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- (54) RECOMBINANT INFLUENZA VIRUSES
WITH STABILIZED HA FOR REPLICATION
IN EGGS

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2760/16051* (2013.01); *C12Y 302/01018*
(2013.01)

(57)

ABSTRACT

Modified influenza virus neuraminidases are described herein that improve viral replication, thus improving the yield of vaccine viruses. Expression of such modified neuraminidases by influenza virus may also stabilize co-expressed hemagglutinins so that the hemagglutinins do not undergo mutation.

Specification includes a Sequence Listing.

A Yokohama 2017/03/28 3

ACCAACAGGAGCTTAAATATTCAGTTGGAAAAGATAAAAGAACTACGGAACTGTGATGCGTCACTCTGCACT
 CGCGA
 GATAGATGACAAAAACACAGTGACCATATGGCCATAATTAGAAGATCACATGGGGAGACAGGAAAAGAAC
 CGTAC
 TTAGGATGAAATGATGATGCGATGAAATTGCCAATGACTGTGACAAMGGATACAGAAATGUGTCCGAGA
 GAAAT
 GACCAAGGACAACTCTATGGAGTAAATGGTGTGATGCTGGATCAGATGCGTGTGTTATCCCTTGGCTGTG
 ACATG
 GTGGAAATAGAAATGGGCCGTGACAAGTAGCGTGTCCATTGCAAAAGTACAGATTTTGACAAAGTGA
 AUGST
 TAAACATGGAAACCTTGGCCCTGTTATTTAGAAATCAAGTCAGATAACGGGAAGAGTAGACACAAACCCCTGG
 TCAT
 GGGACGTCAGTOCAAGGAGGCAAAAGATGTAATTATGSAAGTTGTTTCCAAATGAAAGTGGGGACCCAGGATA
 CTAAC
 ATCAGAATGCGAATTAAATACTAAAGAGAAAAAGAAGAACTGAGATTGCGAAATTCCTCTGTGGTT
 GCGAT
 ACATGTTAGAGAGAACTTGTCCGAAAAGAAGATTTCTCCAGTTGCTGGCGAACAAGCAGTATATACATG
 AGTT
 TCAATGTCAGTCAGGGACGTTGGGACAAATGTCACCTTCAGGTTGAGAAGTGGAGGAAATGAGCTGTCAG
 CAAG
 CCTAATTATGCGAGCCAGGAACATGAGTAAAGAAGGGCGAGTATCAGCGATGACTAGGCATCTTATGGAGATG
 TSCC
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 ATATGC
 AAGGCTCAATGGGATGAGAATCAGTAACTCTTCAGCTTGGGGGTTAACATTAAAGAACAGCGGTCAT
 CAGT
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 TCAAA

A/1028809/2017/06 PB;

AAAAACGCTTGTCTTCTTCTT (SEQ ID NO:4)

5'-GGAAAGCAGGCAACCCATTGAATGGATCTAACATCCGACTCTACTTTCCTAAAGGTTCAGCGCaaaaATGCCA
AAG

Figure 1

A/Yokohama/2017/03 PB2

AGCAAAAGCAGGTCAATTATTCAGTATGGAAAGAATAAAAGAACTACGGAACCTGATGTCGCAGTCTCGCACT
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CGTCAC

TTAGGATGAAATGGATGATGGCAATGAAATACCCAACTGCTGACAAAAGGATAACAGAAATGGTCCGGAGA
GAAAT

GAACAAGGACAAACTCTATGGAGTAAAATGAGTGATGCTGGATCAGATCGAGTGATGGTATCACCTTGGCTGTG
ACATG

GTGGAATAGAAATGGACCCGTGACAAGTACGGTCCATTACCCAAAAGTATAACAAGACTTATTTGACAAAGTCGA
AAGGT

TAAAACATGGAACCTTGGCCCTGTTCAATTAGAAATCAAGTCAAGATAACGCCGAAGAGTAGACACAAACCTGG
TCAT

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CTAAC

ATCAGAACATCGCAATTACAATAACTAAAGAGAAAAAGAAGAACCTCGAGATTGCAAAATTCTCCCTTGATGGTT
GCAT

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AAGTT

TTACATTGACTCAAGGGACGTGTTGGAACAAATGTACACTCCAGGTGGAGAAGTGAGGAATGACGATGTTGAC
CAAAG

CCTAATTATTGCAGCCAGGAACATAGTAAGAAGAGCCGCAGTATCAGCAGATCCACTAGCATTTATTGGAGATG
TGCC

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ATATGC

AAGGCTGCAATGGGATTGAGAATCAGCTCATCCTCAGCTTGGTGGGTTACATTAAAAGAACAGCGGGTCAT
CAGT

CAAAAAAGAGGAAGAAGTGTACAGGCAATCTCAAACATTGAAGATAAGAGTACATGAGGGTATGAGGAGT
TCACAA

TGGTGGGAAAAGAGCAACAGCTATACTCAGAAAAGCAACCAGAAGATTGGTTCAGCTCATAGTGAGTGGAAAGA
GACGAA

Figure 1 cont.

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GACCT
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GTGC
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GCACA
GAGATGTCAATGAGAGGAATAAGAGTCAGCAAAATGGGTGGATGAATACTCCAGTACAGAGAGGGTGGTGGT
TAGCAT
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AACTG
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TCAA
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TTGA
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GAG
ACGTACTTGGACATTGACACCAACCCAGATAATAAGCTTCTCCCTTGCAGCCGCTCCACAAAGCAAAGCAG
AATG
CAGTTCTTCACTGACTGTAAATGTGAGGGGATCAGGGATGAGAATACTGTAAAGGGCAATTCTCCTGTATTCA
ACTA
CAACAAGACCAACTAAAGACTAACAAATTCTGGAAAAGATGCCGGACTTAATTGAAGACCCAGATGAAAGCAC
ATCCG
GAGTGGAGTCGCTGTATTGAGAGGGTTCTCATTAGGTAAAGGAAGACAGAAGATCGGCCAGCATTAGC
ATCAAT
GAACGTGAGTAACCTTGCAAAAGGGAAAAGGCTAATGTGCTAATCGGGCAAGGAGACGTGGTGGTAATGAA
ACGAAA
ACGGGACTCTAGCATACTTACTGACAGCCAGACAGCGACCAAAAGAATTGGATGCCATCAATTATGTTGAAT
AGTTT
AAAAACGACCTTGTCTACT (SEQ ID NO:4)

A/Yokohama/2017/03 PB1

AGCAAAAGCAGGCAAACCATTGAATGGATGTCAATCCGACTCTACTGTTCTAAAGGTTCCAGCGAAAATGCCA
TAAG

Figure 1 cont.

CACCAACATTCCCTTATACTGGAGATCCTCCATACAGCCATGGAACAGGAACAGGGTACACCATGGACACAGTCAC
AGAA
CACACCAATATTCAAGATAAGGGGAAGTGGACGACAATAACAGAAACTGGGCACCCCAACTCAACCCAATTGATG
GACCA
CTACCTGAGGATAATGAGCCAAGTGGATATGCACAAACAGACTGTGTCTGGAGGCTATGGCCTCCTGAAGAA
TCCC
CCCAGGTATCTTGAGAACTCATGCCTGAAACAATGGAAGTCGTTCAACAAACAAGGGTGGACAAACTAACCCA
AGGTC
GCCAGACTTATGATTGGACATTAACAGAAATCAACCGGCAGCAACTGCATTAGCCAACACCATAGAAGTTTAG
ATCG
AATGGACTAACAGCTAATGAATCAGGAAGGCTAATAGATTCTCAAGGATGTGATGGAATCAATGGATAAAGAG
GAAAT
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CAATAG
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GCAGAG
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TTAGC
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ATGTTG
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AAAAT
CAAAACCTCGAATGTTTGGCGATGATTACATATATCACAAAAAATCAACCTGAGTGGTTAGAAACATCCTGA
GCAT
CGCACCAATAATGTTCTAAACAAAATGGCAAGACTGGAAAAGGATACTGTTGAGAGTAAGAGAATGAAACT
CCGAA
CACAAATACCGCAGAAATGCTAGCAAACATTGACCTGAAGTATTCAATGAATCAACAAGGAAGAAAATTGAGA
AAATA
AGGCCTCTTCTAATAGATGGCACAGCATCATTGAGCCTGGGATGATGATGGCATGTTAACATGCTAAGTACG
GTTT
AGGAGTCTGATACTGAATCTGGCAAAAGAAATACCCAAGACAACATACTGGTGGATGGCTCCAATCCTC
CGACG
ATTTGCCCTCATAGTGAATGCACAAATCATGAGGAAATACAAGCAGGAGTGGATAGATTTACAGGACCTGCA
AGTTA

Figure 1 cont.

GTGGGAATCAACATGAGCAAAAGAAGTCCTATATAAATAAAAACAGGGACATTGAATTACAAGCTTTTATC
GATA
TGGATTGTGGCTAATTAGCATGGAGCTGCCAGTTGGAGTGTCTGGAATAAACGAGTCAGCTGATATGAGC
ATTG
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TCATC
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AAGCT
GTGGGATCAAACCAATCAAGGGCAGGACTATTGGTATCAGATGGGGACCAAACCTATAACATATCCGAATCT
TCACA
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TGTC
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CGTGCAACTACACACTCCTGGATTCCAAGAGGAACCGCTATTCTAACACACAAGCCAAAGGGGATTCTTGAG
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GGAAGA
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A/Yokohama/2017/03 PA

AGCAAAAGCAGGTACTGATTGAAATGGAAGATTGTGCGACAATGCTCAACCCGATGATTGTCGAACCTGCA
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TACTGG

Figure 1 cont.

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AATTG
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GGAA
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GTCAAC
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AGCAA
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ACTGA
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TTATT
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TGCA
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ACCAA
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TTTT
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AACT

Figure 1 cont.

GCCATAGGCCAAATTCAAGGCCTATGTTCTGTATGTGAGGACAAACGGAACATCAAAGGTAAAATGAAATGG
GGAAT
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GAAAG
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GGTTCC
ATTGGGAAAGTCTGTAGGACTCTATTGGCTAAGTCAGTGTCAATAGCCTGTATGCATCACCAACATTGGAAGGGAT
TTTC
AGCGGAGTCAGAAAATGCTCCTGTTAGGCTTAGGGACAACCTCGAACCTGGACCTTGATCTTGGG
GGGC
TATATGAAGCAATTGAGGAGTGCCTGATTAATGATCCCTGGTTGCTCAATGCGTCTGGTTCAACTCCTTCCTG
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ID NO:6)

A/Yokohama/2017/03 HA

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AAAAC
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AGTC
CTCATCAGATCCTGATGGAGAAAATGCACACTAATAGATGCTCTATTGGAGACCCCTCAGTGTGATGGCTTCCA
AAAT
AAGAAAATGGGACCTTTGTTAACGCAGCAAAGCCTACAGCAACTGTTACCCATTGATGTGCCGGATTATGCCT
CCCT
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GGAA
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CGGA

Figure 1 cont.

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TTTC
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GGTGGG
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AATCA
ACCAAATCAATGGAAACTGAATAGGTTAACGGAAAACAAACGAGAAATTCCATCAGATTGAAAAAGAATTCT
CAGAA
GTAGAAGGGAGAATTCAAGGACCTCGAGAAATATGTTGAGGACACTAAAATAGATCTCTGGTCATAACGCGGA
GCTTCT
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GCAAC
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CAATC
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CTGAA
GTCAGGATACAAAGATTGGATCCTATGGATTTCCTTGCATATCATGTTTGTGCTCTGTTGCTTGGGGTT
CA
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TCTACT (SEQ ID NO:7)

A/Yokohama/2017/03 NP

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GAACA

Figure 1 cont.

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GGAGAT

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GAAA

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AAAAC

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GGCGAA

TCTGGCGCCAAGCCAACAATGGTGAGGATGCGACAGCTGGCTAACTCACATAATGATCTGGCATTCCAATTGAA
TGAT

GCAACATACCAGAGGACAAGAGCTTGTGAAACCGGAATGGATCCCAGAATGTGCTCTGATGCAGGGCTCG
ACTCT

CCCTAGAAGGTCCGGAGCTGCAGGTGCTGCAGTCAAAGGAATCGGACAATGGTATGGAGCTGATCAGAATGG
TCAAAC

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CAACATT

CTTAAAGGAAAATTCAAACAGCTGCACAAAGAGCAATGGTGGATCAAGTGAGAGAAAGTCGGAACCCAGGAAA
TGCTGA

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CCT

GTGTGTATGGACCTGCAGTATCCAGTGGTACGACTTCGAAAAAGAGGGATATTCTGGTGGAAATAGACCCCTT
TCAAAC

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ATGGC

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CTTT

CAACTAGAGGAGTACAAATTGCTCAAATGAGAACATGGATAATATGGATCGAGCACTCTGAACTGAGAACG
GGTAC

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TACGTT

TTCTGTACAAAGAAACCTCCCATTGAAAAGTCAACCATCATGGCAGCATTCACTGGAAATACGGAGGGAGAACT
TCAG

ACATGAGGGCAGAAATCATAAGAATGATGGAAGGTGCAAAACCAGAAGAAGTGTGTTCCGGGGAGGGAGT
TTTCGAG

Figure 1 cont.

CTCTCAGACGAGAAGGCAACGAACCGATCGTGCCTCTTTGATATGAGTAATGAAGGATCTTATTCTTCGGAG
ACAA

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A/Yokohama/2017/03 NA

AGCAAAAGCAGGAGTAAAGATGAATCCAATCAAAGATAATAACGATTGGCTCTGTTCCCTCACCATTTCCACA
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AC

AACCAAGTGTGCTGTGAACCAACAATAATAGAAAGAACATAACAGAGATAGTGTATCTGACCAACACCACC
ATAGA

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TTTTT

CTAAGGACAATTGATTGGCTTCCGCTGGTGGGACATCTGGGTGACAAGAGAACCTTATGTGTATGCGATCC
TGAC

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CCCC

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AGCT

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TTTAC

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ATCAA

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GGGGA

AAATTGTCATACTAGCACATTATCAGGAAGTGCTCAGCATGTCAGGAGTGCTCCTGTTATCCTGATATCCTGGT
GTC

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GTTTC

CAGTTATGTTGCTCAGGACTTGTGGAGACACACCCAGAAAAACGACAGCTCCAGCAGTAGCCATTGCTTGG
TCCAA

ACAATGAGGAAGGTGGTATGGAGTGAAAGGCTGGCCTTGATGATGGAAATGACGTGTGGATGGAAAGAAC
GATCAGC

Figure 1 cont.

GAGAAGTTACGCTCAGGATATGAAACCTCAAAGTCATTGAAGGGCTGGTCCAACCCTAACCTCAAATTGCAGATAA
ATAG

GCAAGTCATAGTTGACAGAGGTAACAGGTCCGGTTATTCTGGTATTTCTCTGTTGAAGGCAAAAGCTGCATCAAT
CGGT

GCTTTATGTGGAGTTGATAAGGGGAAGAAAACAGGAAACTGAAGTCTTGTGGACCTCAAACAGTATTGTTGT
TTTGT

GGCACCTCAGGTACATATGGAACAGGCTCATGGCCTGATGGGGCGGACATCAATCTCATGCCTATATAAGCTTCG
CAAT

TTTAGAAAAAAACTCCTGTTCTACT (SEQ ID NO:9)

Which encodes MNPNQKHTIGSVSLTISTICFFMQIAILITTVTLHFKOYE
FNSPPNNQVMICERTIERNITEIVYLTNTTIEKEICPKLAEYRNWS
KPQCNITGFAPFSKDN SIRLSAGGDIWVTRPYVSCDPDKCYQFAL
GQGTTINNVHSNDIVHDRTPYRTLLMNELGVPFHGTQVCIAW
SSSSCHDGRAWLHVCVTGDDENATASFLYNGRLADSIWSWSKKIL
RTQESECVCINGTCTVVMTDG SASGKADTKILFIEEGKIVHTSTLS
GSAQHVEECSCYPYRPGVRCVCRDNWKGSNRPIVDINIKDYSIVSS
YVCSQLVGDTPRKNDSSSSSHCLDPNNEEGGHGVKGWAFFDGND
VWMGRTISEKLRLSGYETFKVIEGWSNPNSKLQINRQVIVDRGNRS
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TYGTGSWPDGADINLMP1 (SEQ ID NO:3)

A/Yokohama/2017/03 M

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GGCC

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AATGG

CTAAAGACAAGACCAATTCTGTACCTCTGACTAAGGGGATTCTGGGTTGTGTCACGCTCACCGTGCCCCAGTG
AGCG

AGGACTGCAGCGTAGACGCTTGTCAAAATGCCCTCAATGGGAATGGAGATCAAATAACATGGACAAAGCAGT
TAAAC

TGTATAGGAAACTTAAGAGGGAGATAACGTTCCATGGGCCAAGAAATAGCTCTCAGTTATTCTGCTGGTGCAC
TTGCC

Figure 1 cont.

AGTTGCATGGGCCTCATATAACAATAGGATGGGGCTGTAACCACTGAAGTGGCATTTGCCCTGGTATGTGCAACA
TGTGA
GCAGATTGCTGACTCCCAGCACAGGTCTCATAGGCAAATGGTGGCAACAACCAATCCATTAAAGGCATGAGAA
CAGAA
TGGTTTGCCAGCACTACAGCTAAGGCTATGGAGCAAATGGCTGGATCAAGTGAGCAGGCAGCGGAGGCCATG
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GCTAGTCAGGCCAGGCAAATGGTGCAGGCAATGAGAGGCCATTGGGACTCATCCTAGCTCCAGTACTGGCTAAGA
GATGA
TCTTCTTGAAGAATTGCAGACCTATCAGAAACGAATGGGGTGCAGATGCAACGATTCAAGTGACCCACTGTTGT
TGCC
GCGAGTATCATTGGATCTGCACTTGATATTGTGGATTCTGATCGTCTTTTCAAATGCGTATCGACTCTTC
AA
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AGAATG (SEQ ID NO:1.0)

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A/Yokohama/2017/03 NS

AGCAAAAGCAGGGTGACAAAGACATAATGGATTCCAACACTGTGTCAAGTTCCAGGTAGATTGCTTCTTGGCA
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AGGGG
AAGAGGCAATACTCTGGTCTAGACATCAAAGCAGCCACCCATGTTGGAAAGCAAATTGTAGAAAAGATTCTGAA
AGAAG
AATCTGATGAGGCACCTAAATGACCATGGTCTCCACACCTGCTCGCGATACATAACTGACATGACTATTGAGGA
ATTG
TCAAGAAACTGGTCATGCTAATGCCAAGCAGAAAGTGGAGGACCTCTTGATCAGAATGGACCCAGGCAATC
ATGGA
GAAAAACATCATGTTGAAAGCGAATTCACTGAGTGTGATTTTGACCGACTAGAGACCATAGTATTACTAAGGGCTTC
ACCG
AAGAGGGAGCAATTGTTGGCGAAATCTCACCATGGCTTCTTCCAGGACATACTATTGAGGATGTCAAAATGC
AATT

Figure 1 cont.

GGGGTCCTCATCGGAGGACTTGAATGGAATGATAACACAGTTGAGTCTCTAAAAATCTACAGAGATTGCTTGG
AGAAG

CAGTAATGAGAATGGGGACCTCCACTTACTCCAAAACAGAAACGGAAATGGCGAGAACAGCTAGGTCAAAAG
TTTGAA

GAGATAAGATGGCTGATTGAAGAAGTGAGACACAGACTAAAAACAACGTAAAATAGCTTGAACAAATAACATTC
ATGCA

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CCCT

TGTTTCTACT (SEQ ID NO:11)

Figure 2

MNPQNQKIIITIGSVSLTISTICFFMQIAILITTVTLHFKQYEFNSPPNNQVMLCEPTIIEERNVTEIVYLTNTTIEKEI
CPKPAEYRNWSKPQCGITGFAPPDKDNLRLSAGGDIWVTREPYVSCDPDKCYQFALGQGTTLNNSNNTVRDRTP
YRTLLMNELGVPFHGLGKQVCIAWSSSSCHDGKAWLWVCITGDDNMATASPIYNGRLVDVVWSKDLIRKTQESECV
CINGTCTVVMTDGSASGKADTKILFIEEGKIVHTSKLSGSAQHVEECSCSYPRYPGVRCVCRDNWKGSNRPTVDINIK
DHSIVSSYVCSCGLVGDTPRKNDSSSSGHCLDPNNEEGGHGVKGWAFDDGNDVWMGRTINETSRLGYETIFKVVEGWSN
PKSKLQINRQVIVDRGDRSGYSGIFSVEGKSCINRCFYVELIRGRKEETEVLWTSNSIVVFCGTSGTYGTGSWPDGA
DLNLMPI (SEQ ID NO:2)

Figure 3

>Y2017M3L4-NA (32A, 147N, 329D, 347Q, del46-50aa)
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GTGTATCTGACCAACACCACCATAGAGAAGGAAATATGCCCAAAACTAGCAGAAATACAGA
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CCTGACAAGTGTATCAATTGGCCTTGGACAGGGACAACACTAAACAACTGCAATTCA
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GGAAAAGCATGGCTGCATGTTGTAAACGGGGATGATGAAATGCAACTGCTAGCTTC
ATTTACAATGGGAGGCTTGCAGATAGTATTGTTCATGGTCAAAAAAATCCTCAGGACC
CAGGAGTCAGAATGGCTTGTATCAATGAACTTGTACAGTAGTAATGACTGATGGGAGT
GCTTCAGGAAAAGCTGATACTAAAATACTATTGAGGAGGGAAAATGTTCAACT
AGCACATTATCAGGAAGTGTCAAGCATGTCAGGAGTGTCTCTGTTATCCTCGATATCCT
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GGTCAAGGAGTCAAAGGCTGGGCTTGTGATGAAATGACGTGTGGATGGGAAGAACG
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YVSCDPDKCYQFALGQQGTTLNNSVHSNNVHDRTPYR
TLLMNELGYPFHILGTQVCIAWSSSSCHDGKAWLHV
CVTGDDENATASEFIYNGRLADSIIVSWSKKILRTQESE
CVCINGTCTVVMTDGSASGKADTKILFIEGKIVHTS
TLSGSAQRVEECSCYPRYRGVRVCVDRDNWKGSNRPI
VDINIKDYSIVSSYVCSGLVGDTPRKDDSSSSSHCLD
PNNEEGGQGVKGWAEDDGNDVWMGRТИSEKLRSGY
ETEKVIEGWNPNSKLQINRQVIMDRGNRSGYSGIFS
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>Y2017M3L4HA
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Figure 3 cont.

GACCTTTGTTAACCGAGCAAAGCCTACAGCAACTGTTACCCCTATGATGTGCCGGAT
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GATGCTCCAGCAGAACAAAGCATTGACAAATAGTAAACCGGGAGACATACTTTG
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CCAGATCTAAAAGCACTCAAGCAGCAATCAACCAATCAATGGAAACTGAATAGGTTA
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ACTTATGACCATGATGTTACAGAGATGAAAGCATTAAACACCGGTTCCAGATCAAAGGT
GTTGAGCTGAAGTCAGGATACAAAGATTGGATCTTGTGACATGGATTTCCCTGCCATATCATGT
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>Y2017M3L4-M (M1-23Q)
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GAGGCCATGGAGATTGCTAGTCAGGCCAGGCAAATGGTGCAGGCAATGAGAGGCCATTGGG
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CAGAAACGAATGGGGGTGCAAGATGCAACGATTCAAGTGACCCACTGTTGTTGCCCGAG
TATCATTGGGATCTGCACATTGCAATTGCGATTCTGATCGTCTTTTCAATGCGT
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>Y2017M3L4-NP (101N)
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ATCCAAATGTCACGAACTAAACTCAGTGATTATGAAAGGGGGTTGATCCAGAACAGC
TTGACAATAGAGAAAATGGTGCCTCTGCTTTGATGAAAGAAGGAATAATATCTGGAA
GAACACCCCTAGGCCGGGGAAAGATCTTAAGAAAACCTGGGGGGCCATATAACAGGAGAGTA
AATGGAAAATGGATGAGGGAACTCGTCCCTTATGACAAAGAAGAAATAAGGCGAATCTGG
CGCCAAGCCAACAATGGTGGAGGATGCCACAGCTGGTCTAACTCACATAATGATCTGGCAT

Figure 3 cont.

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GCTGCAGTCAAAGGAATCGGGACAATGGTGTGGAGCTGATCAGAATGGTCAAACGGGG
ATCAACGATCGAAATTCTGGAGAGGTGAGAATGGGGAAAACAAGAAGTGCCTATGAG
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CAAGTGAGAGAAAGTCGGAACCCASGAAATGCTGAGATCGAAGATCTCATATTTGGCA
AGATCTGATTGATATTGAGAGGATCAGTTGCTCACAAATCTGCCTACCTGCCGTG
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AATCCAGCACACAAAGAGTCAGCTGGTATGGATGGCATGCCATTCTGCCTGCAATTGAAGAT
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GAAAAGTCACCATCATGGCAGCATTCACTGGAAATACGGAGGGAAAGAAACTTCAGACATG
AGGGCAGAAATCATAAGAATGATGGAAGGTGCAAAACCAAGAAGAAGTGTGTTCCGGGG
AGGGGAGTTTCGAGCTCTCAGACGAGAACGAAACGAAACCCGATCGTGCCTCTTGT
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AAAATACCCCTGTTCTACT (SEQ ID NO:15)

>Y2017M3L4-NS

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CAGGCAATCATGGAGAAAACATCATGTTGAAAGCGAATTCTGATGTTTGACCGA
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TTGCTTGGAGAAGCAGTAATGAGAATGGGGACCTCCACTTACTCCAAACAGAACCG
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>Y2017M3L4-PB1

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Figure 3 cont.

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GCTGATATGAGCATTGGAGTAACAGTGAATAAGAACAACTGATAAAACAATGACCTGG
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>Y2017M3L4-PA
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Figure 3 cont.

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ATGAGACGTTGCCTCCTTCAGTCAGTCAGCAGATCGAGAGCATGATTGAAGCCGAGTCC
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>M3L4-PB2 (1471)

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Figure 3 cont.

>M3L4-PB2 (147I, 344L)
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ACCACTAAAAGACTAACAAATTCTGGAAAAGATGCCGACTTTAATTGAAAGACCCAGAT
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GACAGAAGATAACGGGCAAGCAGTCAATGAACTGAGTAACTTGCAAAAGGGGAA
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Figure 3 cont.

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GAACAAAATGTACACTCCAGGTGGAGAAGTGAGGAATGACGATGTTGACCAAAGCCTAATT
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GTCAACAGAGCAAATCAGCGGTTGAACCCCCTGCACTAGCTTTAAGGCATTTCAGAAA
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GGTCTGAGTCGGTTTGGTCAATACTTATCAATGGATCATCAGAAATTGGGAAGCTGTC
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Figure 4

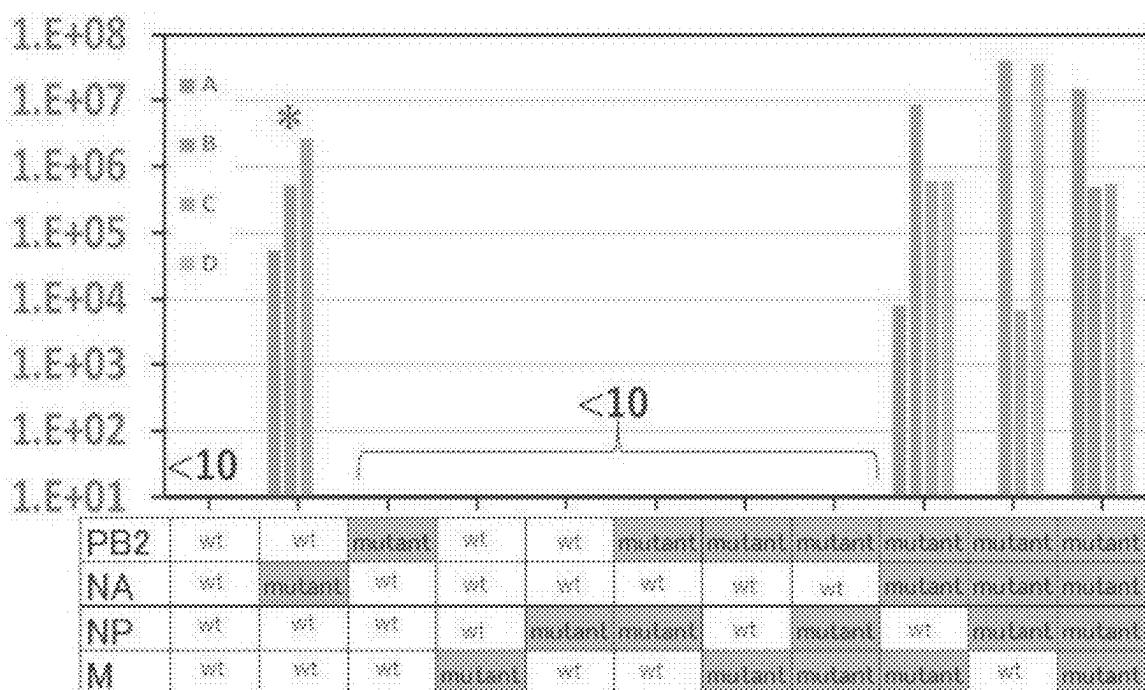


Figure 5

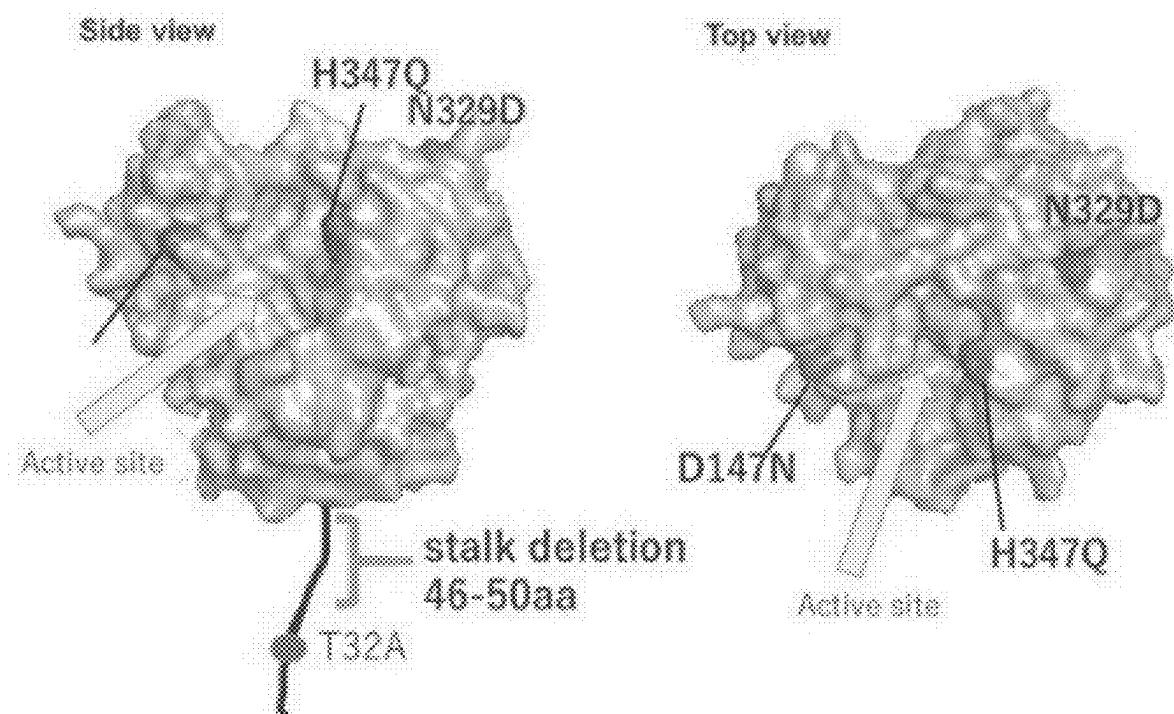


Figure 6

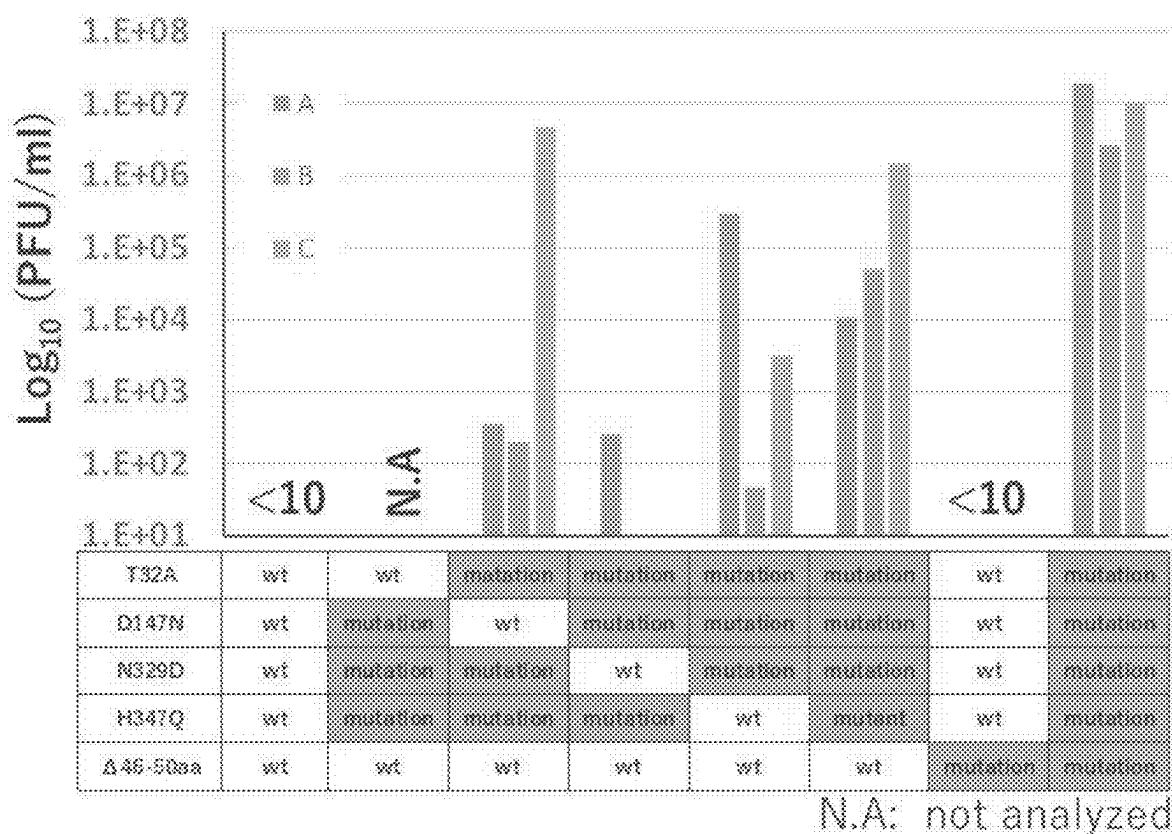
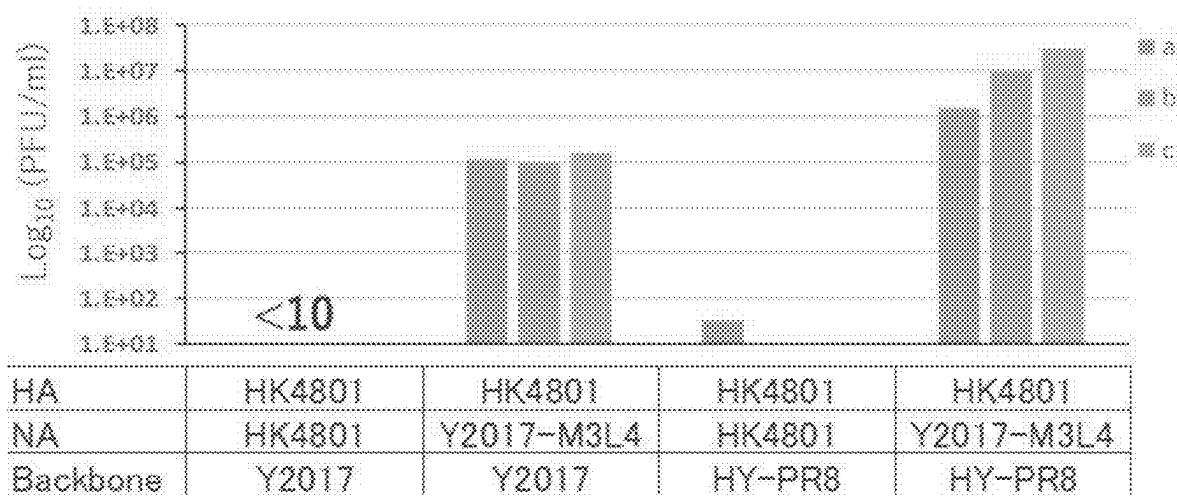


Figure 7



Yokohama/2017/2003 NA

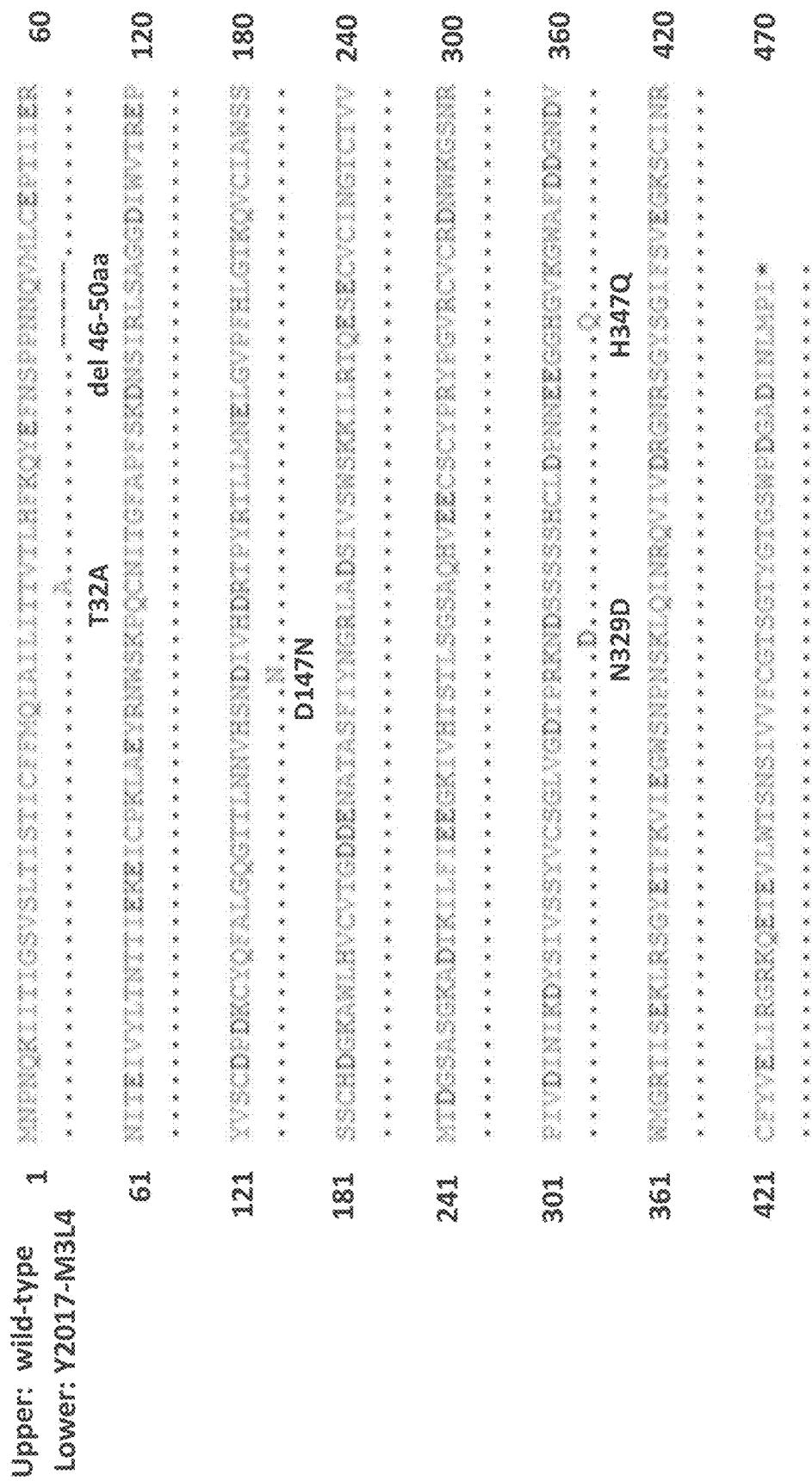


Figure 8

Figure 9

N3 (Accession No. AAO62039.1)

1 mnpngkiiti gvvnttisti alligvgnli fntvihekig dhqtvihttt ttpaipncsd
61 tiitynnntvi nnittiatea erifkplpli cpfrqffpfh kdnairlgen kdvivtrepy
121 vscndndncws falaaggaliq tkhengtikd rtpyrsliqf pigtapvign ykeiciawss
181 sscfdgkewm hvcmtnndnd asaqiyagr mtdsikawkr dilrtqesec qcldgtcvva
241 vtdgpaansa dhrvywireg rivkyenvpk tkighleecs cyvdidvyci crdnwkgsnr
301 pwmrinneti letgyvcskf hsdtprpadp stvscdspsn vngpgvkgl qfkvgndvwl
361 grtmstsgs gfeiikvaeg winsphaks vtqtlvsnd wsgysgsfiv ktkacfqpcf
421 yvelirgrpn knddvswtsn sivfcldn epgsgnwpdg snigfnpk (SEQ ID NO:30)

N4 (Accession No. AAO62043.1)

1 mnpngkiiti gsvsiillti glliqitslc siwfshynqv tqtheqpcsn nttnyyneft
61 vnytnvqnny ttviepsapd vvhysgrdl cpirgwapis kdnigirigsr gevfvirepf
121 iscsisecrt ffltgalln dkhsngtvkd rspftrilm sc pigvapspsn srfevawsa
181 tacsdgpjwi tlgitgpdat avavikyngi itdtlkswkg nimrtqesec vcqdefcytl
241 itdgpsdaqa fykilkirkg kivsmkdvdta tgfhfeecsc ypsgtdiecv crdnwrgsnr
301 pwirfnasdld yqigiyvesgi fgdnprpvdg tgscnspvnn gkqrgygvkgl sfrygdgvwi
361 grtkslasrs qfemvwdang wvstdkdsng vqdiidndnw sgysqfsfir gettgrnctv
421 pcfvwemirg qpkektiwts gssiafcgvn sdttgswpd gallpdfidk (SEQ ID NO:31)

N6 (Accession No. AAO62070.1)

1 mnpngkiici satgmtlsvv slligianlg lnighykmg dtpdvnipnm netnstattii
61 nnhtqnnftn ithniivnkne egtflnlthp lcevnswhil skdnairige dahilvtrep
121 ylscdpqgcr mfalsqqgtl rgrhangtih drspfralis wemqapspv nvrvecigws
181 stschdgisr msicmsgann nasavwygg rpvteipswa gnirltqese cvchkgicpv
241 vmtdgpannr aatkiiyfke gkiqkieela gntqhieecs cygavgvikc icrdnwkgan
301 rpvitidpem mthtkskylcs kiltdtsrpn dptngncdap itggspdpgv kgfafldren
361 swlgrtiskd srsgyemlkv pnaetdtqsg pishqvivoo quwsgysgaf idywankecf
421 npcfyveir grpkevvliw tsnsivalcg skerlgswws hdgaciyyf (SEQ ID NO:32)

N7 (Accession No. AIK26357.1)

1 mnpngklfal sgvaialsil nlligisnvg lnvslhlkgs sdqdknwtct svtqnnttli
61 entyvnnttv idketgtakp nymlinkslic kvegwwvak dnairfgese qiiivtrepyv
121 scdplgckmy alhqggttira khngtihdr tafrglistp lgsppvvsns dflcvwgssst
181 schdgigrmt icvqgnndna tatvyydrri tttktwagn ilrtqesecv chngtcvvim
241 tdgsasssqay tkvlyfhkgl vikeealkgs arhieecscy ghnskvtcvc rdnwqganrp
301 vieidmname htsgyictgv ltdtsrpsdk smgdcnnpit gspgapgvkg fgfldssntw
361 lgrtisprsr sgfemalkipn aetdpnskit ergeivdnrrn wsgysgsfid ywdessecyn

Figure 9 cont.

421 pcfyvelirg rpeeakyvgw tsnsliaalc spisvsgsgsf pdgaqiqyfs (SEQ ID NO:33)

N8 (Accession No. AIK26315.1)

1 mnpnqkiity gsvslqlvvvi nillhivsit vtvivilpgng nnknncnetvi reynetvrie
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121 scsptecrtf fitqgsilnd khsngtvkdr spyrtimsve igqspnvyqa rfeavawsat
181 achdgkkwmt igvtgpaka vavvhyggip tdvinswagd iirtqessct cijgecywvm
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301 vlvispdlsy ragylcaglp sdtprgedsq ftgsctspvg nqgygvkgfg frqgndvwmmg
361 rtisrtsrsg feilkvrngw vqnskeqikr qvvvdnlkwq qysqsfslpv eltkrnclvp
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N9 (Accession No. ALH21371)

1 mnpnqkilct sataiiigai avligiaolg lniglblkpg cnchshsqpet tntsequinn
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121 dpdeacrlyal sqggttirgkh snqtihdrsq yraliswpls spptvynsr ecigwsstsc
181 hdgksrmsic isgpnnnasa vvwynrrpvt eintwarni rtqesecvch ngvcvvftd
241 gsatgpadtr iyyfkegkli kwesitgtak hieecscyge rtgitctcrd nwqgsnrpvi
301 qidpvamtht eqyicspvit dprpndpni gkcnndpypgn nnngvkgfsy ldgantwlgr
361 tistasrsgy emlkvpnalt ddrskpiqqq tivlnadwsg ysgsfimdywa egdcyracfy
421 velirgrpkedkywwisnsi vsmcsstei gqwnwpdgak icyfl (SEQ ID NO:35)

Figure 10

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HADLSAKEAQDVIMEVVFPNEVGARILTSESQLTITKEK
KEELQDCKISPLMVAYMLERELVRKTRFLPVAGGTSSV
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Figure 10 cont.

EQAVDICKAAMGLRISSSFSGGTFKRTSGSSVKREEE
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NTRANQRLNPMHQLLRFQDKDAKVLFQNWGVEPIDNVM
GMIGILPDMDTPSIEMSMRGVRISKMGVDEYSSTERVVV
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Figure 10 cont.

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NO:40) which encodes

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Figure 10 cont.

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agggacaatc tggAACCTGG gacctttgtt ctggggggcc tatatgaagc aattgaggag
tgcctaatta atgatccctg gtttttgtt aatgtttctt gttcaactc ctcccttaca
catgcattga gttagttgtg gcaatgttac tatgttcttccatactgtc caaaaaaaaat
ccttgtttctt act (SEQ ID NO:41) which encodes

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RFIEIGVTRREVHIYYLEKANKIKSEKTHIHIFSFTGEEM
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SERGEETIEERFEITGTMRKLA DQSLPPNFSSLNFRAY
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CLINDPWVLLNASWFNSFLTHALS

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agagcatccg tcggaaaaat gatgggtgca attggacgt totacatcca aatgtgcaca
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Figure 10 cont.

MASQGTKRSYEQMETDGERQNATEIRASVGKMIIGGIGRFYIQMCTELKLSDYEGRLIQNSLTIERMVLASFDERRNKYLEEHPSAGKDPKKTGGPIYRRVNGKWMRELILYDKEEIRRIWRQANNGDDATAGLTHMMIWHSNLNDATYQRTRALVRTGMDPRMCSDLMQGSTLPRRSGAAGAAVKGVGTMVMELVRMIKRGINDRNFWRGENGRKTRIAYERMNCNILKGKFQTAAQKAMMDQVRESRNPGNNAEFEDLTFLARSALILRGSVAHKSCLPACVYGPAVASGYDFEREGYSLVGIDPFRLLQNSQVYSLIRPNENPAHKSQLVWMACHSAAFEDELRLSFIKGTKVVPRGKLSTRGVQIASNENMETMESSTLELRSRYWAIRTRSGGNTNQQRASAGQISIQPTFSVQRNLPFDRTTVMAAFTGNTEGRTSMDRTEIIRM MESARPEDVSFQGRGVFELSDEKAASPIVPSFDMSNEGSYFFGDNAEELYDN

ggcaaaagca ggttagatatt gaaaaga:gag tctttctaacc gaggtcgaaa cgtacgttc
ctcttatcatc cggcaggcc ccctcaaaagc cgagatcgca cagagacttg aagatgttt
tgcagggaaag aacaccgatc ttggaggttt catgaaatgg ctaaagacaa gaccaatcct
gtcacctctg actaagggya ttttaggatt tgttttcacg ctcacccgtgc ccagtggcgg
aggactgcag cgttagacgtt ttgtccaaaa tgccttaat gggAACGGGG atccaaataaa
catggacaaa gcagttaaac tgtataggas gctcaagagg gagataaacat tccatggggc
caaagaaaatc tcactcagtt atttgtctgg tycacttgcc agtgtatgg gcttcaatata
caacaggatg ggggtgtga ccactgaagt ggcatttggc ctggatgtg caacctgtgt
acagattgtt gactcccago atccggtctca taggcaaatg gtgacaacaa ccaacccact
aatcagacat gaaaacagaa gggtggatcg agtgagcaag tgggttttgc cagcaactaca gctaaaggctt
gggtgcagcg atgaaaacca tcttttttgcagg aatttgcagg cctatcaagaa acgaatgggg
gtgatctctc cgctattgcc gcaaatatca ttgggatctt gcaacttgata ttgttgtttc
ttgtatgttttgcatttacq gtcgccttiaa atacccqactq aaaggagggc

Figure 10 cont.

cttctacggaa aggagtgccta aagtctatga gggaaagaata tcgaaaaggaa cagcagagtg
ctgtggatgc tgaccgtatgt cattttgtca gcatagagct ggagtaaaaaa actaacattgt
ttctact (SEQ ID NO:43) which encodes

MSLLTEVETYVLSIIPSGPLKAEIAQRLEDVFAGKNTDL
EVLMEWLKTRPILSPLTKGILGFVFTLTVPSERGLQRRR
FVQNALNGNDPNNMDKAVKLYRKLKREITFHGAKEIS
LSYSAGALASCMGLIYNRMGAVTTEVAFGLVCATCEQI
ADSQHRSHRQMVTTNPLIRHENRMVLASTTAKAMEQ
MAGSSEQAAEAMEVASQARQMVKQAMRTIGTHPSSSA
LKNDLLENLQAYQKRMGVQMQRFK

agcaaaaagca gggtgacaaa gacataatgg atccaaacac tgbgtcaagg tttaggttag
attgtttctt ttggcatgtc cycaaacqag ttgcagacca agaacttagt gatgccccat 60
tccttgcatacg gcttcgcga gatcagaat ccctaagagg aagggggcagc actcttggc 120
tggacatcga gacagccaca cgtgctgaa agcagatgt ggagcggatt ctgaaaagaag 180
aatccatgtga ggcacttaaa atgaccatgg cctctgtacc tgctgcgt tacctaaccg 240
acatgactctt gagggaaatg tcaaggaaat ggtccatgtc catacccaag cagaaaagtgg 300
caggcccctctt ttgtatcaga atggaccagg cgatcatgaa taaaaacatc atactgaaag 360
cgaacttcag tgbgattttt gaccggctgg agactctaaat attgtctaagg gctttcacccg 420
aagagggagc aattgttggc gaaaatttcac cattgccttc tottccagga catabctgtg 480
aggatgtcaa aaatgcagtt ggagtcctca tggaggact tgaatggaaat gataacacag 540
tttcgagtc tggaaactcta cagagattog ctggagaag cagtaatgg aatggggagac 600
ctccactcac tccaaaacag aaacgagaaa tggcgggaaac aattaggta caagtttgsa 660
gaaataagat qgttgtattga agaagtgaga cacaaactga aggttaacaga gaatagttt 720
gagcaastaa catttatgca agccttacat ctattgcttgg aagtggagca agagataaga 780
actttctcat ttoagottat
tttataataaa aaaaacaccct
tgtttctact
(SEQ ID NO:44)

Figure 11

N1

1 mnnpnqkiiti gsvcmtíigma nliiqignii siwihsiql gnqnqietcn qsvityennt
61 wvnqtyvnis ntnfaaggsv vsvklagnss lcpvsgwaiy skdnsvrigs kgdvvirep
121 fiscsplecr tffltqgall ndkhsngtik drspyrtilms cpigevpspy nsrfesvaws
181 asachdginw ltigisqpdn gavavlkyng iitdtikswr nnlirtqese cacvngscft
241 vmtdgpsnqq asykifriek gkivksvemn apnyhyeeecs cypdsseitc vcrdnwhgsn
301 rpwvfnqnli eyqiqyicsg ifgdnprpnd ktgscgpvss ngangvkqfs fkyqngvwig
361 rtksissrng femiwdpngw tgtdmnfsik qdivginews gysgsfvqhp eltglcdcirp
421 cfwvelirgr pkentiwtsg ssisfcgvns dtvgwswpdg aelpftidk

N7

1 mnnpnqkifal sgvaialsil nlligisnvg lnvslhlkgs sdqdknwtct svtqnntlli
61 entyvnnttv idketgtakp nyimlinksic kvegwvvvak dnairfgece qiivtrepvyv
121 scdplicckmy alhqggttirn khsngtihdr tafrglistp lqspvvvsns dflcvgwst
181 schdgigrmt icvqgnndna tatvyydrri tttixtwagn ilrtqesecv chngtcvvim
241 tdgsassqay tkvlyfhkgl vikeealkgs arhieecscy ghnskvtcvc rdnwqganrp
301 vieidmname htsqylctgv ltdtsrpsdk smgdccnnpit gspgapgvkg fgfldssntw
361 lgrtisprsr sgfemlkpn aetdpnskit erqeivdnnn wsgysgsfid ywdessecyn
421 pcfyvelirg rpeeakyvgw tsnslialcg spisvgsgsf pdgaqiqyfs

N9

mnnpnqkilict sataiiigai avligianlg lnighhlkpg cncshsqpet tntsqtiiinn
61 yynetnitni qmeertsrnf nnltkglcti nswhiygkdn avrigessdv lvtrepvyvsc
121 dpdecrfyal sqggtirgkh sngtihdrsq yraliswpis spptvynsrv ecigwsstsc
181 hdgksrmsic isgpnnnasa vvwynrrpva eintwarnil rtqesecvch ngvcpvvftd
241 gsatgpadtr iyyfkegkil kwesltgtak hieecscyge rtgitctcd nwqgsnrpvi
301 qidpvamtht sqyicspvlt dnprpndpni gkcndpypgn nnngvkqfsy ldgantwlgr
361 tistasrsgy emlkvpnalt ddrskpiqqq tivlnadwsg ysgsfmdywa egdcyracfy
421 velirgrpke dkvwntsni vsmcsstei gqwnwpdgak ieyfl

N2

1 mnnpnqkiiti gsvsltisti cffmqialli ttvthfkqy efnsppnnqv mlceptiier
61 niteivyltn ttiekeicpk laeyrnwskp qcniitgfapf skdnsirlsa ggdiwvtrep
121 yvsccdpdkcy qfalgggttl nnvhhsndivh drtpyrtlim nelgvpfhlg tkqvciaawss
181 sschdgkawl hvcttgddens atasfiyngr ladsivswsk kilrtqesec vcingtctvv
241 mtdgsasgka dtkilfieeg kivhtstlsg saqhveecsc yprypgvrcv crdnwkgsnr

Figure 11 cont.

301 pivdinikdy sivssyvcsg lvgdtpknd sssshcldp nneegghgvk gwafddgndv
361 wmgrtisekl rsgyetfkvi egwsnpnskl qinrqvivdr qnrsgysgif svegkscinr
421 cfyvelirgr kqetevlwts nsivvfctgs gtytgswpd gadinlmpi

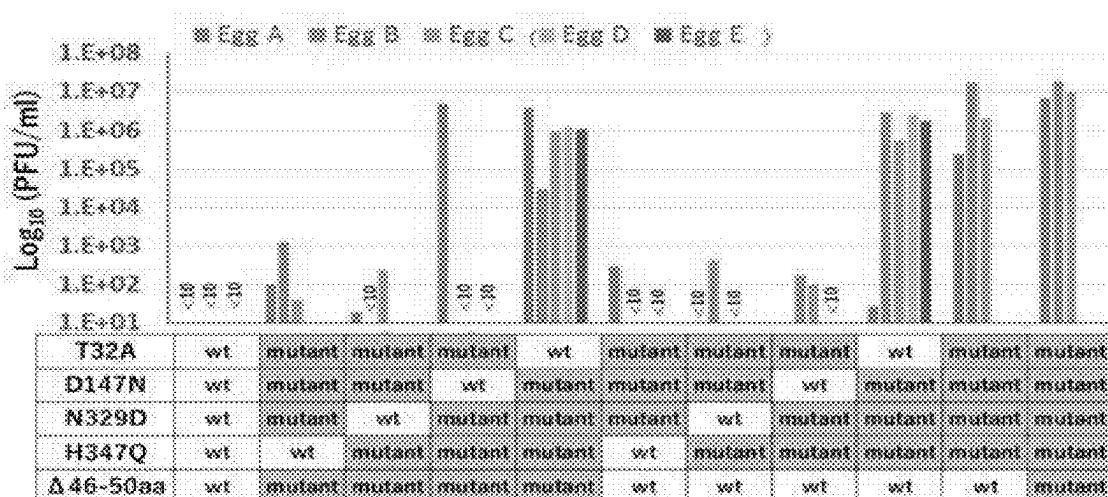


Figure 12

Passage 1			Passage 2			Passage 3		
egg	virus titer (pfu/ml)	HA mutation	egg	virus titer (pfu/ml)	HA mutation	egg	virus titer (pfu/ml)	HA mutation
A	2.6x10 ⁸	none	A1	6.6x10 ⁷	none	A1a	5.3x10 ⁷	none
			A2	3.5x10 ⁷	none	A1b	1.2x10 ⁸	none
			A3	2.8x10 ⁷	none	A1c	3.7x10 ⁷	none
B	3.7x10 ⁷	none	B1	1.15x10 ⁸	none	B2a	5.8x10 ⁷	none
			B2	4.85x10 ⁷	none	B2b	1.0x10 ⁸	none
			C1	2.65x10 ⁷	none	B3a	3.0x10 ⁷	none
C	9.0x10 ⁶	none	C2	6.45x10 ⁷	none	B3b	5.5x10 ⁷	none
			C3	1.6x10 ⁸	none	C2a	3.8x10 ⁷	none
						C2b	3.4x10 ⁸	none
						C3a	3.9x10 ⁷	none
						C3b	3.9x10 ⁷	none

Figure 13

AM: amniotic cavity		AM1		AM1A1		AM1A2		AM1A3		AM1A4		AM1A5		
		Bter	mutation	Bter	mutation	Bter	mutation	Bter	mutation	Bter	mutation	Bter	mutation	
phage	HA	HA	phage	HA	HA	phage	HA	HA	phage	HA	HA	phage	HA	HA
AI: allantoic cavity												a	11×10^7	none
												b	26×10^7	none
												c	32×10^7	none
												d	11×10^7	none
Virus generated by reverse genetics												b	11×10^7	none
MA: A/HK/4801/2014												a	75×10^7	none
MA: A/HK/4801/2014MA(T148K, N329X, 347X)												b	18×10^7	none
backbone: High Yield-PR8												c	16×10^7	none
												d	13×10^7	none
Hier: 4×10^7 (pfu/ml)		1×10^7	ad	ad	3×10^7	none	3×10^7					a	25×10^7	none
												b	13×10^7	none
												c	13×10^7	none
												d	18×10^7	none
													32×10^7	none
													80×10^7	none
												b	17×10^7	none
												c	59×10^7	none
												d	27×10^7	none

Figure 14

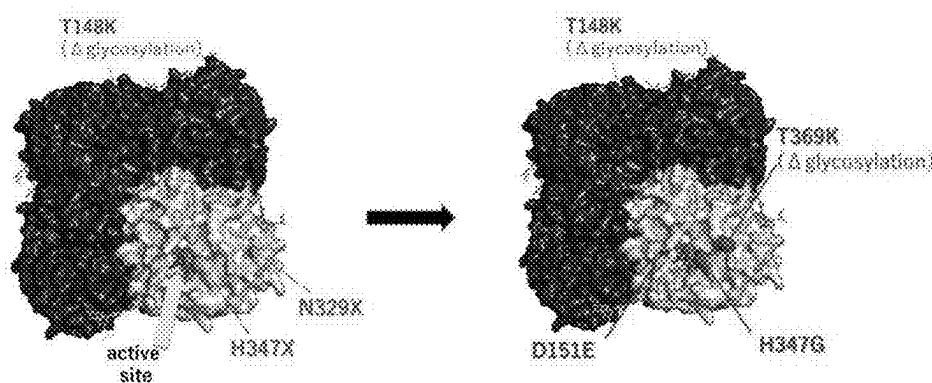
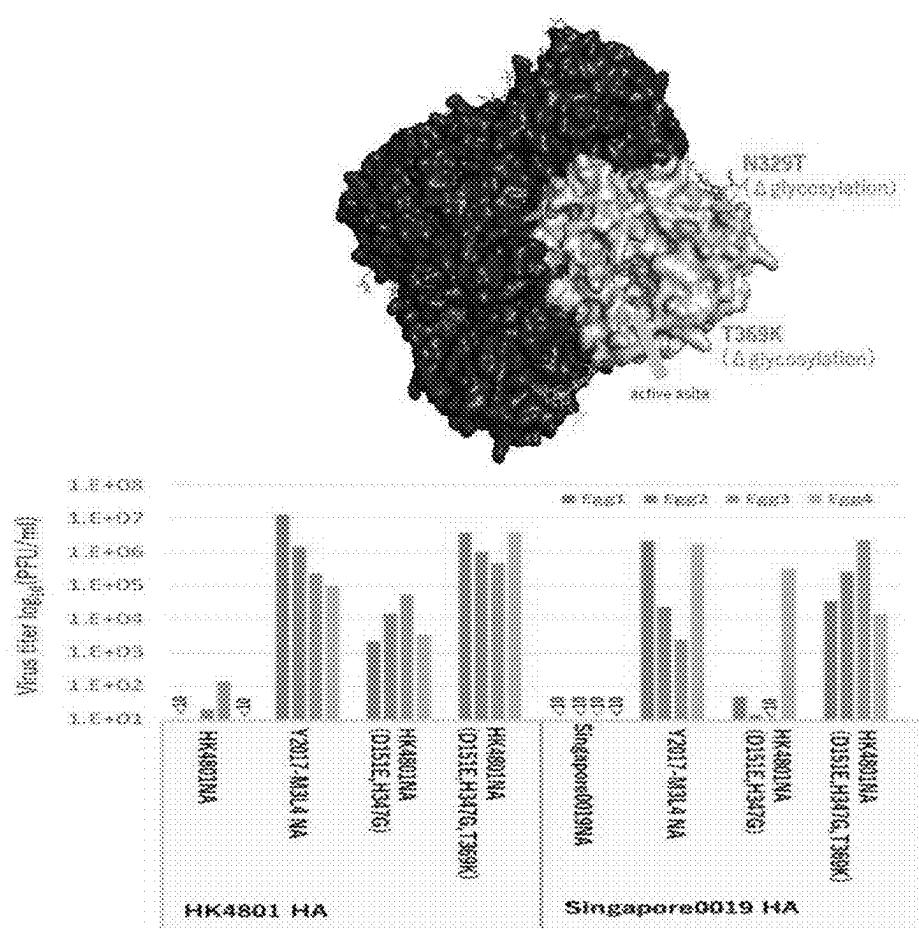


Figure 15



Figures 16 and 17

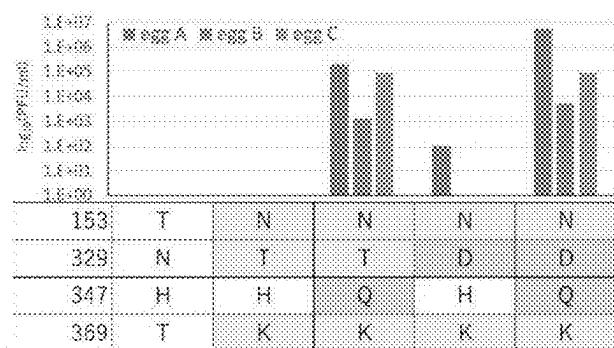


Figure 18

	Passage 1			Passage 2			Passage 3		
	virus titer (pfu/ml)	HA mutation	egg	virus titer (pfu/ml)	HA mutation	egg	virus titer (pfu/ml)	HA mutation	
A	2.6x10 ⁶	none	A1	6.6x10 ⁶	none		A1a	5.3x10 ⁷	none
			A2	3.5x10 ⁷	none		A1b	1.2x10 ⁸	none
			A3	2.8x10 ⁷	none		A1c	3.7x10 ⁷	none
	3.7x10 ⁷	none	B1	1.15x10 ⁸	none		A2a	5.8x10 ⁷	none
			B2	4.85x10 ⁷	none		A2b	1.0x10 ⁸	none
			C1	2.65x10 ⁷	none		A3a	3.0x10 ⁷	none
	9.0x10 ⁵	none	C2	6.45x10 ⁷	none		A3b	5.5x10 ⁷	none
			C3	1.6x10 ⁶	none		B1a	4.3x10 ⁶	none
							B1b	1.6x10 ⁸	none
							B2a	2.1x10 ⁷	none
							B2b	4.3x10 ⁷	none
							C1a	5.3x10 ⁷	none
							C1b	9.3x10 ⁶	none
							C2a	3.8x10 ⁷	none
							C3a	3.4x10 ⁸	none
							C3b	3.9x10 ⁸	none

Fig. 19

Fig. 20

Figure 21.

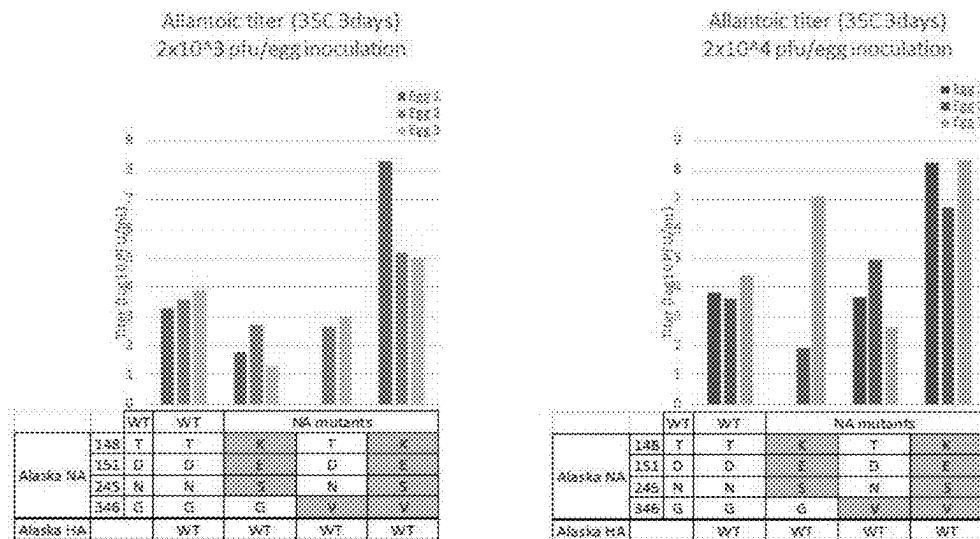
	Passage	Inoculation			Egg 1			Egg 2			Egg 3		
		WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT
K189E/N158K/A212T	Harvested	4.3	2.6	0.2	1.3	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
	Inoculation	6.0	5.0	5.0	5.0	5.0	5.0	4.0	4.0	4.0	4.0	4.0	4.0
P5	Harvested	8.5	4.0	0.5	4.0	3.0	3.0	2.2	2.2	2.2	1.8	1.8	1.8
	Inoculation	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0
P6	Harvested	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0
	Inoculation	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0
P7	Harvested	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0
	Inoculation	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0
P8	Harvested	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0
	Inoculation	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0
P9	Harvested	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0
	Inoculation	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0
P10	Harvested	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0
	Inoculation	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0
P11	Harvested	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0
	Inoculation	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0

Inoculation <- Titer (\log_{10} PFU/egg)
Egg 1|Egg 2|Egg 3 <- Titer (\log_{10} PFU/ml)

Figure 22. HA/NA mutations (HA-K189E/N158K/A212T mutant virus)

	Passage	HA		NA				
		149	151	235	346	N	S	G
K189E/N158K/A212T	E4	No mutation	K	E	H			
	E6	No mutation	K	E	S	V		
	E7	No mutation	K	E	S	V		
	E10	No mutation	K	E	S	V		

Figure 23.



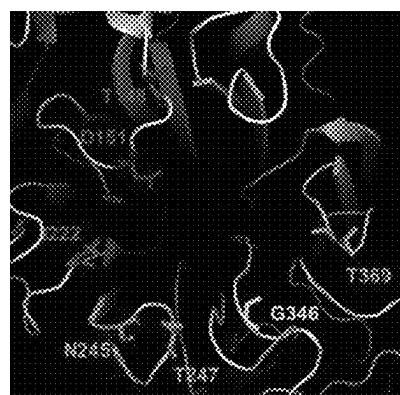


Figure 24.

**RECOMBINANT INFLUENZA VIRUSES
WITH STABILIZED HA FOR REPLICATION
IN EGGS**

**CROSS-REFERENCE TO RELATED
APPLICATIONS**

[0001] This application is a continuation of U.S. application Ser. No. 17/546,967, filed Dec. 9, 2021, which is a continuation of U.S. application Ser. No. 16/170,321, filed Oct. 25, 2018, which application claims the benefit of the filing date of U.S. application Ser. No. 62/577,049, filed on Oct. 25, 2017, and U.S. application Ser. No. 62/633,400, filed on Feb. 21, 2018, the disclosures of which are incorporated by reference herein.

STATEMENT OF GOVERNMENT FUNDING

[0002] This invention was made with government support under HHSO100201500033C awarded by the Biomedical Advanced Research and Development Authority. The government has certain rights in the invention.

**INCORPORATION BY REFERENCE OF
SEQUENCE LISTING**

[0003] This application contains a Sequence Listing which has been submitted electronically in ST26 format and hereby incorporated by reference in its entirety. Said ST26 file, created on Feb. 26, 2025, is named 800101US3.xml and is 121,962 byte in size

BACKGROUND

[0004] Influenza is a major respiratory disease in some mammals including horses and is responsible for substantial morbidity and economic losses each year. In addition, influenza virus infections can cause severe systemic disease in some avian species, leading to death. The segmented nature of the influenza virus genome allows for reassortment of segments during virus replication in cells infected with two or more influenza viruses. The reassortment of segments, combined with genetic mutation and drift, can give rise to a myriad of divergent strains of influenza virus over time. The new strains exhibit antigenic variation in their hemagglutinin (HA) and/or neuraminidase (NA) proteins, and in particular the gene coding for the HA protein has a high rate of variability. The predominant current practice for the prevention of flu is vaccination. Most commonly, inactivated virus vaccines are used. 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.

[0005] There are four general types of influenza viruses, Type A, Type B, Type C and Type D, 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. All the known HA and NA subtypes (H1 to H18 and N1 to N11) have been isolated from aquatic birds, which are thought to act as a natural reservoir for influenza.

[0006] Most influenza vaccines are produced in embryonated chicken eggs. However, the WHO-recommended influenza vaccine strains often do not replicate efficiently in embryonated chicken eggs, requiring serial passages in eggs in order to allow for adaptation of the virus. During adaptation and amplification in eggs, the hemagglutinin (HA) protein of influenza viruses often acquires egg-adapting mutations. These egg-adapting mutations in HA often alter the antigenicity of the viruses, resulting in vaccine viruses that are no longer optimally matched to the circulating virus strains.

SUMMARY

[0007] As described herein, an influenza virus was passaged 7 times in eggs (in triplicate) to study the mutations that occurred in the 6 non-immunogenic viral segments during adaptation. Surprisingly, the virus acquired no HA mutations and instead had mutations in the NA, PB2, NP, and M1 proteins. The NA mutations were identical in all three experiments, and they included a deletion and 4 amino acid mutations. The NA mutations were tested alone and it was found that they, e.g., alone or in various combinations, were responsible for the effect, which permitted efficient growth in eggs without HA mutations.

[0008] The present disclosure thus relates to influenza mutations that prevent the acquisition of antigenicity-compromising mutations in the hemagglutinin (HA) segment of influenza virus during growth in eggs. The mutations in the neuraminidase (NA) protein of human influenza viruses were found to ‘stabilize’ the HA during egg-passages, e.g., in the presence of the mutations in NA, the HA protein did not acquire egg-adapting mutations. Those NA mutations may also increase the vaccine virus yield.

[0009] The disclosure provides isolated recombinant, e.g., reassortant, influenza viruses with selected amino acid residues or deletions at specified positions in NA. In one embodiment, the NA is selected to not encode a threonine at residue 32. In one embodiment, the NA is selected to not encode an aspartic acid at position 147. In one embodiment, the NA is selected to not encode an asparagine at residue 329. In one embodiment, the NA is selected to not encode a threonine at residue 329. In one embodiment, the NA is selected to not encode a histidine at residue 347. In one embodiment, the NA is selected to not encode an arginine or an asparagine at residue 347. In one embodiment, the NA is selected to not encode a NA having a threonine at position 148. In one embodiment, the NA is selected to not encode a NA having an aspartic acid at position 151. In one embodiment, the NA is selected to not encode a NA having an asparagine at position 245. In one embodiment, the NA is selected to not encode a NA having a glycine at position 346. In one embodiment, the NA is selected to have a deletion of one or more of residues 46 to 50. The numbering for NA is based on N2. In one embodiment, the disclosure provides an isolated recombinant reassortant influenza virus having six “internal” viral segments from a vaccine influenza virus, e.g., PR8UW, a NA viral segment with one or more of the specified residues at particular positions or a deletion of specified residues, or any combination thereof, and a HA viral segment, e.g., any of H1-H18, e.g., from a circulating influenza virus. Also provided are compositions comprising the recombinant influenza virus, pharmaceutical compositions such as vaccines.

[0010] Thus, for vaccine viruses that are to be grown or passaged in cells, e.g., in eggs, replacement of the residue at position 32, 147, 329, 347, or a deletion of one or more of residues 46 to 50, or any combination thereof, in NA, e.g., by mutation, or selection of a NA viral segment for a NA to not encode a threonine at residue 32, to not encode an aspartic acid at position 147, to not encode an asparagine at residue 329, to not encode a histidine at residue 347, or to have a deletion of one or more of residues 46 to 50, or any combination thereof, wherein the numbering is based on N2, may result in stabilization of HA and/or higher viral titers. In one embodiment, for vaccine viruses that are to be grown or passaged in cells, e.g., in eggs, replacement of the residue at position 147, 329, 347, or a deletion of one or more of residues 46 to 50, or any combination thereof, in NA, e.g., by mutation, or selection of a NA viral segment for a NA to not encode an aspartic acid at position 147, to not encode an asparagine at residue 329, to not encode a histidine at residue 347, 369, or any combination thereof, or optionally not encode a threonine at residue 369, or any combination thereof, wherein the numbering is based on N2, may result in stabilization of HA and/or higher viral titers. In one embodiment, for vaccine viruses that are to be grown or passaged in cells, e.g., in eggs, replacement of the residue at position 148, 151, 245, 346, or any combination thereof, in NA, e.g., by mutation, or selection of a NA viral segment for a NA to not encode a threonine at residue 148, to not encode an aspartic acid at position 151, to not encode an asparagine at residue 245, to not encode a glycine at residue 346, or any combination thereof, wherein the numbering is based on N2, may result in stabilization of HA and/or higher viral titers. In one embodiment, for vaccine viruses that are to be grown or passaged in cells, e.g., in eggs, replacement of the residue at position 148, 151, 347, or any combination thereof, in NA, e.g., by mutation, or selection of a NA viral segment for a NA to not encode a threonine at residue 148, to not encode an aspartic acid at position 151, to not encode a histidine at residue 347, or any combination thereof, wherein the numbering is based on N2, may result in stabilization of HA and/or higher viral titers.

[0011] In one embodiment, the disclosure provides an isolated recombinant influenza virus comprising PA, PB1, PB2, NP, NS, M, and HA viral segments and a NA viral segment that encodes an NA selected to not encode a threonine at residue 32, to not encode an aspartic acid at position 147, to not encode an asparagine at residue 329, to not encode a histidine at residue 347, or to have a deletion of one or more of residues 46 to 50, or any combination thereof, wherein the numbering is based on N2, wherein the recombinant influenza virus has enhanced replication in avian eggs or has a reduction in HA mutations when grown in avian eggs relative to a corresponding influenza virus that has a NA that encodes a threonine at residue 32, does not have a deletion of residues 46 or 50, encodes an aspartic acid at position 147, encodes an asparagine at residue 329, encodes a histidine at residue 347, or any combination thereof. In one embodiment, the disclosure provides an isolated recombinant influenza virus comprising PA, PB1, PB2, NP, NS, M, and HA viral segments and a NA viral segment that encodes an NA selected to not encode a threonine at residue 148, to not encode an aspartic acid at position 151, to not encode an asparagine at residue 245, to not encode a glycine at residue 346, to not encode a histidine at residue 347, or any combination thereof, wherein the

numbering is based on N2, wherein the recombinant influenza virus has enhanced replication in avian eggs or has a reduction in HA mutations when grown in avian eggs relative to a corresponding influenza virus that has a NA that encodes a threonine at residue 148, encodes an aspartic acid at position 151, encodes an asparagine at residue 245, encodes a glycine at residue 346, encodes a histidine at residue 347, or any combination thereof. In one embodiment, the isolated recombinant influenza virus is a reassortant. In one embodiment, the NA viral segment encodes a NA that has at least 80%, 85%, 90%, 95%, or 99% amino acid sequence identity to any one of SEQ ID Nos. 1-3, 30-38, 48-50, or 54. In one embodiment, the NA viral segment encodes a NA that has less than 100% amino acid sequence identity to SEQ ID NO:2 or SEQ ID NO:3. In one embodiment, the NA viral segment encodes a N2, N3, N7, or N9 and the positions in N3, N7, or N9 with the specified residue(s) correspond to the specified positions in N2. In one embodiment, the NA viral segment encodes a N1, N4, N5, N6, N8, N10 or N11 and the positions in N1, N4, N5, N6, N8, N10 or N11 with the specified residue(s) correspond to the specified positions in N2. In one embodiment, the residue at position 32 is A, I, G, or L. In one embodiment, the deletion is a deletion of residues 46 to 50. In one embodiment, the residue at position 147 is N or Q. In one embodiment, the residue at position 329 is D or E. In one embodiment, the residue at position 347 is Q, N, S, T, Y, C or W. In one embodiment, the HA is H1, H3, H5, H7, or H9. In one embodiment, the virus is an influenza A virus. In one embodiment, the PA, PB1, PB2, NP, M, and NS viral segments have at least 85% nucleic acid sequence identity to SEQ ID Nos. 24 to 29 or encode a polypeptide having at least 80%, 85%, 90%, 95%, or 99% amino acid sequence identity to a polypeptide encoded by SEQ ID Nos. 24 to 29 or 39-44. In one embodiment, the PB2 has I, A, L, or G at residue 147. In one embodiment, the virus is an influenza B virus.

[0012] Further provided is an isolated recombinant nucleic acid, e.g., a vector such as a viral vector, comprising a nucleic acid sequence that encodes an influenza virus NA selected to not encode a threonine at residue 32, to have a deletion of one or more of residues 46-50, to not encode an aspartic acid at position 147, to not encode an asparagine at residue 329, or to not encode a histidine at residue 347, or any combination thereof, wherein the numbering is based on N2. In one embodiment, the isolated recombinant nucleic acid does not encode a threonine at residue 148, to not encode an aspartic acid at position 151, to not encode an asparagine at residue 245, to not encode a glycine at residue 346, or any combination thereof. In one embodiment, the NA has at least 95% amino acid sequence identity to SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:48, or SEQ ID NO:49. In one embodiment, the NA has less than 100% amino acid sequence identity to SEQ ID NO:2 or SEQ ID NO:3. In one embodiment, the NA is a N2, N3, N7, or N9. In one embodiment, the NA is a N1, N4, N5, N6, N8, N10 or N11. In one embodiment, the residue at position 32 is A, I, G, or L. In one embodiment, the deletion is a deletion of residues 46 to 50. In one embodiment, the residue at position 147 is N or Q. In one embodiment, the residue at position 329 is D or E. In one embodiment, the residue at position 347 is Q, N, S, T, Y, C or W. In one embodiment, the residue at position 148 is K, R or H. In one embodiment, the residue

at position 151 is E, N or Q. In one embodiment, the residue at position 245 is S, T, I, L, A, N, or V.

[0013] Also provided is a method to prepare influenza virus. The method includes contacting a cell with: a vector for vRNA production comprising a promoter operably linked to an influenza virus PA DNA linked to a transcription termination sequence, a vector for vRNA production comprising a promoter operably linked to an influenza virus PB1 DNA linked to a transcription termination sequence, a vector for vRNA production comprising a promoter operably linked to an influenza virus PB2 DNA linked to a transcription termination sequence, a vector for vRNA production comprising a promoter operably linked to an influenza virus HA DNA linked to a transcription termination sequence, a vector for vRNA production comprising a promoter operably linked to an influenza virus NP DNA linked to a transcription termination sequence, a vector for vRNA production comprising a promoter operably linked to an influenza virus NA DNA linked to a transcription termination sequence, a vector for vRNA production comprising a promoter operably linked to an influenza virus M DNA linked to a transcription termination sequence, and a vector for vRNA production comprising a promoter operably linked to an influenza virus NS DNA linked to a transcription termination sequence, wherein the PB1, PB2, PA, NP, NS, and M DNAs in the vectors for vRNA production are from one or more influenza vaccine virus isolates, wherein the NA DNA in the vector for vRNA production encodes an NA selected to not encode a threonine at residue 32, to not encode an aspartic acid at position 147, to not encode an asparagine at residue 329, to not encode a histidine at residue 347, to not encode a threonine at residue 148, to not encode an aspartic acid at position 151, to not encode an asparagine at residue 245, to not encode a glycine at residue 346, or to have a deletion of one or more of residues 46 to 50, or any combination thereof, wherein the numbering for NA residues is that for N2; 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 comprising one or more of: 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, a vector for mRNA production comprising a promoter operably linked to a DNA segment encoding influenza virus NS1, 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 NA has at least 80%, 85%, 90%, 95%, or 99% amino acid sequence identity to SEQ ID NO:1 SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:48 or SEQ ID NO:49. In one embodiment, the NA has less than 100% amino acid sequence identity to SEQ ID NO:2 or SEQ ID NO:3. In one

embodiment, the NA is N2, N3, N7, or N9. In one embodiment, the NA is N1, N4, N5, N6, N8, N10 or N11. In one embodiment, the residue at position 32 is A, I, G, or L. In one embodiment, the deletion is a deletion of residues 46 to 50. In one embodiment, the residue at position 147 is N or Q. In one embodiment, the residue at position 329 is D or E. In one embodiment, the residue at position 346 is S, T, P, Y, W, A, N, I, L, or V. In one embodiment, the residue at position 347 is Q, N, S, T, Y, C or W. In one embodiment, the residue at position 148 is K, R or H. In one embodiment, the residue at position 151 is E, N or Q. In one embodiment, the residue at position 245 is S, T, I, L, A, N, or V.

[0014] In one embodiment, the HA is H1, H3, H5, H7, or H9. In one embodiment, the virus is an influenza A virus. In one embodiment, PA, PB1, PB2, NP, M, and NS viral segments have at least 85%, 85%, 90%, 95%, or 99% nucleic acid sequence identity to SEQ ID Nos. 24 to 29 or 39 to 44 or encode a polypeptide having at least 80%, 85%, 90%, 95%, or 99% amino acid sequence identity to a polypeptide encoded by SEQ ID Nos. 24 to 29 or 39 to 44. In one embodiment, PB2 has I, A, L, or G at residue 147. In one embodiment, HA is H2, H4, H5, H6, H8, or any of H10-H18. In one embodiment, the virus is an influenza B virus.

[0015] Further provided is a method of immunizing an avian or a mammal with a composition having an effective amount of the virus described herein. In one embodiment, the composition comprises at least one other different influenza virus. In one embodiment, the mammal is a human. In one embodiment, the composition is administered intranasally or via injection.

[0016] Thus, the invention provides a method to select for influenza viruses with enhanced replication in cell culture, e.g., in embryonated avian eggs. The method includes providing cells suitable for influenza vaccine production; serially culturing one or more influenza virus isolates in eggs; and isolating serially cultured virus with enhanced growth relative to the one or more isolates prior to serial culture. Also provided is a method to identify a NA that stabilizes HA and/or that confers altered growth of a recombinant influenza virus, e.g., in eggs. The method includes introducing one or more substitutions or deletions as described herein into a NA viral segment to yield a mutant NA viral segment; and optionally identifying whether the mutant NA viral segment, when present in a replication competent recombinant influenza virus, results in enhanced replication of the recombinant influenza virus in eggs and optionally inhibits HA mutations, relative to a corresponding replication competent influenza virus without the one or more substitutions and/or deletions in NA.

[0017] In one embodiment, the disclosure provides isolated influenza type A virus with a characteristic residue(s) and/or deletion, or a combination thereof, in NA described herein. In one embodiment, the isolated influenza type A virus with a characteristic residue(s) and/or deletion, or a combination thereof, has an NA amino acid sequence with at least 80%, e.g., 90%, 92%, 95%, 97% or 99%, including any integer between 80 and 99, contiguous amino acid sequence identity to a polypeptide encoded by one of SEQ ID NOs:1, 2, 3, or 30-38. In one embodiment, the isolated influenza type A virus of the invention with a characteristic residue(s) and/or deletion, or a combination thereof, has an HA from any one of subtypes 1-18 of HA. In one embodiment the characteristic residue is a conservative substitution,

e.g., relative to SEQ ID NO:2 or SEQ ID NO:3. Conservative amino acid substitutions refer to the interchangeability of residues having similar side chains. For example, a group of amino acids having aliphatic side chains is glycine, alanine, valine, leucine, and isoleucine; a group of amino acids having aliphatic-hydroxyl side chains is serine and threonine; a group of amino acids having amide-containing side chains is asparagine and glutamine; a group of amino acids having aromatic side chains is phenylalanine, tyrosine and tryptophan; a group of amino acids having basic side chains is lysine, arginine and histidine; and a group of amino acids having sulfur-containing side chain is cysteine and methionine. In one embodiment, conservative amino acid substitution groups are: threonine-valine-leucine-isoleucine-alanine; phenylalanine-tyrosine; lysine-arginine; alanine-valine; glutamic-aspartic; and asparagine-glutamine.

[0018] In one embodiment, a mutation is introduced into a NA viral segment of an influenza virus isolate, e.g., via recombinant DNA techniques including site-specific mutagenesis, or replacing a portion of the NA coding sequence with a portion that includes the characteristic residue(s) or deletion. In one embodiment, a NA viral segment with a characteristic residue and/or deletion described herein is combined with a HA segment, and internal viral segments of an influenza vaccine virus.

[0019] The disclosure 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 is an influenza virus with 6 internal viral segments from a vaccine virus, a HA viral segment that is from a different (second) viral isolate than the vaccine virus, and a NA viral segment with a characteristic residue(s) and/or deletion, or a combination thereof, as described herein, which is from a different viral source than the HA segment and the vaccine virus; a 6:2 reassortant is an influenza virus with 6 internal viral segments from a vaccine virus, and a NA viral segment having a characteristic residue(s) and/or deletion, or a combination thereof, which segment is from the same source as the HA segment, and a HA viral segment from a different viral isolate than the vaccine virus; and a 7:1 reassortant, in one embodiment, is an influenza virus with 6 internal viral segments and a HA segment from a vaccine virus, and a NA segment that is modified to include the characteristic residue(s) and/or deletion, or a combination thereof, which NA segment is from a different viral source than the vaccine virus.

[0020] In one embodiment of the invention, the plurality includes vectors for vRNA production selected from a vector comprising a promoter operably linked to an influenza virus PA DNA linked to a transcription termination sequence, a vector comprising a promoter operably linked to an influenza virus PB1 DNA linked to a transcription termination sequence, a vector comprising a promoter operably linked to an influenza virus PB2 DNA linked to a transcription termination sequence, a vector comprising a promoter operably linked to an influenza virus HA DNA linked to a transcription termination sequence, a vector comprising a promoter operably linked to an influenza virus NP DNA linked to a transcription termination sequence, a vector comprising a promoter operably linked to an influenza virus NA DNA linked to a transcription termination sequence, a vector comprising a promoter operably linked to an influenza virus M DNA linked to a transcription termination

sequence, and a vector comprising a operably linked to an influenza virus NS DNA linked to a transcription termination sequence. In one embodiment, the DNAs for vRNA production of PB1, PB2, PA, NP, M, and NS, have sequences from an influenza virus that replicates to high titers in cultured mammalian cells such as Vero cells, MDCK cells, or PER.C6® cells, or embryonated eggs, and/or from a vaccine virus, e.g., one that does not cause significant disease in humans. The DNA for vRNA production of NA may be from any NA, e.g., any of N1-N9, 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. For example, the DNAs for vRNA production include influenza B virus PA, PB1, PB2, NP, NS, and M or influenza B virus PA, PB1, PB2, NP, NS, M, and NA, wherein the vRNA for NA has a NA with a characteristic residue and/or deletion as described herein. 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 or HA 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.

[0021] Viruses that may provide the internal genes for reassortants within the scope of the invention include viruses that have high titers, e.g., titers of at least about 10^5 PFU/mL, e.g., at least 10^6 PFU/mL, 10^7 PFU/mL or 10^8 PFU/mL; high titers in embryonated eggs, e.g., titers of at least about 10^7 EID₅₀/mL, e.g., at least 10^8 EID₅₀/mL, 10^9 EID₅₀/mL or 10^{10} EID₅₀/mL; high titers in 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.

[0022] Other reassortants with internal genes from other PR8 isolates or vaccine viruses may be employed in recombinant reassortant viruses.

[0023] 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:24-29 or 39 to 44. 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% or 99%, including any integer between 80 and 99, contiguous amino acid sequence identity to, a polypeptide encoded by one of SEQ ID NOs:24-29 or 39 to 44. In one embodiment, the isolated and/or purified nucleic acid molecule comprises a nucleotide sequence which is substantially the same as, e.g., having at least 50%, e.g., 60%, 70%, 80% or 90%, including any integer between 50 and 100, or more contiguous nucleic acid sequence identity to one of SEQ ID NOs:24-29 and, in one embodiment, also encodes a polypeptide having at least 80%, e.g., 90%, 92%, 95%, 97% or 99%, including any integer between 80 and 99, contiguous amino acid sequence

identity to a polypeptide encoded by one of SEQ ID NOS: 24-29 or 39 to 44. 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 the residues, relative to a polypeptide encoded by one of SEQ ID NOS: 24-29 or 39 to 44. 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: valine-leucine-isoleucine; phenylalanine-tyrosine; lysine-arginine; alanine-valine; glutamic-aspartic; and asparagine-glutamine. In one embodiment, the influenza virus polypeptide has one or more, for instance, 2, 3 or 4, nonconservative amino acid substitutions, relative to a polypeptide encoded by one of SEQ ID NOS:24-29.

[0024] In one embodiment, the nucleic acid a sequence encoding a NA polypeptide which is substantially the same as, e.g., having at least 80%, e.g., 90%, 92%, 95%, 97% or 99%, including any integer between 80 and 99, contiguous amino acid sequence identity to, a polypeptide encoded by one of SEQ ID NOS:1, 3, 30-35, 48-49, or one of Accession Nos. ACP41107.1 (N1) (SEQ ID NO:36) AIK26357.1 (N7) (SEQ ID NO:37), ALH21372.1 (N9) (SEQ ID NO:45), or BAK86313.1 (N2) (SEQ ID NO:50), the sequences of which are incorporated by reference herein. In one embodiment, the isolated and/or purified nucleic acid molecule encodes a polypeptide having at least 80%, e.g., 90%, 92%, 95%, 97% or 99%, including any integer between 80 and 99, contiguous amino acid sequence identity to a polypeptide encoded by SEQ ID NOS:1, 3, 30-35, 48-49, or one of Accession Nos. ACP41107.1 (N1) AIK26357.1 (N7), ALH21372.1 (N9), or BAK86313.1 (N2), the sequences of which are incorporated by reference herein. 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 the residues, relative to a polypeptide encoded by one of SEQ ID NOS:1, 3, 30-35, 48-49, or one of Accession Nos. ACP41107.1 (N1) AIK26357.1 (N7), ALH21372.1 (N9), or BAK86313.1 (N2), the sequences of which are incorporated by reference herein. 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: valine-leucine-isoleucine; phenylalanine-tyrosine; lysine-arginine; alanine-

valine; glutamic-aspartic; and asparagine-glutamine. In one embodiment, the influenza virus polypeptide has one or more, for instance, 2, 3 or 4, nonconservative amino acid substitutions, relative to a polypeptide encoded by one of SEQ ID NOS:1, 3, 30-35, 48-49, or one of Accession Nos. ACP41107.1 (N1) AIK26357.1 (N7), ALH21372.1 (N9), or BAK86313.1 (N2), the sequences of which are incorporated by reference herein.

[0025] 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 (2013), 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.

[0026] A composition or plurality of vectors of the invention may also comprise a heterologous gene or open reading frame of interest, e.g., a foreign gene encoding an immunogenic peptide or protein useful as a vaccine or in gene replacement, for instance may encode an epitope useful in a cancer therapy or vaccine, or a peptide or polypeptide useful in gene therapy. When preparing virus, the vector or plasmid comprising the gene or cDNA of interest may substitute for a vector or plasmid for an influenza viral gene or may be in addition to vectors or plasmids for all influenza viral genes. Thus, another embodiment of the invention comprises a composition or plurality of vectors as described above in which one of the vectors is replaced with, or further comprises, 5' influenza virus sequences optionally including 5' influenza virus coding sequences or a portion thereof, linked to a desired nucleic acid sequence, e.g., a desired cDNA, linked to 3' influenza virus sequences optionally including 3' influenza virus coding sequences or a portion thereof. In one embodiment, the desired nucleic acid sequence such as a cDNA is in an antisense (antigenomic) orientation. The introduction of such a vector in conjunction with the other vectors described above to a host cell permissive for influenza virus replication results in recombinant virus comprising vRNA corresponding to the heterologous sequences of the vector.

[0027] 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.

[0028] 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.

[0029] 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.

[0030] 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:24-29 or 39 to 44, 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:24-29. 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.

[0031] 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.

[0032] The invention also provides a method to prepare influenza virus. The method comprises contacting a cell with a plurality of the vectors of the invention, e.g., sequentially or simultaneously, in an amount effective to yield infectious influenza virus. The invention also includes isolating virus from a cell contacted with the plurality of vectors. Thus, the invention further provides isolated virus, as well as a host cell contacted with the plurality of vectors or virus of the invention. In another embodiment, the invention includes contacting the cell with one or more vectors, either vRNA or protein production vectors, prior to other vectors, either vRNA or protein production vectors. In one embodiment, the promoter for vRNA vectors employed in the method is a RNA polymerase I promoter, a RNA polymerase II promoter, a RNA polymerase III promoter, a T3 promoter or a T7 promoter. In one embodiment, the RNA polymerase I promoter is a human RNA polymerase I promoter. In one embodiment, each vRNA vector employed in the method is

on a separate plasmid. In one embodiment, the vRNA vectors employed in the method are on one plasmid or on two or three different plasmids. In one embodiment, each mRNA vector employed in the method is on a separate plasmid. In one embodiment, the mRNA vectors for PA, PB1, PB2 and NP employed in the method are on one plasmid or on two or three different plasmids.

[0033] 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.

[0034] 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.

[0035] 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.

[0036] Thus, the modified neuraminidase comprises at least one, or at least two, or at least three modifications, wherein the modification comprise one or more amino acids within positions 29-35, one or more amino acids within positions 44-52, one or more amino acids within positions 144-154, one or more amino acid positions within 240-250, one or more amino acids within positions 326-333, one or more amino acid positions within 344-350, one or more amino acid positions within 365-375, or combinations thereof, wherein the numbering is that for N2. In one embodiment, the NA comprises a deletion of at least one proline, asparagine, glutamine, valine, or a combination of a proline, one or more asparagine(s), a glutamine, and a valine within positions 44-52; a substitution (replacement) of a threonine within positions 29-35; a substitution (replacement) of an threonine or an aspartic acid within positions 145-155; a substitution (replacement) of an asparagine within positions 240 to 250 or 326-333; a substitution (replacement) of a histidine within positions 345-350; or a combination thereof.

BRIEF DESCRIPTION OF FIGURES

- [0037] FIG. 1. Nucleotide sequences for the viral segments of A/Yokohama/2017/2003 (SEQ ID Nos. 4-11), and amino acid sequence of the NA of A/Yokohama/2017/2003 (SEQ ID NO:3).
- [0038] FIG. 2. Amino acid sequence for the NA of A/Saitama/103/2014 (SEQ ID NO:2)
- [0039] FIG. 3. Nucleotide sequence of NA viral segment (SEQ ID NO:12) and amino acid sequences for NA of mutant of A/Yokohama/2017/2003 (SEQ ID NO:1), and nucleotide sequence of other viral segments of the mutant (SEQ ID Nos.12-21)
- [0040] FIG. 4. Graph showing titers in eggs of various reassortants with the PB2, M, NA and NP segments of mutant and wild-type A/Yokohama/2017/2003. Virus inoculation: 2×10^3 pfu/egg into allantoic fluid, 72 h incubation at 37° C.
- [0041] FIG. 5. Locations of the NA mutations on the 3D structure of N2 NA.
- [0042] FIG. 6. Graph showing titers in eggs for recombinant viruses with specific mutations found in the mutant of A/Yokohama/2017/2003 ("Y2017-M3L4"). Virus inoculation: 2×10^3 pfu/egg into allantoic fluid, 72 h incubation at 37° C.
- [0043] FIG. 7. Graph of virus titer in eggs for reassortants with two different backbones (PA, PB1, PB2, NP, NS and M) and two different HA and NA combinations (e.g., PB2-1504V, PB1-M40L/G180W, PA-R401K, NP-I116L, NS1-A30P/R118K; and NA of Y2017-M3L4 contains mutations; NA-T32A, D147N, N329D, H347Q and deletion of 46-50aa). Virus inoculation: 2×10^3 pfu/egg into allantoic fluid, 72 h incubation at 37° C.
- [0044] FIG. 8. Amino acid sequence comparison of Yokohama/2017/2003 NA wild-type (SEQ ID NO:3) and Y2017-M3L4 (SEQ ID NO:1).
- [0045] FIG. 9. Exemplary NA sequences for N3, N4, N6, N7, N8, and N9 (SEQ ID Nos. 30-35).
- [0046] FIG. 10. Exemplary sequences for the internal viral segments for a master vaccine strain (SEQ ID Nos. 39-44).
- [0047] FIG. 11. Exemplary NA sequences (SEQ ID Nos. 51-54).
- [0048] FIG. 12. Titers in eggs for various NA mutants.
- [0049] FIG. 13. Titers of HK4801HA, Y2017-M3L4NA and HY-PR8 (PB2 C4U, I504V; PB1 C4U, M40L/G180W; PA C4U, R401K; NP I116L; NS A30P/R118K) and analyses for HA mutations in infected eggs over time.
- [0050] FIG. 14 shows data for viruses passaged in eggs that had certain NA mutants but did not result in substitutions in HA.
- [0051] FIG. 15 is a schematic of the positions of certain NA residues.
- [0052] FIG. 16 is a schematic of the positions of certain NA residues.
- [0053] FIG. 17 shows virus titers for egg passaged isolates (HK4801NA (T148K, D151E, H347G, and T369K)) conferred efficient replication in the allantoic cavity to viruses possessing either HK4801HA or Singapore0019 HA (HY-PR8 backbone).
- [0054] FIG. 18 shows egg titers for different combinations of selected residues at positions 153, 329, 347, and 369 in NA.
- [0055] FIG. 19 summarizes virus titers and HA status over time (HK4801HA, Y2017-M3L4NA and HY-PR8 (PB2

C4U, I504V; PB1 C4U, M40L/G180W; PA C4U, R401K; NP I116L; NS A30P/R118K)).

[0056] FIG. 20 summarizes virus titers and HA status for viruses with different NAs.

[0057] FIG. 21 provides inoculation and harvested virus titers in allantoic passages (HA-K189E/N158K/A212T mutant virus).

[0058] FIG. 22 shows detection of HA status after multiple passages.

[0059] FIG. 23 shows egg titers for viruses with different NAs.

[0060] FIG. 24 is an enlarged view of the NA activity center. Most egg-adapted mutations are located in/around the NA active site.

DETAILED DESCRIPTION

Definitions

[0061] 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.

[0062] 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.

[0063] As used herein, "substantially free" means below the level of detection for a particular infectious agent using standard detection methods for that agent.

[0064] 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.

[0065] 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 disclosure, by the methodology of genetic engineering.

[0066] 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.

[0067] 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.

[0068] 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.

[0069] 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.

[0070] 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.

[0071] 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.

[0072] 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.

[0073] 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

[0074] 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.

[0075] Although influenza B and C viruses are structurally and functionally similar to influenza A virus, there are some differences. For example, influenza B virus does not have a M2 protein with ion channel activity but has BM2 and has a viral segment with both NA and NB sequences. Influenza C virus has only seven viral segments.

Cells that can be Used to Produce Virus

[0076] Any cell, e.g., any avian or mammalian cell, such as avian eggs, a human, e.g., 293T or PER.C6® cells, or canine, 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.

[0077] 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).

[0078] 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

[0079] A vaccine 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.

[0080] 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.

[0081] A subunit vaccine comprises purified glycoproteins. Such a vaccine may be prepared as follows: using viral suspensions fragmented by treatment with detergent, the surface antigens are purified, by ultracentrifugation for example. The subunit vaccines thus contain mainly HA protein, and also NA. The detergent used may be cationic detergent for example, such as hexadecyl trimethyl ammonium bromide (Bachmeyer, 1975), an anionic detergent such as ammonium deoxycholate (Laver & Webster, 1976); or a nonionic detergent such as that commercialized under the name TRITON X100. The hemagglutinin may also be isolated after treatment of the virions with a protease such as bromelin, and then purified. The subunit vaccine may be combined with an attenuated virus of the invention in a multivalent vaccine.

[0082] 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.

[0083] 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.

[0084] 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.

[0085] 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.

[0086] 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.

[0087] 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.

[0088] In one embodiment, such attenuated viruses maintain the genes from the virus that encode antigenic determinants substantially similar to those of the original clinical isolates. This is because the purpose of the attenuated vaccine is to provide substantially the same antigenicity as the original clinical isolate of the virus, while at the same time lacking pathogenicity to the degree that the vaccine causes minimal chance of inducing a serious disease condition in the vaccinated mammal.

[0089] 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 rimantadine); 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

[0090] 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).

[0091] Conventional vaccines generally contain about 0.1 to 200 µg, e.g., 30 to 100 µg, 0.1 to 2 µg, 0.5 to 5 µg, 1 to 10 µg, 10 µg to 20 µg, 15 µg to 30 µg, or 10 to 30 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).

[0092] 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. Suit-

able 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.

[0093] 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.

[0094] 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.

[0095] 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, thiomcarbarzones, methisazone, rifampin, ribavirin, a pyrimidine analog, a purine analog, foscarnet, phosphonoacetic acid, acyclovir, dideoxynucleosides, a protease inhibitor, or ganciclovir.

[0096] 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

[0097] 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.

[0098] 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.

[0099] 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.

[0100] 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.

[0101] 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

[0102] 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.

[0103] 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).

[0104] 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.

[0105] 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.

[0106] 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.

[0107] 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.

[0108] 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.

[0109] 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^{20} , e.g., 10^3 - 10^{12} , 10^2 - 10^{10} , 10^5 - 10^{11} , 10^6 - 10^{15} , 10^2 - 10^{10} , or 10^{15} - 10^{20} plaque forming units (PFU)/kg, or any range or value therein. The dose of one viral isolate vaccine, e.g., in an inactivated vaccine, may range from about 0.1 to 1000, e.g., 0.1 to 10 g, 1 to 20 g, 30 to 100 g, 10 to 50 g, 50 to 200 g, or 150 to 300 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.

[0110] The dosage of immunoreactive HA in each dose of replicated virus vaccine may be standardized to contain a suitable amount, e.g., 0.1 μ g to 1 μ g, 0.5 μ g to 5 μ g, 1 μ g to 10 μ g, 10 μ g to 20 μ g, 15 μ g to 30 μ g, or 30 μ g 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.

[0111] 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 >3 years of age, and 7.5 g per component for children <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 0.1 to 0.5 billion viral particles, 0.5 to 2 billion viral particles, 1 to 50 billion virus particles, 1 to 10 billion viral particles, 20 to 40 billion viral particles, 1 to 5 billion viral particles, or 40 to 80 billion viral particles.

Exemplary Viruses

[0112] Useful modifications of influenza neuraminidase (NA) proteins are described herein that stabilize hemagglutinin (HA) protein during egg-passages of influenza viruses that express those modified neuraminidase proteins. Modified nucleic acids are also described that encode such modified neuraminidase proteins. The modifications can include deletions, substitutions and combinations thereof

within the neuraminidase protein and nucleic acid sequences. Viruses that express such modified neuraminidase proteins exhibit significantly reduced acquisition of antigenicity-compromising mutations in hemagglutinin (HA) during growth of influenza in eggs.

[0113] For example, in some cases the modified neuraminidase can have at least one, or at least two, or at least three modifications. Amino acid positions within influenza neuraminidase proteins that can be modified include, for example, one or more amino acids within positions 29-35, one or more amino acids within positions 44-52, one or more amino acids within positions 144-154, one or more amino acid positions within 240-250, one or more amino acids within positions 326-333, one or more amino acid positions within 344-350, one or more amino acid positions within 365-375, and combinations thereof, based on N2 numbering. For example, the amino acid(s) can be any amino acid within these positions such as any of the amino acids listed in the table below.

Original Residue	Exemplary Substitutions	Alternative Substitutions
Ala (A)	val; leu; ile	Val
Arg (R)	lys; gln; asn	Lys
Asn (N)	gln; his; lys; arg	Gln
Asp (D)	Glu, Asn	Glu; Asn
Cys (C)	Ser	Ser
Gln (Q)	Asn	Asn
Glu (E)	Asp	Asp
Gly (G)	Pro	Pro
His (H)	asn; gln; lys; arg; gln;	Arg; Gln
Ile (I)	leu; val; met; ala; phe norleucine	Leu
Leu (L)	norleucine; ile; val; met; ala; phe	Ile
Lys (K)	arg; gln; asn	Arg
Met (M)	leu; phe; ile	Leu
Phe (F)	leu; val; ile; ala	Leu
Pro (P)	Gly	Gly
Ser (S)	Thr	Thr
Thr (T)	Ser, Ala	Ser, Als
Trp (W)	Tyr	Tyr
Tyr (Y)	trp; phe; thr; ser	Phe
Val (V)	ile; leu; met; phe; ala; norleucine	Leu

In some cases, a selected amino acid within positions 29-35, positions 44-52, positions 144-154, positions 326-333, positions within 344-350, positions within 365-375, can have a conservative substitution. However, in other cases, the selected amino acid within positions 29-35, positions 44-52, positions 144-150, positions 326-333, positions within 344-350, positions within 365-375, can have a non-conservative substitution.

[0114] For example, a modified neuraminidase can have a deletion of at least one proline, asparagine, glutamine, valine, or a combination of a proline, one or more asparagine (s), a glutamine, and a valine within positions 44-52 of the modified neuraminidase. A modified neuraminidase can have a substitution (replacement) of a threonine within positions 29-35, where the replacement is any amino acid. A modified neuraminidase can have a substitution (replacement) of a threonine or an aspartic acid within positions 145-154 or 365 to 375, where the replacement is any amino acid. A modified neuraminidase can have a substitution (replacement) of an asparagine within positions 326-333, where the replacement is any amino acid. A modified

neuraminidase can have a substitution (replacement) of a histidine within positions 345-350, where the replacement is any amino acid. Exemplary substitutions (replacements) for various types of amino acids are provided in the table above.

[0115] One example of an influenza A virus (A/Yokohama/2013/2003(H3N2)) neuraminidase protein sequence is provided below

(SEQ ID NO : 55)

1	MNPQNQKIIITI GSVSLTISTI CFFMQIAILII TTVTLHFQY
41	E FNSP <u>PNNQV</u> MLCEPTIIER NITEIVYLTN TTIEKEICPK
81	LAEYRNWSKP QCNITGFAPP SKDNSIRLSA GGDIWVTREP
121	YVSCDPDKCY QFALGQGTTL NNVHSND <u>I</u> VH DRTPYRTLLM
161	NELGVPFHLG TKQVCIAWSS SSCHDGKAWL HVCVTGDEN
201	ATASFIYNGR LADSIVSWSK KILRTQESEC VCINGTCTVV
241	MTDGSASGKA DTKILFIEEG KIVHTSTLSG SAQHVEECSC
281	YPRYPGVRCV CRDNWKGSNR PIVDINIKDY SIVSSYVCSG
321	L VGDTPR <u>KND</u> SSSSHCLDP NNEEGGH <u>GVK</u> GWAFDDGNDV
361	WMGRТИSEKL RSGYETFKVI EGWSNPNSKL QINRQVIVDR
401	GNRSGYSGIF SVEGKSCINR CFYVELIRGR KQETEVWLTS
441	NSIVVFCGTS GTYGTGSWP <u>D</u> GADINLMP <u>I</u>

Amino acids that can be modified to improve the stability of co-expressed HA are highlighted in bold and with underlining within the sequence shown above. A nucleic acid that encodes such an influenza A virus (A/Yokohama/2013/2003 (H3N2)) neuraminidase protein sequence is shown below

(SEQ ID NO : 56)

1	AGCAAAAGCA GGAGTAAAGA TGAATCCAAA TCAAAGATA
41	ATAACGATTG GCTCTGTTTC CCTCACCAATT TCCACAATAT
81	GCTTCTTCAT GCAAATTGC <u>A</u> ATCCTGATAAA CTACTGTAAC
121	ATTGCATTTC AAGCAATATG AATTCAACTC CCCCCAAC
161	AACCAAGTGA TGCTGTGTGA ACCAACATA ATA <u>GAAAGAA</u>
201	ACATAACAGA GATA <u>GTGTAT</u> CTGACCAACA CCACCATAGA
241	GAAGGAAATA TGCCCCAAC TAGCAGAA <u>T</u> CAGAAATTGG
281	TCAAAGCCGC AATGTAACAT TACAGGATT <u>T</u> GCACCTTTT
321	CTAAGGACAA TTCGATT <u>CG</u> CTTTCCGCTG GTGGGGACAT
361	CTGGGTGACA AGAGAAC <u>CTT</u> ATGTGTCATG CGATCCTGAC
401	AAGTGTATC AATTG <u>CCCT</u> TGGACAGGG <u>A</u> ACAACACTAA
441	ACAACGTGCA TTCAAATGAC ATAGTACATG ATAGGACCCC
481	TTATCGGACC CTATTGAT <u>G</u> ATGAGT <u>GGG</u> TGTTCCATT
521	CATCTGGGG <u>A</u> CCAAGCAAGT GTGCATAGCA TGGTCCAGCT
561	CAAGTTGTCA CGATGGAAAA GCATGGCTGC ATGTTGTGT
601	AACGGGGAT GATGAAAATG CAACTGCTAG CTTCATTAC
641	AATGGGAGGC TTGCAGATAG TATTGTTCA TGGTCCAAAA

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681 AAATCCTAG GACCAGGAG TCAGAATGCG TTTGTATCAA
721 TGAAACTTGT ACAGTAGTAA TGACTGATGG GAGTGCTTC
761 GGAAAAGCTG ATACTAAAAT ACTATTCAATT GAGGAGGGGA
801 AAATTGTTCA TACTAGCACA TTATCAGGAA GTGCTCAGCA
841 TGTCGAGGAG TGCTCCTGTT ATCCTCGATA TCCTGGTGTG
881 AGATGTGTCT GCAGAGACAA CTGAAAGGC TCCAATAGGC
921 CCATCGTAGA TATAAACATA AAGGATTATA GGATTGTTTC
961 GAGTTATGTG TGCTCAGGAC TTGTTGGAGA GACACCCAGA
1001 AAAAACGACA GCTCCAGCAG TAGGCCATTGC TTGGATCCAA
1041 ACAATGAGGA AGGTGGTCAT GGAGTGAAAG GCTGGGCCTT
1081 TGATGATGGA AATGACGTGT GGATGGGAAG AACGATCAGC
1121 GAGAAGTTAC GCTCAGGATA TGAAACCTTC AAAGTCATTG
1161 AAGGCTGGTC CAACCCCTAAC TCGAAATTGC AGATAAATAG
1201 GCAAGTCATA GTTGACAGAG GTAACAGGTC CGGTTATTCT
1241 GGTATTTCT CTGTTGAAGG GAAAAGCTGC ATCAATCGGT
1281 GCTTTATGT GGAGTTGATA AGGGGAAGAA AACAGGAAAC
1321 TGAAGTCTTG TGGACCTCAA ACAGTATTGT TGTTGTTGT
1361 GGCACCTAG GTACATATGG AACAGGCTCA TGGCCTGATG
1401 GGGCGGACAT GAATCTCATG CCTATATAAG CTTTCGGAAT
1441 TTTAGAAAAA AACTCCTTGT TTCTACT

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Modifications at the specified positions in neuraminidase can confer enhanced growth of the virus.

[0116] Another example of an influenza A virus (A/Yokohama/47/2002(H1N2))) neuraminidase sequence is shown below, with positions of modifications highlighted in bold and with underlining.

(SEQ ID NO: 57)			
10	20	30	40
MNP NQKIIITI	GSVSLTIATI	CFLMQIAILV	<u>TTVTLHFKQY</u>
50	60	70	80
ECNSPP <u>NNQV</u>	MLCEPTIIER	NITEIVYLTN	TTIEKEICPK
90	100	110	120
LAEYRNWSKP	QCNIITGFAPP	SKDNSTIRLSA	GGDIWVTREP
130	140	150	160
YVSCDPDKCY	QFALGQGTTL	NNGHS <u>ND</u> TVH	DRTPYRTLLM
170	180	190	200
NELGVPFHLG	TKQVCIAWSS	SSCHDGKAHL	HVCVTGDDGN
210	220	230	240
ATASFIYNGR	LVDSIGSWSK	KILRTQESEC	VCINGTCVV
250	260	270	280
MTDGSASGKA	DTKILFIEEG	KIVHTSLLSG	SAQHVEECSC
290	300	310	320
YPRYPGVRCV	CRDNWKGSNR	PIVDINVKYD	SIVSSYVCVG
330	340	350	360
LVGDTPR <u>KND</u>	SSSSSHCLDP	NNEEGG <u>HG</u> VK	GWAFDDGNDV

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370 380 390 400
WMGRTISEKL RSGYETFKVI EGWSKPNSKL QINRQVIVDR
410 420 430 440
GNRSGYSGIF SVEGKSCINR CFYVELIRGR NQETEVLWTS
450 460
NSIVVFCGTS GTYGTGSWPD GADINLMPI

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Amino acids that can be modified to improve the stability of co-expressed HA are highlighted in bold and with underlining within the sequence shown above.

[0117] In some cases, in one or more modifications can also be introduced into HA, PA, PB1, PB2, NP, M1, M2, NS2, PB1-F2, PA-X, and/or NS1 proteins (and nucleic acids encoding such proteins).

[0118] Enhanced growth of the virus when passaged through embryonated chicken eggs or cultured cells is observed when the modified NA proteins are expressed and such expression can result in significantly higher viral titers. Thus, the invention provides a method for making influenza viruses with enhanced replication in cell culture or in embryonated chicken eggs. The method includes providing cells suitable for influenza vaccine production; modifying nucleic acids encoding the neuraminidase; and isolating virus strains with enhanced growth relative to the one or more unmodified viral isolates. In some cases, a method for making influenza viruses with enhanced replication in cell culture can involve, serially culturing one or more influenza virus isolates in embryonated chicken eggs; and isolating serially cultured virus with enhanced growth relative to the one or more isolates prior to serial culture. In some cases, the viruses can be grown or passaged within cells in culture, e.g., MDCK or Vero cells.

[0119] The modified neuraminidases can be expressed in a variety of influenza strains. For example, A/Puerto Rico/8/34 (H1N1), "PR8," virus often serves as the genetic backbone for generation of inactivated influenza vaccines. Some vaccine strains based on PR8 backbone can replicate to relatively low titers in eggs and cell culture, resulting in delayed vaccine production and vaccine shortage. However, expression of the modified neuraminidases described herein can improve replication of the PR8 (and other) influenza strains.

[0120] In one embodiment of the invention, vectors for vRNA production can include a vector comprising a promoter operably linked to a modified NA DNA linked to a transcription termination sequence, a vector comprising a promoter operably linked to an influenza virus PB1 DNA linked to a transcription termination sequence, a vector comprising a promoter operably linked to an influenza virus PB2 DNA linked to a transcription termination sequence, a vector comprising a promoter operably linked to an influenza virus HA DNA linked to a transcription termination sequence, a vector comprising a promoter operably linked to an influenza virus NP DNA linked to a transcription termination sequence, a vector comprising a promoter operably linked to an influenza virus NA DNA linked to a transcription termination sequence, a vector comprising a promoter operably linked to an influenza virus M DNA linked to a transcription termination sequence, and a vector comprising a promoter operably linked to an influenza virus NS DNA linked to a transcription termination sequence. In one embodiment, the DNAs for vRNA production of PB1, PB2, PA, NP, M, and

NS, have sequences from an influenza virus that replicates to high titers in cultured mammalian cells such as MDCK cells, Vero cells or PER.C6® cells or embryonated eggs, and/or from a vaccine virus, e.g., one that does not cause significant disease in humans. The DNA for vRNA production of NA may be from any NA, e.g., any of N1-N11, 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). Vectors for mRNA production can include a vector encoding a modified NA, 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.

[0121] 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 (modified) 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, (modified) NA, and NS ("7") may be employed.

[0122] The neuraminidases that can be modified can have sequences that vary from those described herein. However, in some cases, the modified neuraminidases can have substantially the same activity as a corresponding polypeptide described by sequence herein. 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 activity, or a detectable protein level that is about 80%, 90% or more protein level, of the corresponding protein described herein. In one embodiment, the nucleic acid encodes 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 sequences described herein. In one embodiment, the isolated and/or purified nucleic acid molecule comprises a nucleotide sequence which is substantially the same as, e.g., having at least 50%, e.g., 60%, 70%, 80% or 90%, including any integer between 50 and 100, or more contiguous nucleic acid sequence identity to one of the nucleic acid sequences described herein. In one embodiment, a nucleic acid 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 described herein.

[0123] In one embodiment, a modified influenza virus neuraminidase 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 with one of the sequences disclosed herein.

[0124] 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 Wilkins (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.

[0125] A composition or plurality of vectors of the invention may also comprise a heterologous gene or open reading frame of interest, e.g., a foreign gene encoding an immunogenic peptide or protein useful as a vaccine or in gene replacement, for instance, may encode an epitope useful in a cancer therapy or vaccine, or a peptide or polypeptide useful in gene therapy. When preparing virus, the vector or plasmid comprising the gene or cDNA of interest may substitute for a vector or plasmid for an influenza viral gene or may be in addition to vectors or plasmids for all influenza viral genes. Thus, another embodiment of the invention comprises a composition or plurality of vectors as described above in which one of the vectors is replaced with, or further comprises, 5' influenza virus sequences optionally including 5' influenza virus coding sequences or a portion thereof, linked to a desired nucleic acid sequence, e.g., a desired cDNA, linked to 3' influenza virus sequences optionally including 3' influenza virus coding sequences or a portion thereof. In one embodiment, the desired nucleic acid sequence such as a cDNA is in an antisense (antigenomic) orientation. The introduction of such a vector in conjunction with the other vectors described above to a host cell permissive for influenza virus replication results in recombinant virus comprising vRNA corresponding to the heterologous sequences of the vector.

[0126] 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.

[0127] 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.

[0128] 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.

[0129] 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 a modified influenza virus NA DNA linked to a transcription termination sequence, 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 the modified NA, PB1, PB2, PA, NP, NS, and M are from one or more influenza vaccine seed viruses and contain two or more of the characteristic residues at the specified position(s); and a vector for mRNA production comprising a promoter operably linked to a DNA segment encoding influenza virus NA, 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, a vector for mRNA production comprising a promoter operably linked to a DNA

segment encoding influenza virus NS1, or a vector for mRNA production comprising a promoter operably linked to a DNA segment encoding influenza virus NS2. In one embodiment, at least one vector comprises sequences corresponding to those encoding PB1, PB2, PA, NP, M, or NS, or a portion thereof, having substantially the same activity as a corresponding polypeptide described herein or encoded by a nucleic acid described herein. 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.

[0130] 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.

[0131] The invention also provides a method to prepare influenza virus. The method comprises contacting a cell with a plurality of the vectors of the invention, e.g., sequentially or simultaneously, in an amount effective to yield infectious influenza virus. The invention also includes isolating virus from a cell contacted with the plurality of vectors. Thus, the invention further provides isolated virus, as well as a host cell contacted with the plurality of vectors or virus of the invention. In another embodiment, the invention includes contacting the cell with one or more vectors, either vRNA or protein production vectors, prior to other vectors, either vRNA or protein production vectors. In one embodiment, the promoter for vRNA vectors employed in the method is a RNA polymerase I promoter, a RNA polymerase II promoter, a RNA polymerase III promoter, a T3 promoter or a T7 promoter. In one embodiment, the RNA polymerase I promoter is a human RNA polymerase I promoter. In one embodiment, each vRNA vector employed in the method is on a separate plasmid. In one embodiment, the vRNA vectors employed in the method are on one plasmid or on two or three different plasmids. In one embodiment, each mRNA vector employed in the method is on a separate plasmid. In one embodiment, the mRNA vectors for PA, PB1, PB2 and NP employed in the method are on one plasmid or on two or three different plasmids.

EXEMPLARY EMBODIMENTS

[0132] An isolated recombinant influenza virus comprising a selected NA viral segment encoding a plurality of selected residues or a deletion of residues in NA is provided. In one embodiment, the selected NA viral segment does not encode a NA having a threonine at residue 32, does not encode a NA having an aspartic acid at position 147, does not encode a NA having a threonine at position 148, does not encode a NA having an aspartic acid at position 151, does not encode a NA having an asparagine at position 245, does not encode a NA having an asparagine at residue 329, does not encode a NA having a glycine at position 346, does not encode a NA having a histidine at residue 347, or encodes a NA having a deletion of one or more of residues 46 to 50, or any combination thereof, wherein the numbering is based on N2, wherein the recombinant influenza virus has enhanced replication in avian eggs or has a reduction in HA

mutations when grown in avian eggs relative to a corresponding influenza virus that has a NA that encodes a threonine at residue 32, does not have a deletion of residues 46 to 50, encodes an aspartic acid at position 147, encodes a threonine at residue 148, encodes an aspartic acid at residue 151, encodes an asparagine at residue 245, encodes an asparagine at residue 329, encodes a histidine at residue 347, or any combination thereof. In one embodiment, the selected NA viral segment does not have an aspartic acid at position 147, does not have an asparagine at residue 329, and does not have an arginine or a histidine at residue 347. In one embodiment, the selected NA viral segment does not have a threonine at position 148, does not have an aspartic acid at position 151, and does not have an asparagine at position 245. In one embodiment, the selected NA viral segment does not have an aspartic acid at position 147, does not have an asparagine at residue 329, and does not have an arginine or a histidine at residue 347. In one embodiment, the selected NA viral segment does not have a threonine at position 148, does not have an aspartic acid at position 151, and does not have an asparagine at position 245. In one embodiment, the selected NA viral segment has at least two of: N or Q at position 147, D or E at residue 329, or Q or G at residue 347. In one embodiment, the selected NA viral segment has at least two of: K, R or H at position 148, E or Q at position 151, or S, I, T, V or G at position 245. In one embodiment, the selected NA viral segment has N or Q at position 147, D or E at residue 329, and Q or G at residue 347. In one embodiment, the selected NA viral segment has K, R or H at position 148, E or Q at position 151, and S, I, T, V or G at position 245. In one embodiment, the isolated recombinant influenza virus is a reassortant. In one embodiment, the NA viral segment encodes a NA that has at least 90% amino acid sequence identity to SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:48, SEQ ID NO:49, SEQ ID NO:50 or SEQ ID NO:54. In one embodiment, the NA viral segment encodes a NA that has at least 90% amino acid sequence identity to SEQ ID NO:2. In one embodiment, the NA viral segment encodes a N2, N3, N7, or N9. In one embodiment, the NA viral segment encodes a N1, N4, N5, N6, N8, N10 or N11. In one embodiment, the residue at position 32 is A, I, G, or L. In one embodiment, the deletion is a deletion of residues 46 to 50. In one embodiment, the residue at position 147 is N or Q. In one embodiment, the residue at position 148 is K, R or H. In one embodiment, the residue at position 151 is E, N or Q. In one embodiment, the residue at position 245 is S, T, I, L, A, N, W, Y, P, V, or G. In one embodiment, the residue at position 329 is D or E. In one embodiment, the residue at position 346 is S, T, P, Y, W, A, N, I, L, or V. In one embodiment, the residue at position 347 is G, Q, S, T, Y, C or W. In one embodiment, the residue at position 147 is N or Q, the residue at position 329 is D or E, the residue at position 347 is G, Q, S, T, Y, C or W, or any combination thereof. In one embodiment, the residue at position 147 is N or Q, the residue at position 329 is D or E, the residue at position 347 is G or Q, or any combination thereof. In one embodiment, the residue at position 148 is K, R or H, the residue at position 151 is E, N or Q, the residue at position 245 is S, T, I, L, A, W, Y, P, V, or G, or any combination thereof. In one embodiment, the residue at position 148 is K, R or H, the residue at position 151 is E, N or Q, the residue at position 245 is S, T, I, L, A, or V, or any combination thereof. In one embodiment, the selected NA viral segment does not encode a NA having an aspartic acid at position

147, does not encode a NA having a threonine at position 148, does not encode a NA having an aspartic acid at position 151, does not encode a NA having an asparagine at position 245, does not encode a NA having an asparagine or threonine at residue 329, does not encode a NA having a glycine at position 346, does not encode a NA having a histidine, arginine or an asparagine at residue 347, or any combination thereof. In one embodiment the selected NA viral segment does not encode a NA having an aspartic acid at position 147, does not encode a NA having an asparagine at residue 329, does not encode a NA having a histidine, arginine or asparagine at residue 347, or any combination thereof. In one embodiment, the selected NA viral segment does not encode a NA having a threonine at position 148, does not encode a NA having an aspartic acid at position 151, does not encode a NA having an asparagine at position 245, does not encode a NA having a glycine at position 346, or any combination thereof. In one embodiment, the virus has HA H1, H3, H7, or H9. In one embodiment, the virus is an influenza A virus. In one embodiment, the virus comprises PA, PB1, PB2, NP, M, and NS viral segments with at least 85% nucleic acid sequence identity to SEQ ID Nos. 24 to 29 or 39 to 44 or encode a polypeptide having at least 80% amino acid sequence identity to a polypeptide encoded by SEQ ID Nos. 24 to 29 or 39 to 44. In one embodiment, the virus comprises PB2 having I, A, L, or G at residue 147.

[0133] In one embodiment, an isolated recombinant nucleic acid is provided comprising a nucleic acid sequence for an influenza virus NA viral segment that encodes a NA having a plurality of selected residues or a deletion of residues, wherein the NA viral segment does not encode a NA having a threonine at residue 32, does not encode a NA having an aspartic acid at position 147, does not encode a NA having a threonine at position 148, does not encode a NA having an aspartic acid at position 151, does not encode a NA having an asparagine at position 245, does not encode a NA having an asparagine or a threonine at residue 329, does not encode a NA having a histidine, arginine or asparagine at residue 347, or encodes a NA having a deletion of one or more of residues 46 to 50, or any combination thereof, wherein the numbering is based on N2. In one embodiment, the NA has at least 90% amino acid sequence identity to SEQ ID NO:1 or SEQ ID NO:3. In one embodiment, the NA has at least 90% amino acid sequence identity to SEQ ID NO:2. In one embodiment, the NA is a N2, N3, N7, or N9. In one embodiment, the NA is a N1, N4, N5, N6, N8, N10 or N11. In one embodiment, the residue at position 32 is A, I, G, or L. In one embodiment, the residue at position 147 is N or Q. In one embodiment, the residue at position 329 is D or E. In one embodiment, the residue at position 151 is E, N or Q. In one embodiment, the residue at position 148 is K, R or H. In one embodiment, the residue at position 245 is S, T, I, L, A, W, Y, P, V, or G. In one embodiment, the residue at position 347 is G, Q, S, or T.

[0134] In one embodiment, a method to prepare influenza virus is provided. The method includes contacting a cell with: a vector for vRNA production comprising a promoter operably linked to an influenza virus PA DNA linked to a transcription termination sequence, a vector for vRNA production comprising a promoter operably linked to an influenza virus PB1 DNA linked to a transcription termination sequence, a vector for vRNA production comprising a promoter operably linked to an influenza virus PB2 DNA linked to a transcription termination sequence, a vector for

vRNA production comprising a promoter operably linked to an influenza virus HA DNA linked to a transcription termination sequence, a vector for vRNA production comprising a promoter operably linked to an influenza virus NP DNA linked to a transcription termination sequence, a vector for vRNA production comprising a promoter operably linked to an influenza virus NA DNA linked to a transcription termination sequence, a vector for vRNA production comprising a promoter operably linked to an influenza virus M DNA linked to a transcription termination sequence, and a vector for vRNA production comprising a promoter operably linked to an influenza virus NS DNA linked to a transcription termination sequence, wherein the PB1, PB2, PA, NP, NS, and M DNAs in the vectors for vRNA production are from one or more influenza vaccine virus isolates, wherein the NA DNA in the vector for vRNA production encodes a NA having a plurality of selected residues or a deletion of residues, wherein the NA does not encode a NA having a threonine at residue 32, does not encode a NA having an aspartic acid at position 147, does not encode a NA having a threonine at position 148, does not encode a NA having an aspartic acid at position 151, does not encode a NA having an asparagine at position 245, does not encode a NA having an asparagine at residue 329, does not encode a NA having a glycine at position 346, does not encode a NA having a histidine at residue 347, or encodes a NA having a deletion of one or more of residues 46 to 50, or any combination thereof, wherein the numbering for NA residues is that for N2; 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 NA has at least 90% amino acid to SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:48, or SEQ ID NO:49. In one embodiment, the NA is N2, N3, N7, or N9. In one embodiment, the residue at position 147 is N or Q. In one embodiment, the residue at position 329 is D or E. In one embodiment, the residue at position 347 is Q, N, S, T, Y, C or W. In one embodiment, the residue at position 151 is E, N or Q. In one embodiment, the residue at position 148 is K, R or H. In one embodiment, the residue at position 245 is S, T, I, L, A, N, W, Y, P, V, or G. In one embodiment, the virus HA is H1, H3, H7, or H9. In one embodiment, The PA, PB1, PB2, NP, M, and NS viral segments have at least 85% nucleic acid sequence identity to SEQ ID Nos. 24 to 29 or 39 to 44 or encode a polypeptide having at least 80% amino acid sequence identity to a polypeptide encoded by SEQ ID Nos. 24 to 29 or 39 to 44. In one embodiment, HA is H2, H4, H5, H6, H8, or any of H10-H18. In one embodiment, virus prepared by the method is isolated. In one embodiment, virus is passaged through avian eggs.

[0135] In one embodiment, a method of immunizing an avian or a mammal is provided. The method includes administering to the avian or the mammal, e.g., a bovine, ovine, caprine, feline, canine, equine or human, a composition having an effective amount of the virus described above. In one embodiment, the composition comprises at least one other different influenza virus. In one embodiment, the composition is administered intranasally or via injection.

[0136] The invention will be described by the following non-limiting examples.

Example 1

[0137] Exemplary viral sequences for a master vaccine strain (PR8UW)

HA
 AGCAAAAGCAGGGAAAATAAAACACCAAATGAAGGCAAACCTACTGG
 TCCTGTTATGTGCACTTGCAGCTGCAGATGCAGACACAATATGTATAGGCTAC
 CATGCGAACAAATTCAACCGACACTGTTGACACAGTACTCGAGAAGAAATGTGA
 CAGTGACACACTCTGTTAACCTGCTCGAAGACAGCCACAACGGAAAATATG
 TAGATTAAGGAAATGCCCAACTACAATTGGGAAATGTAACATGCCCGGA
 TGGCTCTGGGAAACCCAGAATGCGACCCACTGCTTCAGTGAGATCATGGT
 CCTACATTGTAGAAACCCAAACTCTGAGAATGGAATATGTTATCCAGGAGA
 TTTCATCGACTATGAGGAGCTGAGGGAGCAATTGAGCTCAGTGTACATCATTC
 GAAAGATTGAAATTTCCCAAAGAAAGCTCATGGCCAACCACAAACACAA
 ACGGAGTAACGGCAGCATGCTCCATGAGGGAAAAGCAGTTTACAGAAA
 TTGCTATGGCTGACGGAGAAGGAGGGCTCATACCCAAAGCTGAAAAATTCT
 TATGTGAACAAAAAGGGAAAAGAAGTCCTGTACTGTGGGTATTACATCACC
 (SEQ ID NO: 22)

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CGCCTAACAGTAAGGAACAACAGAATCTCTATCAGAAATGAAAATGCTTATGT
 CTCTGTAGTAGCTCAAATTATAACAGGAGATTACCCGGAAATAGCAGAA
 AGACCCAAAGTAAGAGATCAAGCTGGGAGGATGAACATTACTGGACCTTGC
 TAAAACCCGGAGACACAATAATTTGAGGCAAATGAAATCTAATAGCAC
 AATGTATGCTTCGCACTGAGTAGAGGCTTGGGTCCGGCATCATCACCTCAA
 ACGCATCAATGCATGAGTGTAAACAGAAGTGTCAAACACCCCTGGGAGCTAT
 AACACAGCAGTCTCCCTTACCAAGAATACACCCAGTCACAATAGGAGAGTGC
 CCAAAATACGTCAAGGAGTGCCAAATTGAGGATGGTTACAGGACTAAGGAAC
 ATTCCGTCATTCAATCCAGAGGTCTATTGAGGCCATTGCCGTTTATTGA
 AGGGGGATGGACTGGAATGATGATGGATGGTATGGTTATCATCATCAGAAAT
 GAACAGGGATCAGGCTATGCAGCGGATCAAAAAGCACACAAAATGCCATT
 AACGGGATTACAAAACAAGGTGAAACACTGTTATCGAGAAAATGAACATTCAAT
 TCACAGCTGTGGTAAAGAATTCAACAAATTAGAAAAAGGATGGAAAATTT
 AAATAAAAAGTGTGATGGATTCTGGACATTGGACATATAATGCAGAA
 TTGTTAGTTCTACTGGAAAATGAAAGGACTCTGGATTCCATGACTCAAATGT
 GAAAGAATCTGTATGAGAAAGTAAAAGCCAATTAAAGAATAATGCCAAAGA
 AATCGGAAATGGATGTTGAGTTCTACCACAAGTGTGACAATGAATGCATG
 GAAAAGTGTAAAGGAAATGGACTTATGATTATCCAAATATTCAAAGAGTCAA
 AGTTGAACAGGAAAAGGTAGTGGAGTGAATTGGAAATCAATGGGATCT
 ATCAGATTCTGGCGATCTACTCAACTGTCGCCAGTTCACTGGTGCTTGGTC
 TCCCTGGGGCAATCAGTTCTGGATGTGTTCAATGGATGTTGCAGTCAG
 AATATGCATCTGAGATTAGAATTTCAGAGATATGAGGAAAACACCCCTGTT
 TCTACT
 NA
 (SEQ ID NO: 23)
 AGCAAAAGCAGGGTTAAAATGAATCCAAATCAGAAAATAAACCATG
 GATCAATCTGCTGGTAGTCGGACTATTAGCCTAATTGCAAATAGGGAA
 TATAATCTCAATATGGATTAGCCATTCAATTCAAATGGAAGTCAAAACCAT
 ACTGGAATATGCAACCAAAACATCATTACCTATAAAAATGCACCTGGTAA
 AGGACACAACTCAGTGTATTAACCGGCAATTCACTCTCTGGCCATCCGT
 GGGTGGGCTATATACAGCAAAGACAATAGCATAAGAATTGGTCCAAAGGA
 GACGTTTTGTCTAGAGAGGCCATTATTCATGTTCTCACTGGAAATGCAG
 GACCTTTCTGACCCAAGGTGCCTACTGAATGACAAGCATTCAAGTGGGA
 CTGTTAAGGACAGAAGCCCTTATAGGGCTTAATGAGCTGCCGTGCGGTGA
 AGCTCCGTCCCCGTACAATTCAAGATTGAATCGGTGCTGGTCAGCAAGTG
 CATGTCATGGCATGGCTGGCTAACAACTGGAATTTCAGGTCCAGATAA
 TGGAGCAGTGGCTGTATTAACACGGCATAAAACTGAAACCATAAAA
 AGTTGGAGGAAGAAAATTGAGGACACAAGAGTCTGAATGTCCTGTGTA

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ATGGTTCATTTTACTATAATGACTGATGGCCCGAGTGATGGCTGGCTCG
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GGTGACAACCCCGTCCCGAAGATGGAACAGGCAGCTGGTCCAGTGTATG
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CCTGAGCTGACAGGGCTAGACTGTATGAGGCCGTGCTCTGGGTTGAATTAA
TCAGGGGACGACCTAAAGAAAAAACATCTGAACTAGTGGAGCAGCATTTC
TTTTGTGGCGTGAATAGTGTACTGTAGATTGGCTTGCCAGACGGTGCTG
AGTTGCCATTCACTGACAAGTAGTCTGTTCAAAAACCTTGTTCAC
T

PA

(SEQ ID NO: 24)

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CAATCCGATG ATTGTCGAGC TTGCGGAAAA AACAAATGAAA GAGTATGGGG
AGGACCTGAA AATCGAAACA AACAAATTG CAGCAATATG CACTCACTTG
GAAGTATGCT TCATGTATTC AGATTTCAC TTCATCAATG AGCAAGGGGA
GTCAATAATC GTAGAACTTG GTGATCCAAA TGCACTTTG AAGCACAGAT
TTGAAATAAT CGAGGGAAAGA GATGCAACAA TGGCCTGGAC AGTAGTAAAC
AGTATTTGCA ACACTACAGG GGCTGAGAAA CCAAAGTTTC TACCAGATTT
GTATGATTAC AAGGAGAATA GATTCACTGA AATTGGAGTA ACAAGGAGAG
AAGTTCACT ATACTATCTG GAAAAGGCCA ATAAAATTAA ATCTGAGAAA
ACACACATCC ACATTTCTC GTTCACTGGG GAAGAAATGG CCACAAAGGC
AGACTACACT CTCGATGAAG AAAGCAGGGC TAGGATCAAA ACCAGACTAT
TCACCATAAG ACAAGAAATG GCCAGCAGAG GCCTCTGGGA TTCCCTTCGT
CAGTCCGAGA GAGGAGAAGA GACAATTGAA GAAAGGTTTG AAATCACAGG
AACAAATGCGC AAGCTGCCG ACCAAAGTCT CCCGCCAAC TTCTCCAGCC
TTGAAAATT TAGAGCCTAT GTGGATGGAT TCGAACCGAA CGGCTACATT
GAGGGCAAGC TGTCTCAAAT GTCCAAAGAA GTAAATGCTA GAATTGAAACC
TTTTTGAAA ACAACACCAAC GACCACTTAG ACTTCCGAAT GGGCCTCCCT
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GAGGACCCAA GTCATGAAGG AGAGGGAAATA CCGCTATATG ATGCAATCAA
ATGCAATGAGA ACATTCTTG GATGGAAGGA ACCCAATGTT GTAAACACAC
ACGAAAAGGG AATAAATCCA AATTATCTTC TGTCATGGAA GCAAGTACTG
GCAGAACTGC AGGACATTGA GAATGAGGAG AAAATTCCAA AGACTAAAAA
TATGAAGAAA ACAAGTCAGC TAAAGTGGGC ACTTGGTGAG AACATGGCAC

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 GAGAGTATGA TTGAAGCTGA GTCCTCTGTC AAAGAGAAAG ACATGACCAA
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 TGCCCTGATTA ATGATCCCTG GGTTTGCTT AATGCTTCTT GGTTCAACTC
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 CCATACTGTC CAAAAAAAGTA CCTTGTCTT ACT

PB1

(SEQ ID NO: 25)

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 GTGTTCAAGATCAAATGCCCTACGGCCAATGAGTCTGGAGGCTCATAGACT
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 CTCATTTCAAGAGAAAGAGACGGGTGAGAGACAATATGACTAAGAAAATGAT
 AACACAGAGAACATGGTAAAAGAAGCAGAGATTGAACAAAAGGAGTTA
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 CATAACAGAACAGTCGGGATATCCAGTATGGTGGAGGCTATGGTTCCAG
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 PB2
 (SEQ ID NO: 26)
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 GAGCAAGGAC AAACCTTATG GAGTAAAATG AATGATGCCG GATCAGACCG
 AGTGATGGTA TCACCTCTGG CTGTGACATG GTGGAATAGG AATGGACCAA
 TAACAAATAC AGTTCAATTAT CCACAAATCT ACACAAACTTA TTTTGAAAGA
 GTCGAAAGGC TAAAGCATGG AACCTTGGC CCTGTCCATT TTAGAAACCA

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GAAGAAAGAA GAACTCCAGG ATTGCAAAAT TTCTCCTTTG ATGGTTGCAT
ACATGTTGGA GAGAGAACTG GTCCGCAAA CGAGATTCC CCCAGTGGCT
GGTGGAACAA GCAGTGTGTA CATTGAAGTG TTGCATTGAA CTCAAGGAAC
ATGCTGGAA CAGATGTATA CTCCAGGAGG GGAAGTGAGG AATGATGATG
TTGATCAAAG CTTGATTATT GCTGCTAGGA ACATAGTGAG AAGAGCTGCA
GTATCAGCAG ATCCACTAGC ATCTTATTG GAGATGTGCC ACAGCACACA
GATTGGTGGAA ATTAGGATGG TAGACATCCT TAGGCAGAAC CCAACAGAAC
AGCAAGCCGT GGATATATGC AAGGCTGCAA TGGGACTGAG AATTAGCTCA
TCCTTCAGTT TTGGTGGATT CACATTTAAG AGAACAAAGCG GATCATCAGT
CAAGAGAGAG GAAGAGGGTC TTACGGGCAA TCTTCAAACAA TTGAAGATAA
GAGTGCATGA GGGATATGAA GAGTCACAA TGGTTGGAG AAGAGCAACA
GCCATACTCA GAAAAGCAAC CAGGAGATTG ATTCAAGCTGA TAGTGAGTGG
GAGAGACGAA CAGTCGATTG CCGAAGCAAT AATTGTGGCC ATGGTATTTC
CACAAGAGGA TTGTATGATA AAAGCAGTCA GAGGTGATCT GAATTCGTC
AATAGGGCGA ATCAACGATT GAATCCTATG CATCAACTTT TAAGACATT
TCAGAAGGAT GCGAAAGTGC TTTTCAAAA TTGGGGAGTT AACCTATCG
ACAATGTGAT GGGATATTGC CCGACATGAC TCCAAGCATC
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CTCCAGCACG GAGAGGGTAG TGGTGAGCAT TGACCGTTT TTGAGAATCC
GGGACCAACG AGGAAATGTA CTACTGTCTC CCGAGGAGGT CAGTGAAACA
CAGGGAACAG AGAAACTGAC AATAACTTAC TCATCGTCAA TGATGTGGGA
GATTAATGGT CCTGAATCAG TGTTGGTCAA TACCTATCAA TGGATCATCA
GAAACTGGGA AACTGTTAAA ATTCAAGTGGT CCCAGAACCC TACAATGCTA
TACAATAAAAA TGGAAATTGAA ACCATTCAG TCTTTAGTAC CTAAGGCCAT
TAGAGGCCAA TACAGTGGGT TTGTAAGAAC TCTGTTCCAA CAAATGAGGG
ATGTGCTTGG GACATTGAT ACCGCACAGA TAATAAAACT TCTTCCCTC
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GAATGTGAGG GGATCAGGAA TGAGAATACT TGTAAGGGC AATTCTCCCG
TATTCAACTA TAACAAGGCC ACGAAGAGAC TCACAGTTCT CGGAAAGGAT
GCTGGCACTT TAACTGAAGA CCCAGATGAA GGCACAGCTG GAGTGGAGTC
CGCTGTTCTG AGGGGATTCC TCATTCTGGG CAAAGAACAG AAGAGATATG
GGCCAGCACT AAGCATCAAT GAACTGAGCA ACCTTGCAGA AGGAGAGAAC
GCTAATGTGC TAATTGGCA AGGAGACGTG GTGTTGGTAA TGAAACGGAA
ACGGGACTCT AGCATACTTA CTGACAGCCA GACAGCGACC AAAAGAATTG
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NP

(SEQ ID NO: 27)

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 GCCAGAATGC CACTGAAATC AGAGGCATCCG TCGGAAAAAT GATTGGTGG
 ATTGGACGAT TCTACATCCA AATGTGCACC GAACTCAAC TCAGTGATTA
 TGAGGGACGG TTGATCCAAA ACAGCTTAAC AATAGAGAGA ATGGTGCTCT
 CTGCTTTGGA CGAAAGGAGA AATAAATACC TTGAAGAACCA TCCCAGTGC
 GGGAAAGATC CTAAGAAAAC TGGAGGACCT ATATACAGGA GAGTAAACGG
 AAAGTGGATG AGAGAACTCA TCCTTTATGA CAAAGAAGAA ATAAGGC
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 ATGATGATCT GGCATTCCAA TTTGAATGAT GCAACTTATC AGAGGACAAG
 AGCTCTTGTT CGCACCGGAA TGGATCCCAG GATGTGCTCT CTGATGCAAG
 GTTCAACTCT CCCTAGGAGG TCTGGAGCCG CAGGTGCTGC AGTCAAAGGA
 GTTGGAACAA TCGTGATGGA ATTGGTCAGA ATGATCAAAC GTGGGATCAA
 TGATCGGAAC TTCTGGAGGG GTGAGAATGG ACGAAAAACAA AGAATTGCTT
 ATGAAAAGAAT GTGCAACATT CTCAAAGGGAA AATTCAAAC TGCTGCACAA
 AAAGCAATGA TGGATCAAGT GAGAGAGAGC CGGAACCCAG GGAATGCTGA
 GTTCGAAGAT CTCACTTTTC TAGCACGGTC TGCACTCATA TTGAGAGGGT
 CGGTTGCTCA CAAGTCTGC CTGCTGCCT GTGTGTATGG ACCTGCC
 GCCAGTGGT ACGACTTTGA AAGGGAGGGA TACTCTCTAG TCGGAATAGA
 CCCTTCAGA CTGCTTCAAA ACAGCCAAGT GTACAGCCTA ATCAGACCAA
 ATGAGAATCC AGCACACAAG AGTCAACTGG TGTGGATGGC ATGCCATTCT
 GCGCATTG AAGATCTAAG AGTATTAAGC TTCATCAAAG GGACGAAGGT
 GCTCCCAAGA GGGAGCTTT CCACTAGAGG AGTTCAAATT GCTTCCAATG
 AAAATATGGA GACTATGGAA TCAAGTACAC TTGAACGTGAG AAGCAGGTAC
 TGGGCCATAA GGACCAGAAG TGGAGGAAAC ACCAATCAAC AGAGGGCATC
 TGCAGGGCAA ATCAGCATAAC AACCTACGTT CTCAGTACAG AGAAATCTCC
 CTTTGACAG ACAACCAATT ATGGCAGCAT TCAATGGGAA TACAGAGGG
 AGAACATCTG ACATGAGGAC CGAAATCATA AGGATGATGG AAAGTGC
 ACCAGAAGAT GTGTCTTCC AGGGCGGGGG AGTCTTCAG CTCTCGAC
 AAAAGGCAGC GAGCCCGATC GTGCCTTCCT TTGACATGAG TAATGAAGGA
 TCTTATTCTC TCGGAGACAA TGCAAGAGGAG TACGACAATT AAAGAAAAAT
 ACCCTTGTTT CTACT

M

(SEQ ID NO: 28)

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 TTTTAGGATT TGTGTTCACG CTCACCGTGC CCAGTGAGCG AGGACTGCAG

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TTTTTCAAA TGCATTTACC GTCGCTTTAA ATACGGACTG AAAGGAGGGC
CTTCTACGGA AGGAGTGCCA AAGTCTATGA GGGAAAGATA TCGAAAGGAA
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GGAGTAAAAA ACTACCTTGT TTCTACT

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NS

(SEQ ID NO: 29)

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AGAACTAGGC GATGCCCAT TCCTTGATCG GCTTCGCCGA GATCAGAAAT
CCCTAAGAGG AAGGGGCAGT ACTCTCGGTC TGGACATCAA GACAGCCACA
CGTGCTGGAA AGCAGATAGT GGAGCGGATT CTGAAAGAAG AATCCGATGA
GGCACTTAAA ATGACCATGG CCTCTGTACC TGCCTCGCGT TACCTAACTG
ACATGACTCT TGAGGAAATG TCAAGGGACT GGTCCATGCT CATAACCAAG
CAGAAAGTGG CAGGCCCTCT TTGTATCAGA ATGGACCAGG CGATCATGGA
TAAGAACATC ATACTGAAAG CGAACTTCAG TGTGATTTT GACCGGCTGG
AGACTCTAAAT ATTGCTAAGG GCTTCACCG AAGAGGGAGC AATTGTTGGC
GAAATTTCAC CATTGCCTTC TCTTCCAGGA CATACTGCTG AGGATGTCAA
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TTCGAGTCTC TGAAACTCTA CAGAGATTG CTTGGAGAAG CAGTAATGAG
AATGGGAGAC CTCCACTCAC TCCAAACAG AAACGAGAAA TGGCGGGAAC
AATTAGGTCA GAAGTTGAA GAAATAAGAT GGTTGATTGA AGAAGTGAGA
CACAAACTGA AGATAACAGA GAATAGTTT GAGCAAATAA CATTATGCA
AGCCTTACAT CTATTGCTTG AAGTGGAGCA AGAGATAAGA ACTTTCTCGT
TTCAGCTTAT TTAGTACTAA AAAACACCCCT TGTTCCTACT

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Example 2

Neuraminidase Modifications

Materials

- [0138] Viruses: Y2017: A/Yokohama/2017/2003 (H3N2)
- [0139] HK4801: A/Hong Kong/4801/2014(H3N2)
- [0140] Y2017-M3L4: Y2017 passaged 7 times in eggs
- [0141] HY-PR8: high yield PR8 (H1N1)

Results

[0142] Y2017 virus was passaged 7 times in eggs (3 times in the amniotic cavity, followed by 4 times in the allantoic cavity). A progeny virus, Y2017-M3L4, grew efficiently in the allantoic cavity (10^7 to about 10^8 PFU/mL), whereas the original Y2017 virus did not grow at all (<10 PFU/mL).

[0143] Mutations observed in Y2017-M3L4 virus were as follows:

TABLE 1

	PB2	NA	NP	M1
eggA	T147I, V344L and T174I, V344L, E358K	del 46-50aa, T32A, D147N, N329D, H347Q	none	E23Q
eggB	T147I	del 46-50aa, T32A, D147N, N329D, H347Q	D101N	none
eggC	T147I	del 46-50aa, T32A, D147N, N329D, H347Q	D101N	none

[0144] A comparison of the growth ability of mutant Y2017 viruses, generated by reverse genetics, in allantoic fluid revealed that NA mutations were responsible for the high growth of Y2017-M3L4 virus (FIG. 4). A plasmid with PB2-T147I was used for virus generation (PB2-T147I, V344L and PB2-T147I, V344L, E358K were not analyzed). Mutations were not observed in the HA gene of the virus possessing a mutated NA segment and its other genes from wild-type Y2017 after replication in allantoic fluid (FIG. 4).

[0145] FIG. 5 shows the location of the NA mutations in Y2017-M3L4 in a 3D model.

[0146] Comparison of the growth ability of Y2017 viruses with NA mutations revealed that NA-D147N, N329D, and H347Q generally contributed to the increased growth ability in allantoic fluid (FIG. 6).

[0147] The NA of Y2017-M3L4 allowed virus possessing HK4801HA to replicate efficiently in the allantoic cavity and the HY-PR8 backbone further enhanced the growth of this virus (FIG. 7).

[0148] In summary, described herein are influenza virus mutations that inhibit (e.g., prevent) the acquisition of antigenicity-compromising mutations in the hemagglutinin (HA) protein of influenza during growth in eggs and/or allow for enhanced replication. In one embodiment, the mutations are within the neuraminidase (NA) viral segment of human influenza viruses, and the mutant NA proteins stabilize the HA protein during egg-passages. Thus, in the presence of the mutant NA proteins, the HA protein does not acquire egg-adapting mutations. In some cases, the respective mutations in NA can also increase the yield of vaccine viruses.

Example 3

[0149] Analysis of the growth capability of NA mutant viruses revealed that NA-D147N, N329D, and H347Q contribute to the increased growth capability of the viruses in allantoic fluid (FIG. 12). HA mutations were not observed in the virus possessing HK4801HA, Y2017-M3L4NA, and the HY-PR8 backbone (FIG. 13) after 3 passages in the allantoic cavity.

[0150] By passaging an HY-PR8 backbone virus possessing HK4801NA (T148K and the saturated mutations N329X and H347X) and HK4801HA in eggs, a virus possessing HK4801NA (T148K, D151E, H347G, and T369K) emerged that replicated efficiently in the allantoic cavity (FIG. 14; 4M=T148K, D151E, H347G, and T369K). HA mutations were not observed during passages in eggs (1x in the amniotic cavity then 5x in the allantoic cavity).

[0151] HK4801NA (T148K, D151E, H347G, and T369K) conferred efficient replication in the allantoic cavity to HY-PR8 backbone viruses possessing either HK4801HA or Singapore0019HA. Virus inoculation: 2×10^3 pfu/egg into allantoic fluid, 72 h incubation at 37° C. (FIG. 16).

[0152] The HA coding nucleic acid sequence and NA coding nucleic acid and amino acid sequences for Singapore0019 are as follows:

A/Singapore/INFINH-16-0019/2016 (H3N2) HA
 atgaagactatcattgtttgagctacatttatgtctggtttcgtcaaaaaattccctggaaatgacaatagecacggcaacgcgtg
 (SEQ ID NO: 46)
 gccttggcaccatgcagttaccaaacggaaacgtatgtggaaaacaaatcacaatgacogaattgaaggtaactatgtctactggat
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 tgattaacagcacagggaatctaattgtcctagggtaacttcaaaatcgaagtggaaaagctcaataatgagatcagatgca

- continued

A/Singapore/INFINH-16-0019/2016 (H3N2) NA

(SEQ ID NO: 47)

which encodes

(SEQ ID NO: 48)

M N P N Q K I I T I G S V S L T I S T I C F F M Q I A I L I T T V T L H F K
Q Y E F N S P P N N Q V M L C E P T I I E R N I T E I V Y L T N T T I E K
E I C P K P A E Y R N W S K P Q C G I T G F A P F S K D N S I R L S A G
G D I W V T R E P Y V S C D P D K C Y Q F A L G Q G T T L N N V H S N
N T V K D R T P Y R T L L M N E L G V P F H L G T K Q V C I A W S S S
S C H D G K A W L H V C I T G D D K N A T A S F I Y N G R L I D S V V
S W S K D I L R T Q E S E C V C I N G T C T V V M T D G N A T G K A D
T K I L F I E E G K I V H R T S K L S G S A Q H V E E C S C Y P R Y P G V
R C V C R D N W K G S N R P I V D I N I K D H S I V S S Y V C S G L V G
D T P R K N D S S S S S H C L N P N N E E G G H G V K G W A F D D G

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N D V W M G R T I N E T S R L G Y E T F K V V E G W S N P K S K L Q I
N R Q V I V D R G D R S G Y S G I F S V E G K S C I N R C F Y V E L I R
G R K E E T E V L W T S N S I V V F C G T S G T Y G T G S W P D G A D
L N L M H I.
```

[0153] NA mutations T153N, N329T, and T369K allowed A/Saitama/102/2014 (H3N2) to replicate efficiently in the allantoic cavity (Kuwahara et al., 2018). Therefore, the effect of introducing NA-T153N, N329T (or D), T369K, and H347Q into HK4801NA(T148K) was examined. FIG. 18 reports on virus titers for different combinations of NA residues identified in screenings. FIGS. 19 and 20 report on virus titers for viruses with different combinations of selected NA residues.

Example 4

[0154] A/Alaska/232/2015_HY-PR8 (H3N2) WT/mutant virus were passaged in eggs and HA and NA segments sequenced. Alaska WT (a more recent H3N2 virus where WT has 245N, prior to 2015 H3N2 WT viruses had 245S), HA-R142S, -K189E viruses did not get mutations in HA, even after 3 amniotic and 10 allantoic passages. HA-K189E/N158K/A212T mutant did not get mutations in HA, but had some mutations in NA which exhibited improved growth in eggs since p6 (FIG. 21). The difference of NA mutations between p4 (normal growth) (NA-N245S mutation, virus grows more than 1000 fold better than with NA-245N) and p6 (better growth) was G346V (FIG. 22). Therefore, G346V may also contribute to adaptation to eggs.

The NA for A/Alaska/232/2015 has the following sequence:

```
(SEQ ID NO: 49)
mnpnqkiiti gsvsltisti cffmqaili ttvtlhfkqy
efnsppnnqv mlceptiier niteivyltn ttilkeicpk
paeyrnwskp qcgitgfapf skdnsirlsa ggdiwvtrep
yvscdpdkcy qfalgqggtt nvhhsnnntvr drtpyrtllm
nelgvphlg tkqvciaawss sschdgkawl hvcitgddkn
atasfiyngr lvdsvvswsk dilrtqesec vcingtctvv
mtdgnatgka dtkilfieeg kivhtsklsg saqhveecsc
yprypgvrvcv crdnwkgsnr pivdinikdh sivssyvcsg
lvgdptrknd ssssshclnp nneegghgvk gwafddgndv
wmgrtinets rlygetfkvv egwsnpkskl qinrqvivdr
gdrsgygsif svegkscinr cfyvelirgr keetevlwts
nsivvfcgts gtygtgswpd gadlnlmhi.
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[0155] NA pH21 plasmids were constructed: Alaska NA-T148K/D151E/N245S (found in E4); Alaska NA-G346V; and Alaska NA-T148K/D151E/N245S/G346V (found in E6). Mutant NAs were combined with WT Alaska HA or HY-PR8 backbone. Eggs were inoculated with the same dosage of WT/mutant Alaska viruses and harvested viruses titrated (FIG. 23). NA-T148K/D151E/N245S/G346V mutant virus grew to a higher titer than WT virus but

the single mutation G346V did not increase virus growth compared to WT. These results suggested that a combination of G346V and one (or two to three) other mutations, e.g., 3 mutations such as T148K, D151E and N245S, may be important for virus Alaska virus to grow efficiently in eggs. Harvested virus samples with high titer (>5 Log 10 PFU/mL) were sequenced however none had additional mutations in HA and NA.

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- [0177]** 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

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organism = Influenza virus

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GKAWLHVCTV GDDENTASAF IYNGRLADSI VWSKKILRT QESECVCING TCTVVMTDGS 240
ASGKADTKIL FIEEGKIVHT STLSGSAQHV EECSCYPYRPP GVRCVCRDNW KGSNRPIVDI 300
NIKDYSIVSS YVCGLVGDT PRKDSSSS HCLDPNNEBG QGVKGWAFD DGNDVWMGRT 360
ISEKLRSGYE TFKVIEGWSN PNSKLQINRQ VIVDRGNRSQ YSGIFSVEGK SCINRCFYVE 420
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WMGRTINSEKL RSGYETFKV EGWSNPNSKL QINRQVIVDR GNRSGYSGIF SVEGKSCINR 420
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MTDGSASGKA DTKILFIEEG KIVHTSTSLSA SAQHVEECSC YPRYPGVRCV CRDNWKGSNR 300
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WMGRTISEKL RSGYETFKVI EGWSNPNSKL QINRQVIVDR GNRSGYSGIF SVEGKSCINR 420
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mol_type = other DNA
organism = Influenza virus

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	organism = Influenza virus					
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tgcaacttgt	tggagaaattt	tttcccttagt	agttcatatc	ggagacatgt	tggattttct	2160
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ggacggattt	agaaggaaaga	gttctctgag	atcatgaaga	tctgttccac	cattgaagaa	2280
ctcagacggc	aaaaataatgt	aatttagctt	gtccttcatg	aaaaatggc	ttgtttctac	2340
t						2341

SEQ ID NO: 6	moltype = DNA	length = 2233				
FEATURE	Location/Qualifiers					
source	1..2233					
	mol_type = other DNA					
	organism = Influenza virus					
SEQUENCE: 6						
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aacaaatttc	cagcaatgt	cactcaat	gaggtatgtt	tcatgtatc	agattttcat	180
ttccatcaat	aacaaggcga	atcaatgt	gtagaacttg	atgtatcaaa	tgcactgtt	240
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 gaggaaatgg ccacaaaggc agactacat ctcgcgagg aaagcagggc taggattaag 540
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 cagtccgaaa gaggcgaaga aacaattgg aaaaaattt aatctcagg aactatcgct 660
 aggcttcggcc accaaatgtccc acggccaa ttctctggcc ttgaaattt tagacccat 720
 gtggatggat tgcgaaatggc cggctcgat gaggcgaagc ttcttcaat gtccaaagaa 780
 gtgtatgccc aaattgaaac ttcttctgaa acaacaccaa gaccatcaa acttccgat 840
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 gcccccaatttgc aatacatggc aagcatgggg aggaatttt tcacacggcgggttccat 1380
 tggtagagccca ctggatcat aatgggggggttatacttataacttccctgtcaatgtca 1440
 ttcttctgtgcg caatggacat ttcttcaatgttggatcaatggatggatggatgg 1500
 gagggaaatggc gaaaaacccat ttttatatggat ttccatcataa agggaaatgc tccatgg 1560
 aatgacacatggc atgtggtaaa ctttggatggc atggagtttt ctctcaatgtc cccgagactt 1620
 gagccacataatggggatggaa atactgtgtc cttggatgtatggatgtt actaaagaatgt 1680
 gcccattttgc aatatttcaatggc tggatgttgc ttgtatgtatggatggatggatgg 1740
 gtcaatggatggc aatggggatggatggatggatggatggatggatggatggatggatgg 1800
 gagagcatgttggaaatggatggatggatggatggatggatggatggatggatggatgg 1860
 gagaataatcatggatggatggatggatggatggatggatggatggatggatggatggatgg 1920
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 cccacatggatggatggatggatggatggatggatggatggatggatggatggatggatgg 2100
 tggatggatggatggatggatggatggatggatggatggatggatggatggatggatggatgg 2160
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 cccatggatggatggatggatggatggatggatggatggatggatggatggatggatggatgg 2280
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SEQ ID NO: 7 moltype = DNA length = 1762
FEATURE Location/Qualifiers
source 1..1762
mol_type = other DNA
organism = Influenza virus

SEQ ID NO: 8 moltype = DNA length = 1565
FEATURE Location/Qualifiers
source 1..1565
mol_type = other DNA
organism = Influenza virus

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SEQUENCE: 8

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accaaacgggt ctatgaaca gatggaaact gatggggatc gccagaatgc aactgagatt 120
agggcatccg tcgggaagat gattgatgaa atggggagat tctacatcca aatgtgcact 180
gaacttaaac tcagtgatta tgaagggcggt ttgatccaga acagctgac aatagagaaa 240
atgggtctct ctgctttga tgaaaagaagg aataaaatatic tggaaagaaca ccccaagcgcg 300
ggggaaagatc ctaagaaaac tggggggccc atatacaggaa gatggatgg aaaaatggatg 360
agggaactcg tcctttatga caaagaagaa ataaggcgaat tctggcgccca agccaacaat 420
ggtgaggatg cgacagctgg tctaactcac ataatgatct ggcatcttcaa tttgaatgat 480
gcaacatacc agaggacaac agcttggttt cgaaaccggaa tggatccccag aatgtgcct 540
ctgatgcagg ctgcgactct ccctaaagg tccggagctg cagggtgtgc agtcaaaagga 600
atccggacaa tgggtatgaa gctgtatcata atggtaacaa gggggatcaaa cgatcgaat 660
ttctggagag gtgagaatgg gcgaaaaaca agaagtgcctt atgagagaat gtgcaacatt 720
cttaaaggaa aatttcaaa acgtgcacaa agagcaatgg tggatcaatgt gagagaaatg 780
cggaaccagg gaaatgctca gatcgaatgg ctcatattnn tggcaagatc tgcattgata 840
tttagagggat cagttgtca caaatcttcg ctacctgcct gtgttatgg acctgcgat 900
tccagtgggtt acgacttcga aaaagaggga tattccctgg tgggaataga cccttcaaa 960
ctacttcaaa atagcaactt atacagccca atcagaccta acgagaatcc agcacacaag 1020
agtcaatgg tattggatggc atgcatttcgatcgttgcattt aagatggatgg attgtttagc 1080
ttcatcaagag ggacaaaatgt atctccacga gggaaactttt caacttagagg aatgtcaat 1140
gcttcaatg agaacatggta taatatggga tgcagcactt ttgaactgag aagcgggtac 1200
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agaatgtgg aaggtgcaaa accagaagaa gtgtcggttcc gggggggggg agtggatgg 1440
cttcagacg agaaggcaac gaacccgttccgttcc ttgatgtgg taatgtgg 1500
tcttattttt tggagggacaa tgcagaagag tgcacaaat aaggaaaaat acccttggttt 1560
ctactt 1565

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SEQ ID NO: 9

FEATURE	moltype = DNA length = 1467
source	Location/Qualifiers
	1 .. 1467
	mol_type = other DNA
	organism = Influenza virus

SEQUENCE: 9

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attgcatttc aagcaatatg aattcaactc ccccccacaa acccaatgttgc tgcgtgtgt 180
accaaaatata atagaaaaaa acataacaga gatagtgtat ctgaccaaca ccacccataga 240
gaaggaaataa tgcccccaac tagcagaatgg tcaaaaggccgca aatgtacat 300
tacaggattt gcacccctt ctaaggacaa ttccatttgc ctttccgtgc gtggggacat 360
ctgggtgaca agagaacctt atgtgtcatg cgatcctgc aagtgttatac aatttgcct 420
tggacaggaa acaacactt acaactgtca ttcaaatgttca atagtgatcatg ataggacccc 480
ttatcgacc ttatcgatgaa atgatggggg ttttccattt catctggggg ccaagcaatg 540
gtgcatacga tgggtccatgc caagtgtca cgtatggaaa gcatgggtgc atgtttgtt 600
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tattgtttca tgggtccaaa aaatcttgcg gaccggggg tcaaatgttca 720
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gtcaatgttgc tggatggggg gtaacatggc cgggttatttttgc gatgttttgc ttgttgggg 1260
caaaatgttgc atcaatcggtt gctttatgtt gggatgttca ggggggggg aacaggaaac 1320
tgcgtatgtt tggacccatgc acgtatgttgc tggatgttttgc ggcacccatgc gatcatatgg 1380
aacagggttca tggccatgtt gggccggatcat gatctcgatg cttatataaag ctttcgttcaat 1440
tttagaaaaaa aactccctgtt ttctactt 1467

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SEQ ID NO: 10

FEATURE	moltype = DNA length = 1027
source	Location/Qualifiers
	1 .. 1027
	mol_type = other DNA
	organism = Influenza virus

SEQUENCE: 10

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cttcatcgatccatcgcc ccctccaaaggc cgagatcgccg cagagacttgc aagatgttct 120
tgcgtggaaa aacacatggc ttggggatgttgc tggatgttgc gatgttttgc 180
gtcacccatgc actaaaggaa ttctgggggtt tggatgttgc ctcacccgtgc ccagtggcg 240
aggactgtcgatcgttgc tggatgttgc tggatgttgc gatgttttgc 300
catggacaaa gcaatgttcaactt gatgtatggaa acttaagagg gatgtatgttgc tccatggggc 360
caataggatg ggggtgttgc caactgttgc ggcattttggc tggatgttgc caatgttgc 420
gcagatgttgc gactcccgac acgtatgttgc tggatgttgc tccatggggc 480

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aataaggcat	gagaacagaaa	tggtttggc	cagcaactaca	gctaaggctta	tggagccaaat	600
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ggtcaggcgg	atggagccgg	ttgggactca	ttccgtactcc	agtactggtc	taaggatgat	720
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SEQ ID NO: 12 moltype = DNA length = 1433
FEATURE Location/Qualifiers
source 1..1433
mol_type = other DNA
organism = Influenza virus

SEQUENCE: 12
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gaattcaact ccccccatgt gtgtgaacca acaataatag aaagaaaat aacagagata 180
gtgtatctga ccaacaccac catagagaag gaaatatgc ccaaacttagc agaatacaga 240
aattggtc aa gccgcata taacattaca ggattgcac ctttttctaa ggacaattcg 300
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SEQ ID NO: 13          moltype = DNA    length = 1733
FEATURE                 Location/Qualifiers
source                  1..1733
                        mol_type = other DNA
                        organism = Influenza virus

SEQUENCE: 13
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gacccttttg ttgaacgcgaa acggccctac agcaactgtt accttttaga tggccggatc 360
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aacgtgacta	tgccaaacaa	tgaaaaattt	gacaaatttg	acatttgggg	ggttc当地ac	600
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gagaacacaa	ctagaggcat	atttggcga	atgcgggtt	tcatagaaaa	tggttgggag	1080
ggaatgggtt	acgggttgtt	cggtttcagg	catcaaaat	ctggggcac	aggacaagca	1140
gcagatctca	aaagcactca	agcagcaatc	aaccaatata	atgggaaact	gaataggtt	1200
atcgaaaaaa	caaacagagaa	atccatcag	atggaaaaaa	aattctcaga	agtagaaagg	1260
agaatttcagg	acetcgagaa	atatgtttag	gacactaa	tagatctcg	gtcataacaac	1320
gcggagatcc	ttgttgcctt	ggagaaccaa	catacaattt	atctaactga	ctcaaaat	1380
aacaaactgt	ttgaaaagaa	aaagaagca	ctgagggaaa	atgctgagga	tatggcaat	1440
ggttggttca	aaatataccaa	caaataatgt	aatgcctgc	tagatctat	cagaaatgg	1500
acttcatgacc	atgtatgtata	cagagatgaa	gcattaaaca	accgggttca	gatcaaaggt	1560
gttgagatcga	atcaggata	caaagattgg	atccatatgg	tttccttgc	cataatcatgt	1620
tttttgcgtt	gtgttgcgtt	gttgggttco	atcatgtggg	cctgcca	aggcaacatt	1680
aggtgcaaca	tttgcatttgc	agtgcattaa	ttaaaaacac	ccttgcatttct	act	1733

SEQ ID NO: 14 moltype = DNA length = 1002
 FEATURE Location/Qualifiers
 source 1..1002
 mol_type = other DNA
 organism = Influenza virus

SEQUENCE: 14
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 gctctcatgg aatggctaaa gacaagacca attctgtatc ctctgactaa ggggattctg 180
 gggtttgcgt tcaacgttcac cgtgtccact gggcgaggac tgcagcgttag acgctttgtc 240
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 gaagtggoat ttggcctgtt atgtcacaaca tttgagcaga ttgtctgactc ccagcacagg 480
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SEQ ID NO: 15 moltype = DNA length = 1520
 FEATURE Location/Qualifiers
 source 1..1520
 mol_type = other DNA
 organism = Influenza virus

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FEATURE Location/Qualifiers
source 1..864
mol_type = other DNA
organism = Influenza virus
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FEATURE Location/Qualifiers
source 1..2317
mol_type = other DNA
organism = Influenza virus
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 SEQ ID NO: 18 moltype = DNA length = 2209
FEATURE Location/Qualifiers
source 1..2209
mol_type = other DNA

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organism = Influenza virus

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SEQ ID NO: 19          moltype = DNA length = 2314
FEATURE                Location/Qualifiers
source                 1..2314
                      mol_type = other DNA
                      organism = Influenza virus

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SEQ ID NO: 20	moltype = DNA	length = 2314				
FEATURE	Location/Qualifiers					
source	1..2314					
	mol_type = other DNA					
	organism = Influenza virus					
SEQUENCE: 20						
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SEQ ID NO: 21	moltype = DNA	length = 2314				
FEATURE	Location/Qualifiers					
source	1..2314					
	mol_type = other DNA					
	organism = Influenza virus					
SEQUENCE: 21						
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tcatcccca	gctttgggg	gttacattt	aaaagacaa	cgccgtcatc	agtccaaaaa	1020
gaggagaac	tgcttacagg	caatctcaa	acattgaaga	taagataca	taagggttat	1080
gaggatgttca	caatgggtgg	gaaaagagac	acagotatac	tcagaaaaagc	aaccagaaga	1140
ttggttcagc	tcatagttag	tggagagac	gaacagtc当地	tagccgaagc	aataattgtg	1200
gcccattgtt	tttcacaaga	ggatggcatg	ataaaagacg	tttagggttg	cctggatttc	1260
gtcaacagac	caaatcagcgt	gttgaacccc	atgcatacgc	ttttaaggca	ttttcagaaaa	1320
gtgcgaaag	tgcttttca	gaatggggg	attgagcaca	tcgacatgt	aatgggaatg	1380
gttggagat	taccagat	gactccaagc	acagagatgt	caatgagagg	aataagatc	1440
agccaaaatgg	gtgtggatg	atactccatg	acagagaggg	tgttgtgtt	cattgatcg	1500
tttttggagat	ttcgagacca	acgcgggaa	gttattat	ctcttgaaga	ggtgtatgt	1560
acacaggaa	ctcgagact	gacaataact	tatcatctg	cgatgtgt	ggagatataac	1620
ggtcctgat	cggttttgt	caatactt	caatggatca	tcagaaaaatt	ggaagctgtc	1680
aaaatccat	ggtctcggaa	tcctgtcaatg	ttgtatcaaca	aatggat	tgaccattt	1740
caatctttag	tccccaaaggc	cattagaagc	caatacactg	ggtttgtcag	aacttatttc	1800
caacaaaatg	gagacgtact	tgggacattt	gacacccccc	agataaaaa	gctttccccc	1860
tttgcagccg	ctccacaaaa	gcaaaagcaga	atgcaggat	cttcaactgac	tgttaatgt	1920
agggggatcg	ggatgagaat	acttggtaagg	ggcaatttctc	ctgtatccaa	ctacaacaaag	1980
accataaaaa	gactaataat	tctcgaaat	gatggccgca	ctttaattgt	agaccccgat	2040
gaaaggacat	ccggactgtg	gtccgtgtt	ttqaggggt	tttccattat	aggtaaggaa	2100
gacagaagat	acggggccagc	attaaggatc	aatgaactga	gtaaccttgc	aaaaggggaa	2160
aaggctatgt	tgctaatccg	gcaaggagac	gtgttgttgg	taatgaaacg	aaaacgggac	2220
tctagatcat	ttaatgtacg	ccagacacgc	accaaaaagaa	ttcggatggc	catcaattaa	2280
tgttataatq	ttttaaaaaac	accccttttgc	tact			2314

SEQ ID NO: 22 moltype = DNA length = 1775
FEATURE Location/Qualifiers
source 1..1775
mol_type = other DNA
organism = Influenza virus

SEQUENCE: 22
 organism: *Thiobacillus vivax*
 agcaaaaagca ggggaaaata aaaacaacca aaatgaaggc aaacctactg gtcctgttat 60
 gtgcacttgc agtcacatg gcacagacaa tatgtatagg ctaccatcg aacaattcaa 120
 ccgacactgt tgacacatg ctccgagaaga atgtgacagt gacacactt gttAACCTG 180
 tcgaagacag ccacaaacgaa aataatcgta gattaaaagg aataggcccc caataatgg 240
 gggaaatgtaa catcgccgga tggctcttgg gaaaccggaa atgcgaccca ctgcgttccag 300
 tgagatcatg gtcctacatt gttagaacac caaactctgaa gaatggtaa tgttatccag 360
 gagatttcatg cgactatggag gagctggggg agcaatgtgg ctcaatgtca tcatcgaaa 420
 gattcgaaat atttcccaaa agaaatgtcc ggcccaatcca caacacaaaac ggatgtacgg 480
 cagcatgctc ccatgagggg aaaagcagtt ttacagaaaa tttgtatgg ctgacggaga 540
 aggaggggctc atacccaaag ctgaaaaattt cttatgtgaa caaaaaaggaa aaagaatgtcc 600
 ttgtacttgttgggttattcat cacccgccta acagtaaggaa acaaaatggaa ctccatcgaa 660
 atgaaaatgtc ttatgtctt gtatgtactt caaaatattaa caggagattt acccccggaa 720
 tagcggaaatg accccaaatgtt agatgtcaag ctggggggat gaacttattc tgacccgttc 780
 taaaacccgg agacacaata atatttgagg caaatggaaa tctaatacgca ccaatgtatg 840
 ctttcgactt gaggatggc ttgggttccg gcatcatcac ctccaaacgca tcaatgtatg 900
 agtgtaaacac gaaatgttccaa acacccttgg gaggatcttgg cagcgttcc ctttaccaga 960
 atataccaccg agtcacaata ggagatgtgg caaaatgttccg caggatgtcc aaatgtggaa 1020
 tggttacagg actaaggaaac attccgttca ttcaatccatgg tttttttttt ggagccattt 1080
 ccgggttttat tgaagggggaa tggactggaa tgatgtatgg atggatgtatgg tttttttttt 1140
 agaatgtcaaa gggatgtccg gatgtccggg atccaaaatgg cacaatggaaa gttttttttt 1200
 ggatttacaaa caagggttgcac actgtttatcc agaaaaatggaa ctttccatcc acatgttgc 1260
 gtaaagaattt caacaatggaa gaaaaatggaa tggaaaattt aaataaaaaa gttgtatgtt 1320
 gatttctggaa cattttggaca tataatgttccg aatttggatgtt tttttttttt gatgtttttt 1380
 ctctggatattt ccatgtacttca aatgttccgaa atctgtatgg gaaaatggaaa agccatggaa 1440
 agaaataatgtc caaaatggaa gggatgttccg gttttttttt tttttttttt tttttttttt 1500
 atgttgcattttt aagtgttccg aatgttccgaa atctgtatgg gttttttttt tttttttttt 1560
 agttgttccg gggaaaatggaa gatgttccg aatttggatgtt tttttttttt tttttttttt 1620
 tgggtttttttt ctcaactgtc gcccgttcc tttttttttt tttttttttt tttttttttt 1680
 gttttttttt gttttttttt gttttttttt gttttttttt tttttttttt tttttttttt 1740
 tcacqaaatgttccg qqqqaaaac accctttttt tttttttttt tttttttttt tttttttttt 1750

SEQ ID NO: 23 moltype = DNA length = 1413
FEATURE Location/Qualifiers
source 1..1413
mol_type = other DNA
organism = Influenza virus

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SEQUENCE: 23
          organism = influenza virus

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gtctggtagt cggactaatt agcctaataat tgcaaataagg gaatataatc tcaatatgg 120
ttagccattc aattcaaaact ggaagtcaaa accataactgg aatatgcaac caaaacatca 180
ttacctataaa aaatagcacc tgggttaaagg acacaacttc agtgatattt accggcaatt 240
catctctttg tccccatccgt gggtgggcata tatacagcaa agacaataatgc ataagaattg 300
gttccaaagg agacgtttt gtctaaagag agccctttt ttcatgttct cacttggaat 360
cqacqacacctt ttttctqacc caaqtqccct tactqataa caaqcattca aqtqgqactq 420

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ttaaggacag	aaggccttat	agggcottaa	tgagtcgccc	tgtcggtgaa	gctccgtccc	480
cgtacaattc	aagatttggaa	tcgggtgttt	ggtcagcaag	tgcatacgcat	gatggcatgg	540
gtgtggctaa	aactcggaaat	tcaggctccag	ataatggago	agtggctgtt	ttaaaaatata	600
acggccataat	aactgaaacc	ataaaaaatgt	ggagaaggaa	aatatggagg	acacaaggat	660
ctgaatgtgc	ctgtgttaat	ggttcatgtt	ttactataat	gactgatggc	ccgagtgtat	720
ggctggccctc	gtacaaaatt	ttcaagatcg	aaaaggggaa	ggttactaaa	tcaatagagt	780
tgaatgcac	taatttcac	tatggagat	ttctcggttta	ccctgtatccc	ggcaaaatgt	840
ttgtgtgtgt	cagagacaat	ttggcatgtt	cgaacccggcc	atgggtgtct	tttcgtatcaa	900
acctggattat	tcaaatagga	tacatctgc	gtgggggtttt	cggtgaaacaa	cccgctcccc	960
aagatggAAC	aggcagctgt	ggtccagttt	atgttgcgtt	agcaaaacgg	gtaaaggat	1020
tttcatataat	gtatgttat	ggtgttttgg	taggaaggac	caaaatgtcc	agtttccatgc	1080
atgggttta	gatgttgggg	cttccatata	gtggacaca	gactgtatgt	aatgttctgt	1140
tgaggcaaga	tggtgtggca	atgactgtt	ggtcagggtt	tagcggaaatgt	ttcggttcaac	1200
atccctgagct	gacagggtca	gactgtatga	ggccgtgtt	ctgggttggaa	ttaatcagggg	1260
gacgacccata	agaaaaaaaca	atctggacta	gtgcggacaa	cattttttttt	tgtgcgtgt	1320
atagtatata	tgtatgttgg	tcttggccca	acggtgctgt	gttgcatttcc	agcatttgacaa	1380
agtatgtctgt	tcaaaaaact	ccttggttttt	act			1413

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SEQ ID NO: 24          moltype = DNA  length = 2233
FEATURE                Location/Qualifiers
source                 1..2233
                      mol_type = other DNA
                      organism = Influenza virus
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SEQUENCE: 24
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 atttgtcgagc ttgcggaaaa aacaatggaaa gagtatgggg aggacactgaa aatcgaaaca 120
 aacaatattgc cagaatataat cactcacttg gaagtatgt ctatgttattc agatatttcac 180
 ttcatcaatgc agcaaggcga gtcaataatc gttagaacttg gtgatccaaa tgcaactttt 240
 aagcacagat ttgaaataat cgaggaaaga gatcgacaaat tggccgtggac agtagtaaac 300
 agtatttgcga acactacagg cggtcgaaaa ccaaagggttc taccagattt gtatgattac 360
 aaggagataa gattcatcga aattggatca acaaggagag aagttccat atatacttgc 420
 gaaaaggcca ataaattaa atctggatca aacacatcc acattttctc gttcaactggg 480
 gaagaaatgg ccacaaagc agactacact ctcgatgaag aaagcaggc taggatcaa 540
 accagactat tcaccataatg acaaaatgg gccacgacgg gccttctggg ticctttcg 600
 cagtcggaga gaggagaaga gacaatggaa gaaagggttg aataatcagg aacaatgcgc 660
 aagcttgcgc accaaatgtt cccggcgaa ttctccagcc ttgaaattt tagagccat 720
 gtggatggat tcgaaccgaa cggctacatt gagggcaagc tgtctcaat gtccaaagaa 780
 gtaaatgtca gaatttgcggaa tttttgtaaa acaacaccac gaccacttag actttcgat 840
 gggctccctt gtttcggat gtcacaaatc ctgtgtatgg atgccttaaa attaaatggc 900
 gaggacccaa gtcatgggg agaggggataa ccgtatataatg atgcataatca atgcataatgaga 960
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 tatgatgttgc atgaaccaga attggatgtc cttgcacatgg ggattcggaa tgatgttac 1260
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 gctccatgtt aacacatgtc aagatggaa aggaattttt tcaatcgcgg ggtgtctcc 1380
 tgcacatggca cagaatataat aatggggaa gtgtatcatca atactgcctt gcttaatgtca 1440
 tttttgtcgac caatggatca ttccatataatccatgtca taacaaatgtc tagaaatggaa 1500
 gagggaaaggc gaaagaccaa cttgtatgtt ttcatcataa aaggaagatc ccacttaagg 1560
 aatgcacccg acgtgtttaa ctttgtggc atggatgtt ctcttcactgc cccaaatgtt 1620
 gaaccatataatggggaaa gtactgtttt cttggatggat gagatgttgc tataaaggat 1680
 gccatggcc acgttgcggc gccatgttgc ttgtatgtca gcaacaatggg acactccaaa 1740
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 gagatgttgc ttgaagctgc gtcctctgtc aaagagaaaatgg acatgcacca aagatgttctt 1860
 gagaacaaatc cagaacatgc gcccattggg gagttccccca aaggatggaa ggaatgttgc 1920
 atttggggaaat ttcgtggac ttatggatca aagtgcgtt tcaacatgtt gtatgcatcc 1980
 ccacaacttag aaggattttc agtgcataatc agaaaaactgc ttcttcatgtt tcaggtctt 2040
 agggacaacc tggaaacctgg gaccttgcgat cttggggggc tatatgttgc aatttgggg 2100
 tgcctgttgc atgtatccctg ggttttgcgtt aatgttgc tggatccactc cttccatcaca 2160
 catgcatttgc gttatgttgc gcaatgttgc tttatgttgc tcaatgttgc caaaaaaaaatg 2220
 ctttgttctt act 2233

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SEQ ID NO: 25          moltype = DNA    length = 2341
FEATURE                Location/Qualifiers
source                 1..2341
                      mol_type = other DNA
                      organism = Influenza virus
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SEQUENCE: 25
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gggacaggaca caggatatac catggatact gtcaacagga cacatcgta ctcaaaaaag 180
ggaagatggta caacaacac cgaacttggta gcacccggaaac tcaaccggat tgatggcc 240
ctggccaaag aacaatgaaacc aactgggtatt gcccacaaact atttggttatt ggaggccatg 300
gttttccttg aggaatccca tactgggtatt ttgtaaaaact cgtgttattga aacgtggag 360
gttggtcagc aaacacgagt agacaagctg acacaaggcc gacagaccta tgactggact 420

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ctaaatagaa	accaacactgc	tgcaacagca	ttggccaaca	caatagaagt	gttcagatca	480
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tcaatgaaca	aagaagaaat	ggggatcaca	actcattttc	agagaaagag	acgggtgaga	600
gacaatatgaa	ctaagaaaat	gatacacag	agaacaatgg	gtaaaaaaggaa	gcagagattt	660
aacaaaaggaa	gttatctaatt	tagagcattt	accctgaaca	caatgaccaa	agatgcttag	720
agagggaaagc	taaaaacggag	agcaattgc	accccaggga	tgcaaaataag	ggggtttgta	780
tacttttgtt	agacactggc	aaggagtata	tgtgagaaac	ttgacaatac	agggttgc	840
gttggaggca	atgagaagaa	agcaagttt	gcaaatgtt	taaggaagat	gatgaccaat	900
tctcaggaca	ccgaaacttc	tttceaccatc	actggagata	acaccaaata	gaacgaaaat	960
cagaatcttc	ggatgtttt	ggccatgtat	acatataatgaa	ccagaaatca	gccccaaatgg	1020
ttcagaaata	tcttaatgt	tgtctcaata	atgttctca	acaaaatggc	gagactggga	1080
aaagggtata	tgttgtgagag	caagagtat	aaactttagaa	ctcaataacc	tgcaaaaatg	1140
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acaagttttt	tctatcgat	tgggttgg	gccaatttca	gcatggact	tcccgat	1560
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aaaacacatgg	agatgtatgc	tgttgcaaca	acacactt	ggatccccaa	aagaatcga	2040
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tgcattttat	ttgaaaaattt	tttccccag	atgttgcata	gaagaccat	cgggatatcc	2160
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ggaaaggataa	aaagaagaag	gttcaactg	atcatgaga	tctgttccac	cattgaagag	2280
ctcagacggc	aaaaatgtt	aatttatgtt	gtccttcat	aaaaatg	ttgtttctac	2340
t						2341

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SEQ ID NO: 26      moltype = DNA  length = 2341
FEATURE          Location/Qualifiers
source           1..2341
mol_type = other DNA
organism = Influenza virus

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SEQUENCE: 26
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aaqaagtaca catcaagga acaggagaac aacccagc
ttaggatgaa atggatgatg 180
gcaatgaaat atccaattac agcagacaa
aggataacgg  aaatgatttc tgagagaaat 240
gagcaaggac aaacctttagt gtagaaaatg aatgatgc
gatcagaccc 300
tcacctctgg ctgtgacatg gtggatagg aatggaccaa taacaaatac agttcattat
360
ccaaaaatct aaaaaactt ttttggaaaga gtggaaaggc taaagcatgg aacccatggc 420
ctctgtccatt ttgaaatcaactt agtcaaaatc cgtcgaggat ttgacataaa tcctggccat
480
gcagatctca gtgccaaggaa ggcacaggat gtaatcatgg aagttttt cccta
acgaa 540
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acgca taaccaaaa gaagaagaa 600
gaactccagg attgcaatattt ttctcttgc atggttgc
atcatgttgc gagagaactg 660
gtccgcaggaa cggatcttccc cccagggtt ggtggacaa
gcagtgttgc cattgttgc 720
ttgcatttgc ctcaaggaac atgttggaa
cagatgtata tcctcaggagg ggaatgttgc 780
aatgtatgtt ttgatcaag cttgttattt gctgttgc
acatgttgc aagagctca 840
gtatcagcag atccatgttgc atcttgc
gatgttgc acagcacaca gattttgg 900
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ttgggttgc acatgttgc 1020
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gaagagggtt ttacggccaa tcttcaaaaca 1080
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gcaatgttgc tttttcaaaa ttggggatgttgc 1380
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caggaaacag 1620
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tttgcgttgc 1680
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tttgcgttgc 1800
tacagtgttgc ttgttgc
tttgcgttgc 1860
accgcacaga taataaaact tttttttt
tttgcgttgc caccaaagca aagtagaaatgttgc 1920
cagttcttgc cttttttt
tttgcgttgc 1980
aattcttgc tttttttt
tttgcgttgc 2040
gttggacttca tttttttt
tttgcgttgc 2100
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tttgcgttgc 2341

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caggcccctt	ttgtatcaga	atggaccagg	cgatcatgga	taagaacatc	atactgaaag	420
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aagaggggc	aatttgtggc	gaaatttca	cattgccttc	tcttcaggaa	catactgctg	540
aggatgtcaa	aatatcgat	ggagtcctca	tcggaggact	tgaatggat	gataaacacag	600
ttcgagtctc	tgaaactcta	cagagattcg	cttggagaag	cagtagatgag	aatggagac	660
ctccactcac	tccaaaacag	aaacgagaaa	tggcgggaaac	aattaggtaa	gaagtttgaa	720
gaaataagat	ggttgattga	agaagtttca	cacaaactgaa	agataacaga	gaatagttt	780
gagcaaataa	cattatgca	agccttat	ctattgttt	aagtggagca	agagataaga	840
acttctcg	ttcagttt	tttagtactaa	aaaacaccc	tgtttctact		890

SEQ ID NO: 30 moltype = AA length = 468

FEATURE Location/Qualifiers
source 1..468
 mol_type = protein
 organism = Influenza virus

SEQUENCE: 30

MNPNQKIIITI	GVVNTTLSTI	ALLIGVGNLI	FNTVIHEKIG	DHQTVIHPTT	TPPAIPNCSD	60
TIIYNNNTVI	NNITTTITEA	ERLFLKPPPLP	CPFRGFFFH	KDNAIRLGEN	KDVIVTREPY	120
VSCDNDKNCWMS	PLALAQQALLG	TKHSNGTICKD	RTPYRSLIQF	PIGTAPVLGN	YKEICIAWSS	180
SSCFDGKEWMS	HVCMTGNDND	ASAQIYAGR	MTDSIKSWKR	DILRTOQSEC	QCIDGTCVVA	240
VTDGPAANSA	DHRVYWIREG	RIVKYENVPK	TKIQHLEEC	CYVDIDVYCI	CRDNWKGSNR	300
PWMRINNETI	LETGYVCSKF	HSDTPRPADP	STVSCDPSN	VNGGPGVKGF	GFKVGNDDVWL	360
GRTMSTSGRS	GFEIIKVAEG	WINSPPNHAKS	VTQTLVSNND	WSGYSGSFIV	KTKACFQPCF	420
YVELIRGRPN	KNDDVSWTSN	SIVTFGLDN	EPGSGNWPDG	SNIGFMPK		468

SEQ ID NO: 31 moltype = AA length = 470

FEATURE Location/Qualifiers
source 1..470
 mol_type = protein
 organism = Influenza virus

SEQUENCE: 31

MNPNQKIIITI	GSVSIILTTI	GLLLQITSLC	SIWESHYNQV	TQTHEQPCSN	NTTNYYNETF	60
VNVNTNVQNNY	TTVIEPSAPD	VVHYSSGRDL	CPIRGWAPLS	KDNGIRIGSR	GEVFVIREPF	120
ISCSISERC	FFLTQGALLN	DKHSNGTVKD	RSPFRTLMS	PIGVAPSPSN	SRFESVAWSA	180
TACSDGPQWL	TLGITGPDAT	AVAVLQGI	ITDTLKSWKG	NIMRTQESEC	VCQDEFCYTL	240
ITDGPSDAQ	FYKILKIRKG	KIVSMKDVDA	TGFPHFECCS	YPSGTDIECV	CRDNWRGSNR	300
PWIRFNSDL	YQIGYVCGSI	FGDNPRPVDG	TGSCNSPVNN	GKGRYGVKGF	SFRYGDGVVI	360
GRTKSLESRS	GFEMVWDANG	WVSTDKDNG	VQDIIDNDW	SGYSGSFIR	GETTGRNCTV	420
PCFWVEMIRG	QPKEKTIWTS	GSSIAFCGVN	SDTTGWSWPD	GALLPDFIDK		470

SEQ ID NO: 32 moltype = AA length = 470

FEATURE Location/Qualifiers
source 1..470
 mol_type = protein
 organism = Influenza virus

SEQUENCE: 32

MNPNQKIIICI	SATGMLTSVV	SLLIGIANLG	LNIGLHYKMG	DTPDVNIPNM	NETNSTTII	60
NNHTQNNFTN	ITNIIVNKNE	EGTFLNLTKP	LCEVNSWHIL	SKDNAIRIGE	DAHILVTREP	120
YLSCDPGCR	MFALSQGTTL	RGRHANGTIH	DRSPFRALIS	WEMGQAPS	VNRVECIGWS	180
STSCHDGISR	MSICMSGANN	NASAVVWYGG	RPVTEIPSWA	GNILRTQESE	CVCHKGICPV	240
VMTDGPANR	AATKIIYFKE	GKIQKIEELA	GNTQHIEECS	CYGAVGVIKIC	ICRDNWKGAN	300
RPVITIDPEM	MTHTSKYLCS	KILTDTSRPN	DPTNGNCDAP	ITGQSPDPGV	KGFAFLDREN	360
SWLGRRTISKD	SRSGYBMLKV	PNAETDTQSQ	PISHQVIVNN	QNWSGYSGAF	IDYWAKECF	420
PCFYVELIR	GRPKESSLVW	TSNSIVALCG	SKERLGWSW	HDGAEIYFK		470

SEQ ID NO: 33 moltype = AA length = 470

FEATURE Location/Qualifiers
source 1..470
 mol_type = protein
 organism = Influenza virus

SEQUENCE: 33

MNPNQKLFA	SGVAIALSIL	NLLIGISNVG	LNVLSHLKG	SDQDKNWTCT	SVTQNNTTLI	60
ENTYVNNNTV	IDKETGTAKP	NYLMLNKS	KVEGWVVVAK	DNAIRGESE	QIIVTREPYV	120
SCDPLGCKM	ALHQGTTIRN	KHSNGTIDHR	TAFRGLISTP	LGSPPVVSNS	DFLCVGWSST	180
SCHDGIGRM	TCVQGNNDNA	ATAVYYDRRL	TTTIKTWAGN	ILRTQESEC	CHNGTCVIM	240
TDGSASSQAY	TKVLYPHKGL	VIKEEALKGS	ARHIEECSCY	GHNSKVT	VCV RDNNQGANRP	300
VIEIDMNAME	HTSQYLC	LTDTSRPSDK	SMGDCNNPIT	GSPGAPGVKG	FGFLDSSNTW	360
LGRTISPRSR	SGFEMLKIPN	AETDPNSKIT	ERQEIVDNNN	WSGYSGSFID	YWDESSECYN	420
PCFYVELIRG	RPEEAKYVGW	TSNSLIALCG	SPISVGSGSF	PDGAQIQYFS		470

SEQ ID NO: 34 moltype = AA length = 470

FEATURE Location/Qualifiers
source 1..470
 mol_type = protein
 organism = Influenza virus

SEQUENCE: 34

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MNPNQKIIITV	GSVSLGLVVL	NILLHIVSIT	VTVLVLPNG	NNKNCNETVI	REYNETVRIE	60
KVYQWHNTNV	IEYIEKPESG	HFMNNTTEALC	DAKGFAFPFSK	DNGIRIGSRG	HVFVIREPFV	120
SCSPTECRTF	FLTQGSLLND	KHSNGTVKDR	SPYRTLMSVE	IGQSPNVYQA	RFEAVAWSAT	180
ACHDGKKWMT	IGVTGPDAKA	VAVVHYGGIP	TDVINSWAGD	ILRTQESSCT	CIQGECYVWM	240
TDGPANRQAQ	YRAFKAKQGK	IVGQTEISFN	GSHIEECSCY	PNEGKVECVC	RDNWTGTNRP	300
VLVISPDLSY	RAGYLCAGLP	SDTPRGEDSQ	FTGSCTSPVG	NQGYGVKGFG	FRQGNDVWMG	360
RTISRTSRSG	FEILKVRNGW	VQNSKEQIKR	QVVVDNLKWS	YSGSFTLPV	ELTKRNCLVP	420
CFWVEMIRGK	PEEKTIWTSS	SSIIVMCGVDH	EIAWDWSHDG	AILPFDIDKM		470
 SEQ ID NO: 35	moltype = AA	length = 465				
FEATURE	Location/Qualifiers					
source	1..465					
	mol_type = protein					
	organism = Influenza virus					
 SEQUENCE: 35						
MNPNQKILCT	SATAIIIGAI	AVLIGIANLG	LNLIGLHLKPG	CNCNSHSQPET	TNTSQTINN	60
YYNETNITNI	QMEERTSRNF	NNLTKGLCTI	NSWHYIGKDNN	AVRIGESSDV	LVTREPYVSC	120
DPDECRCYAL	SOGTTIRGKH	SNGTIIHDRSQ	YRALISWPPLS	SPPTVYNSRV	ECIGWSSTSC	180
HDGKSRMSIC	ISGPNNNASA	VVWYNRRPVT	EINTWARNIL	RTQESECVCH	NGVCPVVFDT	240
GSATGPADTR	IYYFKEKGKIL	KWESLTGTAK	HIEECSCYGE	RTGITCTCRD	NWQGSNRPVI	300
QIDPVAMHTH	SQYICSPVLT	DNPRPNPDNPNI	GKCNPDYPGN	NNNGVKGSY	LDGANTWLGR	360
TISTASRSGY	EMLKVPNALT	DDRSKPIQQQ	TIVLNADWSSG	YSGSFMDYWA	EGDCYRACFY	420
VELIRGRPK	DKVWWTSNSI	VSMCSSTEFL	GQWNWPDGAK	IEYFL		465
 SEQ ID NO: 36	moltype =	length =				
 SEQUENCE: 36						
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 SEQ ID NO: 37	moltype =	length =				
 SEQUENCE: 37						
000						
 SEQ ID NO: 38	moltype =	length =				
 SEQUENCE: 38						
000						
 SEQ ID NO: 39	moltype = DNA	length = 2341				
FEATURE	Location/Qualifiers					
source	1..2341					
	mol_type = other DNA					
	organism = Influenza virus					
 SEQUENCE: 39						
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aagaagttaca	catcaggaaag	acaggagaag	aacccagcac	tttagatggaa	atggatgttg	180
gcaatggaaat	atccaaattac	agcagacaag	aggataacgg	aatatgttcc	tgagaaaaat	240
gagcaaggac	aaactttatg	gagttaaaatg	aatgtatggc	gatcagacccg	agtgtatggta	300
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cctgtccatt	tttagaaacca	agtcaaaata	cgtcgagag	ttgacataaa	tcctggcatt	480
gcagatctca	gtgccaagga	ggcaggatgt	gtaatatcatgg	aagttgttt	ccctaaacgaa	540
gtggggagcc	ggataactaac	atcggaaatcg	caactaaacga	taaccaaaga	gaagaaagaa	600
gaactccagg	attgcaaaat	ttcttcattt	atgtgttgc	acatgttgg	gagagaactg	660
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ttgcatttgc	ctcaaggaac	atgtgtggaa	caagatgtata	ctccaggagg	ggaagtgt	780
aatgtatgt	ttgtatggaa	cttgcatttt	gtgtgttgc	acatgtgtgg	aagagctgca	840
gtatcggcag	accactatgc	atctttatgg	gatgtgttgc	acagcacaca	gattttgg	900
attaggatgg	tagacatcct	taagcagaac	ccaacagaag	agcaagccgt	ggatatatgc	960
aaggctgcggaaa	ttggggactgg	aattgtctca	tccttcattt	ttgtgttgcatt	cacattaag	1020
agaacaacgc	gatcatcgat	caagagag	gaagagggtgc	ttacggggca	tcttcacaaa	1080
ttaaagatggaa	gatgtatgt	ggggatcttgc	gggttccaaa	ttgtttgggg	aagagcaaca	1140
gccataactca	ggaaaaccaac	caggagatttgc	attcgtctca	tagtgcgttgc	gagagacaa	1200
cagtctgg	ccggaaacat	aattttggcc	atgttatttt	cacaagagga	tttgtatgata	1260
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gagagggttgc	ttgttgcgtat	tgaccgggttgc	tttgcgttgc	ggggaccaacg	aggaaatgt	1560
ctactcttc	ccggaggatgt	cgttgcacaa	caaggaaacag	agaaaactgtac	aataacttac	1620
tcatcgat	ttgtgtggaa	gatgtatgttgc	cttgcattatgc	tgttggtcaaa	tacattatca	1680
tggatcatgc	gaaactgggg	aactgtttaaa	attcgtgttgc	cccgaaaccc	tacaatgtca	1740
tacaataaaa	ttgaatattgt	accatttcgat	tctttatgtac	ctaaggccat	tagaggccaa	1800
tacagtgggtt	ttgttgcgtat	tttttttttttt	tttttttttttt	tttttttttttt	tttttttttttt	1860
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cagtcttcct	catttactgt	gaatgtgttgc	ggatcaggaa	tgagaatact	tgttggggc	1980
aatttcttcgt	tattcaacta	caacaaggcc	acgaagagac	tcacagtctc	cgggaaatgt	2040

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gctggcactt taacccaaga cccagatgaa ggcacagctg gagttggagtc cgctgttctg 2100
aggggattcc tcattctggg caaagaagac aggagatatg ggccacgatt aagcatcaat 2160
gaactgagca accttgcgaa aggagagaaag gctaattgtgc taatttggca aggagacgtg 2220
gttggtgtt tgaaaacgaaa acggggactt aacgatacta ctgcagaccca gacagcgacc 2280
aaaaagaattc ggatggccat caattatgtt cgaatagtt aaaaacgacc ttgtttctac 2340
t

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SEQ ID NO: 40          moltype = DNA  length = 2341
FEATURE                Location/Qualifiers
source                 1..2341
                      mol_type = other DNA
                      organism = Influenza virus
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SEQ ID NO: 41          moltype = DNA  length = 2233
FEATURE                Location/Qualifiers
source                 1..2233
                      mol_type = other DNA
                      organism = Influenza virus
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aaaattccaa	agactaaaaaa	tatgaaaaaaaaa	acaagtca	taaagtgggc	acttgggtgag	1140
acatggoc	cagaaaaagg	agactttgac	gactgtaaag	atgttaggtt	tttgaagcaa	1200
tatgatagt	atgaaccaga	attgaggctcg	cttgcaggta	ggattcagaa	tgagtcaac	1260
aaggcatcg	aactgacaga	ttcaagctgg	atagagcttg	atgagatgg	agaagatgtg	1320
gttccaattt	aacacattgc	aagcatgaga	aggaattatt	tcacatcaga	ggtgtctcac	1380
tgcagagoca	cagaatacat	aatgaagggg	gtgtacatca	atactgccc	acttaatgca	1440
tcttgcgc	caatggatga	tttccaatta	attccaatga	taagcaagtg	tagaactaa	1500
gagggaaagg	gaaagaccaa	cttggatgg	ttcatcataa	aaggaaagtc	ccacttaagg	1560
aatgacacccg	acgtggtaaa	cttggagcc	atggagttt	ctctactga	cccaagactt	1620
gaaccacaca	aatggggaaa	gtactgttt	cttggatag	gagatgtct	tctaagaagt	1680
gccataggcc	agggttcaag	gccccatgtt	ttgtatgtg	ggacaaatgg	aaccta	1740
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ccacaactag	aggatttc	agctgaatca	agaaaactgc	ttcttatcgt	tcaggcttt	2040
agggacaatc	tggaaacctgg	gacccttgc	cttgggggg	tatgtaa	agtgaggag	2100
tgcctaata	atgatccctg	gggtttgtt	atgtcttct	ggttcaactc	cttcttaca	2160
catgcattga	gttagttgt	gcagtgcac	tatttgcata	ccatactgtc	caaaaagta	2220
ccttgttct	act					2233

SEQ ID NO: 42 moltype = DNA length = 1565
 FEATURE Location/Qualifiers
 source 1..1565
 mol_type = other DNA
 organism = Influenza virus

SEQUENCE: 42

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agagcatcg	tcggaaaaat	gattgggtgg	atggacat	tctacatca	aatgtgcaca	180
gaacttaaac	tca	tgaggagccg	ttgatccaa	acagcttac	aatagagaga	240
atggtgc	ctgc	cgaaaggaga	aataaatacc	tggagaaca	tcccagtgc	300
ggggaaaggat	ctaa	tggggaccc	atatacagaa	gagtaaaacgg	aaagtggatg	360
agagaactca	tccttta	caaaaagaa	ataaggcga	tctggcgc	agctataat	420
ggtgacat	caacggctgg	tctgactca	atgtatgtt	ggcattccaa	tttgaatgt	480
gcaacttata	agaggacaag	ggcttgcgtt	cgccacggaa	ttgatcccag	gatgtgc	540
ctgtatcg	gttcaact	ccctagg	tctggagcc	cagggtgc	agtc	600
gttggaaatc	ttgtatgt	atggcatt	atgtcaaa	gttggatca	tgatcgaa	660
tctggagg	gttggaaatgg	acgaaaaaca	agaattgtt	atgaaagaa	gtcaacatt	720
ctcaaaagg	aaatttcaaa	tgctgcacaa	aaagcaat	tggatca	gagagagac	780
cgaaaccc	ggaatgtca	gttgcacat	tttgcac	tttgcac	tttgcac	840
tttggagg	gttgcacat	caagtc	tttgcac	tttgcac	tttgcac	900
gcccgtgg	acgacttgc	aaagaggaa	tacttct	tttgcac	tttgcac	960
ctgttcaaa	acagccaa	gttgcacat	tttgcac	tttgcac	tttgcac	1020
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tttgcacat	tttgcacat	tttgcac	tttgcac	tttgcac	tttgcac	1140
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atggcagat	tttgcacat	tttgcac	tttgcac	tttgcac	tttgcac	1380
aggatgttgc	aaatgtca	tttgcacat	tttgcac	tttgcac	tttgcac	1440
ctctcgac	aaaaggc	tttgcacat	tttgcac	tttgcac	tttgcac	1500
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ctact						1565

SEQ ID NO: 43 moltype = DNA length = 1027
 FEATURE Location/Qualifiers
 source 1..1027
 mol_type = other DNA
 organism = Influenza virus

SEQUENCE: 43

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tgcaggaaag	acacccgatc	tttgcagg	tttgcagg	tttgcagg	tttgcagg	180
gtcacctctg	actaagggg	tttgcagg	tttgcagg	tttgcagg	tttgcagg	240
aggactgc	cgtagacg	tttgcagg	tttgcagg	tttgcagg	tttgcagg	300
catggacaa	cgatgttgc	tttgcagg	tttgcagg	tttgcagg	tttgcagg	360
caaagaaatc	tca	tttgcagg	tttgcagg	tttgcagg	tttgcagg	420
caacaggat	ggggctgt	ccactg	tttgcagg	tttgcagg	tttgcagg	480
acagattgc	gactccc	atcggt	tttgcagg	tttgcagg	tttgcagg	540
aatcagatc	gagaacagaa	tttgcagg	tttgcagg	tttgcagg	tttgcagg	600
ggctggatc	agtgtacaa	tttgcagg	tttgcagg	tttgcagg	tttgcagg	660
gggtcagac	atgacaa	tttgcagg	tttgcagg	tttgcagg	tttgcagg	720
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cttcatcgaa aggagtgcga aagtctatga gggaaagaata tcgaaaggaa cagcagatgt 960  
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tttctact 1027
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SEQ ID NO: 44          moltype = DNA    length = 890
FEATURE                Location/Qualifiers
source                 1..890
                        mol_type = other DNA
                        organism = Influenza virus
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SEQ ID NO: 45 moltype = length =
SEQUENCE: 45
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SEQ ID NO: 46          moltype = DNA  length = 1701
FEATURE              Location/Qualifiers
source               1..1701
                     mol_type = other DNA
                     organism = Influenza virus
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SEQUENCE: 46

	Organism = influenza virus	60
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ggaaatgacca	atacgacggc aaacgtctgc cttggccacc atgcgttact aaaaacggaaacg	120
atagtaaaaa	caatcacaaa tgccgttacta ggaaatgtacta atgcgttacta gttgggttcag	180
aattcccaa	taggtgtatc atgcgtacgt cctcatcaga tccttgatgg agagaactcgc	240
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tgtcccagat	atgtttaagca tagcactctg aaattggccaa caggaatgcg aaatgttacca	1020
gagaaaacaaa	cttagaggccat atttggccca ataggggtt tcatatggaaa tggttggggag	1080
ggaatgttgg	atgggttggta cggtttcagg catcaaaattt ctgagggaaagg aggacaagaa	1140
cgacatctca	aaagcaacta agacgacatc gatcaatca atggggactt gataatgttgc	1200
atcgaaaaaa	ccaacggaaa attccatccatc attgaaaaag aatttcaga agttagaaggaa	1260
agagttcaag	accttggaaa atatgttgcg gacactaaaa tagatctctg gtcatacaaa	1320
ggccgggttc	ttgttgtccctt ggaaaccaa catacaattt atctaaactgtt ctcggaaat	1380
acaacaaatgtt	ttggaaaaaaac aaaaacggca ctggggggaaa atgttgcggaaat tttggggaaa	1440
gggttgggttca	aaatataccca caaatgttgcg aatgttgcgatc tagaatcaat aagaatggaa	1500
actttatgacc	acaatgttgcg aagggttgcgaa gcattgttgcg accgggttccca gatcaagggg	1560
gttgggttgcg	agtcagggttgcg caaagatgttgcg atcttatgttgc ttcccttgc catatcatgtt	1620
tttttgttgcg	ttgttgttgcg gttgggggttgc atcatgttgcggg cttgtccaaa gggcaacatt	1680
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SEQ ID NO: 47 moltype = DNA length = 1410
FEATURE Location/Qualifiers
source 1..1410
mol_type = other DNA
organism = Influenza virus

SEQUENCE: 47
organism = influenza virus
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gaattcaact	cccccccaa	caaccaagt	atgtctgt	aaccaacaat	aatagaaaaga	180
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cccgacaaat	acagaat	gtc	caatgtgc	ttaaggagg	tgcac	300
tctaaggaca	attcgat	gttcc	ggat	tctgggt	tgacagg	360
tatgttcat	gcatc	tttgc	tttgc	ttggac	aagagaacct	420
aacaacgtgc	attcaaa	cacagt	atgttc	tttgc	ttggacaggd	480
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aaaatcg	tat	tttgc	tttgc	tttgc	tttgc	840
tatcc	ctcgat	atcc	tttgc	tttgc	tttgc	900
cccatcgat	atataa	aaagg	tttgc	tttgc	tttgc	960
cttgttgc	gacac	tttgc	tttgc	tttgc	tttgc	1020
aacaatcgat	aaagg	tttgc	tttgc	tttgc	tttgc	1080
tggatggg	gaa	tttgc	tttgc	tttgc	tttgc	1140
ggagg	tttgc	tttgc	tttgc	tttgc	tttgc	1200
gggtat	tttgc	tttgc	tttgc	tttgc	tttgc	1260
tgtctt	tttgc	tttgc	tttgc	tttgc	tttgc	1320
aaacatgtt	tttgc	tttgc	tttgc	tttgc	tttgc	1380
ggggccgac	tttgc	tttgc	tttgc	tttgc	tttgc	1440

SEQ ID NO: 48 moltype = AA length = 469
FEATURE Location/Qualifiers
source 1..469
mol_type = protein
organism = Influenza virus

SEQUENCE:	48	Organism = Influenza virus				
MNPNPKIITI	GSVSLTISTI	CCFMQIALI	TTVTLHKFQY	EFNNSPNNQV	MLCEPTIIER	60
NITEIVYLNN	TTIEKEICPK	PAEYRNNWSKP	QCIGTGFAPF	SKDNSSIRLSA	GGDIWVTREP	120
YVSCDPDKCY	QFALGQQGTTL	NNHVSNNNTVR	DRTPYRTLNN	NELGVPHFLG	TQKVCIAWSS	180
SCHDGGAKWL	HVCITGDDKN	ATASFIYNGR	LIDSVVWSWK	DILRTQSEC	VINCINGCTVV	240
MTDGNATGKA	DTKILKIFIEG	KIVHTSKLSG	SAQHVEECSC	YPRYPGVRCV	CRDNWKGSNR	300
PIVDINIKDH	SIVSSYYCSLV	LGUDTPRKRND	SSSSSHCLNP	NNEEGHHGVK	GWAFDDGNDV	360
WMGRNTINES	RLGYETFKVV	EGWSNPKSKL	QINRQVIVDR	RDRGSGYSGIF	SVEGKSCINR	420
CFYVELIRGR	KEETEVWLTS	NSIVVFCGT	GTYGTGSGWPD	GADLNLMHI		469

SEQ ID NO: 49 moltype = AA length = 469
FEATURE Location/Qualifiers
source 1..469
mol_type = protein
organism = Influenza virus

SEQUENCE:	49	Organism	- Influenza virus			
MNPQNKIITI	GSVSLTISTI	CFFMQIAILII	TTVTLHFKQY	EFNSPPNNQV	MLCEPTIIER	60
NITEIVYLNN	TTIEKEICKP	PAEYRNWSKP	QCCTGFAFP	SKDNISRLSA	GGDVIWTRP	120
YVSCDPDKCY	QFALGQGTTL	NNHVSNNNTVR	DRTPYRTLNN	NELGVPFHLG	TKQVCIAWSS	180
SCHDGHAKWL	HVCITGDDKN	ATASFIYNGR	LVDHSVWSK	DILRTOQSEC	VICNGTCTVV	240
MTDGNATGKA	DTKILFIEEG	KIVHTSKLSG	SAQHVEECSC	YPRYPGVRCV	CRDNWKGSNR	300
PIVDINIKDH	SIVSSYVCSL	LGUDTPRKRND	SSSSSHCLNP	NNEEGHGKV	GWAFDDGNDV	360
WMGRNTINES	RLGYETFKV	EGWSNPKSKL	QINRQVWDPR	GDRSGYSGIF	SVEGKSCINR	420
CFYVELIRGR	KEETEVWLTS	NSIVFFCGTS	GTYGTGSPWD	GADLNLMHI		469

SEQ ID NO: 50 moltype = length =
SEQUENCE: 50
000

SEQ ID NO: 51 moltype = AA length = 469
FEATURE Location/Qualifiers
source 1..469
mol_type = protein
organism Influenza virus

SEQUENCE:	51	Organism = influenza virus
MNPNQKIIITI	GSVCMТИГMA	NLILQIGNII SIWISHSIQL GNQNQIETCN QSVITYENNT
WVNQTYVNIS	NTNFQAAGQSV	VSVVKLAGNSS LCPVSGWAIY SKDNNSVRIGS KGDFVFI
FISCSPLECR	TFFLTQGALL	DRSPYRTLMS CPIGEVPSPY NSRFESVAWS
ASACHDGINW	LTIGISGPDN	GAVAVLKYGNG IITDTIKSWR NNILRTQESE CACVNGSCFT
VMTDGPSNQH	ASYKIFRKEG	GKIVKSVEVN APNYHYEECS CYPDSSEITEC VCRDNNWHGSN
RPWVFSFNQLI	EYQIGYICSG	IFGDNPRPND KTGSCKPVSS NGANGVKGFS FKYGNGVWIG
RTKSISRRNG	FEMIWDPNWG	TGTDDNNFSIK QDINGINIES CGYSGSFVQHP Q ELTGLDCIRP
CFWVVELIRGR	PKENTIWTSG	SSISFCGVNS DTVGWSWPDG AELPFTIDK
		469

SEQ ID NO: 52 moltype = AA length = 470
FEATURE Location/Qualifiers
source 1..470
mol_type = protein

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	organism = Influenza virus	
SEQUENCE: 52		
MNPNQKLFAL SGVIAILSIL NLLIGISNVG LNVLSHLKG S DQDKNWTCT SVTQNNTTLI ENTVYVNNTTV IDKETGTAKP NYMLMNLNSLC KVEGVVVVAK DNAIRGESE QIIVTREPYV SCDPLGCKMY ALHQGTTIRN KHSNGTIHDR TAFRGLISTP LGSPPVVSNS DFLCVGWSST SCHDGINCRM TCVQGNNDNA TATVYYDRRL TTTIKTWAGN ILRTQSECVC CHNGTCVV TDGSASSQAY TKVLYFHKGK VIKEEALKGS ARHIEECSCY GHNSKVTVCV RDNWQGANRP VIEIDDMNAME HTSQYLCTGV LTDTSRPSKD SMGCDNNPTI GSPGPAGPVKG FGFLDSNTW LGRTISPRSR SGFEMLKIPN AETDPNSKIT ERQEIVDNNN WSGYSGSFID YWDESSECYN PCFYVELIRG RPEEAKYVGW TSNSLIALCG SPISVGSGSF PDGAQIQYFS	60 120 180 240 300 360 420 470	
SEQ ID NO: 53	moltype = AA length = 465	
FEATURE	Location/Qualifiers	
source	1..465	
	mol_type = protein	
	organism = Influenza virus	
SEQUENCE: 53		
MNPNQKILCT SATAIIIGAI AVLIGIANLG LNIGLHLKPG CNCSHSQPET TNTSQTIINN YNEYNTNTINN QMEERTSRNF NNLTGKGLCTI NSWWHYKGDN AVRIGEESDV LVTREPYV DPDECFRYAL SQGTTIRGKH SNGTIHDRSQ YRALISWP LS SPPTVYNSRV ECIGWSST HDGKSRSMIC ISGPNNNNSA VWWYNNRRPA EINTWARNL RTQSECVCV NGVCVFPFTD GSATGPADTR IYYFKEKGKIL KWESLTGTAK HIEECSCYGE RTGITCTRD NWQGSNRPVI QIDPVAMTHT SQYICSPVLT DNPRPNPDPNI GKCNPDYPYGN NNNGVKGFSY LDGANTWLGR TISTASRSGY EMLKVPNAILT DDRSKPIQGQ TIVLNADWWS YSGSFMDYWA EGDCYRACFY VELIRGRPK E DKVWWTSNSI VSMCSSTEPL GQWNWPDGAK IEYFL	60 120 180 240 300 360 420 470	
SEQ ID NO: 54	moltype = AA length = 469	
FEATURE	Location/Qualifiers	
source	1..469	
	mol_type = protein	
	organism = Influenza virus	
SEQUENCE: 54		
MNPNQKIIIT GSVSLTISTI CFFMQIAILIT TTVTLHFQY EFNSPPNNQV MLCEPTIIER NITEIVYLTN TTIEKEICPK LAEYRNWSKP QCNIITGFAPF SKDNSTIRLSA GGDIVW YVSCDPDKCY QFALGQGTTL NNVHSNDIVH DRTPYRTLLM NELGVPFHLS TKQVCI SSCHDGAWL HVCVTFGDDEN ATASFIYNGR LADSIVWSWK KILRTOSESEC V MTDGSASGKA DTKILFIEEG KIVHTSTLSQ SAQHVEECSC YPRYPGVRCV CRDN PIVDINIKDY SIVSSYVCSC LVGDTPRKND SSSSHCLDP NNEEGGHGVK GWAFDDGNDV WMGRTISEKL RSGYETFKVI EGWSNPNSKL QINRQVIVDR GNRSGYSGIF SVEGK CFYVELIRGR KQETEVLWTS NSIVVFCGTS GTYGTGSWPD GADINLMPI	60 120 180 240 300 360 420 470	
SEQ ID NO: 55	moltype = AA length = 469	
FEATURE	Location/Qualifiers	
source	1..469	
	mol_type = protein	
	organism = Influenza virus	
SEQUENCE: 55		
MNPNQKIIIT GSVSLTISTI CFFMQIAILIT TTVTLHFQY EFNSPPNNQV MLCEPTIIER NITEIVYLTN TTIEKEICPK LAEYRNWSKP QCNIITGFAPF SKDNSTIRLSA GGDIVW YVSCDPDKCY QFALGQGTTL NNVHSNDIVH DRTPYRTLLM NELGVPFHLS TKQVCI SSCHDGAWL HVCVTFGDDEN ATASFIYNGR LADSIVWSWK KILRTOSESEC V MTDGSASGKA DTKILFIEEG KIVHTSTLSQ SAQHVEECSC YPRYPGVRCV CRDN PIVDINIKDY SIVSSYVCSC LVGDTPRKND SSSSHCLDP NNEEGGHGVK GWAFDDGNDV WMGRTISEKL RSGYETFKVI EGWSNPNSKL QINRQVIVDR GNRSGYSGIF SVEGK CFYVELIRGR KQETEVLWTS NSIVVFCGTS GTYGTGSWPD GADINLMPI	60 120 180 240 300 360 420 470	
SEQ ID NO: 56	moltype = DNA length = 1467	
FEATURE	Location/Qualifiers	
source	1..1467	
	mol_type = other DNA	
	organism = Influenza virus	
SEQUENCE: 56		
agaaaaaggca ggtagaaaaaga tgaatccaa tcaaaagata ataacgatttgcctgttt cctccattt ctcacaatat gtttcttcat gcaaattggc atccgtataa ctactgttaac attgcattt aagcaaatatg aattcaactc ccccccaaac aaccaagtga tgctgtgtga accaacaata atagaaagaa acataacaga gatagtgtat ctgaccaaca ccaccataga gaaggaaata tgccccaaac tagcagaata cagaaattgg tcaaaaggccg aatgtacat tacaggattt gcacattttt ctaaggacaa ttccatggc ttccatggcgttggggacat ctgggtgaca agagaacattt atgttgcatg cgatccgtac aagtgttatac aatttgcct tggacaggaa acaacactaa acaacgtca ttcaaaatggc atagatcatg ataggacccc ttatcgacc ctattgtatc atggatggg ttgcattt catctggggc ccaagcaagt gtgcatagca tggccagttt caagttgtca cgtatggaaa gcatggcgtc atgttttgtt aacggggatg gatggaaaatg caactgtcatg cttccatttac aatggggaggc ttgcagatgg tattgttca tggccaaaaaa aaatcctcatg gaccggagg tcagaatgtc ttgtatcaa tggatctgtt acatgttagaa tggatgtatc gatgttccatg ggaaaaatgtc atataaaaaat atattcatt gaggagggaa aaattgttca tactagacaa ttatcaggaa gtgcgtcagca	60 120 180 240 300 360 420 480 540 600 660 720 780 840	

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tgtcgaggag tgctcctgtt atcctcgata tcctgggtgc agatgtgtct gcagagacaa 900
ctcgaaaaaggc tccaataggc ccatcgtaga tataaacata aaggattata gcattgttc 960
cagtttatgtg tgctcaggac ttgtggaga cacaccaga aaaaacgaca gctccagcag 1020
tagccatgtc ttggatccaa acaatggaga aggtggcat ggagtgaag gctgggcctt 1080
tgatgtatggaa aatgacgtgt ggatggaaag aacgatcagc gagaagttac gctcaggata 1140
tgaaaccttc aaagtccatgg aaggctggtc caaccctaactccaaatgc agataaataag 1200
gcaagtctata gttgacagag gtaacagtc cgggttattctt ggtattttctt ctgttgaagg 1260
caaagctgc atcaatcggt gctttatgtt ggagttgata agggggaaac aacagggaaac 1320
tgaagtcttg tggacactcaa acagtattgt ttttttttttggcacatcg gtacatatgg 1380
aacaggtcata tggcctgtatggcggacat caatctcatg cctataataag ctttcgcaat 1440
tttagaaaaaa aactccttgtt ttctact 1467
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SEQ ID NO: 57 moltype = AA length = 469
FEATURE Location/Qualifiers
source 1..469
mol_type = protein
organism = Influenza virus
SEQUENCE: 57
MNPNQKIIITI GSVSLTIATI CFLMQIAILV TTVTLHFKQY ECNSPPNNQV MLCEPTIIER 60
NITEIVLTN TTIEKEICPK LAEYRNWSKP QCNIITGFAPF SKDNISRSLA GGDWVTRP 120
YVSCDPDKCY QFALGQQGTTL NNGHSNDTVH DRTPYRTLLM NELGVPHLG TKQVCIAWSS 180
SSCHDGKAHL HVCVTGDDGN ATASFIYNGR LVDSIGSWSK KILRTOSEC VCINGTCVV 240
MTDGSASGKA DTKLIFIEEG KIVHTSLLSG SAQHVEECSE YPRYPGVRCV CRDNWKGSNR 300
PIVDINVHDY SIVSSYVCSSG LVGDTPRKND SSSSSHCLDP NNEEGHHGVK GWAFDGDNDV 360
WMGRTISEKL RSGYETFKVI EGWSKPNDSL QINRQVIVDR GNRSGYSGIF SVEGKSCINR 420
CFYVELIRGR NQETEVLWTS NSIVVFCGTS GTYGTGSWPD GADINLMPI 469
```

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SEQ ID NO: 58 moltype = AA length = 759
FEATURE Location/Qualifiers
source 1..759
mol_type = protein
organism = Influenza virus
SEQUENCE: 58
MERIKELRLN MSQSRTREIL TKTTVDHMAI IKKYTSRQE KNPALRMKWM MAMKYPITAD 60
KRITEMIPER NEQGQTLWSK MNDAGSDRVM VSPLAVTWWN RNPGPMNTNVH YPKIYKTYFE 120
RVERLKHCFT GPVHFRNQVK IRRVDINPQ HADLSAKEAQ DVIMEVVFVN EVGARILTSE 180
SQLTTKEKK EELQDCKISP LMVAYMLERE LVRKTRFLPV AGGTSSVYIE VLHLTQGTCW 240
EQMYTPGGEV KNDDVDQSLI IAARNIVRRA AVSADPLASL LEMCHSTQIG GIRMVLDILKQ 300
NPTEEQAVDI CKAAMGLRIS SSFSGGGFTF KRTSGSSVKK EEEVLTGNLQ TLKIRVHEGS 360
EEFTMVGRRA TAILRKATRR LIQLIVSGRD EQSIAEAIIV AMVFSQEDCM IKAVRGDLNF 420
VNFRANQRNLP MHQLLRHDFQK DAKVLFQNWG VEPIDNVVMG IGILPDMPSTS IEMSMRGVRI 480
SKMGVDEYSS TERVVVSIDR FLRVRDQRGN VLLSPEEVSE TGQTEKLITIT YSSSMMWIEIN 540
GPESVSLVNTY QWIIRNWETV KIQWSQNPTM LYNKMEFEPF QSLVPKAIRG QYSGFVRTLF 600
QQMQRDVLGTF DTQAIKLLP FAAAAPPQSR MFQSSFTVNV RGSGMRILVR GNSPVFNYNK 660
ATKRLTVLGK DAGLTLEDPD EGTAEVESAV LRGFLILGKE DRRYGPALSI NELSNLAKGE 720
KANVLIGQGD VVLMVMKRKD SSIILTDSQTA TKRIRMAIN 759
```

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SEQ ID NO: 59 moltype = AA length = 757
FEATURE Location/Qualifiers
source 1..757
mol_type = protein
organism = Influenza virus
SEQUENCE: 59
MDVNPTLFL KVPAQNAIST TFPYTGDPY SHGTGTGYTM DTVNRTHQYS EKGRWTTNT 60
TGAQPNLDP GPLPEDNEPS GYAQTDCVLE AMAFLEESH P GIFTSCIET MEVVQQTTRVD 120
KLTQGRQTYD WTLNRNQPA TA LANTIEVF RSNGLTANES GRLIDFLKDV MESMKKEEMG 180
ITTHFQRKRR VRDNMTKKM TQRTIGKRKQ RLNKRGYLIR ATLNTMTKD AERGKLKRR 240
IATPGQMIRG FVYFVETLAR SICEKLEQSG LPVGGNEKKA KLANVVRKMM TNSQDTELSF 300
TITGDNTKWN ENQNPRMFLA MITYMTRNQP EWFRNVLSSA PIMFSNKMAR LGKGYMFESK 360
SMKLRQKIPA EMLASIDLKY FNDSTRKKIE KIRPLLIEGT ASLSPGMMMG MFNMNLSTVLG 420
VSILNLQOKR YTKTYYWWDG LQSSDDFAI VNAPNHEGIQ AGVDRFYRTC KLLGINMSKK 480
KSYINRTGTF EFTSFFYRYG FVANFSMELP SFGVSGINES ADMSIGVTI KNNMINNDLG 540
PATAQMALQL PIKDYRRTYR CHRGDTQIQT RRSFEIKKLW EQTRSAGLL VSDGGPNLYN 600
IRNLHIEPEVC LKWELMDEDY QGRLCNPMLP FVSHKEIESM NNAVMMPAHG PAKNMEYDAV 660
ATTHSWIPKR NRSILNTSQR GVLEDEQMYQ RCCNLFEKFF PSSSYRRPVG ISSMVEAMVS 720
RARIDARIDF ESGRICKEEF TEIMKICSTI EELRRQK 757
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SEQ ID NO: 60 moltype = AA length = 716
FEATURE Location/Qualifiers
source 1..716
mol_type = protein
organism = Influenza virus
SEQUENCE: 60
MEDFVRQCFN PMIVELAEGT MKEYGEDLKI ETNKFAAICL HLEVCFCMYSDFHFINEQGES 60
IIVELGDPNA LLKHRFEIIE GRDRTMAWTV VNSICNTTGAEKPKFLPDLY DYKENRFIEI 120
GVTRREVHIY YLEKANKIKS EKTHIIFSF TGEEMATRAD YTLDEESRAR IKTRLFTIRQ 180
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EMASRGLWDS	PRQSERGEET	IEERFEITGT	MRKLADQSLP	PNFSSLENFR	AYVDGFEPNG	240
YIEGKLSQMS	KEVNARIEPF	LKTTPRPLRL	PNGPPCSQRS	KFLLMDALKL	SIEDPSHEGE	300
GIPLYDAIKC	MRTFFGWKEP	NVVKPHEKG	NPNYLLSWKQ	VLAELQDIEN	EEKIPKTKNM	360
KKTSQKWL	GENMAPEKVD	FDDCKDVGDL	KQYDSDEPEL	RSLASWIQNE	FNKACELTDS	420
SWIELDEIGE	DVAPIEHIAS	MRRNYFTSEV	SHCRATEYIM	KGVYINTALL	NASCAAMDDF	480
QLIPMISKCR	TKEGRKTNL	YGFIIKGRSH	LRNDTDVVNF	VSMEFSLTDP	RLEPHKWEKY	540
CVLEIGDMLL	RSAIGQVSRP	MFLYVRTNGT	SKIKMKWGME	MRRCLLQSLQ	QIESMIEAES	600
SVEKEKDMLKE	FFENKSETWP	IGESPKGVEE	SSIGKVCRTL	LAKSVFNLSY	ASPQLEGFS	660
ESRKLLLIVQ	ALRDNLEPGT	FDLGGLYEAI	EECLINDPWV	LLNANSWFNSF	LTHALS	716
SEQ ID NO: 61		moltype = AA	length = 498			
FEATURE		Location/Qualifiers				
source		1..498				
		mol_type = protein				
		organism = Influenza virus				
SEQUENCE: 61						
MASQGTKRSY	EQMETDGERQ	NATEIRASVG	KMIGGIGRKY	IQMCTELKLS	DYEGRLLIQLNS	60
LTIERNVLSA	FDERRNKYLE	EHPSAGKDPK	KTGGPIYRRV	NGKWMRELIL	YDKEEIRRIW	120
RQANNGDDAT	AGLTHMMIWH	SNLNDATYQR	TRALVRTGM	PRMCISLMQGS	TLPRRSGAAG	180
AAVKGVGTMV	MELVRMIKRG	INDRNFWRGE	NGRKTRIAYE	RMCNILKGKF	QTAAQKAMMD	240
QVRESRNPGN	AEEFDLTFLA	RSALILRGSV	AHKSCLPACV	YGPAVASGYD	FEREGRYSLVG	300
IDPFRLLQNS	QVYSLIRPNE	NPAHSQLVW	MACHSAAFED	LRVLSFIKGT	KVVPRGKLST	360
RGVQIASNEN	METMESSTLE	LRSRYWAIRT	RSGGNTNQQR	ASAGQSIQP	TFSVQRNLPF	420
DRITVMAFT	GNTTEGRTSDM	RTEIIRMMES	ARPEDVSPQG	RGVFELSDEK	AASPIVPSFD	480
MSNEGSYFFG	DNAEYDN					498
SEQ ID NO: 62		moltype = AA	length = 252			
FEATURE		Location/Qualifiers				
source		1..252				
		mol_type = protein				
		organism = Influenza virus				
SEQUENCE: 62						
MSLLTEVETY	VLSIIPSGPL	KAEIAQRLED	VFAKGKNTDLE	VLMEWLKTRP	ILSPLTKGIL	60
GFVFVTLTVPS	ERGLQRRRFV	QNALNNGNDP	NNMDKAVKLY	RKLKREITFH	GAKEISLSYS	120
AGALASCML	IYNRNGAVTT	EVAFLVCAT	CEQIADSQHR	SHRQMVTTN	PLIRHENRMV	180
LASTTAKAME	QMAGSSEQAA	EAMEVASQAR	QMVGQAMRTIG	THPSSSAGLK	NDLLENLQAY	240
QKRMGVQMQR	PK					252

1. An isolated recombinant influenza virus comprising a selected NA viral segment encoding a plurality of selected residues or a deletion of residues in NA, wherein the selected NA viral segment does not encode a NA having a threonine at residue 32, does not encode a NA having an aspartic acid at position 147, does not encode a NA having a threonine at position 148, does not encode a NA having an aspartic acid at position 151, does not encode a NA having an asparagine at position 245, does not encode a NA having an asparagine at residue 329, does not encode a NA having a glycine at position 346, does not encode a NA having a histidine at residue 347, or encodes a NA having a deletion of one or more of residues 46 to 50, or any combination thereof, wherein the numbering is based on N2, wherein the recombinant influenza virus has enhanced replication in avian eggs or has a reduction in HA mutations when grown in avian eggs relative to a corresponding influenza virus that has a NA that encodes a threonine at residue 32, does not have a deletion of residues 46 to 50, encodes an aspartic acid at position 147, encodes a threonine at residue 148, encodes an aspartic acid at residue 151, encodes an asparagine at residue 245, encodes an asparagine at residue 329, encodes a glycine at residue 346, encodes a histidine at residue 347, or any combination thereof.

2. The isolated recombinant influenza virus of claim 1 wherein the NA viral segment encodes a NA that has at least 90% amino acid sequence identity to SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:48, SEQ ID NO:49, SEQ ID NO:50 or SEQ ID NO:54.

3. The isolated recombinant influenza virus of claim 1 wherein the NA viral segment encodes a N2, N3, N7, or N9.

4. The isolated recombinant influenza virus of claim 1 wherein the residue at position 32 is A, I, G, or L; the deletion is a deletion of residues 46 to 50; the residue at position 147 is N or Q; the residue at position 148 is K, R or H; the residue at position 151 is E, N or Q; the residue at position 245 is S, T, I, L, A, N, W, Y, P, V, or G; the residue at position 329 is D or E; the residue at position 346 is S, T, P, Y, W, A, N, I, L, or V; the residue at position 347 is G, Q, S, T, Y, C or W; or any combination thereof.

5. The isolated recombinant influenza virus of claim 1 wherein the residue at position 147 is N or Q, the residue at position 329 is D or E, the residue at position 347 is G or Q, or any combination thereof.

6. The isolated recombinant influenza virus of claim 1 wherein the residue at position 148 is K, R or H, the residue at position 151 is E, N or Q, the residue at position 245 is S, T, I, L, A, or V, or any combination thereof.

7. The isolated recombinant influenza virus of claim 1 wherein the selected NA viral segment does not encode a NA having an aspartic acid at position 147, does not encode a NA having an asparagine at residue 329, does not encode a NA having a histidine, arginine or asparagine at residue 347, or any combination thereof.

8. The isolated recombinant influenza virus of claim 1 wherein the selected NA viral segment does not encode a NA having a threonine at position 148, does not encode a NA having an aspartic acid at position 151, does not encode a NA having an asparagine at position 245, does not encode a NA having a glycine at position 346, or any combination thereof.

9. The isolated recombinant influenza virus of claim **1** which comprises PA, PB1, PB2, NP, M, and NS viral segments having at least 85% nucleic acid sequence identity to SEQ ID Nos. 24 to 29 or 39 to 44 or encoding a polypeptide having at least 80% amino acid sequence identity to a polypeptide encoded by SEQ ID Nos. 24 to 29 or 39 to 44.

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 encodes a NA having a plurality of selected residues or a deletion of residues, wherein the NA does not encode a NA having a threonine at residue 32, does not encode a NA having an aspartic acid at position 147, does not encode a NA having a threonine at position 148, does not encode a NA having an aspartic acid at position 151, does not encode a NA having an asparagine at position 245, does not encode a NA having an asparagine at residue 329, does not encode a NA having a glycine at position 346, does not encode a NA having a histidine at residue 347, or encodes a NA having a deletion of one or more of residues 46 to 50, or any combination thereof, wherein the numbering for NA residues is that for N2; 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 NA has at least 90% amino acid sequence identity to SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:48, SEQ ID NO:49, or SEQ ID NO:54.

12. The method of claim **10** wherein the residue at position 147 is N or Q; the residue at position 329 is D or E; the residue at position 347 is Q, N, S, T, Y, C or W; the residue at position 151 is E, N or Q; the residue at position 148 is K, R or H; the residue at position 245 is S, T, I, L, A, N, W, Y, P, V, or G.

13. The method of any one of claim **10** wherein the virus comprises PA, PB1, PB2, NP, M, and NS viral segments having at least 85% nucleic acid sequence identity to SEQ ID Nos. 24 to 29 or 39 to 44 or encoding a polypeptide having at least 80% amino acid sequence identity to a polypeptide encoded by SEQ ID Nos. 24 to 29 or 39 to 44.

14. Isolated virus prepared by the method of claim **10**.

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

16. The method of claim **15** wherein the composition comprises at least one other different influenza virus.

17. The method of claim **15** wherein the mammal is a human.

18. The method of claim **15** wherein the composition is administered intranasally.

19. The method of claim **15** wherein the composition is administered via injection.

20. A method comprising passaging the virus of claim **1** in eggs.

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