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(12) **United States Patent**
Kawaoka et al.(10) **Patent No.:** **US 11,390,649 B2**(45) **Date of Patent:** **Jul. 19, 2022**(54) **INFLUENZA VIRUS REPLICATION FOR VACCINE DEVELOPMENT**(71) Applicant: **Wisconsin Alumni Research Foundation (WARF)**, Madison, WI (US)(72) Inventors: **Yoshihiro Kawaoka**, Middleton, WI (US); **Gabriele Neumann**, Madison, WI (US); **Jihui Ping**, Nanjing (CN)(73) Assignee: **Wisconsin Alumni Research Foundation (WARF)**, Madison, WI (US)

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Primary Examiner — Barry A Chestnut(74) *Attorney, Agent, or Firm* — Schwegman Lundberg & Woessner, P.A.(57) **ABSTRACT**

An isolated recombinant influenza virus is provided having PA, PB1, PB2, NP, NS, M, NA and HA viral segments, wherein the PB1 viral segment encodes a PB1 with a residue other than isoleucine at position 711 or the M viral segment encodes a M1 with a residue other than methionine at position 128, wherein the recombinant influenza virus has enhanced replication relative to a corresponding influenza virus having a PB1 viral segment that encodes a PB1 with an isoleucine at position 711 or having a M viral segment that encodes a M1 with methionine at position 128, as well as methods of making and using the virus.

20 Claims, 64 Drawing Sheets**Specification includes a Sequence Listing.**

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PR8(CAMBRIDGE)

PB2

AGCGAAAGCAGGTCAATTATATTCAATATGGAAAGAATAAAAGAACTAAGAAATCTAATGTCGCAGTCTCGCACCCGCGAGATA
CTCACAAAAACCACCGTGGACCATATGGCCATAATCAAGAAGTACACATCAGGAAGACAGGAGAAGAACCAGCACTTAGGATG
AAATGGATGATGGCAATGAAATATCCAATTACAGCAGACAAGAGGATAACGGAAATGATTCTTGAGAGAAATGAGCAAGGACAA
ACTTTATGGAGTAAAATGAATGATGCCGGATCAGACCGAGTGATGGTATCACCTCTGGCTGTGACATGGTGGAATAGGAATGGA
CCAATGACAAATACAGTTTATTATCCAAAAATCTACAAAACCTTATTTTGAAAGAGTCGAAAGGCTAAAGCATGGAACCTTTGGC
CCTGTCCATTTTAGAAACCAAGTCAAAATACGTCGGAGAGTTGACATAAATCCTGGTTCATGCAGATCTCAGTGCCAAGGAGGCA
CAGGATGTAATCATGGAAGTTGTTTTCCCTAACGAAGTGGGAGCCAGGATACTAACATCGGAATCGCAACTAACGATAACCAAA
GAGAAGAAAGAAGAACTCCAGGATTGCAAAATTTCTCCTTTGATGGTTGCATACATGTTGGAGAGAGAAGTGGTCCGCAAAACG
AGATTCCTCCAGTGGCTGGTGGAAACAGCAGTGTGTACATTGAAGTGTTCATTTGACTCAAGGAACATGCTGGGAACAGATG
TATACTCCAGGAGGGGAAGTGAAGAATGATGATGTTGATCAAAGCTTGATTATTGCTGCTAGGAACATAGTGAGAAGAGCTGCA
GTATCAGCAGACCCACTAGCATCTTTATTGGAGATGTGCCACAGCACACAGATTGGTGGAAATTAGGATGGTAGACATCCTTAAG
CAGAACCACAAGAGAGCAAGCCGTGGATATATGCAAGGCTGCAATGGGACTGAGAATTAGCTCATCCTTCAGTTTTGGTGGG
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CTGATAGTGAGTGGGAGAGACGAACAGTCGATTGCCGAAGCAATAATTGTGGCCATGGTATTTTCAAGAGGATTGTATGATA
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GTGAGCATTGACCGGTTCTTGAGAGTCAGGGACCAACGAGGAAATGTACTACTGTCTCCCGAGGAGGTGAGTGAAACACAGGGA
ACAGAGAAACTGACAATAACTTACTCATCGTCAATGATGTGGGAGATTAATGGTCCTGAATCAGTGTTGGTCAATACCTATCAA
TGGATCATCAGAAACTGGGAAACTGTAAAATTCAGTGGTCCCAGAACCTACAATGCTATACAATAAAATGGAATTTGAACCA
TTTCAGTCTTTAGTACCTAAGGCCATTAGAGGCCAATACAGTGGGTTTGTAAGAACTCTGTTCCAACAAATGAGGGATGTGCTT
GGGACATTTGATACCGCACAGATAATAAACTTCTTCCCTTCGCAGCCGCTCCACCAAAGCAAAGTAGAATGCAGTTCTCCTCA
TTTACTGTGAATGTGAGGGGATCAGGAATGAGAATACTTGTAAGGGGCAATTCTCCTGTATTCAACTACAAACAGGCCACGAAG
AGACTCACAGTTCTCGAAAGGATGCTGGCACTTTAACCAGAACCCAGATGAAGGCACAGCTGGAGTGGAGTCCGCTGTTCTG
AGGGGATTCTCATTCTGGGCAAGAAGACAGGAGATATGGGCCAGCATTAAAGCATCAATGAACTGAGCAACCTTGCAGAAAGGA
GAGAAGGCTAATGTGCTAATTGGGCAAGGAGACGTGGTGTGGTAATGAAACGAAACGGGACTCTAGCATACTTACTGACAGC
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Fig. 1A

(SEQ ID NO:11)

PR8(CAMBRIDGE)

PB1

AGCGAAAGCAGGCAAACCATTTGAATGGATGTCAATCCGACCTTACTTTTCTTAAAAGTGCCAGCACAAAATGCTATAAGCACA
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TACTCAGAAAAGGGAAGATGGACAACAAACACCGAAACTGGAGCACCGCAACTCAACCCGATTGATGGGCCACTGCCAGAAGAC
AATGAACCAAGTGGTTATGCCCAAACAGATTGTGTATTGGAAGCAATGGCTTTCTTGAGGAATCCCATCCTGGTATTTTTGAA
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TTAAATAGAAAACAGCCTGCTGCAACAGCATTGGCCAACACAATAGAAGTGTTGAGATCAAATGGCCTCACGGCCAATGAGTCA
GGAAGGCTCATAGACTTCCTTAAGGATGTAATGGAGTCAATGAAAAAGAAGAAATGGGGATCACTCACTCATTTCAGAGAAAG
AGACGGGTGAGAGACAATATGACTAAGAAAATGATAACACAGAGAAACAATAGGTAAAAGGAAACAGAGATTGAACAAAAGGGGT
TATCTAATTAGAGCATTGACCCTGAACACAATGACCAAAGATGCTGAGAGAGGGAAGCTAAAACGGAGAGCAATTGCAACCCCA
GGGATGCAAATAAGGGGGTTTGTATACTTTGTTGAGACACTGGCAAGGAGTATATGTGAGAACTTGAACAATCAGGGTTGCCA
GTTGGAGGCAATGAGAAGAAAGCAAAGTTGGCAAATGTTGTAAGGAAGATGATGACCAATTCTCAGGACACCGAACTTTCTTTC
ACCATCACTGGAGATAACACCAAATGGAACGAAAATCAGAATCCTCGGATGTTTTTGGCCATGATCACATATATGACCAGAAAT
CAGCCCGAATGGTTGAGAAATGTTCTAAGTATTGCTCCAATAATGTTCTCAACAAAATGGCGAGACTGGGAAAAGGGTATATG
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CTTCAATCCTCTGACGATTTTGCTCTGATTGTGAATGCACCCAATCATGAAGGGATTCAAGCCGGAGTCGACAGGTTTTATCGA
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TGCAATTTATTTGAAAAATTCTTCCCAGCAGTTCATACAGAAGACCAGTCGGGATATCCAGTATGGTGGAGGCTATGGTTTCC
AGAGCCCGAATTGATGCACGGATTGATTTGGAATCTGGAAGGATAAAGAAAGAAGAGTTCACTGAGATCATGAAGATCTGTTCC
ACCATGGAAGAGCTCAGACGGCAAAAATAGTGAATTTAGCTTGTCTTCATGAAAAAATGCCTTGTTTCTACT

Fig. 1B

(SEQ ID NO:10)

PR8(CAMBRIDGE)

PA

AGCGAAAGCAGGTA CTGATTCAAAATGGAAGATTTTGTGCGACAATGCTTCAATCCGATGATTGTCGAGCTTGCGGAAAAAACA
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TCAGATTTCCACTTCATCAATGAGCAAGGCGAGTCAATAATCGTAGAACTTGGTGATCCTAATGCACTTTTGAAGCACAGATTT
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TTTCTACCAGATTTGTATGATTACAAGGAAAAATAGATTCATCGAAATTGGAGTAACAAGGAGAGAAAGTTCACATATACTATCTG
GAAAAGGCCAATAAAATTAAATCTGAGAAAAACACATCCACATTTTCTCGTTCACTGGGGAAGAAATGGCCACAAGGGCCGAC
TACACTCTCGATGAAGAAAGCAGGGCTAGGATCAAAACCAGGCTATTCAACATAAGACAAGAAATGGCCAGCAGAGGCCTCTGG
GATTCCCTTTCGT CAGTCCGAGAGAGGAGAAGAGACAATTGAAGAAAGGTTTGAATCACAGGAACAATGCGCAAGCTTGCCGAC
CAAAGTCTCCCGCCGAACCTTCTCCAGCCTTGAAAATTTTAGAGCCTATGTGGATGGATTGGAACCGAACGGCTACATTGAGGGC
AAGCTGTCTCAAATGTCCAAAGAAGTAAATGCTAGAATTGAACCTTTTTTGAAAACAACACCACGACCACTTAGACTTCCGAAT
GGGCCTCCCTGTTCTCAGCGGTCCAAATTCCTGCTGATGGATGCCTTAAATTAAGCATTGAGGACCCAAGTCATGAAGGAGAG
GGAATACCGCTATATGATGCAATCAAATGCATGAGAACATTCCTTGGATGGAAGGAACCCAATGTTGTTAAACCACACGAAAAG
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TGCAGGACTTTATTAGCAAAGTCGGTATTTAACAGCTTGTATGCATCTCCACA ACTAGAAGGATTTTCAGCTGAATCAAGAAAA
CTGCTTCTTATCGTTCAGGCTCTTAGGGACAATCTGGAACCTGGGACCTTTGATCTTGGGGGGCTATATGAAGCAATTGAGGAG
TGCCTAATTAATGATCCCTGGGTTTTGCTTAATGCTTCTTGGTTCAACTCCTTCTTACACATGCATTGAGTTAGTTGTGGCAG
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(SEQ ID NO:12)

Fig. 1C

NP

PR8(CAMBRIDGE)

AGCAAAAGCAGGGTAGATAATCACTCACTGAGTGACATCAAAATCATGGCGTCCCAAGGCACCAAAACGGTCTTACGAACAGATG
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GGAGTCTTCGAGCTCTCGGACGAAAAGGCAGCGAGCCCGATCGTGCCTTCCTTTGACATGAGTAATGAAGGATCTTATTTCTTC
GGAGACAATGCAGAGGAGTACGACAATTAAGAAAAATACCCTTGTTTCTACT

(SEQ ID NO:13)

Fig. 1D

PR8(CAMBRIDGE)

M

AGCAAAAGCAGGTAGATATTGAAAGATGAGTCTTCTAACCGAGGTCGAAACGTACGTTCTCTCTATCATCCCCTCAGGCCCCCT
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AAGACCAATCCTGTACCTCTGACTAAGGGGATTTTAGGATTTGTGTTACGCTCACCGTGCCAGTGAGCGAGGACTGCAGCG
TAGACGCTTTGTCCAAAATGCCCTTAATGGGAACGGGGATCCAAATAACATGGACAAAGCAGTTAAACTGTATAGGAAGCTCAA
GAGGGAGATAACATTCATGGGGCCAAAGAAATCTCACTCAGTTATTCTGCTGGTGCACTTGCCAGTTGTATGGGCCTCATATA
CAACAGGATGGGGGCTGTGACCACTGAAGTGGCATTGCGCTGGTATGTGCAACCTGTGAACAGATTGCTGACTCCAGCATCG
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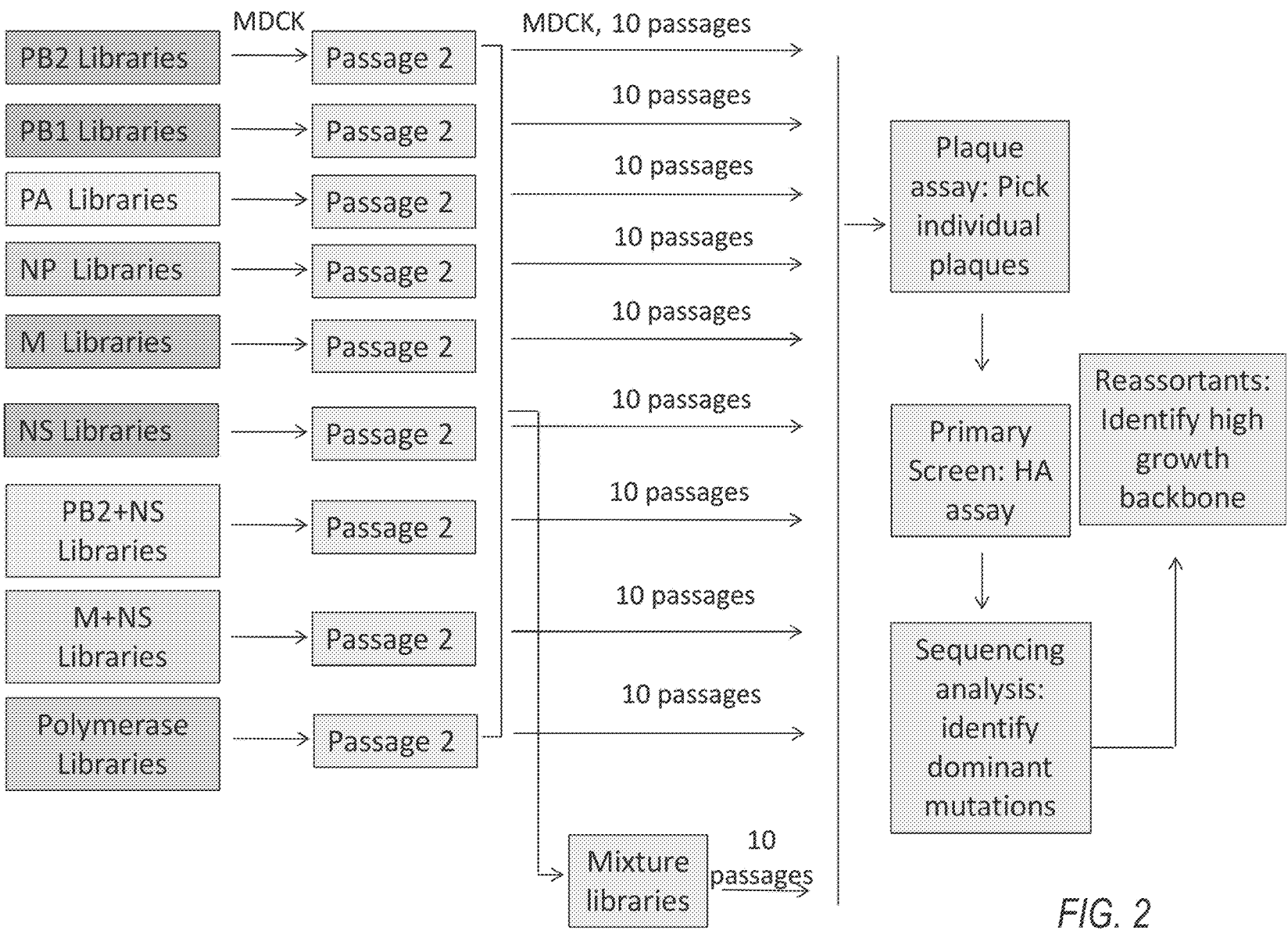
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GCACTCTTGGTCTGGACATCGAGACAGCCACACGTGCTGGAAAGCAGATAGTGGAGCGGATTCTGAAAGAAGAATCCGATGAGG
CACTTAAAATGACCATGGCCTCTGTACCTGCGTCGCGTTACCTAACCGACATGACTCTTGAGGAAAATGTCAAGGGAATGGTCCA
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CAAAACAGAAACGAGAAATGGCGGGAACAATTAGGTGAGAAGTTTGAAGAAATAAGATGGTTGATTGAAGAAGTGAGACACAAA
CTGAAGGTAAACAGAGAATAGTTTTGAGCAAATAACATTTATGCAAGCCTTACATCTATTGCTTGAAGTGGAGCAAGAGATAAGA
ACTTCTCATTTGAGCTTATTTAATAATAAAAAACACCCTTGTTTCTACT

(SEQ ID NO:15)

Fig. 1E



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%

FIG. 3

Recombinant viruses generated from dominant mutations

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

FIG. 4

Growth curve – PB2 mutants (MOI=0.001)

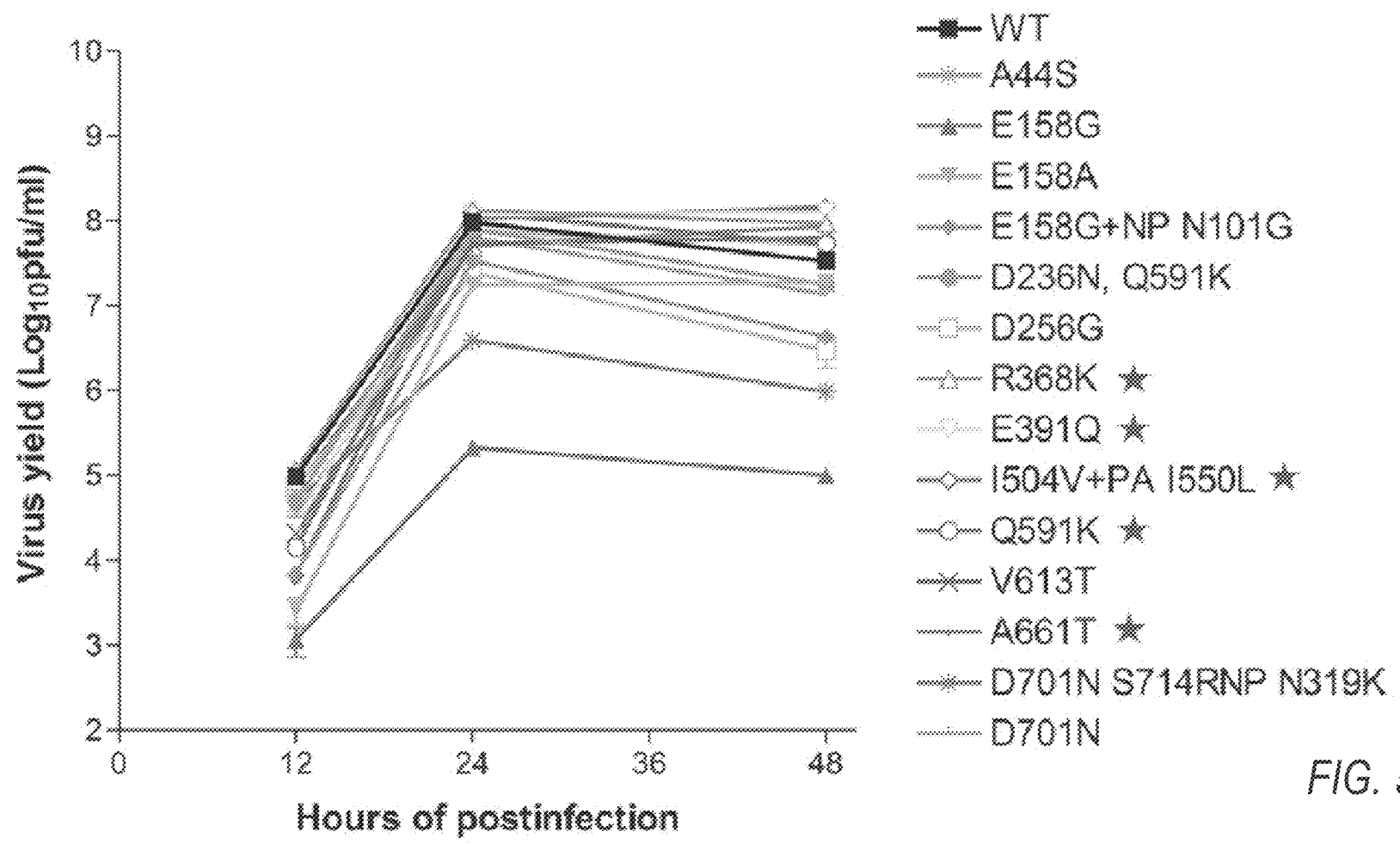


FIG. 5A

PB1 Mutants

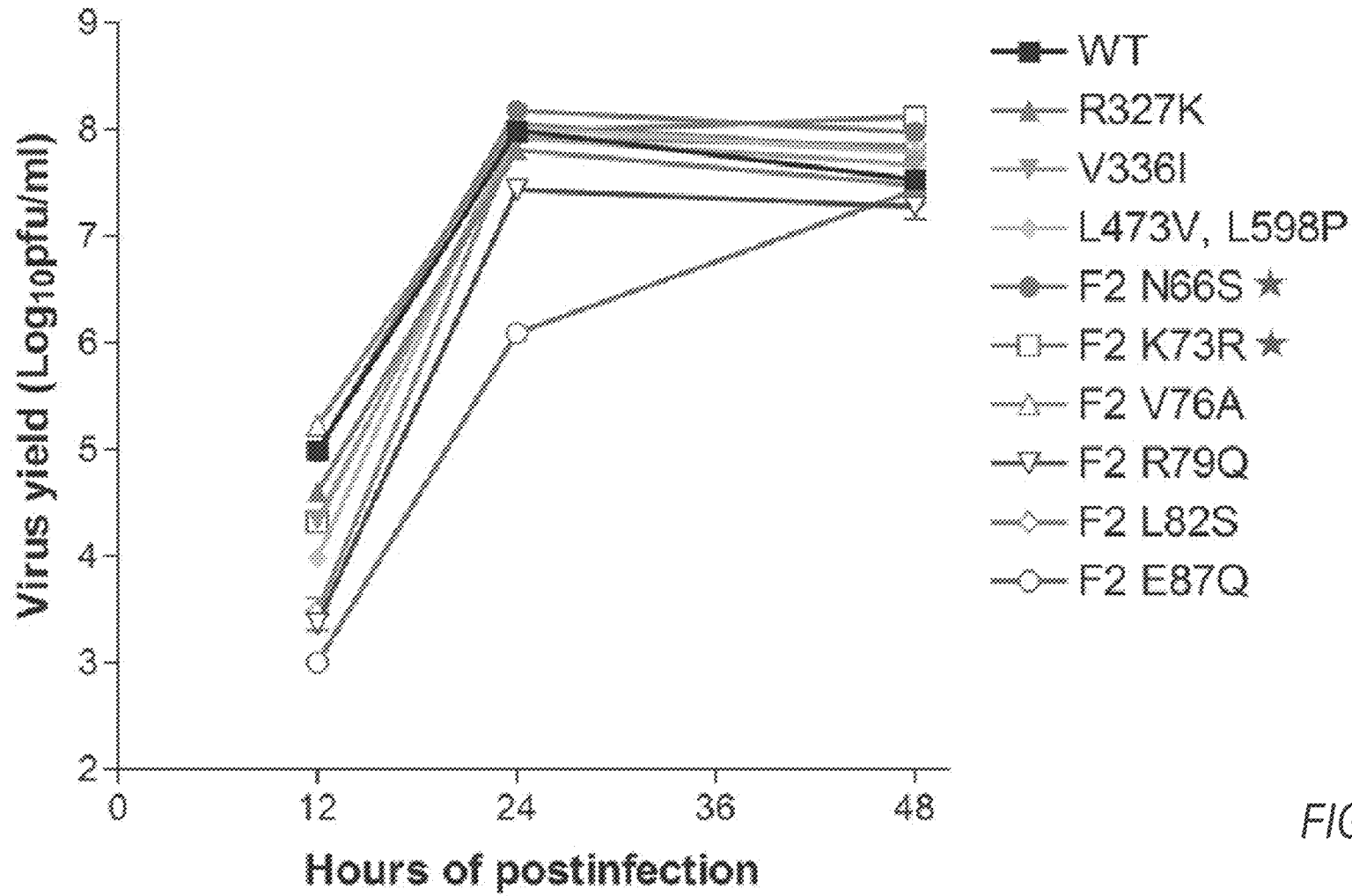


FIG. 5B

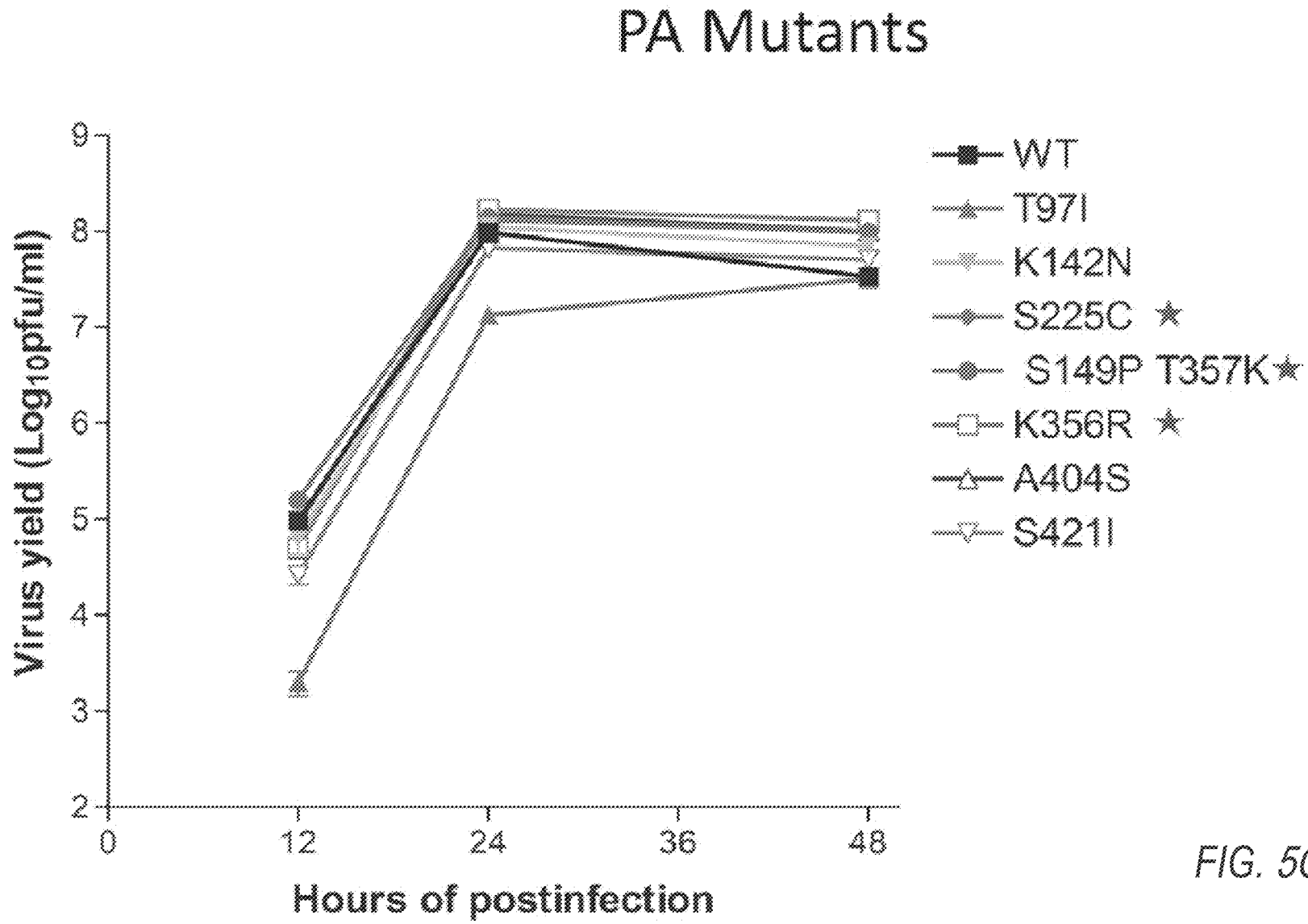


FIG. 5C

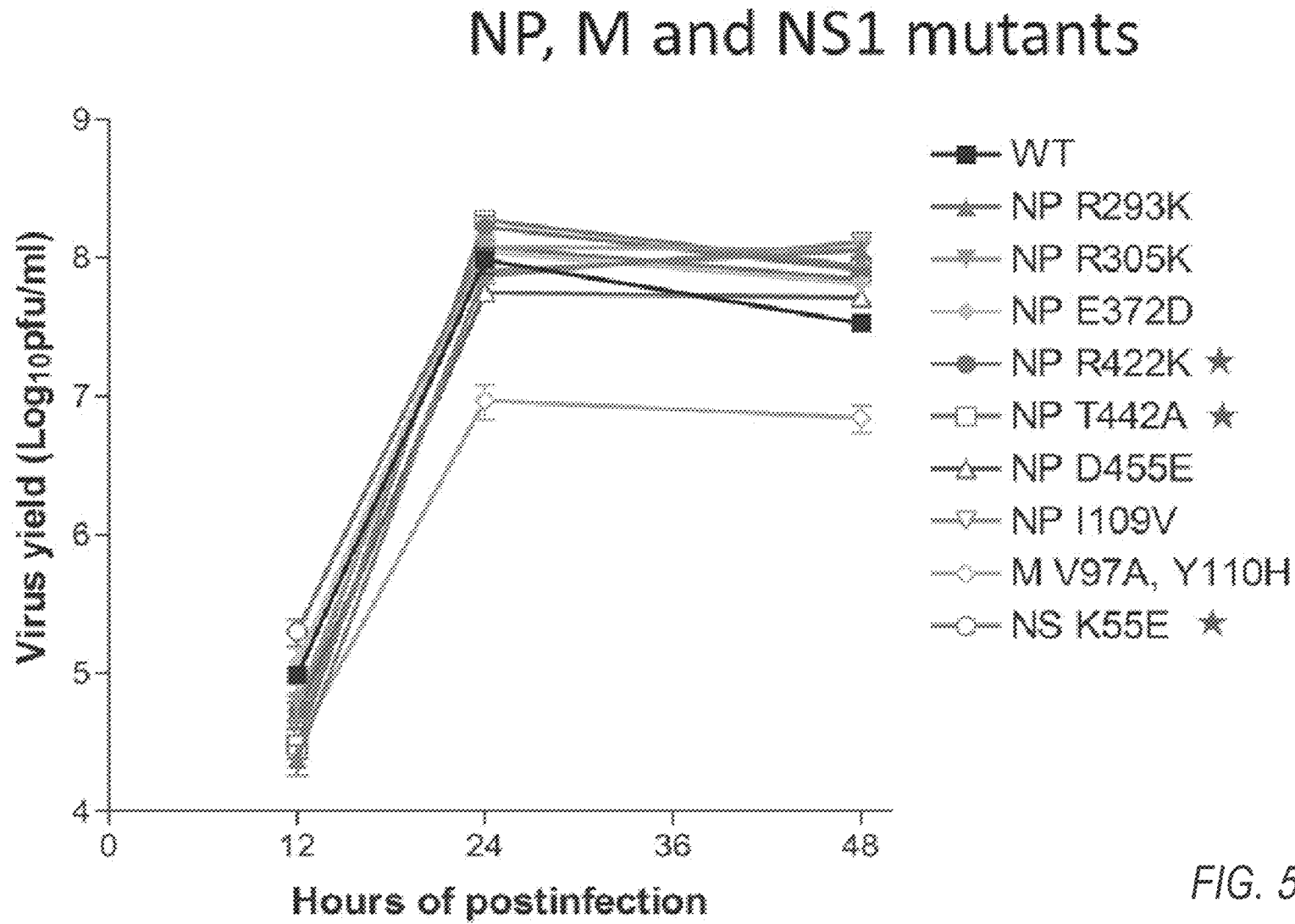


FIG. 5D

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 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>

FIG. 6

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

FIG. 7A

Growth characteristics (MOI=0.001)

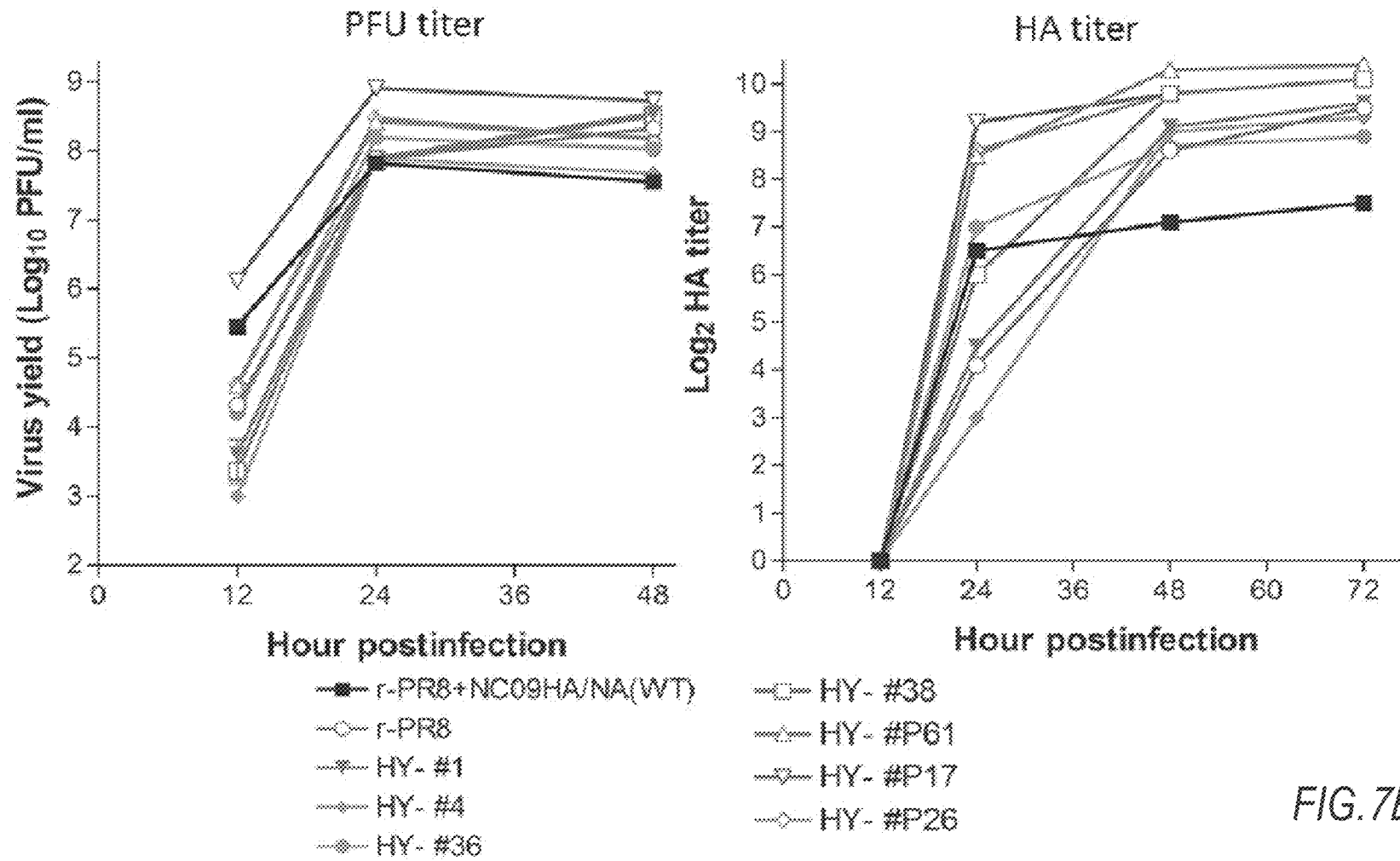


FIG. 7B

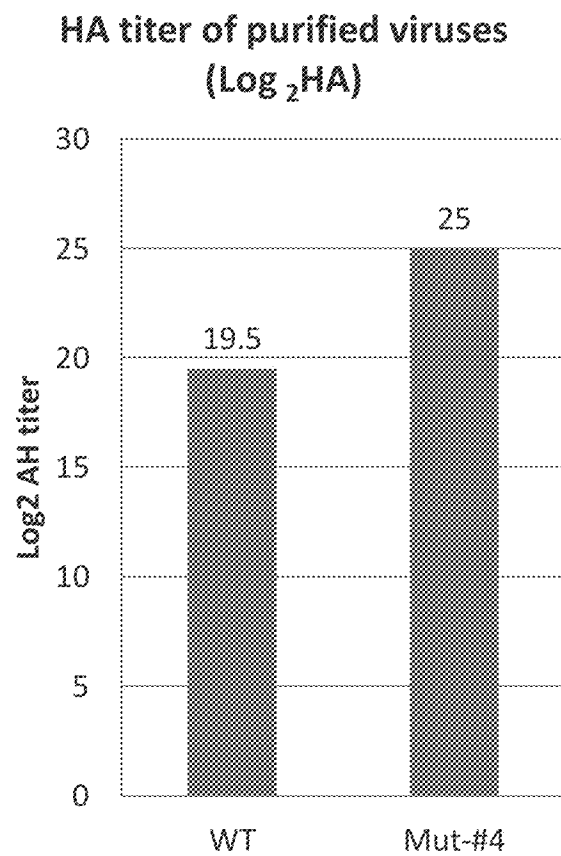


FIG. 8A

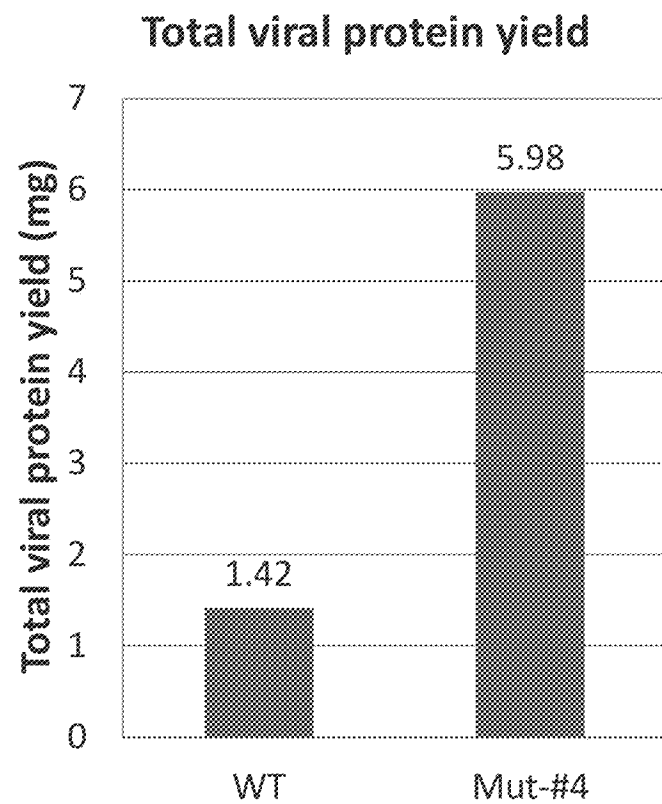
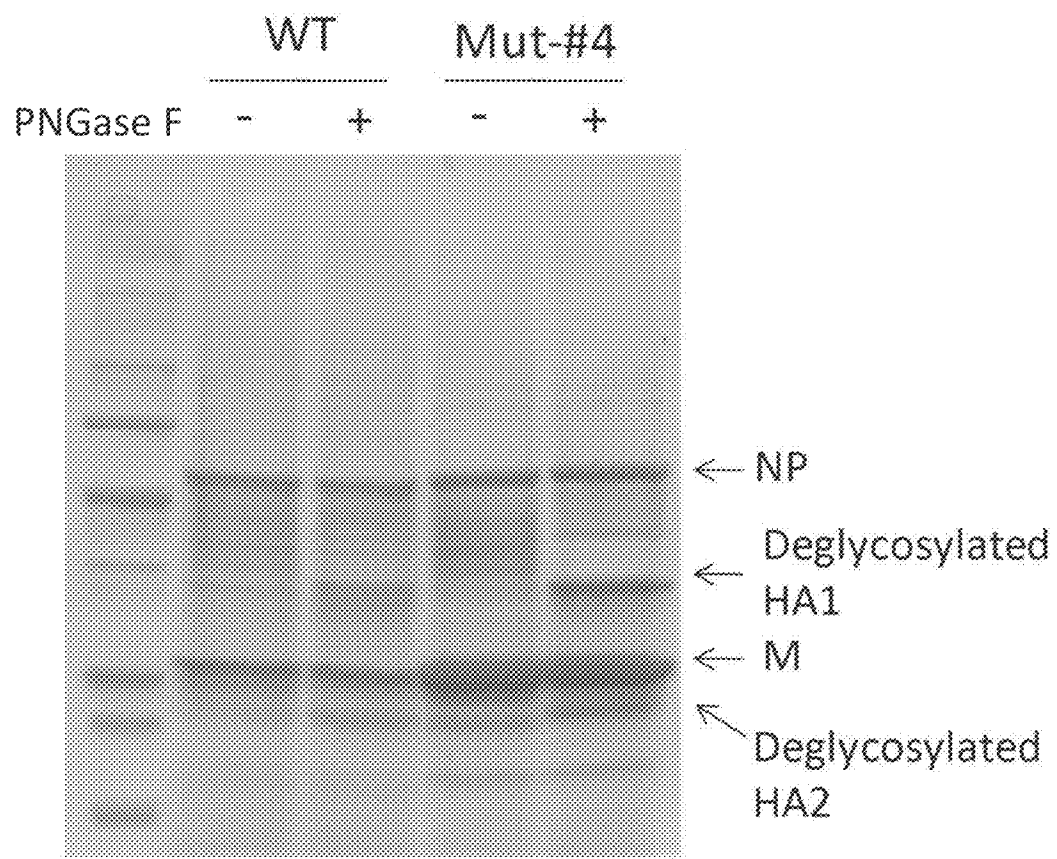


FIG. 8B

Total viral protein yield: 4.2 fold

SDS-PAGE analysis



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

FIG. 8C

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

FIG. 9A

Growth kinetics (MOI=0.001)

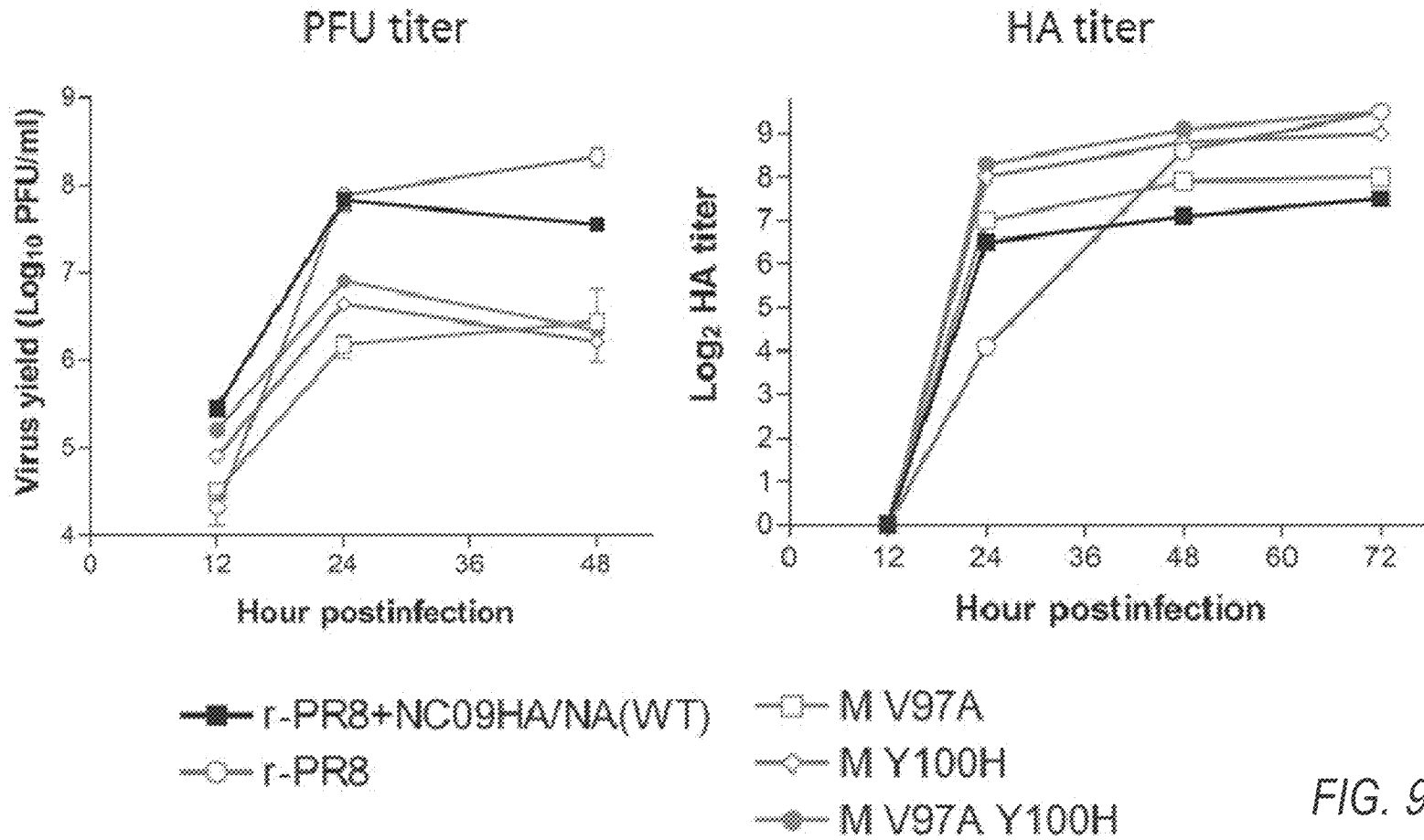


FIG. 9B

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%

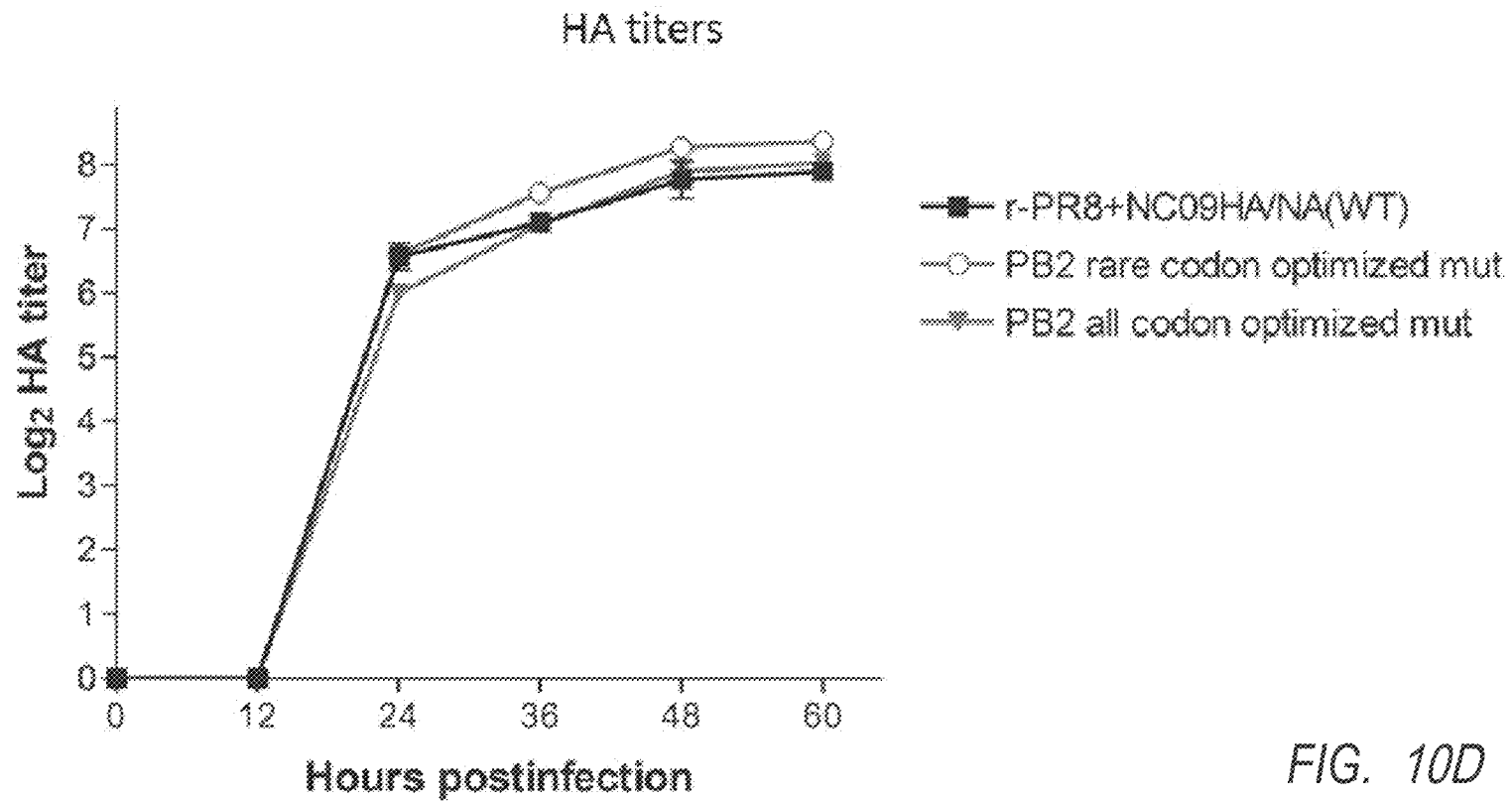
GENETIC CODE 1: STANDARD

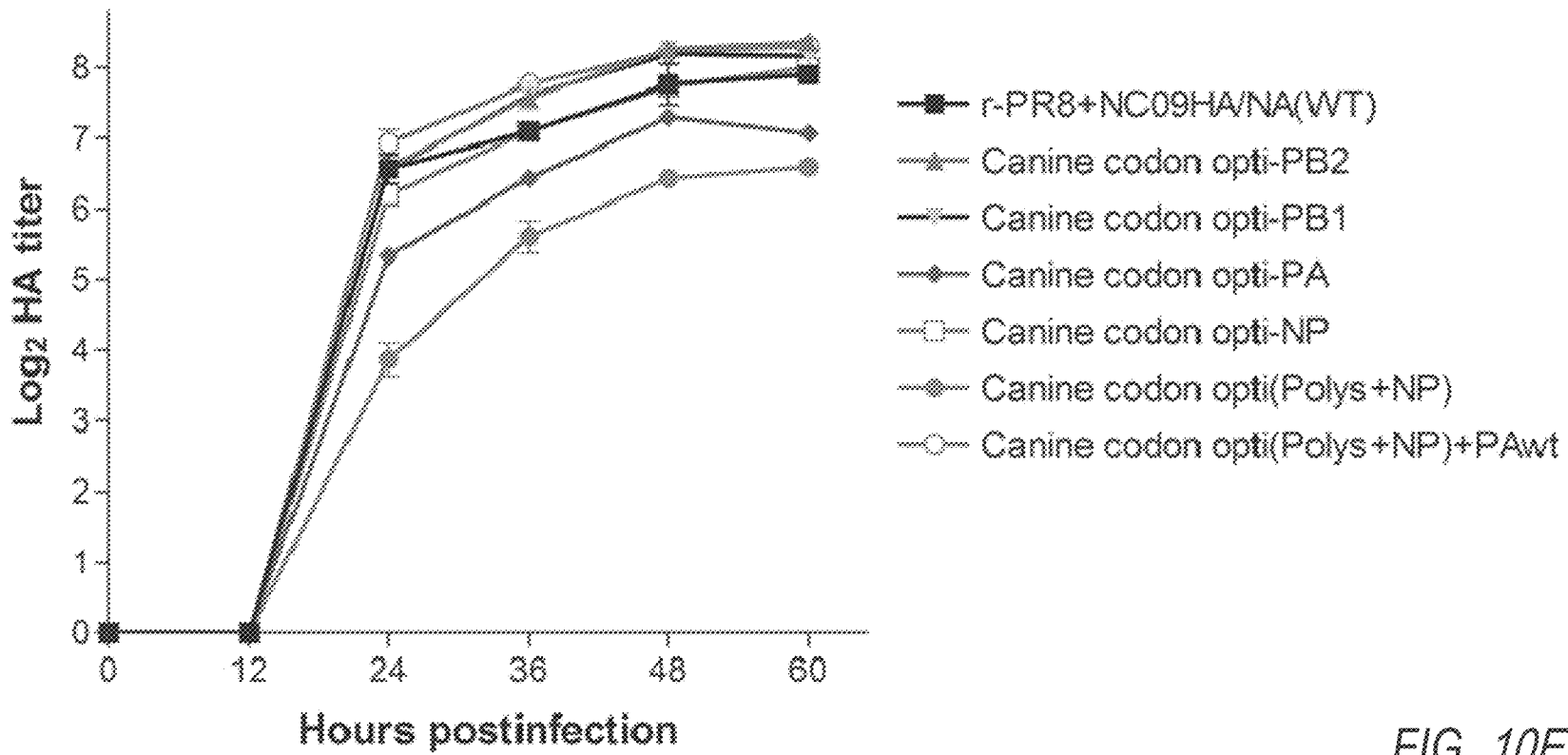
Fig. 10A

Fig. 10B

Fig. 10C

Growth kinetics in MDCK cells

*FIG. 10D*



PR8-UW PB2:

AGCGAAAGCAGGTCAATTATATTCAATATGGAAAGAATAAAAGAACTACGAAATCTAATGTCGCAGTCTCGCACCCGCGA
GATACTCACAAAAACCACCGTGGACCATATGGCCATAATCAAGAAGTACACATCAGGAAGACAGGAGAAGAAGCCAGCAC
TTAGGATGAAATGGATGATGGCAATGAAATATCCAATTACAGCAGACAAGAGGATAACGGAAATGATTCCTGAGAGAAAT
GAGCAAGGACAACTTTATGGAGTAAATGAATGATGCCGGATCAGACCGAGTGATGGTATCACCTCTGGCTGTGACATG
GTGGAATAGGAATGGACCAATAACAAATACAGTTCATTATCCAAAAATCTACAAAACCTATTTTGAAAGAGTCGAAAGGC
TAAAGCATGGAACCTTTGGCCCTGTCCATTTTAGAAACCAAGTCAAAATACGTCGGAGAGTTGACATAAATCCTGGTCAT
GCAGATCTCAGTGCCAAGGAGGCACAGGATGTAATCATGGAAGTTGTTTTCCCTAACGAAGTGGGAGCCAGGATACTAAC
ATCGGAATCGCAACTAACGATAACCAAGAGAAGAAAGAAGAACTCCAGGATTGCAAAATTTCTCCTTGATGGTTGCAT
ACATGTTGGAGAGAGAACTGGTCCGCAAAACGAGATTCCTCCAGTGGCTGGTGGAAACAAGCAGTGTGTACATTGAAGTG
TTGCATTTGACTCAAGGAACATGCTGGGAACAGATGTATACTCCAGGAGGGGAAGTGAGGAATGATGATGTTGATCAAAG
CTTGATTATTGCTGCTAGGAACATAGTGAGAAGAGCTGCAGTATCAGCAGATCCACTAGCATCTTTATTGGAGATGTGCC
ACAGCACACAGATTGGTGGAAATTAGGATGGTAGACATCCTTAGGCAGAACCCAACAGAAGAGCAAGCCGTGGATATATGC
AAGGCTGCAATGGGACTGAGAATTAGCTCATCCTTCAGTTTTGGTGGATTACATTTAAGAGAACAAGCGGGATCATCAGT
CAAGAGAGAGGAAGAGGTGCTTACGGGCAATCTTCAAACATTGAAGATAAGAGTGCATGAGGGATATGAAGAGTTCACAA
TGGTTGGGAGAAGAGCAACAGCCATACTCAGAAAAGCAACCAGGAGATTGATTAGCTGATAGTGAGTGGGAGAGACGAA
CAGTCGATTGCCGAAGCAATAATTGTGGCCATGGTATTTTACAAAGAGGATTGTATGATAAAAGCAGTCAGAGGTGATCT
GAATTCGTCATAGGGCGAATCAACGATTGAATCCTATGCATCAACTTTTAAGACATTTTCAGAAGGATGCGAAAGTGC
TTTTTCAAATTTGGGGAGTTGAACCTATCGACAATGTGATGGGAATGATTGGGATATTGCCCGACATGACTCCAAGCATC
GAGATGTCAATGAGAGGAGTGAGAATCAGCAAAATGGGTGTAGATGAGTACTCCAGCACGGAGAGGGTAGTGGTGAGCAT
TGACCGTTTTTTGAGAATCCGGGACCAACGAGGAAATGTACTACTGTCTCCCGAGGAGGTCAGTGAAACACAGGGAACAG
AGAACTGACAATAACTTACTCATCGTCAATGATGTGGGAGATTAATGGTCTGAATCAGTGTGGTCAATACCTATCAA
TGGATCATCAGAACTGGGAACTGTAAAATTCAGTGGTCCAGAACCTACAATGCTATACAATAAAATGGAATTTGA
ACCATTTAGTCTTTAGTACCTAAGGCCATTAGAGGCCAATACAGTGGGTTTGTAAAGAACTCTGTTCCAACAAATGAGGG
ATGTGCTTGGGACATTTGATACCGCACAGATAATAAACTTCTTCCCTTCGCAGCCGCTCCACCAAAGCAAAGTAGAATG
CAGTTCTCCTCATTTACTGTGAATGTGAGGGGATCAGGAATGAGAATACTTGTAAAGGGCAATTCCTCTGTATTCAACTA
TAACAAGGCCACGAAGAGACTCACAGTCTCGGAAAGGATGCTGGCACTTTAACTGAAGACCCAGATGAAGGCACAGCTG
GAGTGGAGTCCGCTGTTCTGAGGGGATTCCTCATTCTGGGCAAAGAAGACAAGAGATATGGGCCAGCACTAAGCATCAAT
GAACTGAGCAACCTTGCGAAAGGAGAGAAGGCTAATGTGCTAATTGGGCAAGGAGACGTGGTGTGGTAATGAAACGGAA
ACGGGACTCTAGCATACTTACTGACAGCCAGACAGCGACCAAAAGAATTCCGGATGGCCATCAATTAGTGCGAATAGTTT
AAAAACGACCTTGTCTTCTACT (SEQ ID NO:3)

FIG. 10F

Canine codon optimized PR8-PB2:

AGCGAAAGCAGGTCAATTATATTCAATATGGAAAGAATAAAAGAACTACGAAATCTAATGTCGCAGTCTCGCACCCGCGA
GATACTCACAAAAACCACCGTGGACCATATGGCCATAATCAAGAAGTACACATCAGGAAGACAGGAGAAGAACCCAGCAC
TGAGGATGAAATGGATGATGGCAATGAAATATCCAATTACAGCAGACAAGAGGATCACCGAAATGATTCTGAGAGAAAT
GAGCAGGGACAGACTCTGTGGAGTAAATGAATGATGCCGGATCAGACCGAGTGATGGTGTACCTCTGGCTGTGACATG
GTGGAATAGGAATGGACCAATCACAAATACAGTGCATTATCCAAAAATCTACAAAACCTTATTTTGAAAGAGTCGAAAGGC
TGAAGCATGGAACCTTTGGCCCTGTCCATTTTAGAAACCAGGTCAAAATCCGGCGGAGAGTGACATCAATCCTGGTCAT
GCAGATCTCAGTGCCAAGGAGGCACAGGATGTGATCATGGAAGTGGTGTCCCTAACGAAGTGGGAGCCAGGATTCTGAC
ATCCGAATCCAGCTGACCATTACCAAAGAGAAGAAAGAAGAACTCCAGGATTGCAAAATTTCTCCTCTGATGGTGGCAT
ACATGCTGGAGAGAGAACTGGTCCGCAAAACAAGATTCTCCAGTGGCTGGTGGAAACAAGCAGTGTGTACATTGAAGTG
CTGCATCTGACTCAGGGAACATGCTGGGAACAGATGTATACTCCAGGAGGGGAAGTGAGGAATGATGATGTGGATCAGAG
CCTGATTATTGCTGCTAGGAACATTGTGAGAAGAGCTGCAGTGTGAGCAGATCCACTGGCATCTCTGCTGGAGATGTGCC
ACAGCACACAGATTGGTGGGAATTAGGATGGTGGACATCCTGAGGCAGAACCAACAGAAGAGCAGGCCGTGGATATTTGC
AAGGCTGCAATGGGACTGAGAATTAGCTCATCTTCAGTTTTGGTGGATTACATTTAAGAGAACAAGCGGATCATCAGT
CAAGAGAGAGGAAGAGGTGCTGACCGCAATCTGCAGACACTGAAGATCAGAGTGCATGAGGGATATGAAGAGTTCACAA
TGGTGGGGAGAAGAGCAACAGCCATCCTCAGAAAAGCAACCAGGAGACTGATTCAGCTGATCGTGAGTGGGAGAGACGAA
CAGTCCATTGCCGAAGCAATTATTGTGGCCATGGTGTTCACAGGAGGATTGTATGATTAAAGCAGTCAGAGGTGATCT
GAATTCGTCATAGGGCCAATCAGCGACTGAATCCTATGCATCAGCTGCTGAGACATTTTCAGAAGGATGCCAAAGTGC
TGTTTCAGAATTGGGGAGTGGAACCTATCGACAATGTGATGGGAATGATTGGGATCCTGCCCAGCATGACTCCAAGCATC
GAGATGTCAATGAGAGGAGTGAGAATCAGCAAAATGGGTGTGGATGAGTACTCCAGCACCGAGAGGGTCTGGTGAGCAT
TGACAGATTTCTGAGAATCCGGGACCAGCGAGGAAATGTGCTCCTGTCTCCGAGGAGGTGAGTGAACACAGGGAACAG
AGAAACTGACAATTACTTACTCATCCTCAATGATGTGGGAGATTAATGGTCCTGAATCAGTGTGGTCAATACCTATCAG
TGGATCATCAGAACTGGGAACTGTGAAAATTCAGTGGTCCAGAACCTACAATGCTGTACAATAAAATGGAAATTTGA
ACCATTTAGTCTCTGGTGCCTAAGGCCATTAGAGGCCAGTACAGTGGGTTTGTGAGAACTCTGTTCCAGCAGATGAGGG
ATGTGCTGGGGACATTTGATACCGCACAGATTATTAAGTGTGCTGCCCTTCGAGCCGCTCCACCAAAGCAGAGTAGAATG
CAGTTCTCCTCATTTACTGTGAATGTGAGGGGATCAGGAATGAGAATCCTGGTGAGGGGCAATTCCTCTGTGTTCAACTA
TAACAAGGCCACCAAGAGACTCACAGTGTCTGGAAAGGATGCTGGCACTCTGACTGAAGACCCAGATGAAGGCACAGCTG
GAGTGGAGTCCGCTGTGCTGAGGGGATTCCTCATTCTGGGCAAGAAGACAAGAGATATGGGCCAGCACTGAGCATCAAT
GAACTGAGCAACCTGGCCAAAGGAGAGAAGGCTAATGTGCTAATTGGGCAAGGAGACGTGGTGTGGTAAATGAAACGGAA
ACGGGACTCTAGCATACTTACTGACAGCCAGACAGCGACCAAAAGAATTCGGATGGCCATCAATTAGTGTGAATAGTTT
AAAAACGACCTTGTCTTACT (SEQ ID NO:16)

FIG. 10G

PR8-UW PB1:

AGCGAAAGCAGGCAAACCATTTGAATGGATGTCAATCCGACCTTACTTTTCTTAAAAGTGCCAGCACAAAATGCTATAAG
CACAACTTTCCCTTATACTGGAGACCTCCTTACAGCCATGGGACAGGAACAGGATACACCATGGATACTGTCAACAGGA
CACATCAGTACTCAGAAAAGGGAAGATGGACAACAAACACCGAACTGGAGCACCGCAACTCAACCCGATTGATGGGCCA
CTGCCAGAAGACAATGAACCAAGTGGTTATGCCCAAACAGATTGTGTATTGGAGGCGATGGCTTTCCTTGAGGAATCCCA
TCCTGGTATTTTTGAAAACTCGTGATTGAAACGATGGAGGTTGTTTCAGCAAACACGAGTAGACAAGCTGACACAAGGCC
GACAGACCTATGACTGGACTCTAAATAGAAACCAACCTGCTGCAACAGCATTGGCCAACACAATAGAAGTTCAGATCA
AATGGCCTCACGGCCAATGAGTCTGGAAGGCTCATAGACTTCCTTAAGGATGTAATGGAGTCAATGAACAAAGAAGAAAT
GGGGATCACAACTCATTTTCAGAGAAAAGAGACGGGTGAGAGACAATATGACTAAGAAAATGATAACACAGAGAACAATGG
GTAAAAAGAAGCAGAGATTGAACAAAAGGAGTTATCTAATTAGAGCATTGACCTGAACACAATGACCAAAGATGCTGAG
AGAGGGAAGCTAAAACGGAGAGCAATTGCAACCCAGGGATGCAATAAGGGGGTTTGTATACTTTGTGAGACACTGGC
AAGGAGTATATGTGAGAAACTTGAACAATCAGGGTTGCCAGTTGGAGGCAATGAGAAGAAAGCAAAGTTGGCAAATGTTG
TAAGGAAGATGATGACCAATTCTCAGGACACCGAACCTTCTTTCACCATCACTGGAGATAACACCAAATGGAACGAAAAT
CAGAATCCTCGGATGTTTTTGGCCATGATCAGATATATGACCAGAAATCAGCCCGAATGGTTCAGAAATGTTCTAAGTAT
TGCTCCAATAATGTTCTCAAACAAAATGGCGAGACTGGGAAAAGGGTATATGTTTGAGAGCAAGAGTATGAAACTTAGAA
CTCAAATACCTGCAGAAATGCTAGCAAGCATCGATTTGAAATATTTCAATGATTCAACAAGAAAGAAGATTGAAAAATC
CGACCGCTCTTAATAGAGGGGACTGCATCATTGAGCCCTGGAATGATGATGGGCATGTTCAATATGTTAAGCACTGTATT
AGGCGTCTCCATCCTGAATCTTGACAAAAGAGATACACCAAGACTACTTACTGGTGGGATGGTCTTCAATCCTCTGACG
ATTTTGCTCTGATTGTGAATGCACCCAATCATGAAGGGATTCAAGCCGGAGTCGACAGGTTTTATCGAACCTGTAAGCTA
CTTGAATCAATATGAGCAAGAAAAAGTCTTACATAAACAGAACAGGTACATTTGAATTCACAAGTTTTTTCTATCGTTA
TGGGTTTGTGCAATTTAGCATGGAGCTTCCAGTTTTGGGGTGTCTGGGATCAACGAGTCAGCGGACATGAGTATTG
GAGTTACTGTATCAAAAACAATATGATAAACAATGATCTTGGTCCAGCAACAGCTCAAATGGCCCTTCAGTTGTTATC
AAAGATTACAGGTACACGTACCGATGCCATATAGGTGACACACAAATACAAACCCGAAGATCATTTGAAATAAAGAACT
GTGGGAGCAAACCCGTTCCAAAGCTGGACTGCTGGTCTCCGACGGAGGCCAAATTTATACAACATTAGAAATCTCCACA
TTCTGAAGTCTGCCTAAATGGGAATTGATGGATGAGGATTACCAGGGGCGTTTATGCAACCCACTGAACCCATTTGTC
AGCCATAAAGAAATTGAATCAATGAACAATGCAGTGATGATGCCAGCACATGGTCCAGCCAAAAACATGGAGTATGATGC
TGTTGCAACAACACACTCTGGATCCCCAAAAGAAATCGATCCATCTTGAATACAAGTCAAAGAGGAGTACTTGAGGATG
AACAAATGTACCAAAGGTGCTGCAATTTATTTGAAAAATCTTCCCCAGCAGTTCATACAGAAGACCAGTCGGGATATCC
AGTATGGTGGAGGCTATGGTTTCCAGAGCCGAATTGATGCACGGATTGATTTGGAATCTGGAAGGATAAAGAAAGAAGA
GTTCACTGAGATCATGAAGATCTGTTCCACCATGGAAGAGCTCAGACGGCAAAAATAGTGAATTTAGCTTGCTTCATG
AAAAATGCCTTGTTTCTACT (SEQ ID NO:2)

FIG. 10H

Canine codon optimized PR8 PB1:

AGCGAAAGCAGGCAAACCATTTGAATGGATGTCAATCCGACCTTACTTTTCTTAAAAGTGCCAGCACAAAATGCTATAAG
CACAACTTTCCCTTATACTGGAGACCCTCCTTACAGCCATGGGACAGGAACAGGATACACCATGGATACTGTCAACAGGA
CACATCAGTACTCAGAAAAGGGAAGATGGACAACAAACACCGAAACTGGAGCACCGCAACTCAACCCGATTGATGGGCCA
CTGCCAGAAGACAATGAACCAAGTGGTTATGCCCAAACAGATTGTGTATTGGAGGCGATGGCTTTCCTTGAGGAATCCCA
TCCTGGTATTTTGAAAACCTCGTGATTGAAACGATGGAGGTTGTTTCAGCAAACACGAGTGGACAAGCTGACACAGGGCC
GACAGACCTATGACTGGACTCTGAATAGAAACCAGCCTGCTGCAACAGCACTGGCCAACACAATCGAAGTGTTCAGATCA
AATGGCCTCACCGCCAATGAGTCTGGAAGGCTCATCGACTTCTGAAGGATGTGATGGAGTCAATGAACAAAGAAGAAAT
GGGGATCACAACCTATTTTCAGAGAAAGAGACGGGTGAGAGACAATATGACTAAGAAAATGATTACACAGAGAACAAATGG
GTAAAAAGAAGCAGAGACTGAACAAAAGGAGTTATCTGATTAGAGCACTGACCCTGAACACAATGACCAAAGATGCTGAG
AGAGGGAAGCTGAAACGGAGAGCAATTGCAACCCAGGGATGCAGATTAGGGGGTTTGTGTACTTTGTGGAGACACTGGC
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TGAGGAAGATGATGACCAATTCTCAGGACACCGAACTGTCTTTCACCATCACTGGAGATAACACCAAATGGAACGAAAA
CAGAATCCTCGGATGTTTCTGGCCATGATCACATATATGACCAGAAATCAGCCCGAATGGTTCAGAAATGTGCTGAGTAT
TGCTCCAATTATGTTCTCAAAACAAAATGGCCAGACTGGGAAAAGGGTATATGTTTGAGAGCAAGAGTATGAACTGAGAA
CTCAGATTCTGCAGAAATGCTGGCAAGCATCGATCTGAAATATTTCAATGATTCAACAAGAAAGAAGATTGAAAAAATC
CGACCCCTCCTGATTGAGGGGACTGCATCACTGAGCCCTGGAATGATGATGGGCATGTTCAATATGCTGAGCACTGTGCT
GGGCGTCTCCATCCTGAATCTGGGACAGAAGAGATACACCAAGACTACTTACTGGTGGGATGGTCTGCAGTCTCTGACG
ATTTTGCTCTGATTGTGAATGCACCAATCATGAAGGGATTGAGGCCGGAGTCGACAGGTTTTATCGAACCTGTAAGCTG
CTGGGAATCAATATGAGCAAGAAAAAGTCTTACATCAACAGAACAGGTACATTTGAATTCACAAGTTTTTCTATCGCTA
TGGGTTTGTGGCAATTTTCAGCATGGAGCTGCCAGTTTTGGGGTGTCTGGGATCAACGAGTCAGCCGACATGAGTATTG
GAGTGACTGTTCATCAAAAACATATGATCAACAATGATCTGGGTCCAGCAACAGCTCAGATGGCCCTGCAGCTGTTTCATC
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GTGGGAGCAGACCCGCTCCAAAGCTGGACTGCTGGTCTCCGACGGAGGCCCAAATCTGTACAACATTAGAAATCTCCACA
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AACAGATGTACCAGAGGTGCTGCAATCTGTTTGAAAAATTTCTCCAGCAGTTCATACAGAAGACCAGTCGGGATCTCC
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GTTCACTGAGATCATGAAGATCTGTTCCACCATTGAAGAGCTCAGACGGCAAAAAATAGTGAATTTAGCTTGCTCTCATG
AAAAAATGCCTTGTCTTCTACT (SEQ ID NO:17)

FIG. 10I

PR8-UW PA:

AGCGAAAGCAGGTACTGATCCAAAATGGAAGATTTTGTGCGACAATGCTTCAATCCGATGATTGTCGAGCTTGCGGAAAA
ACAATGAAAGAGTATGGGGAGGACCTGAAAATCGAAACAAACAAATTTGCAGCAATATGCACTCACTTGGAAGTATGCT
TCATGTATTGAGATTTTCACTTCATCAATGAGCAAGGCGAGTCAATAATCGTAGAACTTGGTGATCCAAATGCACCTTTG
AAGCACAGATTTGAAATAATCGAGGGAAGAGATCGACAATGGCCTGGACAGTAGTAAACAGTATTTGCAACACTACAGG
GGCTGAGAAACCAAAGTTTCTACCAGATTTGTATGATTACAAGGAGAATAGATTTCGAAAATTGGAGTAACAAGGAGAG
AAGTTCACATATACTATCTGAAAAGGCCAATAAAATTAATCTGAGAAAACACATCCACATTTTCTCGTTCACTGGG
GAAGAAATGGCCACAAAGGCAGACTACACTCTCGATGAAGAAAGCAGGGCTAGGATCAAAACCAGACTATTCACCATAAG
ACAAGAAATGGCCAGCAGAGGGCTCTGGGATTCCTTCGTCACTCCGAGAGAGGAGAAGAGACAATTGAAGAAAGGTTTG
AAATCACAGGAACAATGCGCAAGCTTGCCGACCAAAGTCTCCCGCCGAACCTTCTCAGCCTTGAAAATTTAGAGCCTAT
GTGGATGGATTGGAACCGAACGGCTACATTGAGGGCAAGCTGTCTCAAATGTCCAAAGAAGTAAATGCTAGAATTGAACC
TTTTTTGAAAACAACACCACGACCACTTAGACTTCCGAATGGGCCTCCCTGTTCTCAGCGGTCCAAATTCCTGCTGATGG
ATGCCTTAAATTAAGCATTGAGGACCCAAGTCATGAAGGAGAGGGAATACCGCTATATGATGCAATCAAATGCATGAGA
ACATTCTTTGGATGGAAGGAACCCAATGTTGTTAAACCACACGAAAAGGGAATAAATCCAAATTATCTTCTGTCTGGA
GCAAGTACTGGCAGAACTGCAGGACATTGAGAATGAGGAGAAAATTCCAAAGACTAAAAATATGAAGAAAACAAGTCAGC
TAAAGTGGGCACCTTGGTGAGAACATGGCACCAGAAAAGGTAGACTTTGACGACTGTAAAGATGTAGGTGATTTGAAGCAA
TATGATAGTGATGAACCAGAATTGAGGTCGCTTGCAAGTTGGATTGAGATGAGTTTAAACAAGGCATGCGAACTGACAGA
TTCAAGCTGGATAGAGCTCGATGAGATTGGAGAAGATGTGGCTCCAATTGAACACATTGCAAGCATGAGAAGGAATTATT
TCACATCAGAGGTGTCTCACTGCAGAGCCACAGAATACATAATGAAGGGAGTGACATCAATACTGCCTTGCTTAATGCA
TCITGTGCAGCAATGGATGATTTCCAATTAATTCCAATGATAAGCAAGTGTAAGTAAGGAGGGAAGGCGAAAGACCAA
CTTGATGGTTTCATCATAAAAGGAAGATCCCACTTAAGGAATGACACCGACGTGGTAAACTTTGTGAGCATGGAGTTTT
CTCTCACTGACCCAAGACTTGAACCACATAAATGGGAGAAGTACTGTGTTCTTGAGATAGGAGATATGCTTATAAGAAGT
GCCATAGGCCAGGTTTCAAGGCCCATGTTCTTGATGTGAGAACAAATGGAACCTCAAAAATTAATGAATGGGGAAT
GGAGATGAGGCGTTGCCTCCTCCAGTCACCTCAACAAATGAGAGTATGATTGAAGCTGAGTCTCTGTCAAAGAGAGAAAG
ACATGACCAAAGAGTTCTTTGAGAACAAATCAGAAACATGGCCATTGGAGAGTCCCCAAAGGAGTGAGGAAAGTTCC
ATTGGGAAGGTCTGCAGGACTTTATTAGCAAAGTCGGTATTCAACAGCTTGTATGCATCTCCACAAGTGAAGGATTTTC
AGCTGAATCAAGAAAAGTCTTCTTATCGTTCAAGGCTCTAGGGACAACCTGGAACCTGGGACCTTTGATCTTGGGGGGC
TATATGAAGCAATTGAGGAGTGCTGATTAATGATCCCTGGGTTTTGCTTAATGCTTCTTGGTTCAACTCCTTCTTACA
CATGCATTGAGTTAGTTGTGGCAGTGCTACTATTTGCTATCCATACTGTCCAAAAAAGTACCTGTTTCTACT (SEQ ID NO:1)

FIG. 10J

Canine codon optimized PR8 PA:

AGCGAAAGCAGGTACTGATCCAAAATGGAAGATTTTGTGCGACAATGCTTCAATCCGATGATTGTCGAGCTTGCGGAAAA
AACAAATGAAAGAGTATGGGGAGGACCTGAAAATCGAAACAAACAAATTTGCAGCAATATGCACTCACTTGAAGTATGCT
TCATGTATTCAGATTTTCACTTCATCAATGAGCAAGGCGAGTCAATAATCGTAGAACTTGGTGATCCAAATGCACTTTTG
AAGCACAGATTTGAAATAATCGAGGGAAGAGATCGACAATGGCTGGACAGTAGTAAACAGTATTTGCAACACTACAGG
GGCTGAGAAACCAAAGTTTCTACCAGATTTGTATGATTACAAGGAGAATAGATTCATCGAAATTGGAGTAACAAGGAGAG
AAGTTCACATATACTATCTGGAAAAGGCCAATAAAATTAATCTGAGAAAACACACATCCACATTTTCTCGTTCACTGGG
GAAGAAATGGCCACAAGGCAGACTACACTCTCGATGAAGAAAGCAGGGCTAGGATCAAACCAGACTATTACCATAAG
ACAAGAAATGGCCAGCAGAGGCCCTCTGGGATTCCTTTCGTCACTCCGAGAGAGGAGAAGAGACAATTGAAGAAAGGTTTG
AAATCACAGGAACAATGCGCAAGCTTCCGACCAAAGTCTCCGCGCAACTTCTCCAGCCTTGAAAATTTAGAGCCTAT
GTGGATGGATTGGAACCGAACGGCTACATTGAGGGCAAGCTGTCTCAAATGTCCAAAGAAGTAAATGCTAGAATTGAACC
TTTTCTGAAAACAACACCACGACCACTGAGACTGCCAATGGGCCTCCCTGTTCTCAGCGGTCCAAATTCCTGCTGATGG
ATGCCCTGAAACTGAGCATTGAGGACCAAGTCATGAAGGAGAGGGAATTCCCTGTATGATGCAATCAAATGCATGAGA
ACATTCTTTGGATGGAAGGAACCAATGTGGTGAAACCACACGAAAAGGGGAATCAATCCAAATTATCTGCTGTCATGGAA
GCAGGTGCTGGCAGAACTGCAGGACATTGAGAATGAGGAGAAAATCCAAAGACTAAAAATATGAAGAAAACAAGTCAGC
TGAAGTGGGCACTGGGTGAGAATGGCACCAGAAAAGGTGGACTTTGACGACTGTAAAGATGTGGGTGATCTGAAGCAG
TATGATAGTGATGAACCAGAACTGAGGTCCCTGGCAAGTTGGATTGAGATGAGTTTAAACAGGCATGCGAACTGACAGA
TTCAAGCTGGATTGAGCTCGATGAGATTGGAGAAGATGTGGTCCAATTGAACACATTGCAAGCATGAGAAGGAATTATT
TCACATCAGAGGTGTCTCACTGCAGAGCCACAGAATACATCATGAAGGGAGTGTACATCAATACTGCCCTGCTGAATGCA
TCTGTGCGCAATGGATGATTTCCAGCTGATTCCAATGATCAGCAAGTGTAAGTAAGGAGGGGAAGGCGAAAGACCAA
CCTGTATGGTTTCATCATCAAAGGAAGATCCACCTGAGGAATGACACCGACGTGGTGAACCTTTGTGAGCATGGAGTTTT
CTCTCACTGACCCAAGACTGGAACCACATAAATGGGAGAAGTACTGTGTGCTGGAGATTGGAGATATGCTGATCAGAAGT
GCCATTGGCCAGGTGTCAAGGCCCATGTTCTGTATGTGAGAACAAATGGAACCTCAAAAATTAAATGAAATGGGGAAT
GGAGATGAGGCGCTGCCTCTCCAGTCACTGCAGCAGATTGAGAGTATGATTGAAGCTGAGTCTCTGTCAAAGAGAAAAG
ACATGACCAAAGAGTTCTTTGAGAACAAATCAGAAACATGGCCATTGGAGAGTCCCCAAAGGAGTGAGGAAAAGTTCC
ATTGGGAAGGTCTGCAGGACTCTGCTGGCAAAGTCCGTGTTCAACAGCCTGTATGCATCTCCACAGCTGGAAGGATTTTC
AGCTGAATCAAGAAAAGTCTGCTGATCGTGCAGGCTCTGAGGGACAACCTGGAACCTGGGACCTTTGATCTGGGGGGGC
TGTATGAAGCAATTGAGGAGTGCCTGATTAATGATCCCTGGGTGCTGCTGAATGCTTCTGGTTCAACTCCTTCCTTACA
CATGCATTGAGTTAGTTGTGGCAGTGCTACTATTTGCTATCCATACTGTCCAAAAAAGTACCTTGTCTACT (SEQ ID NO:18)

FIG. 10K

PR8-UW NP:

AGCAAAAGCAGGGTAGATAATCACTCACTGAGTGACATCAAAATCATGGCGTCTCAAGGCACCAAACGATCTTACGAACA
GATGGAGACTGATGGAGAACGCCAGAATGCCACTGAAATCAGAGCATCCGTCGGAAAAATGATTGGTGGAAATTGGACGAT
TCTACATCCAAATGTGCACCGAACTCAAACCTCAGTGATTATGAGGGACGGTTGATCCAAAACAGCTTAACAATAGAGAGA
ATGGTGCTCTCTGCTTTTGACGAAAGGAGAAATAAATACCTTGAAGAACATCCCAGTGCGGGGAAAGATCCTAAGAAAAC
TGGAGGACCTATATACAGGAGAGTAAACGGAAAAGTGGATGAGAGAACTCATCCTTTATGACAAAGAAGAAATAAGGCGAA
TCTGGCGCCAAGCTAATAATGGTGACGATGCAACGGCTGGTCTGACTCACATGATGATCTGGCATTCCAATTTGAATGAT
GCAACTTATCAGAGGACAAGAGCTCTTGTTGCGACCGGAATGGATCCCAGGATGTGCTCTCTGATGCAAGGTTCAACTCT
CCCTAGGAGGTCTGGAGCCGCGAGGTGCTGCAGTCAAAGGAGTTGGAACAATGGTGATGGAATTGGTCAGAATGATCAAAC
GTGGGATCAATGATCGGAACCTTCTGGAGGGGTGAGAATGGACGAAAAACAAGAATTGCTTATGAAAGAATGTGCAACATT
CTCAAAGGGAAATTTCAAACCTGCTGCACAAAAAGCAATGATGGATCAAGTGAGAGAGAGCCGGAACCCAGGGAATGCTGA
GTTCGAAGATCTCACTTTTCTAGCACGGTCTGCACTCATATTGAGAGGGTCGGTTGCTCACAAGTCTGCCTGCCTGCCT
GTGTGTATGGACCTGCCGTAGCCAGTGGGTACGACTTTGAAAGGGAGGGATACTCTCTAGTCGGAATAGACCCCTTCAGA
CTGCTTCAAACAGCCAAGTGACAGCCTAATCAGACCAAATGAGAATCCAGCACACAAGAGTCAACTGGTGTGGATGGC
ATGCCATTCTGCCGATTTGAAGATCTAAGAGTATTAAGCTTCATCAAAGGGACGAAGGTGCTCCCAAGAGGGAAGCTTT
CCACTAGAGGAGTTCAAATTGCTTCCAATGAAAAATATGGAGACTATGGAATCAAGTACACTTGAAGTGAAGCAGGTAC
TGGGCCATAAGGACCAGAAGTGGAGGAAACACCAATCAACAGAGGGCATCTGCGGGCCAAATCAGCATACAACCTACGTT
CTCAGTACAGAGAAATCTCCCTTTTGACAGAACAACCATTATGGCAGCATTCAATGGGAATACAGAGGGGAGAACATCTG
ACATGAGGACCGAAATCATAAGGATGATGGAAAGTGCAAGACCAGAAGATGTGTCTTCCAGGGGCGGGGAGTCTTCGAG
CTCTCGGACGAAAAGGCAGCGAGCCCGATCGTGCTTCTTTGACATGAGTAATGAAGGATCTTATTTCTTCGGAGACAA
TGCAGAGGAGTACGACAATTAAGAAAAATACCCTTGTTTCTACT (SEQ ID NO:4)

FIG. 10L

Canine codon optimized NP:

AGCAAAAGCAGGGTAGATAATCACTCACTGAGTGACATCAAAATCATGGCGTCTCAAGGCACCAAACGATCTTACGAACA
GATGGAGACTGATGGAGAACGCCAGAATGCCACTGAAATCAGAGCATCCGTCGGAAAAATGATTGGTGGAATTGGACGAT
TCTACATCCAGATGTGCACCGAACTCAAACCTCAGTGATTATGAGGGACGGCTGATCCAGAACAGCCTGACAATCGAGAGA
ATGGTGCTCTCTGCTTTTGACGAAAGGAGAAATAAATACCTGGAAGAACATCCAGTGCCGGGAAAGATCCTAAGAAAAC
TGGAGGACCTATCTACAGGAGAGTGAAACGGAAAGTGGATGAGAGAACTCATCCTGTATGACAAAGAAGAAATCAGGCGAA
TCTGGCGCCAGGCTAATAATGGTGACGATGCAACCGCTGGTCTGACTCACATGATGATCTGGCATTCCAATCTGAATGAT
GCAACTTATCAGAGGACAAGAGCTCTGGTGCGCACCGGAATGGATCCCAGGATGTGCTCTCTGATGCAGGGTTCAACTCT
CCCTAGGAGGTCTGGAGCCGCAAGTGCTGCAGTCAAAGGAGTGGGAACAATGGTGATGGAAGTGGTCAAGATGATCAAAA
GAGGGATCAATGATCGGAACCTCTGGAGGGGTGAGAATGGACGAAAAACAAGAATTGCTTATGAAAGAATGTGCAACATT
CTCAAAGGGAAATTTCACTGCTGCACAGAAAGCAATGATGGATCAGGTGAGAGAGAGCCGGAACCCAGGGAATGCTGA
GTTTGAAGATCTCACTTTTCTGGCACGGTCTGCACTCATCTGAGAGGGTCCGTGGCTCACAAGTCTGCCTGCCTGCCT
GTGTGTATGGACCTGCCGTGGCCAGTGGGTACGACTTTGAAAGGGAGGGATACTCTCTGGTCGGAATTGACCCTTTCAGA
CTGCTGCAGAACAGCCAGGTGTACAGCCTGATCAGACCAAATGAGAATCCAGCACACAAGAGTCAGCTGGTGTGGATGGC
ATGCCATTCTGCCGATTTGAAGATCTGAGAGTGCTGAGCTTCATCAAAGGGACCAAGGTGCTCCAAGAGGGAAGCTGT
CCACTAGAGGAGTGCAGATTGCTTCCAATGAAAAATGGAGACTATGGAATCAAGTACACTGGAAGTGAAGCAGGTAC
TGGGCCATCAGGACCAAGTGGAGGAAACACCAATCAGCAGAGGGCATCTGCCGGCCAGATCAGCATTAGCCTACCTT
CTCAGTGCAGAGAAATCTCCCTTTGACAGAACCAACATTATGGCAGCATTCAATGGGAATACAGAGGGGAGAACATCTG
ACATGAGGACCGAAATCATCAGGATGATGGAAAGTGCAAGACCAGAAGATGTGTCTTCCAGGGGGCGGGAGTCTTCGAG
CTCTCGGACGAAAAGGCAGCGAGCCCGATCGTGCTTCTTTGACATGAGTAATGAAGGATCTTATTTCTTCGGAGACAA
TGCAGAGGAGTACGACAATTAAGAAAAATACCCTTGTCTTACT (SEQ ID NO:19)

FIG. 10M

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

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

FIG. 11A

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

FIG. 11B

Growth kinetics of 3' C4U mutant

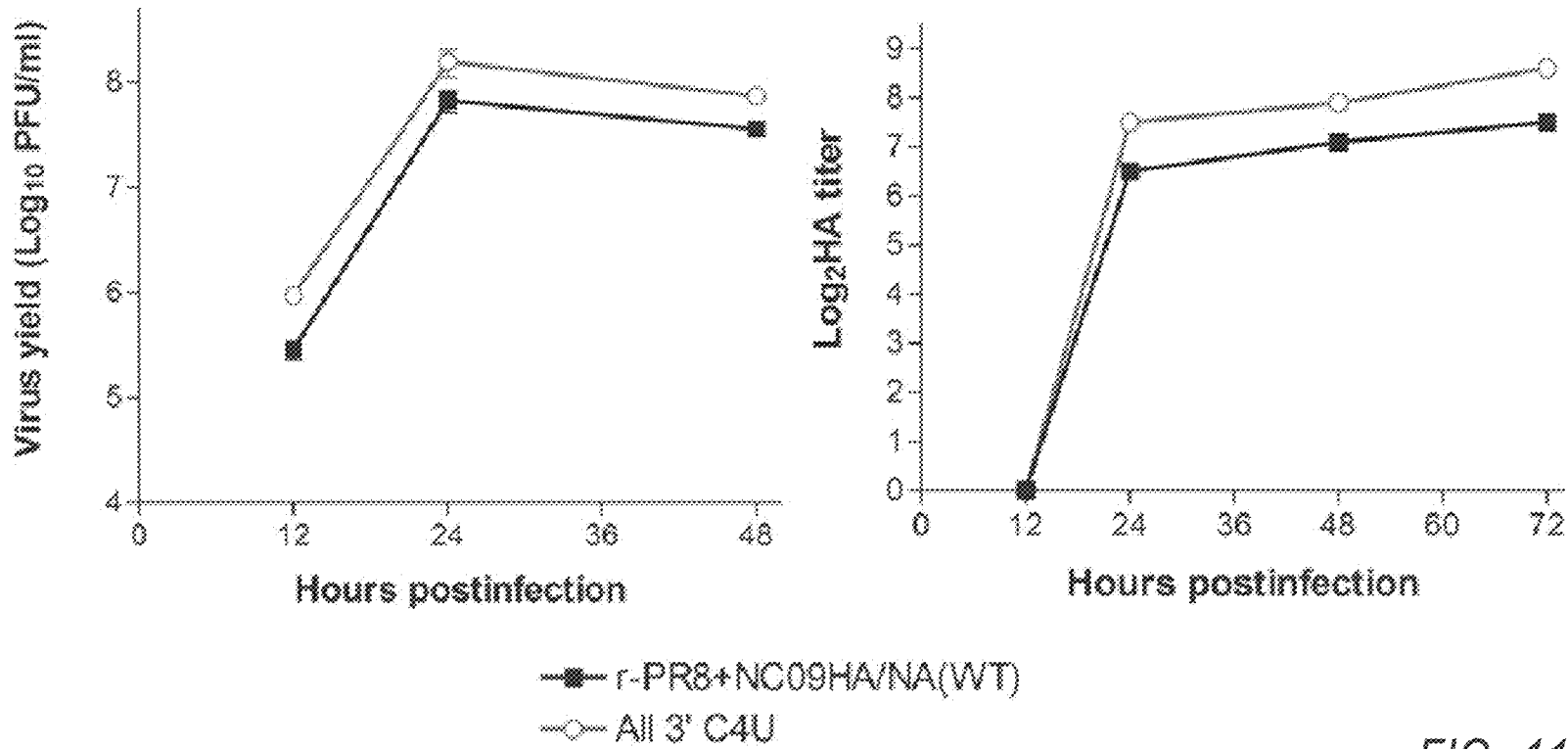


FIG. 11C

Figure 12

HA

atgaacactcaaatcctggattcgtctgattgcgatcattccaacaaatgcagacaaaatctgcctcggacatcatgccgtgtcaaacggaaccaaagtaaa
cacattaactgaaaggaggagtggaagtcgtcaatgcaactgaaacagtggaacgaacaaacatcccaggatctgctcaaaagggaaggaacagtgtagcc
tcgggtcaatgtggactcctggggacaatcactggaccacctcaatgtgaccaatcttagaatttcagccgatttaattattgagaggcgagaaggaaagttag
tctgttatccctgggaaattcgtgaatgagaagctctgaggcaaatctcagagaatcaggcggaattgacaagggaagcaatgggattcacatagtggaat
aagaactaatggagcaaccagtgcatgtaggagatcaggatcttcatctatgcagaatgaaatggctcctgtcaaacacagataatgtgcatctccgcaga
tgactaagtcatataaaaatacaagaaaaagcccagctctaattagtagtggggagccatcattccgtatcaactgcagagcaaaccaagctatatgggagtg
aaacaaactggtagcagtgaggagttctaattatcaacaatctttgtacagagtcaggagcgagaccacaagttaatggcttatctggaagaattgacttcat
tggtaatgctaaatcccaatgatcacgtagctttagttcaatggggcttcatagctccagaccgtgcaagcttctgagaggaaaatctatgggaatccag
agtgaggtagcaggttgatgccaaattgtgaaggaggactgctatcatagtgaggaggcaataataagtaacttgcatttcagaaacatagatagcaggcgagt
gaaaatgtccgagatagttaagcaaggagctgctgtagcaacaggaggagaagaatgttctgagatttcaaaagggaagaggcctatttgggtctatag
gggttctattgaaaatggagtggaaggcctaattgatgttggtatgggttagacaccagaatgcacaggagagggaactgctgcagattacaagcact
caatcggcaattgatcaataacaggaataaaccggcttatagaaaaaaccaaccaacaatttgagttgatagacaatgaattcaatgaggtagagaag
caaatcggtaattgtataaattggaccagagattctataacagaagtgtggtcatacaatgctgaactcttgtagcaatggagaaccagcatacaattgatct
ggctgattcagaatggacaactgtacgaacagagtgaagaagacagctgagagagaatgctgaagaagatggcactggttcttgaataattcacaagtg
gatgatgactgtatggcagtagtagaataacacctatgacacagcaatacagggaagaggcaatgcataatagaalacagattgaccagtcacaacta
agcagcggtctacaaagatgtgatacttggtttagcttcggggcatcatgttcatatcttagccattgtaatgggccttcttcatatgtgtaagaatggaaa
catcggtgtagcattttgtatataa (SEQ ID NO:20)

MNTQILVFAL	IALIPTNADK	ICLGHHAVSN	GTKVNTLTER	GVEVVNAIET	VERTNIPRIC
SKGKRTVDLG	QCGLLGTITG	PPQCDQFLEF	SADLI IERRE	GSDVCYPGKF	VNEEALRQIL
RESGGIDKEA	MGFTYSGIRT	NGATSACRRS	GSSFYAEMKW	LLSNTDNAAF	PQMTKSYKNT
RKSPALIVWG	IHHSVSTAEQ	TKLYGSGNKL	VTVGSSNYQQ	SFVSPGARP	QVNGLSGRID
FWLMLNPND	TVTFSFNGAF	IAPDRASFLR	GKSMGTQSGV	QVDANCEGDC	YHSGGTITISN
LPFQNI DSRA	VGKCPRYVKQ	RSLLLATGMK	NVPEIPKGRG	LFGAIAGFIE	NGWEGLIDGW
YGFRRHQNAQG	EGTAADYKST	QSAIDQITGK	LNRLIEKTNQ	QFELIDNEFN	EVKQIGNVI
NWTRDSITEV	WSYNAELIVA	MENQRTIDLA	USEMDKLYER	VKRQLRENAE	EDGTGCFEIF
HKDDDCMAS	IRNNTYDHSK	YREEAMQNR	QIDPVKLSSG	YKDVILWFSF	GASCFILLAI

VMGLVFICVK NGNMRCTICI (SEQ ID NO:21)

NA

atgaatccaaatcagaagattctatgcacttcagccactgctatcaataataggcgcaatcgagtagctcattggaatagcaaacctaggattgaacataggact
gcactcaaaacccgggtgcaattgtctcacactcacaacctgaacaaacaaacagccaaacaataataaacaactattataatgaacaaacatccacaa
catccaaatgggaagagagaacaagcagggaattcaataacttaactaaagggtctgtactataaattcatggcacatatatgggaagacaatgcagtaaga
attggagagagctcggtatgttttagtcacaagagaacctatgtttcatgagaccagatgaatgcaggttctatgctctcagccaagggaacaacaatcagagg
gaaacactcaaacgggaacaatacacgataggtcccagtagtcgcgcctgabaagctggccactatcatcaccgccacagtgtaacacagcagggtggaatg
cattgggtggtcaagtacttagttgccatgatggcaatccaggatgtcaabtgatatcaggaccaaaacaacaatgcatctgcagtagtagttacacagaa
ggcctgttgcaaaattaacacatggggccgaacatactaagaacacaggaatctgaatgtgtatgccaaacggcgtatgccagtaggttaccagtagg
gtctgcccactggacctgcagacacaagaatatactattttaaaggagggaataatgaaatgggaggtctctgactggaactgctaagcatattgaagaatgt
catgttacggggaacgaacaggaattaccgtcacatgcaggggacaattggcagggtcacaatagaccagtgattcagatagaccagtagcaatgacacaca
ctagtcaatatatatgcagtcctgtcttacagacaatccccgaccgaatgacccaatataggtgaatgtaatgaccttatccaggtataataacaatggag
tcaagggtattctacactggatggggctaacacttggtcaggaggagacaataagcacagcctcgagggtctggatagagatgttaaaagtccaatgcaat
gacagatgatagatcaagccattcaaggctcagacaattgtattaaacgtgactggagtggttacagtggtatcttcatggactattgggtggaagggtgact
gctatcgagcgtgttttaltgtggaattgatagtggaagacccaaggagaataaagtgtgtggaccagcaatagtagtagtcatgtgttcagtagcagaat
tctcgggacaatggaactggcctgatgggctaaaatagagtacttctctaa (SEQ ID NO:22)

MNPNQKILCTSATAIHGAJAVLIGIANLGLNIGLHLKPGCNCSHSQPETTNTSQTIIINNYNETNITNIQMEERTSRNFNNLTGKL
CTINSWHYIGKDNVIRIGESSDVLVTREPYVSCDPDECRFYALSQGTITRGKHSNGTIHDSQYRALISWPLSSPPTVYNSRVECI
GWSSTSCHDGRMSICISGPNNNASAVVWYNRRPVAEINTWARNILRTOESECVCNNGVCPVVFDTGSATGPADTRIYYFK

FIG. 12A

EGKILKWESLTGTAKHIEECSCYGERTGITCTCRDNWQGSNRPIQIDPVAMTHTSQYICSPVLTDPNRPNDPNIGKCNDPYPG
NNNNGVKGSYLDGANTWLGRITISTASRSYEMLKVPNALTDORSKPIQGQTIVLNADW5GYSGSFMDYWAEGDCYRACF
Y VELIRGRPKEDKVVWTSNSIVSMCSSTFLGQWNNWPDGAKIEYFL (SEQ ID NO:23)

HA

atgaacactcaaatcctggtattcgtctgattgcatcattccaacaaatgcagacaaaatctgcctcggacatcatgctgtgtcaaacggaaccaaagtaaa
cacattaactgaaagaggagtggaagtcgtcaatgcaactgaaacagtggaacgaacaaacatccccaggatctgctcaaaagggaaggaacagtgacc
tcggicaatgtggactcctggggacaatcactggaccacctcaatgigaccaattcctagaatttcagccgatitaaatgatgagaggcgagaaggaaagtgatg
tctgttatcctgggaaattcgtgaatgaagaagctctgaggcaaatctcagagaatcaggcggaattgacaagggaagcaatgggattcacatagagggaat
aagaactaatggagcaaccagttcatgtaggagatcaggatcttcattctatgcagaaatgaaatggctcctgtcaaacacagataatgctgcattcccgcaga
tgactaagtcataaaaaatacaagaaaaaaccagctctaatagtatgggggatccatcattccggatcaactgcagagcaaaccaagctatatgggagtgga
aaacaactgggtgacagttgggagttctaaitatcaacaatctttgtacgagtcggggagcgagaacacaagttaatggtaacttggaagaattgacttca
ttggctaagtctaaatccaatgatacagtcactttcagttcaatgggggtttcatagctccagaccgtccaagcttctgagagggaatctatgggaatccag
agtgaggtacaggttgatccgattgtgaaggggagctgctattatagtgaggaggaacaataaagtaacttgccatttcagaacatagatagcagggcagtgga
aaaatgtccgagatatgttaagcaaaaggagctgctgctagcaacagggaatgaagaatgttctgagattccaagggaaggagcctatttgggtctatagcg
ggtttcattgaaaatggatgggaaggcctaattgatgggttgatggtttcagacaccagaatgcacaggagagggaactgctgcagattacaaaagcactc
aatcggcaattgatcaataacagggaataaaccggcttatagaaaaaaccacaaacaatttgattgtagacaatgaattcactgaggtagagaagc
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atgatgactgtatgccagcattagaaataacacctatgatcacagcaataacagggaaggaggaatgcaaaatagaatacagattgaccagctcaactaa
gcagcggctcaaaagatgtgatactttgggttagcttggggcattcatgtttcactcttagccattgcaatgggccttgccttcatatgtgtaagaatggaaa
c atcggtgcactatttctatataa (SEQ ID NO:24)

MNTQILVFALIAIPTNADKICLGHHAVSNGTKVNTLTERGVEVVNATETVERTNIPRICSKGKRTVDLGQCGLLGTITGPPQCDQ
FLEFSADLIHERREGSDVCYPGKFVNEELRQILRESGGIDKEAMGFTYSG IRTNGATSSCRRSGSSFYAEMKWLNSNTDNAAFP
QMTKSYKNTRKNPALIVWGIHSGSTAEQTKLYGSGNKLTVTGSSNYQQSFVPSPGARTQVNGQSGRIDFWHMLNPNNDTV
TFSFNGAFIAPDRASFLRGKSMGIQSGVQVQDADCEGDCYSSGGTIIISNLPFQNIDSRAVGKCPRYVKQRSLLATGMKNVPEIP
KGRGLFGAIAAGFIENGWEGLIDGWYGRHQNAQGEQTAADYKSTQSAIDQITGKLNRLIEKTNQQFELIDNEFTEVEKQIGNVI
NWTRDSITEVWSYNAELLVAMENQHTIDLADSEMDKLYERVKRLRENAEEDGTGCFEIFHKDDDCMASIRNNTYDHSKY
R EAMQNRIQIDPVKLSSGYKDVILWFSFGASCILLAIAMGLVFCVKNNGNMRTICI (SEQ ID NO:25)

NA

atgaatccaaatcagaagattctatgcactcagccactgctatcataataggcgcaatcgagtagctcattggaatagcaaacctaggattgaacataggact
gcatctaaaaccgagctgcaattgctcacactcacaacctgaaacaaccaacaagccaacaataataaacaactattataatgaacaacaacatcaccaa
catccaaatggaagagagagaacaagcaggaaattcaataaacttaactaaagggtctgtactataaattcatggcacatatatgggaagacaatgcggtaaga
attggagagagctcggatgttttagtcacaagagaacctatgtttcatgagaccagatgaatgcaggttctatgctctcagccaagggaacaacaatcagagg
aaaacactcaaacggaacaatacacgataggctccagatcgcgccttgatagctggccactatcatcaccgccacagtgatcacacagcaggggtggaatg
cattgggtgtgcaagtactagttgccatgatggcaaatccaggatgtcaaatgtatatcaggaccaacaacaatgcacatcgtcagtagtatgtgtacaacaga
ggcctgttcagaaattaacacatgggcccgaacatactaagaacacaggaaatctgaatgtgtatgccacaacggcgtatgccagtagtgttaccagatgg
gtctgccactggacctgcagacacaagaataactatfttaagagggggaataatgaaatgggagtcctgactggaactgctaagcatattgaagaatgct
catgttacgggggaacgaacaggaattacctgcacatgcaaggacaattggcagggctcaaatagaccagtgattcagatagatccagtagcaatgacacaca
ctagtcagtatatatgcagtcctgtcttacagacaatccccgaccgaatgaaccaatfataggtgaatgtaagacccttatccaggtaataataacaatggag
tcaagggaattctcatcctggatggggctaacacttggctaggaggagacaataagcacagcctcagaggtctggatagagatgttaaaagtccaatgcaat
gacagatgatagatcaaaagccattcaaggctcagacaattgtataaacgctgactggagtggttacagtgatctttcagactattgggtcagggggact
gctatcgagcgtgttttatgtgaattgatactggaagaccaaggaggataaagtggtgggaccagcaatagtagtatgatgtgttcagtagcagaat
tcctgggacaatggaactggcctgatggggctaaaatagagtacttctctaa (SEQ ID NO:26)

MNPNQKILCTSAIIHGAIVLIGIANLGLNIGLHLKPSNCNSHSPETNTSQTIIINYYNETNITNIQMEERTSRNFNNLTGKL
CTINSWHYKDNVAVRIGESSDVLVTREPVVSCDPDECRFYALSQGTIIRG KHSNGTIHDSQYRALISWPLSSPPTVYNSRVECI
GWSSTSCHDGKSRMSICISGPNNNASAVVWYNRRPVAEINTWARNILRTQESCVCHNGVCPVVFTDGSATGPADTRIYYFK

FIG. 12B

EGKILKWESLTGTAKHIEECSCYGERTGITCTCKDNWQGSNRPVIQIDPVAMTHTSQYICSPVLTDNPRPNDPNIGKCNDPYPG
NNNNGVKGFSYLDGANTWLGRTISTASRSGYEMLKVPNALTDDRSKPIQGQTIVLNADWSGYSGSFMDYWAEGDCYRACF
Y VELIRGRPKEKVVWTSNSIVSMCSSTFLGQWNWPDGAKIEYFL (SEQ. ID NO:27)

FIG. 12C

Construct chimeric HA &NA to increase virus replication

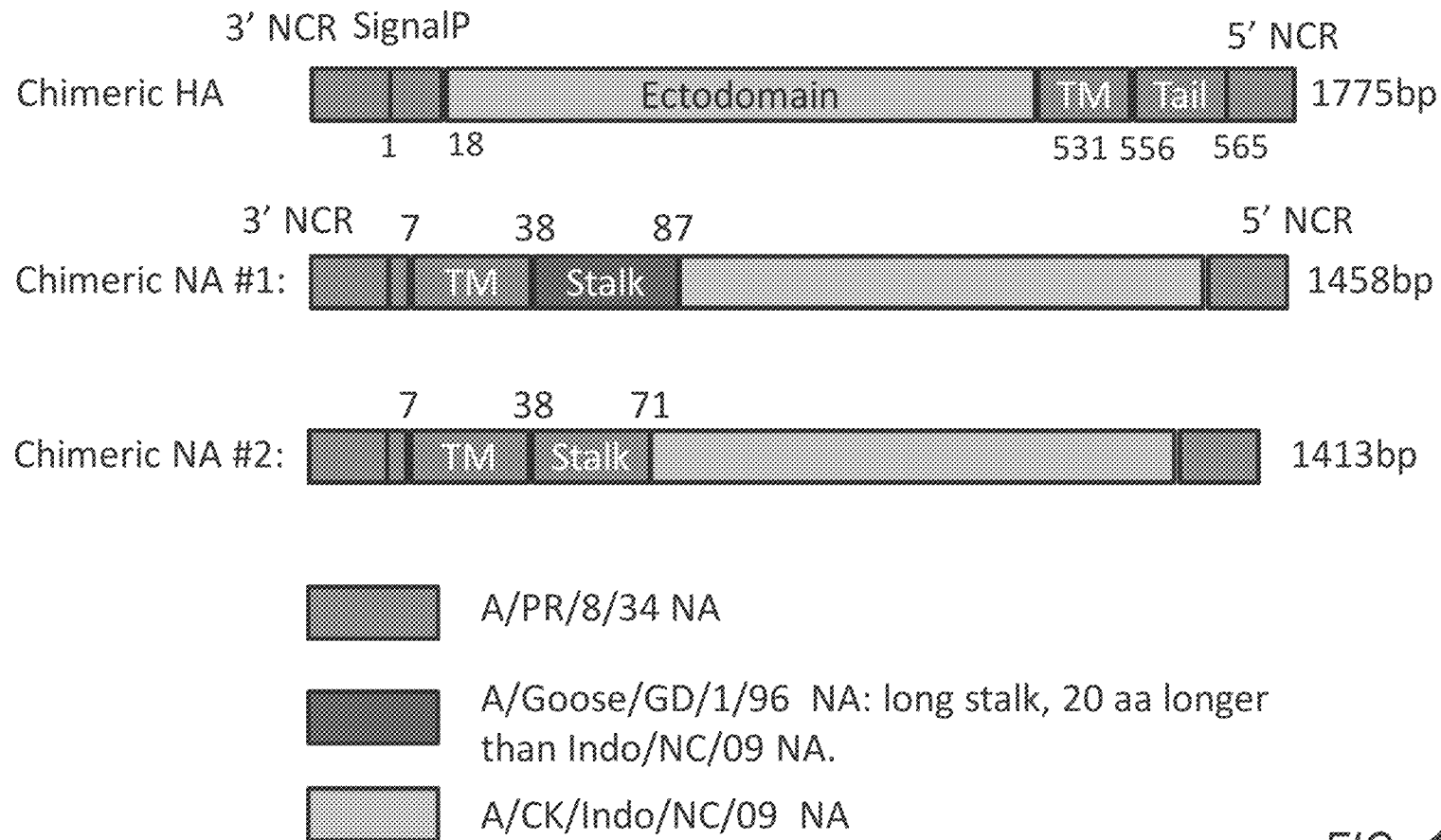


FIG. 13A

Growth kinetics in MDCK cells

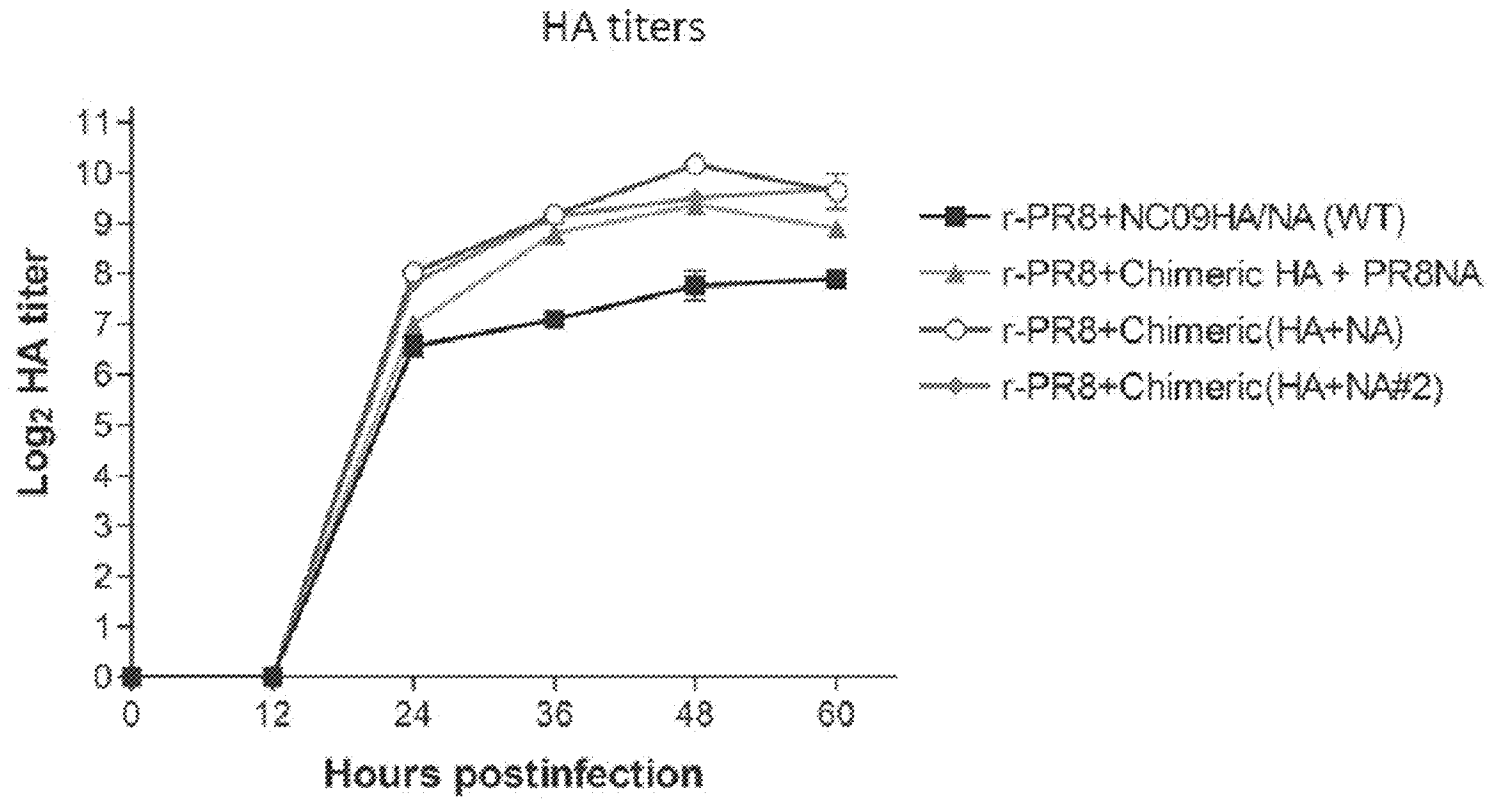
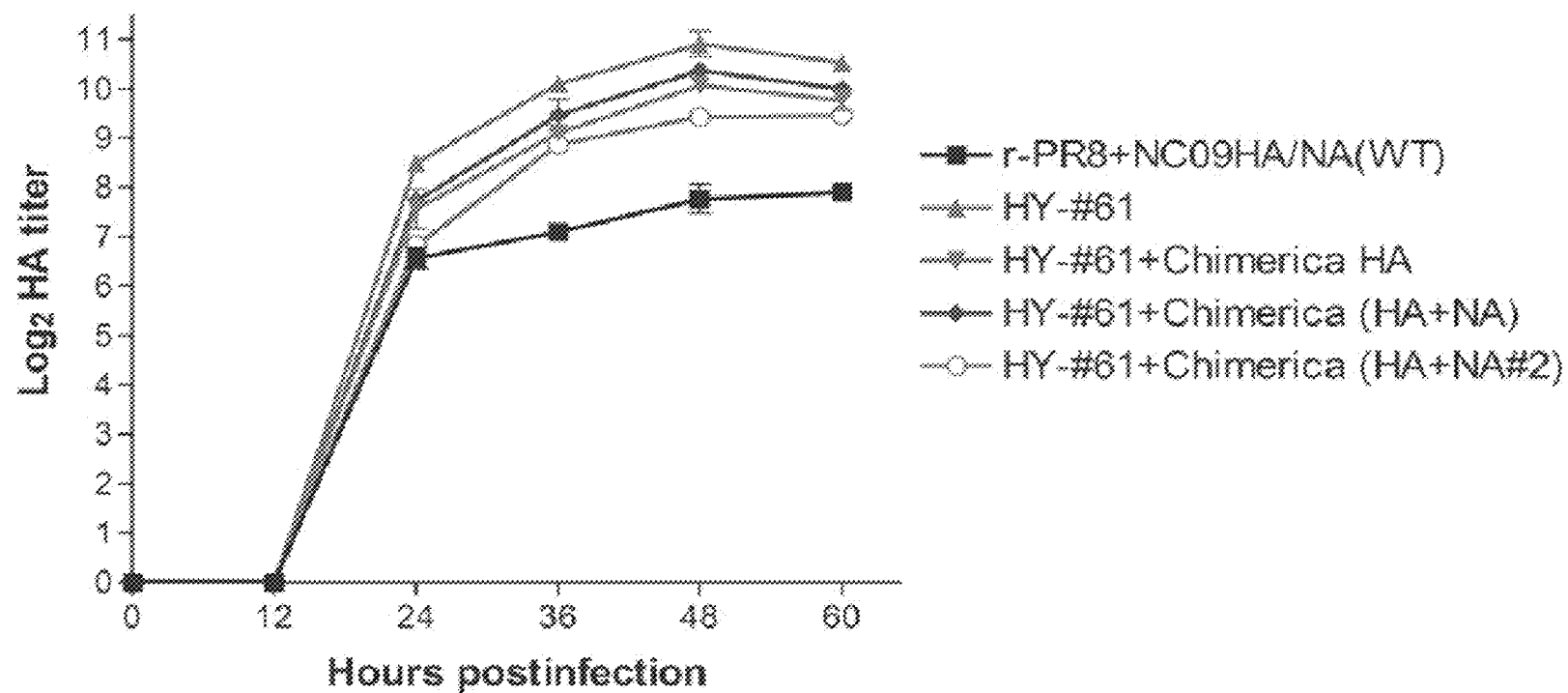


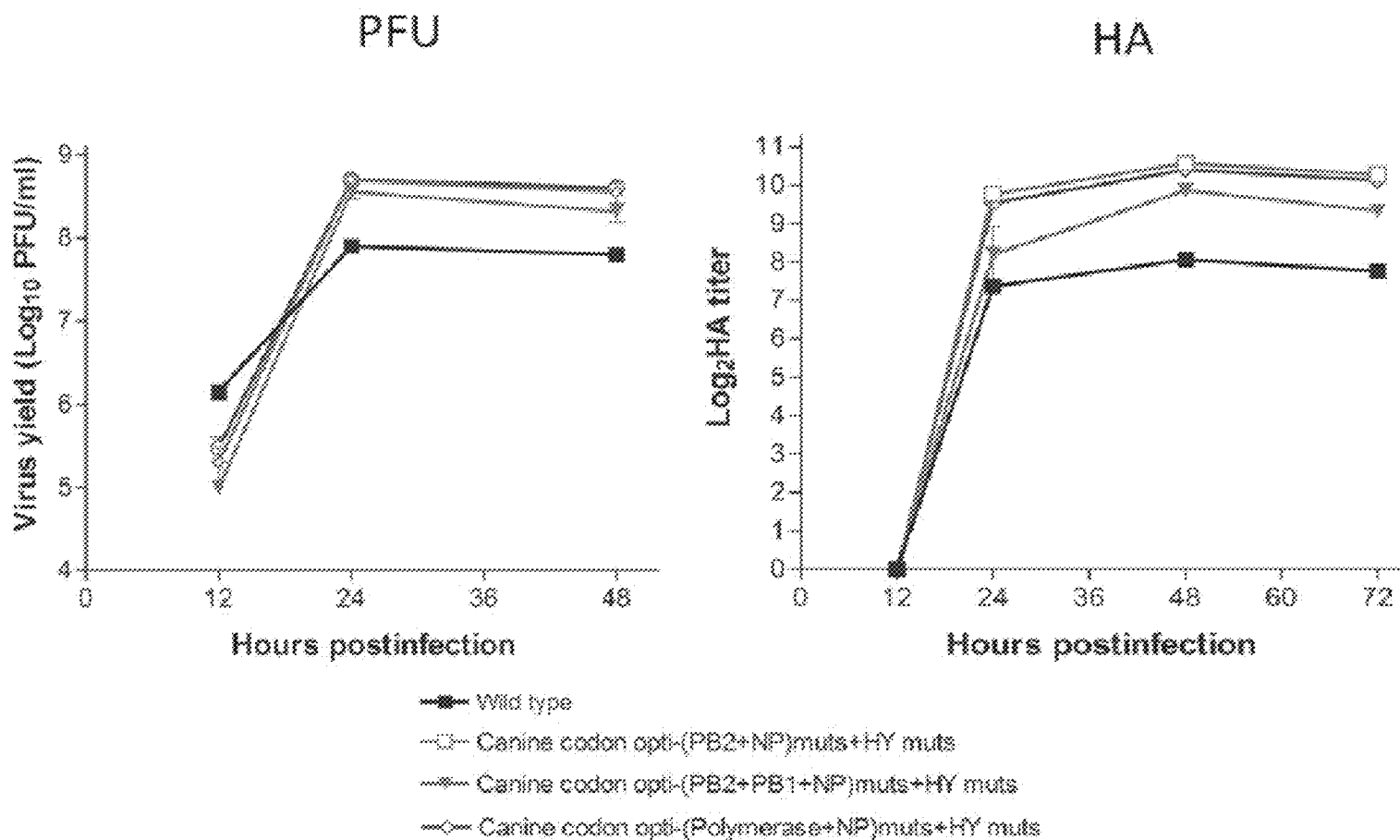
FIG. 13B

Growth kinetics in MDCK cells



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

FIG. 14A



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

FIG. 14B

US 11,390,649 B2



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%

FIG. 16

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

FIG. 17

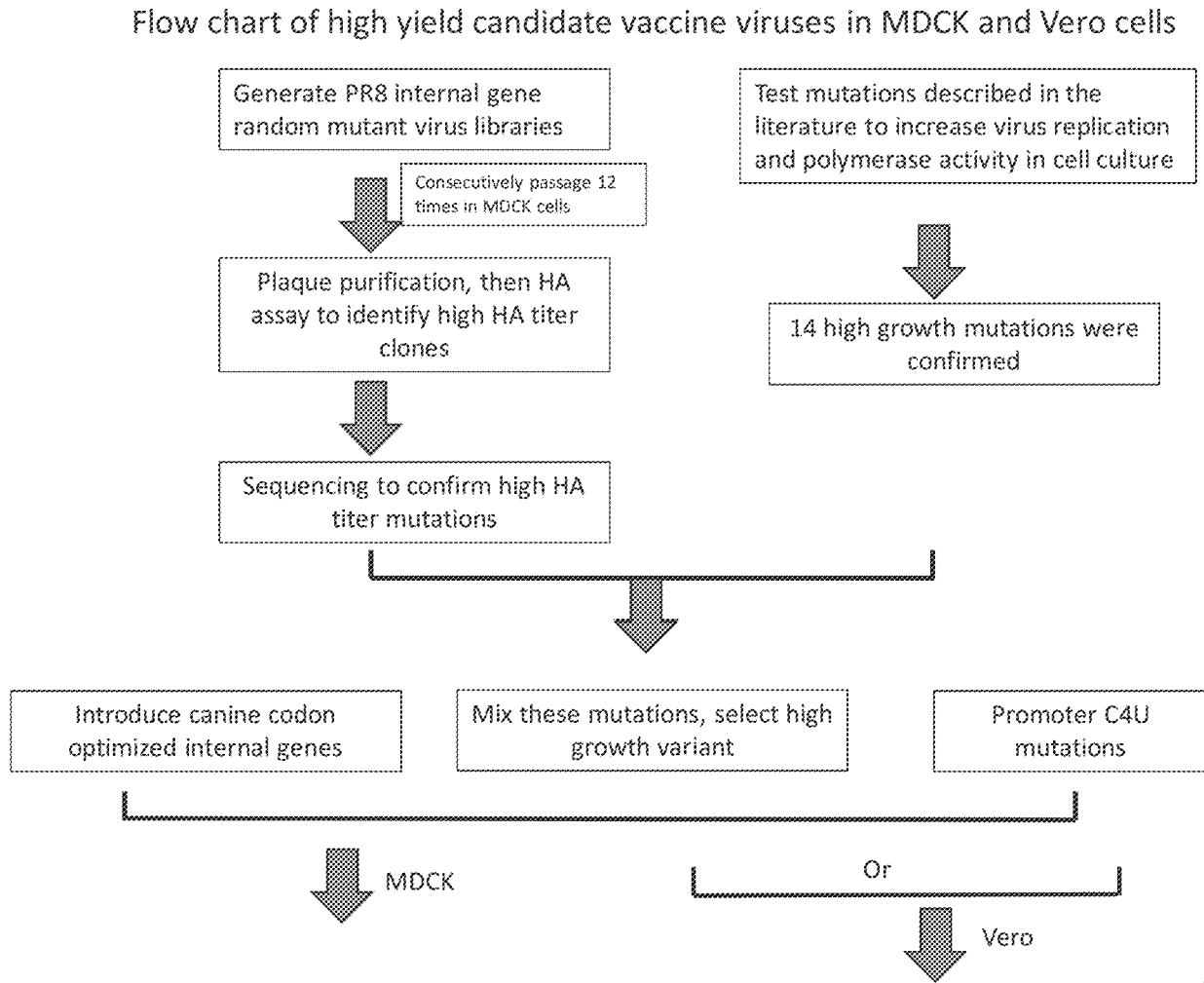


FIG. 18A

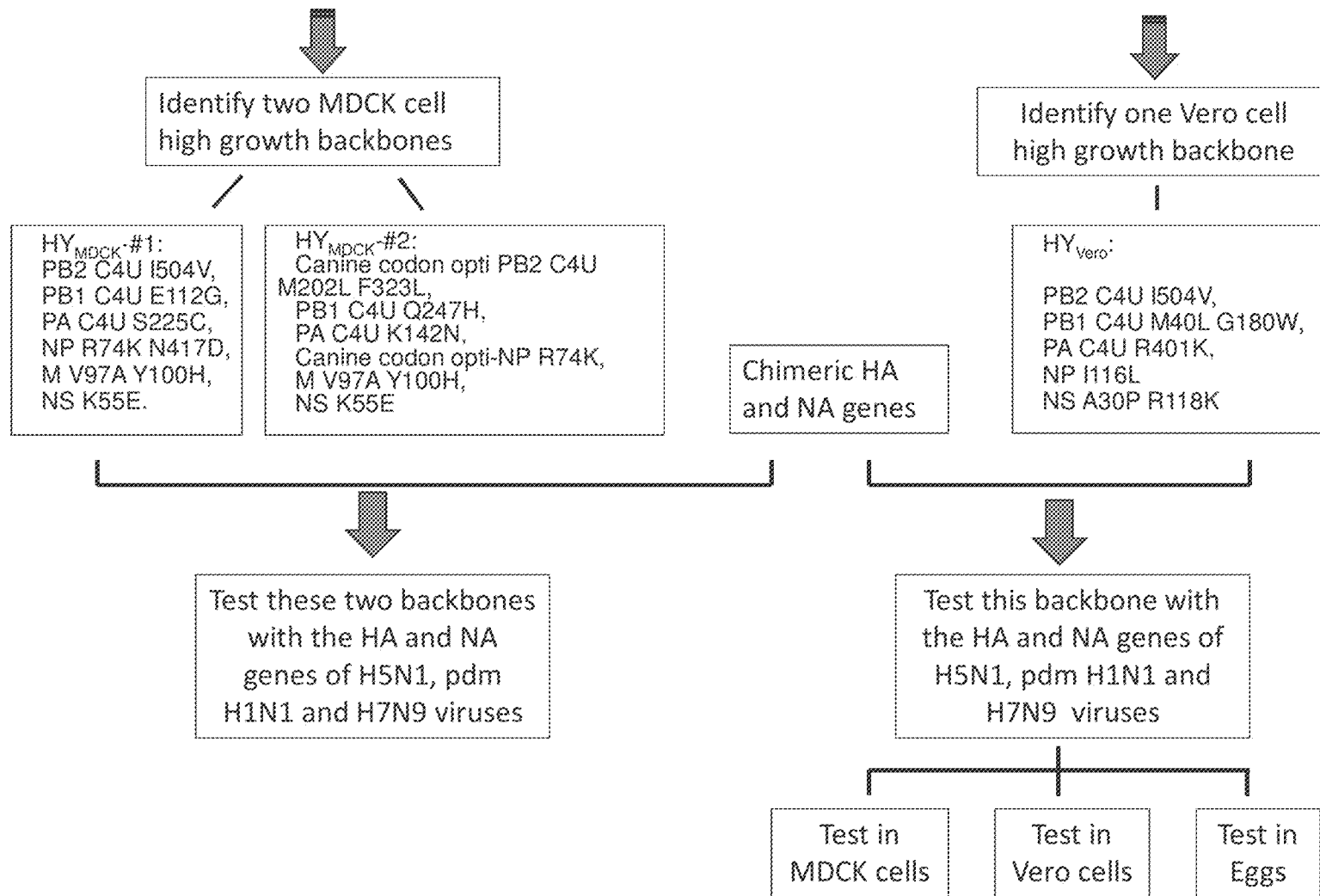


FIG. 18B

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-R61G)					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	V164I		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/California/07/2009 pdmH1N1)			G180E S261G S361R Q621R N654S					MDCK	Viral titer	3.5
										HA titer	2.1
59	A/Chicken/Indonesia/NC/2009, A/Vietnam/1203/2004, A/Hubei/1/2010, A/Egypt/N03072/2010, A/Indonesia/5/2005, A/Anhui/1/2013, X-181, X-223A	C4U I504V	C4U M40L G180W	C4U R401K	I116L			A30P R118K	Vero	Viral titer	5.1~269
										HA titer	2.2~134
									MDCK	Viral titer	1.8~29
										HA titer	1.3~5.5
									Eggs	Viral titer	4.6~172
										HA titer	1.3~17.7
60	A/Chicken/Indonesia/NC/2009	C4U I504V	C4U M40L G180W	C4U R401K	I116L	G1012C, A1013U, U1014A		A30P R118K	Vero	Viral titer	2.8
										HA titer	1.2
									Egg	Viral titer	1.5
										HA titer	1.6

Note: 1. The HA and NA genes of #1-58 come from A/Chicken/Indonesia/NC/2009 (H5N1) or mutant HA and NA of A/Chicken/Indonesia/NC/2009.

2: All the H5N1 HAs used in this study were mutated to remove multibasic cleavage sites to create lowly pathogenicity mutants.

FIG. 19D

- UW-PR8_INDO/05(HA+NA)
- ▲ UW-PR8+PR8-HY PB2_INDO/05(HA+NA)
- ▼ UW-PR8+PR8-HY PB1_INDO/05(HA+NA)
- ◆ UW-PR8+PR8-HY PA_INDO/05(HA+NA)
- UW-PR8+PR8-HY NP_INDO/05(HA+NA)
- UW-PR8+PR8-HY NS_INDO/05(HA+NA)
- △ UW-PR8+PR8-HY PB2+PB1+PA_INDO/05(HA+NA)
- ▽ UW-PR8+PR8-HY PB2+PB1+PA+NP_INDO/05(HA+NA)
- PR8-HY_INDO/05(HA+NA)

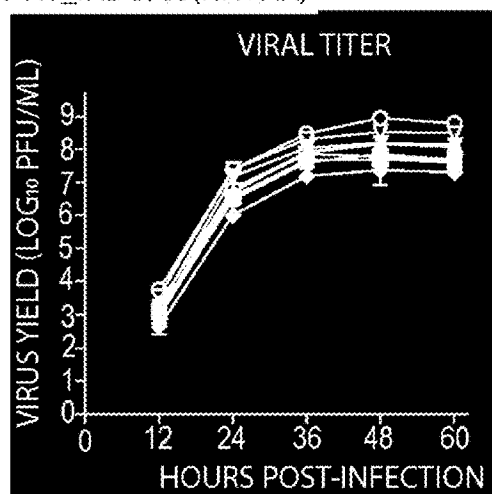


Fig. 20A

- UW-PR8_INDO/05(HA+NA)
- ▲ UW-PR8+PR8-HY PB2_INDO/05(HA+NA)
- ▼ UW-PR8+PR8-HY PB1_INDO/05(HA+NA)
- ◆ UW-PR8+PR8-HY PA_INDO/05(HA+NA)
- UW-PR8+PR8-HY NP_INDO/05(HA+NA)
- UW-PR8+PR8-HY NS_INDO/05(HA+NA)
- △ UW-PR8+PR8-HY PB2+PB1+PA_INDO/05(HA+NA)
- ▽ UW-PR8+PR8-HY PB2+PB1+PA+NP_INDO/05(HA+NA)
- PR8-HY_INDO/05(HA+NA)

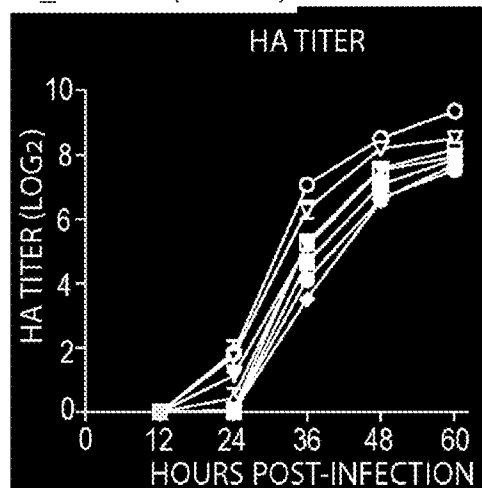


Fig. 20B

- UW-PR8_INDO/05(HA+NA)
- ▲ UW-PR8+PR8-HY PB2_INDO/05(HA+NA)
- ▼ UW-PR8+PR8-HY PB1_INDO/05(HA+NA)
- ◆ UW-PR8+PR8-HY PA_INDO/05(HA+NA)
- UW-PR8+PR8-HY NP_INDO/05(HA+NA)
- UW-PR8+PR8-HY NS_INDO/05(HA+NA)
- △ UW-PR8+PR8-HY PB2+PB1+PA_INDO/05(HA+NA)
- ▽ UW-PR8+PR8-HY PB2+PB1+PA+NP_INDO/05(HA+NA)
- PR8-HY_INDO/05(HA+NA)

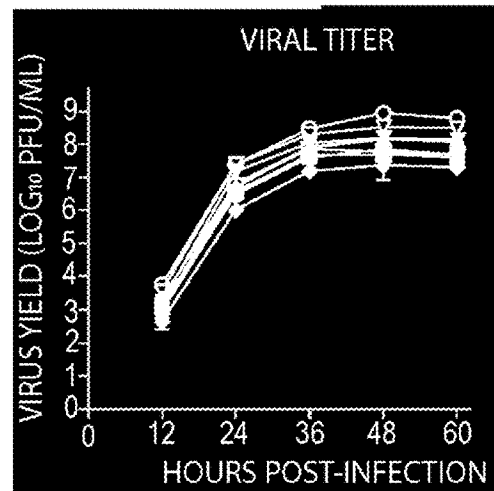


Fig. 20C

- UW-PR8_INDO/05(HA+NA)
- ▲ UW-PR8+PR8-HY PB2_INDO/05(HA+NA)
- ▼ UW-PR8+PR8-HY PB1_INDO/05(HA+NA)
- ◆ UW-PR8+PR8-HY PA_INDO/05(HA+NA)
- UW-PR8+PR8-HY NP_INDO/05(HA+NA)
- UW-PR8+PR8-HY NS_INDO/05(HA+NA)
- △ UW-PR8+PR8-HY PB2+PB1+PA_INDO/05(HA+NA)
- ▽ UW-PR8+PR8-HY PB2+PB1+PA+NP_INDO/05(HA+NA)
- PR8-HY_INDO/05(HA+NA)

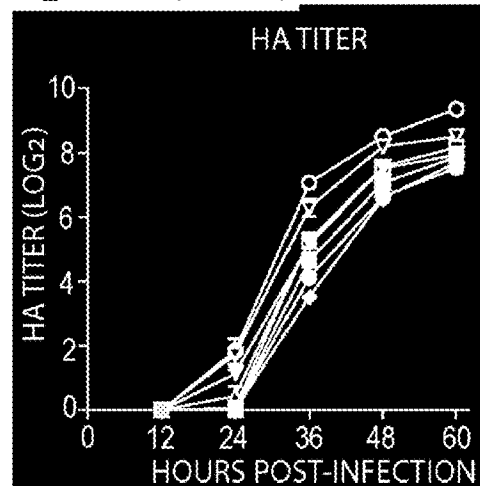


Fig. 20D

- UW-PR8_INDO/05(HA+NA)
- ▲ UW-PR8+PR8-HY PB2_INDO/05(HA+NA)
- ▼ UW-PR8+PR8-HY PB1_INDO/05(HA+NA)
- ◆ UW-PR8+PR8-HY PA_INDO/05(HA+NA)
- UW-PR8+PR8-HY NP_INDO/05(HA+NA)
- UW-PR8+PR8-HY NS_INDO/05(HA+NA)
- ☆ UW-PR8+PR8-HY PB2+PB1+PA_INDO/05(HA+NA)
- ▽ UW-PR8+PR8-HY PB2+PB1+PA+NP_INDO/05(HA+NA)
- PR8-HY_INDO/05(HA+NA)

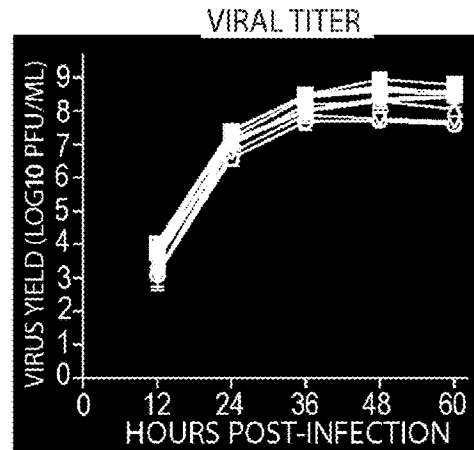


Fig. 20E

- UW-PR8_INDO/05(HA+NA)
- ▲ UW-PR8+PR8-HY PB2_INDO/05(HA+NA)
- ▼ UW-PR8+PR8-HY PB1_INDO/05(HA+NA)
- ◆ UW-PR8+PR8-HY PA_INDO/05(HA+NA)
- UW-PR8+PR8-HY NP_INDO/05(HA+NA)
- UW-PR8+PR8-HY NS_INDO/05(HA+NA)
- ☆ UW-PR8+PR8-HY PB2+PB1+PA_INDO/05(HA+NA)
- ▽ UW-PR8+PR8-HY PB2+PB1+PA+NP_INDO/05(HA+NA)
- PR8-HY_INDO/05(HA+NA)

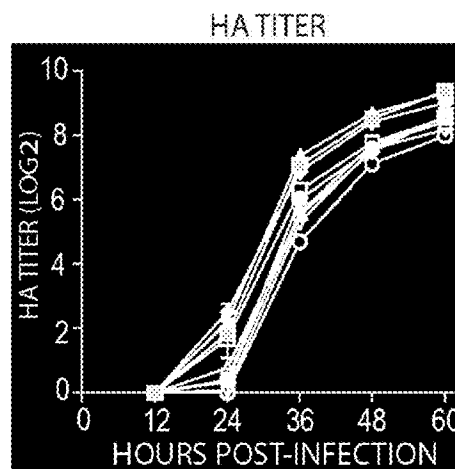
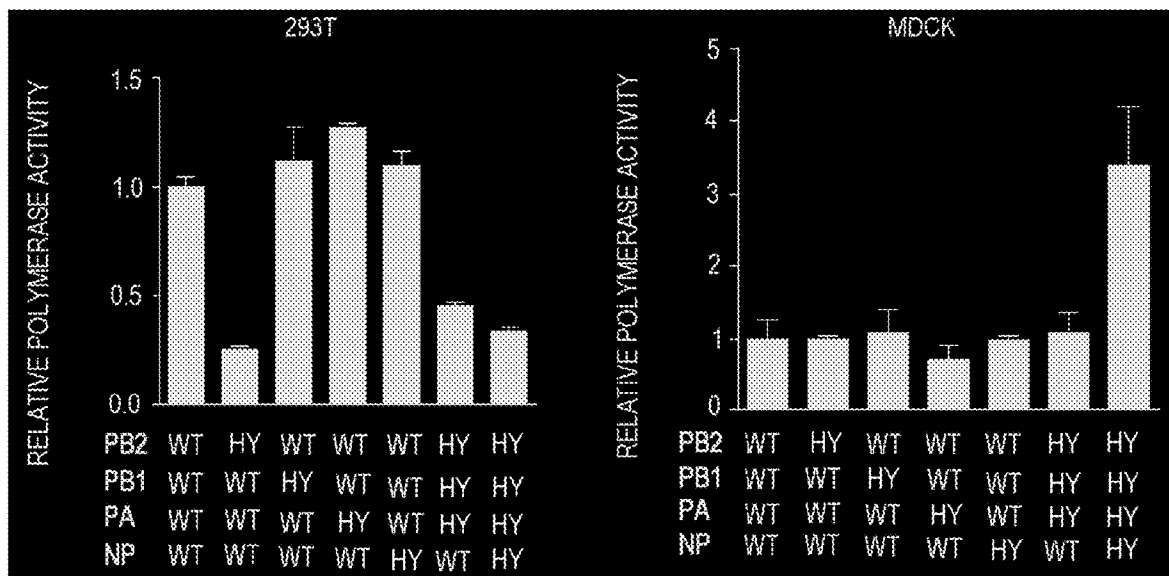
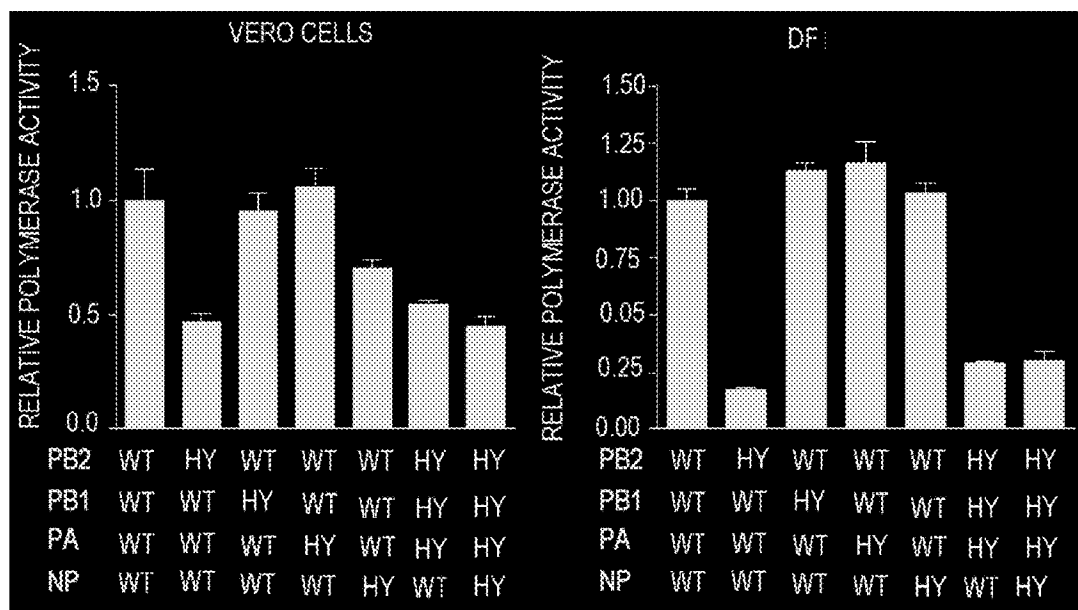
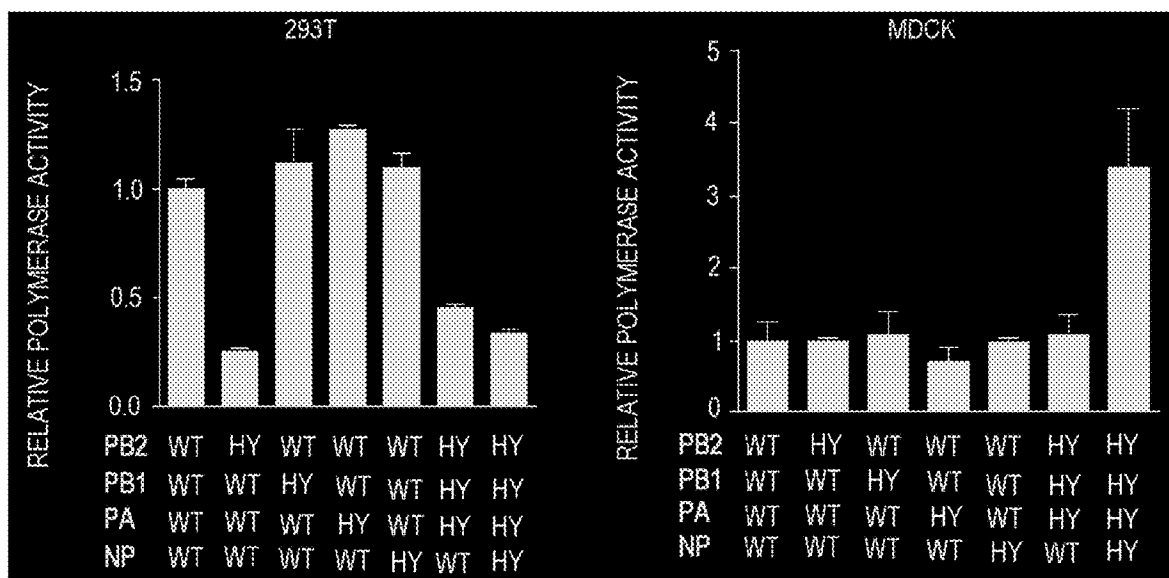
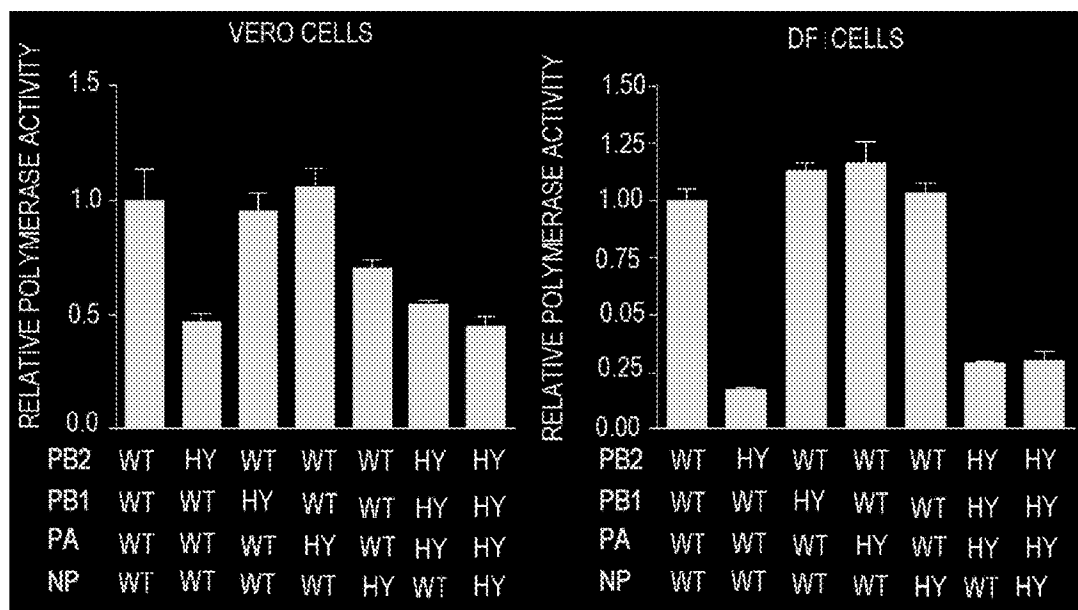


Fig. 20F

*Fig. 21A**Fig. 21B**Fig. 21C**Fig. 21D*

*Fig. 21E**Fig. 21F**Fig. 21G**Fig. 21H*

- UW-PR8_Indo/05(HA+NA)
- ▲ UW-PR8+HY-Vero PB2_Indo/05(HA+NA)
- ▼ UW-PR8+HY-Vero PB1_Indo/05(HA+NA)
- ◆ UW-PR8+HY-Vero PA_Indo/05(HA+NA)
- UW-PR8+HY-Vero NP_Indo/05(HA+NA)
- UW-PR8+HY-Vero NS_Indo/05(HA+NA)
- △ UW-PR8+HY-Vero(PB2+PB1+PA)_Indo/05(HA+NA)
- ▽ UW-PR8+HY-Vero(PB2+PB1+PA+NP)_Indo/05(HA+NA)
- HY-Vero_Indo/05(HA+NA)

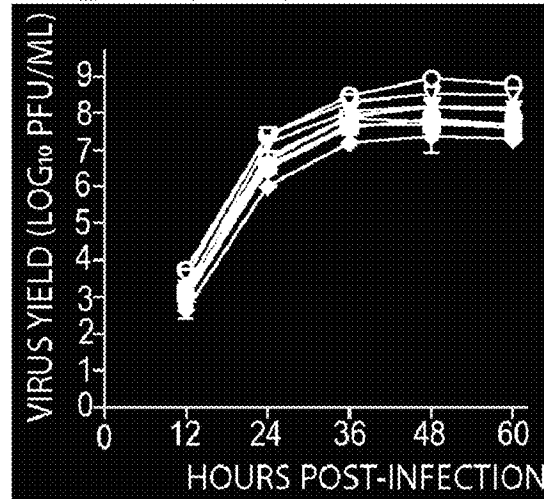


Fig. 22A

- HY-Vero_Indo/05(HA+NA)
- ▲ HY-Vero+UW-PR8 PB2_Indo/05(HA+NA)
- ▼ HY-Vero+UW-PR8 PB1_Indo/05(HA+NA)
- ◆ HY-Vero+UW-PR8 PA_Indo/05(HA+NA)
- HY-Vero+UW-PR8 NP_Indo/05(HA+NA)
- HY-Vero+UW-PR8 NS_Indo/05(HA+NA)
- △ HY-Vero+UW-PR8 (PB2+PB1+PA)_Indo/05(HA+NA)

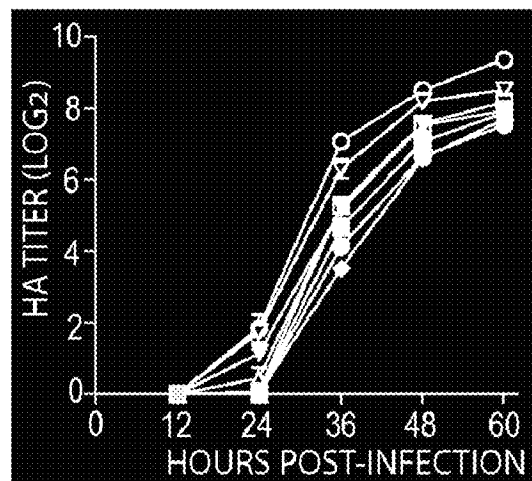


Fig. 22B

- UW-PR8_Indo/05(HA+NA)
- ▲ UW-PR8+HY-Vero PB2_Indo/05(HA+NA)
- ▼ UW-PR8+HY-Vero PB1_Indo/05(HA+NA)
- ◆ UW-PR8+HY-Vero PA_Indo/05(HA+NA)
- UW-PR8+HY-Vero NP_Indo/05(HA+NA)
- UW-PR8+HY-Vero NS_Indo/05(HA+NA)
- △ UW-PR8+HY-Vero(PB2+PB1+PA)_Indo/05(HA+NA)
- ▽ UW-PR8+HY-Vero(PB2+PB1+PA+NP)_Indo/05(HA+NA)
- HY-Vero_Indo/05(HA+NA)

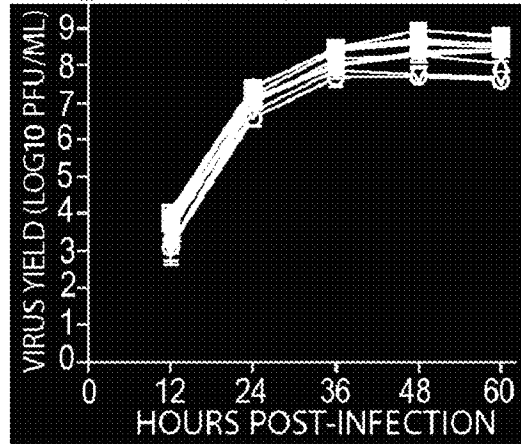


Fig. 22C

- HY-Vero_Indo/05(HA+NA)
- ▲ HY-Vero+UW-PR8 PB2_Indo/05(HA+NA)
- ▼ HY-Vero+UW-PR8 PB1_Indo/05(HA+NA)
- ◆ HY-Vero+UW-PR8 PA_Indo/05(HA+NA)
- HY-Vero+UW-PR8 NP_Indo/05(HA+NA)
- HY-Vero+UW-PR8 NS_Indo/05(HA+NA)
- △ HY-Vero+UW-PR8 (PB2+PB1+PA)_Indo/05(HA+NA)

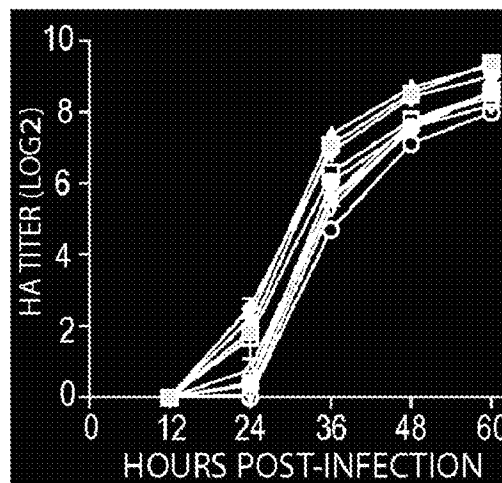
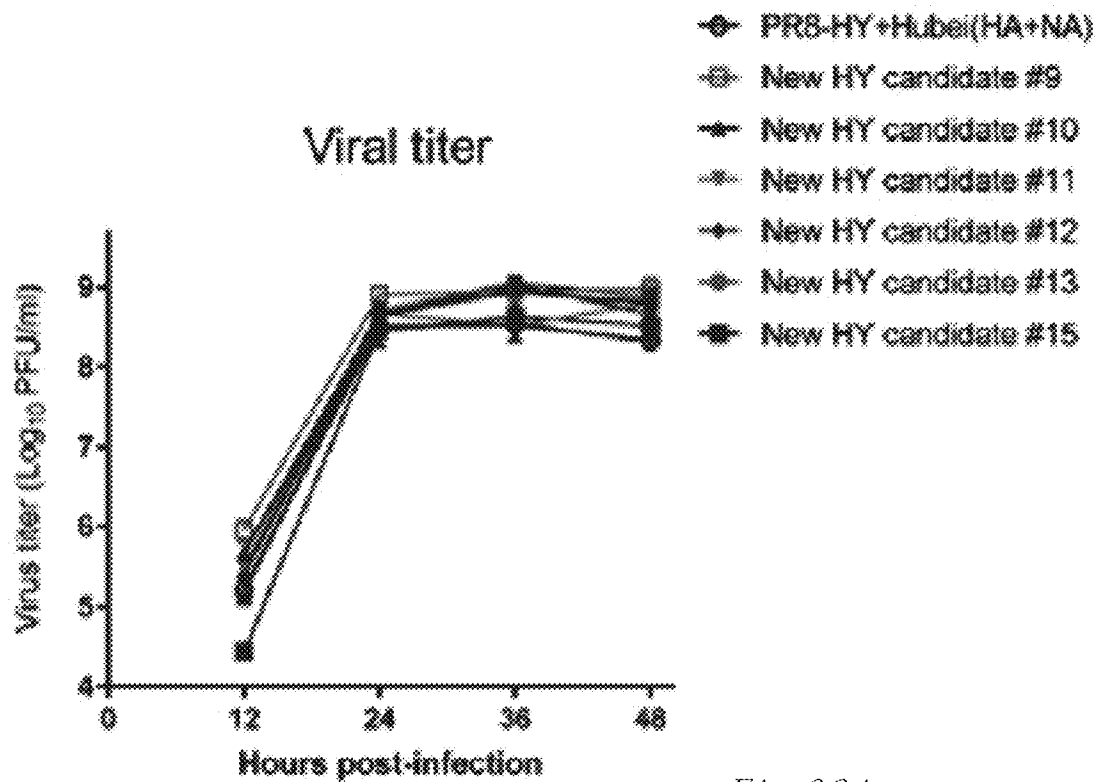
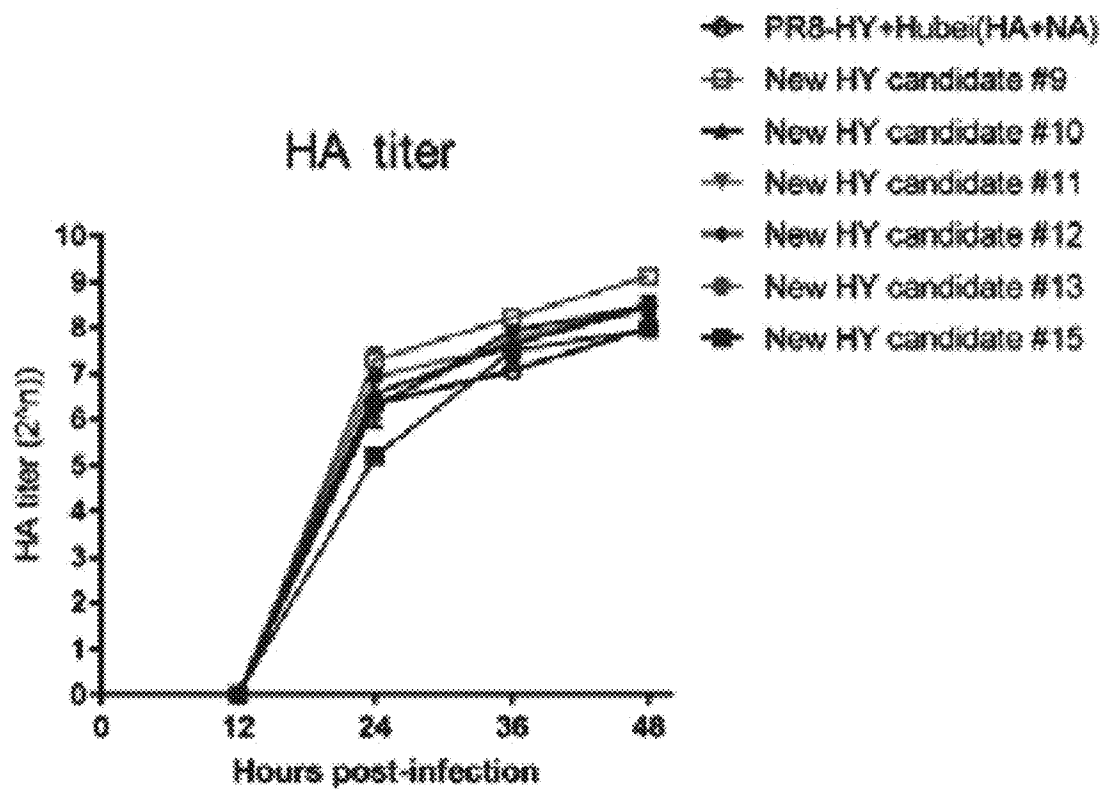
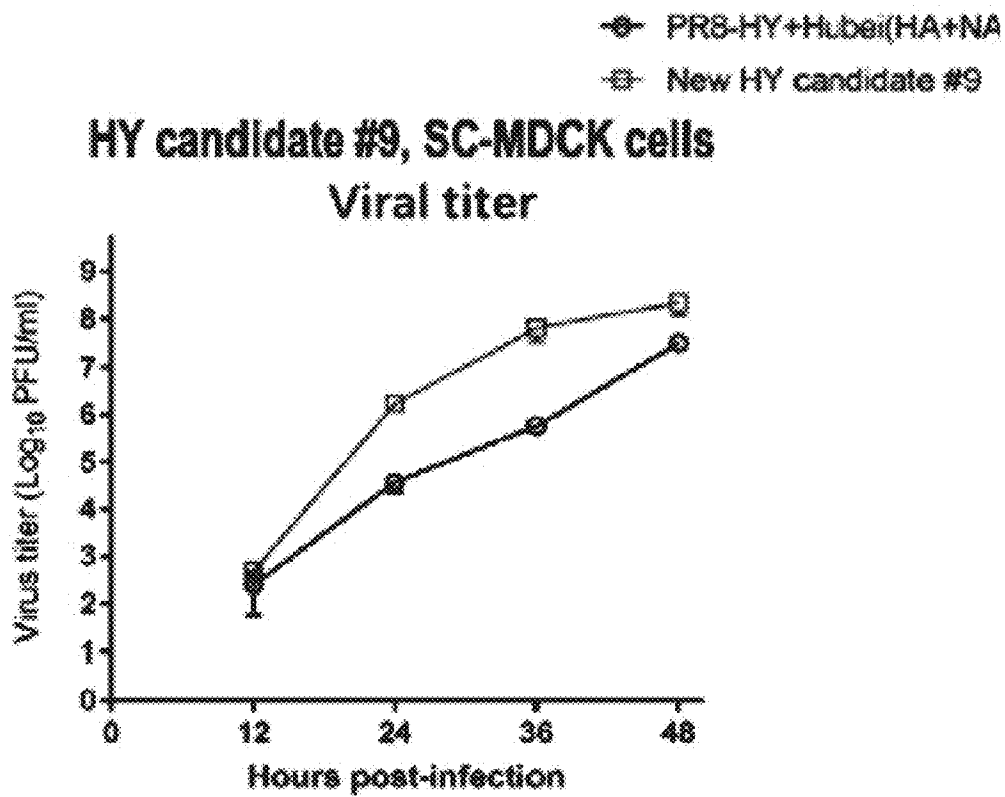
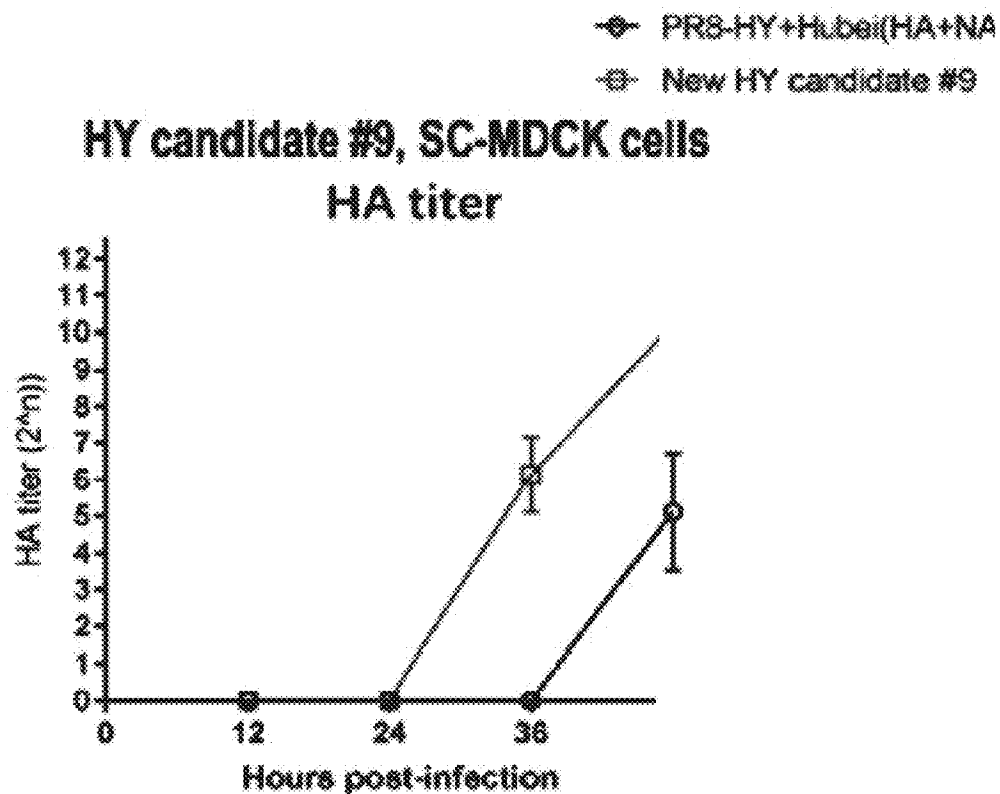
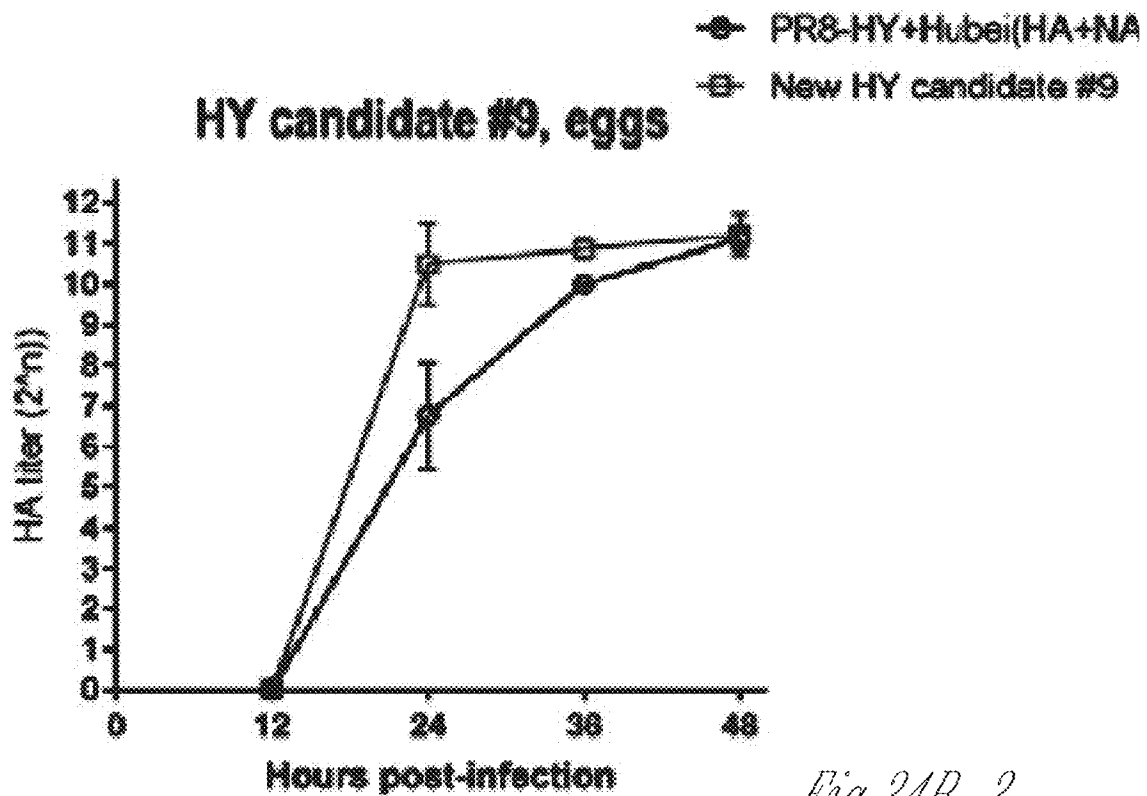
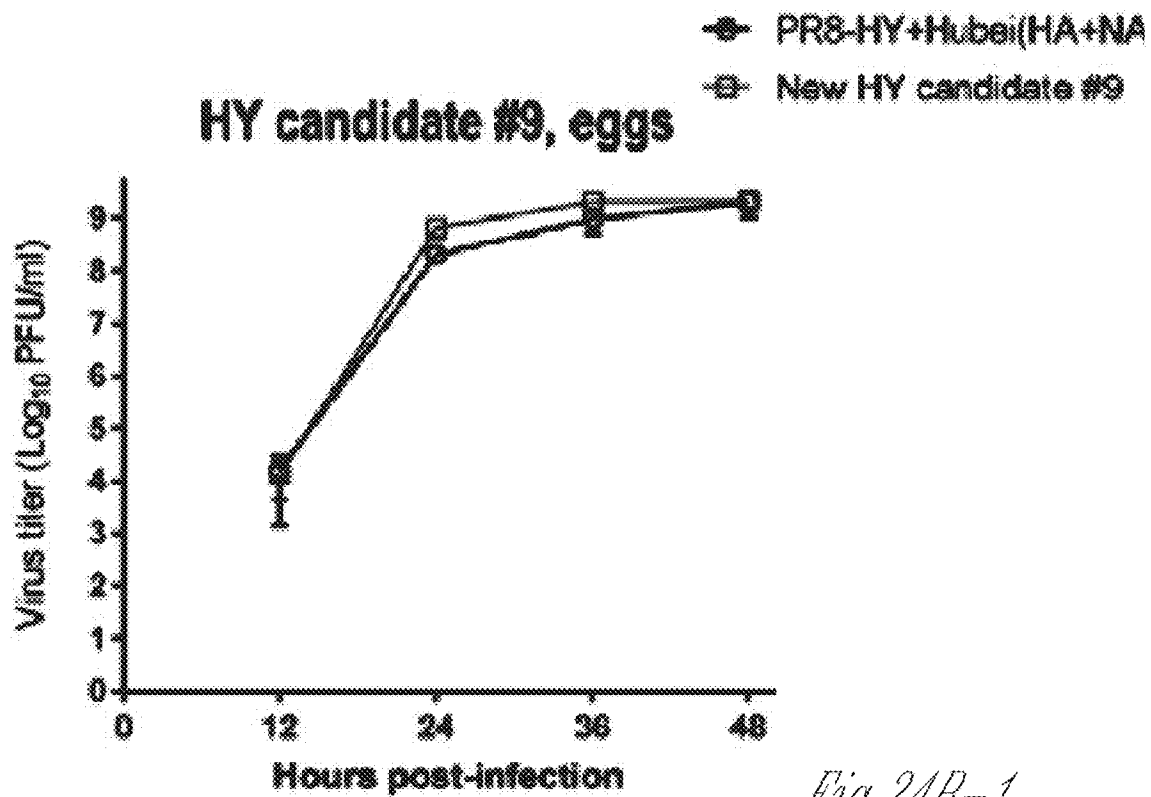
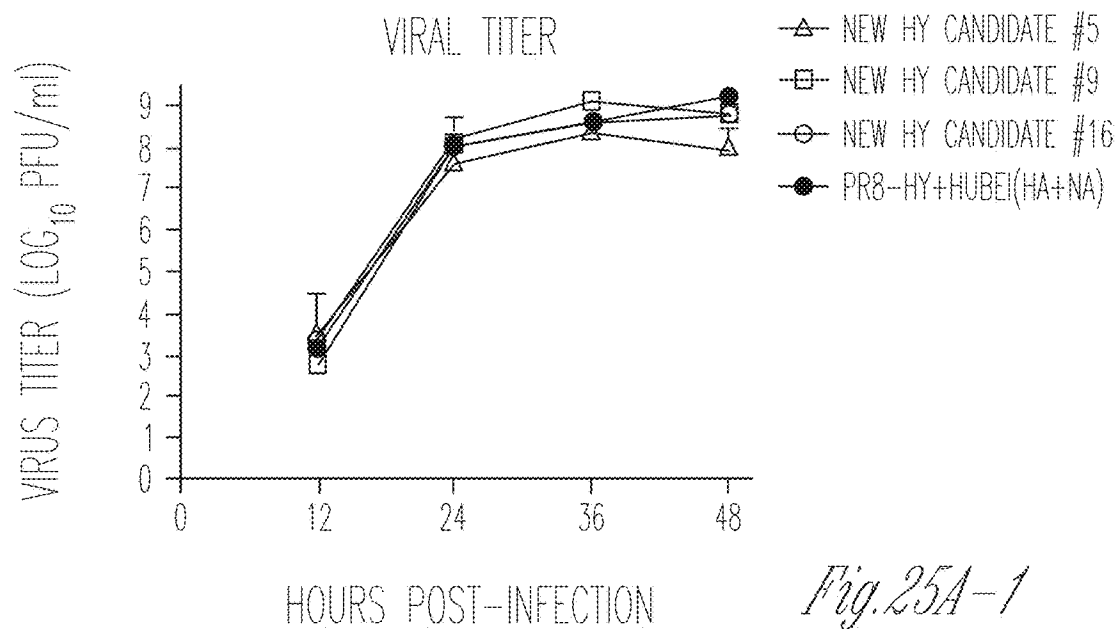
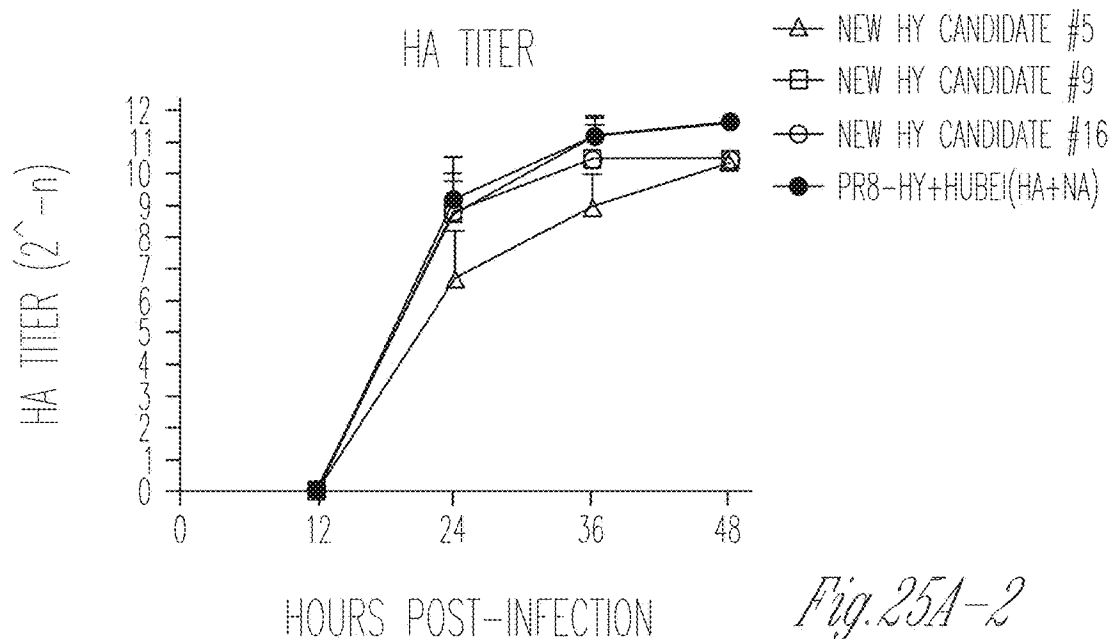


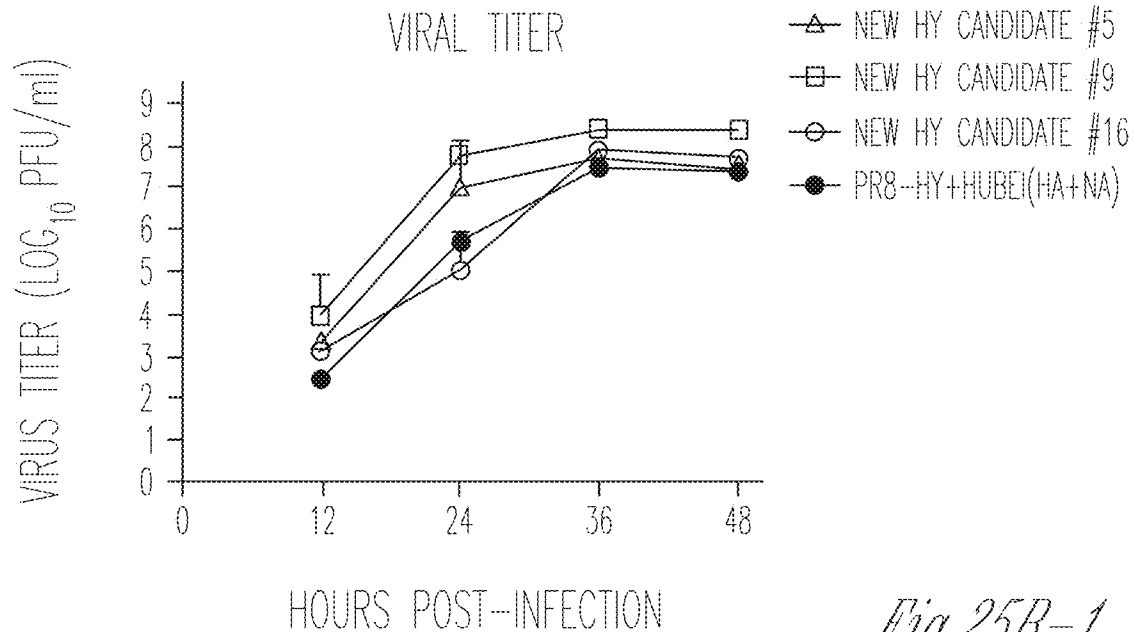
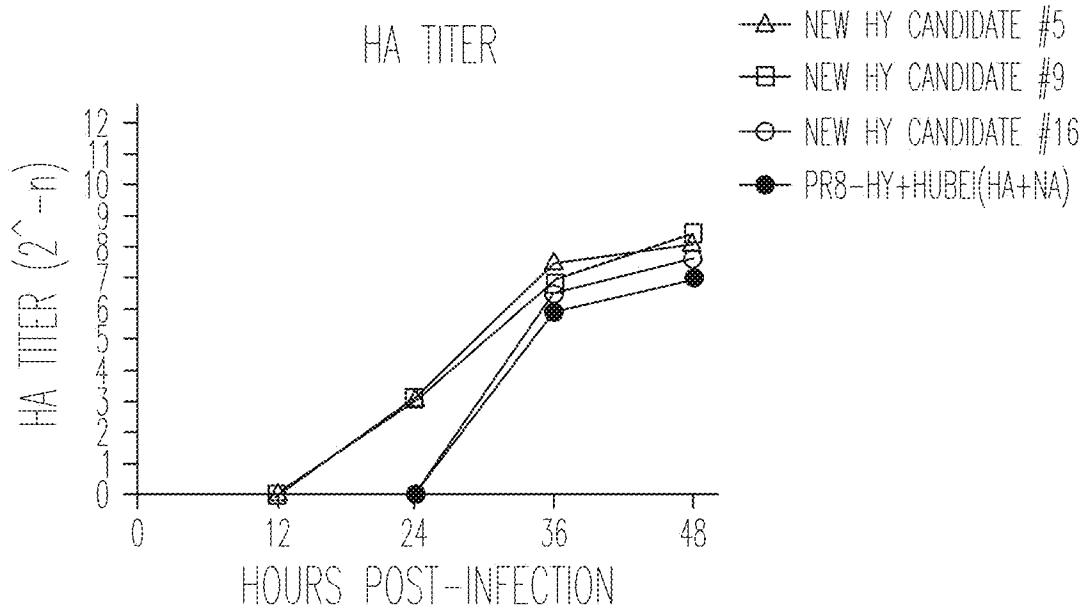
Fig. 22D

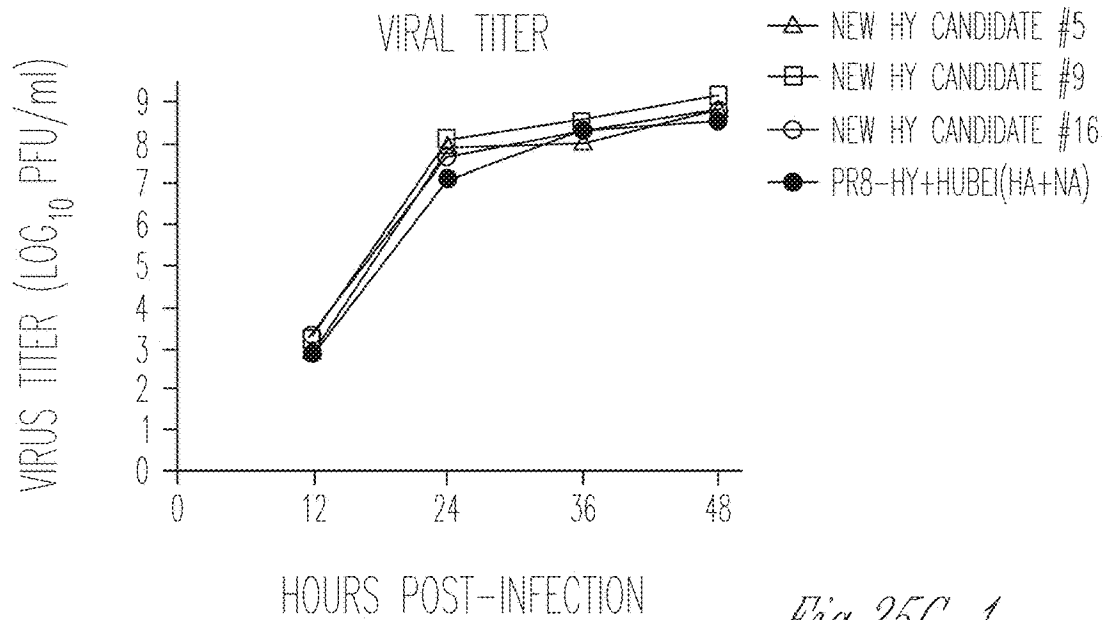
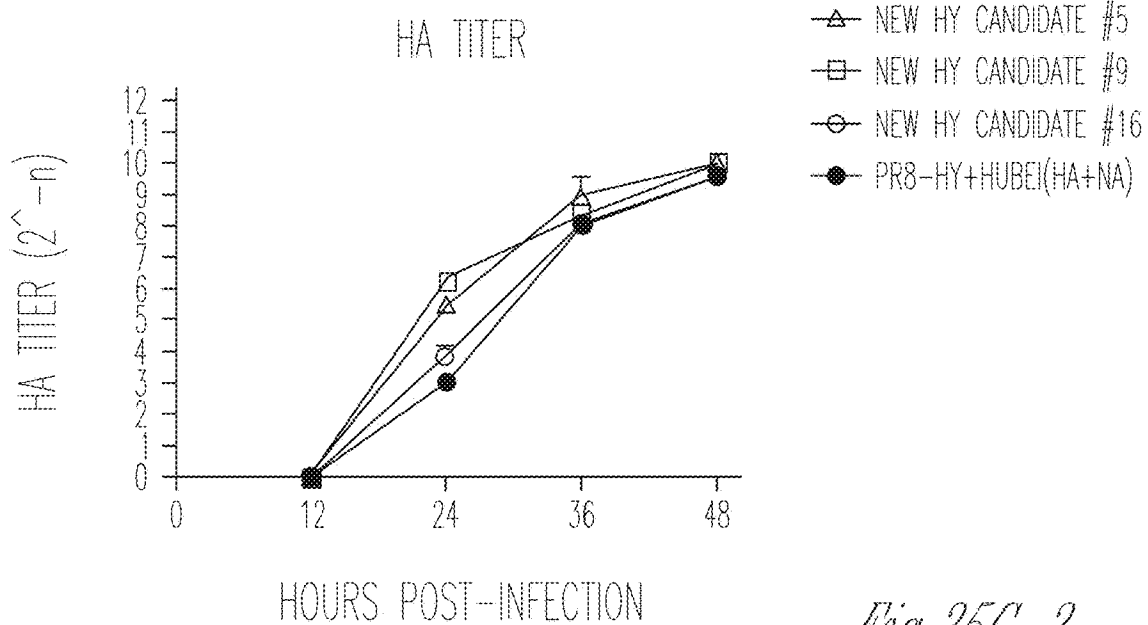
*Fig. 23A**Fig. 23B*

*Fig. 24A-1**Fig. 24A-2*



HY candidate #5,9#,16#,WT,Eggs**HY candidate #5,9#,16#,WT,Eggs**

HY candidate #5,9#,16#,WT,SC-MDCK cells*Fig. 25B-1***HY candidate #5,9#,16#,WT,SC-MDCK cells***Fig. 25B-2*

HY candidate #5,9#,16#,WT,SF-MDCK cells*Fig. 25C-1***HY candidate #5,9#,16#,WT,SF-MDCK cells***Fig. 25C-2*

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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 No. 62/841,491, filed on May 1, 2019, the disclosure of which is incorporated by reference 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 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. The H1 N1 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

Several strategies were employed (including random mutagenesis and the comprehensive testing of potentially growth-enhancing mutations) to develop influenza A/Puerto Rico/8/34 (H1N1; the strain commonly used for the generation of inactivated influenza vaccines) viruses that replicate to high titers in cultured cells and/or embryonated chicken eggs. A number of growth-enhancing mutations were identified that increase the yield of influenza vaccine viruses. Individual growth-enhancing residues in an influenza virus

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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 (see Example 1), 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, M1, and NS1; PA, PB1, PB2, NP and NS1; or PA, PB1, PB2, NP, M1, and NS1, and including HA and NA genes/proteins

of interest, e.g., from annual and pandemic strains, which viruses are produced more efficiently and cost-effectively via cell culture (in MDCK or Vero cells) or in embryonated chicken eggs than parental virus. 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. Recombinant virus may also be obtained by generating influenza viral plus-sense cRNA from a transcription cassette.

In one embodiment, the isolated recombinant influenza virus has PA, PB1, PB2, NP, NS, and M viral segments from a first influenza virus isolate, a heterologous or chimeric influenza virus NA viral segment, and a heterologous or chimeric HA viral segment, wherein the PB1 viral segment encodes a PB1 with a residue other than isoleucine at position 711 or the M viral segment encodes a M1 with a residue other than methionine at position 128, wherein the recombinant influenza virus has enhanced replication, e.g., in MDCK cells, Vero cells or eggs, relative to a corresponding influenza virus having a PB1 viral segment that encodes a PB1 with an isoleucine at position 711 or having a M viral segment that encodes a M1 with methionine at position 128. In one embodiment, the PB1 viral segment encodes a PB1 with a residue other than isoleucine at position 711 and the M viral segment encodes a M1 with a residue other than methionine at position 128. In one embodiment, the PA viral segment encodes a PA with a residue other than phenylalanine at position 105, a residue other than lysine at position 142, a residue other than serine at position 149, a residue other than serine at position 225, a residue other than lysine at position 356, a residue other than threonine at position 357, a residue other than arginine at residue 401, or a residue other than isoleucine at position 550, or any combination thereof. In one embodiment, the PB1 viral segment further encodes a PB1 with a residue other than methionine at position 40, a residue other than glutamic acid at position 112, a residue other than glycine at position 180, or a residue other than glutamine at residue 247, or any combination thereof. In one embodiment, the PB2 viral segment encodes a PB2 a residue other than methionine at position 202, a residue other than phenylalanine at position 323, or a residue other than isoleucine at position 504, or any combination thereof. In one embodiment, the NP viral segment encodes a NP with a residue other than arginine at position 74, a residue other than isoleucine at position 116, or a residue other than asparagine at position 417, or any combination thereof. In one embodiment, the NS viral segment encodes a NS1 with a residue other than alanine at position 30, a residue other than lysine at position 55, or a residue other than arginine at position 118, or any combination thereof. In one embodiment, the recombinant virus has a V, A, L, G, S, T, C, or M at position 711 in PB1, has an A, L, G, V, S, T, I, or C at position 40 in PB1, has an A, L, G, V, S, T, I, or C at position 112 in PB1, has an A, L, W, Y, F, V, S, T, I, or C at position 180 in PB1, has a H, R, K, D, E, D or E at position 247 in PB1, or any combination thereof. In one embodiment, the recombinant virus has a K, H, D, E, Q or N at position 74 in NP, has a L, V, G, A, S, C or T at position 116 in NP, has a D, E, Q, K, or H at position 417 in NP, or any combination thereof. In one embodiment, the recombinant virus has a P, W, F, L, I, V, G, S, C or T at position 30 in NS1, has E, D, Q, or N at position 55, has a K, H, D, E, Q, or N at position 118 in NS1, has a L, I, V, G, A, S, C or T at position 128 in M1, or any combination thereof. In one embodiment, the recombinant virus has a C, L, I, V, G, A, or

T at position 142 in PA, has a C, L, I, V, G, A, or T at position 225 in PA, has a K, H, D, E, N or Q at position 401 in PA, or any combination thereof. In one embodiment, the recombinant virus has a V, A, L, G, S, T, C, or M at position 504 in PB2, has a A, L, G, S, T, I, or C at position 202 in PB2, has a A, L, G, S, T, I, or C at position 323 in PB2, or any combination thereof. In one embodiment, the recombinant virus has a V, A, L, G, S, T, C, or M at position 711 in PB1, has a A, L, G, V, S, T, I, or C at position 40 in PB1, has a A, L, W, Y, F, V, S, T, I, or C at position 180 in PB1; has a L, V, G, A, S, C or T at position 116 in NP; has a P, W, F, L, I, V, G, S, C or T at position 30 in NS1, has a K, H, D, E, Q, or N at position 118 in NS1; has a L, I, V, G, A, S, C or T at position 128 in M1; has a K, H, D, E, N or Q at position 401 in PA; has a V, A, L, G, S, T, C, or M at position 504 in PB2; or any combination thereof. In one embodiment, the recombinant virus has 504V in PB2. In one embodiment, the recombinant virus has 74K, 116L or 417D in NP. In one embodiment, the recombinant virus has 30P, 55E or 118K in NS1. In one embodiment, the recombinant virus has 225C or 401K in PA. In one embodiment, the recombinant virus of has a V, A, L, G, S, T, C, or M at position 711 in PB1. In one embodiment, the recombinant virus has a L, I, V, G, A, S, C or T at position 128 in M1. In one embodiment, the recombinant virus has a U at position 4 in the viral segment for any one of PB1, PB2 or PA. In one embodiment, the recombinant virus has a U at position 4 in the viral segment for PB1, PB2 and PA. 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 P32 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 M1 and/or M2 having the amino acid sequence encoded by SEQ ID NO:5 or M1 and/or M2 with at least 95% amino acid sequence identity to the M1 and/or M2 encoded by SEQ ID NO:5; or a NS1 and/or NS2 having the amino acid sequence encoded by SEQ ID NO:6 or NS1 and/or NS2 with at least 95% amino acid sequence identity to the NS encoded by SEQ ID NO:6 or wherein 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 M1 and/or M2 having the amino acid sequence encoded by SEQ ID NO:14 or M1 and/or M2 with at least 95% amino acid sequence identity to the M1 and/or M2 encoded by SEQ ID NO:14; or a NS1 and/or NS2 having the amino acid sequence encoded by SEQ ID NO:15 or NS1 and/or NS2 with at least 95% amino acid sequence identity to the NS1 and/or NS2 encoded by SEQ ID NO:15. In one embodiment, two of the PA, PB1,

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PB2, NP, NS, and M viral segments have at least one of the alterations disclosed herein. In one embodiment, three of the PA, PB1, PB2, NP, NS, and M viral segments have at least one of the alterations disclosed herein. In one embodiment, four of the PA, PB1, PB2, NP, NS, and M viral segments have at least one of the alterations disclosed herein. In one embodiment, five of the PA, PB1, PB2, NP, NS, and M viral segments have at least one of the alterations disclosed herein. In one embodiment, the PA, PB1, PB2, NP, NS, and M viral segments have at least one of the alterations disclosed herein.

Also provided is 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 NA, and wherein the HA DNA in the vector for vRNA production of HA has sequences for a heterologous HA, wherein the PB1 DNA encodes a PB1 with a residue other than isoleucine at position 711 or the M DNA encodes a M1 with a residue other than methionine at position 128; 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 or a mammalian cell. In one embodiment, the cell is a Vero cell, a human cell or a MDCK cell. The cell is in an avian egg.

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Further provided is a method of using the recombinant virus. In one embodiment, an avian or a mammal is administered an amount of the virus effective to induce an immune response.

In one embodiment, the invention provides an isolated recombinant reassortant influenza virus having six "internal" viral segments from a vaccine influenza virus with two or more of the selected amino acid residues at specified positions described herein, and a NA viral segment selected from a first influenza virus isolate, and a HA viral segment from the same isolate or a different isolate.

In one embodiment, the influenza virus of the invention is a recombinant influenza virus having a particular amino acid residue at specified positions, as disclosed herein, 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. 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 or valine-leucine-isoleucine-alanine; phenylalanine-tyrosine; lysine-arginine; alanine-valine; glutamic acid-aspartic acid; 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 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 other than R401 in PA, M40 or G180 in PB1, 1504 in PB2, I116 in NP, and/or A30 or R118 in NS1, or any combination thereof, as well as a residue other than I711 in PB1 or M128 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 other than R401 in PA, M40 or G180 in PB1, 1504 in PB2, I116 in NP, and/or A30 or R118 in NS1, or any combination thereof, as well as a residue other than I711 in PB1 and M128 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 R401 in PA, M40 or G180 in PB1, I504 in PB2, I116 in NP, and/or A30 or R118 in NS1, or any combination thereof, as well as a conservative substitution relative to I711 in PB1 or a conservative substitution relative to M128 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 R401 in PA, M40 or G180 in PB1, I504 in PB2, I116 in NP, and/or A30 or R118 in NS1, or any combination thereof, as well as a conservative substitution relative to I711 in PB1 and a conservative substitution relative to M128 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 R401 in PA, M40 or G180 in PB1, I504 in PB2, I116 in NP, and/or A30 or R118 in NS1, or any combination thereof, as well as a conservative substitution relative to I711 in PB1 or a non-conservative substitution relative to M128 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 R401 in PA, M40 or G180 in PB1, I504 in PB2, I116 in NP, and/or A30 or R118 in NS1, or any combination thereof, as well as a conservative substitution relative to I711 in PB1 and a non-conservative substitution relative to M128 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, P52, 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 conservative residue other than R401 in PA, a non-conservative residue other than M40 or G180 in PB1, a conservative residue other than I504 in PB2 and I116 in NP, a non-conservative residue at A30 or a conservative residue at R118 in NS1, or any combination thereof, as well as a conservative residue other than I711 in PB1 or a non-conservative residue other than M128 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 conservative residue other than R401 in PA, a non-conservative residue other than M40 or G180 in PB1, a conservative residue other than I504 in PB2 and I116 in NP, a non-conservative residue at A30 or a conservative residue at R118 in NS1, or any combination thereof, as well as a conservative residue other than I711 in PB1 and a non-conservative residue other than M128 in M1.

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.

As described herein, virus libraries with random mutations related to 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, were 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, and/or NS1, that confer enhanced growth of the virus in cultured cells when employed with HA and NA sequences of interest, can result in significantly higher viral titers. Thus, the invention provides a method to select for influenza viruses with enhanced replication in cell culture. The method includes providing cells suitable for influenza vaccine production; serially culturing one or more influenza virus isolates in the cells; and isolating serially cultured virus with enhanced growth relative to the one or more isolates prior to serial culture. In one embodiment, the cells are canine or primate, e.g., human or monkey, cells.

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 mRNA 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 transcrip-

tion 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 mRNA production of NA may be from any NA, e.g., any of N1-N1, and the DNA for vRNA production of HA may be from any HA, e.g., H1-H18. 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 10^8 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

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purified nucleic acid molecule comprises a nucleotide sequence which is substantially the same as, e.g., having at least 50%, e.g., 60%, 70%, 80% or 90%, including any integer between 50 and 100, or more contiguous nucleic acid sequence identity to one of SEQ ID NOs:1-6 or 10-15 and, in one embodiment, also encodes a polypeptide having at least 80%, e.g., 90%, 92%, 95%, 97%, 98%, or 99%, including any integer between 80 and 99, contiguous amino acid sequence identity to a polypeptide encoded by one of SEQ ID NOs:1-6 or 10-15. In one embodiment, the influenza virus polypeptide has one or more, for instance, 2, 5, 10, 15, 20 or more, conservative amino acids substitutions, e.g., conservative substitutions of up to 10% or 20% of 2, 5, 10, 15, 20 or more, of a combination of conservative and non-conservative amino acids substitutions, e.g., conservative substitutions of up to 10% or 20% of the residues, or relative to a polypeptide encoded by one of SEQ IS NOs:1-6 or 10-15, and has a characteristic residue in two or more of PA, PB1, PB2, NP, M1, and/or NS1 the residues, relative to a polypeptide encoded by one of SEQ ID NOs:1-6 or 10-15, and has a characteristic residue, as described herein, in two or more of the viral segments for PA, PB1, PB2, NP, M, and/or NS, such as in PA and PB1 viral segments, PB1 and PB2 viral segments, PA, PB1 and PB2 viral segments, or in PA, PB1, PB2 and NP viral segments, or in PA, PB1, PB2 and M viral segments. 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, at positions including but not limited to 401 in PA, 40 or 180 and 711 in PB1, 504 in PB2, 116 in NP, 30 or 118 in NS1, or 128 in M1, or any combination thereof.

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 18 HA or 11 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

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vectors described above to a host cell permissive for influenza virus replication results in recombinant virus comprising vRNA corresponding to the heterologous sequences of the vector.

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

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

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

In one embodiment, the invention provides a plurality of influenza virus vectors for a reassortant, comprising a vector for vRNA production comprising a promoter operably linked to an influenza virus PA DNA linked to a transcription termination sequence, a vector for vRNA production comprising a promoter operably linked to an influenza virus PB1 DNA linked to a transcription termination sequence, a vector for vRNA production comprising a promoter operably linked to an influenza virus PB2 DNA linked to a transcription termination sequence, a vector for vRNA production comprising a promoter operably linked to an influenza virus HA DNA linked to a transcription termination sequence, a vector for vRNA production comprising a promoter operably linked to an influenza virus NP DNA linked to a transcription termination sequence, a vector for vRNA production comprising a promoter operably linked to an influenza virus NA DNA linked to a transcription termination sequence, a vector for vRNA production comprising a promoter operably linked to an influenza virus M DNA linked to a transcription termination sequence, and a vector for vRNA production comprising a promoter operably linked to an influenza virus NS cDNA linked to a transcription termination sequence, wherein the DNAs for PB1, PB2,

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PA, NP, NS, and M are from one or more influenza vaccine seed viruses and contain two or more of the characteristic residues at the specified position(s); and a vector for mRNA production comprising a promoter operably linked to a DNA segment encoding influenza virus PA, a vector for mRNA production comprising a promoter operably linked to a DNA segment encoding influenza virus PB1, a vector for mRNA production comprising a promoter operably linked to a DNA segment encoding influenza virus PB2, and a vector for mRNA production comprising a promoter operably linked to a DNA segment encoding influenza virus NP, and optionally a vector for mRNA production comprising a promoter operably linked to a DNA segment encoding influenza virus HA, a vector for mRNA production comprising a promoter operably linked to a DNA segment encoding influenza virus NA, a vector for mRNA production comprising a promoter operably linked to a DNA segment encoding influenza virus M1, a vector for mRNA production comprising a promoter operably linked to a DNA segment encoding influenza virus M2, or a vector for mRNA production comprising a promoter operably linked to a DNA segment encoding influenza virus N52. 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,

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PB1, PB2 and NP employed in the method are on one plasmid or on two or three different plasmids.

In one embodiment, the invention provides a method to select for influenza viruses with enhanced replication in cell culture. The method includes providing cells suitable for influenza vaccine production; serially culturing one or more influenza virus isolates in the cells; and isolating serially cultured virus with enhanced growth relative to the one or more isolates prior to serial culture. In one embodiment, the cells are rodent or primate cells.

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

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

The influenza virus of the invention may be 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-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-5D. Growth curves of UW-PR8 viruses possessing previously identified mutations in PB2 (A), PB1 or PB1-F2 (B), PA (C), and NP, M or NS1 (D).

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

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

FIGS. 8A-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-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-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 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 no. 1-4 and 16-19).

FIGS. 11A-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).

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

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

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

FIGS. 19A-19D. Exemplary high yield substitutions (relative to PR8 (UW)).

FIGS. 20A-20F. Growth kinetics and HA titers of reassortant viruses possessing one or several vRNAs of PR8-HY virus.

FIGS. 21A-21H. 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.

FIGS. 22A-22D. HA titer and virus yield for various recombinant viruses and parent reassortant virus in Vero cells.

FIGS. 23A-23B. Growth kinetics analysis of Hubei/1/10 (H5N1) high yield candidates in serum-free (SF)-MDCK cells.

FIGS. 24A1-24B2. Growth kinetics analysis of HY candidate #9 backbone in SC-MDCK cells (A) and in eggs (B). HY candidate #9 was selected from SC-MDCK cells.

FIGS. 25A1-25C2. Growth kinetics analysis of HY candidate #5, #9, #16 backbone in eggs (A), in SC-MDCK cells (B) and in SF-MDCK cells (C).

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 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 (layer & Webster, 1976); or a nonionic detergent such as that commercialized under the name TRITON X100. The hemagglutinin may also be isolated after treatment of the virions with a protease such as 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 (live 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, an isolated influenza virus having PA, PB1, PB2, NP, NS, M, NA and HA viral segments is provided, wherein the PB1 viral segment encodes a PB1 with a residue other than isoleucine at position 711 or the M viral segment encodes a M1 with a residue other than methionine at position 128, wherein the influenza virus has enhanced replication relative to a corresponding influenza virus having a PB1 viral segment that encodes a PB1 with an isoleucine at position 711 or having a M viral segment

that encodes a M1 with methionine at position 128. In one embodiment, the NA viral segment is heterologous to the PA, PB1, PB2, NP, NS, and M viral segments. In one embodiment, the NA and HA viral segments are from the same viral isolate. In one embodiment, the PB1 viral segment encodes a PB1 with a residue other than isoleucine at position 711 and the M viral segment encodes a M1 with a residue other than methionine at position 128. In one embodiment, the PA viral segment encodes a PA with a residue other than phenylalanine at position 105, a residue other than lysine at position 142, a residue other than serine at position 149, a residue other than serine at position 225, a residue other than lysine at position 356, a residue other than threonine at position 357, a residue other than arginine at residue 401, or a residue other than isoleucine at position 550, or any combination thereof. In one embodiment, the PB1 viral segment further encodes a PB1 with a residue other than methionine at position 40, a residue other than glutamic acid at position 112, a residue other than glycine at position 180, or a residue other than glutamine at residue 247, or any combination thereof. In one embodiment, the PB2 viral segment encodes a PB2 a residue other than methionine at position 202, a residue other than phenylalanine at position 323, or a residue other than isoleucine at position 504, or any combination thereof. In one embodiment, the NP viral segment encodes a NP with a residue other than arginine at position 74, a residue other than isoleucine at position 116, or a residue other than asparagine at position 417, or any combination thereof. In one embodiment, the NS viral segment encodes a NS1 with a residue other than alanine at position 30, a residue other than lysine at position 55, or a residue other than arginine at position 118, or any combination thereof.

In one embodiment, the PA viral segment encodes a PA with a residue other than phenylalanine at position 105, a residue other than lysine at position 142, a residue other than serine at position 149, a residue other than serine at position 225, a residue other than arginine at position 256, a residue other than asparagine at position 350, a residue other than lysine at position 356, a residue other than threonine at position 357, a residue other than alanine at position 369, a residue other than arginine at residue 401, or a residue other than isoleucine at position 550, or any combination thereof.

In one embodiment, the PB1 viral segment encodes a PB1 with a residue other than methionine at position 40, a residue other than methionine at position 92, a residue other than asparagine at position 105, a residue other than glutamic acid at position 112, a residue other than glutamic acid at position 178, a residue other than glycine at position 180, a residue other than glutamine at residue 247, a residue other than methionine at position 290, a residue other than methionine at position 325, a residue other than isoleucine at position 711, or any combination thereof.

In one embodiment, the PB2 viral segment encodes a PB2 a residue other than arginine at position 62, a residue other than methionine at position 202, a residue other than alanine at position 221, a residue other than phenylalanine at position 323, a residue other than alanine at position 370, a residue other than methionine at position 467, a residue other than isoleucine at position 504, a residue other than tyrosine at position 704, or a residue other than lysine at position 721, or any combination thereof.

In one embodiment, the NP viral segment encodes a NP with a residue other than arginine at position 74, a residue other than lysine at position 103, a residue other than isoleucine at position 116, a residue other than valine at position 194, or a residue other than asparagine at position

417, or any combination thereof. In one embodiment, the M viral segment encodes a M1 with a residue other than serine at position 126, with a residue other than methionine at position 128, a residue other than methionine at position 244, or a M2 with a residue other than tryptophan at position 15, or any combination thereof.

In one embodiment, the NS viral segment encodes a NS1 with a residue other than alanine at position 30, a residue other than lysine at position 55, a residue other than aspartic acid at position 101, or a residue other than arginine at position 118, or any combination thereof.

In one embodiment, the virus has a V, A, L, G, S, T, C, or M at position 711 in PB1, has an A, L, G, V, S, T, I, or C at position 40 in PB1, has an A, L, G, V, S, T, I, or C at position 112 in PB1, has an A, L, W, Y, F, V, S, T, I, or C at position 180 in PB1, has a H, R, K, D or E at position 247 in PB1, has a V, A, L, G, S, T, C, or I at position 290 in PB1, has a V, A, L, G, S, T, C, or I at position 92 in PB1, has a V, A, L, G, S, T, C, or I at position 325 in PB1, has a H, R, K, D, or E at position 105 in PB1, has a H, R, or K at position 178 in PB1, or any combination thereof.

In one embodiment, the virus has a V, A, L, or G at position 711 in PB1, has an A, L, G, V, or I at position 40 in PB1, has an A, L, G, V, S, T, or I at position 112 in PB1, has a W, Y, or F, at position 180 in PB1, has a H, R, K, D or E at position 247 in PB1, has a V, A, L, G, or I at position 290 in PB1, has a V, A, L, G, or I at position 92 in PB1, has a V, A, L, G, or I at position 325 in PB1, has a H, R, or K at position 105 in PB1, has a R or K at position 178 in PB1, or any combination thereof.

In one embodiment, the virus has a K, H, D, E, Q or N at position 74 in NP, has a R or H at position 103 in NP, has a I, A, L, or G at position 194 in NP, has a L, V, G, A, S, or T at position 116 in NP, has a D, E, Q, K, or H at position 417 in NP, or any combination thereof.

In one embodiment, the virus of has a P, W, F, S or T at position 30 in NS1, has E, D, Q, or N at position 55, has a L, I, V, G, A or T at position 101 in NS1, has a K, H, D, E, Q, or N at position 118 in NS1, or any combination thereof.

In one embodiment, the virus has a E, D, H, R or K at position 142 in PA, has a C, L, I, V, G, A, or T at position 225 in PA, has a L, I, V, S, G, A, or T at position 256 in PA, has a L, I, V, G, A, or T at position 350 in PA, has a L, I, V, G, S, or T at position 356 in PA, has a K, H, D, E, N or Q at position 401 in PA, or any combination thereof.

In one embodiment, the virus has a V, A, L, G, S, or T at position 504 in PB2, has a A, L, G, S, T, I, or C at position 202 in PB2, has a A, L, G, S, T, I, or C at position 323 in PB2, has a V, L, G, or I at position 221 in PB2, has a V, L, G, T or I at position 370 in PB2, has a V, L, G, S, T or I at position 62 in PB2, has a V, L, A, G, S, T, or lat position 467 in PB2, has a K, H, D, E, N or Q at position 704 in PB2, has a R, H, D, E, N or Q at position 721 in PB2, or any combination thereof.

In one embodiment, the virus has a V, A, L, G, or T at position 126 in M1, has a V, A, L, G, T, or Sat position 128 in M1, has a R, K, D, E or H at position 244 in M1, has a A, L, G, S, T, or I at position 15 in M2, or any combination thereof.

In one embodiment, the virus has a V, A, L, G, S, T, C, or M at position 711 in PB1, has a A, L, G, V, S, T, I, or C at position 40 in PB1, has a A, L, W, Y, F, V, S, T, I, or Cat position 180 in PB1; has a A, L, V, G, A, S, C or T at position 116 in NP; has a P, W, F, L, I, V, G, S, C or T at position 30 in NS1, has a K, H, D, E, Q, or N at position 118 in NS1; has a L, I, V, G, A, S, C or T at position 128 in M1; has a K, H, D, E, N or Q at position 401 in PA; has a V, A, L, G,

S, T, C, or M at position 504 in PB2; or any combination thereof. In one embodiment, the virus has a V, A, L, G, S, T, C, or M at position 711 in PB1, has a H, R, K, D, E, D or E at position 247 in PB1; has a K, H, D, E, Q or N at position 74 in NP; has E, D, Q, or N at position 55 in NS1; has a L, I, V, G, A, S, C or T at position 128 in M1; has a C, L, I, V, G, A, or T at position 142 in PA; has a A, L, G, S, T, I, or C at position 202 in PB2, has a A, L, G, S, T, I, or C at position 323 in PB2; or any combination thereof. In one embodiment, the virus has a V, A, L, G, S, T, C, or M at position 711 in PB1, has a A, L, G, V, S, T, I, or C at position 112 in PB1; has a K, H, D, E, Q or N at position 74 in NP, has a D, E, Q, K, or H at position 417 in NP; has E, D, Q, or N at position 55; has a L, I, V, G, A, S, C or T at position 128 in M1; has a C, L, I, V, G, A, or T at position 225 in PA; has a V, A, L, G, S, T, C, or M at position 504 in PB2; or any combination thereof. In one embodiment, the 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 any combination thereof. 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 virus has 202L and/or 323L in PB2.

In one embodiment, the virus has 247H in PB1. In one embodiment, the virus has 55E in NS1. In one embodiment, the virus has 142C in PA. In one embodiment, the virus has 74K in NP. In one embodiment, the virus has 504V in PB2. In one embodiment, the virus has 74K, 116L or 417D in NP.

In one embodiment, the virus has 30P, 55E or 118K in NS1. In one embodiment, the virus has 225C or 401K in PA. In one embodiment, the virus has a V, A, L, G, S, T, C, or M at position 711 in PB1. In one embodiment, the virus has a L, I, V, G, A, S, C or T at position 128 in M1. In one embodiment, the virus has a U at position 4 in the viral segment for any one of PB1, PB2 or PA. 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 M1 and/or M2 having the amino acid sequence encoded by SEQ ID NO:5 or M1 and/or M2 with at least 95% amino acid sequence identity to the M1 and/or M2 encoded by SEQ ID NO:5; or a NS1 and/or NS2 having the amino acid sequence encoded by SEQ ID NO:6 or NS1 and/or NS2 with at least 95% amino acid sequence identity to the NS1 and/or NS2 encoded by SEQ ID NO:6 or wherein 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 M1 and/or M2 having the amino acid sequence encoded by SEQ ID NO:14 or M1 and/or M2 with at least 95% amino acid sequence identity to the M1 and/or M2 encoded by SEQ ID NO:14; or a NS1 and/or NS2 having the amino acid sequence encoded by SEQ ID NO:15 or NS1 and/or NS2 with at least 95% amino acid sequence identity to the NS1 and/or NS2 encoded by SEQ ID NO:15.

A vaccine having the isolated virus is also provided.

In one embodiment, a plurality of influenza virus vectors for preparing a reassortant virus is provided. In one embodiment, the plurality includes but is not limited to 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, wherein the PB1 DNA encodes a PB1 with a residue other than isoleucine at position 711 or the M DNA encodes a M1 with a residue other than methionine at position 128; 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 HA is H1, H3, H5, H7 or H9. In one embodiment, the PA, PB1, and PB2 viral segments, and optionally NP, NS, and M viral segments has a promoter C to a mutation.

Further provided is 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 NA, and wherein the HA DNA in the vector for vRNA production of HA has sequences for a heterologous HA, wherein the PB1 DNA encodes a PB1 with a residue other than isoleucine at position 711 or the M DNA encodes a M1 with a residue other than methionine at position 128; 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 or a mammalian cell. In one embodiment, the cell is 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 virus is isolated. In one embodiment, the PB1 viral segment encodes a PB1 with a residue other than isoleucine at position 711 and the M viral segment encodes a M1 with a residue other than methionine at position 128. In one embodiment, the PA viral segment encodes a PA with a residue other than phenylalanine at position 105, a residue other than lysine at position 142, a residue other than serine at position 149, a residue other than serine at position 225, a residue other than lysine at position 356, a residue other than threonine at position 357, a residue other than arginine at residue 401, or a residue other than isoleucine at position 550, or any combination thereof; or wherein the PB1 viral segment further encodes a PB1 with a residue other than methionine at position 40, a residue other than glutamic acid at position 112, a residue other than glycine at position 180, or a residue other than glutamine at residue 247, or any combination thereof; or wherein the PB2 viral segment encodes a PB2 a residue other than methionine at position 202, a residue other than phenylalanine at position 323, or a residue other than isoleucine at position 504, or any combination thereof or wherein the NP viral segment encodes a NP with a residue other than arginine at position 74, a residue other than isoleucine at position 116, or a residue other than asparagine at position 417, or any combination thereof; or wherein the NS viral segment encodes a NS1 with a residue other than alanine at position 30, a residue other than lysine at position 55, or a residue other than arginine at position 118, or any combination thereof. In one embodiment, the virus has a V, A, L, G, S, T, C, or M at position 711 in PB1, has a A, L, G, V, S, T, I, or C at position 40 in PB1, has a A, L, G, V, S, T, I, or C at position 112 in PB1, has a A, L, W, Y, F, V, S, T, I, or C at position 180 in PB1, has a H, R, K, D, E, D or E at position 247 in PB1, or any combination thereof; or has a K, H, D, E, Q or N at position 74 in NP, has a L, V, G, A, S, C or T at position 116 in NP, has a D, E, Q, K, or H at position 417 in NP, or any combination thereof; or has a P, W, F, L, I, V, G, S, C or T at position 30 in NS1, has E, D, Q, or N at position 55, has a K, H, D, E, Q, or N at position 118 in NS1, has a L, I, V, G, A, S, C or T at position 128 in M1, or any combination thereof; or has a C, L, I, V, G, A, or T at position 142 in PA, has a C, L, I, V, G, A, or T at position 225 in PA, has a K, H, D, E, N or Q at position 401 in PA, or any combination thereof; or has an V, A, L, G, S, T, C, or M at position 504 in PB2, has a A, L, G, S, T, I, or C at position 202 in PB2, has a A, L, G, S, T, I, or C at position 323 in PB2, or any combination thereof. In one embodiment, the virus has a V, A, L, G, S, T, C, or M at position 711 in PB1, has a A, L, G, V, S, T, I, or C at position 40 in PB1, has a A, L, W, Y, F, V, S, T, I, or C at position 180 in PB1; has a L, V, G, A, S, C or T at position 116 in NP; has a P, W, F, L, I, V, G, S, C or T at position 30 in NS1, has a K, H, D, E, Q, or N at position 118 in NS1; has a L, I, V, G, A, S, C or T at position 128 in M1; has a K, H, D, E, N or Q at position 401 in PA; has an V, A, L, G, S, T, C, or M at position 504 in PB2; or any combination thereof. In one embodiment, the virus has a V, A, L, G, S, T, C, or M at position 711 in PB1, has a H, R, K, D, E, D or E at position 247 in PB1; has a K, H, D, E, Q or N at position 74 in NP; has E, D, Q, or N at position 55 in NS1; has a L, I, V, G, A, S, C or T at position 128 in M1; has a C, L, I, V, G, A, or T at position 142 in PA; has a A, L, G, S, T, I, or C at position 202 in PB2, has a A, L, G, S, T, I, or C at position 323 in PB2; or any combination thereof. In one embodiment, the virus has a V, A, L, G, S, T, C, or M at position 711 in PB1, has a A, L, G, V, S, T, I, or

C at position 112 in PB1; has a K, H, D, E, Q or N at position 74 in NP, has a D, E, Q, K, or H at position 417 in NP; has E, D, Q, or N at position 55; has a L, I, V, G, A, S, C or T at position 128 in M1; has a C, L, I, V, G, A, or T at position 225 in PA; has an V, A, L, G, S, T, C, or M at position 504 in PB2; or any combination thereof. In one embodiment, the virus has one or more of 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 any combination thereof. 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 virus has 202L and/or 323L in PB2. In one embodiment, the virus has 247H in PB1. In one embodiment, the virus has 55E in NS1. In one embodiment, the virus has 142C in PA. In one embodiment, the virus has 74K in NP. In one embodiment, the virus has 504V in PB2. In one embodiment, the virus has 74K, 116L or 417D in NP. In one embodiment, the virus has 30P, 55E or 118K in NS1. In one embodiment, the virus has 225C or 401K in PA. In one embodiment, the virus has a V, A, L, G, S, T, C, or M at position 711 in PB1. In one embodiment, the virus has a L, I, V, G, A, S, C or T at position 128 in M1. In one embodiment, at least one of PA, PB1, or PB2 viral segments has a C to U promoter mutation.

In other embodiments, the virus has a residue at position 30 or 31 in PA other than the residue encoded by SEQ ID NO:1 or 10; a residue in PB1 other than R54, T59, G62, A63, E75, D76, E78, P79, S80, R327, M507, L624, V644, I667, N694, L695, E697, F699, F700, P701, S702, Y705, S713, and/or M714; a residue in PB2 other than I57, T58, A59, K61, M66, R368, E391, Q591, E677, D678, and/or P679, in PB2; a residue in NP other than I112, N224, R293, M371, R422 or T442; a residue in M1 other than P90, V97, and/or Y100; or a residue in NS1 other than T49, R140, S161 and/or A223. In one embodiment, the PB1 viral segment and/or the NS viral segment encodes a polypeptide having a residue other than glycine, serine, serine, glutamine or asparagine at position 62, 261, 361, 621, and/or 654 in PB1 or a residue other than arginine at position 81 in F2.

In one embodiment, the isolated recombinant influenza virus has 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

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than leucine or alanine at residue 55 or 265, respectively, e.g., 55S or 265V. In one embodiment, the isolated virus has 142N, 225C, 356R, or 550L in PA; has one or more of 112G, 247H, 507V, or 644A in PB1; has one or more of 202L, 323L or 504V in PB2; has one or more of 74K, 112L, 116L, 417D, or 442A in NP; 97A and/or 100H in M1; and/or 55E and/or 140Q in NS1, or combinations thereof, e.g., has at least one of 202L and/or 323L in PB2, 247H in PB1 or 74K in NP and optionally at least one of 142N in PA1, 55K in NS1 or 97A and/or 100H in M1 or has at least one of 202L and/or 323L in PB2, 247H in PB1 or 74K in NP and at least one of 142N in PA1, 55K in NS1 or 97A and/or 100H in M1. In one embodiment, the virus has at least one of 202L and/or 323L in PB2, 247H in PB1 or 74K in NP and optionally at least one of 142N in PA1, 55K in NS1 or 97A and/or 100H in M1. In one embodiment, the virus has at least one of 202L and/or 323L in PB2, 247H in PB1 or 74K in NP and at least one of 142N in PA1, 55K in NS1 or 97A and/or 100H in M1. In one embodiment, the isolated virus has 202L and/or 323L in PB2, and optionally has 247H in PB1 and optionally 74K in NP. In one embodiment, the isolated virus has 247H in PB1 and optionally 74K in NP. In one embodiment, the isolated virus has 40I, 40L, 112G, 180W, 247H, 507V, or 644A in PB1 and optionally has 202L and/or 323L in PB2, and optionally has 74K, 112L, 116L, 377N, 417D, or 422L in NP, and optionally has 30P, 118K, 161T or 140Q in NS1, and optionally has 142N, 225C, 356R, 401K, or 550L in PA. In one embodiment, the isolated virus has 40I, 40L, 112G, 180W, 247H, 507V, or 644A in PB1. In one embodiment, the isolated virus has 202L and/or 323L in PB2. In one embodiment, the isolated virus has 74K, 112L, 116L, 377N, 417D, or 422L in NP. In one embodiment, the isolated virus has 30P, 118K, 161T or 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,

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and/or 100 any combination with the selected residues for PA, PB1, PB2, and/or NP; and optionally the selected amino acid residues at specified positions in the NS1 is/are at position(s) 49, 30, 55, 161, and/or 223, or in any combination with the selected residues for PA, PB1, PB2, NP, and/or M1.

For any of the exemplary viruses disclosed above, in one embodiment, the PA, PB1, PB2, NP, NS, and M viral segments comprise sequences for at least one of the following: a PB1 having the amino acid sequence encoded by SEQ ID NO:2 or PB1 with at least 95% amino acid sequence identity to the PB1 encoded by SEQ ID NO:2; a PB2 having the amino acid sequence encoded by SEQ ID NO:3 or PB2 with at least 95% amino acid sequence identity to the PB2 encoded by SEQ ID NO:3; a PA having the amino acid sequence encoded by SEQ ID NO:1 or PA with at least 95% amino acid sequence identity to the PA encoded by SEQ ID NO:1; a NP having the amino acid sequence encoded by SEQ ID NO:4 or NP with at least 95% amino acid sequence identity to the NP encoded by SEQ ID NO:4; a M1 and/or M2 having the amino acid sequence encoded by SEQ ID NO:5 or M1 and/or M2 with at least 95% amino acid sequence identity to the M1 and/or M2 encoded by SEQ ID NO:5; or a NS1 and/or NS2 having the amino acid sequence encoded by SEQ ID NO:6 or NS1 and/or NS2 with at least 95% amino acid sequence identity to the NS1 and/or NS2 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 M1 and/or M2 having the amino acid sequence encoded by SEQ ID NO:14 or M1 and/or M2 with at least 95% amino acid sequence identity to the M1 and/or M2 encoded by SEQ ID NO:14; or a NS1 and/or NS2 having the amino acid sequence encoded by SEQ ID NO:15 or NS1 and/or NS2 with at least 95% amino acid sequence identity to the NS1 and/or NS2 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 oper-

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

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

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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.

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 (H1N1; WSN) or PR8 strains is used, for reverse genetics (Horimoto et al., 2006; Neumann et al.,

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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 manufacturers 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 titer (HAU/mL) in each dilution						
	10-2	10-3	10-4	10-5	10-6	10-7	10-8
HA and NA genes							
WSN-HA NA	160	40	40	320	40	640	<1
HK-HAavir NA	400	800	400	400	400	800	<1

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

PA

(SEQ ID NO: 1)

AGCGAAAGCA GGTACTGATC CAAATGGAA GATTTTGTGC GACAATGCTT CAATCCGATG
ATTGTCGAGC TTGCGGAAAA AACATGAAA GAGTATGGGG AGGACCTGAA AATCGAAACA
AACAAATTTG CAGCAATATG CACTCACTTG GAAGTATGCT TCATGTATTC AGATTTTCAC
TTCATCAATG AGCAAGGCGA GTCAATAATC GTAGAACTTG GTGATCCAAA TGCACCTTTG
AAGCACAGAT TTGAAATAAT CGAGGGAAGA GATCGCACAA TGGCCTGGAC AGTAGTAAAC
AGTATTTGCA AACTACAGG GGCTGAGAAA CCAAAGTTTC TACCAGATTG GTATGATTAC
AAGGAGAATA GATTCATCGA AATTGGAGTA ACAAGGAGAG AAGTTCACAT ATACTATCTG
GAAAAGGCCA ATAAAATTAA ATCTGAGAAA ACACACATCC ACATTTTCTC GTTCACTGGG
GAAGAAATGG CCACAAAGGC AGACTACACT CTCGATGAAG AAAGCAGGGC TAGGATCAAA
ACCAGACTAT TCACCATAAG ACAAGAAATG GCCAGCAGAG GCCTCTGGGA TTCTTTTCGT
CAGTCCGAGA GAGGAGAAGA GACAATTGAA GAAAGGTTTG AAATCACAGG AACATGCGC
AAGCTTGCCG ACCAAAGTCT CCCGCCGAAC TTCTCCAGCC TTGAAAATTT TAGAGCCTAT
GTGGATGGAT TCGAACCGAA CGGCTACATT GAGGGCAAGC TGTCTCAAAT GTCCAAAGAA
GTAAATGCTA GAATTGAACC TTTTGTGAAA ACAACACCAC GACCACTTAG ACTTCCGAAT
GGGCTCCCT GTTCTCAGCG GTCCAAATTC CTGCTGATGG ATGCCTTAAA ATTAAGCATT
GAGGACCCAA GTCATGAAGG AGAGGGAATA CCGCTATATG ATGCAATCAA ATGCATGAGA

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AAAATTCCAA AGACTAAAAA TATGAAGAAA ACAAGTCAGC TAAAGTGGGC ACTTGGTGAG
AACATGGCAC CAGAAAAGGT AGACTTTGAC GACTGTAAAG ATGTAGGTGA TTTGAAGCAA
TATGATAGTG ATGAACCAGA ATTGAGGTCG CTGCAAGTT GGATTTCAGAA 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
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AATGACACCG ACGTGGTAAA CTTTGTGAGC ATGGAGTTTT CTCTCACTGA CCCAAGACTT
GAACCACATA AATGGGAGAA GTACTGTGTT CTTGAGATAG GAGATATGCT TATAAGAAGT
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GAGAACAAAT CAGAAACATG GCCCATGGA GAGTCCCCCA AAGGAGTGA GGAAGTTCC
ATTGGGAAGG TCTGCAGGAC TTTATTAGCA AAGTCGGTAT TCAACAGCTT GTATGCATCT
CCACAACCTAG AAGGATTTTC AGCTGAATCA AGAAAACCTG TTCTTATCGT TCAGGCTCTT
AGGGACAACC TGGAACCTGG GACCTTTGAT CTTGGGGGCG TATATGAAGC AATTGAGGAG
TGCCTGATTA ATGATCCCTG GGTTTTGCTT AATGCTTCTT GGTTCAACTC CTTCTTACA
CATGCATTGA GTTAGTTGTG GCAGTGCTAC TATTTGCTAT CCATACTGTC CAAAAAGTA
CCTTGTCTCT ACT

PB1

(SEQ ID NO: 2)

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CATGGATACTGTCAACAGGACACATCAGTACTCAGAAAAGGGAAGATGGACAACAAACACCGAACTGG
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 GGAGGCTATGGTTTCCAGAGCCGAATTGATGCACGATTGATTTCGAATCTGGAAGGATAAAGAAAGA
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 CTTGTCCTTCATGAAAAAATGCCTTGTCTTCTACT

PB2

(SEQ ID NO: 3)

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 GAGCAAGGAC AAACCTTTATG GAGTAAAATG AATGATGCCG GATCAGACCG AGTGATGGTA
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M

(SEQ ID NO: 5)

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NS

(SEQ ID NO: 6)

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HA

(SEQ ID NO: 7)

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NA

(SEQ ID NO: 8)

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 CTGTAGATTGGTCTTGGCCAGACGGTGCTGAGTTGCCATTGACATTGACAAGTAGTCTGTTCAAAAAAC
 TCCTTGTTTCTACT

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. 1), 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 generate "6+2" recombinant viruses. Consecutive passages of the virus in MDCK cells were employed to select for variants with high-growth properties.

TABLE 1

Virus libraries generated				
Internal genes			Titer of	
Gene	Other internal	HA + NA	virus library	
Number library	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	3UW-PR8 genes	NC/09/H5N1	75	
8 PB2 + PB1 + PA + NP	2UW-PR8 genes	NC/09/H5N1	33	
9 PB2 + NS	4UW-PR8 genes	NC/09/H5N1	2×10^2	
10 M + NS	4UW-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 NS1: 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			L182V				
			F323L							
154	Mix	8.5~9	M202L			L182V				
			F323L							
347	Mix	9	M202L			L182V				
			F323L							
94	Mix	8.5	M202L			F252I	I116L	L55S		
			F323L							

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
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	R54I					
981	Mix	8.5~9	M243I 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 G62X 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)					
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) S713C					
1408	PB1	8.5		M40I G180W					S161T

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
582	PB1	8.5~9		M40L, G180W					S161T
545	PB1	8.5		M40L, G180W		K121E (HA2)			
543	PB1	8.5		I667T		K162E			
219	PB1	9		I667T, M714T					
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-PRS virus (see Table 3 for virus stock titers) and tested for replicative ability. FIGS. 5A-D show growth curves for various viruses.

TABLE 3

UW-PRS 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

TABLE 3-continued

UW-PRS viruses possessing mutation(s) identified in the literature		
Gene	Mutation(s)	Virus stock titer (Pfu/ml)
25 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
	F2 L82S	2.7×10^7
	F2 E87Q	1.5×10^6
30 PA	T97I	1.6×10^7
	K142N	3.3×10^7
	S225C	6.7×10^7
	S149P + T357K	3.4×10^8
	K356R	8.5×10^7
35 WT	A404S	5.2×10^7
	S421I	2.7×10^7
	—	2×10^7
	R293K	4.7×10^7
	R305K	7.2×10^7
40 NP	E372D	2.2×10^7
	R422K	1.3×10^8
	T442A	5×10^7
	D455E	2.2×10^7
	I109V	3.9×10^7
M	V97A + Y100H	1.4×10^7
	NS1 K55E	1.6×10^7

45 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

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") Pfu/ml
6			I504V	M507V V644A	I550L	R74K N417D	V97A Y100H	K55E	9~9.5 4.40E+07
7			I505V	E112G	I550L	R74K		S161T	9 1.60E+08
8			M202L F323L	I667T M714T		I116L		R140Q	<1 <1E3
9			M202L F323L	E112G (PB1-F2- R81G)				S161T	8.5 1.30E+08
10			M66R	M40I G180W		R74K		S161T	8~8.5 2.30E+07
12			R368K	PB1 F2 N66S	K356R	R422K		K55E	5.5 9.00E+02
13			E391Q	R327K	S149P T357K	R293K			3 1.60E+06
14			Q591K	PB1 F2 K73R	S225C	R422K		K55E	7.5 2.00E+07
23							V97A		8.5~9 1.50E+07
24							Y100H		9~9.5 2.90E+07
25	NCR 15- 19 nt 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- 19 nt 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- 19 nt 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
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 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 FIG. 8BC 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-B). The V97A/Y100H mutations in M1 may result in particles with a larger surface into which more HA protein can be incorporated. Since inactivated influenza viruses are dosed based on their HA content, variants with high HA content are attractive vaccine candidates.

To identify mutations in the influenza promoter region that provide for enhanced replication, viruses possessing a

'U' at position 4 at the 3' end of all eight vRNA segments were prepared in the UW-PR8 PA, PB1 and PB2 internal genes (the UW-PR8 PB2, PB1, and PA segments possess a 'C' at position 4). The growth curves of the resulting viruses are shown in FIG. 11C.

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

Virus	Gene backbone									Virus stock titer	
	HA									HA	
	HA	NA	PB2	PB1	PA	NP	M	NS		(2")	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 FIG. 14). An exemplary set of backbone mutations are canine codon opti-PB2+C4U+M202L, F323L; PB1: C4U+Q247H; PA: C4U+K142N; NP: Canine codon opti-NP+R74K; M: V97A, Y100H; and NS: K55E.

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

tions 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) viruses: 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 G10120, 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+

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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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Libraries were screened in serum-free (SF) medium, serum-containing (SC) medium, and eggs. To identify a variant that replicates efficiently in all growth media tested, viruses were passaged under changing conditions:

SF to SC to Eggs, SF to Eggs to SC

SC to SF to Eggs, SC to Eggs to SF

Eggs to SF to SC, Eggs to SC to SF

For example, other identified mutations that further improve the replication of influenza virus in cultured cells and/or embryonated chicken eggs are I711V in PB1 and M128L in the M1 protein, and those may be combined with any of the mutations disclosed herein, and in any combination (see FIGS. 23-25 and Tables 8-9). The viral titers for HY candidate #9 were 5.5-fold, 2.4-fold, 2.6-fold, 4.4-fold higher than those of the parental high yield backbone recombinant virus at 12 h, 24 h, 36 h and 48 h. HY candidate #9 contains the following mutations: PB2: C4U I504V; PB1: C4U M40L/G180W/I711V; PA: C4U R401K; NP: I116L; M1: M128L; NS1: A30P/R118K.

TABLE 8

Top 8 high HA titer clones selected from SF-MDCK PR8-HY + Hubei/1/10(H5N1) virus libraries										
Virus stock titer										
Virus	HA		Mutations							
	titer (2 ⁻ⁿ)	PFU/ml	PB2	PB1	PA	NP	M1	NS1	HA (H3 numbering)	NA
WT	—	—	C4U I504V	C4U M40L/G180W	C4U R401K	I116L		A30P/R118K		
SF-14	10	1.44 × 10 ⁹		+I711V			+M128L		K216N	
SF-22	9.4	6.6 × 10 ⁸		+I711V					K216N	
SF-37	9.6	8.7 × 10 ⁸		+I711V					K216N	
SF-46	9.3	8.3 × 10 ⁸	+M467I/K721R						K222T/G326R	
SF-42	9.5	8.7 × 10 ⁸		+I711V	+R256G				K216N	
SF-57	9.6	1 × 10 ⁹							K222T	
SF#15	10	1.24 × 10 ⁹	+Y704H						K222T	
SF-E-85	9.6	9 × 10 ⁸			+N350I/A369T				K222T	

TABLE 9

Top 12 HA titer clones selected from one round of passage of second generation mixed library (SC = serum containing; SF-serum-free).											
Medium	Plaque #	HA titer (2 ⁿ)	HA & NA	PB2	PB1	PA	NP	M1	M2	NS1	NS2
	WT		Detoxified- A/Hubei/1/ 2010(HA + NA)	C4UI504V	C4UM40L/ G180W	C4UR401K	I116L			A30P/ R118K	
SC ↓ Egg ↓ SF	11	7.5		C4UI504V + A221V	C4UM40L/ G180W	C4UR401K	I116L + K103R + V194I	S126G + 244 (mixture) M/R	W15G	A30P/ R118K + D101V	176M
	14	7.5		C4UI504V + A221V	C4UM40L/ G180W + I711V	C4UR401K	I116L + K103R + V194I	M128L + 244 (mixture) M/R	W15G	A30P/ R118K	E820
	23	8.0		C4UI504V + R62S + A370T	C4UM40L/ G180W + I711V	C4UR401K	I116L + K103R + V194I	F62L + 244 (mixture) M/R	W15G	A30P/ R118K	
	27	7.5		C4UI504V + R62S	C4UM40L/ G180W + E178K + M290I	C4UR401K	I116L	S126G	W15G	A30P/ R118K	
	33	7.5		C4UI504V	C4UM40L/ G180W + I711V	C4UR401K + K142E	I116L	S126G + 244 (mixture) M/R	W15G	A30P/ R118K + D101V	I76M

TABLE 9-continued

Top 12 HA titer clones selected from one round of passage of second generation mixed library (SC = serum containing; SF-serum-free).											
Medium	Plaque #	HA titer (2 ⁿ)	HA & NA	PB2	PB1	PA	NP	M1	M2	NS1	NS2
SC ↓ SF ↓ Egg	1	7.5		C4UI504V + R62S + A370T	C4UM40L/ G180W + M92I	C4UR401K + R256G	I116L	S126G + 244 (mixture) M/R	W15G	A30P/ R118K	N92D
	2	7.5		C4UI504V + A221V	C4UM40L/ G180W + M92I	C4UR401K + R256G	I116L	M128L + 244 (mixture) M/R		A30P/ R118K + D101V	I76M
	4	7.5		C4UI504V + R62S	C4UM40L/ G180W + M325V	C4UR401K + R256G	I116L	244(mixture) M/R	W15G	A30P/ R118K	
	5	8.0		C4UI504V + A221V	C4UM40L/ G180W + M92I	C4UR401K + K142E	I116L	S126G + 244 (mixture) M/R	W15G	A30P/ R118K	E82D
	13	8.0		C4UI504V	C4UM40L/ G180W + M92I	C4UR401K + K142E	I116L + K103R + V194I	M128L + 244 (mixture) M/R	W15G	A30P/ R118K	N92D
	24	8.0		C4UI504V + R62S + A370T	C4UM40L/ G180W	C4UR401K + K142E	I116L + K103R + V194I	M128L + 244 (mixture) M/R	W15G	A30P/ R118K	E82D
	25	7.5		C4UI504V	C4UM40L/ G180W + M92I + N105H	C4UR401K	I116L + K103R + V194I	S126G + 244 (mixture) M/R	W15G	A30P/ R118K	N92D

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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.

SEQUENCE LISTING

<160> NUMBER OF SEQ ID NOS: 27

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<211> LENGTH: 2233

<212> TYPE: DNA

<213> ORGANISM: Influenza virus

<400> SEQUENCE: 1

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aacaaatttg cagcaatatg cactcacttg gaagtatgct tcatgtatcc agattttcac      180
ttcatcaatg agcaaggcga gtcaataatc gtagaacttg gtgateccaa tgcacttttg      240
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gaagaaatgg ccacaaaggc agactacact ctcgatgaag aaagcagggc taggatcaaa	540
accagactat tcaccataag acaagaaatg gccagcagag gcctctggga ttcccttcgt	600
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<212> TYPE: DNA

<213> ORGANISM: Influenza virus

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<212> TYPE: DNA

<213> ORGANISM: Influenza virus

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	2340

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t 2341

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 <212> TYPE: DNA
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 <213> ORGANISM: Influenza virus

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atgaaaaatgc ttatgtctct gtagtgactt caaattataa caggagattt acccggaaa	720
tagcagaaag acccaaagta agagatcaag ctgggaggat gaactattac tggaccttgc	780
taaaaccgag agacacaata atatttgagg caaatggaaa tctaatagca ccaatgtatg	840
ctttcgcact gagtagaggc ttgggtccg gcatcatcac ctcaaacgca tcaatgcatg	900
agtgtaacac gaagtgtcaa acaccctgg gagctataaa cagcagcttc ccttaccaga	960
atatacacc agtcacaata ggagagtgc caaaatacgt caggagtgc aaattgagga	1020
tggttacagg actaaggaac attcgtcca ttcaatccag aggtctatgt ggagccattg	1080
ccggttttat tgaaggggga tggactggaa tgatagatgg atggtatggt tatcatcatc	1140
agaatgaaca gggatcaggc tatgcagcgg atcaaaaaag cacacaaat gccattaacg	1200
ggattacaaa caaggtgaac actgttatcg agaaaatgaa cattcaattc acagctgtgg	1260
gtaaagaatt caacaaatta gaaaaagga tggaaaattt aaataaaaaa gttgatgatg	1320
gatttctgga catttggaac tataatgcag aattgttagt tctactggaa aatgaaagga	1380
ctctggattt ccatgactca aatgtgaaga atctgtatga gaaagtaaaa agccaattaa	1440
agaataatgc caaagaaatc ggaaatggat gttttgagtt ctaccacaag tgtgacaatg	1500
aatgcatgga aagtgtgaag aatgggactt atgattatcc caaatattca gaagagtcaa	1560
agttgaacag ggaagggta gatggagtga aattggaatc aatggggatc tatcagattc	1620
tggcgatcta ctcaactgtc gccagttcac tgggtgtttt ggtctccctg ggggcaatca	1680
gtttctggat gtgttctaag ggtctttgc agtgcagaat atgcatctga gattagaatt	1740
tcagagatat gaggaaaaac acccttggtt ctact	1775

<210> SEQ ID NO 8

<211> LENGTH: 1413

<212> TYPE: DNA

<213> ORGANISM: Influenza virus

<400> SEQUENCE: 8

agcaaaagca ggggtttaaa atgaatccaa atcagaaaat aataaccatt ggatcaatct	60
gtctggtagt cggactaatt agcctaatat tgcaaatagg gaataataat tcaatatgga	120
ttagccattc aattcaaact ggaagtcaaa accatactgg aatatgcaac caaaacatca	180
ttacctataa aaatagcacc tgggtaaagg acacaacttc agtgatatta accggcaatt	240
catctctttg tcccatccgt ggggtgggta tatacagcaa agacaatagc ataagaattg	300
gttccaaagg agacgttttt gtcataagag agccctttat ttcatgttct cacttggaat	360
gcaggacott tttctgacc caaggtgcct tactgaatga caagcattca agtgggactg	420

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ttaaggacag aagcccttat agggccttaa tgagctgccc tgtcggtgaa gctccgtccc	480
cgtacaattc aagatttgaa tcggttgctt ggtcagcaag tgcatgtcat gatggcatgg	540
gctggctaac aatcggaatt tcaggccag ataatggagc agtggctgta ttaaaataca	600
acggcataat aactgaaacc ataaaaagt ggaggaagaa aatattgagg acacaagagt	660
ctgaatgtgc ctgtgtaaat ggttcattgt ttactataat gactgatggc ccgagtgatg	720
ggctggcctc gtacaaaatt ttcaagatcg aaaaggggaa gggtactaaa tcaatagagt	780
tgaatgcacc taattctcac tatgaggaat gttcctgtta ccctgatacc ggcaaagtga	840
tgtgtgtgtg cagagacaat tggcatgggt cgaaccggcc atgggtgtct ttcgatcaaa	900
acctggatta tcaaatagga tacatctgca gtgggggttt cggtgacaac ccggtccc	960
aagatggaac aggcagctgt ggtccagtgt atgttgatgg agcaaacgga gtaaagggat	1020
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atgggtttga gatgatttgg gatcctaata gatggacaga gactgatagt aagttctctg	1140
tgaggcaaga tgttgtggca atgactgatt ggtcagggta tagcgggaagt ttcgttcaac	1200
atcctgagct gacagggcta gactgtatga ggccgtgctt ctgggttgaa ttaatcaggg	1260
gacgacctaa agaaaaaaca atctggacta gtgcgagcag catttctttt tgtggcgtga	1320
atagtgtatc ttagatttgg tcttgccag acggtgctga gttgccattc agcattgaca	1380
agtagtctgt tcaaaaaact ccttgtttct act	1413

<210> SEQ ID NO 9

<400> SEQUENCE: 9

000

<210> SEQ ID NO 10

<211> LENGTH: 2342

<212> TYPE: DNA

<213> ORGANISM: Influenza virus

<400> SEQUENCE: 10

agcgaagca ggcaaacat ttgaatggat gtcaatccga ccttactttt cttaaaagt	60
ccagcacaaa atgtataag cacaactttc cttataccg gagaccctcc ttacagccat	120
gggacaggaa caggatacac catggatact gtcaacagga cacatcagta ctcagaaaag	180
ggaagatgga caacaaacac cgaactgga gcaccgcaac tcaaccgat tgatgggcca	240
ctgccagaag acaatgaacc aagtgggtat gcccaaacag attgtgtatt ggaagcaatg	300
gctttccttg aggaatccca tcttggtatt ttgaaaact cgtgtattga aacgatggag	360
gttgttcagc aaacactgag tagacaagct gacacaaggc cgacagacct atgactggac	420
tttaaataga aaccagcctg ctgcaacagc attggccaac acaatagaag tgttcagatc	480
aatggcctc acggccaatg agtcaggaag gctcatagac ttccttaagg atgtaatgga	540
gtcaatgaaa aaagaagaaa tggggatcac aactcatttt cagagaaaga gacgggtgag	600
agacaatatg actaagaaaa tgataacaca gagaacaata ggtaaaagga aacagagatt	660
gaacaaaagg ggttatctaa ttagagcatt gacctgaac acaatgacca aagatgctga	720
gagagggaag ctaaacgga gagcaattgc aacccagggt atgcaataa gggggtttgt	780
atactttgtt gagacactgg caaggagtat atgtgagaaa cttgaacaat cagggttgcc	840
agttggaggc aatgagaaga aagcaaagt ggcaaatgtt gtaaggaaga tgatgaccaa	900

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ttctcaggac accgaacttt ctttcacccat cactggagat aacaccaaat ggaacgaaaa	960
tcagaatcct cggatgtttt tggccatgat cacatatatg accagaaatc agcccgaatg	1020
gttcagaaat gttctaagta ttgctccaat aatgttttca aacaaaatgg cgagactggg	1080
aaaagggtat atgtttgaga gcaagagtat gaaacttaga actcaaatac ctgcagaaat	1140
gctagcaagc attgatttga aatatttcaa tgattcaaca agaaagaaga ttgaaaaaat	1200
cgcaccgctc ttaatagagg ggactgcac attgagccct ggaatgatga tgggcatgtt	1260
caatatgtta agcactgtat taggcgtctc catcctgaat cttggacaaa agagatacac	1320
caagactact tactgggtggg atgggtcttca atcctctgac gattttgctc tgattgtgaa	1380
tgcacccaat catgaaggga ttcaagccgg agtcgacagg ttttatcgaa cctgtaagct	1440
acttgaatc aatatgagca agaaaaagtc ttacataaac agaacaggta catttgaatt	1500
cacaagtttt ttctatcggt atgggtttgt tgccaatttc agcatggagc ttcccagttt	1560
tgggtgtgtc gggatcaacg agtcagcggg catgagtatt ggagttactg tcatcaaaaa	1620
caatatgata aacaatgatc ttggtccagc aacagctcaa atggcccttc agttgttcat	1680
caaagattac aggtacacgt accgatgcca tagaggtgac acacaaatac aaaccggaag	1740
atcatttgaa ataagaaac tgtgggagca aaccggttc aaagctggac tgetggtctc	1800
cgacggaggc ccaaatttat acaacattag aaatctccac attcctgaag tctgcctaaa	1860
atgggaattg atggatgagg attaccaggg gcgtttatgc aacctactga acccatttgt	1920
cagccataaa gaaattgaat caatgaacaa tgcagtgatg atgccagcac atgggtccagc	1980
caaaaacatg gagtatgatg ctgttgcaac aacacactcc tggatcccca aaagaaatcg	2040
atccatcttg aatacaagtc aaagaggagt acttgaagat gaacaaatgt accaaaggty	2100
ctggaattta tttgaaaaat tcttccccag cagttcatac agaagaccag tcgggatatc	2160
cagtatggtg gaggtatagg tttccagagc ccgaattgat gcacggattg atttcgaatc	2220
tggaaggata aagaagaag agttcactga gatcatgaag atctgttcca ccattgaaga	2280
gctcagacgg caaaaatagt gaatttagct tgtccttcat gaaaaaatgc cttgtttcta	2340
ct	2342

<210> SEQ ID NO 11

<211> LENGTH: 2341

<212> TYPE: DNA

<213> ORGANISM: Influenza virus

<400> SEQUENCE: 11

agcgaagca ggtcaattat attcaatatg gaaagaataa aagaactaag aaatctaattg	60
tcgcagtctc gcaccccgga gatactcaca aaaaccaccg tggaccatat ggccataatc	120
aagaagtaca catcaggaag acaggagaag aaccagcac ttaggatgaa atggatgatg	180
gcaatgaaat atccaattac agcagacaag aggataacgg aaatgattcc tgagagaaat	240
gagcaaggac aaactttatg gagtaaaatg aatgatgccg gatcagaccg agtgaaggta	300
tcacctctgg ctgtgacatg gtggaatagg aatggaccaa tgacaaatac agttcattat	360
ccaaaaatct acaaaactta ttttgaaaga gtcgaaaggc taaagcatgg aacctttggc	420
cctgtccatt ttgaaacca agtcaaaaata cgtcggagag ttgacataaa tctgtgtcat	480
gcagatctca gtgccaaagg ggcacaggat gtaatcatgg aagttgtttt ccetaacgaa	540
gtgggagcca ggatactaac atcggaatcg caactaacga taaccaaaga gaagaaagaa	600
gaactccagg attgcaaaat ttctcctttg atggttgcac acatgttgga gagagaactg	660

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gtccgcaaaa cgagattcct ccagtggt ggtggaacaa gcagtgtgta cattgaagtg	720
ttgcatttga ctcaaggaac atgctgggaa cagatgtata ctccaggagg ggaagtgaag	780
aatgatgatg ttgatcaaa cttgattatt gctgctagga acatagtgaag aagagctgca	840
gtatcagcag acccactagc atctttattg gagatgtgcc acagcacaca gattggtgga	900
attaggatgg tagacatcct taagcagaac ccaacagaag agcaagccgt ggatatatgc	960
aaggctgcaa tgggactgag aattagctca tccttcagtt ttggtggatt cacatttaag	1020
agaacaagcg gatcatcagt caagagagag gaagagggtc ttacgggcaa tcttcaaaca	1080
ttgaagataa gagtgcata gggatctgaa gagttcaca tgggtgggag aagagcaaca	1140
gccatactca gaaaagcaac caggagattg attcagctga tagtgagtgg gagagacgaa	1200
cagtcgattg ccgaagcaat aattgtggc atggtatctt cacaagagga ttgtatgata	1260
aaagcagtta gaggtgatct gaatttcgtc aatagggcga atcagcgact gaatcctatg	1320
catcaacttt taagacatct tcagaaggat gcgaaagtgc tttttcaaaa ttggggagtt	1380
gaacctatcg acaatgtgat gggatgatt gggatattgc ccgacatgac tccaagcatc	1440
gagatgtcaa tgagaggagt gagaatcagc aaaatgggtg tagatgagta ctccagcacg	1500
gagagggtag tggtgagcat tgaccggttc ttgagagta gggaccaacg aggaaatgta	1560
ctactgtctc ccgaggaggt cagtgaacaa cagggaacag agaaactgac aataacttac	1620
tcacgtctca tgatgtggga gattaatggt cctgaatcag tgttggtcaa tacctatcaa	1680
tggatcatca gaaactggga aactgttaaa attcagtggt ccgagaaccc tacaatgcta	1740
tacaataaaa tggaaattga accatttcag tcttttagtac ctaaggccat tagaggccaa	1800
tacagtgggt ttgtaagaac tctgttccaa caaatgaggg atgtgcttgg gacatttgat	1860
accgcacaga taataaaaact tcttccttc gcagccgctc caccaaagca aagtagaatg	1920
cagttctcct catttactgt gaatgtgagg ggaatcaggaa tgagaatact tgtaaggggc	1980
aattctcctg tattcaacta caacaaggcc acgaagagac tcacagtctc cggaaggat	2040
gctggcactt taaccgaaga ccagatgaa ggcacagctg gagtggagtc cgctgttctg	2100
aggggattcc tcattctggg caaagaagac aggagatatg ggccagcatt aagcatcaat	2160
gaactgagca accttgcgaa aggagagaag gctaattgtc taattgggca aggagacgtg	2220
gtgttggtaa tgaacgaaa acgggactct agcatactta ctgacagcca gacagcgacc	2280
aaaagaattc ggatggccat caattagtgt cgaatagttt aaaaacgacc ttgtttctac	2340
t	2341

<210> SEQ ID NO 12

<211> LENGTH: 2234

<212> TYPE: DNA

<213> ORGANISM: Influenza virus

<400> SEQUENCE: 12

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attgtcgagc ttgcggaaaa aacaatgaaa gagtatgggg aggacctgaa aatcgaaaca	120
aacaaatttg cagcaatatg cactcacttg gaagtatgct tcatgtattc agatttccac	180
ttcatcaatg agcaaggcga gtcaataatc gtgaacttg gtgaccta tgcacttttg	240
aagcacagat ttgaataat cgaggggaaga gatcgacaaa tggcctggac agtagtaaac	300
agtatttgca aactacagg ggctgagaaa ccaagtttc taccagattt gtatgattac	360

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aaggaaaata gattcatcga aattggagta acaaggagag aagttcacat atactatctg	420
gaaaaggcca ataaaattaa atctgagaaa acacacatcc acattttctc gttcactggg	480
gaagaaatgg ccacaagggc cgactacact ctcgatgaag aaagcagggc taggatcaaa	540
accaggctat tcaccataag acaagaaatg gccagcagag gcctctggga ttccttctgt	600
cagtccgaga gaggagaaga gacaattgaa gaaagggttg aaatcacagg aacaatgcgc	660
aagcttgccg accaaagtct ccgcgcgaac ttctccagcc ttgaaaattt tagagcctat	720
gtggatggat tcgaaccgaa cggtacatt gagggcaagc tgtctcaaat gtccaaagaa	780
gtaaatgcta gaattgaacc ttttttgaaa acaacaccac gaccacttag acttccgaat	840
gggctccctt gttctcagcg gtccaaattc ctgctgatgg atgccttaaa attaagcatt	900
gaggacccaa gtcatgaagg agaggaata ccgctatatg atgcaatcaa atgcatgaga	960
acattctttg gatggaagga acccaatgtt gttaaacacc acgaaaaggg aataaatcca	1020
aattatcttc tgtcatggaa gcaagtactg gcagaactgc aggacattga gaatgaggag	1080
aaaattccaa agactaaaaa tatgaaaaa acaagtcagc taaagtgggc acttggtgag	1140
aacatggcac cagaaaaggt agactttgac gactgtaaag atgtaggatga tttgaagcaa	1200
tatgatagtg atgaaccaga attgaggtcg cttgcaagtt ggattcagaa tgagtccaac	1260
aaggcatcgc aactgacaga ttcaagctgg atagagcttg atgagattgg agaagatgtg	1320
gtccaattg aacacattgc aagcatgaga aggaattatt tcacatcaga ggtgtctcac	1380
tgagagacca cagaatacat aatgaagggg gtgtacatca atactgcctt acttaatgca	1440
tcttgtgcag caatggatga tttccaatta attccaatga taagcaagtg tagaactaag	1500
gaggaagggc gaaagaccaa cttgtatggt ttcatacaa aaggaagatc ccacttaagg	1560
aatgacaccg acgtggtaaa ctttgtgagc atggagtttt ctctcactga cccaagactt	1620
gaaccacaca aatgggagaa gtactgtggt cttgagatag gagatatgct tctaagaagt	1680
gccataggcc aggtttcaag gcccatgttc ttgtatgta ggacaaatgg aacctcaaaa	1740
attaaaaatga aatggggaat ggagatgagg cgttgtctcc tccagtcact tcaacaaatt	1800
gagagtatga ttgaagctga gtcctctgtc aaagagaaag acatgaccaa agagttcttt	1860
gagaacaaat cagaaacatg gcccatgga gagtctccca aaggagtgga ggaaagtctc	1920
attggggaag gtctgcagga ctttattagc aaagtcggta tttaacagct tgtatgcac	1980
tccacaacta gaaggatttt cagctgaatc aagaaaactg cttcttatcg ttcaggtctt	2040
tagggacaat ctggaacctg ggacctttga tcttgggggg ctatatgaag caattgagga	2100
gtgctaatt aatgatccct gggttttgct taatgcttct tggttcaact ccttccttac	2160
acatgcattg agttagtgtg ggcagtgcta ctatttgcta tccatactgt ccaaaaaagt	2220
accttgtttc tact	2234

<210> SEQ ID NO 13

<211> LENGTH: 1565

<212> TYPE: DNA

<213> ORGANISM: Influenza virus

<400> SEQUENCE: 13

agcaaaagca gggtagataa tcactcactg agtgacatca aaatcatggc gtcccaaggc	60
accaaacggg cttacgaaca gatggagact gatggagaac gccagaatgc cactgaaatc	120
agagcatccg tcggaaaaat gattggtgga attggacgat tctacatcca aatgtgcaca	180
gaacttaaac tcagtgatta tgagggacgg ttgatccaaa acagcttaac aatagagaga	240

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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
gcaacttata agaggacaag ggctcttggt cgcaccggaa tggatcccag gatgtgctct	540
ctgatgcaag gttcaactct ccttaggagg tctggagccg cagggtgctgc agtcaaagga	600
gttgaacaa tggatgatga attggtcagg atgatcaaac gtgggatcaa tgatcggaac	660
ttctggaggg gtgagaatgg acgaaaaaca agaattgctt atgaaagaat gtgcaacatt	720
ctcaaaggga aatttcaaac tgcgtcacaa aaagcaatga tggatcaagt gagagagagc	780
cggaaaccag ggaatgctga gttcgaagat ctcacttttc tagcacggtc tgcactcata	840
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gccagtgggt acgactttga aagagaggga tactctctag tcggaataga ccctttcaga	960
ctgcttcaaa acagccaagt gtacagccta atcagaccaa atgagaatcc agcacacaag	1020
agtcaactgg tgtggatggc atgccattct gccgcatttg aagatctaag agtattgagc	1080
ttcatcaaag ggacgaaggt ggccccaga gggaagcttt ccactagagg agttcaaatt	1140
gcttccaatg aaaatatgga gactatggaa tcaagtacac ttgaactgag aagcaggtag	1200
tgggccataa ggaccagaag tggaggaaac accaatcaac agagggcatc tgcgggccaa	1260
atcagcatat aacctacgtt ctcagtacag agaaatctcc cttttgacag aacaaccgtt	1320
atggcagcat tcactgggaa tacagagggg agaacatctg acatgaggac cgaaatcata	1380
aggatgatgg aaagtgaag accagaagat gtgtctttcc aggggcgggg agtcttcgag	1440
ctctcggaag aaaaggcagc gagcccgatc gtgccttctt ttgacatgag taatgaagga	1500
tcttatttct tcggagacaa tgcagaggag tacgacaatt aaagaaaaat acccttgttt	1560
ctact	1565

<210> SEQ ID NO 14

<211> LENGTH: 1027

<212> TYPE: DNA

<213> ORGANISM: Influenza virus

<400> SEQUENCE: 14

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ctctatcatc ccgtcaggcc ccctcaaagc cgagatcgca cagagacttg aagatgtctt	120
tgcagggaag aacaccgatc ttgaggttct catggaatgg ctaaagacaa gaccaatcct	180
gtcacctctg actaaggggg ttttaggatt tgtgttcacg ctccaccgtc ccagtgagcg	240
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caacaggatg ggggctgtga ccactgaagt ggcatttggc ctggtatgtg caacctgtga	480
acagattgct gactcccagc atcggctctc taggcaaatg gtgacaacaa ccaaccact	540
aatcagacat gagaacagaa tggtttttagc cagcactaca gctaaggcta tggagcaaat	600
ggctggatcg agtgagcaag cagcagaggc catggagggt ctagtcagg ctaggcaaat	660
ggtgcaagcg atgagaacca ttgggactca tccatagctcc agtgctgggc tgaaaaatga	720

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tcttcttgaa aatttgcagg cctatcagaa acgaatgggg gtgcagatgc aacggttcaa	780
gtgatcctct cgctattgcc gcaaatatca ttgggatctt gcaattgata ttgtggattc	840
ttgatcgtct ttttttcaaa tgcatttacc gtcgctttaa atacggactg aaaggagggc	900
cttctacgga aggagtgcc aagtctatga gggaagaata tcgaaaggaa cagcagagtg	960
ctgtggatgc tgacgatggc cattttgtca gcatagagct ggagtaaaaa actaccttgt	1020
ttctact	1027

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

<400> SEQUENCE: 15

agcaaaagca gggtgacaaa gacataatgg atccaaacac tgtgtcaagc tttcaggtag	60
attgtcttct ttggcatgct cgcaaacgag ttgcagacca agaactaggt gatgccccat	120
tccttgatcg gcttcgcoga gatcagaaat ccctaagagg aaggggcagc actcttggtc	180
tggacatcga gacagccaca cgtgctggaa agcagatagt ggagcggatt ctgaaagaag	240
aatccgatga ggcacttaaa atgaccatgg cctctgtacc tgcgtcgcgt tacctaaccg	300
acatgactct tgaggaaatg tcaagggaat ggtccatgct cataccaag cagaaagtgg	360
caggccctct ttgtatcaga atggaccagg cgatcatgga taaaaacatc atactgaaag	420
cgaacttcag tgtgatTTTT gaccggctgg agactctaat attgctaagg gctttcaccg	480
aagagggagc aattgttggc gaaatttcac cattgccttc tcttcagga catactgctg	540
aggatgtcaa aaatgcagtt ggagtcctca tcggaggact tgaatggaat gataacacag	600
ttcgagtctc tgaaactcta cagagattcg cttggagaag cagtaatgag aatgggagac	660
ctccactcac tccaaaacag aaacgagaaa tggcgggaac aattaggtca gaagtttgaa	720
gaaataagat ggttgattga agaagtgaga cacaactga aggtaacaga gaatagtttt	780
gagcaataaa catttatgca agccttacat ctattgcttg aagtggagca agagataaga	840
actttctcat ttcagcttat ttaataataa aaaacaccct tgtttctact	890

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

<400> SEQUENCE: 16

agcgaaagca ggtcaattat attcaatatg gaaagaataa aagaactacg aaatctaattg	60
tcgcagtctc gcacccgcga gatactcaca aaaaccaccg tggaccatat ggccataatc	120
aagaagtaca catcaggaag acaggagaag aaccagcac tgaggatgaa atggatgatg	180
gcaatgaaat atccaattac agcagacaag aggatcaccg aaatgattcc tgagagaaat	240
gagcagggac agactctgtg gagtaaaatg aatgatgccg gatcagaccg agtcatggtg	300
tcacctctgg ctgtgacatg gtggaatagg aatggaccaa tcacaaatc agtgcattat	360
ccaaaaatct acaaaactta ttttgaaaga gtcgaaaggc tgaagcatgg aacctttggc	420
cctgtccatt ttgaaacca ggtcaaaatc cggcggagag tggacatcaa tcctggtcat	480
gcagatctca gtgccaaagg ggcacaggat gtgatcatgg aagtgggtgt ccctaacgaa	540
gtgggagcca ggattctgac atccgaatcc cagctgacca ttaccaaaga gaagaaagaa	600
gaactccagg attgcaaaat ttctcctctg atggtggcat acatgctgga gagagaactg	660

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gtccgcaaaa caagattcct cccagtggct ggtggaacaa gcagtgtgta cattgaagtg	720
ctgcatctga ctcagggaac atgctgggaa cagatgtata ctccaggagg ggaagtgagg	780
aatgatgatg tggatcagag cctgattatt gctgctagga acattgtgag aagagctgca	840
gtgtcagcag atccactggc atctctgctg gagatgtgcc acagcacaca gattggtgga	900
attaggatgg tggacatcct gaggcagaac ccaacagaag agcaggccgt ggatatttgc	960
aaggctgcaa tgggactgag aattagctca tccttcagtt ttggtggatt cacatttaag	1020
agaacaagcg gatcatcagt caagagagag gaagagggtc tgaccggcaa tctgcagaca	1080
ctgaagatca gagtgcata gggatatgaa gagttcaca tgggtggggag aagagcaaca	1140
gccatcctca gaaaagcaac caggagactg attcagctga tcgtgagtgg gagagacgaa	1200
cagtccattg ccgaagcaat tattgtggcc atggtgtttt cacaggagga ttgtatgatt	1260
aaagcagtca gaggtgatct gaatttcgtc aatagggcca atcagcgact gaatcctatg	1320
catcagctgc tgagacattt tcagaaggat gccaaagtgc tgtttcagaa ttggggagtg	1380
gaacctatcg acaatgtgat gggaatgatt gggatcctgc ccgacatgac tccaagcatc	1440
gagatgtcaa tgagaggagt gagaatcagc aaaatgggtg tggatgagta ctccagcacc	1500
gagagggctg tggtgagcat tgacagattt ctgagaatcc gggaccagcg aggaaatgtg	1560
ctcctgtctc ccgaggaggt cagtgaacaa cagggaacag agaaactgac aattacttac	1620
tcacctcaa tgatgtggga gattaatggt cctgaatcag tgctggtcaa tacctatcag	1680
tggatcatca gaaactggga aactgtgaaa attcagtggc ccagaaccc tacaatgctg	1740
tacaataaaa tggaaattga accatttcag tctctggtgc ctaaggccat tagaggccag	1800
tacagtgggt ttgtgagaac tctgttcag cagatgaggg atgtgctggg gacatttgat	1860
accgcacaga ttattaaact gctgccttc cgacccgtc caccaaagca gagtagaatg	1920
cagttctcct catttactgt gaatgtgagg ggcacaggaa tgagaatcct ggtgaggggc	1980
aattctcctg tgttcaacta taacaaggcc accaagagac tcacagtgtc cggaaggat	2040
gctggcactc tgactgaaga cccagatgaa ggcacagctg gagtggagtc cgctgtgctg	2100
aggggatcc tcattctggg caaagaagac aagagatatg ggccagcact gagcatcaat	2160
gaactgagca acctggccaa aggagagaag gctaattgtc taattgggca aggagacgtg	2220
gtgttggtaa tgaacaggaa acgggactct agcatactta ctgacagcca gacagcgacc	2280
aaaagaattc ggatggccat caattagtgt cgaatagttt aaaaacgacc ttgtttctac	2340
t	2341

<210> SEQ ID NO 17

<211> LENGTH: 2341

<212> TYPE: DNA

<213> ORGANISM: Influenza virus

<400> SEQUENCE: 17

agcgaaagca ggcaaacat ttgaatggat gtcaatccga ccttactttt cttaaaagtg	60
ccagcacaaa atgtataag cacaactttc ctttatactg gagaccctcc ttacagccat	120
gggacaggaa caggatacac catggatact gtcaacagga cacatcagta ctcagaaaag	180
ggaagatgga caacaaacac cgaaactgga gcaccgcaac tcaaccgat tgatgggcca	240
ctgccagaag acaatgaacc aagtggttat gcccaaacag attgtgtatt ggaggcgatg	300
gctttccttg aggaatccca tcctgggtatt ttgaaaact cgtgtattga aacgatggag	360

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gttggtcagc aaacacgagt ggacaagctg acacagggcc gacagaccta tgactggact	420
ctgaatagaa accagcctgc tgcaacagca ctggccaaca caatcgaagt gttcagatca	480
aatggcctca ccgccaatga gtctggaagg ctcatcgact tctgaagga tgtgatggag	540
tcaatgaaca aagaagaaat ggggatcaca actcattttc agagaaagag acgggtgaga	600
gacaatatga ctaagaaaat gattacacag agaacaatgg gtaaaaagaa gcagagactg	660
aacaaaagga gttatctgat tagagcactg accctgaaca caatgaccaa agatgctgag	720
agagggaagc tgaacaggag agcaattgca accccaggga tgcagattag ggggtttgtg	780
tactttgtgg agacactggc aaggagtatt tgtgagaaac tggaacagtc agggctgcca	840
gtgggaggca atgagaagaa agcaaagctg gcaaagtgg tgaggaagat gatgaccaat	900
tctcaggaca ccgaactgtc ttccaccatc actggagata acaccaaag gaacgaaaat	960
cagaatcctc ggatgtttct ggccatgac acatatatga ccagaaatca gcccgatgg	1020
ttcagaaatg tgctgagtat tgctccaatt atgttctcaa acaaaatggc cagactggga	1080
aaagggtata tgtttgagag caagagtatg aaactgagaa ctgagattcc tgcagaaatg	1140
ctggcaagca tcgatctgaa atatttcaat gattcaacaa gaaagaagat tgaaaaaatc	1200
cgaccctcc tgattgagg gactgcatca ctgagccctg gaatgatgat gggcatgttc	1260
aatatgctga gcaactgtgt gggcgtctcc atcctgaatc tgggacagaa gagatacacc	1320
aagactactt actggtggga tggctctgac tctctgacg attttgcctt gattgtgaat	1380
gcacccaatc atgaagggat tcaggccgga gtcgacaggt tttatcgaac ctgtaagctg	1440
ctgggaatca atatgagcaa gaaaaagtct tacatcaaca gaacaggtac atttgaattc	1500
acaagttttt tctatcgcta tgggtttgtg gccaatcca gcatggagct gccagtttt	1560
ggggtgtctg ggtcaacga gtcagccgac atgagtattg gagtgaactg catcaaaaac	1620
aatatgatca acaatgatct gggccagca acagctcaga tggccctgca gctgttcac	1680
aaagattaca ggtacaccta ccgatgccat atcggtgaca cacagattca gaccgaaga	1740
tcatttgaat tcaagaaact gtgggagcag acccgctcca aagctggact gctggtctcc	1800
gacggaggcc caaatctgta caacattaga aatctccaca ttctgaagt ctgctgaaa	1860
tgggaactga tggatgagga ttaccagggt cgcctgtgca acccaactgaa cccatttgtc	1920
agccataaag aaattgaatc aatgaacaat gcagtgatga tgccagcaca tgggtccagcc	1980
aaaaacatgg agtatgatgc tgtggcaaca acacactcct ggatcccaa aagaaatcga	2040
tccatcctga atacaagtca gagaggagtg ctggaggatg aacagatgta ccagagggtc	2100
tgcaatctgt ttgaaaaatt ctccccagc agttcataca gaagaccagt cgggatctcc	2160
agtatggtgg aggctatggt gtccagagcc cgaattgatg cacggattga tttcgaatct	2220
ggaaggatca agaagaaga gttcactgag atcatgaaga tctgttccac cattgaagag	2280
ctcagacggc aaaaatagtg aatttagctt gtccttcacg aaaaaatgcc ttgtttctac	2340
t	2341

<210> SEQ ID NO 18

<211> LENGTH: 2233

<212> TYPE: DNA

<213> ORGANISM: Influenza virus

<400> SEQUENCE: 18

agcgaagca ggtactgatc caaaatggaa gatthttgtc gacaatgctt caatccgatg	60
attgtcgagc ttgcgaaaa aacaatgaaa gagtatgggg aggacctgaa aatcgaacaa	120

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aacaaatttg cagcaatatg cactcacttg gaagtatgct tcatgtattc agattttcac	180
ttcatcaatg agcaaggcga gtcaataatc gtagaacttg gtgatccaaa tgcacttttg	240
aagcacagat ttgaaataat cgaggggaaga gatcgacaaa tggcctggac agtagtaaac	300
agtattttgca acactacagg ggctgagaaa ccaaagtctc taccagattt gtatgattac	360
aaggagaata gattcatcga aattggagta acaaggagag aagttcacat atactatctg	420
gaaaaggcca ataaaattaa atctgagaaa acacacatcc acattttctc gttcactggg	480
gaagaaatgg ccacaaaggc agactacact ctcgatgaag aaagcagggc taggatcaaa	540
accagactat tcaccataag acaagaaatg gccagcagag gcctctggga ttcctttcgt	600
cagtccgaga gaggagaaga gacaattgaa gaaagggttg aaatcacagg aacaatgcgc	660
aagcttgccg accaaagtct ccgcgcgaac ttctccagcc ttgaaaattt tagagcctat	720
gtggatggat tcgaaccgaa cggtacatt gagggcaagc tgtctcaaat gtccaaagaa	780
gtaaatgcta gaattgaacc ttttctgaaa acaacaccac gaccactgag actgcccaat	840
gggcctccct gttctcagcg gtccaaattc ctgctgatgg atgccctgaa actgagcatt	900
gaggacccaa gtcatgaagg agaggggaatt cccctgtatg atgcaatcaa atgcatgaga	960
acattctttg gatggaagga acccaatgtg gtgaaaccac acgaaaaggg aatcaatcca	1020
aattatctgc tgtcatggaa gcagggtgct gcagaactgc aggacattga gaatgaggag	1080
aaaattccaa agactaaaaa tatgaagaaa acaagtcagc tgaagtgggc actgggtgag	1140
aacatggcac cagaaaagggt ggactttgac gactgtaaaag atgtgggtga tctgaagcag	1200
tatgatagtg atgaaccaga actgaggtcc ctggcaagtt ggattcagaa tgagttaaac	1260
aaggcatgcg aactgacaga ttcaagctgg attgagctcg atgagattgg agaagatgtg	1320
gctccaattg aacacattgc aagcatgaga aggaattatt tcacatcaga ggtgtctcac	1380
tgcagagcca cagaatacat catgaaggga gtgtacatca atactgccct gctgaatgca	1440
tcttgtgcag caatggatga tttccagctg attccaatga tcagcaagtg tagaactaag	1500
gaggggaagg gaaagaccaa cctgtatggt ttcacatca aaggaagatc ccacctgagg	1560
aatgacaccg acgtgggtgaa ctttgtgagc atggagtttt ctctcactga cccaagactg	1620
gaaccacata aatgggagaa gtactgtgtg ctggagattg gagatatgct gatcagaagt	1680
gccattggcc aggtgtcaag gcccatgttc ctgtatgtga gaacaaatgg aacctcaaaa	1740
attaaaaatga aatggggaat ggagatgagg cgctgcctcc tcacgtcact gcagcagatt	1800
gagagtatga ttgaagtga gtcctctgtc aaagagaaaag acatgaccaa agagttcttt	1860
gagaacaaat cagaaacatg gcccatgga gagtcccca aaggagtgga ggaaagtctc	1920
attgggaagg tctgcaggac tctgctggca aagtcctgt tcaacagcct gtatgcatct	1980
ccacagctgg aaggattttc agctgaatca agaaaactgc tgctgatcgt gcaggctctg	2040
agggacaacc tggaacctgg gacctttgat ctgggggggc tgtatgaagc aattgaggag	2100
tgcttgatta atgatccctg ggtgctgctg aatgcttctt gggtcaactc cttecttaca	2160
catgcattga gttagtgtg gcagtgtctac tatttgcctat ccatactgtc caaaaaagta	2220
ccttgtttct act	2233

<210> SEQ ID NO 19

<211> LENGTH: 1565

<212> TYPE: DNA

<213> ORGANISM: Influenza virus

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<400> SEQUENCE: 19

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agcaaaagca gggtagataa tcactcactg agtgacatca aaatcatggc gtctcaaggc    60
accaaacgat cttacgaaca gatggagact gatggagaac gccagaatgc cactgaaatc    120
agagcatccg tcggaaaaat gattggtgga attggacgat tctacatcca gatgtgcacc    180
gaactcaaac tcagtgtatta tgagggacgg ctgatccaga acagcctgac aatcgagaga    240
atggtgctct ctgcttttga cgaaggagga aataaatacc tggaagaaca tcccagtgcc    300
gggaaagatc ctaagaaaac tggaggacct atctacagga gagtgaacgg aaagtggatg    360
agagaactca tcctgtatga caaagaagaa atcaggcgaa tctggcgcca ggctaataat    420
ggtgacgatg caaccgctgg tctgactcac atgatgatct ggcattccaa tctgaatgat    480
gcaacttata agaggacaag agctctggtg cgcaccggaa tggatcccag gatgtgctct    540
ctgatgcagg gttcaactct ccctaggagg tctggagccg caggtgctgc agtcaaagga    600
gtgggaacaa tggatgatga actggtcaga atgatcaaaa gagggatcaa tgatcggaac    660
ttctggaggg gtgagaatgg acgaaaaaca agaattgctt atgaaagaat gtgcaacatt    720
ctcaaaggga aatttcagac tgtgacacag aaagcaatga tggatcagg gagagagagc    780
cggaaaccag ggaatgctga gttcgaagat ctcaactttt tggcacggtc tgcaactatc    840
ctgagagggt ccgtggctca caagtcctgc ctgctgcct gtgtgtatgg acctgccgtg    900
gccagtgggt acgactttga aagggaggga tactctctgg tcggaattga ccctttcaga    960
ctgctgcaga acagccagg gtacagcctg atcagaccaa atgagaatcc agcacacaag   1020
agtcagctgg tgtggatggc atgccattct gccgcatttg aagatctgag agtgctgagc   1080
ttcatcaaag ggaccaaggt gctcccaaga gggaagctgt ccactagagg agtgcagatt   1140
gcttccaatg aaaatatgga gactatggaa tcaagtacac tggaactgag aagcaggtag   1200
tgggccatca ggaccagaag tggaggaaac accaatcagc agagggcatc tgccggccag   1260
atcagcatte agcctacctt ctcaagtgcag agaaatctcc cttttgacag aacaaccatt   1320
atggcagcat tcaatgggaa tacagagggg agaacatctg acatgaggac cgaaatcatc   1380
aggatgatgg aaagtgaag accagaagat gtgtctttcc aggggcgggg agtcttcgag   1440
ctctcggaag aaaaggcagc gagcccgatc gtgccttctt ttgacatgag taatgaagga   1500
tcttatttct tcggagacaa tgcagaggag tacgacaatt aaagaaaaat acccttgttt   1560
ctact

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<210> SEQ ID NO 20

<211> LENGTH: 1684

<212> TYPE: DNA

<213> ORGANISM: Influenza virus

<400> SEQUENCE: 20

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atgaacactc aaatcctggt attcgctctg attgcgatca ttccaacaaa tgcagacaaa    60
atctgcctcg gacatcatgc cgtgtcaaac ggaaccaaag taaacacatt aactgaaaga   120
ggagtggaag tcgtcaatgc aactgaaaca gtggaacgaa caaacatccc caggatctgc   180
tcaaaaggga aaaggacagt tgacctcggc caatgtggac tcctggggac aatcactgga   240
ccacctcaat gtgaccaatt cctagaattt tcagccgatt taattattga gaggcgagaa   300
ggaagtgatg tctgttatcc tgggaaatc gtgaatgaag aagctctgag gcaaattctc   360
agagaatcag gcggaattga caaggaaagc atgggattca catacagtgg aataagaact   420
aatggagcaa ccagtgcatt taggagatca ggatcttcat tctatgcaga aatgaaatgg   480

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ctcctgtcaa acacagataa tgctgcattc ccgccagatg actaagtcac ataaaaatac 540
aagaaaaagc ccagctctaa tagtatgggg gatccatcat tccgtatcaa ctgcagagca 600
aaccaagcta tatgggagtg gaaacaaact ggtgacagtt gggagttcta attatcaaca 660
atctttttgta ccgagtccag gagcgagacc acaagttaat ggtctatctg gaagaattga 720
ctttcattgg ctaatgctaa atcccaatga tacagtcact ttcagtttca atggggcttt 780
catagctcca gaccgtgcaa gcttcctgag aggaaaatct atgggaatcc agagtggagt 840
acaggttgat gccaatgtg aaggggactg ctatcatagt ggagggacaa taataagtaa 900
cttgccattt cagaacatag atagcagggc agttggaaaa tgtccgagat atgttaagca 960
aaggagctcg ctgctagcaa cagggatgaa gaatgttcct gagattccaa agggaagagg 1020
cctatttggg gctatagcgg gtttcattga aaatggatgg gaaggcctaa ttgatgggtg 1080
gtatggtttc agacaccaga atgcacaggg agagggaact gctgcagatt acaaaagcac 1140
tcaatcgcca attgatcaaa taacaggaaa attaaaccgg cttatagaaa aaaccaacca 1200
acaatttgag ttgatagaca atgaattcaa tgaggtagag aagcaaactg gtaatgtgat 1260
aaattggacc agagattcta taacagaagt gtggtcatac aatgctgaac tcttggtagc 1320
aatggagaac cagcatacaa ttgatctggc tgattcagaa atggacaaac tgtacgaacg 1380
agtgaagaag cagctgagag agaatgctga agaagatggc actgggtgct ttgaaatatt 1440
tcacaagtgt gatgatgact gtatggccag tattagaaat aacacctatg atcacagcaa 1500
atacagggaa gaggcaatgc aaaatagaat acagattgac ccagtcaaac taagcagcgg 1560
ctacaagatg 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

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<400> SEQUENCE: 21

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Met Asn Thr Gln Ile Leu Val Phe Ala Leu Ser Lys Gly Lys Arg Thr
1             5             10             15
Val Asp Leu Gly Ile Ala Ile Ile Pro Thr Asn Ala Asp Lys Gln Cys
20            25            30
Gly Leu Leu Gly Thr Ile Thr Gly Ile Cys Leu Gly His His Ala Val
35            40            45
Ser Asn Pro Pro Gln Cys Asp Gln Phe Leu Glu Phe Gly Thr Lys Val
50            55            60
Asn Thr Leu Thr Glu Arg Ser Ala Asp Leu Ile Ile Glu Arg Arg Glu
65            70            75            80
Gly Val Glu Val Val Asn Ala Thr Glu Thr Gly Ser Asp Val Cys Tyr
85            90            95
Pro Gly Lys Phe Val Glu Arg Thr Asn Ile Pro Arg Ile Cys Val Asn
100           105           110
Glu Glu Ala Leu Arg Gln Ile Leu Arg Glu Ser Gly Gly Ile Asp Lys
115           120           125
Glu Ala Met Gly Phe Thr Tyr Ser Gly Ile Arg Thr Asn Gly Ala Thr
130           135           140
Ser Ala Cys Arg Arg Ser Gly Ser Ser Phe Tyr Ala Glu Met Lys Trp

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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
Phe His Trp Leu Met Leu Asn Pro Asn Asp Thr Val Thr Phe Ser Phe			
	245	250	255
Asn Gly Ala Phe Ile Ala Pro Asp Arg Ala Ser Phe Leu Arg Gly Lys			
	260	265	270
Ser Met Gly Ile Gln Ser Gly Val Gln Val Asp Ala Asn Cys Glu Gly			
	275	280	285
Asp Cys Tyr His Ser Gly Gly Thr Ile Ile Ser Asn Leu Pro Phe Gln			
	290	295	300
Asn Ile Asp Ser Arg Ala Val Gly Lys Cys Pro Arg Tyr Val Lys Gln			
	305	310	315
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
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
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

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<211> LENGTH: 1398

<212> TYPE: DNA

<213> ORGANISM: Influenza virus

<400> SEQUENCE: 22

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gcagtaactca ttggaatagc aaacctagga ttgaacatag gactgcatct aaaaccgggc      120
tgcaattgct cacttcaca acctgaaaca accaacacaa gccaaacaat aataaacaac      180
tattataatg aaacaaacat caccaacatc caaatggaag agagaacaag caggaatttc      240
aataacttaa ctaagggtct ctgtactata aattcatggc acatatatgg gaaagacaat      300
gcagtaagaa ttggagagag ctcggtggtt ttagtcacaa gagaacccta tgtttcatgc      360
gaccagatg aatgcagggt ctatgctctc agccaaggaa caacaatcag agggaaacac      420
tcaaacggaa caatacacga taggtcccag tatcgcgccc tgataagctg gccactatca      480
tcaccgcccc cagtgtacaa cagcaggggtg gaatgcattg ggtgggtcaag tactagtgtc      540
catgatggca aatccaggat gtcaatatgt atatcaggac caaacaacaa tgcactctga      600
gtagtatggt acaacagaag gcctgttgca gaaattaaca catgggcccc aaacatacta      660
agaacacagg aatctgaatg tgtatgccac aacggcggtat gccagtagt gttcaccgat      720
gggtctgcca ctggacctgc agacacaaga atatactatt ttaaagaggg gaaaatattg      780
aatgggaggt ctctgactgg aactgctaag catattgaag aatgctcatg ttacggggaa      840
cgaacaggaa ttacctgcac atgcagggac aattggcagg gctcaaatag accagtgtatt      900
cagatagacc cagtagcaat gacacacact agtcaatata tatgcagtc tgttcttaca      960
gacaatcccc gaccgaatga cccaaatata ggtaagtgtg atgaccctta tccaggtaat     1020
aataacaatg gagtcaaggg attctcatac ctggatgggg ctaacacttg gctagggagg     1080
acaataagca cagcctcgag gtctggatac gagatgttaa aagtgccaaa tgcattgaca     1140
gatgatagat caaagcccat tcaaggtcag acaattgtat taaacgctga ctggagtgggt     1200
tacagtggat ctttcatgga ctattgggct gaaggggact gctatcgagc gtgtttttat     1260
gtggagtnga tacgtggaag acccaaggaa gataaagtgt ggtggaccag caatagtata     1320
gtatcgatgt gttccagtac agaattcctg ggacaatgga actggcctga tggggctaaa     1380
atagagtact tcctctaa                                     1398

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<210> SEQ ID NO 23

<211> LENGTH: 464

<212> TYPE: PRT

<213> ORGANISM: Influenza virus

<400> SEQUENCE: 23

```

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

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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
 Val Ala Glu Ile Asn Thr Trp Ala Arg Asn Ile Leu Arg Thr Gln Glu
 210 215 220
 Ser Glu Cys Val Cys His Asn Gly Val Cys Pro Val Val Phe Thr Asp
 225 230 235 240
 Gly Ser Ala Thr Gly Pro Ala Asp Thr Arg Ile Tyr Phe Lys Glu Gly
 245 250 255
 Lys Ile Leu Lys Trp Glu Ser Leu Thr Gly Thr Ala Lys His Ile Glu
 260 265 270
 Glu Cys Ser Cys Tyr Gly Glu Arg Thr Gly Ile Thr Cys Thr Cys Arg
 275 280 285
 Asp Asn Trp Gln Gly Ser Asn Arg Pro Val Ile Gln Ile Asp Pro Val
 290 295 300
 Ala Met Thr His Thr Ser Gln Tyr Ile Cys Ser Pro Val Leu Thr Asp
 305 310 315 320
 Asn Pro Arg Pro Asn Asp Pro Asn Ile Gly Lys Cys Asn Asp Pro Tyr
 325 330 335
 Pro Gly Asn Asn Asn Asn Gly Val Lys Gly Phe Ser Tyr Leu Asp Gly
 340 345 350
 Ala Asn Thr Trp Leu Gly Arg Thr Ile Ser Thr Ala Ser Arg Ser Gly
 355 360 365
 Tyr Glu Met Leu Lys Val Pro Asn Ala Leu Thr Asp Asp Arg Ser Lys
 370 375 380
 Pro Ile Gln Gly Gln Thr Ile Val Leu Asn Ala Asp Trp Ser Gly Tyr
 385 390 395 400
 Ser Gly Ser Phe Met Asp Tyr Trp Ala Glu Gly Asp Cys Tyr Arg Ala
 405 410 415
 Cys Phe Tyr Val Glu Leu Ile Arg Gly Arg Pro Lys Glu Asp Lys Val
 420 425 430
 Trp Trp Thr Ser Asn Ser Ile Val Ser Met Cys Ser Ser Thr Glu Phe
 435 440 445
 Leu Gly Gln Trp Asn Trp Pro Asp Gly Ala Lys Ile Glu Tyr Phe Leu
 450 455 460

<210> SEQ ID NO 24

<211> LENGTH: 1683

<212> TYPE: DNA

<213> ORGANISM: Influenza virus

<400> SEQUENCE: 24

atgaacactc aaatcctggt attcgctctg attgcgatca ttccaacaaa tgcagacaaa

60

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atctgcctcg gacatcatgc tgtgtcaaac ggaaccaaag taaacacatt aactgaaaga 120
ggagtggaag tcgtcaatgc aactgaaaca gtggaacgaa caaacatccc caggatctgc 180
tcaaagggga aaaggacagt tgacctcggt caatgtggac tcctggggac aatcactgga 240
ccacctcaat gtgaccaatt cctagaattt tcagccgatt taattattga gaggcgagaa 300
ggaagtgatg tctgttatcc tgggaaattc gtgaatgaag aagctctgag gcaaattctc 360
agagaatcag gcggaattga caaggaagca atgggattca catacagtgg aataagaact 420
aatggagcaa ccagttcatg taggagatca ggatcttcat tctatgcaga aatgaaatgg 480
ctcctgtcaa acacagataa tgctgcattc ccgcagatga ctaagtcata taaaaatata 540
agaaaaaacc cagctctaata agtatggggg atccatcatt ccgatcaac tgcagagcaa 600
accaagctat atgggagtggt aaacaaactg gtgacagttg ggagttctaa ttatcaacaa 660
tcttttgtac cgagtccggg agcgagaaca caagttaatg gtcaatctgg aagaattgac 720
tttcattggc taatgctaaa tcccaatgat acagtcactt tcagtttcaa tggggctttc 780
atagctccag accgtgcaag ctctctgaga ggaaatctta tgggaatcca gattggagta 840
caggttgatg ccgatttgta aggggactgc tattatagtg gagggacaat aataagtaac 900
ttgccatttc agaacataga tagcagggca gttggaaat gtccgagata tgttaagcaa 960
aggagtctgc tgctagcaac agggatgaag aatgttctct agattccaaa gggaagaggc 1020
ctatttggtg ctatagcggg ttccattgaa aatggatggg aaggcctaata tgatggttgg 1080
tatggtttca gacaccagaa tgcacaggga gagggaactg ctgcagatta caaaagcact 1140
caatcggaac ttgatcaaat aacaggaaaa ttaaaccggc ttatagaaaa aaccaaccaa 1200
caatttgagt tgatagacaa tgaattcact gaggtagaga agcaaatcgg taatgtgata 1260
aattggacca gagattctat aacagaagtg tggtcataca atgctgaact cttggttagca 1320
atggagaacc agcatacaat tgatctggct gattcagaaa tggacaaact gtacgaacga 1380
gtgaaaagac agctgagaga gaatgctgaa gaagatggca ctggttgctt tgaaatattt 1440
cacaagtgtg atgatgactg tatggccagc attagaaata acacctatga tcacagcaaa 1500
tacaggggaag aggcaatgca aaatagaata cagattgacc cagtcaaact aagcagcggc 1560
tacaagatg tgatactttg gtttagcttc ggggcacat gtttcatact tctagccatt 1620
gcaatgggcc ttgtcttcat atgtgtaaag aatggaaaca tgcggtgcac tatttgtata 1680
taa 1683

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

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<400> SEQUENCE: 25

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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

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Pro	Pro	Gln	Cys	Asp	Gln	Phe	Leu	Glu	Phe	Ser	Ala	Asp	Leu	Ile	Ile	
				85									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		
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		
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		
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		
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		
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		
His	Lys	Cys	Asp	Asp	Asp	Cys	Met	Ala	Ser	Ile	Arg	Asn	Asn	Thr	Tyr	

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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

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atgaatccaa atcagaagat tctatgcact tcagccactg ctatcataat aggcgcaatc 60
gcagtactca ttggaatagc aaacctagga ttgaacatag gactgcatct aaaaccgagc 120
tgcaattgct cacactcaca acctgaaaca accaaccacaa gccaaacaat aataaacaac 180
tattataatg aaacaaacat caccaacatc caaatggaag agagaacaag caggaatttc 240
aataaacttaa ctaagggtct ctgtactata aattcatggc acatatatgg gaaagacaat 300
gcggtaagaa ttggagagag ctcggatgtt ttagtcacaa gagaacccta tgtttcatgc 360
gacccagatg aatgcagggt ctatgtcttc agccaaggaa caacaatcag aggaaaaac 420
tcaaacggaa caatacacga taggtcccag tatcgcgccc tgataagctg gccactatca 480
tcaccgcccc cagtgtacaa cagcagggtg gaatgcattg ggtggtcaag tactagtgtc 540
catgatggca aatccaggat gtcaatatgt atatcaggac caaacaacaa tgcatctgca 600
gtagtatggt acaacagaag gctgttgca gaaattaaca catgggcccc aaacatacta 660
agaacacagg aatctgaatg tgtatgccac aacggcgatg gcccagtagt gttcacgat 720
gggtctgcca ctggacctgc agacacaaga atatactatt ttaaagaggg gaaaatattg 780
aatggggagt ctctgactgg aactgctaag catattgaag aatgctcatg ttacggggaa 840
cgaacaggaa ttacctgcac atgcaaggac aattggcagg gctcaaatac accagtgatt 900
cagatagatc cagtagcaat gacacacact agtcagtata tatgcagtcc tgttcttaca 960
gacaatcccc gaccgaatga cccaaatata ggtaagtgtg atgaccctta tccaggtaat 1020
aataacaatg gagtcaaggg attctcatac ctggatgggg ctaacacttg gctagggagg 1080
acaataagca cagcctcgag gtctggatac gagatgttaa aagtgccaaa tgcatgaca 1140
gatgatagat caaagcccat tcaaggtcag acaattgtat taaacgctga ctggagtgg 1200
tacagtggat ctttcatgga ctattgggct gagggggact gctatcgagc gtgtttttat 1260
gtggaattga tacgtggaag acccaaggag gataaagtg ggtggaccag caatagtata 1320
gtatcgatgt gttccagtac agaattctc 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

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

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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			
Ala	Leu	Ser	Gln	Gly	Thr	Ile	Ile	Arg	Gly	Lys	His	Ser	Asn	Gly	Thr
	130					135					140				
Ile	His	Asp	Arg	Ser	Gln	Tyr	Arg	Ala	Leu	Ile	Ser	Trp	Pro	Leu	Ser
145					150					155				160	
Ser	Pro	Pro	Thr	Val	Tyr	Asn	Ser	Arg	Val	Glu	Cys	Ile	Gly	Trp	Ser
			165						170					175	
Ser	Thr	Ser	Cys	His	Asp	Gly	Lys	Ser	Arg	Met	Ser	Ile	Cys	Ile	Ser
			180					185					190		
Gly	Pro	Asn	Asn	Asn	Ala	Ser	Ala	Trp	Trp	Tyr	Asn	Arg	Arg	Pro	Val
	195						200					205			
Ala	Glu	Ile	Asn	Thr	Trp	Ala	Arg	Asn	Ile	Leu	Arg	Thr	Gln	Glu	Ser
	210					215					220				
Glu	Cys	Val	Cys	His	Asn	Gly	Val	Cys	Pro	Trp	Phe	Thr	Asp	Gly	Ser
225					230					235				240	
Ala	Thr	Gly	Pro	Ala	Asp	Thr	Arg	Ile	Tyr	Tyr	Phe	Lys	Glu	Gly	Lys
			245						250					255	
Leu	Lys	Trp	Glu	Ser	Leu	Thr	Gly	Thr	Ala	Lys	His	Ile	Glu	Glu	Cys
		260						265					270		
Ser	Cys	Tyr	Gly	Glu	Arg	Thr	Gly	Ile	Thr	Cys	Thr	Cys	Lys	Asp	Asn
	275						280					285			
Trp	Gln	Gly	Ser	Asn	Arg	Pro	Val	Ile	Gln	Ile	Asp	Pro	Val	Ala	Met
	290					295					300				
Thr	His	Thr	Ser	Gln	Tyr	Ile	Cys	Ser	Pro	Val	Leu	Thr	Asp	Asn	Pro
305					310					315				320	
Arg	Pro	Asn	Asp	Pro	Asn	Ile	Gly	Lys	Cys	Asn	Asp	Pro	Tyr	Pro	Gly
			325						330					335	
Asn	Asn	Asn	Asn	Gly	Val	Lys	Gly	Phe	Ser	Tyr	Leu	Asp	Gly	Ala	Asn
			340					345					350		
Thr	Trp	Leu	Gly	Arg	Thr	Ile	Ser	Thr	Ala	Ser	Arg	Ser	Gly	Tyr	Glu
		355					360					365			
Met	Leu	Lys	Val	Pro	Asn	Ala	Leu	Thr	Asp	Asp	Arg	Ser	Lys	Pro	Ile
	370					375					380				
Gln	Gly	Gln	Thr	Ile	Val	Leu	Asn	Ala	Asp	Trp	Ser	Gly	Tyr	Ser	Gly
385					390					395					400

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Ser	Phe	Met	Asp	Tyr	Trp	Ala	Glu	Gly	Asp	Cys	Tyr	Arg	Ala	Cys	Phe
			405						410					415	
Tyr	Val	Glu	Leu	Ile	Arg	Gly	Arg	Pro	Lys	Glu	Asp	Lys	Val	Trp	Trp
		420						425					430		
Thr	Ser	Asn	Ser	Ile	Val	Ser	Met	Cys	Ser	Ser	Thr	Glu	Phe	Leu	Gly
		435					440					445			
Gln	Trp	Asn	Trp	Pro	Asp	Gly	Ala	Lys	Ile	Glu	Tyr	Phe	Leu		
	450					455					460				

What is claimed is:

1. An isolated recombinant influenza virus having PA, PB1, PB2, NP, NS, M, NA and HA viral segments, wherein the PB1 viral segment encodes a PB1 with a residue other than isoleucine at position 711 or the M viral segment encodes a M1 with a residue other than methionine at position 128, wherein the recombinant influenza virus has enhanced replication relative to a corresponding influenza virus having a PB1 viral segment that encodes a PB1 with an isoleucine at position 711 or having a M viral segment that encodes a M1 with methionine at position 128.

2. The recombinant virus of claim 1 wherein the PB1 viral segment encodes a PB1 with a residue other than isoleucine at position 711 and the M viral segment encodes a M1 with a residue other than methionine at position 128; or the PA viral segment encodes a PA with a residue other than phenylalanine at position 105, a residue other than lysine at position 142, a residue other than serine at position 149, a residue other than serine at position 225, a residue other than lysine at position 356,

a residue other than threonine at position 357, a residue other than arginine at residue 401, or a residue other than isoleucine at position 550, or any combination thereof; or the PB1 viral segment further encodes a PB1 with a residue other than methionine at position 40, a residue other than glutamic acid at position 112, a residue other than glycine at position 180, or a residue other than glutamine at residue 247, or any combination thereof; or the PB2 viral segment encodes a PB2 a residue other than methionine at position 202, a residue other than phenylalanine at position 323, or a residue other than isoleucine at position 504, or any combination thereof; or the NP viral segment encodes a NP with a residue other than arginine at position 74, a residue other than isoleucine at position 116, or a residue other than asparagine at position 417, or any combination thereof; or the NS viral segment encodes a NS1 with a residue other than alanine at position 30, a residue other than lysine at position 55, or a residue other than arginine at position 118, or any combination thereof.

3. The recombinant virus of claim 1 which has a V, A, L, G, S, T, C, or M at position

711 in PB1, has an A, L, G, V, S, T, I, or C at position 40 in PB1, has an A, L, G, V, S, T, I, or C at position 112 in PB1, has an A, L, W, Y, F, V, S, T, I, or C at position 180 in PB1, has a H, R, K, D, E, D or E at position 247 in PB1, or any combination thereof; or which has a K, H, D, E, Q or N at position 74 in NP, has a L, V, G, A, S, C or T at position 116 in NP, has a D, E, Q, K, or H at position 417 in NP, or any

combination thereof; or which has a P, W, F, L, I, V, G, S, C or T at position 30 in NS1, has E, D, Q, or N at position 55, has a K, H, D, E, Q, or N at position 118

in NS1, has a L, I, V, G, A, S, C or T at position 128 in M1, or any combination thereof; or which has a C, L, I, V, G, A, or T at position 142 in PA, has a C, L, I, V, G, A, or T at position 225 in PA, has a K, H, D, E, N or Q at position 401 in PA, or any combination thereof; or which has an V, A, L, G, S, T, C, or M at position 504 in PB2, has a A, L, G, S, T, I, or C at position 202 in PB2, has a A, L, G, S, T, I, or C at position 323 in PB2, or any combination thereof; or which has a V, A, L, G, S, T, C, or M at position 711 in PB1, has an A, L, G, V, S, T, I, or C at position 40 in PB1, has an A, L, W, Y, F, V, S, T, I, or C at position 180 in PB1; has a L, V, G, A, S, C or T at position 116 in NP; has a P, W, F, L, I, V, G, S, C or T at position 30 in NS1, has a K, H, D, E, Q, or N at position 118 in NS1; has a L, I, V, G, A, S, C or T at position 128 in M1; has a K, H, D, E, N or Q at position 401 in PA; has a V, A, L, G, S, T, C, or M at position 504 in PB2; or any combination thereof; or which has a V, A, L, G, S, T, C, or Mat position 711 in PB1, has a H, R, K, D, E, DoE at position 247 in PB1; has a K, H, D, E, Q or N at position 74 in NP; has E, D, Q, or N at position 55 in NS1; has a L, I, V, G, A, S, C or T at position 128 in M1; has a C, L, I, V, G, A, or T at position 142 in PA; has an A, L, G, S, T, I, or C at position 202 in PB2, has a A, L, G, S, T, I, or C at position 323 in PB2; or any combination thereof; or which has a V, A, L, G, S, T, C, or Mat position 711 in PB1, has a A, L, G, V, S, T, I, or C at position 112 in PB1; has a K, H, D, E, Q or N at position 74 in NP, has a D, E, Q, K, or H at position 417 in NP; has E, D, Q, or N at position 55; has a L, I, V, G, A, S, C or T at position 128 in M1; has a C, L, I, V, G, A, or T at position 225 in PA; has an V, A, L, G, S, T, C, or M at position 504 in PB2; or any combination thereof.

4. The recombinant virus of claim 1 which 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 any combination thereof; or which 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 which 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.

5. The recombinant virus of claim 1 which has 504V or 202L and/or 323L in PB2; or which has 40L and 180W or 247H in PB1; or which has 30P and 118K or 55E in NS1; or which has 401K or 142C in PA; or which has 116L or 74K in NP; or which has 504V in PB2, 116L in NP; 30P or 118K

in NS1 and 401K in PA; and which has a V, A, L, G, S, T, C, or Mat position 711 in PB1 or a L, I, V, G, A, S, C or T at position 128 in M1.

6. The recombinant virus of claim 1 which has a U at position 4 in the viral segment for any one of PB1, PB2 or PA.

7. The recombinant virus of claim 1 which has V, A, L, G, or T at position 711 in PB1 and a L, I, V, G, A, or T at position 128 in M1.

8. The recombinant virus of claim 1 wherein 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 M1 and/or M2 having the amino acid sequence encoded by SEQ ID NO:5 or M1 and/or M2 with at least 95% amino acid sequence identity to the M1 and/or M2 encoded by SEQ ID NO:5; or a NS1 and/or NS2 having the amino acid sequence encoded by SEQ ID NO: 6 or NS1 and/or NS2 with at least 95% amino acid sequence identity to the NS1 and/or NS2 encoded by SEQ ID NO: 6 or wherein 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 M1 and/or M2 having the amino acid sequence encoded by SEQ ID NO:14 or M1 and/or M2 with at least 95% amino acid sequence identity to the M1 and/or M2 encoded by SEQ ID NO:14; or a NS1 and/or NS2 having the amino acid sequence encoded by SEQ ID NO:15 or NS1 and/or NS2 with at least 95% amino acid sequence identity to the NS1 and/or NS2 encoded by SEQ ID NO:15.

9. A vaccine having the isolated recombinant virus of claim 1.

10. 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 NA, and wherein the HA DNA in the vector for vRNA production of HA has sequences for a heterologous HA, wherein the PB1 DNA encodes a PB1 with a residue other than isoleucine at position 711 or the M DNA encodes a M1 with a residue other than methionine at position 128; 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.

11. The method of claim 10 wherein the cell is a Vero cell, a human cell or a MDCK cell.

12. The method of claim 10 wherein the PB1 viral segment encodes a PB1 with a residue other than isoleucine at position 711 and the M viral segment encodes a M1 with a residue other than methionine at position 128; or wherein the PA viral segment encodes a PA with a residue other than phenylalanine at position 105, a residue other than lysine at position 142, a residue other than serine at position 149, a residue other than serine at position 225, a residue other than lysine at position 356, a residue other than threonine at position 357, a residue other than arginine at residue 401, or a residue other than isoleucine at position 550, or any combination thereof; or wherein the PB1 viral segment further encodes a PB1 with a residue other than methionine at position 40, a residue other than glutamic acid at position 112, a residue other than glycine at position 180, or a residue other than glutamine at residue 247, or

any combination thereof; or wherein the PB2 viral segment encodes a PB2 a residue other than methionine at position 202, a residue other than phenylalanine at position 323, or a residue other than isoleucine at position 504, or any combination thereof or wherein the NP viral segment encodes a NP with a residue other than arginine at position 74, a residue other than isoleucine at position 116, or a residue other than

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asparagine at position 417, or any combination thereof; or wherein the NS viral segment encodes a NS1 with a residue other than alanine at position 30, a residue other than lysine at position 55, or a residue other than arginine at position 118, or any combination thereof.

13. The method of claim 10 wherein the virus has a V, A, L, G, S, T, C, or Mat position 711 in PB1, has a A, L, G, V, S, T, I, or C at position 40 in PB1, has a A, L, W, Y, F, V, S, T, I, or C at position 180 in PB1; has a L, V, G, A, S, C or T at position 116 in NP; has a P, W, F, L, I, V, G, S, C or Tat position 30 in NS1, has a K, H, D, E, Q, or N at position 118 in NS1; has a L, I, V, G, A, S, C or T at position 128 in M1; has a K, H, D, E, N or Q at position 401 in PA; has a V, A, L, G, S, T, C, or M at position 504 in PB2; or any combination thereof; or wherein the virus has a V, A, L, G, S, T, C, or M at position 711 in PB1, has a H, R, K, D, E, D or E at position 247 in PB1; has a K, H, D, E, Q or N at position 74 in NP; has E, D, Q, or N at position 55 in NS1; has a L, I, V, G, A, S, C or T at position 128 in M1; has a C, L, I, V, G, A, or T at position 142 in PA; has a A, L, G, S, T, I, or C at position 202 in PB2, has a A, L, G, S, T, I, or C at position 323 in PB2; or any combination thereof.

14. The method of claim 10 wherein the virus has a V, A, L, G, S, T, C, or Mat position 711 in PB1, has a A, L, G, V, S, T, I, or C at position 112 in PB1; has a K, H, D, E, Q or N at position 74 in NP, has a D, E, Q, K, or H at position 417 in NP; has E, D, Q, or N at position 55; has a L, I, V, G, A, S, C or T at position 128 in M1; has a C, L, I, V, G, A, or T at position 225 in PA; has an V, A, L, G, S, T, C, or M at position 504 in PB2; or any combination thereof; or wherein the virus has one or more of 142N, 225C, 356R, or 550L in PA; has one or more of 112G, 247H, 507V, or 644A in PB1;

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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 any combination thereof.

15. The method of claim 10 wherein 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; or wherein 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; or wherein the virus has 202L and/or 323L in PB2; or wherein the virus has 247H in PB1; or wherein the virus has 55E in NS1; or wherein the virus has 142C in PA; or wherein the virus has 74K in NP; or wherein the virus has 504V in PB2; or wherein the virus has 74K, 116L or 417D in NP; or wherein the virus has 30P, 55E or 118K in NS1; or wherein the virus has 225C or 401K in PA; or wherein the virus has a V, A, L, G, S, T, C, or Mat position 711 in PB1.

16. The method of claim 10 wherein the virus has a L, I, V, G, A, S, C or T at position 128 in M1.

17. A method of immunizing an avian or a mammal, comprising: administering to the avian or the mammal an effective amount of a composition comprising the recombinant virus of claim 1.

18. The method of claim 17 wherein the mammal is a human.

19. The method of claim 17 wherein the composition is injected.

20. The method of claim 17 wherein the composition is administered to the upper respiratory tract.

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