(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
19 October 2017 (19.10.2017)

WIPO | PCT

(10) International Publication Number
WO 2017/181107 A2

(51) International Patent Classification:
A61K 48/00 (2006.01) *C12N 15/85* (2006.01)

(21) International Application Number:
PCT/US2017/027762

(22) International Filing Date:
14 April 2017 (14.04.2017)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:

62/323,683	16 April 2016 (16.04.2016)	US
62/328,741	28 April 2016 (28.04.2016)	US
62/385,471	9 September 2016 (09.09.2016)	US
62/400,843	28 September 2016 (28.09.2016)	US

(71) Applicant: **OHIO STATE INNOVATION FOUNDATION** [US/US]; 1524 North High Street, Columbus, Ohio 43201 (US).

(72) Inventors: **DONG, Yizhou**; 9949 Glasgow Ct., Dublin, Ohio 43017 (US). **LI, Bin**; 4803 Brodribb Ct, Apt D., Columbus, Ohio 43220 (US).

(74) Agents: **PRATHER, Donald M.** et al.; Meunier Carlin & Curfman LLC, 999 Peachtree St. NE, Suite 1300, Atlanta, Georgia 30309 (US).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:

— *of inventorship (Rule 4.17(iv))*

Published:

— *without international search report and to be republished upon receipt of that report (Rule 48.2(g))*

— *with sequence listing part of description (Rule 5.2(a))*

(54) Title: MODIFIED CPF1 MRNA, MODIFIED GUIDE RNA, AND USES THEREOF

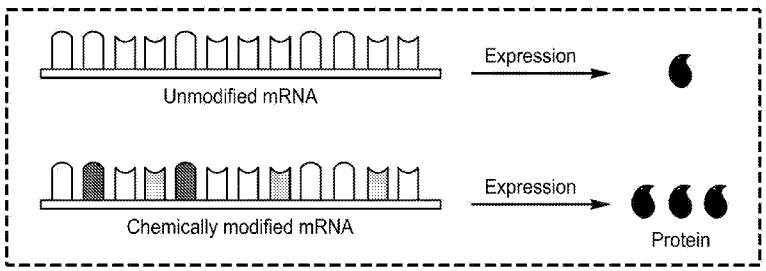


FIG. 2

(57) Abstract: The present disclosure generally relates to systems, methods and compositions for use in Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-Cpf1 genome editing systems. Disclosed herein are modified Cpf1 mRNAs, modified guide RNAs, and combinations thereof, that confer increased levels of genome editing.

WO 2017/181107 A2

MODIFIED CPF1 MRNA, MODIFIED GUIDE RNA, AND USES THEREOF

CROSS REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Patent Application Serial No. 62/323,683, filed April 16, 2016, U.S. Provisional Patent Application Serial No. 62/328,741 filed April 28, 2016, U.S. Provisional Patent Application Serial No. 62/385,471, filed September 9, 2016, and U.S. Provisional Patent Application Serial No. 62/400,843 filed September 28, 2016, each of which are expressly incorporated herein by reference.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH

This invention was made with Government Support under Grant No. 1R01HL136652 awarded by the National Heart, Lung, and Blood Institute. The Government has certain rights to the invention.

FIELD OF THE INVENTION

The present disclosure generally relates to systems, methods and compositions for use in Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-Cpf1 genome editing systems. Disclosed herein are modified Cpf1 mRNAs, modified guide RNAs, and combinations thereof, that confer increased levels of genome editing.

BACKGROUND

The bacterial type II CRISPR-Cas9 genome editing method has recently received a great deal of interest in the field of genome engineering. The co-expression of a single Cas9 protein isolated from Streptococcus pyogenes with a chimeric single guide RNA (sgRNA) can precisely create double stranded breaks (DSBs) in a genome. The Cas9 protein is directed to a precise DNA sequence in the genome by a twenty nucleotide target sequence present in the sgRNA, which guides the Cas9 protein to create the DSB. The presence of a double-stranded break in genomic DNA dramatically increases the rate of homologous recombination.

Recently, an additional genome editing system, termed the CRISPR-Cpf1 system, was identified (Zetsche, B. et al. Cpf1 is a single RNA-guided endonuclease of a class 2 CRISPR-Cas system. Cell. 2015 Oct 22; 163(3):759-71). Cpf1 cleaves DNA in a staggered pattern and leaves sticky ends, as compared to the cleaved blunt DNA ends left by the Cas9 enzyme. In addition, Cpf1 only requires one guide RNA rather than the two (tracrRNA and crRNA) needed by Cas9 for

cleavage (or a chimeric single guide RNA). However, gene editing frequencies are still very low, and thus new methods are needed to improve the efficiency of the CRISPR-Cpf1 gene editing system.

Messenger RNAs (mRNAs) encoding functional proteins have demonstrated their therapeutic potential in fundamental and clinical studies. For example, immunotherapy with mRNA-electroporated dendritic cell provided therapeutic benefit in several cancer clinical trials. mRNAs were also utilized to produce chimeric antigen receptors in T cells for adoptive T-cell therapy, to express functional proteins for protein replacement therapy, and most recently, to make nucleases for gene engineering. Although there have been significant advances in mRNA-based therapeutics in the past decade, instability and immunogenicity of mRNA hinders its therapeutic application in humans.

The systems, methods, and compositions disclosed herein address these and other needs.

SUMMARY

Disclosed herein are systems, methods, and compositions that utilize modified Cpf1 mRNAs, modified guide RNAs, and combinations thereof. These modified RNAs can be used in the CRISPR-Cpf1 genome editing system. These modified Cpf1 mRNAs and modified guide RNAs can incorporate a number of chemical changes to the nucleotides, including changes to the nucleobase, the ribose sugar, and/or the phosphodiester linkage; or these changes can include insertions or deletions into the guide RNA sequence. These modified Cpf1 mRNAs, modified guide RNAs, and combinations thereof, can improve efficiency of the CRISPR/Cpf1 genome editing system, reduce off-target effects, reduce toxicity, increase Cpf1 protein levels, increase Cpf1 nuclease activity, increase guide RNA stability, and/or increase Cpf1 mRNA stability.

In one aspect, disclosed herein is a genome editing system comprising:

a) a guide RNA that hybridizes with a target sequence of a DNA molecule in a eukaryotic cell that contains the DNA molecule, and

b) an mRNA encoding a Cpf1 protein;

wherein the mRNA encoding a Cpf1 protein comprises at least one chemically modified nucleotide.

In one embodiment, the guide RNA hybridizes with the target sequence and the Cpf1 protein cleaves the DNA molecule.

In one embodiment, the at least one chemically modified nucleotide confers increased Cpf1 nuclease activity, increased Cpf1 protein levels, decreased off-target effects, reduced toxicity, and/or increased Cpf1 mRNA stability as compared to a corresponding mRNA encoding a Cpf1

protein not having the chemically modified nucleotide. In one aspect, disclosed herein is a genome editing system comprising:

a) a guide RNA that hybridizes with a target sequence of a DNA molecule in a eukaryotic cell that contains the DNA molecule, and

b) an mRNA encoding a Cpf1 protein;

wherein the guide RNA comprises at least one chemically modified nucleotide; and

wherein the mRNA encoding a Cpf1 protein comprises at least one chemically modified nucleotide.

In one aspect, disclosed herein is a genome editing system comprising:

a) a guide RNA that hybridizes with a target sequence of a DNA molecule in a eukaryotic cell that contains the DNA molecule, and

b) a DNA vector comprising a regulatory element operable in a eukaryotic cell operably linked to a nucleotide sequence encoding a Cpf1 protein; an mRNA encoding a Cpf1 protein; or a Cpf1 protein;

wherein the guide RNA comprises at least one chemically modified nucleotide.

In another aspect, provided herein is a method of RNA-guided genome editing, the method comprising:

introducing into a cell of the subject:

a) a guide RNA that hybridizes with a target sequence of a DNA molecule in a eukaryotic cell that contains the DNA molecule, and

b) an mRNA encoding a Cpf1 protein;

wherein the guide RNA comprises at least one chemically modified nucleotide;

wherein the mRNA encoding a Cpf1 protein comprises at least one chemically modified nucleotide; and

wherein the guide RNA hybridizes with the target sequence and the Cpf1 protein cleaves the DNA molecule.

In another aspect, provided herein is a method of RNA-guided genome editing, the method comprising:

introducing into a cell of the subject:

a) a guide RNA that hybridizes with a target sequence of a DNA molecule in a eukaryotic cell that contains the DNA molecule, and

b) an mRNA encoding a Cpf1 protein;

wherein the mRNA encoding a Cpf1 protein comprises at least one chemically modified nucleotide; and

wherein the guide RNA hybridizes with the target sequence and the Cpf1 protein cleaves the DNA molecule.

In another aspect, provided herein is a method of RNA-guided genome editing, the method comprising:

introducing into a cell of the subject:

a) a guide RNA that hybridizes with a target sequence of a DNA molecule in a eukaryotic cell that contains the DNA molecule, and

b) a DNA vector comprising a regulatory element operable in a eukaryotic cell operably linked to a nucleotide sequence encoding a Cpf1 protein; an mRNA encoding a Cpf1 protein; or a Cpf1 protein;

wherein the guide RNA comprises at least one chemically modified nucleotide; and

wherein the guide RNA hybridizes with the target sequence and the Cpf1 protein cleaves the DNA molecule.

In another aspect, provided herein is a method of increasing Cpf1 protein levels in a cell, the method comprising:

introducing into a cell of the subject:

a) a guide RNA that hybridizes with a target sequence of a DNA molecule in a eukaryotic cell that contains the DNA molecule, and

b) an mRNA encoding a Cpf1 protein;

wherein the mRNA encoding a Cpf1 protein comprises at least one chemically modified nucleotide, and

wherein the at least one chemically modified nucleotide confers increased Cpf1 protein levels as compared to a corresponding mRNA encoding a Cpf1 protein not having the chemically modified nucleotide.

In a further aspect, provided herein is a method of increasing Cpf1 nuclease activity in a cell, the method comprising:

introducing into a cell of the subject:

a) a guide RNA that hybridizes with a target sequence of a DNA molecule in a eukaryotic cell that contains the DNA molecule, and

b) an mRNA encoding a Cpf1 protein;

wherein the mRNA encoding a Cpf1 protein comprises at least one chemically modified nucleotide, and

wherein the at least one chemically modified nucleotide confers increased Cpf1 nuclease activity as compared to a corresponding mRNA encoding a Cpf1 protein not having the chemically modified nucleotide.

In another aspect, provided herein is a method of reducing toxicity of RNA-guided genome editing in a cell, the method comprising:

introducing into a cell of the subject:

a) a guide RNA that hybridizes with a target sequence of a DNA molecule in a eukaryotic cell that contains the DNA molecule, and

b) an mRNA encoding a Cpf1 protein;

wherein the mRNA encoding a Cpf1 protein comprises at least one chemically modified nucleotide, and

wherein the at least one chemically modified nucleotide confers reduced toxicity as compared to a corresponding mRNA encoding a Cpf1 protein not having the chemically modified nucleotide.

In one aspect, disclosed herein is a genome editing system comprising:

a) a guide RNA that hybridizes with a target sequence of a DNA molecule in a eukaryotic cell that contains the DNA molecule, and

b) a DNA vector comprising a regulatory element operable in a eukaryotic cell operably linked to a nucleotide sequence encoding a Cpf1 protein; an mRNA encoding a Cpf1 protein; or a Cpf1 protein;

wherein the guide RNA comprises at least one chemically modified nucleotide, and

wherein the guide RNA hybridizes with the target sequence and the Cpf1 protein cleaves the DNA molecule.

In one embodiment, the at least one chemically modified nucleotide confers increased Cpf1 nuclease activity, decreased off-target effects, reduces toxicity, and/or increased guide RNA stability as compared to a corresponding guide RNA not having the chemical modification.

In another aspect, provided herein is a method of increasing the efficiency of RNA-guided genome editing in a cell, the method comprising:

introducing into a cell of the subject:

a) a guide RNA that hybridizes with a target sequence of a DNA molecule in a eukaryotic cell that contains the DNA molecule, and

b) a DNA vector comprising a regulatory element operable in a eukaryotic cell operably linked to a nucleotide sequence encoding a Cpf1 protein; an mRNA encoding a Cpf1 protein; or a Cpf1 protein;

wherein the guide RNA comprises at least one chemically modified nucleotide, and

wherein the at least one chemically modified nucleotide confers increased Cpf1 nuclease activity as compared to a corresponding guide RNA not having the chemical modification.

In a further aspect, provided herein is a method of decreasing the off-target effects of RNA-guided genome editing in a cell, the method comprising:

introducing into a cell of the subject:

a) a guide RNA that hybridizes with a target sequence of a DNA molecule in a eukaryotic cell that contains the DNA molecule, and

b) a DNA vector comprising a regulatory element operable in a eukaryotic cell operably linked to a nucleotide sequence encoding a Cpf1 protein;

wherein the guide RNA comprises at least one chemically modified nucleotide, and

wherein the at least one chemically modified nucleotide confers decreased off-target effects as compared to a corresponding guide RNA not having the chemical modification.

In another aspect, provided herein is a method of reducing toxicity of RNA-guided genome editing in a cell, the method comprising:

introducing into a cell of the subject:

a) a guide RNA that hybridizes with a target sequence of a DNA molecule in a eukaryotic cell that contains the DNA molecule, and

b) a DNA vector comprising a regulatory element operable in a eukaryotic cell operably linked to a nucleotide sequence encoding a Cpf1 protein; an mRNA encoding a Cpf1 protein; or a Cpf1 protein;

wherein the guide RNA comprises at least one chemically modified nucleotide, and

wherein the at least one chemically modified nucleotide confers reducing toxicity as compared to a corresponding guide RNA not having the chemical modification.

BRIEF DESCRIPTION OF THE DRAWINGS

The accompanying figures, which are incorporated in and constitute a part of this specification, illustrate several aspects described below.

FIG. 1. Schematic illustration of AsCpf1-crRNA-target DNA complex and their terminology. crRNA is composed of a 5' handle (in a pseudoknot structure) and a guide segment (consists of a seed region and 3'-termini).

FIG. 2. Illustration showing chemically modified mRNAs improve protein expression.

FIGS. 3A-3D. A panel of chemically modified nucleotides utilized for Cpf1 mRNAs. (FIG. 3A) A schematic showing examples of chemical changes to the chemically modified nucleotides

utilized for Cpf1 mRNAs. (FIG. 3B) A panel showing examples of chemically modified phosphodiester linkages for the chemically modified nucleotides utilized for Cpf1 mRNAs. Boxed phosphodiester linkage shows the natural unit. (FIG. 3C) A panel showing examples of chemically modified ribose sugar moieties for the chemically modified nucleotides utilized for Cpf1 mRNAs. Boxed riboseshows the natural unit. (FIG. 3D) A panel showing examples of chemically modified nucleobases for the chemically modified nucleotides utilized for Cpf1 mRNAs.

FIG. 4. Polyacrylamide gel electrophoresis (PAGE) analysis of modified AsCpf1 mRNAs. Polyacrylamide gel electrophoresis (PAGE) analysis of WT, ψ, $m^1\psi$ and 5moU-modified AsCpf1 mRNAs as well as ψ modified LbCpf1 mRNA.

FIGS. 5A-5B. FLuc expression of chemically modified FLuc mRNAs in a rabbit reticulocyte lysate system. (FIG. 5A) Translation at 30 °C. Fold change was normalized by the unmodified FLuc mRNA. (FIG. 5B) Translation at 37 °C. Fold change was normalized by the unmodified FLuc mRNA; the inserted figure denoted the Pearson correlation analysis for translation at 30 and 37 °C in the rabbit reticulocyte lysate system.

FIG. 6. (a) Relative FLuc expression of chemically modified FLuc mRNAs in Hep 3B and THP-1 cells (normalized by unmodified FLuc mRNA). Five types of modified FLuc mRNAs (ψ, $me^1\psi$, 5meC/ψ, 5hmC/5meU, and 5moC/5meU-FLuc mRNAs) showed superior activity to unmodified FLuc mRNA. **, $p< 0.01$; ***, $p< 0.001$. (b) Significant correlation of luciferase intensity in the rabbit reticulocyte lysate system at 30 °C and in Hep 3B cells was observed ($p = 0.013$; $R^2 = 0.2396$; $r= 0.5845$). (c) No significant correlation was found for luciferase intensity in the rabbit reticulocyte lysate system at 37 °C and in Hep 3B cells. (d) Relative eGFP expression of chemically modified eGFP mRNAs in Hep 3B and THP-1 cells (normalized by unmodified eGFP mRNA). $me^1\psi$, 5moU and ψ-modified eGFP mRNA displayed higher eGFP expression compared to other chemically modified eGFP mRNAs. **, $p< 0.01$; ***, $p< 0.001$.

FIG. 7. Relative mRNA levels at different time intervals (t = 0, 1.5, 3, and 4.5 h). The initial level of unmodified and modified mRNAs was normalized by 18S rRNA using RT-qPCR quantification. 5moU modifications significantly increased stability of eGFP mRNA. *, $p< 0.05$; **, $p< 0.01$; ***, $p< 0.001$.

FIG. 8. Relative FLuc expression of 24 chemically modified FLuc mRNAs in Hep 3B cells (normalized by unmodified FLuc mRNA).

FIGS. 9A-9E. Design of chemically modified crRNAs and Cpf1 mRNA and their gene editing efficiency. (FIG. 9A) A panel of chemically modified crRNAs tested in this study. Unmodified nucleotides are shown in black. Full-length PS modifications are in pink. 2'-O-methyl modifications are highlighted in blue. 2'-F modifications are in red. 2'-O-methyl combined with

PS modifications are in light blue. 2'-F combined with PS modifications are in green. (FIG. 9B) Stem engineering of crRNAs. Deleted bases are highlighted with light blue box denotes, and inserted bases are with pink box. The length of the stem duplex was altered by deletion or insertion (in light orange) of nucleotides. For crSplit, the pseudoknot-like structure was formed by hybridization of two arms of the direct repeat. (FIG. 9C, FIG. 9D) Gene cutting efficiency using modified crRNAs (FIG. 9C) and 5'-handle rearranged crRNAs (FIG. 9D). Unlock nucleotides are shown in purple. Locked nucleotides are shown in orange. Other colors in sequence are the same as that in Fig. 9a. The dotted line denotes deleted nt. Lowercase denotes inserted nt. "T" sign in figure 9d left panel denotes the split site. Indel (%) was determined by the T7E1 cleavage assay, normalized to that of crWT of each independent experiment. (FIG. 9E) Gene cutting efficiency using chemically modified Cpf1 mRNA. Relative gene cutting efficiency was normalized to indel percentage of the treatment with crWT and plasmid encoding Cpf1.

FIG. 10. Increasing gene editing efficiency through combination of chemically modified crRNA and Cpf1 mRNA. (a, b, c) AsCpf1-mediated gene cutting efficiency for the human DNMT1-3 (a), AAVS1 (b) and FANCF-2 (c) locus in HEK293T, Hep3B and U87 cells. (d) LbCpf1-mediated gene cutting efficiency for the human *DNMT1-3* in HEK293T, Hep3B and U87 cells. Indel percentage at each locus was determined using the T7E1 assay by quantification of the uncut (solid arrow) and cut DNA bands (hollow arrow), and expressed as the mean ± S.D. from three biological replicates (**, $P < 0.01$; ***, $P < 0.001$; *t* test, double-tailed).

FIGs. 11A-11C. Targeted deep sequencing analysis of on-target and off-target gene cutting using chemically modified crRNA and AsCpf1 mRNA. (FIG. 11A) Indel at on-target and predicted top four off-target sites analyzed by deep sequencing at genomic on- and off-target locus. Indel was plotted as the mean of three biological replicates. (FIG. 11B) Representative top ten high-frequent on-target mutagenesis aligned to the target site of *DNMT1-3* induced by crWT + AsCpf1 plasmid (top) and cr3'5F + AsCpf1 mRNA (bottom). The unmodified sequence of *DNMT1-3* was termed as 'WT' at the top. Deletions were marked as dotted lines. Numbers on the left referred to the size of deletions. The read ratio of each mutated site was listed on the right side. (FIG. 11C) Plot of representative mutations (insertions, deletions, and substitutions). Size distribution (top panel) and position distribution (bottom panel) of all reads for crWT + AsCpf1 plasmid (left panel) and cr3'5F + Cpf1 mRNA (right panel).

FIG. 12. Targeted deep sequencing analysis of on-target and off-target gene cutting using chemically modified crRNA and LbCpf1 mRNA. (a) Indel at on-target and predicted top four off-target sites analyzed by deep sequencing at genomic on- and off-target locus. Indel was plotted as the mean of three biological replicates. (b) Representative top ten high-frequent on-target

mutagenesis aligned to the target site of *DNMT1-3* induced by crWT + LbCpf1 plasmid (top) and cr3'5F + Cpf1 mRNA (bottom).The unmodified sequence of *DNMT1-3* was termed as 'WT' at the top. Deletions were marked as dotted lines. Numbers on the left referred to the size of deletions. The read ratio of each mutated site was listed on the right side. (c) Plot of representative mutations (insertions, deletions, and substitutions). Size distribution (top panel) and position distribution (bottom panel) of all reads for crWT + Cpf1 plasmid (left panel) and cr3'5F + Cpf1 mRNA (right panel).

FIG. 13. A panel of chemically modified nucleotides utilized for crRNAs and AsCpf1 mRNA. Blue box: natural units.

FIG. 14. T7E1 cleavage assay measuring the off-target effect. The size of predicted T7E1 fragments was shown at the bottom of gels. *, nonspecific bands.

FIG. 15. Chemically modified crRNAs. Unmodified nucleotides are shown in black. 2'-H substitutions are in pink. Unlocked nt are highlighted in purple. Locked nt are highlighted in yellow. For crl21F, 2'-F modifications were applied to 5, 6, 9, 10, 12-14, 16-19, 29-32, 37 and 39-43 positions. For crl10F, 2'-F modifications were applied to 29-32, 37 and 39-43 positions. The 5' uridine was seen as position 1.

FIG. 16. Loop engineering of crRNAs. (a) Nucleotides of the loop were altered according to crRNAs from other 15 Cpf1-family orthologs. crRNAs were termed by combining initial of Cpf1 protein and crRNA; crRNAs of PbCpf1, PeCpf1 and LiCpf1 share the same loop UUUU; crRNAs of Lb2Cpf1, PcCpf1 and PmCpf1 share the same loop UAUU. Nucleotides difference between AscrRNA and other crRNAs in the loop were heighted in red. (b) Gene cutting efficiency of loop engineered crRNAs in the presence of ψ-modified AsCpf1 mRNA. Red nucleotides denote sequence difference in the loop between AscrRNA and other crRNAs. Gene cutting efficiency was determined by the T7E1 cleavage assay, and normalized to that of the treatment with AscrRNA and ψ-modified AsCpf1 mRNA. N.D., Not detectable. Data were expressed as the mean ± S.D. from three biological replicates (*, $P < 0.05$, Lb2crRNA versus to AscrRNA; t test, double-tailed).

FIG. 17. Schematic diagram depicting chemical modifications applied to CRISPR-Cpf1 crRNAs. For Ψ-modified crRNA, all U bases of wide-type crRNA can be substituted with pseudoU (Ψ). For 5moU-modified crRNA, all U bases can be substituted with 5moU. For 5meC-modified crRNA, all C bases can be substituted with 5meC. For m^1A-modified crRNA, all A bases can be substituted with m^1A.

FIG. 18. Gene cutting efficiency of chemically modified crRNAs. Indel is determined by the T7E1 cleavage assay, and normalized to that of the treatment with crWT and plasmid encoding AsCpf1.

DETAILED DESCRIPTION OF THE INVENTION

Disclosed herein are systems, methods, and compositions that utilize modified Cpf1 mRNAs, modified guide RNAs, and combinations thereof. These modified RNAs can be used in the CRISPR-Cpf1 genome editing system. These modified Cpf1 mRNAs and modified guide RNAs can incorporate a number of chemical changes to the nucleotides, including changes to the nucleobase, the ribose sugar, and/or the phosphodiester linkage; or these changes can include insertions or deletions into the guide RNA sequence. These modified Cpf1 mRNAs, modified guide RNAs, and combinations thereof, can improve efficiency of the CRISPR/Cpf1 genome editing system, reduce off-target effects, reduce toxicity, increase Cpf1 protein levels, increase Cpf1 nuclease activity, increase guide RNA stability, and/or increase Cpf1 mRNA stability.

Reference will now be made in detail to the embodiments of the invention, examples of which are illustrated in the drawings and the examples. This invention may, however, be embodied in many different forms and should not be construed as limited to the embodiments set forth herein.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood to one of ordinary skill in the art to which this invention belongs. The following definitions are provided for the full understanding of terms used in this specification.

Terminology

As used herein, the article "a," "an," and "the" means "at least one," unless the context in which the article is used clearly indicates otherwise.

The term "nucleic acid" as used herein means a polymer composed of nucleotides, e.g. deoxyribonucleotides or ribonucleotides.

The terms "ribonucleic acid" and "RNA" as used herein mean a polymer composed of ribonucleotides.

The terms "deoxyribonucleic acid" and "DNA" as used herein mean a polymer composed of deoxyribonucleotides.

The term "oligonucleotide" denotes single- or double-stranded nucleotide multimers of from about 2 to up to about 100 nucleotides in length. Suitable oligonucleotides may be prepared by the phosphoramidite method described by Beaucage and Carruthers, *Tetrahedron Lett.*, 22:1859-1862 (1981), or by the triester method according to Matteucci, et al., *J. Am. Chem. Soc.*, 103:3185 (1981), both incorporated herein by reference, or by other chemical methods using either a commercial automated oligonucleotide synthesizer or VLSIPS™ technology. When oligonucleotides are referred to as "double-stranded," it is understood by those of skill in the art

that a pair of oligonucleotides exist in a hydrogen-bonded, helical array typically associated with, for example, DNA. In addition to the 100% complementary form of double-stranded oligonucleotides, the term "double-stranded," as used herein is also meant to refer to those forms which include such structural features as bulges and loops, described more fully in such biochemistry texts as Stryer, *Biochemistry*, Third Ed., (1988), incorporated herein by reference for all purposes.

The term "polynucleotide" refers to a single or double stranded polymer composed of nucleotide monomers. In some embodiments, the polynucleotide is composed of nucleotide monomers of generally greater than 100 nucleotides in length and up to about 8,000 or more nucleotides in length.

The term "polypeptide" refers to a compound made up of a single chain of D- or L-amino acids or a mixture of D- and L-amino acids joined by peptide bonds.

The term "complementary" refers to the topological compatibility or matching together of interacting surfaces of a probe molecule and its target. Thus, the target and its probe can be described as complementary, and furthermore, the contact surface characteristics are complementary to each other.

The term "hybridization" refers to a process of establishing a non-covalent, sequence-specific interaction between two or more complementary strands of nucleic acids into a single hybrid, which in the case of two strands is referred to as a duplex.

The term "anneal" refers to the process by which a single-stranded nucleic acid sequence pairs by hydrogen bonds to a complementary sequence, forming a double-stranded nucleic acid sequence, including the reformation (renaturation) of complementary strands that were separated by heat (thermally denatured).

The term "melting" refers to the denaturation of a double-stranded nucleic acid sequence due to high temperatures, resulting in the separation of the double strand into two single strands by breaking the hydrogen bonds between the strands.

The term "target" refers to a molecule that has an affinity for a given probe. Targets may be naturally-occurring or man-made molecules. Also, they can be employed in their unaltered state or as aggregates with other species.

The term "promoter" or "regulatory element" refers to a region or sequence determinants located upstream or downstream from the start of transcription and which are involved in recognition and binding of RNA polymerase and other proteins to initiate transcription. Promoters need not be of bacterial origin, for example, promoters derived from viruses or from other organisms can be used in the compositions, systems, or methods described herein. The term

"regulatory element" is intended to include promoters, enhancers, internal ribosomal entry sites (IRES), and other expression control elements (e.g. transcription termination signals, such as polyadenylation signals and poly-U sequences). Such regulatory elements are described, for example, in Goeddel, Gene Expression Technology: Methods in Enzymology 185, Academic Press, San Diego, Calif. (1990). Regulatory elements include those that direct constitutive expression of a nucleotide sequence in many types of host cell and those that direct expression of the nucleotide sequence only in certain host cells (e.g., tissue-specific regulatory sequences). A tissue-specific promoter may direct expression primarily in a desired tissue of interest, such as muscle, neuron, bone, skin, blood, specific organs (e.g. liver, pancreas), or particular cell types (e.g. lymphocytes). Regulatory elements may also direct expression in a temporal-dependent manner, such as in a cell-cycle dependent or developmental stage-dependent manner, which may or may not also be tissue or cell-type specific. In some embodiments, a vector comprises one or more pol III promoter (e.g. 1, 2, 3, 4, 5, or more pol I promoters), one or more pol II promoters (e.g. 1, 2, 3, 4, 5, or more pol II promoters), one or more pol I promoters (e.g. 1, 2, 3, 4, 5, or more pol I promoters), or combinations thereof. Examples of pol III promoters include, but are not limited to, U6 and H1 promoters. Examples of pol II promoters include, but are not limited to, the retroviral Rous sarcoma virus (RSV) LTR promoter (optionally with the RSV enhancer), the cytomegalovirus (CMV) promoter (optionally with the CMV enhancer) [see, e.g., Boshart et al, Cell, 41:521-530 (1985)], the SV40 promoter, the dihydrofolate reductase promoter, the β-actin promoter, the phosphoglycerol kinase (PGK) promoter, and the EF1α promoter. Also encompassed by the term "regulatory element" are enhancer elements, such as WPRE; CMV enhancers; the R-U5′ segment in LTR of HTLV-I (Mol. Cell. Biol., Vol. 8(1), p. 466-472, 1988); SV40 enhancer; and the intron sequence between exons 2 and 3 of rabbit β-globin (Proc. Natl. Acad. Sci. USA., Vol. 78(3), p. 1527-31, 1981). It will be appreciated by those skilled in the art that the design of the expression vector can depend on such factors as the choice of the host cell to be transformed, the level of expression desired, etc.

The term "recombinant" refers to a human manipulated nucleic acid (e.g. polynucleotide) or a copy or complement of a human manipulated nucleic acid (e.g. polynucleotide), or if in reference to a protein (i.e, a "recombinant protein"), a protein encoded by a recombinant nucleic acid (e.g. polynucleotide). In embodiments, a recombinant expression cassette comprising a promoter operably linked to a second nucleic acid (e.g. polynucleotide) may include a promoter that is heterologous to the second nucleic acid (e.g. polynucleotide) as the result of human manipulation (e.g., by methods described in Sambrook et al., *Molecular Cloning—A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., (1989) or Current Protocols

in Molecular Biology Volumes 1-3, John Wiley & Sons, Inc. (1994-1998)). In another example, a recombinant expression cassette may comprise nucleic acids (e.g. polynucleotides) combined in such a way that the nucleic acids (e.g. polynucleotides) are extremely unlikely to be found in nature. For instance, human manipulated restriction sites or plasmid vector sequences may flank or separate the promoter from the second nucleic acid (e.g. polynucleotide). One of skill will recognize that nucleic acids (e.g. polynucleotides) can be manipulated in many ways and are not limited to the examples above.

The term "expression cassette" refers to a nucleic acid construct, which when introduced into a host cell, results in transcription and/or translation of a RNA or polypeptide, respectively. In embodiments, an expression cassette comprising a promoter operably linked to a second nucleic acid (e.g. polynucleotide) may include a promoter that is heterologous to the second nucleic acid (e.g. polynucleotide) as the result of human manipulation (e.g., by methods described in Sambrook et al., *Molecular Cloning—A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., (1989) or Current Protocols in Molecular Biology Volumes 1-3, John Wiley & Sons, Inc. (1994-1998)). In some embodiments, an expression cassette comprising a terminator (or termination sequence) operably linked to a second nucleic acid (e.g. polynucleotide) may include a terminator that is heterologous to the second nucleic acid (e.g. polynucleotide) as the result of human manipulation. In some embodiments, the expression cassette comprises a promoter operably linked to a second nucleic acid (e.g. polynucleotide) and a terminator operably linked to the second nucleic acid (e.g. polynucleotide) as the result of human manipulation. In some embodiments, the expression cassette comprises an endogenous promoter. In some embodiments, the expression cassette comprises an endogenous terminator. In some embodiments, the expression cassette comprises a synthetic (or non-natural) promoter. In some embodiments, the expression cassette comprises a synthetic (or non-natural) terminator.

The terms "identical" or percent "identity," in the context of two or more nucleic acids or polypeptide sequences, refer to two or more sequences or subsequences that are the same or have a specified percentage of amino acid residues or nucleotides that are the same (i.e., about 60% identity, preferably 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%,94%, 95%, 96%, 97%, 98%, 99% or higher identity over a specified region when compared and aligned for maximum correspondence over a comparison window or designated region) as measured using a BLAST or BLAST 2.0 sequence comparison algorithms with default parameters described below, or by manual alignment and visual inspection (see, e.g., NCBI web site or the like). Such sequences are then said to be "substantially identical." This definition also

refers to, or may be applied to, the compliment of a test sequence. The definition also includes sequences that have deletions and/or additions, as well as those that have substitutions. As described below, the preferred algorithms can account for gaps and the like. Preferably, identity exists over a region that is at least about 10 amino acids or 20 nucleotides in length, or more preferably over a region that is 10-50 amino acids or 20-50 nucleotides in length. As used herein, percent (%) amino acid sequence identity is defined as the percentage of amino acids in a candidate sequence that are identical to the amino acids in a reference sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity. Alignment for purposes of determining percent sequence identity can be achieved in various ways that are within the skill in the art, for instance, using publicly available computer software such as BLAST, BLAST-2, ALIGN, ALIGN-2 or Megalign (DNASTAR) software. Appropriate parameters for measuring alignment, including any algorithms needed to achieve maximal alignment over the full-length of the sequences being compared can be determined by known methods.

For sequence comparisons, typically one sequence acts as a reference sequence, to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are entered into a computer, subsequence coordinates are designated, if necessary, and sequence algorithm program parameters are designated. Preferably, default program parameters can be used, or alternative parameters can be designated. The sequence comparison algorithm then calculates the percent sequence identities for the test sequences relative to the reference sequence, based on the program parameters.

One example of an algorithm that is suitable for determining percent sequence identity and sequence similarity are the BLAST and BLAST 2.0 algorithms, which are described in Altschul et al. (1977) *Nuc. Acids Res.* 25:3389-3402, and Altschul et al. (1990) *J. Mol. Biol.* 215:403-410, respectively. Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence, which either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold (Altschul et al. (1990) *J. Mol. Biol.* 215:403-410). These initial neighborhood word hits act as seeds for initiating searches to find longer HSPs containing them. The word hits are extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Cumulative scores are calculated using, for nucleotide sequences, the parameters M (reward score for a pair of matching residues; always >0) and N (penalty score for mismatching residues; always <0). For amino acid sequences, a scoring matrix is used to calculate the

cumulative score. Extension of the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T, and X determine the sensitivity and speed of the alignment. The BLASTN program (for nucleotide sequences) uses as defaults a wordlength (W) of 11, an expectation (E) or 10, M=5, N=−4 and a comparison of both strands. For amino acid sequences, the BLASTP program uses as defaults a wordlength of 3, and expectation (E) of 10, and the BLOSUM62 scoring matrix (see Henikoff and Henikoff (1989) *Proc. Natl. Acad. Sci. USA* 89:10915) alignments (B) of 50, expectation (E) of 10, M=5, N=−4, and a comparison of both strands.

The BLAST algorithm also performs a statistical analysis of the similarity between two sequences (see, e.g., Karlin and Altschul (1993) *Proc. Natl. Acad. Sci. USA* 90:5873-5787). One measure of similarity provided by the BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. For example, a nucleic acid is considered similar to a reference sequence if the smallest sum probability in a comparison of the test nucleic acid to the reference nucleic acid is less than about 0.2, more preferably less than about 0.01.

The phrase "codon optimized" as it refers to genes or coding regions of nucleic acid molecules for the transformation of various hosts, refers to the alteration of codons in the gene or coding regions of polynucleic acid molecules to reflect the typical codon usage of a selected organism without altering the polypeptide encoded by the DNA. Such optimization includes replacing at least one, or more than one, or a significant number, of codons with one or more codons that are more frequently used in the genes of that selected organism.

Nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For example, DNA for a presequence or secretory leader is operably linked to DNA for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, "operably linked" means that the DNA sequences being linked are near each other, and, in the case of a secretory leader, contiguous and in reading phase. However, operably linked nucleic acids (e.g. enhancers and coding sequences) do not have to be contiguous. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, the synthetic oligonucleotide adaptors or linkers are used in accordance with conventional practice. In embodiments, a promoter is operably linked with a coding sequence

when it is capable of affecting (e.g. modulating relative to the absence of the promoter) the expression of a protein from that coding sequence (i.e., the coding sequence is under the transcriptional control of the promoter).

The term "nucleobase" refers to the part of a nucleotide that bears the Watson/Crick base-pairing functionality. The most common naturally-occurring nucleobases, adenine (A), guanine (G), uracil (U), cytosine (C), and thymine (T) bear the hydrogen-bonding functionality that binds one nucleic acid strand to another in a sequence specific manner.

As used throughout, by a "subject" (or a “host”) is meant an individual. Thus, the "subject" can include, for example, domesticated animals, such as cats, dogs, etc., livestock (e.g., cattle, horses, pigs, sheep, goats, etc.), laboratory animals (e.g., mouse, rabbit, rat, guinea pig, etc.) mammals, non-human mammals, primates, non-human primates, rodents, birds, reptiles, amphibians, fish, and any other animal. The subject can be a mammal such as a primate or a human.

The terms “guide RNA”, “gRNA”, “CRISPR RNA”, or “crRNA” are used interchangeably throughout the specification. This crRNA (or guide RNA) consists of a 5’-handle and a guide segment. Cpf1 protein interacts with the pseudoknot structure formed by the 5’-handle of crRNA (or guide RNA). The guide segment possesses complementary binding with the target DNA sequences.

Genome Editing Systems

In one aspect, disclosed herein is a genome editing system comprising:

a) a guide RNA that hybridizes with a target sequence of a DNA molecule in a eukaryotic cell that contains the DNA molecule, and

b) an mRNA encoding a Cpf1 protein;

wherein the mRNA encoding a Cpf1 protein comprises at least one chemically modified nucleotide, and

wherein the guide RNA hybridizes with the target sequence and the Cpf1 protein cleaves the DNA molecule.

In one aspect, disclosed herein is a genome editing system comprising:

a) a guide RNA that hybridizes with a target sequence of a DNA molecule in a eukaryotic cell that contains the DNA molecule, and

b) a DNA vector comprising a regulatory element operable in a eukaryotic cell operably linked to a nucleotide sequence encoding a Cpf1 protein; an mRNA encoding a Cpf1 protein; or a Cpf1 protein;

wherein the guide RNA comprises at least one chemically modified nucleotide, and

wherein the guide RNA hybridizes with the target sequence and the Cpf1 protein cleaves the DNA molecule.

In one aspect, disclosed herein is a genome editing system comprising:

a) a guide RNA that hybridizes with a target sequence of a DNA molecule in a eukaryotic cell that contains the DNA molecule, and

b) a DNA vector comprising a regulatory element operable in a eukaryotic cell operably linked to a nucleotide sequence encoding a Cpf1 protein;

wherein the guide RNA comprises at least one chemically modified nucleotide, and

wherein the guide RNA hybridizes with the target sequence and the Cpf1 protein cleaves the DNA molecule.

In one aspect, disclosed herein is a genome editing system comprising:

a) a guide RNA that hybridizes with a target sequence of a DNA molecule in a eukaryotic cell that contains the DNA molecule, and

b) an mRNA encoding a Cpf1 protein,

wherein the guide RNA comprises at least one chemically modified nucleotide, and

wherein the guide RNA hybridizes with the target sequence and the Cpf1 protein cleaves the DNA molecule.

In one aspect, disclosed herein is a genome editing system comprising:

a) a guide RNA that hybridizes with a target sequence of a DNA molecule in a eukaryotic cell that contains the DNA molecule, and

b) a Cpf1 protein,

wherein the guide RNA comprises at least one chemically modified nucleotide, and

wherein the guide RNA hybridizes with the target sequence and the Cpf1 protein cleaves the DNA molecule.

In one embodiment, the at least one chemically modified nucleotide confers increased Cpf1 nuclease activity, decreased off-target effects, and/or increased guide RNA stability as compared to a corresponding guide RNA not having the chemical modification.

In one aspect, disclosed herein is a genome editing system comprising:

a) a guide RNA that hybridizes with a target sequence of a DNA molecule in a eukaryotic cell that contains the DNA molecule, and

b) an mRNA encoding a Cpf1 protein;

wherein the guide RNA comprises at least one chemically modified nucleotide; and

wherein the mRNA encoding a Cpf1 protein comprises at least one chemically modified nucleotide.

In one aspect, disclosed herein is a genome editing system comprising:

a) a guide RNA that hybridizes with a target sequence of a DNA molecule in a eukaryotic cell that contains the DNA molecule, and

b) a DNA vector comprising a regulatory element operable in a eukaryotic cell operably linked to a nucleotide sequence encoding a Cpf1 protein; an mRNA encoding a Cpf1 protein; or a Cpf1 protein;

wherein the guide RNA comprises at least one chemically modified nucleotide.

In one aspect, disclosed herein is a nucleic acid comprising:

an mRNA encoding a Cpf1 protein;

wherein the mRNA encoding a Cpf1 protein comprises at least one chemically modified nucleotide.

In one embodiment, the at least one chemically modified nucleotide confers increased Cpf1 nuclease activity, increased Cpf1 protein levels, increased Cpf1 translation, decreased off-target effects, reduced toxicity, and/or increased Cpf1 mRNA stability as compared to a corresponding mRNA encoding a Cpf1 protein not having the chemically modified nucleotide.

In one embodiment, the mRNA encoding a Cpf1 protein is encoded by the DNA sequence SEQ ID NO:1, wherein the mRNA encoding a Cpf1 protein comprises at least one chemically modified nucleotide. In one embodiment, the mRNA encoding a Cpf1 protein is encoded by a nucleic acid which is at least 50%, at least 60%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97.5%, or at least 99% identical to the DNA sequence SEQ ID NO:1.

In one embodiment, the mRNA encoding a Cpf1 protein is encoded by the DNA sequence SEQ ID NO:2, wherein the mRNA encoding a Cpf1 protein comprises at least one chemically modified nucleotide. In one embodiment, the mRNA encoding a Cpf1 protein is encoded by a nucleic acid which is at least 50%, at least 60%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97.5%, or at least 99% identical to the DNA sequence SEQ ID NO:2.

In some embodiments, the at least one chemically modified nucleotide comprises a chemically modified nucleobase, a chemically modified ribose, a chemically modified phosphodiester linkage, or a combination thereof.

In some embodiments, the mRNA encoding a Cpf1 protein comprises at least one chemically modified nucleotide selected from a chemically modified nucleobase, a chemically modified ribose, a chemically modified phosphodiester linkage, or a combination thereof.

In some embodiments, the guide RNA comprises at least one chemically modified nucleotide selected from a chemically modified nucleobase, a chemically modified ribose, a chemically modified phosphodiester linkage, or a combination thereof.

In some embodiments, the mRNA encoding a Cpf1 protein comprises at least one chemically modified nucleotide selected from a chemically modified nucleobase, a chemically modified ribose, a chemically modified phosphodiester linkage, or a combination thereof; and the guide RNA comprises at least one chemically modified nucleotide selected from a chemically modified nucleobase, a chemically modified ribose, a chemically modified phosphodiester linkage, or a combination thereof.

In some embodiments, the mRNA encoding a Cpf1 protein comprises a chemically modified nucleobase and the guide RNA comprises a chemically modified ribose.

In some embodiments, the mRNA encoding a Cpf1 protein comprises a pseudouridine (Ψ) and the guide RNA comprises a chemically modified ribose.

In some embodiments, the mRNA encoding a Cpf1 protein comprises a chemically modified nucleobase and the guide RNA comprises a 2′-Fluoro (2′-F).

In some embodiments, the mRNA encoding a Cpf1 protein comprises a pseudouridine (Ψ) and the guide RNA comprises a 2′-Fluoro (2′-F).

In some embodiments, the chemically modified nucleotides in the mRNA encoding a Cpf1 protein and the chemically modified nucleotides in the guide RNA can be the same type of modification. In some embodiments, the chemically modified nucleotides in the mRNA encoding a Cpf1 protein can be different than the chemically modified nucleotides in the guide RNA.

In one aspect, disclosed herein is a genome editing system comprising:

a) an engineered guide RNA that hybridizes with a target sequence of a DNA molecule in a eukaryotic cell that contains the DNA molecule, and

b) a DNA vector comprising a regulatory element operable in a eukaryotic cell operably linked to a nucleotide sequence encoding a Cpf1 protein; an mRNA encoding a Cpf1 protein; or a Cpf1 protein;

wherein the engineered guide RNA comprises at least one nucleotide insertion or deletion.

In one aspect, disclosed herein is a genome editing system comprising:

a) an engineered guide RNA that hybridizes with a target sequence of a DNA molecule in a eukaryotic cell that contains the DNA molecule, and

b) an mRNA encoding a Cpf1 protein;

wherein the engineered guide RNA comprises at least one nucleotide insertion or deletion; and wherein the mRNA encoding a Cpf1 protein comprises at least one chemically modified nucleotide.

In some embodiments, the engineered guide RNA comprises at least one nucleotide insertion. In some embodiments, the engineered guide RNA comprises at least four nucleotide

insertions. In some embodiments, the engineered guide RNA comprises from four to twelve nucleotide insertions.

In some embodiments, the engineered guide RNA comprises at least one nucleotide deletion. In some embodiments, the engineered guide RNA comprises at least two nucleotide deletion. In some embodiments, the engineered guide RNA comprises from two to eight nucleotide deletion.

In one embodiment, the guide RNA is split into at least two RNAs. In one embodiment, the guide RNA is split at the stem loop into two RNAs.

Methods

In one aspect, provided herein is a method of RNA-guided genome editing, the method comprising:

introducing into a cell of the subject:

a) a guide RNA that hybridizes with a target sequence of a DNA molecule in a eukaryotic cell that contains the DNA molecule, and

b) an mRNA encoding a Cpf1 protein;

wherein the mRNA encoding a Cpf1 protein comprises at least one chemically modified nucleotide; and

wherein the guide RNA hybridizes with the target sequence and the Cpf1 protein cleaves the DNA molecule.

In another aspect, provided herein is a method of RNA-guided genome editing, the method comprising:

introducing into a cell of the subject:

a) a guide RNA that hybridizes with a target sequence of a DNA molecule in a eukaryotic cell that contains the DNA molecule, and

b) an mRNA encoding a Cpf1 protein;

wherein the guide RNA comprises at least one chemically modified nucleotide;

wherein the mRNA encoding a Cpf1 protein comprises at least one chemically modified nucleotide; and

wherein the guide RNA hybridizes with the target sequence and the Cpf1 protein cleaves the DNA molecule.

In another aspect, provided herein is a method of RNA-guided genome editing, the method comprising:

introducing into a cell of the subject:

a) a guide RNA that hybridizes with a target sequence of a DNA molecule in a eukaryotic cell that contains the DNA molecule, and

b) a DNA vector comprising a regulatory element operable in a eukaryotic cell operably linked to a nucleotide sequence encoding a Cpf1 protein; an mRNA encoding a Cpf1 protein; or a Cpf1 protein;

wherein the guide RNA comprises at least one chemically modified nucleotide; and

wherein the guide RNA hybridizes with the target sequence and the Cpf1 protein cleaves the DNA molecule.

In another aspect, provided herein is a method of increasing Cpf1 protein levels in a cell, the method comprising:

introducing into a cell of the subject:

a) a guide RNA that hybridizes with a target sequence of a DNA molecule in a eukaryotic cell that contains the DNA molecule, and

b) an mRNA encoding a Cpf1 protein;

wherein the mRNA encoding a Cpf1 protein comprises at least one chemically modified nucleotide, and

wherein the at least one chemically modified nucleotide confers increased Cpf1 protein levels as compared to a corresponding mRNA encoding a Cpf1 protein not having the chemically modified nucleotide.

In another aspect, provided herein is a method of increasing Cpf1 protein levels in a cell, the method comprising:

introducing into a cell of the subject:

an mRNA encoding a Cpf1 protein;

wherein the mRNA encoding a Cpf1 protein comprises at least one chemically modified nucleotide, and

wherein the at least one chemically modified nucleotide confers increased Cpf1 protein levels as compared to a corresponding mRNA encoding a Cpf1 protein not having the chemically modified nucleotide.

In some embodiments, the method further comprises: introducing into a cell of the subject a guide RNA that hybridizes with a target sequence of a DNA molecule in a eukaryotic cell that contains the DNA molecule.

In a further aspect, provided herein is a method of increasing Cpf1 nuclease activity in a cell, the method comprising:

introducing into a cell of the subject:

a) a guide RNA that hybridizes with a target sequence of a DNA molecule in a eukaryotic cell that contains the DNA molecule, and

b) an mRNA encoding a Cpf1 protein;

wherein the mRNA encoding a Cpf1 protein comprises at least one chemically modified nucleotide, and

wherein the at least one chemically modified nucleotide confers increased Cpf1 nuclease activity as compared to a corresponding mRNA encoding a Cpf1 protein not having the chemically modified nucleotide.

In a further aspect, provided herein is a method of increasing Cpf1 nuclease activity in a cell, the method comprising:

introducing into a cell of the subject:

an mRNA encoding a Cpf1 protein;

wherein the mRNA encoding a Cpf1 protein comprises at least one chemically modified nucleotide, and

wherein the at least one chemically modified nucleotide confers increased Cpf1 nuclease activity as compared to a corresponding mRNA encoding a Cpf1 protein not having the chemically modified nucleotide.

In some embodiments, the method further comprises: introducing into a cell of the subject a guide RNA that hybridizes with a target sequence of a DNA molecule in a eukaryotic cell that contains the DNA molecule.

In another aspect, provided herein is a method of reducing toxicity of RNA-guided genome editing in a cell, the method comprising:

introducing into a cell of the subject:

a) a guide RNA that hybridizes with a target sequence of a DNA molecule in a eukaryotic cell that contains the DNA molecule, and

b) an mRNA encoding a Cpf1 protein;

wherein the mRNA encoding a Cpf1 protein comprises at least one chemically modified nucleotide, and

wherein the at least one chemically modified nucleotide confers reduced toxicity as compared to a corresponding mRNA encoding a Cpf1 protein not having the chemically modified nucleotide.

In another aspect, provided herein is a method of increasing Cpf1 mRNA levels, the method comprising:

transcribing a nucleic acid encoding a Cpf1 protein;

wherein the nucleic acid encoding a Cpf1 protein comprises at least one chemically modified nucleotide, and

wherein the at least one chemically modified nucleotide confers increased Cpf1 mRNA levels as compared to a corresponding mRNA encoding a Cpf1 protein not having the chemically modified nucleotide.

In another aspect, provided herein is a method of increasing Cpf1 protein levels, the method comprising:

translating an mRNA encoding a Cpf1 protein;

wherein the mRNA encoding a Cpf1 protein comprises at least one chemically modified nucleotide, and

wherein the at least one chemically modified nucleotide confers increased Cpf1 protein levels as compared to a corresponding mRNA encoding a Cpf1 protein not having the chemically modified nucleotide.

In another aspect, provided herein is a method of increasing the efficiency of RNA-guided genome editing in a cell, the method comprising:

introducing into a cell of the subject:

a) a guide RNA that hybridizes with a target sequence of a DNA molecule in a eukaryotic cell that contains the DNA molecule, and

b) a DNA vector comprising a regulatory element operable in a eukaryotic cell operably linked to a nucleotide sequence encoding a Cpf1 protein; an mRNA encoding a Cpf1 protein; or a Cpf1 protein;

wherein the guide RNA comprises at least one chemically modified nucleotide, and

wherein the at least one chemically modified nucleotide confers increased Cpf1 nuclease activity as compared to a corresponding guide RNA not having the chemical modification.

In a further aspect, provided herein is a method of decreasing the off-target effects of RNA-guided genome editing in a cell, the method comprising:

introducing into a cell of the subject:

a) a guide RNA that hybridizes with a target sequence of a DNA molecule in a eukaryotic cell that contains the DNA molecule, and

b) a DNA vector comprising a regulatory element operable in a eukaryotic cell operably linked to a nucleotide sequence encoding a Cpf1 protein; an mRNA encoding a Cpf1 protein; or a Cpf1 protein;

wherein the guide RNA comprises at least one chemically modified nucleotide, and

wherein the at least one chemically modified nucleotide confers decreased off-target effects as compared to a corresponding guide RNA not having the chemical modification.

In a further aspect, provided herein is a method of reducing toxicity of RNA-guided genome editing in a cell, the method comprising:

introducing into a cell of the subject:

a) a guide RNA that hybridizes with a target sequence of a DNA molecule in a eukaryotic cell that contains the DNA molecule, and

b) a DNA vector comprising a regulatory element operable in a eukaryotic cell operably linked to a nucleotide sequence encoding a Cpf1 protein; an mRNA encoding a Cpf1 protein; or a Cpf1 protein;

wherein the guide RNA comprises at least one chemically modified nucleotide, and

wherein the at least one chemically modified nucleotide confers reduced toxicity as compared to a corresponding guide RNA not having the chemical modification.

Previous studies have reported that chemical modifications can improve the stability and potency of various RNAs including siRNA, miRNA and antisense nucleic acids. Recently, chemical modifications were incorporated into guide RNAs for the CRISPR-Cas9 system comprising 2'O-methyl, 3'phosphorothioate, or 3'thioPACE at three terminal nucleotides at both the 5' and 3' ends of gRNAs (Hendel, A. *Nature Biotechnology* 2015, 33: 985-989). In some embodiments, the guide RNA comprises at least one chemically modified nucleotide.

In some embodiments, the at least one chemically modified nucleotide comprises a chemically modified nucleobase, a chemically modified ribose, a chemically modified phosphodiester linkage, or a combination thereof.

In some embodiments, the mRNA encoding a Cpf1 protein comprises at least one chemically modified nucleotide selected from a chemically modified nucleobase, a chemically modified ribose, a chemically modified phosphodiester linkage, or a combination thereof.

In some embodiments, the guide RNA comprises at least one chemically modified nucleotide selected from a chemically modified nucleobase, a chemically modified ribose, a chemically modified phosphodiester linkage, or a combination thereof.

In some embodiments, the mRNA encoding a Cpf1 protein comprises at least one chemically modified nucleotide selected from a chemically modified nucleobase, a chemically modified ribose, a chemically modified phosphodiester linkage, or a combination thereof; and the guide RNA comprises at least one chemically modified nucleotide selected from a chemically modified nucleobase, a chemically modified ribose, a chemically modified phosphodiester linkage, or a combination thereof.

In some embodiments, the mRNA encoding a Cpf1 protein comprises a chemically modified nucleobase and the guide RNA comprises a chemically modified ribose.

In some embodiments, the mRNA encoding a Cpf1 protein comprises a pseudouridine (Ψ) and the guide RNA comprises a chemically modified ribose.

In some embodiments, the mRNA encoding a Cpf1 protein comprises a chemically modified nucleobase and the guide RNA comprises a 2′-Fluoro (2′-F).

In some embodiments, the mRNA encoding a Cpf1 protein comprises a pseudouridine (Ψ) and the guide RNA comprises a 2′-Fluoro (2′-F).

In one aspect, disclosed herein is a method of RNA-guided genome editing, the method comprising:

introducing into a cell of the subject:

a) an engineered guide RNA that hybridizes with a target sequence of a DNA molecule in a eukaryotic cell that contains the DNA molecule, and

b) a DNA vector comprising a regulatory element operable in a eukaryotic cell operably linked to a nucleotide sequence encoding a Cpf1 protein; an mRNA encoding a Cpf1 protein; or a Cpf1 protein;

wherein the engineered guide RNA comprises at least one nucleotide insertion or deletion; and

wherein the guide RNA hybridizes with the target sequence and the Cpf1 protein cleaves the DNA molecule.

In one aspect, disclosed herein is a method of RNA-guided genome editing, the method comprising:

introducing into a cell of the subject:

a) an engineered guide RNA that hybridizes with a target sequence of a DNA molecule in a eukaryotic cell that contains the DNA molecule, and

b) an mRNA encoding a Cpf1 protein;

wherein the engineered guide RNA comprises at least one nucleotide insertion or deletion; and

wherein the mRNA encoding a Cpf1 protein comprises at least one chemically modified nucleotide; and

wherein the guide RNA hybridizes with the target sequence and the Cpf1 protein cleaves the DNA molecule.

In some embodiments, the engineered guide RNA comprises at least one nucleotide insertion. In some embodiments, the engineered guide RNA comprises at least four nucleotide insertions. In some embodiments, the engineered guide RNA comprises from four to twelve nucleotide insertions.

In some embodiments, the engineered guide RNA comprises at least one nucleotide deletion. In some embodiments, the engineered guide RNA comprises at least two nucleotide deletion. In some embodiments, the engineered guide RNA comprises from two to eight nucleotide deletion.

Chemically modified nucleobases

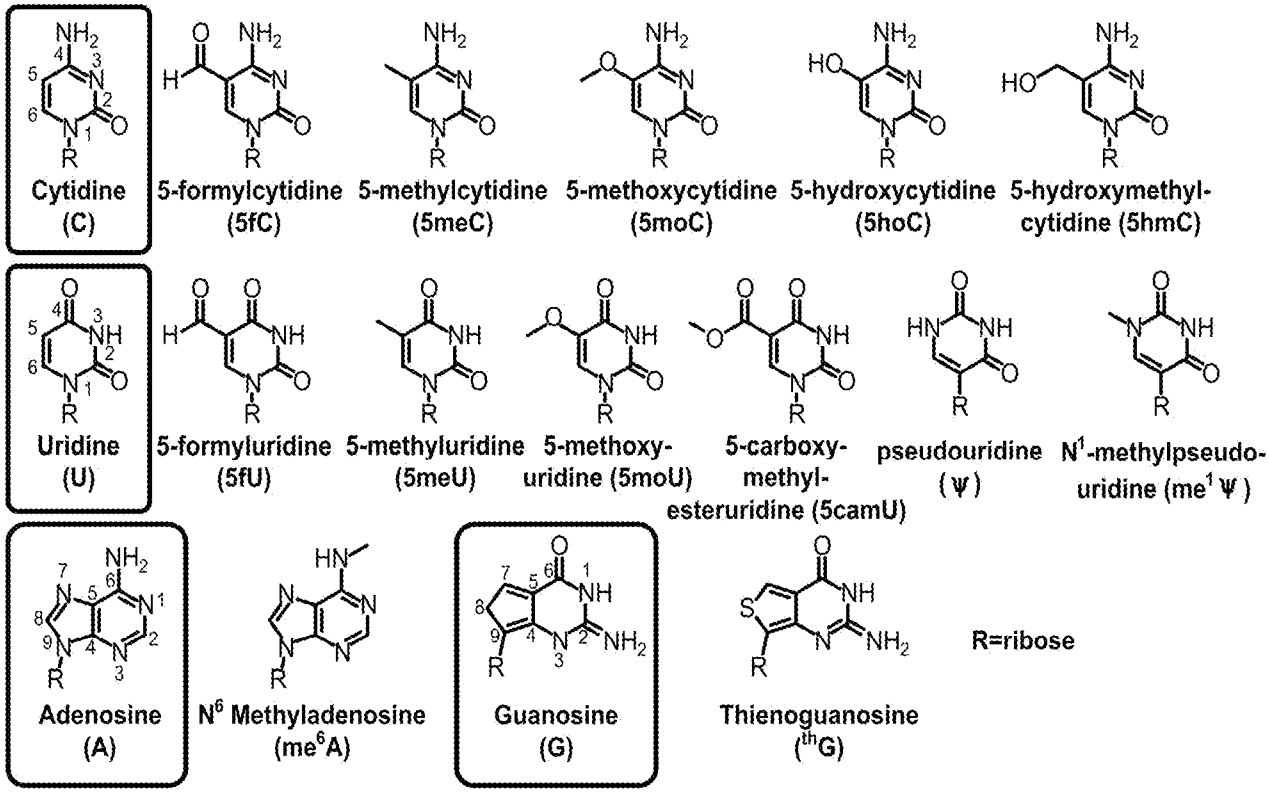
In one embodiment, the at least one chemically modified nucleotide is a chemically modified nucleobase. In some embodiments, the mRNA encoding a Cpf1 protein comprises a chemically modified nucleobase. In some embodiments, the guide RNA comprises a chemically modified nucleobase.

In one embodiment, the chemically modified nucleobase is selected from 5-formylcytidine (5fC), 5-methylcytidine (5meC), 5-methoxycytidine (5moC), 5-hydroxycytidine (5hoC), 5-hydroxymethylcytidine (5hmC), 5-formyluridine (5fU), 5-methyluridine (5-meU), 5-methoxyuridine (5moU), 5-carboxymethylesteruridine (5camU), pseudouridine (Ψ), N^1-methylpseudouridine ($me^1\Psi$), N^6-methyladenosine (me^6A), or thienoguanosine (^{th}G).

In some embodiments, the chemically modified nucleobase is selected from 5-methoxyuridine (5moU), pseudouridine (Ψ), and N^1-methylpseudouridine ($me^1\Psi$). In some embodiments, the chemically modified nucleobase is 5-methoxyuridine (5moU). In some embodiments, the chemically modified nucleobase is pseudouridine (Ψ). In some embodiments, the chemically modified nucleobase is N^1-methylpseudouridine ($me^1\Psi$).

In some embodiments, the at least one chemically modified nucleobase comprises N^1-methylpseudouridine ($me^1\Psi$) and 5-methylcytidine (5meC). In some embodiments, the at least one chemically modified nucleobase comprises pseudouridine (Ψ) and 5-methylcytidine (5meC). In some embodiments, the at least one chemically modified nucleobase comprises 5-methyluridine (5-meU) and 5-methoxycytidine (5moC). In some embodiments, the at least one chemically modified nucleobase comprises 5-methyluridine (5-meU) and 5-hydroxymethylcytidine (5hmC).

The structures of these modified nucleobases are shown below:

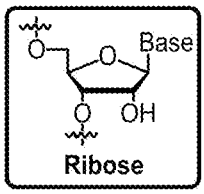


Chemically modified ribose moieties

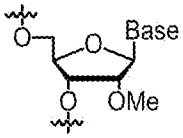
In one embodiment, the at least one chemically modified nucleotide is a chemically modified ribose. In some embodiments, the mRNA encoding a Cpf1 protein comprises a chemically modified ribose. In some embodiments, the guide RNA comprises a chemically modified ribose.

In one embodiment, the chemically modified ribose is selected from 2′-*O*-methyl (2′-*O*-Me), 2′-Fluoro (2′-F), 2′-deoxy-2′-fluoro-beta-D-arabino-nucleic acid (2′F-ANA), 4′-S, 4′-SFANA, 2′-azido, UNA, 2′-*O*-methoxy-ethyl (2′-*O*-ME), 2′-*O*-Allyl, 2′-*O*-Ethylamine, 2′-*O*-Cyanoethyl, Locked nucleic acid (LAN), Methylene-cLAN, N-MeO-amino BNA, or N-MeO-aminooxy BNA. In one embodiment, the chemically modified ribose is selected from 2′-O-methyl (2′-O-Me) or 2′-Fluoro (2′-F).

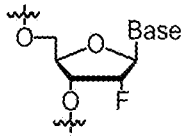
The structures of these modified riboses are shown below:



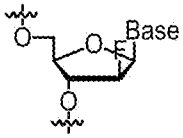
Ribose



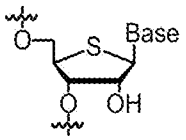
2'-*O*-methyl (2'-*O*-Me)



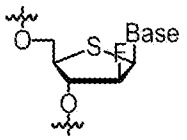
2'-Fluoro (2'-F)



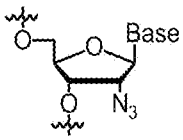
2'-deoxy-2'-fluoro-beta-D-arabino-nucleic acid (2'F-ANA)



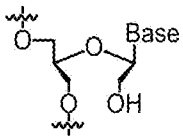
4'-S



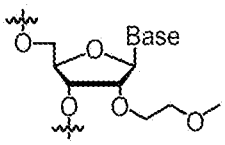
4'-SFANA



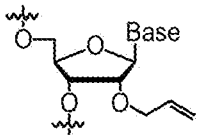
2'-azido



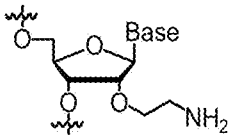
UNA



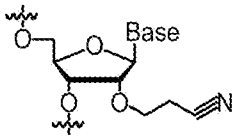
2'-*O*-methoxy-ethyl (2'-*O*-ME)



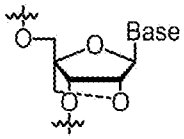
2'-*O*-Allyl



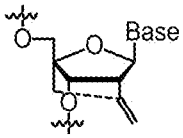
2'-*O*-Ethylamine



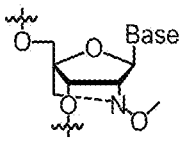
2'-*O*-Cyanoethyl



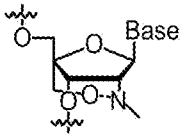
Locked nucleic acid (LAN)



Methylene-cLAN



N-MeO-amino BNA



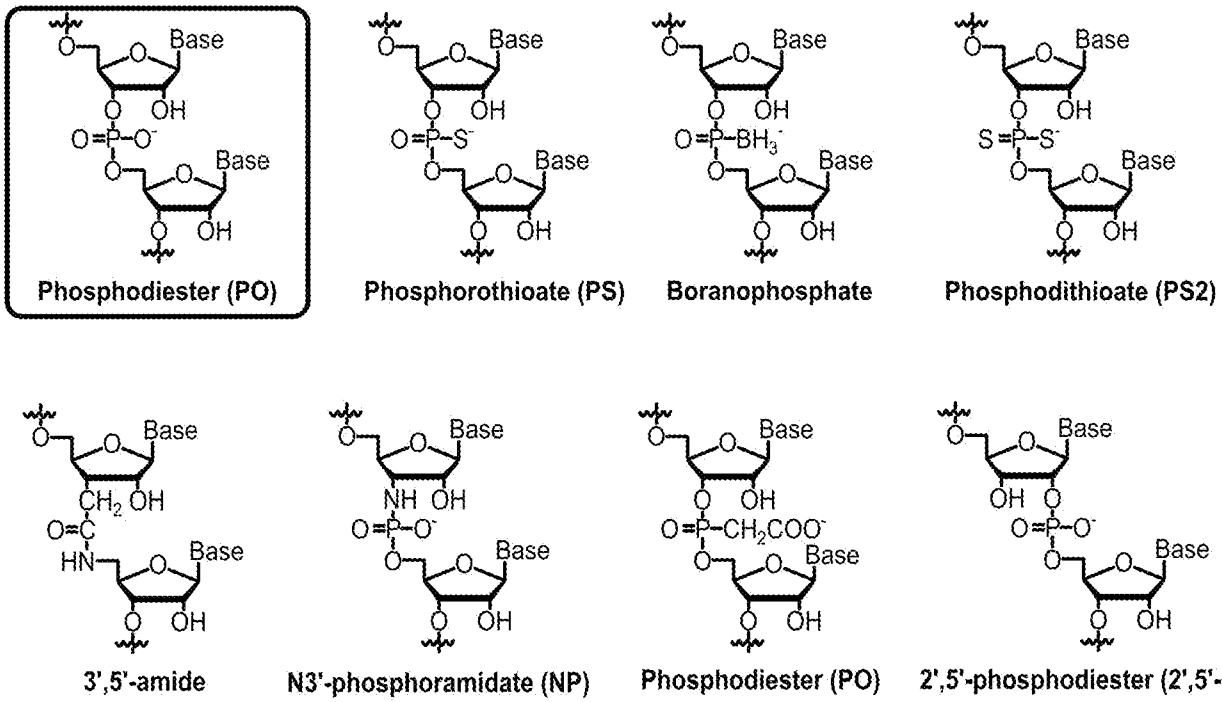
N-MeO-aminooxy BNA

Chemically modified phosphodiester backbone

In one embodiment, the at least one chemically modified nucleotide is a chemically modified phosphodiester linkage. In some embodiments, the mRNA encoding a Cpf1 protein comprises a chemically modified phosphodiester linkage. In some embodiments, the guide RNA comprises a chemically modified phosphodiester linkage.

In one embodiment, the chemically modified phosphodiester linkage is selected from Phosphorothioate (PS), Boranophosphate, phosphodithioate (PS2), 3′,5′-amide, N3′-phosphoramidate (NP), Phosphodiester (PO), or 2′,5′-phosphodiester (2′,5′-PO). In one embodiment, the chemically modified phosphodiester linkage is phosphorothioate.

The structures of these modified phosphodiester linkages are shown below:



In one aspect, the invention provides a kit comprising one or more of the components described herein. In some embodiments, the kit comprises instructions for using the kit. In some embodiments, the kit comprises:

a) a guide RNA that hybridizes with a target sequence of a DNA molecule in a eukaryotic cell that contains the DNA molecule, and

b) an mRNA encoding a Cpf1 protein;

wherein the mRNA encoding a Cpf1 protein comprises at least one chemically modified nucleotide, and

wherein the guide RNA hybridizes with the target sequence and the Cpf1 protein cleaves the DNA molecule.

In one embodiment, the invention provides a method of modifying a target polynucleotide in a eukaryotic cell. In one embodiment, the invention provides a method of modifying expression of a polynucleotide in a eukaryotic cell.

In some embodiments, an enzyme coding sequence encoding a Cpf1 protein is codon optimized for expression in particular cells, such as eukaryotic cells. In some embodiments, the DNA sequence encoding a Cpf1 protein is similar, or shares substantial identity with SEQ ID NO:1. In one embodiment, the mRNA encoding a Cpf1 protein is encoded by SEQ ID NO:1. In one embodiment, the mRNA encoding a Cpf1 protein is encoded by a nucleic acid which is at least 50%, at least 60%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97.5%, or at least 99% identical to SEQ ID NO:1.

In some embodiments, an enzyme coding sequence encoding a Cpf1 protein is codon optimized for expression in particular cells, such as eukaryotic cells. In some embodiments, the DNA sequence encoding a Cpf1 protein is similar, or shares substantial identity with SEQ ID NO:2. In one embodiment, the mRNA encoding a Cpf1 protein is encoded by SEQ ID NO:2. In one embodiment, the mRNA encoding a Cpf1 protein is encoded by a nucleic acid which is at least 50%, at least 60%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97.5%, or at least 99% identical to SEQ ID NO:2.

In general, a guide RNA sequence is any polynucleotide sequence having sufficient complementarity with a target polynucleotide sequence to hybridize with the target sequence and direct sequence-specific binding of a CRISPR-Cpf1 complex to the target sequence. In some embodiments, the degree of complementarity between a guide sequence and its corresponding target sequence, when optimally aligned using a suitable alignment algorithm, is about or more than about 50%, 60%, 75%, 80%, 85%, 90%, 95%, 97.5%, 99%, or more. In some embodiments, the guide RNA comprises at least one chemically modified nucleotide.

Methods of Treatment

The chemically modified Cpf1 mRNA can be used to correct a mutation in a genome. For example, the guide RNAs can be designed to correct mutations that cause hemophilia (due to mutations in the genes encoding Factor VIII (F8; hemophilia A) or Factor IX (F9; hemoglobin B). In one aspect, the CRISPR-Cpf1 system, including the chemically modified Cpf1 mRNA, may be used to correct genetic mutations causing hemophilia.

In one aspect, disclosed herein is a method of treating hemophilia in a subject comprising:

introducing into a cell of the subject:

a) a guide RNA that hybridizes with a target sequence of a DNA molecule in a eukaryotic cell that contains the DNA molecule,

b) an mRNA encoding a Cpf1 protein, and

c) an isolated nucleic acid comprising a donor sequence comprising a nucleic acid encoding a truncated FVIII polypeptide;

wherein the cell of the subject contains a genetic mutation in the F8 gene;

wherein the mRNA encoding a Cpf1 protein comprises at least one chemically modified nucleotide,

wherein the guide RNA hybridizes with the target sequence,

wherein the target sequence is in the F8 gene,

wherein the Cpf1 protein creates a double stranded break in the DNA molecule,

wherein the nucleic acid encoding the truncated FVIII polypeptide is flanked by nucleic acid sequences homologous to the nucleic acid sequences upstream and downstream of the double stranded break in the DNA molecule, and

wherein the resultant repaired gene, upon expression, confers improved coagulation functionality to the encoded FVIII protein of the subject compared to the non-repaired F8 gene.

In one aspect, disclosed herein is a method of treating hemophilia in a subject comprising: introducing into a cell of the subject:

a) a guide RNA that hybridizes with a target sequence of a DNA molecule in a eukaryotic cell that contains the DNA molecule,

b) a DNA vector comprising a DNA vector comprising a regulatory element operable in a eukaryotic cell operably linked to a nucleotide sequence encoding a Cpf1 protein, and

c) an isolated nucleic acid comprising a donor sequence comprising a nucleic acid encoding a truncated FVIII polypeptide;

wherein the guide RNA comprises at least one chemically modified nucleotide,

wherein the guide RNA hybridizes with the target sequence,

wherein the target sequence is in the F8 gene,

wherein the Cpf1 protein creates a double stranded break in the DNA molecule,

wherein the nucleic acid encoding the truncated FVIII polypeptide is flanked by nucleic acid sequences homologous to the nucleic acid sequences upstream and downstream of the double stranded break in the DNA, and

wherein the resultant repaired gene, upon expression, confers improved coagulation functionality to the encoded FVIII protein of the subject compared to the non-repaired F8 gene.

In one aspect, disclosed herein is a method of treating hemophilia in a subject comprising: introducing into a cell of the subject:

a) a guide RNA that hybridizes with a target sequence of a DNA molecule in a eukaryotic cell that contains the DNA molecule,

b) an mRNA encoding a Cpf1 protein, and

c) an isolated nucleic acid comprising a portion of the wild-type F9 gene;

wherein the cell of the subject contains a genetic mutation in the F9 gene;

wherein the mRNA encoding a Cpf1 protein comprises at least one chemically modified nucleotide,

wherein the guide RNA hybridizes with the target sequence,

wherein the target sequence is in the F9 gene,

wherein the Cpf1 protein creates a double stranded break in the DNA molecule,

wherein the nucleic acid encoding the nucleic acid encoding a wild-type portion of the F9 gene is flanked by nucleic acid sequences homologous to the nucleic acid sequences upstream and downstream of the double stranded break in the DNA molecule, and

wherein the resultant repaired gene, upon expression, confers improved coagulation functionality to the encoded FIX protein of the subject compared to the non-repaired F9 gene.

In additional embodiments, the CRISPR-Cpf1 system can be used to repair point mutations, truncations, deletions, inversions, or other genetic mutations that are identified as the causal mutation for a genetic disease.

Cpf1 protein

In one embodiment, the Cpf1 protein is encoded by SEQ ID NO:1. This sequence can be codon optimized, can differ due to the degeneracy of the genetic code, can be similar, or share substantial identity, to SEQ ID NO:1, but still retain nuclease activity.

AsCpf1 sequence (SEQ ID NO:1)

ATGGCCCCAAAGAAGAAGCGGAAGGTCGGTATCCACGGAGTCCCAGCAGCCACACA
GTTCGAGGGCTTTACCAACCTGTATCAGGTGAGCAAGACACTGCGGTTTGAGCTGAT
CCCACAGGGCAAGACCCTGAAGCACATCCAGGAGCAGGGCTTCATCGAGGAGGACA
AGGCCCGCAATGATCACTACAAGGAGCTGAAGCCCATCATCGATCGGATCTACAAGA
CCTATGCCGACCAGTGCCTGCAGCTGGTGCAGCTGGATTGGGAGAACCTGAGCGCCG
CCATCGACTCCTATAGAAAGGAGAAAACCGAGGAGACAAGGAACGCCCTGATCGAG
GAGCAGGCCACATATCGCAATGCCATCCACGACTACTTCATCGGCCGGACAGACAAC
CTGACCGATGCCATCAATAAGAGACACGCCGAGATCTACAAGGGCCTGTTCAAGGCC
GAGCTGTTTAATGGCAAGGTGCTGAAGCAGCTGGGCACCGTGACCACAACCGAGCA
CGAGAACGCCCTGCTGCGGAGCTTCGACAAGTTTACAACCTACTTCTCCGGCTTTTAT
GAGAACAGGAAGAACGTGTTCAGCGCCGAGGATATCAGCACAGCCATCCCACACCG
CATCGTGCAGGACAACTTCCCCAAGTTTAAGGAGAATTGTCACATCTTCACACGCCTG
ATCACCGCCGTGCCCAGCCTGCGGGAGCACTTTGAGAACGTGAAGAAGGCCATCGG
CATCTTCGTGAGCACCTCCATCGAGGAGGTGTTTTCCTTCCCTTTTTATAACCAGCTGC
TGACACAGACCCAGATCGACCTGTATAACCAGCTGCTGGGAGGAATCTCTCGGGAGG
CAGGCACCGAGAAGATCAAGGGCCTGAACGAGGTGCTGAATCTGGCCATCCAGAAG
AATGATGAGACAGCCCACATCATCGCCTCCCTGCCACACAGATTCATCCCCCTGTTTA
AGCAGATCCTGTCCGATAGGAACACCCTGTCTTTCATCCTGGAGGAGTTTAAGAGCG
ACGAGGAAGTGATCCAGTCCTTCTGCAAGTACAAGACACTGCTGAGAAACGAGAAC
GTGCTGGAGACAGCCGAGGCCCTGTTTAACGAGCTGAACAGCATCGACCTGACACA
CATCTTCATCAGCCACAAGAAGCTGGAGACAATCAGCAGCGCCCTGTGCGACCACTG
GGATACACTGAGGAATGCCCTGTATGAGCGGAGAATCTCCGAGCTGACAGGCAAGAT
CACCAAGTCTGCCAAGGAGAAGGTGCAGCGCAGCCTGAAGCACGAGGATATCAACC
TGCAGGAGATCATCTCTGCCGCAGGCAAGGAGCTGAGCGAGGCCTTCAAGCAGAAA
ACCAGCGAGATCCTGTCCCACGCACACGCCGCCCTGGATCAGCCACTGCCTACAACC
CTGAAGAAGCAGGAGGAGAAGGAGATCCTGAAGTCTCAGCTGGACAGCCTGCTGGG
CCTGTACCACCTGCTGGACTGGTTTGCCGTGGATGAGTCCAACGAGGTGGACCCCGA
GTTCTCTGCCCGGCTGACCGGCATCAAGCTGGAGATGGAGCCTTCTCTGAGCTTCTAC
AACAAGGCCAGAAATTATGCCACCAAGAAGCCCTACTCCGTGGAGAAGTTCAAGCTG

AACTTTCAGATGCCTACACTGGCCTCTGGCTGGGACGTGAATAAGGAGAAGAACAAT
GGCGCCATCCTGTTTGTGAAGAACGGCCTGTACTATCTGGGCATCATGCCAAAGCAG
AAGGGCAGGTATAAGGCCCTGAGCTTCGAGCCCACAGAGAAAACCAGCGAGGGCTT
TGATAAGATGTACTATGACTACTTCCCTGATGCCGCCAAGATGATCCCAAAGTGCAGC
ACCCAGCTGAAGGCCGTGACAGCCCACTTTCAGACCCACACAACCCCCATCCTGCTG
TCCAACAATTTCATCGAGCCTCTGGAGATCACAAAGGAGATCTACGACCTGAACAAT
CCTGAGAAGGAGCCAAAGAAGTTTCAGACAGCCTACGCCAAGAAAACCGGCGACCA
GAAGGGCTACAGAGAGGCCCTGTGCAAGTGGATCGACTTCACAAGGGATTTTCTGTC
CAAGTATACCAAGACAACCTCTATCGATCTGTCTAGCCTGCGGCCATCCTCTCAGTATA
AGGACCTGGGCGAGTACTATGCCGAGCTGAATCCCCTGCTGTACCACATCAGCTTCCA
GAGAATCGCCGAGAAGGAGATCATGGATGCCGTGGAGACAGGCAAGCTGTACCTGTT
CCAGATCTATAACAAGGACTTTGCCAAGGGCCACCACGGCAAGCCTAATCTGCACAC
ACTGTATTGGACCGGCCTGTTTTCTCCAGAGAACCTGGCCAAGACAAGCATCAAGCT
GAATGGCCAGGCCGAGCTGTTCTACCGCCCTAAGTCCAGGATGAAGAGGATGGCACA
CCGGCTGGGAGAGAAGATGCTGAACAAGAAGCTGAAGGATCAGAAAACCCCAATCC
CCGACACCCTGTACCAGGAGCTGTACGACTATGTGAATCACAGACTGTCCCACGACC
TGTCTGATGAGGCCAGGGCCCTGCTGCCCAACGTGATCACCAAGGAGGTGTCTCACG
AGATCATCAAGGATAGGCGCTTTACCAGCGACAAGTTCTTTTTCCACGTGCCTATCAC
ACTGAACTATCAGGCCGCCAATTCCCCATCTAAGTTCAACCAGAGGGTGAATGCCTAC
CTGAAGGAGCACCCCGAGACACCTATCATCGGCATCGATCGGGGCGAGAGAAACCTG
ATCTATATCACAGTGATCGACTCCACCGGCAAGATCCTGGAGCAGCGGAGCCTGAAC
ACCATCCAGCAGTTTGATTACCAGAAGAAGCTGGACAACAGGGAGAAGGAGAGGGT
GGCAGCAAGGCAGGCCTGGTCTGTGGTGGGCACAATCAAGGATCTGAAGCAGGGCT
ATCTGAGCCAGGTCATCCACGAGATCGTGGACCTGATGATCCACTACCAGGCCGTGGT
GGTGCTGGAGAACCTGAATTTCGGCTTTAAGAGCAAGAGGACCGGCATCGCCGAGA
AGGCCGTGTACCAGCAGTTCGAGAAGATGCTGATCGATAAGCTGAATTGCCTGGTGC
TGAAGGACTATCCAGCAGAGAAAGTGGGAGGCGTGCTGAACCCATACCAGCTGACA
GACCAGTTCACCTCCTTTGCCAAGATGGGCACCCAGTCTGGCTTCCTGTTTTACGTGC
CTGCCCCATATACATCTAAGATCGATCCCCTGACCGGCTTCGTGGACCCCTTCGTGTGG
AAAACCATCAAGAATCACGAGAGCCGCAAGCACTTCCTGGAGGGCTTCGACTTTCTG
CACTACGACGTGAAAACCGGCGACTTCATCCTGCACTTTAAGATGAACAGAAATCTG
TCCTTCCAGAGGGGCCTGCCCGGCTTTATGCCTGCATGGGATATCGTGTTCGAGAAGA
ACGAGACACAGTTTGACGCCAAGGGCACCCCTTTCATCGCCGGCAAGAGAATCGTGC
CAGTGATCGAGAATCACAGATTCACCGGCAGATACCGGGACCTGTATCCTGCCAACG
AGCTGATCGCCCTGCTGGAGGAGAAGGGCATCGTGTTCAGGGATGGCTCCAACATCC
TGCCAAAGCTGCTGGAGAATGACGATTCTCACGCCATCGACACCATGGTGGCCCTGA
TCCGCAGCGTGCTGCAGATGCGGAACTCCAATGCCGCCACAGGCGAGGACTATATCA
ACAGCCCCGTGCGCGATCTGAATGGCGTGTGCTTCGACTCCCGGTTTCAGAACCCAG
AGTGGCCCATGGACGCCGATGCCAATGGCGCCTACCACATCGCCCTGAAGGGCCAGC
TGCTGCTGAATCACCTGAAGGAGAGCAAGGATCTGAAGCTGCAGAACGGCATCTCCA
ATCAGGACTGGCTGGCCTACATCCAGGAGCTGCGCAACAAGCGTCCTGCTGCTACTA
AGAAAGCTGGTCAAGCTAAGAAAAAGAAATAA(SEQ ID NO:1).

In one embodiment, the Cpf1 protein is encoded by SEQ ID NO:2. This sequence can be codon optimized, can differ due to the degeneracy of the genetic code, can be similar, or share substantial identity, to SEQ ID NO:2, but still retain nuclease activity.

LbCpf1 sequence (SEQ ID NO:2)

ATGGCCCCAAAGAAGAAGCGGAAGGTCGGTATCCACGGAGTCCCAGCAGCCAGCAA
GCTGGAGAAGTTTACAAACTGCTACTCCCTGTCTAAGACCCTGAGGTTCAAGGCCAT
CCCTGTGGGCAAGACCCAGGAGAACATCGACAATAAGCGGCTGCTGGTGGAGGACG
AGAAGAGAGCCGAGGATTATAAGGGCGTGAAGAAGCTGCTGGATCGCTACTATCTGT
CTTTTATCAACGACGTGCTGCACAGCATCAAGCTGAAGAATCTGAACAATTACATCAG
CCTGTTCCGGAAGAAAACCAGAACCGAGAAGGAGAATAAGGAGCTGGAGAACCTGG
AGATCAATCTGCGGAAGGAGATCGCCAAGGCCTTCAAGGGCAACGAGGGCTACAAG
TCCCTGTTTAAGAAGGATATCATCGAGACAATCCTGCCAGAGTTCCTGGACGATAAGG
ACGAGATCGCCCTGGTGAACAGCTTCAATGGCTTTACCACAGCCTTCACCGGCTTCTT
TGATAACAGAGAGAATATGTTTTCCGAGGAGGCCAAGAGCACATCCATCGCCTTCAG
GTGTATCAACGAGAATCTGACCCGCTACATCTCTAATATGGACATCTTCGAGAAGGTG
GACGCCATCTTTGATAAGCACGAGGTGCAGGAGATCAAGGAGAAGATCCTGAACAGC
GACTATGATGTGGAGGATTTCTTTGAGGGCGAGTTCTTTAACTTTGTGCTGACACAGG
AGGGCATCGACGTGTATAACGCCATCATCGGCGGCTTCGTGACCGAGAGCGGCGAGA
AGATCAAGGGCCTGAACGAGTACATCAACCTGTATAATCAGAAAACCAAGCAGAAGC
TGCCTAAGTTTAAGCCACTGTATAAGCAGGTGCTGAGCGATCGGGAGTCTCTGAGCTT
CTACGGCGAGGGCTATACATCCGATGAGGAGGTGCTGGAGGTGTTTAGAAACACCCT
GAACAAGAACAGCGAGATCTTCAGCTCCATCAAGAAGCTGGAGAAGCTGTTCAAGA
ATTTTGACGAGTACTCTAGCGCCGGCATCTTTGTGAAGAACGGCCCCGCCATCAGCAC
AATCTCCAAGGATATCTTCGGCGAGTGGAACGTGATCCGGGACAAGTGGAATGCCGA
GTATGACGATATCCACCTGAAGAAGAAGGCCGTGGTGACCGAGAAGTACGAGGACG
ATCGGAGAAAGTCCTTCAAGAAGATCGGCTCCTTTTCTCTGGAGCAGCTGCAGGAGT
ACGCCGACGCCGATCTGTCTGTGGTGGAGAAGCTGAAGGAGATCATCATCCAGAAGG
TGGATGAGATCTACAAGGTGTATGGCTCCTCTGAGAAGCTGTTCGACGCCGATTTTGT
GCTGGAGAAGAGCCTGAAGAAGAACGACGCCGTGGTGGCCATCATGAAGGACCTGC
TGGATTCTGTGAAGAGCTTCGAGAATTACATCAAGGCCTTCTTTGGCGAGGGCAAGG
AGACAAACAGGGACGAGTCCTTCTATGGCGATTTTGTGCTGGCCTACGACATCCTGCT
GAAGGTGGACCACATCTACGATGCCATCCGCAATTATGTGACCCAGAAGCCCTACTCT
AAGGATAAGTTCAAGCTGTATTTTCAGAACCCTCAGTTCATGGGCGGCTGGGACAAG
GATAAGGAGACAGACTATCGGGCCACCATCCTGAGATACGGCTCCAAGTACTATCTGG
CCATCATGGATAAGAAGTACGCCAAGTGCCTGCAGAAGATCGACAAGGACGATGTGA
ACGGCAATTACGAGAAGATCAACTATAAGCTGCTGCCCGGCCCTAATAAGATGCTGCC
AAAGGTGTTCTTTTCTAAGAAGTGGATGGCCTACTATAACCCCAGCGAGGACATCCAG
AAGATCTACAAGAATGGCACATTCAAGAAGGGCGATATGTTTAACCTGAATGACTGTC
ACAAGCTGATCGACTTCTTTAAGGATAGCATCTCCCGGTATCCAAAGTGGTCCAATGC
CTACGATTTCAACTTTTCTGAGACAGAGAAGTATAAGGACATCGCCGGCTTTTACAGA
GAGGTGGAGGAGCAGGGCTATAAGGTGAGCTTCGAGTCTGCCAGCAAGAAGGAGGT
GGATAAGCTGGTGGAGGAGGGCAAGCTGTATATGTTCCAGATCTATAACAAGGACTTT
TCCGATAAGTCTCACGGCACACCCAATCTGCACACCATGTACTTCAAGCTGCTGTTTG
ACGAGAACAATCACGGACAGATCAGGCTGAGCGGAGGAGCAGAGCTGTTCATGAGG
CGCGCCTCCCTGAAGAAGGAGGAGCTGGTGGTGCACCCAGCCAACTCCCCTATCGCC
AACAAGAATCCAGATAATCCCAAGAAAACCACAACCCTGTCCTACGACGTGTATAAG
GATAAGAGGTTTTCTGAGGACCAGTACGAGCTGCACATCCCAATCGCCATCAATAAGT
GCCCCAAGAACATCTTCAAGATCAATACAGAGGTGCGCGTGCTGCTGAAGCACGACG
ATAACCCCTATGTGATCGGCATCGATAGGGGCGAGCGCAATCTGCTGTATATCGTGGTG
GTGGACGGCAAGGGCAACATCGTGGAGCAGTATTCCCTGAACGAGATCATCAACAAC
TTCAACGGCATCAGGATCAAGACAGATTACCACTCTCTGCTGGACAAGAAGGAGAAG
GAGAGGTTCGAGGCCCGCCAGAACTGGACCTCCATCGAGAATATCAAGGAGCTGAA
GGCCGGCTATATCTCTCAGGTGGTGCACAAGATCTGCGAGCTGGTGGAGAAGTACGA
TGCCGTGATCGCCCTGGAGGACCTGAACTCTGGCTTTAAGAATAGCCGCGTGAAGGT

GGAGAAGCAGGTGTATCAGAAGTTCGAGAAGATGCTGATCGATAAGCTGAACTACAT
GGTGGACAAGAAGTCTAATCCTTGTGCAACAGGCGGCGCCCTGAAGGGCTATCAGAT
CACCAATAAGTTCGAGAGCTTTAAGTCCATGTCTACCCAGAACGGCTTCATCTTTTAC
ATCCCTGCCTGGCTGACATCCAAGATCGATCCATCTACCGGCTTTGTGAACCTGCTGA
AAACCAAGTATACCAGCATCGCCGATTCCAAGAAGTTCATCAGCTCCTTTGACAGGAT
CATGTACGTGCCCGAGGAGGATCTGTTCGAGTTTGCCCTGGACTATAAGAACTTCTCT
CGCACAGACGCCGATTACATCAAGAAGTGGAAGCTGTACTCCTACGGCAACCGGATC
AGAATCTTCCGGAATCCTAAGAAGAACAACGTGTTCGACTGGGAGGAGGTGTGCCTG
ACCAGCGCCTATAAGGAGCTGTTCAACAAGTACGGCATCAATTATCAGCAGGGCGATA
TCAGAGCCCTGCTGTGCGAGCAGTCCGACAAGGCCTTCTACTCTAGCTTTATGGCCCT
GATGAGCCTGATGCTGCAGATGCGGAACAGCATCACAGGCCGCACCGACGTGGATTT
TCTGATCAGCCCTGTGAAGAACTCCGACGGCATCTTCTACGATAGCCGGAACTATGAG
GCCCAGGAGAATGCCATCCTGCCAAAGAACGCCGACGCCAATGGCGCCTATAACATC
GCCAGAAAGGTGCTGTGGGCCATCGGCCAGTTCAAGAAGGCCGAGGACGAGAAGCT
GGATAAGGTGAAGATCGCCATCTCTAACAAGGAGTGGCTGGAGTACGCCCAGACCAG
CGTGAAGCACAAGCGTCCTGCTGCTACTAAGAAAGCTGGTCAAGCTAAGAAAAAGA
AATAA(SEQ ID NO:2).

EXAMPLES

The following examples are set forth below to illustrate the systems, methods, compositions and results according to the disclosed subject matter. These examples are not intended to be inclusive of all aspects of the subject matter disclosed herein, but rather to illustrate representative systems, methods, compositions and results. These examples are not intended to exclude equivalents and variations of the present invention which are apparent to one skilled in the art.

Example 1. Chemical modification of Cpf1 mRNA

Synthesis of chemically modified Cpf1 mRNA.

Chemically modified mRNAs encoding AsCpf1 (*Acidaminococcus sp.* BV3L6 CRISPR from Prevotella and Francisella 1) protein including but not limited to PS backbone modification, ψ, 5moU, $me^1ψ$, 5hmC, 5meU, 5meC and 5moC base modification, 2'-*O*-Me, 2'-F sugar modification, as well as their combinations (Fig. 2) are synthesized using a commercially available *in vitro* transcription kit. All mRNA were verified by polyacrylamide gel electrophoresis (PAGE) (Figure 4). These Cpf1 mRNAs contained a complete replacement of the wild-type uridine nucleotides with either ψ, 5moU, or $me^1ψ$ (Fig. 3).

Co-delivery of AsCpf1 mRNA & guide RNA (gRNA) and extraction of genomic DNA

293T cells were seeded on a 24-well plate at a density of 100,000 cells per well. After overnight culture, cells were treated with chemically modified AsCpf1 mRNA expressing AsCpf1

protein (500 or 1000 ng) and gRNA (15 or 30 pmol, PAGE-grade) using LipofectamineMAX, Lipofectamine 3000 or mRNA-in reagent. 48 hr after treatment, genomic DNA (gDNA) from 293T cells was extracted using the DNeasy Blood & Tissue Kit (QIAGEN) following the manufacturer's instructions, and quantified by Nanodrop 2000. Unmodified AsCpf1 mRNA served as a control.

T7E1 Assay

On-target or off-target sites were amplified using Q5 High-Fidelity DNA Polymerase (New England Biolabs) and specific primers (Integrated DNA Technologies, Supplementary). The PCR products (10 uL) were then hybridized in NEBuffer 2 (New England Biolabs) in a T100 thermal cycler (Bio-Rad). Subsequently, the annealed PCR products were subjected to T7 Endonuclease I (New England Biolabs) digestion, and analysis on a 2% agarose gel to determine the efficiency of genome editing (indel %) with the following formula: $100 \times (1 - (1 - \text{fraction cleaved})^{1/2})$.

Cpf1 protein

In one embodiment, the Cpf1 protein is encoded by SEQ ID NO:1. This sequence can be codon optimized, can differ due to the degeneracy of the genetic code, can be similar, or share substantial identity, to SEQ ID NO:1, but still retain nuclease activity.

In one embodiment, the Cpf1 protein is encoded by SEQ ID NO:2. This sequence can be codon optimized, can differ due to the degeneracy of the genetic code, can be similar, or share substantial identity, to SEQ ID NO:2, but still retain nuclease activity.

In one embodiment, all of the uridines (corresponding to the thymidines (T) in the DNA sequence) in the Cpf1 mRNA have been replaced by pseudouridine (See Fig. 3). In one embodiment, all of the uridines (corresponding to the thymidines (T) in the DNA sequence) in the Cpf1 mRNA have been replaced by N_1-methylpseudouridine ($me^1\psi$) (See Fig. 3). In one embodiment, all of the uridines (corresponding to the thymidines (T) in the DNA sequence) in the Cpf1 mRNA have been replaced by 5-methoxyuridine (5moU) (See Fig. 3).

Synthesis of DNMT1 guide RNA (gRNA).

Guide RNAs (gRNAs) targeting human *DNMT1* locus were synthesized using automated solid-phase DNA/RNA synthesizer. The sequence of unmodified gRNA for CRISPR-AsCpf1 is 5'-UAAUUUCUACUCUUGUAGAUCUGAUGGUCCAUGUCUGUUACUC-3' (SEQ ID NO:3) (gWT).

Example 2. Effects of Chemically Modified Messenger RNA on Protein Expression.

Messenger RNAs (mRNAs) encoding functional proteins have demonstrated their therapeutic potential in fundamental and clinical studies (Sahin, U., et. al. (2014) *Nat. Rev. Drug Discov. 13*, 759-780; Islam, M. A., et. al. (2015) *Biomater. Sci. 3*, 1519-1533; McIvor, R. S. (2011) *Mol. Ther. 19*, 822-823; Pascolo, S. (2008) *Handb. Exp. Pharmacol.* 221-235; Tavernier, G., et. al. (2011) *J. Control Release 150*, 238-247)). For example, immunotherapy with mRNA-electroporated dendritic cell provided therapeutic benefit in several cancer clinical trials (Pascolo, S. (2008) *Handb. Exp. Pharmacol.* 221-235; Anguille, S., et. al. (2014) *Lancet Oncol. 15*, e257-267)). mRNAs were also utilized to produce chimeric antigen receptors in T cells for adoptive T-cell therapy, (Zhao, Y., et. al. (2010) *Cancer Res. 70*, 9053-9061)) to express functional proteins for protein replacement therapy, (Li, B., et. al. (2015) *Nano Lett. 15,* 8099-8107; Kauffman, et. al. (2015) *Nano Lett. 15*, 7300-7306; Zangi, L., et. al. (2013) *Nat. Biotechnol. 31*, 898-907; Kormann, M. S., et. al. (2011) *Nat. Biotechnol. 29*, 154-157; Thess, A., et. al. (2015) *Mol. Ther. 23*, 1456-1464)) and most recently, to make nucleases for gene engineering (Hendel, A., et. al. (2015) *Nat. Biotechnol. 33*, 985-989; Wang, J., et. al. (2015) *Nat. Biotechnol. 33,* 1256-1263)). Although there have been significant advances in mRNA-based therapeutics in the past decade, instability and immunogenicity of mRNA hinders its therapeutic application in humans (Sahin, U., et. al. (2014) *Nat. Rev. Drug Discov. 13*, 759-780; Pascolo, S. (2008) *Handb. Exp. Pharmacol.* 221-235; Tavernier, G., et. al. (2011) *J. Control Release 150*, 238-247; Weissman, D., and Kariko, K. (2015) *Mol. Ther. 23*, 1416-1417; Kariko, K., et. al. (2004) *J. Biol. Chem. 279*, 12542-12550)).

In order to address these issues, numerous strategies for mRNA modification can be investigated to improve translation efficiency and reduce immunogenicity, including modifications at the 5' cap, 5' and 3'-untranslated regions, the coding region, and the poly(A) tail (Sahin, U., et. al. (2014) *Nat. Rev. Drug Discov. 13*, 759-780). Incorporation of chemically modified nucleotides into mRNAs (Figure 2) has also been reported in the literature (Sahin, U., et. al. (2014) *Nat. Rev. Drug Discov. 13*, 759-780). For instance, Karikó *et al.* showed that pseudouridine (ψ) modified mRNA increased expression of encoded erythropoietin (Kariko, K., et. al. (2012) *Mol. Ther. 20*, 948-953; Kariko, K., et. al. (2008) *Mol. Ther. 16*, 1833-1840). Kormann *et al.* reported that combination of 2-thiouridine (s2U) and 5-methylcytidine (5meC) in modified mRNAs extended expression of encoded protein to four weeks (Kormann, M. S., et. al. (2011) *Nat. Biotechnol. 29*, 154-157). Recently, Zangi *et al.* achieved induction of vascular regeneration using modified (5meC and ψ) mRNA encoding human vascular endothelial growth factor-A (Zangi, L., et. al. (2013) *Nat. Biotechnol. 31*, 898-907). Uchida *et al.* stated that combination of 5meC and ψ in mRNAs augmented protein expression (Uchida, S., et. al. (2015) *Pharmaceutics 7*, 137-151). Most

recently, Andries *et al.* reported that N_1-methylpseudouridine ($me^1\psi$) modified mRNA enhanced luciferase expression and reduced immunogenicity (Andries, O., et. al. (2015) *J. Control Release 217*, 337-344). These studies demonstrate the importance of chemically modified nucleotides on mRNA structure and function. However, knowledge of the structure-activity relationship on chemically modified mRNAs remains limited. Disclosed herein is the synthesis and evaluation of a library of chemically modified mRNAs. A correlation analysis for protein expression of mRNAs at various conditions including translation temperature, cell types, and coding sequences was performed (Figure 2). From these experiments, $me^1\psi$, 5-methoxyuridine (5moU) and ψ were shown to have the largest increases in improving protein expression.

In order to investigate the effects of chemical modifications on mRNAs, a library of 24 firefly Luciferase mRNAs (FLuc mRNAs) using chemically modified nucleotides was synthesized (See Figure 3). Single modifications and combinations of modifications were incorporated. Next, FLuc expression of mRNAs was evaluated in a rabbit reticulocyte lysate system at 30 °C according to the manufacturer's protocol. As shown in Figure 5a, eight of twenty four FLuc mRNAs showed higher luciferase intensity compared to unmodified FLuc mRNA (three single modifications: ψ, 5moU, $me^1\psi$; and five combination modifications: 5hmC/5meU, 5meC/ψ, 5moC/5meU, 5meC/5moU, and 5meC/$me^1\psi$). Among them, ψ-modified FLuc mRNA displayed the highest increase of translation efficiency.

Next, the effects of temperature on mRNA translation was analyzed by conducting the experiment at 37 °C. Analysis of the results indicated significant correlation for translation at 30 and 37 °C ($p<0.0001$; $R^2 = 0.69$; Pearson correlation coefficient $r = 0.8316$, Figure 5b), but rank order of the top modified mRNAs showed dramatic changes. For instance, FLuc expression of 5hmC/5meU-modified mRNA was over 2-fold higher than that of ψ-modified mRNA at 37 °C compared to that at 30 °C (Fig. 5). These results suggested that temperature did not affect the overall trend for a set of modified mRNAs, while it did have an important impact on individual mRNAs. Consequently, the top six modified mRNAs (ψ, 5moU, $me^1\psi$, 5hmC/5meU, 5meC/ψ and 5moC/5meU) in both experiments were selected for further studies.

In order to study protein expression in cells, FLuc mRNAs were transfected in Hep 3B cells (a hepatocellular carcinoma cell line). As shown in Figure 6a, ψ and $me^1\psi$-modified FLuc mRNAs were favorable modifications in Hep 3B cells compared to unmodified and other modified FLuc mRNAs. To explore mRNA translation in a different cell line, similar experiments were conducted in THP-1 cells (an acute monocytic leukemia cell line). Interestingly, five types of modified FLuc mRNAs (ψ, $me^1\psi$, 5meC/ψ, 5hmC/5meU, and 5moC/5meU-FLuc mRNAs) showed superior activity to unmodified FLuc mRNA. In particular, 5hmC/5meU-FLuc mRNA was

over four-fold better than unmodified FLuc mRNA in THP-1 cells, while it was less efficient than unmodified FLuc mRNA in Hep 3B cells (Fig. 6a). These results indicate that mRNAs translation was dependent on cell types to a certain extent. In this study, ψ-modified FLuc mRNA was the most potent in both Hep 3B and THP-1 cells (over 10 and 40-fold higher than unmodified FLuc mRNA, respectively). In order to explore the correlation between the cell-free rabbit reticulocyte lysate system and mRNA translation in cells, luciferase intensity was quantified for the whole library of modified FLuc mRNAs in Hep 3B cells (Fig. 8). A significant correlation was observed for luciferase intensity in the rabbit reticulocyte lysate system at 30 °C and in Hep 3B cells. However, no significant correlation was found for luciferase intensity in the rabbit reticulocyte lysate system at 37 °C and in Hep 3B cells (Fig. 6b and 6c).

In order to examine the effects of chemical modification on mRNAs with a different coding sequence, a library of 24 mRNAs encoding enhanced green fluorescent protein (eGFP mRNAs) with the same chemical modifications as FLuc mRNAs was prepared by *in vitro* transcription as mentioned above. Next, Hep 3B and THP-1 cells were transfected with modified eGFP mRNAs using the unmodified eGFP mRNA as a control. As shown in Figure 6d, ψ, $me^1ψ$ and 5moU-modified eGFP mRNAs were the preferred modifications in Hep 3B cells. Consistent with previous observation, 5hmC/5meU-modified eGFP mRNA was also more favorable in THP-1 cells compared to that in Hep 3B cells. The results further indicated that effects of chemically modified mRNAs may depend on cell types. In addition, 5moU-modified eGFP mRNA showed much higher translation when encoding eGFP in comparison to encoding FLuc. More importantly, lead eGFP mRNAs were identified consisting of full substitution of $me^1ψ$-, 5moU-, and ψ-modified nucleotides, protein expression of which ranked in the top three in cells lines tested. In order to further explore the effects in primary cells, similar experiments were conducted in primary rat hepatocytes. Consistently, $me^1ψ$-, 5moU-, and ψ-modified eGFP mRNAs were more potent than unmodified eGFP mRNA. These results indicated that coding sequences of mRNA are capable of affecting the protein expression of chemically modified mRNAs.

To study the stability of chemically modified mRNAs, Hep 3B cells were transfected with unmodified, $me^1ψ$-, 5moU-, or ψ-modified eGFP mRNAs. After 1h treatment (t = 0), total RNA was collected at different time intervals (t = 0, 1.5, 3, and 4.5 h) and reversely transcribed into complementary DNA (cDNA). The amount of each eGFP mRNA was calculated by quantitative PCR (qPCR) analysis in normalization of endogenous 18S rRNA. Relative mRNA levels were in percentage of their corresponding mRNAs at t = 0. As shown in Figure 7, over 50% unmodified, $me^1ψ$-, or ψ-modified eGFP mRNAs were degraded, while approximately 75% 5moU-modified eGFP mRNA remained at t = 4.5 h. These results suggested 5moU modifications significantly

increased stability of eGFP mRNA.

In summary, two sets of chemically modified mRNAs (FLuc and eGFP mRNAs) were designed and synthesized, which incorporated full substitution of one or two types of chemically modified nucleotides. The effects of the chemically modified mRNAs on protein expression were investigated by varying conditions including temperature in the rabbit reticulocyte lysate system, cell types, and coding sequences. The results indicated that temperature doesn't affect the trend for a large set of mRNAs screening in the rabbit reticulocyte lysate. Yet, when conducting high throughput screening of chemically modified mRNAs, it is necessary to strictly control assay temperature in the rabbit reticulocyte lysate system in order to predict the top modified mRNAs since significant correlation of luciferase intensity in the rabbit reticulocyte lysate system at 30 °C and in Hep 3B cells was observed. Additionally, cell types and coding sequences play important roles in protein expression of different chemically modified mRNAs. Moreover, 5moU modifications significantly increased the stability of eGFP mRNA compared to unmodified and other modified mRNAs. Therefore, the chemical modification of mRNA may require specific design and screening for particular therapeutic applications or biological studies. Because numerous enzymes play essential roles in mRNA translation and protein expression, further mechanistic studies may elucidate mRNA-enzyme interactions involved in multiple signaling pathways. For example, Karikó *et al* reported that ψ-modified mRNA enhances translation by reducing the activation of RNA-dependent protein kinase (PKR) (Anderson, B. R., et. al. (2010) *Nucleic Acids Res. 38*, 5884-5892). Lastly, N_1-methylpseudouridine ($me^1\psi$), 5-methoxyuridine (5moU), and pseudouridine (ψ) were identified as exhibiting the largest increases on protein expression.

Methods and Protocols

Materials.

Rabbit reticulocyte lysate kit and Bright-Glo reagent were purchased from Promega Corporation. Eagle's Minimum Essential Medium (EMEM) was purchased from Corning Incorporated. RPMI-1640 Medium and Hep 3B cells were purchased from American Type Culture Collection. RNeasy Mini Kit was from QIAGEN. Dulbecco's Modified Eagle Medium (DMEM), Opti-MEM medium, fetal bovine serum, Lipofectamine 2000, High Capacity cDNA Reverse Transcription Kits and SYBR Green PCR Master Mix were purchased from Life technologies. Antarctic phosphatase was purchased from New England Biolabs.

Synthesis of chemically modified mRNA.

Chemically modified mRNAs encoding firefly Luciferase and green fluorescent protein respectively were synthesized using an in vitro transcription from the corresponding plasmid DNA template by TriLink BioTechnologies. Chemically modified nucleotides were completely substituted for their natural counterparts while synthesizing the mRNAs. The transcripts were then further modified for mammalian systems with 5' cap and 3' poly(A) tail structures. mRNAs were purified using commercially available silica-based spin columns. mRNAs were dephosphorylated with Antarctic phosphatase and repurified by silica-based spin column.

mRNA translation in rabbit reticulocyte lysate system.

Two microliters of FLuc mRNA (1 μg/μL) was added to 48 μL of rabbit reticulocyte lysate reaction assembly and the mixture was incubated at either 30 °C or 37 °C for 90 min. After incubation, 50 μL of the substrate consisting of 25 μL of Bright-Glo and 25 μL of PBS was added to 2.5 μL of the reaction mixture. Luminescence intensity was measured immediately after 5 minutes dark incubation.

mRNA translation in cells.

Hep 3B cells were cultured in Eagle's Minimum Essential Medium (EMEM), THP-1 cells in RPMI-1640 Medium and primary rat hepatocytes in Dulbecco's Modified Eagle Medium (DMEM). All medium supplemented with 10% fetal bovine serum. For FLuc mRNA translation, Hep 3B and THP-1 cells were seeded in 96-well white plates at a density of 10^4 cells/well. Following overnight culture, 50 ng of modified FLuc mRNA was complexed with Lipofectamine 2000 in Opti-MEM medium, and the mixture was added to each well after 5 min incubation. Twenty four hours post-transfection, 100 μL of Bright-Glo reagent was added to each well, and luminescence intensity was measured immediately after 5 minutes dark incubation. For eGFP mRNA translation, Hep 3B, THP-1 cells and primary rat hepatocytes were seeded in 6-well plates at a density of 2.0×10^5/well. After overnight culture, 2 μg of modified FLuc mRNA was complexed with Lipofectamine 2000 in Opti-MEM medium, and the mixture was added to each well after 5 min incubation. After 24 hours treatment, cells were harvested and the green fluorescence intensity was determined on a BD LSR II flow cytometer (BD Biosciences, San Jose, CA).

RNA isolation, cDNA synthesis and quantitative PCR (qPCR).

Hep 3B cells were seeded in 6-well plates at a density of 3×10^5 cells/well and cultured

overnight. 1 μg of eGFP mRNA complexed with Lipofectamine 2000 in Opti-MEM medium was tansfected into cells. After one hour incubation, cells were rinsed twice to remove the residual RNA and incubated with fresh medium. At desired time points (t = 0, 1.5, 3 and 4.5 h), total RNAs were extracted with RNeasy Mini Kit, reverse transcribed into complementary DNA (cDNA) by High Capacity cDNA Reverse Transcription Kits, and quantified with gene-specific primers (Table 1) and SYBR Green PCR Master Mix in a StepOne Plus Real Time PCR system (Applied Biosystems, Foster City, CA) according the manufacturer's instruction. The expression of the target gene versus that of the reference gene (18S rRNA) was calculated using formula $2^{-\Delta\Delta Ct}$, where C_t was the cycle threshold value. The relative eGFP mRNA levels at different time points were normalized to the initial (t = 0) eGFP mRNAs.

Table 1. List of gene-specific oligonucleotide primer sets used for SYBR Green real-time qPCR.

Name	Forward primer sequence	Reverse primer sequence
18S rRNA	5'-GCT CTA GAA TTA CCA CAG TTA TC-3' (SEQ ID NO:4)	5'-AAA TCA GTT ATG GTT CCT TTG GT-3' (SEQ ID NO:5)
eGFP	5'-ACG TAA ACG GCC ACA AGT TC-3' (SEQ ID NO:6)	5'-AAG TCG TGC TGC TTC ATG TG-3' (SEQ ID NO:7)

References Cited in this Example

(1) Sahin, U., Kariko, K., and Tureci, O. (2014) mRNA-based therapeutics--developing a new class of drugs. *Nat. Rev. Drug Discov. 13*, 759-780.

(2) Islam, M. A., Reesor, E. K., Xu, Y., Zope, H. R., Zetter, B. R., and Shi, J. (2015) Biomaterials for mRNA delivery. *Biomater. Sci. 3*, 1519-1533.

(3) McIvor, R. S. (2011) Therapeutic delivery of mRNA: the medium is the message. *Mol. Ther. 19*, 822-823.

(4) Pascolo, S. (2008) Vaccination with messenger RNA (mRNA). *Handb. Exp. Pharmacol.* 221-235.

(5) Tavernier, G., Andries, O., Demeester, J., Sanders, N. N., De Smedt, S. C., and Rejman, J. (2011) mRNA as gene therapeutic: how to control protein expression. *J. Control Release 150*, 238-247.

(6) Anguille, S., Smits, E. L., Lion, E., van Tendeloo, V. F., and Berneman, Z. N. (2014) Clinical use of dendritic cells for cancer therapy. *Lancet Oncol. 15*, e257-267.

(7) Zhao, Y., Moon, E., Carpenito, C., Paulos, C. M., Liu, X., Brennan, A. L., Chew, A., Carroll, R. G., Scholler, J., Levine, B. L., *et al.* (2010) Multiple injections of electroporated autologous T cells expressing a chimeric antigen receptor mediate regression of human

disseminated tumor. *Cancer Res. 70*, 9053-9061.

(8) Li, B., Luo, X., Deng, B., Wang, J., McComb, D. W., Shi, Y., Gaensler, K. M., Tan, X., Dunn, A. L., Kerlin, B. A., *et al.* (2015) An orthogonal array optimization of lipid-like nanoparticles for mRNA delivery in vivo. *Nano Lett. 15,* 8099-8107.

(9) Kauffman, K. J., Dorkin, J. R., Yang, J. H., Heartlein, M. W., DeRosa, F., Mir, F. F., Fenton, O. S., and Anderson, D. G. (2015) Optimization of lipid nanoparticle formulations for mRNA delivery in vivo with fractional factorial and definitive screening designs. *Nano Lett. 15*, 7300-7306.

(10) Zangi, L., Lui, K. O., von Gise, A., Ma, Q., Ebina, W., Ptaszek, L. M., Spater, D., Xu, H., Tabebordbar, M., Gorbatov, R., *et al.* (2013) Modified mRNA directs the fate of heart progenitor cells and induces vascular regeneration after myocardial infarction. *Nat. Biotechnol. 31*, 898-907.

(11) Kormann, M. S., Hasenpusch, G., Aneja, M. K., Nica, G., Flemmer, A. W., Herber-Jonat, S., Huppmann, M., Mays, L. E., Illenyi, M., Schams, A., *et al.* (2011) Expression of therapeutic proteins after delivery of chemically modified mRNA in mice. *Nat. Biotechnol. 29*, 154-157.

(12) Thess, A., Grund, S., Mui, B. L., Hope, M. J., Baumhof, P., Fotin-Mleczek, M., and Schlake, T. (2015) Sequence-engineered mRNA without chemical nucleoside modifications enables an effective protein therapy in large animals. *Mol. Ther. 23*, 1456-1464.

(13) Hendel, A., Bak, R. O., Clark, J. T., Kennedy, A. B., Ryan, D. E., Roy, S., Steinfeld, I., Lunstad, B. D., Kaiser, R. J., Wilkens, A. B., *et al.* (2015) Chemically modified guide RNAs enhance CRISPR-Cas genome editing in human primary cells. *Nat. Biotechnol. 33*, 985-989.

(14) Wang, J., Exline, C. M., DeClercq, J. J., Llewellyn, G. N., Hayward, S. B., Li, P. W., Shivak, D. A., Surosky, R. T., Gregory, P. D., Holmes, M. C., *et al.* (2015) Homology-driven genome editing in hematopoietic stem and progenitor cells using ZFN mRNA and AAV6 donors. *Nat. Biotechnol. 33,* 1256-1263.

(15) Weissman, D., and Kariko, K. (2015) mRNA: fulfilling the promise of gene therapy. *Mol. Ther. 23*, 1416-1417.

(16) Kariko, K., Ni, H. P., Capodici, J., Lamphier, M., and Weissman, D. (2004) mRNA is an endogenous ligand for Toll-like receptor 3. *J. Biol. Chem. 279*, 12542-12550.

(17) Kariko, K., Muramatsu, H., Keller, J. M., and Weissman, D. (2012) Increased erythropoiesis in mice injected with submicrogram quantities of pseudouridine-containing mRNA encoding erythropoietin. *Mol. Ther. 20*, 948-953.

(18) Kariko, K., Muramatsu, H., Welsh, F. A., Ludwig, J., Kato, H., Akira, S., and

Weissman, D. (2008) Incorporation of pseudouridine into mRNA yields superior nonimmunogenic vector with increased translational capacity and biological stability. *Mol. Ther. 16*, 1833-1840.

(19) Uchida, S., Kataoka, K., and Itaka, K. (2015) Screening of mRNA chemical modification to maximize protein expression with reduced immunogenicity. *Pharmaceutics 7*, 137-151.

(20) Andries, O., Mc Cafferty, S., De Smedt, S. C., Weiss, R., Sanders, N. N., and Kitada, T. (2015) N(1)-methylpseudouridine-incorporated mRNA outperforms pseudouridine-incorporated mRNA by providing enhanced protein expression and reduced immunogenicity in mammalian cell lines and mice. *J. Control Release 217*, 337-344.

(21) Anderson, B. R., Muramatsu, H., Nallagatla, S. R., Bevilacqua, P. C., Sansing, L. H., Weissman, D., and Kariko, K. (2010) Incorporation of pseudouridine into mRNA enhances translation by diminishing PKR activation. *Nucleic Acids Res. 38*, 5884-5892.

Example 3. Genome editing efficiency using a combination of chemically modified CRISPR-Cpf1 crRNAs and mRNAs

CRISPR (clustered, regularly interspaced, short palindromic repeats) is part of adaptive immunity in bacteria and archaea (Makarova, K.S. et al. *Nat Rev Microbiol* 9, 467-477 (2011); Bosley, K.S. et al. *Nat Biotechnol* 33, 478-486 (2015)). CRISPR-associated protein 9 (Cas9) induces double stranded DNA breaks through complexation with two RNA molecules: CRISPR RNA (crRNA) and trans-activating crRNA (Jinek, M. et al. *Science* 337, 816-821 (2012); Cong, L. et al. *Science* 339, 819-823 (2013); Mali, P. et al. *Science* 339, 823-826 (2013)). Recently, CRISPR-Cpf1 from *Acidaminococcus sp.* (AsCpf1) and *Lachnospiraceae* (LbCpf1) (Zetsche, B. et al. *Cell* 163, 759-771 (2015); Dong, D. et al. *Nature* 532, 522-526 (2016); Fonfara, I., et al. *Nature* 532, 517-521 (2016); Yamano, T. et al. *Cell* (2016)), a second class 2 (type V) CRISPR system displayed comparable genome editing capability to Cas9. Genome-wide analysis suggested that Cpf1 may cause fewer off-target cleavages in comparison to Cas9 (Kleinstiver, B.P. et al. *Nat Biotechnol* 34, 869-874 (2016)), (Kim, D. et al. *Nat Biotechnol* 34, 863-868 (2016)). To exert sequence-specific endonuclease activity, Cpf1 is functional through a single crRNA without an additional tracrRNA (Zetsche, B. et al. *Cell* 163, 759-771 (2015); Fonfara, I., et al. *Nature* 532, 517-521 (2016)). As shown in Figure 1, this crRNA (43 nucleotides) consists of a 5'-handle (20 nucleotides) and a guide segment (23 nucleotides) (Zetsche, B. et al. *Cell* 163, 759-771 (2015)). Cpf1 protein interacts with the pseudoknot structure formed by the 5'-handle of crRNA (Yamano, T. et al. *Cell* (2016)). A guide segment possesses complementary binding with the target DNA sequences. This complexes recognize a T-rich protospacer-adjacent motif (PAM) and leads to a

staggered DNA double stranded break (Yamano, T. et al. *Cell* (2016)) (The dotted line denotes the cleavage sites, Fig. 11a).

To increase genome editing efficiency and reduce off-target effects of CRISPR systems, previous studies explored a wide variety of approaches (Slaymaker, I.M. et al. *Science* 351, 84-88 (2016); Kleinstiver, B.P. et al. *Nature* 529, 490-495 (2016)). Among them, chemical modifications of CRISPR-Cas9 demonstrated enhanced activity in a number of human cells (Hendel, A. et al. *Nat Biotechnol* 33, 985-989 (2015); Rahdar, M. et al. *Proc Natl Acad Sci U S A* 112, E7110-7117 (2015)). For example, guide RNAs with three chemically modified nucleotides at both 5'- and 3'-end strongly improved Cas9-mediated genome editing in human primary T cells (Hendel, A. et al. *Nat Biotechnol* 33, 985-989 (2015)). Moreover, incorporation of chemically modified nucleotides was able to remain indel percentage using Cas9 and truncated guide RNAs (Rahdar, M. et al. *Proc Natl Acad Sci U S A* 112, E7110-7117 (2015)). In addition, the structures of guide RNA for Cas9 play important roles in gene cutting (Chen, B. et al. *Cell* 155, 1479-1491 (2013); Dang, Y. et al. *Genome Biol* 16, 280 (2015)). Yet, no literature is known about the effects of chemically modified crRNAs and Cpf1 mRNAs on their genome editing efficiency and off-target effects. In this example, 26 chemically modified crRNAs were investigated, which established comprehensive structure-activity (gene editing efficiency) relationships. A ψ-modification was identified in this example as a favorable chemical alteration for Cpf1 mRNA. Importantly, combination of crRNA and ψ-mRNA significantly increased gene editing efficiency over 300% compared to the positive control group. This combination induced more dramatic improvement of gene cutting efficiency when utilizing LbCpf1. More interestingly, AsCpf1 in complexation with LbCpf1 crRNA was able to effectively achieve genome editing while LbCpf1 in complexation with AsCpf1 crRNA completely lost its function. Overall, our findings offer a promising strategy for broad genome editing applications.

To study the effects of chemically modified crRNAs on gene editing efficiency, three types of chemically modified nucleotides were utilized in this example: phosphorothioate (PS), 2'-*O*-Me-, and 2'-F-modifications (Watts, J.K., et al. *Drug Discov Today* 13, 842-855 (2008)) (Fig. 14). Based on previous findings for CRISPR-Cas9 in the literature (Hendel, A. et al. *Nat Biotechnol* 33, 985-989 (2015); Rahdar, M. et al. *Proc Natl Acad Sci U S A* 112, E7110-7117 (2015)), a full-backbone PS-modified cr42PS and a cr5'&3'3F2PS (three 2'-F modifications with two PS linkages at both 5' and 3' end) was synthesized. These crRNAs targeting the *DNMT1* locus were purified on denaturing polyacrylamide gels and their structures were validated by mass spectrometry analysis (Sequence information and mass spectrometry data are included in Tables 2 and 3). HEK293T cells were treated with synthesized crRNAs and plasmid encoding Cpf1

complexed with Lipofectamine 3000 reagent. Gene editing efficiency was quantified using a T7E1 assay (Kim, D. et al. *Nat Biotechnol* 34, 863-868 (2016)). Relative gene cutting efficiency in figure 9 was normalized to the treatment of unmodified crRNA (crWT) and plasmid encoding AsCpf1. As shown in Figure9c, both crRNAs displayed dramatic reduction of gene editing capability, which suggests that chemical modifications of Cpf1 crRNA are not in the same pattern as Cas9 guide RNAs. Additional experiments were conducted using chemical modifications at the 5'-handle, seed region, and 3'-termini. Five, ten, or twenty nucleotides with 2'-*O*-Me-, and 2'-F-modifications, respectively, were introduced at the 5'-handle of the crRNAs, which led to complete loss of activity. These data indicate that 5'-handle is not favorable for chemical modifications. Then, four modifications were made at the seed region, crS3M3PS, crS3F, crS2F, and crS1F (Fig. 9a). Although all four crRNAs diminished indel percentage, the results inferred interesting structure-activity relationship. The order of efficiency is crS3F < crS2F < crS1F, which indicated that the seed region may tolerate slight modifications. Furthermore, at the 3'-termini (Fig. 9a), five or ten nucleotides with 2'-*O*-Me- and 2'-F-modifications, respectively, were incorporated Interestingly, while 2'-*O*-Me-modifications decreased the activity, five or ten 2'-F-modifications improved the efficiency 119% and 111%, respectively compared to the crWT. In addition to five 2'-F-modifications at the 3'-termini, incorporation of PS modifications hampered the efficiency (Fig. 9c). We also noticed that substitution of some or full RNA bases of crWT with DNA bases (Fig. 15) severely impaired cutting activities, and full A, U or C bases substitution with corresponding m1A, pseudoU (or 5moU) or 5meC modified bases did not enhance their activities (Fig. 18). Lastly, the 5'-handle was modified by split of the crRNAs, deletion or insertion of nucleotides. Current crRNAs with split sequences and deletion of nucleotides exhibited no gene editing function (Fig. 9d). To some extent, insertion of four or six nucleotides retained the activity (Fig. 11e). Moreover, the activity is dependent on inserted base pairs (Fig. 9d, crIns4 vs crIns4'). Collectively, these results revealed the following SAR and design criteria for crRNAs: (I) PS linkage replacement generally hampered gene editing efficiency; (II) 5'-handle is not a preferred region for chemical modifications; current results indicate 5'-handle does not tolerate split of the crRNAs, deletion or insertion of nucleotides; (III) seed region is sensitive to chemical modifications and can only accommodate slight modifications; (IV) 3'-termini is favorable for certain chemical modifications, such as 2'-F modifications; (V) A combination of chemical modifications at both 5'- and 3'-ends of crRNA reduced the activity to some extent.

Meanwhile, the effects of chemically modified AsCpf1 mRNAs on their gene editing efficiency were investigated. Pseudouridine (ψ), N^1-methylpseudouridine ($me^1\psi$), and 5-methoxyuridine (5moU) modified AsCpf1 mRNA was also designed, which were produced via an

in vitro transcription. HEK293T cells were treated with crWT and modified Cpf1 mRNA. As shown in Figure 9e, ψ- and $me^1ψ$- modified AsCpf1 mRNA showed higher activity (165% and 132%, respectively) than plasmid encoding AsCpf1, while 5moU modification decreased the activity. In addition, a recent literature reported that plasmid encoding AsCpf1 with a S1228A mutation improved gene editing efficiency (Yamano, T. et al. *Cell* (2016)). Therefore, AsCpf1 mRNA (S1228A mRNA) with a S1228A mutation and ψ-modification was also constructed. S1228A mRNA displayed comparable activity to original ψ-modified mRNA (Fig. 9e).

Terminology of the Cpf1-family orthologs: Acidaminococcus sp. BV3L6 Cpf1 (AsCpf1); Francisella tularensis subsp. Novicida U112 Cpf1 (FnCpf1); Lachnospiraceae bacterium MC2017 Cpf1 (Lb3Cpf1); Butyrivibrio proteoclasticus Cpf1 (BpCpf1); Parcubacteria bacterium GWC2011_GWC2_44_17 Cpf1 (PbCpf1); Peregrinibacteria bacterium GW2011_GWA_33_10 Cpf1 (PeCpf1); Leptospira inadai Cpf1 (LiCpf1); Smithella sp. SC_K08D17 Cpf1 (SsCpf1); Lachnospiraceae bacterium MA2020 Cpf1 (Lb2Cpf1); Porphyromonas crevioricanis Cpf1 (PcCpf1); Porphyromonas macacae Cpf1 (PmCpf1); Candidatus Methanoplasma termitum Cpf1 (CMtCpf1); Eubacterium eligens Cpf1 (EeCpf1); Moraxella bovoculi 237 Cpf1 (MbCpf1); Prevotella disiens Cpf1 (PdCpf1); Lachnospiraceae bacterium ND2006 Cpf1 (LbCpf1).

Inspired by crRNAs from other 15 Cpf1-family, we then engineered the loop [U(-10)–C(-9)–U(-8)–U(-7)] of 5'-handle by substituting the loop of wild-type AsCpf1 crRNA (crWT here is defined as AscrRNA to distinguish crRNAs from other Cpf1-protein family) with those from crRNAs of other Cpf1 family orthologs to investigate the effects of loop on gene cutting activity (Fig. 16a; crRNAs were termed by combining initial of Cpf1 protein and crRNA; crRNAs of PbCpf1, PeCpf1 and LiCpf1 share the same loop UUUU; crRNAs of Lb2Cpf1, PcCpf1 and PmCpf1 share the same loop UAUU). Interestingly, co-delivery of ψ-modified AsCpf1 mRNA and a panel of loop engineered crRNAs revealed that crRNAs with four-membered loop including FncrRNA, Pb/Pe/LicrRNA and Lb2/Pc/PmcrRNA induced evident cleavage on the target locus. Cytosine at position -9 tolerates nucleotide changes and the order of potency is: adenine (A) > cytosine (C) > guanine (G) > uridine (U). In addition, crRNAs with three-membered (EecrRNA) or five-membered loop (MbcrRNA and LbcrRNA) reduced the activity (Fig. 16b). Meanwhile, if the uridine at position -10 was replaced by G (Lb3crRNA and BpcrRNA) or A (SscrRNA) on the five-membered loop, crRNAs completely lost function (Fig. 16b). These results suggested that U (-10) is a critical position for gene editing, consistent with the findings from the crystal structure of AsCpf1. After sequence alignment, we observed that the whole sequence of AscrRNA was fully matched with that of crRNAs from FnCpf1, Pb/LiCpf1 and Lb2/Pc/Pm Cpf1 expect PeCpf1 (According to the crystal structure of AsCpf1-crRNA-target DNA complex, uridine at position -

20 is not involved in the complexation. Hence, U(-20) was not included for alignment). In other words, AsCpf1 is able to complex with these crRNAs, attaining effective genome editing. These data, to our knowledge, first uncovered the cross complexation between AsCpf1 and crRNAs in the Cpf1 family, which expand the applicability of crRNAs, and are conducive to our understanding of the CRISPR-Cpf1 system and its potential biological and therapeutic applications. Lastly, we investigated the applicability of crRNAs for LbCpf1. We treated cells with loop engineered crRNAs as shown in Fig. 3c in the presence of ψ-modified LbCpf1 mRNA. Except its own LbcrRNA, LbCpf1 only led to reduced indel with Lb2/Pc/PmcrRNA, AscrRNA, and MbcrRNA, while no activity for other crRNAs (Fig. 16c), indicating different crRNA applicability between AsCpf1 and LbCpf1.

To study the combination effects of chemically modified crRNA and AsCpf1 mRNAs, HEK293T cells were treated with the top-performing modified crRNA and AsCpf1 mRNA (cr3'5F and ψ-modified AsCpf1 mRNA). Strikingly, this combination significantly enhanced the gene editing efficiency over 250% compared to the treatment of crWT and plasmid encoding Cpf1 (Fig.10a). We then analyzed the interaction among crRNA, AsCpf1 protein and target DNA. Crystal structure of the AsCpf1 complex indicates that 2'-OH of ribose on crRNA at the following positions plays a trivial role: -16, -15, -12, -11, -9, -8, -7, -5, -4, -3, -2, +1, +6, +8, +9, +10, +11, +12, +17, +19, +20, +21, +22 and +23. Based on this analysis, we designed additional three type of crRNAs: crI21F, crI16M5F and crI16M10PS5F (Fig. 15, Tables 2 and 3), by introducing interspersed modifications at the ribose units without interaction with AsCpf1 and target DNA and avoiding modifications at the seed region (+1 to +8). Interestingly, crI21F was comparable to cr3'5F, whereas crI16M5F and crI16M10PS5F dramatically weakened the activity of crRNA (Fig. 18). Since cr3'5F possesses less modifications than crI21F, we further investigated the applicability of cr3'5F in Hep3B (a human hepatoma cell line) and U87 cells (a human glioblastoma cell line). Consistent with the results in HEK293T cells, combination of cr3'5F and ψ-modified AsCpf1 mRNA 328% gene cutting for Hep3B ($p<0.001$, Fig. 12b) and 293% for U87 cells ($p<0.001$, Fig. 10a) compared to the treatment of crWT and plasmid encoding AsCpf1. To investigate the applicability in different cells, similar experiments were conducted in Hep3B cells, a human hepatoma cell line. Consistent with the results in HEK293T cells, combination of cr3'5F and ψ-modified AsCpf1 mRNA induced 328% gene cutting compared to the treatment of crWT and plasmid encoding Cpf1 (Fig. 10a). In addition to *DNMT1* crRNA, gene cutting efficiency was examined for two target gene, *AAVS1* and *FANCF-2* loci [11]. Regarding *AAVS1* locus (Fig. 10b), the order of potency in HEK293T cells is cr3'5F + ψ-modified AsCpf1 mRNA > crWT + ψ-modified AsCpf1 mRNA > cr3'5F + AsCpf1 plasmid > crWT + AsCpf1 plasmid. cr3'5F + ψ-

modified AsCpf1 mRNA is 2.77-fold more efficient than crWT + AsCpf1 plasmid ($p<0.001$). The enhanced efficiency was also observed in Hep3B and U87 cells (257% and 394% increase, respectively; Fig. 4b). For FANCF-2 locus, the trend is consistent with that for AAVS1 locus (Fig. 10c). These results demonstrate the broad applicability of the chemically modified crRNAs and Cpf1 mRNAs.

Along with AsCpf1, LbCpf1 is another important endonuclease in the Cpf1-family, which displayed genome editing ability in human cells[6, 7]. To investigate whether our strategy is applied to the LbCpf1, we utilized similar chemical modifications for LbCpf1 mRNA as well as its corresponding crRNA (LbCpf1 crRNA). As shown in Figure 10d, the combination of LbCpf1 crWT+LbCpf1 plasmid led to no detectable gene cutting, while the combination of LbCpf1 cr3'5F (Lbcr3'5F) + ψ-modified LbCpf1 mRNA induced remarkable gene cutting activity in all three cell lines tested. Moreover, LbCpf1 cr3'5F was more efficient than LbCpf1 crWT. These results further proved the concept of our chemical modifications to advance CRISPR-Cpf1 mediated genome editing.

Potential off-target effects are one of the major concerns for CRISPR mediated gene editing and may limit its applications (Slaymaker, I.M. et al. *Science* 351, 84-88 (2016); Kleinstiver, B.P. et al. *Nature* 529, 490-+ (2016)). To study off-target effects of chemically modified Cpf1 mRNAs and crRNAs, the top four previously defined off-target sites were selected and a T7EI assay was performed at each genomic site. No detectable indels were observed in both treatment groups (cr3'5F + ψ-modified AsCpf1 mRNA and crWT + plasmid encoding Cpf1; Fig. 14). To further characterize on-target and off-target effects, targeted deep sequencing was conducted using the samples as that in Fig. 10a. CRISPResso was utilized to analyze the deep sequencing data (Pinello, L. et al. *Nat Biotechnol* 34, 695-697 (2016)). Similar to the results from T7E1 assays, combination of cr3'5F and ψ-modified AsCpf1 mRNA induced 311% gene cutting compared to the treatment of plasmid encoding Cpf1 and crWT (Fig. 11a). In addition, no significant difference was observed for off-target gene cutting between cr3'5F + ψ-modified AsCpf1 mRNA and crWT + plasmid encoding Cpf1 (Fig. 11a). The majority of the on-target indels were occurred within the -50 bp of the predicted cleavage site (Fig. 11c). Compatible with the results in the literature (Kim, D. et al. *Nat Biotechnol* 34, 863-868 (2016)), Cpf1 mainly induced deletions at the targeted gene site (Fig. 11b and 11c).

In addition to AsCpf1, we also examined the on-target and off-target effects of LbCpf1 with three biological samples from HEK293T cells used in Fig. 10. As shown in Fig. 12a, Lbcr3'5F + ψ-modified LbCpf1 mRNA caused 46.7% indel compared to minimal effects of LbcrWT + LbCpf1 plasmid. The indel pattern for LbCpf1 was consistent with AsCpf1, while LbCpf1 led to

larger fragment deletion than AsCpf1 (Fig. 12b and 12c). Regarding off-target effects, LbCpf1 exhibited much higher mutagenesis at the position of off-target 4 (OT4) than AsCpf1 under the same condition (Fig. 12a). Similarly, Lbcr3'5F + ψ-modified LbCpf1 mRNA showed comparable off-target effects to that of LbcrWT + LbCpf1 plasmid (Fig. 12a). Taken together, combination of engineered crRNA and Cpf1 mRNA enables us to effectively enhance genome editing efficiency without increasing off-target effects.

In summary, genome editing efficiency and off-targets effects are major challenges for the broad applications of CRISPR systems. To address these issues, an array of crRNA variants including chemically modified crRNAs and pseudoknot rearranged crRNAs were designed, and the structure-activity relationships of crRNAs were elucidated for improved genome editing activity in CRISP-Cpf1 system. In sharp contrast to CRISPR-Cas9, neither phosphorothioate substitutions nor dual-modification at both sides of guide RNA enhanced gene editing efficiency. Moreover, slight modifications at the 5'-handle or seed region severely hampered cleavage activity. Importantly, 2'-F modification at the 3' terminus (cr3'5F) exhibited higher potency compared with wild-type crRNA. Regarding chemically modified Cpf1 mRNA, pseudo-U (ψ) and $me^1ψ$ are favorable modifications compared to the mRNA with unmodified nucleotides. Strikingly, combination of cr3'5F and ψ Cpf1 mRNA synergistically improved the gene cutting efficiency, which improved over 3-fold compared to unmodified crRNA (crWT) and plasmid encoding AsCpf1. This phenomena were not only confirmed in three cell lines and two target gene sites, but also observed in another Cpf1 family protein LbCpf1, demonstrating the broad applicability of this chemical strategy. In addition, it was shown that the cross complexation of Cpf1 and its guide RNA: AsCpf1 was able to effectively achieve genome editing in the presence of LbCpf1 crRNA; nevertheless, LbCpf1 in combination with AsCpf1 crRNA completely lost its function. This finding further expands the current understanding of the CRISPR-Cpf1 system. Furthermore, targeted deep sequencing data suggested that combination of cr3'5F and ψ Cpf1 mRNA did not increase the level of off-target effects.

Methods

Synthesis of crRNAs.

The sequences of the crRNA targeting the *DNMT1-3* locus:

AsCpf1 crRNA: 5'-UAAUUUCUACUCUUGUAGAUCUGAUGGUCCAUGUCUGUUACUC-3' (SEQ ID NO:3);

LbCpf1 crRNA: 5'-AAUUUCUACUAAGUGUAGAUCUGAUGGUCCAUGUCUGUUACUC -

3' (SEQ ID NO:8).

The sequence of unmodified crRNA targeting *AAVS1* locus:

AsCpf1 crRNA: 5'-UAAUUUCUACUCUUGUAGAUCUUACGAUGGAGCCAGAGAGGAU-3' (SEQ ID NO:9).

The sequence of unmodified crRNA targeting *FANCF-2* locus:

AsCpf1 crRNA: 5'-UAAUUUCUACUCUUGUAGAUGUCGGCAUGGCCCCAUUCGCACG-3' (SEQ ID NO:10).

Unmodified crRNA (crWT) and all other crRNA variants including chemically modified crRNAs, stem loop deleted and inserted crRNAs were synthesized using an automated solid-phase DNA/RNA synthesizer. Chemically modified crRNAs consisted of partial or total chemically modified nucleotides including phosphate linkage (PS), 2'-*O*-Me, 2'-F modified, unlocked, locked nucleotides as well as their combinations (Tables 2 and 3 and Fig. 9a, and Fig. 15). Stem-engineered crRNAs were designed by deleting certain number of Watson-Crick base pairs from the stem duplex or inserting additional paired or unpaired bases into the stem duplex of crWT (Fig. 1c and Supplementary Table 1). Split crRNA (crSplit) was generated by incubating equimolar of relevant RNA sequences listed in Table 2 in TE buffer at 95 °C for 30 s, followed by gradient cooling (95-25 °C ramping at 0.1 °C/s). Loop engineered crRNAs were designed by employing the loops from other Cpf1 orthologs (Fig. 16a). The Split crRNA (crSplit) was hybridized by incubating equimolar of relevant RNA sequences listed in Tables 2 and 3 in TE buffer at 95 °C for 30 s, followed by gradient cooling (95-25°C ramping at 0.1°C/s). All crRNAs were purified on denaturing polyacrylamide gels and verified by mass spectrometry (Tables 2 and 3, and Fig. 9b).

AsCpf1 plasmid and mRNAs.

Cpf1 plasmid was a gift from Dr. Feng Zhang. ARCA capped and polyadenylated AsCpf1 mRNA transcripts were purchased from TriLink BioTechnologies (San Diego, CA, USA). For modified mRNAs, uridines were fully substituted with pseudouridine (ψ), N^1-methylpseudouridine ($me^1ψ$), or 5-methoxyuridine (5moU). For S1228A & ψ modified mRNA, serine 1228 was substituted with alanine and uridines were fully substituted with pseudouridine (ψ). These mRNAs were subjected to DNase and phosphatase treatment and silica membrane-based purification for further use. All mRNAs were verified by polyacrylamide gels (Fig. 4).

Co-delivery AsCpf1 plasmid/mRNA and crRNA.

HEK-293T cells were cultured in Dulbecco's modified Eagle's medium (Life

technologies). Hep3B cells were cultured in Eagle's Minimum Essential Medium (EMEM, ATCC). All medium supplemented with 10% FBS, and both cell lines used in this study were maintained at 37 °C with 5% CO_2. After overnight incubation (approximate 60-80% confluence), cells, seeded on 24-well plates at an initial density of approximate 100,000 cells per well were treated with either 500 ng of Cpf1 expression plasmid (transfection with Lipofectamine 3000 according to the protocol provided by Life Technologies) or 500 ng of mRNA (transfection with Lipofectamine 3000). At the same time, crRNAs (38 pmol for *DNMT1-3* and *AAVS1* locus, and 114 pmol for *FANCF-2* locus) were formulated with Lipofectamine 3000 in Opti-MEM I reduced serum medium (Life Technologies) and added to each well following the manufacturer's recommended protocol.

Genomic DNA purification and PCR amplification.

Two days post-treatment, cells were washed with PBS. The genomic DNA (gDNA) was then extracted and purified with a DNeasy Blood & Tissue Kit (QIAGEN) following the manufacturer's instructions. Concentrations of gDNA were determined on a Nanodrop 2000. Genomic regions flanking the on-target as well as previously predicted off-target sites off-target sites for T7E1 assay were amplified using 100 ng of purified gDNA template, Q5 high-fidelity DNA polymerase (New England Biolabs) and specific primers (Integrated DNA Technologies, Table 3) on a T100 thermal cycler (Bio-Rad).

T7E1 Cleavage assay.

The PCR products (10 uL) generated using Q5 high-fidelity DNA polymerase were heteroduplexed in hybridization buffer (50mM NaCl, 10mM Tris-HCl, 10mM $MgCl_2$, 1mM DTT, pH 7.9) (New England Biolabs) by heating to 98 °C for 10 minutes, followed by a 2 °C/s ramp down to 85 C, 1 min at 85 °C, and a 0.1 °C/s ramp down to 25 °C on a T100 thermal cycler (Bio-Rad). Subsequently, the annealed samples were subjected to T7 Endonuclease I (New England Biolabs) digestion for 30 min, separated by a 2% agarose gel and quantitated on ChemiDoc XRS (Bio-Rad) using Quantity One software. The mutation frequency (indel %) was calculated with the following formula: 100 x (1 – (1- fraction cleaved)$^{1/2}$).

MiSeq library preparation and targeted deep sequencing.

To further characterize on-target and off-target effects, genomic segment (200~300 bp) spanning the sites of interest were first amplified using sequencing primers with overhang adapter sequences (Table 4) in the first round of PCR for 25 cycles. After purification, the second limited-

cycle PCR amplification (8 cycles) was performed using the Nextera XT Index Kit (Illumina) to attach multiplexing indices and Illumina P5/P7 sequencing adapters (Tables 5 and 6) to the first round PCR product. Next, libraries were normalized and pooled, and subjected to 2 × 300 paired-end sequencing on an Illumina MiSeq system. The raw deep sequencing data from MiSeq were analyzed with a bioinformatic tool, CRISPResso, with specific parameters.

References Cited in this Example

1. Makarova, K.S. et al. Evolution and classification of the CRISPR-Cas systems. *Nat Rev Microbiol* 9, 467-477 (2011).
2. Bosley, K.S. et al. CRISPR germline engineering--the community speaks. *Nat Biotechnol* 33, 478-486 (2015).
3. Jinek, M. et al. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* 337, 816-821 (2012).
4. Cong, L. et al. Multiplex genome engineering using CRISPR/Cas systems. *Science* 339, 819-823 (2013).
5. Mali, P. et al. RNA-guided human genome engineering via Cas9. *Science* 339, 823-826 (2013).
6. Zetsche, B. et al. Cpf1 Is a Single RNA-Guided Endonuclease of a Class 2 CRISPR-Cas System. *Cell* 163, 759-771 (2015).
7. Dong, D. et al. The crystal structure of Cpf1 in complex with CRISPR RNA. *Nature* 532, 522-526 (2016).
8. Fonfara, I., Richter, H., Bratovic, M., Le Rhun, A. & Charpentier, E. The CRISPR-associated DNA-cleaving enzyme Cpf1 also processes precursor CRISPR RNA. *Nature* 532, 517-521 (2016).
9. Yamano, T. et al. Crystal Structure of Cpf1 in Complex with Guide RNA and Target DNA. *Cell* (2016).
10. Kleinstiver, B.P. et al. Genome-wide specificities of CRISPR-Cas Cpf1 nucleases in human cells. *Nat Biotechnol* 34, 869-874 (2016).
11. Kim, D. et al. Genome-wide analysis reveals specificities of Cpf1 endonucleases in human cells. *Nat Biotechnol* 34, 863-868 (2016).
12. Slaymaker, I.M. et al. Rationally engineered Cas9 nucleases with improved specificity. *Science* 351, 84-88 (2016).
13. Kleinstiver, B.P. et al. High-fidelity CRISPR-Cas9 nucleases with no detectable genome-wide off-target effects. *Nature* 529, 490-+ (2016).

14. Hendel, A. et al. Chemically modified guide RNAs enhance CRISPR-Cas genome editing in human primary cells. *Nat Biotechnol* 33, 985-989 (2015).

15. Rahdar, M. et al. Synthetic CRISPR RNA-Cas9-guided genome editing in human cells. *Proc Natl Acad Sci U S A* 112, E7110-7117 (2015).

16. Chen, B. et al. Dynamic imaging of genomic loci in living human cells by an optimized CRISPR/Cas system. *Cell* 155, 1479-1491 (2013).

17. Dang, Y. et al. Optimizing sgRNA structure to improve CRISPR-Cas9 knockout efficiency. *Genome Biol* 16, 280 (2015).

18. Watts, J.K., Deleavey, G.F. & Damha, M.J. Chemically modified siRNA: tools and applications. *Drug Discov Today* 13, 842-855 (2008).

19. Kariko, K., Muramatsu, H., Keller, J.M. & Weissman, D. Increased erythropoiesis in mice injected with submicrogram quantities of pseudouridine-containing mRNA encoding erythropoietin. *Mol Ther* 20, 948-953 (2012).

20. Andries, O. et al. N(1)-methylpseudouridine-incorporated mRNA outperforms pseudouridine-incorporated mRNA by providing enhanced protein expression and reduced immunogenicity in mammalian cell lines and mice. *J Control Release* 217, 337-344 (2015).

21. Li, B., Luo, X. & Dong, Y. Effects of Chemically Modified Messenger RNA on Protein Expression. *Bioconjug Chem* (2016).

22. Pinello, L. et al. Analyzing CRISPR genome-editing experiments with CRISPResso. *Nat Biotechnol* 34, 695-697 (2016).

Modification pattern and mass spectrometry data of crRNAs used are shown in Tables1 and 2.

Table 2 | Engineered crRNAs and their mass spectrometry data.

No.	AscrRNA	Modification pattern (5'- to -3')	M.W.	
Chemically modified crRNAs targeting *DNMT1-3* locus			**Calcd.**	**Found**
WT	crWT	UAAUUUCUACUCUUGUAGAUCUGAUGG UCCAUGUCUGUUACUC (SEQ ID NO:3)	13552.5	13552.2
1	cr42PS	UAAUUUCUACUCUUGUAGAUCUGAUGG UCCAUGUCUGUUACUC (SEQ ID NO:11)	14227.0	14226.1
2	cr5'&'3F2PS	UAAUUUCUACUCUUGUAGAUCUGAUGG UCCAUGUCUGUUACUC (SEQ ID NO:12)	13628.7	13627.6
3	cr5'20M	*UAAUUUCUACUCUUGUAGAU*CUGAUGG UCCAUGUCUGUUACUC (SEQ ID NO:13)	13832.5	13832.1
4	cr5'10M	*UAAUUUCUA*CUCUUGUAGAUCUGAUGG UCCAUGUCUGUUACUC (SEQ ID NO:14)	13692.5	13691.8
5	cr5'5M	*UAAUU*UCUACUCUUGUAGAUCUGAUGG UCCAUGUCUGUUACUC (SEQ ID NO:15)	13622.5	13621.5

6	cr5'20F	UAAUUUCUACUCUUGUAGAUCUGAUGG UCCAUGUCUGUUACUC (SEQ ID NO:16)	13592.5	13590.6
7	cr5'10F	UAAUUUCUACUCUUGUAGAUCUGAUGG UCCAUGUCUGUUACUC (SEQ ID NO:17)	13572.5	13570.7
8	cr5'5F	UAAUUUCUACUCUUGUAGAUCUGAUGG UCCAUGUCUGUUACUC (SEQ ID NO:18)	13562.5	13560.9
9	crS3M3PS	UAAUUUCUACUCUUGUAGAU*CUG*AUGG UCCAUGUCUGUUACUC (SEQ ID NO:19)	13642.7	13643.4
10	crS3F	UAAUUUCUACUCUUGUAGAUCUGAUGG UCCAUGUCUGUUACUC (SEQ ID NO:20)	13558.5	13557.9
11	crS2F	UAAUUUCUACUCUUGUAGAUCUGAUGG UCCAUGUCUGUUACUC (SEQ ID NO:21)	13556.5	13557.4
12	crS1F	UAAUUUCUACUCUUGUAGAUCUGAUGG UCCAUGUCUGUUACUC (SEQ ID NO:22)	13554.5	13553.6
13	cr3'10M	UAAUUUCUACUCUUGUAGAUCUGAUGG UCCAUG*UCUGUUACUC* (SEQ ID NO:23)	13692.5	13691.2
14	cr3'5M	UAAUUUCUACUCUUGUAGAUCUGAUGG UCCAUGUCUGU*UACUC* (SEQ ID NO:24)	13622.5	13621.2
15	cr3'10F	UAAUUUCUACUCUUGUAGAUCUGAUGG UCCAUGUCUGUUACUC (SEQ ID NO:25)	13572.5	13572.0
16	cr3'5F	UAAUUUCUACUCUUGUAGAUCUGAUGG UCCAUGUCUGUUACUC (SEQ ID NO:26)	13562.5	13561.4
17	cr3'5F4PS	UAAUUUCUACUCUUGUAGAUCUGAUGG UCCAUGUCUGUUACUC (SEQ ID NO:27)	13626.7	13625.8
18	cr3'5U	UAAUUUCUACUCUUGUAGAUCUGAUGG UCCAUGUCUGU**UACUC** (SEQ ID NO:28)	13626.7	13625.8
19	cr3'5L	UAAUUUCUACUCUUGUAGAUCUGAUGG UCCAUGUCUGUUACUC (SEQ ID NO:29)	13626.7	13625.8
20	crI21F	UAAUUUCUACUCUUGUAGAUCUGAUGG UCCAUGUCUGUUACUC (SEQ ID NO:30)	13594.5	13594.2
21	crI16M5F	UAAU*UU*CU*AC*U*CUU*G*UAGA*UCUGAUGG U*CCAU*GUCU*G*UUACUC (SEQ ID NO:31)	13786.5	13783.7
22	crI16M10PS5F	UAAU*UU*CU*AC*U*CUU*G*UAGA*UCUGAUGG U*CCAU*GUCU*G*UUACUC (SEQ ID NO:32)	13947.1	13944.2
Stem-engineered crRNAs targeting *DNMT1-3* locus				
23	crSplit-L	UAAUUUCUACUC (SEQ ID NO:33)	3678.5	3677.8
	crSplit-R	UUGUAGAUCUGAUGGUCCAUGUCUGUU ACUC (SEQ ID NO:34)	9812.1	9811.4
24	crDel2	UAAUU-CUACUCUUGUAG-UCUGAUGG UCCAUGUCUGUUACUC (SEQ ID NO:35)	12917.1	12916.2
25	crDel4	UAAUUUCU--UCUU— AGAUCUGAUGGUC CAUGUCUGUUACUC (SEQ ID NO:36)	12266.7	12266.4
26	crDel8	UAAUUU----UCUU----AUCUGAUGGUCCA UGUCUGUUACUC (SEQ ID NO:37)	10980.9	10980.9
27	crIns4	UAAUUUCUAC*AC*UCUU*GU*GUAGAUCUG AUGGUCCAUGUCUGUUACUC (SEQ ID NO:38)	14838.3	14837.7
28	crIns4'	UAAUUUCUAC*UC*UCUU*GU*GUAGAUCUG AUGGUCCAUGUCUGUUACUC (SEQ ID	14815.3	14813.5

		NO:39)		
29	crIns6'	UAAUUUCUACUGCUCUUGCUGUAGAUC UGAUGGUCCAUGUCUGUUACUC (SEQ ID NO:40)	15465.6	15463.9
30	crIns8	UAAUUUCUACACACUCUUGUGUGUAGA UCUGAUGGUCCAUGUCUGUUACUC (SEQ ID NO:41)	16124.1	16123.5
31	crIns12	UAAUUUCUACACACACUCUUGUGUGUG UAGAUCUGAUGGUCCAUGUCUGUUACU C (SEQ ID NO:42)	17409.8	17408.7
Loop-engineered crRNAs targeting DNMT1-3 locus				
32	FncrRNA	UAAUUUCUACUGUUGUAGAUCUGAUGG UCCAUGUCUGUUACUC (SEQ ID NO:43)	13592.5	13593.0
33	Lb3crRNA	UAAUUUCUACGUUCUGUAGAUCUGAUG GUCCAUGUCUGUUACUC (SEQ ID NO:44)	13897.7	13898.2
34	BpcrRNA	UAAUUUCUACGUAAUGUAGAUCUGAUG GUCCAUGUCUGUUACUC (SEQ ID NO:45)	13944.7	13945.6
35	Pb/Pe/LicrRN A	UAAUUUCUACUUUUGUAGAUCUGAUGG UCCAUGUCUGUUACUC (SEQ ID NO:46)	13553.5	13553.6
36	SscrRNA	UAAUUUCUACACGCGGUAGAUCUGAUG GUCCAUGUCUGUUACUC (SEQ ID NO:47)	13958.7	13959.3
37	Lb2/Pc/PmcrR NA	UAAUUUCUACUAUUGUAGAUCUGAUGG UCCAUGUCUGUUACUC (SEQ ID NO:48)	13576.5	13577.3
38	CMtcrRNA	UAAUUUCUACUCUUUGUAGAUCUGAUG GUCCAUGUCUGUUACUC (SEQ ID NO:49)	13858.7	13859.1
39	EecrRNA	UAAUUUCUACU-UUGUAGAUCUGAUG GUCCAUGUCUGUUACUC (SEQ ID NO:50)	13247.3	13247.4
40	MbcrRNA	UAAUUUCUACUGUUUGUAGAUCUGAUG GUCCAUGUCUGUUACUC (SEQ ID NO:51)	13898.7	13898.6
41	PdcrRNA	UAAUUUCUACUUCGGUAGAUCUGAUGG UCCAUGUCUGUUACUC (SEQ ID NO:52)	13591.5	13592.4
42	LbcrRNA	AAUUUCUACUAAGUGUAGAUCUGAUGG UCCAUGUCUGUUACUC (SEQ ID NO:53)	13638.5	13637.2
Chemically modified crRNAs targeting *AAVS1* locus				
WT	crWT	UAAUUUCUACUCUUGUAGAUCUUACGA UGGAGCCAGAGAGGAU (SEQ ID NO:9)	13763.5	13763.2
1	cr3'5F	UAAUUUCUACUCUUGUAGAUCUUACGA UGGAGCCAGAGAGGAU (SEQ ID NO:54)	13773.5	13772.2
Chemically modified crRNAs targeting *FANCF-2* locus				
WT	crWT	UAAUUUCUACUCUUGUAGAUGUCGGCA UGGCCCCAUUCGCACG (SEQ ID NO:10)	13627.4	13625.6
1	cr3'5F	UAAUUUCUACUCUUGUAGAUGUCGGCA UGGCCCCAUUCGCACG (SEQ ID NO:55)	13637.4	13635.6
No.	**LbcrRNA**	**Modification pattern (5'- to -3')**	**M.W.**	
Chemically modified crRNAs targeting DNMT1-3 locus			**Calcd.**	**Found**
WT	crWT	AAUUUCUACUAAGUGUAGAUCUGAUGG UCCAUGUCUGUUACUC (SEQ ID NO:8)	13638.5	13637.2
1	cr3'5F	AAUUUCUACUAAGUGUAGAUCUGAUGG	13648.5	13648.0

		UCCAUGUCUGUUACUC (SEQ ID NO:56)		

Unmodified nucleotides are shown in black. Full-length PS modifications are underlined. 2'-*O*-methyl modifications are italized. 2'-F modifications are shaded. 2'-*O*-methyl combined with PS modifications are italicized and underlined. 2'-F combined with PS modifications are in shaded and underlined. Unlocked nucleotides are shown in bold. Locked nucleotides are shown in a box. A dash denotes deleted nucleotides. (This applies to all sequences other than sequences 23-42 in the table above).

Underlined letters for sequences 23-31 denotes inserted nucleotides. Underlined letters in the loop for sequences 32-42 above denotes nucleotides difference between AscrRNA and other crRNAs.

No.	**crRNA**	**Modification pattern (5'- to -3')**	**M.W.**	
Chemically modified crRNAs targeting DNMT1-3 locus			**Calcd.**	**Found**
WT	crWT	UAAUUUCUACUCUUGUAGAUCUGAUGG UCCAUGUCUGUUACUC (SEQ ID NO:3)	13552.5	13552.2
1	cr16M5F	UAAU*UU*CU*AC*U*CUU*G*UAGA*UCUGAUGG U*CCAU*GUCU*G*UUACUC (SEQ ID NO:57)	13786.5	13783.7
2	cr16M10PS5F	UAAU*UU*CU*AC*U*CUU*G*UAGA*UCUGAUGG U*CCAU*GUCU*G*UUACUC (SEQ ID NO:58)	13947.1	13944.2
3	cr10PS5F	UAAUUUCUACUCUUGUAGAUCUGAUGG UCCAUGUCUGUUACUC (SEQ ID NO:59)	13723.1	13719.3
4	crI21F	UAAUUUCUACUCUUGUAGAUCUGAUGG UCCAUGUCUGUUACUC (SEQ ID NO:60)	13594.5	13594.2
5	crI10F	UAAUUUCUACUCUUGUAGAUCUGAUGG UCCAUGUCUGUUACUC (SEQ ID NO:61)	13572.5	13572.5
6	crI3F	UAAUUUCUACUCUUGUAGAUCUGAUGG UCCAUGUCUGUUACUC (SEQ ID NO:62)	13558.5	13557.6
7	crI1F	UAAUUUCUACUCUUGUAGAUCUGAUGG UCCAUGUCUGUUACUC (SEQ ID NO:63)	13554.5	13553.8
8	cr21H	UAAUuuCUacUcuuGuagaUCUGAUGGUccau GUCUgUuacuc (SEQ ID NO:64)	13328.5	13328.9
9	cr10H	UAAUUUCUACUCUUGUAGAUCUGAUGG UccauGUCUgUuacuc (SEQ ID NO:65)	13450.5	13450.2
10	cr5H	UAAUUUCUACUCUUGUAGAUCUGAUGG UCCAUGUCUGUuacuc (SEQ ID NO:66)	13500.5	13500.3
11	Ψ-crRNA	**U**AA**U**UU**C**UA**C**UC**U**UG**U**AGA**U**C**U**GA**U**GG **U**CCA**U**GU**C**U**G**U**U**AC**U**C (SEQ ID NO:67)		
12	5meC-crRNA	UAAUUU**C**UA**C**U**C**UUGUAGAU**C**UGAUG GU**CC**AUGU**C**UGUUA**CUC** (SEQ ID NO:68)		
13	5moU-crRNA	**U**AA**U**UU**C**UA**C**UC**U**UG**U**AGA**U**C**U**GA**U**GG **U**CCA**U**GU**C**U**G**U**U**AC**U**C (SEQ ID NO:69)		
14	m1A-crRNA	U**A**AUUUCUA**C**UCUUGU**A**GA**U**CUGA**U**GG UCCA**U**GUCUGUU**A**CUC (SEQ ID NO:70)		
15	cr7PS21F	UAAU*UU*CU*AC*U*CUU*G*UAGA*UCUGAUGG UCCAUGUCUGUUACUC (SEQ ID NO:71)		
16	cr3PS21F	UAAUUUCUACUCUUGUAGAUCUGAUGG U*CCAU*GUCUGUUACUC (SEQ ID NO:72)		
17	cr5'5F+5F	UAAUUUCUACUCUUGUAGAUCUGAUGG		

		UCCAUGUCUGUUACUCGCCUG (SEQ ID NO:73)		

In Table 2, unmodified nucleotides are shown in black. PS modifications are underlined. 2'-*O*-methyl modifications are shown in italics. 2'-F modifications are shaded. 2'-*O*-methyl combined with PS modifications are underlined and italicized. 2'-F combined with PS modifications are shaded and underlined. Lowercase denotes DNA base. For Ψ-crRNA, 5meC-crRNA, 5moU-crRNA and m1A-crRNA, correspondent modified nucleotides are in bold (Figure 15 and Figure 17). The gene cutting efficiency of these chemically modified crRNAs were determined by T7E1 cleavage assay and the results are shown in Figure 18.

Table 3 | A list of genomic locus and primers used for T7E1 assay.

Locus	Genome	Location	Protospacer(5'-3')	Mismatch (bp)	Strand
DNMT1-3 On target	Homo sapiens	NC_000019.10	**TTTC**CTGATGGTCCATG TCTGTTACTC (SEQ ID NO:74)	0	-
DNMT1-3 OT1	Homo sapiens	NC_000007.14	**TTTC**CTGCTGGTCCATG TCTAATACTC (SEQ ID NO:75)	3	-
DNMT1-3 OT2	Homo sapiens	NC_000016.10	**TTTT**CTGATGGTCCATA CCTGTTACAC (SEQ ID NO:76)	3	+
DNMT1-3 OT3	Homo sapiens	NC_000011.10	**TTTT**CTTATTGTACATG TCTGTAACTC (SEQ ID NO:77)	4	-
DNMT1-3 OT4	Homo sapiens	NC_000023.11	**TTTC**CTGATGGTCACA CCTGTTACAC (SEQ ID NO:78)	4	+
AAVS1 On target	Homo sapiens	NC_000019.10	**TTTG**CTTACGATGGAGC CAGAGAGGAT (SEQ ID NO:79)	0	-
FANCF-2 On target	Homo sapiens	NC_000011.10	**TTTG**GTCGGCATGGCCC CATTCGCACG (SEQ ID NO:80)	0	-

Locus	Forward primer	Reverse primer	Amplicon (bp)	Predicted fragment (bp)
DNMT1-3 On target	CTGGGACTCAGGCG GGTCAC (SEQ ID NO:81)	CCTCAGCCAGAAG TCCCGTGC (SEQ ID NO:82)	827	358 + 469
DNMT1-3 OT1	AGGAAAGCCATGCC AGAGACTCA (SEQ ID NO:83)	CACCGCCACTCTG TTTCCAAG (SEQ ID NO:84)	800	341 + 459

DNMT1-3 OT2	GTTGGGACATGAAG GTCAAGTGTG (SEQ ID NO:85)	TTTGTCTCCTGTTG CCTTCAGGCC (SEQ ID NO:86)	788	296 + 492
DNMT1-3 OT3	GAGGCATAGCAAGG TCATGCCTTT (SEQ ID NO:87)	TGCTTCCCTTGGTG GAGCTG (SEQ ID NO:88)	923	424 + 499
DNMT1-3 OT4	TTTCCATGTAGGCCC ATGCCC (SEQ ID NO:89)	CCAGGTTACCAGC AACAGATCTC (SEQ ID NO:90)	800	313 + 487
AAVS1 On target	GGGCTGGCTACTGGC CTTAT (SEQ ID NO:91)	ATGGCATCTTCCA GGGGTCC (SEQ ID NO:92)	700	314 + 386
FANCF-2 On target	AGCTCCGCCTGGGTC TTCAT (SEQ ID NO:93)	GCGGAGACGTTCA TGACTGG (SEQ ID NO:94)	800	341 + 459
For Table 3: PAM is in bold; Mismatched bases are underlined.				

Table 4. List of primers used for targeted for target deep sequencing.

Locus	Forward Primer for 1st stage PCR	Reverse Primer for 1st stage PCR
DNMT1-3 On target	TCGTCGGCAGCGTCAGATGTGT ATAAGAGACAGTCCCTCACTCC TGCTCGGTGAA (SEQ ID NO:95)	GTCTCGTGGGCTCGGAGATGTG TATAAGAGACAGAAGTCACTCT GGGGAACACGCC (SEQ ID NO:96)
DNMT1-3 OT1	TCGTCGGCAGCGTCAGATGTGT ATAAGAGACAGACCTTTTGGGC GTGGAGAAGGG (SEQ ID NO:97)	GTCTCGTGGGCTCGGAGATGTG TATAAGAGACAGGGGAGGGGGT CAGCATGAAAGG (SEQ ID NO:98)
DNMT1-3 OT2	TCGTCGGCAGCGTCAGATGTGT ATAAGAGACAGCTCCCCCACCC CCTAGGAAAGT (SEQ ID NO:99)	GTCTCGTGGGCTCGGAGATGTG TATAAGAGACAGCCCTTTCTGGT GGAGTGTCCCC (SEQ ID NO:100)
DNMT1-3 OT3	TCGTCGGCAGCGTCAGATGTGT ATAAGAGACAGTGAAGGTATAG GAGAGGTTTTGGGCT (SEQ ID NO:101)	GTCTCGTGGGCTCGGAGATGTG TATAAGAGACAGAGACGACCTT AGATGGAGTGTTGTGT (SEQ ID NO:102)
DNMT1-3 OT4	TCGTCGGCAGCGTCAGATGTGT ATAAGAGACAGTGCCAGTGGAA GGAGGGAGTGT (SEQ ID NO:103)	GTCTCGTGGGCTCGGAGATGTG TATAAGAGACAGTGCCCAGGAA GTTGCTTCTCCC (SEQ ID NO:104)

* Shaded nucleotides indicate the Illumina overhang adapter sequences.

Table 5 | AsCpf1: A list of index sequences used for the 2nd round of PCR of targeted deep sequencing.					
Index No.	Sample name	Index 1	Sequence	Index 2	Sequence
1	AscrWT/AsCpf1 plasmid_1_OT1	N701	TAAGGCGA	N517	GCGTAAGA
2	AscrWT/AsCpf1 plasmid_2_OT1	N701	TAAGGCGA	N502	CTCTCTAT
3	AscrWT/AsCpf1 plasmid_3_OT1	N701	TAAGGCGA	N503	TATCCTCT
4	AscrWT/AsCpf1 plasmid_1_OT2	N701	TAAGGCGA	N504	AGAGTAGA
5	AscrWT/AsCpf1 plasmid_2_OT2	N701	TAAGGCGA	N505	GTAAGGAG

6	AscrWT/AsCpf1 plasmid_3_OT2	N701	TAAGGCGA	N506	ACTGCATA
7	AscrWT/AsCpf1 plasmid_1_OT3	N701	TAAGGCGA	N507	AAGGAGTA
8	AscrWT/AsCpf1 plasmid_2_OT3	N701	TAAGGCGA	N508	CTAAGCCT
9	AscrWT/AsCpf1 plasmid_3_OT3	N702	CGTACTAG	N517	GCGTAAGA
10	AscrWT/AsCpf1 plasmid_1_OT4	N702	CGTACTAG	N502	CTCTCTAT
11	AscrWT/AsCpf1 plasmid_2_OT4	N702	CGTACTAG	N503	TATCCTCT
12	AscrWT/AsCpf1 plasmid_3_OT4	N702	CGTACTAG	N504	AGAGTAGA
13	Ascr3'5F/AsCpf1 ψ mRNA_1_OT1	N702	CGTACTAG	N505	GTAAGGAG
14	Ascr3'5F/AsCpf1 ψ mRNA_2_OT1	N702	CGTACTAG	N506	ACTGCATA
15	Ascr3'5F/AsCpf1 ψ mRNA_3_OT1	N702	CGTACTAG	N507	AAGGAGTA
16	Ascr3'5F/AsCpf1 ψ mRNA_1_OT2	N702	CGTACTAG	N508	CTAAGCCT
17	Ascr3'5F/AsCpf1 ψ mRNA_2_OT2	N703	AGGCAGAA	N517	GCGTAAGA
18	Ascr3'5F/AsCpf1 ψ mRNA_3_OT2	N703	AGGCAGAA	N502	CTCTCTAT
19	Ascr3'5F/AsCpf1 ψ mRNA_1_OT3	N703	AGGCAGAA	N503	TATCCTCT
20	Ascr3'5F/AsCpf1 mRNA_2_OT3	N703	AGGCAGAA	N504	AGAGTAGA
21	Ascr3'5F/AsCpf1 mRNA_3_OT3	N703	AGGCAGAA	N505	GTAAGGAG
22	Ascr3'5F/AsCpf1 ψ mRNA_1_OT4	N703	AGGCAGAA	N506	ACTGCATA
23	Ascr3'5F/AsCpf1 ψ mRNA_2_OT4	N703	AGGCAGAA	N507	AAGGAGTA
24	Ascr3'5F/AsCpf1 ψ mRNA_3_OT4	N703	AGGCAGAA	N508	CTAAGCCT
25	AscrWT/AsCpf1 plasmid_1_On target	N704	TCCTGAGC	N517	GCGTAAGA
26	AscrWT/AsCpf1 plasmid_2_On target	N704	TCCTGAGC	N502	CTCTCTAT
27	AscrWT/AsCpf1 plasmid_3_On target	N704	TCCTGAGC	N503	TATCCTCT
28	Ascr3'5F/AsCpf1 ψ mRNA_1_On target	N704	TCCTGAGC	N504	AGAGTAGA
29	Ascr3'5F/AsCpf1 ψ mRNA_2_On target	N704	TCCTGAGC	N505	GTAAGGAG
30	Ascr3'5F/AsCpf1 ψ mRNA_3_On target	N704	TCCTGAGC	N506	ACTGCATA

Table 6 | LbCpf1: A list of index sequences used for the 2nd round of PCR of targeted deep sequencing.

Index No.	Sample name	Index 1	Sequence	Index 2	Sequence
1	LbcrWT/LbCpf1 plasmid_1_OT1	N701	TAAGGCGA	N517	GCGTAAGA
2	LbcrWT/LbCpf1 plasmid_2_OT1	N701	TAAGGCGA	N502	CTCTCTAT
3	LbcrWT/LbCpf1 plasmid_3_OT1	N701	TAAGGCGA	N503	TATCCTCT
4	LbcrWT/LbCpf1 plasmid_1_OT2	N701	TAAGGCGA	N504	AGAGTAGA
5	LbcrWT/LbCpf1 plasmid_2_OT2	N701	TAAGGCGA	N505	GTAAGGAG
6	LbcrWT/LbCpf1 plasmid_3_OT2	N701	TAAGGCGA	N506	ACTGCATA
7	LbcrWT/LbCpf1 plasmid_1_OT3	N701	TAAGGCGA	N507	AAGGAGTA
8	LbcrWT/LbCpf1 plasmid_2_OT3	N701	TAAGGCGA	N508	CTAAGCCT
9	LbcrWT/LbCpf1 plasmid_3_OT3	N702	CGTACTAG	N517	GCGTAAGA
10	LbcrWT/LbCpf1 plasmid_1_OT4	N702	CGTACTAG	N502	CTCTCTAT
11	LbcrWT/LbCpf1 plasmid_2_OT4	N702	CGTACTAG	N503	TATCCTCT
12	LbcrWT/LbCpf1 plasmid_3_OT4	N702	CGTACTAG	N504	AGAGTAGA
13	Lbcr3'5F/LbCpf1 ψ mRNA_1_OT1	N702	CGTACTAG	N505	GTAAGGAG

14	Lbcr3'5F/LbCpf1 ψ mRNA_2_OT1	N702	CGTACTAG	N506	ACTGCATA
15	Lbcr3'5F/LbCpf1 ψ mRNA_3_OT1	N702	CGTACTAG	N507	AAGGAGTA
16	Lbcr3'5F/LbCpf1 ψ mRNA_1_OT2	N702	CGTACTAG	N508	CTAAGCCT
17	Lbcr3'5F/LbCpf1 ψ mRNA_2_OT2	N703	AGGCAGAA	N517	GCGTAAGA
18	Lbcr3'5F/LbCpf1 ψ mRNA_3_OT2	N703	AGGCAGAA	N502	CTCTCTAT
19	Lbcr3'5F/LbCpf1 ψ mRNA_1_OT3	N703	AGGCAGAA	N503	TATCCTCT
20	Lbcr3'5F/LbCpf1 ψ mRNA_2_OT3	N703	AGGCAGAA	N504	AGAGTAGA
21	Lbcr3'5F/LbCpf1 ψ mRNA_3_OT3	N703	AGGCAGAA	N505	GTAAGGAG
22	Lbcr3'5F/LbCpf1 ψ mRNA_1_OT4	N703	AGGCAGAA	N506	ACTGCATA
23	Lbcr3'5F/LbCpf1 ψ mRNA_2_OT4	N703	AGGCAGAA	N507	AAGGAGTA
24	Lbcr3'5F/LbCpf1 ψ mRNA_3_OT4	N703	AGGCAGAA	N508	CTAAGCCT
25	LbcrWT/LbCpf1 plasmid_1_On target	N704	TCCTGAGC	N517	GCGTAAGA
26	LbcrWT/LbCpf1 plasmid_2_On target	N704	TCCTGAGC	N502	CTCTCTAT
27	LbcrWT/LbCpf1 plasmid_3_On target	N704	TCCTGAGC	N503	TATCCTCT
28	Lbcr3'5F/LbCpf1 ψ mRNA_1_On target	N704	TCCTGAGC	N504	AGAGTAGA
29	Lbcr3'5F/LbCpf1 ψ mRNA_2_On target	N704	TCCTGAGC	N505	GTAAGGAG
30	Lbcr3'5F/LbCpf1 ψ mRNA_3_On target	N704	TCCTGAGC	N506	ACTGCATA
31	Untreated_1_OT1	N705	GGACTCCT	N517	GCGTAAGA
32	Untreated_2_OT1	N705	GGACTCCT	N502	CTCTCTAT
33	Untreated_3_OT1	N705	GGACTCCT	N503	TATCCTCT
34	Untreated_1_On target	N705	GGACTCCT	N504	AGAGTAGA
35	Untreated_2_On target	N705	GGACTCCT	N505	GTAAGGAG
36	Untreated_3_On target	N705	GGACTCCT	N506	ACTGCATA

Amplicons used for targeted MiSeq analysis.

DNMT1-3 on-target sense (245 bp) (SEQ ID NO:105)

>NC_000007.14 Homo sapiens chromosome 7, GRCh38.p7 Primary Assembly

AAGTCACTCTGGGGAACACGCCCGGTGTCACGCCACTTGACAGGCGAGTAACAGAC
ATGGACCATCAGGAAACATTAACGTACTGATGTTAACAGCTGACCCAATAAGTGGC
AGAGTGCTAAGGGAACGTTCACGGAGACTGAACACTCCTCAAACGGTCCCCAGAGG
GTTCTAGACCCAGAGGCTCAAGTGAGCAGCTGAGGCAGGTGCCTGCTGAGCCAAAT
TCACCGAGCAGGAGTGAGGGA

DNMT1-3 off-target 1 (OT1, 243 bp) (SEQ ID NO:106)

>NC_000007.14 Homo sapiens chromosome 7, GRCh38.p7 Primary Assembly

GGGAGGGGGTCAGCATGAAAGGAGCAGTGTGAAATGGACAATTCACACACCTTCCA
TGTTTCTCAACAATGTCAGTCCCTGCTGTTGCCACGGCCGCTATGGTCTTCTCACAAC
AAGAAGAGTATTAGACATGGACCAGCAGGAAAGCACACGTTCACAGGCACGTGCCC
ATGCTCACAAACACACACATGTGTGCATGCACACATGCACACTCCCTACACCCCTTC
TCCACGCCCAAAAGGT

DNMT1-3 off-target 2 (OT2, 230 bp) (SEQ ID NO:107)

>NC_000016.10 Homo sapiens chromosome 16, GRCh38.p7 Primary Assembly

CTCCCCCACCCCCTAGGAAAGTCAGGTGATGGTTCAGCAAGTATCACATCGCCTCTG
TAAAGGTGATAAACTGGCTGCCAGGGCCAGGGAGAGGCCATTTTCTGATGGTCCAT
ACCTGTTACACTAAAGTGTTAATTGAATGCAGATGCCAGGGAGGAGCAACTTCCAG
GGCATGTGCATCAAGAGACAAAACAGTGGAATATGTCCTGGGGACACTCCACCAGA
AAGGG

DNMT1-3 off-target 3 (OT3, 226 bp) (SEQ ID NO:108)

>NC_000011.10 Homo sapiens chromosome 11, GRCh38.p7 Primary Assembly

AGACGACCTTAGATGGAGTGTTGTGTATTTCAAACAGAGTTACCATTGTGCTTTATC
GTGATCAGTCCCCTTCTTGACACGTGAGAGTTACAGACATGTACAATAAGAAAATTA
GGAAAATTTCGGACAAAAACATCTGAATATATAAGAATTTGAATTGAATTTCCTATC
TCTCTTATTAAAAACAAACATAAACCTTAAGCCCAAAACCTCTCCTATACCTTCA

DNMT1-3 off-target 4 (OT2, 230 bp) (SEQ ID NO:109)

>NC_000023.11 Homo sapiens chromosome X, GRCh38.p7 Primary Assembly

TGCCAGTGGAAGGAGGGAGTGTTACAGGTAGTTAAGCAGGCATGAGCTGGGCTGGA
GAGGGCTGTCCTCCACCCACTAGGAATGTCAGGTGATGGCTCAGCAATTATCACATT
GACTCTCTAAAAGTGACAAATTGGCAGCCAGTGCCAGGGAGAAGCCATTTCCTGAT
GGTCCACACCTGTTACACTAAAGGGTTAATTGAATGCAGATGCCAGGGAGAAGCAA
CTTCCTGGGCA

Sequencing data analysis.

CRISPResso was utilized to analyze the deep sequencing data. 22 nt (T rather than U) from 3' of the PAM (TTTN) is designated as crRNA sequences. The amplicon containing the PAM was used for alignment. To quantify mutation events and avoid false positives, the reading size is set up as 10 (10 nt before and after each side of the predicted cleavage site). Minimum average reading quality (phred33 scale) and minimum single bp quality (phred33 scale) is greater than 30 and 20 (recommended values), respectively. Default values were used for other parameters.

Those skilled in the art will appreciate that numerous changes and modifications can be made to the preferred embodiments of the invention and that such changes and modifications can be made without departing from the spirit of the invention. It is, therefore, intended that the appended claims cover all such equivalent variations as fall within the true spirit and scope of the invention.

CLAIMS

We claim:

1. A genome editing system comprising:

a) a guide RNA that hybridizes with a target sequence of a DNA molecule in a eukaryotic cell that contains the DNA molecule, and

b) an mRNA encoding a Cpf1 protein;

wherein the mRNA encoding a Cpf1 protein comprises at least one chemically modified nucleotide.

2. The system of claim 1, wherein the at least one chemically modified nucleotide comprises a chemically modified nucleobase, a chemically modified ribose, a chemically modified phosphodiester linkage, or a combination thereof.

3. The system of claim 1, wherein the at least one chemically modified nucleotide is a chemically modified nucleobase.

4. The system of claim 3, wherein the chemically modified nucleobase is selected from 5-formylcytidine (5fC), 5-methylcytidine (5meC), 5-methoxycytidine (5moC), 5-hydroxycytidine (5hoC), 5-hydroxymethylcytidine (5hmC), 5-formyluridine (5fU), 5-methyluridine (5-meU), 5-methoxyuridine (5moU), 5-carboxymethylesteruridine (5camU), pseudouridine (Ψ), N^1-methylpseudouridine ($me^1\Psi$), N^6-methyladenosine (me^6A), or thienoguanosine (^{th}G).

5. The system of claim 4, wherein the chemically modified nucleobase is selected from 5-methoxyuridine (5moU), pseudouridine (Ψ), and N^1-methylpseudouridine ($me^1\Psi$).

6. The system of claim 5, wherein the chemically modified nucleobase is 5-methoxyuridine (5moU).

7. The system of claim 5, wherein the chemically modified nucleobase is pseudouridine (Ψ).

8. The system of claim 5, wherein the chemically modified nucleobase is N^1-methylpseudouridine ($me^1\Psi$).

9. The system of claim 1, wherein the at least one chemically modified nucleotide is a chemically modified ribose.

10. The system of claim 9, wherein the chemically modified ribose is selected from 2′-*O*-methyl (2′-*O*-Me), 2′-Fluoro (2′-F), 2′-deoxy-2′-fluoro-beta-D-arabino-nucleic acid (2′F-ANA), 4′-S, 4′-SFANA, 2′-azido, UNA, 2′-*O*-methoxy-ethyl (2′-*O*-ME), 2′-*O*-Allyl, 2′-*O*-Ethylamine, 2′-*O*-Cyanoethyl, Locked nucleic acid (LAN), Methylene-cLAN, N-MeO-amino BNA, or N-MeO-aminooxy BNA.

11. The system of claim 10, wherein the chemically modified ribose is selected from 2′-O-methyl (2′-O-Me) or 2′-Fluoro (2′-F).

12. The system of claim 1, wherein the at least one chemically modified nucleotide is a chemically modified phosphodiester linkage.

13. The system of claim 12, wherein chemically modified phosphodiester linkage is selected from Phosphorothioate (PS), Boranophosphate, phosphodithioate (PS2), 3′,5′-amide, N3′-phosphoramidate (NP), Phosphodiester (PO), or 2′,5′-phosphodiester (2′,5′-PO).

14. The system of claim 13, wherein the chemically modified phosphodiester linkage is phosphorothioate.

15. The system of any one of claims 1-14, wherein the at least one chemically modified nucleotide confers increased Cpf1 protein levels as compared to a corresponding mRNA encoding a Cpf1 protein not having the chemically modified nucleotide.

16. The system of any one of claims 1-14, wherein the at least one chemically modified nucleotide confers increased Cpf1 nuclease activity as compared to a corresponding mRNA encoding a Cpf1 protein not having the chemically modified nucleotide.

17. The system of any one of claims 1-14, wherein the at least one chemically modified nucleotide confers reduced toxicity as compared to a corresponding mRNA encoding a Cpf1 protein not having the chemically modified nucleotide.

18. A method of increasing Cpf1 protein levels in a cell, the method comprising:

introducing into a cell of the subject:

a) a guide RNA that hybridizes with a target sequence of a DNA molecule in a eukaryotic cell that contains the DNA molecule, and

b) an mRNA encoding a Cpf1 protein;

wherein the mRNA encoding a Cpf1 protein comprises at least one chemically modified nucleotide, and

wherein the at least one chemically modified nucleotide confers increased Cpf1 protein levels as compared to a corresponding mRNA encoding a Cpf1 protein not having the chemically modified nucleotide.

19. The method of claim 18, wherein the at least one chemically modified nucleotide comprises a chemically modified nucleobase, a chemically modified ribose, a chemically modified phosphodiester linkage, or a combination thereof.

20. The method of claim 18, wherein the at least one chemically modified nucleotide is a chemically modified nucleobase.

21. The method of claim 20, wherein the chemically modified nucleobase is selected from 5-formylcytidine (5fC), 5-methylcytidine (5meC), 5-methoxycytidine (5moC), 5-hydroxycytidine (5hoC), 5-hydroxymethylcytidine (5hmC), 5-formyluridine (5fU), 5-methyluridine (5-meU), 5-

methoxyuridine (5moU), 5-carboxymethylesteruridine (5camU), pseudouridine (Ψ), N^1-methylpseudouridine ($me^1Ψ$), N^6-methyladenosine (me^6A), or thienoguanosine (^{th}G).

22. The method of claim 21, wherein the chemically modified nucleobase is selected from 5-methoxyuridine (5moU), pseudouridine (Ψ), and N^1-methylpseudouridine ($me^1Ψ$).

23. The method of claim 22, wherein the chemically modified nucleobase is 5-methoxyuridine (5moU).

24. The method of claim 22, wherein the chemically modified nucleobase is pseudouridine (Ψ).

25. The method of claim 22, wherein the chemically modified nucleobase is N^1-methylpseudouridine ($me^1Ψ$).

26. The method of claim 18, wherein the at least one chemically modified nucleotide is a chemically modified ribose.

27. The method of claim 26, wherein the chemically modified ribose is selected from 2′-*O*-methyl (2′-*O*-Me), 2′-Fluoro (2′-F), 2′-deoxy-2′-fluoro-beta-D-arabino-nucleic acid (2′F-ANA), 4′-S, 4′-SFANA, 2′-azido, UNA, 2′-*O*-methoxy-ethyl (2′-*O*-ME), 2′-*O*-Allyl, 2′-*O*-Ethylamine, 2′-*O*-Cyanoethyl, Locked nucleic acid (LAN), Methylene-cLAN, N-MeO-amino BNA, or N-MeO-aminooxy BNA.

28. The method of claim 27, wherein the chemically modified ribose is selected from 2′-O-methyl (2′-O-Me) or 2′-Fluoro (2′-F).

29. The method of claim 18, wherein the at least one chemically modified nucleotide is a chemically modified phosphodiester linkage.

30. The method of claim 29, wherein chemically modified phosphodiester linkage is selected from Phosphorothioate (PS), Boranophosphate, phosphodithioate (PS2), 3′,5′-amide, N3′-phosphoramidate (NP), Phosphodiester (PO), or 2′,5′-phosphodiester (2′,5′-PO).

31. The method of claim 30, wherein the chemically modified phosphodiester linkage is phosphorothioate.

32. A method of increasing Cpf1 nuclease activity in a cell, the method comprising:

introducing into a cell of the subject:

a) a guide RNA that hybridizes with a target sequence of a DNA molecule in a eukaryotic cell that contains the DNA molecule, and

b) an mRNA encoding a Cpf1 protein;

wherein the mRNA encoding a Cpf1 protein comprises at least one chemically modified nucleotide, and

wherein the at least one chemically modified nucleotide confers increased Cpf1 nuclease activity as compared to a corresponding mRNA encoding a Cpf1 protein not having the chemically modified nucleotide.

33. The method of claim 32, wherein the at least one chemically modified nucleotide comprises a chemically modified nucleobase, a chemically modified ribose, a chemically modified phosphodiester linkage, or a combination thereof.

34. The method of claim 32, wherein the at least one chemically modified nucleotide is a chemically modified nucleobase.

35. The method of claim 34, wherein the chemically modified nucleobase is selected from 5-formylcytidine (5fC), 5-methylcytidine (5meC), 5-methoxycytidine (5moC), 5-hydroxycytidine (5hoC), 5-hydroxymethylcytidine (5hmC), 5-formyluridine (5fU), 5-methyluridine (5-meU), 5-methoxyuridine (5moU), 5-carboxymethylesteruridine (5camU), pseudouridine (Ψ), N^1-methylpseudouridine ($me^1\Psi$), N^6-methyladenosine (me^6A), or thienoguanosine (^{th}G).

36. The method of claim 35, wherein the chemically modified nucleobase is selected from 5-methoxyuridine (5moU), pseudouridine (Ψ), and N^1-methylpseudouridine ($me^1\Psi$).

37. The method of claim 36, wherein the chemically modified nucleobase is 5-methoxyuridine (5moU).

38. The method of claim 36, wherein the chemically modified nucleobase is pseudouridine (Ψ).

39. The method of claim 36, wherein the chemically modified nucleobase is N^1-methylpseudouridine ($me^1\Psi$).

40. The method of claim 32, wherein the at least one chemically modified nucleotide is a chemically modified ribose.

41. The method of claim 40, wherein the chemically modified ribose is selected from 2′-*O*-methyl (2′-*O*-Me), 2′-Fluoro (2′-F), 2′-deoxy-2′-fluoro-beta-D-arabino-nucleic acid (2′F-ANA), 4′-S, 4′-SFANA, 2′-azido, UNA, 2′-*O*-methoxy-ethyl (2′-*O*-ME), 2′-*O*-Allyl, 2′-*O*-Ethylamine, 2′-*O*-Cyanoethyl, Locked nucleic acid (LAN), Methylene-cLAN, N-MeO-amino BNA, or N-MeO-aminooxy BNA.

42. The method of claim 41, wherein the chemically modified ribose is selected from 2′-O-methyl (2′-O-Me) or 2′-Fluoro (2′-F).

43. The method of claim 32, wherein the at least one chemically modified nucleotide is a chemically modified phosphodiester linkage.

44. The method of claim 43, wherein chemically modified phosphodiester linkage is selected from Phosphorothioate (PS), Boranophosphate, phosphodithioate (PS2), 3′,5′-amide, N3′-

phosphoramidate (NP), Phosphodiester (PO), or 2′,5′-phosphodiester (2′,5′-PO).

45. The method of claim 44, wherein the chemically modified phosphodiester linkage is phosphorothioate.

46. A method of reducing toxicity of RNA-guided genome editing in a cell, the method comprising:

introducing into a cell of the subject:

a) a guide RNA that hybridizes with a target sequence of a DNA molecule in a eukaryotic cell that contains the DNA molecule, and

b) an mRNA encoding a Cpf1 protein;

wherein the mRNA encoding a Cpf1 protein comprises at least one chemically modified nucleotide, and

wherein the at least one chemically modified nucleotide confers reduced toxicity as compared to a corresponding mRNA encoding a Cpf1 protein not having the chemically modified nucleotide.

47. The method of claim 46, wherein the at least one chemically modified nucleotide comprises a chemically modified nucleobase, a chemically modified ribose, a chemically modified phosphodiester linkage, or a combination thereof.

48. The method of claim 47, wherein the at least one chemically modified nucleotide is a chemically modified nucleobase.

49. The method of claim 48, wherein the chemically modified nucleobase is selected from 5-formylcytidine (5fC), 5-methylcytidine (5meC), 5-methoxycytidine (5moC), 5-hydroxycytidine (5hoC), 5-hydroxymethylcytidine (5hmC), 5-formyluridine (5fU), 5-methyluridine (5-meU), 5-methoxyuridine (5moU), 5-carboxymethylesteruridine (5camU), pseudouridine (Ψ), N^1-methylpseudouridine ($me^1\Psi$), N^6-methyladenosine (me^6A), or thienoguanosine (^{th}G).

50. The method of claim 49, wherein the chemically modified nucleobase is selected from 5-methoxyuridine (5moU), pseudouridine (Ψ), and N^1-methylpseudouridine ($me^1\Psi$).

51. The method of claim 50, wherein the chemically modified nucleobase is 5-methoxyuridine (5moU).

52. The method of claim 50, wherein the chemically modified nucleobase is pseudouridine (Ψ).

53. The method of claim 50, wherein the chemically modified nucleobase is N^1-methylpseudouridine ($me^1\Psi$).

54. The method of claim 46, wherein the at least one chemically modified nucleotide is a chemically modified ribose.

55. The method of claim 54, wherein the chemically modified ribose is selected from 2′-*O*-methyl (2′-*O*-Me), 2′-Fluoro (2′-F), 2′-deoxy-2′-fluoro-beta-D-arabino-nucleic acid (2′F-ANA), 4′-S, 4′-SFANA, 2′-azido, UNA, 2′-*O*-methoxy-ethyl (2′-*O*-ME), 2′-*O*-Allyl, 2′-*O*-Ethylamine, 2′-*O*-Cyanoethyl, Locked nucleic acid (LAN), Methylene-cLAN, N-MeO-amino BNA, or N-MeO-aminooxy BNA.

56. The method of claim 55, wherein the chemically modified ribose is selected from 2′-O-methyl (2′-O-Me) or 2′-Fluoro (2′-F).

57. The method of claim 46, wherein the at least one chemically modified nucleotide is a chemically modified phosphodiester linkage.

58. The method of claim 57, wherein chemically modified phosphodiester linkage is selected from Phosphorothioate (PS), Boranophosphate, phosphodithioate (PS2), 3′,5′-amide, N3′-phosphoramidate (NP), Phosphodiester (PO), or 2′,5′-phosphodiester (2′,5′-PO).

59. The method of claim 58, wherein the chemically modified phosphodiester linkage is phosphorothioate.

60. The method of claim 58, wherein the chemically modified phosphodiester linkage is phosphorodithioate.

61. A genome editing system comprising:

a) a guide RNA that hybridizes with a target sequence of a DNA molecule in a eukaryotic cell that contains the DNA molecule, and

b) an mRNA encoding a Cpf1 protein;

wherein the guide RNA comprises at least one chemically modified nucleotide; and

wherein the mRNA encoding a Cpf1 protein comprises at least one chemically modified nucleotide.

62. The system of claim 61, wherein the mRNA encoding a Cpf1 protein comprises at least one chemically modified nucleotide selected from a chemically modified nucleobase, a chemically modified ribose, a chemically modified phosphodiester linkage, or a combination thereof.

63. The system of claim 62, wherein the mRNA encoding a Cpf1 protein comprises a chemically modified nucleobase.

64. The system of claim 63, wherein the chemically modified nucleobase is selected from 5-formylcytidine (5fC), 5-methylcytidine (5meC), 5-methoxycytidine (5moC), 5-hydroxycytidine (5hoC), 5-hydroxymethylcytidine (5hmC), 5-formyluridine (5fU), 5-methyluridine (5-meU), 5-methoxyuridine (5moU), 5-carboxymethylesteruridine (5camU), pseudouridine (Ψ), N^1-methylpseudouridine ($me^1\Psi$), N^6-methyladenosine (me^6A), or thienoguanosine (^{th}G).

65. The system of claim 64, wherein the chemically modified nucleobase is selected from 5-

methoxyuridine (5moU), pseudouridine (Ψ), and N^1-methylpseudouridine ($me^1\Psi$).

66. The system of claim 65, wherein the chemically modified nucleobase is 5-methoxyuridine (5moU).

67. The system of claim 65, wherein the chemically modified nucleobase is pseudouridine (Ψ).

68. The system of claim 65, wherein the chemically modified nucleobase is N^1-methylpseudouridine ($me^1\Psi$).

69. The system of claim 62, wherein the mRNA encoding a Cpf1 protein comprises a chemically modified ribose.

70. The system of claim 69, wherein the chemically modified ribose is selected from 2′-*O*-methyl (2′-*O*-Me), 2′-Fluoro (2′-F), 2′-deoxy-2′-fluoro-beta-D-arabino-nucleic acid (2′F-ANA), 4′-S, 4′-SFANA, 2′-azido, UNA, 2′-*O*-methoxy-ethyl (2′-*O*-ME), 2′-*O*-Allyl, 2′-*O*-Ethylamine, 2′-*O*-Cyanoethyl, Locked nucleic acid (LAN), Methylene-cLAN, N-MeO-amino BNA, or N-MeO-aminooxy BNA.

71. The system of claim 70, wherein the chemically modified ribose is selected from 2′-O-methyl (2′-O-Me) or 2′-Fluoro (2′-F).

72. The system of claim 71, wherein the chemically modified ribose is 2′-Fluoro (2′-F).

73. The system of claim 62, wherein the mRNA encoding a Cpf1 protein comprises a chemically modified phosphodiester linkage.

74. The system of claim 73, wherein chemically modified phosphodiester linkage is selected from Phosphorothioate (PS), Boranophosphate, phosphodithioate (PS2), 3′,5′-amide, N3′-phosphoramidate (NP), Phosphodiester (PO), or 2′,5′-phosphodiester (2′,5′-PO).

75. The system of claim 74, wherein the chemically modified phosphodiester linkage is phosphorothioate.

76. The system of any one of claims 61 to 75, wherein the guide RNA comprises at least one chemically modified nucleotide selected from a chemically modified nucleobase, a chemically modified ribose, a chemically modified phosphodiester linkage, or a combination thereof.

77. The system of claim 76, wherein the guide RNA comprises a chemically modified nucleobase.

78. The system of claim 77, wherein the chemically modified nucleobase is selected from 5-formylcytidine (5fC), 5-methylcytidine (5meC), 5-methoxycytidine (5moC), 5-hydroxycytidine (5hoC), 5-hydroxymethylcytidine (5hmC), 5-formyluridine (5fU), 5-methyluridine (5-meU), 5-methoxyuridine (5moU), 5-carboxymethylesteruridine (5camU), pseudouridine (Ψ), N^1-methylpseudouridine ($me^1\Psi$), N^6-methyladenosine (me^6A), or thienoguanosine (^{th}G).

79. The system of claim 78, wherein the chemically modified nucleobase is selected from 5-

methoxyuridine (5moU), pseudouridine (Ψ), and N^1-methylpseudouridine ($me^1\Psi$).

80. The system of claim 79, wherein the chemically modified nucleobase is 5-methoxyuridine (5moU).

81. The system of claim 79, wherein the chemically modified nucleobase is pseudouridine (Ψ).

82. The system of claim 79, wherein the chemically modified nucleobase is N^1-methylpseudouridine ($me^1\Psi$).

83. The system of any one of claims 61 to 75, wherein the guide RNA comprises a chemically modified ribose.

84. The system of claim 83, wherein the chemically modified ribose is selected from 2′-*O*-methyl (2′-*O*-Me), 2′-Fluoro (2′-F), 2′-deoxy-2′-fluoro-beta-D-arabino-nucleic acid (2′F-ANA), 4′-S, 4′-SFANA, 2′-azido, UNA, 2′-*O*-methoxy-ethyl (2′-*O*-ME), 2′-*O*-Allyl, 2′-*O*-Ethylamine, 2′-*O*-Cyanoethyl, Locked nucleic acid (LAN), Methylene-cLAN, N-MeO-amino BNA, or N-MeO-aminooxy BNA.

85. The system of claim 84, wherein the chemically modified ribose is selected from 2′-O-methyl (2′-O-Me) or 2′-Fluoro (2′-F).

86. The system of claim 84, wherein the chemically modified ribose is 2′-Fluoro (2′-F).

87. The system of any one of claims 61 to 75, wherein the guide RNA comprises a chemically modified phosphodiester linkage.

88. The system of claim 87, wherein chemically modified phosphodiester linkage is selected from Phosphorothioate (PS), Boranophosphate, phosphodithioate (PS2), 3′,5′-amide, N3′-phosphoramidate (NP), Phosphodiester (PO), or 2′,5′-phosphodiester (2′,5′-PO).

89. The system of claim 88, wherein the chemically modified phosphodiester linkage is phosphorothioate.

90. A method of RNA-guided genome editing, the method comprising:

introducing into a cell of the subject:

a) a guide RNA that hybridizes with a target sequence of a DNA molecule in a eukaryotic cell that contains the DNA molecule, and

b) an mRNA encoding a Cpf1 protein;

wherein the guide RNA comprises at least one chemically modified nucleotide;

wherein the mRNA encoding a Cpf1 protein comprises at least one chemically modified nucleotide; and

wherein the guide RNA hybridizes with the target sequence and the Cpf1 protein cleaves the DNA molecule.

91. The method of claim 90, wherein the mRNA encoding a Cpf1 protein comprises at least one chemically modified nucleotide selected from a chemically modified nucleobase, a chemically

modified ribose, a chemically modified phosphodiester linkage, or a combination thereof.

92. The method of claim 91, wherein the mRNA encoding a Cpf1 protein comprises a chemically modified nucleobase.

93. The method of claim 92, wherein the chemically modified nucleobase is selected from 5-formylcytidine (5fC), 5-methylcytidine (5meC), 5-methoxycytidine (5moC), 5-hydroxycytidine (5hoC), 5-hydroxymethylcytidine (5hmC), 5-formyluridine (5fU), 5-methyluridine (5-meU), 5-methoxyuridine (5moU), 5-carboxymethylesteruridine (5camU), pseudouridine (Ψ), N^1-methylpseudouridine ($me^1\Psi$), N^6-methyladenosine (me^6A), or thienoguanosine (^{th}G).

94. The method of claim 93, wherein the chemically modified nucleobase is selected from 5-methoxyuridine (5moU), pseudouridine (Ψ), and N^1-methylpseudouridine ($me^1\Psi$).

95. The method of claim 94, wherein the chemically modified nucleobase is 5-methoxyuridine (5moU).

96. The method of claim 94, wherein the chemically modified nucleobase is pseudouridine (Ψ).

97. The method of claim 94, wherein the chemically modified nucleobase is N^1-methylpseudouridine ($me^1\Psi$).

98. The method of claim 91, wherein the mRNA encoding a Cpf1 protein comprises a chemically modified ribose.

99. The method of claim 98, wherein the chemically modified ribose is selected from 2′-*O*-methyl (2′-*O*-Me), 2′-Fluoro (2′-F), 2′-deoxy-2′-fluoro-beta-D-arabino-nucleic acid (2′F-ANA), 4′-S, 4′-SFANA, 2′-azido, UNA, 2′-*O*-methoxy-ethyl (2′-*O*-ME), 2′-*O*-Allyl, 2′-*O*-Ethylamine, 2′-*O*-Cyanoethyl, Locked nucleic acid (LAN), Methylene-cLAN, N-MeO-amino BNA, or N-MeO-aminooxy BNA.

100. The method of claim 99, wherein the chemically modified ribose is selected from 2′-O-methyl (2′-O-Me) or 2′-Fluoro (2′-F).

101. The method of claim 100, wherein the chemically modified ribose is 2′-Fluoro (2′-F).

102. The method of claim 91, wherein the mRNA encoding a Cpf1 protein comprises a chemically modified phosphodiester linkage.

103. The method of claim 102, wherein chemically modified phosphodiester linkage is selected from Phosphorothioate (PS), Boranophosphate, phosphodithioate (PS2), 3′,5′-amide, N3′-phosphoramidate (NP), Phosphodiester (PO), or 2′,5′-phosphodiester (2′,5′-PO).

104. The method of claim 103, wherein the chemically modified phosphodiester linkage is phosphorothioate.

105. The method of any one of claims 90 to 104, wherein the guide RNA comprises at least one chemically modified nucleotide selected from a chemically modified nucleobase, a chemically modified ribose, a chemically modified phosphodiester linkage, or a combination thereof.

106. The method of claim 105, wherein the guide RNA comprises a chemically modified nucleobase.

107. The method of claim 106, wherein the chemically modified nucleobase is selected from 5-formylcytidine (5fC), 5-methylcytidine (5meC), 5-methoxycytidine (5moC), 5-hydroxycytidine (5hoC), 5-hydroxymethylcytidine (5hmC), 5-formyluridine (5fU), 5-methyluridine (5-meU), 5-methoxyuridine (5moU), 5-carboxymethylesteruridine (5camU), pseudouridine (Ψ), N^1-methylpseudouridine ($me^1\Psi$), N^6-methyladenosine (me^6A), or thienoguanosine (^{th}G).

108. The method of claim 107, wherein the chemically modified nucleobase is selected from 5-methoxyuridine (5moU), pseudouridine (Ψ), and N^1-methylpseudouridine ($me^1\Psi$).

109. The method of claim 108, wherein the chemically modified nucleobase is 5-methoxyuridine (5moU).

110. The method of claim 108, wherein the chemically modified nucleobase is pseudouridine (Ψ).

111. The method of claim108, wherein the chemically modified nucleobase is N^1-methylpseudouridine ($me^1\Psi$).

112. The method of any one of claims 90 to 104, wherein the guide RNA comprises a chemically modified ribose.

113. The method of claim 112, wherein the chemically modified ribose is selected from 2′-*O*-methyl (2′-*O*-Me), 2′-Fluoro (2′-F), 2′-deoxy-2′-fluoro-beta-D-arabino-nucleic acid (2′F-ANA), 4′-S, 4′-SFANA, 2′-azido, UNA, 2′-*O*-methoxy-ethyl (2′-*O*-ME), 2′-*O*-Allyl, 2′-*O*-Ethylamine, 2′-*O*-Cyanoethyl, Locked nucleic acid (LAN), Methylene-cLAN, N-MeO-amino BNA, or N-MeO-aminooxy BNA.

114. The method of claim 113, wherein the chemically modified ribose is selected from 2′-O-methyl (2′-O-Me) or 2′-Fluoro (2′-F).

115. The method of claim 113, wherein the chemically modified ribose is 2′-Fluoro (2′-F).

116. The method of any one of claims 90 to 104, wherein the guide RNA comprises a chemically modified phosphodiester linkage.

117. The method of claim 116, wherein chemically modified phosphodiester linkage is

selected from Phosphorothioate (PS), Boranophosphate, phosphodithioate (PS2), 3′,5′-amide, N3′-phosphoramidate (NP), Phosphodiester (PO), or 2′,5′-phosphodiester (2′,5′-PO).

118. The method of claim 117, wherein the chemically modified phosphodiester linkage is phosphorothioate or phosphodithioate (PS2).

119. The method of claim 117, wherein the chemically modified phosphodiester linkage is phosphorothioate.

120. The method of claim 117, wherein the chemically modified phosphodiester linkage is phosphodithioate (PS2).

121. A genome editing system comprising:

a) a guide RNA that hybridizes with a target sequence of a DNA molecule in a eukaryotic cell that contains the DNA molecule, and

b) a DNA vector comprising a regulatory element operable in a eukaryotic cell operably linked to a nucleotide sequence encoding a Cpf1 protein; an mRNA encoding a Cpf1 protein; or a Cpf1 protein;

wherein the guide RNA comprises at least one chemically modified nucleotide, and

wherein the guide RNA hybridizes with the target sequence and the Cpf1 protein cleaves the DNA molecule.

122. The system of claim 121, wherein the at least one chemically modified nucleotide comprises a chemically modified ribose, a chemically modified phosphodiester linkage, a chemically modified nucleobase, or a combination thereof.

123. The system of claim 121, wherein the at least one chemically modified nucleotide is a chemically modified ribose.

124. The system of claim 123, wherein the chemically modified ribose is selected from 2′-*O*-methyl (2′-*O*-Me), 2′-Fluoro (2′-F), 2′-deoxy-2′-fluoro-beta-D-arabino-nucleic acid (2′F-ANA), 4′-S, 4′-SFANA, 2′-azido, UNA, 2′-*O*-methoxy-ethyl (2′-*O*-ME), 2′-*O*-Allyl, 2′-*O*-Ethylamine, 2′-*O*-Cyanoethyl, Locked nucleic acid (LAN), Methylene-cLAN, N-MeO-amino BNA, or N-MeO-aminooxy BNA.

125. The system of claim 124, wherein the chemically modified ribose is selected from 2′-O-methyl (2′-O-Me) or 2′-Fluoro (2′-F).

126. The system of claim 121, wherein the at least one chemically modified nucleotide is a chemically modified nucleobase.

127. The system of claim 126, wherein the chemically modified nucleobase is selected from 5-formylcytidine (5fC), 5-methylcytidine (5meC), 5-methoxycytidine (5moC), 5-hydroxycytidine (5hoC), 5-hydroxymethylcytidine (5hmC), 5-formyluridine (5fU), 5-

methyluridine (5-meU), 5-methoxyuridine (5moU), 5-carboxymethylesteruridine (5camU), pseudouridine (Ψ), N^1-methylpseudouridine ($me^1\Psi$), N^6-methyladenosine (me^6A), or thienoguanosine (^{th}G).

128. The system of claim 121, wherein the at least one chemically modified nucleotide is a chemically modified phosphodiester linkage.

129. The system of claim 128, wherein chemically modified phosphodiester linkage is selected from Phosphorothioate (PS), Boranophosphate, phosphodithioate (PS2), 3′,5′-amide, N3′-phosphoramidate (NP), Phosphodiester (PO), or 2′,5′-phosphodiester (2′,5′-PO).

130. The system of claim 129, wherein the chemically modified phosphodiester linkage is phosphorothioate.

131. The system of any one of claims 121-130, wherein the at least one chemically modified nucleotide confers increased Cpf1 nuclease activity as compared to a corresponding guide RNA not having the chemical modification.

132. The system of any one of claims 121-130, wherein the at least one chemically modified nucleotide confers reduced toxicity as compared to a corresponding guide RNA not having the chemical modification.

133. The system of any one of claims 121-130, wherein the at least one chemically modified nucleotide confers decreased off-target effects as compared to a corresponding guide RNA not having the chemical modification.

134. A method of increasing the efficiency of RNA-guided genome editing in a cell, the method comprising:

introducing into a cell of the subject:

a) a guide RNA that hybridizes with a target sequence of a DNA molecule in a eukaryotic cell that contains the DNA molecule, and

b) a DNA vector comprising a regulatory element operable in a eukaryotic cell operably linked to a nucleotide sequence encoding a Cpf1 protein; an mRNA encoding a Cpf1 protein; or a Cpf1 protein;

wherein the guide RNA comprises at least one chemically modified nucleotide, and

wherein the at least one chemically modified nucleotide confers increased Cpf1 nuclease activity as compared to a corresponding guide RNA not having the chemical modification.

135. The method of claim 134, wherein the at least one chemically modified nucleotide comprises a chemically modified ribose, a chemically modified phosphodiester linkage, a chemically modified nucleobase, or a combination thereof.

136. The method of claim 134, wherein the at least one chemically modified nucleotide

is a chemically modified ribose.

137. The method of claim 136, wherein the chemically modified ribose is selected from 2′-*O*-methyl (2′-*O*-Me), 2′-Fluoro (2′-F), 2′-deoxy-2′-fluoro-beta-D-arabino-nucleic acid (2′F-ANA), 4′-S, 4′-SFANA, 2′-azido, UNA, 2′-*O*-methoxy-ethyl (2′-*O*-ME), 2′-*O*-Allyl, 2′-*O*-Ethylamine, 2′-*O*-Cyanoethyl, Locked nucleic acid (LAN), Methylene-cLAN, N-MeO-amino BNA, or N-MeO-aminooxy BNA.

138. The method of claim 137, wherein the chemically modified ribose is selected from 2′-O-methyl (2′-O-Me) or 2′-Fluoro (2′-F).

139. The method of claim 134, wherein the at least one chemically modified nucleotide is a chemically modified nucleobase.

140. The method of claim 139, wherein the chemically modified nucleobase is selected from 5-formylcytidine (5fC), 5-methylcytidine (5meC), 5-methoxycytidine (5moC), 5-hydroxycytidine (5hoC), 5-hydroxymethylcytidine (5hmC), 5-formyluridine (5fU), 5-methyluridine (5-meU), 5-methoxyuridine (5moU), 5-carboxymethylesteruridine (5camU), pseudouridine (Ψ), N^1-methylpseudouridine ($me^1\Psi$), N^6-methyladenosine (me^6A), or thienoguanosine (^{th}G).

141. The method of claim 134, wherein the at least one chemically modified nucleotide is a chemically modified phosphodiester linkage.

142. The method of claim 141, wherein the chemically modified phosphodiester linkage is selected from Phosphorothioate (PS), Boranophosphate, phosphodithioate (PS2), 3′,5′-amide, N3′-phosphoramidate (NP), Phosphodiester (PO), or 2′,5′-phosphodiester (2′,5′-PO).

143. The method of claim 142, wherein the chemically modified phosphodiester linkage is phosphorothioate.

144. A method of decreasing the off-target effects of RNA-guided genome editing in a cell, the method comprising:

introducing into a cell of the subject:

a) a guide RNA that hybridizes with a target sequence of a DNA molecule in a eukaryotic cell that contains the DNA molecule, and

b) a DNA vector comprising a regulatory element operable in a eukaryotic cell operably linked to a nucleotide sequence encoding a Cpf1 protein; an mRNA encoding a Cpf1 protein; or a Cpf1 protein;

wherein the guide RNA comprises at least one chemically modified nucleotide, and

wherein the at least one chemically modified nucleotide confers decreased off-target effects as compared to a corresponding guide RNA not having the chemical modification.

145. The method of claim 144, wherein the at least one chemically modified nucleotide comprises a chemically modified ribose, a chemically modified phosphodiester linkage, a chemically modified nucleobase, or a combination thereof.

146. The method of claim 144, wherein the at least one chemically modified nucleotide is a chemically modified ribose.

147. The method of claim 146, wherein the chemically modified ribose is selected from 2′-*O*-methyl (2′-*O*-Me), 2′-Fluoro (2′-F), 2′-deoxy-2′-fluoro-beta-D-arabino-nucleic acid (2′F-ANA), 4′-S, 4′-SFANA, 2′-azido, UNA, 2′-*O*-methoxy-ethyl (2′-*O*-ME), 2′-*O*-Allyl, 2′-*O*-Ethylamine, 2′-*O*-Cyanoethyl, Locked nucleic acid (LAN), Methylene-cLAN, N-MeO-amino BNA, or N-MeO-aminooxy BNA.

148. The method of claim 147, wherein the chemically modified ribose is selected from 2′-O-methyl (2′-O-Me) or 2′-Fluoro (2′-F).

149. The method of claim 144, wherein the at least one chemically modified nucleotide is a chemically modified nucleobase.

150. The method of claim 149, wherein the chemically modified nucleobase is selected from 5-formylcytidine (5fC), 5-methylcytidine (5meC), 5-methoxycytidine (5moC), 5-hydroxycytidine (5hoC), 5-hydroxymethylcytidine (5hmC), 5-formyluridine (5fU), 5-methyluridine (5-meU), 5-methoxyuridine (5moU), 5-carboxymethylesteruridine (5camU), pseudouridine (Ψ), N^1-methylpseudouridine ($me^1\Psi$), N^6-methyladenosine (me^6A), or thienoguanosine (^{th}G).

151. The method of claim 144, wherein the at least one chemically modified nucleotide is a chemically modified phosphodiester linkage.

152. The method of claim 151, wherein the chemically modified phosphodiester linkage is selected from Phosphorothioate (PS), Boranophosphate, phosphodithioate (PS2), 3′,5′-amide, N3′-phosphoramidate (NP), Phosphodiester (PO), or 2′,5′-phosphodiester (2′,5′-PO).

153. The method of claim 152, wherein the chemically modified phosphodiester linkage is phosphorothioate.

154. A method of reducing toxicity of RNA-guided genome editing in a cell, the method comprising:

introducing into a cell of the subject:

a) a guide RNA that hybridizes with a target sequence of a DNA molecule in a eukaryotic cell that contains the DNA molecule, and

b) a DNA vector comprising a regulatory element operable in a eukaryotic cell operably linked to a nucleotide sequence encoding a Cpf1 protein; an mRNA encoding a Cpf1 protein; or a Cpf1 protein;

wherein the guide RNA comprises at least one chemically modified nucleotide, and

wherein the at least one chemically modified nucleotide confers reduced toxicity as compared to a corresponding guide RNA not having the chemical modification.

155. The method of claim 154, wherein the at least one chemically modified nucleotide comprises a chemically modified ribose, a chemically modified phosphodiester linkage, a chemically modified nucleobase, or a combination thereof.

156. The method of claim 154, wherein the at least one chemically modified nucleotide is a chemically modified ribose.

157. The method of claim 156, wherein the chemically modified ribose is selected from 2′-*O*-methyl (2′-*O*-Me), 2′-Fluoro (2′-F), 2′-deoxy-2′-fluoro-beta-D-arabino-nucleic acid (2′F-ANA), 4′-S, 4′-SFANA, 2′-azido, UNA, 2′-*O*-methoxy-ethyl (2′-*O*-ME), 2′-*O*-Allyl, 2′-*O*-Ethylamine, 2′-*O*-Cyanoethyl, Locked nucleic acid (LAN), Methylene-cLAN, N-MeO-amino BNA, or N-MeO-aminooxy BNA.

158. The method of claim 157, wherein the chemically modified ribose is selected from 2′-O-methyl (2′-O-Me) or 2′-Fluoro (2′-F).

159. The method of claim 154, wherein the at least one chemically modified nucleotide is a chemically modified nucleobase.

160. The method of claim 159, wherein the chemically modified nucleobase is selected from 5-formylcytidine (5fC), 5-methylcytidine (5meC), 5-methoxycytidine (5moC), 5-hydroxycytidine (5hoC), 5-hydroxymethylcytidine (5hmC), 5-formyluridine (5fU), 5-methyluridine (5-meU), 5-methoxyuridine (5moU), 5-carboxymethylesteruridine (5camU), pseudouridine (Ψ), N^1-methylpseudouridine ($me^1\Psi$), N^6-methyladenosine (me^6A), or thienoguanosine (^{th}G).

161. The method of claim 154, wherein the at least one chemically modified nucleotide is a chemically modified phosphodiester linkage.

162. The method of claim 161, wherein the chemically modified phosphodiester linkage is selected from Phosphorothioate (PS), Boranophosphate, phosphodithioate (PS2), 3′,5′-amide, N3′-phosphoramidate (NP), Phosphodiester (PO), or 2′,5′-phosphodiester (2′,5′-PO).

163. The method of claim 162, wherein the chemically modified phosphodiester linkage is phosphorothioate.

164. A genome editing system comprising:

a) an engineered guide RNA that hybridizes with a target sequence of a DNA molecule in a eukaryotic cell that contains the DNA molecule, and

b) a DNA vector comprising a regulatory element operable in a eukaryotic cell operably linked to a nucleotide sequence encoding a Cpf1 protein; an mRNA encoding a Cpf1 protein; or a Cpf1 protein;

wherein the engineered guide RNA comprises at least one nucleotide insertion or deletion.

165. The system of claim 164, wherein the engineered guide RNA hybridizes with the target sequence and the Cpf1 protein cleaves the DNA molecule.

166. The system of claim 164, wherein the engineered guide RNA comprises at least one nucleotide insertion.

167. The system of claim 164, wherein the engineered guide RNA comprises at least four nucleotide insertions.

168. The system of claim 164, wherein the engineered guide RNA comprises from four to twelve nucleotide insertions.

169. The system of claim 164, wherein the engineered guide RNA comprises at least one nucleotide deletion.

170. The system of claim 164, wherein the engineered guide RNA comprises at least two nucleotide deletion.

171. The system of claim 164, wherein the engineered guide RNA comprises from two to eight nucleotide deletion.

172. A method of RNA-guided genome editing, the method comprising:

introducing into a cell of the subject:

a) an engineered guide RNA that hybridizes with a target sequence of a DNA molecule in a eukaryotic cell that contains the DNA molecule, and

b) a DNA vector comprising a regulatory element operable in a eukaryotic cell operably linked to a nucleotide sequence encoding a Cpf1 protein; an mRNA encoding a Cpf1 protein; or a Cpf1 protein;

wherein the engineered guide RNA comprises at least one nucleotide insertion or deletion; and

wherein the guide RNA hybridizes with the target sequence and the Cpf1 protein cleaves the DNA molecule.

173. The method of claim 172, wherein the engineered guide RNA comprises at least one nucleotide insertion.

174. The method of claim 172, wherein the engineered guide RNA comprises at least four nucleotide insertions.

175. The method of claim 172, wherein the engineered guide RNA comprises from four to twelve nucleotide insertions.

176. The method of claim 172, wherein the engineered guide RNA comprises at least one nucleotide deletion.

177. The method of claim 172, wherein the engineered guide RNA comprises at least two nucleotide deletion.

178. The method of claim 172, wherein the engineered guide RNA comprises from two to eight nucleotide deletion.

179. A genome editing system comprising:

a) an engineered guide RNA that hybridizes with a target sequence of a DNA molecule in a eukaryotic cell that contains the DNA molecule, and

b) an mRNA encoding a Cpf1 protein;

wherein the engineered guide RNA comprises at least one nucleotide insertion or deletion; and

wherein the mRNA encoding a Cpf1 protein comprises at least one chemically modified nucleotide.

180. The system of claim 179, wherein the engineered guide RNA hybridizes with the target sequence and the Cpf1 protein cleaves the DNA molecule.

181. The system of claim 179, wherein the engineered guide RNA comprises at least one nucleotide insertion.

182. The system of claim 179, wherein the engineered guide RNA comprises at least four nucleotide insertions.

183. The system of claim 179, wherein the engineered guide RNA comprises from four to twelve nucleotide insertions.

184. The system of claim 179, wherein the engineered guide RNA comprises at least one nucleotide deletion.

185. The system of claim 179, wherein the engineered guide RNA comprises at least two nucleotide deletion.

186. The system of claim 179, wherein the engineered guide RNA comprises from two to eight nucleotide deletion.

187. A method of RNA-guided genome editing, the method comprising:

introducing into a cell of the subject:

a) an engineered guide RNA that hybridizes with a target sequence of a DNA molecule in a eukaryotic cell that contains the DNA molecule, and

b) an mRNA encoding a Cpf1 protein;

wherein the engineered guide RNA comprises at least one nucleotide insertion or deletion; and

wherein the mRNA encoding a Cpf1 protein comprises at least one chemically modified nucleotide; and

wherein the guide RNA hybridizes with the target sequence and the Cpf1 protein cleaves the DNA molecule.

188. The method of claim 187, wherein the engineered guide RNA comprises at least one nucleotide insertion.

189. The method of claim 187, wherein the engineered guide RNA comprises at least four nucleotide insertions.

190. The method of claim 187, wherein the engineered guide RNA comprises from four to twelve nucleotide insertions.

191. The method of claim 187, wherein the engineered guide RNA comprises at least one nucleotide deletion.

192. The method of claim 187, wherein the engineered guide RNA comprises at least two nucleotide deletion.

193. The method of claim 187, wherein the engineered guide RNA comprises from two to eight nucleotide deletion.

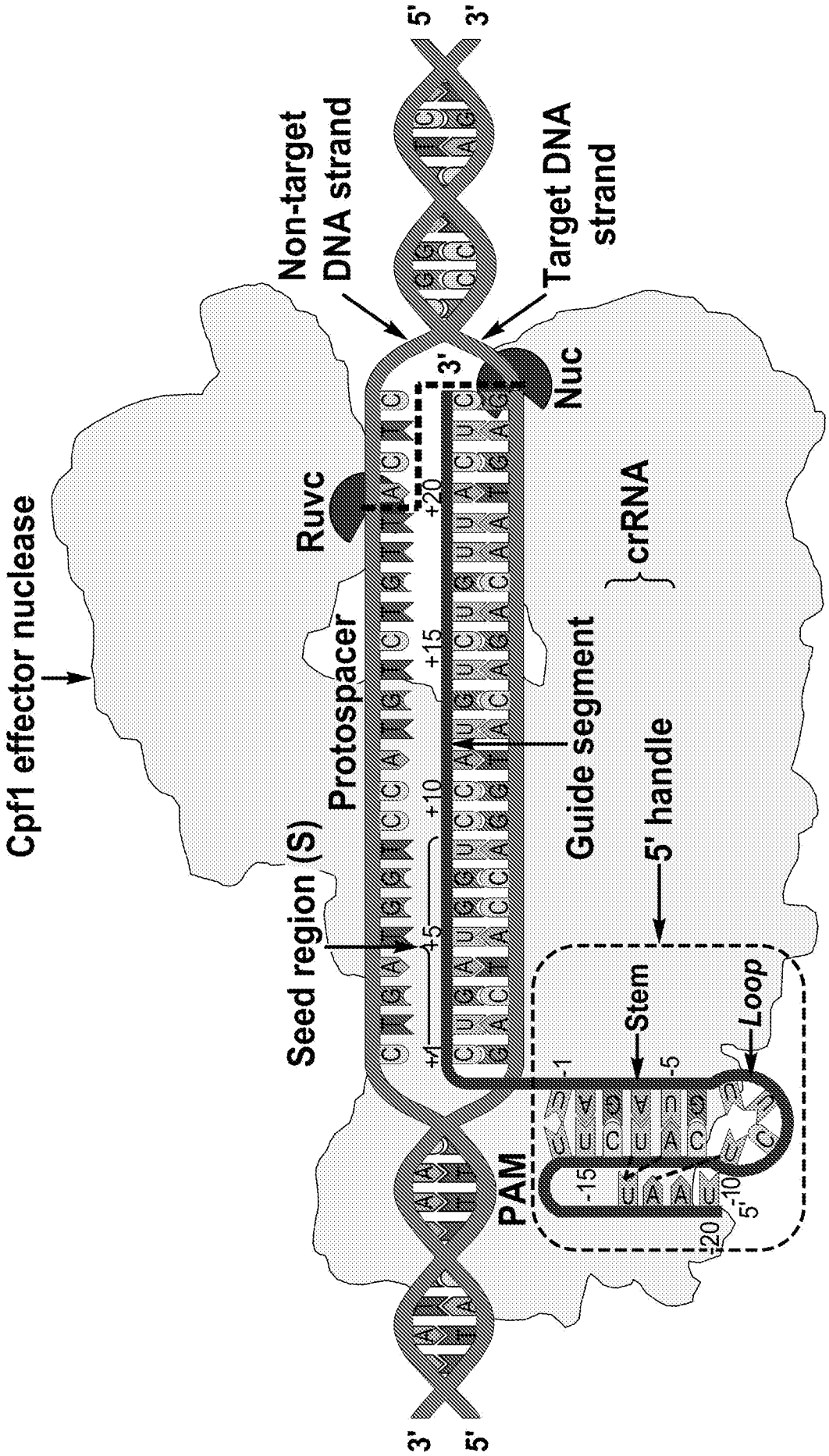

Cpf1 effector nuclease
Non-target DNA strand
Target DNA strand
5'
3'
Ruvc
Nuc
Protospacer
Seed region (S)
Guide segment
crRNA
5' handle
Stem
Loop
PAM
+1
+5
+10
+15
+20
-1
-5
-10
-15
-20

FIG. 1

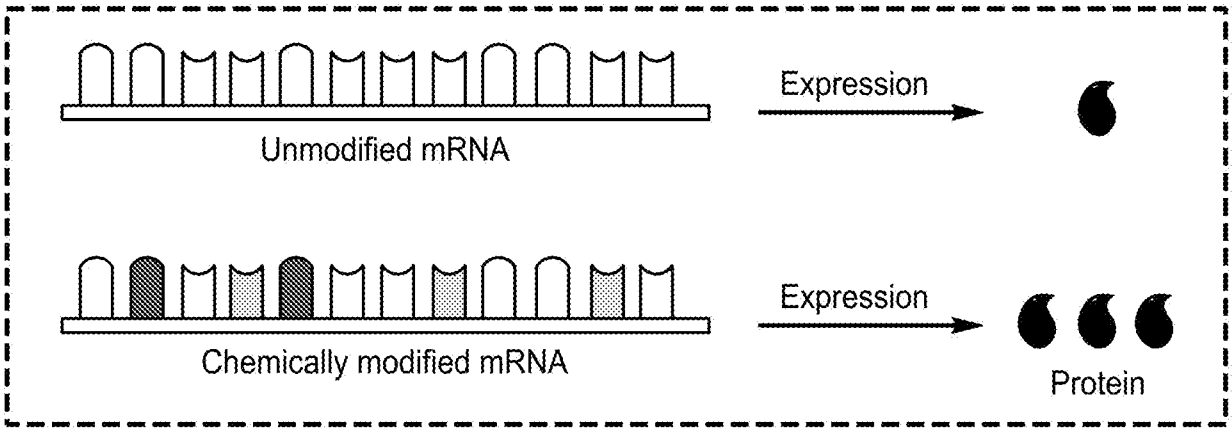

Unmodified mRNA
Expression
Chemically modified mRNA
Expression
Protein

FIG. 2

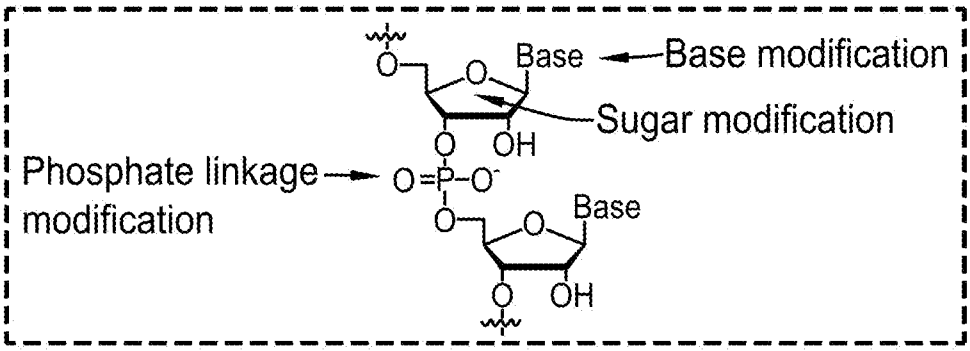

Base
Base modification
Sugar modification
Phosphate linkage modification
O=P-O
Base
OH
OH

FIG. 3A

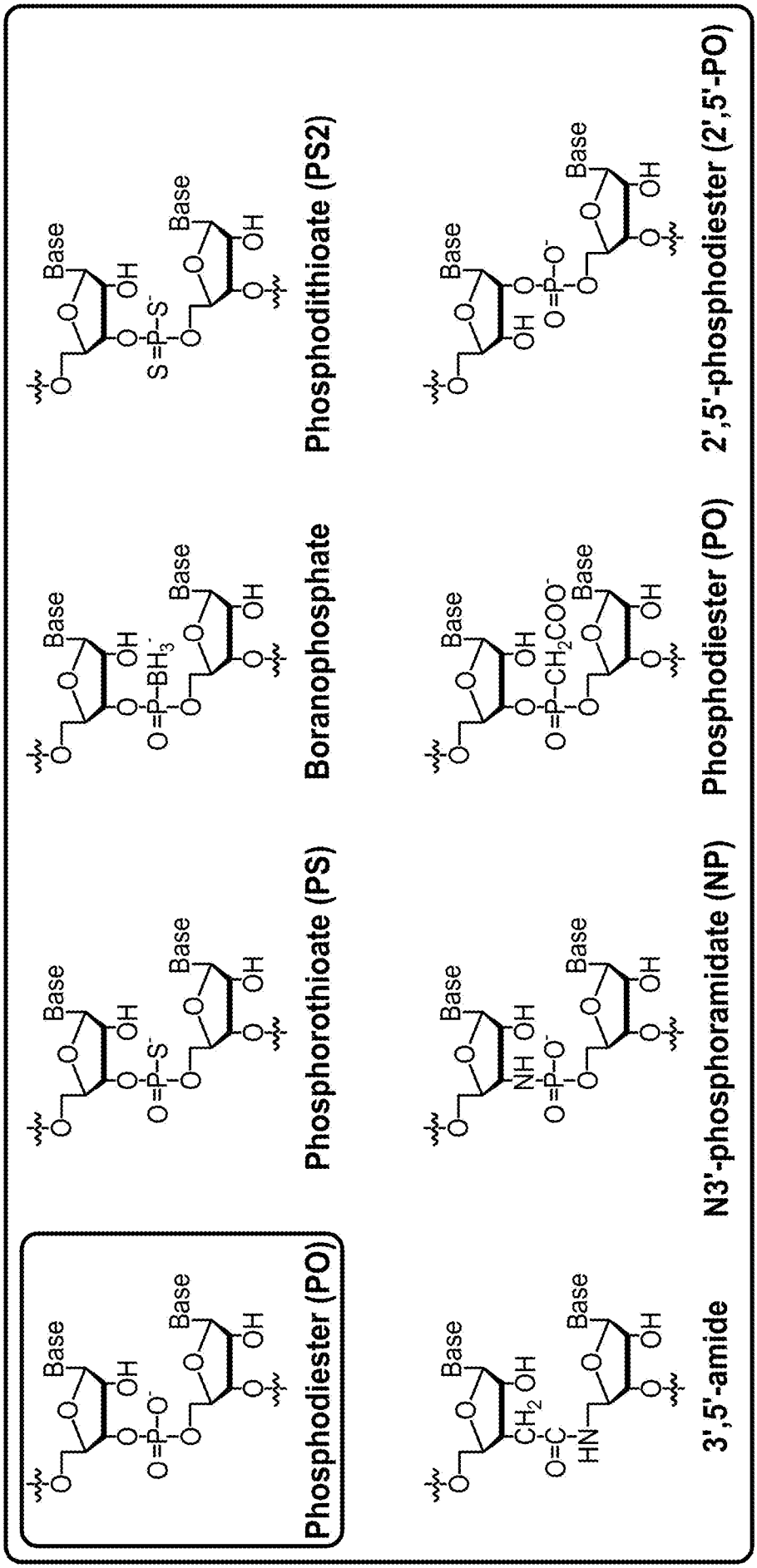

Phosphodiester (PO)
Phosphorothioate (PS)
Boranophosphate
Phosphodithioate (PS2)
3',5'-amide
N3'-phosphoramidate (NP)
Phosphodiester (PO)
2',5'-phosphodiester (2',5'-PO)

FIG. 3B

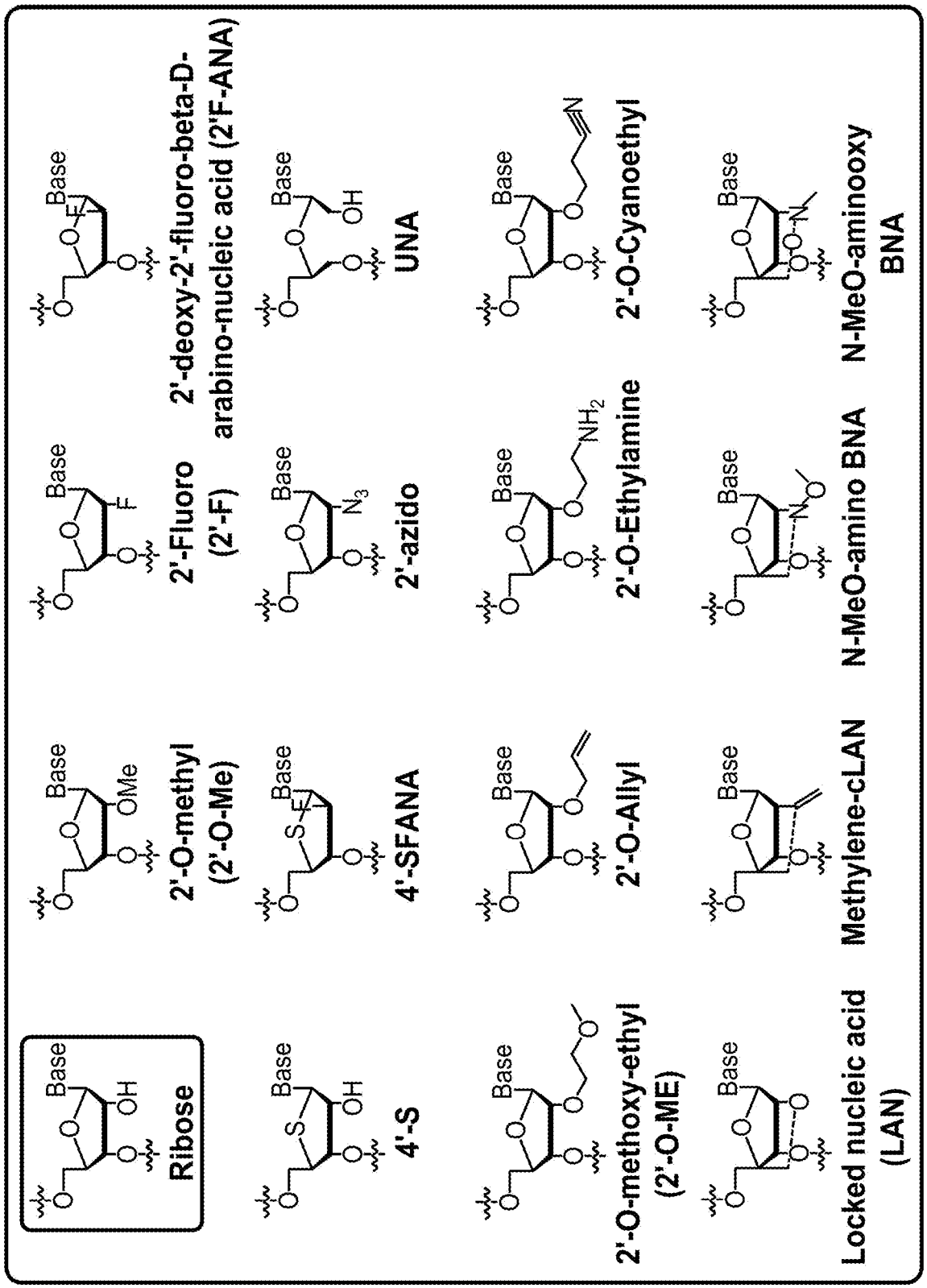

Ribose
2'-O-methyl (2'-O-Me)
2'-Fluoro (2'-F)
2'-deoxy-2'-fluoro-beta-D-arabino-nucleic acid (2'F-ANA)
4'-S
4'-SFANA
2'-azido
UNA
2'-O-methoxy-ethyl (2'-O-ME)
2'-O-Allyl
2'-O-Ethylamine
2'-O-Cyanoethyl
Locked nucleic acid (LAN)
Methylene-cLAN
N-MeO-amino BNA
N-MeO-aminooxy BNA

FIG. 3C

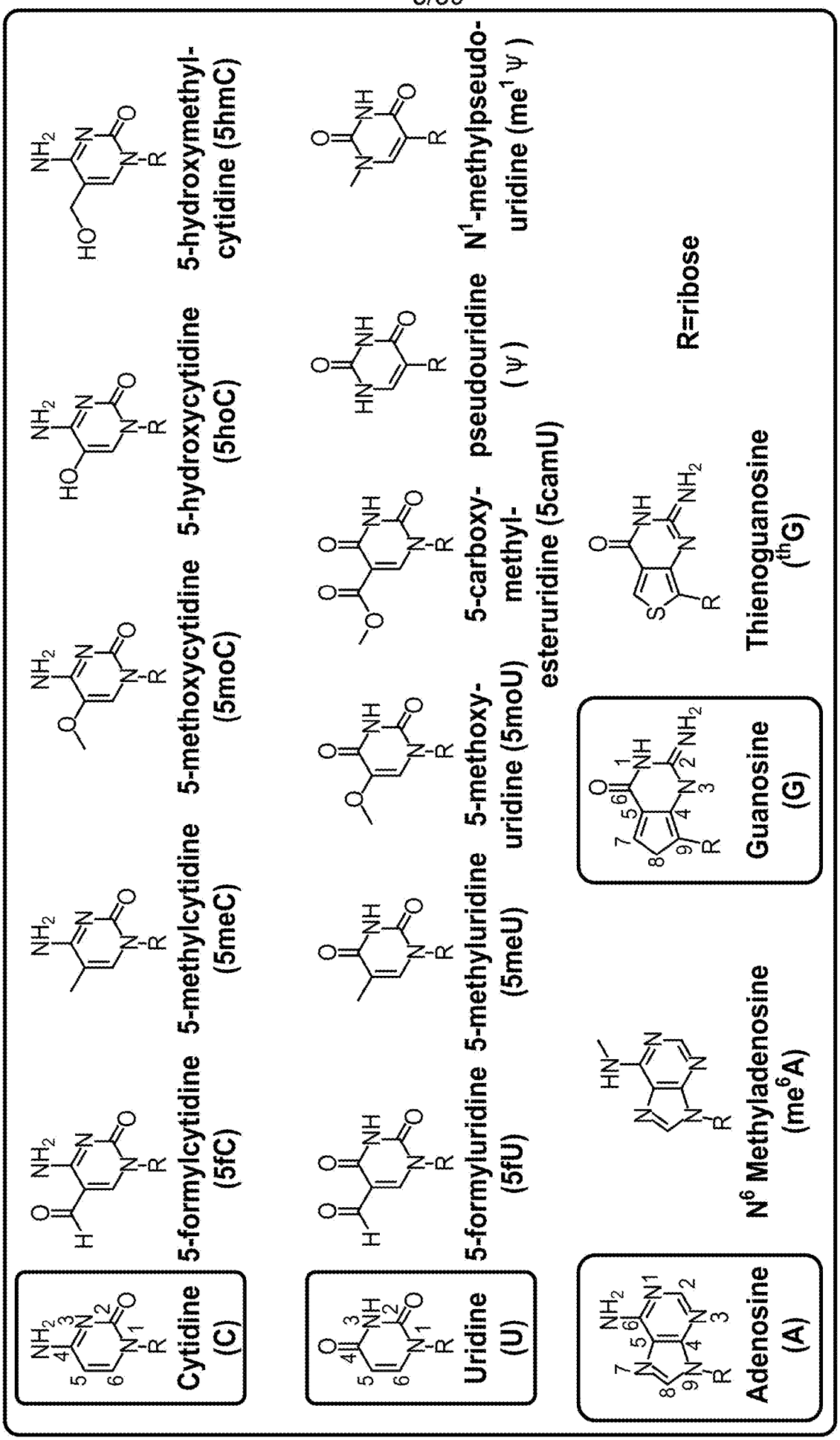

Cytidine (C)
5-formylcytidine (5fC)
5-methylcytidine (5meC)
5-methoxycytidine (5moC)
5-hydroxycytidine (5hoC)
5-hydroxymethyl-cytidine (5hmC)
Uridine (U)
5-formyluridine (5fU)
5-methyluridine (5meU)
5-methoxy-uridine (5moU)
5-carboxy-methyl-esteruridine (5camU)
pseudouridine (ψ)
N1-methylpseudo-uridine (me1ψ)
Adenosine (A)
N6 Methyladenosine (me6A)
Guanosine (G)
Thienoguanosine (thG)
R=ribose

FIG. 3D

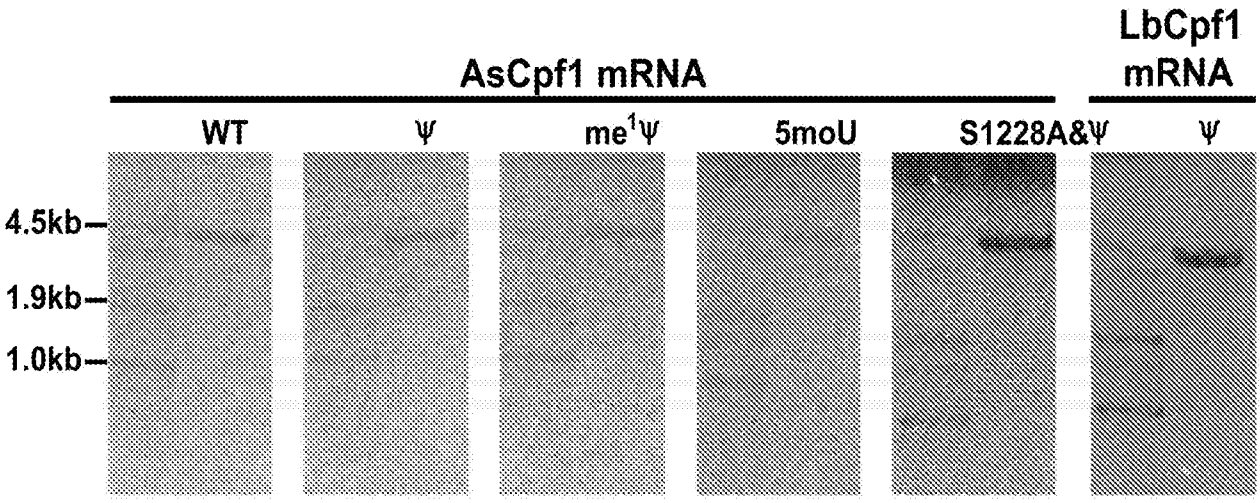

AsCpf1 mRNA
LbCpf1 mRNA
WT
Ψ
me1Ψ
5moU
S1228A&Ψ
Ψ
4.5kb
1.9kb
1.0kb

FIG. 4

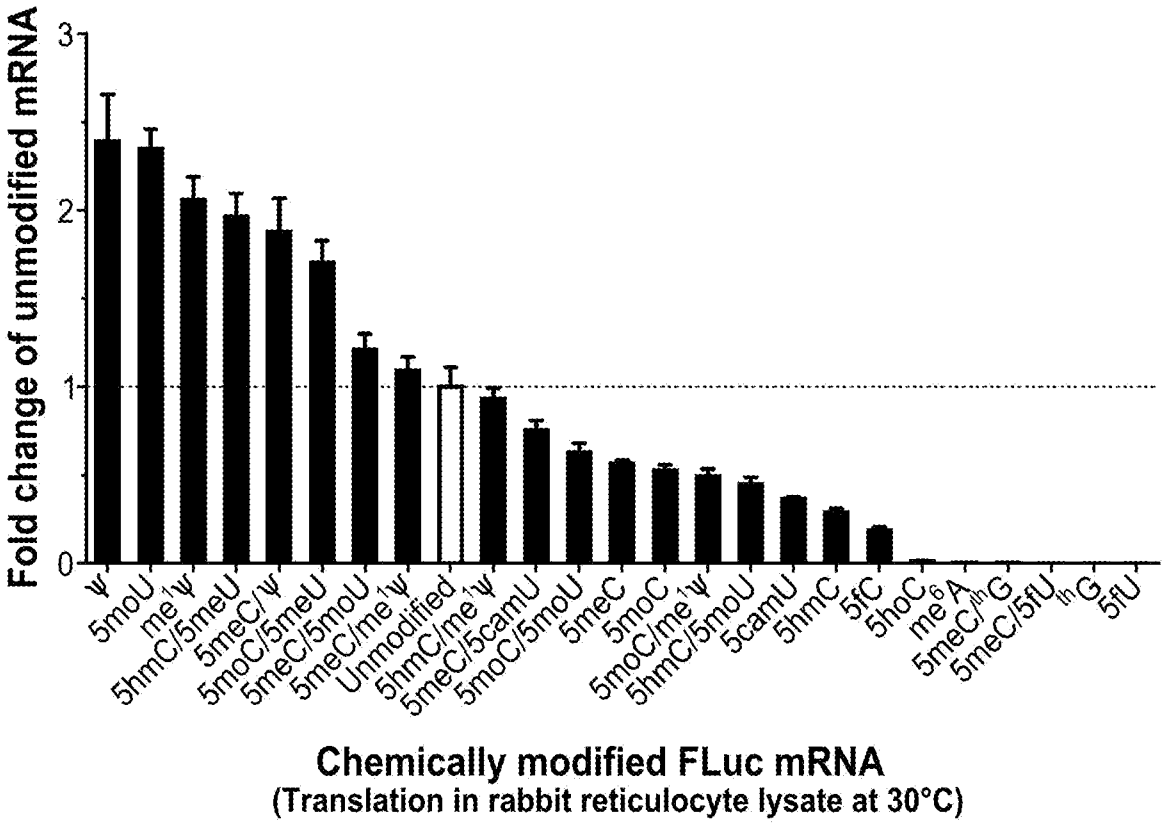

Fold change of unmodified mRNA
3
2
1
0
Ψ
5moU
me1Ψ
5hmC/5meU
5meC/Ψ
5moC/5meU
5meC/5moU
5meC/me1Ψ
Unmodified
5hmC/me1Ψ
5meC/5camU
5moC/5moU
5meC
5moC
5moC/me1Ψ
5hmC/5moU
5camU
5hmC
5fC
5hoC
me6A
5meC/inG
5meC/5fU
inG
5fU
Chemically modified FLuc mRNA
(Translation in rabbit reticulocyte lysate at 30°C)

FIG. 5A

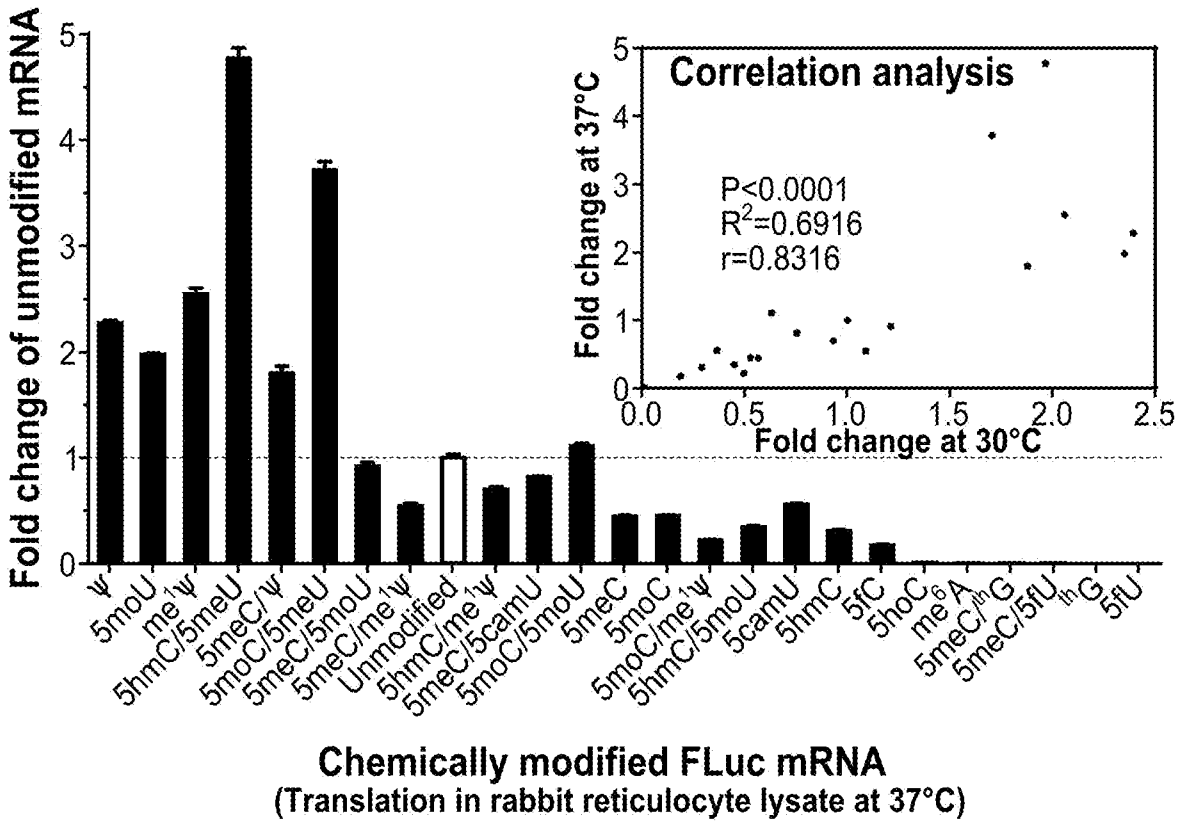

Fold change of unmodified mRNA
Correlation analysis
P<0.0001
R2=0.6916
r=0.8316
Fold change at 37°C
Fold change at 30°C
Ψ
5moU
me1Ψ
5hmC/5meU
5meC/Ψ
5moC/5meU
5meC/5moU
5meC/me1Ψ
Unmodified
5hmC/me1Ψ
5meC/5camU
5moC/5moU
5meC
5moC
5moC/me1Ψ
5hmC/5moU
5camU
5hmC
5fC
5hoC
me6A
5meC/thG
5meC/5fU
thG
5fU
Chemically modified FLuc mRNA
(Translation in rabbit reticulocyte lysate at 37°C)

FIG. 5B

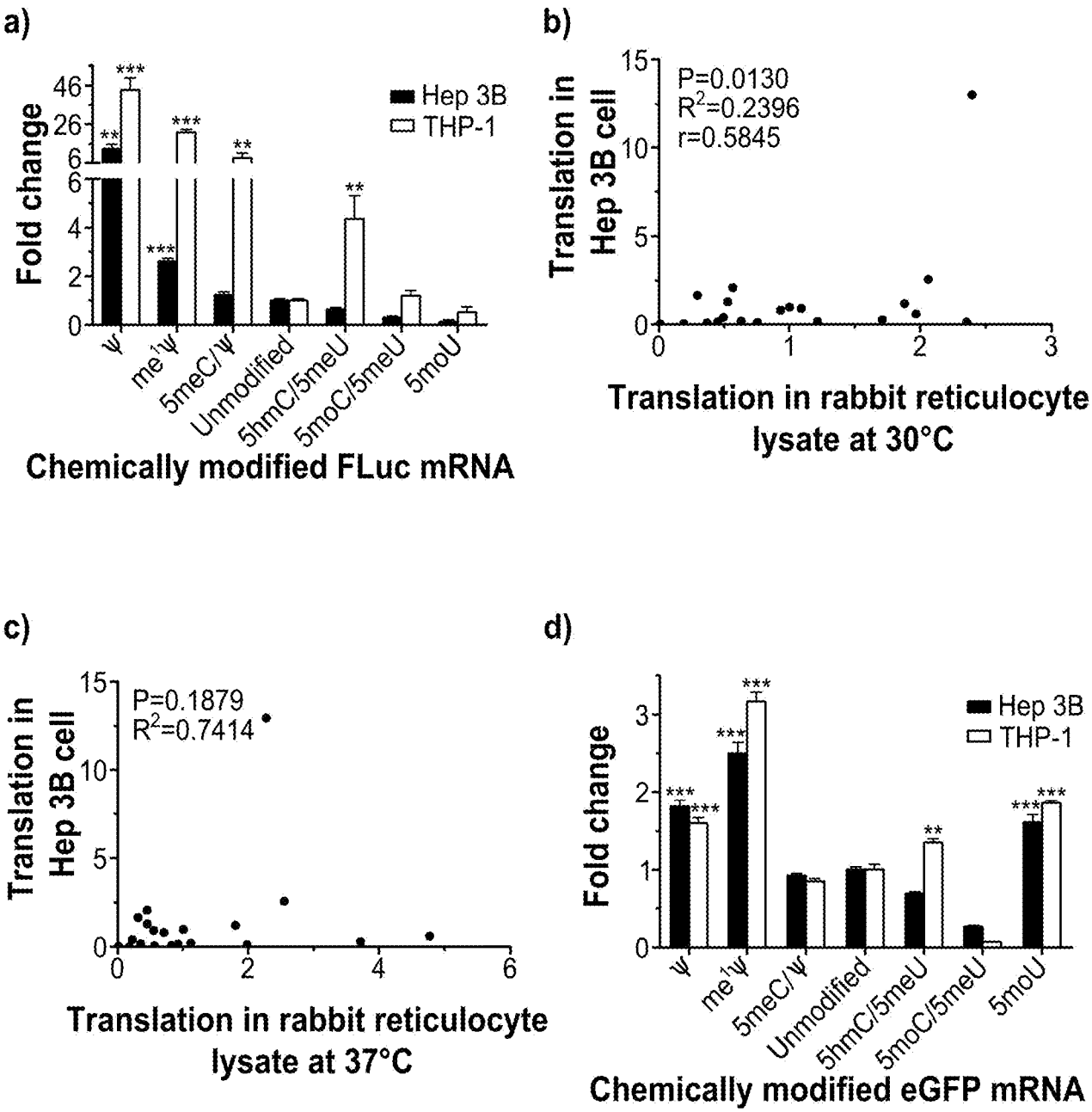

a)
Fold change
Hep 3B
THP-1
Ψ
me1Ψ
5meC/Ψ
Unmodified
5hmC/5meU
5moC/5meU
5moU
Chemically modified FLuc mRNA
b)
Translation in Hep 3B cell
P=0.0130
R2=0.2396
r=0.5845
Translation in rabbit reticulocyte lysate at 30°C
c)
Translation in Hep 3B cell
P=0.1879
R2=0.7414
Translation in rabbit reticulocyte lysate at 37°C
d)
Fold change
Hep 3B
THP-1
Chemically modified eGFP mRNA

FIG. 6

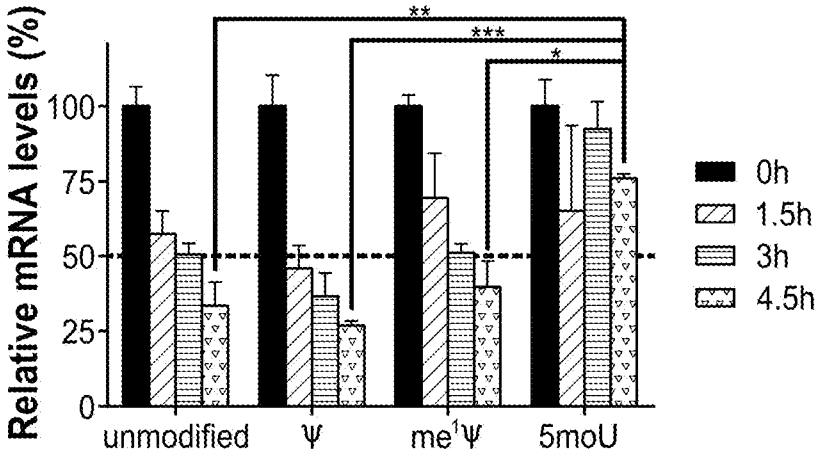

Relative mRNA levels (%)
100
75
50
25
0
**
***
*
0h
1.5h
3h
4.5h
unmodified
Ψ
me1Ψ
5moU

FIG. 7

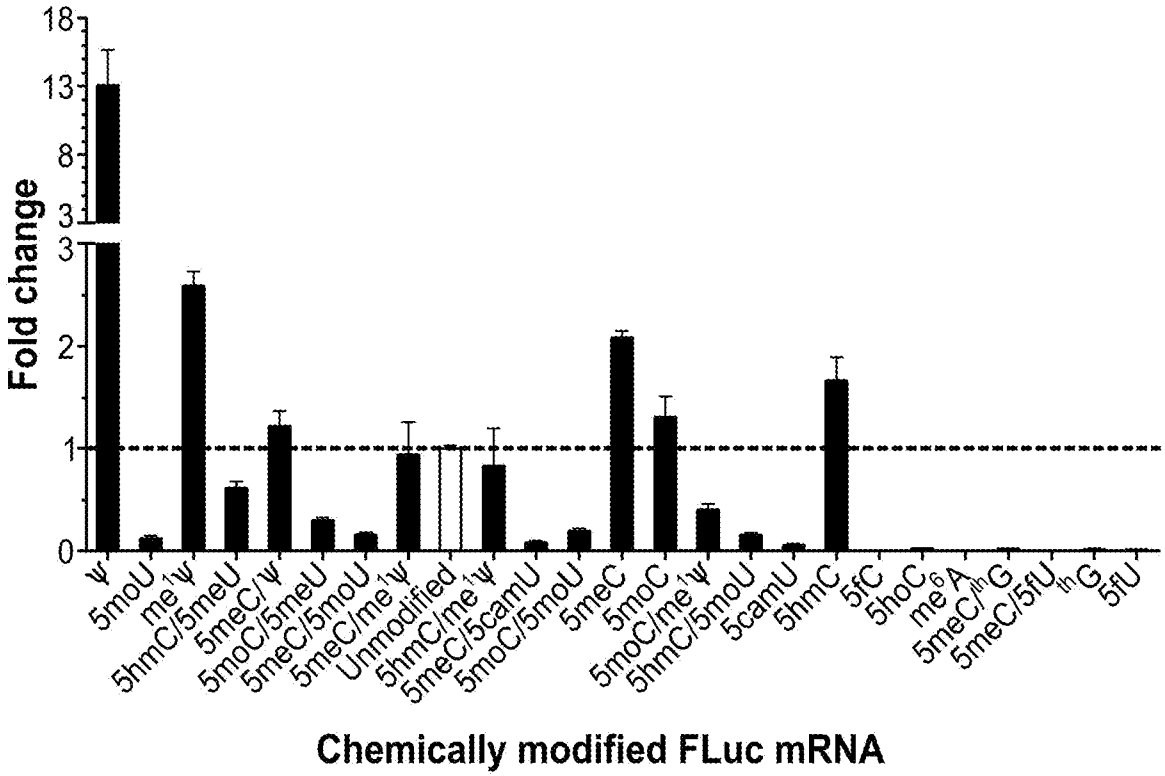

Fold change
18
13
8
3
3
2
1
0
Ψ
5moU
me1Ψ
5hmC/5meU
5meC/Ψ
5moC/5meU
5meC/5moU
5meC/me1Ψ
Unmodified
5hmC/me1Ψ
5meC/5camU
5moC/5moU
5meC
5moC
5moC/me1Ψ
5hmC/5moU
5camU
5hmC
5fC
5hoC
me6A
5meC/thG
5meC/5fU
thG
5fU
Chemically modified FLuc mRNA

FIG. 8

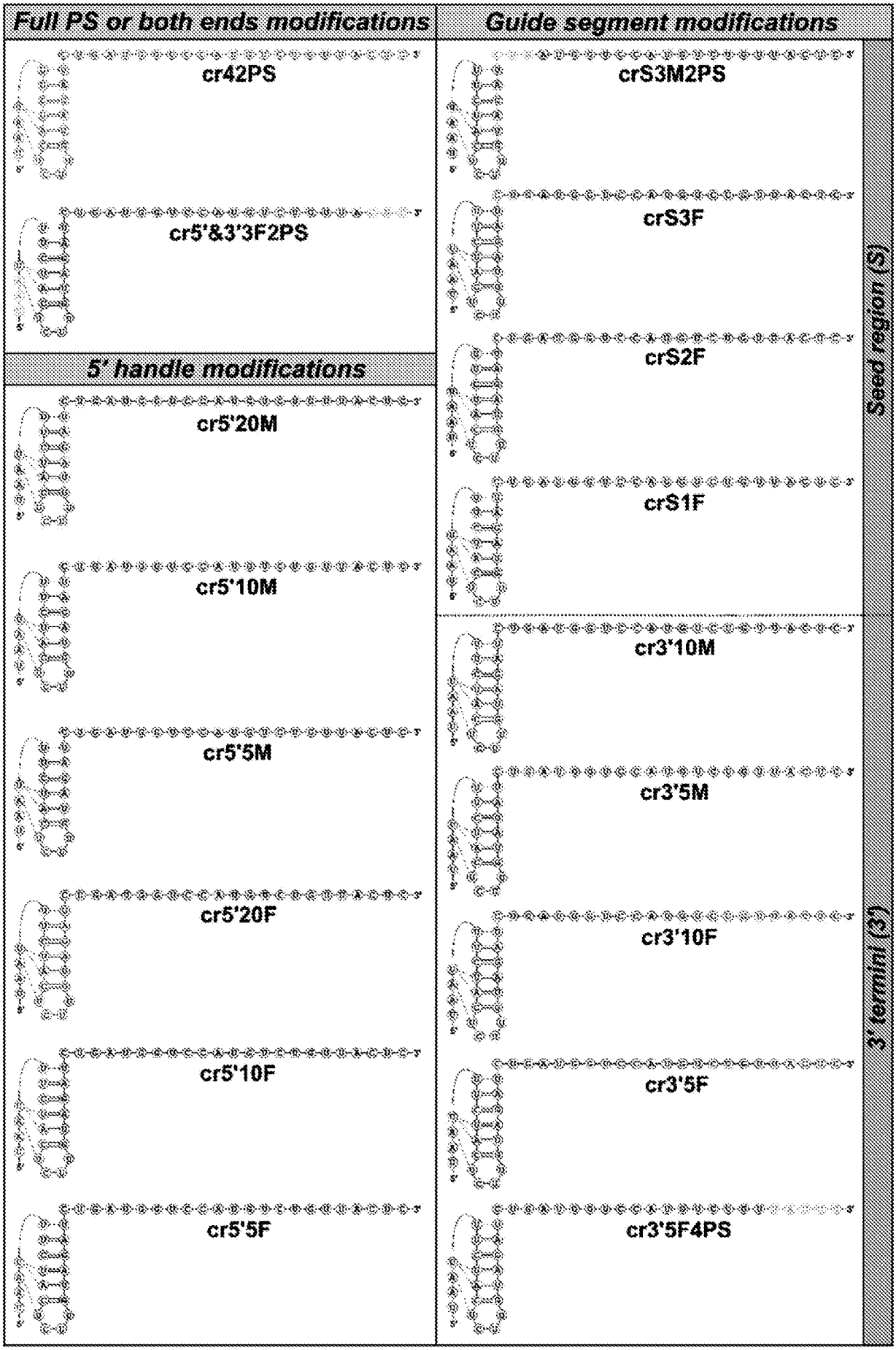

Full PS or both ends modifications
cr42PS
cr5'&3'3F2PS
5' handle modifications
cr5'20M
cr5'10M
cr5'5M
cr5'20F
cr5'10F
cr5'5F
Guide segment modifications
crS3M2PS
crS3F
crS2F
crS1F
cr3'10M
cr3'5M
cr3'10F
cr3'5F
cr3'5F4PS
Seed region (S)
3' terminal (3')

FIG. 9A

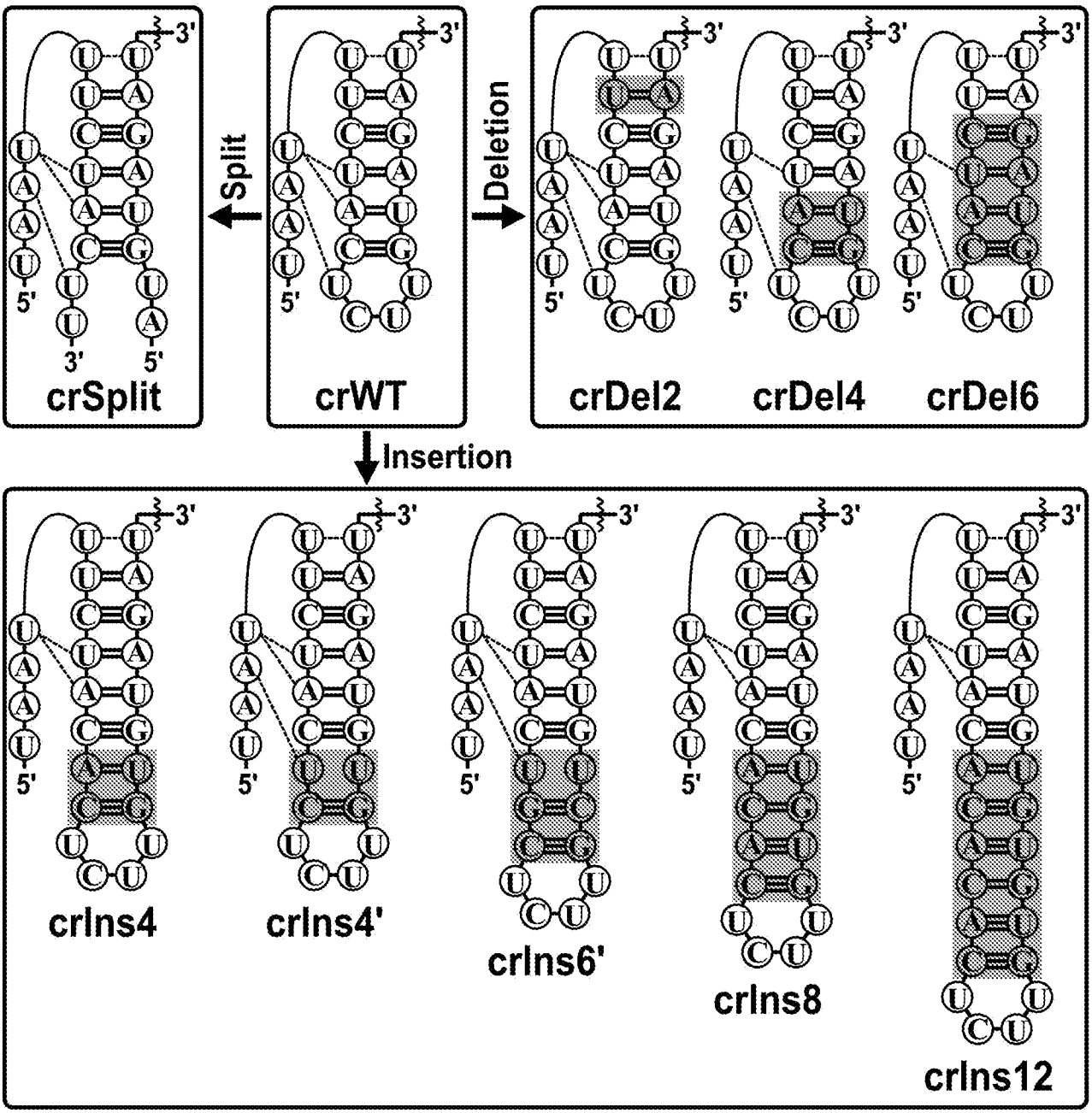
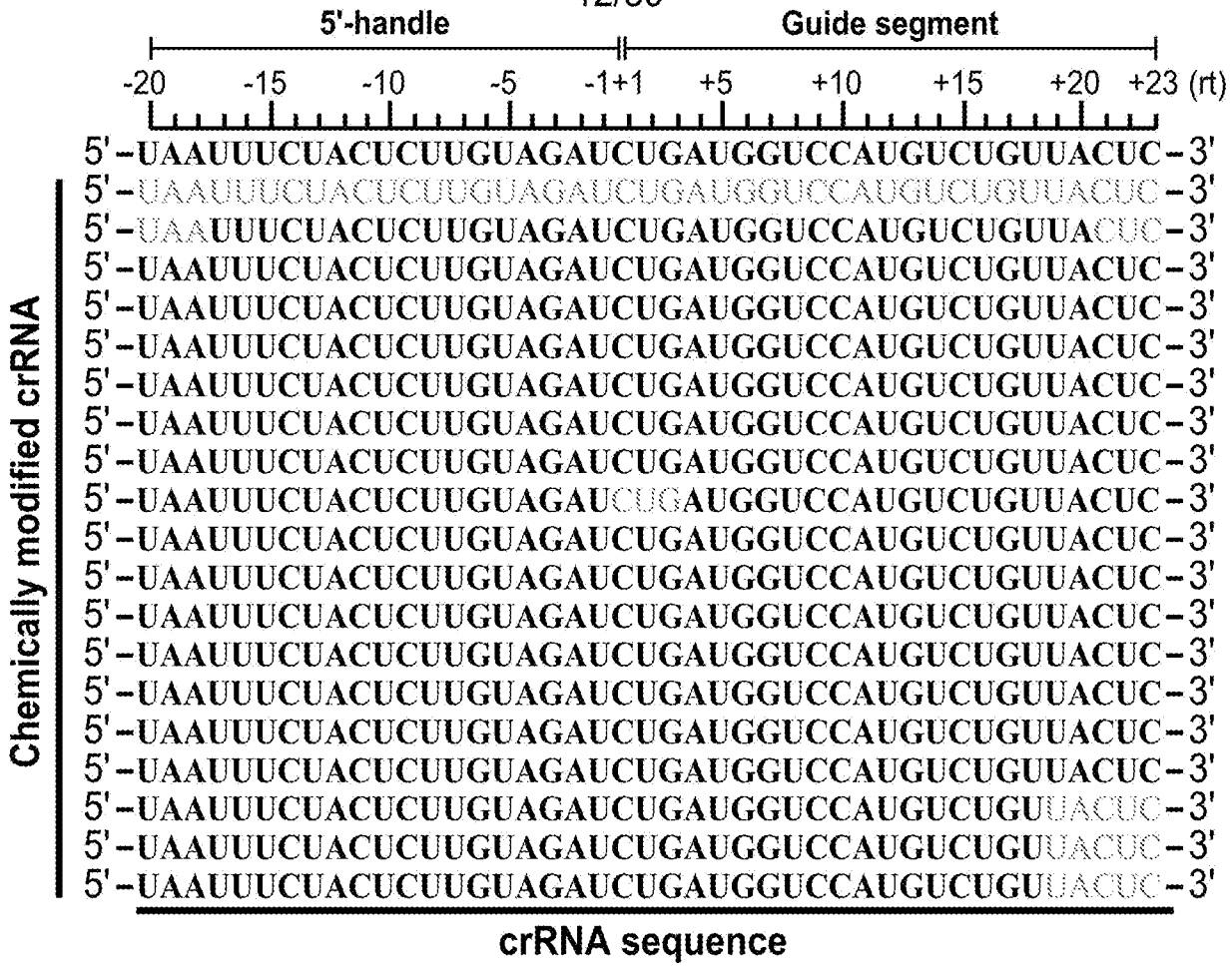

Split
Deletion
Insertion
crSplit
crWT
crDel2
crDel4
crDel6
crIns4
crIns4'
crIns6'
crIns8
crIns12
5'
3'

FIG. 9B


5'-handle
Guide segment
-20 -15 -10 -5 -1+1 +5 +10 +15 +20 +23 (rt)
Chemically modified crRNA
5'-UAAUUUCUACUCUUGUAGAUCUGAUGGUCCAUGUCUGUUACUC-3'
crRNA sequence

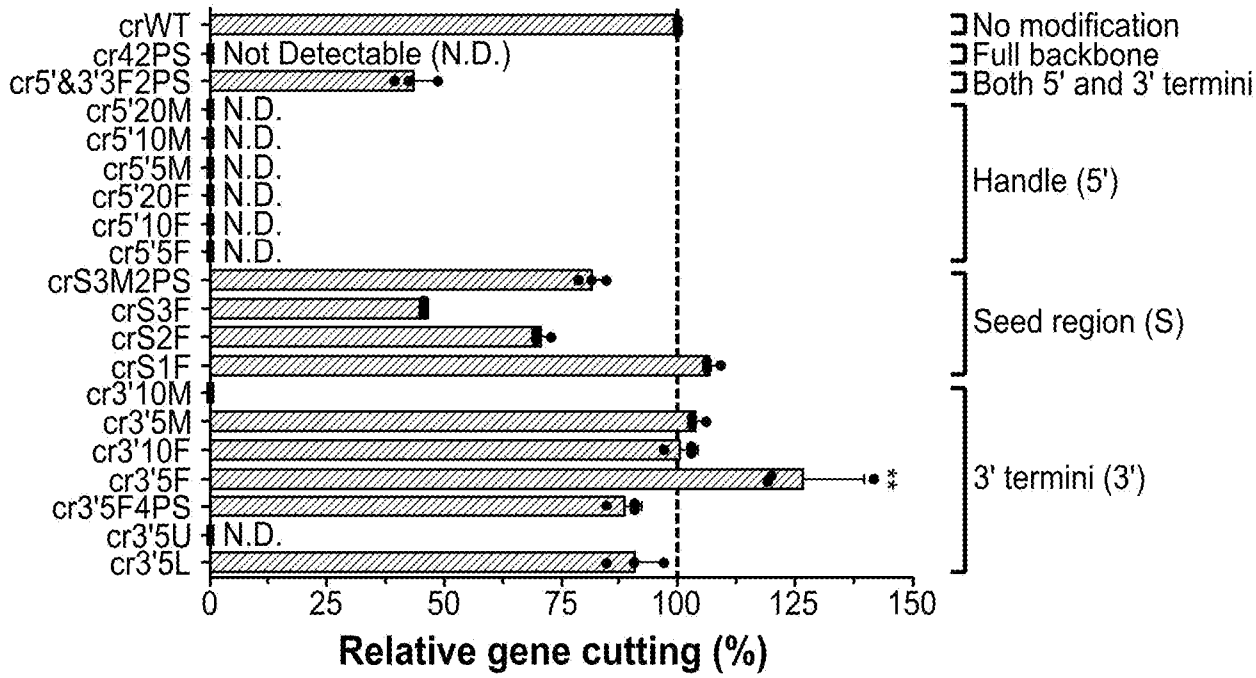
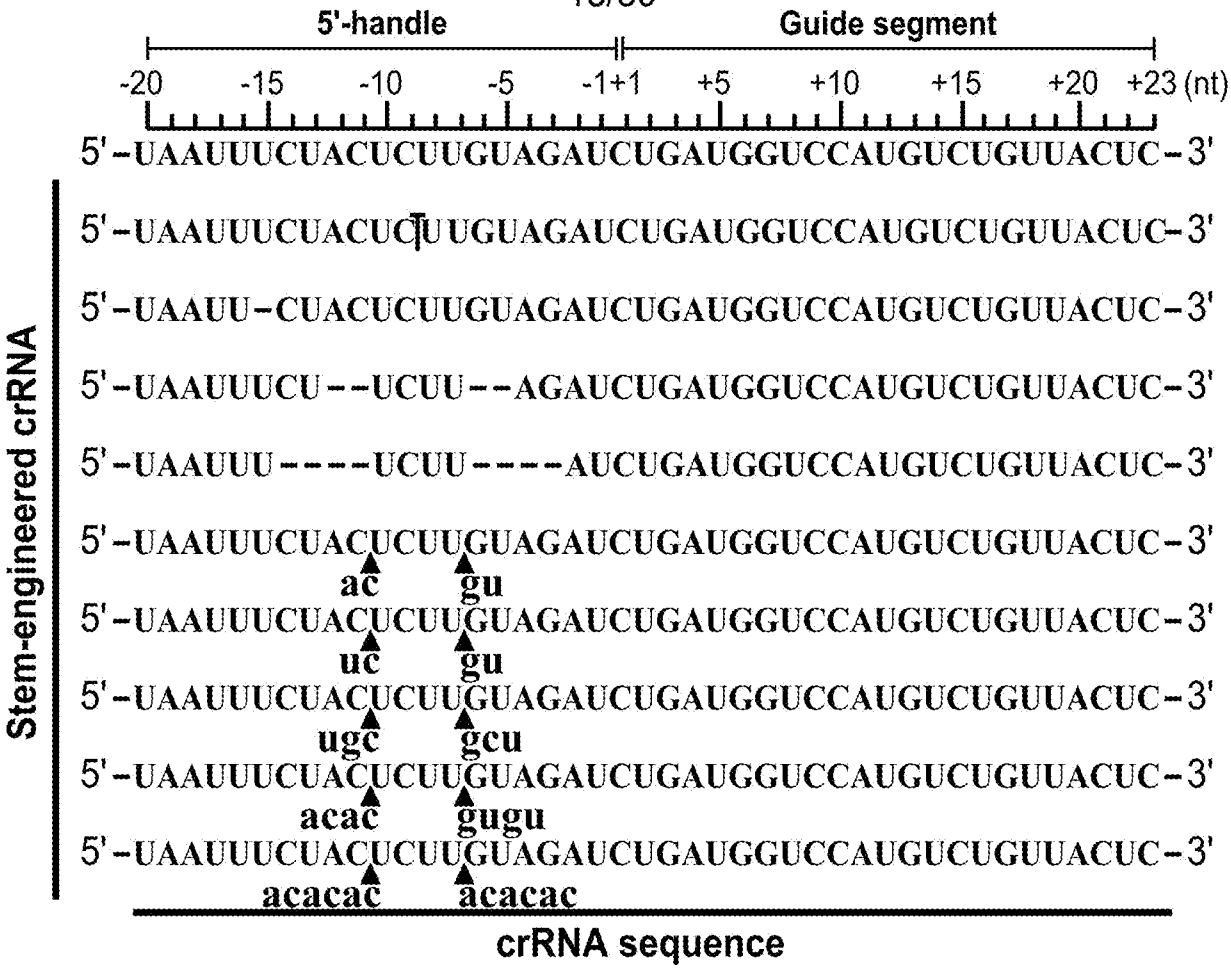

crWT
cr42PS
cr5'&3'3F2PS
cr5'20M
cr5'10M
cr5'5M
cr5'20F
cr5'10F
cr5'5F
crS3M2PS
crS3F
crS2F
crS1F
cr3'10M
cr3'5M
cr3'10F
cr3'5F
cr3'5F4PS
cr3'5U
cr3'5L
Not Detectable (N.D.)
N.D.
0 25 50 75 100 125 150
Relative gene cutting (%)
No modification
Full backbone
Both 5' and 3' termini
Handle (5')
Seed region (S)
3' termini (3')

FIG. 9C


5'-handle
Guide segment
-20 -15 -10 -5 -1+1 +5 +10 +15 +20 +23 (nt)
Stem-engineered crRNA
5'-UAAUUUCUACUCUUGUAGAUCUGAUGGUCCAUGUCUGUUACUC-3'
5'-UAAUUUCUACUCTUGUAGAUCUGAUGGUCCAUGUCUGUUACUC-3'
5'-UAAUU-CUACUCUUGUAGAUCUGAUGGUCCAUGUCUGUUACUC-3'
5'-UAAUUUCU--UCUU--AGAUCUGAUGGUCCAUGUCUGUUACUC-3'
5'-UAAUUU----UCUU----AUCUGAUGGUCCAUGUCUGUUACUC-3'
5'-UAAUUUCUACUCUUGUAGAUCUGAUGGUCCAUGUCUGUUACUC-3'
ac gu
5'-UAAUUUCUACUCUUGUAGAUCUGAUGGUCCAUGUCUGUUACUC-3'
uc gu
5'-UAAUUUCUACUCUUGUAGAUCUGAUGGUCCAUGUCUGUUACUC-3'
ugc gcu
5'-UAAUUUCUACUCUUGUAGAUCUGAUGGUCCAUGUCUGUUACUC-3'
acac gugu
5'-UAAUUUCUACUCUUGUAGAUCUGAUGGUCCAUGUCUGUUACUC-3'
acacac acacac
crRNA sequence

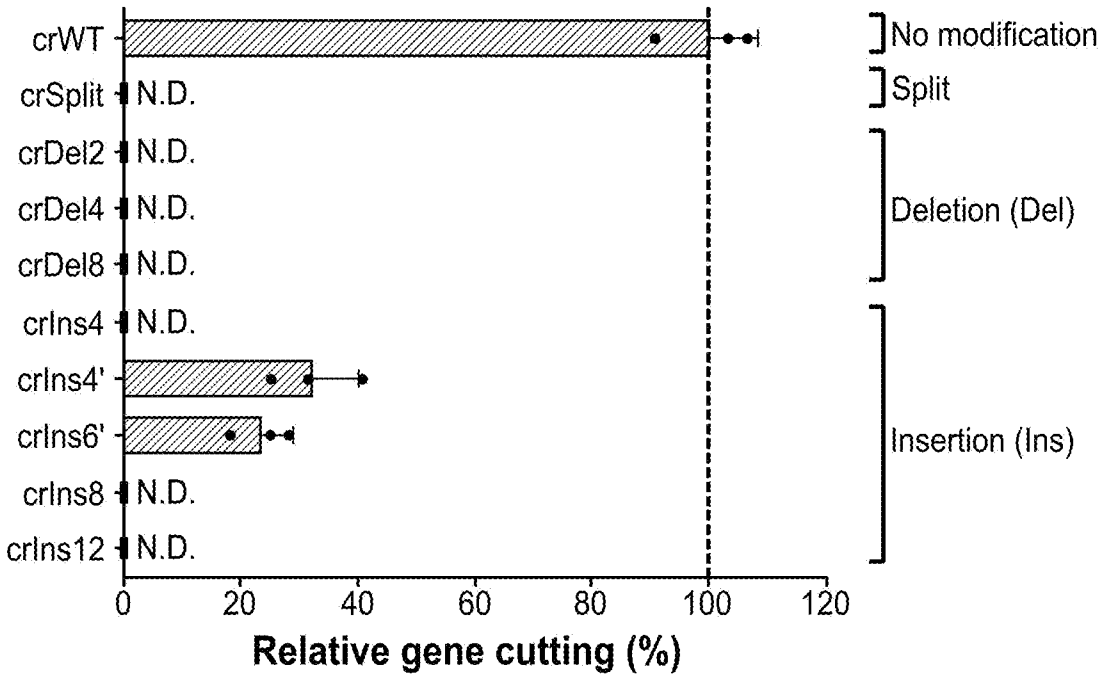

crWT
crSplit N.D.
crDel2 N.D.
crDel4 N.D.
crDel8 N.D.
crIns4 N.D.
crIns4'
crIns6'
crIns8 N.D.
crIns12 N.D.
No modification
Split
Deletion (Del)
Insertion (Ins)
0 20 40 60 80 100 120
Relative gene cutting (%)

FIG. 9D

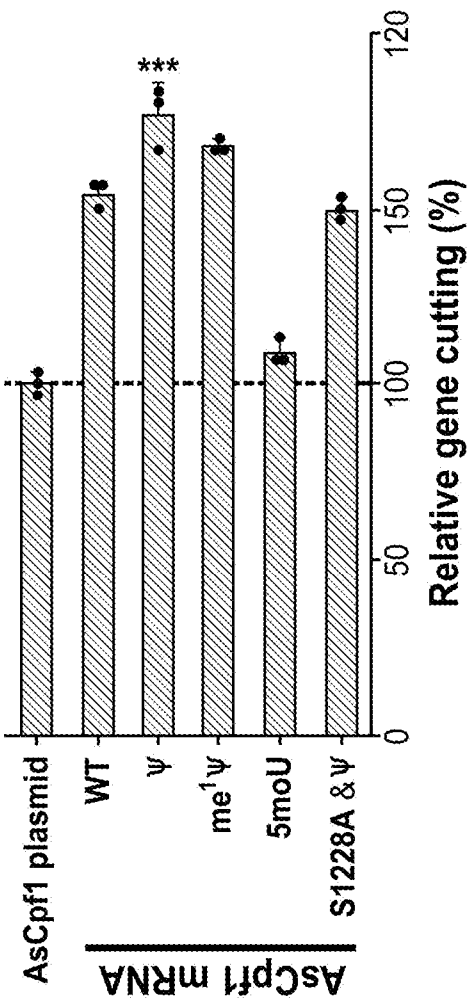

AsCpf1 mRNA
AsCpf1 plasmid
WT
ψ
me1ψ
5moU
S1228A & ψ
***
0
50
100
150
120
Relative gene cutting (%)

FIG. 9E

a
DNMT1-3 Locus: >NC_000019.10, GRCh38.p7
HEK293T
crWT+plasmid
cr3'5F+mRNA (ψ)
Hep3B
crWT+plasmid
cr3'5F+mRNA (ψ)
U87
crWT+plasmid
cr3'5F+mRNA (ψ)
1000 bp
500 bp
300 bp
19±1
48±3
14±1
46±3
15±1
44±4
Indel (%)
***

FIG. 10

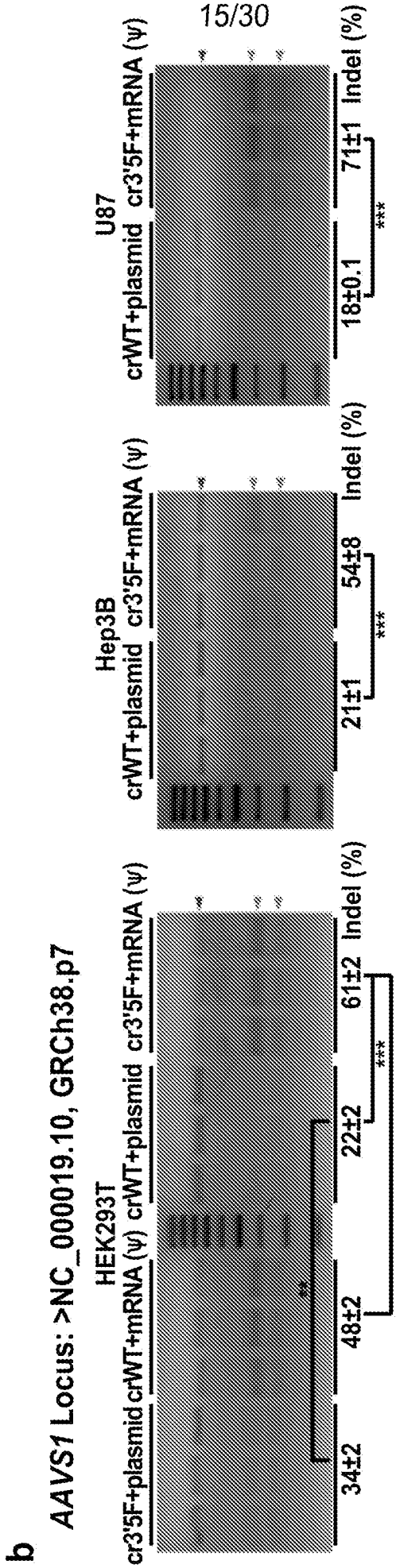
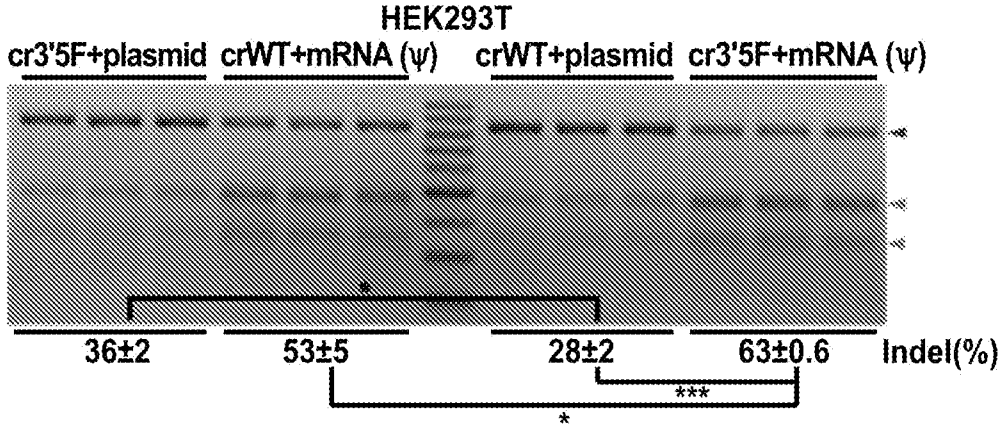


FIG. 10 continued

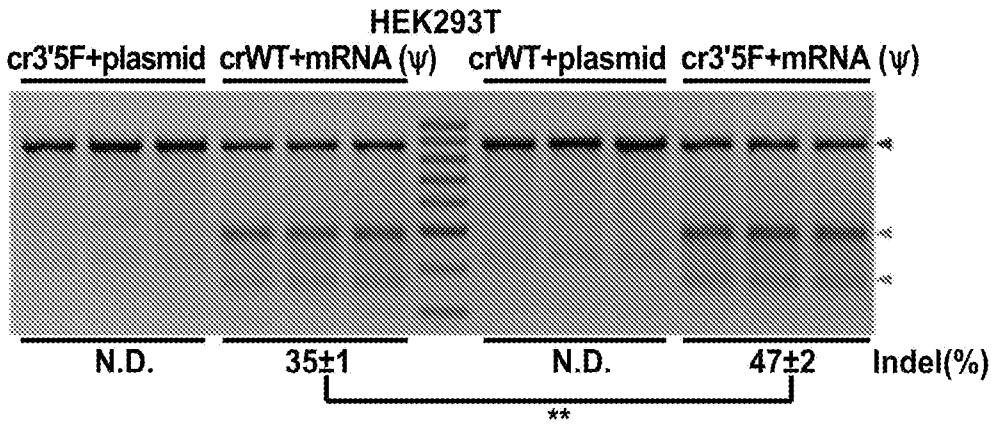
c

FANCF-2 Locus: >NC_000011.10, GRCh38.p7



d

DNMT1-3 Locus: >NC_000019.10, GRCh38.p7



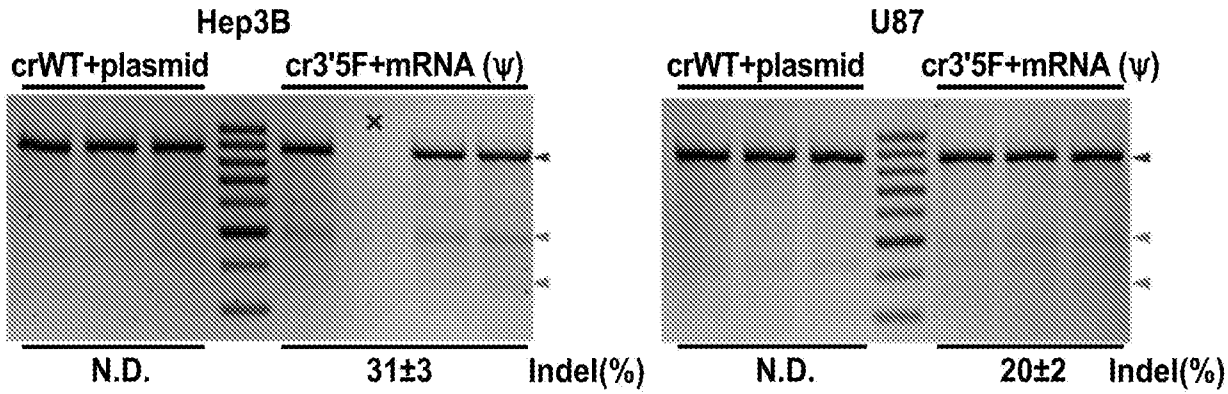


FIG. 10 continued

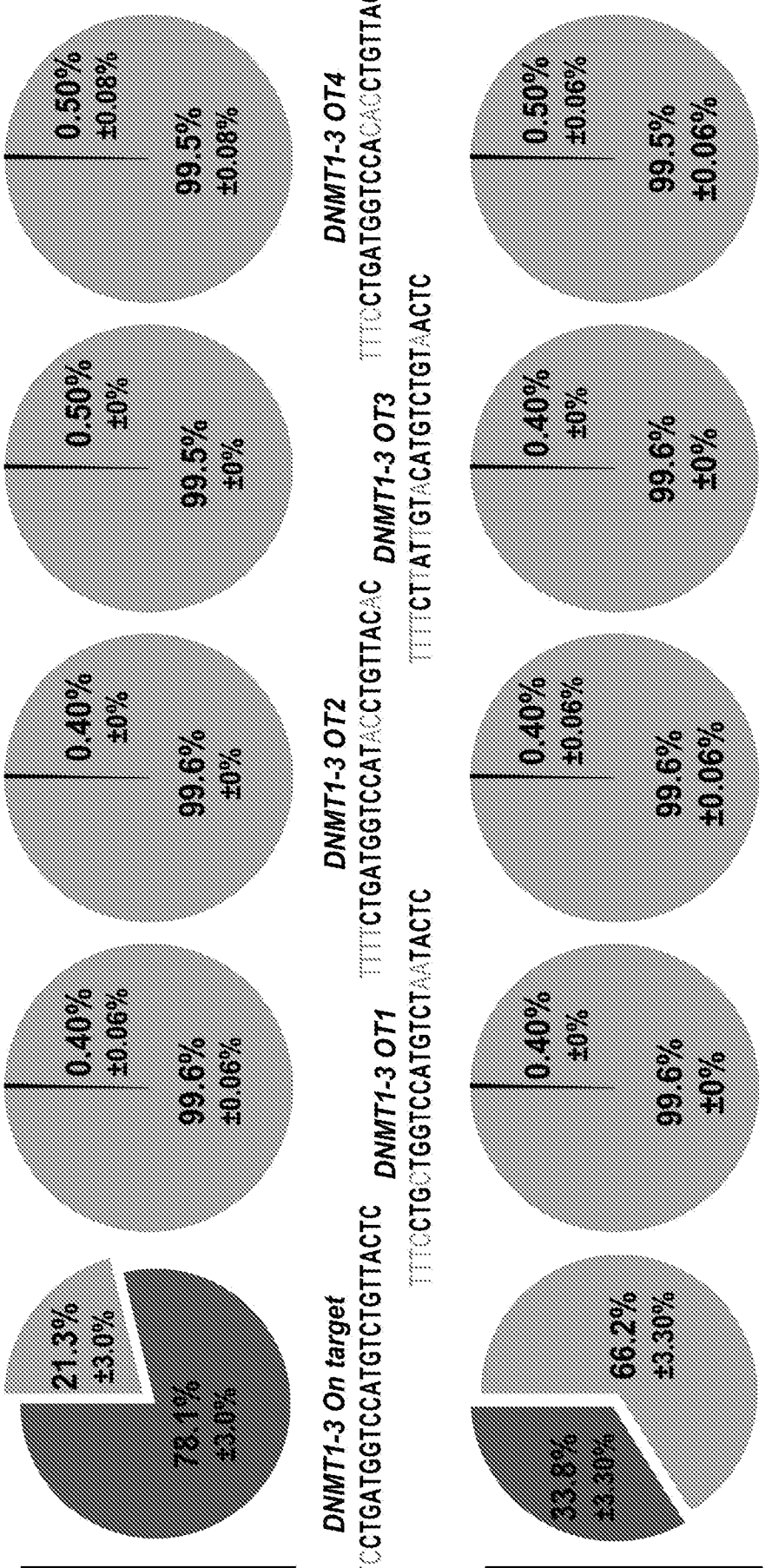

crWT + plasmid
cr3'5F + ΨmRNA
DNMT1-3 On target
TTTCCTGATGGTCCATGTCTGTTACTC
DNMT1-3 OT1
TTTCCTGCTGGTCCATGTCTAATACTC
DNMT1-3 OT2
TTTCCTGATGGTCCATACCTGTTACAC
DNMT1-3 OT3
TTTCTTATTGTACATGTCTGTAACTC
DNMT1-3 OT4
TTTCCTGATGGTCCACACCTGTTACAC
21.3% ±3.0%
78.1% ±3.0%
0.40% ±0.06%
99.6% ±0.06%
0.40% ±0%
99.6% ±0%
0.50% ±0%
99.5% ±0%
0.50% ±0.08%
99.5% ±0.08%
33.8% ±3.30%
66.2% ±3.30%
0.40% ±0%
99.6% ±0%
0.40% ±0.06%
99.6% ±0.06%
0.40% ±0%
99.6% ±0%
0.50% ±0.06%
99.5% ±0.06%
Indel
No indel
On target
Off target

FIG. 11A

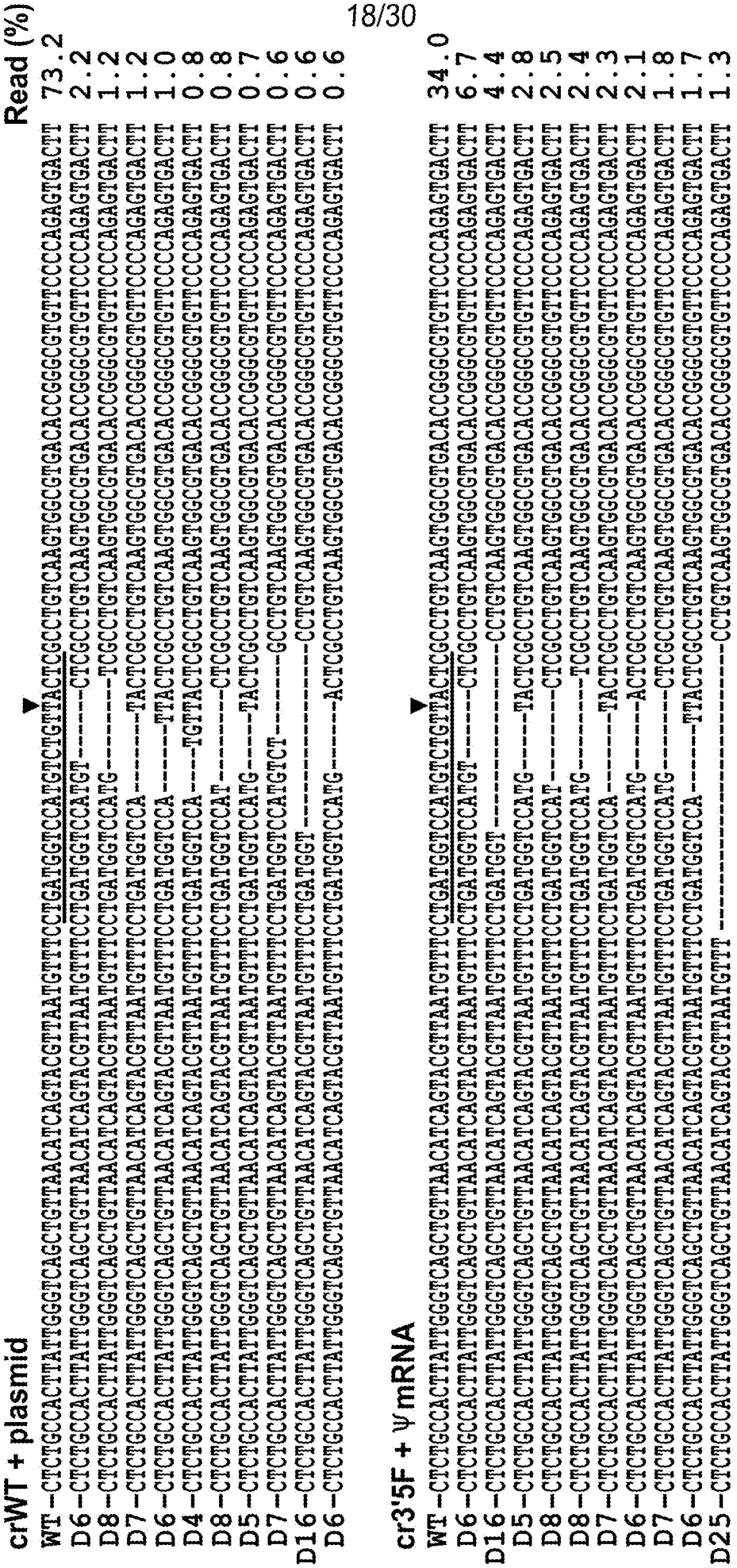

crWT + plasmid
Read (%)
WT -CTCTGCCACTTATTGGGTCAGCTGTTAACATCAGTACGTTAATGTTTCCTGATGGTCCATGTCTGTTACTCGCCTGTCAAGTGGCGTGACACCGGGCGTGTTCCCCAGAGTGACTT 73.2
D6 -CTCTGCCACTTATTGGGTCAGCTGTTAACATCAGTACGTTAATGTTTCCTGATGGTCCATGT-------CTCGCCTGTCAAGTGGCGTGACACCGGGCGTGTTCCCCAGAGTGACTT 2.2
D8 -CTCTGCCACTTATTGGGTCAGCTGTTAACATCAGTACGTTAATGTTTCCTGATGGTCCATG--------TCGCCTGTCAAGTGGCGTGACACCGGGCGTGTTCCCCAGAGTGACTT 1.2
D7 -CTCTGCCACTTATTGGGTCAGCTGTTAACATCAGTACGTTAATGTTTCCTGATGGTCCA---------TACTCGCCTGTCAAGTGGCGTGACACCGGGCGTGTTCCCCAGAGTGACTT 1.2
D6 -CTCTGCCACTTATTGGGTCAGCTGTTAACATCAGTACGTTAATGTTTCCTGATGGTCCA------TTACTCGCCTGTCAAGTGGCGTGACACCGGGCGTGTTCCCCAGAGTGACTT 1.0
D4 -CTCTGCCACTTATTGGGTCAGCTGTTAACATCAGTACGTTAATGTTTCCTGATGGTCCA----TGTTACTCGCCTGTCAAGTGGCGTGACACCGGGCGTGTTCCCCAGAGTGACTT 0.8
D8 -CTCTGCCACTTATTGGGTCAGCTGTTAACATCAGTACGTTAATGTTTCCTGATGGTCCAT---------CTCGCCTGTCAAGTGGCGTGACACCGGGCGTGTTCCCCAGAGTGACTT 0.8
D5 -CTCTGCCACTTATTGGGTCAGCTGTTAACATCAGTACGTTAATGTTTCCTGATGGTCCATG------TACTCGCCTGTCAAGTGGCGTGACACCGGGCGTGTTCCCCAGAGTGACTT 0.7
D7 -CTCTGCCACTTATTGGGTCAGCTGTTAACATCAGTACGTTAATGTTTCCTGATGGTCCATGTCT---------GCCTGTCAAGTGGCGTGACACCGGGCGTGTTCCCCAGAGTGACTT 0.6
D16 -CTCTGCCACTTATTGGGTCAGCTGTTAACATCAGTACGTTAATGTTTCCTGATGGT----------CCTGTCAAGTGGCGTGACACCGGGCGTGTTCCCCAGAGTGACTT 0.6
D6 -CTCTGCCACTTATTGGGTCAGCTGTTAACATCAGTACGTTAATGTTTCCATG-------ACTCGCCTGTCAAGTGGCGTGACACCGGGCGTGTTCCCCAGAGTGACTT 0.6
cr3'5F + Ψ mRNA
WT -CTCTGCCACTTATTGGGTCAGCTGTTAACATCAGTACGTTAATGTTTCCTGATGGTCCATGTCTGTTACTCGCCTGTCAAGTGGCGTGACACCGGGCGTGTTCCCCAGAGTGACTT 34.0
D6 -CTCTGCCACTTATTGGGTCAGCTGTTAACATCAGTACGTTAATGTTTCCTGATGGTCCATGT-------CTCGCCTGTCAAGTGGCGTGACACCGGGCGTGTTCCCCAGAGTGACTT 6.7
D16 -CTCTGCCACTTATTGGGTCAGCTGTTAACATCAGTACGTTAATGTTTCCTGATGGT----------CCTGTCAAGTGGCGTGACACCGGGCGTGTTCCCCAGAGTGACTT 4.4
D5 -CTCTGCCACTTATTGGGTCAGCTGTTAACATCAGTACGTTAATGTTTCCTGATGGTCCATG------TACTCGCCTGTCAAGTGGCGTGACACCGGGCGTGTTCCCCAGAGTGACTT 2.8
D8 -CTCTGCCACTTATTGGGTCAGCTGTTAACATCAGTACGTTAATGTTTCCTGATGGTCCAT---------CTCGCCTGTCAAGTGGCGTGACACCGGGCGTGTTCCCCAGAGTGACTT 2.5
D8 -CTCTGCCACTTATTGGGTCAGCTGTTAACATCAGTACGTTAATGTTTCCTGATGGTCCATG--------TCGCCTGTCAAGTGGCGTGACACCGGGCGTGTTCCCCAGAGTGACTT 2.4
D7 -CTCTGCCACTTATTGGGTCAGCTGTTAACATCAGTACGTTAATGTTTCCTGATGGTCCA---------TACTCGCCTGTCAAGTGGCGTGACACCGGGCGTGTTCCCCAGAGTGACTT 2.3
D6 -CTCTGCCACTTATTGGGTCAGCTGTTAACATCAGTACGTTAATGTTTCCTGATGGTCCATG------ACTCGCCTGTCAAGTGGCGTGACACCGGGCGTGTTCCCCAGAGTGACTT 2.1
D7 -CTCTGCCACTTATTGGGTCAGCTGTTAACATCAGTACGTTAATGTTTCCTGATGGTCCATG------CTCGCCTGTCAAGTGGCGTGACACCGGGCGTGTTCCCCAGAGTGACTT 1.8
D6 -CTCTGCCACTTATTGGGTCAGCTGTTAACATCAGTACGTTAATGTTTCCTGATGGTCCA------TTACTCGCCTGTCAAGTGGCGTGACACCGGGCGTGTTCCCCAGAGTGACTT 1.7
D25 -CTCTGCCACTTATTGGGTCAGCTGTTAACATCAGTACGTTAATGTTT ----------CCTGTCAAGTGGCGTGACACCGGGCGTGTTCCCCAGAGTGACTT 1.3

FIG. 11B

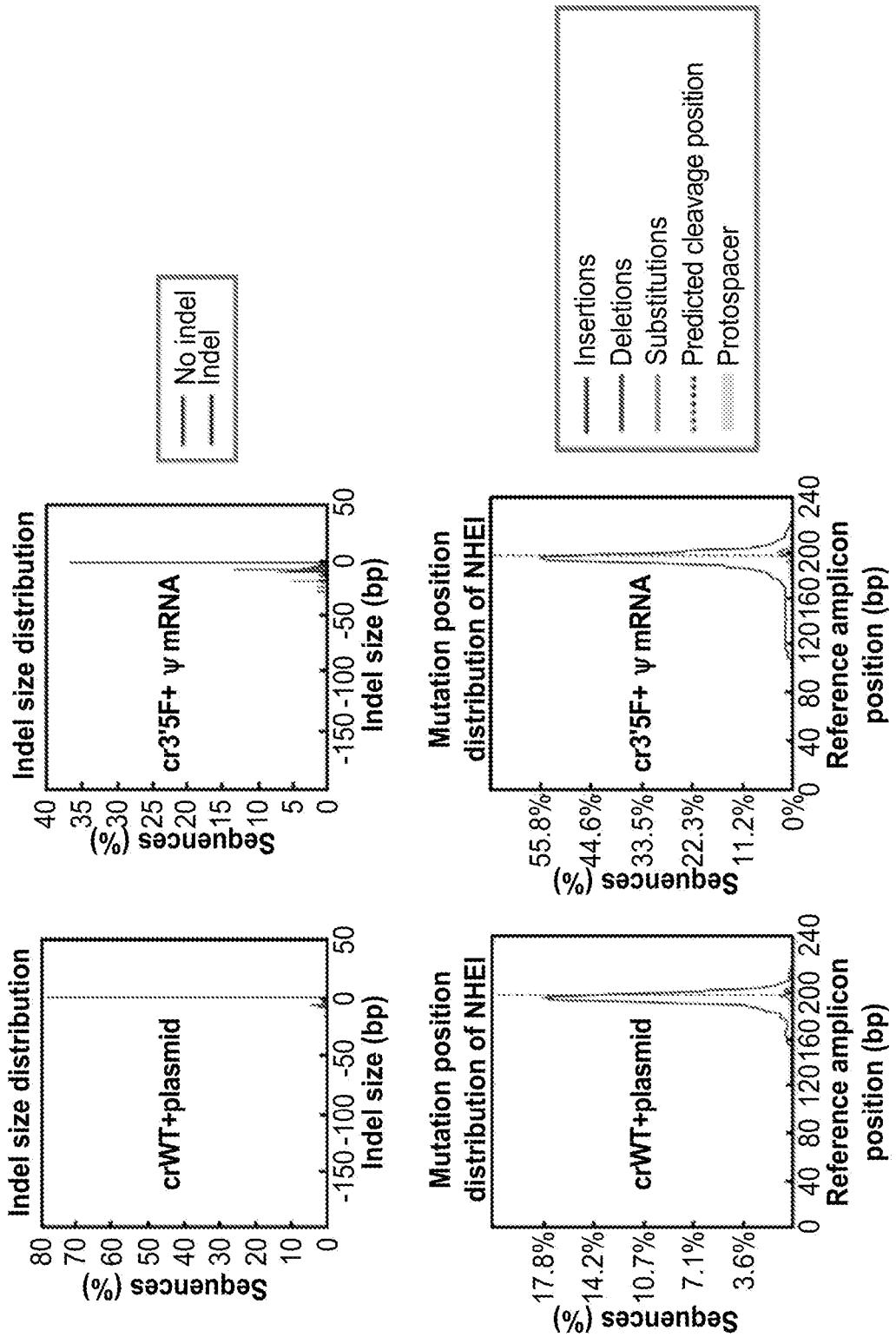

No indel
Indel
Insertions
Deletions
Substitutions
Predicted cleavage position
Protospacer
Indel size distribution
crWT+plasmid
Sequences (%)
Indel size (bp)
Mutation position distribution of NHEI
Reference amplicon position (bp)
cr3'5F+ ψ mRNA

FIG. 11C

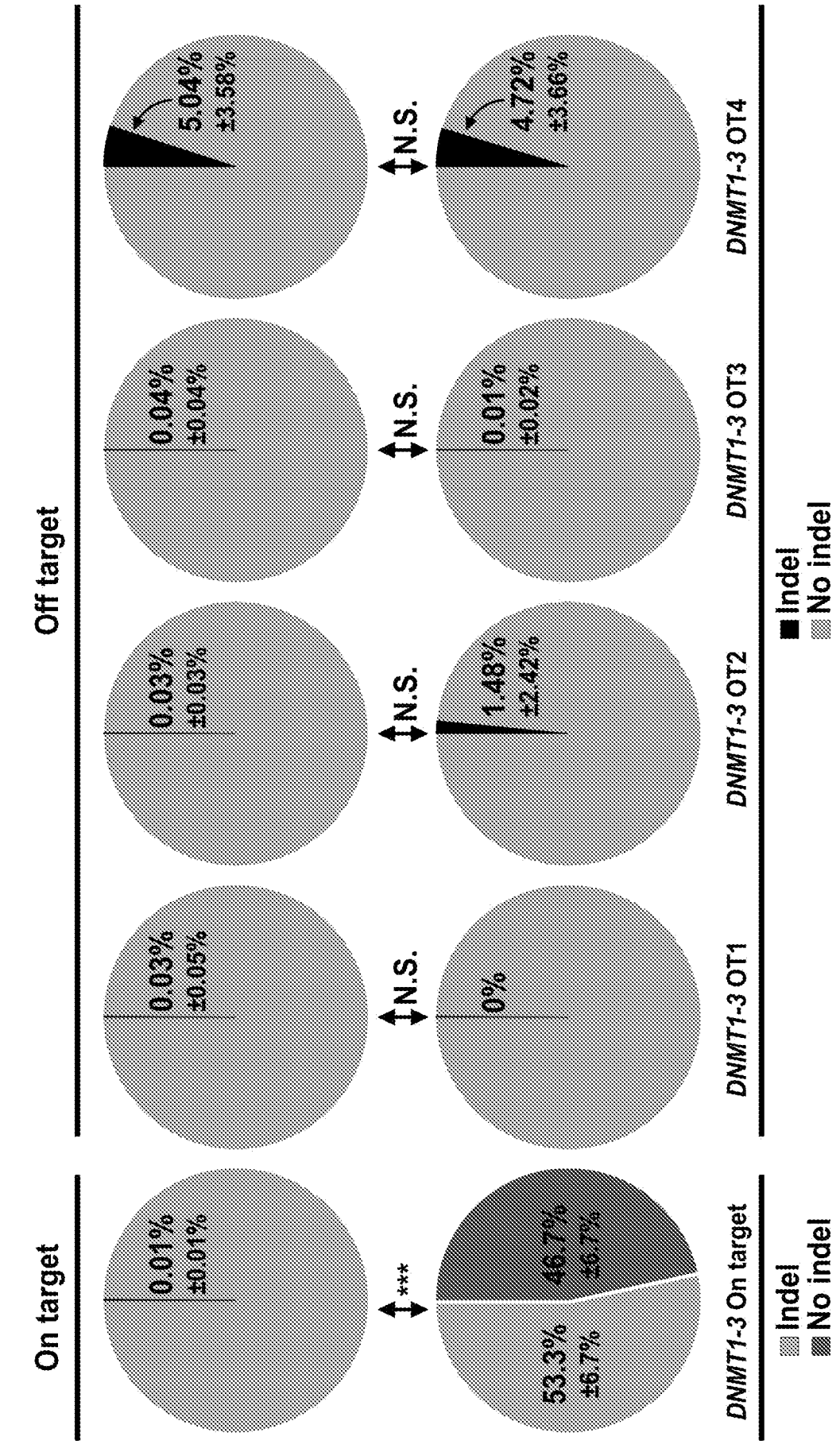

a
crWT + LbCpf1 plasmid
cr3'5F + LbCpf1 mRNA(Ψ)
On target
Off target
0.01%
±0.01%
***
53.3%
±6.7%
46.7%
±6.7%
0.03%
±0.05%
N.S.
0%
0.03%
±0.03%
N.S.
1.48%
±2.42%
0.04%
±0.04%
N.S.
0.01%
±0.02%
5.04%
±3.58%
N.S.
4.72%
±3.66%
DNMT1-3 On target
DNMT1-3 OT1
DNMT1-3 OT2
DNMT1-3 OT3
DNMT1-3 OT4
Indel
No indel
Indel
No indel

FIG. 12

b

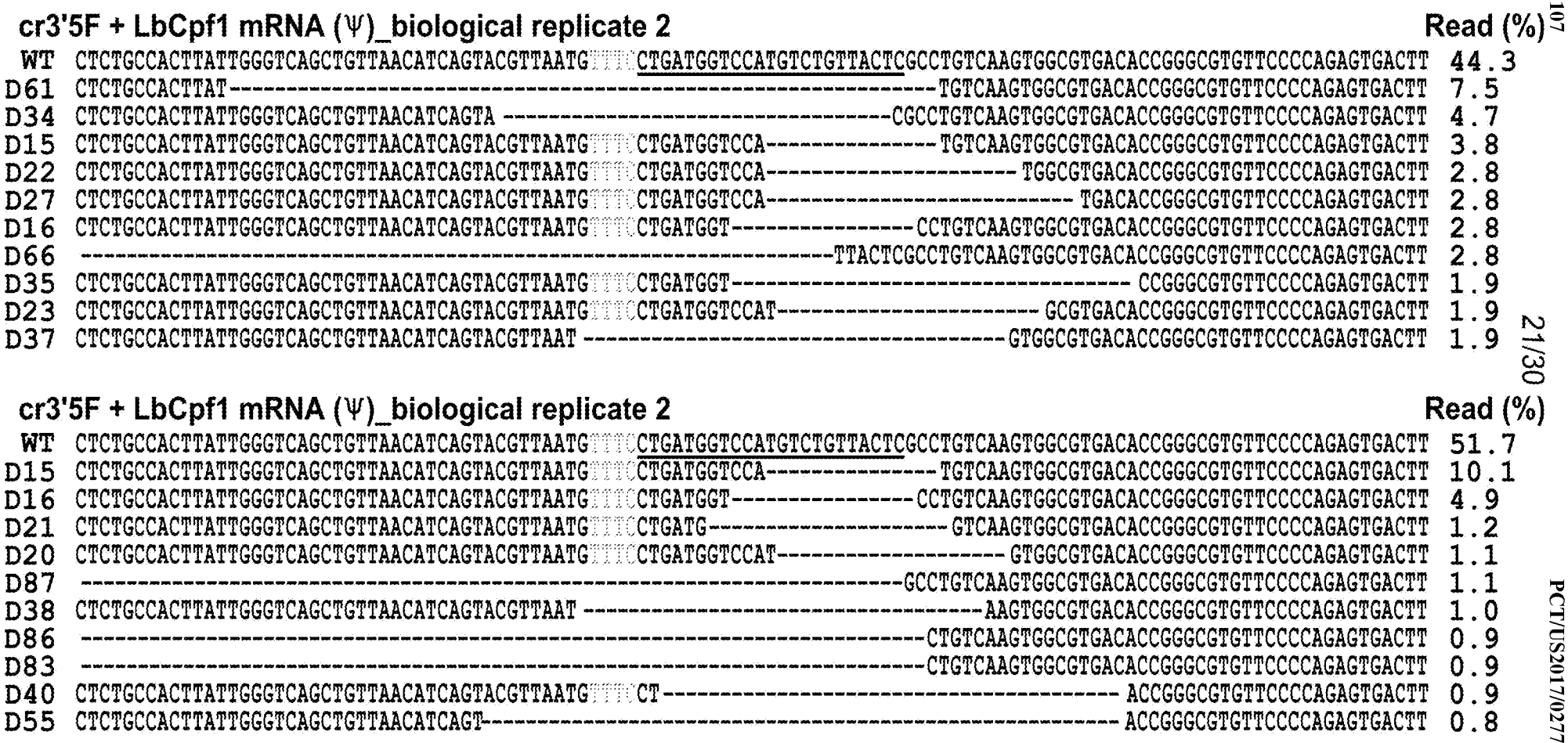


FIG. 12 continued

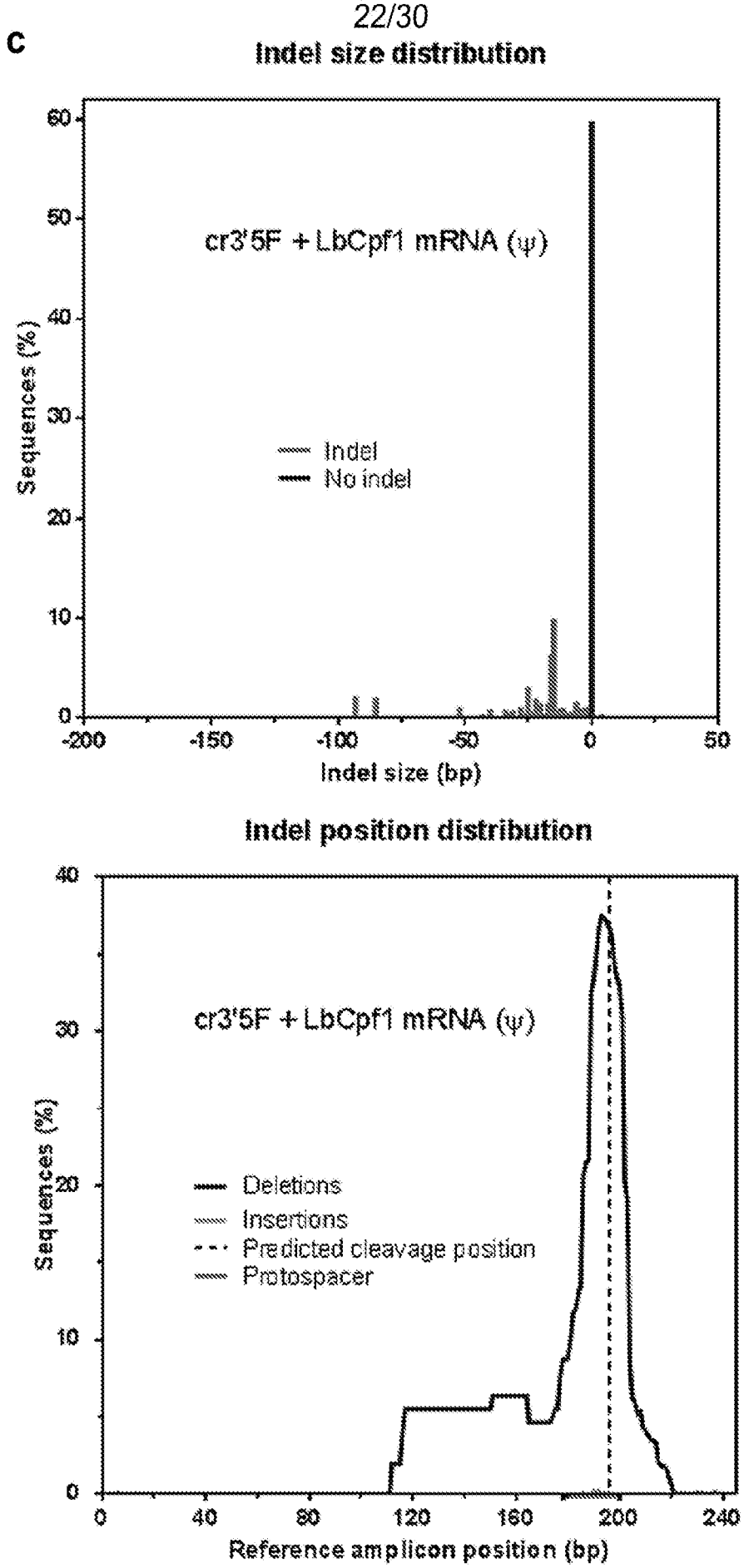
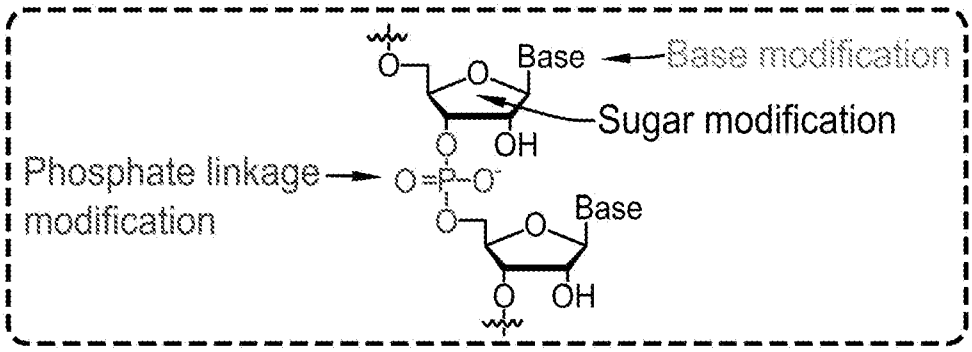


FIG. 12 continued


Base
Base modification
Sugar modification
OH
Phosphate linkage modification
O=P-O
Base
OH

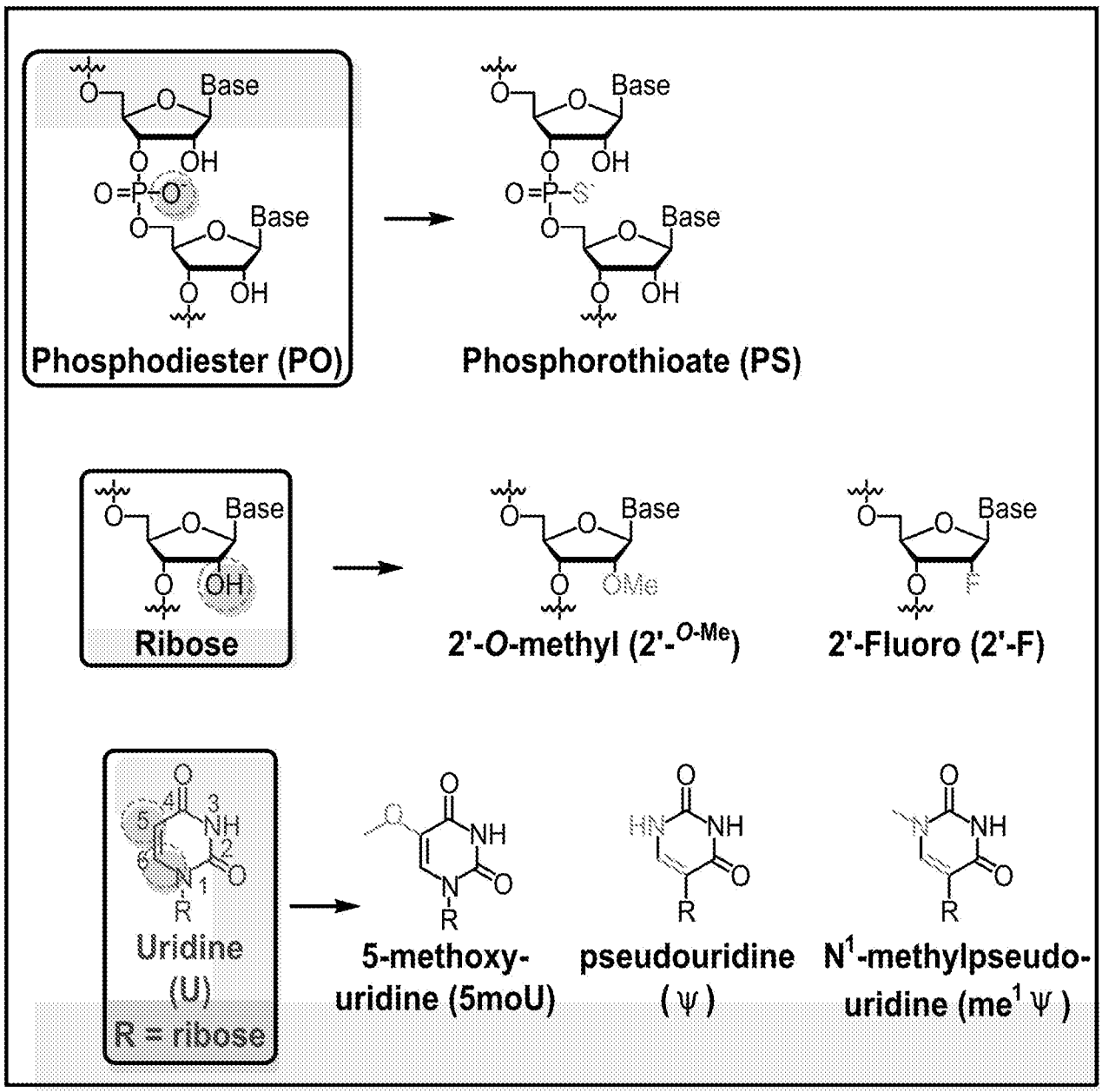

Base
OH
O=P-O
Base
OH
Phosphodiester (PO)
O=P-S
Phosphorothioate (PS)
Ribose
OMe
2'-O-methyl (2'-O-Me)
F
2'-Fluoro (2'-F)
Uridine
(U)
R = ribose
5-methoxy-uridine (5moU)
pseudouridine (ψ)
N1-methylpseudo-uridine (me1ψ)

FIG. 13

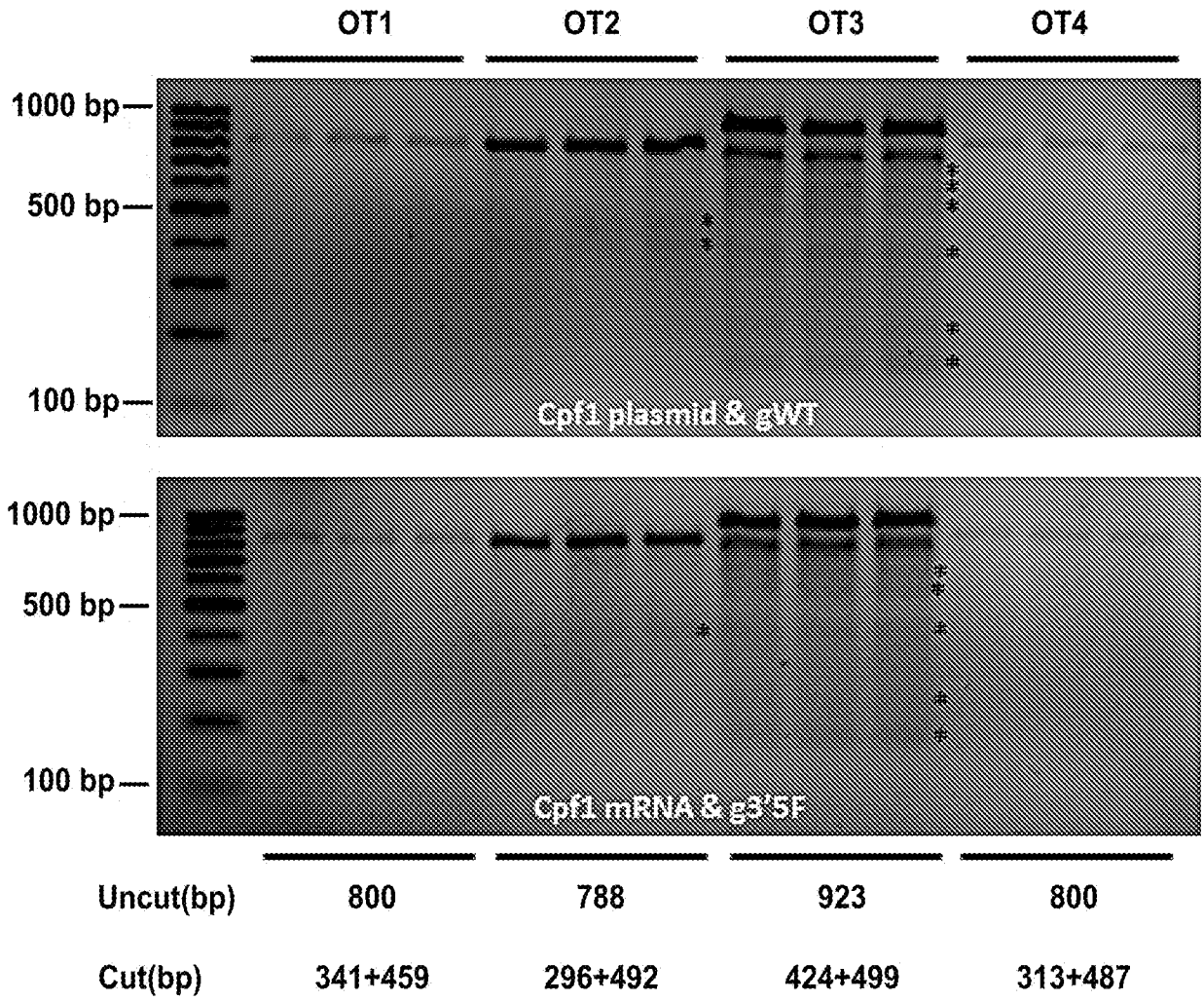

OT1
OT2
OT3
OT4
1000 bp
500 bp
100 bp
Cpf1 plasmid & gWT
1000 bp
500 bp
100 bp
Cpf1 mRNA & g3'5F
Uncut(bp) 800 788 923 800
Cut(bp) 341+459 296+492 424+499 313+487

FIG. 14

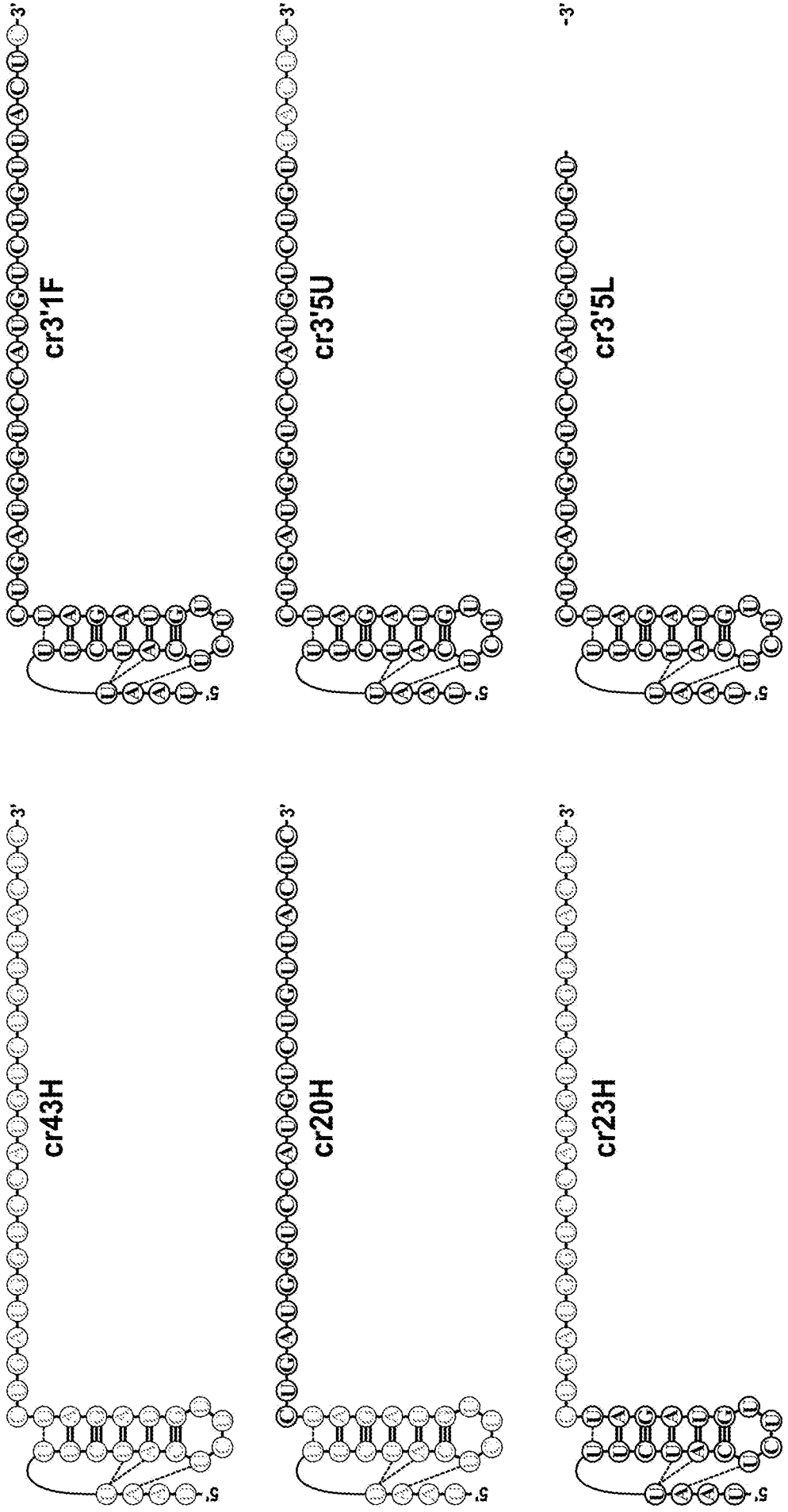


FIG. 15

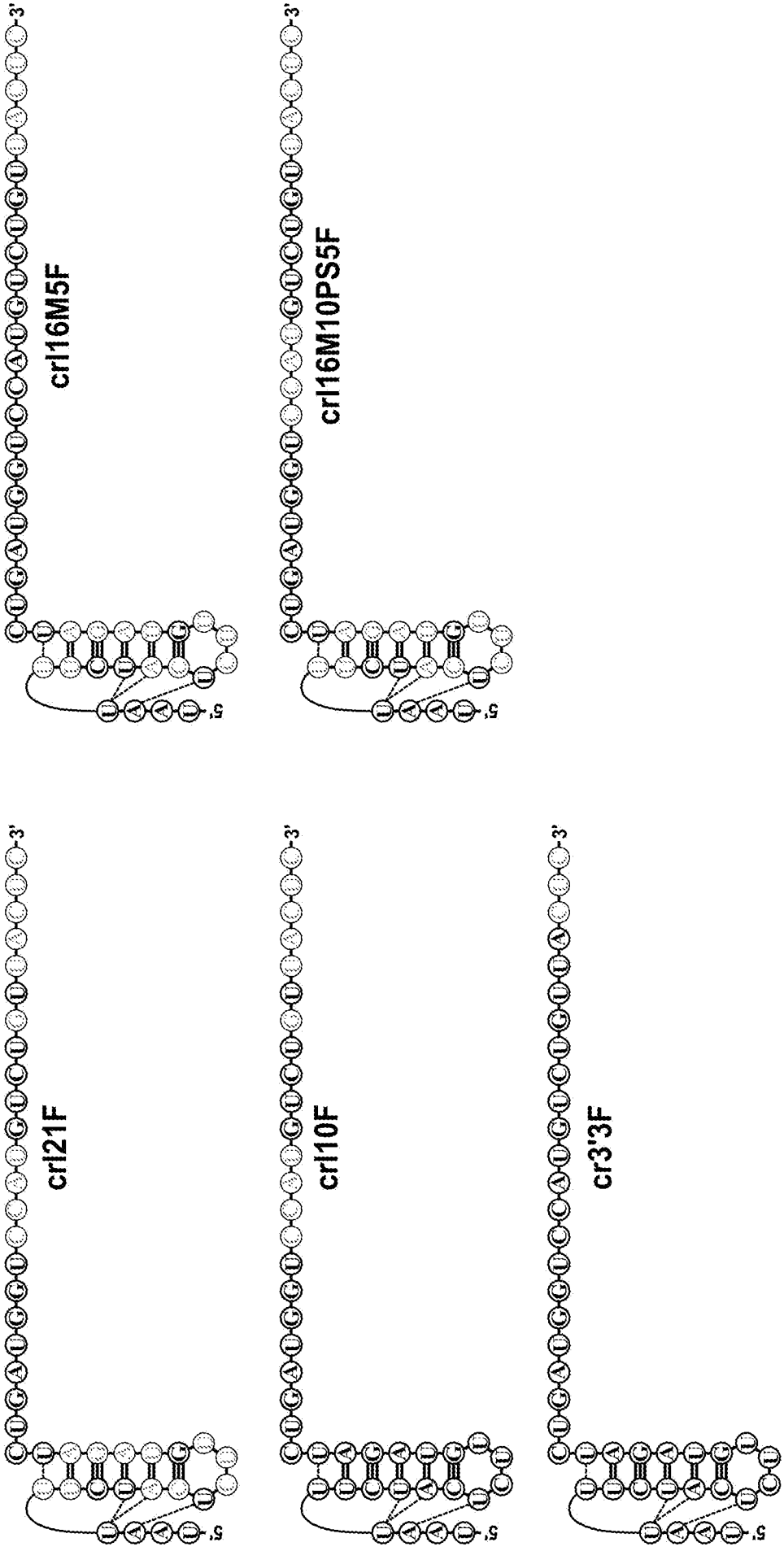


FIG. 15 continued

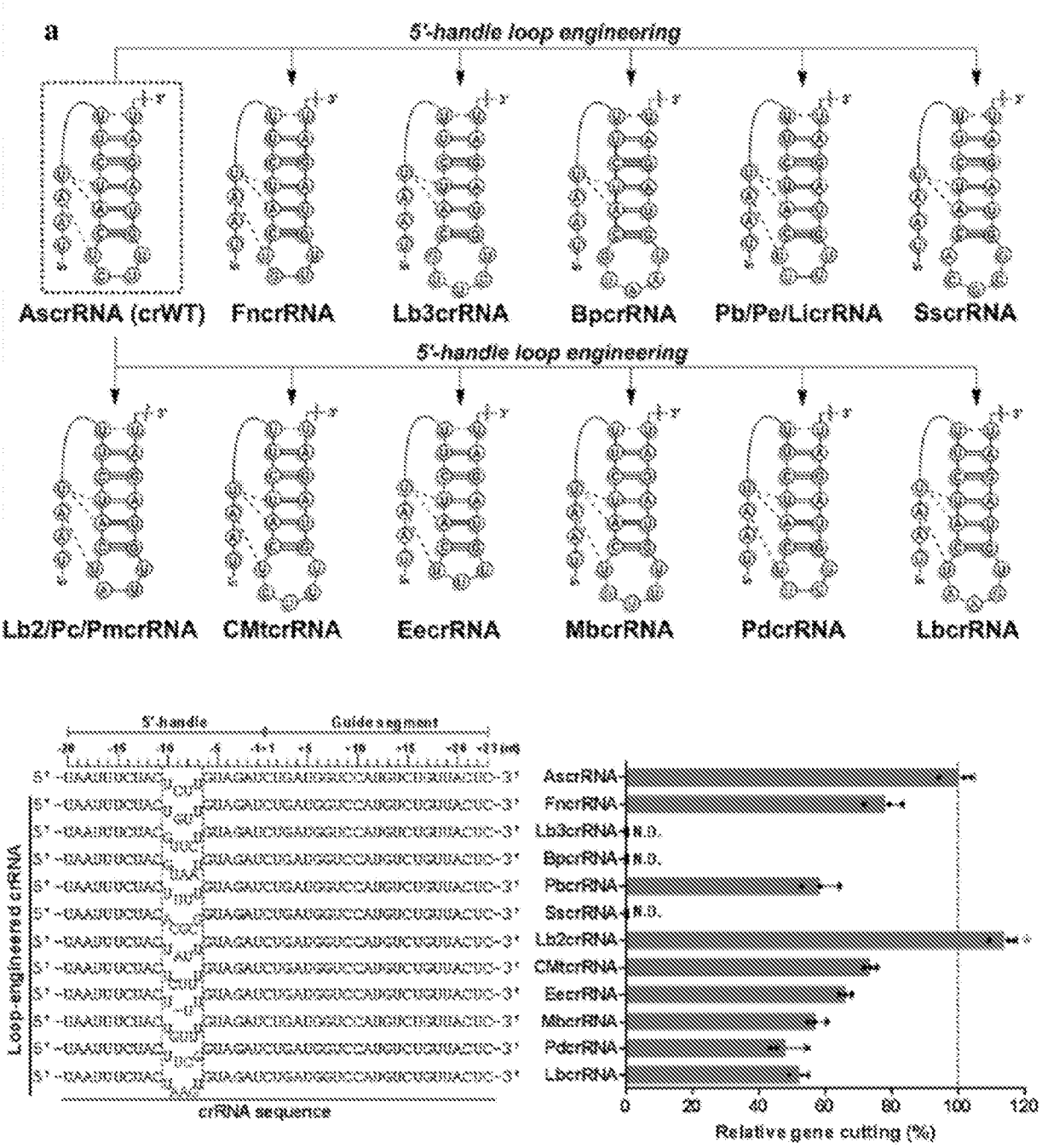

a
5'-handle loop engineering
AscrRNA (crWT)
FncrRNA
Lb3crRNA
BpcrRNA
Pb/Pe/LicrRNA
SscrRNA
5'-handle loop engineering
Lb2/Pc/PmcrRNA
CMtcrRNA
EecrRNA
MbcrRNA
PdcrRNA
LbcrRNA
5' handle
Guide segment
Loop-engineered crRNA
crRNA sequence
AscrRNA
FncrRNA
Lb3crRNA
BpcrRNA
PbcrRNA
SscrRNA
Lb2crRNA
CMtcrRNA
EecrRNA
MbcrRNA
PdcrRNA
LbcrRNA
N.D.
N.D.
N.D.
0
20
40
60
80
100
120
Relative gene cutting (%)

FIG. 16

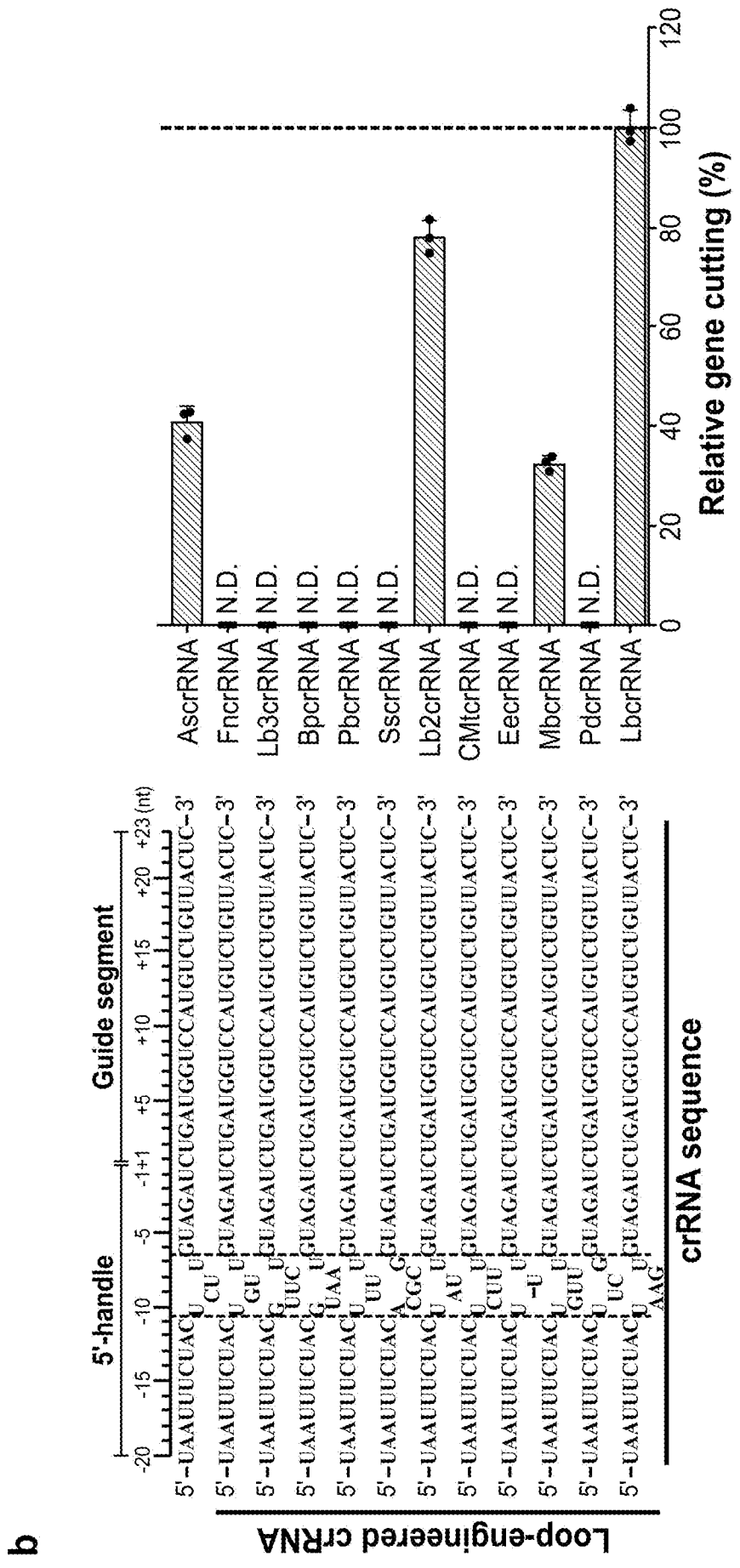


FIG. 16 continued

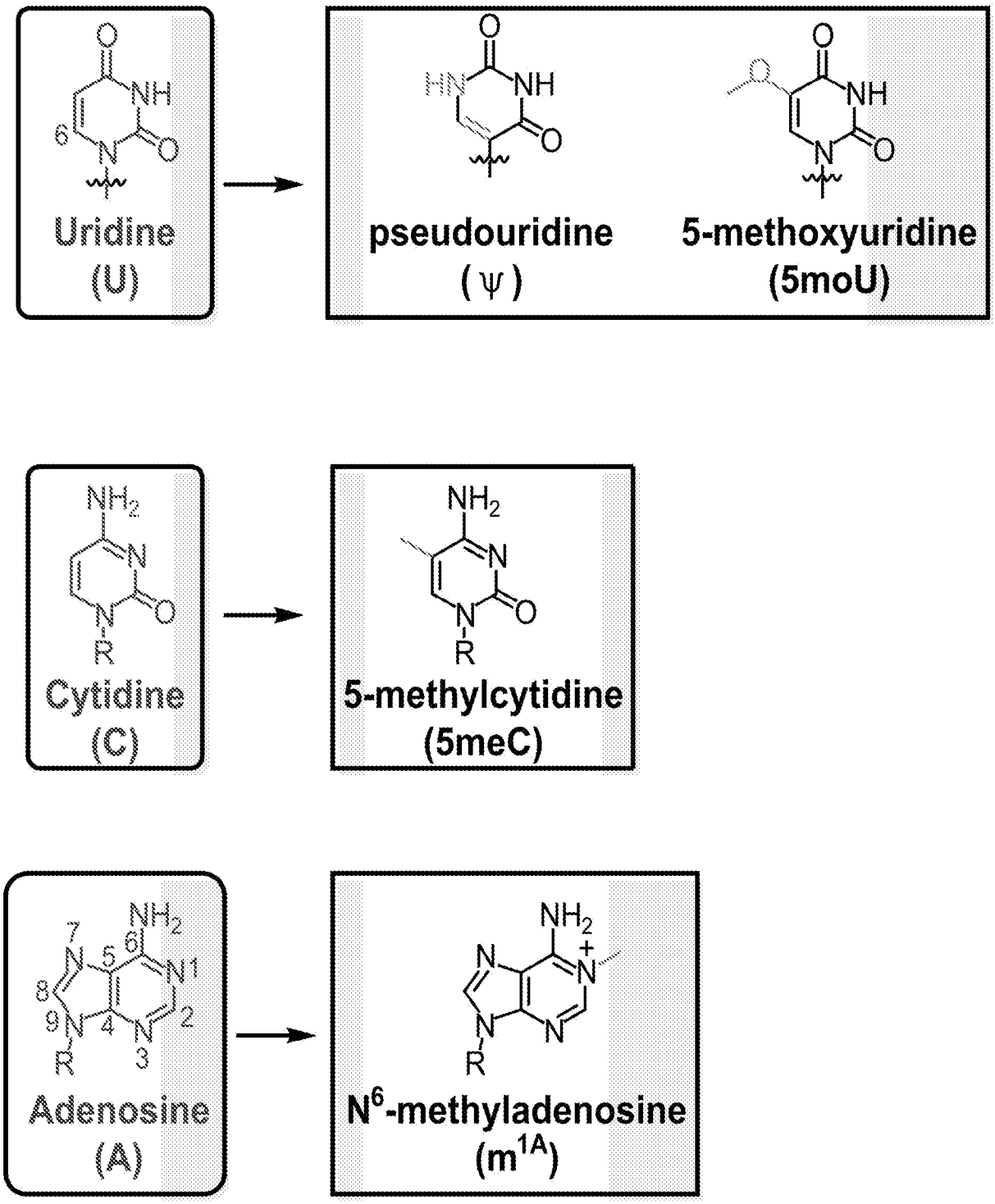

Uridine
(U)
pseudouridine
(ψ)
5-methoxyuridine
(5moU)
Cytidine
(C)
5-methylcytidine
(5meC)
Adenosine
(A)
N6-methyladenosine
(m1A)

FIG. 17

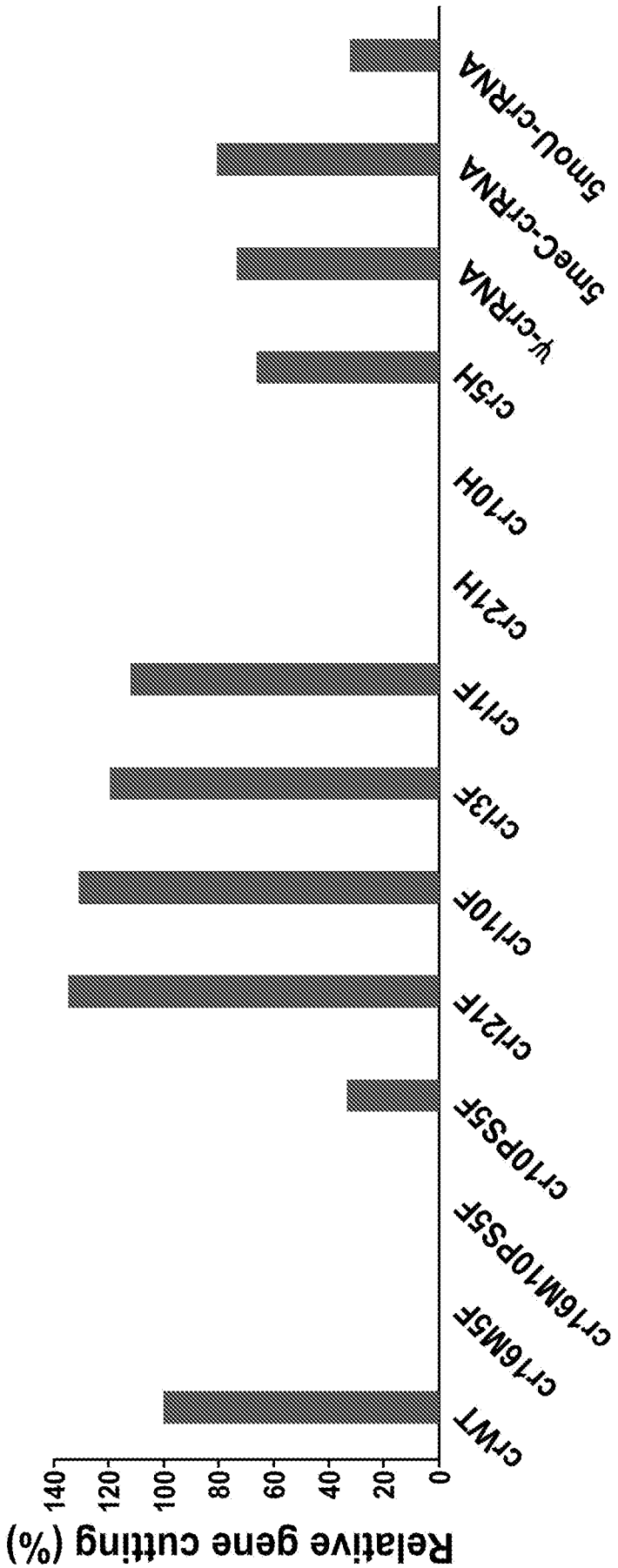

Relative gene cutting (%)
0
20
40
60
80
100
120
140
crWT
cr16M5F
cr16M10PS5F
cr10PS5F
cr12F
cr10F
cr13F
cr11F
cr21H
cr10H
cr5H
Ψ-crRNA
5meC-crRNA
5moU-crRNA

FIG. 18