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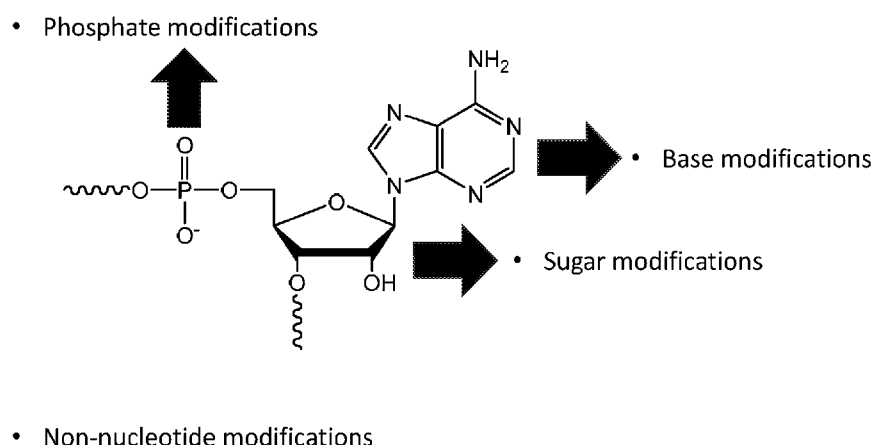


FIG. 1

(57) Abstract: Provided herein are modified prime editing guide RNA (PEgRNA) and nicking guide RNA (ngRNA) sequences and compositions comprising the same, as well as using the same in methods for gene editing.



CHEMICAL MODIFICATIONS IN PEgRNAs and ngRNAs**CROSS REFERENCE TO RELATED APPLICATIONS**

[001] The present application claims priority to and the benefits of U.S. Provisional Application No. 63/601,415, filed November 21, 2023, the contents of which are herein incorporated by reference in their entirety.

SEQUENCE LISTING

[002] This application includes and incorporates by reference in its entirety a Sequence Listing XML in the required .xml format. The Sequence Listing XML file that has been electronically filed contains the information of the nucleotide and/or amino acid sequences disclosed in the patent application using the symbols and format in accordance with the requirements of 37 C.F.R. §§1.832 through 1.834.

[003] The Sequence Listing XML filed herewith serves as the electronic copy required by §1.834(b)(1).

[004] The Sequence Listing XML is identified as follows: “272059-557691_SL.xml” (1,154,386 bytes in size), which was created on November 21, 2024.

FIELD OF THE INVENTION

[005] The present disclosure describes modified PEgRNA and ngRNA sequences and compositions comprising the same, as well as using the same in methods for gene editing.

BACKGROUND

[006] Great progress has been achieved in developing Cas enzyme-based gene therapy as a therapeutic agent, especially with the introduction and enhancement of prime editing for altering single nucleotides or larger segments of genomic DNA. However, the stability, specificity and affinity, immunogenicity, pharmacokinetics, and delivery of the prime editing system are still pivotal issues that need to be addressed to implement prime editing therapeutics and expand to multiple therapeutic areas. RNA molecules used for prime editing such as prime editing guide RNA (PEgRNA) and nicking guide RNA (ngRNA) are intrinsically unstable and prone to degradation, although some chemical modifications have been reported to improve their stability. To obtain the best PEgRNA and ngRNA therapeutic efficacy, it is necessary to optimize their chemistry to reduce immunogenicity and improve stability and safety profiles.

SUMMARY

[007] Provided herein are modified prime editing guide RNA (PEgRNA) and prime editor nicking guide RNA (ngRNA) sequences and compositions comprising the same, as well as using the same in methods for gene editing.

[008] In certain aspects, the disclosure provides a modified PEgRNA comprising, 5' to 3':

- (a) a spacer that comprises a region of complementarity to a search target sequence in a target strand of a double-stranded target DNA;
- (b) a guide RNA (gRNA) core capable of binding to a Cas protein wherein the gRNA core comprises, in 5' to 3' order, a tetraloop, a first stem loop (SL1), a second stem loop (SL2), and a third stem loop (SL3);
- (c) an extension arm comprising:
 - (i) an editing template that comprises an intended edit compared to the double-stranded target DNA, and
 - (ii) a primer binding site (PBS) that comprises a region of complementarity to a region upstream of a nick site in a non-target strand of the double-stranded target DNA; and
- (d) wherein the PEgRNA comprises 5 or more nucleotide modifications.

[009] In another aspect, the disclosure provides a modified ngRNA comprising, 5' to 3':

- (a) a spacer that comprises a region of complementarity to a search target sequence in a target strand of a double-stranded target DNA;
- (b) a guide RNA (gRNA) core capable of binding to a Cas protein wherein the gRNA core comprises, in 5' to 3' order, a tetraloop, a first stem loop (SL1), a second stem loop (SL2), and a third stem loop (SL3); and
- (c) wherein the ngRNA comprises 5 or more nucleotide modifications.

[0010] In some embodiments, a nucleotide modification is located in the 3' end.

[0011] In some embodiments, a nucleotide modification is located in the 5' end.

[0012] In some embodiments, wherein nucleotide modifications are located in the 3' end and the 5' end.

[0013] In some embodiments, a nucleotide modification is located in the spacer.

[0014] In some embodiments, a nucleotide modification is located in the gRNA core.

[0015] In some embodiments, a nucleotide modification is located in the extension arm.

[0016] In some embodiments, a nucleotide modification is located in the tetraloop.

[0017] In some embodiments, a nucleotide modification is located in the SL1.

- [0018] In some embodiments, a nucleotide modification is located in the SL2.
- [0019] In some embodiments, a nucleotide modification is located in the SL3.
- [0020] In some embodiments, at least 2 or more nucleotide modifications are located in the 5' end, 3' end, tetraloop, SL2, or SL3.
- [0021] In some embodiments, nucleotide modifications are located in the tetraloop, SL1, SL2, and SL3.
- [0022] In some embodiments, nucleotide modifications are located in the tetraloop, SL2, and SL3.
- [0023] In some embodiments, nucleotide modifications are located in the 5' end, 3' end, tetraloop, SL2, and SL3.
- [0024] In some embodiments, at least 2 or more nucleotide modifications occur within the gRNA.
- [0025] In some embodiments, the gRNA core comprises a nucleotide modification at nucleotide 25, 37, 38, 46, 64, 65, 67, or 68.
- [0026] In some embodiments, the gRNA core comprises a nucleotide modification at nucleotide 25.
- [0027] In some embodiments, the gRNA core comprises a nucleotide modification at nucleotide 37 and/or 38.
- [0028] In some embodiments, the gRNA core comprises a nucleotide modification at nucleotide 65.
- [0029] In some embodiments, the gRNA core comprises a nucleotide modification at nucleotide 68.
- [0030] In some embodiments, the PEgRNA or ngRNA comprises 10 or more nucleotide modifications.
- [0031] In some embodiments, the PEgRNA or ngRNA comprises 20 or more nucleotide modifications.
- [0032] In some embodiments, the PEgRNA or ngRNA comprises 30 or more nucleotide modifications.
- [0033] In some embodiments, the PEgRNA or ngRNA comprises 40 or more nucleotide modifications.
- [0034] In some embodiments, the PEgRNA or ngRNA comprises 50 or more nucleotide modifications.
- [0035] In some embodiments, the PEgRNA or ngRNA comprises 60 or more nucleotide modifications.

[0036] In some embodiments, the PEGRNA or ngRNA comprises 70 or more nucleotide modifications.

[0037] In some embodiments, the PEGRNA or ngRNA comprises 80 or more nucleotide modifications.

[0038] In some embodiments, the PEGRNA or ngRNA comprises 90 or more nucleotide modifications.

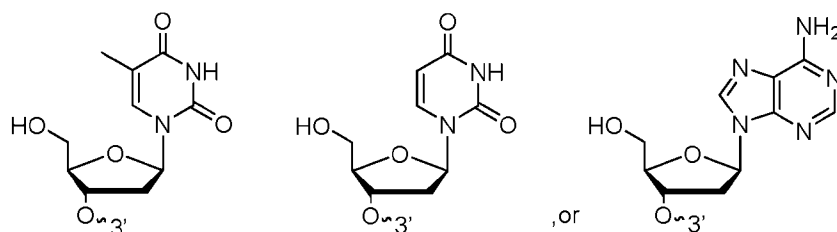
[0039] In some embodiments, the PEGRNA or ngRNA comprises 100 or more modified nucleotides.

[0040] In some embodiments, a base modification is selected from the group consisting of N⁶-methyladenosine (m⁶A), N⁶-methyl-2'-O-methyladenosine (2'OMe-m⁶A), xanthine, allyaminouracil, allyaminothymidine, hypoxanthine, digoxigeninated adenine, digoxigeninated cytosine, digoxigeninated guanine, digoxigeninated uracil, 6-chloropurineriboside, N⁶-methyladenine, methylpseudouracil, 2-thiocytosine, 2-thiouracil, 5-methyluracil, 4-thiothymidine, 4-thiouracil, 5,6-dihydro-5-methyluracil, 5,6-dihydrouracil, 5-[(3-Indolyl)propionamide-N-allyl]uracil, 5-aminoallylcytosine, 5-aminoallyluracil, 5-bromouracil, 5-bromocytosine, 5-carboxycytosine, 5-carboxymethylesteruracil, 5-carboxyuracil, 5-fluorouracil, 5-formylcytosine, 5-formyluracil, 5-hydroxycytosine, 5-hydroxymethylcytosine, 5-hydroxymethyluracil, 5-hydroxyuracil, 5-iodocytosine, 5-iodouracil, 5-methoxycytosine, 5-methoxyuracil, 5-methylcytosine, 5-methyluracil, 5-propargylaminocytosine, 5-propargylaminouracil, 5-propynylcytosine, 5-propynyluracil, 6-azacytosine, 6-azauracil, 6-chloropurine, 6-thioguanine, 7-deazaadenine, 7-deazaguanine, 7-deaza-7-propargylaminoadenine, 7-deaza-7-propargylaminoguanine, 8-azaadenine, 8-azidoadenine, 8-chloroadenine, 8-oxoadenine, 8-oxoguanine, araadenine, aracytosine, araguanine, arauracil, biotin-16-7-deaza-7-propargylaminoguanine, biotin-16-aminoallylcytosine, biotin-16-aminoallyluracil, cyanine 3-5-propargylaminocytosine, cyanine 3-6-propargylaminouracil, cyanine 3-aminoallylcytosine, cyanine 3-aminoallyluracil, cyanine 5-6-propargylaminocytosine, cyanine 5-6-propargylaminouracil, cyanine 5-aminoallylcytosine, cyanine 5-aminoallyluracil, cyanine 7-aminoallyluracil, dabcy-5-3-aminoallyluracil, desthiobiotin-16-aminoallyluracil, desthiobiotin-6-aminoallylcytosine, isoguanine, N¹-ethylpseudouracil, N¹-methoxymethylpseudouracil, N¹-methyladenine, N¹-methylpseudouracil, N¹-propylpseudouracil, N²-methylguanine, N⁴-biotin-OBEA-cytosine, N⁴-methylcytosine, N⁶-methyladenine, O⁶-methylguanine, pseudoisocytosine, pseudouracil, thienocytosine, thienoguanine, thienouracil, xanthosine, 3-deazaadenine, 2,6-diaminoadenine, 2,6-daminoguanine, 5-carboxamide-uracil, 5-ethynyluracil, N⁶-isopentenyladenine (i⁶A), 2-

methyl-thio- N⁶-isopentenyladenine (ms2i⁶A), 2-methylthio- N⁶-methyladenine (ms2m⁶A), N⁶-(cis-hydroxyisopentenyl)adenine (io⁶A), 2-methylthio- N⁶-(cis-hydroxyisopentenyl)adenine (ms2io⁶A), N⁶-glycinylicarbamoyladenine (g⁶A), N⁶-threonylicarbamoyladenine (t⁶A), 2-methylthio- N⁶-threonyl carbamoyladenine (ms2t⁶A), N⁶-methyl- N⁶-threonylicarbamoyladenine (m⁶t⁶A), N⁶-hydroxynorvalylcarbamoyladenine (hn⁶A), 2- methylthio- N⁶-hydroxynorvalyl carbamoyladenine (ms2hn⁶A), N⁶, N⁶-dimethyladenine (m⁶2A), and N⁶-acetyladenine (ac⁶A).

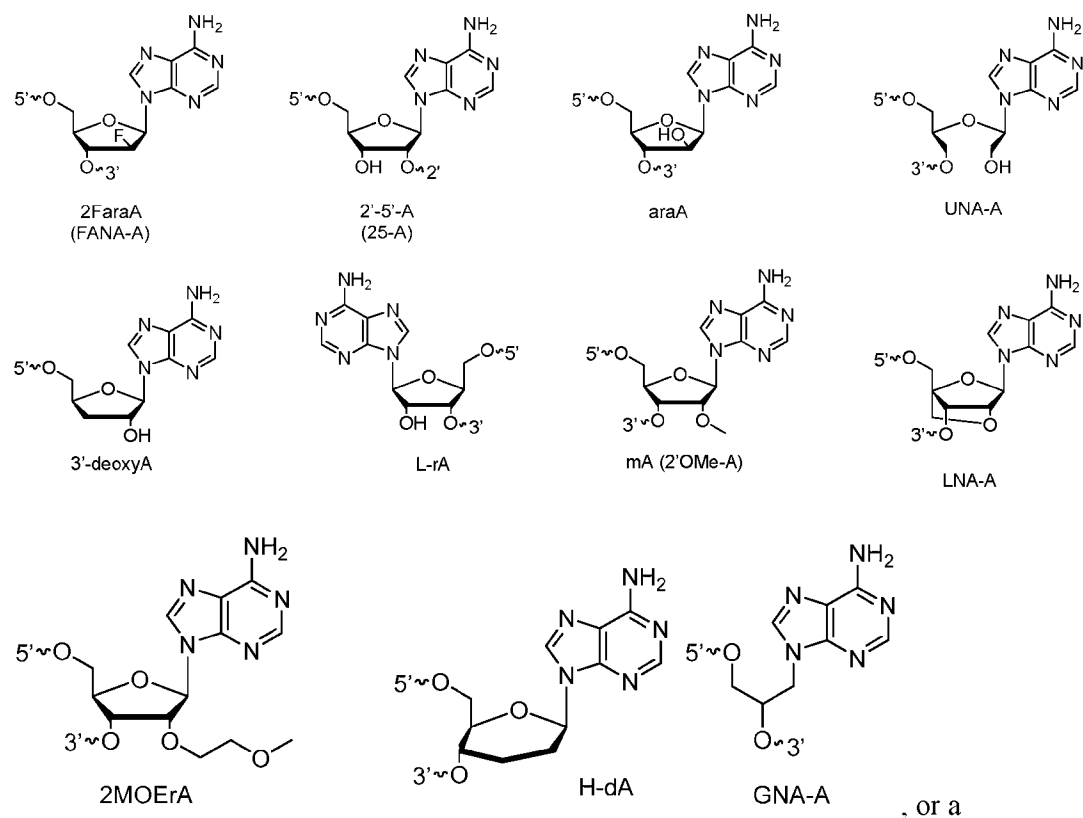
[0041] In some embodiments, the modification comprises an inverted nucleotide located in the 3' end.

[0042] In some embodiments, the inverted nucleotide comprises



[0043] In some embodiments, a nucleotide modification is a sugar modification.

[0044] In some embodiments, the sugar modification comprises



combination thereof.

[0045] In some embodiments, the sugar modification is selected from the group consisting of 2'-thioribose, 2', 3'-dideoxyribose, 2'-amino-2'-deoxyribose, 2' deoxyribose, 2'-azido-2'-deoxyribose, 2'-fluoro-2'-deoxyribose, 2'-O-methoxyethyl (MOE), 2'-O-methylribose, 2'-O-methyldeoxyribose, 3'-amino- 2',3'-dideoxyribose, 3'-azido-2', 3'-dideoxyribose, 3'-deoxyribose, 3'-O-(2-nitrobenzyl)-2'-deoxyribose, 3'-O-methylribose, 5'-aminoribose, 5'-thioribose, 5-nitro-l-indolyl-2'-deoxyribose, 5'-biotin-ribose, 2'-O,4'-C-methylene-linked ribose, 2'-O,4'-C-amino-linked ribose, and 2'-O,4'-C-thio-linked ribose.

[0046] In some embodiments, a nucleotide modification is a phosphate modification.

[0047] In some embodiments, a phosphate modification comprises phosphorothioate, a stereospecific phosphorothioate, or a combination thereof.

[0048] In some embodiments, the phosphate modification is selected from the group consisting of phosphorothioate (PS), a stereospecific phosphorothioate, phosphorodithioate, thiophosphate, 5'-O-methylphosphonate, 3'-O- methylphosphonate, 5'-hydroxyphosphonate, hydroxyphosphanate, phosphoroselenoate, selenophosphate, phosphoramidate, carbophosphonate, methylphosphonate, phenylphosphonate, ethylphosphonate, H-phosphonate, guanidinium ring, triazole ring, boranophosphate (BP), methylphosphonate, and guanidinopropyl phosphoramidate.

[0049] In some embodiments, the nucleotide modification comprises a phosphate modification, a base modification, a sugar modification, or a combination thereof.

[0050] In some embodiments, a modified PEgRNA sequence is selected from the group consisting of Tables 5 or 6.

[0051] In some embodiments, the nucleotide modification comprises a non-nucleotide modification comprises a hairpin a C₂-C₁₂ linker, an ethylene glycol linker, 2'-5' linkages, or a combination thereof.

[0052] Another aspect of the disclosure provides a method for producing a modified PEgRNA or modified ngRNA, the method comprising ligating a coding nucleotide sequence with the modified nucleic acid sequence disclosed herein.

[0053] Another aspect of the disclosure provides a method for producing a modified PEgRNA or modified ngRNA, the method comprising ligating a sequence encoding a prime editor with the modified nucleic acid sequence disclosed herein.

[0054] In some embodiments, the ligation comprises a self-templated enzymatic ligation. In some embodiments, the self-templated enzymatic ligation is splint-mediated.

[0055] In some embodiments, the ligation comprises a templated enzymatic ligation.

[0056] In some embodiments, the enzymatic ligation is catalyzed by a nucleic acid ligase to generate a natural phosphodiester linkage between the coding nucleotide sequence and the modified nucleic acid sequence of any one of the foregoing claims, or between the sequence encoding a prime editor with the modified nucleic acid sequence of any one of the foregoing claims, or between two fragments of the nucleotide sequences containing the chemical modifications of any one of the foregoing claims.

[0057] In some embodiments, the nucleic acid ligase is T4 RNA Ligase I, T4 RNA Ligase II, or T4 DNA Ligase.

[0058] Another aspect of the disclosure provides a prime editing system comprising a modified PEgRNA or modified ngRNA disclosed herein or one or more polynucleotides encoding the modified PEgRNA or modified ngRNA.

[0059] Another aspect of the disclosure provides a lipid nanoparticle comprising the modified PEgRNA or modified ngRNA disclosed herein.

[0060] Another aspect of the disclosure provides a lipid nanoparticle comprising the prime editing system disclosed herein.

[0061] Another aspect of the disclosure provides a method for editing a gene, the method comprising contacting the gene with the modified PEgRNA, modified ngRNA, prime editing system, lipid nanoparticle, or as disclosed herein.

INCORPORATION BY REFERENCE

[0062] All publications, patents, and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication, patent, or patent application was specifically and individually indicated to be incorporated by reference.

BRIEF DESCRIPTION OF THE DRAWINGS

[0063] The novel features of the methods and compositions provided herein are set forth with particularity in the appended claims. A better understanding of the features and advantages of the methods and compositions provided herein will be obtained by reference to the following detailed description that sets forth illustrative embodiments, in which the principles of the methods and compositions provided herein are utilized, and the accompanying drawings of which:

[0064] **FIG. 1** shows exemplified modifications in a PEgRNA or ngRNA (nucleotide and non-nucleotide modifications).

[0065] **FIG. 2** depicts a PEgRNA architectural overview in an exemplary schematic of PEgRNA designed for a prime editor.

[0066] **FIG. 3** is a schematic showing the spacer and gRNA core part of an exemplary guide RNA, in two separate molecules (crRNA/tracrRNA). The rest of the PEgRNA structure is not shown.

[0067] **FIG. 4** is a schematic showing modification sites in exemplified PEgRNAs.

[0068] **FIG. 5** shows prime editing efficiency for targeted end modifications and gRNA chemical modifications of the exemplified PEgRNAs.

[0069] **FIG. 6** shows prime editing efficiency and Indel qualifications for exemplified PEgRNA No. 1-PEgRNA No. 6 molecules and visual representation of the modifications within the tested PEgRNAs is also shown.

[0070] **FIG. 7A** shows metabolite cleavage or nuclease degradation profiles of exemplary PEgRNA No. 1 with specific site modifications, and the respective liquid chromatography mass spectrometry (LCMS) profiles after incubation in human S9 fractions.

[0071] **FIG. 7B** shows metabolite cleavage or nuclease degradation profiles of exemplary PEgRNA No. 2 with specific site modifications, and the respective liquid chromatography mass spectrometry (LCMS) profiles after incubation in human S9 fractions.

[0072] **FIG. 7C** shows metabolite cleavage or nuclease degradation profiles of exemplary PEgRNA No. 3 with specific site modifications, and the respective liquid chromatography mass spectrometry (LCMS) profiles after incubation in human S9 fractions.

[0073] **FIG. 7D** shows metabolite cleavage or nuclease degradation profiles of exemplary PEgRNA No. 6 with specific site modifications, and the respective liquid chromatography mass spectrometry (LCMS) profiles after incubation in human S9 fractions.

[0074] **FIG. 8** shows exemplified site-specific modifications in a PEgRNA or ngRNA (nucleotide and non-nucleotide modifications)

[0075] **FIG. 9** shows *in-vitro* half-life stability in human liver S9 for modifications present in the PEgRNAs No. 1, 2, 6.

[0076] **FIG. 10A** depicts exemplified modifications (i.e. sugar modifications) in a PEgRNA or ngRNA.

[0077] **FIG. 10A** depicts exemplified modifications (i.e. phosphate modifications) in a PEgRNA or ngRNA.

[0078] **FIG. 10B** depicts exemplified modifications (i.e. base modifications and inverted nucleotide) in a PEgRNA or ngRNA.

[0079] FIG. 11 shows results for shows a UV-Vis melt (T_m), curves for RNP structure formation using (Differential Scanning Fluorimetry, DSF) and *in-vitro* half-life stability related to melting temperatures for RNP stability (T_M – melting temperature) for exemplary modifications to PEgRNA No. 2, 4, 5, and 6.

[0080] FIG. 12 shows *in vivo* prime editing efficiency of PEgRNAs P2, P31, P32, P33, P34, P35, P36, P37, P38, and P1.

[0081] FIG. 13 shows a detection method and assay to identify metabolites using *in vitro* assays human liver cells, fractions S9 and detected with LCMS techniques.

DETAILED DESCRIPTION

[0082] Provided herein, in some embodiments, are modified prime editing guide RNA (PEgRNA) and modified prime editor nicking guide RNA (ngRNA) sequences and compositions comprising the same, as well as using the same in methods for gene editing.

[0083] The following description and examples illustrate embodiments of the present disclosure in detail. It is to be understood that this disclosure is not limited to the particular embodiments described herein and as such can vary. Those of skill in the art will recognize that there are numerous variations and modifications of this disclosure, which are encompassed within its scope. Although various features of the present disclosure can be described in the context of a single embodiment, the features can also be provided separately or in any suitable combination. Conversely, although the present disclosure can be described herein in the context of separate embodiments for clarity, the present disclosure can also be implemented in a single embodiment.

Definitions

[0084] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as is commonly understood by one of ordinary skill in the art.

[0085] The terminology used herein is for the purpose of describing particular embodiments only and is not intended to be limiting. As used herein, the singular forms “a”, “an” and “the” are intended to include the plural forms as well, unless the context clearly indicates otherwise. Furthermore, to the extent that the terms “including”, “includes”, “having”, “has”, “with”, or variants thereof as used herein mean “comprising”.

[0086] Unless otherwise specified, the words “comprising”, “comprise”, “comprises”, “having”, “have”, “has”, “including”, “includes”, “include”, “containing”, “contains” and “contain” are inclusive or open-ended and do not exclude additional, unrecited elements or method steps.

[0087] Reference to “some embodiments”, “an embodiment”, “one embodiment”, or “other embodiments” means that a particular feature or characteristic described in connection with the embodiments is included in at least one or more embodiments, but not necessarily all embodiments, of the present disclosure.

[0088] The term “about” or “approximately” means within an acceptable error range for the particular value as determined by one of ordinary skill in the art, which will depend in part on how the value is measured or determined, *i.e.*, the limitations of the measurement system. For example, “about” can mean within 1 standard deviation, per the practice in the art.

Alternatively, “about” can mean a range of up to 20%, up to 10%, up to 5%, or up to 1% of a given value. Alternatively, particularly with respect to biological systems or processes, the term can mean within an order of magnitude, preferably within 5-fold, and more preferably 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 should be assumed.

[0089] The term “substantially” as used herein may refer to a value approaching 100% of a given value. In some embodiments, the term may refer to an amount that may be at least about 70%, 80%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.9%, or 99.99% of a total amount. In some embodiments, the term may refer to an amount that may be about 100% of a total amount.

[0090] The terms “protein” and “polypeptide” can be used interchangeably to refer to a polymer of two or more amino acids joined by covalent bonds (*e.g.*, an amide bond) that can adopt a three-dimensional conformation. In some embodiments, a protein or polypeptide comprises at least 10 amino acids, 15 amino acids, 20 amino acids, 30 amino acids or 50 amino acids joined by covalent bonds (*e.g.*, amide bonds). In some embodiments, a protein comprises at least two amide bonds. In some embodiments, a protein comprises multiple amide bonds. In some embodiments, a protein comprises an enzyme, enzyme precursor proteins, regulatory protein, structural protein, receptor, nucleic acid binding protein, a biomarker, a member of a specific binding pair (*e.g.*, a ligand or aptamer), or an antibody. In some embodiments, a protein may be a full-length protein (*e.g.*, a fully processed protein having certain biological function). In some embodiments, a protein may be a variant or a fragment of a full-length protein. For example, in some embodiments, a Cas9 protein domain comprises an H840A amino acid substitution compared to a naturally occurring *S. pyogenes* Cas9 protein. A variant of a protein or enzyme, for example a variant reverse transcriptase, comprises a polypeptide having an amino acid sequence that is about 60% identical, about

70% identical, about 80% identical, about 90% identical, about 95% identical, about 96% identical, about 97% identical, about 98% identical, about 99% identical, about 99.5% identical, or about 99.9% identical to the amino acid sequence of a reference protein.

[0091] In some embodiments, a protein comprises one or more protein domains or subdomains. As used herein, the term “polypeptide domain”, “protein domain”, or “domain” when used in the context of a protein or polypeptide, refers to a polypeptide chain that has one or more biological functions, *e.g.*, a catalytic function, a protein-protein binding function, or a protein-DNA function. In some embodiments, a protein comprises multiple protein domains. In some embodiments, a protein comprises multiple protein domains that are naturally occurring. In some embodiments, a protein comprises multiple protein domains from different naturally occurring proteins. For example, in some embodiments, a prime editor may be a fusion protein comprising a Cas9 protein domain of *S. pyogenes* and a reverse transcriptase protein domain of Moloney murine leukemia virus. A protein that comprises amino acid sequences from different origins or naturally occurring proteins may be referred to as a fusion, or chimeric protein.

[0092] The term “polynucleotide,” “oligonucleotide,” or “nucleic acid molecule” can be any polymeric form of nucleotides, including DNA, RNA, a hybridization thereof, or RNA-DNA chimeric molecules. In some embodiments, a polynucleotide comprises cDNA, genomic DNA, mRNA, tRNA, rRNA, or microRNA. In some embodiments, a polynucleotide is double stranded, *e.g.*, a double-stranded DNA in a gene. In some embodiments, a polynucleotide is single-stranded or substantially single-stranded, *e.g.*, single-stranded DNA or an mRNA. In some embodiments, a polynucleotide is a cell-free nucleic acid molecule. In some embodiments, a polynucleotide circulates in blood. In some embodiments, a polynucleotide is a cellular nucleic acid molecule. In some embodiments, a polynucleotide is a cellular nucleic acid molecule in a cell circulating in blood.

[0093] Polynucleotides can have any three-dimensional structure. The following are nonlimiting examples of polynucleotides: a gene or gene fragment (for example, a probe, primer, EST or SAGE tag), an exon, an intron, intergenic DNA (including, without limitation, heterochromatic DNA), messenger RNA (mRNA), transfer RNA (tRNA), ribosomal RNA (rRNA), a ribozyme, cDNA, a recombinant polynucleotide, a branched polynucleotide, a plasmid, a vector, isolated DNA, isolated RNA, sgRNA, , ngRNA, guide RNA, a nucleic acid probe, a primer, an snRNA, a long non-coding RNA, a snoRNA, a siRNA, a miRNA, a tRNA-derived small RNA (tsRNA), an antisense RNA, an shRNA, or a small rDNA-derived RNA (srRNA).

[0094] In some embodiments, a polynucleotide comprises deoxyribonucleotides, ribonucleotides or analogs thereof. In some embodiments, a polynucleotide comprises 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.

[0095] In some embodiments, a polynucleotide is composed of a specific sequence of four nucleotide bases: adenine (A); cytosine (C); guanine (G); thymine (T); and uracil (U) for thymine when the polynucleotide is RNA. In some embodiments, the polynucleotide may comprise one or more other nucleotide bases, such as inosine (I), which is read by the translation machinery as guanine (G).

[0096] As used herein, the terms “modified” or “modification” refers to chemical modification with respect to the A, C, G, T and U nucleotides. In some embodiments, modifications may be on the nucleoside base and/or sugar portion of the nucleosides that comprise the polynucleotide. In some embodiments, the modification may be on the internucleoside linkage (*e.g.*, phosphate backbone). In some embodiments, multiple modifications are included in the modified nucleic acid molecule. In some embodiments, a single modification is included in the modified nucleic acid molecule.

[0097] In some embodiments, the terms “modified” or “modification” refers to RNA modifications with respect incorporation of inverted nucleotides in the PEGRNA or ngRNA sequences, or non-nucleotide modifications wherein PEGRNA or ngRNA sequences of nucleotides can be interrupted by non-nucleotide components, or further modified after polymerization, such as by conjugation with a labeling component.

[0098] The term “complement, “complementary,” or “complementarity” as used herein, refers to the ability of two polynucleotide molecules to base pair with each other.

Complementary polynucleotides may base pair via hydrogen bonding, which may be Watson Crick, Hoogsteen or reversed Hoogsteen hydrogen bonding. For example, an adenine on one polynucleotide molecule will base pair to a thymine or uracil on a second polynucleotide molecule and a cytosine on one polynucleotide molecule will base pair to a guanine on a second polynucleotide molecule. Two polynucleotide molecules are complementary to each other when a first polynucleotide molecule comprising a first nucleotide sequence can base pair with a second polynucleotide molecule comprising a second nucleotide sequence. For instance, the two DNA molecules 5'-ATGC-3' and 5'-GCAT-3' are complementary, and the

complement of the DNA molecule 5'-ATGC-3' is 5'-GCAT-3'. A percentage of complementarity indicates the percentage of nucleotides in a polynucleotide molecule which can base pair with a second polynucleotide molecule (*e.g.*, 5, 6, 7, 8, 9, 10 out of 10 being 50%, 60%, 70%, 80%, 90%, and 100% complementary, respectively). "Perfectly complementary" means that all the contiguous nucleotides of a polynucleotide molecule will base pair with the same number of contiguous nucleotides in a second polynucleotide molecule. "Substantially complementary" as used herein refers to a degree of complementarity that can be 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, or 99% over all or a portion of two polynucleotide molecules. In some embodiments, the portion of complementarity may be a region of 10, 15, 20, 25, 30, 35, 40, 45, 50, or more nucleotides. "Substantial complementary" can also refer to a 100% complementarity over a portion of two polynucleotide molecules. In some embodiments, the portion of complementarity between the two polynucleotide molecules is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, or 99% of the length of at least one of the two polynucleotide molecules or a functional or defined portion thereof.

[0099] The term "subject" and its grammatical equivalents as used herein may refer to a human or a non-human. A subject may be a mammal. A human subject may be male or female. A human subject may be of any age. A subject may be a human embryo. A human subject may be a newborn, an infant, a child, an adolescent, or an adult. A human subject may be up to about 100 years of age. A human subject may be in need of treatment for a genetic disease or disorder.

[00100] The terms "treatment" or "treating" and their grammatical equivalents may refer to the medical management of a subject with an intent to cure, ameliorate, or ameliorate a symptom of, a disease, condition, or disorder. Treatment may include active treatment, that is, treatment directed specifically toward the improvement of a disease, condition, or disorder. Treatment may include causal treatment, that is, treatment directed toward removal of the cause of the associated disease, condition, or disorder. In addition, this treatment may include palliative treatment, that is, treatment designed for the relief of symptoms rather than the curing of the disease, condition, or disorder. Treatment may include supportive treatment, that is, treatment employed to supplement another specific therapy directed toward the improvement of the disease, condition, or disorder. In some embodiments, a condition may be pathological. In some embodiments, a treatment may not completely cure or prevent a disease, condition, or disorder. In some embodiments, a treatment ameliorates, but does not completely cure or prevent a disease, condition, or disorder. In some embodiments, a subject

may be treated for 12 hours, 24 hours, 2 days, 3 days, 4 days, 5 days, 6 days, 7 days, 2 weeks, 3 weeks, 4 weeks, 2 months, 3 months, 4 months, 5 months, 6 months, 1 year, 2 years, 3 years, 4 years, 5 years, 6 years, indefinitely, or life of the subject.

[00101] The term “ameliorate” and its grammatical equivalents means to decrease, suppress, attenuate, diminish, arrest, or stabilize the development or progression of a disease.

[00102] The terms “prevent” or “preventing” means delaying, forestalling, or avoiding the onset or development of a disease, condition, or disorder for a period of time. Prevent also means reducing risk of developing a disease, disorder, or condition. Prevention includes minimizing or partially or completely inhibiting the development of a disease, condition, or disorder. In some embodiments, a composition, *e.g.*, a pharmaceutical composition, prevents a disorder by delaying the onset of the disorder for 12 hours, 24 hours, 2 days, 3 days, 4 days, 5 days, 6 days, 7 days, 2 weeks, 3 weeks, 4 weeks, 2 months, 3 months, 4 months, 5 months, 6 months, 1 year, 2 years, 3 years, 4 years, 5 years, 6 years, indefinitely, or life of a subject.

[00103] The term “effective amount” or “therapeutically effective amount” may refer to a quantity of a composition, for example a composition comprising a construct, that can be sufficient to result in a desired activity upon introduction into a subject as disclosed herein. An effective amount of the prime editing compositions can be provided to the target gene or cell, whether the cell is *ex vivo* or *in vivo*.

[00104] An effective amount can be the amount to induce, for example, at least about a 2-fold change (increase or decrease) or more in the amount of target nucleic acid modulation (*e.g.*, expression of a gene to produce functional a protein) observed relative to a negative control. An effective amount or dose can induce, for example, about 2-fold increase, about 3-fold increase, about 4-fold increase, about 5-fold increase, about 6-fold increase, about 7-fold increase, about 8-fold increase, about 9-fold increase, about 10-fold increase, about 25-fold increase, about 50-fold increase, about 100-fold increase, about 200-fold increase, about 500-fold increase, about 700-fold increase, about 1000-fold increase, about 5000-fold increase, or about 10,000-fold increase in target gene modulation (*e.g.*, expression of a target gene to produce a functional protein).

[00105] The amount of target gene modulation may be measured by any suitable method known in the art. In some embodiments, the “effective amount” or “therapeutically effective amount” is the amount of a composition that is required to ameliorate the symptoms of a disease relative to an untreated patient. In some embodiments, an effective amount is the amount of a composition sufficient to introduce an alteration in a gene of interest in a cell (*e.g.*, a cell *in vitro* or *in vivo*).

[00106] For purposes of this disclosure, the chemical elements are identified in accordance with the Periodic Table of the Elements, CAS version, Handbook of Chemistry and Physics, 75th Ed. Additionally, general principles of organic chemistry are described in “Organic Chemistry,” Thomas Sorrell, University Science Books, Sausalito: 1999, and “March’s Advanced Organic Chemistry,” 5th Ed., Ed.: Smith, M.B. and March, J., John Wiley and Sons, New York: 2001, the entire contents of which are hereby incorporated by reference.

Modified PEgRNAs and ngRNAs

[00107] An aspect of the disclosure provides a modified prime editing guide RNA (PEgRNA) comprising, 5’ to 3’:

- (a) a spacer that comprises a region of complementarity to a search target sequence in a target strand of a double-stranded target DNA
- (b) a guide RNA (gRNA) core capable of binding to a Cas protein wherein the gRNA core comprises, in 5’ to 3’ order, a tetraloop, a first stem loop (SL1), a second stem loop (SL2), and a third stem loop (SL3);
- (c) an extension arm comprising:
 - (i) an editing template that comprises an intended edit compared to the double-stranded target DNA, and
 - (ii) a primer binding site (PBS) that comprises a region of complementarity to a region upstream of a nick site in a non-target strand of the double-stranded target DNA;
- (d) wherein the PEgRNA comprises 5 or more nucleotide modifications.

[00108] Another aspect of the disclosure provides a modified prime editor nicking guide RNA (ngRNA) comprising, 5’ to 3’:

- (a) a spacer that comprises a region of complementarity to a search target sequence in a target strand of a double-stranded target DNA;
- (b) a guide RNA (gRNA) core capable of binding to a Cas protein wherein the gRNA core comprises, in 5’ to 3’ order, a tetraloop, a first stem loop (SL1), a second stem loop (SL2), and a third stem loop (SL3); and
- (c) wherein the ngRNA comprises 5 or more nucleotide modifications.

[00109] In some embodiments, the nucleotide modification is located in the 3’ end.

[00110] In some embodiments, the nucleotide modification is located in the 5’ end.

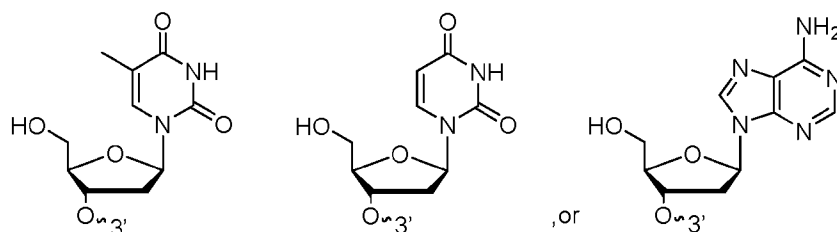
- [00111] In some embodiments, nucleotide modifications are located in the 3' end and the 5' end.
- [00112] In some embodiments, the nucleotide modification is located in the spacer.
- [00113] In some embodiments, the nucleotide modification is located in the gRNA core.
- [00114] In some embodiments, the nucleotide modification is located in the extension arm.
- [00115] In some embodiments, the nucleotide modification is located in the tetraloop.
- [00116] In some embodiments, the nucleotide modification is located in the SL1.
- [00117] In some embodiments, the nucleotide modification is located in the SL2.
- [00118] In some embodiments, the nucleotide modification is located in the SL3.
- [00119] In some embodiments, at least 2 or more nucleotide modifications are located in the 5' end, 3' end, tetraloop, SL2, or SL3.
- [00120] In some embodiments, the nucleotide modifications are located in the tetraloop, SL1, SL2, and SL3.
- [00121] In some embodiments, nucleotide modifications are located in the tetraloop, SL2, and SL3.
- [00122] In some embodiments, the nucleotide modifications are located in the 5' end, 3' end, tetraloop, SL2, and SL3.
- [00123] In some embodiments, at least 2 or more nucleotide modifications occur within the gRNA.
- [00124] In some embodiments, the gRNA core comprises a nucleotide modification at nucleotide 25, 37, 38, 46, 64, 65, 67, or 68.
- [00125] In some embodiments, the gRNA core comprises a nucleotide modification at nucleotide 25.
- [00126] In some embodiments, the gRNA core comprises a nucleotide modification at nucleotide 37 and/or 38.
- [00127] In some embodiments, the gRNA core comprises a nucleotide modification at nucleotide 65.
- [00128] In some embodiments, the gRNA core comprises a nucleotide modification at nucleotide 68.
- [00129] In some embodiments, the PEgRNA comprises 10 or more nucleotide modifications.

- [00130] In some embodiments, the PEgRNA comprises 20 or more nucleotide modifications.
- [00131] In some embodiments, the PEgRNA comprises 30 or more nucleotide modifications.
- [00132] In some embodiments, the PEgRNA comprises 40 or more nucleotide modifications.
- [00133] In some embodiments, the PEgRNA comprises 50 or more nucleotide modifications.
- [00134] In some embodiments, the PEgRNA comprises 60 or more nucleotide modifications.
- [00135] In some embodiments, the PEgRNA comprises 70 or more nucleotide modifications.
- [00136] In some embodiments, the PEgRNA comprises 80 or more nucleotide modifications.
- [00137] In some embodiments, the PEgRNA comprises 90 or more nucleotide modifications.
- [00138] In some embodiments, the PEgRNA comprises 100 or more modified nucleotides.
- [00139] In some embodiments, a nucleotide modification comprises a phosphate modification, a base modification, a sugar modification, or a combination thereof.
- [00140] In some embodiments, a nucleotide modification comprises a base modification.
- [00141] In some embodiments, the base modification is selected from the group consisting of N⁶-methyladenosine (m⁶A), N⁶-methyl-2'-O-methyladenosine (2'OMe-m⁶A), xanthine, allyaminouracil, allyaminothymidine, hypoxanthine, digoxigeninated adenine, digoxigeninated cytosine, digoxigeninated guanine, digoxigeninated uracil, 6-chloropurineriboside, N6-methyladenine, methylpseudouracil, 2-thiocytosine, 2-thiouracil, 5-methyluracil, 4-thiothymidine, 4-thiouracil, 5,6-dihydro-5-methyluracil, 5,6-dihydrouracil, 5-[(3-Indolyl)propionamide-N-allyl]uracil, 5-aminoallylcytosine, 5-aminoallyluracil, 5-bromouracil, 5-bromocytosine, 5-carboxycytosine, 5-carboxymethylesteruracil, 5-carboxyuracil, 5-fluorouracil, 5-formylcytosine, 5-formyluracil, 5-hydroxycytosine, 5-hydroxymethylcytosine, 5-hydroxymethyluracil, 5-hydroxyuracil, 5-iodocytosine, 5-iodouracil, 5-methoxycytosine, 5-methoxyuracil, 5-methylcytosine, 5-methyluracil, 5-propargylaminocytosine, 5-propargylaminouracil, 5-propynylcytosine, 5-propynyluracil, 6-

azacytosine, 6-azauracil, 6- chloropurine, 6-thioguanine, 7-deazaadenine, 7-deazaguanine, 7-deaza-7-propargylaminoadenine, 7- deaza-7-propargylaminoguanine, 8-azaadenine, 8-azidoadenine, 8-chloroadenine, 8-oxoadenine, 8- oxoguanine, araadenine, aracytosine, araguanine, arauracil, biotin- 16-7-deaza-7- propargylaminoguanine, biotin- 16-aminoallylcytosine, biotin- 16-aminoallyluracil, cyanine 3-5- propargylaminocytosine, cyanine 3-6-propargylaminouracil, cyanine 3-aminoallylcytosine, cyanine 3-aminoallyluracil, cyanine 5-6-propargylaminocytosine, cyanine 5-6-propargylaminouracil, cyanine 5-aminoallylcytosine, cyanine 5-aminoallyluracil, cyanine 7-aminoallyluracil, dabcy1-5-3 - aminoallyluracil, desthiobiotin- 16-aminoallyl-uracil, desthiobiotin-6-aminoallylcytosine, isoguanine, N¹ -ethylpseudouracil, N¹-methoxymethylpseudouracil, N¹-methyladenine, N¹-methylpseudouracil, N¹-propylpseudouracil, N²-methylguanine, N⁴-biotin-OBFA-cytosine, N⁴-methylcytosine, N⁶- methyladenine, O⁶-methylguanine, pseudoisocytosine, pseudouracil, thienocytosine, thienoguanine, thienouracil, xanthosine, 3-deazaadenine, 2,6-diaminoadenine, 2,6-daminoguanine, 5-carboxamide- uracil, 5-ethynyluracil, N⁶-isopentenyladenine (i⁶A), 2-methyl-thio- N⁶-isopentenyladenine (ms2i⁶A), 2-methylthio- N⁶-methyladenine (ms2m⁶A), N⁶-(cis-hydroxyisopentenyl)adenine (io⁶A), 2-methylthio- N⁶-(cis-hydroxyisopentenyl)adenine (ms2io⁶A), N⁶-glyciny1carbamoyladenine (g⁶A), N⁶-threony1carbamoyladenine (t⁶A), 2-methylthio- N⁶-threony1 carbamoyladenine (ms2t⁶A), N⁶-methyl- N⁶-threony1carbamoyladenine (m⁶t⁶A), N⁶-hydroxynorvalylcarbamoyladenine (hn⁶A), 2- methylthio- N⁶-hydroxynorvalyl carbamoyladenine (ms2hn⁶A), N⁶, N⁶-dimethyladenine (m⁶2A), and N⁶-acetyladenine (ac⁶A), or a combination thereof.

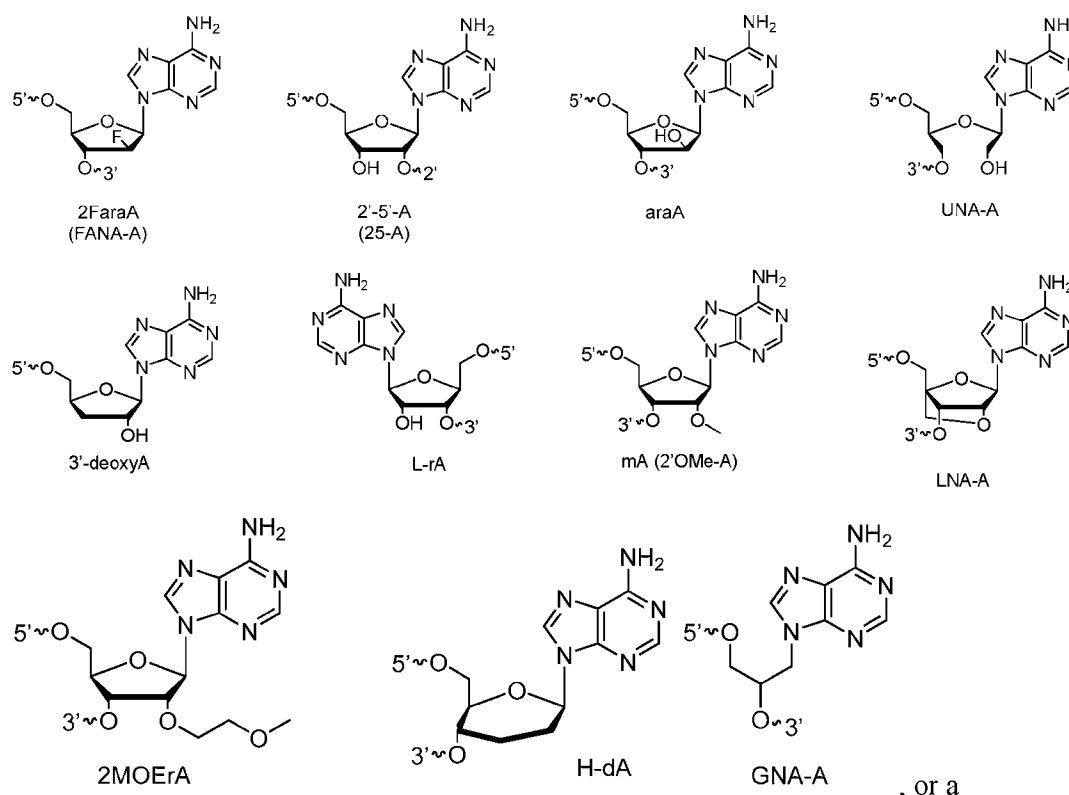
[00142] In some embodiments, the modification comprises an inverted nucleotide located in the 3' end.

[00143] In some embodiments, the inverted nucleotide comprises



[00144] In some embodiments, the nucleotide modification comprises a sugar modification.

[00145] In some embodiments, the sugar modification comprises



combination thereof. In some embodiments, the sugar modification is selected from the structures above with substituted bases (e.g. guanine, cytosine, thymine, and uracil is used in place of adenine).

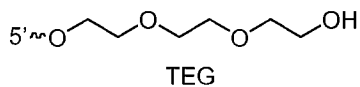
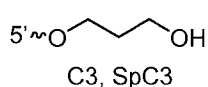
[00146] In some embodiments, the sugar modification is selected from the group consisting of 2'-thioribose, 2', 3'-dideoxyribose, 2'-amino-2'-deoxyribose, 2'-deoxyribose, 2'-azido-2'-deoxyribose, 2'-fluoro-2'-deoxyribose, 2'-O-methoxyethyl (MOE), 2'-O-methylribose, 2'-O-methyldeoxyribose, 3'-amino-2',3'-dideoxyribose, 3'-azido-2', 3'-dideoxyribose, 3'-deoxyribose, 3'-O-(2-nitrobenzyl)-2'-deoxyribose, 3'-O-methylribose, 5'-aminoribose, 5'-thioribose, 5-nitro-l-indolyl-2'-deoxyribose, 5'-biotin-ribose, 2'-O,4'-C-methylene-linked ribose, 2'-O,4'-C-amino-linked ribose, and 2'-O,4'-C-thio-linked ribose.

[00147] In some embodiments, the nucleotide modification comprises a phosphate modification.

[00148] In some embodiments, the phosphate modification comprises phosphorothioate, a stereospecific phosphorothioate, phosphorodithioate, thiophosphate, 5'-O-methylphosphonate, 3'-O-methylphosphonate, 5'-hydroxyphosphonate, hydroxyphosphanate, phosphoroselenoate, selenophosphate, phosphoramidate, carbophosphonate, methylphosphonate, phenylphosphonate, ethylphosphonate, H-

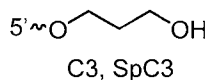
phosphonate, guanidinium ring, triazole ring, boranophosphate (BP), methylphosphonate, guanidinopropyl phosphoramidate, or a combination thereof.

[00149] In some embodiments, the non-nucleotide modification comprises a hairpin, a C₂-C₁₂ linker, an ethylene glycol linker, 2'-5' linkages, or a combination thereof. In some embodiments, the non-nucleotide modification comprises a hairpin,

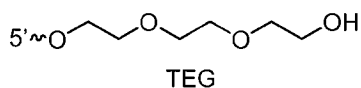


, Tetra ethylene glycol, 2'-5'

linkages, or a combination thereof. In some embodiments, the non-nucleotide modification comprises a hairpin. In some embodiments, the non-nucleotide modification comprises a C₂-C₆ linker. In some embodiments, the non-nucleotide modification comprises a C₂ linker, a C₃ linker, a C₄ linker, a C₅ linker, a C₆ linker, a C₇ linker, a C₈ linker, a C₉ linker, a C₁₀ linker, a C₁₁ linker, or a C₁₂ linker. In some embodiments, the non-nucleotide modification comprises



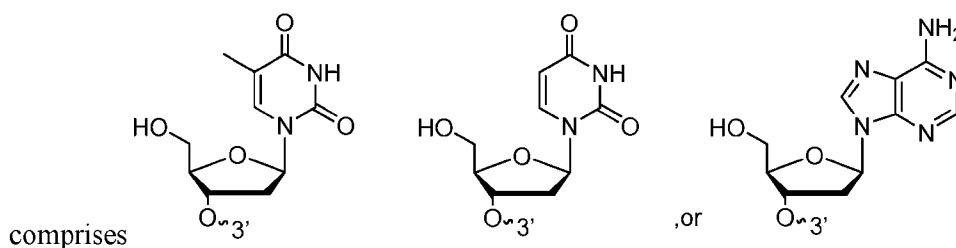
. In some embodiments, the non-nucleotide modification comprises an ethylene glycol linker. In some embodiments, the ethylene glycol linker comprises from one to twelve ethylene glycol moieties. In some embodiments, the ethylene glycol linker comprises a single ethylene glycol moiety (e.g., -OCH₂CH₂-). In some embodiments, the ethylene glycol linker comprises a di-, tri-, tetra-, penta-, hexa-, hepta-, octa-, nona-, or deca-ethylene glycol linker. In some embodiments, the ethylene glycol linker comprises 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, or 12 ethylene glycol moieties. In some embodiments, the non-nucleotide



modification comprises . In some embodiments, the non-nucleotide modification comprises tetra-ethylene glycol.

[00150] In some embodiments, the non-nucleotide modification comprises an abasic deoxyribonucleotide, an abasic ribonucleotide, an abasic 2'-substituted ribonucleotide or a combination thereof.

[00151] In some embodiments, the modification comprises an inverted nucleotide



[00152] In some embodiments, the prime editor comprises a Cas protein and a DNA polymerase.

[00153] In some embodiments, the modified nucleic acid sequence comprises about 15 nucleotides to about 180 nucleotides. In some embodiments, the modified nucleic acid sequence comprises about 15 nucleotides to about 150 nucleotides. In some embodiments, the the modified nucleic acid sequence comprises about 15 nucleotides to about 120 nucleotides. In some embodiments, the the modified nucleic acid sequence comprises about 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 105, 110, 115, 120, 125, 130, 135, 140, 145, 150, 155, 160, 165, 170, 175, or 180 nucleotides.

[00154] In some embodiments, the modified nucleic acid sequence comprises about 5 to about 180 unmodified nucleotides. In some embodiments, the modified nucleic acid sequence comprises about 5 to about 150 unmodified nucleotides. In some embodiments, the modified nucleic acid sequence comprises about 5 to about 120 unmodified nucleotides. In some embodiments, the modified nucleic acid sequence comprises about 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 105, 110, 115, 120, 125, 130, 135, 140, 145, 150, 155, 160, 165, 170, 175, or 180 unmodified nucleotides.

[00155] In some embodiments, the modified nucleic acid sequence comprises from 1 to about 25 modifications. In some embodiments, the modified nucleic acid sequence comprises from 1 to about 20 modifications. In some embodiments, the modified nucleic acid sequence comprises from about 5 to about 25 modifications. In some embodiments, the modified nucleic acid sequence comprises from about 5 to about 25 modifications. In some embodiments the modified nucleic acid sequence comprises from 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25 modifications.

[00156] In some embodiments, the modified nucleic acid sequence further comprises a deoxyribose sugar at the 3' end.

[00157] In some embodiments, the modifications are nucleotide modifications.

[00158] In some embodiments, the nucleotide modifications comprise a phosphate modification, a base modification, a sugar modification, or a combination thereof.

[00159] In some embodiments, the nucleotide modifications are the same.

[00160] In some embodiments, the nucleotide modifications are different.

[00161] In some embodiments, the modifications are non-nucleotide modifications.

[00162] In some embodiments, the non-nucleotide modifications are the same.

[00163] In some embodiments, the non-nucleotide modifications are different.

[00164] In some embodiments, the modifications are a combination of nucleotide and non-nucleotide modifications.

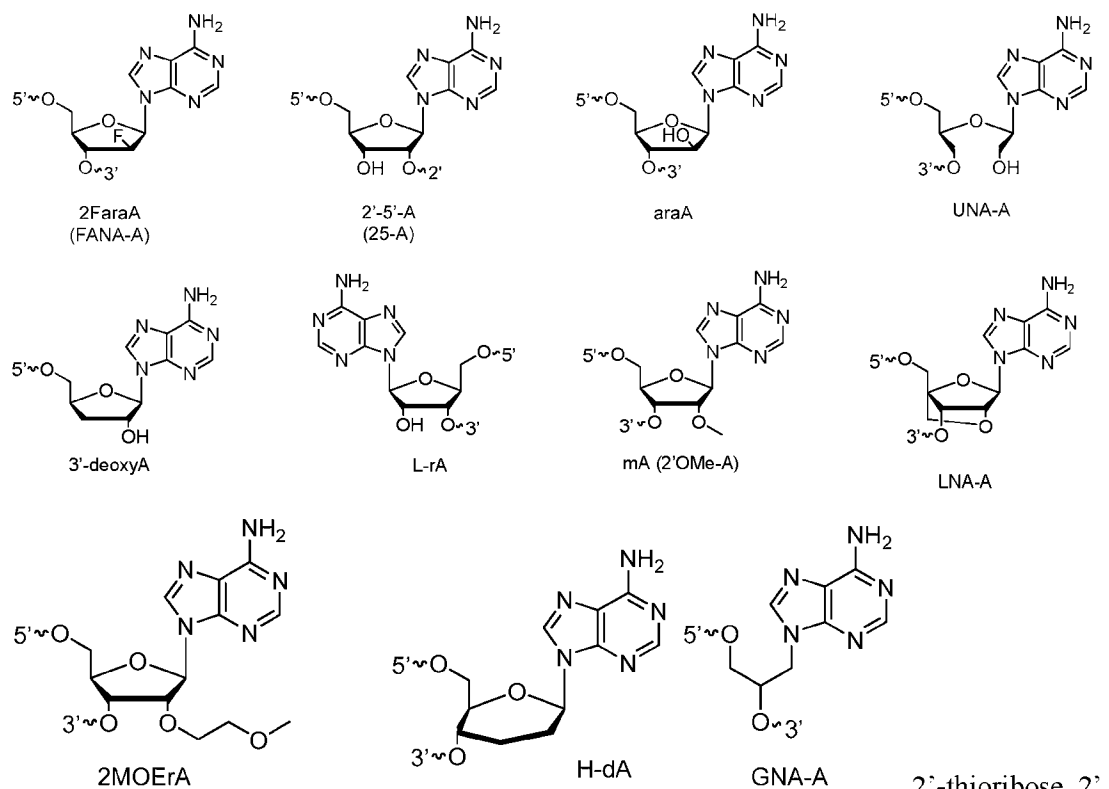
[00165] In some embodiments, the nucleotide modifications comprise a base modification.

[00166] In some embodiments, the base modification is selected from the group consisting of N⁶-methyladenosine (m⁶A), N⁶-methyl-2'-O-methyladenosine (2'OMe-m⁶A), xanthine, allyaminouracil, allyaminothymidine, hypoxanthine, digoxigeninated adenine, digoxigeninated cytosine, digoxigeninated guanine, digoxigeninated uracil, 6-chloropurineriboside, N⁶-methyladenine, methylpseudouracil, 2-thiocytosine, 2-thiouracil, 5-methyluracil, 4-thiothymidine, 4-thiouracil, 5,6-dihydro-5-methyluracil, 5,6-dihydrouracil, 5-[(3-Indolyl)propionamide-N-allyl]uracil, 5-aminoallylcytosine, 5-aminoallyluracil, 5-bromouracil, 5-bromocytosine, 5-carboxycytosine, 5-carboxymethylesteruracil, 5-carboxyuracil, 5-fluorouracil, 5-formylcytosine, 5-formyluracil, 5-hydroxycytosine, 5-hydroxymethylcytosine, 5-hydroxymethyluracil, 5-hydroxyuracil, 5-iodocytosine, 5-iodouracil, 5-methoxycytosine, 5-methoxyuracil, 5-methylcytosine, 5-methyluracil, 5-propargylaminocytosine, 5-propargylaminouracil, 5-propynylcytosine, 5-propynyluracil, 6-azacytosine, 6-azauracil, 6-chloropurine, 6-thioguanine, 7-deazaadenine, 7-deazaguanine, 7-deaza-7-propargylaminoadenine, 7-deaza-7-propargylaminoguanine, 8-azaadenine, 8-azidoadenine, 8-chloroadenine, 8-oxoadenine, 8-oxoguanine, araadenine, aracytosine, araguanine, arauracil, biotin-16-7-deaza-7-propargylaminoguanine, biotin-16-aminoallylcytosine, biotin-16-aminoallyluracil, cyanine 3-5-propargylaminocytosine, cyanine 3-6-propargylaminouracil, cyanine 3-aminoallylcytosine, cyanine 3-aminoallyluracil, cyanine 5-6-propargylaminocytosine, cyanine 5-6-propargylaminouracil, cyanine 5-aminoallylcytosine, cyanine 5-aminoallyluracil, cyanine 7-aminoallyluracil, dabcy1-5-3-aminoallyluracil, desthiobiotin-16-aminoallyluracil, desthiobiotin-6-aminoallylcytosine, isoguanine, N¹-ethylpseudouracil, N¹-methoxymethylpseudouracil, N¹-methyladenine, N¹-methylpseudouracil, N¹-propylpseudouracil, N²-methylguanine, N⁴-biotin-OBEA-cytosine, N⁴-methylcytosine, N⁶-methyladenine, O⁶-methylguanine, pseudoisocytosine, pseudouracil, thienocytosine, thienoguanine, thienouracil, xanthosine, 3-deazaadenine, 2,6-diaminoadenine, 2,6-daminoguanine, 5-carboxamide-uracil, 5-ethynyluracil, N⁶-isopentenyladenine (i⁶A), 2-methylthio-N⁶-isopentenyladenine (ms2i⁶A), 2-methylthio-N⁶-methyladenine (ms2m⁶A), N⁶-(cis-hydroxyisopentenyl)adenine (io⁶A), 2-methylthio-N⁶-(cis-hydroxyisopentenyl)adenine (ms2io⁶A), N⁶-glyciny carbamoyl adenine (g⁶A), N⁶-threonyl carbamoyl adenine (t⁶A), 2-methylthio-N⁶-threonyl carbamoyl adenine (ms2t⁶A), N⁶-

methyl- N⁶-threonylcarbamoyladenine (m⁶t⁶A), N⁶-hydroxynorvalylcarbamoyladenine (hn⁶A), 2- methylthio- N⁶-hydroxynorvalyl carbamoyladenine (ms2hn⁶A), N⁶, N⁶-dimethyladenine (m⁶2A), and N⁶-acetyladenine (ac⁶A).

[00167] In some embodiments, the nucleotide modifications comprise a sugar modification.

[00168] In some embodiments, the sugar modification comprises



, 2'-thioribose, 2', 3'-dideoxyribose, 2'-amino-2'-deoxyribose, 2'-deoxyribose, 2'-azido-2'-deoxyribose, 2'-fluoro-2'-deoxyribose, 2'-O-methoxyethyl (MOE), 2'-O-methylribose, 2'-O-methyldeoxyribose, 3'-amino-2',3'-dideoxyribose, 3'-azido-2', 3'-dideoxyribose, 3'-deoxyribose, 3'-O-(2-nitrobenzyl)-2'-deoxyribose, 3'-O-methylribose, 5'-aminoribose, 5'-thioribose, 5-nitro-l-indolyl-2'-deoxyribose, 5'-biotin-ribose, 2'-O,4'-C-methylene-linked, 2'-O,4'-C-amino-linked ribose, 2'-O,4'-C-thio-linked ribose, abasic nucleotides, abasic nucleosides, abasic 2' substituted nucleotides, or a combination thereof.

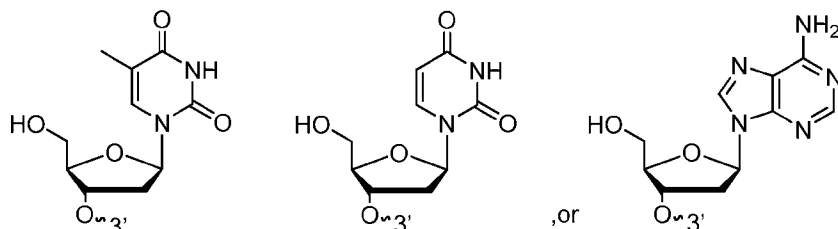
[00169] In some embodiments, the nucleotide modifications comprise a phosphate modification.

[00170] In some embodiments, the phosphate modification comprises phosphorothioate, a stereospecific phosphorothioate, phosphorodithioate, thiophosphate, 5'-O-methylphosphonate, 3'-O-methylphosphonate, 5'-hydroxyphosphonate,

hydroxyphosphanate, phosphoroselenoate, selenophosphate, phosphoramidate, carbophosphonate, methylphosphonate, phenylphosphonate, ethylphosphonate, H-phosphonate, guanidinium ring, triazole ring, boranophosphate (BP), methylphosphonate, guanidinopropyl phosphoramidate, or a combination thereof for a combination thereof.

[00171] In some embodiments, the nucleotide modifications comprise a hairpin, a C₂-C₁₂ linker, an ethylene glycol linker, 2'-5' linkages, or a combination thereof.

[00172] In some embodiments, the inverted nucleotide comprises



[00173] Another aspect of the disclosure provides a method for producing a modified PEgRNA or ngRNA, the method comprising ligating one or more modified nucleotide sequences with one or more modified nucleic acid sequences disclosed herein.

[00174] Another aspect of the disclosure provides a method for producing a modified PEgRNA or ngRNA, the method comprising ligating a nucleotide sequence encoding a prime editor with one or more modified nucleic acid sequences disclosed herein.

[00175] In some embodiments, the ligation comprises a self-templated enzymatic ligation. In some embodiments, the self-templated enzymatic ligation is splint-mediated.

[00176] In some embodiments, the ligation comprises a templated enzymatic ligation.

[00177] In some embodiments, the enzymatic ligation is catalyzed by a nucleic acid ligase to generate a natural phosphodiester linkage between the coding nucleotide sequence and the modified nucleic acid sequence of any one of the foregoing claims, or between the sequence encoding a prime editor with the modified nucleic acid sequence of any one of the foregoing claims, or between two fragments of the nucleotide sequences containing the chemical modifications of any one of the foregoing claims.

Ligation

[00178] Free 3' ends and phosphorylated 5' ends are brought into proximity by way of sequence complementarity resulting in base pairing hybridization either between the RNA fragments directly (self-templated) or via an additional complementary DNA splint (splinted). Subsequent enzymatic catalysis by a nucleic acid ligase can generate a natural phosphodiester linkage between fragments, yielding the full-length PEgRNA or ngRNA. In

some embodiments, a nucleic acid ligase is T4 RNA Ligase I, T4 RNA Ligase II, or T4 DNA Ligase.

[00179] Self-templated Ligation: In some embodiments, two functionalized, single-stranded RNA fragments with complementary base pairs are hybridized for enzymatic ligation (e.g., ligase-mediated conjugation). In some embodiments, the fragments take natural advantage of the sequence complementarity driven structure of any region within the RNA to help facilitate a very specific ligation reaction between the fragments. Once these fragments are annealed under controlled buffered conditions, the RNA duplex forms a native secondary structure mimic to the full length RNA, resulting in a net increase in the effective local concentration of the terminal 3'-OH and 5'-monophosphate functional ends required for ligase activity. Subsequent ligase catalysis forms a phosphodiester bond between the RNA fragments which covalently seals the nick and generates a full-length RNA.

[00180] Template Ligation: In some embodiments, two functionalized, single-stranded RNA fragments are conjugated with ligase, similarly to self-templated ligation. In addition, a short, complementary nucleic acid splint facilitates a trimeric secondary structure formation during templated ligation. The nucleic acid splint is designed such that it bears proximal sequence complementarity of both the 3' end of the upstream RNA fragment and 5' end of the downstream RNA fragment to facilitate a splinted trimeric structure.

Consequently, the RNA fragments may or may not require complementarity at the ligation site, and so the advantage of templated ligation for RNA synthesis is that the splinted approach enables ligation at RNA locations which lack secondary structure and anywhere in the sequence without extensions. In some embodiments, the splint is a DNA strand. In some embodiments, the splint is a modified nucleic acid strand.

ngRNA for Prime editing

[00181] As used herein, includes single guide RNA (sgRNA) or nicking guide RNA (ngRNA) are designed to have (i) a guide RNA sequence domain (designated as a nucleotide spacer sequence as disclosed) at the 5' end, which is complementary to a target DNA sequence and (ii) guide core RNA that's binds to DNA nickase enzyme or other polypeptides for form ribonucleoproteins (RNP). ngRNAs are used to guide the DNA nickase enzyme (e.g. Cas9 protein) to specific sites in the genome for targeted single stranded cleavage of DNA.

PEgRNA for Prime editing

[00182] The term "prime editing guide RNA", or "PEgRNA", refers to a guide polynucleotide that comprises one or more intended nucleotide edits for incorporation into the target double stranded DNA. In some embodiments, the PEGRNA associates with and

directs a prime editor to incorporate the one or more intended nucleotide edits into the target gene via prime editing.

[00183] In some embodiments, a PEgRNA comprises a spacer that is complementary or substantially complementary to a search target sequence on a target strand of the target gene. In some embodiments, the PEgRNA comprises a gRNA core that associates with a DNA binding domain, e.g., a CRISPR-Cas protein domain, of a prime editor. In some embodiments, the PEgRNA comprises an editing template. In some embodiments, the PEgRNA comprises a primer binding site (PBS). In some embodiments, a PEgRNA comprises an extension arm that comprises an editing template and a PBS.

[00184] As used herein in a PEgRNA sequence, or fragments thereof such as a spacer, PBS, or RTT sequence, unless indicated otherwise, it should be appreciated that the letter “T” or “thymine” indicates a nucleobase in a DNA sequence that encodes the PEgRNA or guide RNA sequence, and is intended to refer to a uracil (U) nucleobase of the PEgRNA or guide RNA or any chemically modified uracil nucleobase known in the art, such as 5-methoxyuracil.

[00185] In some embodiments, the PEgRNA or naRNA may comprise a first polynucleotide comprising the spacer and a first portion of a gRNA core referred to as a crRNA. In some embodiments, the PEgRNA comprise a second polynucleotide comprising a second portion of the gRNA core and the extension arm, wherein the second portion of the gRNA core may also be referred to as a trans-activating crRNA, or tracr RNA. In some embodiments, the crRNA portion and the tracr RNA portion of the gRNA core are at least partially complementary to each other. In some embodiments, the partially complementary portions of the crRNA and the tracr RNA form a lower stem, a bulge, and an upper stem. In some embodiments, the partially complementary portions of the crRNA and the tracr RNA form a lower stem, a bulge, and an upper stem, as exemplified in Fig. 3. Together, the crRNA:tracrRNA duplex forms the lower stem, bulge, and upper stem modules. The crRNA contains the spacer module, and the tracrRNA contains the nexus and terminal hairpins.

Spacers

[00186] As discussed above, PEgRNAs comprises a spacer complementary or substantially complementary to a separate search target sequence. In some embodiments, a PEgRNA anneals with a separate search target sequence through its spacer. In some embodiments, a PEgRNA comprises a spacer complementary to a search target sequence on a strand of a double stranded target DNA, e.g., a double stranded target gene.

[00187] In some embodiments, the PEGRNA complexes with and directs a prime editor to bind the double stranded target DNA at the position corresponding to the search target sequence.

[00188] In some embodiments, a spacer comprises a region that has substantial complementarity to a search target sequence on a target strand, or strand, of a double stranded target DNA. In some embodiments, the first spacer is substantially complementary to the search target sequence.

[00189] Exemplary sequences for PEG-Seq spacers are provided below:

Table 1

sgRNA	Sequence	Spacer
BCL11A_sgRNA	mC*mU*mA*ACAGUUGC UUUUAUCACGUUUUAGAGCUAG AAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCmU*mU*mU*U (SEQ ID NO.: 1)	CTAACAG TTGCTTT TATCAC (SEQ ID NO.: 10)
EMX1_sgRNA	mG*mA*mG*UCCGAGCAGAAGAAGAAGUUUUAGAGCUAG AAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCmU*mU*mU*U (SEQ ID NO.: 2)	GAGTCCG AGCAGAA GAAGAA (SEQ ID NO.: 11)
FANCF_sgRNA	mG*mG*mA*AUCCCUUCUGCAGCACCGUUUUAGAGCUAGAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCmU*mU*mU*U (SEQ ID NO.: 3)	GGAATCC CTTCTGC AGCACC (SEQ ID NO.: 12)
HBB_sgRNA	mG*mU*mA*ACGGCAGACUUCUCCUCGUUUUAGAGCUAG AAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCmU*mU*mU*U (SEQ ID NO.: 4)	GTAACGG CAGACTT CTCCTC (SEQ ID NO.: 13)
HEK3_sgRNA	mG*mG*mC*CCAGACUGAGCACGUGAGUUUUAGAGCUAG AAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCmU*mU*mU*U (SEQ ID NO.: 5)	GGCCCAG ACTGAGC ACGTGA (SEQ ID NO.: 14)

HEK 4_sg RNA	mG*mG*mC*ACUGCGGCUGGAGGUGGGUUUUAGAGCUAG AAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUU GAAAAAGUGGCACCGAGUCGGUGCmU*mU*mU*U (SEQ ID NO.: 6)	GGCACTG CGGCTGG AGGTGG (SEQ ID NO.: 15)
HEX A_sg RNA	mU*mA*mC*CUGAACCGUAUAUCCUAGUUUUAGAGCUAG AAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUU GAAAAAGUGGCACCGAGUCGGUGCmU*mU*mU*U (SEQ ID NO.: 7)	TACCTGA ACCGTAT ATCCTA (SEQ ID NO.: 16)
PCS K9_s gRNA	mC*mC*mA*CCGCUGCGCCAAGGUGCGUUUUAGAGCUAGA AAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUG AAAAAGUGGCACCGAGUCGGUGCmU*mU*mU*U (SEQ ID NO.: 8)	CCACCGC TGCGCCA AGGTGC (SEQ ID NO.: 17)
RNF2 _sgR NA	mG*mU*mC*AUCUUAGUCAUUACCUGGUUUUAGAGCUAG AAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUU GAAAAAGUGGCACCGAGUCGGUGCmU*mU*mU*U (SEQ ID NO.: 9)	GTCATCT TAGTCAT TACCTG (SEQ ID NO.: 18)

[00190] In some embodiments, a spacer sequence may further comprise additional nucleotides beside a region of complementarity to genomic search target sequence. For example, in some embodiments, a spacer sequence (as well as the full PEgRNA sequence) may comprise an additional G at the 5' end, for example, wherein the 5' most nucleotide of the spacer (or the PEgRNA) is not a G.

[00191] In some embodiments, the gRNA core comprises the sequence
GUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUU
GAAAAAGUGGCACCGAGUCGGUGC (SEQ ID NO.: 19), or
GUUUGAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUU
GAAAAAGUGGGACCGAGUCGGUCC (SEQ ID NO.: 20), or
GUUUAAGAGCUAUGCUGGAAACAGCAUAGCAAGUUAAAAUAAGGCUAGUCCG
UUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGC (SEQ ID NO.: 21). In some
embodiments, the gRNA core comprises the sequence
GUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUU
GAAAAAGUGGCACCGAGUCGGUGC (SEQ ID NO.: 19). In some embodiments, the
gRNA core comprises the sequence

AGAGCUAGAAAUAGCAAGUUA AAAUAAGGCUAGUCCGUUAUCAACUUGAAAA
AGUGGCACCGAGUCG (SEQ ID NO.: 22).

Guide RNA

[00192] A guide RNA core (also referred to herein as the gRNA core, gRNA scaffold, or gRNA backbone sequence) of a PEgRNA may contain a polynucleotide sequence that binds to a DNA binding domain (e.g., Cas9) of a prime editor. The gRNA core may interact with a prime editor as described herein, for example, by association with a DNA binding domain, such as a Cas9 nickase of the prime editor. One of skill in the art will recognize that different prime editors having different DNA binding domains from different DNA binding proteins may require different gRNA core sequences specific to the DNA binding protein. In some embodiments, the gRNA core is capable of binding to a Cas9-based prime editor. In some embodiments, the gRNA core is capable of binding to a Cpf1-based prime editor. In some embodiments, the gRNA core is capable of binding to a Cas12b-based prime editor.

[00193] In some embodiments, the gRNA core comprises regions and secondary structures involved in binding with specific CRISPR Cas proteins. For example, in a Cas9 based prime editing system, the gRNA core of a PEgRNA may comprise one or more regions of a base paired “lower stem” adjacent to the spacer and a base paired “upper stem” following the lower stem, where the lower stem and upper stem may be connected by a “bulge” comprising unpaired RNAs. The gRNA core may further comprise a “nexus” distal from the spacer, followed by a hairpin structure, e.g., at the 3' end. In some embodiments, the gRNA core comprises modified nucleotides as compared to a wild type gRNA core in the lower stem, upper stem, and/or the hairpin. For example, nucleotides in the lower stem, upper stem, and/or the hairpin regions may be modified, deleted, or replaced. In some embodiments, RNA nucleotides in the lower stem, upper stem, and/or the hairpin regions may be replaced with one or more DNA sequences. In some embodiments, the gRNA core comprises unmodified or wild type RNA sequences in the nexus and/or the bulge regions. In some embodiments, the gRNA core does not include long stretches of A-T pairs, for example, a GUUUU-AAAAC (SEQ ID NO.: 23) pairing element.

[00194] A guide RNA core (also referred to herein as the gRNA core, gRNA scaffold, or gRNA backbone sequence) of a PEgRNA or ngrRNA may contain a polynucleotide sequence that binds to a DNA binding domain (e.g., Cas9) of a prime editor. The gRNA core may interact with a prime editor as described herein, for example, by association with a DNA binding domain, such as a Cas9 nickase of the prime editor.

[00195] One of skill in the art will recognize that different prime editors having different DNA binding domains from different DNA binding proteins may require different gRNA core sequences specific to the DNA binding protein. In some embodiments, the gRNA core is capable of binding to a Cas9-based prime editor. In some embodiments, the gRNA core is capable of binding to a Cpf1-based prime editor. In some embodiments, the gRNA core is capable of binding to a Cas12b-based prime editor.

[00196] In some embodiments, the gRNA core comprises regions and secondary structures involved in binding with specific CRISPR Cas proteins. For example, in a Cas9 based prime editing system, the gRNA core of a PEGRNA may comprise one or more regions of a base paired “lower stem” adjacent to the spacer and a base paired “upper stem” following the lower stem, where the lower stem and upper stem may be connected by a “bulge” comprising unpaired RNAs. The gRNA core may further comprise a “nexus” distal from the spacer, followed by a hairpin structure, e.g., at the 3' end. In some embodiments, the gRNA core comprises modified nucleotides as compared to a wild type gRNA core in the lower stem, upper stem, and/or the hairpin. For example, nucleotides in the lower stem, upper stem, and/or the hairpin regions may be modified, deleted, or replaced. In some embodiments, RNA nucleotides in the lower stem, upper stem, and/or the hairpin regions may be replaced with one or more DNA sequences. In some embodiments, the gRNA core comprises unmodified or wild type RNA sequences in the nexus and/or the bulge regions. In some embodiments, the gRNA core does not include long stretches of A-T pairs, for example, a GUUUU-AAAAC (SEQ ID NO.: 23) pairing element.

[00197] In some embodiments, the gRNA core comprises the sequence,
 GUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUU
 GAAAAAGUGGCACCGAGUCGGUGC (SEQ ID NO.: 19), or
 GUUUGAGAGCUAGAAAUAGCAAGUUUAAAAUAAGGCUAGUCCGUUAUCAACUU
 GAAAAAGUGGGACCGAGUCGGUCC (SEQ ID NO.: 20), or
 GUUUUAGAGCUAUGCUGGAAACAGCAUAGCAAGUUUAAAAUAAGGCUAGUCCG
 UUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGC (SEQ ID NO.: 21). In some
 embodiments, the gRNA core comprises the sequence,
 GUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUU
 GAAAAAGUGGCACCGAGUCGGUGC (SEQ ID NO.: 19). In some embodiments, the
 gRNA core comprises the sequence,
 AGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAA
 AGUGGCACCGAGUCG (SEQ ID NO.: 22).

[00198] Any gRNA core sequences known in the art are also contemplated in the prime editing compositions described herein. In some embodiments, one or more nucleotides in the gRNA core is DNA.

Prime Editor

[00199] The term “prime editor (PE)” refers to the polypeptide or polypeptide components involved in prime editing, or any polynucleotide(s) encoding the polypeptide or polypeptide components. In various embodiments, a prime editor includes a polypeptide domain having DNA binding activity and a polypeptide domain having DNA polymerase activity. In some embodiments, the polypeptide domain having DNA binding activity is a polypeptide domain having programmable DNA binding activity. In some embodiments, the prime editor further comprises a polypeptide domain having nuclease activity. In some embodiments, the polypeptide domain having DNA binding activity comprises a nuclease domain or nuclease activity. In some embodiments, the polypeptide domain having nuclease activity comprises a nickase, or a fully active nuclease. In some embodiments, the polypeptide domain having DNA polymerase activity comprises a template-dependent DNA polymerase, for example, a DNA-dependent DNA polymerase or an RNA-dependent DNA polymerase. In some embodiments, the DNA polymerase is a reverse transcriptase. In some embodiments, the prime editor comprises additional polypeptides or polypeptide domains involved in prime editing, for example, a polypeptide domain having 5' endonuclease activity, *e.g.*, a 5' endogenous DNA flap endonucleases (*e.g.*, FEN1), for helping to drive the prime editing process towards the edited product formation. In some embodiments, the prime editor further comprises an RNA-protein recruitment polypeptide, for example, a MS2 coat protein.

[00200] A prime editor may be engineered. In some embodiments, the polypeptide components of a prime editor do not naturally occur in the same organism or cellular environment. In some embodiments, the polypeptide components of a prime editor may be of different origins or from different organisms. In some embodiments, a prime editor comprises a DNA binding domain and a DNA polymerase domain that are derived from different species. In some embodiments, a prime editor comprises a Cas polypeptide and a reverse transcriptase polypeptide that are derived from different species. For example, a prime editor may comprise a *S. pyogenes* Cas9 polypeptide and a Moloney murine leukemia virus (M-MLV) reverse transcriptase polypeptide.

[00201] In some embodiments, polypeptide domains of a prime editor may be fused or linked by a peptide linker to form a fusion protein.

[00202] The term “prime editor complex” is used interchangeably with the term “prime editing complex” and refers to a complex comprising one or more prime editor components (e.g., a polypeptide domain having DNA binding activity and a polypeptide domain having DNA polymerase activity) complexed with a PEgRNA.

[00203] In some embodiments, a prime editor comprises a M-MLV RT variant, wherein the M-MLV RT variant consists of the following amino acid sequence:
 TLNIEDEYRLHETSKEPDVSLGSTWLSDFPQAWAETGGMGLAVRQAPLIPLKATSTP
 VSIKQYPMSQEARLGIKPHIQRLLDQGILVPCQSPWNTPLL PVKKPGTNDYRPVQDLR
 EVNKRVEDIHPTVPNPYNLLSGLPPSHQWYTVLDLKDAFFCLRLHPTSQPLFAFEWR
 DPEMGISGQLTWTRL PQGFKNSPTLFNEALHRDLADFRIQHPDLILLQYVDDLLLAAT
 SELDCQQGTRALLQTLGNLGYRASAKKAQICQKQVKYLGYLLKEGQRWLTEARKE
 TVMGQPTPKTPRQLREFLGKAGFCRLFIPGFAEMAAPLYPLTKPGTLFNWGPDQKKA
 YQEIKQALLTAPALGLPDLTKPFELFVDEKQGYAKGVLTQKLGPWRRPVAYLSKKL
 DPVAAGWPPCLRMVAAIAVLTKDAGKLTMGQPLVILAPHAVEALVKQPPDRWLSN
 ARMTHYQALLLDTDRVQFGPVVALNPATLLPLPEEGLQHNCLDNSR LIN (SEQ ID
 NO.: 24).

[00204] In some embodiments, a prime editor comprises a eukaryotic RT, for example, a yeast, drosophila, rodent, or primate RT. In some embodiments, the prime editor comprises a Group II intron RT, for example, a *Geobacillus stearothermophilus* Group II Intron (GSI-IIIC) RT or a Eubacterium rectale group II intron (Eu.re.I2) RT. In some embodiments, the prime editor comprises a retron RT.

Programmable DNA Binding Domain

[00205] In some embodiments, the DNA-binding domain of a prime editor is a programmable DNA binding domain. A programmable DNA binding domain refers to a protein domain that is designed to bind a specific nucleic acid sequence, e.g., a target DNA or a target RNA. In some embodiments, the DNA-binding domain is a polynucleotide programmable DNA-binding domain that can associate with a guide polynucleotide (e.g., a PEgRNA) that guides the DNA-binding domain to a specific DNA sequence, e.g., a search target sequence in a target gene. In some embodiments, the DNA-binding domain comprises a Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) Associated (Cas) protein. A Cas protein may comprise any Cas protein described herein or a functional fragment or functional variant thereof. In some embodiments, a DNA-binding domain may also comprise a zinc-finger protein domain. In other cases, a DNA-binding domain comprises a transcription activator-like effector domain (TALE). In some embodiments, the DNA-

binding domain comprises a DNA nuclease. For example, the DNA-binding domain of a prime editor may comprise an RNA-guided DNA endonuclease, *e.g.*, a Cas protein. In some embodiments, the DNA-binding domain comprises a zinc finger nuclease (ZFN) or a transcription activator like effector domain nuclease (TALEN), where one or more zinc finger motifs or TALE motifs are associated with one or more nucleases, *e.g.*, a Fok I nuclease domain.

[00206] In some embodiments, the DNA-binding domain comprise a nuclease activity. In some embodiments, the DNA-binding domain of a prime editor comprises an endonuclease domain having single strand DNA cleavage activity. For example, the endonuclease domain may comprise a FokI nuclease domain. In some embodiments, the DNA-binding domain of a prime editor comprises a nuclease having full nuclease activity. In some embodiments, the DNA-binding domain of a prime editor comprises a nuclease having modified or reduced nuclease activity as compared to a wild type endonuclease domain. For example, the endonuclease domain may comprise one or more amino acid substitutions as compared to a wild type endonuclease domain. In some embodiments, the DNA-binding domain of a prime editor has nickase activity. In some embodiments, the DNA-binding domain of a prime editor comprises a Cas protein domain that is a nickase with single stranded DNA nicking activities. In some embodiments, compared to a wild type Cas protein, the Cas nickase comprises one or more amino acid substitutions in a nuclease domain that reduces or abolishes its double strand nuclease activity but retains DNA binding activity. In some embodiments, the Cas nickase comprises an amino acid substitution in a HNH domain. In some embodiments, the Cas nickase comprises an amino acid substitution in a RuvC domain.

[00207] In some embodiments, the DNA-binding domain comprises a CRISPR associated protein (Cas protein) domain.

[00208] A Cas protein may be a Class 1 or a Class 2 Cas protein. A Cas protein can be a type I, type II, type III, type IV, type V Cas protein, or a type VI Cas protein. Non-limiting examples of Cas proteins include Cas9, Cas12a (Cpf1), Cas12e (CasX), Cas12d (CasY), Cas12b1 (C2c1), Cas12b2, Cas12c (C2c3), C2c4, C2c8, C2c5, C2c10, C2c9, Cas14a, Cas14b, Cas14c, Cas14d, Cas14e, Cas14f, Cas14g, Cas14h, Cas14u, Cns2, Cas Φ , and homologs, functional fragments, or modified versions thereof. A Cas protein can be a chimeric Cas protein that is fused to other proteins or polypeptides. A Cas protein can be a chimera of various Cas proteins, for example, comprising domains of Cas proteins from different organisms.

[00209] A Cas protein, *e.g.*, Cas9, can be from any suitable organism. In some aspects, the organism is *Streptococcus pyogenes* (*S. pyogenes*). In some aspects, the organism is *Staphylococcus aureus* (*S. aureus*). In some aspects, the organism is *Streptococcus thermophilus* (*S. thermophilus*). In some embodiments, the organism is *Staphylococcus lugdunensis*.

[00210] A Cas protein, *e.g.*, Cas9, may comprise one or more domains. Non-limiting examples of Cas domains include, guide nucleic acid recognition and/or binding domain, nuclease domains (*e.g.*, DNase or RNase domains, RuvC, HNH), DNA binding domain, RNA binding domain, helicase domains, protein-protein interaction domains, and dimerization domains. In various embodiments, a Cas protein comprises a guide nucleic acid recognition and/or binding domain that can interact with a guide nucleic acid, and one or more nuclease domains that comprise catalytic activity for nucleic acid cleavage.

[00211] In some embodiments, a prime editor comprises a Cas nickase that can bind to the target gene in a sequence-specific manner and generate a single-strand break at a protospacer within double-stranded DNA in the target gene, but not a double-strand break. For example, the Cas nickase can cleave the edit strand (*i.e.*, the PAM strand) or the non-edit strand of the target gene, but may not cleave both. In some embodiments, a prime editor comprises a Cas nickase comprising two nuclease domains (*e.g.*, Cas9), with one of the two nuclease domains modified to lack catalytic activity or deleted. In some embodiments, the Cas nickase of a prime editor comprises a nuclease inactive RuvC domain and a nuclease active HNH domain. In some embodiments, the Cas nickase of a prime editor comprises a nuclease inactive HNH domain and a nuclease active RuvC domain. In some embodiments, a prime editor comprises a Cas9 nickase having an amino acid substitution in the RuvC domain. In some embodiments, the Cas9 nickase comprises a D10X amino acid substitution compared to a wild type *S. pyogenes* Cas9, wherein X is any amino acid other than D. In some embodiments, a prime editor comprises a Cas9 nickase having an amino acid substitution in the HNH domain. In some embodiments, the Cas9 nickase comprises a H840X amino acid substitution compared to a wild type *S. pyogenes* Cas9, wherein X is any amino acid other than H.

[00212] In some embodiments, a prime editor comprises a Cas protein, *e.g.*, Cas9, containing modifications that allow altered PAM recognition. In prime editing using a Cas-protein-based prime editor, a “*protospacer* adjacent motif (PAM)”, PAM sequence, or PAM-like motif, may be used to refer to a short DNA sequence immediately following the protospacer on the PAM strand of the target gene. In some embodiments, the PAM is

recognized by the Cas nuclease in the prime editor during prime editing. In certain embodiments, the PAM is required for target binding of the Cas protein. The specific PAM sequence required for Cas protein recognition may depend on the specific type of the Cas protein. A PAM can be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more nucleotides in length. In some embodiments, a PAM is between 2-6 nucleotides in length. In some embodiments, the PAM can be a 5' PAM (*i.e.*, located upstream of the 5' end of the protospacer). In other embodiments, the PAM can be a 3' PAM (*i.e.*, located downstream of the 5' end of the protospacer). In some embodiments, the Cas protein of a prime editor recognizes a canonical PAM, for example, a SpCas9 recognizes 5'-NGG-3' PAM. In some embodiments, the Cas protein of a prime editor has altered or non-canonical PAM specificities.

Prime Editing

[00213] Another aspect of the present disclosure includes a prime editing system comprising: (a) a prime editing guide RNA (PEgRNA), single guide RNA (sgRNA), nicking guide RNA (ngRNA) or one or more polynucleotides encoding the PEgRNA; and (b) a modified mRNA disclosed herein or the modified mRNA produced by the methods described herein. In some embodiments, the modified mRNA encodes a prime editor. In some embodiments, the prime editor comprises a DNA binding domain and a DNA polymerase domain. In some embodiments, the DNA binding domain is a CRISPR associated (Cas) protein domain.

[00214] The term “prime editing” refers to programmable editing of a target DNA using a prime editor complexed with a PEgRNA to incorporate an intended nucleotide edit into the target DNA through target-primed DNA synthesis. A target polynucleotide, *e.g.*, a target gene of prime editing may comprise a double stranded DNA molecule having two complementary strands: a first strand that may be referred to as a “target strand” or a “non-edit strand”, and a second strand that may be referred to as a “non-target strand,” or an “edit strand.” In some embodiments, in a prime editing guide RNA (PEgRNA), a spacer sequence is complementary or substantially complementary to a specific sequence on the target strand, which may be referred to as a “search target sequence”. In some embodiments, the spacer sequence anneals with the target strand at the search target sequence. The target strand may also be referred to as the “non-Protospacer Adjacent Motif (non-PAM strand).” In some embodiments, the non-target strand may also be referred to as the “PAM strand”. In some embodiments, the PAM strand comprises a protospacer sequence and optionally a protospacer adjacent motif (PAM) sequence. In prime editing using a Cas-protein-based prime editor, a PAM sequence refers to a short DNA sequence immediately adjacent to the

protospacer sequence on the PAM strand of the target gene. A PAM sequence may be specifically recognized by a programmable DNA binding protein, e.g., a Cas nickase or a Cas nuclease. In some embodiments, a specific PAM is characteristic of a specific programmable DNA binding protein, e.g., a Cas nickase or a Cas nuclease. A protospacer sequence refers to a specific sequence in the PAM strand of the target gene that is complementary to the search target sequence. In a PEGRNA, a spacer sequence may have a substantially identical sequence as the protospacer sequence on the edit strand of a target gene, except that the spacer sequence may comprise Uracil (U) and the protospacer sequence may comprise Thymine (T).

[00215] In some embodiments, the double stranded target DNA comprises a nick site on the PAM strand (or non-target strand). As used herein, a “nick site” refers to a specific position in between two nucleotides or two base pairs of the double stranded target DNA. In some embodiments, the position of a nick site is determined relative to the position of a specific PAM sequence. In some embodiments, the nick site is the particular position where a nick will occur when the double stranded target DNA is contacted with a nickase, for example, a Cas nickase, that recognizes a specific PAM sequence. In some embodiments, the nick site is upstream of a specific PAM sequence on the PAM strand of the double stranded target DNA. In some embodiments, the nick site is downstream of a specific PAM sequence on the PAM strand of the double stranded target DNA. In some embodiments, the nick site is 3 base pairs upstream of the PAM sequence, and the PAM sequence is recognized by a *Streptococcus pyogenes* Cas9 nickase, a *P. lavamentivorans* Cas9 nickase, a *C. diphtheriae* Cas9 nickase, a *N. cinerea* Cas9, a *S. aureus* Cas9, or a *N. lari* Cas9 nickase. In some embodiments, the nick site is 3 base pairs upstream of the PAM sequence, and the PAM sequence is recognized by a Cas9 nickase, wherein the Cas9 nickase comprises a nuclease active HNH domain and a nuclease inactive RuvC domain. In some embodiments, the nick site is 2 base pairs upstream of the PAM sequence, and the PAM sequence is recognized by a *S. thermophilus* Cas9 nickase.

[00216] In some embodiments, a PEGRNA complexes with and directs a prime editor to bind to the search target sequence of the target gene. In some embodiments, the bound prime editor generates a nick on the edit strand (PAM strand) of the target gene at the nick site. In some embodiments, a primer binding site (PBS) of the PEGRNA anneals with a free 3' end formed at the nick site, and the prime editor initiates DNA synthesis from the nick site, using the free 3' end as a primer. Subsequently, a single-stranded DNA encoded by the editing template of the PEGRNA is synthesized. In some embodiments, the newly synthesized

single-stranded DNA comprises one or more intended nucleotide edits compared to the endogenous target gene sequence. In some embodiments, the editing template of a PEGRNA is complementary to a sequence in the edit strand except for one or more mismatches at the intended nucleotide edit positions in the editing template partially complementary to the editing template may be referred to as an “editing target sequence”. Accordingly, in some embodiments, the newly synthesized single stranded DNA has identity or substantial identity to a sequence in the editing target sequence, except for one or more insertions, deletions, or substitutions at the intended nucleotide edit positions.

[00217] In some embodiments, the newly synthesized single-stranded DNA equilibrates with the editing target on the edit strand of the target gene for pairing with the target strand of the target gene. In some embodiments, the editing target sequence of the target gene is excised by a flap endonuclease (FEN), for example, FEN1. In some embodiments, the FEN is an endogenous FEN, for example, in a cell comprising the target gene. In some embodiments, the FEN is provided as part of the prime editor, either linked to other components of the prime editor or provided in *trans*. In some embodiments, the newly synthesized single stranded DNA, which comprises the intended nucleotide edit, replaces the endogenous single stranded editing target sequence on the edit strand of the target gene. In some embodiments, the newly synthesized single stranded DNA and the endogenous DNA on the target strand form a heteroduplex DNA structure at the region corresponding to the editing target sequence of the target gene. In some embodiments, the newly synthesized single-stranded DNA comprising the nucleotide edit is paired in the heteroduplex with the target strand of the target DNA that does not comprise the nucleotide edit, thereby creating a mismatch between the two otherwise complementary strands. In some embodiments, the mismatch is recognized by DNA repair machinery, *e.g.*, an endogenous DNA repair machinery. In some embodiments, through DNA repair, the intended nucleotide edit is incorporated into the target gene.

[00218] In some embodiments, the Cas protein domain has nickase activity. In some embodiments, the Cas protein domain is a Cas9. In some embodiments, the Cas9 comprises a mutation in an HNH domain. In some embodiments, the Cas9 comprises a H840A mutation in the HNH domain. In some embodiments, the Cas protein domain is a Cas12b. In some embodiments, the Cas protein domain is a Cas12a, Cas12b, Cas12c, Cas12d, Cas12e, Cas14a, Cas14b, Cas14c, Cas14d, Cas14e, Cas14f, Cas14g, Cas14h, Cas14u, or a Casp.

[00219] In some embodiments, the DNA polymerase domain is a reverse transcriptase. In some embodiments, the reverse transcriptase is a retrovirus reverse transcriptase. In some

embodiments, the reverse transcriptase is a Moloney murine leukemia virus (M-MLV) reverse transcriptase. In some embodiments, the DNA polymerase and the DNA binding domain are fused or linked to form a fusion protein.

[00220] In some embodiments, a PEgRNA comprises a gRNA core that comprises a modified direct repeat compared to the sequence of a naturally occurring CRISPR-Cas guide RNA scaffold, for example, a Cas9 gRNA scaffold. In some embodiments, the PEgRNA comprises a “flip and extension (F+E)” gRNA core, wherein one or more base pairs in a direct repeat is modified. In some embodiments, the PEgRNA comprises a first direct repeat (the first paring element or the lower stem), wherein a Uracil is changed to a Adenine (such that in the stem region, a U-A base pair is changed to a A-U base pair). In some embodiments, the PEgRNA comprises a first direct repeat wherein the fourth U-A base pair in the stem is changed to a A-U base pair. In some embodiments, the PEgRNA comprises a first direct repeat wherein one or more U-A base pair is changed to a G-C or C-G base pair. For example, in some embodiments, the PEgRNA comprises a first direct repeat comprising a modification to a GUUUU-AAAAC (SEQ ID NO.: 23) pairing element, wherein one or more of the U-A base pairs is changed to a A-U base pair, a G-C base pair, or a C-G base pair. In some embodiments, the PEgRNA comprises an extended first direct repeat.

[00221] In some embodiments, polynucleotides encoding polypeptide components of a prime editing composition are codon optimized by replacing at least one codon (*e.g.*, about or more than about 1, 2, 3, 4, 5, 10, 15, 20, 25, 50, or more codons) of the native sequence with codons that are more frequently or most frequently used in the genes of that host cell while maintaining the native amino acid sequence. In some embodiments, a polynucleotide encoding a polypeptide component of a prime editing composition are operably linked to one or more expression regulatory elements, for example, a promoter, a 3' UTR, a 5' UTR, or any combination thereof. In some embodiments, a polynucleotide encoding a prime editing composition component is a messenger RNA (mRNA). In some embodiments, the mRNA comprises a Cap at the 5' end and/or a poly A tail at the 3' end.

[00222] In some embodiments, a prime editing system comprises a PEgRNA, and a nuclease that recognizes the PAM sequence “NG.” In some embodiments, a PAM motif on the edit strand comprises an “NG” motif, wherein N is any nucleotide.

[00223] In some embodiments, a prime editing system comprises a PEgRNA, and a nuclease that recognizes the PAM sequence “NAG.” In some embodiments, a PAM motif on the edit strand comprises an “NAG” motif, wherein N is any nucleotide.

[00224] In some embodiments, a prime editing system comprises a PEgRNA, and a nuclease that recognizes the PAM sequence “NGA.” In some embodiments, a PAM motif on the edit strand comprises an “NGA” motif, wherein N is any nucleotide.

[00225] In some embodiments, a prime editing system comprises a PEgRNA, and a nuclease that recognizes the PAM sequence “NNGG.” In some embodiments, a PAM motif on the edit strand comprises an “NNGG” motif, wherein N is any nucleotide.

[00226] In some embodiments, a prime editing system comprises a PEgRNA, and a nuclease that recognizes the PAM sequence “NNGRRT.” In some embodiments, a PAM motif on the edit strand comprises an “NNGRRT” motif, wherein N is any nucleotide and R is A or G. Provided herein in some embodiments are example sequences for PEgRNA spacers, PBS, and editing templates for a prime editing system comprising a nuclease that recognizes the PAM sequence “NGG.” In some embodiments, a PAM motif on the edit strand comprises an “NGG” motif, wherein N is any nucleotide.

[00227] In some embodiments, a prime editing system comprises a PEgRNA, and a nuclease recognizes the PAM motif NGG, wherein the comprising a spacer, a PBS sequence, an RTT sequence, and a gRNA core sequence. In some embodiments, the PEgRNA is part of a prime editing system that recognizes the PAM motif NGG and comprises, contiguously from 5' to 3': a spacer sequence, a gRNA core sequence, a RTT sequence, and a PBS sequence. In certain embodiments, one or more of the components (e.g., spacer, PBS or RTT) of a 5' PEgRNA can operate as part of a single or stand-alone PEgRNA. In certain embodiments, one or more of the components of a 3' PEgRNA can operate as part of a single or stand-alone PEgRNA.

[00228] In some embodiments, the prime editing compositions provided herein are capable of incorporating one or more intended nucleotide edits without generating a significant proportion of indels. The term “indel(s)”, as used herein, refers to the insertion or deletion of a nucleotide base within a polynucleotide, for example, a target gene. Such insertions or deletions can lead to frame shift mutations within a coding region of a gene. Indel frequency is calculated using the formula: indel frequency = (numerator/denominator)%, wherein the denominator is the same as in editing efficiency calculation, and the numerator is the number of reads of the RTT insert subtracted by the number of reads of exact match of the expected edited sequence as designed by the editing templates.

Delivery

[00229] The prime editing systems comprising the modified mRNA described herein can be delivered to a cellular environment with any approach known in the art. Components of a prime editing composition can be delivered to a cell by the same mode or different modes. For example, in some embodiments, a prime editor can be delivered as a polypeptide or a polynucleotide (DNA or RNA) encoding the polypeptide. In some embodiments, a PEgRNA can be delivered directly as an RNA or as a DNA encoding the PEgRNA.

[00230] In some embodiments, the polynucleotide encoding one or more prime editing composition components is a part of, or is encoded by, a vector. In some embodiments, the vector is a viral vector. In some embodiments, the vector is a non-viral vector. Exemplary delivery methods are shown in Table 2 below.

Table 2: Exemplary delivery methods

Delivery	Vector/Mode	Delivery into Non-Dividing Cells	Duration of Expression	Genome Integration	Type of Molecule Delivered
Physical	(e.g., electroporation, particle gun, Calcium phosphate transfection)	YES	Transient	NO	Nucleic Acids and Proteins
Viral	Retrovirus	NO	Stable	YES	RNA
	Lentivirus	YES	Stable	YES/NO with modification	RNA
	Adenovirus	YES	Transient	NO	DNA
	Adeno-Associated Virus (AAV)	YES	Stable	NO	DNA
	Vaccinia Virus	YES	Very Transient	NO	DNA
	Herpes Simplex Virus	YES	Stable	NO	DNA
Non-Viral	Cationic	YES	Transient	Depends on what is delivered	Nucleic acids and Proteins
	Polymeric Nanoparticles	YES	Transient	NO	Nucleic Acids
Biological	Attenuated Bacteria	YES	Transient	NO	Nucleic Acids
Non-Viral Delivery Vehicles	Engineered Bacteriophages	YES	Transient	NO	Nucleic Acids
	Mammalian Virus-like Particles	YES	Transient	NO	Nucleic Acids

Delivery	Vector/Mode	Delivery into Non-Dividing Cells	Duration of Expression	Genome Integration	Type of Molecule Delivered
	Biological liposomes: Erythrocyte Ghosts and Exosomes	YES	Transient	NO	Nucleic Acids

[00231] In some embodiments, a prime editing system, for example, prime editor polypeptide components and PEgRNA/ngRNA are introduced to a target cell by nanoparticles. In some embodiments, the prime editor polypeptide components and the PEgRNA and/or ngRNA form a complex in the nanoparticle. Any suitable nanoparticle design can be used to deliver genome editing system components or nucleic acids encoding such components. In some embodiments, the nanoparticle is inorganic. In some embodiments, the nanoparticle is organic. In some embodiments, a prime editing composition is delivered to a target cell, *e.g.*, a hepatocyte, in an organic nanoparticle, *e.g.*, a lipid nanoparticle (LNP) or polymer nanoparticle.

[00232] In some embodiments, LNPs are formulated from cationic, anionic, neutral lipids, or combinations thereof. In some embodiments, neutral lipids, such as the fusogenic phospholipid DOPE or the membrane component cholesterol, are included to enhance transfection activity and nanoparticle stability. In some embodiments, LNPs are formulated with hydrophobic lipids, hydrophilic lipids, or combinations thereof. Lipids may be formulated in a wide range of molar ratios to produce an LNP. Any lipid or combination of lipids that are known in the art can be used to produce an LNP. Exemplary lipids used to produce LNPs are provided in **Table 3** below.

[00233] In some embodiments, components of a prime editing system form a complex prior to delivery to a target cell. For example, a PEgRNA can form a complex prior to delivery to the target cell. In some embodiments, a prime editing polypeptide (*e.g.*, a prime editor fusion protein) and a guide polynucleotide (*e.g.*, a PEgRNA or ngRNA) form a ribonucleoprotein (RNP) for delivery to a target cell. In some embodiments, the RNP comprises a prime editor fusion protein in complex with a PEgRNA. RNPs may be delivered to cells using known methods, such as electroporation, nucleofection, or cationic lipid-mediated methods, or any other approaches known in the art. In some embodiments, delivery of a prime editing composition or complex to the target cell does not require the delivery of foreign DNA into the cell. In some embodiments, the RNP comprising the prime editing

complex is degraded over time in the target cell. Exemplary lipids for use in nanoparticle formulations and/or gene transfer are shown in Table 3 below.

[00234] Table 3: Exemplary lipids for nanoparticle formulation or gene transfer

Lipid	Abbreviation	Feature
1,2-Dioleoyl-sn-glycero-3-phosphatidylcholine	DOPC	Helper
1,2-Dioleoyl-sn-glycero-3-phosphatidylethanolamine	DOPE	Helper
Cholesterol		Helper
N41-(2,3-Dioleoyloxy)propyl-N,N,N-trimethylammonium chloride	DOTMA	Cationic
1,2-Dioleoyloxy-3-trimethylammonium-propane	DOGS	Cationic
Diocetadecylamidoglycylspermine		
N-(3-Aminopropyl)-N,N-dimethyl-2,3-bis(dodecyloxy)-1-propanaminium bromide	GAP-DLRIE	Cationic
Cetyltrimethylammonium bromide	CTAB	Cationic
6-Lauroxyhexyl omithinate	LHON	Cationic
1-(2,3-Dioleoyloxypropyl)-2,4,6-trimethylpyridinium	2Oc	Cationic
2,3-Dioleoyloxy-N-P(spenninecarboxamido-ethyl)-N,N-dimethyl-1-propanatninium trifluoroacetate	DOSPA	Cationic
1,2-Dioleoyl-3-trimethylammonium-propane	DOPA	Cationic
N-(2-Hydroxyethyl)-N,N-dimethyl-2,3-bis(tetradecyloxy)-1-propanaminium bromide	MDRIE	Cationic
Dimyristooxypropyl dimethyl hydroxyethyl ammonium bromide	DMRI	Cationic
3β-[N-(N', N'-Dimethylaminoethane)-carbamoyle]cholesterol	DC-Chol	Cationic
Bis-guanidium-tren-cholesterol	BGTC	Cationic
1,3-Dioleoyl-2-(6-carboxy-spermyl)-propylamide	DOSPER	Cationic
Dimethyloctadecylammonium bromide	DDAB	Cationic
Diocetadecylamidoglycylspermidin	DSL	Cationic
rac-[(2,3-Dioctadecyloxypropyl)(2-hydroxyethyl)]-dimethylammonium chloride	CLIP-1	Cationic
rac-[2(2,3-Dihexadecyloxypropyloxymethyloxy)ethyl]trimethylammonium bromide	CLIP-6	Cationic
Ethyl dimyristoylphosphatidylcholine	EDMPC	Cationic
1,2-Distearyloxy-N,N-dimethyl-3-aminopropane	DSDMA	Cationic
1,2-Dimyristoyl-trimethylammonium propane	DMTAP	Cationic
O,O'-Dimyristyl-N-lysyl aspartate	DMKE	Cationic
1,2-Distearoyl-sn-glycero-3-ethylphosphocholine	DSEPC	Cationic
N-Palmitoyl D-erythro-sphingosyl carbamoyle-spermine	CCS	Cationic
N-t-Butyl-N0-tetradecyl-3-tetradecylaminopropionamidine	diC14-amidine	Cationic
Octadecenolyoxy[ethyl-2-heptadecenyl-3 hydroxyethyl]imidazolinium chloride	DOTIM	Cationic
N1 -Cholesterylloxycarbonyl-3,7-diazanonane-1,9-diamine	CDAN	Cationic
2-(3-Bis(3-amino-propyl)-amino)propylamino)-Nditetradecylcarbamoyle-ethyl-acetamide	RPR209120	Cationic
1,2-dilinoleyloxy-3-dimethylaminopropane	DLinDMA	Cationic

Lipid	Abbreviation	Feature
2,2-dilinoleyl 1-4-dimethylaminoethyl-[1,3]-dioxolane	DLin-KC2-DMA	Cationic
dilinoleyl-methyl-4-dimethylaminobutyrate	DLin-MC3-DMA	Cationic

[00235] Exemplary polymers for use in nanoparticle formulations and/or gene transfer are shown in Table 4 below.

[00236] **Table 4: Exemplary lipids for nanoparticle formulation or gene transfer**

Polymer	Abbreviation
Poly(ethylene)glycol	PEG
Polyethylenimine	PEI
Dithiobis (succinimidylpropionate)	DSP
Dimethyl-3,3'-dithiobispropionimide	DTBP
Poly(ethylene imine)biscarbamate	PEIC
Poly(L-lysine)	PLL
Histidine modified PLL	
Poly(N-vinylpyrrolidone)	PVP
Poly(propylenimine)	PPI
Poly(amidoamine)	PAMAM
Poly(amidoethylenimine)	SS PAEI
Triethylenetetramine	TETA
Poly(β -aminoester)	
Poly(4-hydroxy-L-proline ester)	PHP
Poly(allylamine)	
Poly(α -[4-aminobutyl]-L-glycolic acid)	PAGA
Poly(D,L-lactic-co-glycolic acid)	PLGA
Poly(N-ethyl-4-vinylpyridinium bromide)	
Poly(phosphazene)s	PPZ
Poly(phosphoester)s	PPE
Poly(phosphoramidate)s	PPA
Poly(N-2-hydroxypropylmethacrylamide)	pHPMA
Poly (2-(dimethylamino)ethyl methacrylate)	pDMAEMA
Poly(2-aminoethyl propylene phosphate)	PPE-EA
Chitosan	
Galactosylated chitosan	
N-dodacylated chitosam	
Histone	
Collagen	
Dextran-spermine	D-SPM

Pharmaceutical compositions

[00237] Disclosed herein are pharmaceutical compositions comprising any of the prime editing composition components, for example, prime editors, fusion proteins,

polynucleotides encoding prime editor polypeptides, PEgRNAs, and/or prime editing complexes described herein.

[00238] The term “pharmaceutical composition”, as used herein, refers to a composition formulated for pharmaceutical use. In some embodiments, the pharmaceutical composition further comprises a pharmaceutically acceptable carrier. In some embodiments, the pharmaceutical composition comprises additional agents, *e.g.*, for specific delivery, increasing half-life, or other therapeutic compounds.

[00239] In some embodiments, a pharmaceutically-acceptable carrier comprises any vehicle, such as a liquid or solid filler, diluent, excipient, manufacturing aid (*e.g.*, lubricant, talc magnesium, calcium or zinc stearate, or steric acid), or solvent encapsulating material, involved in carrying or transporting the compound from one site (*e.g.*, the delivery site) of the body, to another site (*e.g.*, organ, tissue or portion of the body). A pharmaceutically acceptable carrier is “acceptable” in the sense of being compatible with the other ingredients of the formulation and not injurious to the tissue of the subject (*e.g.*, physiologically compatible, sterile, physiologic pH, etc.)

[00240] Disclosed herein are pharmaceutical compositions comprising the modified mRNA, the prime editing system, or the LNP or RNP described herein.

[00241] The term “pharmaceutical composition”, as used herein, refers to a composition formulated for pharmaceutical use. In some embodiments, the pharmaceutical composition further comprises a pharmaceutically acceptable carrier. In some embodiments, the pharmaceutical composition comprises additional agents, *e.g.*, for specific delivery, increasing half-life, or other therapeutic compounds.

[00242] In some embodiments, a pharmaceutically-acceptable carrier comprises any vehicle, such as a liquid or solid filler, diluent, excipient, manufacturing aid (*e.g.*, lubricant, talc magnesium, calcium or zinc stearate, or steric acid), or solvent encapsulating material, involved in carrying or transporting the compound from one site (*e.g.*, the delivery site) of the body, to another site (*e.g.*, organ, tissue or portion of the body). A pharmaceutically acceptable carrier is “acceptable” in the sense of being compatible with the other ingredients of the formulation and not injurious to the tissue of the subject (*e.g.*, physiologically compatible, sterile, physiologic pH, etc.)

[00243] Formulations of the pharmaceutical compositions described herein can be prepared by any method known or hereafter developed in the art of pharmacology. In general, such preparatory methods include the step of bringing the active ingredient(s) into association with an excipient and/or one or more other accessory ingredients, and then, if necessary

and/or desirable, shaping and/or packaging the product into a desired single- or multi-dose unit. Pharmaceutical formulations can additionally comprise a pharmaceutically acceptable excipient, which, as used herein, includes any and all solvents, dispersion media, diluents, or other liquid vehicles, dispersion or suspension aids, surface active agents, isotonic agents, thickening or emulsifying agents, preservatives, solid binders, lubricants and the like, as suited to the particular dosage form desired.

Methods of Editing

[00244] The methods and compositions disclosed herein can be used to edit a target gene or sequence of interest by editing.

[00245] In some embodiments, contacting the target gene with the prime editing composition results in binding of the PEgRNA to a strand of the target gene. In some embodiments, contacting the target gene with the prime editing composition results in binding of the PEgRNA to a search target sequence on the strand of the target gene upon contacting with the PEgRNA. In some embodiments, contacting the target gene with the prime editing composition results in binding of the prime editor to the target gene, upon the contacting of the PE composition with the target gene. In some embodiments, components of a prime editing composition form a complex prior to delivery to a target cell. For example, a prime editor fusion protein and a PEgRNA can form a complex prior to delivery to the target cell. In some embodiments, a prime editing polypeptide (*e.g.*, a prime editor fusion protein) and a guide polynucleotide (*e.g.*, a PEgRNA) form a ribonucleoprotein (RNP) for delivery to a target cell. In some embodiments, the RNP comprises a prime editor fusion protein in complex with a PEgRNA. RNPs may be delivered to cells using known methods, such as electroporation, nucleofection, or cationic lipid-mediated methods, or any other approaches known in the art. In some embodiments, delivery of a prime editing composition or complex to the target cell does not require the delivery of foreign DNA into the cell. In some embodiments, the RNP comprising the prime editing complex is degraded over time in the target cell.

[00246] The methods and compositions disclosed herein can be used to edit a target gene of interest by prime editing.

[00247] In some embodiments, the prime editing method comprises contacting a target gene, with the prime editing system or lipid nanoparticle described herein. For example, in a method for editing a gene comprises contacting a PEgRNA and a prime editor (PE) comprising a DNA binding domain and a DNA polymerase domain, wherein the PEgRNA

directs the prime editor to incorporate the intended nucleotide edit in the gene, thereby editing the gene.

[00248] In some embodiments, a method for editing a gene comprises contacting the gene with the prime editing system described herein, wherein the PEgRNA directs the prime editor to incorporate the intended nucleotide edit in the gene, thereby editing the gene.

[00249] In some embodiments, the editing efficiency is at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, at least 99.5%, at least 99.9% equivalent as compared to editing with an unmodified control PEgRNA.

[00250] In some embodiments, the prime editor synthesizes a single stranded DNA encoded by the editing template, wherein the single stranded DNA replaces the editing target sequence and results in incorporation of the intended nucleotide edit into a region corresponding to the editing target sequence.

[00251] In some embodiments, the subject is a human.

[00252] In some embodiments, the method further comprising administering the cell to the subject after incorporation of the intended nucleotide edit.

EXAMPLES

[00253] **EXAMPLE 1 – Examples of Chemically Modified PEgRNA and ngRNA Sequences**

[00254] FIG. 1 discloses possible PEgRNA or ngRNA modifications in nucleotides for both nucleotide and non-nucleotide modifications. The modified PEgRNAs or ngRNAs synthesis can be achieved by *in-vitro* transcription (IVT) or solid phase synthesis on automated synthesizers. These methods utilize synthesis of short oligonucleotides of about 10-120 nucleotides with desired chemical modifications.

[00255] Solid phase synthesis of long RNA oligonucleotides (>100 nt length) suffers from low yield and purity of the final product. The coupling efficiency is not quantitative, thus limiting the synthesis yield for long-RNA oligonucleotides. These non-quantitative couplings generate shorter impurities, N-X (X nucleotides shorter than the full-length product (FLP; N), X= 1, 2, etc.) species. Impurities with longer length called N+X (X nucleotides longer than the FLP, X= 1, 2, etc.) species can also be generated during the coupling step. It is difficult to remove shorter and longer impurities that have close lengths compared to the FLP due to the similarity of the chemical/physical properties of the impurities and the FLP.

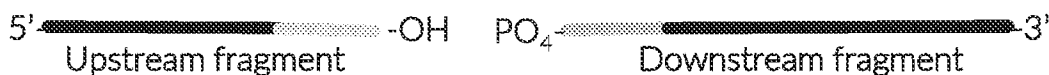
This is particularly problematic for long RNAs > 100-nt, because just several nucleotides shorter or longer becomes a trivial difference for long oligonucleotides (> 100-nt length) compared to much shorter oligonucleotides (20-nt length). Applicant has developed alternate synthesis and purification methods for long-RNA synthesis to overcome these synthesis challenges.

[00256] The methods developed included synthesizing long RNAs, such as PEgRNAs or ngRNAs, via split synthesis using functionalized fragments of RNA. The functionalized fragments can be joined together via enzymatic ligation or chemical conjugation.

[00257] An important factor to consider in long RNA split synthesis methods is choosing the appropriate split site for chemical ligations. In PEgRNAs, due to the additional chemical structure formed after chemical ligation, it is important the chemical structure avoids interfering with the (a) Cas9 nuclease interaction and (b) its activity, by disturbing the gRNA/Cas9 complex. A crystal structure analysis of gRNA/Cas9 complex has showed that the TL and SL2 in the gRNA core have minimal interactions with the Cas9 protein. Therefore, TL and SL2 are considered as the potential split sites for chemical ligations. Other potential split sites include the junction between the gRNA core and the RTT and the end of the PBS and extensions of the 3' end.

[00258] Additionally, the PEgRNAs or ngRNAs may be enzymatically ligated between two or more oligoribonucleotides synthesized by solid phase synthesis which may or may not contain chemically modifications of the sugar, phosphodiester backbone, or nucleobase canonical structures. The PEgRNAs or ngRNAs can be generated from one 5' fragment, and one or more 3' fragments which are 5' terminally modified with a free monophosphate to facilitate the ligation by way of a nucleotide ligase enzyme. Free 3' ends and phosphorylated 5' ends are brought into proximity by way of sequence complementarity resulting in base pairing hybridization either between the RNA fragments directly (self-templated) or via an additional complementary DNA splint (splinted). Subsequent enzymatic catalysis by a nucleic acid ligase, such as, but not limited to, T4 RNA Ligase I, T4 RNA Ligase II, or T4 DNA Ligase can generate a natural phosphodiester linkage between fragments, yielding the full-length PEgRNA. Other ligase variants can also be used to ligate the fragments.

[00259] Scheme A below shows a potential fragment functionalization for enzymatic split synthesis, where "Upstream" or 5' end RNA fragments have a free 3' OH available for ligation, while "Downstream" or 3' end RNA fragments are terminally functionalized with a 5'-monophosphate.



Scheme A.

Schematic of upstream and downstream fragments are shown. The light shaded segments are complementary and facilitate self-templated ligation.

[00260] In another approach using self-templated enzymatic ligation, two functionalized, single-stranded RNA fragments with complementary base pairs are hybridized for ligase-mediated conjugation. In here, the full-length PEgRNA is split into two or more fragments that take natural advantage of the sequence complementarity driven structure of either the Tetraloop or Stem-loop 2 features within the scaffold of a PEgRNA to help facilitate a very specific ligation reaction between the fragments. Once these fragments are annealed under controlled buffered conditions, the RNA duplex can form a native secondary structure mimic to the full length PEgRNA, resulting in a net increase in the effective local concentration of the terminal 3'-OH and 5'-monophosphate functional ends required for ligase activity. Subsequent ligase catalysis forms a phosphodiester bond between the RNA fragments which covalently seals the nick and generates a full-length PEgRNA. Accordingly, during self-templated ligation two RNA fragments with significant complementarity are enzymatically conjugated. The first step is to anneal the fragments, bringing the reactive functional groups together and creating a nicked double-stranded substrate for ligase catalysis. In the presence of ligase, a phosphodiester bond is formed between the 5'-monophosphate and native 3'-hydroxyl of the fragments, yielding a full-length PEgRNA.

[00261] EXAMPLE 2 – Editing Efficiency

[00262] Editing efficiency of the modified PEgRNA or ngRNA compositions and methods described herein can be measured by calculating the percentage of edited target genes in a population of cells introduced with the prime editing composition. In some embodiments, the editing efficiency is determined after 1 hour, 2 hours, 6 hours, 12 hours, 24 hours, 36 hours, 48 hours, 72, hours, 3 days, 4 days, 5 days, 7 days, 10 days, or 14 days of exposing a target gene to a prime editing composition. In some methods, the population of cells introduced with the prime editing composition is *ex vivo*. In some methods, the population of cells introduced with the prime editing composition is *in vitro*.

[00263] To evaluate the editing efficiency of different PEgRNAs, plasmids encoding a prime editor fusion protein and PEgRNA were transfected into primary hepatocyte cells with Lipofectamine transfection reagents. Next generation sequencing-based methods or ddPCR-based methods may be developed to determine editing efficiency at 72 hours after

transfection. PEgRNAs Nos. 1, 2, 4, 5, and 6 successfully tested with top editing efficiency reaching over 50% (FIG. 5). The chemical modifications of these PEgRNAs are shown in FIG. 8. Chemical modifications to PEgRNA and ngRNA were evaluated and compared to control sequences for editing efficiency as measured by percent editing of the target DNA. Five PEgRNAs and their results are displayed in FIG. 5. As demonstrated, PEgRNA1 showed reproducible editing efficiency, and exemplary PEgRNA6 with novel 3'-end modifications showed better editing than the PEgRNA2. Tested PEgRNAs with modifications as indicated in pink, orange, and blue are site specific chemical modifications that were incorporated into the PEgRNAs (FIG. 4). The novel end modifications in the 3' end in exemplary PEgRNAs 4, 5, 6 increased prime editing compared with PEgRNA3, all of which contain the chemically modified gRNAs, crRNAs and tracrRNAs. These PEgRNAs were screened in primary hepatocytes, suggesting that novel end modifications to 3' could contribute to improved prime editing.

[00264] Based on editing efficiency experiments, PEgRNAs were prioritized to further evaluation.

[00265] **EXAMPLE 3 – Site-Specific Chemical Modifications in PEgRNAs Reduces Metabolic Cleavage by Endonucleases**

[00266] The PEgRNAs were identified of metabolites using *in vitro* assays human liver cells, fractions S9 and detected with LCMS techniques. The detection method and assay are disclosed in FIG. 13.

[00267] Metabolite cleavage sites by endonucleases were identified in PEgRNAs Nos.: 1, 2, 3, 6 through metabolic profiling by liquid chromatography mass spectrometry (LCMS) after 4 hours incubation in human S9 fraction. The incorporation of site-specific gRNA modifications in TL, SL2, and SL3 of PEgRNA No.3 stabilized and eliminated cleavage sites 5'-U37 or A38-3' within that region that were found for example in PEgRNA No.1. This was observed by comparing metabolite peaks in the LCMS profiles found in FIG. 7A and FIG. 7B. The incorporation of site-specific 2'-MOE modifications in PEgRNA No.3 stabilized and eliminated cleavage sites 5'-U37, A38-3', A65-3, A68-3' within that region that were found for example in PEgRNA No.1. This was observed by comparing metabolite peaks in the LCMS profiles found in FIG. 7A and FIG. 7C. Newly identified metabolites (i.e., cleavage sites) were visible in the PEgRNA No.3 LCMS profile (A46 and A26).

[00268] **EXAMPLE 4 – Nuclease Degradation and Half-Life of Chemical Modifications**

[00269] Novel end modifications were examined for the potential to improve PEgRNA stability as determined by half-life (FIG. 9). These 3' end modifications (i.e. LNA-T*LNA-T*LNA-T*U-3') improved the half-life of exemplary PEgRNA No.6 by approximately two-fold compared to the parent PEgRNA No.2, both of which have consistent the gRNA modifications. Compared to the PEgRNA No.1 parent without the gRNA modifications, PEgRNA No.6 had half-life improvements by approximately 4 hours. The new end modifications therefore improve prime editing by increasing the stability and availability of prime editing compositions.

[00270] **EXAMPLE 5 – Thermal Melting Studies of PEgRNA and Biophysical Characterization of Chemically Modified PEgRNA and ngRNAs**

[00271] UV-Vis melting (T_m) experiments were performed by monitoring PEgRNAs formation of RNPs by absorbance at 260nm in phosphate-buffered saline (PBS) against a 0.2°C/min rise in temperature (FIG. 11). Selected PEgRNAs were used to study the impact and RNP formation maintenance with substituting single 'U' residues with 2'-O-Me-U modifications in the gRNA and scaffold. The melting temperature T_m -2 (the melting of the potential duplex between protospacer and RTT-PBS) was not affected much by a single 2'-O-Me-U substitution. Slight stabilization of the secondary structures in the scaffold region was observed in the PEgRNAs. Additionally, the data is supports the notion that the 3' sequence modifications in the PEgRNAs does not greatly alter the specificity of the RNP and formation.

[00272] **EXAMPLE 6 - Editing Efficiency of Additional PEgRNAs**

[00273] Additional chemically modified PEgRNA and ngRNA sequences are shown Table 5 and Table 6.

[00274] **Table 5**

SEQ ID NO.:	PEgRNA Name	Sequence (5'-3')
25	P1	mC*mC*mC*AUACCUUGGAGCAACGG GUUUUAGAGCUAGAAAUAGCAAGUUAUUAAAGGCUAGUCCG UUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCGCCACCUU CGGCUAACGUUGCUCUCCAAGGU*mU*mU*mU
26	P2	mC*mC*mC*AUACCUUGGAGCAACGG GUUUUAGAmGmCmUmAmGmAmAmUmAmGmCAAGUUAUUAA AUAAGGCUAGUCCGUUAUCAmAmCmUmUmGmAmAmAmAmAm GmUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCGCCACC UUCGGCUAACGUUGCUCUCCAAGGU*mU*mU*mU
27	P3	mC*mC*mC*AmUACCUUGGAGCAACGG GUUUUmUAGAGCmUAGAAAmUAGCAAGUUAUUAAAGGCUAG UCCGUUAUmCAACUUGAAAAAGUGGCACCGAGUCGGUGCGCC ACCUUCGGCUAACGUUGCUCmCAAGGU*mU*mU*mU

28	P4	mC*mC*mC*AmUACCUUGGAGCAACGG GUUUmUAGAGCmUAGAAAmUAGCAAGUAAAAUAAGGCUAG UCCGUUAUmCAACUUGAAAAAGUGGCACCGAGUCGGUGCGCC ACCUUCGGCUAACGUUGCUCCAAGGULNA-T*LNA-T*LNA- T*mU
29	P5	mC*mC*mC*AmUACCUUGGAGCAACGG GUUUmUAGAmGmCmUmAmGmAmAmUmAmGmCAAGUUA AAUAAGGCUAGUCCGUUAUmCAACUUGAAAAAGUGGCACCGA GUCGGUGCGCCACCUUCGGCUAACGUUGCUCmCAAGGUUmU*m U*mU*mU
30	P6	mC*mC*mC*AmUACCUUGGAGCAACGG GUUUmUAGAmGmCmUmAmGmAmAmUmAmGmCAAGUUA AAUAAGGCUAGUCCGUUAUmCAmAmCmUmUmGmAmAmAmAm AmGmUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCGCC ACCUUCGGCUAACGUUGCUCmCAAGGUUmU*mU*mU*mU
31	P7	mC*mC*mC*AmUACCUUGGAGCAACGG GUUUmUAGAmGmCmUmAmGmAmAmUmAmGmCAAGUUA AAUAAGGCUAGUCCGUUAUmCAmAmCmUmUmGmAmAmAmAm AmGmUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCGCC ACCUUCGGCUAACGUUGCUCmCAAGGULNA-T*LNA-T*LNA- T*mU
32	P8	mC*mC*mC*AUACCUUGGAGCAACGGGUUUUAGAGCUCGAAAG AGCAAGUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAG UGGCACCGAGUCGGUGCGCCACCUUCGGCUAACGUUGCUCCA AGGUUmU*mU*mU*mU
33	P9	mC*mC*mC*AUACCUUGGAGCAACGGGUUUUUGGAGCUAGAAA UAGCAAGUCAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAA GUGGCACCGAGUCGGUGCGCCACCUUCGGCUAACGUUGCUC AAGGUUmU*mU*mU*mU
34	P10	mC*mC*mC*AUACCUUGGAGCAACGGGUUUUUGGAGCUCGAAAG AGCAAGUCAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAG UGGCACCGAGUCGGUGCGCCACCUUCGGCUAACGUUGCUCCA AGGUUmU*mU*mU*mU
35	P11	mC*mC*mC*AUACCUUGGAGCAACGGGUUUUAGAGCGAGAAA UUGCAAGUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAA GUGGCACCGAGUCGGUGCGCCACCUUCGGCUAACGUUGCUC AAGGUUmU*mU*mU*mU
36	P12	mC*mC*mC*AUACCUUGGAGCAACGG GUUUUAGAGCUAGAAAUAGCAAGUAAAAUAAGGCUAGUCCG UUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCGCCACCU CGGCUAACGUUGCUCCAAGGULNA-T*LNA-T*LNA-T*LNA-T
37	P13	mC*mC*mC*AUACCUUGGAGCAACGG GUUUmUAGAGCmUAGAAAmUAGCAAGUAAAAUAAGGCUAG UCCGUUmUAUmCAACUUGAAAAAGUGGCACCGAGUCGGUGCGC CACCUUCGGCUAACGUUGCUCCAAGGULNA-T*LNA-T*LNA- T*LNA-T
38	P14	mC*mC*mC*AUACCUUGGAGCAACGG GUUUmUAGAGCmUAGAAAmUAGCAAGUAAAAUAAGGCUAG UCCGUaraUAUmCAACUUGAAAAAGUGGCACCGAGUCGGUGCG CCACCUUCGGCUAACGUUGCUCCAAGGULNA-T*LNA-T*LNA- T*LNA-T
39	P15	mC*mC*mC*AUACCUUGGAGCAACGGGUUUUmUAGAGCmUAGA AAmUAGCAAGUAAAAUAAGGCUAGUCCGUN1-Me-Pseudo- UAUmCAACUUGAAAAAGUGGCACCGAGUCGGUGCGCCACCU CGGCUAACGUUGCUCCAAGGULNA-T*LNA-T*LNA-T*LNA-T

40	P16	mC*mC*mC*AUACCUUGGAGCAACGG GUUUUmUAGAGCmUAGAAAmUAGCAAGUAAAAUAAGGCUAG UCCGU(5MerU)AUmCAACUUGAAAAAGUGGCACCGAGUCGGUG CGCCACCUUCGGCUAACGUUGCUCUCAAGGULNA-T*LNA- T*LNA-T*LNA-T
41	P17	mC*mC*mC*AUACCUUGGAGCAACGGGUUUUmUAGAGCmUAGA AAmUAGCAAGUAAAAUAAGGCUAGUCCGUL-RNA- UAUmCAACUUGAAAAAGUGGCACCGAGUCGGUGCGCCACCUU CGGCUAACGUUGCUCUCAAGGULNA-T*LNA-T*LNA-T*LNA-T
42	P18	mC*mG*mG*GCUGGAGCUGUUCGCGCGUUUUAGAGCUAGAAA UAGCAAGUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAA GUGGCACCGAGUCGGUGCUGCAAGAUGCCAGCGGGGCGAACA GCUCCAGmU*mU*mU*mU
43	P19	mC*mG*mG*GCUGGAGCUGUUCGCGCGUUUmUAGAGCmUAGA AAmUAGCAAGUAAAAUAAGGCUAGUCCGUUAUCAACUUGAA AAAGUGGCACCGAGUCGGUGCUGCAAGAUGCCAGCGGGGCGA AmCAGCUCmCAGmU*mU*mU*mU
44	P20	mC*mG*mG*GCUGGAGCUGUUCGCGCGUUUmUAGAGCmUAGA AAmUAGCAAGUAAAAUAAGGCUAGUCCGUUAUmCAACUUG AAAAAGUGGCACCGAGUCGGUGCUGCAAGAUGCCAGCGGGGC GAACAGCUCCAGLNA-T*LNA-T*LNA-T*mU
45	P21	mC*mG*mG*GCUGGAGCUGUUCGCGCGUUUUAGAmGmCmUmA mGmAmAmAmUmAmGmCAAGUAAAAUAAGGCUAGUCCGUUA UCAmAmCmUmUmGmAmAmAmAmGmUmGmGmCmAmCmCm GmAmGmUmCmGmGmUmGmCUGCAAGAUGCCAGCGGGGCGAA CAGCUCCAGmU*mU*mU*mU
46	P22	mC*mG*mG*GCUGGAGCUGUUCGCGCGUUUmUAGAmGmCmUm AmGmAmAmAmUmAmGmCAAGUAAAAUAAGGCUAGUCCGUU AUmCAmAmCmUmUmGmAmAmAmAmAmGmUmGmGmCmAmCm CmGmAmGmUmCmGmGmUmGmCUGCAAGAUGCCAGCGGGGCG AAmCAGCUCmCAGmU*mU*mU*mU
47	P23	mC*mG*mG*GCUGGAGCUGUUCGCGCGUUUmUAGAmGmCmUm AmGmAmAmAmUmAmGmCAAGUAAAAUAAGGCUAGUCCGUU AUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUGCAAGAUGC CAGCGGGGCGAAmCAGCUCmCAGmU*mU*mU*mU
48	P24	mC*mG*mG*GCUGGAGCUGUUCGCGCGUUUmUAGAmGmCmUm AmGmAmAmAmUmAmGmCAAGUAAAAUAAGGCUAGUCCGUU AUmCAmAmCmUmUmGmAmAmAmAmAmGmUmGmGmCmAmCm CmGmAmGmUmCmGmGmUmGmCUGCAAGAUGCCAGCGGGGCG AAmCAGCUCmCAGLNA-T*LNA-T*LNA-T*mU
49	P25	mC*mG*mG*GCUGGAGCUGUUCGCGCGUUUUAGAGCUAGAAA UAGCAAGUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAA GUGGCACCGAGUCGGUGCUGCAAGAUGCCAGCGGGGCGAACA GCUCCAGLNA-T*LNA-T*LNA-T*LNA-T
50	P26	mC*mG*mG*GCUGGAGCUGUUCGCGCGUUUmUAGAGCmUAGA AAmUAGCAAGUAAAAUAAGGCUAGUCCGUUmUAUmCAACU GAAAAAGUGGCACCGAGUCGGUGCUGCAAGAUGCCAGCGGGG CGAACAGCUCCAGLNA-T*LNA-T*LNA-T*LNA-T
51	P27	mC*mG*mG*GCUGGAGCUGUUCGCGCGUUUmUAGAGCmUAGA AAmUAGCAAGUAAAAUAAGGCUAGUCCGUUaraUAUmCAACU GAAAAAGUGGCACCGAGUCGGUGCUGCAAGAUGCCAGCGGGG CGAACAGCUCCAGLNA-T*LNA-T*LNA-T*LNA-T
52	P28	mC*mG*mG*GCUGGAGCUGUUCGCGCGUUUmUAGAGCmUAGA AAmUAGCAAGUAAAAUAAGGCUAGUCCGU(NMP- U)AUmCAACUUGAAAAAGUGGCACCGAGUCGGUGCUGCAAGA

		UGCCAGCGGGGCGAACAGCUCCAGLNA-T*LNA-T*LNA-T*LNA-T
53	P29	mC*mG*mG*GCUGGAGCUGUUCGCGCGUUUmUAGAGCmUAGA AAmUAGCAAGUUAAAAUAAGGCUAGUCCGUL-RNA- UUmCAACUUGAAAAAGUGGCACCGAGUCGGUGCUGCAAGAU GCCAGCGGGGCGAACAGCUCCAGLNA-T*LNA-T*LNA-T*LNA-T
54	P30	mC*mG*mG*GCUGGAGCUGUUCGCGCGUUUmUAGAGCmUAGA AAmUAGCAAGUUAAAAUAAGGCUAGUCCGU(5MerU)AUmCAAC UUGAAAAAGUGGCACCGAGUCGGUGCUGCAAGAUGCCAGCGG GGCGAACAGCUCCAGLNA-T*LNA-T*LNA-T*LNA-T
55	P31	mC*mC*mC*AUACCUUGGAGCAACGG GUUUUAGAmGmCmUmAmGmAmAmUmAmGmCAAGUUAAA AUAAGGCUAGUCCGUUAUCAmAmCmUmUmGmAmAmAmAm GmUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCGCCACC UUCGGCUAACGUUGCUCUCCAAGGmUmU*mU*mU
56	P32	mC*mC*mC*AUACCUUGGAGCAACGG GUUUUAGAmGmCmUmAmGmAmAmUmAmGmCAAGUUAAA AUAAGGCUAGUCCGUUAUCAmAmCmUmUmGmAmAmAmAm GmUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCGCCACC UUCGGCUAACGUUGCUCUCCAAGGmUmU*mU
57	P33	mC*mC*mC*AUACCUUGGAGCAACGG GUUUUAGAmGmCmUmAmGmAmAmUmAmGmCAAGUUAAA AUAAGGCUAGUCCGUUAUCAmAmCmUmUmGmAmAmAmAm GmUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCGCCACC UUCGGCUAACGUUGCUCUCCAAGmGmUmU
58	P34	mC*mC*mC*AUACCUUGGAGCAACGG GUUUUAGAmGmCmUmAmGmAmAmUmAmGmCAAGUUAAA AUAAGGCUAGUCCGUUAUCAmAmCmUmUmGmAmAmAmAm GmUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCGCCACC UUCGGCUAACGUUGCUCUCCAAGmGmUmU
59	P35	mC*mC*mC*AUACCUUGGAGCAACGG GUUUUAGAmGmCmUmAmGmAmAmUmAmGmCAAGUUAAA AUAAGGCUAGUCCGUUAUCAmAmCmUmUmGmAmAmAmAm GmUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCGCCACC UUCGGCUAACGUUGCUCUCCAAGGmU*mU*mU*mU
60	P36	mC*mC*mC*AUACCUUGGAGCAACGG GUUUUAGAmGmCmUmAmGmAmAmUmAmGmCAAGUUAAA AUAAGGCUAGUCCGUUAUCAmAmCmUmUmGmAmAmAmAm GmUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCGCCACC UUCGGCUAACGUUGCUCUCCAAGmG*mU*mU*mU
61	P37	mC*mC*mC*AUACCUUGGAGCAACGG GUUUUAGAmGmCmUmAmGmAmAmUmAmGmCAAGUUAAA AUAAGGCUAGUCCGUUAUCAmAmCmUmUmGmAmAmAmAm GmUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCGCCACC UUCGGCUAACGUUGCUCUCCAAGmG*mU*mU
62	P38	mC*mC*mC*AUACCUUGGAGCAACGG GUUUUAGAmGmCmUmAmGmAmAmUmAmGmCAAGUUAAA AUAAGGCUAGUCCGUUAUCAmAmCmUmUmGmAmAmAmAm GmUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCGCCACC UUCGGCUAACGUUGCUCUCCAAGmG*mU
63	P39	mC*mC*mC*AUACCUUGGAGCAACGGGUUUUfUAGAGfCfUAGAA AfUAGfCAAGUUAAAAfUAAGGfCfUAGfUfCfCGUUAfUfCAAfCfUf UGAAAAAGfUGGfCfCfCfGAGfUfCfGGfUfCfGCCACCUUCGGCU AACGUUGCUCUCCAAGGUmU*mU*mU*mU

64	P40	mC*mC*mC*AUACCUUGGAGCAACGGGUUUfUAGAGfCfUAGAA AfUAGfCAAGUUA AAAfU AAGGfCfUAGfUfCfCGUfU AfUfCAAfCfU fUGAAAAAGfUGGfCAfCfCGAGfUCGGfUGfC GCCACCUUCGGCUAACGUUGCUCCAAGGU mU*mU*mU*mU
65	P41	mC*mC*mC*AUACCUUGGAGCAACGGGUUUfU*AGAGfC*fU*AG AAAfU*AGfC*AAGUUA AAAfU AAGGfC*fU*AGfU*fC*fC*GUU Af U*fC*AAfC*fU*fU*GAAAAAGfU*GGfC*AfC*fC*GAGfU*fC*GGfU *GfC*GCCACCUUCGGCUAACGUUGCUCCAAGGU mU*mU*mU*mU U
66	P42	mC*mC*mC*AUACCUUGGAGCAACGGGUUUfU*AGAGfC*fU*AG AAAfU*AGfC*AAGUUA AAAfU AAGGfC*fU*AGfU*fC*fC*GUfUA fU*fC*AAfC*fU*fU*GAAAAAGfU*GGfC*AfC*fC*GAGfU*fC*GGfU *GfC*GCCACCUUCGGCUAACGUUGCUCCAAGGU mU*mU*mU*mU U
67	P43	mC*mC*mC*AUACCUUGGAGCAACGGGUUUfU*AGAGfC*fU*AG AAAfU*AGfC*AAGUUA AAAfU AAGGfC*fU*AGfU*fC*fC*GUfU* AfU*fC*AAfC*fU*fU*GAAAAAGfU*GGfC*AfC*fC*GAGfU*fC*GGf U*fC*GCCACCUUCGGCUAACGUUGCUCCAAGGU mU*mU*mU*mU* mU
68	P44	mC*mC*mC*AUACCUUGGAGCAACGGGUUUfUAGAGfC*fU*AGA AAfU*AGfCAAGUUA AAAfU AAGGfC*fU*AGfUfCfC*GUfUAfUfC *AAfC*fU*fU*GAAAAAGfU*GGfCAfC*fC*GAGfU*fC*GGfU*GfC* GCCACCUUCGGCUAACGUUGCUCCAAGGU mU*mU*mU*mU
69	P45	mC*mC*mC*AUACCUUGGAGCAACGGmGUUUmUmAmGmAmGm CmUmAmGmAmAmAmUmAmGmCmAmAGUUmAAmAmAmUmAm AmGmGmCmUmAGmUmCmCGUUmUmCmAmAmCmUmUmGmA mAmAmAmAmGmUmGmGmCmAmCmCmGmAmGmUmCmGmGmU mGmCGCCACCUUCGGCUAACGUUGCUCCAAGGU mU*mU*mU*mU* mU-
70	P46	mC*mC*mC*AUACCUUGGAGCAACGGmGUUUmUmAmGmAmGm CmUmAmGmAmAmAmUmAmGmCmAmAGUUmAAmAmAmUmAm AmGmGmCmUmAGmUmCmCGU(NMPU)AmUmCmAmAmCmUmU mGmAmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGmUmCmG mGmUmGmCGCCACCUUCGGCUAACGUUGCUCCAAGGUU*U*U* U-
71	P47	mC*mC*mC*AUACCUUGGAGCAACGGmGUUUmUmAmGmAmGm CmUmAmGmAmAmAmUmAmGmCmAmAGUUmAAmAmAmUmAm AmGmGmCmUmAGmUmCmCGU(araU)AmUmCmAmAmCmUmUmG mAmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGmUmCmGmG mUmGmCGCCACCUUCGGCUAACGUUGCUCCAAGGU mU*mU*mU* U*mU
72	P48	mC*mC*mC*AUACCUUGGAGCAACGGmGUUUmUmAmGmAmGm CmUmAmGmAmAmAmUmAmGmCmAmAGUUmAAmAmAmUmAm AmGmGmCmUmAGmUmCmCGU(5MerU)AmUmCmAmAmCmUmU mGmAmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGmUmCmG mGmUmGmCGCCACCUUCGGCUAACGUUGCUCCAAGGU mU*mU *mU*mU
73	P49	mC*mC*mC*AUACCUUGGAGCAACGGfGUUUfUfAfGfAfGfCfUfAf GfAfAfAfUfAfGfCfAfAGUUF AAfAfAfUfAfAfGfGfCfUfAfGfUfCfCGU U AfUfCfAfAfCfUfUfGfAfAfAfAfGfUfGfGfCfAfCfCfGfAfGfUfCfGf GfUfGfCGCCACCUUCGGCUAACGUUGCUCCAAGGU mU*mU*mU* *mU
74	P50	mC*mC*mC*AUACCUUGGAGCAACGGGUUUfUfAfGfAfGfCfUfAf GfAfAfAfUfAfGfCfAfAGUUF AAfAfAfUfAfAfGfGfCfUfAfGfUfCfCGU (NMP-

		U)AfUfCfAfAfCfUfUfGfAfAfAfAfAfGfUfGfGfCfAfCfCfGfAfGfUfCfGfGfUfGfCfGCCACCUUCGGCUAACGUUGCUCCAAGGU ^{mU} * ^{mU} * ^{mU}
75	P51	mC*mC*mC*AUACCUUGGAGCAACGGfGUUuUfAfGfAfGfCfUfAfGfAfAfUfAfGfCfAfAGUuUfAAfAfAfUfAfAfGfGfCfUfAfGfUfCfCfGU(araU)AfUfCfAfAfCfUfUfGfAfAfAfAfAfGfUfGfGfCfAfCfCfGfAfGfUfCfGfGfUfGfCfGCCACCUUCGGCUAACGUUGCUCCAAGGU ^{mU} * ^{mU} * ^{mU}
76	P52	mC*mC*mC*AUACCUUGGAGCAACGGfGUUuUfAfGfAfGfCfUfAfGfAfAfUfAfGfCfAfAGUuUfAAfAfAfUfAfAfGfGfCfUfAfGfUfCfCfGU(5MerU)AfUfCfAfAfCfUfUfGfAfAfAfAfAfGfUfGfGfCfAfCfCfGfAfGfUfCfGfGfUfGfCfGCCACCUUCGGCUAACGUUGCUCCAAGGU ^{mU} * ^{mU} * ^{mU}
77	P53	mC*mC*mC*AUACCUUGGAGCAACGGfGUUuUfAfGfAfGmCmUfAfGfAfAfAmUfAfGmCfAfAGUuUfAAfAfAmUfAfAfGfGmCmUfAGmUmCmCGUUmUmCAfAmCmUmUfGfAfAfAfAfGmUfGfGmCfAmCmCfGfAfGmUmCfGfGmUfGmCGCCACCUUCGGCUAACGUUGCUCCAAGGU ^{mU} * ^{mU} * ^{mU}
78	P54	mC*mC*mC*AUACCUUGGAGCAACGGfGUUuUfAfGfAfGmCmUfAfGfAfAfAmUfAfGmCfAfAGUuUfAAfAfAmUfAfAfGfGmCmUfAGmUmCmCGU(NMP-U)AmUmCAfAmCmUmUfGfAfAfAfAfGmUfGfGmCfAmCmCfGfAfGmUmCfGfGmUfGmCGCCACCUUCGGCUAACGUUGCUCCAAGGU ^{mU} * ^{mU} * ^{mU}
79	P55	mC*mC*mC*AUACCUUGGAGCAACGGfGUUuUfAfGfAfGmCmUfAfGfAfAfAmUfAfGmCfAfAGUuUfAAfAfAmUfAfAfGfGmCmUfAGmUmCmCGU(araU)AmUmCAfAmCmUmUfGfAfAfAfAfGmUfGfGmCfAmCmCfGfAfGmUmCfGfGmUfGmCGCCACCUUCGGCUAACGUUGCUCCAAGGU ^{mU} * ^{mU} * ^{mU}
80	P56	mC*mC*mC*AUACCUUGGAGCAACGGfGUUuUfAfGfAfGmCmUfAfGfAfAfAmUfAfGmCfAfAGUuUfAAfAfAmUfAfAfGfGmCmUfAGmUmCmCGU(5MerU)AmUmCAfAmCmUmUfGfAfAfAfAfGmUfGfGmCfAmCmCfGfAfGmUmCfGfGmUfGmCGCCACCUUCGGCUAACGUUGCUCCAAGGU ^{mU} * ^{mU} * ^{mU}
81	P57	mC*mC*mC*AUACCUUGGAGCAACGGmGUUuUfUmAmGmAmGfCfUmAmGmAmAmAfUmAmGfCmAmAGUUmAAmAmAfUmAmAmGmGfCfUmAGfUfCfCGUuAfUfCmAmAfCfUfUmGmAmAmAmAmGfUmGmGfCmAfCfCmGmAmGfUfCmGmGfUmGfCGCCACCUUCGGCUAACGUUGCUCCAAGGU ^{mU} * ^{mU} * ^{mU}
82	P58	mC*mC*mC*AUACCUUGGAGCAACGGmGUUuUfUmAmGmAmGfCfUmAmGmAmAmAfUmAmGfCmAmAGUUmAAmAmAfUmAmAmGmGfCfUmAGfUfCfCGU(NMP-U)AfUfCmAmAfCfUfUmGmAmAmAmAmGfUmGmGfCmAfCfCmGmAmGfUfCmGmGfUmGfCGCCACCUUCGGCUAACGUUGCUCCAAGGU ^{mU} * ^{mU} * ^{mU}
83	P59	mC*mC*mC*AUACCUUGGAGCAACGGmGUUuUfUmAmGmAmGfCfUmAmGmAmAmAfUmAmGfCmAmAGUUmAAmAmAfUmAmAmGmGfCfUmAGfUfCfCGU(araU)AfUfCmAmAfCfUfUmGmAmAmAmAmAmGfUmGmGfCmAfCfCmGmAmGfUfCmGmGfUmGfCGCCACCUUCGGCUAACGUUGCUCCAAGGU ^{mU} * ^{mU} * ^{mU}
84	P60	mC*mC*mC*AUACCUUGGAGCAACGGmGUUuUfUmAmGmAmGfCfUmAmGmAmAmAfUmAmGfCmAmAGUUmAAmAmAfUmAmAmGmGfCfUmAGfUfCfCGU(5MerU)AfUfCmAmAfCfUfUmGmAmAmAmAmAmGfUmGmGfCmAfCfCmGmAmGfUfCmGmGfUmGfCGCCACCUUCGGCUAACGUUGCUCCAAGGU ^{mU} * ^{mU} * ^{mU}

85	P61	mC*mC*mC*AUACCUUGGAGCAACGGGUUUUAGAmGmCmUmA mGmAmAmAmUmAmGmCAAGUUAAAAUAAGGCUAGUCCGUUA UCAACUmUmGmAmAmAmAAGUGGCACcmGmAmGmUmCGGUG CGCCACCUUCGGCUAACGUUGCUCCAAGGUmU*mU*mU*mU
86	P62	mC*mC*mC*AUACCUUGGAGCAACGGGUUUUmUAGAmGmCmUm AmGmAmAmAmUmAmGCAAGUUAAAAUAAGGCUAGUCCGUUA UmCAACUmUmGmAmAmAmAAGUGGCACcmGmAmGmUmCGGU GCGCCACCUUCGGCUAACGUUGCUCCAAGGUmU*mU*mU*mU
87	P63	mC*mC*mC*AUACCUUGGAGCAACGGGUUUUmUAGAmGmCmUm AmGmAmAmAmUmAmGmCAAGUUAAAAUAAGGCUAGUCCGU(NMP- U)AUmCAACUmUmGmAmAmAmAAGUGGCACcmGmAmGmUmC GGUGCGCCACCUUCGGCUAACGUUGCUCCAAGGUmU*mU*mU* mU
88	P64	mC*mC*mC*AUACCUUGGAGCAACGGGUUUUmUAGAmGmCmUm AmGmAmAmAmUmAmGmCAAGUUAAAAUAAGGCUAGUCCGU(a raU)AUmCAACUmUmGmAmAmAmAAGUGGCACcmGmAmGmUm CGGUGCGCCACCUUCGGCUAACGUUGCUCCAAGGUmU*mU*m U*mU
89	P65	mC*mC*mC*AUACCUUGGAGCAACGGGUUUUmUAGAmGmCmUm AmGmAmAmAmUmAmGmCAAGUUAAAAUAAGGCUAGUCCGU(5 MerU)AUmCAACUmUmGmAmAmAmAAGUGGCACcmGmAmGmU mCGGUGCGCCACCUUCGGCUAACGUUGCUCCAAGGUmU*mU* mU*mU

* indicates phosphorothioate; 3'-idT-5' = inverted deoxythymidine; 3'-idA-5' = inverted deoxyadenosine; 3'-idU-5' = inverted deoxyuridine; mA = 2'O methyl adenosine; mC = 2'O methyl cytosine; mU = 2'O methyl uridine; mG = 2'O methyl guanosine; LNA-T = locked nucleic acid thymidine; NMP-U = N1-methyl-pseudouridine; riboT = ribothymidine; fA = 2' fluoro adenosine; fC = 2' fluoro cytosine; fU = 2' fluoro uridine; fG = 2' fluoro guanosine; araU = arabinoside; 5MerU = 5' methyl uridine.

[00275] Table 6

SEQ ID NO.:	ngRNA Name	Modified Sequence
90	N1	C*U*A*ACGUUGCUCCAAGGUAUGUUUUAGAGCUAGA AAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAAC UUGAAAAAGUGGCACCGAGUCGGUGCLNA-T*LNA- T*LNA-T*LNA-T
91	N2	C*C*G*CGCGCGGAAAAAGCCGGUUUUAGAGCUAGAA AUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACU UGAAAAAGUGGCACCGAGUCGGUGC LNA-T*LNA- T*LNA-T*LNA-T

* indicates phosphorothioate; LNA-T = locked nucleic acid thymidine.

[00276] FIG. 12 shows evaluation of Pcsk9 editing efficiency with PEGRNAs P2, P31, P32, P33, P34, P35, P36, P37, P38, and P1.

OTHER EMBODIMENTS

[00277] The above examples are to be understood as illustrative examples. Further examples are envisaged, which include combinations of features as indicated in the following embodiments, which lists various envisaged claim dependencies for the claims originally filed with this application. Hence, in addition to the description above, these embodiments provide basis for examples having a combination of features of claims filed herewith.

[00278] Embodiment 1: A modified PEGRNA comprising, 5' to 3':

- (a) a spacer that comprises a region of complementarity to a search target sequence in a target strand of a double-stranded target DNA;
- (b) a guide RNA (gRNA) core capable of binding to a Cas protein wherein the gRNA core comprises, in 5' to 3' order, a tetraloop, a first stem loop (SL1), a second stem loop (SL2), and a third stem loop (SL3);
- (c) an extension arm comprising:
 - (i) an editing template that comprises an intended edit compared to the double-stranded target DNA, and
 - (ii) a primer binding site (PBS) that comprises a region of complementarity to a region upstream of a nick site in a non-target strand of the double-stranded target DNA; and
- (d) wherein the PEGRNA comprises 5 or more nucleotide modifications.

[00279] Embodiment 2: In another aspect, the disclosure provides a modified ngRNA comprising, 5' to 3':

- (a) a spacer that comprises a region of complementarity to a search target sequence in a target strand of a double-stranded target DNA;
- (b) a guide RNA (gRNA) core capable of binding to a Cas protein wherein the gRNA core comprises, in 5' to 3' order, a tetraloop, a first stem loop (SL1), a second stem loop (SL2), and a third stem loop (SL3); and
- (c) wherein the ngRNA comprises 5 or more nucleotide modifications.

[00280] Embodiment 3: a nucleotide modification is located in the 3' end.

[00281] Embodiment 4: a nucleotide modification is located in the 5' end.

[00282] Embodiment 5: nucleotide modifications are located in the 3' end and the 5' end.

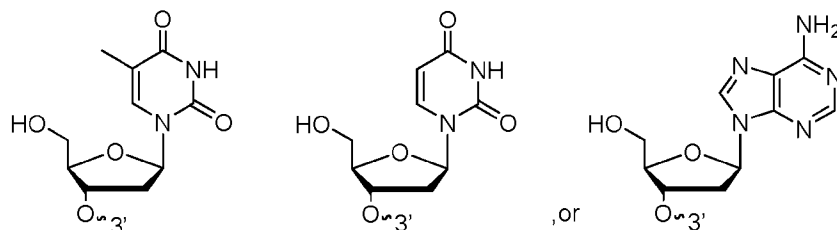
- [00283] Embodiment 6: a nucleotide modification is located in the spacer.
- [00284] Embodiment 7: a nucleotide modification is located in the gRNA core.
- [00285] Embodiment 8: a nucleotide modification is located in the extension arm.
- [00286] Embodiment 9: a nucleotide modification is located in the tetraloop.
- [00287] Embodiment 10: a nucleotide modification is located in the SL1.
- [00288] Embodiment 11: a nucleotide modification is located in the SL2.
- [00289] Embodiment 12: a nucleotide modification is located in the SL3.
- [00290] Embodiment 13: at least 2 or more nucleotide modifications are located in the 5' end, 3' end, tetraloop, SL2, or SL3.
- [00291] Embodiment 14: nucleotide modifications are located in the tetraloop, SL1, SL2, and SL3.
- [00292] Embodiment 15: nucleotide modifications are located in the tetraloop, SL2, and SL3.
- [00293] Embodiment 16: nucleotide modifications are located in the 5' end, 3' end, tetraloop, SL2, and SL3.
- [00294] Embodiment 17: at least 2 or more nucleotide modifications occur within the gRNA.
- [00295] Embodiment 18: the gRNA core comprises a nucleotide modification at nucleotide 25, 37, 38, 46, 64, 65, 67, or 68.
- [00296] Embodiment 19: the gRNA core comprises a nucleotide modification at nucleotide 25.
- [00297] Embodiment 20: the gRNA core comprises a nucleotide modification at nucleotide 37 and/or 38.
- [00298] Embodiment 21: the gRNA core comprises a nucleotide modification at nucleotide 65.
- [00299] Embodiment 22: the gRNA core comprises a nucleotide modification at nucleotide 68.
- [00300] Embodiment 23: the PEgRNA or ngRNA comprises 10 or more nucleotide modifications.
- [00301] Embodiment 24: the PEgRNA or ngRNA comprises 20 or more nucleotide modifications.
- [00302] Embodiment 25: the PEgRNA or ngRNA comprises 30 or more nucleotide modifications.

- [00303] Embodiment 26: the PEgRNA or ngRNA comprises 40 or more nucleotide modifications.
- [00304] Embodiment 27: the PEgRNA or ngRNA comprises 50 or more nucleotide modifications.
- [00305] Embodiment 28: the PEgRNA or ngRNA comprises 60 or more nucleotide modifications.
- [00306] Embodiment 29: the PEgRNA or ngRNA comprises 70 or more nucleotide modifications.
- [00307] Embodiment 30: the PEgRNA or ngRNA comprises 80 or more nucleotide modifications.
- [00308] Embodiment 31: the PEgRNA or ngRNA comprises 90 or more nucleotide modifications.
- [00309] Embodiment 32: the PEgRNA or ngRNA comprises 100 or more modified nucleotides.
- [00310] Embodiment 33: a base modification is selected from the group consisting of N⁶-methyladenosine (m⁶A), N⁶-methyl-2'-O-methyladenosine (2'OMe-m⁶A), xanthine, allyaminouracil, allyaminothymidine, hypoxanthine, digoxigeninated adenine, digoxigeninated cytosine, digoxigeninated guanine, digoxigeninated uracil, 6-chloropurineriboside, N6-methyladenine, methylpseudouracil, 2-thiocytosine, 2-thiouracil, 5-methyluracil, 4-thiothymidine, 4-thiouracil, 5,6-dihydro-5-methyluracil, 5,6-dihydrouracil, 5-[(3-Indolyl)propionamide-N-allyl]uracil, 5-aminoallylcytosine, 5-aminoallyluracil, 5-bromouracil, 5-bromocytosine, 5-carboxycytosine, 5-carboxymethylesteruracil, 5-carboxyuracil, 5-fluorouracil, 5-formylcytosine, 5-formyluracil, 5-hydroxycytosine, 5-hydroxymethylcytosine, 5-hydroxymethyluracil, 5-hydroxyuracil, 5-iodocytosine, 5-iodouracil, 5-methoxycytosine, 5-methoxyuracil, 5-methylcytosine, 5-methyluracil, 5-propargylaminocytosine, 5-propargylaminouracil, 5-propynylcytosine, 5-propynyluracil, 6-azacytosine, 6-azauracil, 6-chloropurine, 6-thioguanine, 7-deazaadenine, 7-deazaguanine, 7-deaza-7-propargylaminoadenine, 7-deaza-7-propargylaminoguanine, 8-azaadenine, 8-azidoadenine, 8-chloroadenine, 8-oxoadenine, 8-oxoguanine, araadenine, aracytosine, araguanine, arauracil, biotin-16-7-deaza-7-propargylaminoguanine, biotin-16-aminoallylcytosine, biotin-16-aminoallyluracil, cyanine 3-5-propargylaminocytosine, cyanine 3-6-propargylaminouracil, cyanine 3-aminoallylcytosine, cyanine 3-aminoallyluracil, cyanine 5-6-propargylaminocytosine, cyanine 5-6-propargylaminouracil, cyanine 5-aminoallylcytosine, cyanine 5-aminoallyluracil, cyanine 7-aminoallyluracil, dabcy1-5-3 -

aminoallyluracil, desthiobiotin- 16-aminoallyl-uracil, desthiobiotin-6-aminoallylcytosine, isoguanine, N¹-ethylpseudouracil, N¹-methoxymethylpseudouracil, N¹-methyladenine, N¹-methylpseudouracil, N¹-propylpseudouracil, N²-methylguanine, N⁴-biotin-OBEA-cytosine, N⁴-methylcytosine, N⁶-methyladenine, O⁶-methylguanine, pseudoisocytosine, pseudouracil, thienocytosine, thienoguanine, thienouracil, xanthosine, 3-deazaadenine, 2,6-diaminoadenine, 2,6-daminoguanine, 5-carboxamide-uracil, 5-ethynyluracil, N⁶-isopentenyladenine (i⁶A), 2-methylthio- N⁶-isopentenyladenine (ms2i⁶A), 2-methylthio- N⁶-methyladenine (ms2m⁶A), N⁶-(cis-hydroxyisopentenyl)adenine (io⁶A), 2-methylthio- N⁶-(cis-hydroxyisopentenyl)adenine (ms2io⁶A), N⁶-glycylcarbamoyladenine (g⁶A), N⁶-threonylcarbamoyladenine (t⁶A), 2-methylthio- N⁶-threonyl carbamoyladenine (ms2t⁶A), N⁶-methyl- N⁶-threonylcarbamoyladenine (m⁶t⁶A), N⁶-hydroxynorvalylcarbamoyladenine (hn⁶A), 2- methylthio- N⁶-hydroxynorvalyl carbamoyladenine (ms2hn⁶A), N⁶, N⁶-dimethyladenine (m⁶2A), and N⁶-acetyladenine (ac⁶A).

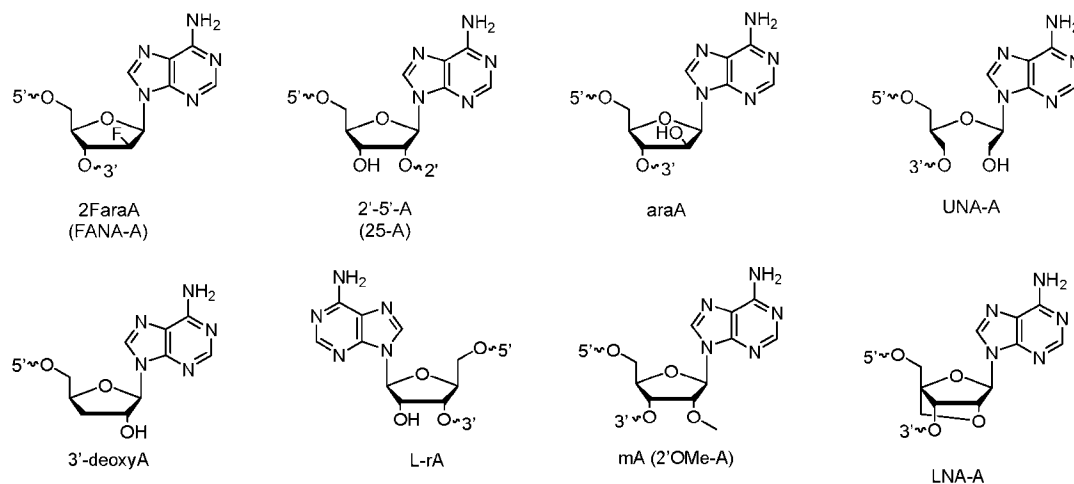
[00311] Embodiment 34: the modification comprises an inverted nucleotide located in the 3' end.

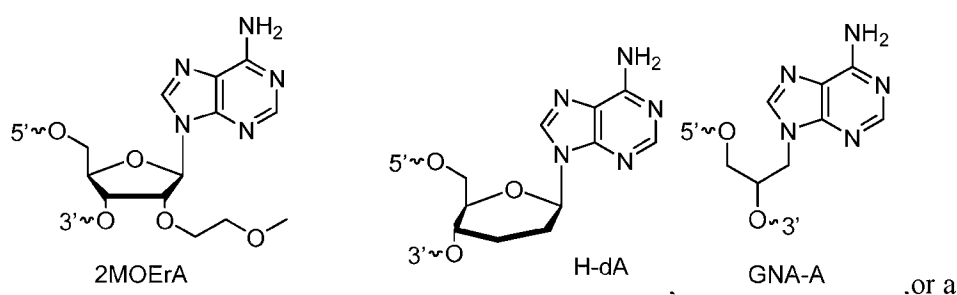
[00312] Embodiment 35: the inverted nucleotide comprises



[00313] Embodiment 36: a nucleotide modification is a sugar modification.

[00314] Embodiment 37: the sugar modification comprises





combination thereof.

[00315] Embodiment 38: the sugar modification is selected from the group consisting of 2'-thioribose, 2', 3'-dideoxyribose, 2'-amino-2'-deoxyribose, 2' deoxyribose, 2'-azido-2'- deoxyribose, 2'-fluoro-2'-deoxyribose, 2'-O-methoxyethyl (MOE), 2'-O-methylribose, 2'-O-methyldeoxyribose, 3'-amino- 2',3'-dideoxyribose, 3'-azido-2', 3'-dideoxyribose, 3'-deoxyribose, 3'-O-(2-nitrobenzyl)-2'-deoxyribose, 3'-O-methylribose, 5'-aminoribose, 5'-thioribose, 5-nitro-1-indolyl-2'-deoxyribose, 5'-biotin-ribose, 2'-O,4'-C-methylene-linked ribose, 2'-O,4'-C-amino-linked ribose, and 2'-O,4'-C-thio-linked ribose.

[00316] Embodiment 39: a nucleotide modification is a phosphate modification.

[00317] Embodiment 40: a phosphate modification comprises phosphorothioate, a stereospecific phosphorothioate, or a combination thereof.

[00318] Embodiment 41: the phosphate modification is selected from the group consisting of phosphorothioate (PS), a stereospecific phosphorothioate, phosphorodithioate, thiophosphate, 5'-O-methylphosphonate, 3'-O- methylphosphonate, 5'-hydroxyphosphonate, hydroxyphosphanate, phosphoroselenoate, selenophosphate, phosphoramidate, carbophosphonate, methylphosphonate, phenylphosphonate, ethylphosphonate, H-phosphonate, guanidinium ring, triazole ring, boranophosphate (BP), methylphosphonate, and guanidinopropyl phosphoramidate.

[00319] Embodiment 42: the nucleotide modification comprises a phosphate modification, a base modification, a sugar modification, or a combination thereof.

[00320] Embodiment 43: a modified PEgRNA sequence is selected from the group consisting of Tables 5 or 6.

[00321] Embodiment 44: the non-nucleotide modification comprises a hairpin, a C₂-C₁₂ linker, an ethylene glycol linker, 2'-5' linkages, or a combination thereof.

[00322] Embodiment 45: a method for producing a modified PEgRNA or modified ngRNA, the method comprising ligating a coding nucleotide sequence with the modified nucleic acid sequence of any of the claims disclosed herein.

[00323] Embodiment 46: a method for producing a modified PEgRNA or modified ngRNA, the method comprising ligating a sequence encoding a prime editor with the modified nucleic acid sequence of any of the claims disclosed herein.

[00324] Embodiment 47: the ligation comprises a self-templated enzymatic ligation, optionally the self-templated enzymatic ligation is splint-mediated.

[00325] Embodiment 48: the ligation comprises a templated enzymatic ligation.

[00326] Embodiment 49: the enzymatic ligation is catalyzed by a nucleic acid ligase to generate a natural phosphodiester linkage between the coding nucleotide sequence and the modified nucleic acid sequence of any one of the foregoing claims, or between the sequence encoding a prime editor with the modified nucleic acid sequence of any one of the foregoing claims, or between two fragments of the nucleotide sequences containing the chemical modifications of any one of the foregoing claims.

[00327] Embodiment 50: the nucleic acid ligase is T4 RNA Ligase I, T4 RNA Ligase II, or T4 DNA Ligase.

[00328] Embodiment 51: a prime editing system comprising a modified PEgRNA or modified ngRNA of any of the claims disclosed herein or one or more polynucleotides encoding the modified PEgRNA or modified ngRNA.

[00329] Embodiment 52: a lipid nanoparticle comprising the modified PEgRNA or modified ngRNA of any one of the claims disclosed herein.

[00330] Embodiment 53: a lipid nanoparticle comprising the prime editing system disclosed herein.

[00331] Embodiment 54: a method for editing a gene, the method comprising contacting the gene with the modified PEgRNA, modified ngRNA, prime editing system, lipid nanoparticle, or any of the disclosed claims described herein.

[00332] It is to be understood that while the disclosure has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the disclosure, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.

WHAT IS CLAIMED IS:

1. A modified prime editing guide RNA (PEgRNA) comprising, 5' to 3':
 - (a) a spacer that comprises a region of complementarity to a search target sequence in a target strand of a double-stranded target DNA;
 - (b) a guide RNA (gRNA) core capable of binding to a Cas protein wherein the gRNA core comprises, in 5' to 3' order, a tetraloop, a first stem loop (SL1), a second stem loop (SL2), and a third stem loop (SL3);
 - (c) an extension arm comprising:
 - (i) an editing template that comprises an intended edit compared to the double-stranded target DNA, and
 - (ii) a primer binding site (PBS) that comprises a region of complementarity to a region upstream of a nick site in a non-target strand of the double-stranded target DNA; and
 - (d) wherein the PEgRNA comprises 5 or more nucleotide modifications.
2. The modified PEgRNA of claim 1, wherein a nucleotide modification is located in the 3' end.
3. The modified PEgRNA of any one of the forgoing claims, wherein a nucleotide modification is located in the 5' end.
4. The modified PEgRNA of any one of the forgoing claims, wherein nucleotide modifications are located in the 3' end and the 5' end.
5. The modified PEgRNA of any one of the forgoing claims, wherein a nucleotide modification is located in the spacer.
6. The modified PEgRNA of any one of the forgoing claims, wherein a nucleotide modification is located in the gRNA core.
7. The modified PEgRNA of any one of the forgoing claims, wherein a nucleotide modification is located in the extension arm.
8. The modified PEgRNA of any one of the forgoing claims, wherein a nucleotide modification is located in the tetraloop.
9. The modified PEgRNA of any one of the forgoing claims, wherein a nucleotide modification is located in the SL1.
10. The modified PEgRNA of any one of the forgoing claims, wherein a nucleotide modification is located in the SL2.
11. The modified PEgRNA of any one of the forgoing claims, wherein a nucleotide modification is located in the SL3.

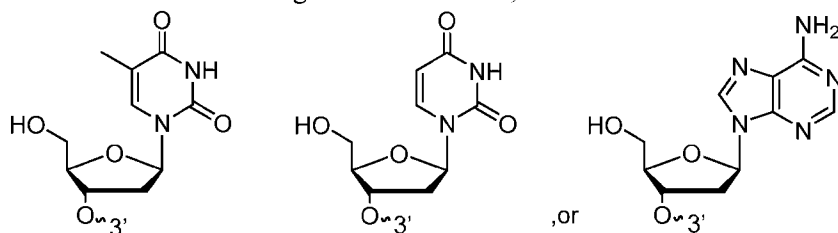
12. The modified PEgRNA of any one of the forgoing claims, wherein at least 2 or more nucleotide modifications are located in the 5' end, 3' end, tetraloop, SL2, or SL3.
13. The modified PEgRNA of any one of the forgoing claims, wherein nucleotide modifications are located in the tetraloop, SL1, SL2, and SL3.
14. The modified PEgRNA of any one of the forgoing claims, wherein nucleotide modifications are located in the tetraloop, SL2, and SL3.
15. The modified PEgRNA of any one of the forgoing claims, wherein nucleotide modifications are located in the 5' end, 3' end, tetraloop, SL2, and SL3.
16. The modified PEgRNA of any one of the forgoing claims, wherein at least 2 or more nucleotide modifications occur within the gRNA.
17. The modified PEgRNA of any one of the forgoing claims, wherein the gRNA core comprises a nucleotide modification at nucleotide 25, 37, 38, 46, 64, 65, 67, or 68.
18. The modified PEgRNA of any one of the forgoing claims, wherein the gRNA core comprises a nucleotide modification at nucleotide 25.
19. The modified PEgRNA of any one of the forgoing claims, wherein the gRNA core comprises a nucleotide modification at nucleotide 37 and/or 38.
20. The modified PEgRNA of any one of the forgoing claims, wherein the gRNA core comprises a nucleotide modification at nucleotide 65.
21. The modified PEgRNA of any one of the forgoing claims, wherein the gRNA core comprises a nucleotide modification at nucleotide 68.
22. The modified PEgRNA of any one of the forgoing claims, wherein the PEgRNA comprises 10 or more nucleotide modifications.
23. The modified PEgRNA of any one of the forgoing claims, wherein the PEgRNA comprises 20 or more nucleotide modifications.
24. The modified PEgRNA of any one of the forgoing claims, wherein the PEgRNA comprises 30 or more nucleotide modifications.
25. The modified PEgRNA of any one of the forgoing claims, wherein the PEgRNA comprises 40 or more nucleotide modifications.
26. The modified PEgRNA of any one of the forgoing claims, wherein the PEgRNA comprises 50 or more nucleotide modifications.
27. The modified PEgRNA of any one of the forgoing claims, wherein the PEgRNA comprises 60 or more nucleotide modifications.
28. The modified PEgRNA of any one of the forgoing claims, wherein the PEgRNA comprises 70 or more nucleotide modifications.

29. The modified PEgRNA of any one of the forgoing claims, wherein the PEgRNA comprises 80 or more nucleotide modifications.
30. The modified PEgRNA of any one of the forgoing claims, wherein the PEgRNA comprises 90 or more nucleotide modifications.
31. The modified PEgRNA of any one of the forgoing claims, wherein the PEgRNA comprises 100 or more modified nucleotides.
32. The modified PEgRNA of any one of the forgoing claims, wherein a nucleotide modification comprises a phosphate modification, a base modification, a sugar modification, or a combination thereof.
33. The modified PEgRNA of any one of the forgoing claims, wherein a nucleotide modification comprises a base modification.
34. The modified PEgRNA of any one of the forgoing claims, wherein the base modification is selected from the group consisting of N⁶-methyladenosine (m⁶A), N⁶-methyl-2'-O-methyladenosine (2'OMe-m⁶A), xanthine, ally aminouracil, ally aminothymidine, hypoxanthine, digoxigeninated adenine, digoxigeninated cytosine, digoxigeninated guanine, digoxigeninated uracil, 6- chloropurineriboside, N6-methyladenine, methylpseudouracil, 2-thiocytosine, 2-thiouracil, 5- methyluracil, 4-thiothymidine, 4-thiouracil, 5,6-dihydro-5-methyluracil, 5,6-dihydrouracil, 5-[(3- Indolyl)propionamide-N-allyl]uracil, 5-aminoallylcytosine, 5-aminoallyluracil, 5-bromouracil, 5- bromocytosine, 5-carboxycytosine, 5-carboxymethylesteruracil, 5-carboxyuracil, 5-fluorouracil, 5- formylcytosine, 5-formyluracil, 5-hydroxycytosine, 5-hydroxymethylcytosine, 5- hydroxymethyluracil, 5-hydroxyuracil, 5-iodocytosine, 5-iodouracil, 5-methoxycytosine, 5- methoxyuracil, 5-methylcytosine, 5-methyluracil, 5-propargylaminocytosine, 5- propargylaminouracil, 5-propynylcytosine, 5-propynyluracil, 6-azacytosine, 6-azauracil, 6- chloropurine, 6-thioguanine, 7-deazaadenine, 7-deazaguanine, 7-deaza-7-propargylaminoadenine, 7- deaza-7-propargylaminoguanine, 8-azaadenine, 8-azidoadenine, 8-chloroadenine, 8-oxoadenine, 8-oxoguanine, araadenine, aracytosine, araguanine, arauracil, biotin- 16-7-deaza-7-propargylaminoguanine, biotin- 16-aminoallylcytosine, biotin- 16-aminoallyluracil, cyanine 3-5- propargylaminocytosine, cyanine 3-6-propargylaminouracil, cyanine 3-aminoallylcytosine, cyanine 3-aminoallyluracil, cyanine 5-6-propargylaminocytosine, cyanine 5-6-propargylaminouracil, cyanine 5-aminoallylcytosine, cyanine 5-aminoallyluracil, cyanine 7-aminoallyluracil, dabcy1-5-3 - aminoallyluracil, desthiobiotin- 16-aminoallyluracil, desthiobiotin-6-aminoallylcytosine, isoguanine, N¹-ethylpseudouracil, N¹-methoxymethylpseudouracil, N¹-methyladenine, N¹-methylpseudouracil, N¹-

propylpseudouracil, N²-methylguanine, N⁴-biotin-OBEA-cytosine, N⁴-methylcytosine, N⁶-methyladenine, O⁶-methylguanine, pseudoisocytosine, pseudouracil, thienocytosine, thienoguanine, thienouracil, xanthosine, 3-deazaadenine, 2,6-diaminoadenine, 2,6-daminoguanine, 5-carboxamide- uracil, 5-ethynyluracil, N⁶-isopentenyladenine (i⁶A), 2-methyl-thio- N⁶-isopentenyladenine (ms2i⁶A), 2-methylthio- N⁶-methyladenine (ms2m⁶A), N⁶-(cis-hydroxyisopentenyl)adenine (io⁶A), 2-methylthio- N⁶-(cis-hydroxyisopentenyl)adenine (ms2io⁶A), N⁶-glycincylcarbamoyladenine (g⁶A), N⁶-threonylcarbamoyladenine (t⁶A), 2-methylthio- N⁶-threonyl carbamoyladenine (ms2t⁶A), N⁶-methyl- N⁶-threonylcarbamoyladenine (m⁶t⁶A), N⁶-hydroxynorvalylcarbamoyladenine (hn⁶A), 2- methylthio- N⁶-hydroxynorvalyl carbamoyladenine (ms2hn⁶A), N⁶, N⁶-dimethyladenine (m⁶2A), and N⁶-acetyladenine (ac⁶A).

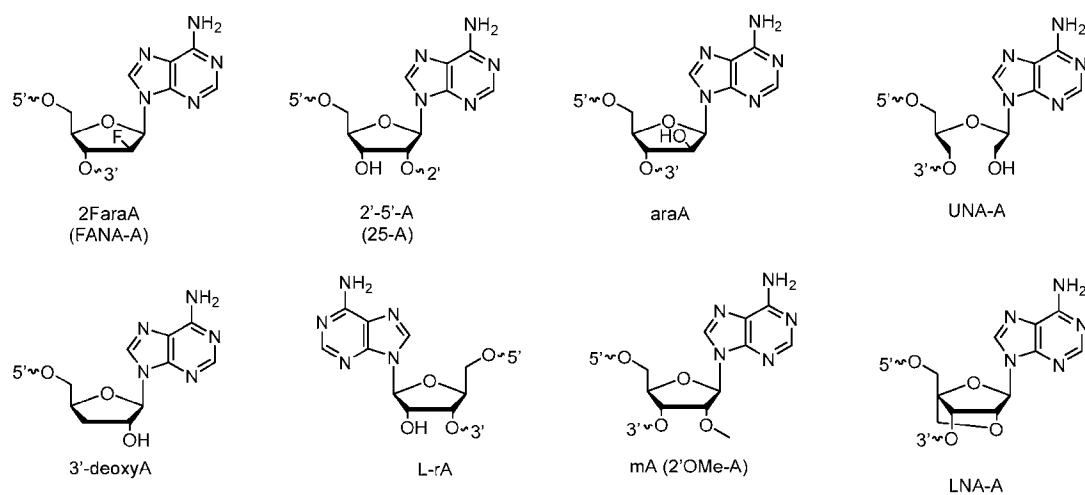
35. The modified PEGRNA of any one of the forgoing claims, wherein the modification comprises an inverted nucleotide located in the 3' end.

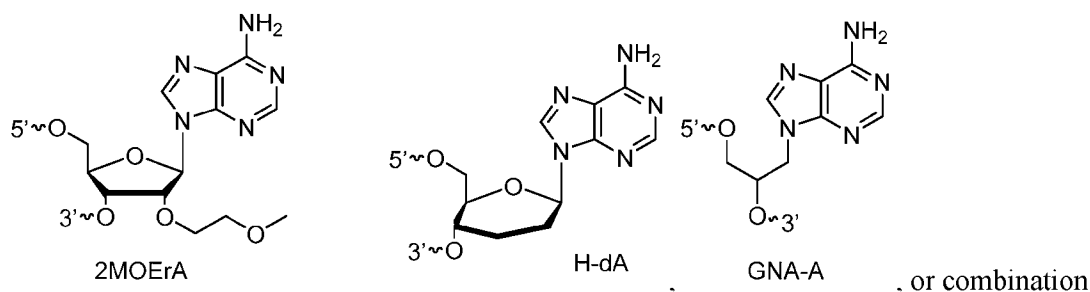
36. The modified PEGRNA of claim 34, wherein the inverted nucleotide comprises



37. The modified PEGRNA of any one of the forgoing claims, wherein a nucleotide modification is a sugar modification.

38. The modified PEGRNA of any one of the forgoing claims, wherein the sugar modification comprises





thereof.

39. The modified PEgRNA of any one of the forgoing claims, wherein the sugar modification is selected from the group consisting of 2'-thioribose, 2', 3'-dideoxyribose, 2'-amino-2'-deoxyribose, 2'-deoxyribose, 2'-azido-2'-deoxyribose, 2'-fluoro-2'-deoxyribose, 2'-O-methoxyethyl (MOE), 2'-O-methylribose, 2'-O-methyldeoxyribose, 3'-amino-2',3'-dideoxyribose, 3'-azido-2', 3'-dideoxyribose, 3'-deoxyribose, 3'-O-(2-nitrobenzyl)-2'-deoxyribose, 3'-O-methylribose, 5'-aminoribose, 5'-thioribose, 5-nitro-1-indolyl-2'-deoxyribose, 5'-biotin-ribose, 2'-O,4'-C-methylene-linked ribose, 2'-O,4'-C-amino-linked ribose, and 2'-O,4'-C-thio-linked ribose, or combination thereof.

40. The modified PEgRNA of any one of the forgoing claims, wherein a nucleotide modification is a phosphate modification.

41. The modified PEgRNA of any one of the forgoing claims, wherein a phosphate modification comprises phosphorothioate, a stereospecific phosphorothioate, or a combination thereof.

42. The modified PEgRNA of any one of the forgoing claims, wherein the phosphate modification is selected from the group consisting of phosphorothioate (PS), a stereospecific phosphorothioate, phosphorodithioate, thiophosphate, 5'-O-methylphosphonate, 3'-O-methylphosphonate, 5'-hydroxyphosphonate, hydroxyphosphanate, phosphoroselenoate, selenophosphate, phosphoramidate, carbophosphonate, methylphosphonate, phenylphosphonate, ethylphosphonate, H-phosphonate, guanidinium ring, triazole ring, boranophosphate (BP), methylphosphonate, and guanidinopropyl phosphoramidate, or combination thereof.

43. A modified PEgRNA sequence selected from the group consisting of Tables 5 or 6.

44. A modified prime editor nicking guide RNA (ngRNA) comprising, 5' to 3':

(a) a spacer that comprises a region of complementarity to a search target sequence in a target strand of a double-stranded target DNA;

(b) a guide RNA (gRNA) core capable of binding to a Cas protein wherein the gRNA core comprises, in 5' to 3' order, a tetraloop, a first stem loop (SL1), a second stem loop (SL2), and a third stem loop (SL3); and

wherein the ngRNA comprises 5 or more nucleotide modifications.

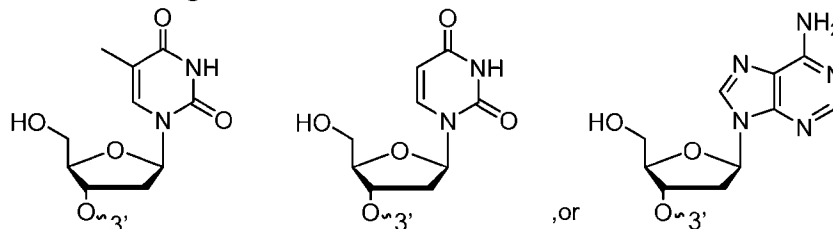
45. The modified ngRNA of claim 46, wherein a nucleotide modification is located in the 3' end.
46. The modified ngRNA of any one of the forgoing claims, wherein a nucleotide modification is located in the 5' end.
47. The modified ngRNA of any one of the forgoing claims, wherein nucleotide modifications are located in the 3' end and the 5' end.
48. The modified ngRNA of any one of the forgoing claims, wherein a nucleotide modification is located in the spacer.
49. The modified ngRNA of any one of the forgoing claims, wherein a nucleotide modification is located in the gRNA core.
50. The modified ngRNA of any one of the forgoing claims, wherein a nucleotide modification is located in the tetraloop.
51. The modified ngRNA of any one of the forgoing claims, wherein a nucleotide modification is located in the SL1.
52. The modified ngRNA of any one of the forgoing claims, wherein a nucleotide modification is located in the SL2.
53. The modified ngRNA of any one of the forgoing claims, wherein a nucleotide modification is located in the SL3.
54. The modified ngRNA of any one of the forgoing claims, wherein at least 2 or more nucleotide modifications are located in the 5' end, 3' end, tetraloop, SL2, or SL3.
55. The modified ngRNA of any one of the forgoing claims, wherein nucleotide modifications are located in the tetraloop, SL1, SL2, and SL3.
56. The modified ngRNA of any one of the forgoing claims, wherein nucleotide modifications are located in the tetraloop, SL2, and SL3.
57. The modified ngRNA of any one of the forgoing claims, wherein nucleotide modifications are located in the 5' end, 3' end, tetraloop, SL2, and SL3.
58. The modified ngRNA of any one of the forgoing claims, wherein at least 2 or more nucleotide modifications occur within the gRNA.
59. The modified ngRNA of any one of the forgoing claims, wherein the gRNA core comprises a nucleotide modification at nucleotide 25, 37, 38, 46, 64, 65, 67, or 68.

60. The modified ngRNA of any one of the forgoing claims, wherein the gRNA core comprises a nucleotide modification at nucleotide 25.
61. The modified ngRNA of any one of the forgoing claims, wherein the gRNA core comprises a nucleotide modification at nucleotide 37 and/or 38.
62. The modified ngRNA of any one of the forgoing claims wherein the gRNA core comprises a nucleotide modification at nucleotide 65.
63. The modified ngRNA of any one of the forgoing claims, wherein the gRNA core comprises a nucleotide modification at nucleotide 68.
64. The modified ngRNA of any one of the forgoing claims, wherein the ngRNA comprises 10, 20, 30, 40, 50, 60, 70, 80, 90, 100 or more nucleotide modifications.
65. The modified ngRNA of any one of the forgoing claims, wherein a nucleotide modification comprises a phosphate modification, a base modification, a sugar modification, or a combination thereof.
66. The modified ngRNA of any one of the forgoing claims, wherein a nucleotide modification comprises a base modification.
67. The modified ngRNA of any one of the forgoing claims, wherein the base modification is selected from the group consisting of N⁶-methyladenosine (m⁶A), N⁶-methyl-2'-O-methyladenosine (2'OMe-m⁶A), xanthine, allyaminouracil, allyaminothymidine, hypoxanthine, digoxigeninated adenine, digoxigeninated cytosine, digoxigeninated guanine, digoxigeninated uracil, 6- chloropurineriboside, N6-methyladenine, methylpseudouracil, 2-thiocytosine, 2-thiouracil, 5- methyluracil, 4-thiothymidine, 4-thiouracil, 5,6-dihydro-5-methyluracil, 5,6-dihydrouracil, 5-[(3- Indolyl)propionamide-N-allyl]uracil, 5-aminoallylcytosine, 5-aminoallyluracil, 5-bromouracil, 5- bromocytosine, 5-carboxycytosine, 5-carboxymethylesteruracil, 5-carboxyuracil, 5-fluorouracil, 5- formylcytosine, 5-formyluracil, 5-hydroxycytosine, 5-hydroxymethylcytosine, 5- hydroxymethyluracil, 5-hydroxyuracil, 5-iodocytosine, 5-iodouracil, 5-methoxycytosine, 5- methoxyuracil, 5-methylcytosine, 5-methyluracil, 5-propargylaminocytosine, 5- propargylaminouracil, 5-propynylcytosine, 5-propynyluracil, 6-azacytosine, 6-azauracil, 6- chloropurine, 6-thioguanine, 7-deazaadenine, 7-deazaguanine, 7-deaza-7-propargylaminoadenine, 7- deaza-7-propargylaminoguanine, 8-azaadenine, 8-azidoadenine, 8-chloroadenine, 8-oxoadenine, 8-oxoguanine, araadenine, aracytosine, araguanine, arauracil, biotin- 16-7-deaza-7-propargylaminoguanine, biotin- 16-aminoallylcytosine, biotin- 16-aminoallyluracil, cyanine 3-5- propargylaminocytosine, cyanine 3-6-propargylaminouracil, cyanine 3-aminoallylcytosine, cyanine 3-aminoallyluracil, cyanine 5-6-propargylaminocytosine,

cyanine 5-6-propargylaminouracil, cyanine 5-aminoallylcytosine, cyanine 5-aminoallyluracil, cyanine 7-aminoallyluracil, dabcyl-5-3 - aminoallyluracil, desthiobiotin- 16-aminoallyl-uracil, desthiobiotin-6-aminoallylcytosine, isoguanine, N¹ -ethylpseudouracil, N¹-methoxymethylpseudouracil, N¹-methyladenine, N¹-methylpseudouracil, N¹-propylpseudouracil, N²-methylguanine, N⁴-biotin-OBEA-cytosine, N⁴-methylcytosine, N⁶-methyladenine, O⁶-methylguanine, pseudoisocytosine, pseudouracil, thienocytosine, thienoguanine, thienouracil, xanthosine, 3-deazaadenine, 2,6-diaminoadenine, 2,6-daminoguanine, 5-carboxamide- uracil, 5-ethynyluracil, N⁶-isopentenyladenine (i⁶A), 2-methyl-thio- N⁶-isopentenyladenine (ms2i⁶A), 2-methylthio- N⁶-methyladenine (ms2m⁶A), N⁶-(cis-hydroxyisopentenyl)adenine (io⁶A), 2-methylthio- N⁶-(cis-hydroxyisopentenyl)adenine (ms2io⁶A), N⁶-glycinylicarbamoyladenine (g⁶A), N⁶-threonylicarbamoyladenine (t⁶A), 2-methylthio- N⁶-threonyl carbamoyladenine (ms2t⁶A), N⁶-methyl- N⁶-threonylicarbamoyladenine (m⁶t⁶A), N⁶-hydroxynorvalylcarbamoyladenine (hn⁶A), 2- methylthio- N⁶-hydroxynorvalyl carbamoyladenine (ms2hn⁶A), N⁶, N⁶-dimethyladenine (m⁶2A), and N⁶-acetyladenine (ac⁶A).

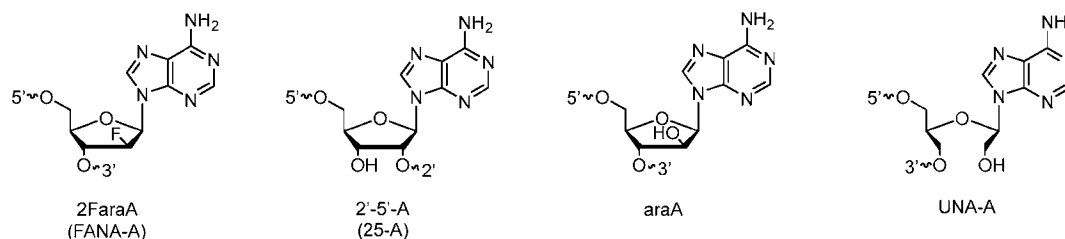
68. The modified ngRNA of any one of the forgoing claims, wherein the modification comprises an inverted nucleotide located in the 3' end.

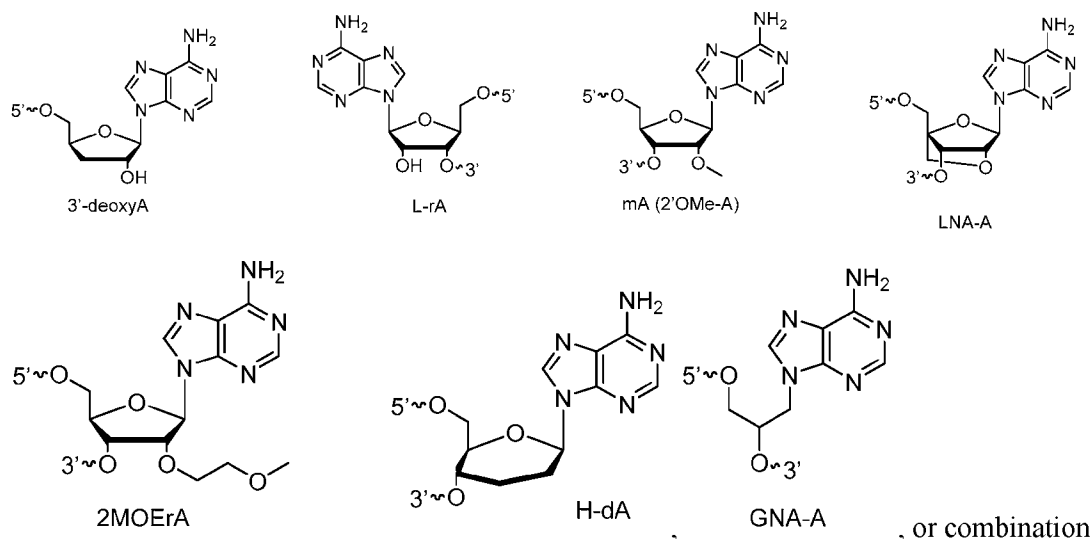
69. The modified ngRNA of claim 68, wherein the inverted nucleotide comprises



70. The modified ngRNA of any one of the forgoing claims, wherein a nucleotide modification is a sugar modification.

71. The modified ngRNA of any one of the forgoing claims, wherein the sugar modification comprises





thereof.

72. The modified ngRNA of any one of the forgoing claims, wherein the sugar modification is selected from the group consisting of 2'-thioribose, 2', 3'-dideoxyribose, 2'-amino-2'-deoxyribose, 2'-deoxyribose, 2'-azido-2'-deoxyribose, 2'-fluoro-2'-deoxyribose, 2'-O-methoxyethyl (MOE), 2'-O-methylribose, 2'-O-methyldeoxyribose, 3'-amino-2', 3'-dideoxyribose, 3'-azido-2', 3'-dideoxyribose, 3'-deoxyribose, 3'-O-(2-nitrobenzyl)-2'-deoxyribose, 3'-O-methylribose, 5'-aminoribose, 5'-thioribose, 5-nitro-1-indolyl-2'-deoxyribose, 5'-biotin-ribose, 2'-O,4'-C-methylene-linked ribose, 2'-O,4'-C-amino-linked ribose, and 2'-O,4'-C-thio-linked ribose.

73. The modified ngRNA of any one of the forgoing claims, wherein a nucleotide modification is a phosphate modification.

74. The modified ngRNA of any one of the forgoing claims, wherein a phosphate modification comprises phosphorothioate, a stereospecific phosphorothioate, or a combination thereof.

75. The modified ngRNA of any one of the forgoing claims, wherein the phosphate modification is selected from the group consisting of phosphorothioate (PS), phosphorodithioate, thiophosphate, 5'-O-methylphosphonate, 3'-O-methylphosphonate, 5'-hydroxyphosphonate, hydroxyphosphanate, phosphoroselenoate, selenophosphate, phosphoramidate, carbophosphonate, methylphosphonate, phenylphosphonate, ethylphosphonate, H-phosphonate, guanidinium ring, triazole ring, boranophosphate (BP), methylphosphonate, guanidinopropyl phosphoramidate, or combination thereof.

76. The modified ngRNA of any one of the foregoing claims, wherein a nucleotide modification comprises a phosphate modification, a base modification, a sugar modification, or a combination thereof.
77. A method for producing a modified PEgRNA or modified ngRNA, the method comprising ligating a coding nucleotide sequence with the modified nucleic acid sequence of any one of the foregoing claims.
78. A method for producing a modified PEgRNA or modified ngRNA, the method comprising ligating a sequence encoding a prime editor with the modified nucleic acid sequence of any one of the foregoing claims.
79. The method of claim 80, wherein the ligation comprises a self-templated enzymatic ligation, optionally the self-templated enzymatic ligation is splint-mediated.
80. The method of claim 79, wherein the ligation comprises a templated enzymatic ligation.
81. The method of claims 79 or 81, wherein the enzymatic ligation is catalyzed by a nucleic acid ligase to generate a natural phosphodiester linkage between the coding nucleotide sequence and the modified nucleic acid sequence of any one of the foregoing claims, or between the sequence encoding a prime editor with the modified nucleic acid sequence of any one of the foregoing claims, or between two fragments of the nucleotide sequences containing the chemical modifications of any one of the foregoing claims.
82. The method of claim 83, wherein the nucleic acid ligase is T4 RNA Ligase I, T4 RNA Ligase II, or T4 DNA Ligase.
83. A prime editing system comprising a modified prime editing guide RNA (PEgRNA) or ngRNA of any of the foregoing claims or one or more polynucleotides encoding the modified PEgRNA or modified ngRNA.
84. A lipid nanoparticle comprising the modified PEgRNA or ngRNA of any one of the foregoing claims or the prime editing system of claim 83.
85. A method for editing a gene, the method comprising contacting the gene with the modified PEgRNA or ngRNA of any one of the foregoing claims, the prime editing system of claim 83 or the lipid nanoparticle of claim 84.

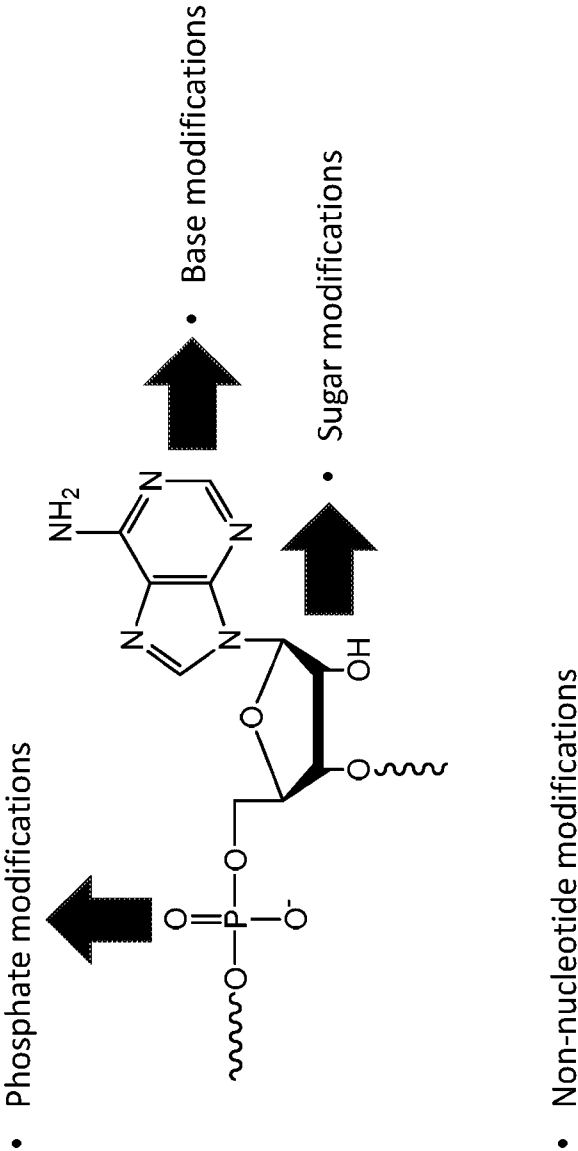


FIG. 1

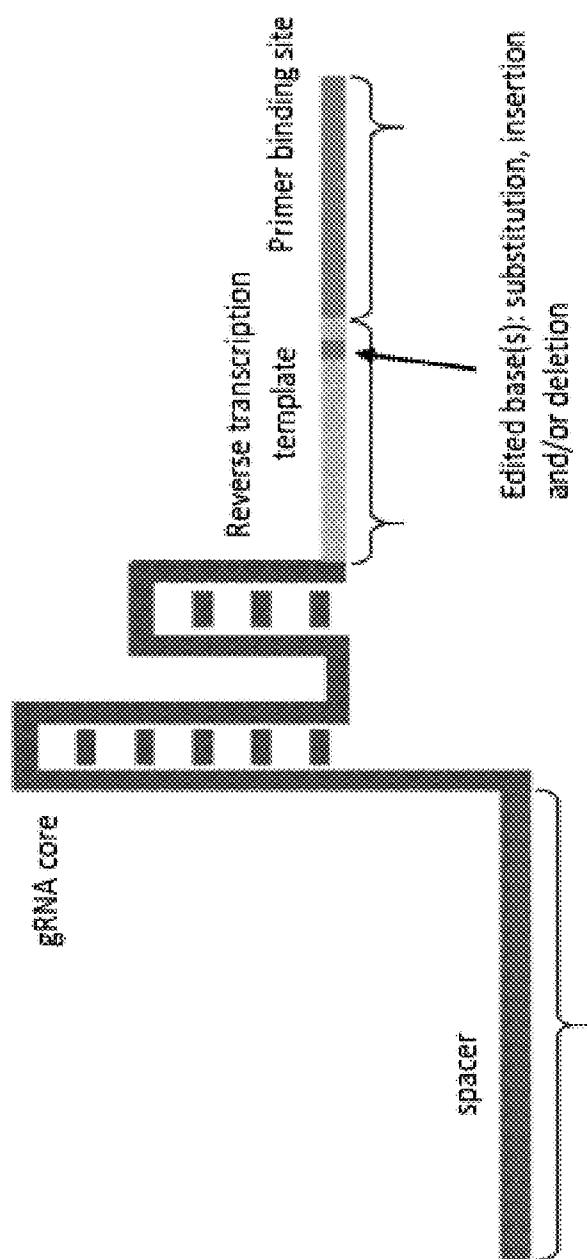


FIG. 2

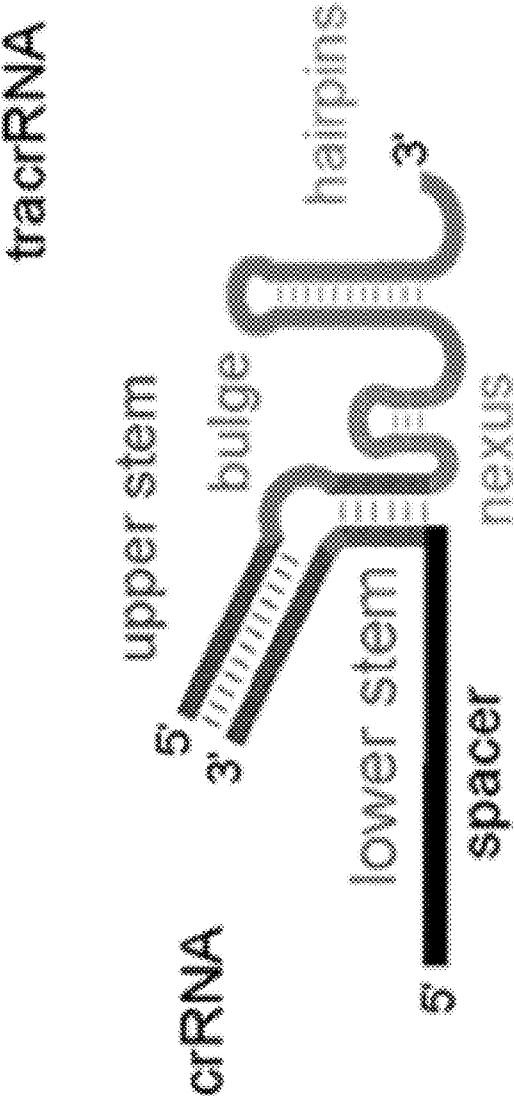


FIG. 3

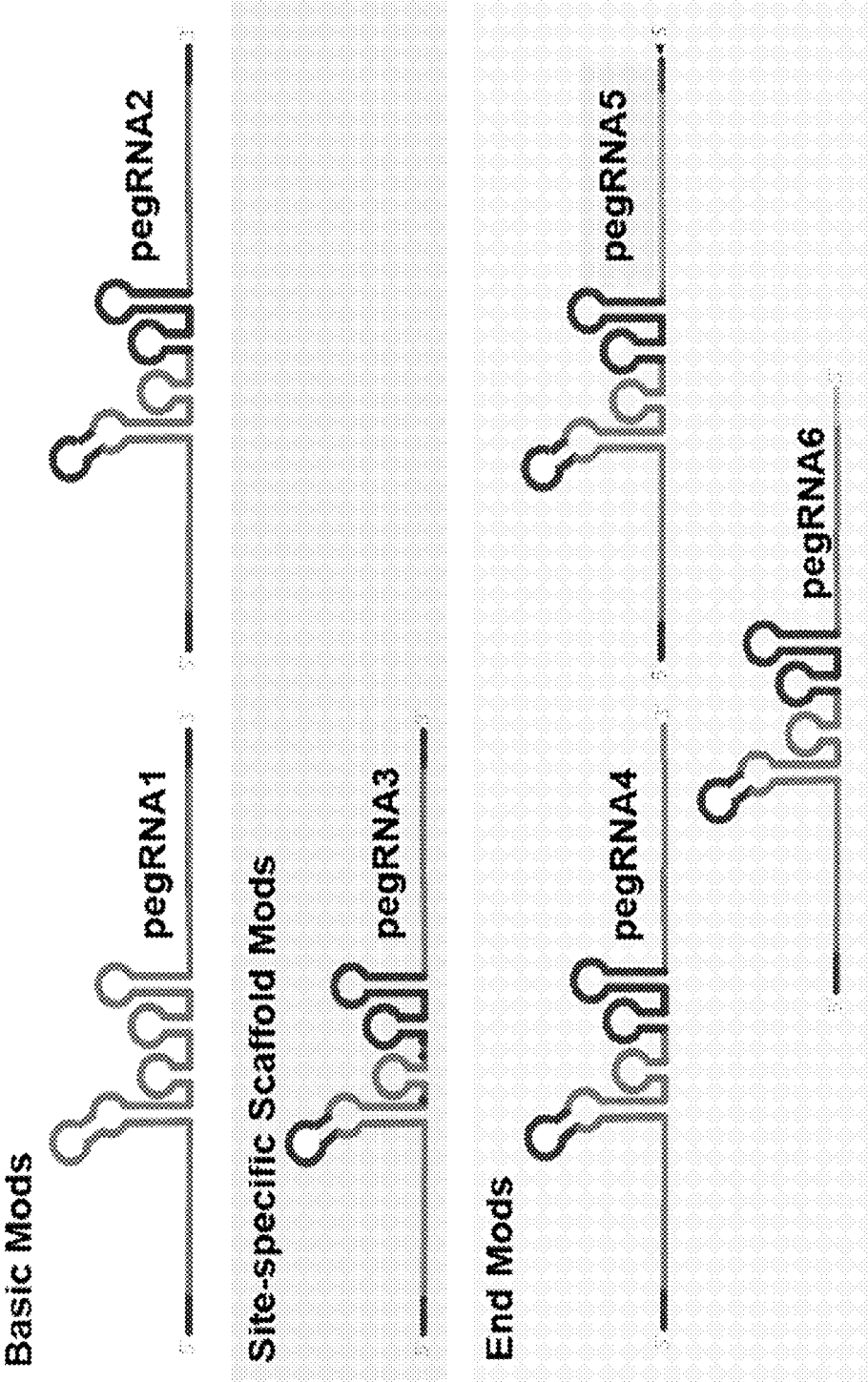


FIG. 4

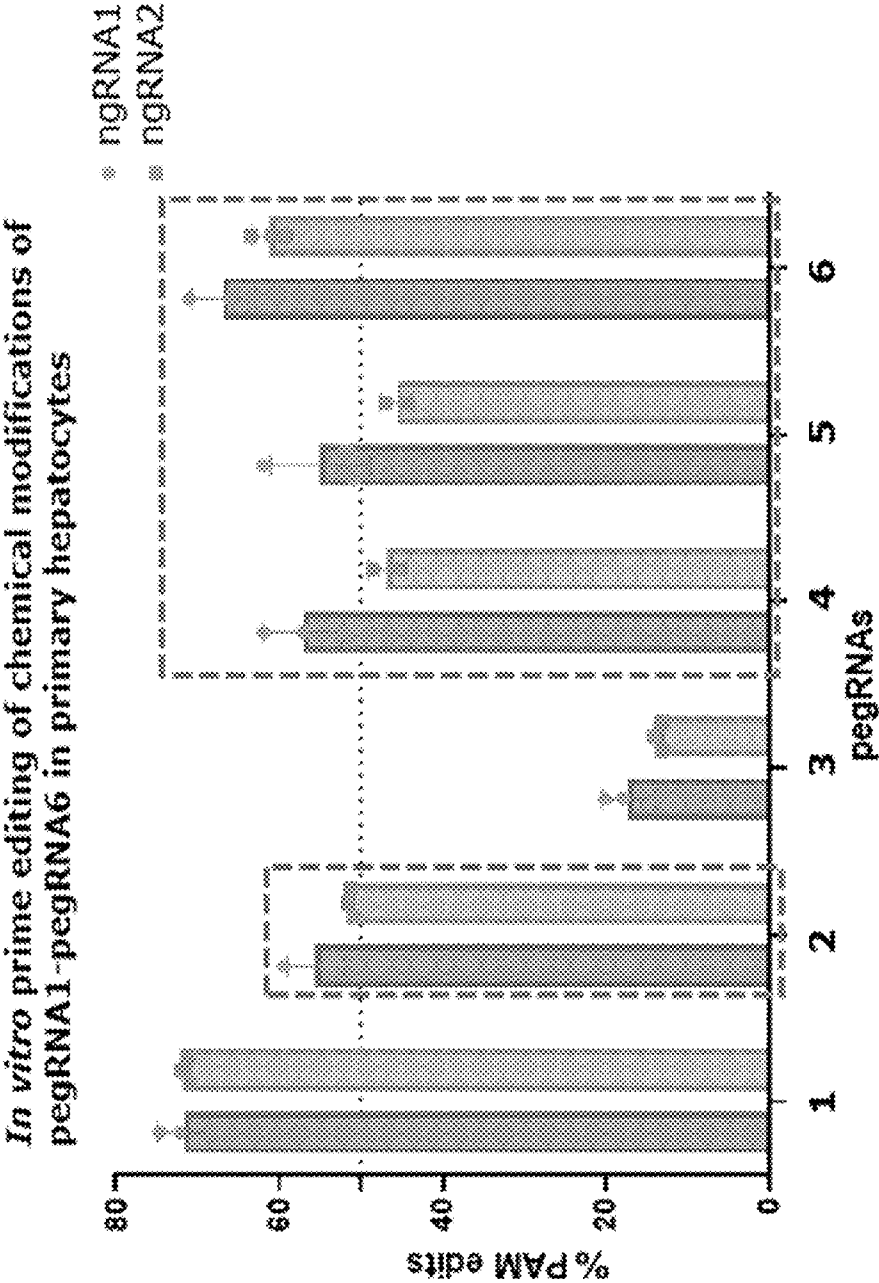


FIG. 5

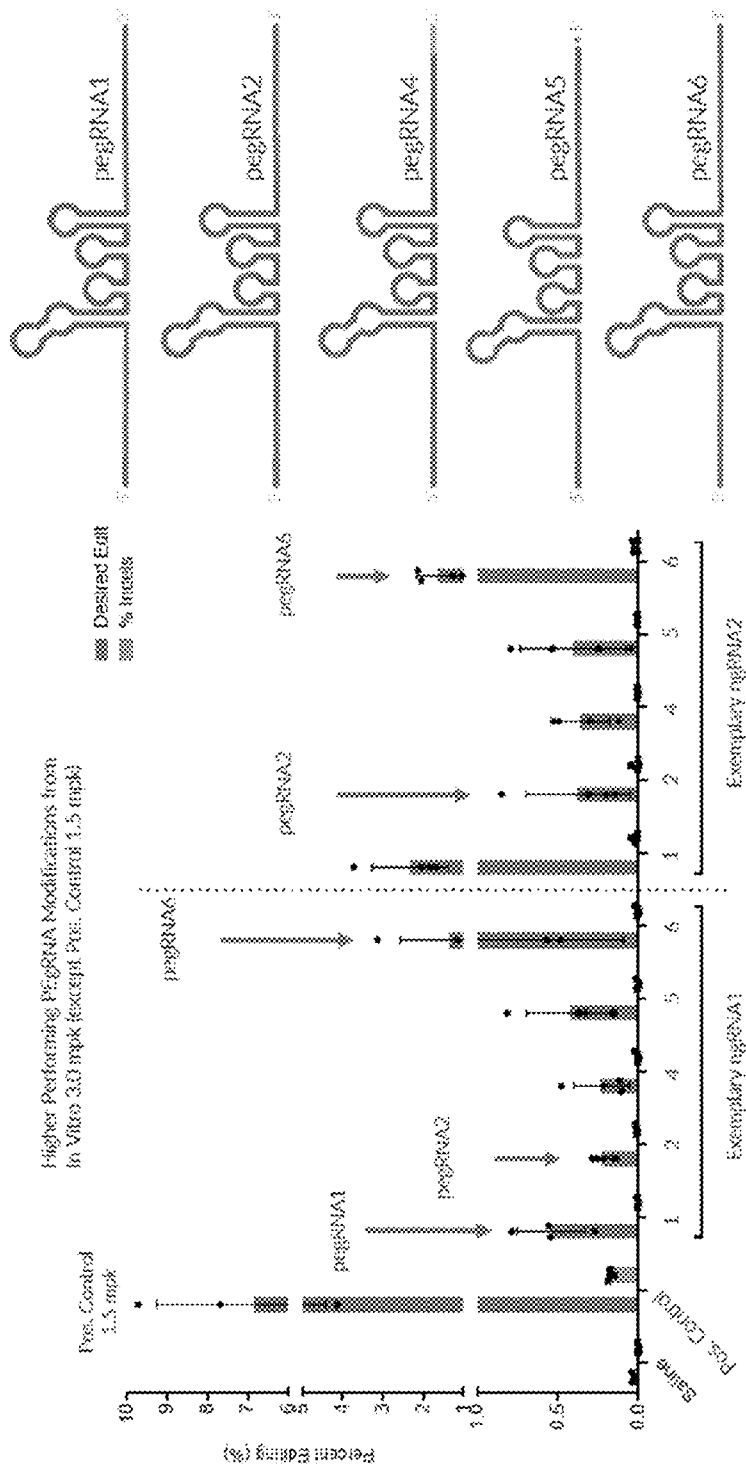


FIG. 6

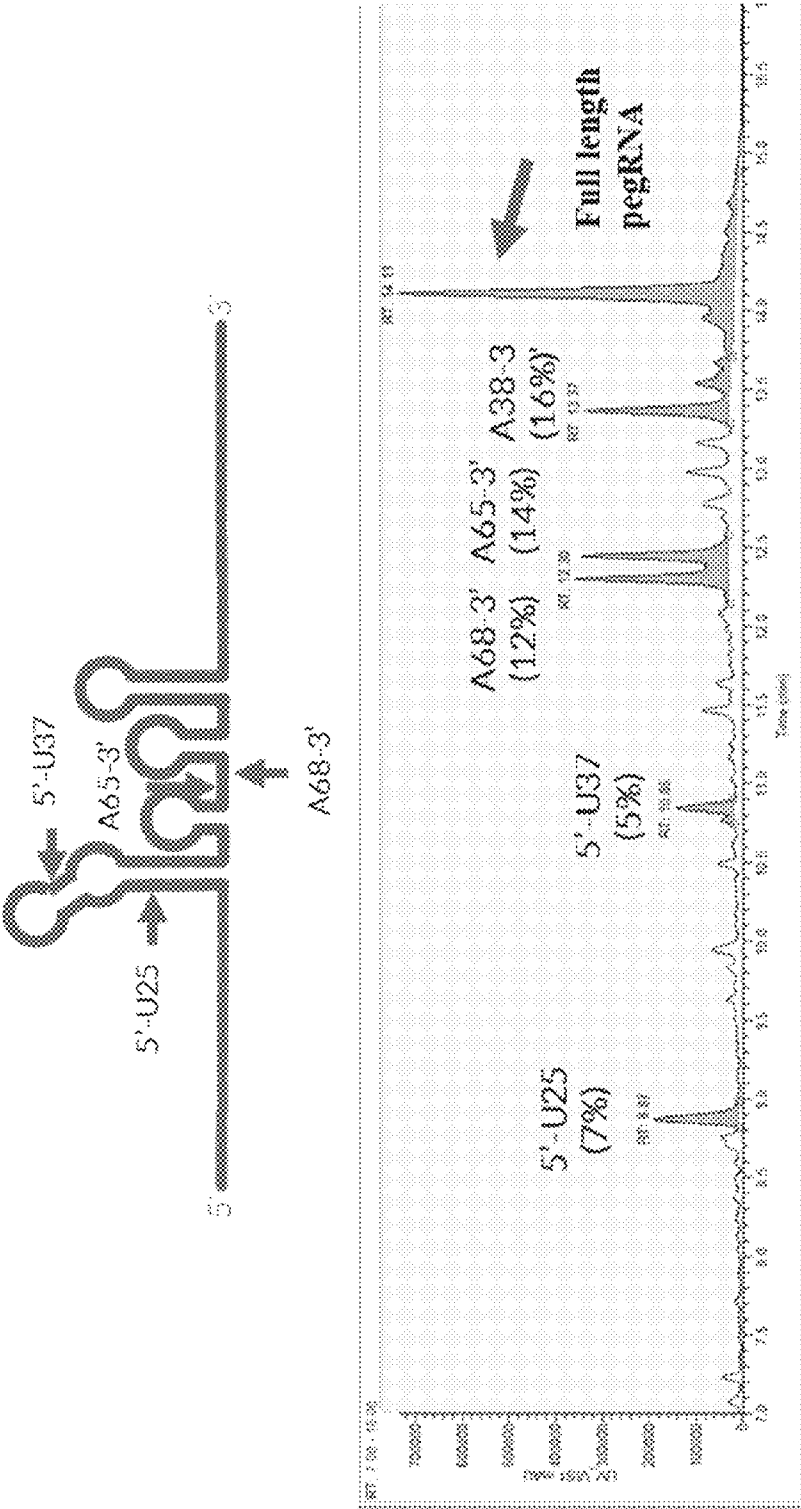


FIG. 7A

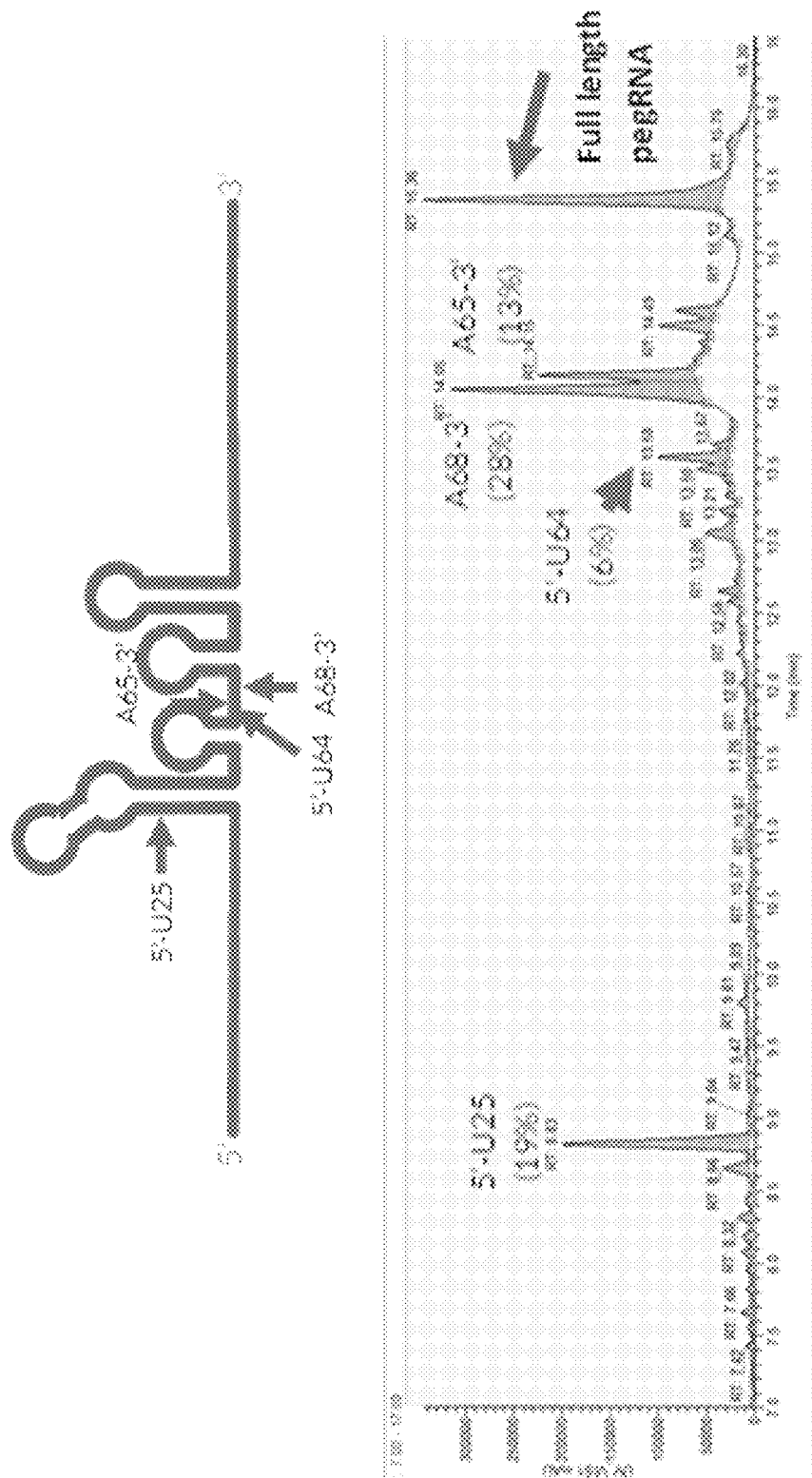


FIG. 7B

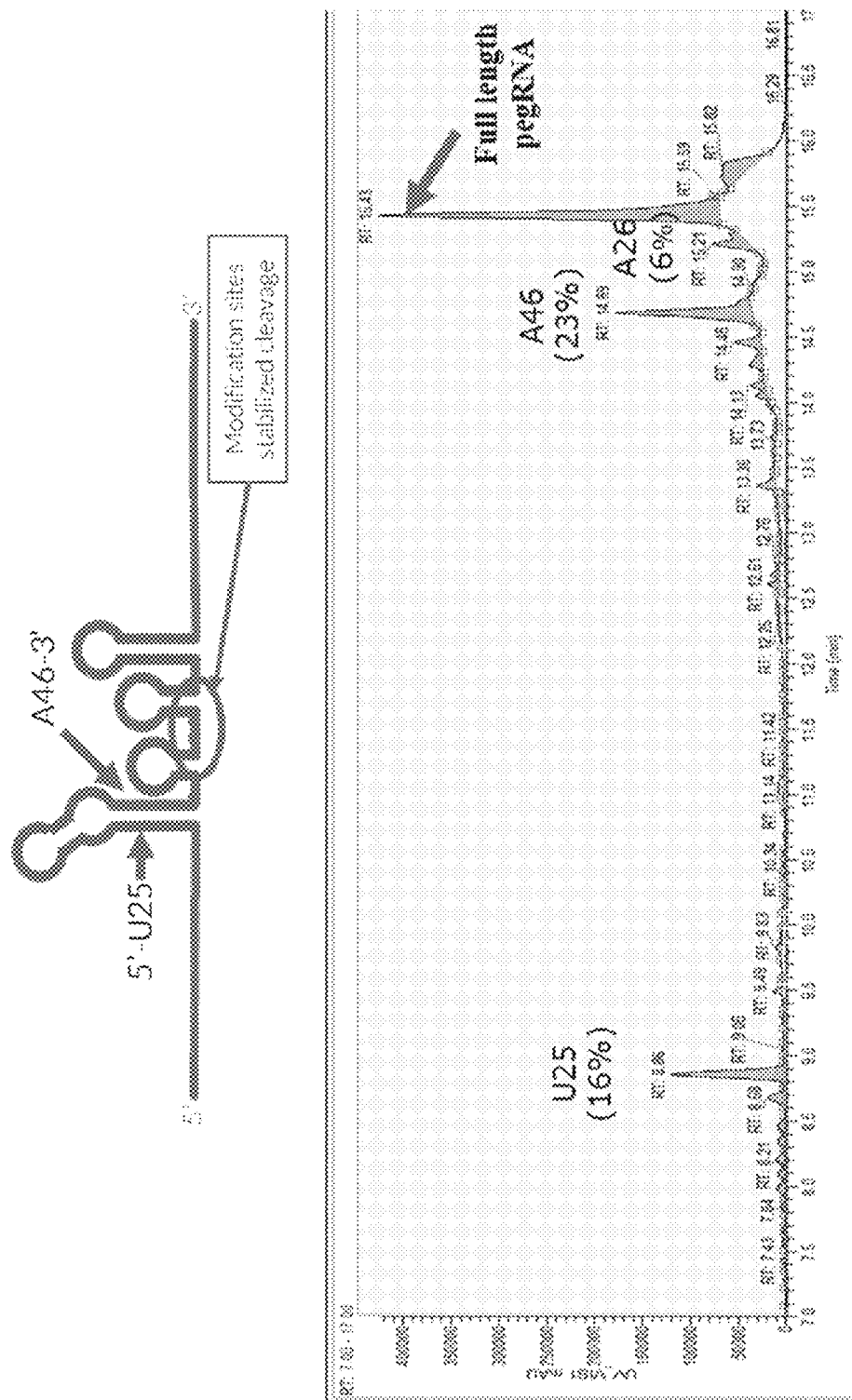


FIG. 7C

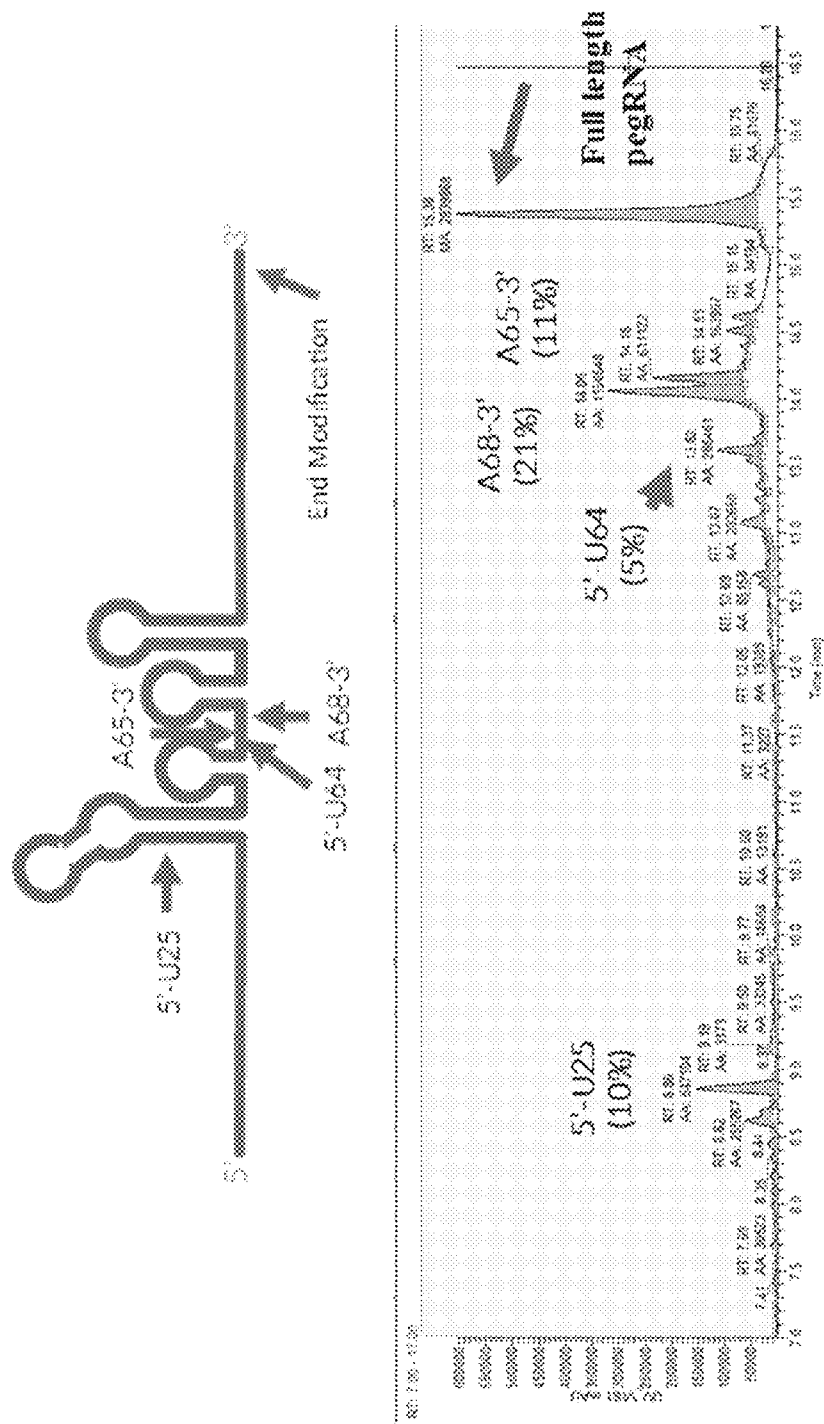


FIG.7D

pegRNA end mods
pegRNA2 – gRNA mods---mU*mU*mU-3'
pegRNA4 – gRNA mods---(2'MOE-T)*(2'MOE-T)*(2'MOE-T)*mU-3'
pegRNA5 – gRNA mod---mU*mU*-3'-3'-idT-5'
pegRNA6 – gRNA mods---LNA-T* LNA-T* LNA-T*mU-3'
pegRNA1 – gRNA w/o mods---mU*mU*mU-3'
pegRNA3 – gRNA mods and cite specific mods mU50, mU64, mC67

mU = 2'-O-Me-U, (2'-MOE)T = 2'-MOE, LNA-T = LNA, idT = inverted dT,
* = phosphorothioate linkage

FIG. 8

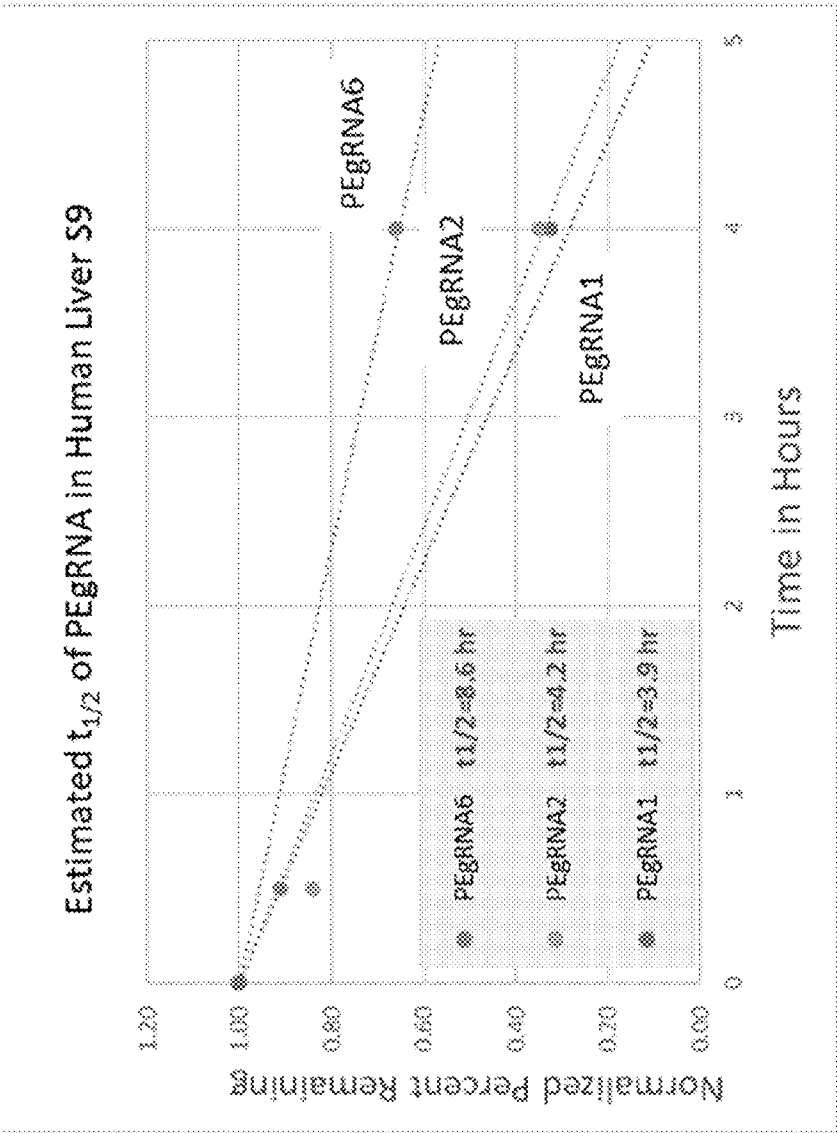


FIG. 9

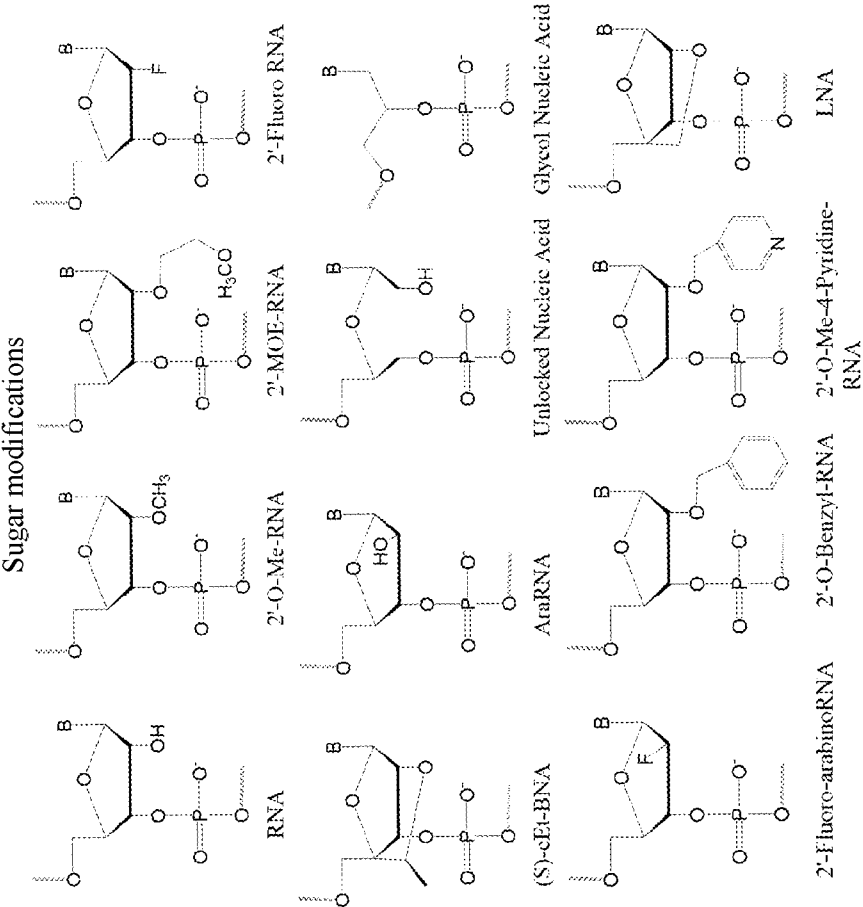


FIG. 10A

Phosphate Backbone Modifications

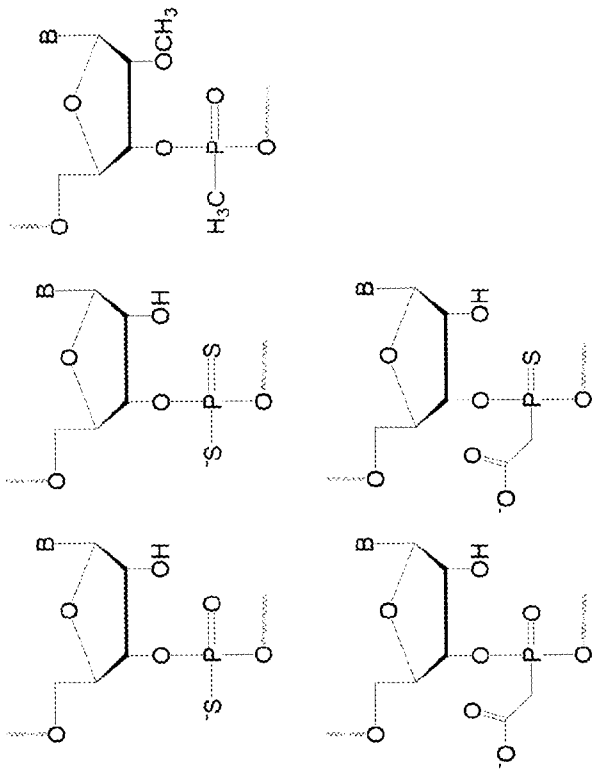
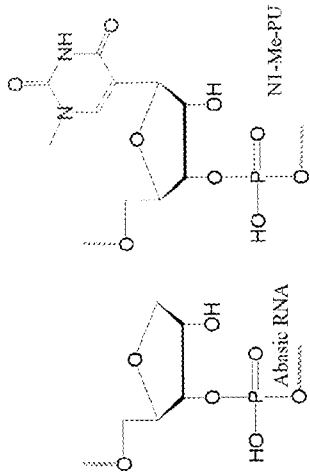


FIG. 10B

Base Modifications



Inverted Nucleotide at 3'-end

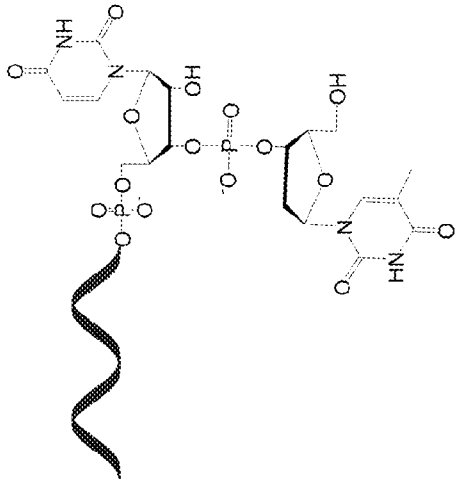


FIG. 10C

3' modifications effect on RNP structure/stability by DSF analysis

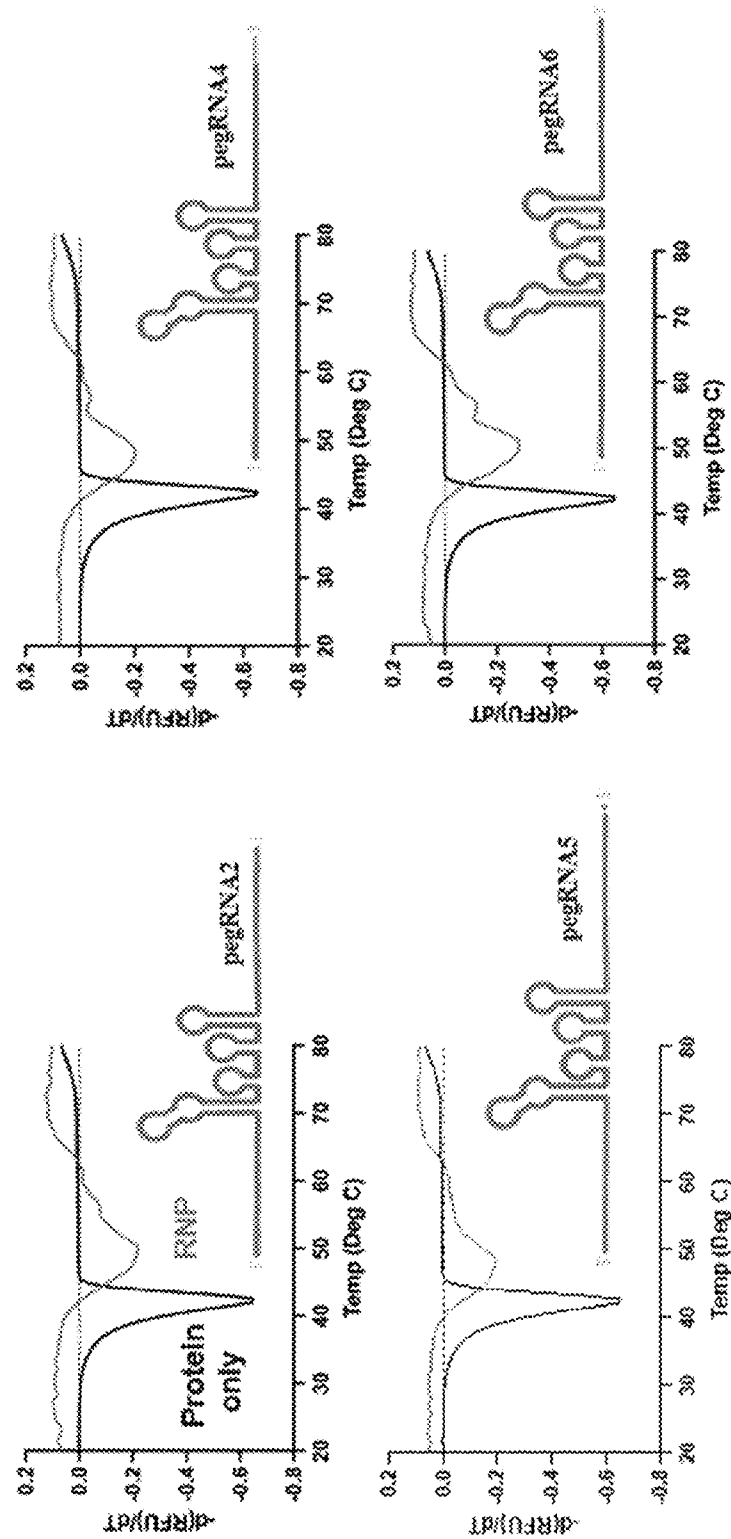


FIG. 11

- DSF measures protein unfolding indirectly using fluorescent dye as sample is heated at constant rate
- Melting temperature (T_m) allows for measurement of relative stability
- Higher T_m = demonstrates greater stable

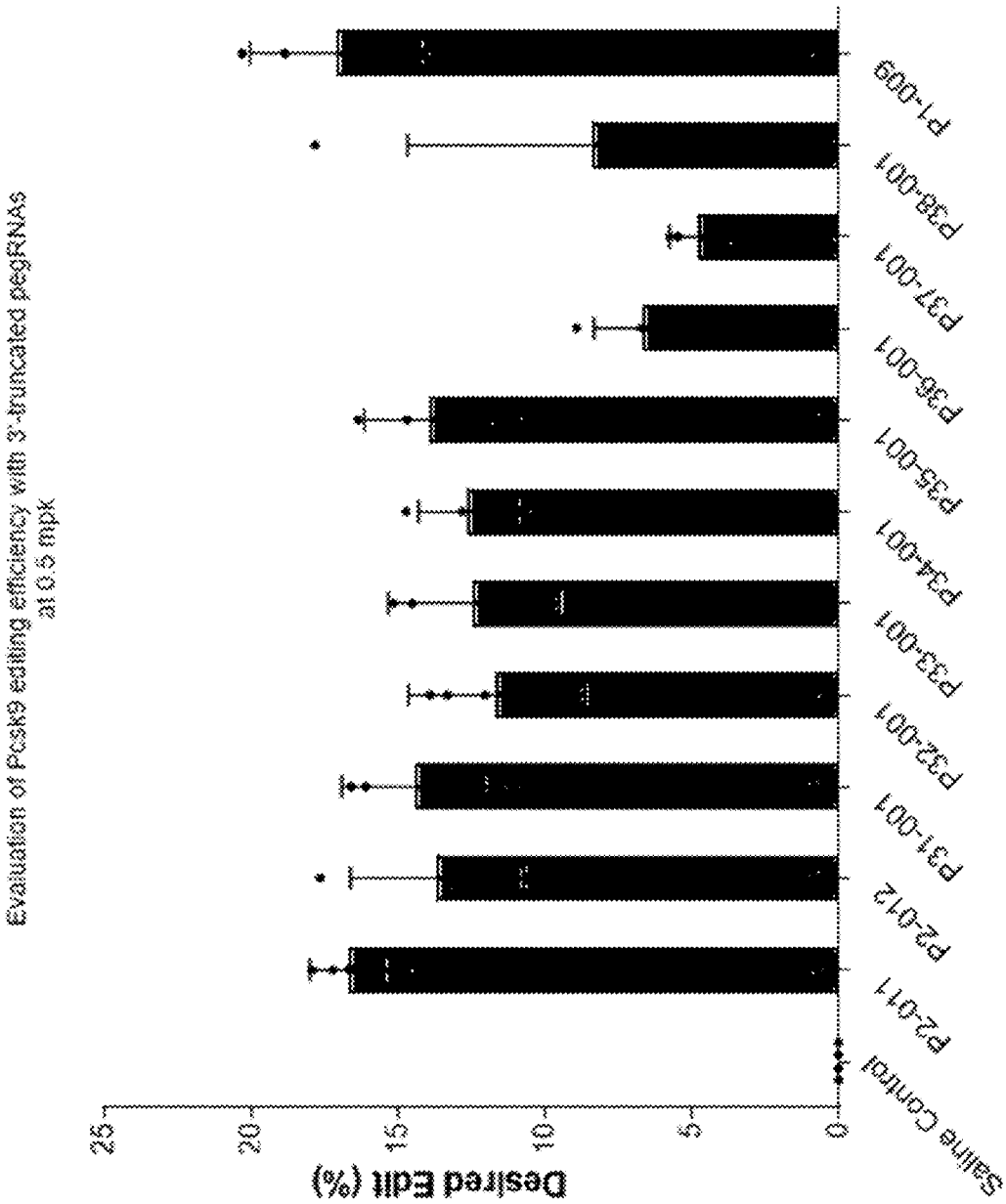


FIG. 12

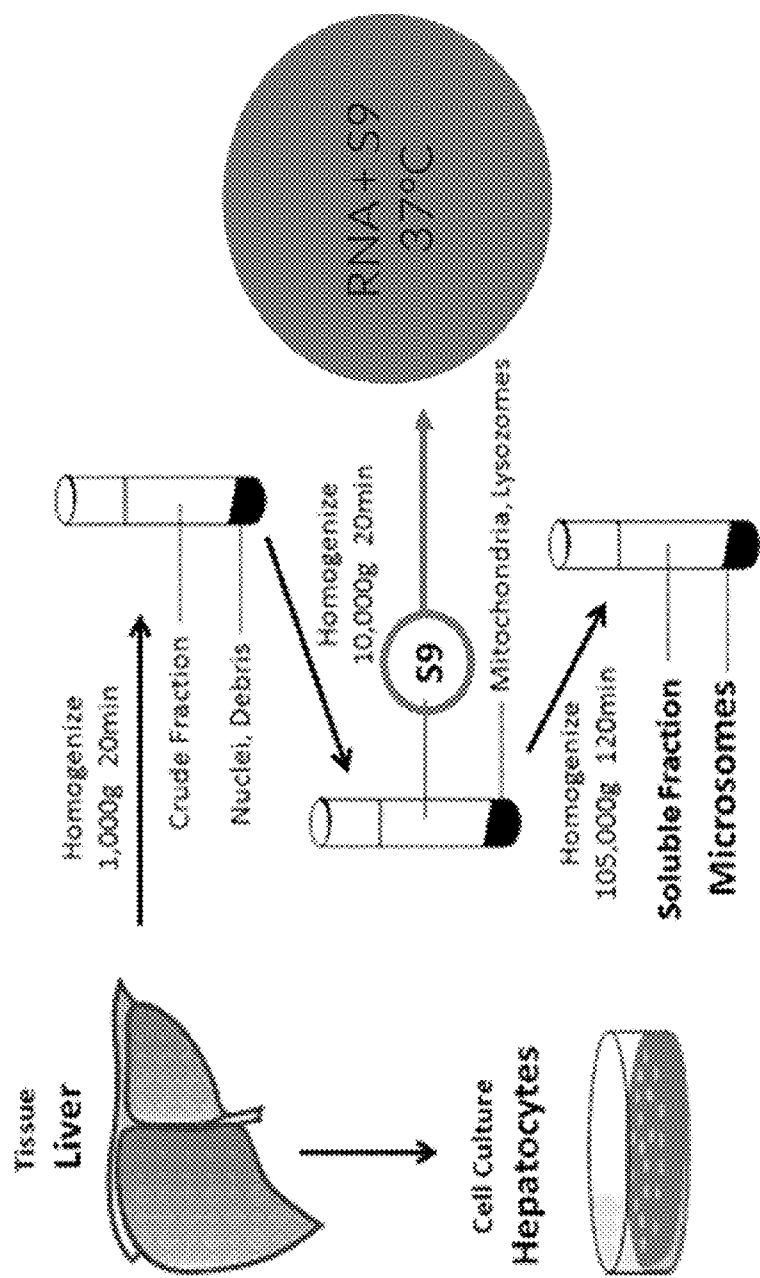


FIG. 13