

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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. 11A, 11B and 11C. 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).

FIGS. 12A-12C. 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. 13A and 13B. A) Schematic of chimeric HA and NA genes to increase virus titer. B) Growth kinetics of chimeric viruses.

FIGS. 14A and 14B. 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. 18A and 18B. Overview of generation of viruses with enhanced growth in MDCK cells and Vero cells.

FIGS. 19A, 19B, 19C and 19D. 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 deter-

gent 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 bromelain, 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, thiosemicarbazones, methisazone, rifampin, ribavirin, a pyrimidine analog, a purine analog, foscarnet, 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 μ g, 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 μ g 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 μ g or any range or value therein, or the amount recommended by the U.S. Public Health Service (PHS), which is usually 15 μ g per component for older children (greater than or equal to 3 years of age), and 7.5 μ g 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 contain 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

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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 140Q 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.

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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, 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% CO₂. 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} EID₅₀/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 dilution						
	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)

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AGCGAAAGCA GGTACTGATC CAAATGGAA GATTTTGTGC GACAATGCTT CAATCCGATG
ATTGTCGAGC TTGCGGAAAA AACAAATGAAA GAGTATGGGG AGGACCTGAA AATCGAAACA
AACAAATTTG CAGCAATATG CACTCACTTG GAAGTATGCT TCATGTATT C AGATTTTCAC
TTCATCAATG AGCAAGGCGA GTCAATAATC GTAGAACTTG GTGATCCAAA TGCACTTTTG
AAGCACAGAT TTGAAATAAT CGAGGGAAGA GATCGCACAA TGGCCTGGAC AGTAGTAAAC
AGTATTTGCA AACTACAGG GGCTGAGAAA CCAAAGTTTC TACCAGATTT GTATGATTAC
AAGGAGAATA GATTTCATCGA AATTGGAGTA ACAAGGAGAG AAGTTCACAT ATACTATCTG
GAAAAGGCCA ATAAATTAAT ATCTGAGAAA ACACACATCC ACATTTTCTC GTTCACTGGG
GAAGAAATGG CCACAAAGGC AGACTACACT CTCGATGAAG AAAGCAGGGC TAGGATCAAA
ACCAGACTAT TCACCATAAG ACAAGAAATG GCCAGCAGAG GCCTCTGGGA TTCCTTTCGT
CAGTCCGAGA GAGGAGAAGA GACAATTGAA GAAAGGTTTG AAATCACAGG AACAAATGCGC
AAGCTTGCCG ACCAAAGTCT CCCGCCGAAC TTCTCCAGCC TTGAAAATTT TAGAGCCTAT
GTGGATGGAT TCGAACCGAA CGGCTACATT GAGGGCAAGC TGCTCTCAAT GTCCAAAGAA
GTAAATGCTA GAATTGAACC TTTTGTGAAA ACAACACCAC GACCACTTAG ACTTCCGAAT
GGGCCTCCCT GTTCTCAGCG GTCCAAATTC CTGCTGATGG ATGCCTTAAA ATTAAGCATT
GAGGACCCAA GTCATGAAGG AGAGGGAATA CCGCTATATG ATGCAATCAA ATGCATGAGA
ACATTCTTTG GATGGAAGGA ACCCAATGTT GTTAAACCAC ACGAAAAGGG AATAAATCCA

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AATTATCTTC TGTTCATGGAA GCAAGTACTG GCAGAAGTGC 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 GCCCATGTTT TGTATGTGA GAACAAATGG AACCTCAAAA
 ATTAAATGA AATGGGGAAT GGAGATGAGG CGTTGCCTCC TCCAGTCACT TCAACAAATT
 GAGAGTATGA TTGAAGCTGA GTCCTCTGTC AAAGAGAAAG ACATGACCAA AGAGTTCTTT
 GAGAACAAAT CAGAAACATG GCCCATGGA GAGTCCCCCA AAGGAGTGA GGAAAGTTCC
 ATTGGGAAGG TCTGCAGGAC TTTATTAGCA AAGTCGGTAT TCAACAGCTT GTATGCATCT
 CCACAAC TAG AAGGATTTTC AGCTGAATCA AGAAAAGTGC TTCTTATCGT TCAGGCTCTT
 AGGGACAACC TGGAACCTGG GACCTTTGAT CTTGGGGGCG TATATGAAGC AATTGAGGAG
 TGCTTGATTA ATGATCCCTG GGTTTTGCTT AATGCTTCTT GGTTCAACTC CTTCTTACA
 CATGCATTGA GTTAGTTGTG GCAGTGCTAC TATTTGCTAT CCATACTGTC CAAAAAGTA
 CTTGTTTCT ACT

PB1

(SEQ ID NO: 2)

AGCGAAAGCA GGCAACCAT TTGAATGGAT GTCAATCCGA CCTTACTTTT CTTAAAAGTG
 CCAGCACAAA ATGCTATAAG CACAACCTTC CCTTACTG GAGACCTCC TTACAGCCAT
 GGGACAGGAA CAGGATACAC CATGGATACT GTCAACAGGA CACATCAGTA CTCAGAAAAG
 GGAAGATGGA CAACAAACAC CGAAACTGGA GCACCGCAAC TCAACCCGAT TGATGGGCCA
 CTGCCAGAAG ACAATGAACC AAGTGGTTAT GCCCAAACAG ATTGTGTATT GGAGGCGATG
 GCTTTCCTTG AGGAATCCCA TCCTGGTATT TTTGAAAAGT CGTGTATTGA AACGATGGAG
 GTTGTTTCAGC AAACACGAGT AGACAAGCTG ACACAAGGCC GACAGACCTA TGAAGTGGCT
 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 TAAACCGGAG AGCAATTGCA ACCCCAGGGA TGCAATAAAG GGGGTTTGTA
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 GTTGGAGGCA ATGAGAAGAA AGCAAGTTG GCAAAATGTTG TAAGGAAGAT GATGACCAAT
 TCTCAGGACA CCGAACTTTC TTTCAACATC ACTGGAGATA ACACCAAATG GAACGAAAAAT
 CAGAAATCCTC GGATGTTTTT GGCCATGATC ACATATATGA CCAGAAATCA GCCCGAATGG
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 AATATGTTAA GCACTGTATT AGGCGTCTCC ATCCTGAATC TTGGACAAAA GAGATACACC
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 GGGGTGTCTG GGATCAACGA GTCAGCGGAC ATGAGTATTG GAGTTACTGT CATCAAAAAAC
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 AGTATGGTGG AGGCTATGGT TTCCAGAGCC CGAATTGATG CACGGATTGA TTTCGAATCT
 GGAAGGATAA AGAAAGAAGA GTTCACTGAG ATCATGAAGA TCTGTTCCAC CATTGAAGAG
 CTCAGACGGC AAAAATAGTG AATTAGCTT GTCCTTCATG AAAAAATGCC TTGTTTCTAC T

PB2

(SEQ ID NO: 3)

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 GAGCAAGGAC AAAGTTTATG GAGTAAATG AATGATGCCG GATCAGACCG AGTATGGTA
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 GCAGATCTCA GTGCCAAGGA GGCACAGGAT GTAATCATGG AAGTTGTTTT CCCTAACGAA
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GTGTTGGTAA TGAACCGAA ACGGGACTCT AGCATACTTA CTGACAGCCA GACAGCGACC
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NP

(SEQ ID NO: 4)

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GAACTCAAAC TCAGTGATTA TGAGGGACGG TTGATCCAAA ACAGCTTAAC AATAGAGAGA
ATGGTGCTCT CTGCTTTTGA CGAAAGGAGA AATAAATACC TTGAAGAACA TCCAGTGCG
GGGAAAGATC CTAAGAAAAC TGGAGGACCT ATATACAGGA GAGTAAACGG AAAGTGGATG
AGAGAACTCA TCCTTTATGA CAAAGAAGAA ATAAGGCGAA TCTGGCGCCA AGCTAATAAT
GGTGACGATG CAACGGCTGG TCTGACTCAC ATGATGATCT GGCATTCCAA TTTGAATGAT
GCAACTTATC AGAGGACAAG AGCTCTTGTT CGCACCGGAA TGGATCCCAG GATGTGCTCT
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
TTGAGAGGGT CGGTTGCTCA CAAGTCTGC CTGCCTGCCT GTGTGTATGG ACCTGCCGTA
GCCAGTGGGT ACGACTTTGA AAGGGAGGGA TACTCTCTAG TCGGAATAGA CCCTTTCAGA
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 TGGGCCATAA GGACCAGAAG TGGAGGAAAC ACCAATCAAC AGAGGGCATC TCGGGGCCAA
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 ATGGCAGCAT TCAATGGGAA TACAGAGGGG AGAACATCTG ACATGAGGAC CGAAATCATA
 AGGATGATGG AAAGTGCAAG ACCAGAAGAT GTGTCTTTCC AGGGGCGGGG AGTCTTCGAG
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 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 TGTGTTACG CTCACCGTGC CCAGTGAGCG
 AGGACTGCAG CGTAGACGCT TTGTCCAAAA TGCCCTTAAT GGAACGGGG ATCCAAATAA
 CATGGACAAA GCAGTTAAAC TGTATAGGAA GCTCAAGAGG GAGATAACAT TCCATGGGGC
 CAAAGAAATC TCACTCAGTT ATTCTGCTGG TGCACCTGCC AGTTGTATGG GCCTCATATA
 CAACAGGATG GGGGCTGTGA CCACTGAAGT GGCATTTGGC CTGGTATGTG CAACCTGTGA
 ACAGATTGCT GACTCCCAGC ATCGGTCTCA TAGGCAAATG GTGACAACAA CCAATCCACT
 AATCAGACAT GAGAACAGAA TGGTTTTAGC CAGCACTACA GCTAAGGCTA TGGAGCAAAT
 GGCTGGATCG AGTGAGCAAG CAGCAGAGGC CATGGAGGTT GCTAGTCAGG CTAGACAAAT
 GGTGCAAGCG ATGAGAACCA TTGGGACTCA TCCTAGCTCC AGTGCTGGTC TGAAAAATGA
 TCTTCTTGAA AATTTGCAGG CCTATCAGAA ACGAATGGGG GTGCAGATGC AACGGTTCAA
 GTGATCCTCT CACTATTGCC GCAAATATCA TTGGGATCTT GCACTTGACA TTGTGGATTG
 TTGATCGTCT TTTTTCAAA TGCAATTACC GTCGCTTTAA ATACGGACTG AAAGGAGGGC
 CTCTACGGA AGGAGTGCCA AAGTCTATGA GGAAGAATA TCGAAAGGAA CAGCAGAGTG
 CTGTGGATGC TGACGATGGT CATTTTGTCA GCATAGAGCT GGAGTAAAAA ACTACCTTGT 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
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 ACATGACTCT TGAGGAAATG TCAAGGGACT GGTCCATGCT CATACCCAAG CAGAAAGTGG
 CAGGCCCTCT TTGTATCAGA ATGGACCAGG CGATCATGGA TAAGAACATC ATACTGAAAG
 CGAATTCTAG TGTGATTTTT GACCGGCTGG AGACTCTAAT ATTGCTAAGG GCTTTCACCG
 AAGAGGGAGC AATTGTTGGC GAAATTTTAC CATTGCCTTC TCTTCCAGGA CATACTGCTG
 AGGATGTCAA AAATGCAGTT GGAGTCCTCA TCGGAGGACT TGAATGGAAT GATAACACAG

TTCGAGTCTC TGAAACTCTA CAGAGATTCG CTTGGAGAAG CAGTAATGAG AATGGGAGAC
CTCCACTCAC TCCAAAACAG AAACGAGAAA TGGCGGGAAC AATTAGGTCA GAAGTTTGAA
GAAATAAGAT GGTGATTGA AGAAGTGAGA CACAACTGA AGATAACAGA GAATAGTTTT
GAGCAAATAA CATTTATGCA AGCCTTACAT CTATTGCTTG AAGTGGAGCA AGAGATAAGA
ACTTTCTCGT TTCAGCTTAT TTAGTACTAA AAAACACCCT TGTTTCTACT

HA

(SEQ ID NO: 7)

AGCAAAGCAGGGGAAAATAAAAACAACCAAATGAAGGCAACCTACTGGTCTGTTATGTGCACTTGC
AGCTGCAGATGCAGACACAATATGTATAGGCTACCATGCGAACAATTCAACCGACACTGTTGACACAGTA
CTCGAGAAGAATGTGACAGTGACACACTCTGTTAACCTGCTCGAAGACAGCCACAACGGAAAACCTATGTA
GATTAAGGAATAGCCCCACTACAATTGGGGAAATGTAACATCGCCGGATGGCTCTTGGGAAACCCAG
AATGCGACCCACTGCTTCCAGTGAGATCATGGTCTTACATTGTAGAAACACCAAACCTGAGAATGGAAT
ATGTTATCCAGGAGATTTTCATCGACTATGAGGAGCTGAGGGAGCAATTGAGCTCAGTGTATCATTCGAA
AGATTGGAATATTTCCCAAAGAAAGCTCATGGCCCAACCACAACACAACCGAGTAACGGCAGCATGCT
CCCATGAGGGGAAAAGCAGTTTTTACAGAAATTGCTATGGCTGACGGAGAAGGAGGGCTCATACCCAA
AGCTGAAAAATCTTATGTGAACAAAAAGGGAAGAAGTCCTTGTAAGGGGTATTTCATCACC CGC
TAACAGTAAGGAACAACAGAATCTCTATCAGAATGAAATGCTTATGTCTCTGTAGTGACTTCAAATTATA
ACAGGAGATTTACCCCGGAAATAGCAGAAAGACCCAAAGTAAGAGATCAAGCTGGGAGGATGAACTATT
ACTGGACCTTGCTAAACCCCGAGACACAATAATTTGAGGCAATGGAAATCTAATAGCACCAATGTA
TGCTTTCGCACTGAGTAGAGGCTTTGGGTCCGGCATCATCACCTCAAACGCATCAATGCATGAGTGTAAC
ACGAAGTGTCAAACACCCCTGGGAGCTATAAACAGCAGTCTCCCTTACCAGAATATACCCAGTCACAA
TAGGAGAGTGCCCAAAATACGTCAGGAGTGCCAAATTGAGGATGGTTACAGGACTAAGGAACATTCCGT
CCATTCAATCCAGAGTCTATTTGGAGCCATTGCCGGTTTTATTGAAGGGGATGGACTGGAATGATAGA
TGGATGGTATGGTTATCATCATCAGAATGAACAGGGATCAGGCTATGCAGCGGATCAAAAAGCACACAA
AATGCCATTAAACGGGATTACAACAAGGTGAACACTGTTATCGAGAAAATGAACATTCAATTACAGCTGT
GGGTAAGAATTCAACAAATTAGAAAAAGGATGGAAAATTTAAATAAAAAAGTTGATGATGGATTTCTGG
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AATGTGAAGAATCTGTATGAGAAAGTAAAAAGCCAATTAAAGAATAATG0CAAAGAAATCGGAAATGGAT
GTTTTGAGTTCTACCACAAGTGTGACAATGAATGCATGGAAAGTGAAGAAATGGGACTTATGATTATCC
CAAATATTGAGAAGAGTCAAAGTTGAACAGGGAAAAGGTAGATGGAGTGAATTGGAAATCAATGGGGATC
TATCAGATTCTGGCGATCTACTCAACTGTCCGCAGTTCACTGGTGCTTTTGGTCTCCCTGGGGGCAATCA
GTTTCTGGATGTTCTAATGGATCTTTGCAGTGCAGAAATATGCATCTGAGATTAGAATTCAGAGATATG
AGGAAAAACACCCTTGTTTCTACT

NA

(SEQ ID NO: 8)

AGCAAAGCAGGGGTTTAAATGAATCCAAATCAGAAAATAATAACCATTGGATCAATCTGTCTGGTAGTC
GGACTAATTAGCCTAATATTGCAAATAGGGAATATAATCTCAATATGATTAGCCATTCAATTCAAACCTGG
AAGTCAAAACCATACTGGAATATGCAACCAAACATCATTACCTATAAAAAAGCACCTGGGTAAAGGACA
CAACTTCAGTGATATTAACCGCAATTCTCTCTTGTCCCATCCGTGGGTGGGCTATATACAGCAAAGA
CAATAGCATAAGAATTGGTTCCAAAGGAGACGTTTTTGTGCATAAGAGAGCCCTTTATTTTCATGTTCTCACT

- continued

TGGAATGCAGGACCTTTTCTGACCCAAGGTGCCTTACTGAATGACAAGCATTCAAGTGGGACTGTTAA
 GGACAGAAGCCCTTATAGGGCCTTAATGAGCTGCCCTGTCGGTGAAGCTCCGTCCCCGTACAATCAAG
 ATTTGAATCGGTGCTTGGTCAGCAAGTGCATGTCATGATGGCATGGGCTGGCTAACAAATCGGAATTTCA
 GTCCAGATAATGGAGCAGTGGCTGTATTAAAAATACAACGGCATAATAACTGAAACCATAAAAAAGTTGGA
 GGAAGAAAAATATTGAGGACACAAGAGTCTGAATGTGCCTGTGTAATGGTTTATGTTTACTATAATGACT
 GATGGCCCGAGTGATGGGCTGGCCTCGTACAAAATTTTCAAGATCGAAAGGGGAAGGTTACTAAATCA
 ATAGAGTTGAATGCACCTAATTTCTCACTATGAGGAATGTTCTGTTACCTGATACCGCAAGTGATGT
 GTGTGTGCAGAGACAATTGGCATGGTTCGAACCGGCCATGGGTGTCTTTCGATCAAAACCTGGATTATC
 AAATAGGATACATCTGCAGTGGGGTTTTCGGTGACAACCCGCGTCCCGAAGATGGAACAGGCAGCTGTG
 GTCCAGTGTATGTTGATGGAGCAAACGGAGTAAAGGATTTTCATATAGGTATGTAATGGTGTGTTGGAT
 AGGAAGGACCAAAAGTCACAGTTCAGACATGGGTTTGAGATGATTTGGGATCCTAATGGATGGACAGA
 GACTGATAGTAAGTTCTCTGTGAGCAAGATGTTGTGGCAATGACTGATTGGTCAGGGTATAGCGGAAG
 TTTCGTTCAACATCTGAGCTGACAGGGCTAGACTGTATGAGGCCGTGCTTCTGGGTGAATTAATCAGG
 GGACGACCTAAAGAAAAACAATCTGGACTAGTGCAGCAGCATTTCTTTTGTGGCGTGAATAGTGATA
 CTGTAGATTGGTCTTGCCAGACGGTGTGAGTTGCCATTGAGCATTGACAAGTAGTCTGTTCAAAAAAC
 TCCTTGTCTTACT

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} EID₅₀/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. 1A, 1B, 1C, 1D, 1E), 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 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 gen-

erate "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	Gene library	Internal genes		HA + NA	Titer of virus library (pfu/ml)
		Other internal genes			
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		M148I (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	8~8.5		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
571	PB1	9~9.5		E112G (PB1-F2-R81G)					
572	PB1	8.5		E112G (PB1-F2-R81G)					
573	PB1	8.5		E112G (PB1-F2-R81G)					
1404	PB1	8.5	I57V T58G A59V K61Q E677D D678E P679M	E112G (PB1-F2-R81G)					
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. 5A, 5B, 5C and 5D 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	8.3×10^6
	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
	F2 R79Q	6.2×10^6

TABLE 3-continued

UW-PR8 viruses possessing mutation(s) identified in the literature		
Gene	Mutation(s)	Virus stock titer (Pfu/ml)
PA	F2 L82S	2.7×10^7
	F2 E87Q	1.5×10^6
	T97I	1.6×10^7
	K142N	3.3×10^7
	S225C	6.7×10^7
NP	S149P + T357K	3.4×10^8
	K356R	8.5×10^7
	A404S	5.2×10^7
	S421I	2.7×10^7
	R293K	4.7×10^7
55	R305K	7.2×10^7
	E372D	2.2×10^7
	R422K	1.3×10^8
	T442A	5×10^7
	D455E	2.2×10^7
60	I109V	3.9×10^7
	M V97A + Y100H	1.4×10^7
	NS1 K55E	1.6×10^7

65 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 (detoxified)	Indo/NC/09	UW-PR8	UW-PR8	UW-PR8	UW-PR8	UW-PR8	UW-PR8	7	3.00E+07
1			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	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 (detoxified)	Indo/NC/09						A30P	6.5~7	1.00E+07
27								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)	Indo/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 (detoxified)	Indo/NC/09	M202L F323L	M507V V644A	R401K	T442A	Y100H	R140Q	9	3.20E+07
36			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	I112G	I550L	R74K	Y100H	K55E	9.2	7.50E+07

TABLE 4-continued

High-growth candidates identified in approaches 1 and 2 were tested in various combinations.										
#	HA	NA	Gene origin						Virus stock titer	
			PB2	PB1	PA	NP	M	NS	HA (2 ⁿ)	Pfu/ml
P17			I504V	E112G (PB1-F2-R81G)	S225C	R74K N417D	V97A Y100H	K55E	9.5~10	5.80E+08
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

¹Mutation in the HA gene noncoding region;²A 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

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. 11C.

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.										
Viruses	HA	Gene backbone							Virus stock titer	
		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").

50

protein evaluated. FIG. 8A shows HA titer of wild type (UW-PR8) and #4, FIG. 8B shows viral protein for wild type (UW-PR8) and #4, and FIGS. 8B and 8C 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. 9A and 9B). 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

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. 10A), 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. 10C) and in another approach, codon optimization was performed for so-called 'rare' codons (FIG. 10B) (used at significantly lower frequency compared to the codon used most frequently for a given amino acid) (see SEQ ID NO:25 in FIG. 10F). Analyses were carried out using the "Graphical Codon Usage Analyser" (www.gcua.de). The titers of those viruses are shown in Table 6 (see also FIGS. 10B and 10C).

65

TABLE 6

Titers of viruses encoding codon-optimized PB2 genes.										
Virus	Gene backbone								Virus stock titer	
	HA	NA	PB2	PB1	PA	NP	M	NS	HA (2 nd)	pfu/ml
Wild type	WT	WT	WT	WT	WT	WT	WT	WT	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. 10D). Other viral segments were codon optimized and titers of viruses with those segments or combinations of optimized segments were determined (FIG. 10E).

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

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. 14A and 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-PR8 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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tccttgatcg gcttcgccga gatcagaaat ccctaagagg aaggggcagt actctcggtc	180
tggacatcaa gacagccaca cgtgctggaa agcagatagt ggagcggatt ctgaaagaag	240
aatccgatga ggcaactaaa atgaccatgg cctctgtacc tgcgtcgcgt tacctaactg	300
acatgactct tgaggaaatg tcaagggact ggtccatgct catacccaag cagaaagtgg	360
caggccctct ttgtatcaga atggaccagg cgatcatgga taagaacatc atactgaaag	420
cgaacttcag tgtgatthtt gaccggctgg agactctaatt attgctaagg gctttcaccg	480
aagagggagc aattgttggc gaaatttcac cattgccttc tcttccagga catactgctg	540
aggatgtcaa aaatgcagtt ggagtctca tcggaggact tgaatggaat gataacacag	600
ttcgagtctc tgaactctca cagagattcg cttggagaag cagtaatgag aatgggagac	660
ctccactcac tccaaaacag aaacgagaaa tggcgggaac aattaggtea gaagtttgaa	720
gaaataagat ggttgattga agaagtgaga cacaaactga agataacaga gaatagthtt	780
gagcaataaa catthtatga 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
cagcatgtct ccatgagggg aaaagcagtt ttacagaaa ttgctatgg ctgacggaga	540
aggagggtct atacccaaag ctgaaaaatt cttatgtgaa caaaaaagg aaagaagtcc	600
ttgtactgtg gggatttcat caccgccta acagtaagga acaacagaat ctctatcaga	660
atgaaaaatc ttatgtctct gtagtgactt caaattataa caggagattt accccggaaa	720

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tagcagaaaag acccaaaagta agagatcaag ctgggaggat gaactattac tggaccttgc	780
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agtgtaacac gaagtgtcaa acaccctgg gagctataaa cagcagtctc ccttaccaga	960
atatacacc agtcacaata ggagagtgcc caaaatacgt caggagtgcc aaattgagga	1020
tggttacagg actaaggaaac attccgtcca ttcaatccag aggtctatgt ggagccattg	1080
ccggttttat tgaaggggga tggactggaa tgatagatgg atggtatggt tatcatcatc	1140
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ggattacaaa caaggtgaac actgttatcg agaaaatgaa cattcaattc acagctgtgg	1260
gtaaagaatt caacaaatta gaaaaaagga tggaaaattt aaataaaaaa gttgatgatg	1320
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agaataatgc caaagaaatc ggaatggat gttttgagtt ctaccacaag tgtgacaatg	1500
aatgcatgga aagtgtgaag aatgggactt atgattatcc caaatattca gaagagtcaa	1560
agttgaacag ggaagagga gatggagtga aattggaatc aatggggatc tatcagattc	1620
tggcgatcta ctcaactgtc gccagttcac tgggtctttt ggtctccctg ggggcaatca	1680
gtttctggat gtgttctaata 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 ggggtgggcta tatacagcaa agacaatagc ataagaattg	300
gttccaaagg agacgttttt gtcataagag agccctttat ttcattgtct cacttggaat	360
gcaggacctt ttttctgacc caaggtgcct tactgaatga caagcattca agtgggactg	420
ttaaggacag aagcccttat agggccttaa tgagctgccc tgtcggtgaa gctccgtccc	480
cgtacaattc aagatttgaa tcggttgctt ggtcagcaag tgcattgcat gatggcatgg	540
gctggctaac aatcggaatt tcagggtccag ataatggagc agtggctgta ttaaaataca	600
acggcataat aactgaaacc ataaaaagtt ggaggaagaa aatatgagg acacaagagt	660
ctgaatgtgc ctgtgtaaat ggttcattgt ttactataat gactgatggc ccgagtgatg	720
ggctggcctc gtacaaaatt ttcaagatcg aaaaggggaa gggtactaaa tcaatagagt	780
tgaatgcacc taattctcac tatgaggaat gtccctgtta ccctgatacc ggcaaaagtga	840
tgtgtgtgtg cagagacaat tggcatgggt cgaaccggcc atgggtgtct ttcgatcaaa	900
acctggatta tcaaatagga tacatctgca gtggggtttt cggtgacaac ccgcgtccc	960
aagatggaac aggcagctgt ggtccagtgt atgttgatgg agcaaacgga gtaagggat	1020
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atgggtttga gatgatttgg gatcctaata 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
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<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

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gggacaggaa caggatacac catggatact gtcaacagga cacatcagta ctcagaaaag 180
ggaagatgga caacaaacac cgaaactgga gcaccgcaac tcaaccgat tgatgggcca 240
ctgccagaag acaatgaacc aagtggttat gcccacacag attgtgtatt ggaagcaatg 300
gctttccttg aggaatccca tcttggattt ttgaaaaact cgtgtattga aacgatggag 360
gttgttcagc aaacactgag tagacaagct gacacaaggc cgacagacct atgactggac 420
tttaaataga aaccagcctg ctgcaacagc attggccaac acaatagaag tggtcagatc 480
aaatggcctc acggccaatg agtcaggaag gctcatagac ttccttaagg atgtaatgga 540
gtcaatgaaa aaagaagaaa tggggatcac aactcatttt cagagaaaag gacgggtgag 600
agacaatatg actaagaaaa tgataacaca gagaacaata ggtaaaagga aacagagatt 660
gaacaaaagg gggtatctaa ttagagcatt gaccctgaac acaatgacca aagatgctga 720
gagaggggaag ctaaaacgga gagcaattgc aaccccaggg atgcaaataa gggggtttgt 780
atactttgtt gagacactgg caaggagtat atgtgagaaa cttgaacaat cagggttgcc 840
agttggaggc aatgagaaga aagcaaagt ggcaaatgtt gtaaggaaga tgatgaccaa 900
ttctcaggac accgaacttt ctttcacat cactggagat aacaccaaag ggaacgaaaa 960
tcagaatcct cggatgtttt tggccatgat cacatatatg accagaaatc agcccgaatg 1020
gttcagaaat gttctaagta ttgctccaat aatgttctca aacaaaatgg cgagactggg 1080
aaaaggggat atgtttgaga gcaagagtat gaaacttaga actcaaatac ctgcagaaat 1140
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tggggtgtct	gggatcaacg	agtcagcggg	catgagtatt	ggagttactg	tcatcaaaaa	1620
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caaagattac	aggtagacgt	accgatgcc	tagaggtag	acacaaatac	aaacccgaag	1740
atcatttgaa	ataaagaaac	tgtgggagca	aacccgttcc	aaagctggac	tgctggtctc	1800
cgacggaggc	ccaaatttat	acaacattag	aaatctccac	attcctgaag	tctgcctaaa	1860
atgggaattg	atggatgagg	attaccaggg	gcgtttatgc	aacccactga	accattttgt	1920
cagccataaa	gaaattgaat	caatgaacaa	tgcagtgatg	atgccagcac	atgggtccagc	1980
caaaaacatg	gagtatgatg	ctgttgcaac	aacacactcc	tggatcccca	aaagaaatcg	2040
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ctggaattta	tttgaataat	tcttccccag	cagttcatac	agaagaccag	tcgggatatc	2160
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<210> SEQ ID NO 11

<211> LENGTH: 2341

<212> TYPE: DNA

<213> ORGANISM: Influenza virus

<400> SEQUENCE: 11

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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	atcggaaatc	caactaacga	taaccaaaga	gaagaaagaa	600
gaactccagg	attgcaaaat	ttctcctttg	atggttgcat	acatgttgga	gagagaactg	660
gtccgcaaaa	cgagattcct	cccagtggtc	ggtggaacaa	gcagtgtgta	cattgaagtg	720
ttgcatttga	ctcaaggaac	atgctgggaa	cagatgtata	ctccaggagg	ggaagtgaag	780
aatgatgatg	ttgatcaaag	cttgattatt	gctgctagga	acatagttag	aagagctgca	840
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aaggctgcaa	tgggactgag	aattagctca	tccttcagtt	ttggtggatt	cacatttaag	1020
agaacaagcg	gatcatcagt	caagagagag	gaagaggtgc	ttacgggcaa	tcttcaaaca	1080
ttgaagataa	gagtgcata	gggatctgaa	gagttcaca	tggttgggag	aagagcaaca	1140
gccatactca	gaaaagcaac	caggagattg	attcagctga	tagtgagtgg	gagagacgaa	1200
cagtcgattg	ccgaagcaat	aattgtgggc	atggatattt	cacaagagga	ttgtatgata	1260

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aaagcagtta gaggtgatct gaatttcgtc aatagggcga atcagcgact gaatcctatg	1320
catcaacttt taagacattt tcagaaggat gcgaaagtgc tttttcaaaa ttggggaggt	1380
gaacctatcg acaatgtgat gggaatgatt gggaatttgc ccgacatgac tccaagcatc	1440
gagatgtcaa tgagaggagt gagaatcagc aaaatgggtg tagatgagta ctccagcacg	1500
gagagggtag tggtagcat tgaccggttc ttgagagtca gggaccaacg aggaaatgta	1560
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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 ggcagcatt aagcatcaat	2160
gaactgagca accttgcaa aggagagaag gctaattgtc taattgggca aggagacgtg	2220
gtgttggtaa tgaacgaaa acgggactct agcatactta ctgacagcca gacagcgacc	2280
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<210> SEQ ID NO 12

<211> LENGTH: 2234

<212> TYPE: DNA

<213> ORGANISM: Influenza virus

<400> SEQUENCE: 12

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aacaaatttg cagcaatatg cactcacttg gaagtatgct tcatgtattc agatttccac	180
ttcatcaatg agcaaggcga gtcaataatc gtagaacttg gtgacctaa tgcacttttg	240
aagcacagat ttgaataat cgagggaaga gatcgacaa tggcctggac agtagtaaac	300
agtatttgca aactacagg ggctgagaaa ccaaagtctc taccagattt gtatgattac	360
aaggaaaata gattcatcga aattggagta acaaggagag aagttcacat atactatctg	420
gaaaaggcca ataaaattaa atctgagaaa acacacatcc acattttctc gttcactggg	480
gaagaaatgg ccacaagggc cgactacact ctgatgaag aaagcagggc taggatcaaa	540
accaggctat tcaccataag acaagaaatg gccagcagag gcctctggga ttctttctgt	600
cagtccgaga gaggagaaga gacaattgaa gaaaggtttg aaatcacagg aacaatgcgc	660
aagcttgccg accaaagtct ccgcccgaac ttctccagcc ttgaaaattt tagagcctat	720
gtggatggat tcgaaccgaa cggtacatt gagggcaagc tgtctcaaat gtccaaagaa	780
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gggcctcctt gttctcagcg gtccaaatc ctgctgatgg atgcctaaa attaaagcatt	900
gaggacccaa gtcataagg agagggaata ccgctatatg atgcaatcaa atgcatgaga	960
acattctttg gatggaagga acccaatggt gttaaaccac acgaaaaggg aataaatcca	1020

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tgacagacca	cagaatacat	aatgaagggg	gtgtacatca	atactgcctt	acttaatgca	1440
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gaggggaaggc	gaaagaccaa	cttgatgggt	ttcatcataa	aaggaagatc	ccacttaagg	1560
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gtgcctaatt	aatgatccct	gggttttgct	taatgcttct	tggttcaact	ccttccttac	2160
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accttggttc	tact					2234

<210> SEQ ID NO 13

<211> LENGTH: 1565

<212> TYPE: DNA

<213> ORGANISM: Influenza virus

<400> SEQUENCE: 13

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agagcatccg	tcgaaaaaat	gattggtgga	attggacgat	tctacatcca	aatgtgcaca	180
gaacttaaac	tcagtgatta	tgagggacgg	ttgatccaaa	acagcttaac	aatagagaga	240
atggtgctct	ctgcttttga	cgaaggaga	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
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gttgaacaa	tggtgatgga	attggtcagg	atgatcaaac	gtgggatcaa	tgatcggaac	660
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ctcaaaggga	aatttcaaac	tgctgcacaa	aaagcaatga	tggatcaagt	gagagagagc	780
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ctgcttcaaa	acagccaagt	gtacagccta	atcagaccaa	atgagaatcc	agcacacaag	1020
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ttcatcaaa	ggacgaaggt	gggcccaaga	gggaagcttt	ccactagagg	agttcaaatt	1140
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tgggccataa	ggaccagaag	tggaggaaac	accaatcaac	agagggcatc	tgcgggcca	1260
atcagcatac	aacctacgtt	ctcagtacag	agaaatctcc	cttttgacag	aacaaccgtt	1320
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ctctcggacg	aaaaggcagc	gagcccgatc	gtgccttcct	ttgacatgag	taatgaagga	1500
tcttatttct	tcggagacaa	tgacagaggag	tacgacaatt	aaagaaaaat	acccttgttt	1560
ctact						1565

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

<400> SEQUENCE: 14

agcaaaagca	ggtagatatt	gaaagatgag	tcttctaacc	gaggtcgaaa	cgtacgttct	60
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tgcagggaag	aacaccgatc	ttgaggttct	catggaatgg	ctaaagacaa	gaccaatcct	180
gtcacctctg	actaagggga	ttttaggatt	tgtgttcacg	ctcacctgtc	ccagtgagcg	240
aggatgcag	cgtagacgct	ttgtccaaaa	tgcccttaat	gggaacgggg	atccaaataa	300
catggacaaa	gcagttaaac	tgtataggaa	gctcaagagg	gagataacat	tccatggggc	360
caaagaaatc	tactcagtt	attctgctgg	tgcacttgcc	agttgtatgg	gcctcatata	420
caacaggatg	ggggctgtga	ccactgaagt	ggcatttggc	ctggtatgtg	caacctgtga	480
acagattgct	gactcccagc	atcgggtctca	taggcaaatg	gtgacaacaa	ccaacccact	540
aatcagacat	gagaacagaa	tggtttttagc	cagcactaca	gctaaggcta	tggagcaaat	600
ggctggatcg	agtgagcaag	cagcagaggc	catggagggt	gctagtcagg	ctaggcaaat	660
ggtgcaagcg	atgagaacca	ttgggactca	tcctagctcc	agtgctggtc	tgaaaaatga	720
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ttgatcgtct	ttttttcaaa	tgcatttacc	gtcgctttaa	atacggactg	aaaggaggggc	900
cttctacgga	aggagtgcc	aagtctatga	gggaagaata	tcgaaaggaa	cagcagagtg	960
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ttctact						1027

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

<400> SEQUENCE: 15

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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 tgaggaaaatg tcaagggaat ggtccatgct catacccaag cagaaagtgg 360
caggccctct ttgtatcaga atggaccagg cgatcatgga taaaacatc atactgaaag 420
cgaacttcag tgtgattttt gaccggctgg agactctaatt attgctaagg gctttcaccg 480
aagaggggagc aattgttggc gaaatttcac cattgccttc tcttcaggga cactactgtg 540
aggatgtcaa aaatgcagtt ggagtccca 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
gagcaataaa catttatgca agccttacat ctattgcttg aagtggagca agagataaga 840
actttctcat ttcagcttat ttaataataa aaaacaccct tgtttctact 890

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<210> SEQ ID NO 16

<211> LENGTH: 2341

<212> TYPE: DNA

<213> ORGANISM: Influenza virus

<400> SEQUENCE: 16

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aagaagtaca catcaggaag acaggagaag aaccacgac tcgagatgaa atggatgatg 180
gcaatgaaat atccaattac agcagacaag aggatcacg aaatgattcc tgagagaaat 240
gagcagggac agactctgtg gagtaaaatg aatgatgccg gatcagaccg agtgatgggtg 300
tcacctctgg ctgtgacatg gtggaatagg aatggaccaa tcacaaatac agtgcattat 360
ccaaaaatct acaaaaactta ttttgaaaga gtcgaaaggc tgaagcatgg aacctttggc 420
cctgtccatt ttagaaacca ggtcaaaatc cggcggagag tggacatcaa tcctggtcac 480
gcagatctca gtgccaagga ggcacaggat gtgatcatgg aagtgggtgt ccctaacgaa 540
gtgggagcca ggattctgac atccgaatcc cagctgacca ttaccaaaga gaagaaagaa 600
gaactccagg attgcaaaat ttctcctctg atggtggcat acatgctgga gagagaactg 660
gtccgcaaaa caagattcct ccagtggtg ggtggaacaa gcagtgtgta cattgaagtg 720
ctgcatctga ctcagggaac atgctgggaa cagatgtata ctccaggagg ggaagtggag 780
aatgatgatg tggatcagag cctgattatt gctgctagga acattgtgag aagagctgca 840
gtgtcagcag atccactggc atctctgctg gagatgtgcc acagcacaca gattggtgga 900
attaggatgg tggacatcct gaggcagaac ccaacagaag agcaggccgt ggatatttgc 960
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agaacaagcg gatcatcagt caagagagag gaagaggtgc tgaccggcaa tctgcagaca 1080
ctgaagatca gagtgcata gggatatgaa gagttcacia tgggtgggag 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
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gaacctatcg	acaatgtgat	gggaatgatt	gggatcctgc	ccgacatgac	tccaagcatc	1440
gagatgtcaa	tgagaggagt	gagaatcagc	aaaatgggtg	tggatgagta	ctccagcacc	1500
gagaggggtcg	tggtagcat	tgacagattt	ctgagaatcc	gggaccagcg	aggaaatgtg	1560
ctcctgtctc	ccgaggaggt	cagtgaacaa	caggaacag	agaaactgac	aattacttac	1620
tcctcctcaa	tgatgtggga	gattaatggt	cctgaatcag	tgctgggtcaa	tacctatcag	1680
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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	ggtagggggc	1980
aattctcctg	tgttcaacta	taacaaggcc	accaagagac	tcacagtgtc	cggaaaggat	2040
gctggcactc	tgactgaaga	cccagatgaa	ggcacagctg	gagtggagtc	cgctgtgctg	2100
aggggattcc	tcattctggg	caaagaagac	aagagatatg	ggccagcact	gagcatcaat	2160
gaactgagca	acctggccaa	aggagagaag	gctaattgtc	taattgggca	aggagacgtg	2220
gtgttggtaa	tgaaccgaa	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

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gggacaggaa	caggatacac	catggatact	gtcaacagga	cacatcagta	ctcagaaaaag	180
ggaagatgga	caacaacac	cgaactgga	gcaccgcaac	tcaaccgat	tgatgggcca	240
ctgccagaag	acaatgaacc	aagtggttat	gcccacacag	attgtgtatt	ggaggcgatg	300
gctttccttg	aggaatccca	tcttgggtatt	tttgaaaaact	cgtgtattga	aacgatggag	360
gttgttcagc	aaacacgagt	ggacaagctg	acacaggggc	gacagaccta	tgactggact	420
ctgaatagaa	accagcctgc	tgcaacagca	ctggccaaca	caatcgaagt	gttcagatca	480
aatggcctca	ccgccaatga	gtctggaagg	ctcatcgact	tcctgaagga	tgtgatggag	540
tcaatgaaca	aagaagaaat	ggggatcaca	actcattttc	agagaaagag	acgggttgaga	600
gacaatatga	ctaagaaaat	gattacacag	agaacaatgg	gtaaaaagaa	gcagagactg	660
aacaaaagga	gttatctgat	tagagcactg	accctgaaca	caatgaccaa	agatgctgag	720
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gtgggaggca	atgagaagaa	agcaaagctg	gcaaatgtgg	tgaggaagat	gatgaccaat	900
tctcaggaca	ccgaactgtc	tttcaccatc	actggagata	acaccaaagt	gaacgaaaat	960
cagaatcctc	ggatgtttct	ggccatgatc	acatatatga	ccagaaatca	gcccgaatgg	1020

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ttcagaaatg tgctgagtat tgctccaatt atgtttctcaa acaaaatggc cagactggga 1080
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cgacccctcc tgattgaggg gactgcatca ctgagccctg gaatgatgat gggcatgttc 1260
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aatatgatca acaatgatct gggccagca acagctcaga tggccctgca gctgttcac 1680
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gacggaggcc caaatctgta caacattaga aatctccaca ttctgaagt ctgcctgaaa 1860
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agtatggtgg aggctatggt gtccagagcc cgaattgatg cacggattga tttcgaatct 2220
ggaaggatca agaaagaaga gttcactgag atcatgaaga tctgttccac cattgaagag 2280
ctcagacggc aaaaatagtg aatttagctt gtccttcacg 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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aacaatttg cagcaatatg cactcacttg gaagtatgct tcatgtattc agattttcac 180
ttcatcaatg agcaaggcga gtcaataatc gtagaacttg gtgatccaaa tgcacttttg 240
aagcacagat ttgaaataat cgagggaaga gatcgacaaa tggcctggac agtagtaaac 300
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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 ttctttctgt 600
cagtccgaga gaggagaaga gacaattgaa gaaaggtttg aaatcacagg aacaatgcgc 660
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gtggatggat tcgaaccgaa cggctacatt gagggcaagc tgtctcaaat gtccaaagaa	780
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gggcctccct gttctcagcg gtccaaattc ctgctgatgg atgccctgaa actgagcatt	900
gaggacccaa gtcatgaagg agaggggaatt ccctgtatg atgcaatcaa atgcatgaga	960
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aaaattccaa agactaaaaa tatgaagaaa acaagtcagc tgaagtgggc actgggtgag	1140
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tatgatatgt atgaaccaga actgaggtcc ctggcaagtt ggattcagaa tgagttaa	1260
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tcgagagcca cagaatacat catgaaggga gtgtacatca atactgcct gctgaatgca	1440
tcttgtgcag caatggatga tttccagctg attccaatga tcagcaagt tagaactaag	1500
gaggggaaggc gaaagaccaa cctgtatggt ttcacatca aaggaagatc ccacctgagg	1560
aatgacaccc acgtgggtgaa ctttgtgagc atggagtttt ctctcactga cccaagactg	1620
gaaccacata aatgggagaa gtactgtgtg ctggagattg gagatatgt gatcagaagt	1680
gccattggcc aggtgtcaag gccatgttc ctgtatgtga gaacaaatgg aacctcaaaa	1740
attaaaatga aatggggaat ggagatgagg cgtgcctcc tcagtcact gcagcagatt	1800
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ccacagctgg aaggattttc agctgaatca agaaaactgc tgcctgatcg gcaggctctg	2040
agggacaacc tggaacctgg gacctttgat ctgggggggc tgtatgaagc aattgaggag	2100
tgctgatta atgatccctg ggtgctgctg aatgcttctt ggttcaactc cttccttaca	2160
catgcattga gttagtgtg gcagtgtac 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

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accaaacgat cttacgaaca gatggagact gatggagaac gccagaatgc cactgaaatc	120
agagcatccg tcggaaaaat gattggtgga attggacgat tctacatcca gatgtgcacc	180
gaactcaaac tcagtgatta tgagggacgg ctgatccaga acagcctgac aatcgagaga	240
atggtgctct ctgcttttga cgaaggaga aataaatacc tggaagaaca tcccagtgcc	300
gggaaagatc ctaagaaaac tggaggacct atctacagga gagtgaacgg aaagtggatg	360
agagaactca tcctgtatga caaagaagaa atcaggcgaa tctggcgcca ggctaataat	420
ggtgacgatg caaccgctgg tctgactcac atgatgatct ggcatccaa tctgaatgat	480
gcaacttatc agaggacaag agctctgggt cgcaccggaa tggatcccag gatgtgctct	540
ctgatgcagg gttcaactct cctaggagg tctggagccg cagggtgctg agtcaaagga	600

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ctcaaaggga aatttcagac tgctgcacag aaagcaatga tggatcaggt gagagagagc	780
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gccagtgggt acgactttga aaggaggga tactctctgg tcggaattga ccctttcaga	960
ctgtgcaga acagccaggt gtacagcctg atcagaccaa atgagaatcc agcacacaag	1020
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ttcatcaaa ggaccaaggt gctcccaaga gggaagctgt ccactagagg agtgcagatt	1140
gcttccaatg aaaatatgga gactatggaa tcaagtacac tggaactgag aagcaggtac	1200
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atggcagcat tcaatgggaa tacagagggg agaacatctg acatgaggac cgaaatcatc	1380
aggatgatgg aaagtgaag accagaagat gtgtctttcc aggggcgggg agtcttcgag	1440
ctctcgagc 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

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ggagtgggaag tcgtcaatgc aactgaaaca gtggaacgaa caaacatccc caggatctgc	180
tcaaaaggga aaaggacagt tgacctcggg caatgtggac tcctggggac aatcactgga	240
ccacctcaat gtgaccaatt cctagaattt tcagccgatt taattattga gaggcgagaa	300
ggaagtgatg tctgttatcc tgggaaatc gtgaatgaag aagctctgag gcaaattctc	360
agagaatcag gcggaattga caaggaagca atgggattca catacagtgg aataagaact	420
aatggagcaa ccagtgcag taggagatca ggatcttcat tctatgcaga aatgaaatgg	480
ctcctgtcaa acacagataa tgctgcattc ccgccagatg actaagtcac 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
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cttgccattt cagaacatag atagcagggc agttggaaaa tgtccgagat atgttaagca	960
aaggagtctg ctgctagcaa cagggatgaa gaatgttct gagattccaa agggaagagg	1020
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acaatttgag ttgatagaca atgaattcaa tgaggtagag aagcaaatcg gtaatgtgat 1260
aaattggacc agagattcta taacagaagt gtggtcatac aatgctgaac tcttggtagc 1320
aatggagaac cagcatacaa ttgatctggc tgattcagaa atggacaaac tgtacgaacg 1380
agtgaagaaga cagctgagag agaatgctga agaagatggc actggttgct ttgaaatatt 1440
tcacaagtgt gatgatgact gtatggccag tattagaaat aacacctatg atcacagcaa 1500
atacagggaa gaggcaatgc aaaatagaat acagattgac ccagtcaaac taagcagcgg 1560
ctacaagat gtgatacttt gggttagctt cggggcatca tgtttcatac ttctagccat 1620
tgtaatgggc cttgtcttca tatgtgtaaa gaatggaaac atgcggtgca ctatttgat 1680
ataa 1684

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<210> SEQ ID NO 21

<211> LENGTH: 560

<212> TYPE: PRT

<213> ORGANISM: Influenza virus

<400> SEQUENCE: 21

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      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

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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

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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 ctaagggtct ctgtactata aattcatggc acatatatgg gaaagacaat      300
gcagtaagaa ttggagagag ctcggatggt ttagtcacaa gagaacccta tgtttcatgc      360
gaccagatg  aatgcaggtt ctatgctctc agccaaggaa caacaatcag agggaaacac      420
tcaaacggaa caatacagc taggtcccag tatcgcgccc tgataagctg gccactatca      480

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tcaccgcccc cagtgtacaa cagcaggggtg gaatgcattg ggtgggtcaag tactagtgtgc 540
catgatggca aatccaggat gtcaatatgt atatcaggac caaacaacaa tgcattctgca 600
gtagtatggt acaacagaag gctgtgtgca gaaattaaca catggggccc aaacatacta 660
agaacacagg aatctgaatg tgtatgccac aacggcggtat gccagtagt gttcacccgat 720
gggtctgcca ctggacctgc agacacaaga atatactatt ttaaagaggg gaaaatattg 780
aaatgggagt ctctgactgg aactgctaag catattgaag aatgctcatg ttacgggggaa 840
cgaacaggaa ttacctgcac atgcaggagc aattggcagg gctcaaatac accagtgtatt 900
cagatagacc cagtagcaat gacacacact agtcaatata tatgcagtc tttctttaca 960
gacaatcccc gaccgaatga cccaaatata ggtaagtgt atgaccctta tccaggtaat 1020
aataacaatg gagtcaaggg attctcatal ctggatgggg ctaacacttg gctagggagg 1080
acaataagca cagcctcgag gtctggatac gagatgttaa aagtgcctaa tgcattgaca 1140
gatgatagat caaagcccat tcaaggctcag acaattgtat taaacgctga ctggagtgggt 1200
tacagtggat ctttcatgga ctattgggct gaagggggact gctatcgagc gtgtttttat 1260
gtggagttag tacgtggaag acccaaggaa gataaagtgt ggtggaccag caatagtata 1320
gtatcgatgt gttccagtac agaattctg ggacaatgga actggcctga tggggctaaa 1380
atagagtact tctcttaa 1398

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

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<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 attgcatca ttccaacaaa tgcagacaaa	60
atctgcctcg gacatcatgc tgtgtcaaac ggaaccaaag taaacacatt aactgaaaga	120
ggagtgggaag tcgtcaatgc aactgaaaca gtggaacgaa caaacatccc caggatctgc	180
tcaaaagggg aaaggacagt tgacctcggg caatgtggac tcctggggac aatcactgga	240
ccacctcaat gtgaccaatt cctagaatct 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 cagctctaata agtatggggg atccatcatt ccggatcaac tgcagagcaa	600
accaagctat atgggagtgg aaacaaactg gtgacagttg ggagttctaa ttatcaacaa	660

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tcttttgtac cgagtcctggg 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 tgtaagcaa	960
aggagtctgc tgctagcaac agggatgaag aatgttcctg agattccaaa gggaagaggc	1020
ctatttggtg ctatagcggg tttcattgaa aatggatggg aaggcctaata tgatggttgg	1080
tatggtttca gacaccagaa tgcacaggga gagggaaactg ctgcagatta caaaagcact	1140
caatcgga caa ttgatcaaat aacaggaaaa ttaaaccggc ttatagaaaa aaccaaccaa	1200
caatttgagt tgatagacaa tgaattcact gaggtagaga agcaaatcgg taatgtgata	1260
aattggacca gagattctat aacagaagtg tggtcataca atgctgaact cttggtagca	1320
atggagaacc agcatacaat tgatctggct gattcagaaa tggacaaaact gtacgaacga	1380
gtgaaaagac agctgagaga gaatgctgaa gaagatggca ctggttgctt tgaaatattt	1440
cacaagtgtg atgatgactg tatggccagc attagaaata acacctatga tcacagcaaa	1500
tacaggggaag aggcaatgca aaatagaata cagattgacc cagtcaaaact aagcagcggc	1560
tacaaagatg tgatactttg gtttagcttc ggggcatcat gtttcatact tctagccatt	1620
gcaatggggc ttgtcttcat atgtgtaaag aatggaaaaca 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 aaacaacat caccaacatc caaatggaag agagaacaag caggaatttc 240
aataacttaa ctaaagggtc ctgtactata aattcatggc acatatatgg gaaagacaat 300
gcggttaagaa ttggagagag ctcggatggt ttagtcacaa gagaacccta tgtttcatgc 360
gaccagatg aatgcagggt ctatgctctc agccaaggaa caacaatcag aggaaaacac 420
tcaaacggaa caatacacga taggtcccag tatcgcgccc tgataagctg gccactatca 480
tcaccgccc cagtgtacaa cagcagggtg gaatgcattg ggtggtaag tactagtgc 540
catgatggca aatccaggat gtcaatatgt atatcaggac caaacaacaa tgcattctgca 600
gtagtatggt acaacagaag gcctgttgca gaaattaaca catgggccc aaacatacta 660
agaacacagg aatctgaatg tgtatgccac aacggcgat gccagtagt gttcaccgat 720
gggtctgcca ctggacctgc agacacaaga atatactatt ttaaagagg gaaaatttg 780
aaatgggagt ctctgactgg aactgctaag catattgaag aatgctcatg ttacggggaa 840
cgaacaggaa ttacctgcac atgcaaggac aattggcagg gctcaaata accagtgatt 900
cagatagatc cagtagcaat gacacacact agtcagtata tatgcagtc tgttcttaca 960
gacaatcccc gaccgaatga ccaaatata ggtaagtgt atgacctta tccaggtaat 1020
aataacaatg gagtcaaggg attctcatc ctggatgggg ctaacacttg gctagggagg 1080
acaataagca cagcctcgag gtcctggatac gagatgttaa aagtgccaa tgcattgaca 1140
gatgatagat caaagcccat tcaaggctag acaattgtat taaacgctga ctggagtgg 1200
tacagtggat ctttcatgga ctattgggct gagggggact gctatcgagc gtgtttttat 1260
gtggaattga tacgtggaag acccaaggag gataaagtgt ggtggaccag caatagtata 1320
gtatcgatgt gttccagtac agaattcctg ggacaatgga actggcctga tggggctaaa 1380
atagagtact tcctctaa 1398

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<210> SEQ ID NO 27

<211> LENGTH: 462

<212> TYPE: PRT

<213> ORGANISM: Influenza virus

<400> SEQUENCE: 27

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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 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

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202L and/or 323L in PB2 and has 247H in PB1, or has 202L and/or 323L in PB2, has 247H in PB1, and has 74K in NP.

5. The isolated virus of claim 1, further comprising least one of the PA, PB1, PB2, NP, NS, and M viral segments has a C to U promoter mutation.

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

7. The isolated virus of claim 1, wherein the NA viral segment and the HA viral segment are from the same influenza virus isolate.

8. The isolated 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.

9. The isolated 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: 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.

10. The isolated virus of claim 9, which 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.

11. The isolated virus of claim 1, wherein the HA viral segment encodes a HA with one or more of 136D, 162E, 179L, 182V, 184I, 252I, 449E, or 476I.

12. The isolated virus of claim 1, wherein the NA viral segment encodes a NA with 55S, and/or 265V.

13. 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 segment encodes a polypeptide having a residue other than glycine, serine, serine, glutamine or asparagine at position 62, 261, 361, 621, and/or 654, respectively, in PB1 or a residue other than arginine at position 81 in F2, 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.

14. The method of claim 13, wherein the cell is an avian cell or a mammalian cell.

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

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16. The method of claim 13, 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. 5

17. The method of claim 13, further comprising isolating the virus.

18. The method of claim 13, wherein at least one of PA, PB1, or PB2 viral segments has a C to U promoter mutation. 10

19. A vector for vRNA or mRNA expression of a) influenza virus PB1 having at least 85% amino acid sequence identity to a polypeptide encoded by SEQ ID NO:2 and an alanine at residue 62, a glycine at residue 261, an arginine at residue 361, an arginine at residue 621, and/or a serine at residue 654, or a combination thereof; or b) influenza virus 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 d) influenza virus NA having a serine at position 55 or a valine at position 265, or both. 15 20

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