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(54) **Title**: METHODS AND COMPOSITIONS RELATED TO INCREASING THE FIDELITY OF INFLUENZA A VIRUS FOR VACCINE DEVELOPMENT

(57) **Abstract**: Disclosed are compositions and methods for related to mutant influenza viruses with increased fidelity.

METHODS AND COMPOSITIONS RELATED TO INCREASING THE FIDELITY OF INFLUENZA A VIRUS FOR VACCINE DEVELOPMENT

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I. BACKGROUND

1. Yearly vaccination against influenza has been recently recommended by the CDC for all persons. Two different types of vaccination are currently available, the Live Attenuated Influenza Vaccine (LAIV) and the Trivalent Inactivated Vaccine (TIV). Both are multivalent and cover both Influenza A and B viruses believed to circulate in the population the following year. The comparative efficacy of LAIV and TIV has recently been examined in two meta-analyses - and LAIV has been shown to possess efficacy of 83% in children, while the efficacy of TIV was between 59-65%. LAIV has the added benefit of being administered through nasal spray in contrast to the injection based TIV. The combination of greater efficacy and nasal administration rather than intramuscular injection has led LAIV to be the preferred vaccine for children 2-8 years in Canada, Germany and the UK. Nevertheless, current live attenuated influenza vaccines (LAIV) are not sufficiently attenuated or stable for administration to children under the age of 2, pregnant women, persons with compromised immunity, persons with asthma, or persons at high risk for complications from influenza. However, these same groups of people are at high risk for complications from influenza. What are needed are new stable attenuated influenza viral strains for use in vaccines.

II. SUMMARY

2. Disclosed are methods and compositions related to mutant influenza viruses with increased fidelity. In one aspect, the disclosed mutant influenza viruses can increase the stability of existing Influenza virus vaccines.

3. In one aspect, disclosed herein are modified influenza A viruses comprising one or more mutations in the influenza RNA polymerase, wherein the one or more mutations causes an increased fidelity of the polymerase.

4. Also disclosed, in one aspect, are the modified influenza A virus of any preceding aspect, wherein the one or more mutations of the influenza RNA polymerase comprises at least one mutation in the PB1 and/or PA subunit of the RNA polymerase.

5. Also disclosed, in one aspect, are pharmaceutical composition comprising the modified influenza virus of any preceding aspect and methods of immunizing a subject against

influenza virus; inducing an immune response to influenza virus in a subject; and/or inhibiting an influenza virus infection in a subject comprising administering to the subject the influenza virus and/or composition of any preceding aspect.

6. Also disclosed are recombinant nucleic acids encoding an influenza RNA polymerase with increased transcriptional fidelity wherein the RNA polymerase comprises at least one mutation selected from the PB1 and/or PA subunits of the RNA polymerase as well as vectors comprising the recombinant nucleic acid.

7. In one aspect, also disclosed are methods of increasing the immunogenicity of an attenuated influenza vaccine comprising obtaining an attenuated influenza vaccine viral strain and generating a mutation in the PB1 and/or PA subunit of the RNA polymerase of the influenza virus, wherein the mutation increases the fidelity of the RNA polymerase; and wherein an increase in fidelity of the polymerase increases the stability of the virus strain in a subject and reduces the amount mutant viruses formed during transcription of the virus thereby increasing the abundance of a single strain and as a result increases the immune response to the immunizing strain.

III. BRIEF DESCRIPTION OF THE DRAWINGS

8. The accompanying drawings, which are incorporated in and constitute a part of this specification, illustrate several embodiments and together with the description illustrate the disclosed compositions and methods.

9. Figure 1 shows the selected residues for initial mutation. The crystal structure of 4WSB was analyzed using PyMol and the desired mutations are visualized with respect to the nucleotide channel. Charge altering entrance mutants (PB1 - 45R, 308K, 387K, and 391K) are highlighted in yellow and internal helix displacing mutations (PB1 -43V; PA - 663R and 664K)(nonmutated residue indicated) are highlighted in red. PA is depicted in green, PB1 in can, and PB2 in pink. Figure 1A shows the full crystal structure. Figure 1B shows a view up through the nucleotide channel. Figure 1C shows a closer view of the mutations in respect to the channel entrance.

10. Figure 2 shows the temperature sensitivity of mutant viruses. Figure 2A shows PA R663K is temperature sensitive by both transcription and replication based minigenomes. Confluent monolayers of 293T cells were co-transfected with pDZ plasmids encoding the control (guassia luciferase driven by CMV IE promoter). After 24hrs at 33, 37, or 39°C, cells were lysed with Passive Lysis Buffer (Promega) and luciferase levels were measured according to the manufacurer's instructions (Promega). Experiments were performed in triplicate and were independently repeated twice. Statistics were performed on GraphPad Prism (one-way ANOVA

followed by Tukey's posttest). Figure 2B shows K387D and K391D have increased titers at 96 and 72 hours respectively. Confluent monolayers of A549 cells were infected at an MOI of 0.001 at 37°C with the indicated viruses. 10% of the supernatant was harvested and replaced at the indicated time points. Samples were clarified by centrifugation and stored at -80°C. Viruses were tittered by $TCID_{50}$ on A549 cells and read by hemagglutination assay 5 days post infection. Statistics were performed on GraphPad Prism (two-way ANOVA followed by Bonferroni's post test). Figure 2C shows that R663K is temperature sensitive. Assay was performed as in B with the following exception: after 1hr infection samples were placed at either 33, 37, or 39°C. Statistics were performed on GraphPad Prism (two-way ANOVA followed by Bonferroni's post test).

11. Figures 3 shows the results of minigenome assays. Briefly, 293T cells were grown on poly D—lysine coated plates and transfected via PEI with 200ng of each polymerase component. A luciferase reporter gene expressing the RNA of the coding region of firefy luciferase flanked by the non coding3' and 5' sequence of influenza NP was used . The production of Renilla from a constituivitely active promoter was used as a control for cell density and transfection efficiency. Guanosine and Ribavirin were each added 3hrs pretransfection and maintained for the course of the experiment. All activities were initially assayed as fold induction or the increase in activity over a –PB1 control. Activities were then then normalized to the 100% in the presence of no drug and the % retained titer was then calculated.

12. Figure 4 shows virus drug resistance. A549 and MDCK cells were infected at a multiplicity of infection (MOI) of 0.1 for 1h, washed once with PBS, and then cultured at 37°C in DMEM containing 0.3% BSA and TPCK treated-trypsin at 1 μg/ml. The indicated drugs were added 3 hour pre-infection and maintained. Supernatant was harvested at 48 hours post infection and viral titers were determined via TCID-50 measurements as described by Reed and Muench. (1) Data is displayed as the decrease in titer from no drug.

13. Figure 5 shows that nucleotide imbalance drives the increased activity of the mutant polymerase. Cells were incubated as in Figure 2 with the following alterations. 293T cells were pretreated with 40μM guanosine (A) or 40μM guanosine and increasing concentrations of ribavirin (B). Fold induction was then normalized to activity at 0μM guanosine (A) or 0μM ribavirin/40μM guanosine (B). Statistics were performed on GraphPad Prism (one-way ANOVA followed by Tukey's post test). ** $p<0.001$.

14. Figure 6 shows the sequencing of viruses. MDCK cells were infected at an MOI of 0.05 with the indicated viruses and incubated at 37°C. 48 hours later supernatant was harvested a

stock aliquotted for titering and RNA purified. Segment 8 was amplified and inserted in to a vector for clonal sanger sequencing. Only unique mutations detected in both the F and R reverse reads were counted.

IV. DETAILED DESCRIPTION

15. Before the present compounds, compositions, articles, devices, and/or methods are disclosed and described, it is to be understood that they are not limited to specific synthetic methods or specific recombinant biotechnology methods unless otherwise specified, or to particular reagents unless otherwise specified, as such may, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only and is not intended to be limiting.

A. Definitions

16. As used in the specification and the appended claims, the singular forms "a," "an" and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a pharmaceutical carrier" includes mixtures of two or more such carriers, and the like.

17. Ranges can be expressed herein as from "about" one particular value, and/or to "about" another particular value. When such a range is expressed, another embodiment includes from the one particular value and/or to the other particular value. Similarly, when values are expressed as approximations, by use of the antecedent "about," it will be understood that the particular value forms another embodiment. It will be further understood that the endpoints of each of the ranges are significant both in relation to the other endpoint, and independently of the other endpoint. It is also understood that there are a number of values disclosed herein, and that each value is also herein disclosed as "about" that particular value in addition to the value itself. For example, if the value "10" is disclosed, then "about 10" is also disclosed. It is also understood that when a value is disclosed that "less than or equal to" the value, "greater than or equal to the value" and possible ranges between values are also disclosed, as appropriately understood by the skilled artisan. For example, if the value "10" is disclosed the "less than or equal to 10"as well as "greater than or equal to 10" is also disclosed. It is also understood that the throughout the application, data is provided in a number of different formats, and that this data, represents endpoints and starting points, and ranges for any combination of the data points. For example, if a particular data point "10" and a particular data point 15 are disclosed, it is understood that greater than, greater than or equal to, less than, less than or equal to, and equal to 10 and 15 are considered disclosed as well as between 10 and 15. It is also understood that each

unit between two particular units are also disclosed. For example, if 10 and 15 are disclosed, then 11, 12, 13, and 14 are also disclosed.

18. In this specification and in the claims which follow, reference will be made to a number of terms which shall be defined to have the following meanings:

19. "Optional" or "optionally" means that the subsequently described event or circumstance may or may not occur, and that the description includes instances where said event or circumstance occurs and instances where it does not.

20. Throughout this application, various publications are referenced. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this pertains. The references disclosed are also individually and specifically incorporated by reference herein for the material contained in them that is discussed in the sentence in which the reference is relied upon.

B. Compositions

21. Disclosed are the components to be used to prepare the disclosed compositions as well as the compositions themselves to be used within the methods disclosed herein. These and other materials are disclosed herein, and it is understood that when combinations, subsets, interactions, groups, etc. of these materials are disclosed that while specific reference of each various individual and collective combinations and permutation of these compounds may not be explicitly disclosed, each is specifically contemplated and described herein. For example, if a particular influenza virus, influenza virus polymerase, influenza virus polymerase PB1 subunit, or influenza virus polymerase PA subunit is disclosed and discussed and a number of modifications that can be made to a number of molecules including the influenza virus, influenza virus polymerase, influenza virus polymerase PB1 subunit, or influenza virus polymerase PA subunit are discussed, specifically contemplated is each and every combination and permutation of influenza virus, influenza virus polymerase, influenza virus polymerase PB1 subunit, or influenza virus polymerase PA subunit and the modifications that are possible unless specifically indicated to the contrary. Thus, if a class of molecules A, B, and C are disclosed as well as a class of molecules D, E, and F and an example of a combination molecule, A-D is disclosed, then even if each is not individually recited each is individually and collectively contemplated meaning combinations, A-E, A-F, B-D, B-E, B-F, C-D, C-E, and C-F are considered disclosed. Likewise, any subset or combination of these is also disclosed. Thus, for example, the sub-group of A-E, B-F, and C-E would be considered disclosed. This concept applies to all aspects of this application including, but not limited to, steps in methods of making and using the disclosed compositions. Thus, if there are a variety of additional steps that can be

performed it is understood that each of these additional steps can be performed with any specific embodiment or combination of embodiments of the disclosed methods.

22. The influenza viral polymerase is a RNA-dependent RNA polymerase (RdRP) consisting of three subunits PB1, PB2, and PA and is responsible for the transcription and replication of the viral genome. RNA viruses have long been assumed to have polymerases high error rate as epitomized by HIV-1. The large number of errors of the RNA polymerase results in inaccurate replication of the RNA transcript. This high error rate of the polymerase is referred to as low fidelity; where fidelity is a relative measure of the ability of a polymerase to accurately copy the nucleic acid template. A polymerase with low fidelity has a high error rate and a polymerase with high fidelity has a small error rate. This compounded with a poor to nonexistent proofreading ability of the polymerase can lead to mutations in immunogenic epitopes and ultimately viral escape. Hence, the majority of newly manufactured influenza viruses are mutants; which causes antigenic drift, that is, a slow change in the antigens on the viral surface over time.

23. As noted above, the low fidelity of the influenza virus polymerase is largely responsible for antigenic drift of an influenza virus. The antigenic drift makes preventing, inhibiting, or treating influenza particularly troublesome as mutants which have changes at antigenic epitopes can escape host immune responses. Also, due to the low fidelity of the polymerase, live attenuated influenza vaccine (LAIV) can be unstable. Fidelity altering mutations are normally associated with effects on the binding of either viral template nucleic acids or incoming nucleotides, and several high fidelity virus mutants are shown herein to be attenuated in vivo, likely due to their inability to respond to selective pressures in the host. As a result, alterations to fidelity have been used to create novel vaccine candidate described herein. In one aspect, disclosed herein are modified influenza A viruses comprising one or more mutations in the influenza RNA polymerase, wherein the one or more mutations causes an increased fidelity of the polymerase.

24. As used throughout, any influenza A virus can be modified to comprise a PB1 or PA subunit with one or more mutations disclosed herein. For example, the influenza A virus can be selected from the group consisting of an H2N2 virus, an H3N2 virus, an H1N1 virus, an H9N2 virus and an H5N1 virus. Optionally, the influenza A virus can be selected from the group consisting of A/Puerto Rico/9/1934 (H1N1), A/ Ann Arbor/6/60, A/California/04/2009, A/bat/Guatemala/060/2010(H17N10), A/Wisconsin/22/201 1 and A/Quail/Hong Kong/Gl/97. The influenza A virus can also be an avian influenza A virus. These include, but are not limited to, A/Chi cken/Nanchang/3- 120/01 H3N2, A/Hong Kong/485/ 1997(H5N1), A/Anhui/ 1 /2013

(H7N9) and A/Shanghai/ 1/2013 (H7N9). It is understood and herein contemplated that the PB1 and PA polymerase subunits have strain variants. Accordingly, all mutation locations disclosed herein are based on the position of a reference PB1 or PA sequence from A/Puerto Rico/9/1934 (H1N1) referred to herein as PR8 strain. It is understood and herein contemplated that the skilled artisan can easily identify the corresponding residue from any other influenza viral strain.

25. In addition to the disclosed mutations to PB1 and PA, resassortant influenza A viruses comprising one or more genomic segments from one or more influenza A viruses are also contemplated. More specifically, the virus includes genetic and/or polypeptide components derived from more than one parental viral strain or source. For example, a 7: 1 reassortant includes 7 viral genomic segments (or gene segments) derived from a first parental virus and a single complementary viral genomic segment, e.g., encoding hemagglutinin or neuraminidase, from a second parental virus. A 6:2 reassortant includes 6 genomic segments, most commonly the 6 internal genes from a first parental virus, and two complementary segments, e.g., hemagglutinin and neuraminidase, from a different parental virus. Optionally, reassortant viruses are produced by introducing vectors including the six internal genes of a viral strain selected for its favorable properties regarding vaccine production, in combination with the genome segments encoding the surface antigens (HA and NA) of a selected, e.g., pathogenic strain. For example, the HA segment can be selected from an HI , H3 or B strain, as is routinely performed for vaccine production. Similarly, the HA segment can be selected from other pathogenic strains such as an H2 strain (e.g., H2N2), an H5 strain (e.g., H5N1), an H7 strain (e.g., H7N7) or an H9 strain (H9N2). In certain modified viruses, the internal gene segments are derived from the influenza PR8 strain.

26. Herein a “mutation” refers to a non-naturally occurring change in the nucleic acid or amino acid sequence and is produced by human intervention (e.g., by mutagenesis of cloned DNA, RNA, cDNA, or amino acid sequences), such as induced point mutation, deletion, insertion and substitution mutants. Amino acid sequence mutations typically fall into one or more of three classes: substitutional, insertional or deletional mutations.

27. Not every mutation in PB1, PB2, or PA will affect the polymerase activity in a manner that will increase fidelity. Specifically contemplated are mutations of the RNA polymerase subunits that increase fidelity. The increase in fidelity can be due to a difference in the side chain in the mutated residue which constricts the entry to the nucleotide channel or constricts the channel itself or alters the charge at the entry of or within the channel. The increase in fidelity can also occur due to conformational change in the binding pocket which alters which amino acid side chains are exposed and changes contact residues thus constricting

the channel or changing charges. It is understood and herein contemplated that mutations that result in a change in the size of a side chain can also negatively affect fidelity by preventing or significantly effecting the rate of transcription or replication if too large (low to no fidelity because little to no transcription or replication), or increases access to the template binding pocket such that transcription and replication have lower fidelity. It is also that a mutation that results in a change in charge, hydrophobicity, or polarity can reduce binding of the template strand significantly enough to stop transcription or replication or decrease fidelity. Moreover, mutations that are located in regions of the subunit spatially removed from the template groove may not have an effect on fidelity.

28. The mutation(s) of the modified viruses disclosed herein can be in any subunit of the influenza viral polymerase, for example, the PB1, PB2, and/or PA subunit. Thus, in one aspect, disclosed herein are herein are modified influenza A viruses comprising one or more mutations in the influenza RNA polymerase, wherein the one or more mutations of the influenza RNA polymerase comprises at least one mutation in the PB1 and/or PA subunit of the RNA polymerase, and wherein the one or more mutations causes an increased fidelity of the polymerase.

29. In one aspect, the one or more mutations of the influenza RNA polymerase can comprise a mutation in the PB1 subunit. In one aspect, the one or more mutations can be located at a residue corresponding to residues 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 77, 78, 79, 80, 228, 229, 230, 231, 232, 233, 234, 235, 236, 237, 238, 239, 240, 306, 307, 308, 309, 310, 311, 383, 384, 385, 386, 387, 388, 389, 390, 391, 392, 393, 394, 395, 480, and/or 481 of SEQ ID NO: 4. For example, disclosed herein are modified influenza viruses comprising at least one mutation at one or more residues of the PB1 subunit, wherein the mutated residue corresponds to residues 43, 45, 235, 237, 239, 308, 387, 391, 480, and/or 481 of SEQ ID NO: 4, and wherein the mutation increases fidelity of the influenza RNA polymerase.

30. PB1 is a 757 amino acid enzyme and is the minimum structural component of the influenza viral RNA polymerase and is necessary for functionality. The secondary structure of the PB1 subunit is comprised of alpha helices, loops, beta sheets, and a beta stem loop. In one aspect, disclosed herein are modified influenza viruses comprising at least one mutation at one or more residues of an alpha helix of the PB1 subunit of the influenza RNA polymerase. It is understood and herein contemplated that PB1 has a number of alpha helices in its secondary structure. For example, there is an alpha helix located at the residues of the PB1 subunit corresponding to residues 36 to 50 of SEQ ID NO: 4, as well as, an alpha helix located at the residues of the PB1 subunit corresponding to residues 383 to 395 of SEQ ID NO: 4. Thus, the

modified influenza virus can comprise a RNA polymerase with one or more mutations corresponding to residues 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 383, 384, 385, 386, 387, 388, 389, 390, 391, 392, 393, 394, and/or 395 of the PB1 subunit of the influenza virus RNA polymerase as set forth in SEQ ID NO: 4. However, the mutation is not a Valine to Isoleucine substitution at a residue corresponding to residue 43 of SEQ ID NO: 4 when only one fidelity increasing mutation is present. For example, disclosed herein are modified influenza viruses comprising one or more mutations in the influenza RNA polymerase, wherein the one or more mutations of the influenza RNA polymerase comprises at least one mutation in the PB1 or PA subunit of the RNA polymerase, wherein the influenza RNA polymerase comprises a mutation at one or more residues of an alpha helix of the PB1 subunit of the influenza RNA polymerase as set forth in SEQ ID NO: 4, wherein the influenza RNA polymerase comprises a mutation at one or more residues of the alpha helix corresponding to residues 36 to 50 of the alpha helix of the PB1 subunit as set forth in SEQ ID NO: 4, wherein the one or more mutations causes an increased fidelity of the polymerase, wherein when one or more mutations is a single substitution at a residue corresponding to residue 43 of SEQ ID NO: 4, the substitution at residue 43 is not a Valine to Isoleucine substitution.

31. Also disclosed are modified influenza viruses comprising at least one mutation at one or more residues of a loop of the PB1 subunit of the influenza RNA polymerase as set forth in SEQ ID NO: 4, wherein the one or more mutations causes an increased fidelity of the polymerase. It is understood and herein contemplated that PB1 has a number of loops in its secondary structure. For example, there is are loops located at the residues of the PB1 subunit corresponding to residues 77 to 80, 228 to 240, and 306 to 311 as set forth in SEQ ID NO: 4. Thus, the modified influenza virus can comprise a RNA polymerase with one or more mutations in one or more loops located at a residue corresponding to residues 77, 78, 79, 80, 228, 229, 230, 231, 232, 233, 234, 235, 236, 237, 238, 239, 240, 306, 307, 308, 309, 310, and/or 311 of the PB1 subunit of the influenza virus RNA polymerase as set forth in SEQ ID NO: 4. In one aspect, disclosed herein are modified influenza viruses comprising at least one mutation at one or more residues of a loop of the PB1 subunit of the influenza RNA polymerase as set forth in SEQ ID NO: 4, wherein the mutation is at one or more residues corresponding to residues 235, 237, 239, and/or 308 as set forth in SEQ ID NO: 4.

32. It is also contemplated herein that the particular mutation of the modified influenza virus polymerase may not be associated with any known secondary structure of PB1. For example, the mutation can be at a residue corresponding to residues 480 and/or 481 of the PB1 subunit of the influenza virus RNA polymerase as set forth in SEQ ID NO: 4. Thus, in one

aspect, disclosed herein are modified influenza viruses comprising at least one mutation at one or more residues of PB1, wherein the mutation is at a residue corresponding to residues 480 and/or 481 of PB1.

33. It is contemplated herein that any of the PB1 mutations disclosed herein can be used in combination with any of the other PB1 mutations disclosed herein as well as any of the PA mutations disclosed. For example, the modified influenza viruses can comprise an RNA polymerase comprising one or more mutations of the PB1 subunit wherein the mutations comprise a mutation in an alpha helix corresponding to residues 36 to 50 as set forth in SEQ ID NO: 4 and a mutation in an alpha helix corresponding to residues 383 to 395 as set forth in SEQ ID NO: 4; a mutation in an alpha helix corresponding to residues 36 to 50 as set forth in SEQ ID NO: 4 and a mutation in a loop corresponding to residues 77 to 80 as set forth in SEQ ID NO: 4; a mutation in an alpha helix corresponding to residues 36 to 50 as set forth in SEQ ID NO: 4 and a mutation in a loop corresponding to residues 228 to 240 as set forth in SEQ ID NO: 4; a mutation in an alpha helix corresponding to residues 36 to 50 as set forth in SEQ ID NO: 4 and a mutation in a loop corresponding to residues 306 to 311 as set forth in SEQ ID NO: 4; a mutation in an alpha helix corresponding to residues 36 to 50 as set forth in SEQ ID NO: 4 and a mutation corresponding to residues 480 and/or 481 as set forth in SEQ ID NO: 4; a mutation in an alpha helix corresponding to residues 383 to 395 as set forth in SEQ ID NO: 4 and a mutation in a loop corresponding to residues 77 to 80 as set forth in SEQ ID NO: 4; a mutation in an alpha helix corresponding to residues 383 to 395 as set forth in SEQ ID NO: 4 and a mutation in a loop corresponding to residues 228 to 240 as set forth in SEQ ID NO: 4; a mutation in an alpha helix corresponding to residues 383 to 395 as set forth in SEQ ID NO: 4 and a mutation in a loop corresponding to residues 306 to 311 as set forth in SEQ ID NO: 4; a mutation in an alpha helix corresponding to residues 383 to 395 as set forth in SEQ ID NO: 4 and a mutation corresponding to residues 480 and/or 481 as set forth in SEQ ID NO: 4; a mutation in a loop corresponding to residues 77 to 80 as set forth in SEQ ID NO: 4 and a mutation in a loop corresponding to residues 228 to 240 as set forth in SEQ ID NO: 4; a mutation in a loop corresponding to residues 77 to 80 as set forth in SEQ ID NO: 4 and a mutation in a loop corresponding to residues 306 to 311 as set forth in SEQ ID NO: 4; a mutation in a loop corresponding to residues 77 to 80 as set forth in SEQ ID NO: 4 and a mutation corresponding to residues 480 and/or 481 as set forth in SEQ ID NO: 4; a mutation in a loop corresponding to residues 228 to 240 as set forth in SEQ ID NO: 4 and a mutation in a loop corresponding to residues 306 to 311 as set forth in SEQ ID NO: 4; a mutation in a loop corresponding to residues 228 to 240 as set forth in SEQ ID NO: 4 and a mutation corresponding to residues 480 and/or 481 as set forth in SEQ ID NO: 4; and a mutation

in a loop corresponding to residues 306 to 311 as set forth in SEQ ID NO: 4 and a mutation corresponding to residues 480 and/or 481 as set forth in SEQ ID NO: 4.

34. It is further contemplated herein that the multiple mutations can occur within the same secondary structure. For example, disclosed herein are modified influenza viruses comprising an RNA polymerase comprising one or more mutations in the alpha helix corresponding to residues 36 to 50 of the PB1 subunit as set forth in SEQ ID NO: 4, one or more mutations in the alpha helix corresponding to residues 383 to 395 of the PB1 subunit as set forth in SEQ ID NO: 4; one or more mutations in the loop corresponding to residues 77 to 80 of the PB1 subunit as set forth in SEQ ID NO: 4, one or more mutations in the loop corresponding to residues 228 to 240 of the PB1 subunit as set forth in SEQ ID NO: 4, and/or one or more mutations in the loop corresponding to residues 306 to 311 of the PB1 subunit as set forth in SEQ ID NO: 4.

35. In one aspect, the one or more mutations of the influenza RNA polymerase can comprise a mutation in the PA subunit. In one aspect, the one or more mutations can be located at a residue corresponding to residues 654, 655, 656, 657, 658, 659, 660, 661, 662, 663, 664, 665, 666 ,667, 668, 669, 670, 671, 672, 673, 674, 682, 683, 684, 685, 686, 687, 688, 689, 690, 691, 692, and/or 693 of the PA subunit of the influenza virus RNA polymerase as set forth in SEQ ID NO: 3. For example, disclosed herein are modified influenza viruses comprising at least one mutation at one or more residues of the PA subunit, wherein the mutated residue corresponds to residues 661, 663, 664, and/or 691 of SEQ ID NO: 3, and wherein the mutation increases fidelity of the influenza RNA polymerase.

36. PA is a 716 amino acid enzyme. PA comprises the endonuclease activity of the RNA polymerase and is promotes the formation of the trimeric polymerase complex. The secondary structure of the PA subunit is comprised of alpha helices, loops, and beta sheets. In one aspect, disclosed herein are modified influenza viruses comprising at least one mutation at one or more residues of an alpha helix of the PA subunit of the influenza RNA polymerase as set forth in SEQ ID NO: 3. It is understood and herein contemplated that PA has a number of alpha helices in its secondary structure. For example, there is an alpha helix located at the residues of the PA subunit corresponding to residues 654 to 674 of SEQ ID NO: 3, as well as, an alpha helix located at the residues of the PA subunit corresponding to residues 682 to 693 of SEQ ID NO: 3. Thus, the modified influenza virus can comprise a RNA polymerase with one or more mutations corresponding to residues 654, 655, 656, 657, 658, 659, 660, 661, 662, 663, 664, 665, 666 ,667, 668, 669, 670, 671, 672, 673, 674, 682, 683, 684, 685, 686, 687, 688, 689, 690, 691, 692, and/or

693 of the PA subunit as set forth in SEQ ID NO: 3, wherein the one or more mutations causes an increase in the fidelity of the RNA polymerase.

37. It is contemplated herein that any of the PA mutations disclosed herein can be used in combination with any of the other PA mutations disclosed herein as well as any of the PB1 mutations disclosed. For example, the modified influenza viruses can comprise an RNA polymerase comprising one or more mutations of the PA subunit wherein the mutations comprise one or more mutations in an alpha helix corresponding to residues 654 to 674 as set forth in SEQ ID NO: 3 and/or one or more mutations in an alpha helix corresponding to residues 682 to 693 as set forth in SEQ ID NO: 3. For example, the one or more mutations can comprise a mutation at any combination of two or more residues two or more corresponding to residues 661, 663, 664, and/or 691 of SEQ ID NO: 3, wherein the one or more mutations causes an increase in the fidelity of the RNA polymerase.

38. As stated throughout this disclosure, the disclosed modified influenza virus can comprise any combination mutation in the PB1 and PA subunits disclosed herein. Thus, in one aspect, disclosed herein are modified influenza viruses comprising one or more mutations in the PB1 subunit of the RNA polymerase further comprising one or more mutations at one or more residues of the PA subunit of the RNA polymerase, wherein the mutations of the RNA polymerase causes an increase in fidelity of the polymerase. For example, disclosed herein are modified influenza viruses comprising one or more mutations of the PB1 subunit of the RNA polymerase including but not limited to a mutation in the alpha helix corresponding to residues 36 to 50 of SEQ ID NO: 4, a mutation in the alpha helix corresponding to residues 383 to 395 of SEQ ID NO: 4, a mutation in the loop corresponding to residues 77 to 80 of SEQ ID NO: 4, a mutation in the loop corresponding to residues 228 to 240 of SEQ ID NO: 4, a mutation in the loop corresponding to residues 306 to 311 of SEQ ID NO: 4, and/or a mutation in the residues corresponding to residues 480 and/or 481 as set forth in SEQ ID NO: 4, further comprising one or more mutations in the alpha helix corresponding to residues 654 to 674 of SEQ ID NO: 3, and/or a mutation in the alpha helix corresponding to residues 682 to 693 of SEQ ID NO: 3, wherein the mutations of the RNA polymerase causes an increase in fidelity of the polymerase. Also disclosed are modified influenza viruses comprising one or more mutations of the PB1 subunit of the influenza virus RNA polymerase including, but not limited to, a mutation at a residue corresponding to 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 77, 78, 79, 80, 228, 229, 230, 231, 232, 233, 234, 235, 236, 237, 238, 239, 240, 306, 307, 308, 309, 310, 311, 383, 384, 385, 386, 387, 388, 389, 390, 391, 392, 393, 394, 395, 480, and/or 481 as set forth in SEQ ID NO: 4 further comprising one or more mutations of the PA subunit of the influenza

virus RNA polymerase including but not limited to a mutation at a residue corresponding to 654, 655, 656, 657, 658, 659, 660, 661, 662, 663, 664, 665, 666 ,667, 668, 669, 670, 671, 672, 673, 674, 682, 683, 684, 685, 686, 687, 688, 689, 690, 691, 692, and/or 693 as set forth in SEQ ID NO: 3.

39. As discussed herein there are numerous variants of the PB1 protein and PA protein that are known and herein contemplated. In addition, to the known functional PB1 and PA strain variants there are derivatives of the PB1 and PA proteins which also function in the disclosed methods and compositions. Protein variants and derivatives are well understood to those of skill in the art and in can involve amino acid sequence modifications. For example, amino acid sequence modifications typically fall into one or more of three classes: substitutional, insertional or deletional variants. Insertions include amino and/or carboxyl terminal fusions as well as intrasequence insertions of single or multiple amino acid residues. Insertions ordinarily will be smaller insertions than those of amino or carboxyl terminal fusions, for example, on the order of one to four residues. Immunogenic fusion protein derivatives, such as those described in the examples, are made by fusing a polypeptide sufficiently large to confer immunogenicity to the target sequence by cross-linking in vitro or by recombinant cell culture transformed with DNA, RAN, or cDNA encoding the fusion. Deletions are characterized by the removal of one or more amino acid residues from the protein sequence. Typically, no more than about from 2 to 6 residues are deleted at any one site within the protein molecule. These variants ordinarily are prepared by site specific mutagenesis of nucleotides in the DNA, RNA, or cDNA encoding the protein, thereby producing DNA, RNA, or cDNA encoding the variant, and thereafter expressing the DNA, RNA, or cDNA in recombinant cell culture. Techniques for making substitution mutations at predetermined sites in DNA having a known sequence are well known, for example M13 primer mutagenesis and PCR mutagenesis.

40. Amino acid substitutions are typically of single residues, but can occur at a number of different locations at once for example in one, two, three, four, five, six, seven or more amino acids of the polypeptide sequence set forth as SEQ ID NOs: 1, 2, 3, or 4; insertions usually will be on the order of about from 1 to 10 amino acid residues; and deletions will range about from 1 to 30 residues. Deletions or insertions preferably are made in adjacent pairs, i.e. a deletion of 2 residues or insertion of 2 residues. Substitutions, deletions, insertions or any combination thereof may be combined to arrive at a final construct. The mutations must not place the sequence out of reading frame and preferably will not create complementary regions that could produce secondary mRNA structure. Substitutional variants are those in which at least one residue has been removed and a different residue inserted in its place. Such substitutions

generally are made in accordance with the following Tables 1 and 2 and are referred to as conservative substitutions.

TABLE 1: Amino Acid Abbreviations

Amino Acid	Abbreviations	
Alanine	Ala	A
allosoleucine	AIle	
Arginine	Arg	R
asparagine	Asn	N
aspartic acid	Asp	D
Cysteine	Cys	C
glutamic acid	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isolelucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
phenylalanine	Phe	F
proline	Pro	P
pyroglutamic acid	pGlu	
Serine	Ser	S
Threonine	Thr	T
Tyrosine	Tyr	Y
Tryptophan	Trp	W
Valine	Val	V

TABLE 2: Amino Acid Substitutions

Original Residue	Exemplary Conservative Substitutions, others are known in the art.
Ala	Ser, Gly, Cys
Arg	Lys; Gln, Met, Ile
Asn	Gln; His, Glu, Asp
Asp	Glu, Asn, Gln
Cys	Ser, Net, Thr
Gln	Asn, Lys, Glu, Asp
Glu	Asp, Asn, Gln
Gly	Pro, Ala
His	Asn; Gln
Ile	Leu; Val, Met
Leu	Ile; Val, Met
Lys	Arg; Gln, Met, Ile
Met	Leu; Ile, Val
Phe	Met; Leu; Tyr, Trp, His
Ser	Thr, Met, Cys
Thr	Ser, Met, Val
Trp	Tyr, Phe
Tyr	Trp; Phe, His
Val	Ile; Leu, Met

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41. Substantial changes in function or immunological identity are made by selecting substitutions that are less conservative than those in Table 2, i.e., selecting residues that differ more significantly in their effect on maintaining (a) the structure of the polypeptide backbone in the area of the substitution, for example as a sheet or helical conformation, (b) the charge or

hydrophobicity of the molecule at the target site or (c) the bulk of the side chain. The substitutions which in general are expected to produce the greatest changes in the protein properties will be those in which (a) a hydrophilic residue, e.g. seryl or threonyl, is substituted for (or by) a hydrophobic residue, e.g. leucyl, isoleucyl, phenylalanyl, valyl or alanyl; (b) a cysteine or proline is substituted for (or by) any other residue; (c) a residue having an electropositive side chain, e.g., lysyl, arginyl, or histidyl, is substituted for (or by) an electronegative residue, e.g., glutamyl or aspartyl; or (d) a residue having a bulky side chain, e.g., phenylalanine, is substituted for (or by) one not having a side chain, e.g., glycine, in this case, (e) by increasing the number of sites for sulfation and/or glycosylation.

42. For example, the replacement of one amino acid residue with another that is biologically and/or chemically similar is known to those skilled in the art as a conservative substitution. For example, a conservative substitution would be replacing one hydrophobic residue for another, or one polar residue for another. The substitutions include combinations such as, for example, Gly, Ala; Val, Ile, Leu; Asp, Glu; Asn, Gln; Ser, Thr; Lys, Arg; and Phe, Tyr. Such conservatively substituted variations of each explicitly disclosed sequence are included within the mosaic polypeptides provided herein.

43. In one aspect contemplated herein are modified influenza viruses comprising one or more mutations at one or more residues of the alpha helix of the PB1 subunit of the influenza virus RNA polymerase corresponding to residues 36 to 50 as set forth in SEQ ID NO: 4 (TGYTMDTVNRTHQYSE SEQ ID NO: 7), wherein the one or more mutations causes an increase in the fidelity of the RNA polymerase, wherein the mutation is a substitution corresponding to residue 43, and wherein the substitution is a Valine to another hydrophobic non-polar amino acid residue including, but not limited to Glycine (V43G), Alanine (V43A), Leucine (V43L), Methionine (V43M), Isoleucine (V43I), Proline (V43P), Phenylalanine (V43F), and Tryptophan (V43W), wherein the substitution is not Valine to Isoleucine when the modified influenza virus comprises only one fidelity increasing substitution. For example, disclosed herein are modified influenza viruses comprising a Valine to Leucine substation at the residue corresponding to residue 43 (V43L) as set forth in SEQ ID NO: 4.

44. Also disclosed herein are modified influenza viruses comprising one or more mutations at one or more residues of the alpha helix of the PB1 subunit of the influenza virus RNA polymerase corresponding to residues 36 to 50 of SEQ ID NO: 4 (TGYTMDTVNRTHQYSE SEQ ID NO: 7), wherein the one or more mutations causes an increase in the fidelity of the RNA polymerase, wherein the mutation is a substitution corresponding to residue 45, and wherein the substitution is a Arginine to another charged amino

acid including, but not limited to Lysine (R45K), Histidine (R45H), Aspartic Acid (R45D), and Glutamic acid (R45E). Alternatively, the substitution can be a Arginine to Glutamine (R45Q) substitution. For example, disclosed herein are modified influenza virus comprising a Arginine to Lysine or Arginine to Aspartic Acid substitution at the PB1 residue corresponding to residue 45 as set forth in SEQ ID NO: 4.

45. Also disclosed herein are modified influenza viruses comprising one or more mutations at one or more residues of the alpha helix of the PB1 subunit of the influenza virus RNA polymerase corresponding to residues 383 to 395 of SEQ ID NO: 4 (DSTRKKIEKIRPL SEQ ID NO: 8), wherein the one or more mutations causes an increase in the fidelity of the RNA polymerase, wherein the mutation is a substitution corresponding to residue 387 or 391, and wherein the substitution is a Lysine to another charged amino acid including, but not limited to Arginine (K387R and K391R), Histidine (K387H and K391H), Aspartic Acid (K387D and K391D), and Glutamic acid (K387E).

46. Also disclosed herein are modified influenza viruses comprising one or more mutations at one or more residues of the loop of the PB1 subunit of the influenza virus RNA polymerase corresponding to residues 77 to 80 of SEQ ID NO: 4 (NEPS SEQ ID NO: 9), wherein the one or more mutations causes an increase in the fidelity of the RNA polymerase.

47. Also disclosed herein are modified influenza viruses comprising one or more mutations at one or more residues of the loop of the PB1 subunit of the influenza virus RNA polymerase corresponding to residues 228 to 240 of SEQ ID NO: 4 (TKDAERGKLKRRA SEQ ID NO: 10), wherein the one or more mutations causes an increase in the fidelity of the RNA polymerase, wherein the mutation is a substitution corresponding to residue 235or 237, and wherein the substitution is a Lysine to another charged amino acid including, but not limited to Arginine (K235R and K237R), Histidine (K235H and K237H), Aspartic Acid (K235D and K237D), and Glutamic acid (K235E and K237E).

48. Also disclosed herein are modified influenza viruses comprising one or more mutations at one or more residues of the alpha helix of the PB1 subunit of the influenza virus RNA polymerase corresponding to residues 228 to 240 of SEQ ID NO: 4 (TKDAERGKLKRRA SEQ ID NO: 10), wherein the one or more mutations causes an increase in the fidelity of the RNA polymerase, wherein the mutation is a substitution corresponding to residue 239, and wherein the substitution is a Arginine to another charged amino acid including, but not limited to Lysine (R239K), Histidine (R239H), Aspartic Acid (R239D), and Glutamic acid (R239E). Alternatively, the substitution can be an Arginine to Glutamine (R239Q) substitution.

49. Also disclosed herein are modified influenza viruses comprising one or more mutations at one or more residues of the loop of the PB1 subunit of the influenza virus RNA polymerase corresponding to residues 306 to 311 of SEQ ID NO: 4 (NTKWNE SEQ ID NO: 11), wherein the one or more mutations causes an increase in the fidelity of the RNA polymerase, wherein the mutation is a substitution corresponding to residue 308, and wherein the substitution is a Lysine to another charged amino acid including, but not limited to Arginine (K308R), Histidine (K308H), Aspartic Acid (K308D), and Glutamic acid (K308E).

50. Also disclosed herein are modified influenza viruses comprising one or more mutations at one or more residues of the PB1 subunit of the influenza virus RNA polymerase corresponding to residues 480 or 481of SEQ ID NO: 4, wherein the one or more mutations causes an increase in the fidelity of the RNA polymerase, wherein the mutation is a substitution corresponding to residue 480 or 481, and wherein the substitution is a Lysine to another charged amino acid including, but not limited to Arginine (K480R and K481R), Histidine (K480H and K481H), Aspartic Acid (K480D and K481D), and Glutamic acid (K480E and K481E).

51. Also disclosed herein are modified influenza viruses comprising one or more mutations at one or more residues of the alpha helix of the PA subunit of the influenza virus RNA polymerase corresponding to residues 654-674of SEQ ID NO: 3 (QLEGFSAESRKLLLIVQALRD SEQ ID NO: 12), wherein the one or more mutations causes an increase in the fidelity of the RNA polymerase, wherein the mutation is a substitution corresponding to residue 661, and wherein the substitution is a Glutamic Acid to another charged amino acid including, but not limited to Arginine (E661R), Histidine (E661H), Aspartic Acid (E661D), and Lysine (E661K) or is a Glutamic Acid to Glutamine (E661Q) substitution.

52. Also disclosed herein are modified influenza viruses comprising one or more mutations at one or more residues of the alpha helix of the PA subunit of the influenza virus RNA polymerase corresponding to residues 654-674of SEQ ID NO: 3 (QLEGFSAESRKLLLIVQALRD SEQ ID NO: 12), wherein the one or more mutations causes an increase in the fidelity of the RNA polymerase, wherein the mutation is a substitution corresponding to residue 663, and wherein the substitution is a Arginine to another charged amino acid including, but not limited to Lysine (R663K), Histidine (R663H), Aspartic Acid (R663D), and Glutamic acid (R663E). Alternatively, the substitution can be an Arginine to Glutamine (R663Q) substitution. For example, disclosed herein are modified influenza virus comprising a Arginine to Lysine (R663K) or Arginine to Glutamic Acid (R663E) substitution at the PA residue corresponding to residue 663 of SEQ ID NO: 3.

53. Also disclosed herein are modified influenza viruses comprising one or more mutations at one or more residues of the alpha helix of the PA subunit of the influenza virus RNA polymerase corresponding to residues 654-674of SEQ ID NO: 3 (QLEGFSAESRKLLLIVQALRD SEQ ID NO: 12), wherein the one or more mutations causes an increase in the fidelity of the RNA polymerase, wherein the mutation is a substitution corresponding to residue 664, and wherein the substitution is a Lysine to another charged amino acid including, but not limited to Arginine (K664R), Histidine (K664H), Aspartic Acid (K664D), and Glutamic acid (K664E). For example, disclosed herein are modified influenza virus comprising a Lysine to Glutamic Acid (K665E) substitution at the PA residue corresponding to residue 664 of SEQ ID NO: 3.

54. Also disclosed herein are modified influenza viruses comprising one or more mutations at one or more residues of the alpha helix of the PA subunit of the influenza virus RNA polymerase corresponding to residues 682 to 693of SEQ ID NO: 3 (DLGGLYEAIEEC SEQ ID NO: 13), wherein the one or more mutations causes an increase in the fidelity of the RNA polymerase, wherein the mutation is a substitution corresponding to residue 691, and wherein the substitution is a Glutamic Acid to another charged amino acid including, but not limited to Arginine (E661R), Histidine (E661H), Aspartic Acid (E661D), and Lysine (E661K) or is a Glutamic Acid to Glutamine (E661Q) substitution.

55. Substitutional or deletional mutagenesis can be employed to insert sites for N-glycosylation (Asn-X-Thr/Ser) or O-glycosylation (Ser or Thr). Deletions of cysteine or other labile residues also may be desirable. Deletions or substitutions of potential proteolysis sites, e.g. Arg, is accomplished for example by deleting one of the basic residues or substituting one by glutaminyl or histidyl residues.

56. Certain post-translational derivatizations are the result of the action of recombinant host cells on the expressed polypeptide. Glutaminyl and asparaginyl residues are frequently post-translationally deamidated to the corresponding glutamyl and asparyl residues. Alternatively, these residues are deamidated under mildly acidic conditions. Other post-translational modifications include hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl or threonyl residues, methylation of the o-amino groups of lysine, arginine, and histidine side chains (T.E. Creighton, Proteins: Structure and Molecular Properties, W. H. Freeman & Co., San Francisco pp 79-86 [1983]), acetylation of the N-terminal amine and, in some instances, amidation of the C-terminal carboxyl.

57. It is understood that one way to define the variants and derivatives of the disclosed proteins herein is through defining the variants and derivatives in terms of homology/identity to

specific known sequences. For example, SEQ ID NO: 6 sets forth a particular nucleic acid sequence of PB1 and SEQ ID NO: 4 sets forth a particular sequence of a PB1 protein. Specifically disclosed are variants of PB1 and PA and other proteins herein disclosed which have at least, 70% or 75% or 80% or 85% or 90% or 95% homology to the stated sequence. Those of skill in the art readily understand how to determine the homology of two proteins. For example, the homology can be calculated after aligning the two sequences so that the homology is at its highest level.

58. Another way of calculating homology can be performed by published algorithms. Optimal alignment of sequences for comparison may be conducted by the local homology algorithm of Smith and Waterman *Adv. Appl. Math.* 2: 482 (1981), by the homology alignment algorithm of Needleman and Wunsch, *J. MoL Biol.* 48: 443 (1970), by the search for similarity method of Pearson and Lipman, *Proc. Natl. Acad. Sci. U.S.A.* 85: 2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI), or by inspection.

59. The same types of homology can be obtained for nucleic acids by for example the algorithms disclosed in Zuker, M. *Science* 244:48-52, 1989, Jaeger et al. *Proc. Natl. Acad. Sci. USA* 86:7706-7710, 1989, Jaeger et al. *Methods Enzymol.* 183:281-306, 1989.

60. It is understood that the description of conservative mutations and homology can be combined together in any combination, such as embodiments that have at least 70% homology to a particular sequence wherein the variants are conservative mutations.

61. As this specification discusses various proteins and protein sequences it is understood that the nucleic acids that can encode those protein sequences are also disclosed. This would include all degenerate sequences related to a specific protein sequence, i.e. all nucleic acids having a sequence that encodes one particular protein sequence as well as all nucleic acids, including degenerate nucleic acids, encoding the disclosed variants and derivatives of the protein sequences. Thus, while each particular nucleic acid sequence may not be written out herein, it is understood that each and every sequence is in fact disclosed and described herein through the disclosed protein sequence. For example, one of the many nucleic acid sequences that can encode the protein sequence set forth in SEQ ID NO: 4 is set forth in SEQ ID NO:6 and one of the many nucleic acid sequences that can encode the protein sequence set forth in SEQ ID NO: 3 is set forth in SEQ ID NO: 5. In addition, for example, a disclosed conservative derivative of SEQ ID NO: 4 where the valine (V) at position 43 is changed to a leucine (L). It is understood that for this mutation all of the nucleic acid sequences that encode this particular derivative of

the PB1 or PA are also disclosed including for example degenerate nucleic acid sequences that encode the particular polypeptide set forth in SEQ ID NOs: 1, 2, 3, and 4. It is also understood that while no amino acid sequence indicates what particular DNA, cDNA, or RNA sequence encodes that protein within a vector, cell, or organism, where particular variants of a disclosed protein are disclosed herein, the known nucleic acid sequence that encodes that protein in the particular PB1 and PA are also known and herein disclosed and described.

62. It is understood that there are numerous amino acid and peptide analogs which can be incorporated into the disclosed compositions. For example, there are numerous D amino acids or amino acids which have a different functional substituent then the amino acids shown in Table 1 and Table 2. The opposite stereo isomers of naturally occurring peptides are disclosed, as well as the stereo isomers of peptide analogs. These amino acids can readily be incorporated into polypeptide chains by charging tRNA molecules with the amino acid of choice and engineering genetic constructs that utilize, for example, amber codons, to insert the analog amino acid into a peptide chain in a site specific way.

63. Molecules can be produced that resemble peptides, but which are not connected via a natural peptide linkage. For example, linkages for amino acids or amino acid analogs can include CH_2NH--, --CH_2S--, --CH_2--CH_2 --, --CH=CH-- (cis and trans), --$COCH_2$ --, --$CH(OH)CH_2$--, and --CHH_2SO—(These and others can be found in Spatola, A. F. in *Chemistry and Biochemistry of Amino Acids, Peptides, and Proteins*, B. Weinstein, eds., Marcel Dekker, New York, p. 267 (1983); Spatola, A. F., *Vega Data* (March 1983), Vol. 1, Issue 3, Peptide Backbone Modifications (general review); Morley, *Trends Pharm Sci* (1980) pp. 463-468; Hudson, D. et al., Int J Pept Prot Res 14:177-185 (1979) (--CH_2NH--, CH_2CH_2--); Spatola et al. *Life Sci* 38:1243-1249 (1986) (--CH H_2--S); Hann *J. Chem. Soc Perkin Trans.* I 307-314 (1982) (--CH--CH--, cis and trans); Almquist et al. *J. Med. Chem.* 23:1392-1398 (1980) (--$COCH_2$--); Jennings-White et al. *Tetrahedron Lett* 23:2533 (1982) (--$COCH_2$--); Szelke et al. European Appln, EP 45665 CA (1982): 97:39405 (1982) (--$CH(OH)CH_2$--); Holladay et al. *Tetrahedron.* Lett 24:4401-4404 (1983) (--$C(OH)CH_2$--); and Hruby *Life Sci* 31:189-199 (1982) (--CH_2--S--); each of which is incorporated herein by reference. A particularly preferred non-peptide linkage is --CH_2NH--. It is understood that peptide analogs can have more than one atom between the bond atoms, such as b-alanine, g-aminobutyric acid, and the like.

64. Amino acid analogs and analogs and peptide analogs often have enhanced or desirable properties, such as, more economical production, greater chemical stability, enhanced pharmacological properties (half-life, absorption, potency, efficacy, etc.), altered specificity (e.g., a broad-spectrum of biological activities), reduced antigenicity, and others.

65. D-amino acids can be used to generate more stable peptides, because D amino acids are not recognized by peptidases and such. Systematic substitution of one or more amino acids of a consensus sequence with a D-amino acid of the same type (e.g., D-lysine in place of L-lysine) can be used to generate more stable peptides. Cysteine residues can be used to cyclize or attach two or more peptides together. This can be beneficial to constrain peptides into particular conformations.

66. In one aspect, also disclosed herein are recombinant nucleic acids encoding an influenza RNA polymerase with increased transcriptional fidelity wherein the RNA polymerase comprises at least one mutation selected from the PB1 or PA subunits of the RNA polymerase. It is understood that the disclosed recombinant nucleic acids can encode any of the PB1 or PA mutant polymerase subunits disclosed herein. As used throughout, the term recombinant means that the material (e.g., a nucleic acid or protein) has been artificially or synthetically (non-naturally) altered by human intervention. It is understood that, when referring to a virus, e.g., an influenza A virus, the virus is recombinant when it is produced by the expression of a recombinant nucleic acid.

67. For example, disclosed herein are recombinant nucleic acids wherein the mutation of the PB1 or PA subunit of the influenza RNA polymerase comprises a mutation of at least one residue selected from one or more residues of the alpha helix corresponding to residues 36 to 50 or residues 383 to 395 of the PB1 subunit of the influenza RNA polymerase as set forth in SEQ ID NO: 4; one or more residues of the loop formed by the amino acids corresponding to residues 77-80 of the PB1 subunit of the influenza RNA polymerase as set forth in SEQ ID NO: 4; one or more residues of the loop formed by the amino acids corresponding to residues 228-240 of the PB1 subunit of the influenza RNA polymerase as set forth in SEQ ID NO: 4; one or more residues of the loop formed by the amino acids corresponding to residues 306-311 of the PB1 subunit of the influenza RNA polymerase as set forth in SEQ ID NO: 4; residues 480 or 481 of the PB1 subunit of the influenza RNA polymerase as set forth in SEQ ID NO: 4; and one or more residues of the alpha helix corresponding to residues 654 to 674 or residues 682 to 693 of the PA subunit of the influenza virus RNA polymerasse as set forth in SEQ ID NO: 3. For example, also disclosed are recombinant nucleic acids encoding one or more mutations of the PB1 subunit of the RNA polymerase including but not limited to a mutation at a residue corresponding to 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 77, 78, 79, 80, 228, 229, 230, 231, 232, 233, 234, 235, 236, 237, 238, 239, 240, 306, 307, 308, 309, 310, 311, 383, 384, 385, 386, 387, 388, 389, 390, 391, 392, 393, 394, 395, 480, and/or 481 of SEQ ID NO: 4 and/or comprising one or more mutations of the PA subunit of the RNA polymerase including

but not limited to a mutation at a residue corresponding to 654, 655, 656, 657, 658, 659, 660, 661, 662, 663, 664, 665, 666 ,667, 668, 669, 670, 671, 672, 673, 674, 682, 683, 684, 685, 686, 687, 688, 689, 690, 691, 692, and/or 693 of SEQ ID NO: 3.

68. In one aspect contemplated herein recombinant nucleic acids encoding one or more mutations at one or more residues of the alpha helix of the PB1 subunit of the influenza virus RNA polymerase corresponding to residues 36 to 50 of SEQ ID NO: 4 (TGYTMDTVNRTHQYSE SEQ ID NO: 7), wherein the one or more mutations causes an increase in the fidelity of the RNA polymerase, wherein the mutation is a substitution corresponding to residue 43, and wherein the substitution is a Valine to another hydrophobic non-polar amino acid residue including, but not limited to Glycine (V43G), Alanine (V43A), Leucine (V43L), Methionine (V43M), Isoleucine (V43I), Proline (V43P), Phenylalanine (V43F), and Tryptophan (V43W), wherein the substitution is not Valine to Isoleucine when the modified influenza virus comprises only one fidelity increasing substitituon. For example, disclosed herein are recombinant nucleic acids encoding a Valine to Leucine substitution at the residue corresponding to residue 43 (V43L) as set forth in SEQ ID NO: 4.

69. Also disclosed herein are recombinant nucleic acids encoding one or more mutations at one or more residues of the alpha helix of the PB1 subunit of the influenza virus RNA polymerase corresponding to residues 36 to 50 of SEQ ID NO: 4 (TGYTMDTVNRTHQYSE SEQ ID NO: 7), wherein the one or more mutations causes an increase in the fidelity of the RNA polymerase, wherein the mutation is a substitution corresponding to residue 45, and wherein the substitution is a Arginine to another charged amino acid including, but not limited to Lysine (R45K), Histidine (R45H), Aspartic Acid (R45D), and Glutamic acid (R45E). Alternatively, the substitution can be a Arginine to Glutamine (R45Q) substitution. For example, disclosed herein are modified influenza virus comprising a Arginine to Lysine or Arginine to Aspartic Acid substitution at the PB1 residue corresponding to residue 45 of SEQ ID NO: 4.

70. Also disclosed herein are recombinant nucleic acids encoding one or more mutations at one or more residues of the alpha helix of the PB1 subunit of the influenza virus RNA polymerase corresponding to residues 383 to 395 of SEQ ID NO: 4 (DSTRKKIEKIRPL SEQ ID NO: 8), wherein the one or more mutations causes an increase in the fidelity of the RNA polymerase, wherein the mutation is a substitution corresponding to residue 387 or 391, and wherein the substitution is a Lysine to another charged amino acid including, but not limited to Arginine (K387R and K391R), Histidine (K387H and K391H), Aspartic Acid (K387D and K391D), and Glutamic acid (K387E).

71. Also disclosed herein are recombinant nucleic acids encoding one or more mutations at one or more residues of the loop of the PB1 subunit of the influenza virus RNA polymerase corresponding to residues 77 to 80 of SEQ ID NO: 4 (NEPS SEQ ID NO: 9), wherein the one or more mutations causes an increase in the fidelity of the RNA polymerase.

72. Also disclosed herein are recombinant nucleic acids encoding one or more mutations at one or more residues of the loop of the PB1 subunit of the influenza virus RNA polymerase corresponding to residues 228 to 240 of SEQ ID NO: 4 (TKDAERGKLKRRA SEQ ID NO: 10), wherein the one or more mutations causes an increase in the fidelity of the RNA polymerase, wherein the mutation is a substitution corresponding to residue 235or 237, and wherein the substitution is a Lysine to another charged amino acid including, but not limited to Arginine (K235R and K237R), Histidine (K235H and K237H), Aspartic Acid (K235D and K237D), and Glutamic acid (K235E and K237E).

73. Also disclosed herein are recombinant nucleic acids encoding one or more mutations at one or more residues of the alpha helix of the PB1 subunit of the influenza virus RNA polymerase corresponding to residues 228 to 240 of SEQ ID NO: 4 (TKDAERGKLKRRA SEQ ID NO: 10), wherein the one or more mutations causes an increase in the fidelity of the RNA polymerase, wherein the mutation is a substitution corresponding to residue 239, and wherein the substitution is a Arginine to another charged amino acid including, but not limited to Lysine (R239K), Histidine (R239H), Aspartic Acid (R239D), and Glutamic acid (R239E). Alternatively, the substitution can be an Arginine to Glutamine (R239Q) substitution.

74. Also disclosed herein are recombinant nucleic acids encoding one or more mutations at one or more residues of the loop of the PB1 subunit of the influenza virus RNA polymerase corresponding to residues 306 to 311 of SEQ ID NO: 4 (NTKWNE SEQ ID NO: 11), wherein the one or more mutations causes an increase in the fidelity of the RNA polymerase, wherein the mutation is a substitution corresponding to residue 308, and wherein the substitution is a Lysine to another charged amino acid including, but not limited to Arginine (K308R), Histidine (K308H), Aspartic Acid (K308D), and Glutamic acid (K308E).

75. Also disclosed herein are recombinant nucleic acids encoding one or more mutations at one or more residues of the PB1 subunit of the influenza virus RNA polymerase corresponding to residues 480 or 481of SEQ ID NO: 4, wherein the one or more mutations causes an increase in the fidelity of the RNA polymerase, wherein the mutation is a substitution corresponding to residue 480 or 481, and wherein the substitution is a Lysine to another charged amino acid including, but not limited to Arginine (K480R and K481R), Histidine (K480H and K481H), Aspartic Acid (K480D and K481D), and Glutamic acid (K480E and K481E).

76. Also disclosed herein are recombinant nucleic acids encoding one or more mutations at one or more residues of the alpha helix of the PA subunit of the influenza virus RNA polymerase corresponding to residues 654-674of SEQ ID NO: 3 (QLEGFSAESRKLLLIVQALRD SEQ ID NO: 12), wherein the one or more mutations causes an increase in the fidelity of the RNA polymerase, wherein the mutation is a substitution corresponding to residue 661, and wherein the substitution is a Glutamic Acid to another charged amino acid including, but not limited to Arginine (E661R), Histidine (E661H), Aspartic Acid (E661D), and Lysine (E661K) or is a Glutamic Acid to Glutamine (E661Q) substitution.

77. Also disclosed herein are recombinant nucleic acids encoding one or more mutations at one or more residues of the alpha helix of the PA subunit of the influenza virus RNA polymerase corresponding to residues 654-674of SEQ ID NO: 3 (QLEGFSAESRKLLLIVQALRD SEQ ID NO: 12), wherein the one or more mutations causes an increase in the fidelity of the RNA polymerase, wherein the mutation is a substitution corresponding to residue 663, and wherein the substitution is a Arginine to another charged amino acid including, but not limited to Lysine (R663K), Histidine (R663H), Aspartic Acid (R663D), and Glutamic acid (R663E). Alternatively, the substitution can be an Arginine to Glutamine (R663Q) substitution. For example, disclosed herein are modified influenza virus comprising a Arginine to Lysine (R663K) or Arginine to Glutamic Acid (R663E) substitution at the PA residue corresponding to residue 663 of SEQ ID NO: 3.

78. Also disclosed herein are recombinant nucleic acids encoding one or more mutations at one or more residues of the alpha helix of the PA subunit of the influenza virus RNA polymerase corresponding to residues 654-674of SEQ ID NO: 3 (QLEGFSAESRKLLLIVQALRD SEQ ID NO: 12), wherein the one or more mutations causes an increase in the fidelity of the RNA polymerase, wherein the mutation is a substitution corresponding to residue 664, and wherein the substitution is a Lysine to another charged amino acid including, but not limited to Arginine (K664R), Histidine (K664H), Aspartic Acid (K664D), and Glutamic acid (K664E). For example, disclosed herein are recombinant nucleic acids encoding a Lysine to Glutamic Acid (K665E) substitution at the PA residue corresponding to residue 664 of SEQ ID NO: 3.

79. Also disclosed herein are recombinant nucleic acids encoding one or more mutations at one or more residues of the alpha helix of the PA subunit of the influenza virus RNA polymerase corresponding to residues 682 to 693of SEQ ID NO: 3 (DLGGLYEAIEEC SEQ ID NO: 13), wherein the one or more mutations causes an increase in the fidelity of the RNA polymerase, wherein the mutation is a substitution corresponding to residue 691, and wherein

the substitution is a Glutamic Acid to another charged amino acid including, but not limited to Arginine (E661R), Histidine (E661H), Aspartic Acid (E661D), and Lysine (E661K) or is a Glutamic Acid to Glutamine (E661Q) substitution.

80. The recombinant nucleic acids disclosed herein can be in any vector that can be used for the production of influenza virus in a host cell. Accordingly, in one aspect, disclosed herein are vectors comprising any recombinant nucleic acid disclosed herein. The vector can direct the in vivo or in vitro synthesis of any of the polypeptides described herein, including, but not limited to PBl and/or PA polymerase subunits. One or more of the vectors described herein can be part of a multi-vector system used to produce an influenza A virus. The vector is contemplated to have the necessary functional elements that direct and regulate transcription of the inserted nucleic acid. These functional elements include, but are not limited to, a promoter, regions upstream or downstream of the promoter, such as enhancers that may regulate the transcriptional activity of the promoter, an origin of replication, appropriate restriction sites to facilitate cloning of inserts adjacent to the promoter, antibiotic resistance genes or other markers which can serve to select for cells containing the vector or the vector containing the insert, RNA splice junctions, a transcription termination region, or any other region which may serve to facilitate the expression of the inserted gene or hybrid gene (See generally, Sambrook et al. (2001)). The vector, for example, can be a plasmid. The vectors can contain genes conferring hygromycin resistance, ampicillin resistance, gentamicin resistance, neomycin resistance or other genes or phenotypes suitable for use as selectable markers.

81. As used throughout, a host cell is a cell that contains one or more of the nucleic acids disclosed herein, including any of the nucleic acids in a vector, and supports the replication and/or expression of the nucleic acids, and optionally production of one or more encoded products including a polypeptide and/or a virus. Host cells can be prokaryotic cells, such as E. coli, or eukaryotic cells, such as yeast, insect, amphibian, avian or mammalian cells, including human cells. Examples of host cells include, but are not limited to, Vera (African green monkey kidney) cells, Per.C6 cells (human embryonic retinal cells), BHK (baby hamster kidney) cells, primary chick kidney (PCK) cells, Madin-Darby Canine Kidney (MDCK) cells, Madin-Darby Bovine Kidney (MDBK) cells, 293 cells (e.g., 293T cells), CEK cells, primary human lung cells, bronchial epithelial cells , COS cells (e.g., COS1, COS7 cells) and any other mammalian or avian cells that can be used to produce or propagate an influenza virus. The term host cell encompasses combinations or mixtures of cells including, but not limited to mixed cultures of different cell types or cell lines.

82. There are a variety of molecules disclosed herein that are nucleic acid based, including for example the recombinant nucleic acids that encode, for example PB1 or PA, as well as various functional nucleic acids. As used herein, nucleic acid refers to single or multiple stranded molecules which can be DNA or RNA, or any combination thereof, including modifications to those nucleic acids. For example, the nucleic acid can be a cDNA. The nucleic acid may represent a coding strand or its complement, or any combination thereof. The nucleic acid can be directly cloned into an appropriate vector, or if desired, can be modified to facilitate the subsequent cloning steps. Such modification steps are routine, an example of which is the addition of oligonucleotide linkers which contain restriction sites to the termini of the nucleic acid. General methods are set forth in in Sambrook et al. (2001) Molecular Cloning - A Laboratory Manual (3rd ed.) Vol. 1-3, Cold Spring Harbor Laboratory, Cold Spring Harbor Press, NY, (Sambrook).

83. The disclosed nucleic acids are made up of for example, nucleotides, nucleotide analogs, or nucleotide substitutes. Non-limiting examples of these and other molecules are discussed herein. It is understood that for example, when a vector is expressed in a cell, that the expressed mRNA will typically be made up of A, C, G, and U. Likewise, it is understood that if, for example, an antisense molecule is introduced into a cell or cell environment through for example exogenous delivery, it is advantageous that the antisense molecule be made up of nucleotide analogs that reduce the degradation of the antisense molecule in the cellular environment.

84. A nucleotide is a molecule that contains a base moiety, a sugar moiety and a phosphate moiety. Nucleotides can be linked together through their phosphate moieties and sugar moieties creating an internucleoside linkage. The base moiety of a nucleotide can be adenin-9-yl (A), cytosin-1-yl (C), guanin-9-yl (G), uracil-1-yl (U), and thymin-1-yl (T). The sugar moiety of a nucleotide is a ribose or a deoxyribose. The phosphate moiety of a nucleotide is pentavalent phosphate. An non-limiting example of a nucleotide would be 3'-AMP (3'-adenosine monophosphate) or 5'-GMP (5'-guanosine monophosphate).

85. A nucleotide analog is a nucleotide which contains some type of modification to either the base, sugar, or phosphate moieties. Modifications to the base moiety would include natural and synthetic modifications of A, C, G, and T/U as well as different purine or pyrimidine bases, such as uracil-5-yl (.psi.), hypoxanthin-9-yl (I), and 2-aminoadenin-9-yl. A modified base includes but is not limited to 5-methylcytosine (5-me-C), 5-hydroxymethyl cytosine, xanthine, hypoxanthine, 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 2-thiouracil, 2-thiothymine and

2-thiocytosine, 5-halouracil and cytosine, 5-propynyl uracil and cytosine, 6-azo uracil, cytosine and thymine, 5-uracil (pseudouracil), 4-thiouracil, 8-halo, 8-amino, 8-thiol, 8-thioalkyl, 8-hydroxyl and other 8-substituted adenines and guanines, 5-halo particularly 5-bromo, 5-trifluoromethyl and other 5-substituted uracils and cytosines, 7-methylguanine and 7-methyladenine, 8-azaguanine and 8-azaadenine, 7-deazaguanine and 7-deazaadenine and 3-deazaguanine and 3-deazaadenine. Additional base modifications can be found for example in U.S. Pat. No. 3,687,808, Englisch et al., Angewandte Chemie, International Edition, 1991, 30, 613, and Sanghvi, Y. S., Chapter 15, *Antisense Research and Applications*, pages 289-302, Crooke, S. T. and Lebleu, B. ed., CRC Press, 1993. Certain nucleotide analogs, such as 5-substituted pyrimidines, 6-azapyrimidines and N-2, N-6 and O-6 substituted purines, including 2-aminopropyladenine, 5-propynyluracil and 5-propynylcytosine. 5-methylcytosine can increase the stability of duplex formation. Often time base modifications can be combined with for example a sugar modification, such as 2'-O-methoxyethyl, to achieve unique properties such as increased duplex stability.

86. Nucleotide analogs can also include modifications of the sugar moiety. Modifications to the sugar moiety would include natural modifications of the ribose and deoxy ribose as well as synthetic modifications. Sugar modifications include but are not limited to the following modifications at the 2' position: OH; F; O-, S-, or N-alkyl; O-, S-, or N-alkenyl; O-, S- or N-alkynyl; or O-alkyl-O-alkyl, wherein the alkyl, alkenyl and alkynyl may be substituted or unsubstituted C_1 to C_{10}, alkyl or C_2 to C_{10} alkenyl and alkynyl. 2' sugar modifications also include but are not limited to $-O[(CH_2)_n O]_m CH_3$, $-O(CH_2)_n OCH_3$, $-O(CH_2)_n NH_2$, $-O(CH_2)_n CH_3$, $-O(CH_2)_n -ONH_2$, and $-O(CH_2)_n ON[(CH_2)_n CH_3)]_2$, where n and m are from 1 to about 10.

87. Other modifications at the 2' position include but are not limited to: C_1 to C_{10} lower alkyl, substituted lower alkyl, alkaryl, aralkyl, O-alkaryl or O-aralkyl, SH, SCH_3, OCN, Cl, Br, CN, CF_3, OCF_3, $SOCH_3$, $SO_2 CH_3$, ONO_2, NO_2, N_3, NH_2, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalkylamino, substituted silyl, an RNA cleaving group, a reporter group, an intercalator, a group for improving the pharmacokinetic properties of an oligonucleotide, or a group for improving the pharmacodynamic properties of an oligonucleotide, and other substituents having similar properties. Similar modifications may also be made at other positions on the sugar, particularly the 3' position of the sugar on the 3' terminal nucleotide or in 2'-5' linked oligonucleotides and the 5' position of 5' terminal nucleotide. Modified sugars would also include those that contain modifications at the bridging ring oxygen, such as CH_2 and S. Nucleotide sugar analogs may also have sugar mimetics such as cyclobutyl moieties in place of the pentofuranosyl sugar.

88. Nucleotide analogs can also be modified at the phosphate moiety. Modified phosphate moieties include but are not limited to those that can be modified so that the linkage between two nucleotides contains a phosphorothioate, chiral phosphorothioate, phosphorodithioate, phosphotriester, aminoalkylphosphotriester, methyl and other alkyl phosphonates including 3'-alkylene phosphonate and chiral phosphonates, phosphinates, phosphoramidates including 3'-amino phosphoramidate and aminoalkylphosphoramidates, thionophosphoramidates, thionoalkylphosphonates, thionoalkylphosphotriesters, and boranophosphates. It is understood that these phosphate or modified phosphate linkage between two nucleotides can be through a 3'-5' linkage or a 2'-5' linkage, and the linkage can contain inverted polarity such as 3'-5' to 5'-3' or 2'-5' to 5'-2'. Various salts, mixed salts and free acid forms are also included.

89. It is understood that nucleotide analogs need only contain a single modification, but may also contain multiple modifications within one of the moieties or between different moieties.

90. A Watson-Crick interaction is at least one interaction with the Watson-Crick face of a nucleotide, nucleotide analog, or nucleotide substitute. The Watson-Crick face of a nucleotide, nucleotide analog, or nucleotide substitute includes the C2, N1, and C6 positions of a purine based nucleotide, nucleotide analog, or nucleotide substitute and the C2, N3, C4 positions of a pyrimidine based nucleotide, nucleotide analog, or nucleotide substitute.

91. A Hoogsteen interaction is the interaction that takes place on the Hoogsteen face of a nucleotide or nucleotide analog, which is exposed in the major groove of duplex DNA. The Hoogsteen face includes the N7 position and reactive groups (NH2 or O) at the C6 position of purine nucleotides.

92. It is understood that as discussed herein the use of the terms homology and identity mean the same thing as similarity. Thus, for example, if the use of the word homology is used between two non-natural sequences it is understood that this is not necessarily indicating an evolutionary relationship between these two sequences, but rather is looking at the similarity or relatedness between their nucleic acid sequences. Many of the methods for determining homology between two evolutionarily related molecules are routinely applied to any two or more nucleic acids or proteins for the purpose of measuring sequence similarity regardless of whether they are evolutionarily related or not.

93. In general, it is understood that one way to define any known variants and derivatives or those that might arise, of the disclosed genes and proteins herein, is through defining the variants and derivatives in terms of homology to specific known sequences. This identity of

particular sequences disclosed herein is also discussed elsewhere herein. In general, variants of genes and proteins herein disclosed typically have at least, about 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99 percent homology to the stated sequence or the native sequence. Those of skill in the art readily understand how to determine the homology of two proteins or nucleic acids, such as genes. For example, the homology can be calculated after aligning the two sequences so that the homology is at its highest level.

94. Another way of calculating homology can be performed by published algorithms. Optimal alignment of sequences for comparison may be conducted by the local homology algorithm of Smith and Waterman *Adv. Appl. Math.* 2: 482 (1981), by the homology alignment algorithm of Needleman and Wunsch, *J. MoL Biol.* 48: 443 (1970), by the search for similarity method of Pearson and Lipman, *Proc. Natl. Acad. Sci. U.S.A.* 85: 2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI), or by inspection.

95. It is understood that any of the methods typically can be used and that in certain instances the results of these various methods may differ, but the skilled artisan understands if identity is found with at least one of these methods, the sequences would be said to have the stated identity, and be disclosed herein.

96. For example, as used herein, a sequence recited as having a particular percent homology to another sequence refers to sequences that have the recited homology as calculated by any one or more of the calculation methods described above. For example, a first sequence has 80 percent homology, as defined herein, to a second sequence if the first sequence is calculated to have 80 percent homology to the second sequence using the Zuker calculation method even if the first sequence does not have 80 percent homology to the second sequence as calculated by any of the other calculation methods. As another example, a first sequence has 80 percent homology, as defined herein, to a second sequence if the first sequence is calculated to have 80 percent homology to the second sequence using both the Zuker calculation method and the Pearson and Lipman calculation method even if the first sequence does not have 80 percent homology to the second sequence as calculated by the Smith and Waterman calculation method, the Needleman and Wunsch calculation method, the Jaeger calculation methods, or any of the other calculation methods. As yet another example, a first sequence has 80 percent homology, as defined herein, to a second sequence if the first sequence is calculated to have 80 percent

homology to the second sequence using each of calculation methods (although, in practice, the different calculation methods will often result in different calculated homology percentages).

97. It is understood and herein contemplated that the mutations to the PB1 and/or PA subunit of the influenza viral polymerase can be used to increase the immunogenicity of an influenza vaccine including but not limited to trivalent influenza vaccines, and live attenuated influenza vaccines, as well as, influenza vaccine strains that have proven to be genetically unstable including, but not limited to, influenza strains comprising the PB2 K637E, N265S, E65G, P112S, N556D, and/or Y658H substitutions.

98. Therefore, in one aspect, disclosed herein are methods of increasing the immunogenicity of an attenuated influenza vaccine comprising obtaining an attenuated influenza vaccine viral strain and generating a mutation in the PB1 or PA subunit of the RNA polymerase of the influenza virus, wherein the mutation increases the fidelity of the RNA polymerase; and wherein an increase in fidelity of the polymerase increases the stability of the virus strain in a subject and reduces the amount mutant viruses formed during transcription of the virus thereby increasing the abundance of a single strain and as a result increases the immune response to the immunizing strain. For example, disclosed herein are methods of increasing the immunogenicity of an attenuated influenza vaccine, wherein the mutation of the PB1 or PA subunit of the influenza RNA polymerase comprises a mutation at least one residue selected from one or more residues of the alpha helix corresponding to residues 36 to 50 or residues 383 to 395 of the PB1 subunit as set forth in SEQ ID NO: 4; at mutation at one or more residues of the loop formed by the amino acids corresponding to residues 77-80 as set forth in SEQ ID NO: 4; a mutation at one or more residues of the loop formed by the amino acids corresponding to residues 228-240 as set forth in SEQ ID NO: 4; a mutation at one or more residues of the loop formed by the amino acids corresponding to residues 306-311 as set forth in SEQ ID NO: 4; a mutation at residues 480 or 481 as set forth in SEQ ID NO: 4; a mutation one or more residues of the alpha helix corresponding to residues 654 to 674 and/or a mutation at residues corresponding to 682 to 693 of the PA subunit as set forth in SEQ ID NO: 3.

99. In one aspect, disclosed herein are pharmaceutical compositions comprising any of the modified influenza viruses or recombinant nucleic acids disclosed herein. For example, disclosed herein are pharmaceutical compositions comprising one or more modified influenza viruses comprising one or more mutations in the PB1 subunit of the RNA polymerase further comprising one or more mutations at one or more residues of the PA subunit of the RNA polymerase, wherein the mutations of the RNA polymerase causes an increase in fidelity of the polymerase. For example, disclosed herein are pharmaceutical compositions comprising one or

more modified influenza viruses comprising one or more mutations of the PB1 subunit of the RNA polymerase including but not limited to a mutation in the alpha helix corresponding to residues 36 to 50 of SEQ ID NO: 4, the alpha helix corresponding to residues 383 to 395 of SEQ ID NO: 4, the loop corresponding to residues 77 to 80 of SEQ ID NO: 4, the loop corresponding to residues 228 to 240 of SEQ ID NO: 4, the loop corresponding to residues 306 to 311 of SEQ ID NO: 4, and/or residues corresponding to residues 480 and/or 481 as set forth in SEQ ID NO: 4 and/or comprising one or more mutations in the alpha helix corresponding to residues 654 to 674 of SEQ ID NO: 3, and/or the alpha helix corresponding to residues 682 to 693 of SEQ ID NO: 3, wherein the mutations of the RNA polymerase causes an increase in fidelity of the polymerase. Also disclosed are pharmaceutical compositions comprising one or more modified influenza viruses comprising one or more mutations of the PB1 subunit of the RNA polymerase including but not limited to a mutation at a residue corresponding to 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 77, 78, 79, 80, 228, 229, 230, 231, 232, 233, 234, 235, 236, 237, 238, 239, 240, 306, 307, 308, 309, 310, 311, 383, 384, 385, 386, 387, 388, 389, 390, 391, 392, 393, 394, 395, 480, and/or 481 of SEQ ID NO: 4 and/or comprising one or more mutations of the PA subunit of the RNA polymerase including but not limited to a mutation at a residue corresponding to 654, 655, 656, 657, 658, 659, 660, 661, 662, 663, 664, 665, 666 ,667, 668, 669, 670, 671, 672, 673, 674, 682, 683, 684, 685, 686, 687, 688, 689, 690, 691, 692, and/or 693 of SEQ ID NO: 3.

100. Also disclosed herein are pharmaceutical compositions comprising one or more recombinant nucleic acids encoding one or more mutations of the PB1 subunit of the RNA polymerase including but not limited to a mutation in the alpha helix corresponding to residues 36 to 50 of SEQ ID NO: 4, the alpha helix corresponding to residues 383 to 395 of SEQ ID NO: 4, the loop corresponding to residues 77 to 80 of SEQ ID NO: 4, the loop corresponding to residues 228 to 240 of SEQ ID NO: 4, the loop corresponding to residues 306 to 311 of SEQ ID NO: 4, and/or residues corresponding to residues 480 and/or 481 as set forth in SEQ ID NO: 4 and/or encoding one or more mutations in the alpha helix corresponding to residues 654 to 674 of SEQ ID NO: 3, and/or the alpha helix corresponding to residues 682 to 693 of SEQ ID NO: 3, wherein the mutations of the RNA polymerase causes an increase in fidelity of the polymerase.

101. Also disclosed are pharmaceutical compositions comprising one or more recombinant nucleic acids encoding one or more mutations of the PB1 subunit of the RNA polymerase including but not limited to a mutation at a residue corresponding to 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 77, 78, 79, 80, 228, 229, 230, 231, 232, 233, 234, 235, 236, 237, 238, 239, 240, 306, 307, 308, 309, 310, 311, 383, 384, 385, 386, 387, 388, 389, 390,

391, 392, 393, 394, 395, 480, and/or 481 of SEQ ID NO: 4 and/or encoding one or more mutations of the PA subunit of the RNA polymerase including but not limited to a mutation at a residue corresponding to 654, 655, 656, 657, 658, 659, 660, 661, 662, 663, 664, 665, 666 ,667, 668, 669, 670, 671, 672, 673, 674, 682, 683, 684, 685, 686, 687, 688, 689, 690, 691, 692, and/or 693 of SEQ ID NO: 3.

102. As described above, the compositions can also be administered *in vivo* in a pharmaceutically acceptable carrier. By "pharmaceutically acceptable" is meant a material that is not biologically or otherwise undesirable, i.e., the material may be administered to a subject, along with the nucleic acid or vector, without causing any undesirable biological effects or interacting in a deleterious manner with any of the other components of the pharmaceutical composition in which it is contained. The carrier would naturally be selected to minimize any degradation of the active ingredient and to minimize any adverse side effects in the subject, as would be well known to one of skill in the art.

103. The compositions may be administered orally, parenterally (e.g., intravenously), by intramuscular injection, by intraperitoneal injection, transdermally, subcutaneuously, extracorporeally, topically or the like, including topical intranasal administration or administration by inhalant or nebulization, or installation via bronchoscopy. As used herein, "topical intranasal administration" or "intranasally" means delivery of the compositions into the nose and nasal passages through one or both of the nares and can comprise delivery by a spraying mechanism or droplet mechanism, or through aerosolization of the nucleic acid or vector. Optionally, the composition is administered by oral inhalation, nasal inhalation or intranasal mucosal administration. That is, administration of the compositions by inhalant can be through the nose or mouth via delivery by a spraying or droplet mechanism. Delivery can also be directly to any area of the respiratory system (e.g., lungs) via intubation. The exact amount of the compositions required will vary from subject to subject, depending on the species, age, weight and general condition of the subject, the severity of the allergic disorder being treated, the particular nucleic acid or vector used, its mode of administration and the like. Thus, it is not possible to specify an exact amount for every composition. However, an appropriate amount can be determined by one of ordinary skill in the art using only routine experimentation given the teachings herein.

104. Parenteral administration of the composition, if used, is generally characterized by injection. Injectables can be prepared in conventional forms, either as liquid solutions or suspensions, solid forms suitable for solution of suspension in liquid prior to injection, or as emulsions. A more recently revised approach for parenteral administration involves use of a

slow release or sustained release system such that a constant dosage is maintained. See, e.g., U.S. Patent No. 3,610,795, which is incorporated by reference herein.

105. The materials may be in solution, suspension (for example, incorporated into microparticles, liposomes, or cells). These may be targeted to a particular cell type via antibodies, receptors, or receptor ligands. The following references are examples of the use of this technology to target specific proteins to tumor tissue (Senter, et al., *Bioconjugate Chem.*, 2:447-451, (1991); Bagshawe, K.D., *Br. J. Cancer*, 60:275-281, (1989); Bagshawe, et al., *Br. J. Cancer*, 58:700-703, (1988); Senter, et al., *Bioconjugate Chem.*, 4:3-9, (1993); Battelli, et al., *Cancer Immunol. Immunother.*, 35:421-425, (1992); Pietersz and McKenzie, *Immunolog. Reviews*, 129:57-80, (1992); and Roffler, et al., *Biochem. Pharmacol*, 42:2062-2065, (1991)). Vehicles such as "stealth" and other antibody conjugated liposomes (including lipid mediated drug targeting to colonic carcinoma), receptor mediated targeting of DNA through cell specific ligands, lymphocyte directed tumor targeting, and highly specific therapeutic retroviral targeting of murine glioma cells *in vivo*. The following references are examples of the use of this technology to target specific proteins to tumor tissue (Hughes et al., *Cancer Research*, 49:6214-6220, (1989); and Litzinger and Huang, *Biochimica et Biophysica Acta*, 1104:179-187, (1992)). In general, receptors are involved in pathways of endocytosis, either constitutive or ligand induced. These receptors cluster in clathrin-coated pits, enter the cell via clathrin-coated vesicles, pass through an acidified endosome in which the receptors are sorted, and then either recycle to the cell surface, become stored intracellularly, or are degraded in lysosomes. The internalization pathways serve a variety of functions, such as nutrient uptake, removal of activated proteins, clearance of macromolecules, opportunistic entry of viruses and toxins, dissociation and degradation of ligand, and receptor-level regulation. Many receptors follow more than one intracellular pathway, depending on the cell type, receptor concentration, type of ligand, ligand valency, and ligand concentration. Molecular and cellular mechanisms of receptor-mediated endocytosis has been reviewed (Brown and Greene, *DNA and Cell Biology* 10:6, 399-409 (1991)).

106. The compositions, including modified influenza viruses and recombinant nucleic acids disclosed herein can be used therapeutically in combination with a pharmaceutically acceptable carrier.

107. Suitable carriers and their formulations are described in *Remington: The Science and Practice of Pharmacy* (19th ed.) ed. A.R. Gennaro, Mack Publishing Company, Easton, PA 1995. Typically, an appropriate amount of a pharmaceutically-acceptable salt is used in the formulation to render the formulation isotonic. Examples of the pharmaceutically-acceptable

carrier include, but are not limited to, saline, Ringer's solution and dextrose solution. The pH of the solution is preferably from about 5 to about 8, and more preferably from about 7 to about 7.5. Further carriers include sustained release preparations such as semipermeable matrices of solid hydrophobic polymers containing the antibody, which matrices are in the form of shaped articles, e.g., films, liposomes or microparticles. It will be apparent to those persons skilled in the art that certain carriers may be more preferable depending upon, for instance, the route of administration and concentration of composition being administered.

108. Pharmaceutical carriers are known to those skilled in the art. These most typically would be standard carriers for administration of drugs to humans, including solutions such as sterile water, saline, and buffered solutions at physiological pH. The compositions can be administered intramuscularly or subcutaneously. Other compounds will be administered according to standard procedures used by those skilled in the art.

109. Pharmaceutical compositions may include carriers, thickeners, diluents, buffers, preservatives, surface active agents and the like in addition to the molecule of choice. Pharmaceutical compositions may also include one or more active ingredients such as antimicrobial agents, antiinflammatory agents, anesthetics, and the like.

110. The pharmaceutical composition may be administered in a number of ways depending on whether local or systemic treatment is desired, and on the area to be treated. Administration may be orally, parenterally (e.g., intravenously), by intramuscular injection, by intraperitoneal injection, transdermally, subcutaneuously, extracorporeally, topically (including ophthalmically, vaginally, rectally, intranasally), or the like, including topical intranasal administration or administration by inhalant or nebulization, or installation via bronchoscopy. As used herein, "topical intranasal administration" or “intranasally” means delivery of the compositions into the nose and nasal passages through one or both of the nares and can comprise delivery by a spraying mechanism or droplet mechanism, or through aerosolization of the nucleic acid or vector. Optionally, the composition is administered by oral inhalation, nasal inhalation or intranasal mucosal administration. That is, administration of the compositions by inhalant can be through the nose or mouth via delivery by a spraying or droplet mechanism. Delivery can also be directly to any area of the respiratory system (e.g., lungs) via intubation.

111. Preparations for parenteral administration include sterile aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. Parenteral vehicles include sodium chloride solution,

Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's, or fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers (such as those based on Ringer's dextrose), and the like. Preservatives and other additives may also be present such as, for example, antimicrobials, anti-oxidants, chelating agents, and inert gases and the like.

112. Formulations for topical administration may include ointments, lotions, creams, gels, drops, suppositories, sprays, liquids and powders. Conventional pharmaceutical carriers, aqueous, powder or oily bases, thickeners and the like may be necessary or desirable.

113. Compositions for oral administration include powders or granules, suspensions or solutions in water or non-aqueous media, capsules, sachets, or tablets. Thickeners, flavorings, diluents, emulsifiers, dispersing aids or binders may be desirable..

114. Some of the compositions may potentially be administered as a pharmaceutically acceptable acid- or base- addition salt, formed by reaction with inorganic acids such as hydrochloric acid, hydrobromic acid, perchloric acid, nitric acid, thiocyanic acid, sulfuric acid, and phosphoric acid, and organic acids such as formic acid, acetic acid, propionic acid, glycolic acid, lactic acid, pyruvic acid, oxalic acid, malonic acid, succinic acid, maleic acid, and fumaric acid, or by reaction with an inorganic base such as sodium hydroxide, ammonium hydroxide, potassium hydroxide, and organic bases such as mono-, di-, trialkyl and aryl amines and substituted ethanolamines.

115. The disclosed pharmaceutical compositions, modified influenza viruses and recombinant nucleic acids can be used as therapeutic or prophylactic vaccines to elicit a protective or therapeutic immune response against influenza virus. In one aspect, disclosed herein are methods of immunizing a subject against influenza virus comprising administering to the subject any of the pharmaceutical compositions, recombinant nucleic acids, or modified influenza viruses disclosed herein. Also disclosed are methods of inducing an immune response against influenza virus in a subject comprising administering to the subject any of the modified influenza viruses, recombinant nucleic acids, or pharmaceutical compositions disclosed herein.

116. It is understood and herein contemplated that the elicitation of a protective or therapeutic immune response can inhibit an influenza virus infection in a subject. Thus, in one aspect, disclosed herein are methods of inhibiting an influenza virus infection in a subject comprising administering to the subject any of the modified influenza viruses, recombinant nucleic acids, or pharmaceutical compositions disclosed herein.

117. As used herein the terms treatment, treat, or treating refers to a method of reducing one or more of the effects of the infection or one or more symptoms of the infection by eliciting an immune response in the subject. Thus in the disclosed method, treatment can refer to

a 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 100% reduction in the severity of an established infection or a symptom of the infection. For example, a method for treating an infection is considered to be a treatment if there is a 10% reduction in one or more symptoms of the infection in a subject as compared to a control. Thus the reduction can be a 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, or any percent reduction in between 10% and 100% as compared to native or control levels. It is understood that treatment does not necessarily refer to a cure or complete ablation of the infection or disease or symptoms of the infection or disease.

118. As used herein, the terms prevent, preventing, and prevention of an infection, refers to an action, for example, administration of a therapeutic agent (e.g., a composition disclosed herein), that occurs before or at about the same time a subject begins to show one or more symptoms of the infection, which inhibits or delays onset or exacerbation or delays recurrence of one or more symptoms of the infection. As used herein, references to decreasing, reducing, or inhibiting include a change of 10%>, 20%>, 30%>, 40%>, 50%>, 60%>, 70%), 80%), 90%) or greater relative to a control level. For example, the disclosed methods are considered to be a prevention if there is about a 10%> reduction in onset, exacerbation or recurrence of infection, or symptoms of infection in a subject exposed to an infection when compared to control subjects exposed to an infection that did not receive a composition for decreasing infection. Thus, the reduction in onset, exacerbation or recurrence of infection can be about a 10, 20, 30, 40, 50, 60, 70, 80, 90, 100%, or any amount of reduction in between as compared to control subjects. For example, and not to be limiting, if about 10% of the subjects in a population do not become infected as compared to subjects that did not receive preventive treatment, this is considered prevention. Similarly, as used herein, “inhibit” or “inhibition” refers to any decreased change in viral growth, replication, or spread that decreases the virulence of a virus. Inhibition can commence after antigenic exposure. Inhibition can comprise a change of 10%>, 20%>, 30%>, 40%>, 50%>, 60%>, 70%>, 80%>, 90%> or greater relative to a control level. For example, the disclosed methods are considered to be a inhibition if there is about a 10%> reduction in onset, exacerbation, or recurrence of infection, replication rate of a virus, viral spread, or symptoms of infection in a subject exposed to an infection when compared to control subjects exposed to an infection that did not receive a composition for decreasing infection.

119. Effective dosages and schedules for administering the compositions may be determined empirically, and making such determinations is within the skill in the art. The dosage ranges for the administration of the compositions are those large enough to produce the desired effect in which the symptoms of the disorder are effected. The dosage should not be so

large as to cause adverse side effects, such as unwanted cross-reactions, anaphylactic reactions, and the like. Generally, the dosage will vary with the age, condition, sex and extent of the disease in the patient, route of administration, or whether other drugs are included in the regimen, and can be determined by one of skill in the art. The dosage can be adjusted by the individual physician in the event of any counterindications. Dosage can vary, and can be administered in one or more dose administrations daily, for one or several days. Guidance can be found in the literature for appropriate dosages for given classes of pharmaceutical products. For example, guidance in selecting appropriate doses for antibodies can be found in the literature on therapeutic uses of antibodies, e.g., *Handbook of Monoclonal Antibodies*, Ferrone et al., eds., Noges Publications, Park Ridge, N.J., (1985) ch. 22 and pp. 303-357; Smith et al., *Antibodies in Human Diagnosis and Therapy*, Haber et al., eds., Raven Press, New York (1977) pp. 365-389. A typical daily dosage of the antibody used alone might range from about 1 μg/kg to up to 100 mg/kg of body weight or more per day, depending on the factors mentioned above.

120. Following administration of a disclosed composition, such as an the modified influenza virus, vaccine comprising a modified influenza virus, recombinant PB1 and/or recombinant PA, for treating, inhibiting, or preventing an influenza infection, the efficacy of the modified influenza virus, vaccine comprising a modified influenza virus, recombinant PB1 and/or recombinant PA can be assessed in various ways well known to the skilled practitioner. For instance, one of ordinary skill in the art will understand that a composition, such as an the modified influenza virus, vaccine comprising a modified influenza virus, recombinant PB1 and/or recombinant PA, disclosed herein is efficacious in treating or inhibiting an influenza infection in a subject by observing that the composition reduces viral load of a challenge virus or actual infection or prevents a further increase in viral load. Viral loads can be measured by methods that are known in the art, for example, using polymerase chain reaction assays to detect the presence of Influenza nucleic acids or antibody assays to detect the presence of Influenza proteins in a sample (e.g., but not limited to, blood) from a subject or patient, or by measuring the level of circulating anti- Influenza antibody levels in the patient.

121. The compositions such as, the modified influenza virus, vaccine comprising a modified influenza virus, recombinant PB1 and/or recombinant PA, that are disclosed herein may be administered prophylactically to patients or subjects who are at risk for being exposed to Influenza virus or who have been newly exposed to Influenza virus. In subjects who have been newly exposed to Influenza virus but who have not yet displayed the presence of the virus (as measured by PCR or other assays for detecting the virus) in blood or other body fluid, efficacious treatment with a modified influenza virus, vaccine comprising a modified influenza

virus, recombinant PB1 and/or recombinant PA partially or completely inhibits the appearance of the virus in the blood or other body fluid.

122. As used throughout, a subject can be a vertebrate, more specifically a mammal (e.g., a human, horse, cat, dog, cow, pig, sheep, goat, mouse, rabbit, rat, non-human primate, and guinea pig), birds, reptiles, amphibians, fish, and any other animal. The term does not denote a particular age or sex. Thus, adult and newborn subjects, whether male or female, are intended to be covered. As used herein, patient or subject may be used interchangeably and can refer to a subject with or at risk of developing an influenza infection. The term patient or subject includes human and veterinary subjects.

C. Methods of making the compositions

123. The compositions disclosed herein and the compositions necessary to perform the disclosed methods can be made using any method known to those of skill in the art for that particular reagent or compound unless otherwise specifically noted.

1. Nucleic acid synthesis

124. For example, the nucleic acids, such as, the oligonucleotides to be used as primers can be made using standard chemical synthesis methods or can be produced using enzymatic methods or any other known method. Such methods can range from standard enzymatic digestion followed by nucleotide fragment isolation (see for example, Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, 2nd Edition (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989) Chapters 5, 6) to purely synthetic methods, for example, by the cyanoethyl phosphoramidite method using a Milligen or Beckman System 1Plus DNA synthesizer (for example, Model 8700 automated synthesizer of Milligen-Biosearch, Burlington, MA or ABI Model 380B). Synthetic methods useful for making oligonucleotides are also described by Ikuta *et al.*, *Ann. Rev. Biochem.* 53:323-356 (1984), (phosphotriester and phosphite-triester methods), and Narang *et al.*, *Methods Enzymol.*, 65:610-620 (1980), (phosphotriester method). Protein nucleic acid molecules can be made using known methods such as those described by Nielsen *et al.*, *Bioconjug. Chem.* 5:3-7 (1994).

2. Peptide synthesis

125. One method of producing the disclosed proteins, such as SEQ ID NO: 4, is to link two or more peptides or polypeptides together by protein chemistry techniques. For example, peptides or polypeptides can be chemically synthesized using currently available laboratory equipment using either Fmoc (9-fluorenylmethyloxycarbonyl) or Boc (*tert* -butyloxycarbonoyl) chemistry. (Applied Biosystems, Inc., Foster City, CA). One skilled in the art can readily appreciate that a peptide or polypeptide corresponding to the disclosed proteins, for example,

can be synthesized by standard chemical reactions. For example, a peptide or polypeptide can be synthesized and not cleaved from its synthesis resin whereas the other fragment of a peptide or protein can be synthesized and subsequently cleaved from the resin, thereby exposing a terminal group which is functionally blocked on the other fragment. By peptide condensation reactions, these two fragments can be covalently joined via a peptide bond at their carboxyl and amino termini, respectively, to form an antibody, or fragment thereof. (Grant GA (1992) *Synthetic Peptides: A User Guide*. W.H. Freeman and Co., N.Y. (1992); Bodansky M and Trost B., Ed. (1993) Principles of Peptide Synthesis. Springer-Verlag Inc., NY (which is herein incorporated by reference at least for material related to peptide synthesis). Alternatively, the peptide or polypeptide is independently synthesized *in vivo* as described herein. Once isolated, these independent peptides or polypeptides may be linked to form a peptide or fragment thereof via similar peptide condensation reactions.

126. For example, enzymatic ligation of cloned or synthetic peptide segments allow relatively short peptide fragments to be joined to produce larger peptide fragments, polypeptides or whole protein domains (Abrahmsen L et al., *Biochemistry*, 30:4151 (1991)). Alternatively, native chemical ligation of synthetic peptides can be utilized to synthetically construct large peptides or polypeptides from shorter peptide fragments. This method consists of a two step chemical reaction (Dawson et al. Synthesis of Proteins by Native Chemical Ligation. Science, 266:776-779 (1994)). The first step is the chemoselective reaction of an unprotected synthetic peptide--thioester with another unprotected peptide segment containing an amino-terminal Cys residue to give a thioester-linked intermediate as the initial covalent product. Without a change in the reaction conditions, this intermediate undergoes spontaneous, rapid intramolecular reaction to form a native peptide bond at the ligation site (Baggiolini M et al. (1992) *FEBS Lett.* 307:97-101; Clark-Lewis I et al., *J.Biol.Chem.*, 269:16075 (1994); Clark-Lewis I et al., *Biochemistry*, 30:3128 (1991); Rajarathnam K et al., *Biochemistry* 33:6623-30 (1994)).

127. Alternatively, unprotected peptide segments are chemically linked where the bond formed between the peptide segments as a result of the chemical ligation is an unnatural (non-peptide) bond (Schnolzer, M et al. *Science*, 256:221 (1992)). This technique has been used to synthesize analogs of protein domains as well as large amounts of relatively pure proteins with full biological activity (deLisle Milton RC et al., *Techniques in Protein Chemistry IV*. Academic Press, New York, pp. 257-267 (1992)).

3. Process claims for making the compositions

128. Disclosed are processes for making the compositions as well as making the intermediates leading to the compositions. For example, disclosed are nucleic acids in SEQ ID

NO: 5 and/or SEQ ID NO: 6. There are a variety of methods that can be used for making these compositions, such as synthetic chemical methods and standard molecular biology methods. It is understood that the methods of making these and the other disclosed compositions are specifically disclosed.

129. Disclosed are nucleic acid molecules produced by the process comprising linking in an operative way a nucleic acid comprising the sequence set forth in SEQ ID NO: 5 and/or SEQ ID NO: 6 and a sequence controlling the expression of the nucleic acid.

130. Also disclosed are nucleic acid molecules produced by the process comprising linking in an operative way a nucleic acid molecule comprising a sequence having 80% identity to a sequence set forth in SEQ ID NO: 5 and/or SEQ ID NO: 6, and a sequence controlling the expression of the nucleic acid.

131. Disclosed are nucleic acid molecules produced by the process comprising linking in an operative way a nucleic acid molecule comprising a sequence that hybridizes under stringent hybridization conditions to a sequence set forth SEQ ID NO: 5 and/or SEQ ID NO: 6 and a sequence controlling the expression of the nucleic acid.

132. Disclosed are nucleic acid molecules produced by the process comprising linking in an operative way a nucleic acid molecule comprising a sequence encoding a protein set forth in SEQ ID NO: 3 and/or SEQ ID NO: 4 and a sequence controlling an expression of the nucleic acid molecule.

133. Disclosed are nucleic acid molecules produced by the process comprising linking in an operative way a nucleic acid molecule comprising a sequence encoding a protein having 80% identity to a peptide set forth in SEQ ID NO: 3 and/or SEQ ID NO: 4 and a sequence controlling an expression of the nucleic acid molecule.

134. Disclosed are nucleic acids produced by the process comprising linking in an operative way a nucleic acid molecule comprising a sequence encoding a peptide having 80% identity to a peptide set forth in SEQ ID NO: 3 and/or SEQ ID NO: 4, wherein any change from SEQ ID NO: 3 and/or SEQ ID NO: 4 are conservative changes and a sequence controlling an expression of the nucleic acid molecule.

135. Disclosed are cells produced by the process of transforming the cell with any of the disclosed nucleic acids. Disclosed are cells produced by the process of transforming the cell with any of the non-naturally occurring disclosed nucleic acids.

136. Disclosed are any of the disclosed proteins or peptides produced by the process of expressing any of the disclosed nucleic acids. Disclosed are any of the non-naturally occurring disclosed peptides produced by the process of expressing any of the disclosed nucleic acids.

Disclosed are any of the disclosed peptides produced by the process of expressing any of the non-naturally disclosed nucleic acids.

137. The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how the compounds, compositions, articles, devices and/or methods claimed herein are made and evaluated, and are intended to be purely exemplary and are not intended to limit the disclosure. Efforts have been made to ensure accuracy with respect to numbers (e.g., amounts, temperature, etc.), but some errors and deviations should be accounted for. Unless indicated otherwise, parts are parts by weight, temperature is in °C or is at ambient temperature, and pressure is at or near atmospheric.

D. Examples

1. Example 1: Rational Design of High Fidelity Mutant Viruses

138. Disclosed herein is the discovery of several high fidelity virus variants which are attenuated in mice. These mutants were selected using the mutagenic purine analog, ribavirin, and have a single amino acid substitution within a putative nucleotide influx channel in the viral polymerase – indicating the possibility that the nucleotide influx channel may regulate virus fidelity. Disclosed herein is a panel of 11 mutants that map to the putative nucleotide influx channel, and have characterized their replicative properties. By doing so, a number of mutations were identified which confer altered nucleotide selectivity both *in vitro* and *in vivo* – as well as viable virus mutants that mediate protection against challenge with wild-type influenza virus in mice.

139. Novel Influenza A Virus (IAV) polymerase mutants with altered nucleotide selectivity were identified in vitro using the crystal structure of IAV. Through the crystal structure, it was shown that viruses possessing these mutations have altered nucleotide selectivity in cell culture. Herein, the initial mutations at the nucleotide entry channel are analyzed using additional nucleoside analogs in minigenome, viral growth and polymerase activity assays. Second generation of mutations with greater impacts on fidelity are also created and tested herein.

140. IAV was passaged in the presence of ribavirin and selected a novel high fidelity virus. The ribavirin resistance was traced to a V34I mutation in the PB1 segment, which lies far from the active site (>100A) but close to a putative nucleotide entry channel. Based on the enzyme structure, it was hypothesize that the PB1 V43I mutation displaces an alpha helix and extrudes lysine at residue 387 and 391 into the channel which may constrict nucleotide influx (Figure 1).

141. To study this channel further, additional mutations were made that fall into three categories: (i) mutations at PB1 residue 43 that are intended to constrict the channel (ii) mutations that alter the charge at the entry of the channel and (iii) PA mutations intended to constrict the channel (Table 3). First generation mutants were tested for activity in polymerase based minigenome assays and saw that 6 of them (V43I, V43L, R45D, K387D, K391D, and R663K) had greater than 1% activity compared to wt. The activity of these polymerases was examined in closer detail. Minigenome assays were performed with both a vRNA (viral transcription only) and cRNA (requiring replication of the reporter gene as well as viral transcription) based viral specific reporter gene and saw that PA R663K exhibited mild temperature sensitivity with both. (Fig. 2A).

Table 3: Selected Residues for mutation

1st Generation	PB1	V43I, V43L, R45K, R45D, K308D, K387D, K391D
	PA	R663E, R663K, K664E
2nd Generation	PB1	K235, K237, K239, K480, K481
	PA	E661, E691

142. Viruses were rescued with and without these mutations and virus multi-cycle growth kinetics were analyzed in A549 cells at 37ºC. PR8 possessing K387D or K391D exhibited elevated growth at 37ºC compared to WT virus (Fig. 2B). All other first generation mutations did not significantly affect growth kinetics (measured by two-way ANOVA). However, PR8 possessing K at PA 663 was significantly temperature sensitive with reduced viral titers at 39ºC at all time points tested (Fig. 2C). The other mutations did not significantly affect the growth kinetics of PR8.

143. Mice were infected with these mutant viruses to determine if any possessed altered attenuation or impaired immunogenicity. All viruses indeed had improved attenuation (safety) as compared to WT PR8, while retaining levels of immunogenicity that were close to the wild-type virus (Table 4).

Table 4: All of the nucleotide channel viruses are safer than wt PR9 and maintain immunogenicity.

Priming Virus	**LD-50, FFU**	**Geometric mean (SD) HAI titer, reciprocal serum dilution:**
PR8 WT	30	2050 (0)
PR8 PB1 V43I	30	1500 (590)
PR8 PB1 V43L	600	390 (340)
PR8 PB1 K387D	100	680 (360)
PR8 PB1 K391D	150	720 (290)

PR8 PA R663K	300	510 (320)
PBS	--	<16 (0)

C57 BL/6 mice (n=5) were inoculated intranasally with 10, 100, and 1000 FFU of the indicated viruses in 30μL after mild avertin anesthetization as described. Sera was collected at 12 days postinfection. Eight hemagglutinating units (HAU) of WT PR8 were incubated with 2-fold serial dilutions of the indicated sera. Weight loss and clinical signs of distress were measured daily. Mice were euthanized upon losing 30% of their initial body weight or displaying clinical signs of distress. Depicted is the mean lethal dose and the geometric mean of the hemagglutination inhibition of the mice infected with 10 FFU. Mean lethal dose (LD50) was calculated with the survival data from above using the method of Reed and Muench.

144. Studies were initiated with the intention of assessing the effects of the various polymerase mutants on enzyme fidelity. Mutants were tested to determine whether they conferred resistance to the drug, ribavirin, which inhibits influenza virus replication in large part by (i) depleting the cellular GTP pool (through its competition with cellular inosine monophosphate for IMP dehydrogenase) and (ii) promoting viral mutagenesis. Briefly, minigenome assays were conducted in the presence of ribavirin (at 0-75 μM). It was found that polymerase complexes containing PB1 mutations V43I, K387D, and K391D exhibited greater activity than WT in the presence of ribavirin (Figs. 3 and 4), indicating that these mutations convey resistance to ribavirin-mediated inhibition.

145. The effects of the mutations on the nucleotide selectivity of the viral polymerase was also tested. To do this, minigenome assays were conducted in the presence of high levels of exogenous guanosine. Excess guanosine inhibits influenza polymerase activity, likely through the creation of unbalanced intracellular NTP pools (with increased GTP and reduced ATP). Polymerases with greater nucleotide selectivity were expected to have reduced activity under these conditions. Fig. 3 shows the polymerase complex containing the PB1 V43I mutation exhibited a greater reduction of polymerase activity in the presence of excess guanosine, compared to the wild-type polymerase. The PA R663K mutation also conferred a similar phenotype – although this mutation resulted in even greater sensitivity to inhibition by excess guanosine.

146. Finally, a determination was made of whether the inhibitory effects of ribavirin were due to nucleotide bias or direct incorporation into the growing mRNA. To do this, low dose guanosine (40μM) was added to the cells and varied the concentration of ribavirin. PB1 mutations K387D, K391D had greatly increased activity in the presence of low dose guanosine (40μM) (Fig. 5A). However, when the minigenome assays were performed in cells that were treated with 40 μM guanosine plus ribavirin (25 or 50 μM), all of the mutants showed the same response as the wild-type enzyme (Fig. 5B). This shows the mutations are not directly affecting

nucleotide incorporation in the presence of guanosine, but rather that they are affecting the ability of the viral polymerase to utilize limited nucleotide pools.

2. Example 2: Identify the highest fidelity variant of the 1st generation nucleotide entry channel mutants.

147. An initial panel of polymerase mutants (Fig. 2) was created and analyzed with ribavirin and guanosine (Fig. 3). The analysis can be extended by treating cells with methotrexate, favipiravir , 5-fluorouracil and 5-azacytidine (all available from Sigma). These conditions examine both susceptibility to nucleotide bias (methotrexate) and direct competitive inhibition. Initially the effect of the mutagens on polymerase activity is examined as measured by minigenome assay (see Fig. 3 for methods). Briefly, cells are pre-treated with the various mutagens and measure the impact on reporter gene production as normalized to cell transfection and viability controls. Assays are conducted in which the base whose synthesis (or phosphorylation) is impaired by the nucleotide analog (Fig. 5) is added back. To test whether reduction in reporter gene activity is through lethal mutagenesis or reduced mRNA synthesis, RT-PCR is performed to determine the relative levels and lengths of reporter gene mRNA.

148. The impact of these nucleoside analogs on virus growth and viral mutation frequency was tested. Virus replication was analyzed in both Madin Darby Canine Kidney (MDCK) cells and human lung carcinoma (A549) cells. This allowed for the detection of differences at both elevated and reduced rates of replication, as altered by temperature. Briefly, cells were infected with virus at a multiplicity of infection (M.O.I.) of 0.05 and incubated with and without the indicated mutagens. Aliquots of culture supernatants were collected at 8,16, 24, 48, and 72 h.p.i. and titrated on MDCK cells to determine viral titer by plaque assay (PFU/ml) or enumeration of NP-expressing cells by immunofluorescence (FFU/ml).

149. Also clonal sequencing was performed on viruses (wild-type and mutant) following two passages in MDCK cells in the absence of any mutagen, in order to calculate the relative mutational frequency of the wild-type and the high fidelity viruses (Fig. 6). Deep sequencing of virus stocks were completed by paired-end Illumina sequencing (2X125) to a ~100X coverage depth. Sequence reads were mapped to reference influenza A genomes using BWA and SNP/INDELs identified with Samtools+BCFtools.

150. Biochemical assays of the mutated viral polymerases are performed. Briefly, both wild type polymerase as well as polymerases containing the most promising fidelity mutants using baculovirus vectors are produced and purified. These purified heterotrimeric polymerases are used in primer extension assays. The rate of misincorporation with both biased (or omitted) nucleotide pools as well as in the presence of various mutagens (see above) is analyzed.

151. A second generation of single and combination mutants (Table 3) is designed based on the results of the first generation of mutants. Residues are mutated to both to alter the charge (K to E) at residues 235, 237, 239, 480, and 481 of PB1 as well as size (K to A and K to R). Additional residues are mutated at position 77, 78, 79, and 80 of PB1 and residues 661 and 691 of PA. The effects of these mutants is tested in minigenome and viral growth assays as described herein. Virus mutants exhibiting the greatest increase in fidelity are progressed to test the immunogenicity (and relative safety/attenuation) of these mutants by performing a lethal dose analysis in mice as well as determining serum antibody titers and susceptibility to heterologous challenge (see Table 4). Briefly, groups of 4 female 5-7-week old C57BL/6 mice are inoculated intranasally (i.n.) with the three most promising viruses. Weight loss is measured over the course of infection, through day 14 (see Vertebrate Animals section for details, including group size justification).

3. Example 3: Using high fidelity variants of IAV to stabilize attenuating and genetically unstable mutations.

152. The ability of the high fidelity mutants to stabilize 6 previously identified, genetically unstable and attenuating mutations within PB2 (E65G, P112S, N265S, N556D, K627E, Y658H) is tested. All 6 of these mutations have been characterized in various temperature sensitive viruses that genotypically reverted. These mutations range in stability from K627E (lost in one passage in vivo) to N265S (stable in the current live attenuated vaccine upon dozens of passages). This panel of mutations therefore provides an ideal spectrum to characterize the high fidelity candidates. To assess viral fidelity viruses are created that contain this mutant PB2 segment in either conventional mouse adapted H1N1 (PR8), or in mutated derivatives of PR8 that contain the fidelity mutations of interest.

4. Example 4: Determination of the ability of high fidelity viruses to stabilize otherwise unstable mutations.

153. Prior experiments identify novel fidelity variants of IAV, and test their ability to stabilize otherwise unfavorable mutations. To do this, advantage is taken of a cohort of mutations that were used for the rational design of influenza vaccine candidates – but which reverted upon passage *in vitro* and *in vivo*. These mutations (PB2 - E65G, P112S, N265S, N556D, K627E, Y658H) (rev+) are introduced into the genetic background of A/PR/8/34 [H1N1] with and without the fidelity altering mutations; the corresponding viruses are produced using standard reverse genetics methods, as described.

154. Rev+ containing WT and fidelity modified viruses are serially passaged at low MOI (0.01) in both MDCK and A549 cells at the permissive temperature of 33°C. Later, the

viruses are serially passaged in mice. The viruses are tittered before each passage to maintain a consistent MOI from passage to passage. Briefly, groups of 4 female 5-7-week old C57BL/6 mice are inoculated intranasally (i.n.) with rev+ WT and the 3 most promising high fidelity viruses at 0.1 LD_{50}. Weight loss is measured over the course of infection, and at 3 days post infection, lungs are surgically extracted and virus titers determined from homogenized lung tissue.

155. Virus titers for both experiments are determined by infecting triplicate wells of confluent MDCK cells with 10-fold serial dilutions of virus and NP-expressing cells are enumerated to determine virus titer in FFU/ml. Each virus passage is clarified by centrifugation, and aliquots of each passage stored at -80°C.

156. The test viruses are then passaged *in vitro* either until all PB2 mutations have been removed or for 15 passages (whichever comes first), and *in vivo* for 5 passages. Deep sequencing of the PB2 gene from the passaged virus stocks are performed at *in vitro* passages 1, 3, 5 and 15, as well as at *in vivo* passages 1, 3 and 5; results are compared to wild-type PR8 virus harboring the same rev+ mutations (see above). Sequencing methods are performed using any method known and accepted in the art.

157. Each passage is tested for phenotypic reversion through multicycle growth kinetics. The original, unpassaged virus stock is also passaged at elevated temperature (39°C). Viruses that are able to grow at 39°C are then plaque purified and sequenced in their entirety. Using this approach, both true reversion rate and the rate of emergence of second site suppressor mutations is monitored. Overall, fidelity mutants are identified which are capable of stabilizing unfavorable viral mutations.

E. References

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F. Sequences

SEQ ID NO: 1 Amino Acid Sequence of PA from Influenza A virus strain A/little yellow-shouldered bat/Guatemala/060/2010(H17N10)(GenBank Accession NO. CY103891.1)

MENFVRTNFNPMILERAEKTMKEYGENPQNEGNKFAAISTHMEVCFMYSDFHFIDLEGNTIVKE
NDDDNAMLKHRFEIIEGQERNIAWTIVNSICNMTENSKPRFLPDLYDYKTNKFIEIGVTRRKVE
DYYYEKASKLKGENVYIHIFSFDGEEMATDDEYILDEESRARIKTRLFVLRQELATAGLWDSFR

QSEKGEETLEEEFSYPPTFQRLANQSLPPSFKDYHQFKAYVSSFKANGNIEAKLGAMSEKVNAQ
IESFDPRTIRELELPEGKFCTQRSKFLLMDAMKLSVLNPAHEGEGIPMKDAKACLDTFWGWKKA
TIIKKHEKGVNTNYLMIWEQLLESIKEMEGKFLNLKKTNHLKWGLGEGQAPEKMDFEDCKEVPD
LFQYKSEPPEKRKLASWIQSEFNKASELTNSNWIEFDELGNDVAPIEHIASRRRNFFTAEVSQC
RASEYIMKAVYINTALLNSSCTAMEEYQVIPIITKCRDTSGQRRTNLYGFIIKGRSHLRNDTDV
VNFISLEFSLTDPRNEIHKWEKYCVLEIGDMEIRTSISTIMKPVYLYVRTNGTSKIKMKWGMEM
RRCLLQSLQQVESMIEAESAVKEKDMTEPFFRNRENDWPIGESPQGIEKGTIGKVCRVLLAKSV
FNSIYASAQLEGFSAESRKLLLLIQAFRDNLDPGTFDLKGLYEAIEECIINDPWVLLNASWFNS
FLKAVQLSM

SEQ ID NO: 2 Amino Acid Sequence of PB1 from Influenza A virus strain bat/Guatemala/060/2010(H17N10)(GenBank Accession NO. CY103890.1)

MDVNPMLIFLKVPVQNAISTTFPYTGDPPYSHGTGTGYTMDTVIRTHDYSSRGIWKTNSETGAQ
QLNPIDGPLPEDNEPSGYAQTDCVLELIEGLDRSHPGLFETACQETIDAIQQTRVDKLTQGRQT
YDWTLNRNQPAATALANTIEVFRKNGYKLNESGRLIDFLKDVLLSFENDSMEVTTHFQKKKRIR
DNHSKKMITQRTIGKKRVKLTKKNYLIRALTLNTMTKDAERGKLKRRAIATPGMQIRGFVYFVE
LLARNICERLEQSGLPVGGNEKKAKLANVIKKMMAKSTDEELSYTITGDNTKWNENQNPRIFLA
MVLRITAGQPEWFRDLLAVAPIMFSNKVARLGRGYMFESKSMHLRTQISAENLSDINLRYFNED
TKKKIEKIRHLMVEGTASLSPGMMMGMFNMLSTVLGVSVLNLGQREILKRTYWWDGLQSSDDFA
LIINGHFKEDIQQGVNHFYRTCKLVGINMSQKKSYINKTGTFEFTSFFYRYGFVANFSMELPSF
GVAGNNESADMSIGTTVIKTNMINNDLGPATAQMAIQLFIKDYRYTYRCHRGDTNLETRRTKSI
KRLWTETISKAGLLVADGGPNPYNLRNLHIPEVCLKWSLMDPDYRGRLCNPNNPFVHHMEVEST
NLAVVMPAHGPAKSLEYDAVATTHSWTPKRNRSILNTNQRGILEDERIYQKCCQVFEKFFPSST
YRRPIGMASMLDAMLSRARIDARIDLESGRISSQDFSEITNTCKAIEALKRQ

SEQ ID NO: 3 Amino Acid Sequence of PA from Influenza A virus strain A/Puerto Rico/9/1934 (H1N1))(GenBank Accession No. EF190981.1)

MEDFVRQCFNPMIVELAEKTMKEYGEDLKIETNKFAAICTHLEVCFMYSDFHFINEQGESIIVE
LGDPNALLKHRFEIIEGRDRTMAWTVVNSICNTTGAEKPKFLPDLYDYKENRFIEIGVTRREVH
IYYLEKANKIKSEKTHIHIFSFTGEEMATKADYTLDEESRARIKTRLFTIRQEMASRGLWDSFR
QSERGEETIEERFEITGTMRKLADQSLPPNFSSLENFRAYVDGFEPNGYIEGKLSQMSKEVNAR
IEPFLKTTPRPLRLPNGPPCSQRSKFLLMDALKLSIEDPSHEGEGIPLYDAIKCMRTFFGWKEP
NVVKPHEKGINPNYLLSWKQVLAELQDIENEEKIPKTKNMKKTSQLKWALGENMAPEKVDFDDC
KDVGDLKQYDSDEPELRSLASWIQNEFNKACELTDSSWIELDEIGEDVAPIEHIASMRRNYFTS
EVSHCRATEYIMKGVYINTALLNASCAAMDDFQLIPMISKCRTKEGRRKTNLYGFIIKGRSHLR
NDTDVVNFVSMEFSLTDPRLEPHKWEKYCVLEIGDMLIRSAIGQVSRPMFLYVRTNGTSKIKMK
WGMEMRRCLLQSLQQIESMIEAESSVKEKDMTKEFFENKSETWPIGESPKGVEESSIGKVCRTL
LAKSVFNSLYASPQLEGFSAESRKLLLIVQALRDNLEPGTFDLGGLYEAIEECLINDPWVLLNA
SWFNSFLTHALS

SE Q ID NO: 4 Amino Acid Sequence of PB1 from Influenza A virus strain A/Puerto Rico/9/1934 (H1N1)(GenBank Accession No. EF190980.1)

MDVNPTLLFLKVPAQNAISTTFPYTGDPPYSHGTGTGYTMDTVNRTHQYSEKGRWTTNTETGAP
QLNPIDGPLPEDNEPSGYAQTDCVLEAMAFLEESHPGIFENSCIETMEVVQQTRVDKLTQGRQT
YDWTLNRNQPAATALANTIEVFRSNGLTANESGRLIDFLKDVMESMNKEEMGITTHFQRKRRVR
DNMTKKMITQRTMGKKKQRLNKRSYLIRALTLNTMTKDAERGKLKRRAIATPGMQIRGFVYFVE

TLARSICEKLEQSGLPVGGNEKKAKLANVVRKMMTNSQDTELSFTITGDNTKWNENQNPRMFLA
MITYMTRNQPEWFRNVLSIAPIMFSNKMARLGKGYMFESKSMKLRTQIPAEMLASIDLKYFNDS
TRKKIEKIRPLLIEGTASLSPGMMMGMFNMLSTVLGVSILNLGQKRYTKTTYWWDGLQSSDDFA
LIVNAPNHEGIQAGVDRFYRTCKLLGINMSKKKSYINRTGTFEFTSFFYRYGFVANFSMELPSF
GVSGINESADMSIGVTVIKNNMINNDLGPATAQMALQLFIKDYRYTYRCHRGDTQIQTRRSFEI
KKLWEQTRSKVGLLVSDGGPNLYNIRNLHIPEVCLKWELMDEDYQGRLCNPLNPFVSHKEIESM
NNAVMMPAHGPAKNMEYDAVATTHSWIPKRNRSILNTSQRGVLEDEQMYQRCCNLFEKFFPSSS
YRRPVGISSMVEAMVSRARIDARIDFESGRIKKEEFTEIMKICSTIEELRRQK

SEQ ID NO: 5 Nucleic Acid Sequence of PBA from Influenza A virus strain A/Puerto Rico/9/1934 (H1N1))(GenBank Accession No. EF190981.1)

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   1 agcgaaagca ggtactgatc caaaatggaa gattttgtgc gacaatgctt caatccgatg
  61 attgtcgagc ttgcggaaaa aacaatgaaa gagtatgggg aggacctgaa aatcgaaaca
 121 aacaaatttg cagcaatatg cactcacttg gaagtatgct tcatgtattc agattttcac
 181 ttcatcaatg agcaaggcga gtcaataatc gtagaacttg gtgatccaaa tgcacttttg
 241 aagcacagat ttgaaataat cgagggaaga gatcgcacaa tggcctggac agtagtaaac
 301 agtatttgca acactacagg ggctgagaaa ccaaagtttc taccagattt gtatgattac
 361 aaggagaata gattcatcga aattggagta acaaggagag aagttcacat atactatctg
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1201 tatgatagtg atgaaccaga attgaggtcg cttgcaagtt ggattcagaa tgagtttaac
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1441 tcttgtgcag caatggatga tttccaatta attccaatga taagcaagtg tagaactaag
1501 gagggaaggc gaaagaccaa cttgtatggt ttcatcataa aaggaagatc ccacttaagg
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2221 ccttgtttct act
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SEQ ID NO: 6 Nucleic Acid Sequence of PB1 from Influenza A virus strain A/Puerto Rico/9/1934 (H1N1) (GenBank Accession No. EF190980.1)

```
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2341 t
```

SEQ ID NO: 7 Amino Acid sequence of alpha helix at residues 36-50 of PB1.

TGYTMDTVNRTHQYSE

SEQ ID NO: 8 Amino Acid sequence of alpha helix at residues 383-395 of PB1.

DSTRKKIEKIRPL

SEQ ID NO: 9 Amino Acid sequence of loop at residues 77-80 of PB1.

NEPS

SEQ ID NO: 10 Amino Acid sequence of loop at residues 228-240 of PB1.

TKDAERGKLKRRA

SEQ ID NO: 11 Amino Acid sequence of loop at residues 306-311 of PB1.

NTKWNE

SEQ ID NO: 12 Amino Acid sequence of alpha helix at residues 654-674 of PA.

QLEGFSAESRKLLLIVQALRD

SEQ ID NO: 13 Amino Acid sequence of alpha helix at residues 682-693 of PA.

DLGGLYEAIEEC

V. CLAIMS

What is claimed is:

1. A modified influenza A virus comprising one or more mutations in the influenza RNA polymerase, wherein the one or more mutations causes an increased fidelity of the polymerase.

2. The modified influenza A virus of claim 1, wherein the one or more mutations of the influenza RNA polymerase comprises at least one mutation in the PB1 or PA subunit of the RNA polymerase.

3. The modified influenza A virus of claim 2, wherein the influenza RNA polymerase comprises a mutation at one or more residues of an alpha helix of the PB1 subunit of the influenza RNA polymerase.

4. The modified influenza A virus of claim 3, wherein the influenza RNA polymerase comprises a mutation at one or more residues of the alpha helix corresponding to residues 383 to 395 of the PB1 subunit of the influenza RNA polymerase as set forth in SEQ ID NO: 4.

5. The modified influenza A virus of claim 4, wherein the mutation comprises a Lysine to Aspartic acid substitution at a residue corresponding to residue 387 of the PB1 subunit of the influenza RNA polymerase as set forth in SEQ ID NO: 4.

6. The modified influenza A virus of claim 4, wherein the mutation comprises a Lysine to Aspartic acid substitution at a residue corresponding to residue 391 of the PB1 subunit of the influenza RNA polymerase as set forth in SEQ ID NO: 4.

7. The modified influenza A virus of claim 2, wherein the influenza RNA polymerase comprises a mutation at one or more residues of the loop formed by the amino acids corresponding to residues 77-80 of the PB1 subunit of the influenza RNA polymerase as set forth in SEQ ID NO: 4.

8. The modified influenza A virus of claim 2, wherein the influenza RNA polymerase comprises a mutation at one or more residues of the loop formed by the amino acids corresponding to residues 228-240 of the PB1 subunit of the influenza RNA polymerase as set forth in SEQ ID NO: 4.

9. The modified influenza A virus of claim 8, wherein the mutation comprises a substitution at a residue corresponding to residues 235, 237, or 239 of the PB1 subunit of the influenza RNA

polymerase as set forth in SEQ ID NO: 4.

10. The modified influenza A virus of claim 2, wherein the influenza RNA polymerase comprises a mutation at one or more residues of the loop formed by the amino acids corresponding to residues 306-311 of the PB1 subunit of the influenza RNA polymerase as set forth in SEQ ID NO: 4.

11. The modified influenza A virus of claim 10, wherein the influenza RNA polymerase comprises a Lysine to Aspartic acid substitution at a residue corresponding to residue 308 as set forth in SEQ ID NO: 4.

12. The modified influenza A virus of claim 2, wherein the influenza RNA polymerase comprises a substitution at one or more residues corresponding to residues 480 or 481 of the PB1 subunit of the influenza RNA polymerase as set forth in SEQ ID NO: 4.

13. The modified influenza A virus of any of claims 3-12 wherein the influenza RNA polymerase further comprises a mutation at one or more residues of an alpha helix of the PA subunit of the influenza RNA polymerase.

14. The modified influenza A virus of claim 13, wherein the influenza RNA polymerase comprises a mutation at one or more residues of the alpha helix corresponding to residues 654 to 674 of the PA subunit of the influenza RNA polymerase as set forth in SEQ ID NO: 3.

15. The modified influenza A virus of claim 14, wherein the mutation comprises a substitution at a residue selected corresponding to residue 661 or 663 of the PA subunit of the influenza RNA polymerase as set forth in SEQ ID NO: 3.

16. The modified influenza A virus of claim 15, wherein the mutation comprises a substitution at a residue corresponding to residue 661 of the PA subunit of the influenza RNA polymerase as set forth in SEQ ID NO: 3.

17. The modified influenza A virus of claim 15, wherein the mutation comprises an Arginine to Glutamic acid or Arginine to Lysine substitution at a residue corresponding to residue 663 of the PA subunit of the influenza RNA polymerase as set forth in SEQ ID NO: 3.

18. The modified influenza A virus of claim 13, wherein the influenza RNA polymerase comprises a mutation at one or more residues of the alpha helix corresponding to residues 682 to 693 of the PA subunit of the influenza RNA polymerase as set forth in SEQ ID NO: 3..

19. The modified influenza A virus of claim 18, wherein the mutation comprises a substitution at the residue corresponding to residue 691 of the PA subunit of the influenza RNA polymerase as set forth in SEQ ID NO: 3..

20. The modified influenza A virus of claim 2, wherein the influenza RNA polymerase comprises a mutation at one or more residues of an alpha helix of the PA subunit of the influenza RNA polymerase.

21. The modified influenza A virus of claim 20, wherein the influenza RNA polymerase comprises a mutation at one or more residues of the alpha helix corresponding to residues 654 to 674 of the PA subunit of the influenza RNA polymerase as set forth in SEQ ID NO: 3.

22. The modified influenza A virus of claim 21, wherein the mutation comprises a substitution at a residue corresponding to residue 661 or 663 of the PA subunit of the influenza RNA polymerase as set forth in SEQ ID NO: 3.

23. The modified influenza A virus of claim 22, wherein the mutation comprises a substitution at a residue corresponding to residue 661 of the PA subunit of the influenza RNA polymerase as set forth in SEQ ID NO: 3.

24. The modified influenza A virus of claim 22, wherein the mutation comprises an Arginine to Glutamic acid or Arginine to Lysine substitution at a residue corresponding to residue 663 of the PA subunit of the influenza RNA polymerase as set forth in SEQ ID NO: 3.

25. The modified influenza A virus of claim 20, wherein the influenza RNA polymerase comprises a mutation at one or more residues of the alpha helix corresponding to residues 682 to 693 of the PA subunit of the influenza RNA polymerase as set forth in SEQ ID NO: 3

26. The modified influenza A virus of claim 25, wherein the mutation comprises a substitution at the residue corresponding to residue 691 as set forth in SEQ ID NO: 3 of the PA subunit of the influenza RNA polymerase as set forth in SEQ ID NO: 3.

27. A pharmaceutical composition comprising the modified influenza virus of Claim 1.

28. A method of immunizing a subject against influenza virus comprising administering to the subject the pharmaceutical composition of Claim 27.

29. A method of inducing an immune response against influenza virus in a subject comprising administering to the subject the pharmaceutical composition of Claim 27.

30. A method of inhibiting an influenza virus infection in a subject comprising administering to the subject the pharmaceutical composition of Claim 27.

31. A recombinant nucleic acid encoding an influenza RNA polymerase with increased transcriptional fidelity wherein the RNA polymerase comprises at least one mutation selected from the PB1 or PA subunits of the RNA polymerase.

32. The recombinant nucleic acid of Claim 31, wherein the mutation of the PB1 or PA subunit of the influenza RNA polymerase comprises a mutation of at least one residue selected from one or more residues of the alpha helix corresponding to residues 383 to 395 of the PB1 subunit of the influenza RNA polymerase as set forth in SEQ ID NO: 4; one or more residues of the loop formed by the amino acids corresponding to residues 77-80 of the PB1 subunit of the influenza RNA polymerase as set forth in SEQ ID NO: 4; one or more residues of the loop formed by the amino acids corresponding to residues 228-240 of the PB1 subunit of the influenza RNA polymerase as set forth in SEQ ID NO: 4; one or more residues of the loop formed by the amino acids corresponding to residues 306-311 of the PB1 subunit of the influenza RNA polymerase as set forth in SEQ ID NO: 4; residues 480 or 481 of the PB1 subunit of the influenza RNA polymerase as set forth in SEQ ID NO: 4; one or more residues of the alpha helix corresponding to residues 654 to 674 of the PA subunit of the influenza RNA polymerase as set forth in SEQ ID NO: 3; or residues 682 to 693 of the PA subunit of the influenza RNA polymerase as set forth in SEQ ID NO: 3.

33. A vector comprising the nucleic acid of Claim 31.

34. A method of increasing the immunogenicity of an attenuated influenza vaccine comprising obtaining an attenuated influenza vaccine viral strain and generating a mutation in the PB1 or PA subunit of the RNA polymerase of the influenza virus, wherein the mutation increases the fidelity of the RNA polymerase; and wherein an increase in fidelity of the polymerase increases the stability of the virus strain in a subject and reduces the amount mutant viruses formed during transcription of the virus thereby increasing the abundance of a single strain and as a result increases the immune response to the immunizing strain.

35. The method of Claim 34, wherein the mutation of the PB1 or PA subunit of the influenza RNA polymerase comprises a mutation of at least one residue selected from one or more residues of the alpha helix corresponding to residues 383 to 395 of the PB1 subunit of the influenza RNA polymerase as set forth in SEQ ID NO: 4; one or more residues of the loop

formed by the amino acids corresponding to residues 77-80 of the PB1 subunit of the influenza RNA polymerase as set forth in SEQ ID NO: 4; one or more residues of the loop formed by the amino acids corresponding to residues 228-240 of the PB1 subunit of the influenza RNA polymerase as set forth in SEQ ID NO: 4; one or more residues of the loop formed by the amino acids corresponding to residues 306-311 of the PB1 subunit of the influenza RNA polymerase as set forth in SEQ ID NO: 4; residues 480 or 481 of the PB1 subunit of the influenza RNA polymerase as set forth in SEQ ID NO: 4; one or more residues of the alpha helix corresponding to residues 654 to 674 of the PA subunit of the influenza RNA polymerase as set forth in SEQ ID NO: 3; or residues 682 to 693 of the PA subunit of the influenza RNA polymerase as set forth in SEQ ID NO: 3.

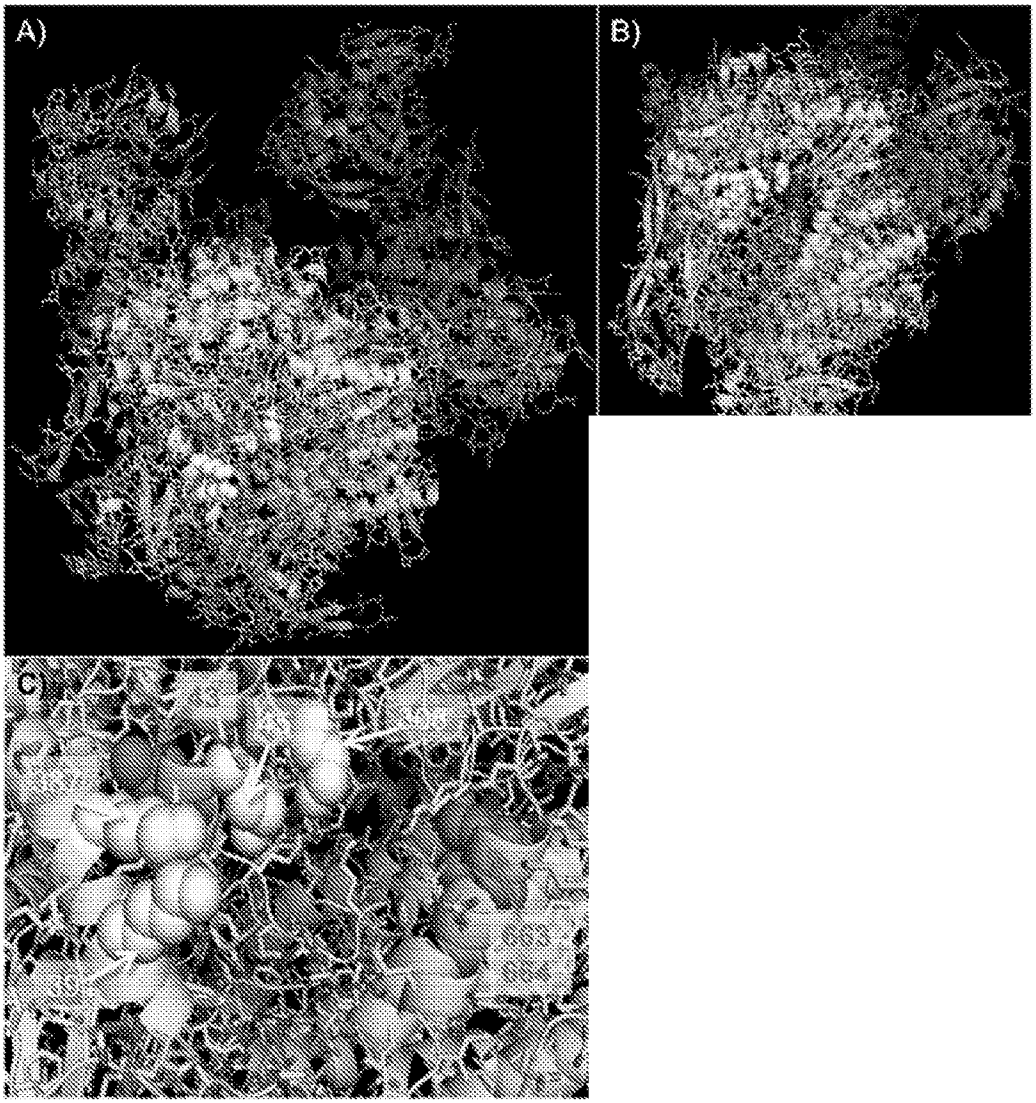


Fig. 1

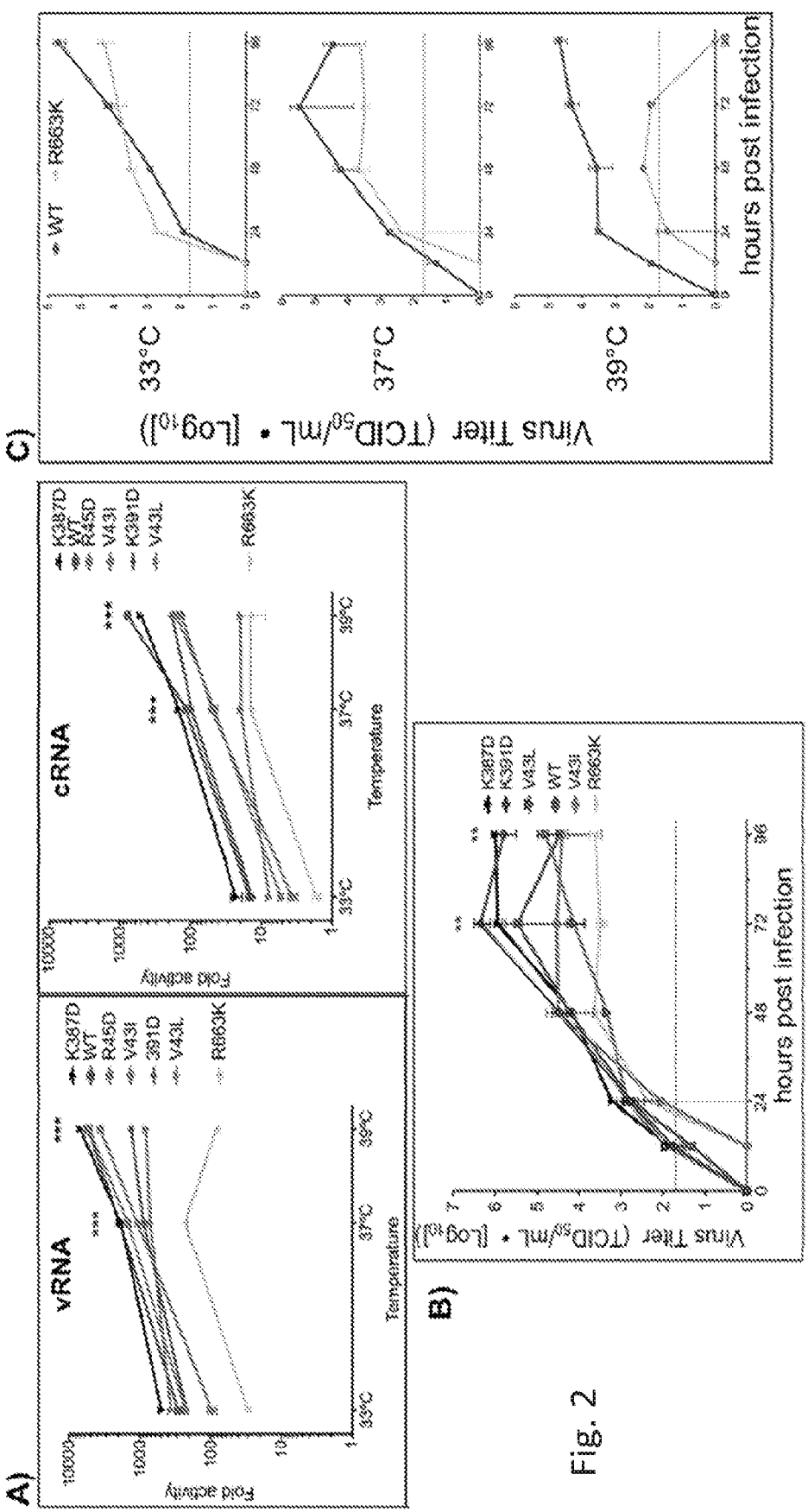
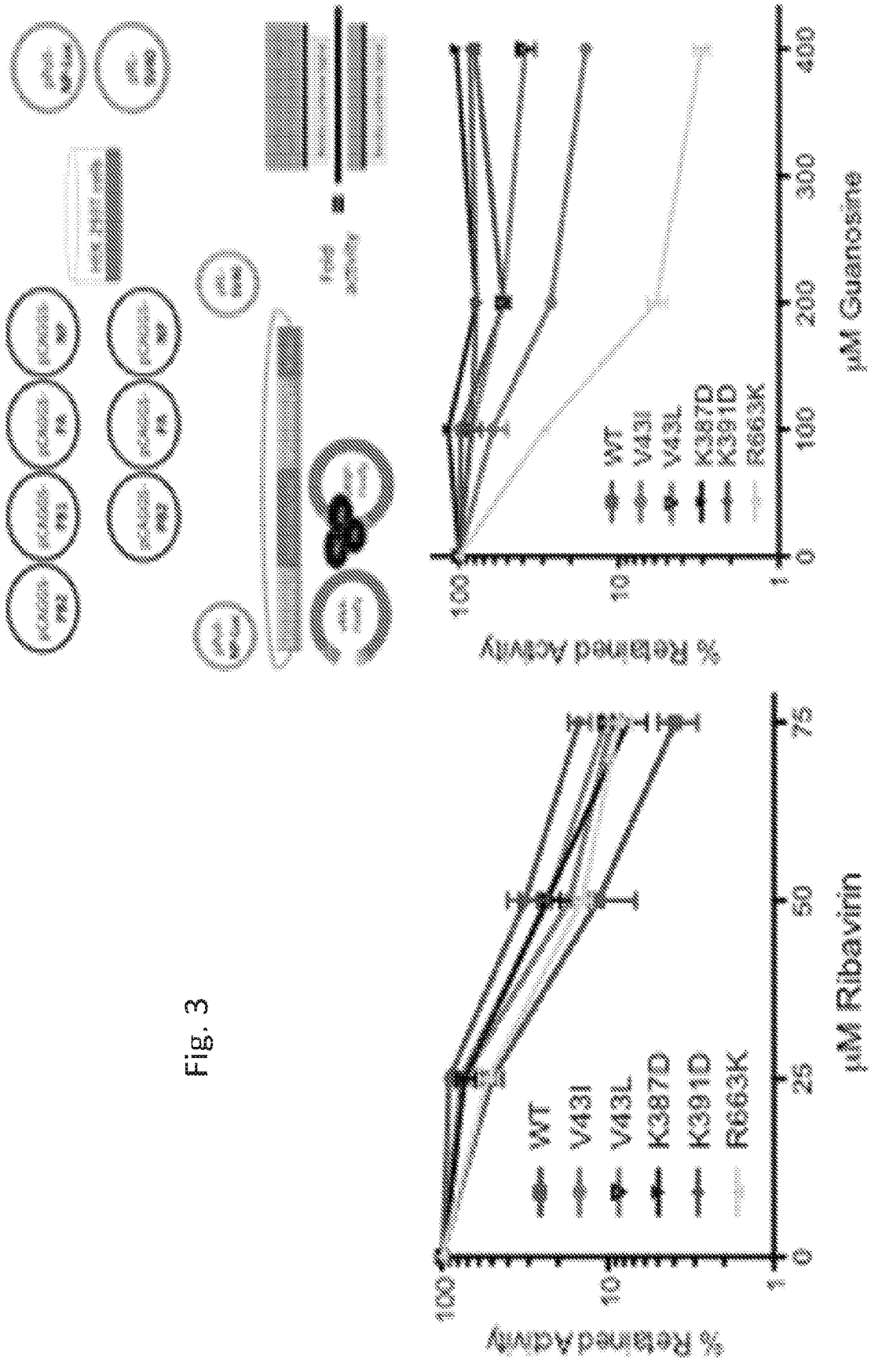

A)
vRNA
cRNA
Fold activity
Temperature
33°C
37°C
39°C
K387D
WT
R45D
V43I
391D
V43L
R663K
B)
Virus Titer (TCID50/mL • [Log10])
hours post infection
C)
33°C
37°C
39°C
WT
R663K

Fig. 2


% Rebind Activity
100
10
1
0
100
200
300
400
μM Guanosine
WT
V43I
V43L
K387Q
K391D
R663K
% Rebind Activity
100
10
1
0
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50
75
μM Ribavirin
WT
V43I
V43L
K387Q
K391D
R663K

Fig. 3

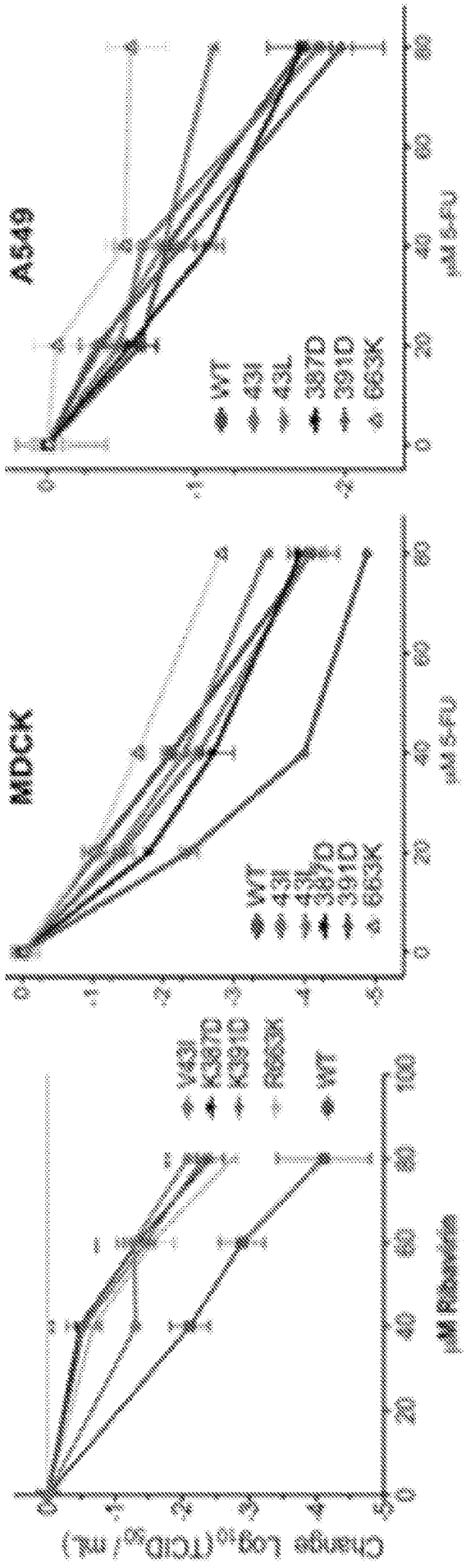

A549
MDCK
WT
43I
43L
387D
391D
663K
μM 6-FU
μM Ribavirin
Change $Log_{10}TCID_{50}$/mL)

Fig. 4

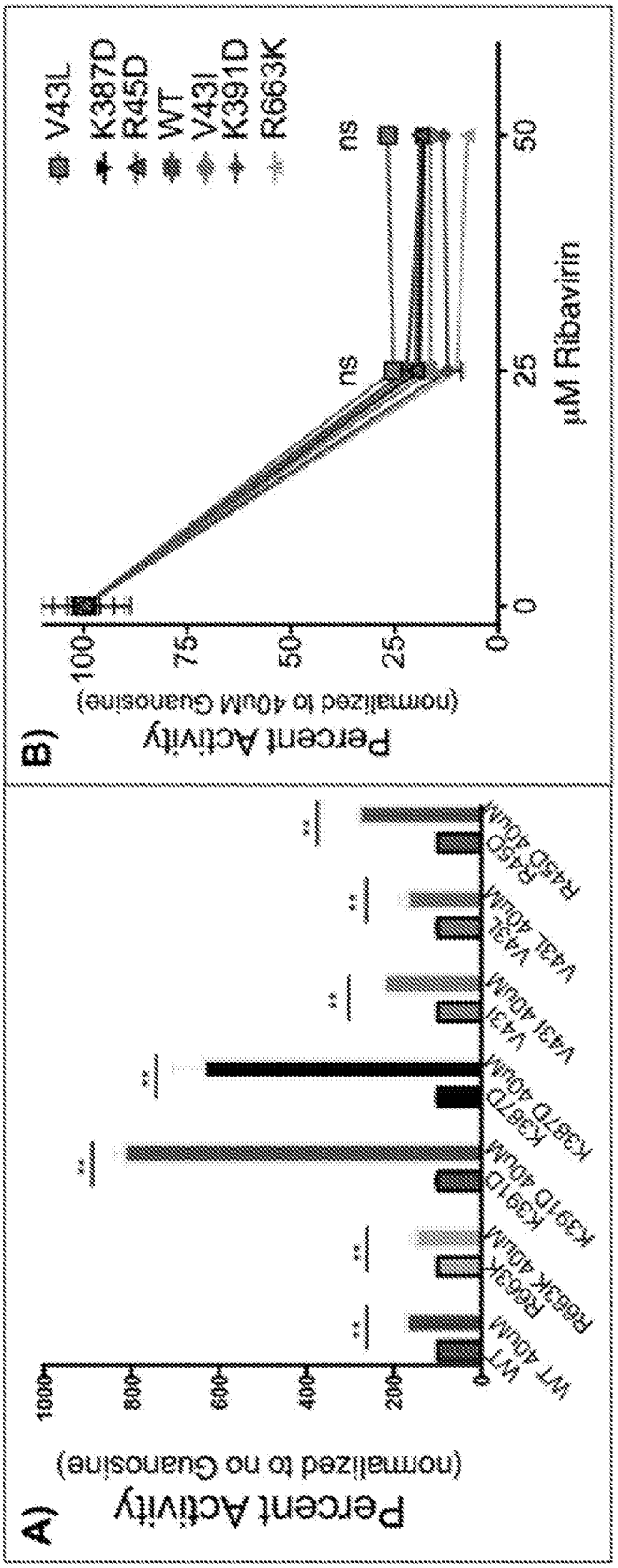
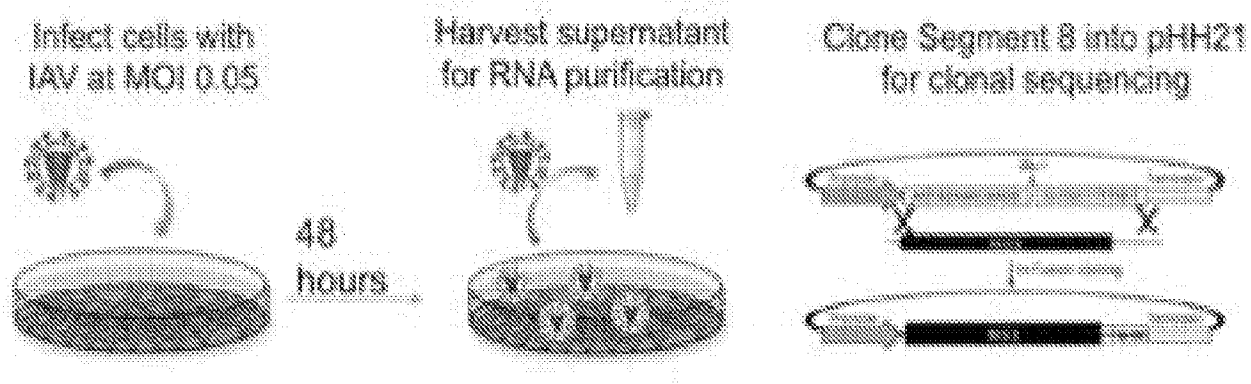

A)
Percent Activity
(normalized to no Guanosine)
1000
800
600
400
200
0
WT 40uM
R663K 40uM
K391D 40uM
K387D 40uM
V43I 40uM
V43L 40uM
R45D 40uM
B)
Percent Activity
(normalized to 40uM Guanosine)
100
75
50
25
0
ns
ns
0
25
50
µM Ribavirin
V43L
K387D
R45D
WT
V43I
K391D
R663K

Fig. 5



Virus	Bases sequenced	1/Error rate	Viral titer
WT	29,971	1070	3.58 e7
43I	15,809	1440	4.32 e7
387D	17,121	1320	1.46 e7
391D	8,959	1790	1.66 e8
663K	13,497	1690	4.28 e7

Fig. 6