(54) Title: ENGINEERING IMMUNE ORTHOGANOL AAV AND IMMUNE STEALTH CRISPR-CAS

FIG. 1A
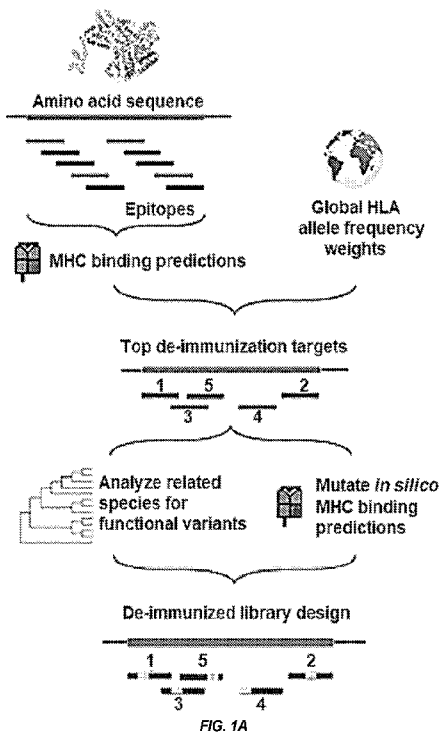
(57) Abstract: Described herein are methods for engineering proteins and
viruses to reduce their immunogenicity, proteins and viruses made by using
said methods, including proteins having Cas9 like activity and viruses having
AAV5 like activity.

[Continued on next page]

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ENGINEERING IMMUNE ORTHOGANOL AAV AND IMMUNE STEALTH CRISPR-CAS

CROSS REFERENCE TO RELATED APPLICATIONS

[0001]   This application claims priority under 35 U.S.C. §119 from Provisional Application Serial No. 63/120,376, filed December 2, 2020, the disclosures of which are incorporated herein by reference in its entirety.

STATEMENT REGARDING FEDERALLY SPONOSORED RESEARCH

[0002]   This invention was made with Government support under RO1HG009285, RO1CA22826, and RO1GM123313 awarded by the National Institutes of Health. The government has certain rights in the invention.

TECHNICAL FIELD

[0003]   Described herein are methods for engineering proteins and viruses to reduce their immunogenicity, proteins and viruses made by using said methods, including proteins having Cas9 like activity and viruses having AAV5 like activity.

INCORPORATION BY REFERENCE OF SEQUENCE LISTING

[0004]   Accompanying this filing is a Sequence Listing entitled "Sequence-Listing_ST25.txt", created on December 2, 2021, and having 491,513 bytes of data, machine formatted on IBM-PC, MS-Windows operating system. The sequence listing is hereby incorporated herein by reference in its entirety for all purposes.

BACKGROUND

[0005]   Immunogenicity is a major concern for protein-based therapeutics, particularly those derived from non-human species. Induction of the immune response can render treatments ineffective and cause serious, even life-threatening side effects. One strategy to overcome this issue is to mutate particularly immunogenic epitopes in the therapeutic target. However, this strategy is hindered by the ability of the adaptive immune system to recognize multiple epitopes across large regions of the antigen. While epitope deletion efforts to date have focused on a few major antibody binding sites, it is not possible to make these studies comprehensive due to the vast possible epitope space. Variant library screening has proven to be an effective approach to protein

engineering but applying it in this case faces several technical
challenges. One problem is the vast mutational space created by the
need for full combinatorial libraries. Fully degenerate libraries
quickly become intractably large as the number of target sites
increases beyond just a few. Narrowing down this space by
intelligent selection of library members is necessary to define a
reasonable mutational landscape to explore and critical for
maximizing the chance of functional hits. Another problem is that
reading out combinatorial mutations scattered across large (>1kb)
regions of the protein is extremely difficult using short read
sequencing. Using short barcodes attached to each variant to
genotype libraries post-screen has proved effective but is limited
by the difficulty of constructing large combinatorial libraries in
which each member has a short, unique barcode. These issues have
generally limited combinatorial library screens to short regions
able to be sequenced directly.

SUMMARY

[0006]   The disclosure provides a method that overcomes the
library size constraints in both the number of unique members and
the length of the mutagenized region. The method comprises selecting
target regions within a protein of interest using software which
predicts HLA-binding and peptide immunogenicity. To generalize
immunogenicity predictions and select appropriate targets, an
approximation of global HLA allele frequencies is generated using
data from the Allele Frequency Net Database. These frequencies were
used to scale immunogenicity predictions such that the top hits are
the peptides likely to be the most immunogenic epitopes for the
largest number of people globally. In order to narrow down the
mutational space associated with fully degenerate combinatorial
libraries, an approach guided by evolution and natural variation was
used. As de-immunizing protein engineering seeks to alter the amino
acid sequence of a protein without disrupting functionality, it
would be extremely useful to narrow down mutations to those less
likely to result in non-functional variants. The method identifies
these mutants by leveraging the large amounts of sequencing data
available to identify low-frequency SNPs that have been observed in
natural environments. Such variants are likely to have limited

effect on protein function, as highly deleterious alleles would likely be immediately selected out of the natural population and not appear in sequencing data. Using these more neutral amino acid substitutions in combinatorial libraries increases the likelihood of functional hits with enough epitope variation to evade immune induction. Once the targets are identified and the mutations defined, the library is assembled piecewise using standard synthesis and assembly methods and apply the screen.

[0007]   In a particular embodiment, the disclosure provides a method for engineering a protein or virus to be less immunoreactive, comprising: identifying target regions of the polynucleotide sequence encoding a protein or virus that are predicted to have human leukocyte antigen (HLA)-binding and/or peptide immunogenicity; identifying single nucleotide polymorphisms (SNPs) or mutations in the targeted region and other regions that are not deleterious to the functioning of the protein; screening a library assembled using standard synthesis and assembly methods by applying the above identifying criteria to find functional variants of the protein or virus; sequencing the functional variants of the protein or virus; mapping genotype to phenotype from the sequences of the functional variants to identify variant candidates that are likely functionally active and have mutations that result in the protein or virus exhibiting less immunogenicity. In another embodiment, the protein is a CRISPR associated protein. In yet another embodiment, the CRISPR associated protein is a Cas9. In a further embodiment, the Cas9 is *Streptococcus pyogenes* Cas9 (SpCas9). In yet a further embodiment, the target regions are identified using a model that predicts human leukocyte antigen (HLA)-binding and peptide immunogenicity. In a certain embodiment, the prediction model is selected from NetMHC, MHCAttnNet, MHCSeqNET, ACME, NetMHCpan EL 4.1, NetMHCstabpan, SMM, SMMPMBEC, PickPocket, Comblib_Sidney2008, NetMHCcons, MHCflurry 2.0, and IConMHC. In another embodiment, the SNPs or mutations are identified by using phylogenetic methods to scan natural variation among naturally occurring SpCas9, mutations generated in the course of research and engineering efforts, and the Cas9 orthologs of closely related bacterial species. In yet another embodiment, the SNPS or mutations are identified, or further

identified, by using immunological prediction of candidate mutations
to ensure significant loss of immunogenicity within the targeted
region in order to preserve function while reducing immunogenicity.
In a further embodiment, the virus is an adeno-associated virus
(AAV). In yet a further embodiment, the AAV is selected from AAV1,
AAV2, AAV5, AAV6, AAV7, and AAV8. In another embodiment, the AAV is
AAV5. In yet another embodiment, the target regions are identified
by aligning conserved sequence regions across AAV serotypes. In a
further embodiment, the SNPs are identified by aligning the
sequences of 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160,
170, 180, 190, 200 or more AAV variants that have been sequenced
from natural or engineered sources. In yet a further embodiment,
the SNPs are located in the target regions. In another embodiment,
wherein long-read sequencing technologies capable of sequencing the
entire sequence of each protein or viral variant in one sequencing
reaction is used to sequence the functional variants of the protein
or virus. In yet another embodiment, the long-read sequencing
technologies is capable of generating 10-15 Gb of sequencing reads
per run. In a further embodiment, a method disclosed herein further
comprises the step of evaluating the immunoreactivity of variant
candidates in one or more immunoassays. In yet a further
embodiment, the one or more immunoassays comprise detecting the
presence of antibodies to the variant candidates (AVA antibodies),
when the variant candidates are administered *in vivo* to an animal.
In another embodiment, the one or more immunoassays comprise an
enzyme-linked immunosorbent assays (ELISAs),
electrochemiluminescence (ECL) assays and/or antigen-binding tests,
wherein the one or more immunoassays utilize AVA antibodies.
The disclosure provides an isolated polypeptide encoded by a
polynucleotide sequence having at least 90%, 95%, 97%, 98%, or 99%
sequence identity to SEQ ID NO:1, wherein the polypeptide has Cas9
like activity and is less immunogenic that SEQ ID NO:2. In one
embodiment, the polynucleotide encodes a polypeptide of SEQ ID NO:2
having one or more mutations selected from the group consisting of:
P28L, L237C, Y286Q, S318(H/C), S368C, F498T, L514(T/G), L616G,
L623Q, L636D, F704A, L727(P/G), L816D, Y1016(K/G), L1245G, I1273Q,
L1282(A/E), and Y1294Q. In particular, the disclosure contemplates

any combination of the foregoing mutations to SEQ ID NO:2, wherein the number of mutations comprises 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, or 18 mutations as recited above. In particular, the disclosure contemplates any combination of the foregoing mutations to SEQ ID NO:2, wherein the number of mutations comprises 1 or more, 2 or more, 3 or more, 4 or more, 5 or more, 6 or more, 7 or more, 8 or more, 9 or more, 10 or more, 11 or more, 12 or more, 13 or more, 14 or more, 15 or more, 16 or more, 17 or more, or 18 mutations as recited above. In addition, the polypeptide may have 1-10 additional conservative mutations at positions other than those set forth above.

The disclosure also provides an isolated polypeptide having a sequence that has at least 90%, 95%, 97%, 98%, or 99% sequence identity to the sequence presented in SEQ ID NO:2 wherein the protein has Cas9 like activity and is immuno-silenced compared to a polypeptide of SEQ ID NO:2. In one embodiment, the polypeptide comprises the sequence of SEQ ID NO:2 and having one or more mutations selected from the group consisting of: P28L, L237C, Y286Q, S318(H/C), S368C, F498T, L514(T/G), L616G, L623Q, L636D, F704A, L727(P/G), L816D, Y1016(K/G), L1245G, I1273Q, L1282(A/E), and Y1294Q. In particular, the disclosure contemplates any combination of the foregoing mutations to SEQ ID NO:2, wherein the number of mutations comprises 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, or 18 mutations as recited above. In particular, the disclosure contemplates any combination of the foregoing mutations to SEQ ID NO:2, wherein the number of mutations comprises 1 or more, 2 or more, 3 or more, 4 or more, 5 or more, 6 or more, 7 or more, 8 or more, 9 or more, 10 or more, 11 or more, 12 or more, 13 or more, 14 or more, 15 or more, 16 or more, 17 or more, or 18 mutations as recited above. In addition, the polypeptide may have 1-10 additional conservative mutations at positions other than those set forth above.

[0008]   The disclosure also provides a virus comprising a viral capsid encoded by a polynucleotide having 90-99% sequence identity to SEQ ID NO:49 and encoding a viral capsid polypeptide having AAV5 like activity and is immuno-silenced compared to a wild-type viral capsid of SEQ ID NO:50. In one embodiment, the viral capsid

comprises a sequence of SEQ ID NO:50 and comprises two or more
mutation selected from the group consisting of R42G, P47L, Y49C,
G55S, N56H, G57S, D59Y, Y89H, L90(P/I), A95V, D96G, E98(K/Q), F99L,
T107A, S108P, Q119(R/E), R123(L/T), V124A, V131A, E132(G/R),
E133(Q/D), G134(V/S), T137A, A214V, S222T, T223(A/K), S267A, Y272H,
F273L, W287R, L290P, I291V, I309V, K312(E/R), N400D, F402L, F413L,
S415T, S416(M/G), Q604R, P606Q, I607T, F627L, L629(P/F), K630E,
H631(R/N), S663G, T664A, R682C, W683R, N684(D/S), T717(S/A), Y719F,
and L720P. In particular, the disclosure contemplates any
combination of the foregoing mutations to SEQ ID NO:50, wherein the
number of mutations comprises 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12,
13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29,
30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46,
47, 48, 49, 50, 51, 52, 53, or 54 mutations as recited above. In
particular, the disclosure contemplates any combination of the
foregoing mutations to SEQ ID NO:50, wherein the number of mutations
comprises 2 or more, 3 or more, 4 or more, 5 or more, 6 or more, 7
or more, 8 or more, 9 or more, 10 or more, 11 or more, 12 or more,
13 or more, 14 or more, 15 or more, 16 or more, 17 or more, 18 or
more, 19 or more, 20 or more, 21 or more, 22 or more, 23 or more, 24
or more, 25 or more, 26 or more, 27 or more, 28 or more, 29 or more,
30 or more, 31 or more, 32 or more, 33 or more, 34 or more, 35 or
more, 36 or more, 37 or more, 38 or more, 39 or more, 40 or more, 41
or more, 42 or more, 43 or more, 44 or more, 45 or more, 46 or more,
47 or more, 48 or more, 49 or more, 50 or more, 51 or more, 52 or
more, 53 or more, or 54 mutations as recited above. In a particular
embodiment, the viral capsid comprises no more than 60 mutations.
In addition, the viral capsid may have 1-10 additional conservative
mutations at positions other than those set forth above.

[0009]   In a certain embodiment, the disclosure also provides for
an AAV system comprising the virus disclosed herein that has AAV5
like activity. In a further embodiment, the AAV system is used for
gene therapy.

DESCRIPTION OF DRAWINGS

[0010]   **Figure 1A-B** provides (**A**) schematic of library design
methodology; (**B**) tables showing the location, codon information,
etc. for the generation of a library of hCas9 constructs using a

method disclosed herein. As indicated in the lower table, there were 56 total constructs generated from a library having the size of 995328.

[0011]   **Figure 2** provides a library construction schematic.

[0012]   **Figure 3** provides Immunogenicity scores of Cas orthologs. As more immunogenic epitopes are mutated total protein immunogenicity decreases rapidly at first and then with diminishing returns at high mutation numbers.

[0013]   **Figure 4** provides Cas9 functional screen schematic.

[0014]   **Figure 5** provides mutation density distribution of Cas9 library before and after screening.

[0015]   **Figure 6** provides HeLa cells transduced with Cas9 and HPRT1 or non-targeting guide were treated with 0-14 μg/mL 6TG. Viable cells are shown by crystal violet staining.

[0016]   **Figure 7** provides replicate correlation of post-screen element frequencies on logarithmic (left) and linear (right) scale. R2 = 0.925.

[0017]   **Figure 8** provides pre- and post-screen allele frequencies at each of the 18 mutation sites. Each site shows enrichment of the wild-type allele, but most sites retain a substantial fraction of mutant alleles.

[0018]   **Figure 9** provides neighbor score as a metric for differentiating true positive hits. Left: Neighbor score is positively correlated with screen score. Right: Divergence between neighbor score and screen score decreases as read coverage increases.

[0019]   **Figure 10** shows a network diagram of screen hits. Nodes are Cas9 variant hits with high coverage in each replicate and enrichment greater than wild-type. Edges between nodes correspond to the distance between genotypes and are weighted as 1/n for n<5 where n is the number of non-shared mutations. Node colors correspond to mutational distance from wild-type. Dark blue = 1, blue = 2, light blue = 3, white = 4, pink = >4, red = tested variant, yellow = variant testing underway. Network layout was computed using the Fruchterman-Reingold algorithm.

[0020]   **Figure 11** shows HDR efficiencies for wildtype and variant Cas as quantified by FACS.

[0021]   **Figure 12** provides NHEJ efficiencies for wildtype and variant Cas as quantified by NGS.

[0022]   **Figure 13** provides Correlation between pLDDT and epistasis in our Cas9 screen (top left), as well as third party double mutant datasets for GFP (top right, DOI: 10.1038/nature17995), human YAP65 WW domain (bottom left, DOI: 10.1073/pnas.1209751109), and yeast Pab1 RRM domain (bottom right, DOI: 10.1261/rna.040709.113).

[0023]   **Figure 14** shows maximum likelihood cladogram of AAVs including the main human serotypes and new tested orthologs.

[0024]   **Figure 15** provides Viral formation titers of AAV orthologs relative to AAV5.

[0025]   **Figure 16** provides tables showing the location, codon information, etc. for the generation of a library of AAV5 constructs using a method disclosed herein. As indicated in the lower table, there were 40 total constructs generated from a library having the size of 2097152.

[0026]   **Figure 17** provides a DNA sequence (SEQ ID NO:49) listing an exemplary AAV5 construct that has modified so as to be less immunoreactive.

[0027]   **Figure 18** provides an AA sequence (SEQ ID NO:50) of an exemplary AAV5 construct that has modified so as to be less immunoreactive.

DETAILED DESCRIPTION

[0028]   As used herein and in 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 cell" includes a plurality of such cells and reference to "the fragment" includes reference to one or more fragments and equivalents thereof known to those skilled in the art, and so forth.

[0029]   Also, the use of "or" means "and/or" unless stated otherwise. Similarly, "comprise," "comprises," "comprising" "include," "includes," and "including" are interchangeable and not intended to be limiting.

[0030]   It is to be further understood that where descriptions of various embodiments use the term "comprising," those skilled in the art would understand that in some specific instances, an embodiment

can be alternatively described using language "consisting essentially of" or "consisting of."

[0031]   Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood to one of ordinary skill in the art to which this disclosure belongs. Although many methods and reagents are similar or equivalent to those described herein, the exemplary methods and materials are disclosed herein.

[0032]   All publications mentioned herein are incorporated herein by reference in full for the purpose of describing and disclosing the methodologies, which might be used in connection with the description herein. Moreover, with respect to any term that is presented in one or more publications that is similar to, or identical with, a term that has been expressly defined in this disclosure, the definition of the term as expressly provided in this disclosure will control in all respects.

[0033]   It should be understood that this disclosure is not limited to the particular methodology, protocols, and reagents, *etc.,* described herein and as such may vary. The terminology used herein is for the purpose of describing particular embodiments or aspects only and is not intended to limit the scope of the present disclosure.

[0034]   Other than in the operating examples, or where otherwise indicated, all numbers expressing quantities of ingredients or reaction conditions used herein should be understood as modified in all instances by the term "about." The term "about" when used to described the present invention, in connection with percentages means $\pm 1\%$. The term "about," as used herein can mean within an acceptable error range for the particular value as determined by one of ordinary skill in the art, which can depend in part on how the value is measured or determined, *e.g.,* the limitations of the measurement system. Alternatively, "about" can mean a range of plus or minus 20%, plus or minus 10%, plus or minus 5%, or plus or minus 1% of a given value. Alternatively, particularly with respect to biological systems or processes, the term can mean within an order of magnitude, within 5-fold, or within 2-fold, of a value. Where particular values are described in the application and claims,

unless otherwise stated the term "about" meaning within an acceptable error range for the particular value can be assumed. Also, where ranges and/or subranges of values are provided, the ranges and/or subranges can include the endpoints of the ranges and/or subranges. In some cases, variations can include an amount or concentration of 20%, 10%, 5%, 1 %, 0.5%, or even 0.1 % of the specified amount.

[0035]   For the recitation of numeric ranges herein, each intervening number there between with the same degree of precision is explicitly contemplated. For example, for the range of 6-9, the numbers 7 and 8 are contemplated in addition to 6 and 9, and for the range 6.0-7.0, the number 6.0, 6.1, 6.2, 6.3, 6.4, 6.5, 6.6, 6.7, 6.8, 6.9, and 7.0 are explicitly contemplated.

[0036]   The term "adeno-associated virus" or "AAV" as used herein refers to a member of the class of viruses associated with this name and belonging to the genus dependoparvovirus, family Parvoviridae. Multiple serotypes of this virus are known to be suitable for gene delivery; all known serotypes can infect cells from various tissue types. Non-limiting exemplary serotypes useful in the methods disclosed herein include any of the 11 or 12 serotypes, *e.g.*, AAV2, AAV5, and AAV8, or variant serotypes such as AAV-DJ. The AAV structural particle is composed of 60 protein molecules made up of VP1, VP2 and VP3. Each particle contains approximately 5 VP1 proteins, 5 VP2 proteins and 50 VP3 proteins ordered into an icosahedral structure. Non-limiting exemplary VP1 sequences useful in the methods disclosed herein are provided below.

[0037]   The term "amino acid" refers to naturally occurring and synthetic amino acids, as well as amino acid analogs and amino acid mimetics that function in a manner similar to the naturally occurring amino acids. Naturally occurring amino acids are those encoded by the genetic code, as well as those amino acids that are later modified, *e.g.*, hydroxyproline, γ-carboxyglutamate, and O-phosphoserine. In some embodiments, an amino acid analog refers to compounds that have the same basic chemical structure as a naturally occurring amino acid, *i.e.*, a carbon that is bound to a hydrogen, a carboxyl group, an amino group, and an R group, *e.g.*, homoserine, norleucine, methionine sulfoxide, methionine methyl sulfonium. Such

analogs have modified R groups (e.g., norleucine) or modified peptide backbones, but retain the same basic chemical structure as a naturally occurring amino acid. In some embodiments, an amino acid mimetics refers to chemical compounds that have a structure that is different from the general chemical structure of an amino acid, but that functions in a manner similar to a naturally occurring amino acid. The terms "non-naturally occurring amino acid" and "unnatural amino acid" refer to amino acid analogs, synthetic amino acids, and amino acid mimetics which are not found in nature. In certain instances one or more D-amino acids can be used in various peptide compositions of the disclosure. The disclosure provides various peptides that are useful for treating various diseases and infections. These peptides can comprise naturally occurring amino acid. In other embodiments, the peptides can comprise non-natural amino acids. The use of non-natural amino acids can improve the peptides stability, decrease degradation and/or improve biological activity. For example, in some embodiments, one or more D-amino acids. In other embodiments, retroinverso peptides are contemplated using various amino acid configurations.

[0038]   Amino acids may be referred to herein by either their commonly known three letter symbols or by the one-letter symbols recommended by the IUPAC-IUB Biochemical Nomenclature Commission. Nucleotides, likewise, may be referred to by their commonly accepted single-letter codes.

[0039]   The term "Cas9" refers to a CRISPR-associated, RNA-guided endonuclease such as *Streptococcus pyogenes* Cas9 (spCas9; see Accession Number Q99ZW2.1, the sequence of which is incorporated herein by reference) and orthologs and biological equivalents thereof. Biological equivalents of Cas9 include, but are not limited to, C2c1 from *Alicyclobacillus acideterrestris* and Cpf1 (which performs cutting/cleaving functions analogous to Cas9) from various bacterial species including *Acidaminococcus spp.* and *Francisella novicida* U112. Cas9 may refer to an endonuclease that causes double stranded breaks in DNA, a nickase variant such as a RuvC or HNH mutant that causes a single stranded break in DNA, as well as other variations such as deadCas-9 ("dCas9"), which lack endonuclease activity. Cas9 may also refer to "split-Cas9" in which

Cas9 is split into two halves – C-terminal Cas9 (C-Cas9) and an N-terminal Cas-9 (N-Cas9) – which can be fused with two intein moieties. *See, e.g.,* U.S. Pat. No. 9,074,199 B1; Zetsche *et al.* (2015) Nat Biotechnol. 33(2):139-42; Wright *et al.* (2015) PNAS 112(10) 2984-89. Non-limiting examples of commercially available sources of SpCas9 comprising plasmids can be found under the following AddGene reference numbers:

42230: PX330; SpCas9 and single guide RNA;

48138: PX458; SpCas9-2A-EGFP and single guide RNA;

62988: PX459; SpCas9-2A-Puro and single guide RNA;

48873: PX460; SpCas9n (D10A nickase) and single guide RNA;

48140: PX461; SpCas9n-2A-EGFP (D10A nickase) and single guide RNA;

62987: PX462; SpCas9n-2A-Puro (D10A nickase) and single guide RNA; and

48137: PX165; SpCas9;

all of which are incorporated herein by reference.

[0040]   As used herein, the term "CRISPR" refers to Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR). CRISPR may also refer to a technique or system of sequence-specific genetic manipulation relying on the CRISPR pathway. A CRISPR recombinant expression system can be programmed to cleave a target polynucleotide using a CRISPR endonuclease and a guideRNA. A CRISPR system can be used to cause double stranded or single stranded breaks in a target polynucleotide. A CRISPR system can also be used to recruit proteins or label a target polynucleotide. In some aspects, CRISPR-mediated gene editing utilizes the pathways of nonhomologous end-joining (NHEJ) or homologous recombination to perform the edits. These applications of CRISPR technology are known and widely practiced in the art. See, *e.g.,* U.S. Pat. No. 8,697,359 and Hsu *et al.* (2014) Cell 156(6): 1262-1278.

[0041]   As used herein, the term "domain" can refer to a particular region of a larger molecule (*e.g.,* a particular region of a protein or polypeptide), which can be associated with a particular function. For example, "a domain which binds to a cognate" can refer to the domain of a protein that binds one or more receptors or other protein moieties. Similarly, a corresponding coding sequence

for a particular polypeptide domain can be referred to as a
polynucleotide domain.

[0042]   The term "encode" as it is applied to polynucleotides can
refer to a polynucleotide which is said to "encode" a polypeptide
if, in its native state or when manipulated by methods well known to
those skilled in the art, it can be transcribed and/or translated to
produce the mRNA for the polypeptide and/or a fragment thereof. In
some cases the antisense strand is the complement of such a nucleic
acid, and the encoding sequence can be deduced therefrom.

[0043]   The terms "equivalent" or "biological equivalent" are
used interchangeably when referring to a particular molecule,
biological, or cellular material and intend those having minimal
homology while still maintaining desired structure or functionality.

[0044]   As used herein, "expression" can refer to the process by
which polynucleotides are transcribed into mRNA and/or the process
by which the transcribed mRNA is subsequently being translated into
peptides, polypeptides, or proteins. If the polynucleotide is
derived from genomic DNA, expression can include splicing of the
mRNA in a eukaryotic cell.

[0045]   As used herein, the term "functional" may be used to
modify any molecule, biological, or cellular material to intend that
it accomplishes a particular, specified effect.

[0046]   The term "gRNA" or "guide RNA" as used herein refers to
the guide RNA sequences used to target specific genes for correction
employing the CRISPR technique. Techniques of designing gRNAs and
donor therapeutic polynucleotides for target specificity are well
known in the art. For example, Doench, J., *et al.* Nature
biotechnology 2014; 32(12): 1262-7, Mohr, S. *et al.* (2016) FEBS
Journal 283: 3232-38, and Graham, D., *et al.* Genome Biol. 2015; 16:
260. gRNA comprises or alternatively consists essentially of, or yet
further consists of a fusion polynucleotide comprising CRISPR RNA
(crRNA) and trans-activating CRIPSPR RNA (tracrRNA); or a
polynucleotide comprising CRISPR RNA (crRNA) and trans-activating
CRIPSPR RNA (tracrRNA). In some aspects, a gRNA is synthetic
(Kelley, M. *et al.* (201) J of Biotechnology 233 (2016) 74-83).

[0047]   "Homology" or "identity" or "similarity" can refer to
sequence similarity between two peptides or between two nucleic acid

molecules. Homology can be determined by comparing a position in
each sequence which can be aligned for purposes of comparison. For
example, when a position in the compared sequence is occupied by the
same base or amino acid, then the molecules are homologous at that
position. A degree of homology between sequences is a function of
the number of matching or homologous positions shared by the
sequences. An "unrelated" or "non-homologous" sequence shares less
than 40% identity, or alternatively less than 25% identity, with one
of the sequences of the disclosure.

[0048]   Homology refers to a percent (%) identity of a sequence
to a reference sequence. As a practical matter, any particular
sequence can be at least 50%, 60%, 70%, 80%, 85%, 90%, 92%, 95%,
96%, 97%, 98% or 99% identical to any sequence described herein.
Whether such particular peptide, polypeptide or nucleic acid
sequence has a particular identity/homology can be determined
conventionally using known computer programs such the Bestfit
program (Wisconsin Sequence Analysis Package, Version 8 for Unix,
Genetics Computer Group, University Research Park, 575 Science
Drive, Madison, Wis. 53711). When using Bestfit or any other
sequence alignment program to determine whether a particular
sequence is, for instance, 95% identical to a reference sequence,
the parameters can be set such that the percentage of identity is
calculated over the full length of the reference sequence and that
gaps in homology of up to 5% of the total reference sequence are
allowed.

[0049]   For example, in a specific embodiment the identity
between a reference sequence (query sequence, *i.e.,* a sequence of
the disclosure) and a subject sequence, also referred to as a global
sequence alignment, can be determined using the FASTDB computer
program based on the algorithm of Brutlag *et al.* (Comp. App. Biosci.
6:237-245 (1990)). In some cases, parameters for a particular
embodiment in which identity is narrowly construed, used in a FASTDB
amino acid alignment, can include: Scoring Scheme=PAM (Percent
Accepted Mutations) 0, k-tuple=2, Mismatch Penalty=1, Joining
Penalty=20, Randomization Group Length=0, Cutoff Score=1, Window
Size=sequence length, Gap Penalty=5, Gap Size Penalty=0.05, Window
Size=500 or the length of the subject sequence, whichever is

shorter. According to this embodiment, if the subject sequence is
shorter than the query sequence due to N- or C-terminal deletions,
not because of internal deletions, a manual correction can be made
to the results to take into consideration the fact that the FASTDB
program does not account for N- and C-terminal truncations of the
subject sequence when calculating global percent identity. For
subject sequences truncated at the N- and C-termini, relative to the
query sequence, the percent identity can be corrected by calculating
the number of residues of the query sequence that are lateral to the
N- and C-terminal of the subject sequence, which are not
matched/aligned with a corresponding subject residue, as a percent
of the total bases of the query sequence. A determination of whether
a residue is matched/aligned can be determined by results of the
FASTDB sequence alignment. This percentage can be then subtracted
from the percent identity, calculated by the FASTDB program using
the specified parameters, to arrive at a final percent identity
score. This final percent identity score can be used for the
purposes of this embodiment. In some cases, only residues to the N-
and C-termini of the subject sequence, which are not matched/aligned
with the query sequence, are considered for the purposes of manually
adjusting the percent identity score. That is, only query residue
positions outside the farthest N- and C-terminal residues of the
subject sequence are considered for this manual correction. For
example, a 90 residue subject sequence can be aligned with a 100
residue query sequence to determine percent identity. The deletion
occurs at the N-terminus of the subject sequence and therefore, the
FASTDB alignment does not show a matching/alignment of the first 10
residues at the N-terminus. The 10 unpaired residues represent 10%
of the sequence (number of residues at the N- and C-termini not
matched/total number of residues in the query sequence) so 10% is
subtracted from the percent identity score calculated by the FASTDB
program. If the remaining 90 residues were perfectly matched the
final percent identity can be 90%. In another example, a 90 residue
subject sequence is compared with a 100 residue query sequence. This
time the deletions are internal deletions so there are no residues
at the N- or C-termini of the subject sequence which are not
matched/aligned with the query. In this case the percent identity

calculated by FASTDB is not manually corrected. Once again, only residue positions outside the N- and C-terminal ends of the subject sequence, as displayed in the FASTDB alignment, which are not matched/aligned with the query sequence are manually corrected for.

[0050]   "Hybridization" can refer to a reaction in which one or more polynucleotides react to form a complex that is stabilized via hydrogen bonding between the bases of the nucleotide residues. The hydrogen bonding can occur by Watson-Crick base pairing, Hoogstein binding, or in any other sequence-specific manner. The complex can comprise two strands forming a duplex structure, three or more strands forming a multi-stranded complex, a single self-hybridizing strand, or any combination of these. A hybridization reaction can constitute a step in a more extensive process, such as the initiation of a PC reaction, or the enzymatic cleavage of a polynucleotide by a ribozyme.

[0051]   Examples of stringent hybridization conditions include: incubation temperatures of about 25°C to about 37°C; hybridization buffer concentrations of about 6x SSC to about 10x SSC; formamide concentrations of about 0% to about 25%; and wash solutions from about 4x SSC to about 8x SSC. Examples of moderate hybridization conditions include: incubation temperatures of about 40°C to about 50°C; buffer concentrations of about 9x SSC to about 2x SSC; formamide concentrations of about 30% to about 50%; and wash solutions of about 5x SSC to about 2x SSC. Examples of high stringency conditions include: incubation temperatures of about 55°C to about 68°C; buffer concentrations of about 1x SSC to about 0.1x SSC; formamide concentrations of about 55% to about 75%; and wash solutions of about 1x SSC, 0.1x SSC, or deionized water. In general, hybridization incubation times are from 5 minutes to 24 hours, with 1, 2, or more washing steps, and wash incubation times are about 1, 2, or 15 minutes. SSC is 0.15 M NaCl and 15 mM citrate buffer. It is understood that equivalents of SSC using other buffer systems can be employed.

[0052]   As used herein, the term "immune orthogonal" refers to a lack of immune cross-reactivity between two or more antigens. In some embodiments, the antigens are proteins (*e.g.*, Cas9). In some embodiments, the antigens are viral antigens associated with a

particular viral vector (*e.g.*, AAV). As is recognized in the art, antigens typically include antigenic determinants having a particular sequence of 3 dimensional structure. Moreover, an antigenic determinant can comprise a domain or subsequence of a larger polypeptide or molecular sequence. In some embodiments, antigens that are immune orthogonal do not share an amino acid sequence of greater than 5, greater than 6, greater than 7, greater than 8, greater than 9, greater than 10, greater than 11, greater than 12, greater than 13, greater than 14, greater than 15, or greater than 16 consecutive amino acids. In some embodiments, antigens that are immune orthogonal do not share any highly immunogenic peptides. In some embodiments, antigens that are immune orthogonal do not share affinity for a major histocompatibility complex (*e.g.*, MHC class I or class II). Antigens that are immune orthogonal are amenable for sequential dosing to evade a host immune system.

[0053]   The term "immunosilent" refers to an epitope or foreign peptide, polypeptide or protein that does not elicit an immune response from a host upon administration. In some embodiments, the peptide, polypeptide or protein does not elicit an adaptive immune response. In some embodiments, the peptide, polypeptide or protein does not elicit an innate immune response. In some embodiments, the peptide, polypeptide or protein does not elicit either an adaptive or an innate immune response. In some embodiments, an immunosilent peptide, polypeptide or protein has reduced immunogenicity.

[0054]   The term "isolated" as used herein can refer to molecules or biologicals or cellular materials being substantially free from other materials. In one aspect, the term "isolated" can refer to nucleic acid, such as DNA or RNA, or protein or polypeptide (*e.g.*, an antibody or derivative thereof), or cell or cellular organelle, or tissue or organ, separated from other DNAs or RNAs, or proteins or polypeptides, or cells or cellular organelles, or tissues or organs, respectively, that are present in the natural source. The term "isolated" also can refer to a nucleic acid or peptide that is substantially free of cellular material, viral material, or culture medium when produced by recombinant DNA techniques, or chemical precursors or other chemicals when chemically synthesized.

Moreover, an "isolated nucleic acid" is meant to include nucleic acid fragments which are not naturally occurring as fragments and may not be found in the natural state. In some cases, the term "isolated" is also used herein to refer to polypeptides which are isolated from other cellular proteins and is meant to encompass both purified and recombinant polypeptides. In some cases, the term "isolated" is also used herein to refer to cells or tissues that are isolated from other cells or tissues and is meant to encompass both cultured and engineered cells or tissues.

[0055]   "Messenger RNA" or "mRNA" is a nucleic acid molecule that is transcribed from DNA and then processed to remove non-coding sections known as introns. In some cases, the resulting mRNA is exported from the nucleus (or another locus where the DNA is present) and translated into a protein. The term "pre-mRNA" can refer to the strand prior to processing to remove non-coding sections. mRNA has "U" in place of "T" in cDNA coding sequences.

[0056]   The term "Major Histocompatibility Complex" (MHC) refers to a family of proteins responsible for the presentation of peptides, including self and non-self (antigenic) to T-cells. T-cells recognize antigenic peptides and trigger a cascade of events which leads to the destruction of pathogens and infected cells. The MHC family is divided into three subgroups: class I, class II, and class III. Class I MHC molecules have β2 subunits that are only recognized by CD8 co-receptors. Class II MHC molecules have β1 and β2 subunits that are only recognized by CD4 co-receptors. In this way MHC molecules chaperone which type of lymphocytes may bind to the given antigen with high affinity, since different lymphocytes express different T-Cell Receptor (TCR) co-receptors. In general, MHC class I molecules bind short peptides, whose N- and C-terminal ends are anchored into pockets located at the ends of a peptide binding groove. While the majority of the peptides are nine amino acid residues in length, longer peptides can be accommodated by the bulging of their central portion, resulting in binding peptides of length 8 to 15. Peptides binding to class II proteins are not constrained in size and can vary from 11 to 30 amino acids long. The peptide binding groove in the MHC class II molecules is open at both ends, which enables binding of peptides with relatively longer

length. The "core" refers to the amino acid residues that contribute the most to the recognition of the peptide. In some embodiments, the core is nine amino acids in length. In addition to the core, the flanking regions are also important for the specificity of the peptide to the MHC molecule.

[0057]   The term "ortholog" is used in reference of another gene or protein and intends a homolog of said gene or protein that evolved from the same ancestral source or which are evolved artificially using molecular biology and genetic engineering. Orthologs may or may not retain the same function as the gene or protein to which they are orthologous. Non-limiting examples of Cas9 orthologs include *S. aureus* Cas9 ("spCas9"), *S. thermophiles* Cas9, *L. pneumophilia* Cas9, *N. lactamica* Cas9, *N. meningitides* Cas9, *B. longum* Cas9, *A. muciniphila* Cas9, and *O. laneus* Cas9.

[0058]   The term "promoter" as used herein refers to any sequence that regulates the expression of a coding sequence, such as a gene. Promoters may be constitutive, inducible, repressible, or tissue-specific, for example. A "promoter" is a control sequence that is a region of a polynucleotide sequence at which initiation and rate of transcription are controlled. It may contain genetic elements at which regulatory proteins and molecules may bind such as RNA polymerase and other transcription factors. Non-limiting exemplary promoters include CMV promoter and U6 promoter.

[0059]   The term "protein", "peptide" and "polypeptide" are used interchangeably and in their broadest sense to refer to a compound of two or more subunit amino acids, amino acid analogs or peptidomimetics. The subunits can be linked by peptide bonds. In another embodiment, the subunit can be linked by other bonds, *e.g.,* ester, ether, *etc.* A protein or peptide can contain at least two amino acids and no limitation is placed on the maximum number of amino acids which can comprise a protein's or peptide's sequence. As mentioned above, the term "amino acid" can refer to either natural and/or unnatural or synthetic amino acids, including glycine and both the D and L optical isomers, amino acid analogs and peptidomimetics. As used herein, the term "fusion protein" can refer to a protein comprised of domains from more than one naturally occurring or recombinantly produced protein, where generally each

domain serves a different function. In this regard, the term "linker" can refer to a peptide fragment that is used to link these domains together - optionally to preserve the conformation of the fused protein domains and/or prevent unfavorable interactions between the fused protein domains which can compromise their respective functions.

[0060]   The terms "polynucleotide" and "oligonucleotide" are used interchangeably and refer to a polymeric form of nucleotides of any length, either deoxyribonucleotides or ribonucleotides or analogs thereof. Polynucleotides can have any three-dimensional structure and can perform any function, known or unknown. The following are non-limiting examples of polynucleotides: a gene or gene fragment (for example, a probe, primer, EST or SAGE tag), exons, introns, messenger RNA (mRNA), transfer RNA, ribosomal RNA, RNAi, ribozymes, cDNA, recombinant polynucleotides, branched polynucleotides, plasmids, vectors, isolated DNA of any sequence, isolated RNA of any sequence, nucleic acid probes and primers. A polynucleotide can comprise modified nucleotides, such as methylated nucleotides and nucleotide analogs. If present, modifications to the nucleotide structure can be imparted before or after assembly of the polynucleotide. The sequence of nucleotides can be interrupted by non-nucleotide components. A polynucleotide can be further modified after polymerization, such as by conjugation with a labeling component. The term also can refer to both double and single stranded molecules. Unless otherwise specified or required, any embodiment of this disclosure that is a polynucleotide can encompass both the double stranded form and each of two complementary single stranded forms known or predicted to make up the double stranded form.

[0061]   The term "polynucleotide sequence" can be the alphabetical representation of a polynucleotide molecule. This alphabetical representation can be input into databases in a computer having a central processing unit and used for bioinformatics applications such as functional genomics and homology searching.

[0062]   Similarly, the term "polypeptide sequence", "peptide sequence" or "protein sequence" can be the alphabetical

representation of a polypeptide molecule. This alphabetical
representation can be input into databases in a computer having a
central processing unit and used for bioinformatics applications
such as functional proteomics and homology searching.

[0063]   As used herein, the term "recombinant expression system"
refers to a genetic construct or constructs for the expression of
certain genetic material formed by recombination.

[0064]   As used herein, the term "recombinant protein" can refer
to a polypeptide or peptide which is produced by recombinant DNA
techniques, wherein generally, DNA encoding the polypeptide or
peptide is inserted into a suitable expression vector which is in
turn used to transform a host cell to produce the heterologous
polypeptide or peptide.

[0065]   The term "sequencing" as used herein, can comprise
bisulfite-free sequencing, bisulfite sequencing, TET-assisted
bisulfite (TAB) sequencing, ACE-sequencing, high-throughput
sequencing, Maxam-Gilbert sequencing, massively parallel signature
sequencing, Polony sequencing, 454 pyrosequencing, Sanger
sequencing, Illumina sequencing, SOLiD sequencing, Ion Torrent
semiconductor sequencing, DNA nanoball sequencing, Heliscope single
molecule sequencing, single molecule real time (SMRT) sequencing,
nanopore sequencing, shot gun sequencing, RNA sequencing, Enigma
sequencing, or any combination thereof.

[0066]   As used herein, the term "subject" is intended to mean
any animal. In some embodiments, the subject may be a mammal; in
further embodiments, the subject may be a bovine, equine, feline,
murine, porcine, canine, human, or rat.

[0067]   As used herein, the terms "transformation" and
"transfection" are intended to refer to a variety of art-recognized
techniques for introducing foreign nucleic acid into a host cell,
including calcium phosphate or calcium chloride co-precipitation,
DEAE-dextran-mediated transfection, lipofection (e.g., using
commercially available reagents such as, for example, LIPOFECTIN®
(Invitrogen Corp., San Diego, CA), LIPOFECTAMINE® (Invitrogen),
FUGENE® (Roche Applied Science, Basel, Switzerland), JETPEI™
(Polyplus-transfection Inc., New York, NY), EFFECTENE® (Qiagen,
Valencia, CA), DREAMFECT™ (OZ Biosciences, France) and the like), or

electroporation. Suitable methods for transforming or transfecting
host cells can be found in Sambrook, *et al.* (Molecular Cloning: A
Laboratory Manual. 2nd, ed., Cold Spring harbor Laboratory, Cold
Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989), and
other laboratory manuals. Standard recombinant DNA and molecular
cloning techniques used herein are well known in the art and are
described in Sambrook, J., Fritsch, E.F. and Maniatis, T., Molecular
Cloning: A Laboratory Manual, 2nd ed.; Cold Spring Harbor Laboratory:
Cold Spring Harbor, N.Y., (1989) and by Silhavy, T.J., Bennan, M.L.
and Enquist, L.W., Experiments with Gene Fusions; Cold Spring Harbor
Laboratory: Cold Spring Harbor, N.Y., (1984); and by Ausubel, F.M.
et. al., Current Protocols in Molecular Biology, Greene Publishing
and Wiley-Interscience (1987) each of which are hereby incorporated
by reference in its entirety. Additional useful methods are
described in manuals including Advanced Bacterial Genetics (Davis,
Roth and Botstein, Cold Spring Harbor Laboratory, 1980), Experiments
with Gene Fusions (Silhavy, Berman and Enquist, Cold Spring Harbor
Laboratory, 1984), Experiments in Molecular Genetics (Miller, Cold
Spring Harbor Laboratory, 1972) Experimental Techniques in Bacterial
Genetics (Maloy, in Jones and Bartlett, 1990), and A Short Course in
Bacterial Genetics (Miller, Cold Spring Harbor Laboratory 1992) each
of which are hereby incorporated by reference in its entirety.

[0068]   The terms "treat", "treating" and "treatment", as used
herein, refers to ameliorating symptoms associated with a disease or
disorder (*e.g.*, cancer, Covid-19 *etc.*), including preventing or
delaying the onset of the disease or disorder symptoms, and/or
lessening the severity or frequency of symptoms of the disease or
disorder.

[0069]   As used herein, the term "vector" can refer to a nucleic
acid construct deigned for transfer between different hosts,
including but not limited to a plasmid, a virus, a cosmid, a phage,
a BAC, a YAC, *etc.* In some embodiments, a "viral vector" is defined
as a recombinantly produced virus or viral particle that comprises a
polynucleotide to be delivered into a host cell, either *in vivo, ex
vivo* or *in vitro*. In some embodiments, plasmid vectors can be
prepared from commercially available vectors. In other embodiments,
viral vectors can be produced from baculoviruses, retroviruses,

adenoviruses, AAVs, *etc.* according to techniques known in the art.
In one embodiment, the viral vector is a lentiviral vector.
Examples of viral vectors include retroviral vectors, adenovirus
vectors, adeno-associated virus vectors, alphavirus vectors and the
like. Infectious tobacco mosaic virus (TMV)-based vectors can be
used to manufacturer proteins and have been reported to express
Griffithsin in tobacco leaves (O'Keefe *et al.* (2009) Proc. Nat.
Acad. Sci. USA 106(15):6099-6104). Alphavirus vectors, such as
Semliki Forest virus-based vectors and Sindbis virus-based vectors,
have also been developed for use in gene therapy and immunotherapy.
See, Schlesinger & Dubensky (1999) Curr. Opin. Biotechnol. 5:434-439
and Ying *et al.* (1999) Nat. Med. 5(7):823-827. In aspects where
gene transfer is mediated by a retroviral vector, a vector construct
can refer to the polynucleotide comprising the retroviral genome or
part thereof, and a gene of interest. Further details as to modern
methods of vectors for use in gene transfer can be found in, for
example, Kotterman *et al.* (2015) Viral Vectors for Gene Therapy:
Translational and Clinical Outlook Annual Review of Biomedical
Engineering 17. Vectors that contain both a promoter and a cloning
site into which a polynucleotide can be operatively linked are well
known in the art. Such vectors are capable of transcribing RNA *in
vitro* or *in vivo* and are commercially available from sources such as
Agilent Technologies (Santa Clara, Calif.) and Promega Biotech
(Madison, Wis.). In one aspect, the promoter is a pol III promoter.

[0070]   Certain vectors are capable of autonomous replication in
a host cell into which they are introduced (*e.g.*, bacterial vectors
having a bacterial origin of replication and episomal mammalian
vectors). Other vectors (*e.g.*, non-episomal mammalian vectors) are
integrated into the genome of a host cell upon introduction into the
host cell, and thereby are replicated along with the host genome.
Moreover, certain vectors are capable of directing the expression of
genes to which they are operatively linked. Such vectors are
referred to herein as "expression vectors." In general, expression
vectors of utility in recombinant DNA techniques are often in the
form of plasmids. In the present specification, "plasmid" and
'Vector" can be used interchangeably. However, the disclosure is
intended to include such other forms of expression vectors, such as

viral vectors (*e.g.,* replication defective retroviruses, adenoviruses and adeno-associated viruses), which serve equivalent functions. Typically, the vector or plasmid contains sequences directing transcription and translation of a relevant gene or genes, a selectable marker, and sequences allowing autonomous replication or chromosomal integration. Suitable vectors comprise a region 5' of the gene which harbors transcriptional initiation controls and a region 3' of the DNA fragment which controls transcription termination. Both control regions may be derived from genes homologous to the transformed host cell, although it is to be understood that such control regions may also be derived from genes that are not native to the species chosen as a production host.

[0071]   Typically, the vector or plasmid contains sequences directing transcription and translation of a gene fragment, a selectable marker, and sequences allowing autonomous replication or chromosomal integration. Suitable vectors comprise a region 5' of the gene which harbors transcriptional initiation controls and a region 3' of the DNA fragment which controls transcription termination. Both control regions may be derived from genes homologous to the transformed host cell, although it is to be understood that such control regions may also be derived from genes that are not native to the species chosen as a production host.

[0072]   Initiation control regions or promoters, which are useful to drive expression of the relevant pathway coding regions in the desired host cell are numerous and familiar to those skilled in the art. Virtually any promoter capable of driving these genetic elements is suitable for the present invention including, but not limited to, lac, ara, tet, trp, IPL, IPR, T7, tac, and trc (useful for expression in Escherichia coli and Pseudomonas); the amy, apr, npr promoters and various phage promoters useful for expression in *Bacillus subtilis*, and *Bacillus licheniformis*; nisA (useful for expression in gram positive bacteria, Eichenbaum *et al.* Appl. Environ. Microbiol. 64(8):2763-2769 (1998)); and the synthetic Pll promoter (useful for expression in *Lactobacillus plantarum*, Rud *et al.,* Microbiology 152:1011-1019 (2006)). Termination control regions may also be derived from various genes native to the preferred hosts.

[0073]   Immunogenicity is a major concern for protein-based therapeutics, particularly those derived from non-human species. Induction of the immune response can render treatments ineffective and cause serious, even life-threatening side effects. One strategy to overcome this issue is to mutate particularly immunogenic epitopes in the therapeutic target. However, this strategy is hindered by the ability of the adaptive immune system to recognize multiple epitopes across large regions of the antigen. While epitope deletion efforts to date have focused on a few major antibody binding sites, it is not possible to make these studies comprehensive due to the vast possible epitope space. Variant library screening has proven to be an effective approach to protein engineering but applying it in this case faces several technical challenges. One problem is the vast mutational space created by the need for full combinatorial libraries. Fully degenerate libraries quickly become intractably large as the number of target sites increases beyond just a few. Narrowing down this space by intelligent selection of library members is necessary to define a reasonable mutational landscape to explore and critical for maximizing the chance of functional hits. Another problem is that reading out combinatorial mutations scattered across large (>1kb) regions of the protein is extremely difficult using short read sequencing. Using short barcodes attached to each variant to genotype libraries post-screen has proved effective but is limited by the difficulty of constructing large combinatorial libraries in which each member has a short, unique barcode. These issues have generally limited combinatorial library screens to short regions able to be sequenced directly.

[0074]   The scale of engineering which would be required to generate an effectively de-immunized Cas9, for example, is not fully understood, as combinatorial de-immunization efforts at the scale of proteins thousands of amino acids long have not yet been possible. Therefore, to roughly estimate these parameters an immunogenicity scoring metric was developed that takes into account all epitopes across a protein and the known diversity of MHC variants in a species weighted by population frequency to generate a single combined score representing the average immunogenicity of a full-

length protein as a function of each of its immunogenic epitopes. Formally, this score is calculated as:

$$I_x = \frac{\sum_i^m \ \sum_j^n \ w_j * (1 - log \ log \ (k_{ij} * \hat{j}))}{y}$$

where I_x = Immunogenicity score of protein x, i = epitopes, j = HLA alleles, $\hat{j}$ = allele specific standardization coefficient, w_j = HLA allele weights, k_{ij} = predicted binding affinity of epitope i to allele j, p_{ij} = percentile rank of epitope i binding to allele j, and y = protein specific scaling factor.

[0075]   The disclosure provides a 3-part strategy to overcome library size constraints in both the number of unique members and the length of the mutagenized region. The disclosure provides a protein engineering platform capable of screening millions of combinatorial variants simultaneously with mutations spread across the full length of arbitrarily large proteins, with computation-guided mutation design to maximize the probability of exploring functional mutation space (**FIG. 1A**). In addition to these advantages, the platform can be applied iteratively to tackle particularly challenging engineering tasks by exploring huge swaths of combinatorial mutation space unapproachable using previous techniques. Furthermore, while this methodology is particularly suited to the unique challenges of protein de-immunization, which is demonstrated here as a proof-of-concept use case, it is also applicable to any potential protein engineering goal, so long as there exists an appropriate screening procedure to select for the desired protein functionality. The specific innovations which enable this technology are threefold: library design, library construction, and sequencing readout linking genotype to phenotype.

[0076]   First, target regions were selected within the protein of interest using software which predicts HLA-binding and peptide immunogenicity. It can be difficult to functionalize these predictions, however, because HLA loci are highly polymorphic, and each HLA allele will have its own particular ligand binding profile. To generalize immunogenicity predictions and select appropriate targets, an approximation of global HLA allele frequencies was created using data from the Allele Frequency Net Database. These frequencies were used to scale immunogenicity predictions such that

the top hits are the peptides likely to be the most immunogenic epitopes for the largest number of people globally.

[0077]   Next, in order to narrow down the vast mutational space associated with fully degenerate combinatorial libraries, an approach guided by evolution and natural variation was utilized. As deimmunizing protein engineering seeks to alter the amino acid sequence of a protein without disrupting functionality, it would be extremely useful to narrow down mutations to those less likely to result in non-functional variants. Mutants were identified by leveraging the large amounts of sequencing data available to identify low-frequency SNPs that have been observed in natural environments. Such variants are likely to have limited effect on protein function, as highly deleterious alleles would likely be immediately selected out of the natural population and not appear in sequencing data. Using these likely neutral amino acid substitutions in combinatorial libraries should substantially increase the likelihood of functional hits with enough epitope variation to evade immune induction. Once the targets are identified and the mutations defined, the library is assembled piecewise using standard synthesis and assembly methods and apply the screen.

[0078]   In order to read out the library containing mutations dispersed along a long sequence, a long read nanopore sequencing system was utilized. This circumvents the limit of short target regions and obviates the need for barcodes altogether by single-molecule sequencing of the entire target gene. The adoption of nanopore sequencing has been limited by its high error rate compared to established short read techniques; however, careful library design can yield multiple nucleotide changes for each single target amino acid change, effectively increasing the sensitivity of nanopore based readouts exponentially with increasing numbers of nucleotide changes per library member. The large majority of amino acid substitutions are amenable to a library design paradigm in which each substitution is encoded by two, rather than one, nucleotide change, due to the degeneracy of the genetic code and the highly permissive third "wobble" position of codons. For example, if the wild-type amino acid leucine is encoded by the codon CTG, typically a substitution to the amino acid proline would be encoded

by the single nucleotide change T to C at the second position, resulting in a CCG codon. However, it is also possible to use any of the other three codons encoding proline, CCT, CCC, and CCA, each of which is two nucleotide changes away from the wild-type sequence. These changes are much easier to reliably detect with error-prone long read nanopore sequencing.

[0079]   Disclosed herein are methods for identifying or modifying a protein sequence to reduce immunogenicity, and optionally be immunosilent. The method comprises, consists of, or consists essentially of identifying targeted regions of a protein associated with HLA binding. The targeted regions can be ranked by HLA allele frequency using data from the Allele Frequency Net Database ([www.]allelefrequencies.net; brackets provided to eliminate hyperlinks). The frequencies are used for immunogenicity predictions, such that the top hits are the peptides likely to be the most immunogenic epitopes for the largest number of people globally. Next, mutational variants are narrowed by identifying mutations that have the least disruption to protein function. These mutations are identified by sequence comparison analysis using various databases available to the public. Using the databases, low frequency SNPs that have been observed in natural environments are then identified. These SNP variants are likely to have limited effect on protein function; as highly deleterious alleles would likely be immediately selected out of the natural population and not appear in sequencing data. The amino acid variant is identified and these substitutions are used and screened for functional activity and for the ability to generate an immune response.

[0080]   It was postulated that the disclosure's library design principles informed by natural variation, and long read nanopore sequencing readouts, would allow for the reliable mapping of genotype to phenotype in large scale combinatorial screens of mutations scattered across the full length of a gene. The information obtained from these screens will allow for evaluation of the effects of epistasis, and allow for the tackling of design problems not amenable to current screening technologies, such as full-length epitope deletion and de-immunization.

[0081]   Disclosed herein are methods for modifying a sequence of a protein or virus to reduce immunogenicity, and optionally be immunosilent. In a particular embodiment, the disclosure provides a method for engineering a protein or virus to be less immunoreactive, comprising one or more of the following steps: identifying target regions of the DNA sequence of protein or virus that are predicted to have human leukocyte antigen (HLA)-binding and/or peptide immunogenicity; identifying single nucleotide polymorphisms (SNPs) or mutations in the targeted region and other regions that are not deleterious to the functioning of the protein; screening a library assembled using standard synthesis and assembly methods by applying the above identifying criteria to find functional variants of the protein or virus; sequencing the functional variants of the protein or virus; and/or mapping genotype to phenotype from the sequences of the functional variants to identify variant candidates that are likely functionally active and have mutations that result in the protein or virus exhibiting less immunogenicity.

[0082]   The disclosure contemplates use of the methods of the disclosure for reducing the immunogenicity of a protein can be applied to a variety of proteins that present a risk of eliciting an immune response. Non-limiting exemplary proteins of interest include cytidine deaminases, which can be used for gene editing via catalysis of DNA base change from C to T (e.g. APOBEC – Conserved across many species e.g. Rat APOBEC3, Rat APOBEC1, Resus Macaque APOBEC3G, human APOBEC1 (A1), AID, APOBEC2 (A2), APOBEC3A (A3A), APOBEC3B (A3B), APOBEC3C (A3C), APOBEC3DE (A3DE), APOBEC3F (A3F), APOBEC3G (A3G), APOBEC3H (A3H) and APOBEC4 (A4)); adenosine deaminases, which can be used for gene editing via catalysis of DNA base change from A to G (e.g. ADA (DNA editor) – Widely conserved across virtually all species and ADAR (RNA editor) – Conserved across most metazoan species); Zing Finger nucleases (ZFNs), which can be used for genome engineering in a similar manner to CRISPR/Cas9 and are engineered site-specific nucleases consisting of: 3-6 repeated zinc finger domains, which is a widely conserved DNA-binding motif and a nuclease domain; transcriptional activator-like effector nucleases (TALENs), which be used for genome engineering in a similar manner to CRISPR/Cas9 and are similar to

ZFNs in that they are engineered site-specific nucleases consisting of: a TAL effector DNA binding domain (generally derived from a species of *Xanthomonas proteobacteria*) and a nuclease domain. The domains of the site-specific enzymes mentioned above (ZFNs and TALENs) are well characterized and subject of extensive engineering to generate the desired specificity. Thus, many variants exist of such proteins. Additional proteins for which HLA-binding affinity analysis is relevant include Cas9 proteins and AAV capsids, both of which are used in CRISPR based gene editing.

[0083]   In a particular embodiment, the methods disclosed herein provide for reducing the immunogenicity of a CRISPR associated protein. Examples of CRISPR associated proteins include, but are not limited to, Cas9, Cas12, Cas13, Cas14. In yet another embodiment, the CRISPR associated protein is a Cas9. In a further embodiment, the Cas9 is *Streptococcus pyogenes* Cas9 (SpCas9). In some embodiments, the Cas9 proteins the orthologs are selected from *S. pyogenes* Cas9 (spCas9), *S. aureus* Cas9 (saCas9), *B. longum* Cas9, *A. muiciniphilia* Cas9, or *O. laneus* Cas9. In order to optimize and broaden the application of CRIPSR based therapeutics the disclosure provides methods to "humanize" the CRISPR associated protein by swapping high immunogenic domains or peptides with less immunogenic counterparts. This is particularly useful to enable the application of CRIPSR based therapeutics for repeat treatments. The disclosure teaches methods and methodology to screen mutations in selected targeted regions of proteins, such as CRISPR associated proteins, in order to reduce immunogenicity.

[0084]   Thus, embodiments of the disclosure relate to a modified CRISPR associated protein that has lower immunogenicity to promote immune evasion. The modified proteins can replace existing wildtype proteins for any application requiring *in vivo* delivery, which would potentially have no loss of efficacy after repetitive use.

[0085]   In some aspects, provided herein are isolated polynucleotides encoding a modified Cas9 protein, wherein the modified Cas9 comprises, consists of, or consists essentially of one or more, two or more, three or more, four or more, five or more, six or more, seven or more, eight or more, nine or more, ten or more, fifteen or more, or twenty or more of the amino acid modifications

in targeted regions to lower the immunogenicity of the protein. In one embodiment, the disclosure provides an engineered immune-silenced Cas9 comprising a sequence of SEQ ID NO:2 and having one or more mutations selected from the group consisting of: P28L, L237C, Y286Q, S318(H/C), S368C, F498T, L514(T/G), L616G, L623Q, L636D, F704A, L727(P/G), L816D, Y1016(K/G), L1245G, I1273Q, L1282(A/E), and/or Y1294Q. In some aspects, provided herein are vectors comprising an isolated polynucleotide encoding an engineered Cas9 comprising one or more mutations selected from the group consisting of: P28L, L237C, Y286Q, S318(H/C), S368C, F498T, L514(T/G), L616G, L623Q, L636D, F704A, L727(P/G), L816D, Y1016(K/G), L1245G, I1273Q, L1282(A/E), and/or Y1294Q. In some embodiments, the vector is an AAV vector, optionally wherein the AAV vector is AAV5.

[0086]   The disclosure provides an isolated polypeptide comprising (i) SEQ ID NO:2 having a mutation(s) selected from P28L, L237C, Y286Q, S318(H/C), S368C, F498T, L514(T/G), L616G, L623Q, L636D, F704A, L727(P/G), L816D, Y1016(K/G), L1245G, I1273Q, L1282(A/E), Y1294Q, and any combination thereof, wherein the polypeptide has Cas9 activity and wherein the polypeptide has reduced immunogenicity compared to SEQ ID NO:2 lacking any one or more of the mutations; (ii) a sequence that is 95%-99% identical to (i); and (iii) related homolog/orthologs having mutations corresponding to the mutations in SEQ ID NO:2 and having Cas9 activity. The disclosure also provided isolated polynucleotides encoding the polypeptide of (i)-(iii) above. The polynucleotides can be RNA or DNA. The polynucleotides can be cloned into a vector for expression and/or delivery to a subject.

[0087]   In yet a further embodiment, the targeted regions to lower the immunogenicity of the protein are identified using a model that predicts human leukocyte antigen (HLA)-binding and peptide immunogenicity. Models for determining HLA-binding affinity and peptide immunogenicity are likewise known in the art and may include computational methods available through software or publicly accessible databases or "wet lab" assays. Examples of computational methods of predicting HLA-binding affinity include, but are not limited to, the MHC prediction models available through the IEDB Analysis Resource ([http://]tools.immuneepitope.org/mhci/ (MHC I)

and [http://]tools.immuneepitope.org/mhcii/ (MHC II)) or NetMHC
([http://www.]cbs.dtu.dk/services/NetMHC/). Other examples of
prediction models include, but are not limited to, NetMHC,
MHCAttnNet, MHCSeqNET, ACME, NetMHCpan EL 4.1, NetMHCstabpan, SMM,
SMMPMBEC, PickPocket, Comblib_Sidney2008, NetMHCcons, MHCflurry 2.0,
and IConMHC. Alternatively or in addition, HLA-binding can be
determined or computational predictions thereof can be validated
using assays, such as, but not limited to, immunoassays, such as
ELISA, microarray, tetramer assay, and peptide-induced MHC
stabilization assay. Using such assays and computational methods
can further be adapted to account for immune response of a specific
subject or patient being treated. Thus, modifications in the
proteins can be optimized to reduce the immunogenicity of the
protein when administered to a particular subject or patient.
Similarly, the comparisons can be host-restricted, such that the
protein is optimized to reduce the immunogenicity of the protein
when administered to a particular host, *e.g.*, a mouse or a human.
Examples of such, include "humanizing" the protein by swapping high
immunogenic domains or peptides with less immunogenic counterparts.

[0088]   In order to narrow down the vast mutational space
associated with fully degenerate combinatorial libraries, an
approach guided by evolution and natural variation is utilized. As
deimmunizing protein engineering seeks to alter the amino acid
sequence of a protein without disrupting functionality, it would be
extremely useful to narrow down mutations to those less likely to
result in non-functional variants. These mutants are identified by
leveraging the large amounts of sequencing data available to
identify low-frequency SNPs and mutations that have been observed in
natural environments. Such variants are likely to have limited
effect on protein function, as highly deleterious alleles would
likely be immediately selected out of the natural population and not
appear in sequencing data. In a particular embodiment, SNPs or
mutations are identified by using phylogenetic methods to scan
natural variation among naturally occurring proteins, mutations
generated in the course of research and engineering efforts, and
protein orthologs from closely related species. In yet another
embodiment, the SNPS or mutations are identified, or further

identified, by using immunological prediction of candidate mutations to ensure significant loss of immunogenicity within the targeted region in order to preserve function while reducing immunogenicity.

[0089]   The disclosure contemplates use of the methods of the disclosure for reducing the immunogenicity of viruses. The methods can be applied to a variety of types of viruses that present a risk of eliciting an immune response, particularly those used in gene therapy or gene delivery. Examples of such viruses, include but are not limited to, retroviruses, adenoviruses, adeno-associated viruses (AAVs), alphaviruses, lentiviruses, pox viruses, and herpes viruses. In a further embodiment, the virus is an AAV. In yet a further embodiment, the AAV is selected from AAV1, AAV2, AAV5, AAV6, AAV7, and AAV8. In another embodiment, the AAV is AAV5.

[0090]   The disclosure provides methods encompassing a step of identifying target regions of proteins and the corresponding polynucleotide coding sequence of a virus that are predicted to have human leukocyte antigen (HLA)-binding and/or peptide immunogenicity target regions. In a further embodiment, the targeted regions are identified by aligning conserved sequence regions across AAV serotypes. In a further embodiment, the SNPs are identified by aligning the sequences of 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200 or more AAV variants that have been sequenced from natural or engineered sources. In yet a further embodiment, the SNPs are located in the target regions.

[0091]   In another embodiment, in order to identify and characterize the function of variant protein or viral candidates that are likely functionally active and have mutations that result in the protein or virus exhibiting less immunogenicity, long-read sequencing technologies capable of sequencing the entire sequence of each protein or viral variant in one sequencing reaction are used. In yet another embodiment, the long-read sequencing technologies is capable of generating 10-15 Gb of sequencing reads per run. Examples of such sequencing technologies include, but are not limited to, Oxford Nanopore's MinIon sequencer.

[0092]   In a further embodiment, a method disclosed herein encompasses the step of evaluating the immunoreactivity of variant candidates in one or more immunoassays. In yet a further

embodiment, the one or more immunoassays comprise detecting the presence of antibodies to the variant candidates (AVA antibodies), when the variant candidates are administered *in vivo* to an animal. In another embodiment, the one or more immunoassays comprise an enzyme-linked immunosorbent assays (ELISAs), electrochemiluminescence (ECL) assays and/or antigen-binding tests, wherein the one or more immunoassays utilize AVA antibodies.

[0093]   The disclosure also provide composition used in various therapies wherein the composition comprises a potentially immunogenic molecule such as a protein or polypeptide. The methods of the disclosure can be used to identify domains that are immunogenic and identify mutations that reduce immunogenicity. For example, Cas9 is a protein used in gene editing *in vivo*, but has been shown to have immunogenic potential. In a particular embodiment, the disclosure provides for a polynucleotide having at least 90%, 95%, 97%, 98%, or 99% sequence identity to the sequence presented in SEQ ID NO:1, wherein the polnucleotide encodes a polypeptide having at least 90% identity to SEQ IDNO:2 having reduced immunogenicity and wherein the protein has Cas9 like activity. In another embodiment, the disclosure also provides for a protein having a polypeptide sequence that has at least 90%, 95%, 97%, 98%, or 99% sequence identity to the sequence presented in SEQ ID NO:2, wherein the protein has Cas9 like activity. In a further embodiment, the protein has less immunogenicity than Cas9.

[0094]   In a certain embodiment, the disclosure further provides for a CRISPR-Cas9 system comprising a protein disclose herein that has Cas9 like activity. In another embodiment, the CRISPR-Cas9 system further comprises RNA that comprise a shot sequence that binds to a specific target sequence of DNA in a genome. It is appreciated by those skilled in the art that RNAs can be generated for target specificity to target a specific gene, optionally a gene associated with a disease, disorder, or condition. Thus, in combination with Cas9, the guide RNAs facilitate the target specificity of the CRISPR/Cas9 system. Further aspects such as promoter choice, may provide additional mechanisms of achieving target specificity – *e.g.,* selecting a promoter for the guide RNA encoding polynucleotide that facilitates expression in a particular

organ or tissue. Accordingly, the selection of suitable RNAs for the particular disease, disorder, or condition is contemplated herein.

[0095]   In a particular embodiment, the disclosure provides for a virus encoded by a polynucleotide sequence having at least 90%, 95%, 97%, 98%, or 99% sequence identity to the sequence presented in Figure 5, wherein the virus has AAV5 like activity. In a certain embodiment, the disclosure also provides for a virus capsid having at least 90%, 95%, 97%, 98%, or 99% sequence identity to the sequence presented in SEQ ID NO:50, wherein the virus has AAV5 like activity. In another embodiment, the virus has less immunogenicity than AAV5.

[0096]   In a certain embodiment, the disclosure also provides for an AAV system comprising a virus disclosed herein that has AAV5 like activity. In a further embodiment, the AAV system is used for gene therapy. Administration of the AAV variant or compositions thereof can be affected in one dose, continuously or intermittently throughout the course of treatment. Administration may be through any suitable mode of administration, including but not limited to: intravenous, intra-arterial, intramuscular, intracardiac, intrathecal, subventricular, epidural, intracerebral, intracerebroventricular, sub-retinal, intravitreal, intraarticular, intraocular, intraperitoneal, intrauterine, intradermal, subcutaneous, transdermal, transmuccosal, and inhalation.

[0097]   Methods of determining the most effective route and dosage of administration are known to those of skill in the art and will vary with the composition used for therapy, the purpose of the therapy and the subject being treated. Single or multiple administrations can be carried out with the dose level and pattern being selected by the treating physician. It is noted that dosage may be impacted by the route of administration. Suitable dosage formulations and methods of administering the agents are known in the art. Non-limiting examples of such suitable dosages may be as low as 1E+9 vector genomes to as much as 1E+17 vector genomes per administration.

[0098]   In a further embodiment, a modified virus and compositions of the disclosure having reduced immunogenicity can be

administered in combination with other treatments, *e.g.* those approved treatments suitable for the particular disease, disorder, or condition.

[0099]   Doses suitable for uses herein may be delivered via any suitable route, *e.g.* intravenous, transdermal, intranasal, oral, mucosal, or other delivery methods, and/or via single or multiple doses. It is appreciated that actual dosage can vary depending on the recombinant expression system used (*e.g.* AAV or lentivirus), the target cell, organ, or tissue, the subject, as well as the degree of effect sought. Size and weight of the tissue, organ, and/or patient can also affect dosing. Doses may further include additional agents, including but not limited to a carrier. Non-limiting examples of suitable carriers are known in the art: for example, water, saline, ethanol, glycerol, lactose, sucrose, dextran, agar, pectin, plant-derived oils, phosphate-buffered saline, and/or diluents. Additional materials, include those disclosed in paragraph [00533] of WO 2017/070605 may be used with the compositions disclosed herein. Paragraphs [00534] through [00537] of WO 2017/070605 also provide non-limiting examples of dosing conventions for CRISPR-Cas systems which can be used herein. In general, dosing considerations are well understood by those in the art.

[00100]   The disclosure also provides for compositions or kits comprising any one or more of the variant proteins and/or variant viruses described herein. In one embodiment, the carrier is a pharmaceutically acceptable carrier. These compositions can be used therapeutically as described herein and can be used in combination with other known therapies and/or according to the method aspects described herein.

[00101]   Briefly, pharmaceutical compositions of the present invention may comprise a variant Cas9 described herein or a polynucleotide encoding said Cas9, optionally comprising a variant AAV described herein, in combination with one or more pharmaceutically or physiologically acceptable carriers, diluents or excipients. Such compositions may comprise buffers such as neutral buffered saline, phosphate buffered saline and the like; carbohydrates such as glucose, mannose, sucrose or dextrans,

mannitol; proteins; polypeptides or amino acids such as glycine; antioxidants; chelating agents such as EDTA or glutathione; adjuvants (*e.g.*, aluminum hydroxide); and preservatives. Compositions of the present disclosure may be formulated for oral, intravenous, topical, enteral, and/or parenteral administration. In certain embodiments, the compositions of the present disclosure are formulated for intravenous administration.

[00102]   The following examples are intended to illustrate but not limit the disclosure. While they are typical of those that might be used, other procedures known to those skilled in the art may alternatively be used.

EXAMPLES

[00103]   Applying the procedure described herein, a library of Cas9 variants were designed based on the SpCas9 backbone containing 23 different mutations across 17 immunogenic epitopes (**Figure 2A**)(e.*g.*, P28L, L237C, Y286Q, S318(H/C), S368C, F498T, L514(T/G), L616G, L623Q, L636D, F704A, L727(P/G), L816D, Y1016(K/G), L1245G, I1273Q, L1282(A/E), and/or Y1294Q; relative to SEQ ID NO:2). Combining these in all possible combinations yields a library of 1,492,992 unique elements. With this design in hand, a library was constructed in a stepwise process. First, the full-length gene was broken up into short blocks of no more than 1000 bp, which overlap by 30 bp on each end. Each block is designed such that it contains no more than 3 or 4 target epitopes to mutagenize. With few epitopes per block and few variant mutations per epitope, it becomes feasible to chemically synthesize each combination of mutations for each block. For example, block 2 of the SpCas9 library contains 3 target epitopes (see, *e.g.*, **FIG. 2B**). One variant mutation was designed to test at two of these sites, and two variant mutations at the final site. These variants plus the wild-type result in a total of 2 * 2 * 3 = 12 combinations. Each of these combinations is then synthesized and mixed at equal ratios to make a degenerate block mix. This is repeated for each of the blocks necessary to complete the full-length protein sequence.

[00104]   Subsequently, each of these block mixes were assembled into a fully combinatorial library of Cas9 sequences using a two-step PCR assembly method. The first annealing-extension step allows

each of the blocks to anneal to and prime their attachment to
neighboring blocks using their 30 bp overlapping ends. DNA
polymerase then extends these attached fragments into full length
Cas9 genes with the order of block assembly being specified by the
unique 30 bp overhangs at each block junction. In the second step,
primers binding to only the far 3' and 5' ends of the Cas9 sequence
are used to amplify the full length Cas9 library in a standard PCR
reaction. The library is then purified and inserted into an
appropriate cloning/expression vector via Gibson assembly.

[00105]   Each of these components of library design and
construction, the selection of target sites, the identification of
high-likelihood functional mutations, the multiple nucleotide
mutation scheme, the subdivision of the protein into blocks, and the
assembly of those blocks into a fully degenerate combinatorial
library, come together to enable the functionality of this protein
engineering platform. To demonstrate the utility of this
methodology, a combinatorial library screen was generated to heavily
de-immunize the *Streptococcus pyogenes* Cas9 nuclease (SpCas9; SEQ ID
NO:1 and 2, polynucleotide and polypeptide, respectively). This
classical Cas9 is currently under clinical development for use in
gene therapy, but being of microbial origin, indeed, from a species
which opportunistically causes human disease, this protein may be
expected to generate an immune response which can inhibit
therapeutic efficacy.

[00106]   After construction of the Cas9 variant library, nanopore
sequencing was applied using the Oxford Nanopore (ONT) MinION
platform. To generate the sequencing library, PCR amplification was
performed on the full-length Cas9 gene from the plasmid library
preparation and the nanopore sequencing adapters were ligated as per
standard ONT protocol. Using a single MinION flow cell, a 1X
sequencing depth of the library was performed, which was sufficient
to serve as a QC check and to ensure library diversity. Although the
low sequencing depth and noisy nature of nanopore reads only allowed
reliable identification of 304,060 unique library elements. The read
count distribution suggests that this is an under-sampling of the
library diversity, and the mutation density distribution, *i.e.*, the
number of detected elements with each possible number of mutations

closely matches theoretical expectation, except for a slight oversampling of sequences with small numbers of mutations. In spite of this, the majority of the pre-screen library consists of Cas9 sequences with significant numbers of mutations, with most falling into a broad peak between 6 and 14 mutations per sequence (**Figure 5**).

[00107]   To identify functional variants still capable of editing DNA, a positive screen targeting the hypoxanthine phosphor-ribosyltransferase 1 (HPRT1) gene was designed and tested. In the context of the screen, HPRT1 converts 6-thioguanine (6TG), an analogue of the DNA base guanine, into 6-thioguanine nucleotides that are cytotoxic to cells via incorporation into the DNA during S-phase. Thus, only cells containing functional Cas9 variants capable of disrupting the HPRT1 gene can survive in 6TG (**Figure 4**).

[00108]   To first identify the optimal 6TG concentration, HeLa cells were transduced with lentivirus particles containing wild-type Cas9 and either a HPRT1-targeting guide RNA (gRNA) or a non-targeting guide. After selection with puromycin, cells were treated with 6TG concentrations ranging from 0-14 µg/mL for one week. Cells were stained with crystal violet at the end of the experiment and imaged. 6 µg/mL was selected as all cells containing non-targeting guide had died while cells containing the HPRT1 guide remained viable (**Figure 6**).

[00109]   To perform the screen, HeLa cells were transduced with lentiviral particles containing variant library or wild-type Cas9 along with the HPRT1-targeting gRNA at 0.3 MOI and at greater than 75-fold coverage of the library elements. Cells were selected using puromycin after two days and 6TG was added once cells reached 75% confluency. After two weeks, genomic DNA was extracted from remaining cells and full-length Cas9 sequences were PCR amplified. Nanopore-compatible sequencing libraries were prepared per manufacturer's instructions and sequenced on the MinION platform. This screening procedure was performed in two replicates.

[00110]   After screening, the library was significantly shifted in the mutation density distribution, suggesting that the majority of the library with large (>4) numbers of mutations resulted in non-functional proteins which were unable to survive the screen.

Meanwhile, wild-type, single, and double mutants were generally enriched as these proteins proved more likely to retain functionality and pass through the screen (**FIG. 5**). Additionally, the two independent replicates of the screen showed strong correlation (R^2 = 0.925) providing further evidence of the robustness of the screen (**FIG. 7**).

[00111]   In addition, the overall frequency of mutations in the pre- and post-screen libraries was analyzed to see if a pattern of mutation effects could be inferred. Although the wild-type allele was enriched at every site in the post-screen sequences, nearly every site retained a significant fraction of mutated alleles, suggesting that the mutations, at least individually, are fairly well-tolerated and do not disrupt Cas9 functionality (**FIG. 8**).

[00112]   In order to select hits from the screen for downstream validation and analysis, a method for differentiating high-support hits likely to be real from noise-driven false positive hits was devised. To do this it was hypothesized that the fitness landscape of the screen mutants is likely to be smooth, *i.e.* variants that contain similar mutations are more likely to have similar fitnesses in terms of editing efficiency compared to randomly selected pairs. This was confirmed by computing a predicted screen score for each variant based on a weighted regression of its nearest neighbors in the screen. This metric correlates well with the actual screen scores and approaches the screen scores even more closely as read coverage increases. This provides good evidence that the fitness landscape is indeed somewhat smooth (**FIG. 9**).

[00113]   Next, a network analysis was performed to try to differentiate noise-driven hits from *bona fide* hits by looking at the degree of connectivity with other hits. The rationale here is that because the fitness landscape is smooth, real hits should reside in broad fitness peaks including many neighbors that also show high screen scores, whereas hits that are less supported by near neighbors are more likely to be spurious as they represent non-smooth fitness peaks (**FIG. 10**).

[00114]   To validate and characterize hits from the screen, two independent methods were applied to quantify editing of the de-immunized Cas9 variants. First, a gene-rescue experiment was

performed using low frequency homology directed repair (HDR) to repair a genetically encoded broken green fluorescent protein (GFP) gene. Upon successful editing and co-transfection with a correct donor GFP copy, a fraction of cells will convert to GFP+. Briefly, a HEK293T cell line containing the GFP DNA sequence with the insertion of a stop codon and a genomic fragment of the AAVS1 locus was developed. Due to the stop codon, this line is naturally nonfluorescent. However, GFP expression can be restored via homologous recombination, where the DNA is edited via a guide that targets the AAVS1 locus fragment and repaired with a GFP donor sequence. GFP+ cells can then be quantified by flow-activated cell sorting (FACS), providing information on editing efficiency. Second, editing was quantified by genomic DNA extraction and Illumina next generation sequencing (NGS) using the CRISPResso package.

[00115]   To specifically validate this network, 20 variants (V1-20) were constructed as detailed in **Table A**. A large majority of the variants were capable of editing, providing further confidence in the network we constructed, and in particular highlighted variants V8 and V12 with high editing capability and 8 and 7 mutations respectively (**FIGs. 11 and 12**). Leveraging the unique combinatorial library design and screening strategy, Cas9 variants were generated with multiple top immunogenic epitopes simultaneously de-immunized while retaining editing functionality.

[00116] Table A. De-Immunized Cas Variant Bits (mutational numbering excludes amino acid 1 (Met) with respect to SEQ ID NO:2.

ID	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
V1	'							L61SG										
V2	'								L62?Q									
V3			Y28SQ	S31TH	S36?C						F70?A	L72?G	L81?D	Y101SG	L124?G	I127?Q	L128IA	Y129Q
V4	P2L			S31TC	S36?C	F49TT					F70?A	L72?D		Y101SK		I127?Q	L128IA	Y129Q
V5			Y28SQ	S31TC		F49TT		L61SG		L68?D	F70?A	L72?D		Y101SG	L124?G		L128IA	
V6										L68?D	F70?A	L72?D	L81?D	Y101SK		I127?Q	L128IA	Y129Q
V7	P2L				S36?C		L51ST		L62?Q			L72?G	L81?D		L124?G		L128IA	
V8					S36?C		L61?T		L62?Q			L72?G	L81?D				L128IA	
V9		L23C	Y28SQ	S31TH	S36?C	F49TT	L51ST	L61SG	L62?Q				L81?D		L124?G	I127?Q		
V10					S36?C								L81?D				L128IA	
V11				S31TH			L61?T		L62?Q	L63?D	F70?A	L72?G	L81?D		L124?G	I127?Q	L128IE	Y129Q
V12		L23C							L62?Q	L63?D			L81?D		L124?G	I127?Q	L128IA	
V13				S31TH	S36?C	F49TT	L61?T			L63?D	F70?A		L81?D			I127?Q	L128IE	Y129Q
V14		L23C				F49TT		L61SG						Y101SG			L128IA	Y129Q
V15				S31TC		F49TT						L72?G		Y101SG				Y129Q
V16					S36?C		L51ST		L62?Q	L63?D		L72?D				I127?Q	L128IA	Y129Q
V17									L62?Q					Y101SG				
V18									L62?Q	L63?D	F70?A			Y101SG	L124?G	I127?Q		

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[00117]   *In silico* Screens. To demonstrate the broad applicability of this combinatorial protein engineering platform, top high-priority immunogenic epitopes were identified, similar to the SpCas9 library, for 10 alternative Cas orthologs (*Staphylococcus aureus* Cas9 (Accession Number: J7RUA5.1; SEQ ID NO:51 and 52, polynucleotide and polypeptide, respectively), *Campylobacter jejuni* Cas9 (Accession Number: YP_002344900.1; SEQ ID NO:53), *Staphylococcus auricularis* Cas9 (Accession Number: WP_107392933.1), CasX (Accession Number: OGP07438.1; SEQ ID NO:54), Cas-Phi (Accession Number: 7LYS_A; SEQ ID NO:55), Cas13d (Accession Number: QMT62609.1), *Acidaminococcus* Cas12a (Accession Number: U2UMQ6.1; SEQ ID NO:56), *Pasteurella pneumotropica* Cas9 (Accession Number: WP_018356570.1), *Brevibacillus lacterosporus* Cas9 (Accession Number: WP_003343632.1), *Neisseria meningitidis* Cas9 (Accession Number: WP_002260677.1) focusing on small Cas9s amenable to *in vivo* use via adeno-associated viral vectors (AAVs), and on Cas orthologs which extend the utility of the CRISPR system beyond the Cas9 case, such as the RNA-targeting Cas13d. (**Table B**; all sequences associated with the accession numbers above are incorporated herein by reference). It will be readily apparent to one of skill in the art that additional immuno-silenced Cas protein constructs can be generated using the information above and in Table B and C in combination with the sequence listings accompanying the application.

[00118]   **Table B.** Top immunogenic epitopes across Cas9 orthologs (Mutational reference amino acids exclude position 1 methionine, *e.g.*, P27L excludes methionine at position 1 of SEQ ID NO:2 Sp-Cas9; or alternatively stated, methionine is amino acid "0").

Source protein	AA position (0-based)	Epitope	Mutation
Sp-Cas9	26	VPSKKFKVL	P27L (*e.g.*, P28L of SEQ ID NO:2)
Sp-Cas9	235	GLFGNLIAL	L236C
Sp-Cas9	277	LLAQIGDQY	Y285Q
Sp-Cas9	316	LSASMIKRY	S317H; S317C; Y324D
Sp-Cas9	366	ASQEEFYKF	S367C
Sp-Cas9	489	SFIERMTNF	F497T; F490N

Sp-Cas9	505	KVLPKHSLLYEYFTVY	L513T; L513G
Sp-Cas9	614	ILEDIVLTL	L615G; L622Q
Sp-Cas9	634	RLKTYAHLF	L635D; R634E
Sp-Cas9	695	LIHDDSLTF	F703A; I696D
Sp-Cas9	718	SLHEHIANL	L726P; L726G
Sp-Cas9	814	YLQNGRDMY	L815D
Sp-Cas9	1007	FVYGDYKVY	Y1015K; Y1015G; Y1015E
Sp-Cas9	1236	YLASHYEKL	L1244G
Sp-Cas9	1264	YLDEIIEQISEFSKRVIL	I1272Q; L1281A; L1281E
Sp-Cas9	1285	NLDKVLSAY	Y1293Q; L1286K
Sp-Cas9	11	GTNSVGWAV	T12N
Sp-Cas9	65	RTARRYTR	T66R
Sp-Cas9	155	LALAHMIKFRGHFL	M160P
Sp-Cas9	441	KILTFRIPYYV	T444E
Sp-Cas9	805	LQNEKLYLY	Q806R
Sp-Cas9	877	KMKNYWRQL	M878D
Sp-Cas9	1030	KATAKYFFYSNIMNFFK	Y1038E
Sp-Cas9	1086	LSMPQVNIV	V1094S
Sp-Cas9	1137	TVAYSVLVV	V1138K; V1145E
Sp-Cas9	1211	RMLASAGEL	L1219N
Sa-Cas9	4	YILGLDIGI	I12G; I12N (*e.g.*, I13G of SEQ 52)
Sa-Cas9	68	KLLFDYNLL	L76S; L76Q
Sa-Cas9	104	FSAALLHLA	A112N
Sa-Cas9	230	EMLMGHCTYF	Y238W
Sa-Cas9	257	ALNDLNNLV	V265S
Sa-Cas9	349	QIAKILTIY	Y357N
Sa-Cas9	388	YTGTHNLSLKAINLI	L396T; L396G
Sa-Cas9	408	HTNDNQIAIFNRL	T409G; I416Q
Sa-Cas9	455	QSIKVINAI	S456G

Sa-Cas9	556	HIIPRSVSFDNSF	F564A
Sa-Cas9	650	YATRGLMNL	L658T
Sa-Cas9	660	RSYFRVNNL	S661D
Sa-Cas9	674	SINGGFTSF	F682K; F682G
Sa-Cas9	706	IIANADFIFK	I707C
Sa-Cas9	735	KQAESMPEI	I743Q
Sa-Cas9	800	IVNNLNGLY	Y808T
Sa-Cas9	916	YLDNGVYKF	F924E; F924G
Sa-Cas9	957	NQAEFIASF	Q958K
Sa-Cas9	972	KINGELYRV	V980Q
Sa-Cas9	992	EVNMIDITY	Y1000E
Sa-Cas9	1014	RIIKTIASKTQSI	K1022C
As-Cas12a	1	TQFEGFTNL	-
As-Cas12a	159	RSFDKFTTYFSGF	-
As-Cas12a	209	RLITAVPSL	-
As-Cas12a	234	TSIEEVFSFPFYNQL	-
As-Cas12a	356	NSIDLTHIF	-
As-Cas12a	472	SLLGLYHLL	-
As-Cas12a	556	FVKNGLYYL	-
As-Cas12a	678	FTRDFLSKY	-
As-Cas12a	716	LLYHISFQR	-
As-Cas12a	760	HTLYWTGLF	-
As-Cas12a	793	RMKRMAHRL	-
As-Cas12a	929	RSLNTIQQF	-
As-Cas12a	970	YLSQVIHEI	-
As-Cas12a	981	LMIHYQAVVV	-
As-Cas12a	1017	MLIDKLNCL	-
As-Cas12a	1034	KVGGVLNPY	-
As-Cas12a	1055	GTQSGFLFYVPAPY	-

As-Cas12a	1131	FMPAWDIVF	-
As-Cas12a	1209	HAIDTMVALIRSVL	-
As-Cas12a	1292	ISNQDWLAY	-
Blat-Cas9	0	MAYTMGIDVGI	-
Blat-Cas9	191	LLVEIHTLF	-
Blat-Cas9	236	KMIGTCTFL	-
Blat-Cas9	252	KASWHFQYF	-
Blat-Cas9	261	MLLQTINHI	-
Blat-Cas9	343	KLNKIFNEV	-
Blat-Cas9	366	TVAYALTFFK	-
Blat-Cas9	404	YTNELIGKV	-
Blat-Cas9	426	KALRKIIPFL	-
Blat-Cas9	439	MTYDKACQA	-
Blat-Cas9	583	HIIPYSRSM	-
Blat-Cas9	669	YITKYLSHFISTNLEF	-
Blat-Cas9	722	AMDAIVIAV	-
Blat-Cas9	735	FIQQVTNYYK	-
Blat-Cas9	831	ITAKKTALVDISY	-
Blat-Cas9	900	RIMENKTLV	-
Blat-Cas9	917	VVYNSSIVR	-
Blat-Cas9	976	SLYPNDLIF	-
Blat-Cas9	1009	EVQEIHAYY	-
Blat-Cas9	1023	STAAIEFIIHDGSYYA	-
Cas13d	5	KSFAKGMGV	-
Cas13d	72	FSHPKGYAVVANNPLY	-
Cas13d	135	YITNAAYAVNNISGL	-
Cas13d	160	STVYTYDEF	-
Cas13d	267	YISTLNYLY	-
Cas13d	278	ITNELTNSF	-

Cas13d	357	KVYTMMDFVIYRY	-
Cas13d	397	FVINLRGSF	-
Cas13d	461	VSAFSKLMYALTMFL	-
Cas13d	495	QSFLKVMPL	-
Cas13d	528	RLIKSFARM	-
Cas13d	553	RILGTNLSY	-
Cas13d	565	KALADTFSL	-
Cas13d	596	ISNKRFHYL	-
Cas13d	702	KIISLYLTVIY	-
Cas13d	798	KLYANYIKY	-
Cas13d	864	EVARYVHAY	-
Cas13d	878	EVNSYFQLYHYI	-
Cas13d	905	KVSEYFDAV	-
Cas13d	925	KLLCVPFGY	-
Cas-Phi	79	KASEAIQRYIYAL	-
Cas-Phi	124	HVQGLNLIFDHTLGR	-
Cas-Phi	180	ATNETGHLL	-
Cas-Phi	192	GINPSFYVY	-
Cas-Phi	201	QTISPQAYR	-
Cas-Phi	223	YVRDPNAPI	-
Cas-Phi	269	VTVPGLSPK	-
Cas-Phi	330	SLNALLDLF	-
Cas-Phi	349	NIVTFTYTLDAC	-
Cas-Phi	361	GTYARKWTL	-
Cas-Phi	381	LTATQTVAL	-
Cas-Phi	396	QTNPISAGI	-
Cas-Phi	428	LLKDISAYR	-
Cas-Phi	492	KMSSNTTFISEALL	-
Cas-Phi	513	QVFFTPAPK	-

Cas-Phi	541	RAYKPRLSV	-
Cas-Phi	564	RTSPEYLKL	-
Cas-Phi	582	RSINYVIEK	-
Cas-Phi	595	TQCQIVIPVIEDL	-
Cas-Phi	647	RTHRSFYVFEVRPER	-
CasX	66	MLLDDYTKMKEAILQV	-
CasX	199	VTKESTHPV	-
CasX	233	GTIASFLSKY	-
CasX	284	HTKEGVDAY	-
CasX	300	RMWVNLNLW	-
CasX	361	RVFWSGVTA	-
CasX	374	TILEGYNYL	-
CasX	404	RQFGDLLLY	-
CasX	435	KIAGLTSHI	-
CasX	525	IQYRNLLAW	-
CasX	541	REFYLLMNY	-
CasX	578	KAKVIDLTF	-
CasX	608	FIWNDLLSL	-
CasX	759	VTHDAVLVF	-
CasX	780	RTFMTERQY	-
CasX	809	YLSKTLAQYTSK	-
CasX	863	ITYYNRYKR	-
CasX	939	LNIARSWLF	-
CasX	947	FLNSNSTEF	-
CasX	965	FVGAWQAFY	-
Cj-Cas9	2	RILAFDIGISSIGWAF	-
Cj-Cas9	63	RLNHLKHLI	-
Cj-Cas9	209	KQREFGFSFSK	-
Cj-Cas9	224	EVLSVAFYK	-

Cj-Cas9	234	ALKDFSHLVGNCSFF	-
Cj-Cas9	262	FMFVALTRIINLLNNL	-
Cj-Cas9	325	GTYFIEFKK	-
Cj-Cas9	398	KALKLVTPL	-
Cj-Cas9	471	KVHKINIEL	-
Cj-Cas9	552	KMLEIDHIYPYSRSF	-
Cj-Cas9	569	SYMNKVLVFT	-
Cj-Cas9	641	YIARLVLNYTKDYLDFL	-
Cj-Cas9	685	LTSALRHTWGF	-
Cj-Cas9	712	IIAYANNSI	-
Cj-Cas9	720	IVKAFSDFK	-
Cj-Cas9	849	YTMDFALKV	-
Cj-Cas9	875	ILMDENYEF	-
Cj-Cas9	911	FTSSTVSLI	-
Cj-Cas9	949	KSIGIQNLK	-
Cj-Cas9	962	YIVSALGEV	-
Nme2-Cas9	0	MAAFKPNPI	-
Nme2-Cas9	17	GIASVGWAM	-
Nme2-Cas9	57	LAMARRLAR	-
Nme2-Cas9	126	WSAVLLHLIKHRGYL	-
Nme2-Cas9	207	LQAELILLF	-
Nme2-Cas9	237	LLMTQRPAL	-
Nme2-Cas9	271	YTAERFIWL	-
Nme2-Cas9	347	MEMKAYHAI	-
Nme2-Cas9	414	ISFDKFVQI	-
Nme2-Cas9	425	KALRRIVPL	-
Nme2-Cas9	583	VEIDHALPFSRTW	-
Nme2-Cas9	671	YVNRFLCQFVADHILLT	-
Nme2-Cas9	693	RVFASNGQI	-

Nme2-Cas9	703	NLLRGFWGL	-
Nme2-Cas9	728	VVACSTVAMQQKITRFV	-
Nme2-Cas9	778	FAQEVMIRV	-
Nme2-Cas9	819	AVHEYVTPLFVSR	-
Nme2-Cas9	965	NQYFIVPIYAWQVAENI	-
Nme2-Cas9	996	YTFCFSLHK	-
Nme2-Cas9	1015	KSKVEFAYY	-
Pp-Cas9	0	MQNNPLNYI	-
Pp-Cas9	14	GIASIGWAV	-
Pp-Cas9	186	GSYTHTFSR	-
Pp-Cas9	195	LDLLAEMELLF	-
Pp-Cas9	219	TLLENLTALLMWQKPAL	-
Pp-Cas9	261	YSAERFVWL	-
Pp-Cas9	304	LTYAQVRAM	-
Pp-Cas9	313	LALSDNAIF	-
Pp-Cas9	419	KALHQILPL	-
Pp-Cas9	479	KVINAVVRLYGSPARI	-
Pp-Cas9	546	ILKMRLYEL	-
Pp-Cas9	631	RVQTSGFSYAK	-
Pp-Cas9	665	YVARFLCNFIADNMLLV	-
Pp-Cas9	697	ALLRHRWGL	-
Pp-Cas9	722	VVACSTVAM	-
Pp-Cas9	829	RLNEGLSVL	-
Pp-Cas9	878	KAFAEPFYK	-
Pp-Cas9	931	YFLVPIYTW	-
Pp-Cas9	965	EMATFQFSL	-
Pp-Cas9	987	TIFGYFNGL	-
Sauri-Cas9	78	YQMIDLNNV	-
Sauri-Cas9	96	RVKGLREPL	-

Sauri-Cas9	109	FAIALLHIA	-
Sauri-Cas9	121	GLHNISVSM	-
Sauri-Cas9	239	KLMGRCTYF	-
Sauri-Cas9	258	YSADLFNAL	-
Sauri-Cas9	403	SLKCIHIVI	-
Sauri-Cas9	420	NQMEIFTRL	-
Sauri-Cas9	467	VINAVINRF	-
Sauri-Cas9	554	LSNPTHYEV	-
Sauri-Cas9	564	HIIPRSVSFDNSL	-
Sauri-Cas9	668	KTYFSTHDY	-
Sauri-Cas9	713	LVIANADFLFKTHKAL	-
Sauri-Cas9	839	KTFEKLMTILNQY	-
Sauri-Cas9	914	KSFRFDIYK	-
Sauri-Cas9	964	DLFVGSFYY	-
Sauri-Cas9	975	LIMYEDELF	-
Sauri-Cas9	1003	ITYKDFCEV	-
Sauri-Cas9	1022	KTIGKRVVL	-
Sauri-Cas9	1034	YTTDILGNL	-

[00119]   To extend and complement the Cas9 screening efforts, the effect of the combinations of mutations on editing functionality, were recapitulated through *in silico* structural analyses using state-of-the-art protein structure prediction software: Google Deepmind's Alphafold. Through predicting the structures of high-confidence double mutants within the screen, we noticed a positive correlation between the position-specific local structure and confidence metric pLDDT, and the epistatic permissiveness of mutations within the screen (**FIG. 13**). This is to say that while pLDDT does not correlate with the effects of a mutation on editing functionality, it does correlate with how well combinations of mutations edit in comparison to the expected editing based on a null, additive model of epistasis. Mutation combinations with high

epistatic scores, *i.e.* mutants with better editing than expected based on the effects of the individual mutations tend to have high pLDDT scores, while double mutants with negative epistasis rarely have high pLDDT scores. To validate this observation, the Alphafold workflow was used to assess this effect in third-party datasets across a variety of proteins (**FIG. 13**).

[00120]   Using this pLDDT - epistasis connection, it is possible to subset de-immunizing mutations to exclude those which have a low likelihood of being epistatically permissive. This is of critical importance to moving further down the spectrum of de-immunization into combinations of mutations disrupting multiple epitopes, as would be needed to circumvent the immune response to Cas9 in a clinical context. Towards this end, single mutants were identified among several clinically-relevant Cas9 orthologs which are less likely to produce negative epistatic effects that will substantially reduce editing efficiency when combined with mutations across other epitopes **(Table C)**. These mutations may contribute to de-immunized versions of these Cas9 orthologs upon further development.

[00121]   **Table C.** High pLDDT mutations predicted to be epistatically permissive: engineered de-immunized Cas9s would bear combinations of these. pLDDT measures are standardized across proteins. (Amino acid position is based upon methionine being position "0").

Source protein	Mutation	pLDDT
Sp-Cas9	S14T	97.21972
Sp-Cas9	L34N	96.29946
Sp-Cas9	A156G	97.70565
Sp-Cas9	I161T	96.70064
Sp-Cas9	L240C	95.5989
Sp-Cas9	F374Q	95.1961
Sp-Cas9	T444E	96.71938
Sp-Cas9	L513D	96.8859
Sp-Cas9	L615N	95.7314
Sp-Cas9	F703T	95.70707

Sp-Cas9	Y813E	96.28403
Sp-Cas9	L815K	95.2872
Sp-Cas9	K877N	96.38619
Sp-Cas9	V1094T	97.93581
Sp-Cas9	V1138F	95.65631
Sp-Cas9	R1211G	97.46792
Sp-Cas9	L1265D	95.5318
Sp-Cas9	I1272N	95.7981
Sp-Cas9	Y1293H	95.3305
Sa-Cas9	I10E	96.04671618970484
Sa-Cas9	L69G	96.4474038803064
Sa-Cas9	L111G	97.5246901174282
Sa-Cas9	Y238D	95.7959234150505
Sa-Cas9	L261Q	95.36556536646381
Sa-Cas9	I353R	97.05582266121893
Sa-Cas9	H392V	96.40816573690797
Sa-Cas9	N410Y	97.39004196840122
Sa-Cas9	I457G	96.99516367772516
Sa-Cas9	F564R	96.04196929055283
Sa-Cas9	Y650G	95.33378157800597
Sa-Cas9	F679H	95.6997935920167
Sa-Cas9	I706E	96.21671919911144
Sa-Cas9	P741Q	96.76471819653675
Sa-Cas9	N803K	95.70463233624467
Sa-Cas9	K923I	95.81887787225756
Sa-Cas9	F924G	96.3942586623205
Sa-Cas9	S964C	97.46403708042462
Sa-Cas9	N974F	96.28983910214497
Sa-Cas9	I1016Y	96.88397168045735

Cj-Cas9	A5C	95.42456861125262
Cj-Cas9	L70F	95.96944171410574
Cj-Cas9	Q210P	95.53016253543593
Cj-Cas9	L235T	95.59476304574478
Cj-Cas9	R269D	95.57444080957478
Cj-Cas9	E330N	95.46899258457661
Cj-Cas9	L400H	97.57050152845376
Cj-Cas9	E478V	95.969187864883
Cj-Cas9	E555T	97.12260803185546
Cj-Cas9	V576W	96.58282786835804
Cj-Cas9	A643L	98.33207173472285
Cj-Cas9	R690V	95.74871521269799
Cj-Cas9	S719N	97.95496295071534
Cj-Cas9	F724S	96.22792344876726
Cj-Cas9	T850N	95.41876846442275
Cj-Cas9	Y881H	96.86255346228734
Cj-Cas9	S917Q	96.2179175861716
Cj-Cas9	Q954D	97.52472596837454
Cj-Cas9	V970H	97.50358598810789
Cas-Phi	Q85Y	96.81295319454516
Cas-Phi	V125L	95.12108676130539
Cas-Phi	T184R	95.1772971119881
Cas-Phi	G192K	96.31102265697412
Cas-Phi	R209V	95.17061366434854
Cas-Phi	D226G	96.73279555432283
Cas-Phi	F338S	96.4409845122545
Cas-Phi	T356A	98.46569344377559
Cas-Phi	A364Q	96.9761260500557
Cas-Phi	A388I	96.4379215589604

Cas-Phi	S401V	95.34453659826832
Cas-Phi	R436L	96.35999339995325
Cas-Phi	N496L	95.26296308688346
Cas-Phi	P518L	95.59765365564213
Cas-Phi	L547S	95.62780934854082
Cas-Phi	T565D	96.87703607854039
Cas-Phi	K590V	96.03570468291413
Cas-Phi	F655E	95.63672115804113
Cas-X	M66V	96.66213349474216
Cas-X	E202Q	96.24421703611794
Cas-X	T234G	95.41585619353467
Cas-X	K286F	97.0668726663819
Cas-X	R300D	97.2909813029547
Cas-X	V362F	96.10786479141233
Cas-X	L411H	96.34639451882234
Cas-X	A437P	96.2646137070106
Cas-X	Q526K	96.16734163961726
Cas-X	E542R	95.86976977015941
Cas-X	S615Q	95.14185165348067
Cas-X	L765C	95.79820047448582
Cas-X	R786P	96.47451095139678
Cas-X	Y817I	97.2015805474868
Cas-X	W945N	97.64293477689652
Cas-X	F955T	95.70627543561199
Cas-X	G967K	97.79349327320138
AsCas12a (Cpf1)	N8W	95.92802445100253
AsCas12a (Cpf1)	L210K	95.10630901001409
AsCas12a (Cpf1)	S235Y	96.11142315459921
AsCas12a (Cpf1)	I363T	95.60480914360495

AsCas12a (Cpf1)	G475N	95.86498924015017
AsCas12a (Cpf1)	F556I	96.03740222311919
AsCas12a (Cpf1)	R680Y	95.47851908471684
AsCas12a (Cpf1)	K795H	96.45630801435865
AsCas12a (Cpf1)	Q936R	95.82214160865102
AsCas12a (Cpf1)	I978T	96.48655903840778
AsCas12a (Cpf1)	V988A	96.70259243879435
AsCas12a (Cpf1)	C1024A	97.018068389001
AsCas12a (Cpf1)	Y1042D	96.87401194635731
AsCas12a (Cpf1)	V1215T	95.68310287241839
AsCas12a (Cpf1)	N1294S	96.90764727328744

[00122]   **Inserting T-regitopes.** A further de-immunization approach beyond the mutation or deletion of MHC-binding cores is to inhibit immune activation at the level of cell signaling. Paramount in facilitating this process is the induction of inhibitory T-reg cells which modulate the activity of other T-cells to promote tolerance of foreign antigens. One canonical pathway in which T-reg induction helps to facilitate tolerance of antigenic diversity is in the potential adaptive response to antibodies themselves. As antibodies are highly polymorphic and undergo substantial mutational remodeling during the process of B-cell activation and maturation. As a result, it might be expected that these neo-antigens may create an adaptive immune response. However, this potential problem is mitigated by certain sequences within the conserved regions of immunoglobulins, termed T-regitopes, which are recognized specifically by regulatory T-cells to promote a tolerogenic response to proteins bearing these T-regitopes. Correspondingly these immune-modulating sequences can be utilized in the context of foreign protein therapeutics to dampen or avoid a problematic immune response.

[00123]   Additionally, a multifaceted approach was used to de-immunizing the AAV capsid itself. Applying similar methodological principles as above, an alignment of the major AAV serotypes was constructed, identifying 21 conserved regions across serotypes which

may constitute cross-reacting T-cell epitopes. By definition, these regions will be the most conserved across AAV serotypes, and presumably the least mutation tolerant. This presents a unique challenge to engineering efforts and predicts that the vast majority of mutants in these regions will be strongly deleterious.

[00124]   To narrow down the space of possible mutations, and to increase the probability of including functional mutations in the library, a large alignment of over 200 AAV variants was also constructed which were sequenced from natural or engineered sources. This larger alignment contains many SNPs which may constitute some of the natural variation among AAVs, some of which occur in the highly conserved regions as identified. As these mutations have been observed to occur in a natural and presumably functional context, they are much more likely to serve as useful library members (**Table D**).

[00125]   **Table D**: Immunogenic and conserved AAV-VP1 epitopes and de-immunizing mutations (methionine is position "0")

Source protein	AA position (0-based)	Epitope	Mutation
AAV5-VP1	41	ARGLVLPGY	P47L; R42G; Y49C
AAV5-VP1	51	YLGPGNGLD	G55S; D59Y; G57S; N56H
AAV5-VP1	84	AGDNPYLKYNHADAEFQE	L90P; Y89H; E98K; A95V; D96G; F99L
AAV5-VP1	106	DTSFGGNLG	T107A; S108P
AAV5-VP1	116	AVFQAKKRVLEP	Q119R; V124A; R123L
AAV5-VP1	129	GLVEEGAKTAPTGK	E133Q; G134V; T137A; V131A; E132G
AAV5-VP1	206	QGADGVGNASGDWHCDSTWMGDRV	A214V; T223A; S222T
AAV5-VP1	263	YFGYSTPWGYFDFNRFH	S267A; Y272H; F273L
AAV5-VP1	283	SPRDWQRLINN	W287R; I291V; L290P
AAV5-VP1	303	RVKIFNIQVKEVT	K312E; I309V
AAV5-VP1	320	TTTIANNLTSTVQVFTD	T328A
AAV5-VP1	395	MLRTGNNFEF	N400D; F402L

AAV5-VP1	408	FEEVPFHSS	S416M; F413L; S415T
AAV5-VP1	599	RDVYLQGPIWAKIP	Q604R; I607T; P606Q
AAV5-VP1	624	MGGFGLKHPPP	L629P; K630E; F627L; H631R
AAV5-VP1	657	SFITQYSTGQV	T664A; S663G
AAV5-VP1	677	KENSKRWNPE	N684D; R682C; W683R
AAV5-VP1	713	RPIGTRYLTR	T717S; Y719F; L720P

[00126]   Given the substantially more challenging prospect of engineering multiple mutations simultaneously in a highly conserved structural protein such as a viral capsid, an orthogonal approach was taken to identifying a potentially immune orthogonal AAV capsid by exploring the utility of highly divergent natural orthologs from various mammalian species which have not yet been thoroughly tested for use as *in vivo* vectors. On this front, 687 AAV capsid sequences were scraped from publicly available sequencing data, filtering this set down to 224 capsids by removing truncated and redundant sequences. From there the list of potential AAVs was narrowed by removing sequences within the main clade of human AAVs that are unlikely to boast novel structural properties (104), removing sequences from non-mammalian hosts to maximize the potential of identifying an AAV with strong transduction potential in the human setting (31), and finally condensing similar sequences to a final set of 23 natural AAV orthologs which we are in the process of constructing and testing for viral formation and transduction efficiency (**FIG. 14**).

[00127]   The capsid sequences of each ortholog were chemically synthesized and split into two blocks from the 5' and 3' end. In order to assemble the two blocks from each ortholog, a process similar to the one used for assembling the Cas9 variants was done. The first step involves the annealing-extension step followed by the addition of primers to amplify the full length ortholog. Then the full length capsid sequences (see, *e.g.*, SEQ ID NO:2-48, which include both polynucleotide coding sequences and polypeptide sequence) are then inserted and cloned into a pAAV RC2 vector via Gibson assembly.

[00128]   To test for viral formation, a triple transfection method was used for AAV production using HEK 293T cells and purified with an iodixanol gradient. Cells were transfected once the confluence reached between 70% and 90% in a 5x15 cm^2 plate. Plates were transfected with 10µg of the plasmid with full length inserted ortholog capsid sequence or pxR-5 used as a control, 10µg of transfer vector, and 10µg of pAd4 helper vector. The virus was collected after 72 hours and purified using an iodixanol-density-gradient ultracentrifugation method. After dialysis and filtration, the virus was quantified by qPCR. Results of viral formation were analyzed by comparing titers of the AAV orthologs to AAV5 titers used as a control. The viral formation of 14 AAV orthologs were tested and have demonstrated 8 AAV orthologs successfully packaging and producing virus (**FIG. 15**).

[00129]   It will be understood that various modifications may be made without departing from the spirit and scope of this disclosure. Accordingly, other embodiments are within the scope of the following claims.

WHAT IS CLAIMED IS:

1.   A method for engineering a protein or virus to be less immunoreactive or to be immuno-silent, comprising:

identifying target regions of the polynucleotide sequence encoding a protein or virus that are predicted to have human leukocyte antigen (HLA)-binding and/or peptide immunogenicity;

identifying single nucleotide polymorphisms (SNPs) or mutations in the targeted region and other regions that are not deleterious to the functioning of the protein to obtain mutational criteria;

screening a library assembled using standard synthesis and assembly methods by applying the above identifying criteria to find one or more functional variants of the protein or virus;

sequencing the one or more functional variants of the protein or virus;

mapping genotype to phenotype from the sequences of the functional variants to identify variant candidates that are likely functionally active and have mutations that result in the protein or virus exhibiting less immunogenicity or are immune-silent.

2.   The method of claim 1, wherein the protein is a CRISPR associated protein.

3.   The method of claim 1, wherein the CRISPR associated protein is a Cas9.

4.   The method of claim 3, wherein the Cas9 is *Streptococcus pyogenes* Cas9 (SpCas9).

5.   The method of any one of claims 1 to 4, wherein the target regions are identified using a model that predicts human leukocyte antigen (HLA)-binding and peptide immunogenicity.

6.   The method of claim 5, wherein the prediction model is selected from NetMHC, MHCAttnNet, MHCSeqNET, ACME, NetMHCpan EL 4.1,

NetMHCstabpan, SMM, SMMPMBEC, PickPocket, Comblib_Sidney2008, NetMHCcons, MHCflurry 2.0, and IConMHC.

7. The method of any one of claims 4 to 6, wherein the SNPs or mutations are identified by using phylogenetic methods to scan natural variation among naturally occurring SpCas9, mutations generated in the course of research and engineering efforts, and the Cas9 orthologs of closely related bacterial species.

8. The method of any one of claims 4 to 7, wherein the SNPS or mutations are identified, or further identified, by using immunological prediction of candidate mutations to ensure significant loss of immunogenicity within the targeted region in order to preserve function while reducing immunogenicity.

9. The method of claim 1, wherein the virus is an adeno-associated virus (AAV).

10. The method of claim 9, wherein the AAV is selected from AAV1, AAV2, AAV5, AAV6, AAV7, and AAV8.

11. The method of claim 10, wherein the AAV is AAV5.

12. The method of any one of claims 9 to 11, wherein the target regions are identified by aligning conserved sequence regions across AAV serotypes.

13. The method of claim 12, wherein the SNPs or mutations are identified by aligning the sequences of 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200 or more AAV variants that have been sequenced from natural or engineered sources.

14. The method of claim 13, wherein the SNPs or mutations are located in the target regions.

15.   The method of any one of the preceding claims, wherein long-
read sequencing technologies capable of sequencing the entire
sequence of each protein or viral variant in one sequencing reaction
is used to sequence the functional variants of the protein or virus.

16.   The method of claim 15, wherein the long-read sequencing
technologies is capable of generating 10-15 Gb of sequencing reads
per run.

17.   The method of any one of the preceding claims, wherein the
method further comprises:
      evaluating the immunoreactivity of variant candidates in one
or more immunoassays.

18.   The method of claim 17, wherein the one or more immunoassays
comprise detecting the presence of antibodies to the variant
candidates (AVA antibodies), when the variant candidates are
administered *in vivo* to an animal.

19.   The method of claim 17 or claim 18, wherein the one or more
immunoassays comprise an enzyme-linked immunosorbent assays
(ELISAs), electrochemiluminescence (ECL) assays and/or antigen-
binding tests, wherein the one or more immunoassays utilize AVA
antibodies.

20.   An isolated polynucleotide having at least 80%, 90%, 95%, 97%,
98%, or 99% sequence identity to SEQ ID NO:1, and encoding a
polypeptide of SEQ ID NO:2 having one or more mutations selected
from the group consisting of: P28L, L237C, Y286Q, S318(H/C), S368C,
F498T, L514(T/G), L616G, L623Q, L636D, F704A, L727(P/G), L816D,
Y1016(K/G), L1245G, I1273Q, L1282(A/E), and/or Y1294Q and wherein
the polypeptide has Cas9 like activity.

21.   An isolated polypeptide having a sequence that has at least
90%, 95%, 97%, 98%, or 99% sequence identity to the sequence
presented in SEQ ID NO:2, wherein the protein has Cas9 like activity
and is immuno-silenced.

22.   The isolated polypeptide of claim 21, wherein the protein has less immunogenicity than wild-type Cas9 of SEQ ID NO:2.

23.   The protein of claim 21, wherein the polypeptide comprises the sequence of SEQ ID NO:2 and having one or more mutations selected from the group consisting of: P28L, L237C, Y286Q, S318(H/C), S368C, F498T, L514(T/G), L616G, L623Q, L636D, F704A, L727(P/G), L816D, Y1016(K/G), L1245G, I1273Q, L1282(A/E), and/or Y1294Q.

24.   A CRISPR-Cas9 system comprising the protein of any one of claims 20 to 23.

25.   The CRISPR-Cas9 system of claim 24, wherein the CRISPR-Cas9 system further comprises RNA that comprise a shot sequence that binds to a specific target sequence of DNA in a genome.

26.   A virus encoded by a polynucleotide sequence having at least 90%, 95%, 97%, 98%, or 99% sequence identity to the sequence of SEQ ID NO:49, wherein the virus has AAV5 like activity and is immuno-silenced.

27.   A AAV viral capsid having at least 90%, 95%, 97%, 98%, or 99% sequence identity to the sequence presented in SEQ ID NO:50, wherein the virus has AAV5 like activity and is immuno-silenced.

28.   The AAV capsid of claim 27, wherein the viral capsid comprises a sequence of SEQ ID NO:50 and comprises two or more mutation selected from the group consisting of R42G, P47L, Y49C, G55S, N56H, G57S, D59Y, Y89H, L90(P/I), A95V, D96G, E98(K/Q), F99L, T107A, S108P, Q119(R/E), R123(L/T), V124A, V131A, E132(G/R), E133(Q/D), G134(V/S), T137A, A214V, S222T, T223(A/K), S267A, Y272H, F273L, W287R, L290P, I291V, I309V, K312(E/R), N400D, F402L, F413L, S415T, S416(M/G), Q604R, P606Q, I607T, F627L, L629(P/F), K630E, H631(R/N), S663G, T664A, R682C, W683R, N684(D/S), T717(S/A), Y719F, and L720P.

29.   The virus of any one of claims 26 to 28, wherein the virus has less immunogenicity than a wild-type AAV5.

30.   An AAV system comprising the virus of any one of claims 26 to 29.

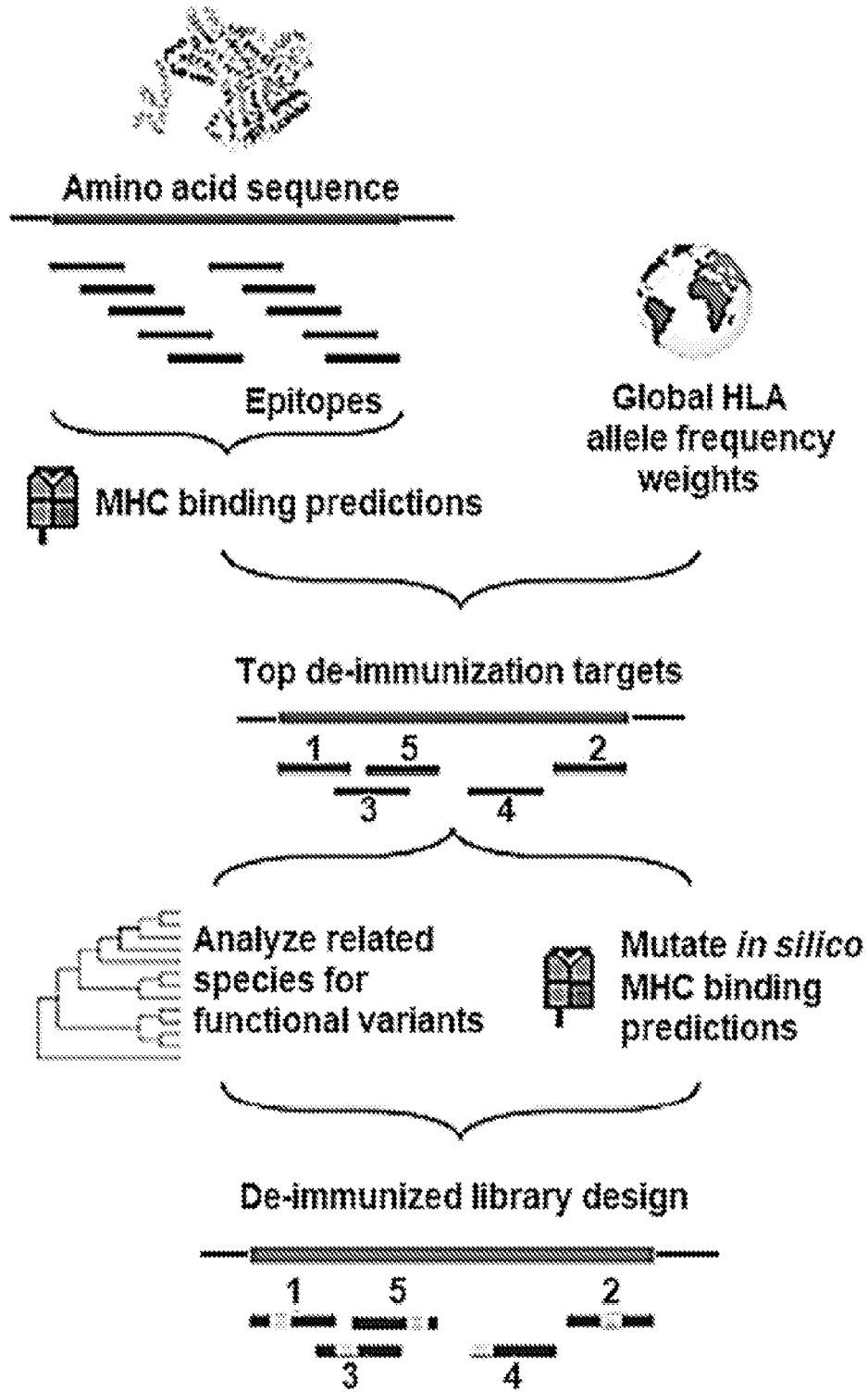31.   The AAV system of claim 30, wherein the AAV system is used for gene therapy.

FIG. 1A

Peptide Locations (Cas9)				Candidate mutations (methionine is position "0")								
start	end	L	sequence	wt	pos	alt1	alt2	wt	cc alt 1	cc alt 2	DNA pos	Block #
26	35	9	VPSKKFKVL	P	27	L			CCG	CTC		82	1
235	244	9	GLFGNLIAL	L	236	C			CTG	TGC		709	2
277	286	9	LLAQIGDQY	Y	285	Q			TAC	CAG		856	2
316	325	9	LSASMIKRY	S	317	H	C		AGC	CAT	TGT	952	2
366	375	9	ASQEEFYKF	S	367	C			AGC	TGT		1102	3
489	498	9	SFIERMTNF	F	497	T			TTT	ACC		1492	3
505	521	16	KVLPKHSLLYEYFTVY	L	513	T	G		CTG	ACC	GGA	1540	3
614	623	9	ILEDIVLTL	L	622	Q			CTT	CAG		1867	4
634	643	9	RLKTYAHLF	L	635	D			TTG	GAC		1906	4
695	704	9	LIHDDSLTF	F	703	A			TTT	GCC		2110	5
718	727	9	SLHEHIANL	L	726	P	G		CTT	CCC	GGA	2179	5
814	823	9	YLQNGRDMY	L	815	D			CTG	GAC		2446	6
1007	1016	9	FVYGDYKVY	Y	1015	K	G		TAC	AAG	GGA	3046	7
1236	1245	9	YLASHYEKL	L	1244	G			CTC	GGA		3733	7
1264	1282	18	YLDEIIEQISEFSKRVIL	I	1272	Q			ATA	CAG		3817	8
				L	1281	A	E		CTC	GCT	GAG	3844	8
1285	1294	9	NLDKVLSAY	Y	1293	Q			TAC	CAG		3880	8

start	end	L	blocks	mutations	# of Constructs
0	680	680	1	1	2
650	1030	380	2	3	12
1000	1630	630	3	3	12
1600	1990	390	4	2	4
1960	2400	440	5	2	6
2370	3000	630	6	1	2
2970	3790	820	7	2	6
3760	4140	380	8	3	12
				total # of constructs	56
				Library size	995328

FIG. 1B

FIG. 2

FIG. 3

**Cas9 variant library
gRNA targetign HPRT1**

Lentivirus library

**Functional
Cas9**

6TG

HPRT1

Apoptosis

**Cell culture
6TG select one week**

**Extract genomic DNA
from viable cells**

**Nanopore sequence
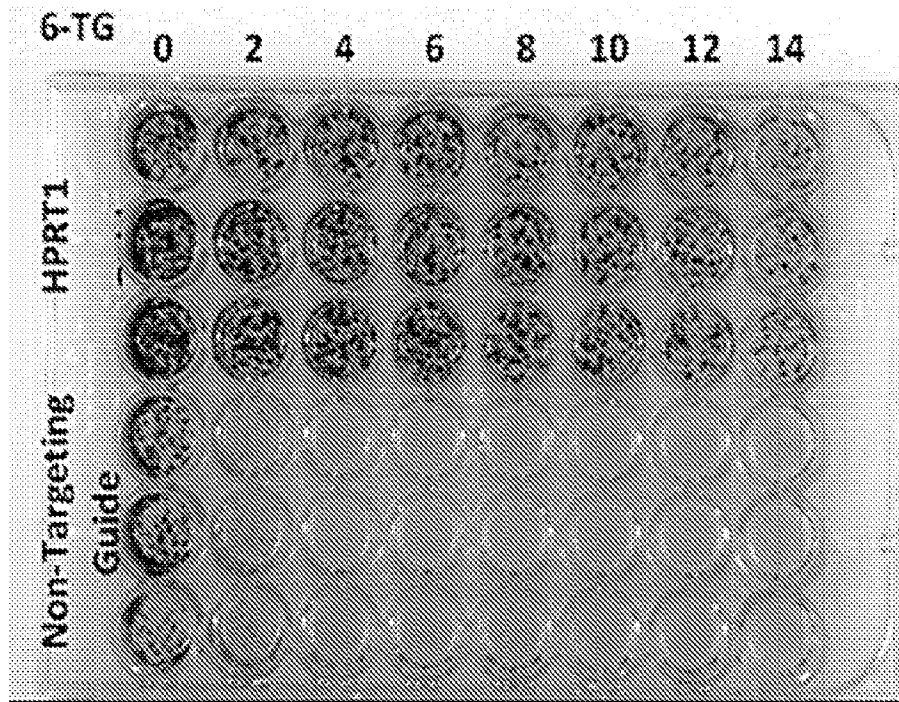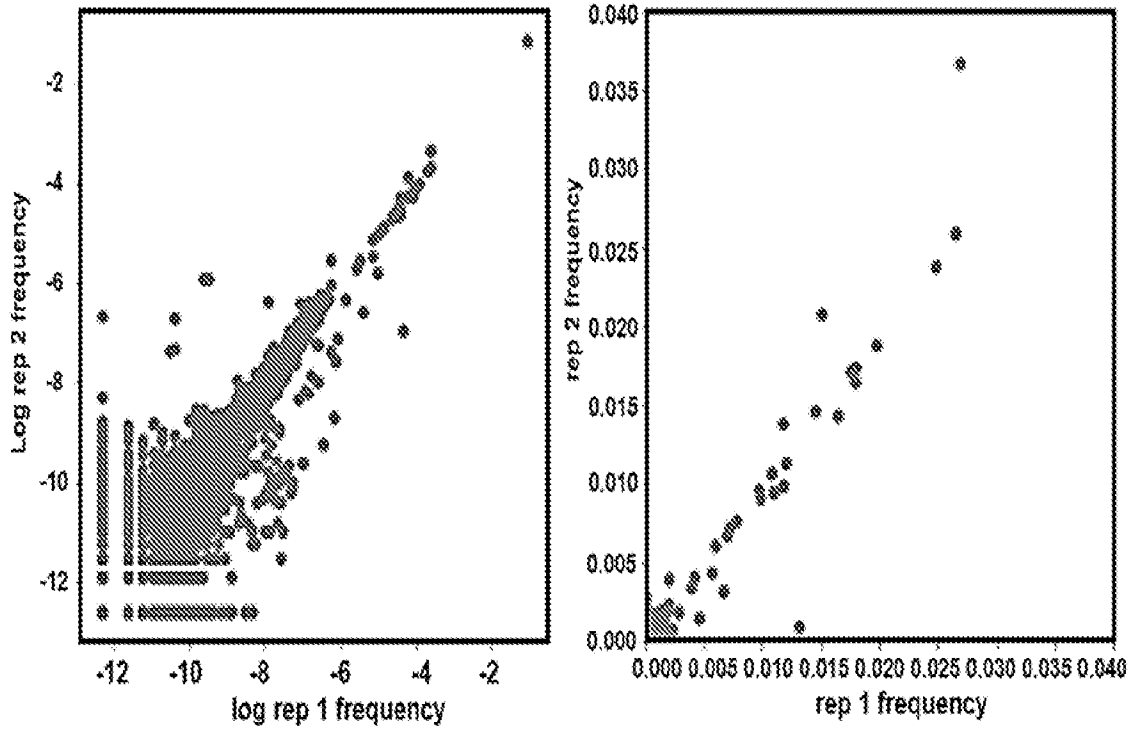to identify hits**
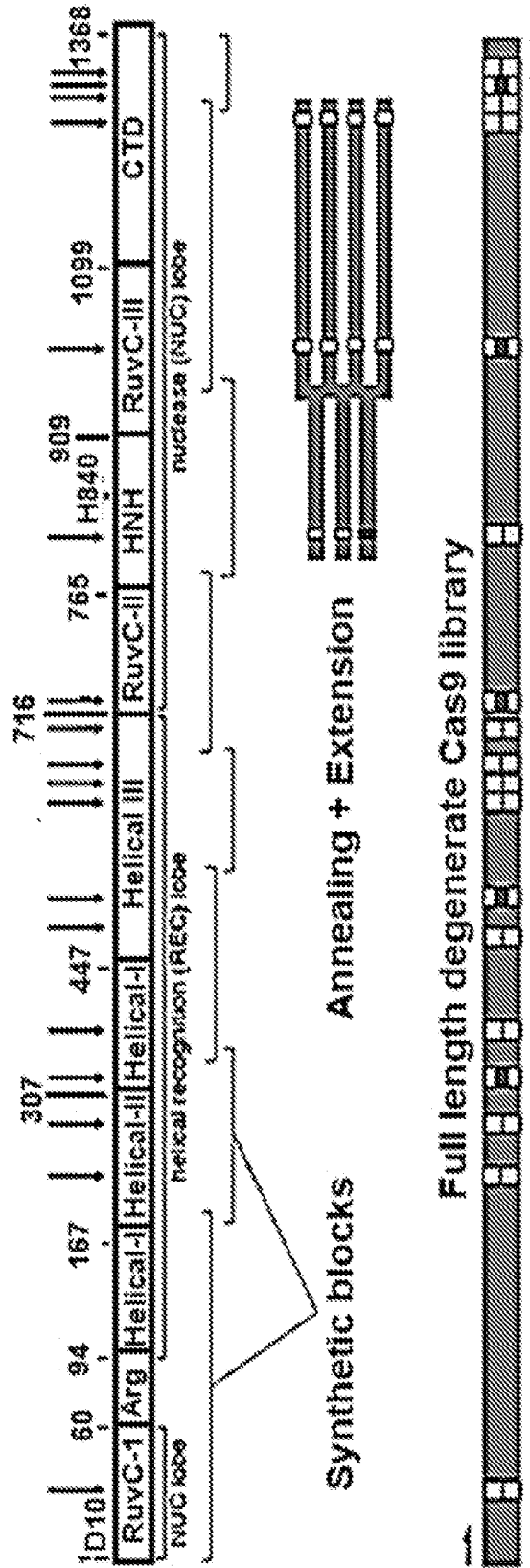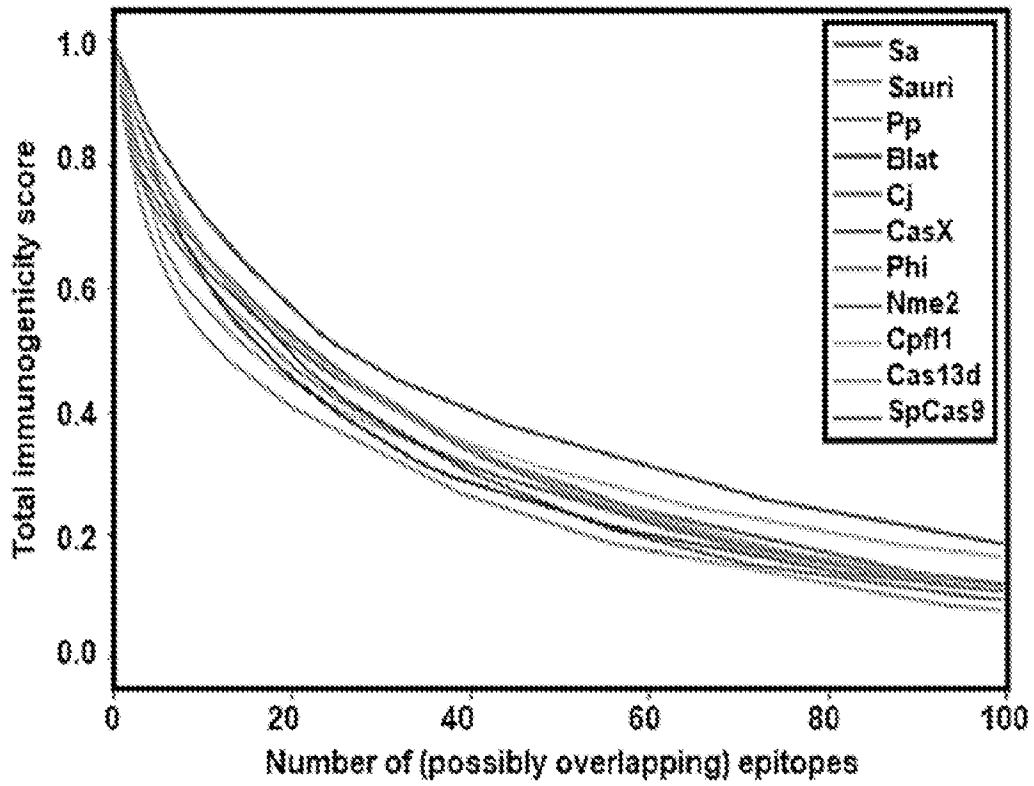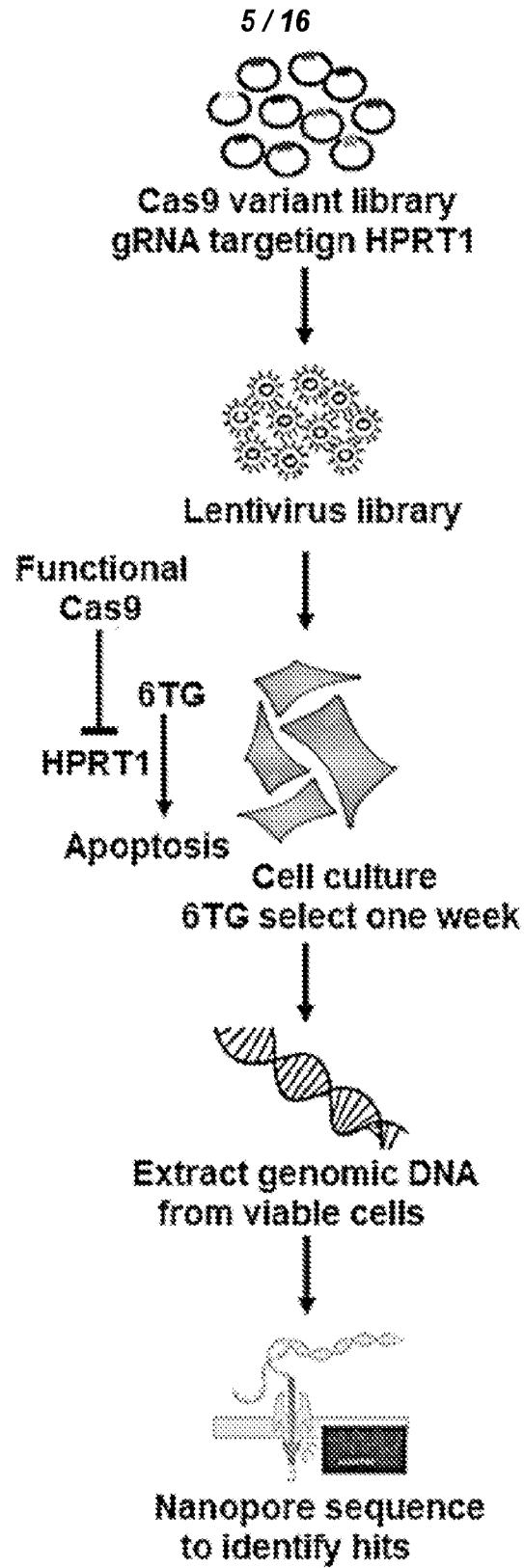
FIG. 4
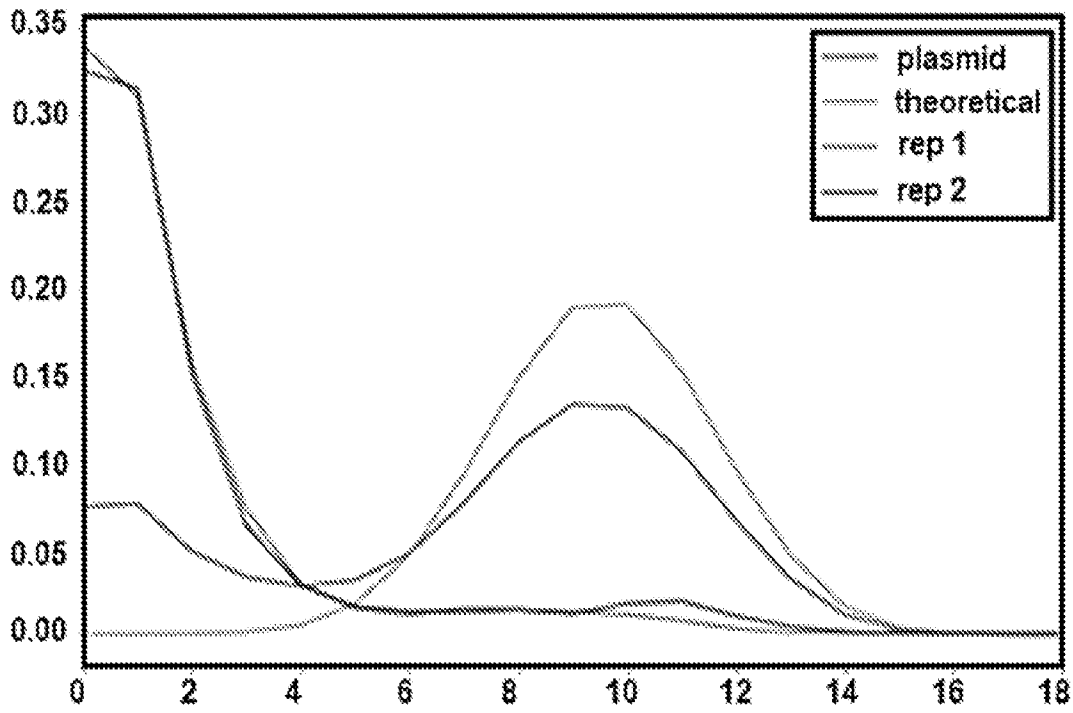
FIG. 5

FIG. 6

FIG. 7
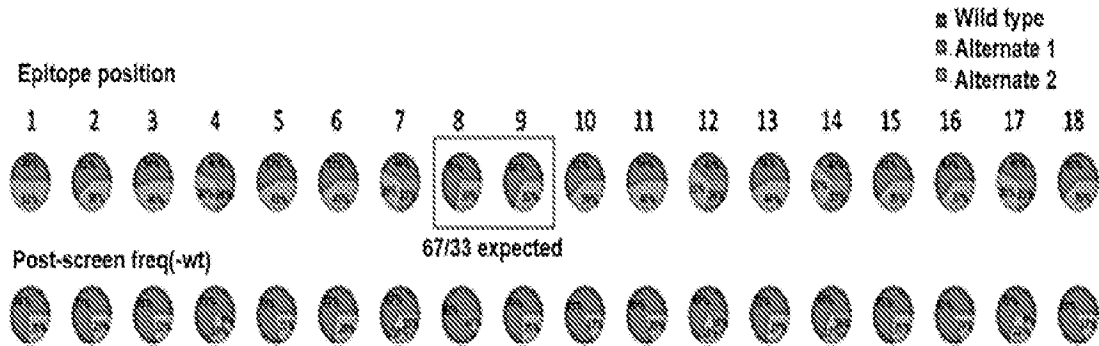

FIG. 8
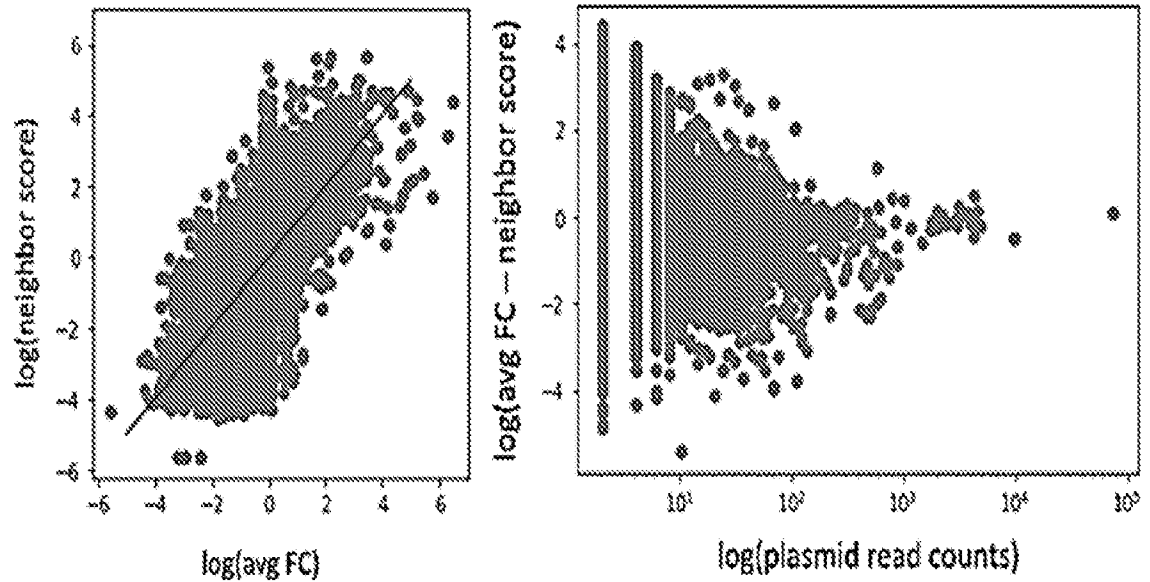

FIG. 9

FIG. 10
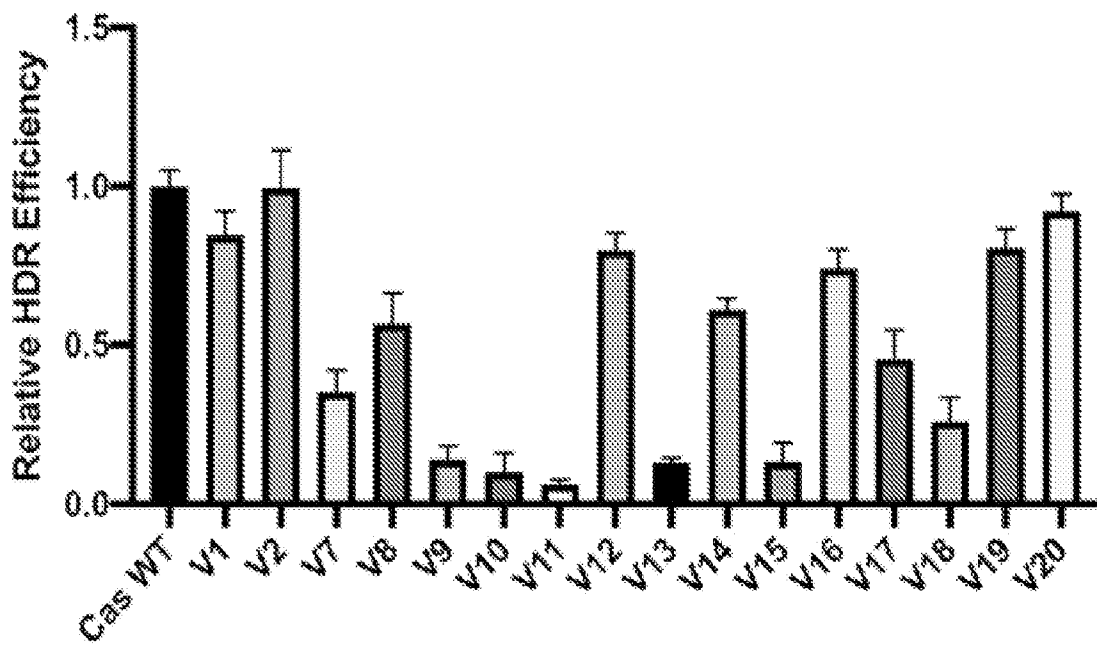
FIG. 11
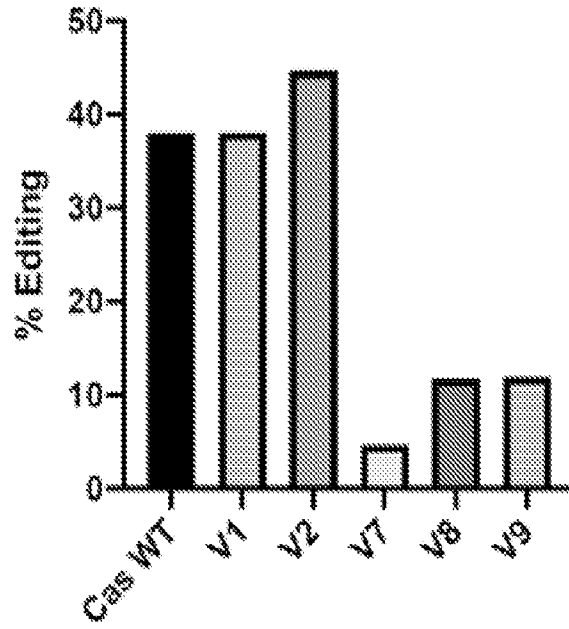
FIG. 12
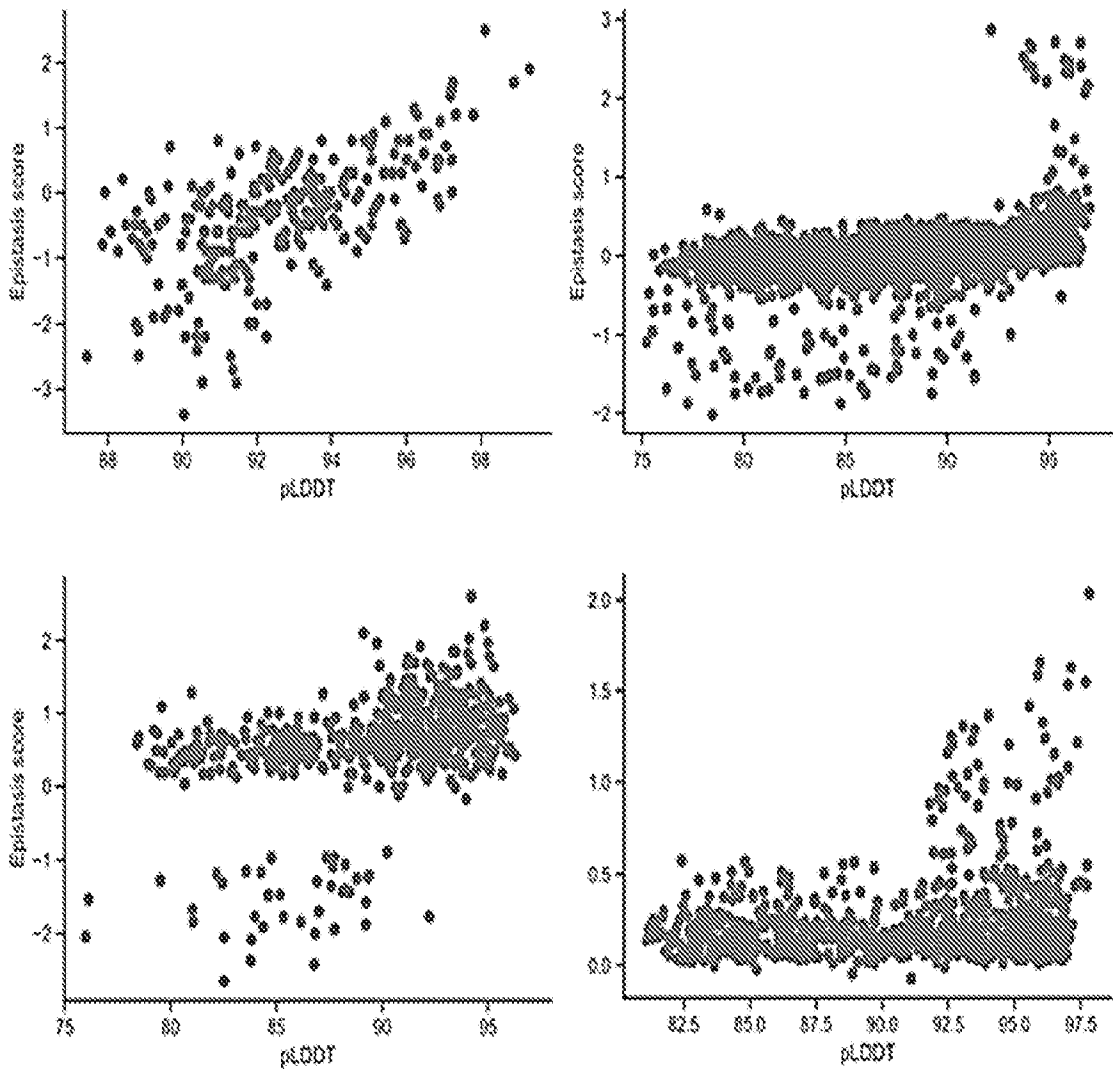
FIG. 13
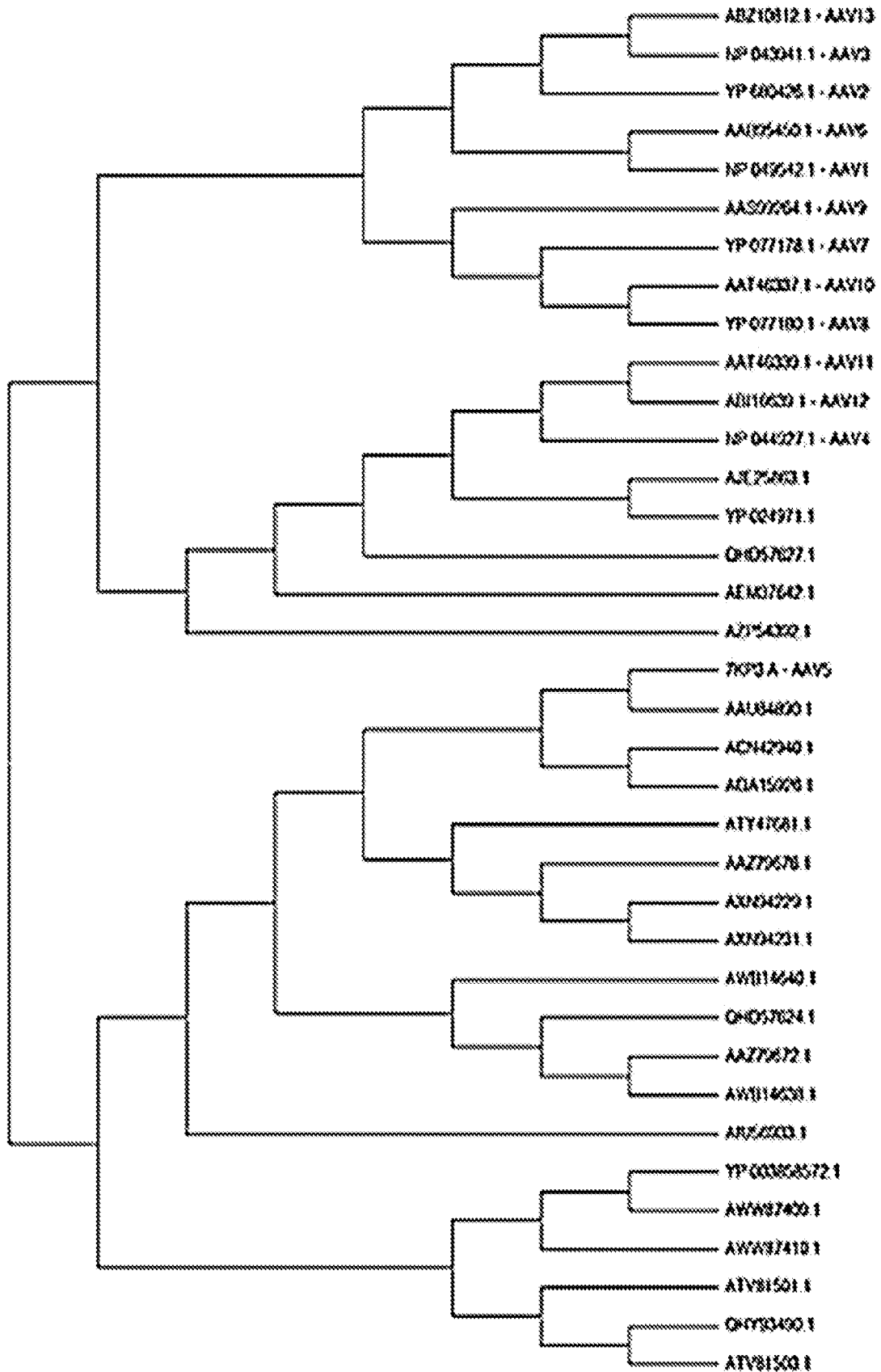
FIG. 14
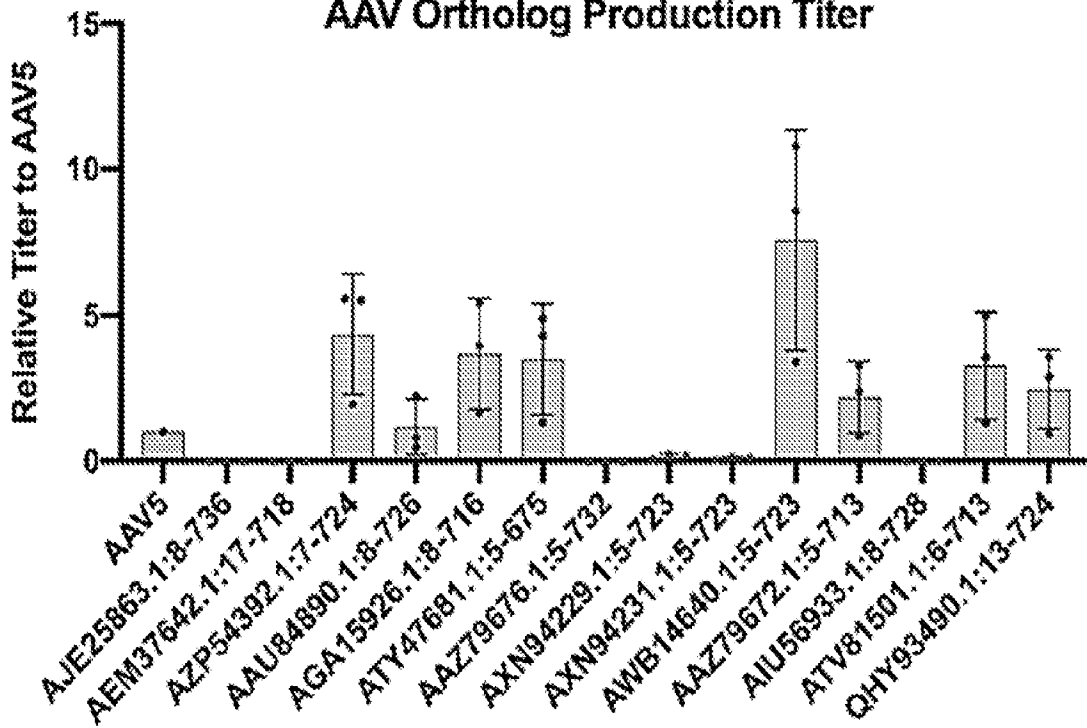
FIG. 15

Peptide Locations								Candidate mutations						
start	end	len	#AAVs	wt	pos	alt1	alt2	wt codon	alt1 codon	alt2 codon	gap position	notes	DNA pos	Block #
41	49	9	3	P	47	L		CCT	TTA		62	1 human isolate	139	1
				R	42	G		CGT			57	1 human isolate	124	
				Y	49	C		TAT			64	1 human isolate	145	
51	59	9	4	G	55	S		GGA	TCT		70	1 human isolate; F is most common allele here, G in 3,4,5,9,13	163	1
				D	59	Y		GAT			74	2 human isolates	175	
				G	57	S		GGT			72	mouse/snake	169	
				N	56	H		AAC			71	1 NHP	166	
84	101	18	14	L	90	P	I	CTC	CCG	ATT	105	2 human isolates P, I in snake, F in bird	268	1
				Y	89	H		TAC			104	1 human isolate	265	
				E	98	K	Q	GAG	AAA	CAA	113	1 human isolate K, Q in rat/snake, R in bird	292	
				A	95	V		GCG			110		283	
				D	96	G		GAC			111		286	
				F	99	L		TTT			114	1 human isolate, rat	295	
106	114	9	14	T	107	A		ACA	GCG		122	showed improvement transduction in CHO cells and mouse liver in AAV5 context	319	2
				S	108	P		TCC			123	1 human isolate	322	
116	127	12	9	Q	119	R	E	CAG	AGA	GAA	134	1 human isolate R, E in mouse/rat	355	2
				V	124	A		GTT	GCG		139	rat A, L/I in other serotypes, required for AAV6	370	
				R	123	L	T	AGG			138	mouse/rat L, snake T	367	

FIG. 16

Peptide Locations				Candidate mutations								notes	DNA pos	Block #
start	end	len	#AAVs	wt	pos	alt1	alt2	wt codon	alt1 codon	alt2 codon	gap position			
129	142	14	6	E	133	Q	D	GAG	CAA	GAY	150	Q in AAV4, but other mut, also seen T, A, D, G	397	2
				G	134	V	S	GGT	GTG	TCC	151	1 human isolate, S in some bird	400	
				T	137	A		ACG			154	1 human isolate, mouse/rat	409	
				V	131	A		GTT			148	2 human isolates	391	
				E	132	G	R	GAA			149	4 human isolates	394	
206	229	24	9	A	214	V		GCC	GTG		252	2 human isolates, S in many serotypes	640	3
				T	223	A	K	ACG	GCC	AAA	261	1 human isolate, Q in some serotypes, K in rat/snake	667	3
				S	222	T		TCC			260	snake	664	
263	279	17	14	S	267	A		AGC	GCG		306	showed improvement transduction in CHO cells and mouse liver in AAV5 context and in HeLa cells in AAV2 context	799	4
				Y	272	H		TAC	CAT		311	1 human isolate	814	4
				F	273	L		TTT			312		817	
283	293	11	14	W	287	R		TGG	CGT		326	2 human isolates	859	4
				I	291	V		ATC			330	1 human isolate, rat	871	
				L	290	P		CTC			329	1 human isolate	868	
303	315	13	13	K	312	E	R	AAA	GAG	CGT	351	R in AAV3, but others; E in 1 human isolate	934	5
				I	309	V		ATT			348	snake	925	
320	336	17	14	T	328	A		ACC	GCG		367	1 human isolate and demonstrated effective in vitro CHO, but not in vivo mouse in AAV5 context	982	5

FIG. 16 (Cont'd)

Peptide Locations								Candidate mutations						
start	end	len	#AAV	wt	pos	alt1	alt2	wt codon	alt1 codon	alt2 codon	gap position	notes	DNA pos	Block #
395	404	10	6	N	400	D		AAC	GAT		440	2 NHP isolates	1198	6
				F	402	L		TTT			442	1 human isolate	1204	
408	416	9	1 AAV-1	S	416	M	G	AGC	ATG	GGT	456		1246	6
				F	413	L		TTC			453		1237	
				S	415	T		TCC			455		1243	
599	612	14	13	Q	604	R		CAA	AGG		668	2 human isolates	1810	7
				I	607	T		ATC			671	1 human isolate	1819	
				P	606	Q		CCC			670	1 human isolate	1816	
624	634	11	12	L	629	P	F	CTC	CCG	TTT	693	1 human isolate, C in bird	1885	7
				K	630	E		AAA			694	1 human isolate	1888	
				F	627	L		TTC			691	1 human isolate	1879	
				H	631	R	N	CAC			695	N in AAV1, R in 1 human isolate	1891	
657	667	11	13	T	664	A		ACC	GCG	AAT	729	A in 1 human isolate, N in bird	1990	8
				S	663	G		AGC			728	1 human isolate	1987	
677	686	10	10	N	684	D	S	AAC	GAT	TCT	749	S in 1 human isolate, D in 1 NHP	2050	8
				R	682	C		AGG			747	1 human isolate	2044	
				W	683	R		TGG			748	1 human and 1 NHP	2047	
713	722	10	10	T	717	S	A	ACC	AGT	GCG	788	S in AAV4 group but others	2149	8
				Y	719	F		TAC			790	in AAV12 but others	2155	
				L	720	P		CTT			791	2 human, 1 NHP, and rat	2158	

FIG. 16 (Cont'd)

block length	blocks	variant #	synthesis
347	1-317: 4 mutations	16	16
235	295-530: 3 mutations	8	mux 8
230	500-730: 2 mutations	4	mux -
215	700-915: 3 mutations	8	mux 8
245	885-1130: 2 mutations	4	mux -
430	1100-1530: 2 mutations	4	4
450	1500-1950: 2 mutations	4	4
325	1920-2245 (2175): 3 mutations	8	mux -

| | | # of constructs: 40 |
| | | Library size: 2097152 |

FIG. 16 (Cont'd)

AAV5 DNA sequence:

```
ATGTCTTTTGTTGATCACCCTCCAGATTGGTTGGAAGAAGTTGGTGAAGGTCTTCGCGAGTTTT
TGGGCCTTGAAGCGGGCCCACCGAAACCAAAACCCAATCAGCAGCATCAAGATCAAGCCCGTGG
TCTTGTGCTGCCTGGTTATAACTATCTCGGACCCGGAAACGGTCTCGATCGAGGAGAGCCTGTC
AACAGGGCAGACGAGGTCGCGCGAGAGCACGACATCTCGTACAACGAGCAGCTTGAGGCGGGAG
ACAACCCCTACCTCAAGTACAACCACGCGGACGCCGAGTTTCAGGAGAAGCTCGCCGACGACAC
ATCCTTCGGGGGAAACCTCGGAAAGGCAGTCTTTCAGGCCAAGAAAAGGGTTCTCGAACCTTTT
GGCCTGGTTGAAGAGGGTGCTAAGACGGCCCCTACCGGAAAGCGGATAGACGACCACTTTCCAA
AAAGAAGAAGGCTCGGACCGAAGAGGACTCCAAGCCTTCCACCTCGTCAGACGCCGAAGCTGG
ACCCAGCGGATCCCAGCAGCTGCAAATCCCAGCCCAACCAGCCTCAAGTTTGGGAGCTGATACA
ATGTCTGCGGGAGGTGGCGGCCCATTGGGCGACAATAACCAAGGTGCCGATGGAGTGGGCAATG
CCTCGGGAGATTGGCATTGCGATTCCACGTGGATGGGGGACAGAGTCGTCACCAAGTCCACCCG
AACCTGGGTGCTGCCCAGCTACAACAACCACCAGTACCGAGAGATCAAAAGCGGCTCCGTCGAC
GGAAGCAACGCCAACGCCTACTTTGGATACAGCACCCCTGGGGGTACTTTGACTTTAACCGCT
TCCACAGCCACTGGAGCCCCCGAGACTGGCAAAGACTCATCAACAACTACTGGGGCTTCAGACC
CCGGTCCCTCAGAGTCAAAATCTTCAACATTCAAGTCAAAGAGGTCACGGTGCAGGACTCCACC
ACCACCATCGCCAACAACCTCACCTCCACCGTCCAAGTGTTTACGGACGACGACTACCAGCTGC
CCTACGTCGTCGGCAACGGGACCGAGGGATGCCTGCCGGCCTTCCCTCCGCAGGTCTTTACGCT
GCCGCAGTACGGTTACGCGACGCTGAACCGCGACAACACAGAAAATCCCACCGAGAGGAGCAGC
TTCTTCTGCCTAGAGTACTTTCCCAGCAAGATGCTGAGAACGGGCAACAACTTTGAGTTTACCT
ACAACTTTGAGGAGGTGCCCTTCCACTCCAGCTTCGCTCCCAGTCAGAACCTGTTCAAGCTGGC
CAACCCGCTGGTGGACCAGTACTTGTACCGCTTCGTGAGCACAAATAACACTGGCGGAGTCCAG
TTCAACAAGAACCTGGCCGGGAGATACGCCAACACCTACAAAAACTGGTTCCCGGGGCCCATGG
GCCGAACCCAGGGCTGGAACCTGGGCTCCGGGGTCAACCGCGCCAGTGTCAGCGCCTTCGCCAC
GACCAATAGGATGGAGCTCGAGGGCGCGAGTTACCAGGTGCCCCCGCAGCCGAACGGCATGACC
AACAACCTCCAGGGCAGCAACACCTATGCCCTGGAGAACACTATGATCTTCAACAGCCAGCCGG
CGAACCCGGGCACCACCGCCACGTACCTCGAGGGCAACATGCTCATCACCAGCGAGAGCGAGAC
GCAGCCGGTGAACCGCGTGGCGTACAACGTCGGCGGGCAGATGGCCACCAACAACCAGAGCTCC
ACCACTGCCCCCGCGACCGGCACGTACAACCTCCAGGAAATCGTGCCCGGCAGCGTGTGGATGG
AGAGGGACGTGTACCTCCAAGGACCCATCTGGGCCAAGATCCCAGAGACGGGGGCGCACTTTCA
CCCCTCTCCGGCCATGGGCGGATTCGGACTCAAACACCCACCGCCCATGATGCTCATCAAGAAC
ACGCCTGTGCCCGGAAATATCACCAGCTTCTCGGACGTGCCCGTCAGCAGCTTCATCACCCAGT
ACAGCACCGGGCAGGTCACCGTGGAGATGGAGTGGGAGCTCAAGAAGGAAAACTCCAAGAGGTG
GAACCCAGAGATCCAGTACACAAACAACTACAACGACCCCCAGTTTGTGGACTTTGCCCCGGAC
AGCACCGGGGAATACAGAACCACCAGACCTATCGGAACCCGATACCTTACCCGACCCCTTTAA
```

FIG. 17

AAV5 AA sequence

```
MSF-VDHPPDWLEEV----GEGL---REFLGLEAGPP-KPKPNQQHQ------DQARGLVLPGYNYLG
PGNGLDRGEPVNRADEVAREHDISYNEQLEAGDNPYLKYNHADAEFQEKLADDTSFGGNLGKAVFQAK
KRVLEPF--GLVEEGAKTAPTGKRIDDHF------PKRKKARTEEDSK-PST--------SSDAE---
-AGPSGSQQLQIPAQPASS—LGADTMSAGGGGPLGDNNQGADGVGNASGDWHCDSTWMGDRVVTKSTR
TWVLPSYNNHQYREIK-SGSVDGSNANAYFGYSTPWGYFDFNRFHSHWSPRDWQRLINNYWGFRPRSL
RVKIFNIQVKEVTVQDSTTTIANNLTSTVQVFTDDDYQLPYVVGNGTEGCLPAFPPQVFTLPQYGYAT
LNRDNTENPTERSSFFCLEYFPSKMLRTGNNFEFTYNFEEVPFHSSFAPSQNLFKLANPLVDQYLYRF
VSTN-------NTGGVQFNKNLAGRYANTYKNWFPGPMGRTQGWNLGSGV-------NRASVSA--FA
TTNRMELEGASYQVPPQPNG—MTNNLQGSNTYALENTMIFNSQPANPGTTATYLEGNMLITSESETQP
VNRVAYNVGGQMA-----TNNQSSTTAPATGTYNLQEIVPGSVWMERDVYLQGPIWAKIPETGAHFHP
SPAMGGFGLKHPPPMMLIKNTPVPGNI-TSFSDVPVSSFITQYSTGQVTVEMEWELKKENSKRWNPEI
QYTNNYNDP--QF----VDFAPDSTGEYRTTRPIGTRYLTRPL---------------
```

FIG. 18

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
IPC -   C12N 9/22, C12N 15/86, C12N 15/10 (2022.01)
CPC -   C07K 14/315, C12N 15/907, G01N 33/6878, C12N 2310/20, C12N 2710/10045

According to International Patent Classification (IPC) or to both national classification and IPC

B.   FIELDS SEARCHED
Minimum documentation searched (classification system followed by classification symbols) See Search History document
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched See Search History document
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2017/081288 A1 (LONZA LTD.) 18 May 2017 (18.05.2017) Abstract; Claim 1; Claim 14; Claim 17; Claim 20; Claim 74; para [00154]; para [0191]; para [00209]; para [00217]; para [00219]	1-6
A	WO 2019/180243 A1 (CHARITE-UNIVERSITATSMEDIZIN BERLIN) 26 September 2019 (26.09.2019) Abstract	1-6

☐ Further documents are listed in the continuation of Box C.   ☐ See patent family annex.

* Special categories of cited documents:	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance	
"D" document cited by the applicant in the international application	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E" earlier application or patent but published on or after the international filing date	
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
19 February 2022	APR 0 7 2022

Name and mailing address of the ISA/US	Authorized officer
Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450	Kari Rodriquez
Facsimile No. 571-273-8300	Telephone No. PCT Helpdesk: 571-272-4300

Form PCT/ISA/210 (second sheet) (July 2019)

| Box No. I | Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet) |

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. ☒ forming part of the international application as filed:

       ☒ in the form of an Annex C/ST.25 text file.

       ☐ on paper or in the form of an image file.

   b. ☐ furnished together with the international application under PCT Rule 13*ter.*1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

   c. ☐ furnished subsequent to the international filing date for the purposes of international search only:

       ☐ in the form of an Annex C/ST.25 text file (Rule 13*ter.*1(a)).

       ☐ on paper or in the form of an image file (Rule 13*ter.*1(b) and Administrative Instructions, Section 713).

2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

Box No. II	Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
   because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
   because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 7-8, 15-19, 30-31
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III	Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
----Please see continuation in first extra sheet ----------------

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
   1-6, limited to a method of engineering SpCas9 to be less immunoreactive or to be immuno-silent

Remark on Protest
☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

Continuation of Box No. III. Observations where unity of invention is lacking.

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I+, Claims 1-6, 9-14, directed to a method for engineering a protein or virus to be less immunoreactive or to be immuno-silent. The method will be searched to the extent that the method encompasses engineering a protein to be less immunoreactive or to be immuno-silent, wherein the protein is Streptococcus pyogenes Cas9 (SpCas9) (note, this is the first claimed protein for the inventive method embodiment). It is believed that claims 1-6 encompass this first named invention, and thus these claims will be searched without fee to the extent that the method encompasses engineering a protein to be less immunoreactive or to be immuno-silent, wherein the protein is Streptococcus pyogenes Cas9 (SpCas9). Additional method(s) comprising additional polypeptide(s) will be searched upon the payment of additional fees. Applicants must specify the claims that encompass any additionally elected method(s) comprising additional polypeptide(s). Applicants must further indicate, if applicable, the claims which encompass the first named invention, if different than what was indicated above for this group. Failure to clearly identify how any paid additional invention fees are to be applied to the "+" group(s) will result in only the first claimed invention to be searched. An exemplary election would be a method for engineering a virus to be less immunoreactive or to be immuno-silent wherein the virus is an adeno-associated virus (AAV) (claims 1, (5-6)(in part), 9-14).

Group II+, claims 20-25, directed to an isolated polynucleotide and encoded polypeptide thereof, wherein the protein has Cas9 like activity and is immuno-silenced. Group II+ will be searched upon payment of additional fees. The isolated polynucleotide and encoded polypeptide may be searched, for example, to encompass isolated polynucleotide having at least 99% sequence identity to SEQ ID NO:1, and encoding a polypeptide of SEQ ID NO:2 having a single mutation selected from the group consisting of: P28L and wherein the polypeptide has Cas9 like activity for an additional fee and election as such. It is believed that claims 20-25 read on this exemplary invention. Additional isolated polynucleotide(s) and encoded polypeptide(s) thereof will be searched upon the payment of additional fees. Applicants must specify the claims that encompass any additionally elected isolated polynucleotide(s) and encoded polypeptide(s) thereof. Failure to clearly identify how any paid additional invention fees are to be applied to the"+" group(s) will result in only the first claimed invention to be searched. Another exemplary election would be an isolated polynucleotide having at least 99% sequence identity to SEQ ID NO:1, and encoding a polypeptide of SEQ ID NO:2 having a single mutation selected from the group consisting of: L237C and wherein the polypeptide has Cas9 like activity (claims 20-25).

Group III+, claims 26-29, directed to a virus encoded by a polynucleotide sequence and a viral capsid wherein the virus has AAV5 like activity and is immuno-silenced. Group III+ will be searched upon payment of additional fees. The virus may be searched, for example, to encompass a virus encoded by a polynucleotide sequence having at least 100% sequence identity to the sequence of SEQ ID NO:49 for an additional fee and election as such. It is believed that claims 26, 29 (in part) read on this exemplary invention. Additional virus(es) and/or capsid(s) will be searched upon the payment of additional fees. Applicants must specify the claims that encompass any additionally elected virus(es) and/or capsid(s). Failure to clearly identify how any paid additional invention fees are to be applied to the"+" group(s) will result in only the first claimed invention to be searched. Another exemplary election would be an AAV viral capsid having at least 100% sequence identity to the sequence presented in SEQ ID NO:50 (claims 27, 29 (in part)).

The inventions listed as Groups I+, II+ and III+ do not relate to a single special technical feature under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Special technical features

The inventions of Groups I+, II+ and III+ each include the special technical feature of a unique amino acid sequence. Each amino acid sequence encodes a unique peptide, and is considered a distinct technical feature.
Additionally,
Group I+ has the special technical feature of a method for engineering a protein or virus to be less immunoreactive or to be immuno-silent, that is not required by Groups II+ and III+.
Group II+ has the special technical feature of an isolated polynucleotide and encoded polypeptide thereof, wherein the protein has Cas9 like activity and is immuno-silenced, that is not required by Groups I+ and III+.
Group III+ has the special technical feature of a virus encoded by a polynucleotide sequence and a viral capsid wherein the virus has AAV5 like activity and is immuno-silenced, that is not required by Groups I+ and II+.

Common technical features

No technical features are shared between the polynucleotide and/or amino acid sequences in each of Groups II+ and III+ and, accordingly, these groups lack unity a priori.

Additionally, even if Groups I+, II+ and III+ were considered to share the technical features of including:
a protein or virus engineered to be less immunoreactive or to be immuno-silent.

However, these shared technical features are previously disclosed by prior art as discussed below.

Group II+ inventions further share the technical features of including:
an isolated polypeptide wherein the protein has Cas9 like activity and is immuno-silenced.

-------please see continuation on next sheet -----------------

Continuation of Box No. III. Observations where unity of invention is lacking.

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However, these shared technical features are previously disclosed by WO 2017/081288 A1 to Lonza Ltd, (hereinafter 'Lonza').

Lonza teaches an isolated polypeptide wherein the protein has Cas9 like activity and is immuno-silenced (Abstract - 'The invention relates to methods of reducing the immunogenicity of CRISPR-associated (Cas) proteins and the modified Cas proteins produced therefrom. In addition, the invention relates to methods for cell and gene therapy, including any and all genetic modifications and alterations of gene expression (and/or genetic elements) made in-vivo or ex- vivo using Cas proteins with reduced immunogenicity.'; Claim 1 - 'A method for reducing the immunogenicity of a CRISPR-associated (Cas) protein, the method comprising introducing one or more amino acid substitutions into one or more residues corresponding to one or more major histocompatibility (MHC) Class I and/or Class II binding sites of the Cas protein to form a recombinant Cas protein.').

Group III+ inventions further share the technical features of including:
an AAV viral capsid wherein the virus has AAV5 like activity and is immuno-silenced.

However, these shared technical features are previously disclosed by US 2009/0317417 A1 to Vandenberghe et al., (hereinafter 'Vandenberghe').

Vandenberghe teaches an AAV viral capsid wherein the virus has AAV5 like activity and is immuno-silenced (Abstract - 'A method of reducing the cellular immune response and/or toxicity of AAV-mediated delivery is described. The method provides for masking or ablating a RxxR motif which induces T-cells, and which is located on select AAV capsids. The method further provides for reducing or eliminating heparin binding to an AAV. Also provided are compositions containing modified AAV capsids and methods of using same.'; para [0024] - 'The reference sequence may be AAV2, or another selected sequence. See, e.g., AAV 1 (U.S. Pat. No. 6,759,237), AAV2, AAV3, AAV4, AAV5'; para [0038] - 'Unless otherwise specified, the AAV ITRs, and other selected AAV components described herein, may be readily selected from among any AAV, including, without limitation, AAV1, AAV2, AAV3, AAV4, AAV5'; para [0059] - 'Additionally, when pseudotyping an AAV vector in a modified AAV, the sequences encoding each of the essential rep proteins may be supplied by different AAV sources (e.g., AAV1, AAV2, AAV3, AAV4, AAV5').

As the technical features were known in the art at the time of the invention, they cannot be considered special technical features that would otherwise unify the groups.

Therefore, Groups I+, II+ and III+ inventions lack unity under PCT Rule 13 because they do not share the same or corresponding special technical feature.

NOTE, continuation of Item 4 above: claims 7-8, 15-19, 30-31 are held unsearchable because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).